Pharmacological Evaluation of NF279 as a P2 Receptor Antagonist

Structure-Activity Relationship Studies of Analogues of the P2 Receptor Antagonists
Suramin and NF023 at Native P2 Receptor Subtypes and Ecto-Nucleotidases

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So eine Arbeit wird eigentlich nie fertig, man muß sie für fertig erklären, wenn man nach Zeit und Umständen das möglichste getan hat.

Johann Wolfgang von Goethe (1749-1832)

Für meine Eltern und Uli

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1. Introduction and Aim of the Thesis

1.1. Introduction

1.1.1. History of purinergic transmission

More than 70 years ago, the effects of extracellular adenosine 5'-triphosphate (ATP), a newly identified and purified biomolecule at that time (Fiske and Subbarow, 1925; Lohmann, 1929) were observed by Drury and Szent-Györgyi (1929). Since then, many pharmacological studies were carried out with extracellular adenine nucleotides in various intact organ systems, isolated tissues, and purified cell preparations. Yet it was not until 1972 that Burnstock introduced the concept of "purinergic nerves" and suggested that ATP might fulfil the criteria generally regarded as necessary for establishing a substance as a neurotransmitter, summarised by Eccles (1964):

- *synthesis and storage of transmitter in nerve terminals*
  Strips of guinea-pig taenia coli (GPTC) were shown to take up large amounts of tritium-labelled adenosine when incubated with tritium-labelled adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP) and ATP. The nucleoside was rapidly converted into and retained largely as [^{3}H]-ATP (Su et al., 1971).
- *release of transmitter during nerve stimulation*
  Spontaneous relaxation of GPTC as well as relaxations induced by nerve stimulation or nicotine, respectively, in the presence of compounds which block adrenergic and cholinergic responses were accompanied by a remarkable increase in release of tritium-labelled material from taenia coli incubated in [^{3}H]-adenosine (Su et al., 1971).
- *postjunctional responses to exogenous transmitters that mimic responses to nerve stimulation*
  Burnstock et al. (1966) characterised ATP and ADP as the most potent inhibitory purine compounds in the gut and observed that the effects of ATP mimic more closely the inhibitory response of the taenia to non-adrenergic nerve-stimulation than to adrenergic nerve stimulation (Burnstock et al., 1970).
- *enzymes that inactivate the transmitter and/or uptake systems for the transmitter or its breakdown products*
  When ATP was added to a perfusion fluid recycled through the vasculature of the stomach, very little ATP remained, but the perfusate contained substantially

increased amounts of adenosine and inosine, as well as some ADP and AMP (Burnstock et al., 1970).

- *drugs that can produce parallel blocking of potentiating effects on the responses of both exogenous transmitter and nerve stimulation*
  Tachyphylaxis to ATP produced in the rabbit ileum resulted in a consistent depression of responses to non-adrenergic inhibitory nerve stimulation, whereas responses to adrenergic nerve stimulation remained unaffected (Burnstock et al., 1970). Lower concentrations of quinidine reduced and finally abolished relaxation of GPTC induced by noradrenaline (NA) and by adrenergic nerve stimulation. Using higher concentrations of the compound, relaxant responses of GPTC to ATP as well as to non-adrenergic inhibitory nerve stimulation were abolished (Burnstock et al., 1970).

Based on these observation it was proposed that ATP and/or adenosine, released as major neurotransmitter or as a co-transmitter from putative purinergic nerves, might mediate the non-adrenergic, non-cholinergic (NANC) signalling observed in the gut.

Although there has been some reluctance to fully accept the idea that nucleotides have the capacity to act as autocrine and paracrine cellular messengers, the purinergic theory was strengthened by a great body of experimental evidence supporting the role of ATP as a transmitter or co-transmitter with noradrenaline, acetylcholine (ACh) and other substances and by the identification of specific receptors mediating the variety of physiological effects induced by purines.

P_1 and P_2 receptors

In 1978, Burnstock proposed a basis for distinguishing two types of purinergic receptors: the P_1 receptor preferentially activated by adenosine, and the P_2 receptor preferentially activated by ATP. This original classification was mainly based on the following criteria:

- the relative potencies of ATP, ADP, AMP and adenosine (P_1: adenosine $\geq$ AMP > ADP $\geq$ ATP; P_2: ATP $\geq$ ADP > AMP $\geq$ adenosine);

- the selective actions of methylxanthines (theophylline, caffeine, and aminophylline), which competitively antagonised adenosine, but not ATP actions;
- the modulation of adenylate cyclase (AC) by adenosine, but not by ATP;
- and the induction of prostaglandin synthesis by ATP, but not by adenosine.

P_{2X} and P_{2Y} purinoceptors

In 1985, a further subdivision of P_2 receptors was introduced by Burnstock and Kennedy. Their definition rested mainly on agonist selectivities:

- potency order of α,β-methylene-ATP (α,β-mATP), β,γ-methylene-ATP (β,γ-mATP) > ATP = 2-methylthio-ATP (2-MeSATP), and antagonism by arylazido-aminopropionyl-ATP (ANAPP$_3$) as well as desensitisation of contractile responses of guinea-pig and rat vas deferens (ratVD) and urinary bladder following administration of α,β-mATP mediated by subtype 1 (designated P_{2X} purinoceptor);
- potency order of 2-MeSATP >> ATP > α,β-mATP = β,γ-mATP, weak antagonism of ANAPP$_3$ and no or poor desensitisation following administration of α,β-mATP of relaxant responses of GPTC and the longitudinal muscle layer of rabbit portal vein mediated by subtype 2 (designated P_{2Y} purinoceptor).

To date, it is obvious that it was a most fortunate historical accident that the pharmacological subdivision was created thus, as it happens to coincide with a fundamental division in molecular structure. The receptors originally identified as P_{2X} receptors for their α,β-mATP-responsiveness are now known to belong to a larger family of ionotropic ATP receptors. The other subclass of this family, originally recognised by preference for 2-MeSATP and being unaffected by α,β-mATP, actually comprises multiple subtypes that belong to the larger family of metabotropic or guanyl nucleotide binding protein (G protein) -coupled receptors.

P_{2T} and P_{2Z} receptors

One year after Burnstock and Kennedy's proposal, in 1986, two additional P2 receptor subtypes on platelets and mast cells (and lymphocytes) that did not seem to fit the P$_{2X}$/P$_{2Y}$ subclassification were defined by Gordon (1986) and were

tentatively termed P_{2T} and P_{2Z} receptors, respectively. The platelet receptor being activated by ADP and blocked by ATP, was designated P_{2T} because of its presence on thrombocytes and megakaryocytes (Born, 1962; Haslam and Cusack, 1981). For activation of the P2 receptor subtype present on mast cells and lymphocytes, designated P_{2Z} (for alphabetical consistency) about 100 μM of ATP was required (for activation of the other subtypes about 1 μM of the most active agonists was sufficient), and it was shown by Cockroft and Gomperts (1979) that the agonist form inducing calcium-dependent histamine secretion was the tetrabasic acid ATP^{4-}.

Pyrimidinoceptors, nucleotide receptors, P_{2U} receptors

A few years later, it became apparent that, similar to ATP, extracellular uracil nucleotides exert effects on many tissues and cells (Seifert and Schultz, 1989; O'Connor et al., 1991). Uridine 5'-triphosphate (UTP) was shown to induce endothelium-dependent relaxation and to stimulate prostacyclin (PGI_2) production in various vascular beds. Although it was obvious that this effect was mediated via phosholipase C (PLC) -linked "purinoceptors" (as described for the classical P_{2Y} purinoceptor), regarding the rank order of agonist potency, these responses could not be attributed to activation of P_{2Y} purinoceptors. These observations led to the definition of a new receptor subtype characterised by the following rank order of agonist potency: UTP = ATP > ADP > α,β-mATP, 2-MeSATP and termed *nucleotide* receptor (P_{2n}) (Davidson et al., 1990; O'Connor et al., 1991). Subsequently, this receptor subtype was referred to as P_{2U} receptor. However, in some systems UTP-sensitive receptors could be distinguished from ATP receptors on the basis of cross-desensitisation experiments, and a rank order of potency of UTP = uridine 5'-diphosphate (UDP) >> uridine-5'-monophosphate (UMP) = cytidine 5'-monophosphate (CMP) was established for this pyrimidine nucleotide receptor (*pyrimidinoceptor*) (von Kügelgen and Starke, 1990).

P_{2S} purinoceptor

The existence of a P2 receptor - the P_{2S} subtype - mediating contractile responses of guinea-pig ileal longitudinal smooth muscle (GPI), at which α,β-mATP and 2-MeSATP have been found to be equipotent, has been proposed but is still questioned (Wiklund and Gustafsson, 1988). Recent findings suggested the

existence of two types of P2 receptors mediating contraction of GPI. An α,β-mATP-sensitive P2 receptor situated somadendritically on cholinergic neurons, the stimulation of which causes ACh-mediated contraction via postjunctional muscarinic M_3 receptors and another $P2Y_1$-like receptor located postsynaptically on the smooth muscle (Kennedy and Humphrey, 1994; Czeche et al. ,1998b; Lambrecht et al., 1999).

P_{2D} and P_4 receptors

There are also hints that adenine dinucleotides (Ap_nA's; "n" indicating the number of phosphates between the two adenosine moieties) may have a role as neurotransmitters or neuromodulators (Pintor and Miras-Portugal, 1995a). The diadenosine polyphosphates Ap_3A and Ap_4A were found to be co-stored with other neurotransmitters in platelets and Ap_4A, Ap_5A as well as Ap_6A were shown to be present in neurosecretory vesicles together with adenine nucleotides, ACh and catecholamines (Flogaard and Klenow, 1982; Rodriguez del Castillo et al., 1988). In several tissues, Ap_nA's and ATP were shown to activate the same P2 receptor subtypes (see Miras-Portugal et al., 1998).

On the basis of binding and displacement studies carried out in rat brain synaptic terminals with [^{3}H]Ap_4A, the presence of high and medium affinity binding sites with K_d values in the nM and μM range have been described. A displacement order of Ap_4A > adenosine 5'-O-2-thiodiphosphate (ADPβS) > β,γ-imido-adenosine-5'-triphosphate (AMP-PNP) > α,β-mATP >> 2-MeSATP which was distinct from the pharmacological profiles of the formerly described ATP receptors was observed for the high affinity binding site. Because of these features, a new subtype was proposed and has been termed P_{2D} receptor (Pintor et al., 1993). Studying the intrasynaptosomal Ca^{2+} increase evoked by Ap_4A and Ap_5A as well as ATP, α,β-mATP and ADPβS in rat midbrain synaptic terminals, the lack of inhibition of responses to the dinucleotides by suramin, the inability to cross-desensitise the polyphosphate responses with ATP or its non-hydrolysable analogues together with their additive responses indicated that adenine dinucleotides and ATP on the one hand and its analogues on the other hand act via different mechanisms and different receptors in this preparation. These receptors, only stimulated by diadenosine polyphosphates, were found in synaptosomes of mouse and guinea-pig as well and were designated *dinucleotide*

or P_4 receptors (Pintor and Miras-Portugal, 1995a, 1995b). However, due to the close structural analogy between diadenosine polyphosphates and ATP it is necessary to be aware of difficulties in clearly identifying their specific physiological receptors and differentiate them from pharmacological actions on other nucleotide receptors. Another factor that should be taken into account is the presence of diadenosine polyphosphatases. These enzymes hydrolyse the Ap_nA's in an asymmetric way producing AMP and the corresponding Ap_{n-1} nucleotide, the breakdown products being active neurotransmitting substances themselves (ATP, ADP) or subject to further breakdown by ecto-nucleotidases, generating adenosine as the final product (Mateo et al., 1997).

P_3 receptors

A distinct P_3 receptor that is activated by both nucleosides and adenine as well as pyrimidine nucleotides, and is antagonised by xanthines as well as α,β-mATP has been proposed. P_3 purinoceptors were originally reported to be located prejunctionally and to inhibit evoked NA release from sympathetic nerves in rat tail artery and ratVD (Shinozuka et al., 1988; Forsyth et al., 1991). They were activated by agonists with a potency order of 2-chloroadenosine (2-Cl-Ado) > β,γ-methylene ATP > ATP = adenosine. Evidence for the existence of receptors distinct from P_1 and P_2 purinoceptors came from several observations: first, the effects of both nucleosides and nucleotides on NA release were blocked by the P_1 receptor antagonist 8-(p-sulphophenyl) theophylline (8-SPT); second, the adenosine uptake inhibitor S-p-nitrobenzyl-6-thioguanosine (NBTG) potentiated, and adenosine deaminase blocked the effect of adenosine on NA overflow but didn't have any effect on the ability of the adenine nucleotides to inhibit NA overflow; third, in ratVD, inhibition of NA release by UTP was also inhibited by 8-SPT. With regard to some more recent data on the inhibitory effect of nucleosides and nucleotides on NA overflow in rat and mouse vas deferens, an alternative explanation to the initial proposal of P_3 receptors might be a mixture of effects mediated via two distinct prejunctional receptors; a P_1 receptor sensitive to nucleosides and nucleotides, which is presumably of the A_1 subtype, and a P2Y-like receptor activated solely by nucleotides (Kurz et al., 1993; von Kügelgen et al., 1994). Similar evidence for separate prejunctional A_1 and P2 receptors has been obtained in rat and mouse atria and the rat iris (Fuder and Muth, 1993; von Kügelgen et al., 1995).

In radioligand binding studies with ratVD membrane preparations using the A_1 receptor antagonist 1,3-$[^3H]$-dipropyl-8-cyclopentylxanthine ($[^3H]$-DPCPX) as radioligand, nucleosides competed for $[^3H]$-DPCPX binding at two distinct sites, whereas nucleotides bound at a single site only. The latter site seemed to correspond with one of the two binding sites predicted by nucleoside competition binding. The identity of these two binding sites for $[^3H]$-DPCPX in ratVD is not quite clear, one appeared to bind only nucleosides and is probably an adenosine A_1 receptor, and the other binds both nucleosides and nucleotides and may most likely be a P_3 receptor or a separate affinity state of the A_1 receptor, respectively (Smith et al., 1997). However, this assumption needs further evaluation before the International Union of Pharmacology (IUPHAR) Receptor Nomenclature Committee will adopt a P_3-category.

In 1994, it was again Burnstock (Abbracchio and Burnstock, 1994) who proposed a new way of defining receptors responsive to nucleotides and established a revised nomenclature, which is still the basis for subclassification of P2 receptors. Based on the predicted structure and the signal transduction mechanism, P2 receptors were divided into two families: the P2X receptors being intrinsic ionic channels permeable to Na^+, K^+ and Ca^{2+} (Benham and Tsien, 1987; Bean, 1992) and the P2Y receptor family belonging to the GTP-binding receptor family (Dubyak, 1991; O'Connor et al., 1991). A detailed analysis of literature led Abbracchio and Burnstock to the proposal that resting on different potency profiles of key agonists the P2Y receptor family comprises seven subtypes (named $P2Y_1$-$P2Y_7$) and the P2X receptor family consists of at least four P2X purinoceptor subclasses ($P2X_1$-$P2X_4$). In contrast to the general P2Y, P2X receptor subdivision, the further classification proposed by Abbracchio and Burnstock (1994) does not correspond with the current definition of P2X and P2Y receptor subtypes (see 1.1.2.2.).

1.1.2. Purinergic Receptors: Subclassification and nomenclature - current state

1.1.2.1. P1/Adenosine receptors

Presynaptically or postsynaptically released ATP is hydrolysed extracellularly to adenosine, which itself is a potent signalling substance acting on adenosine P1 receptors. Furthermore, adenosine can be released (via carrier-mediated mechanisms) from neurons or from the activated postsynaptic cell (Zimmermann, 1994). Biochemical evidence for the existence of at least two distinct adenosine receptors was provided by the demonstration that adenosine analogues inhibited, via A_1 (R_i), or stimulated, via A_2 (R_A), AC activity in several mammalian cell types (van Calker et al., 1979; Londos et al., 1980). The effects of adenosine could be antagonised by methylxanthines and the order of potency of adenosine analogues was different for the two receptor subtypes (van Calker et al., 1979). The $A_1/A_2/A_3$ nomenclature is now generally in use (Fredholm et al., 1994, 1997, 1998).

The adenosine receptor family comprises A_1, A_{2A}, A_{2B} and A_3 adenosine receptors, identified by convergent data from molecular, biochemical, and pharmacological studies, all of which couple to G proteins. Receptors from each of these four distinct subtypes have been cloned from a variety of species including human, rat, mouse, guinea-pig, dog, sheep and rabbit (see table 1.1., for review see Ralevic and Burnstock, 1998).

A subclassification of the A_1 receptor has been proposed which was based on the observation that affinities of a set of agonists and antagonists were higher in the rat and guinea-pig brain than in ratVD and GPI. The high-affinity receptors were suggested to be termed A_{1a} and the low-affinity A_{1b} receptors (Gustafsson et al. 1990). However, there are no cloned equivalents for these putative subtypes and their existence remains equivocal. Additionally, an adenosine binding site in rat striatal membranes was suggested to be a novel "A_4" adenosine receptor (Cornfield et al., 1992). Further studies revealed that the characteristics observed in experiments performed at 4 °C represent binding to a certain state of the A_{2A} receptor (Luthin and Linden, 1995).

A further subclassification of A_2 receptors introduced by Daly and colleagues (1983) was based on the recognition that adenosine-mediated stimulation of AC in rat brain was affected via distinct high affinity binding sites (restricted to striatal membranes) and low affinity binding sites (present throughout the brain). This subdivision was supported in a study which compared the high affinity striatal A_2 binding site with a low affinity A_2 binding site in a human fibroblast cell line (Bruns et al. 1986). A_2 receptors displaying a high affinity for the non-selective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) were termed A_{2A} receptors and those with low affinity were referred to as A_{2B} receptors (Bruns et al., 1986). Final evidence came from the cloning of A_{2A} and A_{2B} receptors which show distinct pharmacological profiles in transfected cells (Maenhaut et al., 1990; Rivkees and Reppert, 1992; Stehle et al., 1992).

A_3, the fourth adenosine receptor subtype was identified relatively late in the history of P1 receptors with the cloning, expression and functional characterisation of a novel adenosine receptor from rat striatum (Zhou et al., 1992).

Generally, adenosine receptor subtypes can be pharmacologically subdivided based on the concentration of the physiological agonist adenosine required for receptor stimulation; A_1 and A_{2A} receptors are "high affinity" adenosine receptors, which are activated by adenosine concentrations of 0.01 - 0.1 μM, while the "low affinity" A_{2B} and A_3 receptors require concentrations of 1 - 10 μM adenosine for activation.

All P1 receptor agonists described so far are structural analogues of the endogenous ligand adenosine. Only minor modifications of the ribose part are permitted to retain adenosine receptor affinity and intrinsic activity. Modifications in the adenine moiety, including substitution at N^6 and C2-positions, yielded analogues with greater stability and higher potency than adenosine. Adenosine receptor antagonists can be divided structurally in xanthine and non-xanthine derivatives. In contrast to many non-xanthine derivatives, xanthine antagonists are well-defined in terms of structure-activity (for more detailed information, the reader is referred to the following reviews: Collis and Hourani, 1993; Coates et al. 1994; Feokistov and Biaggioni, 1996; Jacobson, 1996; Jacobson and Suzuki, 1996; Müller and Stein, 1996).

Table 1.1. Classification of adenosine/P1 receptors, molecular and pharmacological characterisation, localisation (for references see Ralevic and Burnstock, 1998).

	A_1	A_{2A}	A_{2B}	A_3
Molecular characterisation				
Subtype				
Sequences	cow, dog, guinea-pig, human, mouse, rabbit, rat	guinea-pig, human mouse, rat, dog	human, mouse, rat	sheep, human, rabbit, rat
Number of amino acids	326/327/328	409/410/411	328/332	317/318/320
G protein-coupling	$G_{i/o}$	G_s	G_s G_q	G_i G_q
Effects	$\downarrow$ cAMP $\uparrow$ IP$_3$ $\uparrow$ K$^+$ $\downarrow$ Ca^{2+}	$\uparrow$ cAMP	$\uparrow$ cAMP $\uparrow$ IP$_3$	$\downarrow$ cAMP $\uparrow$ IP$_3$
Pharmacological characterisation				
Selective agonists	CPA CCPA	CGS21680 APEC HE-NECA	none	Cl-IB-MECA IB-MECA
Selective antagonists	DPCPX	ZM241385 SCH58261 CSC	none	L-268605 MRS1191

1.1.2.2. P2 receptors

The division of P2 receptors into two main classes, the P2X receptor family of ligand-gated ion channels and the P2Y receptor family of G protein-coupled nucleotide receptors, according to Abbracchio's and Burnstock's proposal (Abbracchio and Burnstock, 1994), is now generally accepted (Fredholm et al., 1997; Harden et al., 1998a; Humphreys et al., 1998). To date seven, mammalian P2X receptors, termed $P2X_{1-7}$, and six P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ have been cloned, characterised pharmacologically and accepted as valid members of the P2 receptor family (von Kügelgen and Wetter, 2000; Hollopeter et al., 2001; Khakh et al., 2001).

The molecular cloning of DNA encoding P2 receptors has enabled the pharmacological selectivity of P2 receptors of defined amino acid sequence to be delineated. This development was particularly important because of the difficulties previously encountered in unambiguously resolving individual members of the P2 class of receptors in pharmacological studies with tissue preparations (Abbracchio and Burnstock, 1994). The existence of at least 14 native nucleotide receptor subtypes could not be clearly demonstrated in traditional pharmacological studies for a number of reasons:

- First, the presence of ecto-enzymes that rapidly hydrolyse nucleoside triphosphates and diphosphates on the extracellular surface of most target cells, can greatly and selectively decrease the apparent P2 receptor potency of susceptible nucleotides under the conditions generally used (Kennedy and Leff, 1995). This problem has been aggravated by the paucity of hydrolysis resistant agonists that can distinguish the various P2 receptor subtypes as well as by the unavailability of selective ecto-nucleotidases inhibitors. To date, 6-N,N-diethyl-D-β,γ-dibromomethylene ATP (ARL 67156, formerly known as FPL 67156) is the only compound displaying selectivity in favour of ecto-nucleotidases inhibition (cf. 1.1.5.7.)
- A second factor is the common co-occurrence of several subtypes of P2X or P2Y, or of both classes, in a single cell or tissue; an overlap that often cannot be resolved in pharmacological measurements. Thus, studies of a response of a tissue to ATP are predictably compromised by (1) rapid conversion of ATP to ADP, which potentially acts on a different receptor subtype, and (2) conversion of ADP to AMP and finally adenosine, which itself can introduce a new set of

complications through activation of P1 receptors. To circumvent at least the latter problem, P1 receptor antagonists of reasonably high selectivity can be utilised to block the potential contribution of these receptors.

- The third and probably most eminent problem is the lack of real discrimination of the pharmacological tools being available. Although some progress has been made in the development of subtype-selective agonists as well as potent subtype-selective antagonists in the last few years (see 1.1.3.), apart from a few exceptions there are neither agonists nor antagonists that discriminate adequately between the families of P2X and P2Y receptors, or between subtypes of receptors within each of these groups. This coincides with scarcity of high-affinity selective or non-selective antagonists which can be applied in radioligand-binding studies.

- Rapid desensitisation is a typical feature of some P2X receptor subtypes, stimulation of which leading to inactivation of ATP-induced inward currents within ms. Hence, precautions such as single dose techniques, minimising the agonist contact time, and allowing sufficiently long dosage intervals have to be taken into consideration.

- Species differences, occurring even among some of the cloned P2 receptor subtypes, display a further problem which seems to become more and more relevant. Studies on recombinant $P2X_4$ and $P2Y_4$ species orthologues revealed fundamental differences in agonist as well as antagonist sensitivities (cf. 1.1.2.2.1.1. and 1.1.2.2.2.1.).

Most of these disadvantages also play a role in studies on the pharmacological properties of cloned P2 receptors in null cell lines. For example, hydrolysis of nucleotides remains a problem in studies with cultured cells, and 1321N1 human astrocytoma cells, which have been widely used for the expression and functional assay of cloned P2Y receptors, express ecto-nucleotidases that rapidly hydrolyse ATP and potentially hydrolyse UTP as well (Lazarowski et al., 1995, 1997b). In addition, a novel enzymatic activity was shown to be present on the extracellular surface of 1321N1 cells: nucleoside diphosphokinase, catalysing the transfer of the γ-phosphate of nucleoside triphosphates to nucleoside diphosphates, can markedly contribute to the observed pharmacological activity of nucleoside di- and triphosphates (Nicholas et al., 1996b; Lazarowski et al., 1997a). Moreover, problems can occur due to the release of nucleotides from cells in response to medium changes or physical stimulation (Lazarowski et al., 1995); this includes

the elevation of basal levels of second messengers, such as inositol phosphates. This phenomenon is particularly important when studying cloned P2Y receptors expressed at high levels (Filtz et al., 1994; Parr et al., 1994; Lazarowski et al., 1995), which may exhibit increased sensitivity to nucleotide-promoted activation of second messenger production due to receptor reserve. Conversely, desensitisation and downregulation of the P2 receptor promoted signalling response can potentially occur as a sequel of such activation, as shown for the P2X$_1$ receptor present in HL-60 cells (Buell et al., 1996c). The modulatory effects of ions, reported for several recombinant homomeric P2X receptors, introduce a further set of complications for the interpretation of data obtained from studies using differential experimental protocols (North and Surprenant, 2000). Finally, nucleotide analogues that are not P2 receptor agonists may appear to be so by reducing the enzymatic breakdown of ATP (or UTP) released from cells. However, some of the above-mentioned problems can be circumvented: purification by high pressure liquid chromatography (HPLC) of nucleotides and their analogues prior to use of this molecules as well as nucleotide-modifying enzymes (e.g. hexokinase to convert nucleoside triphosphates in nucleoside diphosphates generating glucose-6-phosphate or apyrase to remove tri- and diphosphonucleotides from the extracellular medium under conditions where release of cellular nucleotides introduces problems) can be utilised to define precisely the pharmacological selectivity of a P2 receptor subtype (Nicholas et al., 1996b).

The apparent mismatch of some pharmacological data obtained in biological tissues relating to the P2 receptor subtypes classified on the basis of molecular structure may be resolved in part by the development of agonists and antagonists, that selectively activate or block individual receptors without being susceptible to enzymatic breakdown or being inhibitors of nucleotide-degrading enzymes.

1.1.2.2.1. Ionotropic P2 receptors (P2X receptors)

P2X receptors are a unique family of transmembrane proteins, found in a wide variety of cells, including smooth muscle cells, peripheral and central neurons, epithelial cells, and immune cells (Buell et al., 1996a). These receptors are rapidly activating (milliseconds) ligand-gated cationic channels with equal permeability to Na$^+$ and K$^+$, but higher permeability (four times for P2X$_1$, P2X$_3$, and P2X$_4$ receptors, two times for P2X$_2$ receptors) to Ca^{2+} (Valera et al., 1994; Evans

et al., 1995, 1996; Lewis et al., 1995; Soto et al., 1996b; Garcia-Guzman et al., 1997a).

Structure, membrane topology and stoichiometry

Despite the apparent functional similarities of P2X receptor responses to those of other ligand-gated channels such as the nicotinic or glutamate, P2X receptors display no obvious sequence homology with other channel families. P2X receptor subunits (ranging from 379 to 595 amino acids in length) are 26 - 47 % identical to one another at the amino acid level. All seven proteins have similar hydrophobicity profiles, with only two hydrophobic regions sufficiently long to span the membrane, suggesting that P2X receptors may have intracellular N- and C-termini, and two transmembrane domains separated by a large extracellular loop (Valera et al., 1994; Brake et al., 1994; Surprenant et al., 1995). Native and artificially introduced glycosylation sites were used to confirm this topological model (Newboldt et al., 1998; Torres et al., 1998a). Further evidence was provided by mutational studies on P2X subunits: those studies have demonstrated that sensitivity to α,β-mATP can be exchanged between $rP2X_1$ and $rP2X_2$ by swapping the extracellular loop (Werner et al., 1996) and, moreover, the effects of the antagonists suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) can be modified by single point mutations of amino acid residues located in the putative extracellular loop (Buell et al., 1996b; Collo et al., 1996; Garcia-Guzman et al., 1997a).

Recent structure-function studies indicate, that the channel pore is lined by residues that include, at least in part, the second transmembrane domain (Rassendren et al., 1997a; Egan et al., 1998).

The structural domains involved in desensitisation of $P2X_1$ and $P2X_3$ receptors have been localised by engineering chimeric constructs of $rP2X_1$, $rP2X_3$ and $rP2X_2$ subunits. Desensitisation can be introduced in $rP2X_2$ by replacing the putative transmembrane domains in combination with 11 - 15 amino acids towards the N- and C-terminal end with the equivalent $rP2X_1$ or $rP2X_3$ sequence (Werner et al., 1996). The mechanisms governing the desensitisation of P2X receptors are not completely understood although recent data indicates that its kinetics might be a regulated process. It has been proposed that Ca^{2+}, entering through $P2X_3$, will

produce desensitisation through calcineurin-mediated dephosphorylation of N-terminal residues that are phosphorylated under resting conditions (King et al., 1997a).

Similar to other ion channel families, the seven P2X proteins that have been cloned are receptor subunits, not actual receptors, and functional receptors are believed to be formed by oligomeric assembly. Studies using recombinant expression of individual subunits provide evidence that in certain tissues the native P2X response could be due to homo-oligomeric receptors (e.g. the $P2X_1$ receptor and the P2X receptor found in vas deferens) (Valera et al., 1994; Khakh et al., 1995b). On the other hand, a P2X receptor phenotype seen in adult dorsal root sensory neurons appears to result from hetero-oligomeric assembly of $P2X_2$ and $P2X_3$ subunits (Lewis et al., 1995; Cook et al., 1997; Vulchanova et al., 1997). Additional evidence supporting the possible presence of hetero-oligomeric P2X receptors comes from Northern blot, *in situ* hybridisation, and immuno-cytochemical studies suggesting that cell populations in a variety of tissues may express multiple subunit genes (Collo et al., 1996, 1997; Vulchanova et al., 1997; Robertson, 1998; Xiang et al., 1998).

A remaining question is that of the exact stoichiometry of P2X complexes. One piece of evidence, recently proposed, was based on a combination of cross-linking and blue native polyacrylamide gel electrophoresis (PAGE) analysis (Nicke et al., 1998; Nicke, 1999). Cross-linked recombinant P2X receptors were shown to migrate predominantly as trimers. Similar results were obtained with blue native PAGE, a non-denaturating gel electrophoretic method. It was proposed that P2X receptors associate as trimers early during biosynthesis and that this trimeric association is the essential constituent of the receptor complex, the association of trimers into hexamers being also possible (Nicke et al., 1998; Stoop et al., 1999).

The ability of P2X receptor subunits to form stable trimers (or multiples of trimers), in combination with the capacity of hetero-oligomeric assembly of at least some of the cloned subunits yields a large number of potentially existing native P2X receptors, 11 of which have been studied to date.

1.1.2.2.1.1. Cloning of ionotropic P2 receptors

The first two P2X receptor subunits were isolated from ratVD smooth muscle ($rP2X_1$) (Valera et al., 1994) and rat pheochromocytoma PC12 cells ($rP2X_2$, originally called P2XR1) (Brake et al., 1994) by expression cloning. Five additional members of the P2X family were subsequently cloned from rat tissues ($rP2X_3$ to $rP2X_7$) either by screening of diverse libraries, by polymerase chain reaction-techniques (PCR-techniques) or by using a combination of both (for review see Buell et al., 1996a; Soto et al., 1997). The molecular characterisation of P2X receptors has been further expanded with the cloning of various homologous human cDNAs (see Soto et al., 1997, and references therein). Heterologous expression of each of the single cDNAs in *Xenopus laevis* oocytes or mammalian cell lines results in ATP-induced cation-selective currents. However, the efficiency in forming a functional P2X receptor differs among the subunits; for the $rP2X_6$ receptor, currents have been reported in only a small fraction of transfections into a human embryonic kidney cell line (HEK293 cells) (Collo et al., 1996; Soto et al., 1996b) and transiently transfected cell lines expressing $rP2X_5$ produced currents that were 5 - 10 % of the peak currents detected with any other P2X subunit (Collo et al., 1996; Garcia-Guzman et al., 1996). In a recent study, $P2X_6$ receptor subunits failed to give ATP-induced currents when transfected into HEK293 cells, an effect that might be explained by their inability to form homo-oligomeric assemblies (Torres et al., 1999). Similar results were obtained when the cRNA coding for the $P2X_6$ subunit was injected in *Xenopus laevis* oocytes; upon application of ATP (up to 500 μM), no inward current was observed (Soto et al., 1996b; Lê et al., 1998; Khakh et al., 1999b).

Recombinant P2X receptors can be divided into four major groups based on the three following criteria: relative effectiveness of α,β-mATP, sensitivity to suramin and PPADS, and the rate of desensitisation (see table 1.2., Khakh et al., 2001). $P2X_1$ and $P2X_3$ subunits represent the first phenotype. Cells expressing these cDNAs are activated by α,β-mATP and ATP, half-maximal concentrations are close to 1 μM (Valera et al., 1994; Chen et al., 1995; Lewis et al., 1995; Garcia-Guzman et al., 1997b).

Table 1.2. Summary of pharmacological characteristics of P2X receptor subtypes based on studies of recombinant homomers (taken from Humphrey et al., 1998).

Group 1	Group 2	Group 3	Group 4
α,β-mATP-sensitive rapidly desensitising	α,β-mATP-insensitive non desensitising	α,β-mATP-insensitive non desensitising	α,β-mATP-insensitive non desensitising pore forming
suramin-sensitive	suramin-sensitive	suramin-insensitive	suramin-sensitive
$P2X_1$	$P2X_2$	$P2X_4$	$P2X_7$
$P2X_3$	$P2X_5$	$P2X_6$	

$P2X_1$ receptor subunits have been cloned from ratVD, and human and mouse urinary bladder (Valera et al., 1994; Ralevic and Burnstock, 1998). When expressed in *Xenopus* oocytes or in HEK293 cells, rapidly desensitising (in hundreds of ms) inward currents were observed in response to the ATP analogues 2-MeSATP $\geq$ ATP > α,β-mATP >> ADP. In a recent paper, Bianchi and colleagues reported on 2',3'-O-(4-benzoylbenzoyl) ATP (BzATP), which is commonly in use as a $P2X_7$/P2Z receptor agonist, to be a potent (nM) agonist at $hP2X_1$, $rP2X_3$ and $hP2X_3$ receptors. The metabolically stable ADP analogue ADPβS, previously described as a P2Y receptor agonist, was also shown to activate $hP2X_1$ as well as $rP2X_3$ and $hP2X_3$ receptors in the micromolar range (Bianchi et al., 1999). Additionally, the activity of diadenosine polyphosphates (Ap_2A - Ap_6A) at recombinant $P2X_1$ receptors was examined. Ap_4A and Ap_5A were found to be equipotent full agonists at the $hP2X_1$ receptor, Ap_6A acted as a partial agonist, and Ap_2A as well as Ap_3A were inactive (Bianchi et al., 1999). For the $rP2X_1$ receptor, only Ap_6A was a full agonist, both Ap_4A and Ap_5A were partial agonists, and Ap_3A showed weak agonist activity (Wildman et al., 1999a). The P2 antagonists suramin and PPADS readily antagonise the agonist-evoked responses. $P2X_1$ receptor mRNA is expressed in urinary bladder, smooth muscle layers of small arteries and arterioles, and vas deferens, lower levels have been detected in lung and spleen (Valera et al., 1994; Collo et al., 1996). $P2X_1$ receptor mRNA is also present in dorsal root ganglia, trigeminal ganglia, coeliac ganglia, spinal cord, and rat brain (Valera et al., 1994; Collo et al., 1996).

The P2X$_3$ receptor cloned from rat dorsal root ganglion (Chen et al., 1995; Lewis et al., 1995) shows only 43 % amino acid sequence homology with the P2X$_1$ receptor. However, the rank order of agonist potency characterising homomeric hP2X$_3$ and rP2X$_3$ receptors is very similar to that obtained in studies with homomeric P2X$_1$ receptors: 2-MeSATP $\geq$ ATP $>$ α,β-mATP (Lewis et al., 1995; Bianchi et al., 1999). As described for the P2X$_1$ subtype, diadenosine polyphosphates exhibit agonist activity at human and rat P2X$_3$ receptors as well. The hP2X$_3$ is sensitive to activation by submicromolar concentrations of Ap$_4$A, Ap$_5$A and Ap$_6$A, although all responses mediated by diadenosine polyphosphates are submaximal (Bianchi et al., 1999). In oocytes expressing rP2X$_3$ receptors Ap$_4$A, Ap$_5$A and Ap$_6$A were shown to be full agonists and more potent than ATP (Wildman et al., 1999a). Differences described for the agonistic potency of Ap$_3$A at rP2X$_3$, being rather inactive at rP2X$_3$ expressed in 1321N1 human astrocytoma cells and, in contrast, a partial agonist, displaying higher potency than ATP at the rP2X$_3$ expressed in *Xenopus* oocytes, may be determined by the different expression systems applied (Bianchi et al., 1999; Wildman et al., 1999a). In the continuous presence of agonist, the currents elicited by heterologously expressed P2X$_3$ receptors decline rapidly. In contrast to P2X$_1$ receptors (monoexponential decay; τ = 100 - 300 ms) P2X$_3$ receptor desensitisation kinetics are biexponential with decay constants of 50 ms and 1 s (Collo et al., 1996). The P2X$_3$ receptor shows a restricted distribution; it is present in sensory neurons, and is absent in central nervous system neurons and smooth muscle (Chen et al., 1995; Lewis et al., 1995; Collo et al., 1996; Dunn et al., 2001). The human P2X$_3$ receptor transcript has been detected in spinal cord and heart (Garcia-Guzman et al., 1997b).

The second and the third group comprise currents in cells expressing P2X$_2$, P2X$_4$, P2X$_5$ and P2X$_6$ receptors. In these cases, ATP activates the channel with an EC$_{50}$ of about 10 μM (cf. exceptions described above for the P2X$_6$ receptor) but α,β-mATP is ineffective even at concentrations of 100 - 300 μM (Brake et al., 1994; Evans et al., 1995; Buell et al., 1996b; Collo et al., 1996; Séguéla et al., 1996; Soto et al., 1996a). These receptors can be further subdivided according to their antagonist sensitivity: P2X$_2$ and P2X$_5$ receptors are reversibly inhibited by suramin and PPADS ($<$ 30 μM), while P2X$_4$ (and P2X$_6$ receptors) are not blocked by these concentrations of the two antagonists. ATP evokes sustained currents at P2X$_2$ (Brake et al., 1994; Evans et al., 1996) and P2X$_5$ receptors (Collo et al.,

1996; Garcia-Guzman et al., 1996) even during agonist applications lasting tens of seconds (Brake et al., 1994). $P2X_4$ (and $P2X_6$ receptors) show an intermediate phenotype, with about 60 % of the peak current remaining after a 2 s-application of ATP to transfected mammalian cells (Collo et al., 1996).

ATP, 2-MeSATP and ATPγS are approximately equipotent agonists at the recombinant $P2X_2$ receptor, whereas α,β-mATP and β,γ-mATP are inactive either as agonists or antagonists (Brake et al., 1994). In contrast to $P2X_1$ and $P2X_3$ receptors the $rP2X_2$ receptor when expressed in 1321N1 cells is insensitive to activation by diadenosine polyphosphates (Bianchi et al., 1999), although Ap_4A was described to exhibit agonist activity at $rP2X_2$ receptors expressed in *Xenopus* oocytes (Pintor et al., 1996; Wildman et al., 1999a). $P2X_2$ receptor mRNA is present in bladder, brain, spinal cord, superior cervical ganglia, adrenal medulla, intestine, and vas deferens, with highest levels found in the pituitary gland and vas deferens (Brake et al., 1994). Immunohistochemical detection shows a widespread distribution of the $P2X_2$ receptor in brain and spinal cord (Vulchanova et al., 1996). Studies on the genomic organisation of the $rP2X_2$ receptor revealed a large number of exon-intron boundaries present in the coding region, suggesting the existence of several subunit isoforms produced by alternative splicing (Brändle et al., 1997). Accordingly, up to six different splice variants of the rat $P2X_2$ receptor have been identified (Simon et al., 1997; Koshimizu et al., 1998). The first variant, now termed $P2X_{2(b)}$, has a 69 amino acid deletion of the C-terminus, expresses in the nervous system at least as abundantly as the original $P2X_2$ subunit (distinguished by the terminology $P2X_{2(a)}$), and is able to form homo-oligomeric functional channels, as was shown by its expression in *Xenopus* oocytes and mammalian cells (Simon et al., 1997). The $P2X_{2(b)}$ receptor is equally sensitive to agonists - the only difference in properties detected between this short and the $P2X_{2(a)}$ long form is an increased, intermediate rate of desensitisation (Simon et al., 1997). Alternative splicing also occurs in the TM1 region of the $P2X_2$ receptor, generating two additional isoforms, termed $p2x_{2(c)}$ and $p2x_{2(d)}$, respectively. Although functional expression of these two isoforms has not yet been achieved (denoted by the lower case letters), mRNA for the $p2x_{2(c)}$ is present in many structures in the nervous system, whereas the transcript for the $p2x_{2(d)}$ variant has only been found in the nodose ganglion (Simon et al., 1997).

The P2X$_5$ receptor was cloned from rat coeliac ganglia and heart (Collo et al., 1996; Garcia-Guzman et al., 1996). Human homologues of the P2X$_5$ receptor have tentatively been identified (Genbank: U49395/6). However, the full sequence of the hP2X$_5$ receptor has yet to be confirmed (Lê et al., 1997). The rP2X$_5$ receptor is able to form a functional homo-oligomeric channel and has pharmacological properties in terms of agonist potency (ATPγS > ATP = 2-MeSATP >> ADP), antagonist sensitivity, and desensitisation which are very similar to the P2X$_2$ channel, when both are expressed in HEK293 cells (Collo et al., 1996). A major difference to the other known P2X receptor subtypes is that the agonist-evoked peak current obtained for the P2X$_5$ homo-oligomer is always very small, being 10-fold less than that of any other recombinant P2X subtype expressed in HEK293 cells (Collo et al., 1996). Rat P2X$_5$ subunit transcripts have been identified in brain, heart, spinal cord and adrenal medulla, as well as thymus and lymphocytes (Garcia-Guzman et al., 1996; Lê et al., 1997; Bogdanov et al., 1998a). *In situ* hybridisation shows P2X$_5$ mRNA in motoneurons of the ventral horn of the spinal cord. With the exception of trigeminal mesencephalic nucleus neurons, the brain does not express P2X$_5$ mRNA (Collo et al., 1996). Further sequence analysis of the recently identified P2X subunit from chick skeletal muscle, which was tentatively called the chick P2X$_8$ subunit because of its unique functional properties and its 59 % sequence homology to rat and human P2X$_5$ subunits (Bo et al., 2000), indicates that it is a species homologue of P2X$_5$.

The main P2X receptors detected in adult rat central nervous system (CNS) by in situ hybridisation are P2X$_4$ (Bo et al., 1995; Séguéla et al., 1996; Buell et al., 1996b; Soto et al., 1996a) and P2X$_6$ (Collo et al., 1996; Soto et al., 1996b), showing an overlapping distribution in many regions of the brain. This distribution pattern is not present in peripheral tissues: rP2X$_6$ was not detected in RNA isolated from blood vessels and vas deferens (Soto et al., 1996b) where rP2X$_4$ is present (Soto et al., 1996a). P2X$_4$ receptors were originally cloned from rat brain (Bo et al., 1995), and subsequently, both human (Garcia-Guzman et al., 1997a) and murine orthologues (mP2X$_4$) have been isolated (Townsend-Nicholson et al., 1999; Simon et al., 1999). Comparing their pharmacological properties, differences occurred, especially with respect to antagonist sensitivities. Generally, the rank order of agonist potency characterising P2X$_4$ receptors was shown to be similar for rat and human P2X$_4$ receptors expressed in *Xenopus* oocytes (Soto et al., 1996a; Garcia-Guzman et al., 1997a): ATP > 2-MeSATP $\geq$ CTP > dATP;

purine analogues other than ATP exhibited partial agonistic activity at the hP2X$_4$ receptor (Bianchi et al., 1999). AP$_4$A, the most potent member of the diadenosine polyphosphate family, also acts as a partial agonist at human as well as rat P2X$_4$ receptors (Bianchi et al., 1999; Wildman et al., 1999a). In a recent paper, a comparison of the mouse, human and rat orthologues of the P2X$_4$ receptor was performed under identical experimental conditions using HEK293 cells (Jones et al., 2000). In this study, the potency of ATP was shown to be similar at the three species orthologues. In contrast, α,β-mATP acted as a partial agonist at human and mouse P2X$_4$, but was found to block ATP-induced responses at rP2X$_4$ (Jones et al., 2000). The rP2X$_4$ receptor was originally described to be insensitive to the classical P2 receptor antagonists suramin and PPADS (Bo et al., 1995; Buell et al., 1996b). Subsequent studies revealed that all P2X$_4$ orthologues were relatively suramin-insensitive, compared to P2X$_1$, P2X$_2$ and P2X$_3$ subunits (Soto et al., 1996a; Garcia-Guzman et al., 1997a; Bianchi et al., 1999; Jones et al., 2000); the most remarkable pharmacological difference between hP2X$_4$ and mP2X$_4$, and the rP2X$_4$ receptor concerns the antagonistic potency of PPADS. Mouse and human receptors were shown to be sensitive to PPADS when pre-incubated for several minutes, the rat receptor however, exhibited very low sensitivity to PPADS (Soto et al., 1996a; Garcia-Guzman et al., 1997a; Jones et al., 2000). Interestingly, a single point mutation that provides the rat receptor with lysine, instead of glutamate (Glu^{249}) at the position equivalent to that found in the rP2X$_1$ and rP2X$_2$ receptors restores the ability of PPADS to produce slowly reversible inhibition, which seems to be due to the ability of PPADS to form a Schiff's base by virtue of its aldehyde moiety (Buell et al., 1996b). Another interesting feature recently demonstrated for P2X$_4$ receptors is a change in permeability of the ionic channel during ATP applications continued for several seconds. Causing an "activation-history-dependent" increase in permeability, and allowing the passage of fluorescent cations such as the relatively large molecule YO-PRO-1 (Khakh et al., 1999a; Virginio et al., 1999). Those changes in cation permeability in response to ATP application were previously thought to be unique for P2X$_7$ receptors (Surprenant et al., 1996), but have now been demonstrated for P2X$_4$ as well as P2X$_2$ channels.

The P2X$_6$ clone was isolated from rat brain cDNA libraries (Collo et al., 1996; Soto et al., 1996b). The original pharmacological characterisation of rP2X$_6$, based on observations from a small fraction of transfections into HEK293 cells (Collo et al.,

1996), seems to be controversial, especially on the basis of recent findings, indicating that $P2X_6$ subunits are not capable of forming functional homomeric receptors when expressed in HEK293 cells as well as in *Xenopus* oocytes (Soto et al., 1996b; Torres et al., 1999; Khakh et al., 1999b). However, the $P2X_6$ mRNA was found to be widely expressed in both the CNS and in peripheral tissues (Collo et al., 1996; Soto et al., 1996b).

The $P2X_7$ receptor, formerly classified as the P_{2Z} subtype, is the only representative of the fourth group of P2X receptors (see table 1.2.). Electrophysiological experiments in HEK293 cells transfected with the rat or human $P2X_7$ receptor show that brief application of ATP activates an inward cation current (Surprenant et al., 1996; Rassendren et al., 1997b). Key properties of these currents are little rectification, little desensitisation, strong potentiation by reducing the concentration of extracellular Ca^{2+} and/or Mg^{2+} (reviewed by North and Surprenant, 2000), and relative insensitivity to ATP (300 μM) as compared to BzATP (3 - 30 μM). It was particularly the last two of these properties that suggested that the $P2X_7$ receptor might correspond to the P_{2Z} receptor (Surprenant et al., 1996); this interpretation was strongly supported by the findings that activation of $P2X_7$ receptors allowed the uptake of YO-PRO-1 into transfected HEK293 cells, and that prolonged exposure to agonists led to cytolysis. Similarly at P_{2Z} receptors, first described in rat mast cells (Cockroft and Gomperts, 1979) but also present in immune cells (Surprenant et al., 1996; Di Virgilio et al., 1996), high concentrations of ATP led to membrane permeabilisation. There are differences between the properties of the rat and human $P2X_7$ receptors: activation of $hP2X_7$ requires higher agonist concentrations, inward currents decline more quickly when the agonist is removed, and YO-PRO-1 uptake is less for the human compared to the rat receptor under otherwise comparable conditions (Rassendren et al., 1997b). At rat receptors, currents evoked by BzATP are antagonised only poorly by suramin, PPADS and 2',3'-O-(2',4',6')-trinitrophenyl-ATP (TNP-ATP), and irreversibly blocked by 2',3'-dialdehyde-ATP (oxoATP) after pre-incubation for 1 - 2 h (Surprenant et al., 1996; Virginio et al., 1998). The calmodulin inhibitor calmidazolium potently inhibits BzATP activated currents in HEK293 cells expressing $P2X_7$ receptors without affecting the YO-PRO-1 uptake (Virginio et al., 1997). The isoquinolines 1-[N,O-bis(5-isoquinolinesufonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) and N-[1-[N-methyl-p-(5-isoquinolinesulphonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinolinesulphon-

amide (KN-04) inhibit currents evoked by BzATP in HEK293 cells expressing hP2X$_7$, but not in those expressing the rat receptors (Humphreys et al., 1998); a similar species selectivity has first been shown for the respective native P2Z receptors (Gargett and Wiley, 1997). The P2X$_7$ subunit has a C-terminal tail approximately 200 amino acids longer than in any other of the known P2X subunits. Mutagenesis experiments have shown that this long C-terminal tail is involved in the formation of the large pore, though it does not affect the function as an ATP-gated non-selective ion channel (Surprenant et al., 1996). However, recent data, describing pore dilation of P2X$_2$ and P2X$_4$ receptors after prolonged exposure to ATP might indicate that the C-terminal domain is not directly involved in the initial dilation of the ion channel (Virginio et al., 1999). The P2X$_7$ has a much wider distribution than has been previously expected, mRNA for this subtype being not only present in macrophages and other related immune cells, but in heart, liver, pancreas, brain, spinal cord, skeletal muscle, lung, and spleen (Surprenant et al., 1996; Rassendren et al., 1997b). In the brain the P2X$_7$ transcript appears to be present on ependyma and microglia, but not in neurons (Collo et al., 1997).

Like other transmitter-gated ion channels P2X receptors are sensitive to a number of endogenous agents, including Zn^{2+}, Cu^{2+}, H^+, Mg^{2+} and Ca^{2+} (for review see North and Surprenant, 2000) as well as other neurotransmitters or neuro-modulators (Hu and Li, 1996; Wildman et al., 1997). Differential effects of such modulating agents may provide a simple means to discriminate among recombinant P2X receptor subtypes as well as to characterise P2X receptors in native tissues in the absence of subtype-selective antagonists. Effects of H^+ and Zn^{2+} on recombinant rP2X$_{1-4}$ are summarised in table 1.3. (see Wildman et al., 1999c). P2X$_2$ receptors have a unique phenotype with respect to ions. Thus, they are the only P2X receptor subtype at which the response to ATP is increased by acidification of the extracellular solution (King et al., 1997b; Stoop et al., 1997; Ding and Sachs, 1999). In contrast, agonist potency is decreased at P2X$_1$, P2X$_3$ (Wildman et al., 1999c) and P2X$_4$ (Wildman et al., 1999b) in the presence of increasing concentrations of H^+. ATP-induced currents are potentiated by both Zn^{2+} and Cu^{2+} at P2X$_2$ receptors (Wildman et al., 1998; Xiong et al., 1999), allowing P2X$_2$ receptors to be distinguished from P2X$_4$ receptors, which are less sensitive to Cu^{2+} (Xiong et al., 1999). At P2X$_1$ receptors the potency of ATP is decreased in the presence of Zn^{2+}, whereas at P2X$_3$ the actions of Zn^{2+} are complex: ATP-responses are potentiated when Zn^{2+} is co-applied with ATP,

pre-incubation of Zn^{2+} results in a concentration-dependent potentiation followed by inhibition (Wildman et al., 1999c); the same effect occurs at $P2X_2$ receptors, although the magnitude of the Zn^{2+} effect is much greater (Wildman et al., 1998).

Table 1.3. Modulation of recombinant $rP2X_{1-4}$ by H^+ and Zn^{2+} (taken from Wildman et al., 1999c)

	$rP2X_1$	$rP2X_2$	$rP2X_3$	$rP2X_4$
H^+ effect	ATP potency ↓	ATP potency ↑	ATP potency ↓	ATP potency ↓
	ATP efficacy ↔	ATP efficacy ↔	ATP efficacy ↔	ATP efficacy ↔
	time independent	time independent	time independent	time independent
Zn^{2+} effect	ATP potency ↓	ATP potency ↑↓	ATP potency ↑↓	ATP potency ↑
	ATP efficacy ↔	ATP efficacy ↔↓	ATP efficacy ↔↓	ATP efficacy ↓
	time dependent	time dependent	time dependent	time independent
H^+ & Zn^{2+}	ATP potency ↓	ATP potency ↑	ATP potency ↑↓	ATP potency ↑
combined	ATP efficacy ↔	ATP efficacy ↔	ATP efficacy ↔↓	ATP efficacy ↓
	effects additive	H^+ effect	Zn^{2+} effect	effects additive

Symbols: ↑, increase; ↓, decrease; ↔ no change; ↑↓, bi-directional changes, dependent on incubation time; ↔↓, no change initially, but a decrease with prolonged incubation.

1.1.2.2.1.2. Heteromeric ionotropic P2 receptors

The cloning of ATP receptors and their functional expression has proven extremely useful in elucidating the basic properties of these proteins and for providing a template for further study of native P2 receptors. With the molecular identification of seven P2X subunits, understanding of the biophysical and pharmacological properties of these channels has considerably increased. However, discrepancies between heterologously expressed homo-oligomeric P2X subunits and electrophysiological recordings from neuronal preparations likely reflect the existence of native heteromeric phenotypes of P2X receptors. Moreover, results from Northern blots and *in situ* hybridisation data have indicated that the six neuronal P2X subunit genes are transcribed in specific but overlapping populations in the peripheral nervous system as well as the CNS, again suggesting that P2X receptors might co-assemble into heteromultimeric channels. Almost all known ionotropic receptors exist as hetero-oligomers (for review see Barnard, 1996), in which different subunits associate to generate channels with

unique functional properties, allowing for diversity in signalling. The subunit composition of heteromeric P2X receptors is not known. However, using epitope-tagged constructs, physical associations can be shown between some pairs of P2X subunits in heterologous expression systems (Radford et al., 1997; Lê et al., 1998, 1999; Torres et al., 1999). These co-immunoprecipitation studies revealed that the $P2X_7$ subunit is unlikely to assemble with other P2X subunit, whereas others such as $P2X_2$ and $P2X_5$ subunits will readily heteroplymerise with most members of the P2X family (see table 1.4.).

Of those pairs that are now known to co-immunoprecipitate, some have been studied functionally after co-expression. The non-desensitising response to α,β-mATP observed in sensory neurons (Khakh et al., 1995a; Lewis et al., 1995; Cook et al., 1997) can be reproduced in HEK293 cells by co-expression of $P2X_2$ and $P2X_3$ receptor cDNA; because this phenotype could not be accounted for readily by any simple mixing of channels with $P2X_2$ and $P2X_3$ properties, it was concluded that heteromeric channels must be formed (Lewis et al., 1995). This hypothesis was supported by the observation of a high level of co-localisation of $P2X_2$- and $P2X_3$-immunoreactivity in rat nodose and dorsal root ganglia (Vulchanova et al., 1997). Direct evidence for the formation of a $P2X_2P2X_3$ heteromer came from a study showing that in cells co-infected with $P2X_2$ and $P2X_3$ receptors, the two proteins can be cross-immunoprecipitated with antibodies specific for either of the epitope tags introduced at the C-terminus of the proteins (Radford et al., 1997). The heteromeric $P2X_2P2X_3$ receptor shares properties of $P2X_3$ with respect to activation by nucleotide agonists. The rank order of potency of agonists is comparable to the human and rat $P2X_3$ receptors (Lewis et al., 1995; Bianchi et al., 1999). In contrast to their activity at $hP2X_3$, Ap_nA's were significantly less potent as $P2X_2P2X_3$ agonists (Bianchi et al., 1999). Suramin and PPADS readily antagonise $P2X_2P2X_3$-mediated currents (Lewis et al., 1995; Bianchi et al., 1999). The heteromeric receptor is also very sensitive to 8,8'-(carbonylbis(imino-3,1-phenylenecarbonylimino)) bis (1,3,5-naphthalene-trisulphonic acid) (NF023) and to TNP-ATP, implying that for these antagonists, the presence of the $P2X_3$ subunit is sufficient to generate high sensitivity (Soto et al., 1999; Virginio et al., 1998). The effect of pH-changes, however, is similar to that seen for homomeric $P2X_2$ receptors (Evans et al., 1996).

$P2X_4$ and $P2X_6$ subunits are extensively co-expressed throughout the CNS (Collo et al., 1996), and there is evidence for their heteropolymerisation in *Xenopus* oocytes (Lê et al., 1998). The co-injected oocytes were slightly more sensitive to 2-MeSATP and α,β-mATP than were oocytes injected only with the $P2X_4$ subunit (Lê et al., 1998; Khakh et al., 1999b); there was no difference in the effect of zinc or protons between heteromeric $P2X_4P2X_6$ and homomeric $P2X_4$ (Lê et al., 1998). The antagonistic potency of suramin and PPADS is slightly higher at the $P2X_4P2X_6$ heteromer than at homomeric $P2X_4$. Reactive blue 2 (RB2), which was shown to potentiate $P2X_4$ receptor activity, is an antagonist at the $P2X_4P2X_6$ heteromer (Lê et al., 1998). However, in these experiments co-injected oocytes might express both $P2X_4$ homomers and $P2X_4P2X_6$ heteromers which are both activated by ATP, a fact that complicates the interpretation of antagonist sensitivities. Therefore, it remains unclear to what extent this combination can account for the response by many central neurons (Lê et al., 1998).

Results from *in situ* hybridisation, PCR/cloning analysis, and immuno-cytochemistry experiments have implicated the co-existence of $P2X_1$ and $P2X_5$ receptors in heart (Bogdanov et al., 1998a) and spinal cord tissue (Collo et al., 1996). When *Xenopus* oocytes were co-injected with $P2X_1$ and $P2X_5$ subunits, a slowly desensitising response induced by α,β-mATP was observed, whereas homomeric $P2X_5$ receptors are almost insensitive to this agonist and homomeric $P2X_1$ receptors desensitise strongly in the first second of agonist application (Lê et al., 1999). Similar results were obtained from co-injection of HEK293 cells with the cDNAs encoding for the $P2X_1$ and $P2X_5$ isoforms (Torres et al., 1998b; Haines et al., 1999), where biphasic inward currents, consisting of a fast peak current that quickly diminishes to a sustained plateau, were observed in response to ATP $\geq$ 2-MeSATP > ATPγS > α,β-mATP > ADP (Haines et al., 1999). In the same study using ATP as agonist, the $P2X_1P2X_5$ heteromer was shown to be as sensitive to the antagonists suramin and PPADS as the respective homomeric $P2X_1$ and $P2X_5$ receptors, but markedly less sensitive to TNP-ATP than homomeric $P2X_1$ receptors. In contrast, in oocytes co-injected with $P2X_1$ and $P2X_5$, the antagonist TNP-ATP has an inhibitory effect on α,β-mATP-induced currents similar to that observed at the $P2X_1$ receptor in the same study (Lê et al., 1999). Whether these differences are due to different expression systems, the use of different agonists or differences in the interpretation and analysis of the currents (peak vs. plateau) remains to be determined.

Almost recently co-expression of $P2X_2$ and $P2X_6$ receptor subtypes which are present in rat central nervous system (CNS) and frequently co-localise in the same brainstem nuclei resulted in a heteromeric $rP2X_2P2X_6$ receptor which showed a significantly different phenotype from the wildtype $rP2X_2$ receptor (King et al., 2000). Differences included reduction in agonist potencies, a biphasic ATP-evoked inward current, and modulation of channel activity by H^+. The potentiating effect of Zn^{2+} on ATP responses was similar to that observed at $rP2X_2$, whereas the pH-dependent blocking activity of suramin was changed at this heteromeric receptor (King et al., 2000).

Table 1.4. Summary of observed P2X-subunit co-assembly (taken from Torres et al., 1999)

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	-	+	+	-
$P2X_2$		+	+	-	+	+	-
$P2X_3$			+	-	+	-	-
$P2X_4$				+	+	+	-
$P2X_5$					+	+	-
$P2X_6$						-	-
$P2X_7$							+

Co-immunoprecipitation studies (Torres et al., 1999) suggest the existence of further heteromeric receptors. Thus, the lack of novel phenotypes does not necessarily mean that two subunits are not interacting to form a hetero-oligomeric complex, as was originally anticipated from the study of Lewis and colleagues (1995) for the combinations $P2X_1P2X_2$, $P2X_1P2X_4$, and $P2X_3P2X_4$. An alternative explanation is that when two receptor subunits participate to form a complex, one can dominate the phenotype of the resulting channel, and thus, a phenotype resembling one of the respective homomers is observed.

1.1.2.2.1.3. Native ionotropic P2 receptors

ATP-gated ion channels have been studied in a broad range of cell types including smooth muscle, cardiac muscle, platelets, antigen-presenting immune cells, epithelial cells, and peripheral and central neurons (for review see Ralevic and

Burnstock, 1998; Lambrecht, 2000). Three different phenotypes can be observed based on the relative effectiveness of ATP analogues, and biophysical characteristics of the ionic current of native P2X receptors (table 1.5.). Usually selective antagonism is the best way to discriminate among receptors in native tissues, but the limited arsenal of compounds currently available has hampered this approach (see 1.1.3.2.). Comparative studies of agonist potency should ideally be performed in the absence of metabolism, which was not possible for many studies on native P2 receptors as selective, potent inhibitors of ecto-nucleotidases on the one hand, and enzymatically stable agonists on the other hand were not available. Recent studies show that potencies of ATP and 2-MeSATP are decreased markedly by breakdown and that, when this is prevented, e.g. by the use of isolated cells, both agonists are equipotent or even more potent than α,β-mATP at native P2X receptors (Humphrey et al., 1995; Kennedy and Leff, 1995; Khakh et al., 1995b). Therefore, in the absence of nucleotide metabolising enzymes, the original rank order of agonist potency of α,β-mATP $>>$ 2-MeSATP $\geq$ ATP characterising smooth muscle P2X receptors, needed to be redefined. Thus, it was suggested that 2-MeSATP $\geq$ ATP $> \alpha,\beta$-mATP with activity at high nanomolar and low micromolar concentrations and small differences in their potency, is a more accurate description of the pharmacological properties of P2X receptors in native tissues (Kennedy and Leff, 1995).

Accordingly, the first of the three groups of native receptors is represented by response of smooth muscle, cardiac muscle, platelets and sensory neurons of nodose and dorsal root ganglia (reviewed by Evans and Surprenant, 1996; Ralevic and Burnstock, 1998; MacKenzie et al., 1999). In these tissues, α,β-mATP is about equipotent to ATP itself, and concentrations producing halfmaximal effects are in the range of 1 - 3 μM. The currents evoked by these agonists show inward rectification, but they fall into two subgroups depending on the degree to which they decline during continued agonist application. In smooth muscle cells and platelets such desensitisation is profound; the time constant of desensitisation is typically 100 - 300 ms, and largely independent of the concentration of agonist (Evans and Kennedy, 1994; Khakh et al., 1995b). These pharmacological properties are similar to those observed for recombinant $P2X_1$ receptors expressed in heterologous systems (cf. 1.1.2.2.1.1.) and therefore provide further evidence for smooth muscle P2X being the native counterpart of homomeric $P2X_1$ receptors. Currents in neonatal sensory neurons also desensitise, exhibiting

biexponential kinetics with decay constants of 50 ms (fast component) and 1 s (slow component) most similar to those observed at recombinant $P2X_3$ receptors (cf. 1.1.2.2.1.1.); as described for $P2X_3$ homomers desensitisation is strongly dependent on the agonist concentration. In contrast, currents evoked by ATP or α,β-mATP in adult rat sensory neurons or guinea-pig coeliac ganglion cells show little or no desensitisation (Khakh et al., 1995a), a pharmacological profile that does not correspond to any of the homomeric P2X receptors, but was obtained after co-expression of $P2X_2$ and $P2X_3$ receptors in HEK293 cells (see 1.1.2.2.1.2.; Lewis et al., 1995).

Table 1.5. Proposed subtypes of P2X receptors contributing to functional channels in native tissue (for references see text).

Recombinant	Phenotype	Native
$P2X_1$	α,β-mATP-sensitive, concentration-independent desensitisation	smooth muscle
$P2X_3$	α,β-mATP-sensitive, concentration-dependent desensitisation	neonatal sensory ganglia
$P2X_2P2X_3$	α,β-mATP-sensitive, non-desensitising	adult sensory ganglia
$P2X_2$	α,β-mATP-insensitive, non-desensitising	PC12 cells, SCG, submucosal ganglia
$P2X_4$	α,β-mATP-insensitive, non-desensitising, suramin-, PPADS-insensitive	salivary gland

The second group of tissues comprises those where the action of ATP is not mimicked by α,β-mATP. These α,β-mATP-insensitive receptors are present in a diverse range of cells including PC12 cells (Nakazawa et al., 1991), superior cervical ganglion neurons (Khakh et al., 1995a), submucosal enteric neurons (Barajas-Lopez, 1994), outer hair cells of the cochlea (Raybould and Housley, 1997), nucleus tractus solitarius neurons (Ueno et al., 1992), ciliary ganglion (Sun and Stainley, 1996), and epithelial cells including the submandibular gland (Buell et al., 1996b). The effective concentrations of ATP at these receptors with EC_{50} values typically 3 - 30 μM tend to be higher than those at the first class. The currents do not desensitise much during agonist applications of several seconds,

and repeated applications evoke currents with relatively constant peak amplitude. Within this group, the receptors can be further distinguished by their sensitivity to PPADS and suramin. These receptors correspond well in their pharmacological properties to recombinant $P2X_2$ (suramin-, PPADS-sensitive) and $P2X_4$ receptors (largely suramin-, PPADS-insensitive; cf. 1.1.2.2.1.1.).

The third main phenotype, the membrane permeabilising P_{2Z} receptor has been already discussed together with its correlate, the recombinant $P2X_7$ receptor (see 1.1.2.2.1.1.).

1.1.2.2.2. Metabotropic P2 receptors (P2Y receptors)

P2Y receptors couple to heterotrimeric G proteins which in turn activate intracellular second messenger systems (mostly PLC_β, but also PLA_2, PLD and AC) to alter the physiological function of excitable and non-excitable cells. So far, the P2Y receptor family is composed of nine cloned and functionally defined subtypes, six of which ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$) are present in human tissues and are included in the P2Y receptor family by the NC-IUPHAR Committee (Fredholm et al., 1997; Harden et al., 1998a; for review see: Communi and Boeynaems, 1997; King et al., 1998; Ralevic and Burnstock, 1998; von Kügelgen and Wetter, 2000; Hollopeter et al., 2001). The use of lower case was recommended for mammalian orphan receptors or functional non-mammalian P2 receptors without a mammalian orthologue, e.g. cp2y3 for the G-protein-coupled receptor cloned from chick. The first letter indicates species (h, human; c, chick; t, turkey; b, bovine; m, mouse; r, rat; x, *Xenopus laevis*). P2Y receptors can be subdivided based on their agonist profiles: $P2Y_1$, $P2Y_{11}$ and $P2Y_{12}$ receptors are selectively activated by adenine nucleotides, whereas all other P2Y receptors (except $P2Y_6$) display a weak selectivity: uracil nucleotides as well as adenine nucleotides act as agonists on $P2Y_2$, $rP2Y_4$, p2y3, tp2y and p2y8. Additionally, $P2Y_1$, $P2Y_{12}$, $P2Y_6$, and p2y3 receptors show a preference for nucleoside diphosphates and can therefore be distinguished from the triphosphate-preferring $P2Y_2$, $P2Y_4$, $P2Y_{11}$, tp2y and p2y8 receptors (see table 1.6.).

1.1.2.2.2.1. Cloning of metabotropic P2 receptors

The final removal of all earlier doubts concerning the existence of P2 receptors demanded the cloning of their genes. In 1993, the cDNA encoding for a heptahelical protein was isolated from chick brain and, following expression in *Xenopus* oocytes, was shown to be activated by 2-MeSATP ≥ ATP > ADP; UTP, α,β-mATP, and β,γ-mATP were inactive (see table 1.6.). Responses to ATP and 2-MeSATP were antagonised by suramin and RB2 (Webb et al., 1993; Barnard et al., 1994). Activation of this receptor, termed $P2Y_1$, resulted in inositol 1,4,5-triphosphate (IP_3) formation (via PLC) (Simon et al., 1995). Subsequently, orthologues of the $cP2Y_1$ receptor have been cloned from turkey (Filtz et al., 1994), rat (Tokuyama et al., 1995; Pacaud et al., 1996), mouse (Tokuyama et al., 1995), bovine (Henderson et al., 1995) and human tissues (Léon et al., 1995, 1997; Ayyanathan et al., 1996; Janssens et al. 1996; Schachter et al., 1996). Characteristically, the $P2Y_1$ receptor is strongly activated by 2-methylthio-ADP (2-MeSADP), 2-MeSATP, ADP and ADPβS, sensitivity to ATP seems to be variable, even among the cloned orthologues of the $P2Y_1$ receptor (Webb et al., 1993, 1994; Simon et al., 1995; Henderson et al., 1995; see King et al., 1998). Moreover, investigating the purity of commercially available ATP and 2-MeSATP, contamination of solutions with about 10 % nucleoside diphosphates were observed, and after removing contaminating ADP enzymatically (by creatine phosphate/creatine phosphokinase) or by HPLC, both triphosphates appeared to be competitive antagonists rather than agonists at $rP2Y_1$ and $hP2Y_1$ (Hechler et al., 1998c; Léon et al., 1997). However, it remains to be clarified, whether these differences in the relative potency found for ATP and ADP are due to inherent differences in receptor structure or caused by different experimental protocols (Palmer et al., 1998; Dixon, 2000).

The first cloned $P2Y_2$ receptor was from mouse NG108-15 neuroblastoma cells. UTP and ATP were full and equipotent agonists at $mP2Y_2$, whereas ATPγS was less potent, and 2-MeSATP as well as α,β-mATP were reported to act as weak partial agonists (Lustig et al., 1993). This agonist rank order of potency was confirmed and extended for the subsequently cloned $hP2Y_2$ (Parr et al., 1994). ATP and UTP as well as the stable UTP analogue UTPγS were found to be equipotent, full agonists while 2-MeSATP and α,β-mATP were only weakly active (Parr et al., 1994, Lazarowski et al., 1996). In a further study, the polyphosphate

Ap$_4$A was shown to be a potent agonist at hP2Y$_2$ stably expressed in 1321N1 human astrocytoma cells (Lazarowski et al., 1995). Although agonist activity at recombinant P2Y$_2$ receptors has been extensively studied, only little attention has been paid to the blocking activity of P2 receptor antagonists. Charlton and colleagues (1996) have shown that suramin weakly antagonises hP2Y$_2$ receptors, whereas PPADS had no effect on UTP responses. These findings were confirmed in a recent study by Janssens and co-workers (1999), who identified RB2 as the most potent antagonist at hP2Y$_2$.

The P2Y$_4$ receptor, originally believed to be identical with the native pyrimidinoceptor, was cloned from human genomic DNA libraries (Communi et al., 1995; Nguyen et al., 1995). The hP2Y$_4$ is highly selective for UTP, whereas purine nucleotides (ATP, ADP) appeared to be either weak partial agonists or ineffective. Using purified ATP, Nicholas and co-workers reported that ATP is a full agonist at hP2Y$_4$, although 50 times less potent than UTP (Nicholas et al., 1996a, 1996b; Harper et al., 1998). Recently, an orthologue (83 % identity) of the hP2Y$_4$ has been cloned from a rat genomic library. When expressed in *Xenopus* oocytes, the pharmacological profile of rP2Y$_4$ is consistent with P2U receptors, UTP and ATP were equipotent and equiactive agonists (Bogdanov et al., 1998b), as well as with that of the newly cloned turkey receptor tp2y (Boyer et al., 1997). The new form of P2U-like receptor represented by rP2Y$_4$ can be distinguished from P2Y$_2$ on the basis of agonist potency profiles (Nicholas et al., 1996a, 1996b), and antagonist profiles, in that rP2Y$_4$ is blocked by RB2 but is not antagonised by suramin (Bogdanov et al., 1998b). Additional studies comparing human and rat P2Y$_4$ revealed that in contrast to previous observations, ATP acts as an antagonist displaying moderate potency at hP2Y$_4$ receptors (Kennedy et al., 2000). Results obtained for the rat orthologue were confirmed, ATP and UTP, both being potent agonists, and extended to other nucleoside triphosphates including inosine 5'-triphosphate (ITP), guanosine 5'-triphosphate (GTP), cytosine 5'-phosphate and xanthosine 5'-triphosphate (XTP), acting as rP2Y$_4$ agonists, as well (Kennedy et al., 2000). Agonist action of those naturally occurring nucleoside triphosphates on tp2y (Boyer et al., 2000) as well as on xp2y (cloned from *Xenopus laevis*, sometimes referred to as p2y8) (Bogdanov et al., 1997) led to the suggestion that these receptors may represent species homologues of the mammalian P2Y$_4$ receptor (Boeynaems et al., 2000; Boyer et al., 2000; Kennedy et al., 2000).

Species orthologues of the uridine nucleotide-preferring receptor $P2Y_6$ have been cloned from rat and human (Chang et al., 1995; Communi et al., 1996). The human receptor is most potently activated by UDP, and to a lesser extent by UTP, ADP and 2-MeSATP, whereas ATP is only a weak partial agonist (Communi et al., 1996). The more widespread distribution of the $P2Y_6$ receptor, compared to the $P2Y_4$ receptor, suggests that this receptor is more likely to account for endogenous uridine nucleotide-specific responses.

The $P2Y_{11}$ receptor cloned from human placenta, was shown to be coupled to the stimulation of both the phosphoinositide and adenylyl cyclase pathways and therefore being unique among the P2Y receptor subtypes cloned so far (Communi et al., 1997; Kennedy et al., 1999). $P2Y_{11}$ receptors are potently activated by ATPγS, BzATP, ATP and 2-MeSATP, while the dinucleotide derivatives ADP and 2-MeSADP display very weak or, as well as UTP, UDP, AMP and adenosine no agonist activity at all (Communi et al., 1997). In a recent study, the ATP derivative AR-C67085 (2-propylthio-β,γ-dichloromethylene-ATP, formerly FPL 67085, syn. ARL 67085), a potent inhibitor of ADP-induced platelet aggregation (Humphries et al., 1995a), was shown to be the most potent agonist at $hP2Y_{11}$ receptors (Communi et al., 1999). Suramin and RB2 acted as antagonists, whereas PPADS was completely inactive at $P2Y_{11}$ receptors expressed in 1321N1 astrocytoma cells as well as in chinese hamster ovary cells (CHO-K1 cells) (Communi et al., 1999). It was concluded from the authors, that the pharmacological profile of the $P2Y_{11}$ receptor is very similar to the cyclic AMP-coupled P2Y receptor mediating granulocytic differentiation of the HL-60 promyelocytic leukaemia cells (Jiang et al., 1997; Conigrave et al., 1998) and that this receptor is likely to be of the $P2Y_{11}$ subtype (Communi et al., 1999).

Six additional G protein-coupled receptors have been proposed to be members of the P2Y receptor family. The p2y3 receptor is a P2Y receptor originally cloned from a chick brain cDNA library (Webb et al., 1996a). The relatively high amino acid sequence identity between the chick p2y3 and mammalian $P2Y_6$ receptors (65 %) and their similar pharmacological profile, both being potently activated by UDP, indicate that the p2y3 is likely to be the avian homologue of the mammalian $P2Y_6$ receptor (Li et al., 1998).

The proposed p2y5 receptor (Webb et al., 1996b) was originally cloned from activated chick T-lymphocytes as an orphan receptor designated 6H1 receptor (Kaplan et al., 1993), and the human p2y7 receptor was cloned from a human erythroleukemia cell line (Akbar et al., 1996). Both of these G protein-coupled receptors were suggested to be members of the P2Y receptor family largely on the basis of binding studies using [^{35}S]dATPαS, which was subsequently shown not to be a general radioligand for P2Y receptors (Schachter and Harden, 1997). Moreover, these receptors exhibit predicted amino acid sequences that are only 28 - 32 % identical to that of known P2Y receptors, and nucleotide-promoted second messenger signalling was not observed after expression of either of these G protein-coupled receptors (Herold et al., 1997; Li et al., 1997; Yokomizo et al., 1997). The protein originally referred to as the p2y7 receptor has been recently reported to be a receptor for leukotrienes (Yokomizo et al., 1997).

A P2Y receptor cloned from *Xenopus* neural plate, was shown to be activated equipotently by purine and pyrimidine nucleoside triphosphates and was tentatively named P2Y$_8$ (Bogdanov et al., 1997). As a mammalian homologue of this receptor has not been identified, its inclusion as a distinct subtype of the P2Y receptor family does not seem appropriate. Recent studies, however, indicate that based on pharmacological data and a relatively high sequence identity, xp2y8 might represent a species homologue of the mammalian P2Y$_4$ (Boeynaems et al., 2000; Boyer et al., 2000; Kennedy et al., 2000).

Additionally, two sequences (P2Y$_9$, P2Y$_{10}$) related to the p2y5 receptor have been submitted to the GenBank/EMBL™ Data Bank. The P2Y$_9$ sequence is identical to that recently published under the name p2y5-like (Janssens et al., 1997). However, as for the p2y5 receptor, there is no functional evidence that these proteins are nucleotide receptors (Janssens et al., 1997; Schachter and Harden, 1997).

Table 1.6. Pharmacological properties of cloned metabotropic P2 receptors (for references see 1.1.2.2.2.1.)

Subtype	Endogenous ligands	G protein-coupling	Effector	Agonist profile	Antagonists
Adenine nucleotide-selective					
P2Y$_1$	ADP, ATP	G$_{q/11}$	PLC	**h:** 2-MeSADP > ADP > 2-MeSATP > ATP **r:** 2-MeSADP = 2-MeSATP > ADP > ATP	MRS 2179, Suramin, PPADS, Reactive Blue 2
Adenine nucleotide selective, triphosphate-preferring					
P2Y$_{11}$	ATP	G$_{q/11}$	PLC AC	ATPγS $\approx$ BzATP > ATP > 2-MeSATP >> ADP	Reactive Blue 2, Suramin
Adenine nucleotide selective, diphosphate-preferring					
P2Y$_{12}$	ADP	G$_i$	AC	ADP	Clopidogrel, Ticlopidine
Triphosphate-preferring					
P2Y$_2$	ATP, UTP	G$_{i/o}$, G$_{q/11}$	PLC	UTP = ATP $\geq$ ATPγS = UTPγS > Ap$_4$A > ADP, 2-MeSATP, α,β-mATP	Suramin, Reactive Blue 2
P2Y$_4$	UTP, ATP	G$_i$, G$_{q/11}$	PLC	**h:** UTP > GTP > ITP **r:** UTP > ATP > Ap$_4$A $\approx$ ITP $\approx$ GTP > CTP > XTP	Reactive Blue 2, PPADS
Uridine nucleotide selective, diphosphate-preferring					
P2Y$_6$	UDP	G$_{q/11}$	PLC	5BrUDP $\geq$ UDP >> UTP > 2-MeSADP > 2-MeSATP $\geq$ ADP > 5BrUTP >> ATP	Reactive Blue 2, PPADS, Suramin

Table 1.7. Properties of native metabotropic P2 receptors (taken from King et al., 1998).

Subtype	Endogenous ligands	Agonist profile	Antagonists	Tissue distribution
Adenine nucleotide-selective				
P2Y	ATP, ADP	2-MeSATP > ATPγS > ATP $\geq$ ADP >> α,β-mATP, UTP	suramin, RB2, PPADS[a]	endothelium, visceral and vascular smooth muscle, astrocytes, hepatocytes, glioma cells, turkey erythrocytes
P2T	ADP	2-Cl-ADP $\geq$ 2-MeSATP > ADP	AR-C66096, 2-Cl-ATP	platelets
P2D	Ap$_n$A	Ap$_4$A > Ap$_5$A > α,β-mATP > 2-MeSATP		chromaffin cells, brain, synaptosomes
Uridine nucleotide-selective				
Pyrimidino-ceptor	UTP, UDP	UDP = UTP UTP > UDP (human HL-60 cells) UDP > UTP (rat glioma cells)		smooth muscle, superior cervical ganglion neurons, HL-60 cells, glioma cells
Triphosphate-preferring				
P2U	ATP, UTP	UTP = ATP $\geq$ ATPγS = UTPγS > Ap$_4$A >> ADP, 2-MeSATP, α,β-mATP	in some cases: suramin, PPADS	endothelium, vascular smooth muscle, astrocytes, hepatocytes

[a]Antagonism at P2Y receptors coupled to PLC, not at those coupled to AC (Boyer et al., 1994, Webb et al.,1996a)

1.1.2.2.2.2. Native metabotropic P2 receptors

Before the cloning of the first P2Y receptor cDNA's, agonist studies in mammalian tissues indicated that at least three separate metabotropic P2 receptor phenotypes exist (see 1.1.1., table 1.7.): G protein-coupled receptors activated preferentially by adenine nucleotides (P_{2Y}, P_{2T}, P_{2D}), metabotropic P2 receptors activated preferentially by uridine nucleotides (pyrimidinoceptor) and receptors activated equally by adenine and uridine nucleotides (P_{2U}). Some confusion in the literature was caused by the identification of P2U receptors (O'Connor et al., 1991), because they were often loosely termed "pyrimidinoceptors" and separate identity of these and receptors activated preferentially by UTP or UDP (ATP being weakly active or inactive) was often indistinct (Seifert and Schultz, 1989; cf. 1.1.1.).

P2Y receptors were originally identified as receptors that mediate relaxation of the GPTC and the longitudinal layer of the rabbit portal vein, and release of relaxing substances from rat and pig arterial endothelial cells (Burnstock and Kennedy, 1985). Currently, the native P2Y receptor is characterised by a high agonistic potency of alkylthio-ATP derivatives (e.g. 2-MeSATP, 2-MeSADP) and adenosine phosphorothioates (ATPγS, ADPβS). ATP and ADP are usually equiactive, yet less potent than the above, while methylene-phosphonate ATP derivatives (α,β-mATP, β,γ-mATP) as well as pyrimidine nucleotides are either considerably less potent or inactive (Cusack, 1993; Abbrachio and Burnstock, 1994). P2Y receptor-mediated responses have been demonstrated in variety of tissues and cells (for review see: Dubyak and El-Motassim, 1993; Abbracchio and Burnstock, 1994; Boarder et al. 1995; Ralevic and Burnstock, 1998). At present, the correspondence between the recombinant $P2Y_1$ receptor and the native P2Y receptor is controversial (Abbracchio and Burnstock, 1998; King et al., 1998). Moreover, a heterogeneity within pharmacologically characterised P2Y receptor-mediated responses is still under discussion (see Ralevic and Burnstock, 1998; Lambrecht et al., 1999). Coupling of endogenous P2Y receptors to different signal transduction pathways, as described for the PLC-coupled P2Y receptors on turkey erythrocytes and AC-linked P2Y receptors present on rat C6-2B glioma cells (Boyer et al. 1994, 1996b), differences in the relative potencies of ATP and ADP (see Ralevic and Burnstock, 1998) and analogues of ATP (Fischer et al., 1993; Boyer et al., 1995) as well as differences in both, antagonistic properties and potency of PPADS on P2Y receptor-mediated responses (Boyer et al., 1994;

Ziganshin et al., 1994b; Windscheif et al., 1995; Ralevic and Burnstock, 1996; Schachter et al., 1996; Lambrecht et al., 1999) are in favour of a further subdivision of this receptor. The fact, that differences in agonist and antagonist potencies occur, even among the cloned orthologues of the $P2Y_1$ receptor (Boyer et al., 1996a; see section 1.1.2.2.2.1.) may, on the other hand, account for these differences being determined by small structural variations as indicated by sequence homology of 83 % between $tP2Y_1$ and $hP2Y_1$ (Ayyanathan et al., 1996). However, a contribution of P2 receptors that have not yet been cloned to P2Y receptor-mediated responses in native tissues cannot be excluded.

Native P2U receptors, mediating for example PGI_2 release and endo-thelial-dependent relaxation in a variety of vascular preparations and an increase in Cl^- secretion in human airway epithelia, were found to have no preference for either adenine- or uridine-triphosphates, with both ATP and UTP being equiactive agonists, UTPγS and ATPγS equally potent, while 2-MeSATP, ADPβS, α,β-mATP, ADP and UDP are significantly less potent or inactive (O'Connor et al., 1991; Cusack, 1993; Boarder et al., 1995; Ralevic and Burnstock, 1998; King et al., 1998). As for $P2Y_1$ receptors and endogenous P2Y receptors there is controversy about the $P2Y_2$ receptor being the sole equivalent of the P2U receptor. The fact that P2U receptors are either sensitive or insensitive to suramin and PPADS (for review see Ralevic and Burnstock, 1998) may indicate that native P2U receptors which are equieffectively activated by UTP and ATP display a heterogeneous receptor population. In fact, comparing the agonist potencies for recombinant $rP2Y_2$ and $rP2Y_4$, UTP and ATP being equieffective, full agonists at both receptor subtypes, these receptors are two structurally distinct forms of the P2U receptor (Chen et al., 1996; Bogdanov et al., 1998b). Sensitivity to suramin, shown for the human orthologue of $rP2Y_2$ and the fact that $rP2Y_4$ is insensitive to suramin indicate, that P2U receptors can be differentiated by their susceptibility to blockade by suramin.

In some tissues a native P2 receptor, believed to be a uridine nucleotide-selective receptor, could be distinguished from receptors activated by ATP (see 1.1.1., Seifert and Schultz, 1989; von Kügelgen and Starke, 1990). These receptors, termed pyrimidinoceptors, may represent a heterogeneous population as well. The P2 receptor in HL-60 cells is highly selective for UTP, UDP acts as a weak partial agonist, whereas the PLC-coupled P2 receptor in C6-2B rat glioma cells is highly

selective for UDP, while UTP is much less potent (Lazarowski and Harden, 1994). Furthermore, a uridine nucleotide receptor activated by UTP and UDP but not by ATP has been described in neurons of the rat superior cervical ganglion (SCG) (Boehm et al., 1995; Connolly, 1995; Connolly and Harrison, 1995; von Kügelgen et al., 1997). Whether these receptors find their counterparts within the cloned uridine nucleotide-selective mammalian P2Y receptor subtypes (hP2Y$_4$ and P2Y$_6$) is far from clear (for review see: Ralevic and Burnstock, 1998; Communi et al., 1998; King et al., 1998; Communi and Boeynaems, 1997).

Exposure of platelets to ADP results in an increase in intracellular calcium, rapid calcium influx, PLC activation, and in an inhibition of stimulated AC. ADP causes platelets to change shape and activates fibrinogen receptors, leading to platelet aggregation (Hourani and Hall, 1994; Gachet et al., 1997; Kunapuli, 1998a, 1998b). All the physiological and intracellular signalling events triggered by ADP in platelets were initially attributed to a single cell surface receptor, termed P2T (Gordon, 1986). Subsequently, the presence of P2X$_1$ receptors on platelets (MacKenzie et al., 1996), and the identification of mRNA encoding for the P2Y$_1$ receptor in platelets together with the observation, that ATP acts as an antagonist, when this P2Y$_1$ receptor is expressed in Jurkat cells (Léon et al., 1997) led to the proposal of a two receptor model, in which P2Y$_1$ receptors coupled to G$_i$, cause inhibition of AC, and the P2X$_1$ receptor is the receptor mediating rapid calcium influx and shape change (Gachet et al., 1997). Based on the observation that 2-propylthio-d-β,γ-difluoromethylene ATP (ARC 66096MX; formerly FPL 66096), a potent inhibitor of ADP-induced platelet aggregation (Humphries et al., 1994) blocks ADP-induced inhibition of AC, but fails to inhibit ADP-mediated intracellular calcium increases, a three receptor model was proposed (Daniel et al., 1998): one receptor coupled to inhibition of stimulated AC, designated P2T$_{AC}$ by the authors, alternatively termed *P2Y$_{ADP}$*; a second receptor coupled to mobilisation of calcium from intracellular stores through activation of PLC and IP$_3$ formation, designated P2T$_{PLC}$, identical with the P2Y$_1$ receptor; and the third, an ionotropic P2X$_1$ receptor mediating rapid calcium influx. Thus, the concept of the "P2T" receptor was resolved into three P2 receptor subtypes (Hechler et al., 1998b; Jin et al., 1998; Kunapuli et al., 1998a). Inhibition of platelet aggregation by the thienopyridine clopidogrel (Plavix[®]) was reported to be due to the blockade of P2T$_{AC}$ receptors (Hechler et al., 1998a) which seem to act in concert with the PLC-coupled P2Y$_1$ receptors to mediate ADP-induced platelet aggregation (Jin et

al., 1998; Jin and Kunapuli, 1998; Kunapuli et al., 1998a). $P2Y_1$ receptor activation causes a rapid and transient aggregation, whereas the extent of sustained aggregation seems to be determined by the level of $P2T_{AC}$ receptor occupancy (Boeynaems et al., 2000; Jarvis et al., 2000). The function of $P2X_1$ receptors, mediating rapid entry of extracellular calcium, remains to be elucidated.

Although the physiological function of the $P2T_{AC}$ has been extensively studied, the molecular identity of the G_i-linked receptor was elusive until very recently. Displaying only 19 % identity to the $P2Y_1$ receptor and being most closely related to the UDP-glucose receptor (44 % identity) it is conceivable that many effort in cloning of the now termed $P2Y_{12}$ receptor was without success. However, there is convincing evidence that the recently cloned $P2Y_{12}$ receptor is equivalent to the $P2T_{AC}$ receptor (Hollopeter et al., 2001).

1.1.3. P2 receptor ligands

Despite the progress that has been made in the P2 receptor field by cloning and, as far as possible, pharmacological characterisation of recombinant P2X and P2Y receptor subtypes, the development of selective ligands for this receptor class lags far behind most other receptor families. The cloned receptors became available for pharmacological studies only very recently and therefore structure-activity relationship (SAR) studies were performed most thoroughly in classical smooth muscle preparations. In many instances the selectivity and efficacy of a certain compound differed depending on the tissue used and the experimental protocol applied, thus providing a confusing picture for the development of SAR's for the various P2 receptor subtypes. Nevertheless, some principle suggestions of SAR's established for P2 receptor ligands which were based on studies on native smooth muscle P2X and P2Y receptors have been confirmed in recent studies using assays with apparently defined P2 receptor subtypes.

1.1.3.1. Agonists

With the exception of a novel series of recently described theobromine and theophylline analogues (Fischer et al., 1999b), all known agonist ligands for the P2 receptor family are essentially variations on the purine nucleotide

pharmacophore (figure 1.1.). Generally, ATP is the most potent native nucleotide at P2X receptors, whereas at various subtypes of P2Y receptors UTP, and/or ADP and UDP may serve as agonists (see 1.1.2.). Dozens of ATP analogues with modifications at the triphosphate, ribose 2' or 3', purine C-2 or C-8, or at the purine N^6 positions have been synthesised and their pharmacological profile reported in functional assays (for reviews see Cusack, 1993; Fischer et al., 1993; Burnstock et al., 1994; Jacobson and Suzuki, 1996; Jacobson et al., 1998a; Ralevic and Burnstock, 1998). In general, substitution at the C-2 position led to enhancement of potency, and modifications of the triphosphate group generated compounds displaying increased enzymatic stability.

Triphosphate modifications

Modification of the triphosphate group by replacement of the bridging oxygen atoms with methylene units or of the charged oxygen atoms with sulphur resulted in some cases in potent analogues having stability towards ecto-nucleotidases. α,β-mATP, in particular is noteworthy for its potency at native P2X receptors (Cusack, 1993; Burnstock et al., 1994) and its selectivity for recombinant homomeric $P2X_1$ and $P2X_3$ receptors as well as $P2X_2P2X_3$ and $P2X_1P2X_5$ heteromers (Ralevic and Burnstock, 1998; Haines et al., 1999). To date, there is no evidence that α,β-mATP activates P2Y receptors to any degree (Yu et al., 1999).

Replacement of D-ribose by L-ribose in the group of methylenephosphonate analogues generates selective agonists for the P2X receptor family (Cusack, 1993). One of these compounds, L-β,γ-mATP, proved to be a useful agonist for the differentiation of $P2X_1$ receptor-mediated responses from those elicited via other P2X receptor subtypes. It potently activates native smooth muscle P2X (Cusack, 1993; Trezise et al., 1995) and recombinant $P2X_1$ receptors (Evans et al., 1995), but, in contrast, was found to be inactive at native α,β-mATP-sensitive and α,β-mATP-insensitive P2X receptors in neuronal preparations (Evans et al., 1995; Trezise et al., 1995; Rae et al., 1998) as well as at recombinant $P2X_2$ (Evans et al., 1995) and $P2X_4$ receptors (Buell et al., 1996b). Furthermore, L-β,γ-mATP is inactive on P2Y receptors mediating relaxation of GPTC and on native and recombinant P2Y receptors present in chick brain (Hourani et al., 1985; Cusack, 1993; Simon et al., 1995).

A thio-substitution at the terminal phosphate also provides stability to ecto-nucleotidases activity (Cusack, 1993), leading to such analogues as ATPγS and ADPβS, both of which have been explored as radioligands for P2 receptors. ATPγS is a potent agonist at recombinant homomeric $P2X_{1-6}$, heteromeric $P2X_2P2X_3$, and $P2X_1P2X_5$ receptors (Collo et al., 1996; Bianchi et al., 1999; Haines et al., 1999) as well as at recombinant $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors (Erb et al., 1993; Schachter et al., 1996; Sromek and Harden, 1998; Communi et al., 1999). It is only weakly active at $P2X_7$ receptors (Bianchi et al., 1999), and acts as a weak partial agonist at $P2Y_4$ and $P2Y_6$ receptors (Chang et al., 1995; Bogdanov et al., 1998b). ATPγS is almost equipotent to ATP in eliciting relaxation of GPTC and IP_3 formation in turkey erythrocyte membranes both mediated via P2Y receptors, and is more potent than ATP at P2X receptors present in guinea-pig urinary bladder and vas deferens (Burnstock et al., 1994). The phosphorothioate compound ADPβS displayed preference for the P2Y receptor subtype in functional experiments, being markedly more potent than ATP and ADP in the turkey erythrocyte model (Burnstock et al., 1994) and equipotent to ATP in eliciting relaxation of GPTC as well as contraction of guinea-pig vas deferens and urinary bladder (Cusack, 1993; Burnstock et al., 1994). It is more potent than ATP at recombinant $hP2Y_1$, $tP2Y_1$ (Filtz et al., 1994; Schachter et al., 1996), at the P2Y receptor coupled to inhibition of AC in rat C6-2B glioma cells (Filtz et al., 1994), displayed partial agonistic activity at the recombinant $rP2Y_6$ receptor and is a full agonist at its avian orthologue, the chick p2y3 receptor (Chang et al., 1995; Li et al., 1998). At recombinant $P2Y_{11}$ receptors ATP is slightly more potent than ADPβS (Communi et al., 1999). Agonistic action of ADPβS was also described at recombinant $hP2X_1$, $hP2X_3$ and $rP2X_3$ as well as at $hP2X_4$ receptors, although in comparison to ATP, its potency was at least 10 times lower (Bianchi et al., 1999). ADPβS was shown to be inactive as an agonist at recombinant $hP2X_4$ and $hP2X_7$ monomers, the $P2X_2P2X_3$ heteromer (Bianchi et al., 1999), recombinant tp2y receptors (Boyer et al., 1997), and native P2U receptors mediating activation of human neutrophils (Cusack, 1993).

Uri et al. (1994) reported that various negatively charged amino acid derivatives of adenosine (at the 5'-hydroxyl groups) acted as weak agonists at the PLC-linked P2 receptors on C6-2B glioma cells, demonstrating that the triphosphate group could be replaced by a non-phosphate moiety.

Ribose modifications

The potency and selectivity of nucleotide analogues are highly dependent on the 2'- and 3'-ribose substituents (Burnstock et al., 1994; Martin et al., 1995). Generally, removal of either free hydroxyl group resulted in a decrease in potency for P2Y receptors, with the exception of 3'-amino-3'-deoxy ATP which was more potent than ATP at P2Y receptors in turkey erythrocytes. Activities at P2X receptors decreased or remained unchanged by ribose substitutions, except for 3'-benzylamino-3'-deoxy ATP, which was markedly more potent than ATP in guinea-pig vas deferens and urinary bladder and either inactive or a weak agonist in P2Y-systems, thereby displaying selectivity for native P2X receptors (Burnstock et al., 1994). Bulky ether substituents placed at the 2'-position of β,γ-mATP greatly diminished activity, whereas a *p*-methoxybenzyl ether at the 3'-position, resulted in the same potency and efficacy as β,γ-mATP at P2X receptors (Martin et al., 1995). As described for L-β,γ-mATP, replacement of the D-ribose by L-ribose can generate agonists selective for the P2X receptor subtype present in smooth muscle.

Adenine modifications

Numerous nucleotides related to either ATP or α,β-mATP but modified on the adenine base have been synthesised. These include substitutions at the purine C-2 position, at the exocyclic N^6 position, and at the C-8 position.

2-MeSATP is a highly potent P2 receptor agonist. Previously, it was thought to be selective for P2Y receptors; however, its relatively weak activity at native P2X receptors was later shown to be due to its breakdown by ecto-nucleotidases (Kennedy and Leff, 1995). Besides its agonistic action at several P2 receptor subtypes in tissue preparations, 2-MeSATP is equipotent to ATP at recombinant P2X$_{1-3}$ and slightly less potent than ATP at recombinant P2X$_4$ homomers, as well as at the P2X$_2$P2X$_3$ heteromer (Bianchi et al., 1999). Activation of members of the P2Y receptor family was reported for P2Y$_1$ (see comments in 1.1.2.2.2.1.), P2Y$_6$ (Chang et al., 1995) and P2Y$_{11}$ (Communi et al., 1999), and rP2Y$_4$ receptors (Bogdanov et al., 1998b). In contrast, 2-MeSATP was found to be inactive at human P2Y$_4$ (Communi et al., 1995) and mouse and human P2Y$_2$ receptors (Lustig et al., 1993; Lazarowski et al., 1995). Congeners of 2-MeSATP were

synthesised as probes for P2 receptors (Fischer et al., 1993). Chain elongation at this site preserved high potency at P2Y receptors, thus proving that this position within the receptor protein has tolerance for structural variations of the ligand (Fischer et al., 1993). The addition of a thioether chain at the 2-position increased potency not only for 5'-triphosphate derivatives but also for di- and mono-phosphates. Those monophosphate analogues had considerable agonistic potencies at the turkey erythrocyte P2Y receptor and the AC-linked P2Y receptor present in C6-2B glioma cells, at which AMP itself is inactive. However, those derivatives were generally several orders of magnitude less potent than their corresponding 2-thioether triphosphates (Fischer et al., 1993; Boyer et al., 1996b). A further benefit of the presence of a long chain thioether group at the 2-position is the increased stability of the triphosphate group at the 5'-position (Zimmet et al., 1993; Fischer et al., 1999a). Recently, a series of 2-thioether 5'-O-(1-thio-triphosphate) adenosine derivatives have been introduced as $P2Y_1$ receptor agonists, the 2-hexylthio-ATPαS isomers displayed apparent affinities in the nanomolar range and increased stability towards ecto-nucleotidases (Fischer et al., 1999a).

In general, modifications of the ATP purine nucleus at the C-8 or N^6 position were not well-tolerated by smooth muscle P2X receptors as well as native P2Y receptors. 8-Bromo ATP, which was originally claimed to be active at urinary bladder P2X receptors (Welford et al., 1987), was inactive at vas deferens and at vascular P2Y receptors (Burnstock et al., 1994). However, N^6-methyl ATP was equipotent to ATP at GPTC P2Y receptors and inactive in vascular smooth muscle and P2X preparations (Fischer et al., 1993). The N^6 position does not accommodate as wide a range of substituents as does the 2-position. Substituents larger than methyl as well as substitution at the N^6 position with dimethyl abolished potency at P2X as well as P2Y receptors (Burnstock et al., 1994; Boyer et al., 1996b).

Fig. 1.1. *Chemical structure of principle P2 receptor agonists (see page 46).*

ATP

2-Methylthio ATP

α,β-Methylene ATP

β,γ-Methylene ATP

ADPβS

ATPγS

UTP
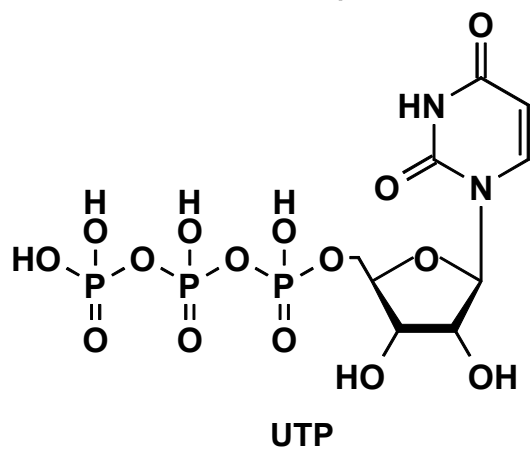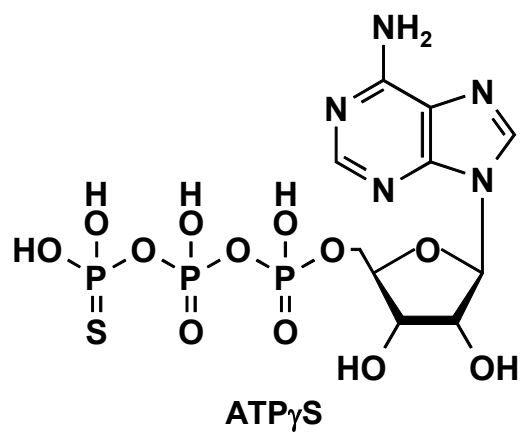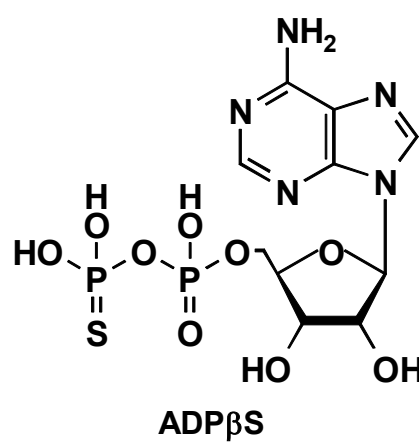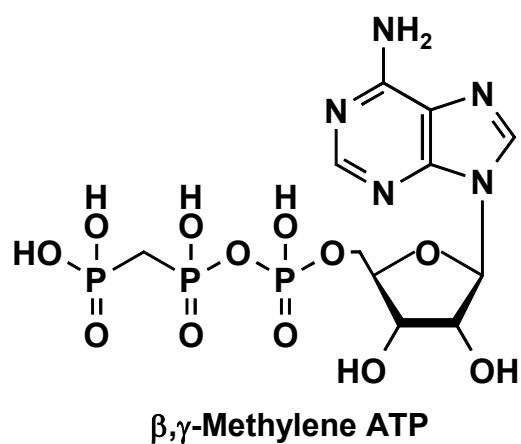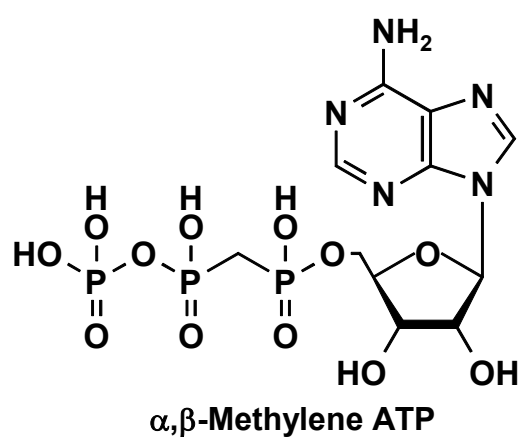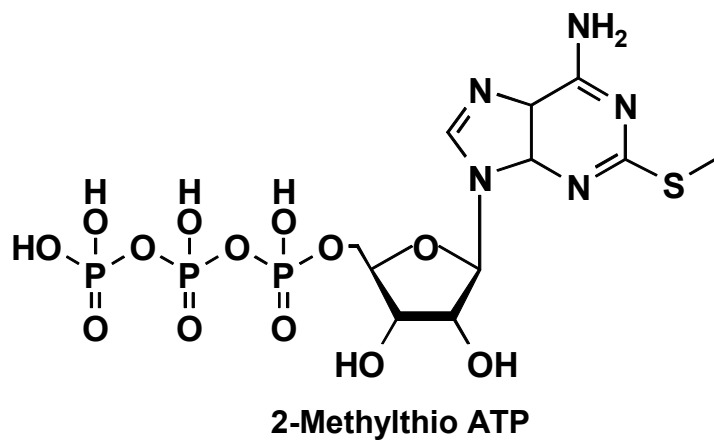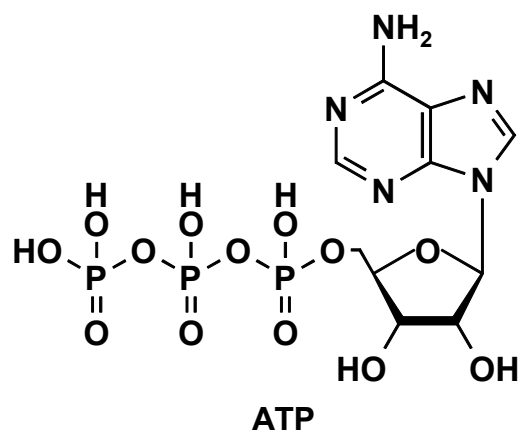
1.1.3.2. Antagonists

Almost all P2 receptor antagonists, including suramin (figure 1.5.), PPADS and RB2 (figure 1.2.), share a common polyaromatic, polysulphonated skeleton. Most of these antagonists have suffered from a serious lack of specificity for the P2 receptor family, selectivity for certain P2 receptor subtypes and potency in blocking P2 receptor-mediated responses.

Histochemical dyes

Among the histochemical dyes displaying P2 receptor-blocking properties are evans blue, RB2, trypan blue and reactive red 2 (figure 1.2.). Evans blue and trypan blue differ only in the position of the sulphonate residues at the naphthalene rings. Both compounds were found to be relatively potent antagonists at the P2X receptor subtype present in ratVD (Bültmann and Starke, 1993; Bültmann et al., 1994; Khakh et al., 1994). However, they display different selectivities in terms of ecto-nucleotidases inhibition and inhibition of P2Y receptor-mediated relaxation of GPTC; evans blue being a potent inhibitor of ATP breakdown in vas deferens tissue, and trypan blue, in contrast, more effective in blocking P2Y receptors present in GPTC (Bültmann et al., 1995; Bültmann et al., 1996a). SAR studies performed with a series of evans blue and trypan blue derivatives confirmed the importance of the substitution pattern of the naphthalene rings for the antagonistic potency of trypan blue derivatives at GPTC P2Y receptors as well as for the selectivity of evans blue derivatives for ratVD P2X receptors (Wittenburg et al., 1996).

Reactive red 2 was introduced as a highly potent competitive, slightly P2Y receptor-selective (GPTC) antagonist. However, potent but non-competitive antagonism at ratVD P2X receptors as well as its property to potently inhibit ATP breakdown in ratVD limit its usefulness (Bültmann and Starke, 1995). RB2 (syn. basilen blue E-3G), which is a mixture of *meta*- and *para*-sulphonic acid isomers (and cibacron blue 3GA the respective *ortho*-isomer) was reported to act as a potent competitive P2Y antagonist in particular at the AC-coupled P2Y receptors of C6-2B glioma cells (Boyer et al., 1994). Among the recombinant receptors $P2X_1$, $P2X_2$ and $P2X_4$, as well as $P2Y_1$, p2y3, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ have been shown to be sensitive to either cibacron blue 3GA or RB2 (Brake et al., 1994;

Simon et al., 1995; Chang et al., 1995; Communi et al., 1996, 1999; Michel et al., 1996; Seguéla et al., 1996; Webb et al., 1996a). Although both compounds were often considered as P2Y-selective antagonists, studying the effect of a series of RB2 derivatives Tuluc and colleagues (1998) reported them to be equipotent antagonists at ratVD P2X and GPTC P2Y receptors.

The anion transport inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS; figure 1.2.) was initially used to block P2Z receptor-mediated responses, and was subsequently shown to act as a non-competitive, slowly reversible antagonist selective for ratVD P2X receptors versus GPTC P2Y receptors, and to inhibit ATP breakdown by ecto-nucleotidases present in ratVD (Bültmann and Starke, 1994a; Bültmann et al., 1996b). DIDS discriminates between subtypes of recombinant P2X receptors, being a potent inhibitor at rat $P2X_1$ receptors, but 100-fold less effective $P2X_2$ receptors (Evans et al., 1995). Evaluation of a series of DIDS analogues revealed that the isothiocyanato residues, which may react with amines to form thioureas, are essential for the interaction of these compounds with P2 receptors (Bültmann et al., 1996b). None of the smaller derivatives was more potent than the parent compound in blocking ratVD P2X receptors, moreover, some structural variations led to a loss of selectivity for the P2X receptor subtype (Bültmann et al., 1996b).

Pyridoxal phosphate derivatives

A diazo derivative of the coenzyme pyridoxal phosphate, PPADS (figure 1.2.), which was shown to be an antagonist at rabbit vas deferens P2X receptors and therefore introduced as P2X-selective compound, is one of the most important and most commonly used P2 receptor antagonists (Lambrecht et al., 1992; for review see Lambrecht, 2000). PPADS has been examined in a wide range of tissues (Lambrecht, 1996; Lambrecht et al., 1996a) and cells, displaying inhibitory potency at recombinant homomeric $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$, heteromeric $P2X_2P2X_3$ and $P2X_1P2X_5$ and $P2Y_1$ receptors, but being weak or ineffective at homomeric $P2X_4$, $P2X_6$ and $P2X_7$, heteromeric $P2X_4P2X_6$ and $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ (Lewis et al., 1995; Soto et al., 1997; Harden et al., 1998b; Lê et al., 1998; Ralevic and Burnstock, 1998; Communi et al., 1999). On the basis of these observations, the original characterisation of PPADS as a selective antagonist for smooth muscle P2X receptors has to be discarded, however, it is still a very useful

tool for P2 receptor characterisation, in being selective for certain P2X receptor subtypes as well as for the $P2Y_1$ receptor. The applicability of PPADS, when used at concentrations > 10 μM is limited by some non-P2 receptor actions; as described for other P2 receptor antagonists, ATP breakdown is inhibited by PPADS (Windscheif et al., 1995; Heine et al., 1999; Lambrecht et al., 2000b). Moreover, antagonistic effects of PPADS (100 μM) have been reported at 5-HT_3 receptors and at nicotinic receptors (Hussy et al., 1994; Khakh et al., 1995a). The pseudo-irreversible nature of antagonism displayed by PPADS at native P2X receptors present in several smooth muscle preparations, including the rabbit, guinea-pig and rat vas deferens (Lambrecht et al., 1992, 1996a, 2000b) and rat mesenteric arterial bed (Windscheif et al., 1994) as well as the slow reversibility of its blocking effects on recombinant $P2X_1$, $P2X_2$ and $P2X_5$ receptors introduce further restraints (Evans et al., 1995; Collo et al., 1996).

An isomer of PPADS, pyridoxalphosphate-6-azophenyl-2',5'-disulphonic acid (iso PPADS), was shown to be a slowly-equilibrating and slowly-reversible P2X antagonist with potency almost similar to PPADS. Pyridoxalphosphate itself, is a non-selective P2 receptor antagonist, being about 10-fold less potent than PPADS (Lambrecht et al., 1996a; Ralevic and Burnstock, 1998).

Of the numerous PPADS analogues investigated so far (Windscheif et al., 1995; Lambrecht et al., 1996a; Jacobson et al., 1998b; Kim et al., 1998; Kim et al., 2001), two derivatives, cyclic pyridoxine-$\alpha^{4,5}$-monophosphate-6-phenylazo-2',5'-disulphonate (MRS 2220; figure 1.2.) and pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulphonate) (PPNDS; figure 1.2.) exhibit advantages both in terms of subtype selectivity, and PPNDS also in terms of antagonistic potency. MRS 2220 was reported to act as a reversible and surmountable antagonist at recombinant $P2X_1$ receptors, being 5-fold less potent at recombinant $P2X_3$, and without effect at $P2X_2$, $P2X_4$ and $P2Y_{1,2,4,6}$ (Jacobson et al., 1998b). However, the $P2X_1$ receptor-blocking potency of MRS 2220 is 100-fold less compared to PPADS. MRS 2220 is particularly interesting because it is the first PPADS analogue that, although lacking the aldehyde group, preserved the P2X receptor-blocking potency. Thus, the Schiff's base formation (Buell et al., 1996b) between ligand and receptor is not necessarily required for recognition of pyridoxalphosphate derivatives.

The naphthylazo derivative PPNDS was found to be more potent than PPADS at native P2X receptors in ratVD as well as at recombinant $P2X_1$ receptors (Lambrecht et al., 2000c). PPNDS was shown to act as a specific, and $P2X_1$ receptor-selective antagonist, being markedly less potent at P2Y receptors of GPI, exhibiting only weak inhibitory potency on ecto-nucleotidases, and being without significant effects on several receptor classes, including α_{1A}, A_1, A_{2B}, H_1, and M_3 (Lambrecht et al., 2000c).

The heteromeric bivalent ligand 6-[(4,6,8-trisulpho-1-naphthyl)iminocarbonyl-1,3-(4-methylphenylene)- iminocarbonyl-1,3- phenylene-azo] -pyridoxal-5'-phosphate (SB9; figure 1.2.) comprising pyridoxal-5'-phosphate, a diazo group as spacer and as second pharmacophore, the monomer of suramin, has been found to be a potent antagonist selective for P2Y receptors present in guinea-pig ileal smooth muscle preparation (Lambrecht et al., 2000b). Inhibitory potency of SB9 at rat vas deferens P2X receptors is about 10-fold lower. Therefore, the compound displays unique properties among the pyridoxal-5'-phosphate and suramin analogues reported to date, which are either non-selective P2 or P2X receptor-preferring antagonists (Lambrecht et al., 2000b).

Nucleotide derivatives

Trinitrophenyl (TNP) -substituted nucleotides have been introduced as potent antagonists, displaying selectivity for homomeric $P2X_1$, $P2X_3$ and heteromeric $P2X_2P2X_3$ receptors (Virginio et al., 1998). TNP-ATP (figure 1.2.) was reported to be markedly less effective as an antagonist in cells expressing homomeric $P2X_2$, $P2X_4$, or $P2X_7$ receptors, however, effects of the TNP-nucleotides on P2Y receptor subtypes as well as on other receptor classes have not been examined, so far. When CRC's were constructed using either ATP or α,β-mATP as agonist in cells expressing $P2X_3$ receptors the antagonism of TNP-ATP was insurmountable, indicating that the compound acts as non-competitive probably allosteric antagonist (Virginio et al, 1998). Non-competitive antagonism was also observed for TNP-ATP at rat mesenteric artery P2X receptors. Surprisingly, compared to results obtained from the dissociated smooth muscle cells, the potency of TNP-ATP was markedly reduced in the whole tissue experiments. (Lewis et al., 1998). This might reflect the breakdown of TNP-ATP by ecto-nucleotidases; as shown in the original study, the breakdown product TNP-adenosine was without

any effect on P2X receptors (Virginio et al., 1998). Thus, although TNP-ATP is a potent antagonist, it is of limited use in whole tissue assays.

Diinosine pentaphosphate (Ip_5I) is a potent antagonist of the tentative P_4 receptor present in rat brain synaptic terminals (Pintor et al., 1999). Antagonistic properties of Ip_5I at guinea-pig vas deferens P2X receptors have also been reported. Furthermore, Ip_5I was found to be ineffective at GPTC P2Y receptors, as well as at P_1 receptors mediating negative inotropy of the guinea-pig driven left atrium (Hoyle et al., 1997). Recently, effects of diinosine polyphosphates were studied at recombinant $P2X_{1-4}$ receptors. Ip_5I was found to be a potent $P2X_1$ receptor-selective antagonist, displaying moderate inhibitory potency at $P2X_3$ receptors, being ineffective at $P2X_2$ and potentiating agonist responses at $P2X_4$ receptors (King et al., 1999). The higher potency of Ip_5I at recombinant $P2X_1$ compared to native guinea-pig vas deferens P2X receptors is probably due to breakdown of Ip_5I by ecto-nucleotidases present in the tissue preparation.

P2T ($P2Y_{12}$) receptor antagonists as inhibitors of ADP-induced platelet aggregation are of special interest. As, in contrast to most other P2 receptor subtypes, ATP acts as an antagonist at these receptors it seemed likely that modifications of the nucleotide pharmacophore might generate potent and selective P2T receptor antagonists. AR-C66096MX (formerly FPL-66096) and its dichloro analogue, 2-propylthio-D-β,γ-dichloromethylene ATP (AR-C67085MX) were introduced, displaying high potency in blocking P2T receptor-mediated ADP-induced platelet aggregation and some 30.000-fold selectivity over native P2X or P2Y receptors (Humphries et al., 1994, 1995b). SAR studies generated 2-(3,3,3-trifluoropropylthio)-N^6-methylthioethyl-D-β,γ-dichloromethylene ATP (AR-C69931MX). This compound exhibits enhanced potency and stability and is currently in phase II of clinical development (Ingall et al., 1999). SAR rules that can be derived from the large number of compounds evaluated are somehow similar to those established for nucleotide analogues being agonists at the other P2 receptor subtypes: introduction of substituents at C-8 reduces potency, replacement of the hydrogen C-2 increases potency, S-propyl being ideal, replacement of the terminal anhydride oxygen in the phosphate chain with β,γ-dihalomethylene enhances enzymatic stability, and monoalkylation of N^6 of the adenine with lipophilic substituents augments potency, in contrast to dialkylation which markedly reduces potency (Ingall et al., 1999).

Recently, a series of adenine nucleotide derivatives in which the 3'-hydroxyl group is replaced by phosphate, including adenosine-3'-phosphate-5'-phosphosulphate (A3P5PS; figure 1.2.), adenosine-3'-phosphate-5' phosphate (A3P5P) and adenosine-2'-phosphate-5' phosphate (A2P5P), has been introduced and proposed to act as competitive antagonists/partial agonists at the turkey erythrocyte P2Y receptors; A3P5PS and A3P5P were devoid of agonist action at the recombinant hP2Y$_1$ receptor (Boyer et al., 1996a). The effects of these 2'- and 3'-phosphate analogues were claimed to be selective for the PLC-coupled P2Y$_1$ receptor, because no agonistic or antagonistic effects on the AC-coupled P2Y receptor of C6-2B glioma cells or on P2Y$_{2,4,6}$ receptors stably expressed in 1321N1 human astrocytoma cells, respectively, were observed (Boyer et al., 1996a). However, further studies on the selectivity and stability of A3P5PS indicated that its usefulness as an antagonist in intact tissues might be limited. It was degraded by ecto-nucleotidases present in ratVD, the only detectable breakdown product being A3P5P (Park et al., 1998). In addition, it was shown to activate α,β-mATP-sensitive receptors present in GPTC as well as to induce P2Y receptor-mediated relaxation of the rat duodenum (Bültmann et al., 1998; Park et al., 1998). Antagonistic effects of A3P5PS were observed in human platelets, inhibiting ADP-induced platelet aggregation and shape change (Park et al., 1998) and in GPTC (inhibition of relaxation induced by either ADPβS, α,β-mATP or noradrenaline) and ratVD (inhibition of contraction induced by α,β-mATP) (Bültmann et al., 1998).

Of the numerous structural analogues of A3P5P that have been synthesised so far some SAR's can be derived for nucleotide analogues displaying agonist as well as antagonistic activity at the P2Y$_1$ receptor subtype. N^6-Methyl-2'-deoxy-adenosine-3',5'-biphosphate (MRS 2179; figure 1.2.) was found to be about 10-fold more potent as an antagonist than A3P5P and without the partial agonistic activity displayed by the parent compound (Camaioni et al., 1998). Therefore it was concluded, that alkylation of the exocyclic amine (methyl- and ethyl substituents are tolerated) is critical for the biological effects of deoxyadenosine biphosphate derivatives, and crucial for pure antagonistic activity (Camaioni et al., 1998). Recently, MRS 2179 was shown to be P2Y$_1$-selective within the P2Y receptor family, displaying neither agonist nor antagonist activity at P2Y$_{2,4,6}$, the AC-linked P2Y receptor of C6-2B glioma cells, as well as the tp2y subtype (Nandanan et al., 2000). However, its selectivity for the P2Y$_1$ receptor is not absolute, since

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MRS 2179 was reported to show considerable antagonistic activities at $P2X_1$ and $P2X_3$ receptors, but not at $P2X_2$ and $P2X_4$ receptors (Nandanan et al., 2000). Further structural variations reflect the ribose moiety which can be substituted by several carbocyclic ring systems (Nandanan et al., 1999, 2000) or even with acyclic aliphatic as well as aromatic chains (Kim et al., 2000) without a total loss of affinity. The balance of agonist versus antagonist properties is highly dependent on ribose modifications, particularly at the 2'-position as substitution generally appears to increase the degree of agonism (Nandanan et al., 1999). The 5'-phosphate group seems to be critical for $P2Y_1$ receptor recognition (Camaioni et al., 1998). 2-Alkylthio-substituted analogues display enhanced agonist properties, the 2-Cl derivative of MRS 2179, however, was shown to be slightly more potent as an antagonist at $tP2Y_1$ (Nandanan et al., 1999). Substituents at the 8-position of the adenine base generally lead to compounds with decreased $P2Y_1$ receptor affinity (Camaioni et al., 1998; Nandanan et al., 1999).

A novel type of $P2Y_1$ receptor antagonists has been introduced recently. Those compounds were synthesised by coupling via a carbonyl linker the adenosine 5'-hydroxyl group to an oligo-aspartate chain. The compounds were reported to act as antagonists at $hP2Y_1$ receptors expressed in 1321N1 human astrocytoma cells, one derivative termed $AdoOC(O)Asp_2$ displayed $P2Y_1$ receptor-blocking potency comparable to A3P5P (Sak et al., 2000). The observation that the phosphate chain can be exchanged by negatively charged peptide fragments is considerably important, because those compounds might be devoid of side effects such as inhibition of ATP-degrading enzymes as well as themselves being resistant to breakdown by ecto-nucleotidases.

Fig. 1.2. Chemical structure of principle P2 receptor antagonists (see pages 54, 55).

Evans blue

Reactive blue 2 (RB2)

Reactive red 2

DIDS

SB9

PPADS

PPNDS

MRS 2179

A3P5PS

MRS 2220

TNP-ATP
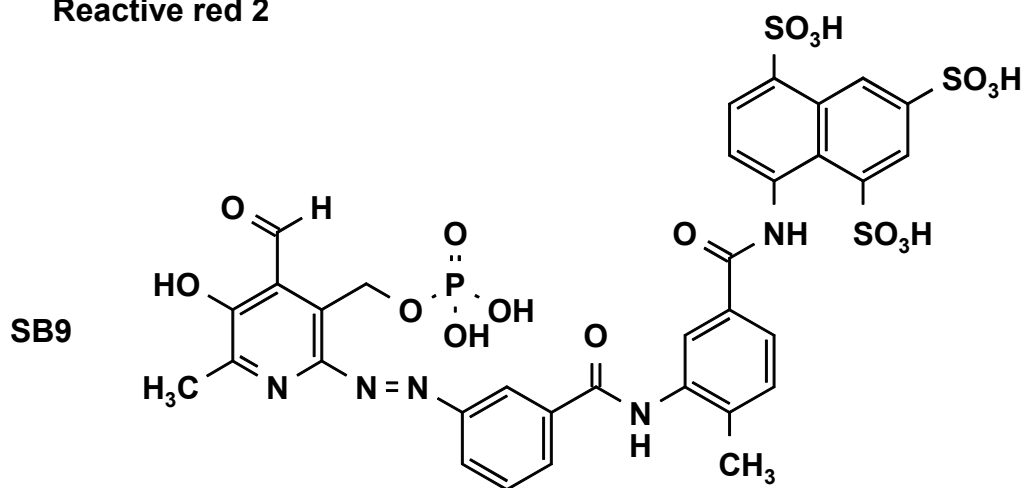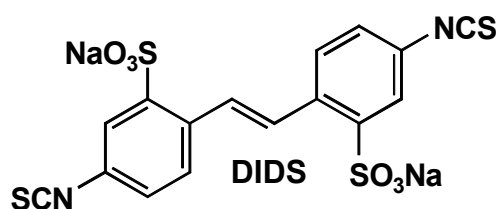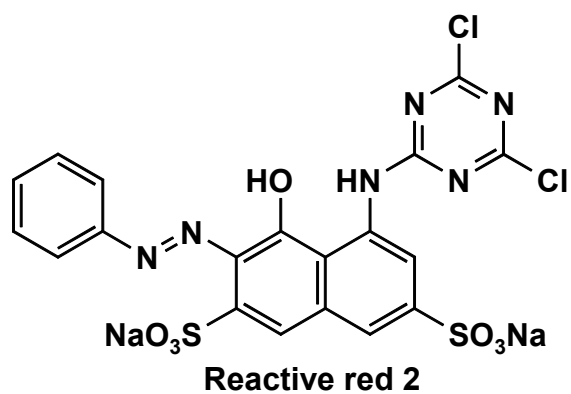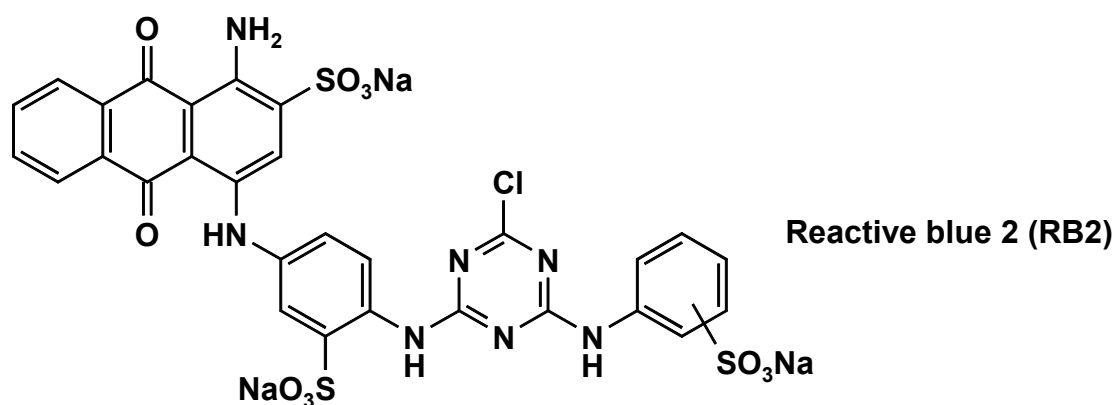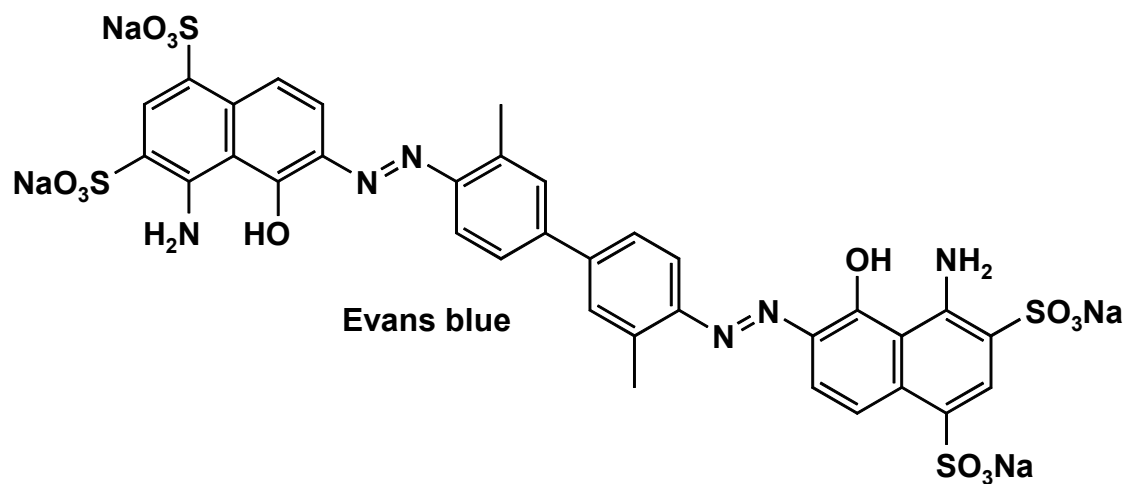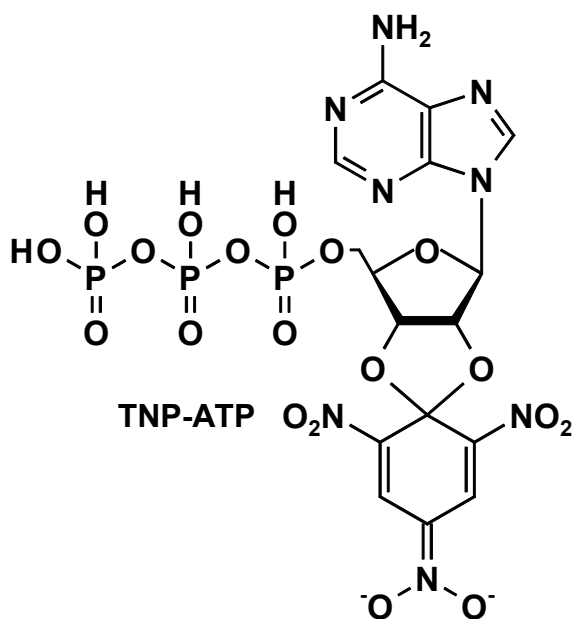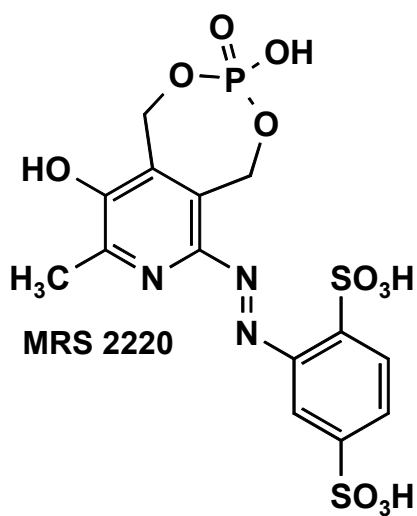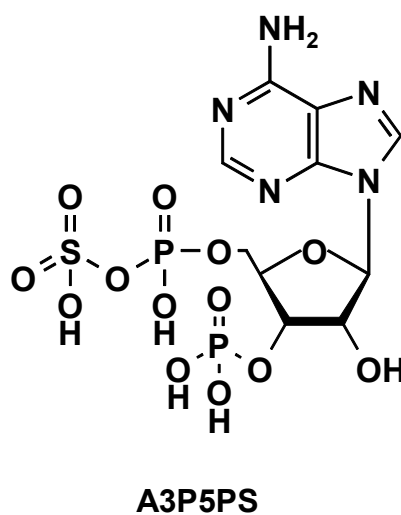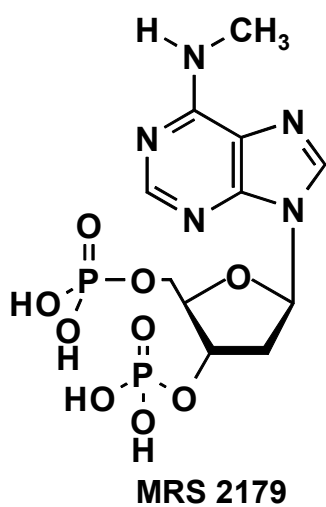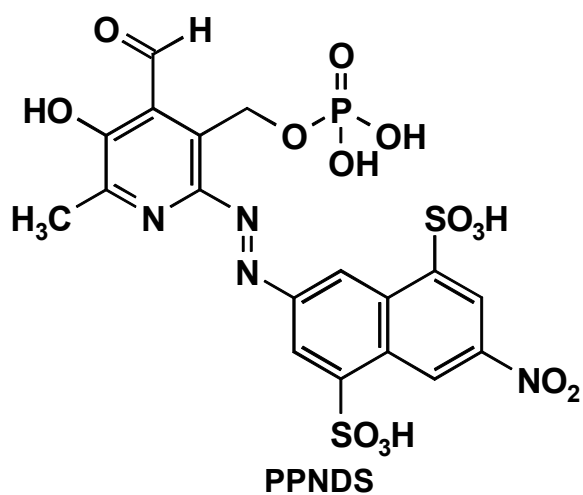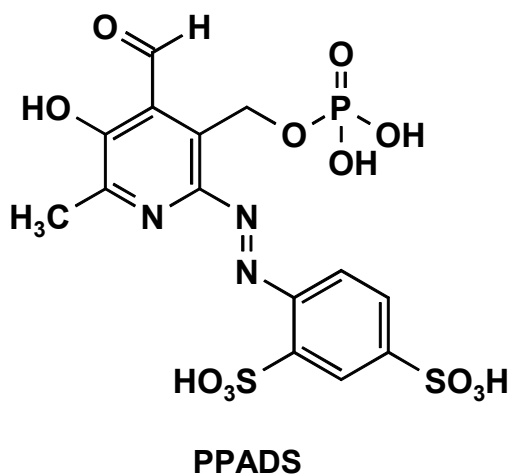
1.1.4. Suramin and derivatives

1.1.4.1. Non-P2 receptor activity

Suramin (synonym: antrypol, Bayer 205, Belganyl, Fourneau 309, Germanin, Moranyl, Naganin, Naganol, Naphuride; figure 1.5.) was originally synthesised in the beginning of the last century by german workers at Farbenfabriken Bayer AG among a series of approximately 2000 compounds that were tested for trypanocidal activity. Since the 1920s the compound has been widely used for the prophylaxis and treatment of early stages of human trypanosomiasis and is still in use for the latter purpose. Van Hoof et al. (1947) observed that congolese patients suffering from trypanosomiasis and onchocerciasis were cured of both diseases following treatment with suramin. To date, suramin remains the only generally available macrofilaricide effective against Onchocerca volvulus (the causative agent of river blindness) (for review see Hawking, 1978; Goa et al., 1991).

Due to the finding that suramin is a competitive inhibitor of retroviral reverse transcriptase (De Clercq, 1979), the RNA-directed DNA polymerase of retroviruses, and, the subsequent demonstration that suramin can block the infectivity and cytopathic effect of the human immunodeficiency virus (HIV) in vitro (Mitsuya et al., 1984), research on suramin has gained considerable impetus. The antiviral action of suramin against a number of other viruses, including the hepatitis B virus, has been tested. However, as established for the treatment of acquired immunodeficiency syndrome (AIDS) it's toxic side effects in addition to a low level of clinical activity limited the usefulness of suramin in the treatment of chronic active hepatitis (Cheson et al., 1987; Loke et al., 1987).

The adrenal toxicity found in patients suffering from AIDS and treated with suramin suggested that this drug might be useful for the treatment of diseases associated with adrenocortical hyperfunction, such as adrenal carcinoma and Cushing's syndrome (Stein et al., 1989). Solid tumours noticeably regressed under suramin therapy in patients with adrenocortical carcinoma, nodular lymphoma and stage D prostate cancer refractory to conventional hormonal manipulation. However, mechanisms underlying suramin's antitumour activity are incompletely understood and are probably multiple (for review see Baghdiguian and Fantini, 1997). Besides the inhibition of steroid hormone production, suramin's

interference with the recognition of a number of growth factors by their membrane receptors as well as inhibition of angiogenesis (Pesenti et al., 1992) may account for the observed antineoplastic effects of suramin. Additionally, the ability of suramin to bind to plasma proteins and thereby inhibiting the synthesis and/or activity of several protein and enzyme systems, including DNA polymerases, complement, and adenosine triphosphate might explain another facet of suramin's antitumour activity (for review see La Rocca et al. 1990).

Among the targets suramin exerts its diverse biological effects are the G protein α-subunits. Butler et al. (1988) characterised suramin as an inhibitor of receptor-G protein-coupling in membranes. Subsequently, three distinct effects of suramin on G_α-subunits were established: first, suramin and suramin analogues (submicromolar to micromolar concentration range) suppress the rate of spontaneous GDP-release from purified G protein α subunits (i.e. suramin inhibits the guanine nucleotide exchange reaction, and is -by definition- a direct G protein inhibitor), second, the effect of suramin on GDP-release is reversed upon addition of an effector, indicating that the binding of an effector and of suramin are mutually exclusive and third, suramin and suramin analogues disrupt ternary complex formation (of agonist-receptor-G-protein). The antagonistic activity of suramin is attributed to its polyanionic nature, but because of its many negative charges it is unlikely to permeate readily the plasma membrane. Therefore and with regard on its many pharmacological actions suramin can only be considered as a lead in the search of more selective G protein inhibitors (Freissmuth et al., 1996; Hohenegger et al., 1998; for review see Höller et al., 1999).

As far as other receptors than P2 are concerned, antagonistic action of suramin has been described for GABA and NMDA receptors, respectively (Nakazawa et al., 1995).

1.1.4.1.1. Structure-activity relationships

Since the structure of suramin was established by Fourneau et al. (1924), a large series of analogues has been prepared. It has been known for many years that small variation in the structure of the suramin molecule considerably reduced the trypanocidal activity. For example NF037 (see figure 3.11.), in which the two

methyl groups of suramin are replaced by two hydrogens, displays only 5 % of the trypanocidal activity of suramin (Fourneau et al., 1924).

In contrast, inhibition of HIV-1 reverse transcriptase is much less sensitive to structural modification and structure-activity relationships are completely different from those of its trypanocidal or antifilarial activity (Jentsch et al., 1987). Braddock et al. (1994) found an absolute requirement of intervening rings between the substituted naphthyl rings but failed to reveal clear SAR's pertaining to the substitution pattern of the naphthyl rings testing the inhibitory potencies of suramin-derived compounds on the fibroblast growth factor (bFGF).

SAR's concerning the inhibitory properties of suramin and its analogues on G protein α-subunits are complicated because of the existence of a large number of different G protein α subunits (more than 20 individual proteins) and the fact that the ability of suramin and of its analogues to disrupt receptor-G protein-coupling is highly dependent on the nature of the receptor-G protein tandem investigated; e.g. suramin is 10-fold more potent than its di-demethylated derivative NF037 in uncoupling the adenosine A_1 receptor, but the two compounds are equipotent in uncoupling the dopamine D2 receptor, although both receptors interact with the same G protein subfamily (Beindl et al., 1996; for review see Freissmuth et al., 1999).

1.1.4.2. Suramin and suramin-derivatives: P2 receptor antagonism

Dunn and Blakeley (1988) were the first to show that suramin (figure 1.5.) antagonises P2X receptor-mediated effects. In many studies, suramin has since been found to be a non-selective antagonist at native P2X and P2Y receptors (Ralevic and Burnstock, 1998), with relatively low potency (pA_2 values approximately 5). Antagonism by suramin is often non-competitive. However, on P2X receptors present in rabbit isolated ear artery suramin was found to be a slowly-equilibrating but competitive antagonist (Leff et al., 1990). Furthermore, suramin inhibits ecto-nucleotidases (Crack et al., 1994; Beukers et al., 1995; Ziganshin et al., 1995; Bültmann et al., 1996c; Heine et al., 1999; Damer et al., 1998; see 1.1.5.7.), an ability which may complicate interpretation of its antagonistic activity when it is used against ligands which are biologically unstable (Crack et al., 1994).

Of the recombinant receptors, suramin was shown to block homomeric $P2X_1$ ($IC_{50} \approx 1\ \mu M$), $P2X_2$ ($IC_{50} \approx 5\ \mu M$), $P2X_3$ ($IC_{50} \approx 3\ \mu M$), $P2X_5$ (30 μM, 94 % inhibition), and $P2X_7$ receptors ($IC_{50} \approx 60\ \mu M$), and to be a weak antagonist at rat $P2X_4$ receptors (Collo et al., 1996; Ralevic and Burnstock, 1998; Jones et al., 2000; Guile et al., 2001). Moreover, antagonism of suramin was reported at heteromeric $P2X_2P2X_3$ ($IC_{50} \approx 6\ \mu M$), $P2X_2P2X_6$ ($IC_{50} \approx 6\ \mu M$) as well as $P2X_1P2X_5$ receptors ($IC_{50} \approx 2\ \mu M$) whereas at heteromeric $P2X_4P2X_6$ receptors suramin is only a poor antagonist as could be expected from its weak activity at the respective homomeric receptors (Lê et al., 1998; Bianchi et al., 1999; Haines et al., 1999; King et al., 2000). Among the recombinant P2Y receptor subtypes, $P2Y_1$, p2y3 and $P2Y_{11}$ receptors were shown to be blocked by suramin (pA_2 values between 5 and 6); it also antagonises $P2Y_2$ receptor-mediated responses, but with an about 10 times lower affinity than that found at the $P2Y_1$ receptor subtype (von Kügelgen and Wetter, 2000). Suramin is inactive at recombinant $hP2Y_4$ and only weakly active at $rP2Y_4$ and $P2Y_6$ orthologues (see von Kügelgen and Wetter, 2000). Additionally, suramin (100 μM) has been demonstrated to abolish p2y8 receptor-mediated responses (Bogdanov et al., 1997). Regarding its antagonistic profile, suramin cannot be considered to be selective for any particular P2 receptor subtype. However, it proved to be a useful tool to differentiate among the recombinant P2X receptor subtypes (see 1.1.2.2.1.1.; table 1.2.; Humphrey et al., 1998) as well as to discriminate between $P2Y_2$ and $P2Y_4$ receptor-mediated responses (King et al., 1998). Moreover, suramin represents the structural lead for a number of compounds that have been analysed for their potentially P2 receptor blocking abilities.

Particularly one derivative, the symmetrical 3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulphonate (NF023; Fig. 3.15.) was found to be a highly specific P2 receptor antagonist displaying selectivity for native $P2X_1$-like receptors in certain vascular and visceral smooth muscle preparations (pA_2 = 5.5 - 6.0; Bültmann et al., 1996c; Lambrecht, 1996; Lambrecht et al., 1996a, 1999; Ziyal, 1996; Ziyal et al., 1996, 1997). It was less potent at $P2Y_1$-like receptors in rat duodenum, guinea-pig taenia coli and ileum, and rat mesenteric arterial bed, and ineffective at UTP-sensitive $P2Y_2$-like receptors in rat and hamster mesenteric arterial beds (Ziyal et al., 1996; Lambrecht 1996) as well as at $P2Y_4$-like receptors in cultured aortic smooth muscle cells from spontaneously hypertensive rats (Harper et al., 1998).

This selectivity profile of NF023 has been confirmed in studies with cloned P2X receptors (Soto et al., 1999). The compound was found to antagonise homomeric rat and human $P2X_1$ receptors with the highest potency (pIC_{50} values 6.6 and 6.7, respectively), $P2X_3$ receptors displayed an intermediate sensitivity with pIC_{50} values of 5.1 and 4.4 for rat and human subtypes, respectively, and $P2X_2$ was the least sensitive subtype (in the presence of 100 μM NF023, the current through $P2X_2$ was blocked by 60 %), whereas human and rat $P2X_4$ receptors were insensitive to NF023 at concentrations up to 100 μM. The heteromeric $P2X_2P2X_3$ receptor was shown to be indistinguishable from the homomultimeric $rP2X_3$ receptor in its sensitivity towards NF023 (Soto et al., 1999; Lambrecht, 2000). Antagonistic activity of NF023 was found to be weak at recombinant $P2Y_1$ receptors (pIC_{50} = 4.6), and the compound was shown to be inactive at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (J.L. Boyer and T.K. Harden, personal communication). Like the parent compound suramin, NF023 inhibits ecto-nucleotidases activity (Beukers et al., 1995; Bültmann et al., 1996c, Lambrecht, 2000), but unlike suramin, it has a high $P2X_1$- versus ecto-nucleotidases-selectivity.

1.1.4.2.1. Structure-activity relationships

Up to 1996, only a few studies dealt with a systematical analysis of structure-activity relationships concerning antagonism of suramin-related compounds at P2 receptor subtypes as well as their inhibitory activity at ecto-nucleotidases. Antagonistic potencies of suramin and the derivatives NF212, NF213 and NF023 on $P2X_1$-like receptor-mediated vasopressor effects induced by α,β-mATP in the pithed rat were found to be markedly different, even between compounds which are closely related, suggesting a clear-cut structure-activity relationship to exist. NF023 was at least as potent as suramin, whereas NF212 and NF213, which differ from suramin with respect to the number and the arrangement of methyl-moieties attached to the aminobenzoyl groups (figure 3.11.) were less potent than the parent compound. The unsymmetrical analogue 8-(2-methyl-3-(3-aminobenzamido)-benzamido)-naphthalene-1,3,5-trisulphonic acid (NF105) was found to be ineffective (Schlicker et al., 1989; Urbanek et al., 1990).

In a binding study performed at turkey erythrocyte membrane P2Y$_1$-like receptors suramin and its desmethyl-derivative NF037 (figure 3.11.) displayed similar affinities, indicating that methyl moieties are not a prerequisite for binding. Binding affinities of the so-called small ureas NF058 (figure 3.15.) and NF023 were markedly decreased, therefore shortening of the chain of the large urea by two aminobenzoyl groups was found to be less favourable for gaining a higher affinity towards P2Y$_1$-like receptors. The even smaller dibenzamide analogues 8-(3-(3-nitrobenzamido)benzamido)-naphthalene-1,3,5-trisulphonic acid (NF018) and 8-(3-(3-aminobenzamido)benzamido)-naphthalene-1,3,5-trisulphonic acid (NF019) were only moderately less potent than suramin, and further simplification of the structure revealed that at least one benzoyl group is necessary to obtain affinity for the receptor. Analysis of benzamide derivatives differing in the substitution pattern of the remaining phenyl ring culminated in 8-(3,5-di-nitro-phenylenecarbonylimino)-1,3,5-naphthalenetrisulphonic acid (XAMR0721), the affinity of which was only reduced to half of that of suramin (van Rhee et al., 1994). However, there are studies that seriously call into question the validity of the [^{35}S]ADPβS-binding assay on turkey erythrocyte membranes indicating that the nucleotide may bind to P2Y receptors and other ATP-binding proteins with almost equal affinity (Harden et al., 1995). Accordingly, caution has to be adopted assessing these observations. The results obtained for XAMR0721 in binding studies could not be confirmed in functional studies at GPTC P2Y receptors where the compound was about 80-fold less potent as an antagonist than suramin (Bültmann et al., 1996a, 1996c).

When suramin and some derivatives were examined for their inhibitory effects on ecto-nucleotidases present on human blood cells, similar potencies were found for the parent compound, the large urea NF031 (see figure 3.13.C.), and NF023, whereas NF058 and the dibenzamide derivative NF018 as well as three benzamide derivatives including XAMR0721 were almost inactive. Therefore, a relation between molecular size and ectoN-inhibitory activity - larger derivatives displaying higher activity - has been proposed (Beukers et al., 1995).

Analysis of a series of suramin derivatives at P2X$_1$-like receptors present in ratVD, GPTC P2Y receptors and ATP-breakdown in ratVD, confirmed results obtained in previous studies (Bültmann et al., 1996c). A novel aspect of SAR's was provided by N-[3-(N-(1,3,5-trisulpho-8-naphthyl) carbamoyl) phenyl]-N'-[3-(N-(1-naphthyl)-

carbamoyl)phenyl]-urea (BSt101), a non-symmetrical derivative of NF023 lacking the three sulphonic acid residues at one of the naphthalene rings. The compound was only slightly less potent than NF023 in blocking $P2X_1$-like receptors, indicating that negative charges on both ends of the molecule, are not a prerequisite for high $P2X_1$-like receptor-blocking potency (Bültmann et al., 1996c).

1.1.5. ATP-degrading enzymes (ecto-nucleotidases)

Many cell types and presumably all tissues have the capacity to metabolise extracellular nucleotides and also nucleosides by surface-located enzymes. A key function of the enzymes presumably is in the inactivation of nucleotides that have been released as signalling substances or during pathological events and in subsequent purine salvage. Nucleotide-degrading enzymes, usually referred to as ecto-nucleotidases, comprise several unrelated enzyme families. Although recently, a uniform nomenclature has been introduced by Zimmermann and colleagues (2000), a somehow confusing accumulation of different designations defining identical enzymes or different enzymes defined by equal designations, respectively, is still persisting in the current literature. Similar to the P2 receptor field, this confusion will be further resolved with the molecular identification and subsequent characterisation of the properties of individual enzymes.
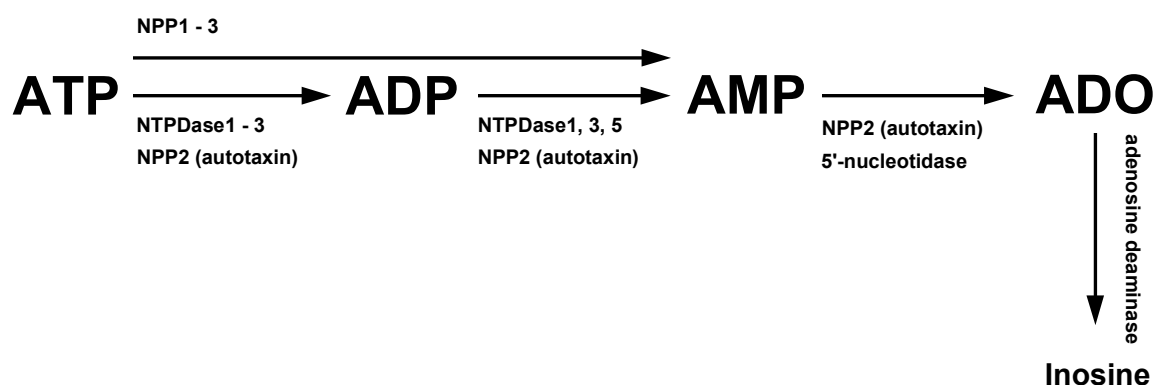
Fig.   1.3.   Extracellular pathways for the degradation of nucleotides.

1.1.5.1. The E-NTPDase family

The E-NTPDase family (ecto-nucleoside triphosphate diphosphohydrolase family) is often also referred to as the ecto-apyrase, NTPase or E-ATPase family.

Members of the E-NTPDase family can hydrolyse extracellular ATP and ADP as well as other nucleoside 5'-di- and triphosphates albeit with varying preference for the individual type of nucleotide. Enzyme activity is activated either by Ca^{2+} or Mg^{2+} and has an alkaline pH optimum. NTPDases are not inhibited by known inhibitors of various intracellular ATPases such as P-type (vanadate), F-type (oligomycin) and V-type ATPases (N-ethyl maleimide) (Ziganshin et al., 1994a; Plesner, 1995; Zimmermann, 1996a,b). The gene family has members not only within vertebrates, related enzymes also exist in invertebrates, plants, yeast and protozoans (Handa and Guidotti, 1996; Zimmermann and Braun, 1999). All of these sequences share a number of highly conserved domains, the "apyrase conserved regions", that presumably are of major relevance for their catalytic activity (Handa and Guidotti, 1996).

According to their presumptive membrane topography members of the E-NTPDase family may be separated into two groups. The first group comprises NTPDase1 - 4 which are highly glycosylated cell-surface located enzymes or integral membrane proteins, respectively, with two putative transmembrane domains, separated by a large extracellular loop, and intracellular N- and C-termini (Maliszewski et al., 1994; Christoforidis et al., 1996). Thus, their membrane topology resembles that of P2X receptors (see figure 1.4.). Enzymatic activity of NTPDase1 was shown to be dependent on homo-oligomeric assembly: although even soluble monomers (mutants lacking both transmembrane regions) were shown to be active, and assembly of mutants into dimers and trimers also results in nucleotide degrading activities, the wild-type NTPDase1 was reported to assemble as a tetramer (Wang et al., 1998).

The lymphocyte surface protein CD39 - now designated NTPDase1 - that has been cloned and sequenced previously (Maliszewski et al., 1994), was shown to be identical to the human ATP diphosphohydrolase (Christoforidis et al., 1995; Kaczmarek et al., 1996; Wang and Guidotti, 1996; Marcus et al., 1997). The enzyme was found to hydrolyse ATP and ADP at a molecular ratio of about 1 : 0.5 to 1 : 0.9. In contrast, NTPDase 2 (CD39L1) revealed high preference for ATP over ADP (1:0.03) hydrolysis and was therefore originally named ecto-ATPase (Chadwick and Frischauf, 1997; Kegel et al., 1997; Kirley, 1997). Another recently sequenced and expressed human enzyme NTPDase3 (HB6) reveals considerable sequence identity to both NTPDase1 and 2. When heterologously expressed it

hydrolyses ATP and ADP at a ratio of about 1 : 0.3 and thus represents a functional intermediate of the other two enzymes (Smith and Kirley, 1998; Zimmermann, 2000). Not all members of this protein family are located to the cell surface, there are soluble forms in plants and protozoa and membrane-bound forms allocated to the Golgi apparatus and to lysosomal/autophagic vacuoles, such as two closely related forms of the NTPDase4 (Zimmermann, 1999, 2000).

The second group comprises the NTPDase5 (CD39L4) and putative NTPdase6 of which only NTPDase5 has been expressed and characterised. It lacks the C-terminal hydrophobic domain and is therefore predicted to have only one transmembrane domain at the N-terminus (Zimmermann, 2000). Soluble nucleo-tidases sequentially degrading ATP to adenosine have been reported to be released from electrically stimulated nerve endings of guinea-pig vas deferens smooth muscle (Todorov et al., 1997; Kennedy et al., 1997). However, the structure of these enzymes is still unknown, a potential candidate may be the recently cloned but functionally as yet unidentified putative NTPDase6 (CD39L2) (Chadwick and Frischauf, 1998; Zimmermann, 1999).
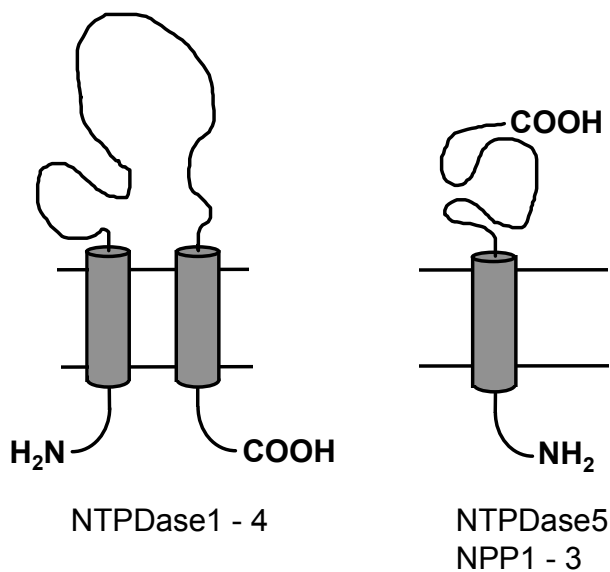
Fig. 1.4. *Presumptive membrane topography of mammalian members of the nucleoside triphosphate diphosphohydrolase- (NTPDase) and of the nucleotide pyrophospha-tase/phosphodiesterase (NPP) family.*

NTPDase1 - 4

NTPDase5
NPP1 - 3

1.1.5.2. The E-NPP family

Members of the ENPP-family (nucleotide pyrophosphatase/phosphodiesterase family) exhibit a broad substrate specificity and possess both, the capacity of hydrolysing phosphodiester bonds of nucleotides and nucleic acids (e.g. hydrolysing cAMP to AMP), and pyrophosphate bonds of nucleotides and

nucleotide sugars, generating pyrophosphate (PP$_i$) from the cleavage of ATP and inorganic phosphate (P$_i$) from ADP (Zimmermann, 1996a; Zimmermann, 1999). Three different types of enzyme (designated NPP1 - 3) have now been cloned from human sources and related sequences were shown to be present in mouse and rat (Zimmermann, 1999). In the current literature, the individual enzymes are referred to as PC-1 (NPP1), PD-Iα, autotaxin (NPP2), which are presumably splice variants of the same NPP2 gene and PD-Iβ, B10 and gp130^{RB13-6} (NPP3), which denominate an identical protein. The membrane glycoprotein PC-1 was the first member of the enzyme family to be identified in molecular terms (van Driel et al., 1985; van Driel and Goding, 1987). Autotaxin, also catalysing the hydrolysis of ATP and GTP to the respective dinucleotides, and AMP to adenosine (a catalytic property shared with ecto-5'-nucleotidase) displays an even wider catalytic capacity than the other members of this family (Clair et al., 1997). Both PC-1 and autotaxin also exist as soluble forms, and the membrane-bound PC-1 protein was reported to occur as a homo-dimer, composed of two subunits consisting of intracellular N-termini, one transmembrane region per subunit and extracellular C-termini (van Driel et al., 1985; van Driel and Goding, 1987). Related sequences have also been identified in plants, yeast and *Caenorhabditis elegans* (Zimmermann and Braun, 1999).

1.1.5.3. Ecto-5'-nucleotidase

Activity of 5'-nucleotidase is present intracellularly and can also be observed at the cell surface. The extracellular form, ecto-5'-nucleotidase is attached to the plasma membrane via a glycosyl-phosphatidylinosotol-anchor and is present in all tissues, although its expression is generally restricted to specific cell types (Zimmermann, 1996a). The protein occurs as a homo-dimer with interchain disulphide bridges. It hydrolyses a variety of nucleoside-5'-monophophates such as AMP, CMP, UMP, IMP and GMP to the respective nucleosides. ATP, ADP and α,β-methylene-ADP (α,β-mADP) act as competitive inhibitors of ecto-5'-nucleotidase (Zimmermann, 1992). Soluble forms of the enzyme exist that at least in part are derived from enzymatic cleavage of the glycosyl-phosphatidylinosotol anchor (Zimmermann, 1992).

1.1.5.4. Alkaline phosphatases

The glycosyl-phosphatidylinosotol-anchored ecto-enzymes alkaline phosphatases are phosphomonoesterases present in essentially all tissues. They are capable of releasing P_i from a large variety of organic molecules including nucleoside 5'-tri-, di- and monophosphates. Its pH optimum is at pH 8.5 and higher, and it is effectively inhibited by free phosphate (Fox, 1978). At least four genes encode alkaline phosphatase isoenzymes in humans, producing a placental, a placental-like, an intestinal isoenzyme and a family of proteins that differ from one another only by postranslational modification and are present throughout the body. A homo-dimeric assembly was described for the enzyme from Escherichia coli (Zimmermann, 1996a). A major question is that of the relative physiological relevance when alkaline phosphatases are co-expressed with other nucleo-tide-degrading enzymes. Because alkaline phosphatases have a considerably higher K_m value for nucleotides and also a much higher pH optimum than members of the NTPDase-family or ecto-5'-nucleotidase, the physiological conditions for the activation of alkaline phosphatase and ecto-nucleotidases may differ (Zimmermann, 1996a).

1.1.5.5. Adenosine deaminase

Adenosine deaminase catalyses the conversion of adenosine and deoxy-adenosine to inosine and deoxyinosine, respectively. It was shown to be present in essentially all tissues (Nagy et al., 1990). The enzyme is mainly cytosolic, but it also occurs on the surface of a variety of cells (Zimmermann, 1996a). Surface-located adenosine deaminase has shown to be associated with a specific binding protein that has been identified as an ecto-peptidase (Kameoka et al., 1993). Furthermore, the association of the enzyme with adenosine A_1 receptors has been described (Ciruela et al., 1996). At present, it is not known how soluble adenosine deaminase can be released from cells; it may be derived from lysed cells or may also be secreted.

1.1.5.6. Nucleoside diphosphokinase

The significance of intracellular nucleoside diphosphokinase (NDPK) in intermediary metabolism, catalysing the transphosphorylation of nucleoside

diphosphates utilising nucleoside triphosphates, is well established (Stoeckler et al., 1978). In contrast, the location and function of NDPK as an extracellular enzyme involved in the transfer of terminal phosphates between extracellular nucleotides has not been addressed until recently, when it was shown to have functional significance in 1321N1 cells (Lazarowski et al., 1997a) and polarised human airway epithelial cells (Lazarowski et al., 1997c). Exogenously applied ATP, as well as ATP released from 1321N1 cells was shown to serve as a γ-phosphate donor for exogenously added nucleoside diphosphates. Thus, observations indicating that UDP acts as an agonist at the P2Y$_4$ receptor expressed in 1321N1 cells likely are due to conversion of UDP to UTP in the presence of released ATP (Lazarowski et al., 1997a). The molecular identity of this extracellular NDPK is not yet clear. Two putative tumour suppressor genes, *nm23*-H1 and *nm23*-H2, have been cloned and shown to encode for proteins (also called NDPK A and NDPK B) that exhibit NDPK activity (Biggs et al., 1990; Kimura et al., 1990). However, whether the ecto-NDPK activity is encoded by this genes has not been determined (Lazarowski et al., 1997a).

1.1.5.7. Inhibitors of ATP degradation

Many types of agents have been used albeit without great specificity or potency as inhibitors of ecto-nucleotidases, i.e. members of the E-NTPDase family, in various animal tissues (for review see Ziganshin et al., 1994a). To date, the ATP analogue ARL 67156 is the only compound displaying a certain specificity for ecto-nucleotidases inhibition over P2 receptor antagonism. ARL 67156 was shown to inhibit ATP degradation in human blood cells (Crack et al., 1995) and the ratVD (Khakh et al., 1995b). ATP-evoked contractions of rabbit ear artery and guinea-pig vas deferens and urinary bladder were enhanced in the presence of ARL 67156, but not responses to the metabolically stable analogue α,β-mATP (Crack et al., 1995; Westfall et al., 1996, 1997). Furthermore, ARL 67156 was reported to potentiate the purinergic component of neurogenic contraction of guinea-pig vas deferens and urinary bladder by up to two-fold (Westfall et al., 1996, 1997). Although, ARL 67156 is a weak P2X receptor antagonist (Crack et al., 1995), if used at 10 - 100 μM, effective inhibition of ecto-nucleotidases should be achieved, with relatively little receptor antagonism. Unfortunately, the compound has not yet been tested in assays applying recombinant, molecularly defined ecto-nucleotidases.

Of the numerous P2 receptor antagonists also displaying inhibitory potency on ATP breakdown (see 1.3.2.), suramin, Evans blue, PPADS, and recently several PPADS analogues have been examined on recombinant ecto-nucleotidases expressed in chinese hamster ovary (CHO) cells (Heine et al., 1997, Hoffmann et al., 2000). Evans blue was shown to be an effective inhibitor of rat NTPDase1, whereas suramin preferentially inhibits rat NTPDase2. PPADS was reported to be the least effective inhibitor, displaying similar activity at both enzymes (Heine et al., 1997). However, studying the inhibitory activity of PPADS analogues selectivity for either NTPDase1 or NTPDase2 was found to be controlled by several structural parameters. In comparison to PPADS inhibitory activity of some of the derivatives was shown to be increased (Hoffmann et al., 2000).

1.1.6. Therapeutic implications

Studies *in vivo* have indicated a number of potential physiological and pathophysiological roles for ATP in smooth muscle as well as neurons and inflammatory cells. Thus, in transgenic mice lacking $P2X_1$ receptors, contraction of the vas deferens in response to sympathetic nerve stimulation is substantially reduced and their fertility impaired by 90 % (Mulryan et al., 2000). It was therefore suggested, that the selective pharmacological blockade of $P2X_1$ receptors should produce a similar effect, and might thus provide means for developing a non-hormonal male contraceptive pill. However, the relative importance of purinergic transmission in the human vas deferens *in vivo* is far from clear. For example, the neurogenic contraction of the rat urinary bladder involves a large purinergic component, while in healthy humans it is almost entirely cholinergic. Thus, there is at present considerable uncertainty in the extrapolation from experimental animals to human clinical practice.

A growing body of evidence suggests that P2X receptors on nerve terminals both in the periphery and spinal dorsal horn are involved in sensory processing and nociception at sites throughout the body including the joints, viscera, and cardiovascular system (see Khakh, 2001). Studies in transgenic mice lacking the $P2X_3$ receptor have shown that formalin-induced pain behaviour is reduced and that their ability to code intensity of non-noxious heat stimulation is absent (Cockayne et al., 2000; Souslova et al., 2000). Interestingly these mice also exhibited a decreased urine voiding frequency and an increased bladder capacity

with a normal intravesical pressure. This suggests an urinary bladder hyporeflexia, whereby the release of ATP on bladder distension no longer excites peripheral primary afferent nerve terminals (Cockayne et al., 2000). It is important to note that $P2X_3$ receptors are not the only detectors of ATP in pain sensation because there is a residual ATP-evoked current in $P2X_3$ knockout mice and a component of ATP-evoked pain that persists *in vivo* (Cockayne et al., 2000; Souslova et al., 2000). The residual response is probably $P2X_2$ receptor-mediated; $P2X_2$ and combined $P2X_2/P2X_3$-receptor knockout mice will be useful to test this hypothesis. Therefore, $P2X_3$ as well as $P2X_2P2X_3$ receptor antagonists might have therapeutic potential as antinociceptive drugs.

Recent studies in a $P2X_7$-deficient mouse have confirmed earlier studies, which suggested that these receptors may play an important role in initiating the processing and release of IL-1β from inflammatory cells (Grahames et al., 1999; Solle et al., 2001). Accordingly, P2 receptor ligands selective for the $P2X_7$ subtype might be useful as anti-inflammatory drugs.

In the urinary bladder of pithed rats PPADS was shown to antagonise the increase in intra-vesical pressure produced by ATP or spinal electrical stimulation (Hegde et al., 1998). In contrast to healthy humans, the contribution of purinergic transmission to the excitatory innervation of the urinary bladder was reported to be enhanced in disease states such as neurogenic bladder, outflow obstruction and interstitial cystitis (Hegde et al. 1998; Bayliss et al., 1999). P2X receptor antagonists may therefore be potentially useful as novel treatment modalities for urge urinary incontinence.

Furthermore, ATP acting as a cotransmitter with noradrenaline (NA) was found to induce vasoconstriction in a great variety of isolated vascular preparations from various species mediated via $P2X_1$-like receptors (Boarder and Hourani, 1998; Lewis et al., 1998; McMillan et al., 1999). Interestingly, in spontaneous hypertensive rats and in a hypertensive rabbit model, a significantly greater cotransmitter role compared with NA has been demonstrated in blood vessels (Burnstock, 1998). If this holds true for human patients suffering from hypertension, P2X receptor antagonists, presumably $P2X_1$ receptor-selective compounds, might have therapeutic potential in pulmonary vasoconstriction and essential hypertension.

P2Y$_1$-like receptors present on pancreatic β-cells were shown to mediate potentiation of glucose-induced insulin secretion (Loubatières-Mariani et al., 1997; Petit et al., 1998). In non-insulin-dependent diabetes (NIDDM) the pancreatic β-cells retain their ability to synthesise and secret insulin, however, their ability to respond to glucose stimulus is diminished. Therefore enzymatically stable P2Y$_1$ receptor agonists which were reported to be highly effective insulin secretagogues in rat isolated pancreas as well as potent agonists at P2Y$_1$ receptors present in turkey erythrocyte membranes (Fischer et al., 1999a), might be useful for the treatment of NIDDM.

The increased resistance to thromboembolism in mice lacking the P2Y$_1$ receptor (Fabre et al., 1999; Léon et al., 1999) suggested that this receptor is a potential target for new antiplatelet drugs. *In vivo* administration of the P2Y$_1$ receptor antagonist MRS 2179 to mice resulted in prolongation of the bleeding time, *ex vivo* inhibition of platelet aggregation to ADP, and resistance to thromboembolism induced by a mixture of collagen and epinephrine or by thromboplastin (Léon et al., 2001). Whether P2Y$_1$ antagonists have therapeutic potential in other thrombosis models remains to be established. Whereas the P2Y$_1$ receptor is mainly involved in shape change and initiation of platelet aggregation, the recently cloned P2Y$_{12}$ (previously referred to as P2T, P2T$_{AC}$ or P2Y$_{ADP}$) is responsible for completion and amplification of the platelet aggregation to ADP and other aggregating agents (see Gachet, 2000). The thienopyridine compounds ticlopidine and clopidogrel are marketed antithrombotic agents that have proved to be efficient in a number of clinical trials evaluating their effects on ischaemic heart diseases, peripheral vascular disease, and stroke (Savi and Herbert, 2000). Similarly, the ATP analogues of the AR-C series, which are potent competitive P2Y$_{12}$ antagonists, were shown to be efficient antithrombotics in animal models, and some of them are in phase II clinical trials for the treatment of acute coronary syndromes (Humphries, 2000).

Cystic fibrosis (CF) is characterised by abnormal fluid transport across many epithelia including airways, pancreas, sweat glands and small intestine. One therapeutic goal is to improve mucociliary clearance, and thereby prevent progressive lung damage caused by infections. The disease is caused by the absence or dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel expressed by epithelial cells, and by an

increase in active Na^+ absorption. One promising treatment is the use of inhaled UTP and amiloride (a K^+-sparing diuretic) (Bennett et al., 1996). UTP (INS-316), by activating $P2Y_2$ receptors in epithelial cells, bypasses the defective Cl^- secretion to activate an alternative Ca^{2+}-dependent Cl^- secretory pathway (Parr et al., 1994). Because extracellular UTP is susceptible to hydrolysis by ecto-nucleotidases and phosphatases present in the airways, stable UTP analogues may have a therapeutic advantage. Therefore, UTPγS and especially several dinucleoside-polyphosphates are the subject of a number of patents claiming their use in lung diseases, CF, and primary ciliary dyskinesia, ear diseases (otitis media) and eye diseases (dry eye) (see Guile et al., 2001; Fujihara et al., 2001). Phase II clinical trials are currently in progress with two compounds, UTP (INS-316) in CF patients and INS-365 (diuridine-tetraphosphate), also as a drug candidate for the treatment of CF and beyond that in dry eye syndrome patients.

$P2Y_2$ receptor antagonists might have therapeutic potential as anti-inflammatory drugs. The majority of compounds described are 4-thiouridine derivatives with a large, semi-planar, hydrophobic aromatic ring system as substituent in 5-position (see Guile et al., 2001).

1.2. Aim of the thesis

P2 receptor pharmacology still lacks specific and selective competitive antagonists to discriminate clearly between receptor subtypes. Although a number of promising compounds have been examined for this purpose, including suramin, PPADS, several histochemical dyes, MRS 2179, Ip_5I and TNP-ATP, none of them can be considered as ideal (Jacobson et al., 1998a; Ralevic and Burnstock, 1998; Lambrecht, 2000; Guile et al., 2001). All compounds are limited in their usefulness by their irreversibility of the antagonism or by the lack of potency, subtype-selectivity and P2 receptor specificity (see 1.1.3.). The ability of most of the antagonists to inhibit ecto-nucleotidases and thereby protect ATP and other metabolically unstable nucleotide agonists from degradation further complicates their use (Humphrey et al., 1995; Kennedy and Leff, 1995). Moreover, studies which systematically analyse structurally related compounds in order to assess structure-activity relationships for a certain series of analogues at P2 receptor subtypes and ecto-nucleotidases, especially for antagonists potentially displaying selectivity for a certain P2X receptor subtype, are almost missing. Definitely one reason for this is the absence of suitable binding assays. To date, only agonist radioligands have been identified for P2 receptors, that have the potential to label binding sites for ATP in tissues and cells in a highly non-specific manner (Humphrey et al., 1995). The usefulness of these radioligands is further compounded by their potential liability. Therefore, a high affinity P2 receptor antagonist either selective for a certain P2 receptor subtype or non-selective would be a valuable pharmacological tool to improve radioligand binding assays, which in turn are critical to the use of high throughput screening to identify novel, non-purine, non-nucleotide pharmacophores.

Subject of this thesis was the pharmacological evaluation of compounds related to the non-selective P2 receptor antagonist suramin (see 1.1.4.2.) with the aim to assess their inhibitory potency at native P2 receptor subtypes and ecto-nucleotidases in relation to suramin and the small urea NF023, which has been characterised as $P2X_1$-receptor-selective antagonist in smooth muscle preparations as well as in studies at cloned P2X receptors (Bültmann et al., 1996c; Lambrecht, 1996; Ziyal et al., 1997; Soto et al., 1999).

To this end, symmetrical small ureas varying in the substitution pattern and substituents of the benzoyl residue/s **(1)**, exchange of benzoyl (I) in large ureas and the benzoyl of small ureas **(2)**, large and small ureas differing in the substitution pattern of the aminonaphthalene sulphonic acid residues combined with varying linkages of the benzoyl residues **(3)**, large and small ureas with modifications of the central urea bridge **(4)**, as well as two small urea derivatives with 7- instead of 6-aminonaphtalene sulphonic acid residues **(5)** (see figure 1.5.) were chosen to be analysed in the present study.
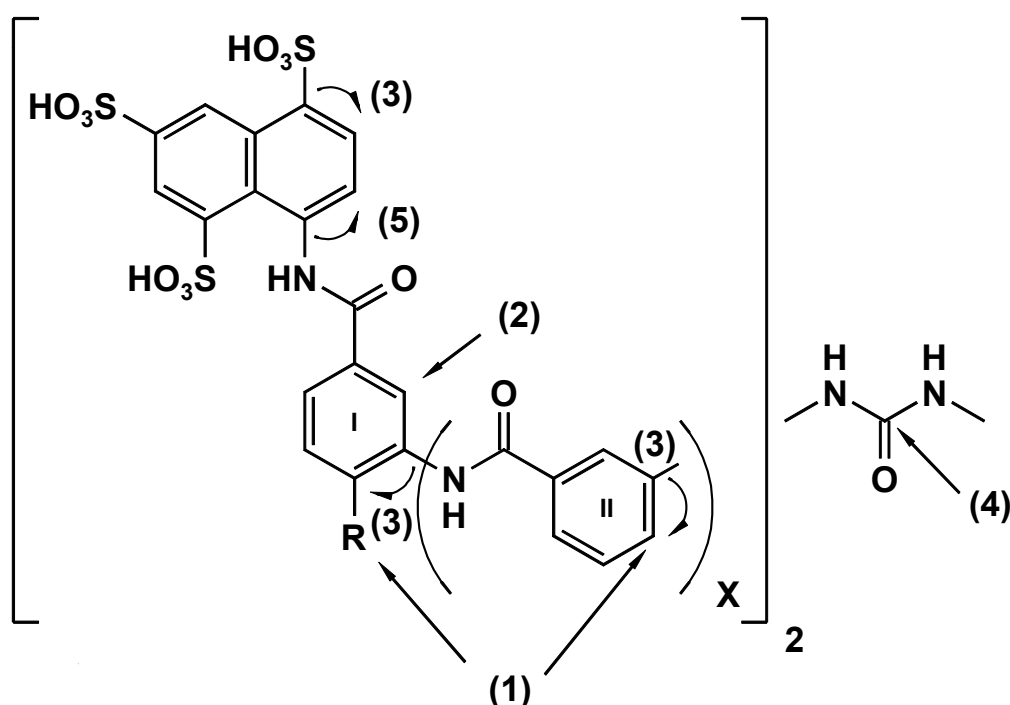
Fig. 1.5. *Structural modifications of small (X = 0) and large (X = 1) ureas related to suramin (R = CH₃,, X = 1) evaluated in the present study.*

To pharmacologically characterise the compounds screening assays had to be developed, in order to examine a larger number of compounds of which only small amounts were available. The following tissues/cells being endowed with different P2 receptor subtypes/ecto-nucleotidases were selected for this purpose:

- rabbit vas deferens (P2X; Windscheif, 1995; Lambrecht et al., 1996b; Ziyal, 1996)
- guinea-pig taenia coli (P2Y; Burnstock and Kennedy, 1985; Cusack, 1993; Dudeck et al., 1995)

- folliculated *Xenopus laevis* oocytes (ecto-nucleotidases; Ziganshin et al., 1995; 1996)

Of the numerous suramin derivatives examined in the screening assays, NF279 (figure 3.13.) displayed an outstanding P2X receptor-blocking potency. Consequently, it was of major interest in this thesis to investigate the P2 receptor subtype selectivity and specificity of this novel antagonist. Moreover, with the aim to identify structural requirements for the interaction with P2 receptors, a series of derivatives representing the missing link between suramin and NF279 as well as the respective small urea analogues, were examined for their inhibitory potencies on native P2X receptors present in rat vas deferens (Khakh et al., 1994; 1995b), guinea-pig ileum (Kennedy and Humphrey, 1994; Czeche et al., 1998b; Lambrecht et al., 2000a) and taenia coli P2Y receptors, and ecto-nucleotidases of folliculated *Xenopus laevis* oocytes.

In vasa deferentia of different species, variation of adrenergic and purinergic transmission with regard to the anatomical origin of the segment (prostatic vs. epididymal) has been reported (Sneddon and Machaly, 1992). In addition, several studies indicated, that contraction of the rat vas deferens induced by either electrical field stimulation or by exogenously applied nucleotides might be mediated via a heterogeneous P2 receptor population (Bültmann and Starke, 1994b, Lambrecht et al., 1996b). Whether these P2 receptors are symmetrically distributed along the length of the rat vas deferens, and as a consequence, prostatic as well as epididymal segments are equally well-suited as pharmacological model for the study of native $P2X_1$-like receptors, was a further subject of this thesis.

2. Materials and Methods

2.1. Drugs and animals

2.1.1. Commercially available drugs

- α,β-Methylene-adenosine-5'-triphosphate dilithium salt (α,β-mATP; Sigma, Deisenhofen, Germany)
- α,β-Methylene-adenosine-5'-diphosphate dilithium salt (α,β-mADP; Sigma, Deisenhofen, Germany)
- β,γ-Methylene-L-adenosine-5'-triphosphate tetrasodium salt (L-β,γ-mATP; Biotrend, Cologne, Germany)
- Adenosine-5'-diphosphate disodium salt (ADP; Sigma, Deisenhofen, Germany)
- Adenosine-5'-monophosphate sodium salt (AMP; Sigma, Deisenhofen, Germany)
- Adenosine-5'-O-(2-thiodiphosphate) dilithium salt (ADPβS; Sigma, Deisenhofen, Germany)
- Adenosine-5'-O-(3-thiotriphosphate) tetralithium salt (ATPγS; Sigma, Deisenhofen, Germany)
- Adenosine-5'-triphosphate disodium salt (ATP; Sigma, Deisenhofen, Germany)
- Ammonium molybdate tetrahydrate (Sigma, Deisenhofen, Germany)
- Carbamoylcholine chloride (carbachol; Sigma, Deisenhofen, Germany)
- 2-Chloroadenosine (2-Cl-Ado; Sigma, Deisenhofen, Germany)
- 2-Chloro-N^6-cyclopentyladenosine (CCPA; Sigma, Deisenhofen, Germany)
- (-)-Cocaine hydrochloride (E. Merck, Darmstadt, Germany)
- 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; Sigma, Deisenhofen, Germany)
- Ethylenediaminetetraacetic acid (EDTA; Sigma, Deisenhofen, Germany)
- Fiske & SubbaRow reducer (formulation of: 1-amino-2-naphthol-4-sulphonic acid sodium sulphite and sodium bisulphite; Sigma, Deisenhofen, Germany)
- Indomethacin (Sigma, Deisenhofen, Germany)
- N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES; Sigma, Deisenhofen, Germany)
- (-)-Nicotine hydrogentartrate (nicotine; Sigma, Deisenhofen, Germany)
- (-)-Noradrenaline hydrochloride (NA; Fluka, Buchs, Switzerland)
- p-Nitrophenylphosphate ammonium salt (PNP; Sigma, Deisenhofen, Germany)
- Pentobarbitone sodium (Nembutal$^®$; Sanofi, Hannover, Germany)
- Suramin (Germanin$^®$, Bayer, Leverkusen, Germany)
- Tetrodotoxin (TTX; Sigma, Deisenhofen, Germany)

- Yohimbine hydrochloride (Sigma, Deisenhofen, Germany)

2.1.2. Gifts

- Adenosine-5'-O-(2-thiodiphosphate) dilithium salt (ADPβS; Dr. H. Woog, Boehringer Mannheim, Mannheim, Germany)
- Arecaidine propargyl ester hydrobromide (APE; synthesised by Dr. U. Moser and Mrs. U. Hermanni, Department of Pharmacology, University of Frankfurt, Frankfurt/Main, Germany)
- 6-N,N-Diethyl-D-β,γ-dibromomethylene-adenosine-5'-triphosphate (ARL 67156, syn. FPL 67156; synthesised by Dr. A. Krayevsky, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia)
- N^6-Methyl-2'-deoxy-adenosine 3',5'-biphosphate (MRS 2179; synthesised by Dr. K. Jacobson and co-workers, National Institutes of Health, Bethesda, Maryland, USA)
- Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; synthesised by Dr. H. G. Bäumert and co-workers, Department of Biochemistry, University of Frankfurt, Frankfurt/Main, Germany)
- All NF compounds examined in this thesis were synthesised by Prof. Dr. P. Nickel and co-workers, Department of Pharmaceutical Chemistry, University of Bonn, Bonn, Germany.

2.1.3. Preparation of stock solutions

In general, drugs were dissolved and diluted in distilled water or in physiological saline (NaCl 0.9 %; w/v). Stock solutions of indomethacin were made up in 96 % ethanol and that of noradrenaline (NA) was stabilised with 0.05 % (w/v) Na$_2$S$_2$O$_5$. In each case, control experiments assured that the final concentration of the vehicle in the bath fluid had no pharmacological effect itself.

2.1.4. Buffer solutions

All components of the bath fluids were commercially available chemicals of analytical grade purity.

Table 2.1.   Composition of bath fluids used in pharmacological experiments on rabbit vas deferens (RVD), guinea-pig taenia coli (GPTC) and rat vas deferens (ratVD) as well as in studies on ATP breakdown in GPTC and *Xenopus laevis* oocytes. The solution oocyte I was used for the storage of oocytes, experiments were performed in oocyte II buffer.

| | Concentration (mM) of buffer components | | | | | |
| | Contraction/ relaxation experiments in smooth muscle preparations | | | Ecto-nucleotidase bioassay | | |
	RVD	GPTC	RatVD	GPTC	Oocytes I (storage)	Oocytes II (exp.)
$CaCl_2$	1.0	2.5	1.3	2.0	1.0	1.8
KCl	4.7	4.7	4.7	5.0	1.0	2.5
KH_2PO_4	1.2		1.2			
$MgCl_2$				2.0	1.0	1.8
$MgSO_4$	0.6	0.6	0.6			
NaCl	118.1	133.5	118.0	135.0	90.0	110.0
$NaHCO_3$	25.0	16.3	25.0			
NaH_2PO_4		0.8				
HEPES					10.0	5.0	5.0
Glucose	11.2	7.8	11.1	10.0		

2.1.5.   Animals

All laboratory animals were housed in an air-conditioned room with a temperature of 23°C, a relative atmospheric humidity of 50 % and an artificial 12-hour light-dark cycle. They were fed a standard diet (rabbits, guinea-pigs: Alltromin[®] 3022, rats: Alltromin[®] 1324) and had free access to food and tap water.

New Zealand white rabbits were purchased from Sawo-Iwanowas (Kissleg/Allgäu, Germany) and Koch (Edingen-Neckarhausen, Germany). Dunkin-Hartley guinea-pigs and wistar rats were delivered by Harlan Winkelmann, Borchen, Germany.

2.2. Methods

2.2.1. Rabbit vas deferens

Preparation. Male New Zealand white rabbits (2.5 - 3.0 kg) were killed by an overdose (120 mg/kg) of pentobarbitone sodium (Nembutal[®]) injected into the ear vein. According to the method described by Eltze (1988), the abdomen was opened and the vasa deferentia were excised from the epididymides to the prostate and cleared of connective tissue. Each vas deferens was divided into six segments of approximately 1.0 cm length. The segments were vertically suspended in 6 ml organ baths containing a modified Krebs solution (for composition see table 2.1.; pH 7.4; 31°C; aerated with 95 % O_2/5 % CO_2). Additionally, yohimbine (1 μM) was added to the buffer in order to block α_2 adrenoceptors. Tissues were loaded with 375 mg tension and were allowed to equilibrate for 30 min with a change of the bath fluid every 10 min before starting the experiment. Mechanical activity was measured isometrically via a force displacement transducer (TF6V5) connected to a DMS DC amplifier (both from W. Fleck, Mainz, Germany), and was registered on a Rikadenki pen recorder (Hellige, Freiburg, Germany).

Agonist studies. Given that two consecutive applications of exogenously applied α,β-methylene ATP (α,β-mATP), at a concentration producing approximately half-maximal effect (1 μM), resulted in a stable response, a concentration-response curve (CRC) was constructed by applying increasing doses of the agonist. Single doses of α,β-mATP were added to the bath fluid with a time interval of 30 min, and had an average contact time of about 20 s. In time-matched control experiments these precautions had been established to be necessary to avoid desensitisation (Ziyal, 1996).

Antagonist studies. After contractions to single doses of exogenously applied α,β-mATP (1 μM), added in 30-min intervals, resulted in a stable response the antagonist was incubated for 60 - 90 min with time inversely related to the concentration tested; suramin and NF023 (< 3 μM) for 90 min, higher concentrations of suramin and its analogues for 60 min. Two antagonist concentrations or one concentration of two different antagonists, respectively, were investigated in a single preparation.

The bath fluid was generally changed approximately every 15 min throughout the experiments. During the equilibration time of antagonists, buffer was replaced according to the above mentioned washing procedure with an immediate readministration of antagonist. When two antagonist concentrations or two different antagonists, respectively, were tested in a single tissue, a washout period of at least 1 h preceded the second incubation of antagonists. One preparation always served as a time-dependent control to assure that during the course of the experiments, there was no change in tissue sensitivity towards single doses of α,β-mATP.

Unless stated otherwise, all experiments were carried out at least three times on preparations from different animals.

2.2.1.1. Data analysis

Concentration-effect relationships presented in this thesis were plotted graphically in the form of $\log_{10}$ concentration of agonist/antagonist versus effect. CRC's were constructed employing computerised curve fitting techniques using a 4 parameter logistic equation on Graphpad software (Version 2.01, Graphpad Software Inc., 1996) based on the logistic function introduced by Parker and Waud (1971).

(1) $E_A = E_{max} [A]^p/([A]^p + EC_{50}^p)$

E_A	=	effect of a given concentration [A] of agonist/ antagonist
E_{max}	=	maximum effect
[A]	=	agonist/antagonist concentration
p	=	slope of the CRC
EC_{50}/IC_{50}	=	concentration of agonist/antagonist producing 50 % of the individual maximum effect

Agonist potencies. Contractions induced by exogenously applied α,β-mATP were quantified in mg tension. Agonist potencies were determined graphically (effect vs. $\log_{10}$ concentration) and expressed as pD_2 values (= -log EC_{50}, i.e. the molar concentration producing 50 % of the individual maximum effect).

(2) $pD_2 = -log\ EC_{50}$

Antagonist potencies. To determine the inhibitory potency of suramin and NF023, IC_{50} values ($= IC_{50}$; i.e. the molar concentration producing 50 % inhibition of the response to a single dose of 1 μM α,β-mATP) were derived from non-linear regression analysis of semilogarithmic concentration-inhibition (% inhibition vs. $\log_{10}$ concentration of antagonist) curves by fitting the logistic function (1) to the pooled data points. The pIC_{50} values were calculated from:

(3) $\qquad pIC_{50} = -\log IC_{50}$

In screening studies performed for the evaluation of the antagonistic properties of small and large ureas, the inhibitory effect of a single dose of the respective derivative (10 μM), on contractions evoked by α,β-mATP (1 μM), was investigated and expressed as inhibition (%):

(4) $\qquad \% \text{ inhibition} = 100 - \dfrac{E_V \times 100}{E_C}$

E_C being the observed effect in controls and E_V the effect observed in the presence of antagonist.

2.2.2. Guinea-pig taenia coli

Preparation. Male Dunkin Hartley guinea-pigs, 250 - 500 g, were killed by a blow to the neck and exsanguination. The taenia was dissected free without opening the caecum and cleared of connective tissue. Segments 1.5 - 2.0 cm long were suspended vertically in 6 ml organ baths containing modified Krebs solution (for composition see table 2.1., 37°C, gassed with 95 % O_2/5 % CO_2). After preloading the taenia strips with 1 g tension they were allowed to equilibrate for 45 - 60 min with a change of bath fluid every 15 min. Mechanical activity was recorded isometrically by a force displacement transducer (TF6V5) connected to a DMS DC amplifier (both from W. Fleck, Mainz, Germany), and was registered on a Rikadenki pen recorder (Hellige, Freiburg, Germany).

Experimental design. After a cumulative concentration-contraction curve to carbachol had been constructed in order to determine the maximal contraction of each strip, a standard tone was induced by a concentration of carbachol giving

2. Materials and Methods

about 50 % of the maximal effect (0.1 μM, occasionally up to 0.3 μM) every 15 min. When the carbachol contractions reached a plateau and single doses of the relaxant agonist ADPβS resulted in stable responses, two cumulative concentration-relaxation curves to ADPβS were determined on each preparation, the first before, the second 60 - 120 min after addition of the antagonists. On several preparations a second concentration of antagonist was incubated and a third CRC was constructed.

Control experiments (same protocol, no incubation of antagonist) were carried out on one preparation per animal. These experiments were considered as time-matched controls.

2.2.2.1. Data analysis

Relaxant responses to exogenously applied ADPβS were expressed as a percentage of the carbachol pre-contraction, and plotted against agonist concentrations in a semilogarithmic manner. EC_{50} values were determined by fitting the logistic function (1) to the pooled data, and pD_2 values were calculated as described above (cf. 2.2.1.1.).

Antagonist potencies. The potencies of antagonists were calculated from the respective rightward shift of the CRC assessed in the presence of antagonist. In that case where only one or two concentrations of an antagonist were tested, its pA_2 value was determined from the individual dose ratios according to the following equation (Tallarida et al., 1979):

(5) $pA_2 = \log (DR-1) + pA_X$

   $pA_X = -\log [\text{Antagonist}]$
   DR = dose ratio, quotient of the agonist's EC_{50} values in the presence
          and in the absence of antagonist

In cases where three or more antagonist concentrations were investigated, pA_2 values were calculated by Arunlakshana-Schild analysis (Arunlakshana and Schild, 1959). Slopes were obtained by linear regression of the experimental data to the following equation using least squares fit:

(6) $\log (DR-1) = m\, pA_X - \log K_D$

        m = slope of the regression line
        K_D = antagonist dissociation constant

If the slope m was found not significantly different from -1.00 (p > 0.05), the antagonism was regarded as purely competitive. The pA_2 value (= -log K_D) was then calculated as the x-intercept of a regression line with unit slope (m = -1.00, "constrained plot") (Tallarida et al., 1979; Tallarida and Murray, 1986).

2.2.3.   Ecto-nucleotidases bioassay

2.2.3.1.   Studies on smooth muscle tissues

Preparation. Guinea-pig taenia coli strips were prepared as described for the organ baths experiments (cf. 2.2.2.) and stored in physiological saline (0.9 % NaCl; w/v).

Experimental protocol. Single pieces of GPTC (2 - 4 mg) were placed in 24-well cell culture dishes filled with 250 μl HEPES-buffer (table 2.1.) and were pre-washed for 15 - 20 min. The pre-wash buffer was changed for 250 μl buffer containing ATP (0.1 or 1 mM, respectively) as substrate, and the tissues were incubated (0.1 mM ATP for 30 min, 1 mM ATP for 15 min) and shaken continuously (first incubation). Incubation was terminated by removing the buffer and adding it to 1 ml of a 2.5 % (w/v) solution of sodium dodecyl sulphate (SDS) in order to avoid further breakdown of ATP by soluble enzymes. Tissues were washed again for 10 - 15 min and then incubated with 200 μl buffer containing a given concentration of a compound, tested for its inhibitory potency, for 30 min (pre-incubation). 50 μl buffer containing ATP (0.5 or 5 mM, giving a final concentration of 0.1 or 1 mM, respectively) and the respective compound (concentration similar to that used for pre-incubation) were added and incubated for 30 or 15 min, respectively (second incubation). When substrate specificity was tested, tissues were incubated with 1 mM of the respective substrate for 15 min after the first incubation with ATP (1 mM, 15-min incubation) and the washing procedures described above. The buffer was again collected in SDS. For the inorganic phosphate (P_i) assay, 1 ml of 1.25 % (w/v) ammonium molybdate

solution in 2 M HCl and 0.1 ml of 16 % (w/v) Fiske and SubbaRow reducing agent (Fiske and SubbaRow, 1925) were added to the samples. The solutions were transferred to cuvettes and left for 30 min at room temperature to develop colour. The blue reaction product for P_i was measured spectrophotometrically at 700 nm in a Hitachi U-2000 spectrophotometer. KH_2PO_4 was used as a phosphate standard.

2.2.3.2. Studies on folliculated *Xenopus laevis* oocytes

Preparation. Ovarian lobes were removed surgically from *Xenopus laevis* females anaesthetised with tricaine (0.2 % w/v) solution and oocytes were separated mechanically from the inner ovarian epithelial layer. Folliculated oocytes were stored at 19 °C for up to 5 days in oocyte ringer solution (ORI) (table 2.1., Oocyte I).

Experimental protocol. Ecto-nucleotidases (ectoN) assays were carried out at 20 ± 1 °C in a modified Ringer buffer solution. Oocytes were placed in 24-well dishes, typically three oocytes per well, in 250 μl buffer solution (table 2.1., Ooycte II). The further experimental protocol was similar to that described above (cf. 2.2.3.1.; Ziganshin et al. 1995). Compounds investigated for their inhibitory properties were pre-incubated for 30 min. When oocytes were broken by handling during assays, these results were discarded and experiments were repeated.

2.2.3.3. Data analysis

The amount of phosphate produced in the presence of a compound tested (second incubation) was calculated as a percentage of that produced in the first incubation. Unless stated otherwise, results were obtained from at least two experiments performed in quadruplicate. Tissues incubated with buffer containing ATP (100 μM) in the first as well as in the second incubation served as time-matched controls in order to determine whether there was a change in enzymatic activity during the time course of the experiment. Background levels of P_i, by contamination with free P_i and the spontaneous breakdown of ATP over the time course of the experiment (without cells), were measured and subtracted to determine the production of P_i by enzymatic activity alone.

2.2.4. Rat vas deferens

Preparation. Wistar rats were killed by asphyxiation with carbon dioxide. Vasa deferentia were removed and freed from connective tissue and adhering fat. Tissues were bisected transversely such that each vas gave two equal preparations, one prostatic and one epididymal, i.e. four preparations per animal. The preparations were mounted vertically in 6 ml organ bath under 750 mg resting tension and immersed in modified Krebs buffer (for composition see table 2.1., pH 7.4, 37°C, aerated with 95 % O_2/5 % CO_2) containing indomethacin (6.0 μM). Tension changes were measured isometrically, via a force displacement transducer (TF6V5) connected to a DMS DC amplifier (both from W. Fleck, Mainz, Germany), and the recordings were displayed on a Rikadenki pen recorder (Hellige, Freiburg, Germany). The tissues were allowed to equilibrate for 30 min, with a change of bath fluid every 10 min. Tension was maintained at 750 mg for the duration of the experiments.

Agonist studies. When consecutive applications of exogenous agonist resulted in a stable response, single dose CRC's were constructed by applying increasing doses of α,β-mATP (0.1 - 300 μM), adenosine-5'-O-(3-thiotriphosphate) (ATPγS; 0.3 - 100 μM), β,γ-methylene-L-ATP (L-β,γ-mATP; 1 - 100 μM) and ADPβS (1 - 300 μM) at 15- to 60-min intervals. Tissues were washed immediately after the responses had peaked (average contact time of approximately 20 s). Studying desensitisation of P2X receptors, 2 to 5 cumulative doses of α,β-mATP (10 μM), L-β,γ-mATP (30 μM) or ADPβS (30 μM), respectively, were applied at 2-min intervals.

Antagonist studies. Contractions were obtained to single doses of α,β-mATP (10 μM), ATPγS (10 μM), L-β,γ-mATP (30 μM) or ADPβS (30 μM), respectively, added in 30-min intervals, in the case of IC_{50} determination, or single dose CRC's of α,β-mATP (15- to 60-min intervals) as well as CRC's to ADPβS (15-min intervals for single doses) were constructed, in the absence and in the presence of antagonists (60 to 120 min exposure). One or two antagonist concentrations were investigated in a single preparation, with a 1-h washout period preceding the incubation of the second antagonist concentration (usually one order of magnitude higher than the first).

The bath fluid was generally changed every 15 min throughout the experiments. During equilibration time of antagonists, buffer was replaced followed by an immediate readministration of antagonist. One preparation (same protocol, no incubation of antagonist) always served as time-dependent control.

2.2.4.1. Data analysis

Analysis of concentration-effect relationships established in ratVD was complicated by the fact that none of the agonists' CRC's did reach a well-defined maximum and, as a consequence, estimation of agonist potency on the basis of the EC_{50} level was impossible.

Agonist potencies. Contractions induced by exogenously applied agonists were quantified in mg tension. Agonist potencies were determined graphically (effect vs. $\log_{10}$ concentration) as pEC_{1000} and pEC_{500} values (i.e. the concentration of agonist developing 1000 mg and 500 mg tension, respectively.).

Antagonist potencies. To determine the inhibitory potency of suramin and the suramin-derived large ureas as well as of NF023 and its analogues, IC_{50} values (i.e. the molar concentration of antagonist producing 50 % inhibition of the response to a single dose of agonist) were derived from non-linear regression analysis of the semilogarithmic concentration-inhibition curves (% inhibition vs. $\log_{10}$ concentration of antagonist) employing computerised curve-fitting techniques based on the 4 parameter logistic function (cf. 2.2.1.1.). pA_2 values were calculated according to equation (5) from dose ratios determined at an appropriate portion of the CRC, obtained in the presence of antagonist, which was parallel to that of the control curve. This was the 1000-mg tension level for analysis of CRC's to α,β-mATP and the 500-mg tension level for CRC's to ADPβS, respectively. Apparent pA_2 values (pA_2*) were calculated according to the equation introduced by Cheng and Prusoff (1973). In those cases where IC_{50} values have been determined using α,β-mATP as agonist, an EC_{50} of 1 μM has been taken for calculation.

(7) pA_2* = IC_{50}/(1+[A]/EC_{50})

      [A]   =   agonist concentration (α,β-mATP 10 μM)

      EC_{50} =   concentration of α,β-mATP producing 50 % of the maximum
                         effect (1 μM)

2.2.5. Statistics

Data are presented as mean $\pm$ S.E.M. with n indicating the number of experiments. Differences between mean values were tested for statistical significance by Student's t-test; $p < 0.05$ was accepted as being significant. Regression lines were calculated using least-squares fit. Calculations were performed on a personal computer employing the programs Pharm/PCS (version 4.1, Pharmacological Calculation System; Tallarida and Murray, 1986) as well as GraphPad Prism (version 2.01, GraphPad Software Inc.).

3. Results

3.1. Screening of suramin- and NF023-related compounds

3.1.1. Rabbit vas deferens

3.1.1.1. Concentration-response curves to α,β-methylene ATP

In order to compare the potency of α,β-methylene ATP (α,β-mATP) and the extent of agonist-induced contraction with regard to the anatomical origin of the tissue (numbering 1 - 6 from prostate to epididymides), single dose CRC's to exogenously applied α,β-mATP (30-min intervals) were constructed. α,β-mATP caused concentration-dependent transient contractions in all segments of rabbit vas deferens (RVD) (figure 3.1.).
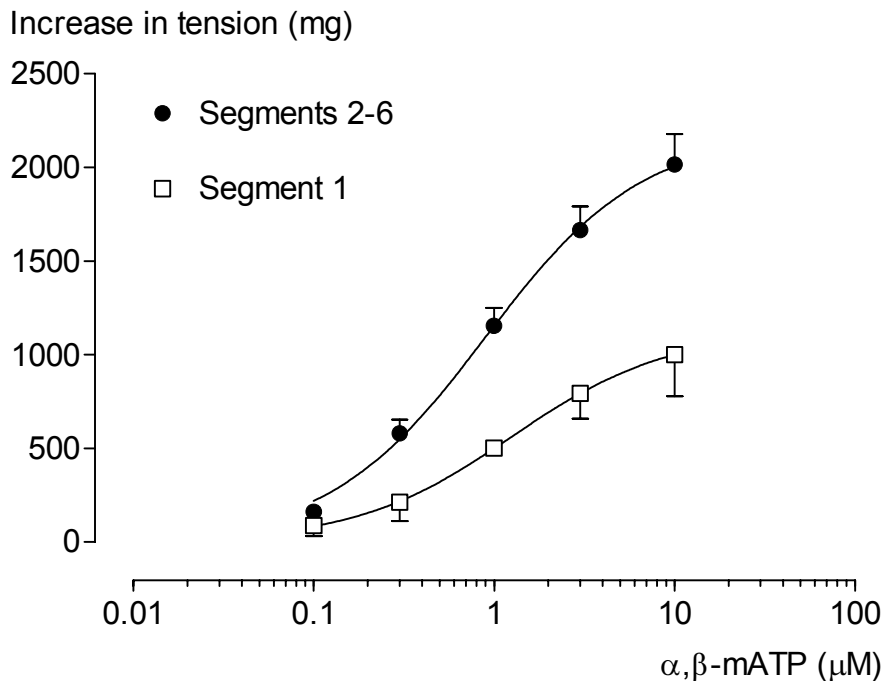
Fig. 3.1. *Concentration-response curves for contractile responses of rabbit vas deferens obtained to single doses of α,β-mATP (30-min intervals) with regard to the anatomical origin of the tissue (numbering 1 - 6 from prostate to epididymides). Data shown are means +/- S.E.M. with n = 3 - 20 for each point. Some error bars were within the width of the symbols.*

At a concentration of 1 μM, segments 2 - 4 generated similar tension, and although segments 1, 5 and 6 were less capable of developing tension, a statistically significant difference was only found for segment 1 (table 3.1.).

Table 3.1. Development of tension (mg) in rabbit vas deferens challenged by exogenous α,β-mATP (1 μM) depending on the segment (1 - 6, counting from the prostate to the epididymides). Data are presented as means $\pm$ S.E.M. from 3 to 4 observations.

Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6
501 ± 31	1340 ± 174	1370 ± 239	1256 ± 219	946 ± 175	865 ± 211

In contrast to the tension generated, differences in the agonist potency of α,β-mATP in RVD were only poor, pD_2 values in segments 2 - 4 being slightly higher than in segments 1, 5 and 6, respectively. Segments 2 - 6 were generally chosen for further investigations on P2X receptors present in RVD (table 3.2.). The overall pD_2 value calculated for α,β-mATP was 6.05 ± 0.13 for segments 2 - 6.

Table 3.2. Potency of α,β-mATP (pD_2 values; mean $\pm$ S.E.M.) in inducing contraction of rabbit vas deferens depending on the segment used (1 - 6, counting from the prostate to the epididymides).

Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6
5.93 ± 0.02	6.12 ± 0.04	6.25 ± 0.09	6.13 ± 0.08	5.91 ± 0.06	5.85 ± 0.08

3.1.1.2. Inhibitory potency of suramin and NF023

In RVD, contractions elicited by α,β-mATP (1 μM) were concentration-dependently blocked by suramin (5, 10, 30, 50 and 100 μM) and NF023 (1, 3, 10 and 30 μM). Analysis of the resulting concentration-inhibition curves (CIC's) (figure 3.2.) yielded pIC_{50} values of 4.75 ± 0.03 for suramin and 4.97 ± 0.03 for NF023.

Inhibition of contraction (%)
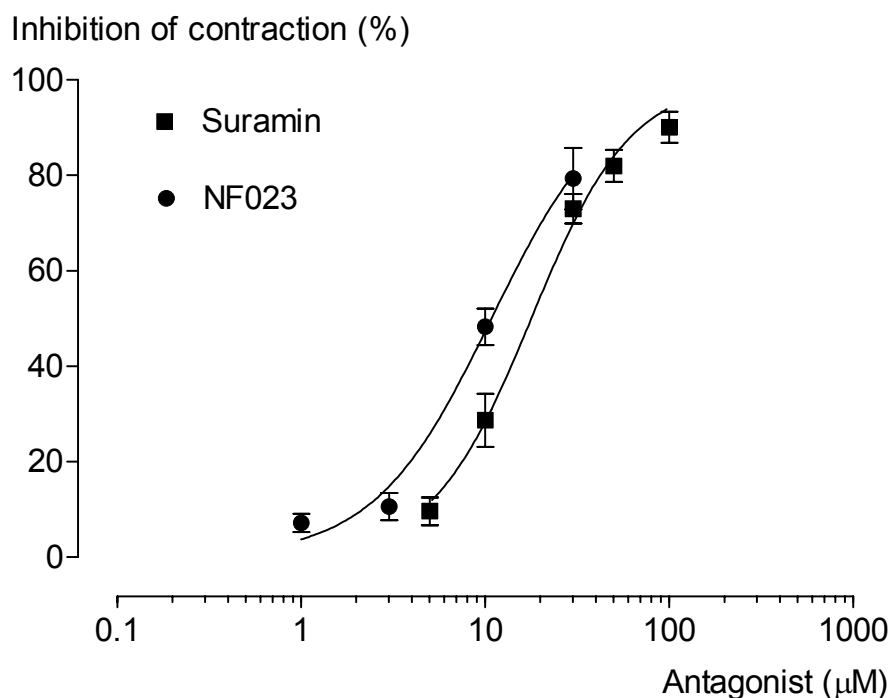
Fig. 3.2. *Inhibitory potency of suramin and NF023 on contractions of rabbit vas deferens induced by α,β-mATP (1 μM). Concentration-inhibition curves for increasing concentrations of antagonists are shown. Data presented are means $\pm$ S.E.M. (n = 3 - 15).*

3.1.1.3. P2X receptor-screening of suramin- and NF023-related compounds

Effects of suramin analogues (10 μM) on contractions of RVD elicited by α,β-mATP (1 μM) were investigated. When responses to α,β-mATP were reduced in the presence of a derivative, the extent of reduction was expressed as inhibition (%) of contraction obtained in the respective control experiment. Results are presented in combination with screening results established in guinea-pig taenia coli and in the ecto-nucleotidases bioassay (cf. 3.1.4.).

3.1.2. Guinea-pig taenia coli

3.1.2.1. Concentration-response curves to carbachol

The potency of the muscarinic agonist carbachol to evoke contraction of guinea-pig taenia coli (GPTC) was investigated in order to determine the appropriate concentration (giving about 50 % of the maximum effect) required to maintain a constant pre-contraction of the tissue. Carbachol, applied in a cumulative fashion, produced concentration-dependent contraction of GPTC yielding a pD_2 value of 6.84 ± 0.04 (figure 3.3.). The following relaxation-experiments were carried out at a standard tone induced by 0.1 - 0.3 μM carbachol.
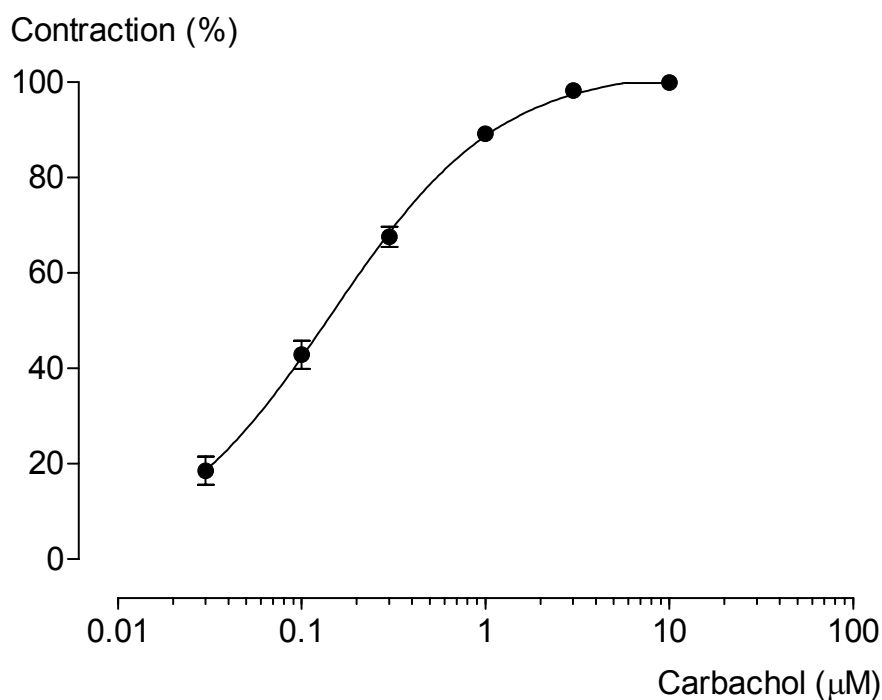
Fig. 3.3. *Concentration-response curve for contractions induced by carbachol in guinea-pig taenia coli. All data points are means $\pm$ S.E.M. with n = 11 - 15 for each point. Some error bars were within the width of the symbols.*

3.1.2.2. Inhibitory potency of suramin and NF023

The adenine nucleotide adenosine-5'-O-2-thiodiphosphate (ADPβS) caused concentration-dependent relaxation of the carbachol-pre-contracted GPTC ($pD_2 = 6.36 \pm 0.05$, n = 14). Increasing concentrations of suramin and NF023 shifted the curve progressively to the right without changing the maximum (figure 3.4. A. and B.). For suramin (30 - 300 μM), the regression line according to Arunlakshana and Schild (1959) was linear with a slope of -0.81 $\pm$ 0.14 (not significantly different from -1.00), resulting in a pA_2 value of 4.45 $\pm$ 0.06 (constrained plot). pA_2 values calculated for NF023 decreased with increasing concentrations of the antagonist (pA_2 values of 4.72 $\pm$ 0.09, 4.40 $\pm$ 0.03 and 4.07 $\pm$ 0.14 for 30, 100 and 300 μM, respectively). In accord with this deviation from competitive antagonism, the plot according to Arunlakshana and Schild was linear with a slope (-0.35 $\pm$ 0.13) significantly different from unity.

3.1.2.3. P2Y receptor-screening of suramin- and NF023-related compounds

Effects of suramin analogues (10 μM) on concentration-relaxation curves to ADPβS in GPTC were investigated. When CRC's to ADPβS were shifted to the right in the presence of a derivative, its antagonistic potency was calculated from the individual dose ratios and expressed as pA_2 value (for results see section 3.1.4.).

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*Fig. 3.4. (page 95) Antagonism of ADPβS-induced relaxation of carbachol-pre-contracted guinea-pig taenia coli by suramin (**A.**) and NF023 (**B.**). Concentration-response curves for ADPβS in the absence and in the presence of increasing concentrations of the antagonists are shown. All points are means +/- S.E.M. with n = 3 - 13 for each point. Some error bars were within the width of the symbols. For suramin, Schild analysis (A., shown in the insert) yielded a slope of -0.81 ±0.14 (not significantly different from -1.00), and a pA2 value (constrained plot) of 4.45 ±0.06 was estimated.*

A.

Relaxation (%)

B.

Relaxation (%)
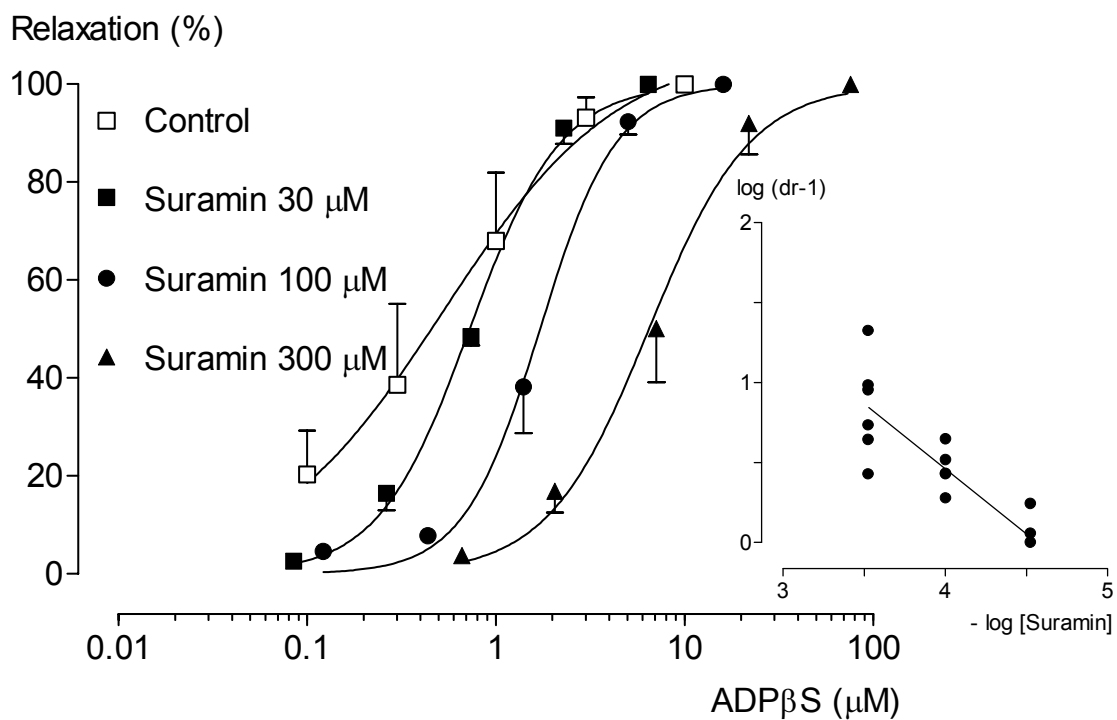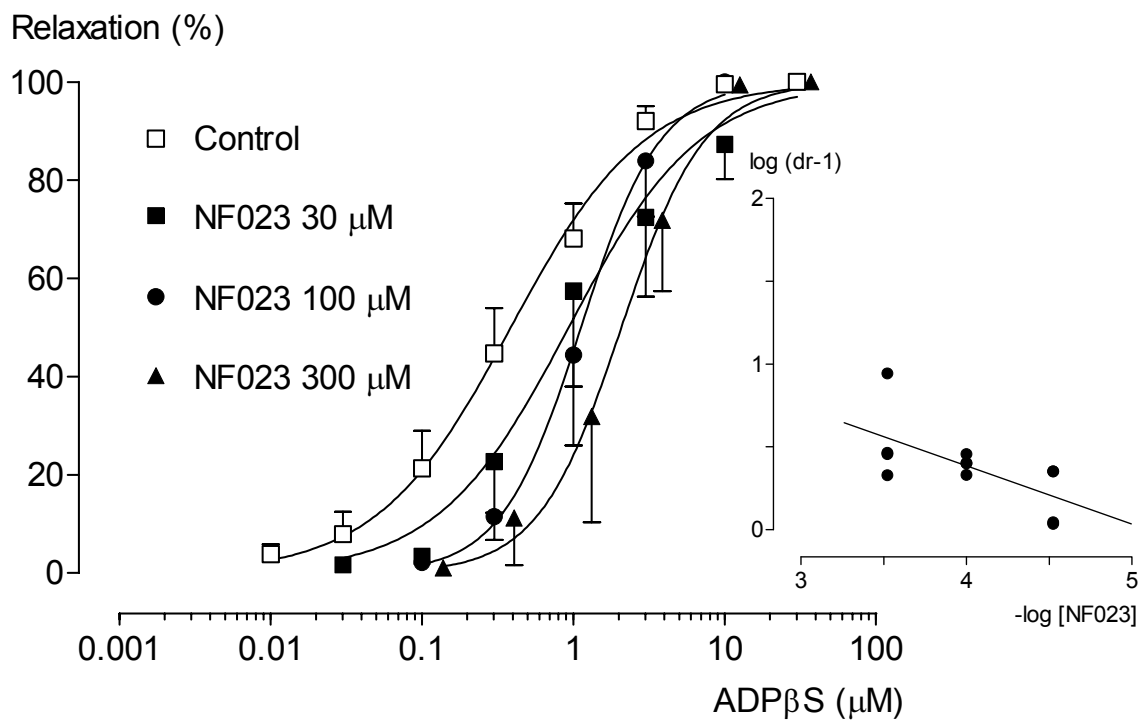
3.1.3. Ecto-nucleotidases bioassay

3.1.3.1. Studies on smooth muscle tissues

3.1.3.1.1. Characterisation of nucleotide-degrading enzymes in guinea-pig taenia coli

Spontaneous ATP breakdown, when incubated in buffer without tissue, as well as the amount of inorganic phosphate (P_i) released from the tissue were negligible (data not shown). Resting ATPase activity was equivalent to 25.2 ± 0.6 nmol P_i mg^{-1} wet tissue (n = 32) in Ringer buffer when ATP (1 mM) was present for 15 min. Over a 15-min incubation period, an average amount of 3.5 mg tissue per well metabolised 35.3 ± 0.8 % of the total amount of ATP added. The rate of hydrolysis of ATP (1 mM) by GPTC was linear for incubation periods up to 30 min duration (figure 3.5.).
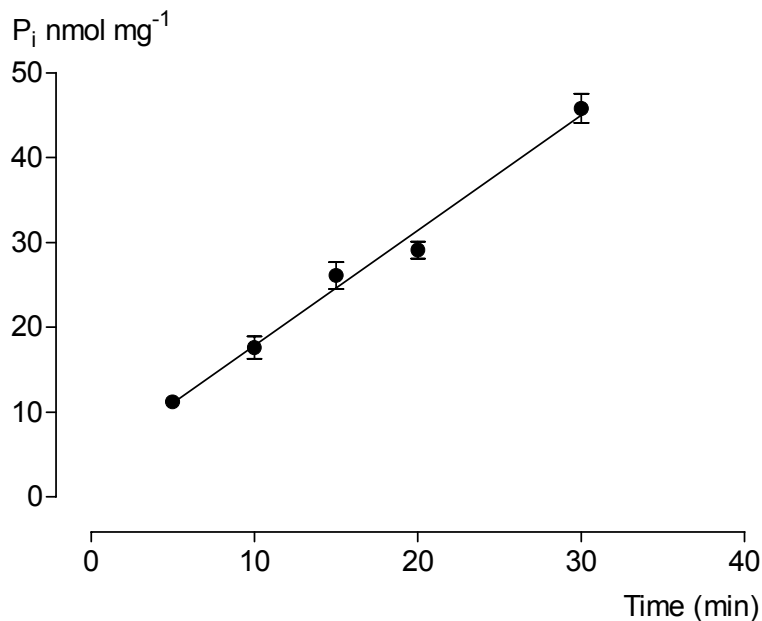
Fig. 3.5. *Time course of ATP (1 mM) hydrolysis determined by the formation of inorganic phosphate (P_i). Data are shown as means $\pm$ S.E.M. (n = 8 - 28). Some error bars were within the width of the symbols.*

The extent of degradation (P_i formation) of ATP, ADP and AMP, used as substrates, at a concentration of 1 mM was: ATP (taken as 100 %) > ADP (44.6 ± 1.7 %, n = 20) $\approx$ AMP (40.2 ± 10.0 %, n = 20) (figure 3.6. A.). Omission of the divalent cations Ca^{2+} and Mg^{2+} (in the presence of 1 mM NaEDTA) diminished hydrolysis of ATP, ADP and AMP to 27.8 ± 1.8 %, 15.6 ± 2.4 % and 16.1 ± 7.0 %,

respectively, (n = 4 - 28) of the respective controls in the presence of Ca^{2+} and Mg^{2+} (figure 3.6. B.).
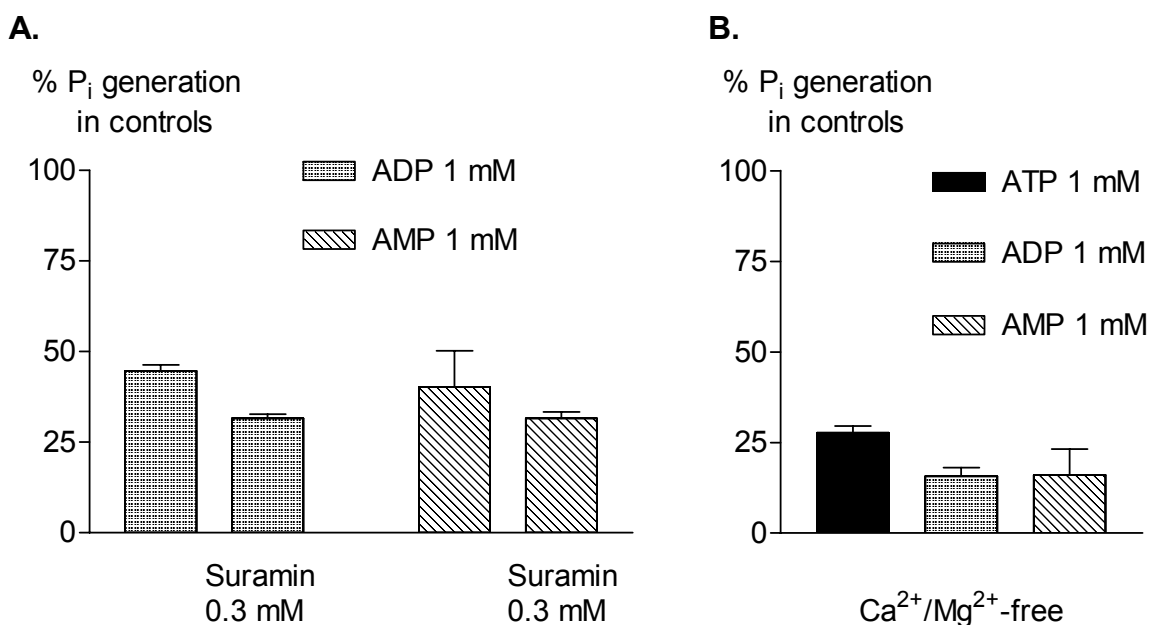
Fig. 3.6. *A. Degradation of ADP and AMP (1 mM, incubated for 15 min) by strips of guinea-pig taenia coli and inhibitory effect of suramin (0.3 mM, 30-min pre-incubation) on the hydrolysis of the nucleotides, respectively. Data are presented as mean percentages of the amount of inorganic phosphate (P_i) produced in the presence of ATP (1 mM, incubated for 15 min) + S.E.M. (n = 8 - 20). B. Degradation of ATP, ADP and AMP in the absence of the divalent cations Ca^{2+} and Mg^{2+} in GPTC (buffer containing 1 mM NaEDTA). Data shown are percentages (mean + S.E.M.) of control incubations in the presence of the divalent cations (n = 4 - 28).*

p-Nitrophenylphosphate (0.5 mM, 15 min), a substrate for the non-specific alkaline phosphatases, was hardly dephosphorylated by GPTC (4.1 ± 0.3 % of P_i formation in controls; n = 12). Furthermore, the 5'-nucleotidase inhibitor α,β-methylene ADP (100 μM) did not affect ATP hydrolysis (100.4 ± 6.0 % of P_i formation in controls; n = 16).

3.1.3.1.2. Inhibitory effects of suramin on nucleotide-degrading enzymes present in guinea-pig taenia coli

Tissues were incubated with ATP in the absence and in the presence of suramin (0.1 and 0.3 mM). In contrast to results obtained for the lower ATP concentration (0.1 mM; 30-min incubation), suramin displayed a concentration-dependent inhibition of P_i generation when degradation of a higher concentration of ATP (1 mM; 15-min incubation) was examined (figure 3.7.).
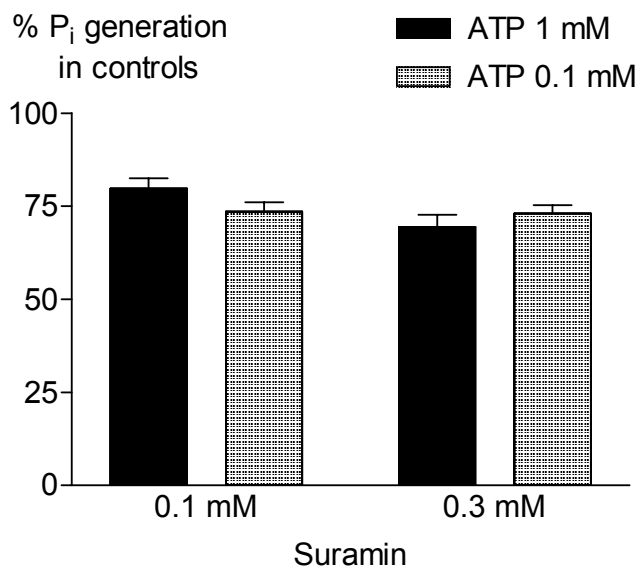
Fig. 3.7. *Inhibitory effect of suramin on degradation of ATP (0.1 mM, incubated for 30 min, and 1 mM, incubated for 15 min) in guinea-pig taenia coli. Data are presented as percentages (means + S.E.M.) of formation of inorganic phosphate (P_i) in the respective control incubations (n = 8 - 30).*

Nevertheless, the extent of inhibition by suramin was considerably low for ATP 0.1 mM (73.6 $\pm$ 2.5 % for 0.1 mM and 73.0 $\pm$ 2.3 % of P_i generation in controls for 0.3 mM of suramin, respectively) as well as for ATP 1 mM (79.8 $\pm$ 2.7 % for 0.1 mM and 69.4 $\pm$ 3.3 % of P_i generation in controls for 0.3 mM of suramin, respectively). Additionally, suramin (1 mM) decreased P_i formation from ATP (1 mM) to 72.4 $\pm$ 4.6 % of controls indicating that the inhibitory effect of suramin was limited, even if the higher ATP concentration was tested (figure 3.8.). Figure 3.6. A. shows the inhibitory effect of suramin on ecto-nucleotidases degrading ADP and AMP. Suramin (0.3 mM) reduced P_i generation of ADP controls (1 mM, 15-min incubation, n = 8) to 70.7 $\pm$ 2.7 % and of AMP controls (1 mM, 15-min incubation, n = 8) to 78.4 $\pm$ 4.4 %, respectively. Thus, the inhibitory activity of suramin on ecto-nucleotidases, degrading ATP, ADP or AMP was almost similar. In contrast, suramin (1 mM) had no effect on P_i formation in the absence of divalent cations (96.9 $\pm$ 6.0 % of P_i generation in controls of ATP 1 mM; n = 8) (figure 3.8.).
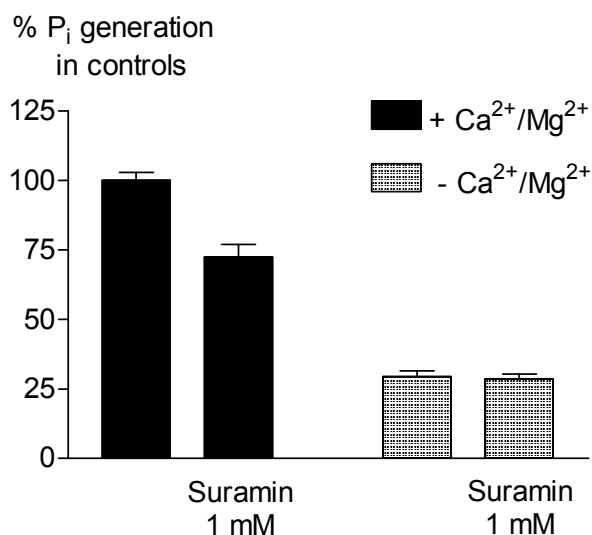
Fig. 3.8. Inhibitory effect of suramin (1 mM) on degradation of ATP (1 mM, incubated for 15 min) in guinea-pig taenia coli in the presence and in the absence of Ca^{2+} and Mg^{2+} (buffer containing 1 mM NaEDTA). Data are presented as percentages (means + S.E.M.) of formation of inorganic phosphate (P_i) in the respective controls (first incubation) (n = 8).

3.1.3.2. Studies on folliculated *Xenopus laevis* oocytes

The suitability of the *Xenopus laevis* oocytes ecto-nucleotidases (ectoN) assay for the screening of the inhibitory activity of a large number of suramin analogues on ATP-degrading enzymes was investigated, testing the inhibitory effects of suramin (0.1 - 1 mM), NF023 (0.1 mM and 0.3 mM) and the ectoN inhibitor ARL 67156 (0.1 mM and 0.3 mM) on ATP breakdown. P_i generation (4.82 $\pm$ 0.13 nmol P_i/oocyte in controls of ATP 0.1 mM incubated for 30 min, n = 24) was progressively decreased in the presence of increasing concentrations of suramin, NF023 and ARL 67156 (second incubation). All compounds inhibited ATP breakdown to approximately a similar extent (table 3.3.; figures 3.9. and 3.10.).
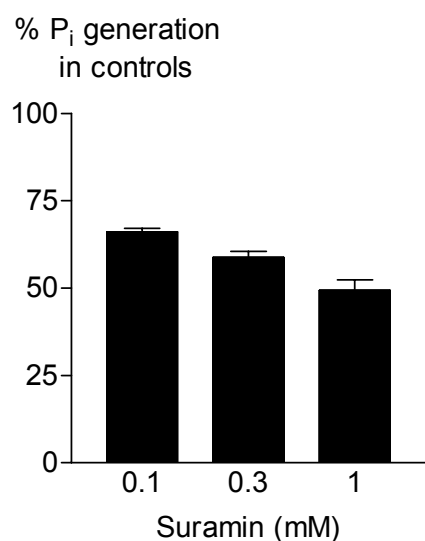
Fig. 3.9. Inhibitory activity of suramin (0.1 - 1 mM) on degradation of ATP (0.1 mM, incubated for 30 min) in Xenopus laevis oocytes. Data are presented as percentages (means + S.E.M.) of formation of inorganic phosphate (P_i) in the respective control experiments (first incubation) (n = 7 - 16).

Table 3.3. Inhibition of ATP breakdown by ecto-nucleotidases present in folliculated *Xenopus laevis* oocytes by suramin, NF023 and ARL 67156. Data presented are means ± S.E.M., obtained from 7 to 31 observations (n. d. = not determined).

Concentration (mM)	% P_i generation in controls		
	Suramin	NF023	ARL 67156
0.1	66.2 ± 1.0	78.5 ± 3.6	81.3 ± 3.1
0.3	58.8 ± 1.8	68.9 ± 2.1	57.1 ± 2.5
1	49.3 ± 2.3	n. d.	n. d.
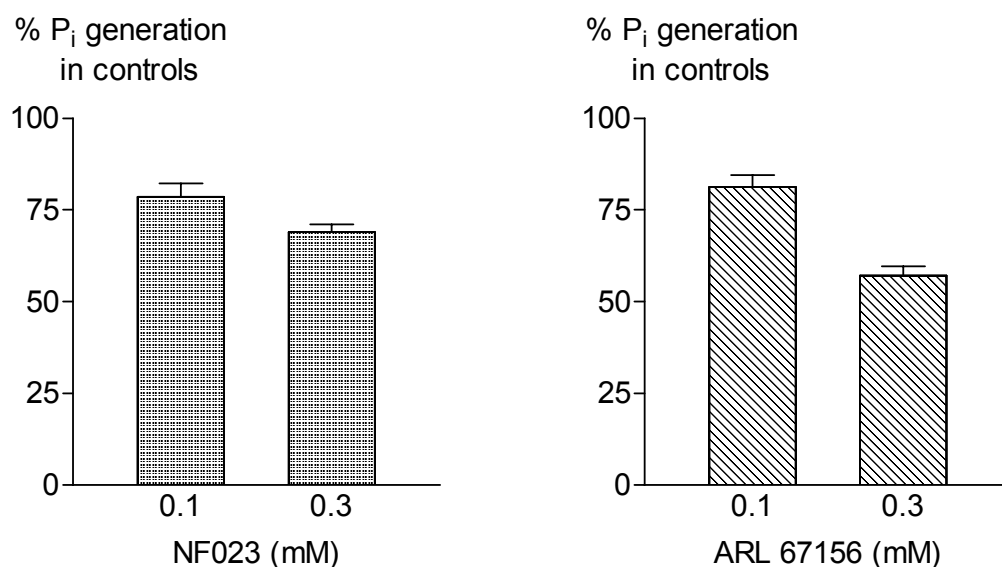
Fig. 3.10. *Inhibitory activity of NF023 and ARL 67156 (0.1 and 0.3 mM) on degradation of ATP (0.1 mM, incubated for 30 min) in Xenopus laevis oocytes. Data are presented as percentages (means + S.E.M.) of formation of inorganic phosphate (P_i) in the respective control experiments (first incubation) (n = 7 - 12).*

3.1.3.3. Ecto-nucleotidases screening of suramin- and NF023-related compounds

Effects of suramin analogues (300 μM) on ATP breakdown in *Xenopus laevis* oocytes were investigated. When the amount P_i generated was decreased in the presence of a derivative (second incubation), its inhibitory activity was expressed

as inhibition (%) of P_i produced in control incubations (first incubation) (for results see next section, 3.1.4.).

3.1.4. Inhibitory activity of suramin- and NF023-related compounds on native P2 receptors and ecto-nucleotidases

To give a comprehensive overview, compounds were divided into two major groups: the suramin-related large ureas and the NF023-derived small ureas. A further subdivision according to structural variations appeared to be appropriate to simplify comparison within the subgroups as well as between the two major families. The inhibitory activity of the derivatives was examined on P2X receptors present in RVD (see 3.1.1.3.), on P2Y receptors mediating relaxation of GPTC (cf. 3.1.2.3.), and on ATP breakdown by ecto-nucleotidases present in *Xenopus laevis* oocytes (cf. 3.1.3.3.). Suramin and NF023 served as reference drugs.

3.1.4.1. Large ureas
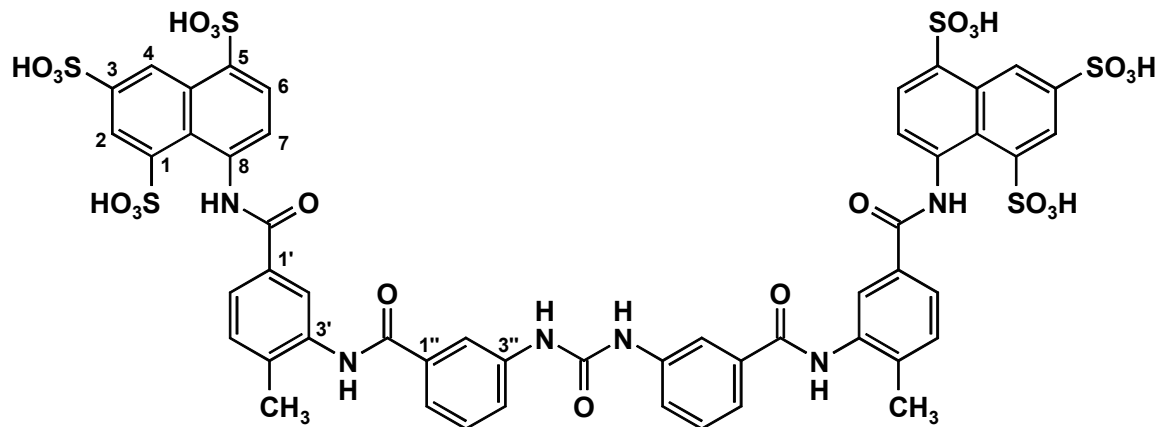
Suramin

3.1.4.1.1. Variations in the substitution pattern of the benzoyl residues
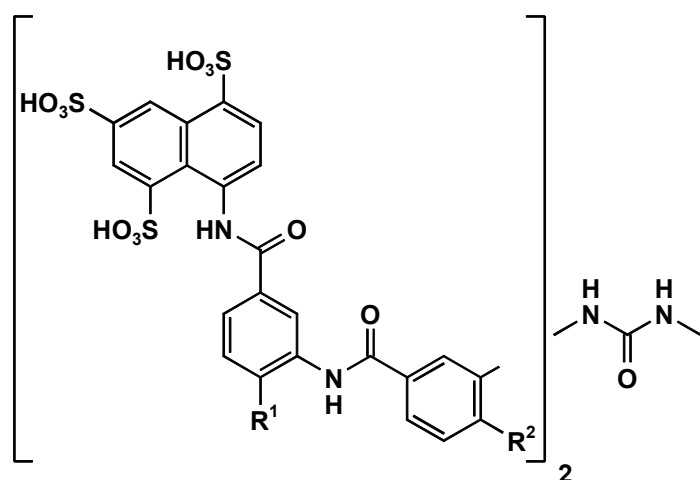
Fig. 3.11. *Principle structure of large ureas I, varying in the substitution pattern of the benzoyl residues (for detailed chemical structure and results see table 3.4. a.).*

Table 3.4.a.-d. (following pages) Screening results of suramin-related large ureas obtained in studies at P2X receptors in rabbit vas deferens (RVD), P2Y receptors in guinea-pig taenia coli (GPTC), and on ATP breakdown by ecto-nucleotidases (ectoN) present in folliculated *Xenopus laevis* oocytes. Data presented are means $\pm$ S.E.M. of at least four observations on different animals, or cells from different animals, in the case of studies on oocytes, respectively. For detailed descriptions of experimental protocols and analysis of results the reader is referred to chapter 2, section 2.2. and chapter 3, sections 3.1.1. to 3.1.3., (n.s. = not significant).

Table 3.4.a.

Large ureas I	R^1	R^2	P2X (RVD)	P2Y (GPTC)	EctoN
			% Inhibition	pA_2 values	% Inhibition
Suramin	**CH_3**	**H**	**26.0 ± 5.6**	**n.s.**	**41.2 ± 1.8**
NF037	H	H	64.7 ± 1.6	4.83 ± 0.23	57.0 ± 5.3
NF157	F	H	16.0 ± 3.3	n.s.	41.4 ± 4.8
NF198	phenyl	H	73.1 ± 3.3	5.07 ± 0.13	56.6 ± 3.3
NF212	CH_3	CH_3	12.1 ± 6.0	4.24 ± 0.31	38.3 ± 2.7
NF213	H	CH_3	21.8 ± 5.4	n.s.	30.6 ± 5.9

3.1.4.1.2. Variations of the benzoyl residues
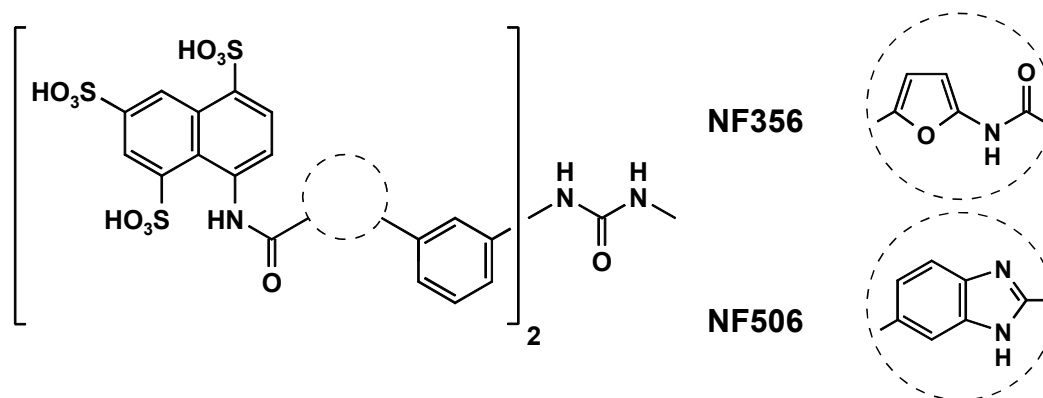
Fig. 3.12. *Chemical structure of NF356 and NF506 (for results see table 3.4.b.).*

Table 3.4.b.

Large ureas II	P2X (RVD)	P2Y (GPTC)	EctoN
	% Inhibition	pA$_2$ values	% Inhibition
NF356	47.0 ± 9.3	5.04 ± 0.12	52.2 ± 2.8
NF506	53.0 ± 13.9	n.s.	62.7 ± 3.0

3.1.4.1.3. Variations in the substitution pattern of the aminonaphthalene sulphonic acid residues in combination with different linkages of the benzoyl residues

A.

B.

C.
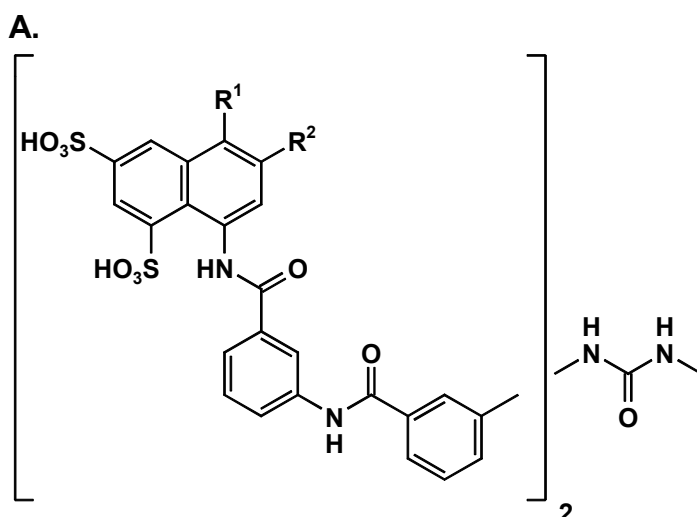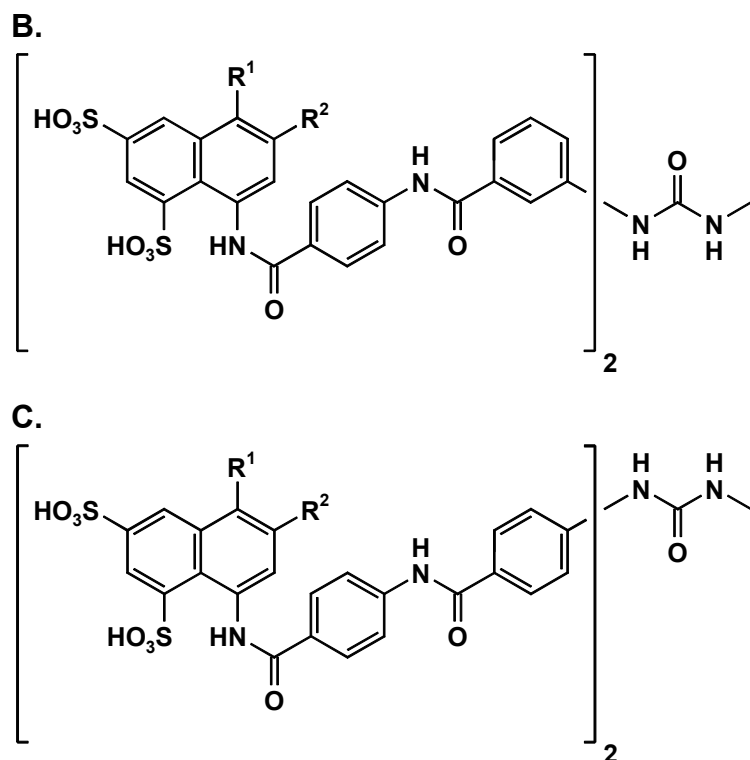
Fig. 3.13. *Principle structure of 1'-3', 1''-3''-linked (**A.**), 1'-4', 1''-3''-linked (**B.**) and 1'-4', 1''-4''-linked (**C.**) large ureas III, varying in the substitution pattern of the aminonaphthalene sulphonic acid residues (for detailed chemical structure and results see table 3.4.c.).*

Table 3.4.c.

Large ureas III	Fig. 3.13.	R^1	R^2	P2X (RVD) % Inhibition	P2Y (GPTC) pA_2 values	EctoN % Inhibition
NF037	**A.**	**SO₃H**	**H**	**64.7 ± 1.6**	**4.83 ± 0.23**	**57.0 ± 5.3**
NF551	A.	H	SO₃H	20.7 ± 8.1	4.60 ± 0.12	49.6 ± 5.4
NF280	B.	SO₃H	H	20.4 ± 7.5	4.60 ± 0.25	43.1 ± 5.8
NF032	B.	H	SO₃H	6.0 ± 3.1	n.s.	28.6 ± 2.4
NF279	C.	SO₃H	H	80.7 ± 7.0	n.s.	66.4 ± 2.6
NF031	C.	H	SO₃H	4.6 ± 2.7	n.s.	52.8 ± 6.6

3.1.4.1.4. Variations of the central urea group
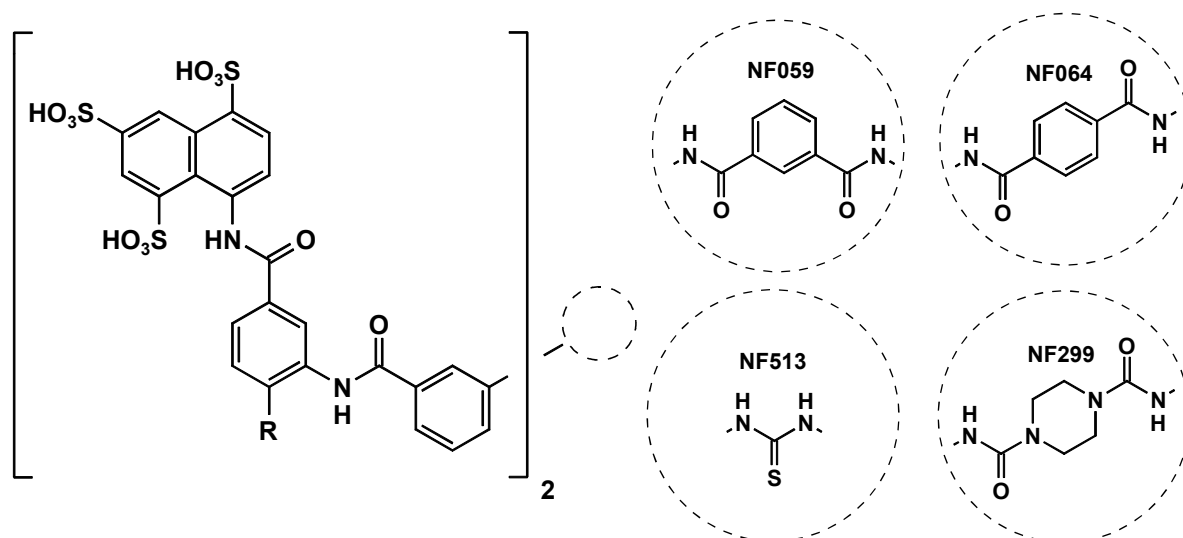
Fig. 3.14. *Chemical structure of suramin-related large ureas IV with central groups differing from the urea-bridged parent compound (for screening results see table 3.4.d.).*

Table 3.4.d.

Large ureas IV	R	P2X (RVD)	P2Y (GPTC)	EctoN
		% Inhibition	pA$_2$ values	% Inhibition
NF059	CH$_3$	9.4 ± 3.7	n.s.	32.5 ± 4.1
NF064	CH$_3$	24.0 ± 4.9	n.s.	47.9 ± 1.5
NF513	H	38.8 ± 5.8	n.s.	50.8 ± 3.4
NF299	CH$_3$	6.8 ± 3.7	n.s.	43.7 ± 5.9

3.1.4.2. Small ureas
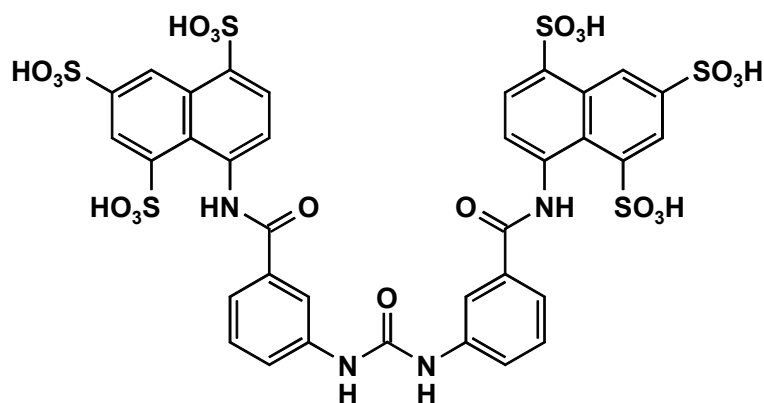
NF023

3.1.4.2.1. Variations of the benzoyl residue substituent
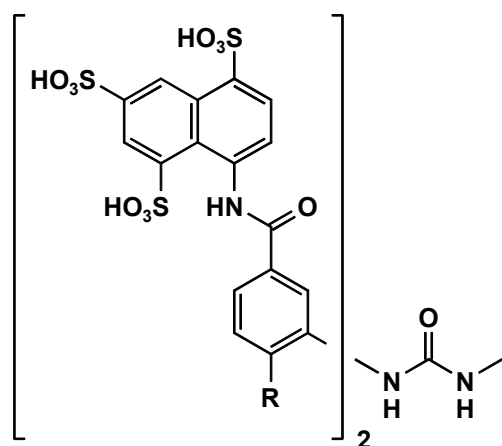
Fig. 3.15. Principle structure of small ureas I, varying in the substitution pattern of the benzoyl residues (for detailed chemical structure and results see table 3.5.a.).

Table 3.5.a.-e. (following pages) Screening results of NF023-related small ureas obtained in studies at P2X receptors in rabbit vas deferens (RVD), P2Y receptors in guinea-pig taenia coli (GPTC) and ATP breakdown by ecto-nucleotidases (ectoN) present in folliculated *Xenopus laevis* oocytes. Data presented are means ± S.E.M. of at least four observations on different animals, or cells from different animals, in the case of studies on oocytes, respectively. For detailed descriptions of experimental protocols and analysis of results the reader is referred to chapter 2, section 2.2. and chapter 3, sections 3.1.1. to 3.1.3.

Table 3.5.a.

Small ureas I	R	P2X (RVD)	P2Y (GPTC)	EctoN
		% Inhibition	pA$_2$ values	% Inhibition
NF023	**H**	**48.3 ± 3.8**	**n.s.**	**31.1 ± 2.1**
NF058	CH$_3$	8.4 ± 6.0	n.s.	35.6 ± 3.3
NF144	C(CH$_3$)$_3$	0	n.s.	n.d.
NF195	phenyl	31.7 ± 2.3	n.s.	39.5 ± 2.5
NF156	F	25.0 ± 11.4	n.s.	34.6 ± 2.1
NF255	Cl	29.2 ± 7.7	n.s.	44.5 ± 2.3

3.1.4.2.2. Exchange of the benzoyl residue
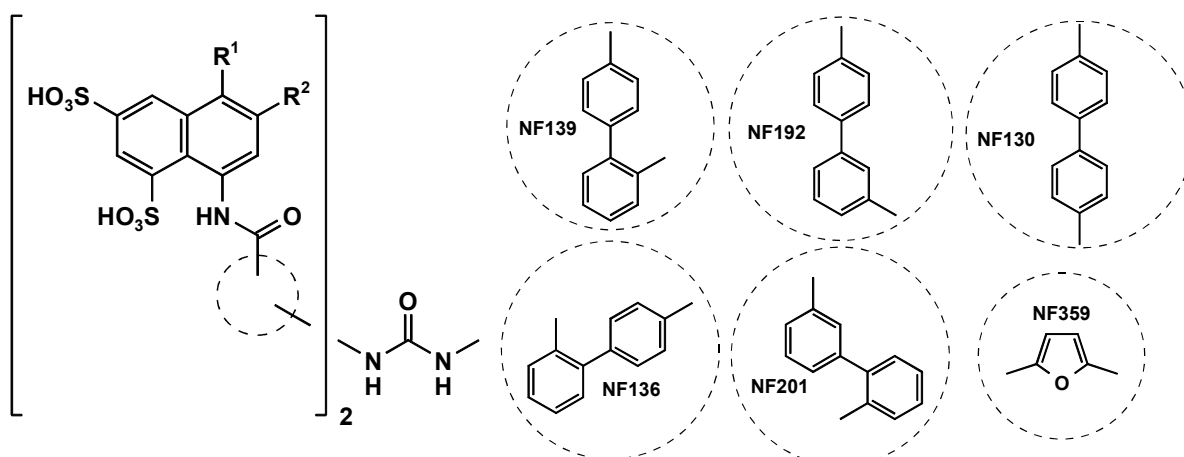
Fig. 3.16. *Chemical structure of small ureas II, in which the benzoyl fragment is exchanged for either isomeric biphenyl residues or a furanyl residue (for results see table 3.5.b.).*

Table 3.5.b.

Small ureas II	R^1	R^2	P2X (RVD)	P2Y (GPTC)	EctoN
			% Inhibition	pA$_2$ values	% Inhibition
NF139	SO$_3$H	H	50.6 ± 3.1	n.s.	33.4 ± 3.7
NF192	SO$_3$H	H	76.9 ± 6.7	5.60 ± 0.09	66.3 ± 2.7
NF130	SO$_3$H	H	16.1 ± 1.4	n.s.	62.7 ± 3.0
NF136	SO$_3$H	H	69.5 ± 6.0	5.56 ± 0.10	51.2 ± 3.0
NF201	SO$_3$H	H	70.1 ± 2.7	4.83 ± 0.13	28.2 ± 3.8
NF359	H	SO$_3$H	4.0 ± 2.2	n.s.	30.6 ± 1.4

3.1.4.2.3. Variations in the substitution pattern of aminonaphthalene sulphonic acid residues in combination with different linkages of the benzoyl residue
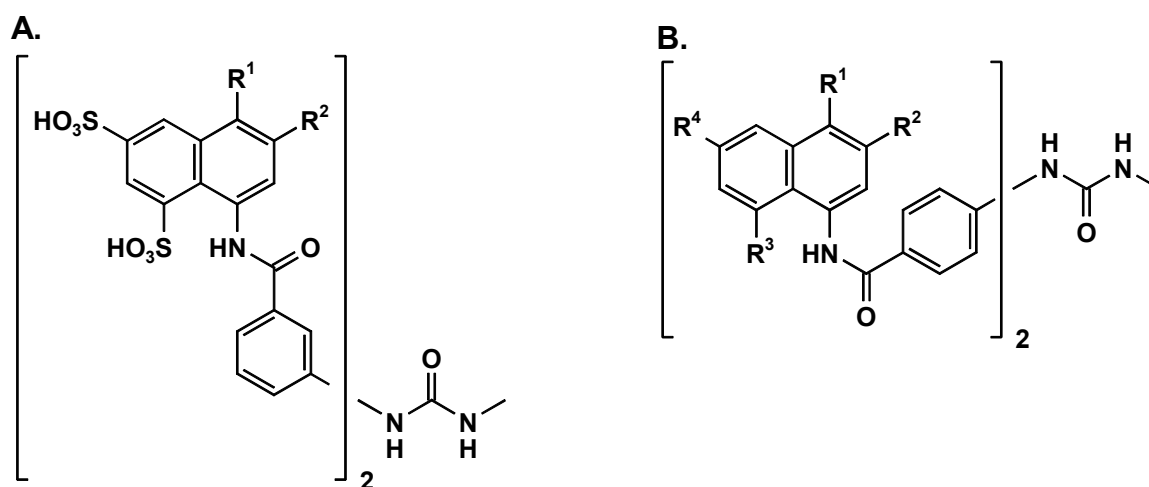
Fig. 3.17. *Principle structure of 1'-3'-linked (A.) and 1'-4'-linked (B.) small ureas III, varying in the substitution pattern of aminonaphthalene sulphonic acid residues (for detailed chemical structure and results see table 3.5.c.).*

Table 3.5.c.

Small ureas III	R^1	R^2	R^3	R^4	P2X (RVD) % Inhibition	P2Y (GPTC) pA$_2$ values	EctoN % Inhibition
NF550 (A.)	H	SO$_3$H	-	-	65.9 ± 6.4	5.43 ± 0.07	43.3 ± 4.0
NF278 (B.)	SO$_3$H	H	SO$_3$H	SO$_3$H	4.7 ± 2.5	n.s.	26.1 ± 3.5
NF013 (B.)	H	SO$_3$H	SO$_3$H	SO$_3$H	3.7 ± 1.5	n.s.	18.3 ± 3.8
NF289 (B.)	SO$_3$H	H	SO$_3$H	H	2.7 ± 1.7	n.s.	12.5 ± 2.0
NF295 (B.)	SO$_3$H	H	H	SO$_3$H	2.8 ± 1.7	n.s.	11.4 ± 2.5

3.1.4.2.4. 1'-4'-linked small ureas connected to 7-aminonaphthalene di- and tri-sulphonic acid residues
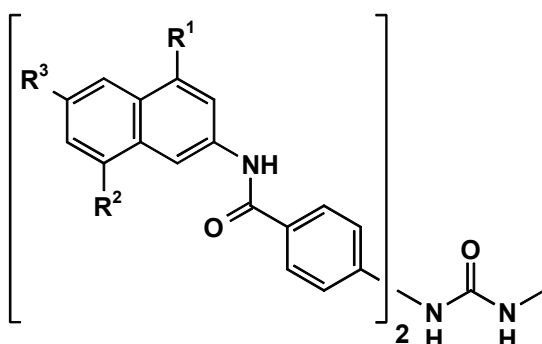
Fig. 3.18. Principle structure of 1'-4'-linked small ureas IV connected to 7-amino-naphthalene di- and trisulphonic acid residues (for results see Table 3.5.d.).

Table 3.5.d.

Small ureas IV	R^1	R^2	R^3	P2X (RVD) % Inhibition	P2Y (GPTC) pA$_2$ values	EctoN % Inhibition
NF347	SO$_3$H	SO$_3$H	SO$_3$H	8.7 ± 4.0	n.s.	36.2 ± 4.5
NF291	SO$_3$H	SO$_3$H	H	1.8 ± 0.7	n.s.	28.3 ± 5.6

## 3.1.4.2.5. Variations of the central urea group
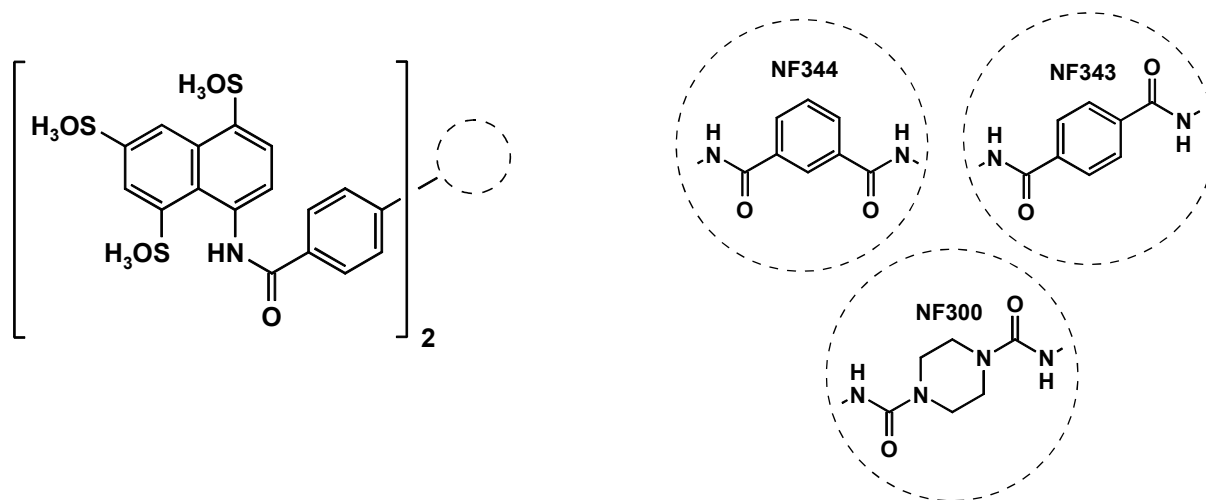
Fig. 3.19. *Chemical structure of NF344, NF343 and NF300 (small ureas V, for results see table 3.5.e.)*

Table 3.5.e.

Small ureas V	P2X (RVD)	P2Y (GPTC)	EctoN
	% Inhibition	pA_2 values	% Inhibition
NF344	0	n.s.	27.2 ± 6.5
NF343	2.4 ± 1.3	n.s.	44.7 ± 4.0
NF300	9.3 ± 3.3	n.s.	27.6 ± 2.7

As shown in the tables 3.4.a.-d. and 3.5.a.-e., the antagonistic properties of large and small ureas at native P2 receptor subtypes and ecto-nucleotidases are controlled by different structural parameters;

- the molecular size of the compounds,
- the position and number of aminonaphthalene sulphonic acid residues
- the nature and substitution pattern of the aromatic rings
- the structure of the bridge linking the moieties of these symmetrical compounds.

As a result, analogues with different receptor-selectivity profiles were obtained. For a detailed structure-activity relationship analysis see 4. Discussion.

3.2. Detailed pharmacological characterisation of large ureas III and small ureas III

Derivatives of large and small ureas type III (for chemical structure see figure 3.13., table 3.4.c. and figure 3.17., table 3.5.c.) were investigated for their inhibitory potencies at $P2X_1$-like receptors present in prostatic segments of rat vas deferens (ratVD), P2Y receptors in GPTC and $P2Y_1$-like receptors in guinea-pig ileal longitudinal smooth muscle (GPI). Additionally, the P2 receptor specificity as well as the concentration-dependent inhibitory potency of the $P2X_1$-selective compounds NF279 and NF031 on ATP-breakdown in *Xenopus laevis* oocytes were examined. The respective parent compounds suramin (large ureas) and NF023 (small ureas) were used as reference drugs.

3.2.1. Inhibitory potency at $P2X_1$-like receptors (rat vas deferens)

The potency of small and large ureas III to inhibit $P2X_1$-like receptor-mediated contraction of prostatic segments of ratVD induced by α,β-mATP (10 μM) was determined. pIC_{50} values were derived from non-linear regression analysis of concentration-inhibition curves obtained for each compound.

3.2.1.1. Large ureas III

α,β-mATP (10 μM) elicited rapid, transient contractions (1499.7 $\pm$ 78.4 mg tension, n = 15) of prostatic segments of ratVD which were reduced and finally abolished by increasing concentrations of the large ureas NF037, NF551, NF280, NF032, NF279, NF031 and suramin. Non-linear regression analysis of the resulting concentration-inhibition curves (figure 3.20.) yielded pIC_{50} values from which apparent pA_2 values (pA_2*) were calculated according to the equation introduced by Cheng and Prusoff (1973) (see 2.2.4.1., table 3.6.).

Among these large ureas, the structural isomers NF279 and NF031 displayed the highest potency in blocking $P2X_1$-like receptor-mediated contractions of ratVD. Thus, the effect of NF279 on CRC's to single doses of α,β-mATP was also examined, for results see section 3.2.1.3.

A.
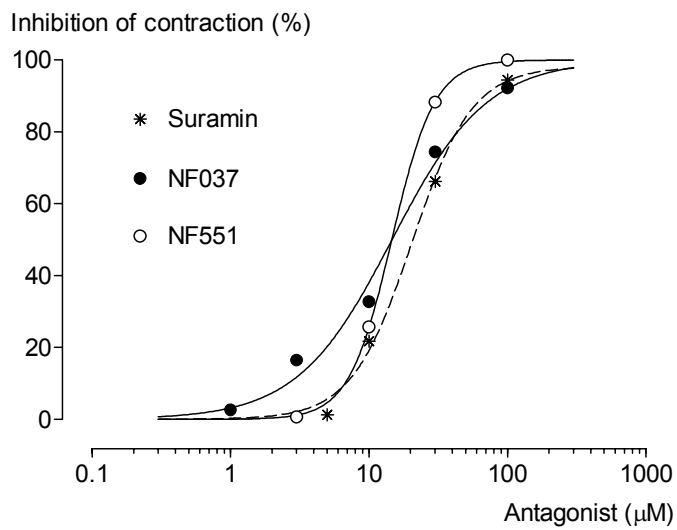
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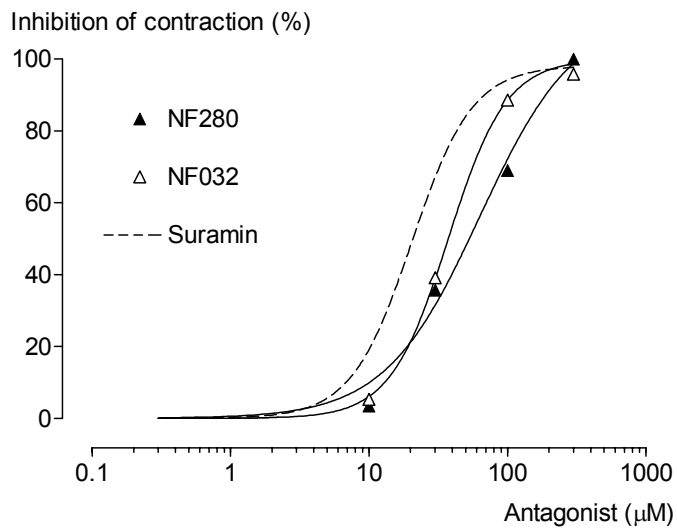
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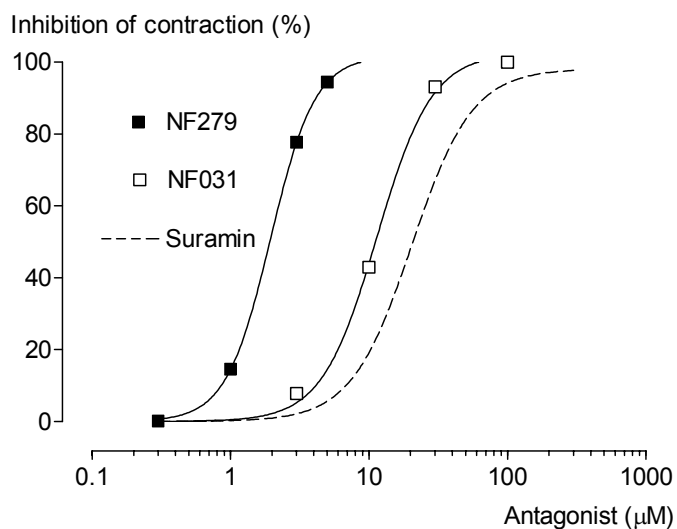
*Fig. 3.20. Inhibitory potency of large ureas III. The graphs show concentration-inhibition curves obtained for the large ureas NF037 and NF551 (**A.**), NF280 and NF032 (**B.**), NF279 and NF031 (**C.**), and the parent compound suramin (broken lines) in prostatic segments of rat vas deferens. Given are mean % inhibition (n = 2 - 5) of contractions induced by single doses of exogenously applied α,β-mATP (10 μM). Error bars are not shown. For corresponding pIC_{50} values see table 3.6.*

Table 3.6. Inhibitory potency (pIC_{50} and apparent pA_2 [pA_2*]) of suramin and its structural analogues on contractions induced by α,β-mATP (10 μM) in prostatic segments of rat vas deferens (for chemical structure see figures 3.11. and 3.13., the corresponding graphs are shown in figure 3.20.). Data are presented as means ± S.E.M. from 3 to 5 observations.

| Compound | Substitution pattern | | pIC_{50} | pA_2* |
	naphthalene -SO$_3$H	linkage of benzoyl moieties		
Suramin	1,3,5	1'-3', 1"-3"	4.68 ± 0.02	5.72 ± 0.02
NF037	1,3,5	1'-3', 1"-3"	4.79 ± 0.17	5.83 ± 0.18
NF551	1,3,6	1'-3', 1"-3"	4.84 ± 0.07	5.88 ± 0.06
NF280	1,3,5	1'-4', 1"-3"	4.31 ± 0.07	5.35 ± 0.07
NF032	1,3,6	1'-4', 1"-3"	4.45 ± 0.05	5.49 ± 0.05
NF279	1,3,5	1'-4', 1"-4"	5.71 ± 0.09	6.75 ± 0.09
NF031	1,3,6	1'-4', 1"-4"	4.96 ± 0.04	6.00 ± 0.04

3.2.1.2. Small ureas III

The potency of NF023-derived small ureas III to inhibit contractions of ratVD to single doses of exogenously applied α,β-mATP (10 μM) was assessed as described for the large ureas III (see 3.2.1.1.). For concentration-inhibition curves see figure 3.21.; the resulting pIC_{50} values as well as apparent pA_2 values are given in table 3.7. The derivatives NF278 and NF013 were without effect when concentrations lower than 100 μM were investigated. Therefore their inhibitory activity was only tested at a single concentration.

Table 3.7. (page 114) Inhibitory potency of NF550 and its parent compound NF023 (pIC_{50} and apparent pA_2) and inhibition (%) of NF278 and NF013 (100 μM), respectively, on contractions of prostatic segments of rat vas deferens induced by α,β-mATP (10 μM). Data shown are means ± S.E.M. (n = 3 - 4). For chemical structures see figures 3.15. and 3.17., the corresponding graphs are shown in figure 3.21.

Compound	Substitution pattern		pIC_{50}	pA_2*	% Inhibition
	naphthalene -SO$_3$H	linkage of benzoyl moiety			
NF023	1,3,5	1'-3'	4.93 ± 0.03	5.97 ± 0.02	
NF550	1,3,6	1'-3'	5.13 ± 0.07	6.17 ± 0.07	
NF278	1,3,6	1'-4'			7.3 ± 3.7
NF013	1,3,5	1'-4'			20.3 ± 6.7
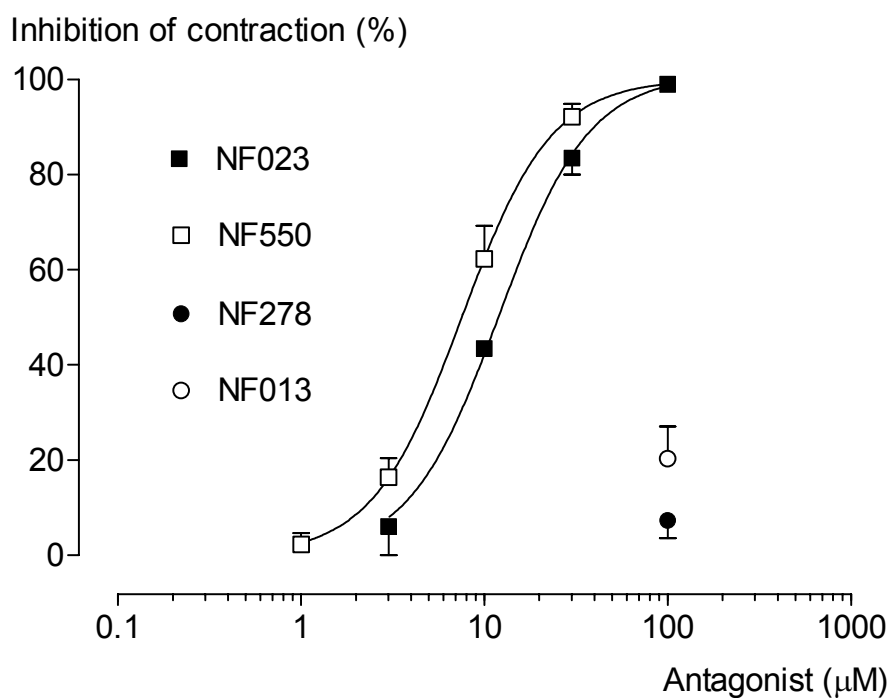
Fig. 3.21. *Inhibitory potency of NF550 and its parent compound NF023 on contractile responses of prostatic segments of rat vas deferens induced by α,β-mATP (10 μM). The graph shows concentration-inhibition curves obtained for NF550 and NF023 and inhibitory activity of 100 μM of NF278 and NF013. Data shown are means +/- S.E.M. (n = 3 - 4) of inhibition (%) of α,β-mATP-induced contractions. Error bars falling within the area of a symbol are not shown (for results see table 3.7.).*

3.2.1.3. Inhibitory properties of NF279

3.2.1.3.1. Effect of NF279 on concentration-response curves to α,β-methylene ATP

As illustrated in figure 3.22., CRC's to single doses of α,β-mATP (15- to 60-min intervals) did not reach a well-defined maximum, thus, the EC_{50} value as an estimate of agonist potency could not be determined. In the absence of antagonist, increasing concentrations of α,β-mATP elicited increasing contractions ($pEC_{1000} = 5.48 \pm 0.06$, n = 9) of prostatic segments of ratVD. CRC's were reproducible over the time course of experimentation, as evidenced by the fact that in time-matched control experiments the first CRC's ($pEC_{1000} = 5.29 \pm 0.11$, n = 2) was almost superimposible by the second CRC's ($pEC_{1000} = 5.17 \pm 0.15$, n = 2; figure 3.22.B.). Incubation of NF279 at 1 μM, 3 μM and 10 μM for 60 min produced concentration-dependent antagonism of contractile responses to α,β-mATP indicated by a rightward shift of the α,β-mATP CRC's in the presence of antagonist (figure 3.22. A.). Additionally, an increase in the slope of the α,β-mATP CRC's, which was not depending on the NF279 concentration tested, was observed.

To test whether the antagonism of NF279 was competitive, dose ratios were calculated from equieffective concentrations of α,β-mATP (read from the 1000 mg tension level of the respective CRC's) in the absence and in the presence of the antagonist. pA_2 values, calculated for each concentration of NF279 from individual dose ratios were significantly different from each other (table 3.8.). To assure that the increase in pA_2 values is not due to non-sufficient pre-incubation of NF279, especially when low concentrations of the antagonist were examined, similar experiments were performed applying NF279 (1 μM) 120 min prior to generation of the second agonist CRC's. However, no difference was found comparing pA_2 values determined for NF279 (1μM) after 60 and 120 min of pre-incubation ($pA_2 = 6.07 \pm 0.08$ for 60 min and 6.01 ± 0.40 for 120 min of pre-incubation of the antagonist), respectively.

A.

Increase in Tension (mg)

B.

Increase in Tension (mg)
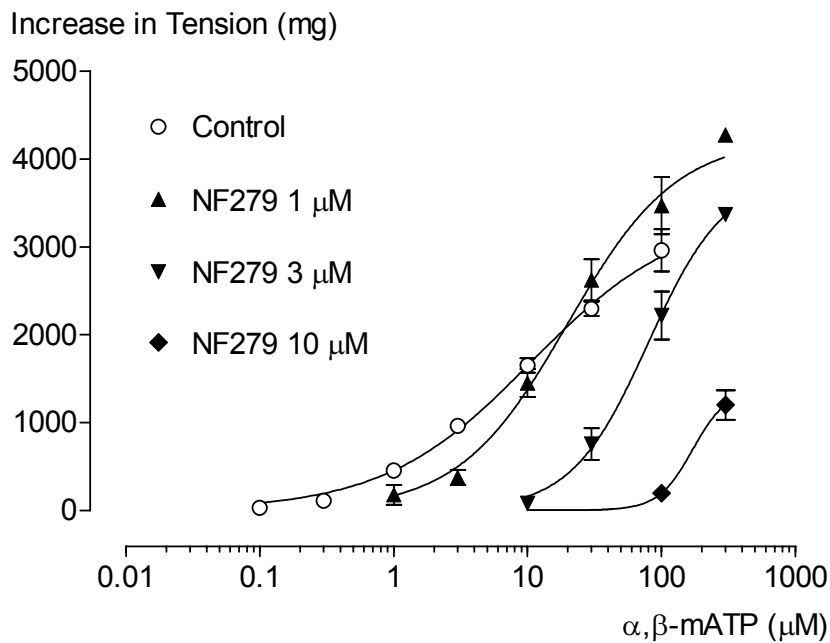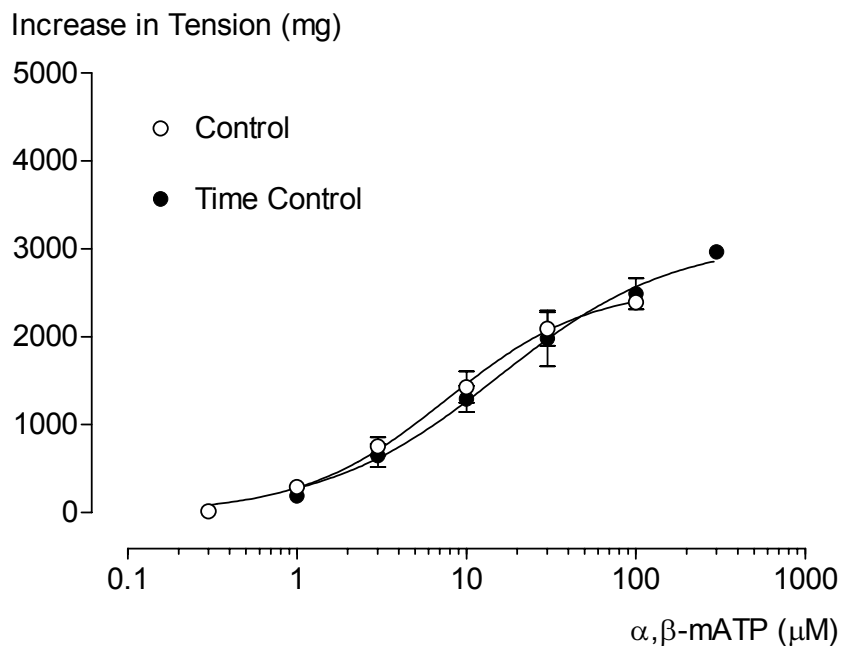
Fig. 3.22. A. *Antagonism of NF279 on α,β-mATP-induced contractions of prostatic segments of rat vas deferens (for corresponding pA_2 values see table 3.8.). Concentration-response curves in the absence and in the presence of increasing concentrations of NF279 are shown.* ***B.*** *The graph shows concentration-response curves obtained in time-matched control experiments. Values are means ± S.E.M.; error bars falling within the area of the symbols are not shown.*

Table 3.8. Antagonism of NF279 (60-min incubation) on α,β-mATP-induced contractions of rat vas deferens. pA_2 estimates (means $\pm$ S.E.M.) were calculated from the individual dose ratios obtained for each antagonist concentration (n = 3).

NF279	1 μM	3 μM	10 μM
pA_2 value	6.07 ± 0.08	6.41 ± 0.07	7.06 ± 0.08

3.2.1.3.2. Time course and reversibility of NF279's inhibitory effect

Investigating the time course of NF279's (3 μM) inhibitory effect to single doses of α,β-mATP (10 μM), a maximum was reached at about 180 min of incubation and the antagonism was almost completely reversed on repeated wash out after 90 min as responses to α,β-mATP totally recovered to their respective pre-NF279 controls (figure 3.23.). Over the same time period (300 min), responses of control tissues to 10 μM α,β-mATP remained unchanged (figure 3.23).
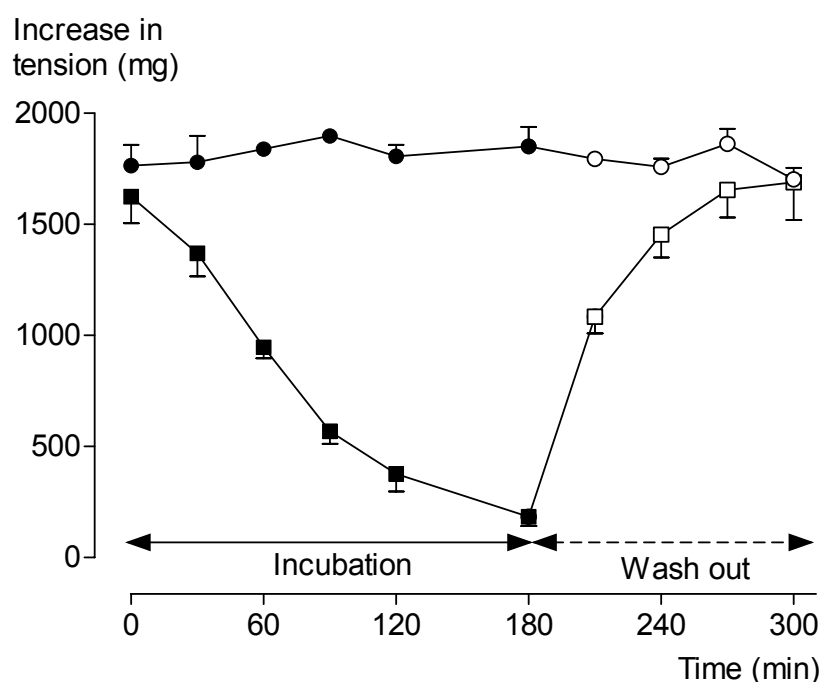
Fig. 3.23. *Kinetics of onset (■)and offset (□) of NF279's (3 μM) antagonistic effect on contractions induced by α,β-mATP (10 μM) in prostatic segments of rat vas deferens; ●, ○ indicate data from time-matched controls. Data shown are mean +/- S.E.M.; error bars exceeding the size of the symbols are not shown (n = 2 - 3).*

3.2.2. Inhibitory potency on P2Y receptors (guinea-pig taenia coli)

The potency of large and small ureas III to inhibit P2Y receptor-mediated relaxation of the carbachol-pre-contracted GPTC induced by ADPβS was determined by calculating pA_2 values from the shift of the agonist's concentration-relaxation curves obtained in the absence and in the presence of large and small ureas III (for potency of ADPβS as well as the pA_2 values obtained for the parent compounds suramin and NF023 see section 3.1.2.2., figure 3.4. A. and B.).

3.2.2.1. Large ureas III

At a concentration of 100 μM all compounds except NF031 (300 μM) caused a rightward shift of the ADPβS CRC's without changing the maximum or altering the contraction induced by carbachol (for resulting pA_2 values see table 3.9.). Increasing concentrations of NF037 shifted the ADPβS CRC's progressively to the right. The regression according to Arunlakshana and Schild (Arunlakshana and Schild, 1959) was linear with a slope of -0.80 ± 0.28 (not significantly different from -1.00) and a pA_2 value of 4.85 ± 0.11 (constrained plot) was estimated (figure 3.24.).

Table 3.9. Inhibitory potency (pA_2 ± S.E.M.) of large ureas III on relaxant responses of carbachol-pre-contracted guinea-pig taenia coli to ADPβS (n indicating the total number of experiments).

Large ureas III	pA_2	n	Concentration tested (μM)
NF037	4.86 ± 0.10	11	10, 30, 100
NF551	4.60 ± 0.08	7	10, 100
NF280	4.87 ± 0.06	7	10, 100
NF032	3.95 ± 0.13	4	100
NF279	4.10 ± 0.29	3	100
NF031	3.30 ± 0.16	3	300
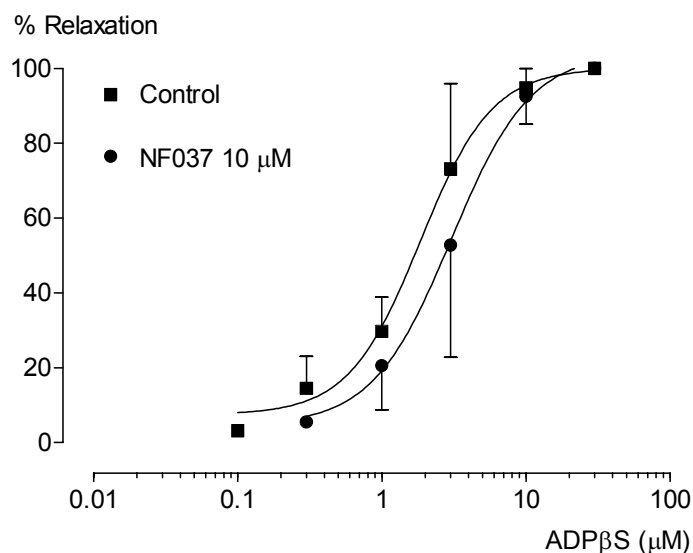
Fig. 3.24. *Concentration-response curves of ADPβS in carbachol-pre-contracted guinea-pig taenia coli in the absence (control) and in the presence of NF037 (10, 30 and 100 µM). Responses (mean +/- S.E.M.) are expressed as percentage of relaxation of the carbachol-induced contraction. Schild analysis (shown in the insert) yielded a slope of -0.80 ± 0.28 (not significantly different from -1.00), and a pA₂ value (constrained plot) of 4.85 ± 0.11 was estimated.*
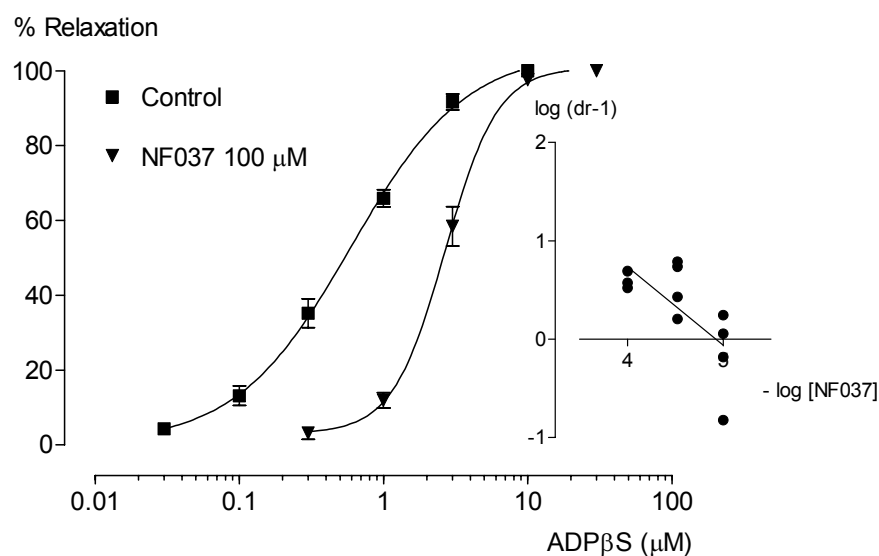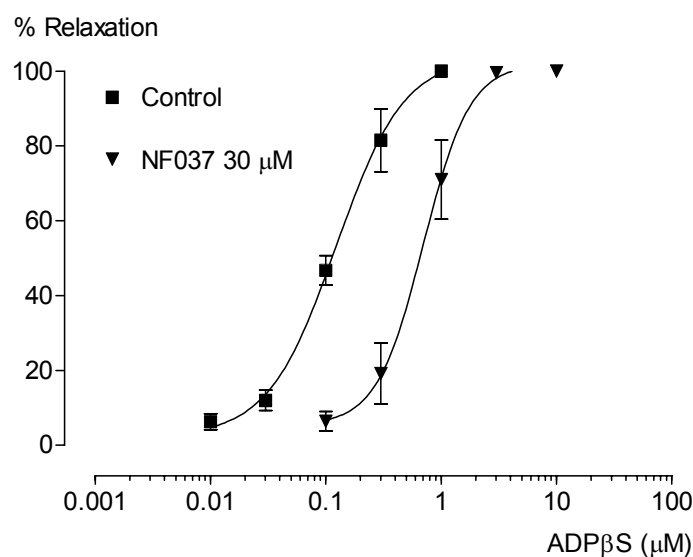
3.2.2.2. Small ureas III

The antagonistic potency of small ureas on P2Y receptors mediating relaxation of the carbachol-pre-contracted GPTC was determined investigating a concentration of 100 μM of NF278 and NF013. pA_2 values of 4.27 $\pm$ 0.15 for NF278 and 4.53 $\pm$ 0.11 for NF013 (means $\pm$ S.E.M., n = 4) were calculated from the individual dose ratios of the ADPβS CRC's generated in the absence and in the presence of the respective compound. As established for the parent compound NF023 (see 3.1.2.2.) pA_2 values determined for NF550 decreased when increasing concentrations of the antagonist were investigated, being 5.43 $\pm$ 0.07 for 10 μM, 4.78 $\pm$ 0.17 for 30 μM and 4.57 $\pm$ 0.10 for 100 μM (n = 3 -4 for each concentration) of NF550 (figure 3.25.).
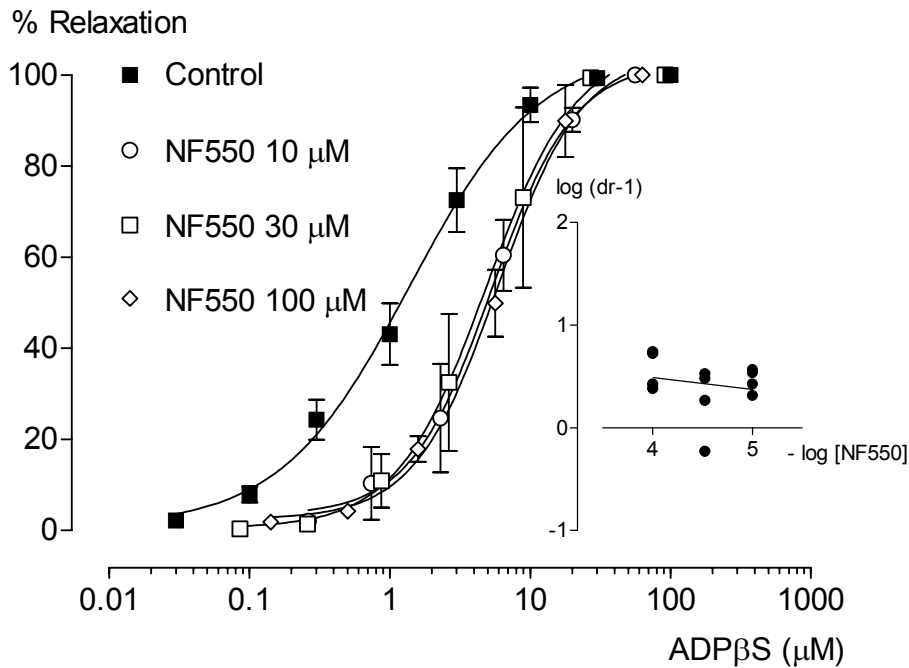
Fig. 3.25. *Limited antagonism of NF550 on P2Y receptors mediating relaxation of carbachol-pre-contracted guinea-pig taenia coli. The graph shows concentration-relaxation curves to ADPβS in the absence (control) and in the presence of increasing concentrations of NF550. Schild analysis (shown in the insert) yielded a slope of -0.11 $\pm$ 0.18 (significantly different from -1.00). Data shown are means $\pm$ S.E.M. of 4 to 16 observations, some error bars are within the width of the symbols.*

3.2.3. Inhibitory potency on P2Y$_1$-like receptors (guinea-pig ileum)

The potencies of large and small ureas to antagonise isometric contractile responses mediated via P2Y$_1$-like receptors present in guinea-pig ileal longitudinal smooth muscle preparation were determined by calculating pA$_2$ values from the shift of the CRC's obtained to ADPβS in the absence and in the presence of the antagonists (for results see tables 3.10.; 3.11.). All experiments on GPI were performed by Sittah Czeche and Matthias Ganso (for experimental design see Czeche et al., 1998b; Lambrecht et al., 1999, 2000b; Braun et al., 2001).

3.2.3.1. Large ureas III

All compounds produced a rightward shift of the CRC's obtained to single doses of ADPβS. pA$_2$ values of suramin, NF037, NF551 and NF280 were very similar; NF279 displayed a moderately, and NF032 as well as NF031 showed a markedly reduced inhibitory activity (table 3.10.).
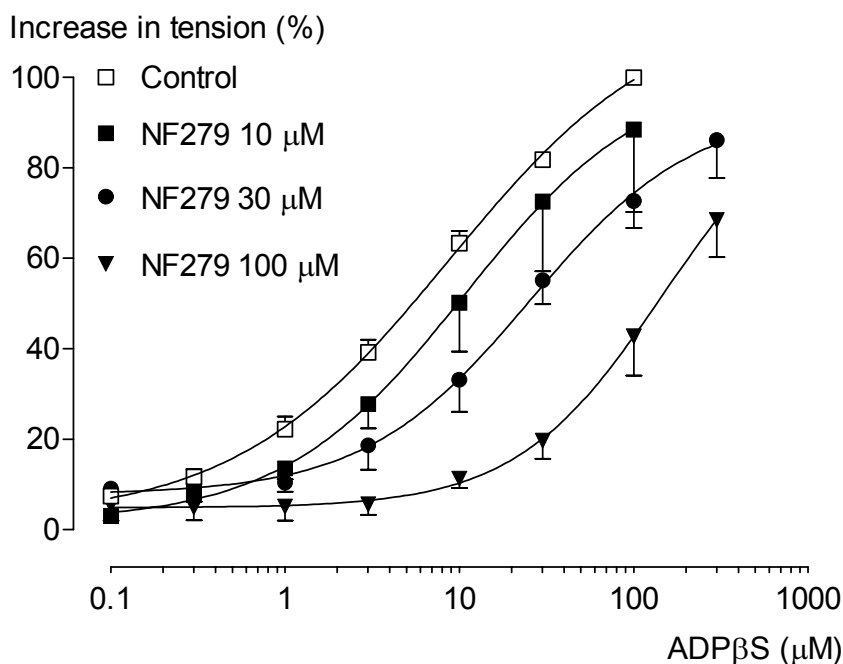
Fig. 3.26. *Antagonism of NF279 on ADPβS-induced contractions of guinea-pig ileal longitudinal muscle (for corresponding pA$_2$ values see table 3.10.). Concentration-response curves in the absence (control) and in the presence of increasing concentrations of NF279 are shown (contractions obtained to 100 μM of ADPβS in the respective control were taken as 100 %).*

Table 3.10. Antagonistic potencies of large ureas III on contractile responses of guinea-pig ileal longitudinal smooth muscle to ADPβS (0.1 - 300 μM). pA_2 values of suramin and NF279 were derived from Schild analysis. The corresponding graphs for NF279 are shown in figure 3.26.

Large ureas III	pA_2	n	Concentration tested (μM)
Suramin	5.68 ± 0.24	12	10, 30, 100
NF037	5.73 ± 0.08	8	10, 30
NF551	5.62 ± 0.02	4	10
NF280	5.72 ± 0.10	4	10
NF032	4.91 ± 0.06	4	100
NF279	5.32 ± 0.08	10	10, 30, 100
NF031	4.62 ± 0.09	5	100

3.2.3.2. Small ureas III

As established for the large ureas III, NF023, NF550, NF278 and NF013 shifted the concentration-response curves of ADPβS to the right. Among the small ureas, NF550 displayed the highest potency in blocking $P2Y_1$-like receptor-mediated contractions of the GPI. NF278 and NF013 were nearly equipotent, and the $P2X_1$-selective compound NF023 showed the lowest $P2Y_1$ receptor-blocking activity (table 3.11).

Table 3.11. Antagonistic potency of small ureas III on contractile responses of guinea-pig ileal longitudinal muscle evoked by ADPβS. The pA_2 value of NF023 was derived from Schild analysis.

Small ureas III	pA_2	n	Concentration tested (μM)
NF023	4.74 ± 0.08	9	100, 300, 1000
NF550	5.87 ± 0.08	13	10, 30
NF278	5.05 ± 0.14	4	30
NF013	5.21 ± 0.13	6	30

3.2.4. Inhibitory potency of NF279 and NF031 on ATP breakdown (*Xenopus laevis* oocytes)

In addition to results obtained for NF279 and NF031 (0.3 mM) from the screening of suramin-related large ureas (cf. 3.1.4.1.3.), the inhibitory activity of each compound was examined at a concentration of 0.1 mM on ATP breakdown in *Xenopus laevis* oocytes.
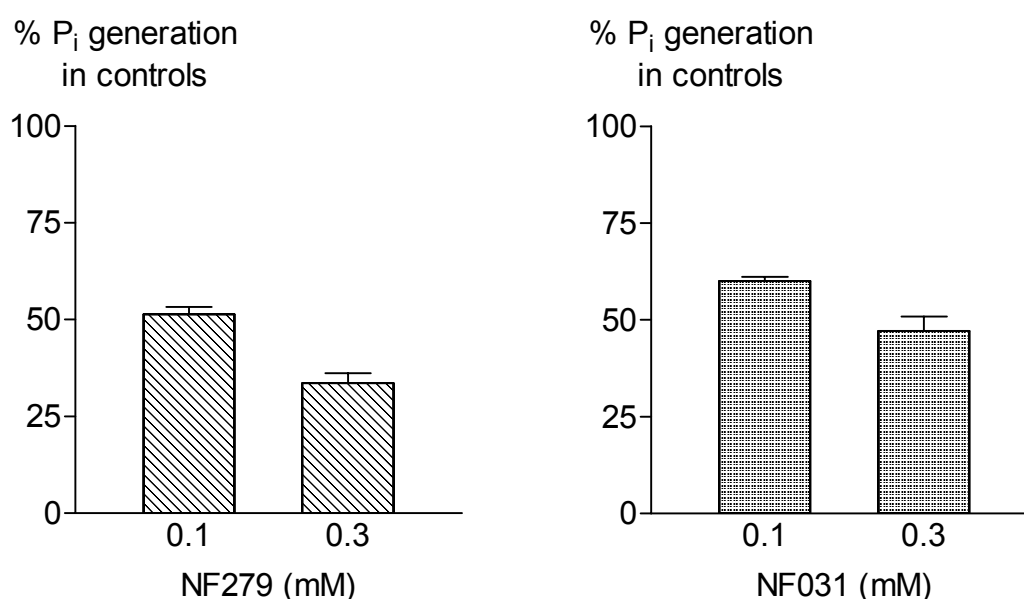
Fig. 3.27. Inhibitory effect of NF279 and NF031 (0.1 and 0.3 mM) on degradation of ATP (0.1 mM, incubated for 30 min) in Xenopus laevis oocytes. Data are presented as percentages (means + S.E.M.) of formation of inorganic phosphate (P_i) in the respective controls (first incubation) (n = 7 - 8).

As described for the parent compound suramin (for results see section 3.1.2.2.), both analogues exhibited a dose-dependent inhibition of ATP-degrading enzymes present in *Xenopus laevis* oocytes. At a concentration of 0.1 mM, the P_i generation was decreased to 51.3 ± 1.9 % (NF279) and 60.0 ± 1.1 % (NF031) of the respective control incubations (figure 3.27.). For 0.3 mM of NF279 and NF031, 33.6 ± 2.6 % and 47.2 ± 3.6 %, respectively, of the P_i generated in the respective controls were determined.

3.2.5. P2 receptor specificity of NF279 and NF031

To determine the P2 receptor specificity of NF279 and NF031, the effects of 100 μM of each compound on responses to various agonists in ratVD (α_{1A}-adrenoceptors; contraction), GPI (adenosine A_1-; relaxation), GPI (histamine H_1- and muscarinic M_3 receptors; contraction) and GPTC (adenosine A_{2B}- and nicotinic acetylcholine receptors; relaxation) were investigated (table 3.12.). NF279 and NF031 had no significant effects on either the potency (table 3.12.) or maximum response to the agonists in the different tissues.

Table 3.12. P2 receptor specificity of NF279 and NF031. Potencies (pD$_2$ values; mean $\pm$ S.E.M.; n = 3 - 4) of the agonists noradrenaline in rat vas deferens (ratVD), 2-chloro-N^6-cyclopentyladenosine (CCPA), histamine and arecaidine propargyl ester (APE) in guinea-pig ileum (GPI), and 2-chloro-adenosine (2-Cl-Ado) and nicotine in guinea-pig taenia coli (GPTC) in the absence (controls) and in the presence of the respective suramin analogue (100 μM). Experimental methods used are described in detail in the literature (see Lambrecht et al., 1999).

Receptor/ agonist/ tissue	Control	NF279 (100 μM)	Control	NF031 (100 μM)
α_{1A}/ noradrenaline /ratVD	5.97 ± 0.32	5.82 ± 0.40	5.85 ± 0.32	5.79 ± 0.30
A_1/ CCPA/ GPI*	8.46 ± 0.07	8.59 ± 0.07	8.45 ± 0.07	8.46 ± 0.06
A_{2B}/ 2-Cl-Ado/ GPTC[+] **	4.56 ± 0.11	4.46 ± 0.21	4.63 ± 0.22	4.43 ± 0.20
H_1/ histamine/ GPI*	6.04 ± 0.07	6.04 ± 0.07	6.11 ± 0.09	6.17 ± 0.09
M_3/ APE/ GPI*	7.53 ± 0.01	7.60 ± 0.05	7.44 ± 0.04	7.49 ± 0.03
N/ nicotine/ GPTC**	4.45 ± 0.07	4.26 ± 0.17	4.57 ± 0.06	4.44 ± 0.11

[+] In the presence of the A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (100 μM).

*/ ** Determined in experiments performed by *S. Czeche and **B. Niebel.

3.3. Regional variation of purinergic responses in rat vas deferens

Differences of inhibitory potencies (pIC_{50} values) of the large urea NF279 assessed in prostatic and epididymal segments of ratVD (for results see 3.3.3.1.) were the starting point for a detailed analysis of agonist and antagonist potencies with regard to the anatomical origin of the segment of ratVD.

3.3.1. Agonists

All agonists applied elicited rapid and transient concentration-dependent contractions of prostatic and epididymal segments of the ratVD (except for ADPβS, which induced contractions of a more sustained type). However, none of the agonists' CRC's did reach a well-defined maximum even at high concentrations (figure 3.28.). For this reason, equieffective concentrations of α,β-mATP, L-β,γ-mATP, ATPγS and ADPβS (inducing 1000 mg of tension of prostatic and epididymal portions of ratVD) were determined to compare their agonistic potencies. α,β-mATP was the most potent agonist in prostatic as well as in epididymal segments. In prostatic portions L-β,γ-mATP and ATPγS were equipotent whereas L-β,γ-mATP was slightly more potent than ATPγS in eliciting contraction of epididymal segments of ratVD. ADPβS, being equieffective in both segments of ratVD, displayed the lowest potencies among the agonists examined (table 3.13.).

Table 3.13. Agonistic potency (pEC_{1000}) of nucleotide analogues in inducing contractions of prostatic and epididymal segments of rat vas deferens. Comparison of equieffective concentrations of the agonists derived from non-linear regression analysis of the concentration-effect curves for either agonist (n = 3 - 6).

Segment	α,β-mATP	L-β,γ-mATP	ATPγS	ADPβS
prostatic	5.72	4.75	4.71	4.20
epididymal	5.37	5.05	4.74	4.18

A.
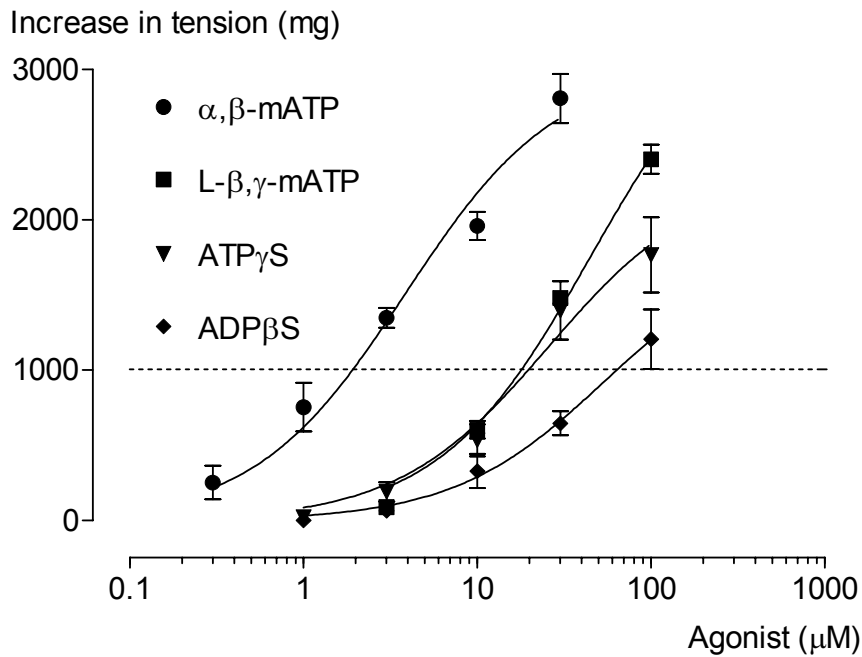
B.
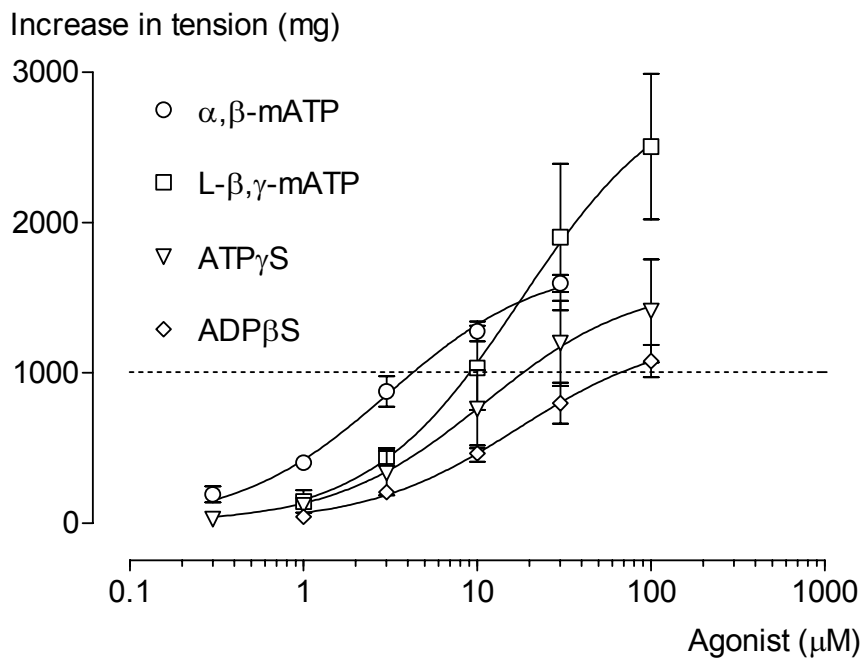
Fig. 3.28. Potency of the nucleotide agonists α,β-mATP, L-β,γ-mATP, ATPγS and ADPβS in eliciting contractions of rat vas deferens. Concentration-response curves of the agonists determined in prostatic (A.) and epididymal (B.) segments are shown. Contraction was measured in mg tension, ranking was based on the 1000 mg-level (dotted line). Error bars falling within the area of a symbol are not shown. Mean values (± S.E.M.) were derived from 3 to 6 individual determinations.

Tetrodotoxin (TTX; 1 μM, pre-incubated for 10 min) had no effect on contractions induced by α,β-mATP (10 μM) or ADPβS (30 μM) in prostatic as well as epididymal segments of ratVD (table 3.14.).

Table 3.14. Effect of tetrodoxin (TTX) on contractile responses (mg tension generated) of prostatic and epididymal segments of ratVD induced by α,β-mATP (10 μM) or ADPβS (30 μM), respectively. Data presented are mean values (± S.E.M.) from 3 individual observations.

Agonist (μM)	prostatic (mg tension)		epididymal (mg tension)	
	Control	TTX (1 μM)	Control	TTX (1 μM)
α,β-mATP (10)	1971 ± 240	2060 ± 209	1529 ± 97	1607 ± 97
ADPβS (30)	599 ± 46	576 ± 86	1057 ± 75	1008 ± 84

3.3.2. Desensitisation studies

To further characterise the P2 receptor subtypes mediating contraction of prostatic and epididymal segments of the ratVD, desensitisation of responses to high doses of α,β-mATP, L-β,γ-mATP and ADPβS was studied. Contractions of both segments of the ratVD completely vanished after 2 to 4 cumulative applications of α,β-mATP (10 μM) or L-β,γ-mATP (30 μM), respectively. In contrast, no desensitisation of contractile responses to ADPβS (30 μM) could be observed. Differences in the time course of desensitisation were obvious when α,β-mATP-induced contractions in prostatic and epididymal segments were compared: contraction of epididymal segments desensitised clearly slower than that of prostatic segments (29.5 ± 2.5 s and 14.0 ± 0.9 s, respectively, time measured when the maximal response decreased to 50 %, n = 4 - 5).

3.3.2.1. Contractions induced by α,β-methylene ATP and L-β,γ-methylene ATP

Contractile responses of ratVD induced by α,β-mATP (10 μM) were decreased to 9.0 $\pm$ 3.7 % and to 31.0 $\pm$ 2.9 % of those obtained in controls of prostatic and epididymal segments (n = 3), respectively, when the tissues were desensitised by 2 to 4 cumulative doses of L-β,γ-mATP (30 μM). In the reverse experiments, using α,β-mATP (10 μM, 2 to 3 cumulative doses) as desensitising agent, contractions to L-β,γ-mATP were nearly abolished, being 1.8 $\pm$ 0.5 % in prostatic and 6.0 $\pm$ 3.3 % in epididymal segments (n = 3).

3.3.2.2. Contractions induced by α,β-methylene ATP and ADPβS

ADPβS-induced contraction of ratVD decreased to 15.6 $\pm$ 2.2 % in prostatic segments after the tissues were desensitised by 2 to 3 cumulative applications of α,β-mATP (10 μM). In contrast, in epididymal portions, desensitisation using α,β-mATP (10 μM) left a residual response to ADPβS (30 μM) of 84.1 $\pm$ 18.5 %.

3.3.3. Antagonists

Inhibitory potencies (pIC_{50} values) of a series of suramin-related compounds and of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on contractions to α,β-mATP in prostatic and epididymal segments of the ratVD were studied. Additionally, the effect of NF279 on CRC's to α,β-mATP in epididymal segments of ratVD was determined and compared to the results obtained for the antagonistic potency of NF279 in prostatic segments (cf. 3.2.1.3.1.).

3.3.3.1. Comparison of pIC_{50} values

Differences in the antagonistic potencies of a series of P2 receptor antagonists were observed testing their inhibitory activity on contractile responses of prostatic and epididymal portions of the rat vas deferens induced by single doses of α,β-mATP (10 μM). Potency estimates (pIC_{50} values) determined in prostatic segments were higher (up to 4.2-fold) than those derived from non-linear regression analysis of concentration-inhibition curves assessed in epididymal portions of ratVD (table 3.15.).

Table 3.15. Differences in the antagonistic potencies of a series of P2 receptor antagonists on contractions of prostatic and epididymal segments of rat vas deferens induced by single doses of α,β-mATP (10 μM). Data are means $\pm$ S.E.M. from at least 3 observations (for the corresponding graphs see figure 3.29.).

Compound	ratVD		IC_{50} [E]/IC_{50} [P]
	prostatic segments	epididymal segments	
	pIC_{50}	pIC_{50}	
Suramin	4.68 ± 0.02	4.53 ± 0.02	1.4
NF037	4.79 ± 0.17	4.24 ± 0.10	3.5
NF279	5.71 ± 0.09	5.12 ± 0.11	3.9
NF031	4.96 ± 0.04	4.59 ± 0.03	2.3
NF023	4.93 ± 0.03	4.51 ± 0.04	2.6
PPADS	5.36 ± 0.06	4.74 ± 0.14	4.2

Inhibition of contraction (%)

Suramin (µM)

Inhibition of contraction (%)

NF037 (µM)

Inhibition of contraction (%)

NF279 (µM)
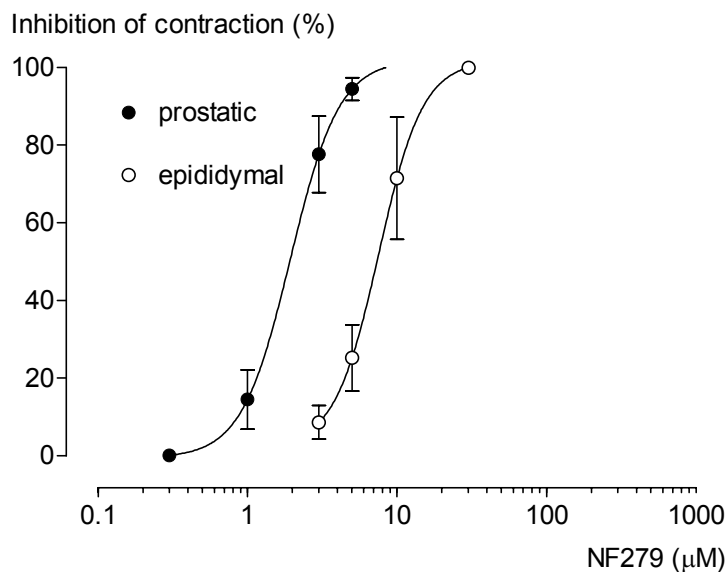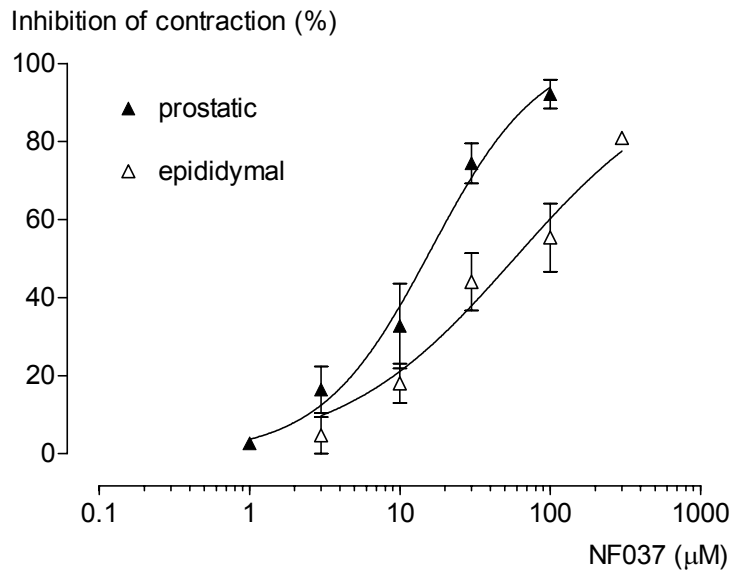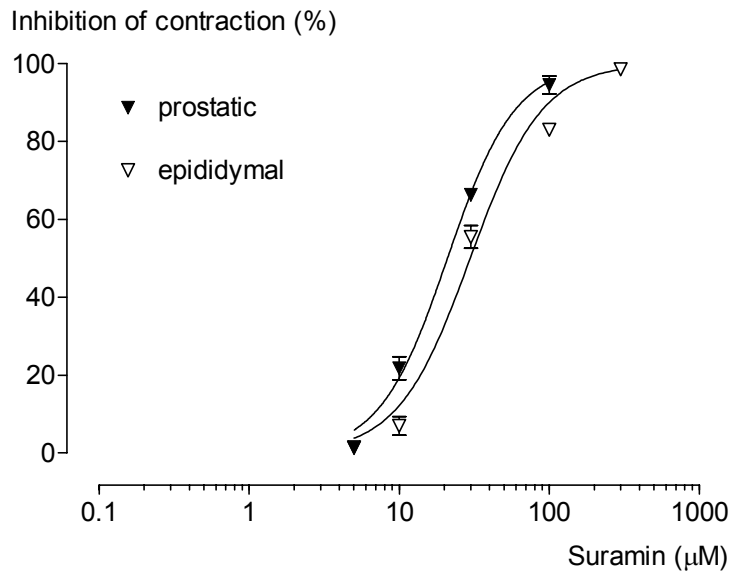
Fig. 3.29. *Concentration-inhibition curves obtained for the large ureas suramin, NF037 NF279 and NF031, the small urea NF023 and the pyridoxal-5-phosphate-derived compound PPADS in prostatic and epididymal segments of rat vas deferens. Given are mean percentages of inhibition ± S.E.M. (n = 3 - 4) of contractions induced by single doses of exogenously applied α,β-mATP (10 µM). The corresponding pIC$_{50}$ values are given in table 3.15.*

Inhibition of contraction (%)

NF031 (μM)

Inhibition of contraction (%)

NF023 (μM)

Inhibition of contraction (%)

PPADS (μM)
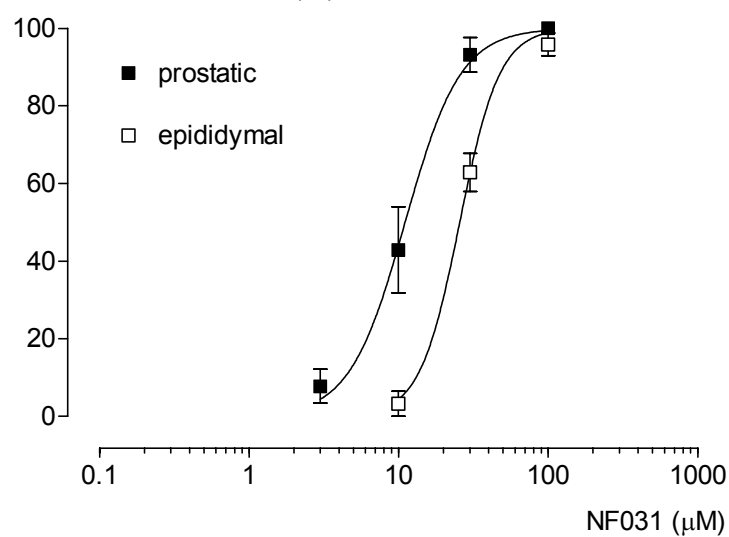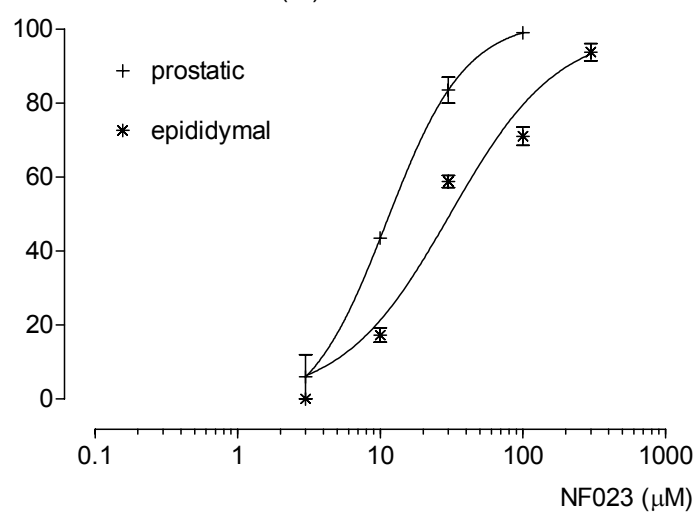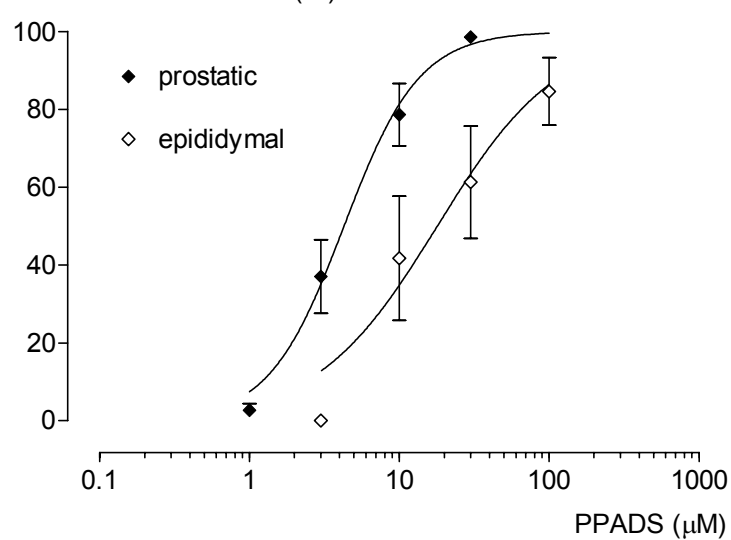
3.3.3.2. Effect of NF279 on concentration-response curves to α,β-methylene ATP in epididymal segments of rat vas deferens

As described for the inhibitory effects of NF279 in prostatic segments of ratVD (cf. 3.2.1.3.1.), increasing concentrations of the antagonist (3, 10 and 30 μM, 60-min exposure) shifted the CRC's to α,β-mATP in epididymal segments progressively to the right. pA_2 values calculated for each concentration of NF279 from the individual dose ratios, which were determined from equieffective concentrations of α,β-mATP (read from the 1000 mg tension level of the respective CRC's) in the absence and in the presence of the antagonist, were lower than those obtained in prostatic segments (table 3.16.).

Table 3.16. Antagonistic potency of NF279 on α,β-mATP-induced contractions of rat vas deferens with respect to the anatomical origin of the segment (60-min pre-incubation). pA_2 values (mean $\pm$ S.E.M.) were calculated from the individual dose ratios for each antagonist concentration (n = 3).

NF279 (μM)	pA_2 value	
	prostatic segments	epididymal segments
1	6.07 ± 0.08	n.d.
3	6.41 ± 0.07	5.70 ± 0.18
10	7.06 ± 0.08	5.93 ± 0.21
30	n.d.	6.41 ± 0.12

Similarly to the results obtained in prostatic segments, there was an increase in pA_2 values when the effect of increasing concentrations of NF279 was investigated on CRC's to α,β-mATP in epididymal segments of ratVD. An enhancement of contractions to α,β-mATP which was observed in the presence of NF279 (3 μM) also occurred in the time-matched controls (without incubation of antagonist; see figure 3.30.B.). However, no corrections were made to the agonist concentration ratios because this effect became relevant for contractions higher than 2000 mg tension. Comparing equieffective concentrations on the 1000 mg tension level no significant difference was detectable ($pEC_{1000} = 5.47 \pm 0.05$ for the overall controls and 5.57 ± 0.09 for the overall time control, respectively).

A.

Increase in tension (mg)

B.

Increase in tension (mg)
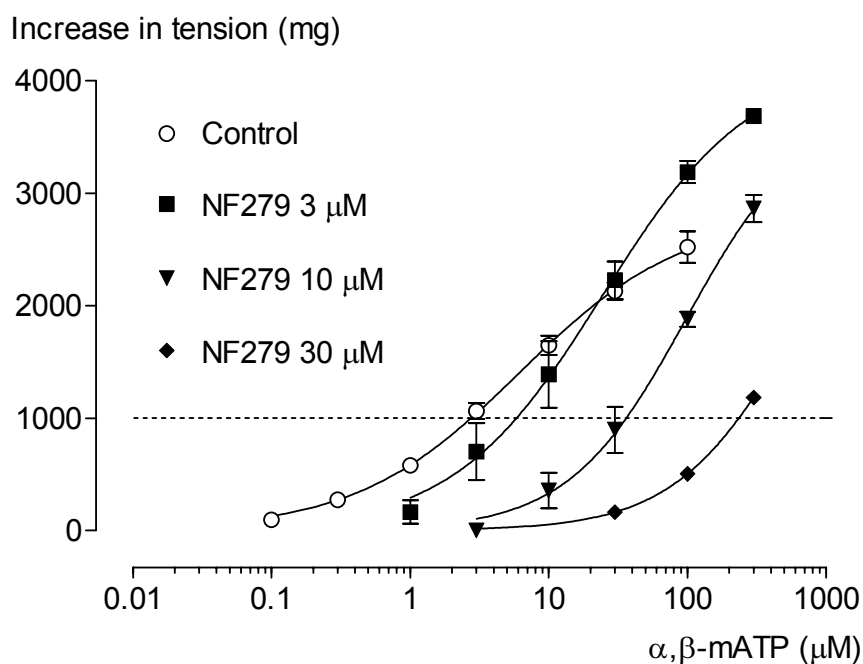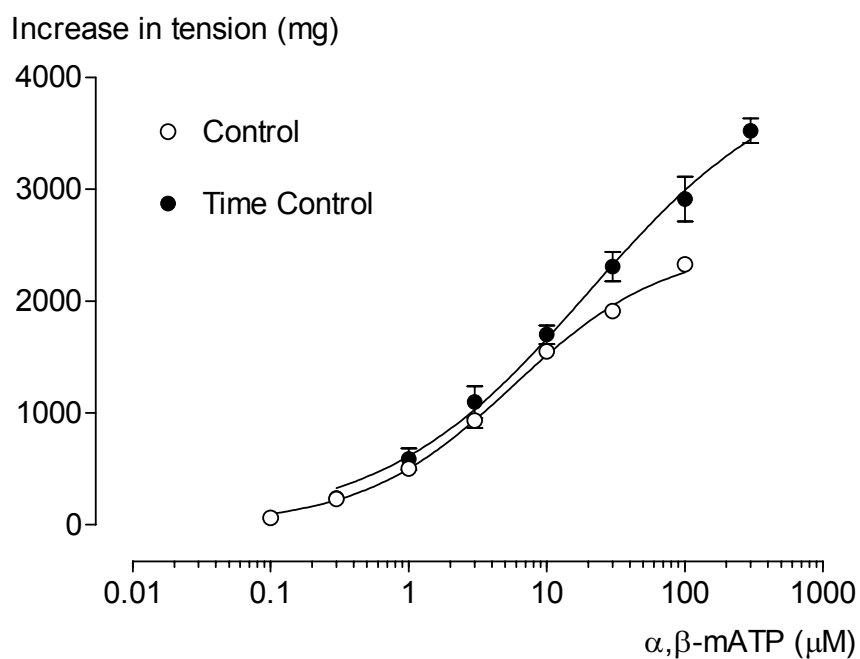
Fig. 3.30. A. *Antagonism of NF279 on α,β-mATP-induced contractions of epididymal segments of rat vas deferens (for corresponding pA_2 values see table 3.16.).* ***B.*** *Time-matched control experiments. Values are means $\pm$ S.E.M.; error bars falling within the area of the symbols are not shown.*

3.3.3.3. Inhibitory potency of NF279 on contractions of rat vas deferens induced by L-β,γ-mATP and ATPγS

The inhibitory potency of NF279 on contractile responses of both segments of ratVD induced by agonists with selectivity profiles distinct from that of α,β-mATP was examined. Concentration-inhibition curves of NF279 were constructed using single doses of exogenously applied L-β,γ-mATP (30 μM) and ATPγS (10 μM), and compared to concentration-inhibition curves of NF279 obtained to single doses of α,β-mATP (10 μM) (cf. 3.2.1.1.; 3.3.3.1.). Because of differences in the potencies of the agonists applied, a comparison on the basis of the resulting pIC_{50} values would have led to misinterpretations. A possibility to transform IC_{50} values into the dissociation constant of an antagonist (designated here as apparent pA_2 value [pA_2*]) was introduced by Cheng and Prusoff (1973). The applicability of the Cheng-Prusoff relationship for the calculation of antagonist dissociation constants from experiments in which a functional response such as muscle contraction is measured was described by Eglen and Whiting (1989). Instead of agonist affinities or as proposed by Eglen and Whiting (1989) EC_{50} values, equieffective concentrations of the agonists (read from the 1000 mg tension level, cf. 3.3.1.) were used for calculation. The IC_{50} values were derived from non-linear regression analysis of the concentration-inhibition curves obtained to increasing concentrations of NF279 in the presence of a single concentration of the respective agonist (for results see table 3.17.).

The resulting pA_2* values should not be considered as the antagonists dissociation constants (i.e. antagonist affinities), they are of use as rough estimates allowing a comparison of the antagonistic potencies of NF279 obtained from the experimental protocol described above. Comparing the antagonistic potency of NF279 on contractions induced by single doses of α,β-mATP (10 μM) in prostatic and epididymal segments of ratVD on the basis of the pA_2* values, differences enhanced (cf. table 3.15.; 3.17.), as could have been expected, regarding the relation between the agonist and the antagonist concentration: the higher the potency of the agonist (i.e. the higher the agonist concentration used to evoke a contractile response) the higher the concentration of antagonist required to inhibit 50 % of the agonist-induced response (i.e. the higher the resulting IC_{50}). pA_2* values obtained for NF279 in prostatic segments using α,β-mATP, L-β,γ-mATP and ATPγS as agonists were similar. In epididymal segments, the

highest potency for NF279 was found when contractions were induced by the P2X$_1$-selective agonist L-β,γ-mATP.

Tab. 3.17. Inhibitory potency (pIC$_{50}$ and apparent pA$_2$ [pA$_2$*]) of NF279. Data (pIC$_{50}$ = means $\pm$ S.E.M.; pA$_2$* = means) were derived from non-linear regression analysis of the antagonist's concentration-inhibition curves. pA$_2$* values were calculated by use of the Cheng-Prusoff relation (Cheng and Prusoff, 1973).

Agonist (μM)	Prostatic segments		Epididymal segments	
	pIC$_{50}$	pA$_2$*	pIC$_{50}$	pA$_2$*
α,β-mATP (10)	5.71 $\pm$ 0.09	6.51	5.12 $\pm$ 0.11	5.64
L-β,γ-mATP (30)	5.91 $\pm$ 0.03	6.34	5.43 $\pm$ 0.05	6.07
ATPγS (10)	6.00 $\pm$ 0.02	6.18	5.57 $\pm$ 0.06	5.76

3.3.4. Contractions of epididymal segments of rat vas deferens induced by ADPβS

Two observations supported the view, that the P2 receptors mediating contractions of epididymal segments of ratVD elicited by ADPβS might be, at least in part, different from the P2 receptor population activated by α,β-mATP: (1) desensitisation using α,β-mATP decreased contractile responses of epididymal segments of ratVD induced by ADPβS to about 80 % of those obtained in control experiments (see 3.3.2.2.). (2) No desensitisation of ADPβS-evoked contraction neither in prostatic nor in epididymal segments was observed. Due to the lack of desensitisation of contraction elicited by ADPβS, the possibility of constructing CRC's in a cumulative instead of single dose fashion was examined. The resulting CRC's (see figure 3.31.) were almost superimposable. As previously described for CRC's obtained to a variety of agonists in ratVD, CRC's to ADPβS did not reach a well-defined maximum as well, thus, agonist potencies could not be determined on the basis of their EC$_{50}$ values. Comparison of equieffective concentrations (read from the 500 mg tension level) yielding pEC$_{500}$ values of 4.84 $\pm$ 0.08 for CRC's to single doses of ADPβS (added in 15-min intervals) and 4.93 $\pm$ 0.11 for CRC's of cumulatively applied ADPβS confirmed that applying the cumulative design no desensitisation of responses occurred. Based on this observation, all further

experiments were carried out constructing CRC's to cumulative applications of ADPβS.
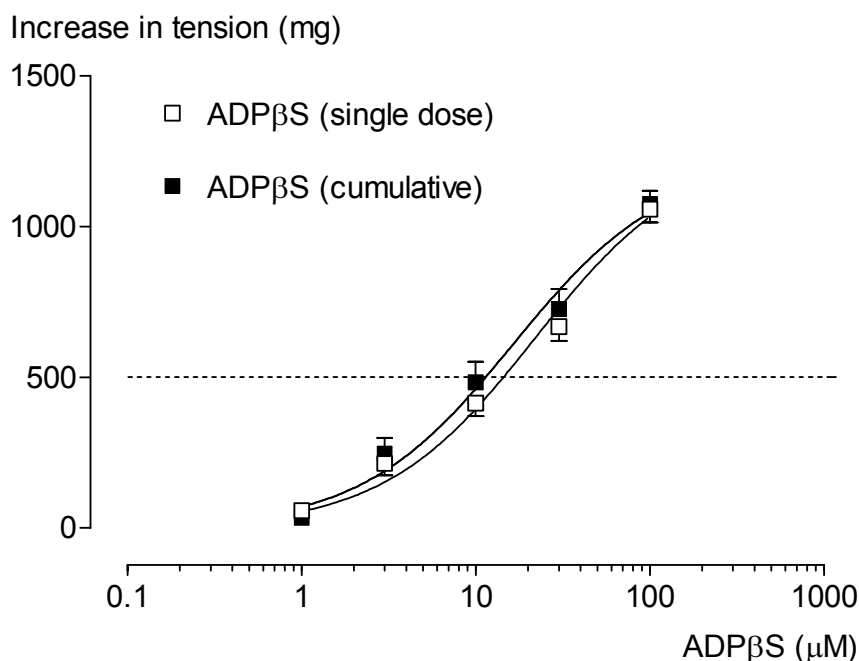
Fig. 3.31. *Comparison of single dose (15-min intervals) and cumulative concentration-response curves to ADPβS in epididymal segments of rat vas deferens. Data shown are means ± S.E.M. from 6 observations, some error bars were within the width of the symbols.*

3.3.4.1. Inhibitory effects of suramin, NF023, NF279 and MRS 2179

In order to identify the P2 receptor subtype(s) mediating contraction induced by ADPβS, the inhibitory effect of a series of P2 receptor antagonists with different selectivity profiles was examined on the agonist's CRC's in epididymal segments of ratVD. MRS 2179 (10 and 100 μM) had no effect on contraction induced by ADPβS (pEC_{500} = 4.90 ± 0.09 in the absence, and pEC_{500} = 4.77 ± 0.08 in the presence of 10 μM as well as 4.88 ± 0.06 in the presence of 100 μM MRS 2179; n = 3; see figure 3.32.A.). In contrast, higher concentrations of suramin, NF023 and NF279 shifted the ADPβS CRC's to the right. Additionally, a marked increase of contraction elicited by ADPβS was observed, especially in the presence of lower concentrations of the antagonists. Because of the complex nature of antagonism and the incompleteness of the CRC's (cf. figure 3.32.B.-D.), quantification of the extent of inhibition by calculating dose ratios from

equieffective concentrations in the absence and in the presence of antagonist, seemed to be not useful. Therefore the antagonists' effects on single doses of ADPβS (30 and 100 μM) were extracted from the CRC's (see figure 3.32.B.-D.) and expressed as a percentage of contractile responses obtained in the respective controls (i.e. in the absence of antagonists) (see figure 3.33.).

A.

Increase in tension (mg)

B.

Increase in tension (mg)
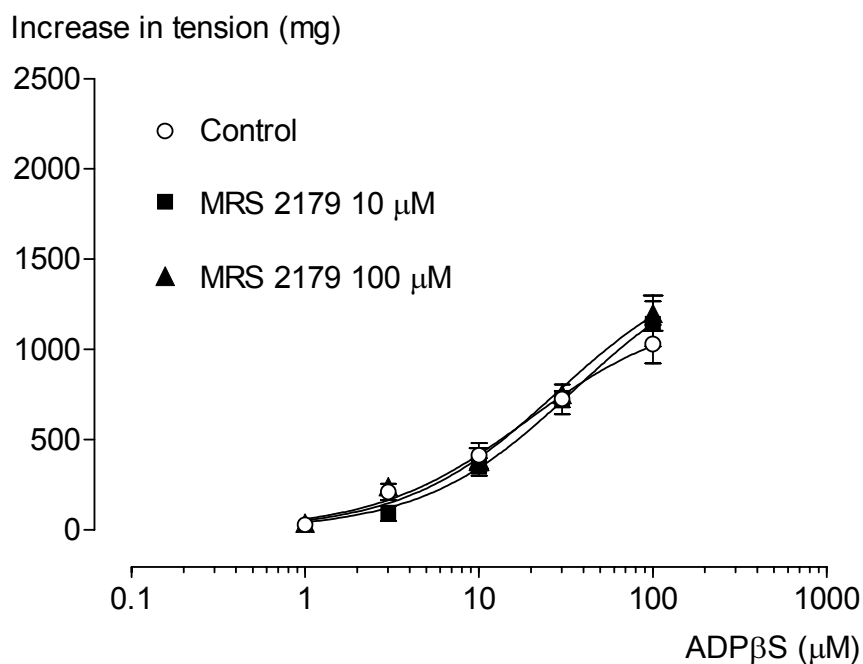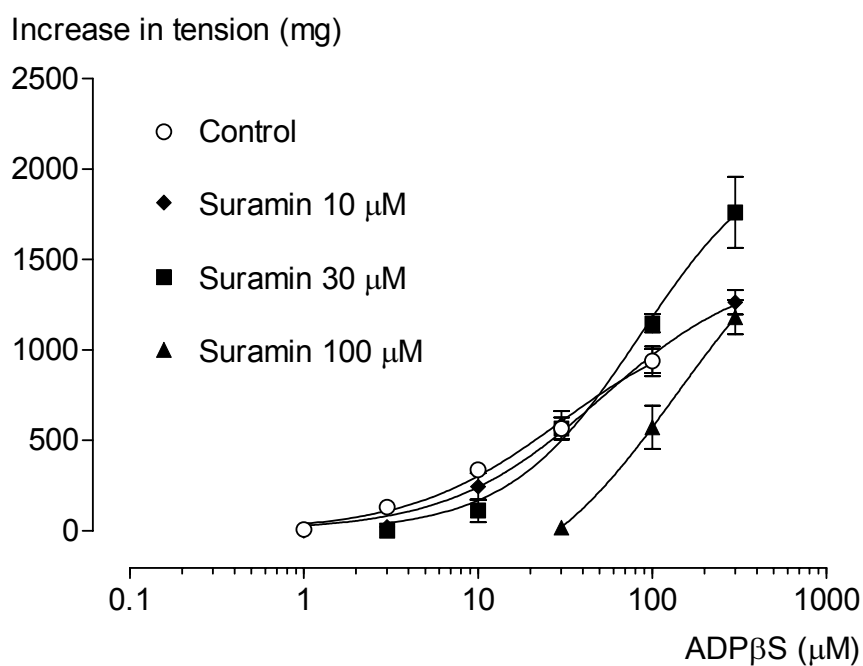
Fig.   3.32.   (legend see page 138)

C.

Increase in tension (mg)

D.

Increase in tension (mg)
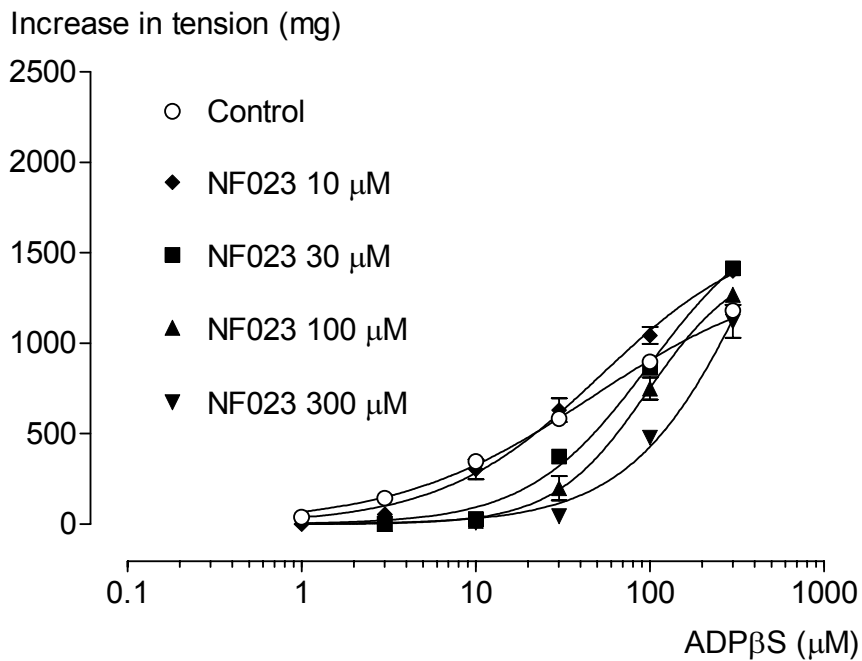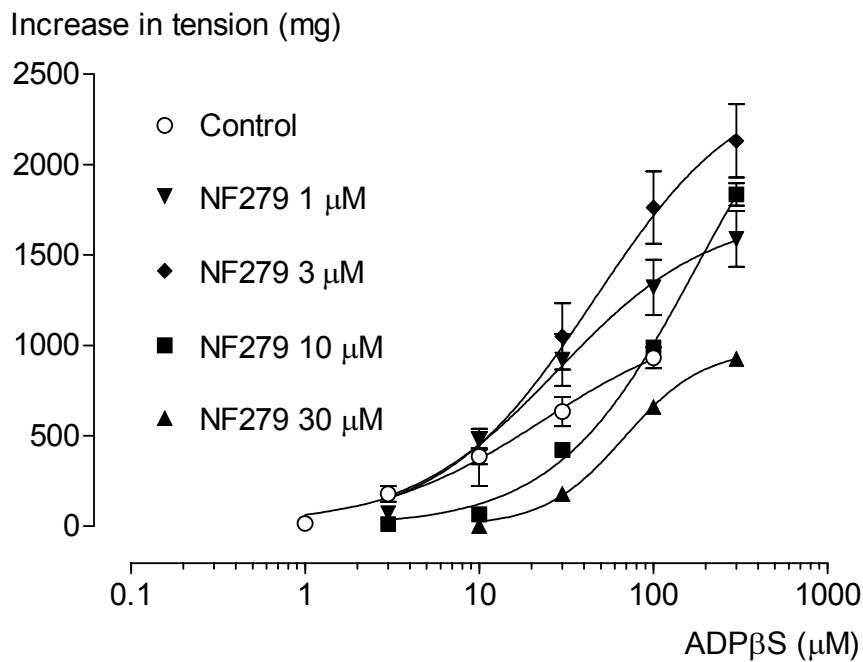
Fig. 3.32. *Antagonism of MRS 2179 (**A.**), suramin (**B.**), NF023 (**C.**) and NF279 (**D.**) on ADPβS-induced contractile responses of epididymal segments of rat vas deferens: concentration-response curves of ADPβS in the absence and in the presence of different concentrations of the antagonists. The data (means ± S.E.M.) were derived from 3 to 4 observations, some error bars were within the area of the symbols.*

As illustrated in figure 3.33., contractions induced by ADPβS (30 μM) were augmented in the presence of 1 and 3 μM of NF279 (129 ± 6 % and 146 ± 6 %, respectively, of contractions induced in the control CRC's) and rather not affected by 10 μM of NF023 (107 ± 10 %) as well as by 10 and 30 μM of suramin (111 ± 5 % and 93 ± 10 %, respectively). Inhibition of ADPβS (30 μM) -induced contractions appeared, when higher concentrations of NF279 (10 μM: 79 ± 3 % and 30 μM: 35 ± 9 %), NF023 (30 μM: 69 ± 9 %; 100 μM: 34 ± 10 % and 300 μM: 7 ± 4 %) and suramin (100 μM: 3 ± 2%) were present. An increase of ADPβS (100 μM) -evoked contractions was found for NF279 (1 μM: 132 ± 3 %, 3 μM: 176 ± 3 % and 10 μM 115 ± 8 %) and to a lesser extent for NF023 (10 μM: 118 ± 1 % and 30 μM: 106 ± 3 %) as well as for suramin (10 μM: 114 ± 5 % and 30 μM: 109 ± 6 %). Responses of epididymal segments of ratVD induced by ADPβS (100 μM) were inhibited using higher concentrations of NF279 (30 μM: 77 ± 2 %), NF023 (100 μM: 84 ± 6 % and 300 μM: 54 ± 4 %) and suramin (100 μM: 69 ± 12 %).

A.
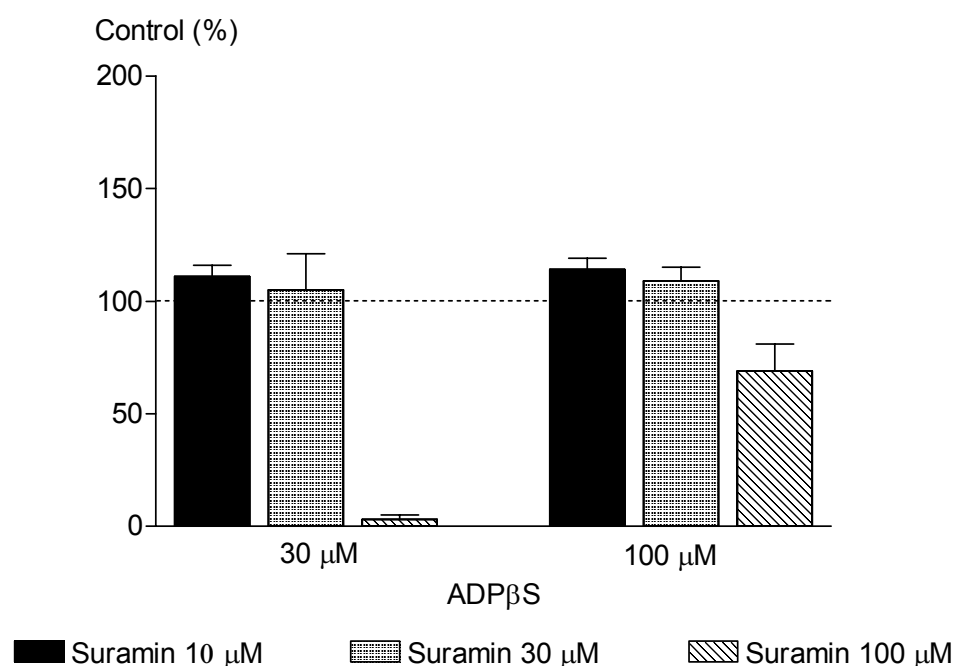
Fig. 3.33. *(legend see p. 140)*

B.
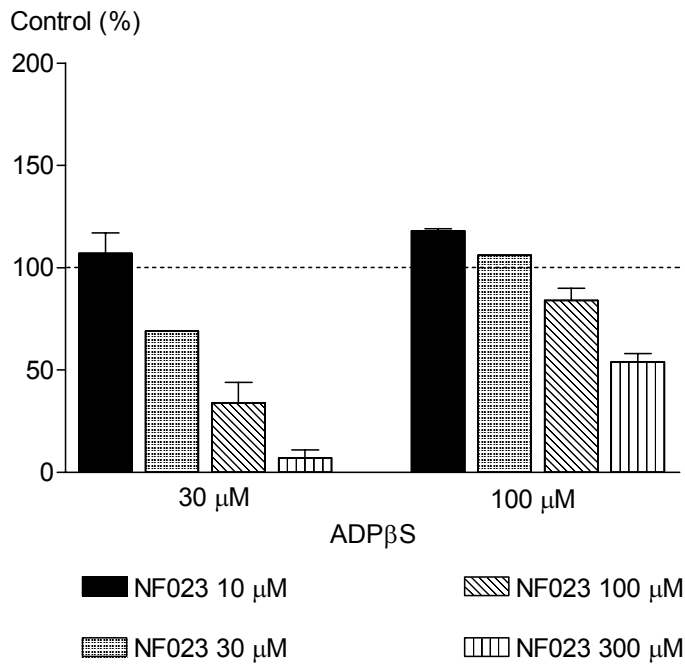
C.
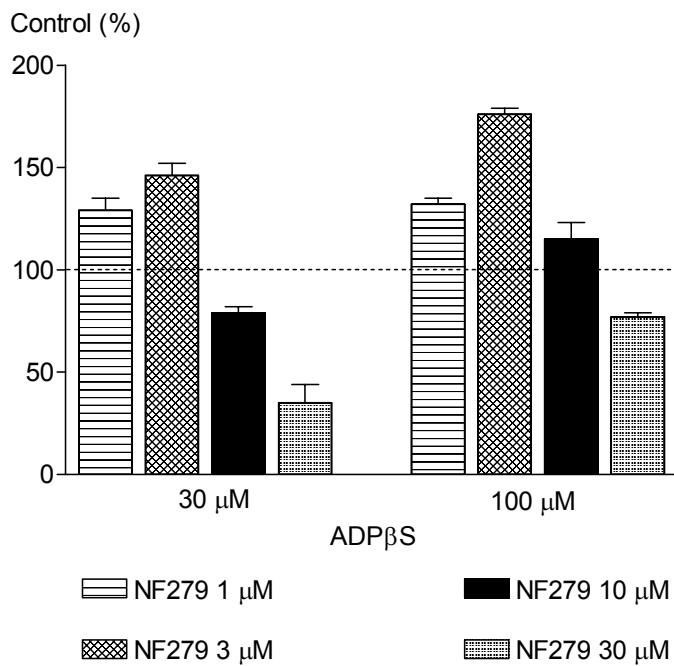
Fig. 3.33. *(pages 139 and 140) Effects of suramin (**A.**), NF023 (**B.**) and NF279 (**C.**) on contractions of epididymal segments of rat vas deferens induced by ADPβS (30 and 100 μM). Responses are expressed as percentage (means + S.E.M.) of contractions obtained in the respective control experiments (n = 3 - 4).*

4. Discussion

4.1. Screening of suramin- and NF023-related compounds

4.1.1. Applicability of the rabbit vas deferens for the evaluation of compounds displaying P2X receptor-blocking potency

Electrical field stimulation of the vas deferens of mouse, rat, rabbit and guinea-pig produces a biphasic contractile response, which is thought to result from the combined action of ATP and NA released as cotransmitters (for review see von Kügelgen and Starke, 1991; Sneddon et al., 1996). The initial phase of the contraction is blocked by either desensitisation with α,β-mATP or by the P2 receptor antagonist suramin and is therefore thought to be mediated by ATP released from sympathetic nerves acting on P2 receptors present on the smooth muscle. The second component of the neurogenic response is blocked by α-adrenoceptor antagonists, and is thought to be mediated by NA acting on α-adrenoceptors. The rank order of agonist potency for eliciting contraction of the RVD, being α,β-mATP >> 2-MeSATP > ATP > UTP (Lambrecht et al., 1992; Windscheif, 1995), indicates the presence of P2X receptors. However, the fact that α,β-mATP was the most potent but least efficacious agonist might suggest that ATP and 2-MeSATP act at an additional P2 receptor subtype present in the RVD smooth muscle. As contractions of RVD were also seen in response to UTP, this second subtype may be a member of the P2Y receptor family; both recombinant $P2Y_2$ and the rat orthologue of the $P2Y_4$ receptor were shown to be sensitive to ATP and UTP (Lazarowski et al., 1995; Bogdanov et al., 1998b). Heterogeneity of P2 receptors has been reported not only for ratVD (Bültmann and Starke, 1994b; Bültmann et al., 1999b), mouse vas deferens (von Kügelgen et al., 1990) and guinea-pig vas deferens (Bailey and Hourani, 1994) but also for the RVD (Lambrecht et al., 1996b). Moreover, in native tissues, the presence of more than one P2 receptor subtype appears to be the rule rather than the exception (see Ralevic and Burnstock, 1998). In the light of these observations, a screening assay for the assessment of P2X receptor-blocking potencies of suramin derivatives based on electrical field stimulation (EFS), resulting in release of the naturally occurring non-selective ligand ATP to induce contraction of the RVD, did not appear to be the appropriate method. The dual effect of the parent compound suramin, to antagonise P2 receptors and inhibit ATP-degrading enzymes with nearly similar potency, thereby producing a phenomenon referred to as "self-cancellation" when its antagonistic effect is examined using agonists,

including ATP, which are not metabolically stable, also argues against those studies applying EFS (Crack et al., 1994). A further problem is, that the amount of ATP being released by nerve stimulation might be affected by several factors, including the frequency of stimulation and the effect (inhibitory as well as potentiating) of other transmitter substances which are known to be co-released with ATP (von Kügelgen and Starke, 1991). Finally, breakdown products of ATP, such as adenosine, were shown to be active in RVD. Adenosine has been shown to act at A_1 receptors mediating inhibition of both the purinergic and adrenergic component of the mechanical response (Windscheif, 1995).

To circumvent the problems discussed above, the metabolically stable nucleotide agonist α,β-mATP, which was reported to be selective for P2X receptors (Bianchi et al., 1999), was applied to evoke contraction of the RVD. CRC's, constructed in order to establish whether differences in the sensitivity towards α,β-mATP exist with regard to the anatomical origin of the segments, yielded similar potency estimates but marked differences in the tension generated for the prostatic segment compared to the segments 2 - 6 (tables 3.1. and 3.2.). A regional variation of purinergic and adrenergic responses in RVD with NA being more effective in eliciting contractions of the prostatic end while contractions evoked by ATP were significantly lower in prostatic compared to the epididymal segments was reported by Windscheif (1995). However, whether those differences are due to differential sensitivity of the segments (i.e. postjunctional receptor distribution, post-receptor activation events) has not been addressed. In contrast, regardless of the species studied, the opposite, NA being more potent in epididymal than in prostatic portions, and ATP and α,β-mATP being more potent in the prostatic end, has been observed (Sneddon and Machaly, 1992). The reason for this discrepancy is unclear.

The selectivity of α,β-mATP for recombinant homomeric $P2X_1$ and $P2X_3$ as well as heteromeric $P2X_2P2X_3$ and $P2X_1P2X_5$ receptors, being inactive at all other P2 receptor subtypes that have been cloned so far, is well established (Ralevic and Burnstock, 1998; Lambrecht, 2000; North and Surprenant, 2000). Comparison of the rank order of potency estimates for suramin, PPADS, and NF023 antagonising α,β-mATP-induced contractions of RVD and inhibiting $P2X_1$, $P2X_3$ and $P2X_2P2X_3$ receptor-mediated currents elicited by ATP or α,β-mATP, respectively, resulting in PPADS > NF023 > suramin for the P2X receptor in RVD and the same

profile for the rP2X$_1$ receptors, clearly indicates that the properties of the α,β-mATP-sensitive P2 receptor present in RVD correspond well with those of recombinant P2X$_1$ receptors (Lambrecht et al., 1999; Lambrecht, 2000; Soto et al., 1999; see table 4.1.).

Table 4.1. Comparison of pIC$_{50}$ and pA$_2$ values determined at P2X receptors mediating contraction to α,β-mATP in rabbit vas deferens and recombinant rat P2X receptor subtypes (data taken from Lambrecht et al., 1996b, 2000b; Bianchi et al., 1999; Soto et al., 1999; [a]Lambrecht and Niebel, personal communication).

Tissue/Receptor	pA$_2$/pIC$_{50}$		
	PPADS	**Suramin**	**NF023**
RVD	6.34/5.55[a]	5.10/4.75	5.68/4.97
rP2X$_1$	n.d./7.06	n.d./ > 5	5.96/6.62
rP2X$_3$	n.d./6.11	n.d./5.44	n.d./5.07
rP2X$_2$P2X$_3$	n.d./5.90	n.d./6.09	n.d./5.85

In order to investigate a large number of compounds, of which only very small amounts were available, the pIC$_{50}$ method was further simplified, testing the effect of 10 μM of the derivatives, a concentration similar to the IC$_{50}$ assessed for NF023 (see table 4.1.), on contractions evoked by 1 μM α,β-mATP. Compared to vasa deferentia of other species, the RVD offers a further advantage, allowing to test 8 different compounds in parallel, because of the number of segments obtained per animal. This screening assay therefore proved to be a valid method to examine the P2X$_1$ receptor-blocking potency of suramin- and NF023-derivatives.

4.1.2. Applicability of the guinea-pig taenia coli for the evaluation of compounds displaying P2Y receptor-blocking potency

The guinea-pig taenia coli has been the prototype tissue for the study of native P2Y receptors (Burnstock and Kennedy, 1985; Cusack, 1993). However, recent investigations indicate that besides the P2Y receptor, relaxation of the carbachol-pre-contracted tissue is also mediated by a yet unidentified P2 receptor subtype sensitive to α,β-mATP (Windscheif et al., 1995; Dudeck et al., 1995;

Bültmann et al., 1996a; Lambrecht et al., 1996b, 1999). The rank order of agonist potency obtained for nucleotides eliciting relaxation of the carbachol-pre-contracted GPTC, 2-MeSATP > ADPβS > α,β-mATP > ATP > adenosine (Dudeck et al., 1995) confirms the presence of a heterogeneous P2 receptor population. Further indications were the biphasic Schild plots observed for PPADS blocking α,β-mATP- as well as ATP- and 2-MeSATP-induced relaxation of GPTC (Windscheif et al., 1995). In addition, differences in antagonist potency reported for PPADS, DIDS, and the suramin-derived compounds NF023 and 8-(3,5-dinitro-phenylenecarbonylimino)-1,3,5-naphthalenetrisulfonic acid (XAMR0721), all of these antagonists being more potent in blocking responses elicited by α,β-mATP compared to those induced by ADPβS (Dudeck et al., 1995, Bültmann et al., 1996a, Lambrecht et al., 1996b), are in favour of a mixed P2 receptor population mediating relaxation of GPTC. The capacity of some of the antagonists to inhibit ecto-nucleotidases as well, should not be relevant for studies applying the metabolically stable nucleotide analogues α,β-mATP and ADPβS (Welford et al., 1986) and therefore a contribution of the inhibition of ATP-degrading enzymes to the differences observed seems to be very unlikely. Based on the selectivity of α,β-mATP for $P2X_1$, $P2X_3$ and heteromeric $P2X_2P2X_3$ and $P2X_1P2X_5$ receptors (North and Surprenant, 2000) the presence of at least one of these proteins in GPTC must be anticipated. However, the biphasic Schild plot obtained for PPADS blocking α,β-mATP-induced relaxation might indicate a heterogeneity even within the α,β-mATP-sensitive receptors in GPTC (Windscheif et al., 1995). Another surprising finding is the slow onset of the relaxant response of GPTC to α,β-mATP (Windscheif et al., 1995), a feature which is usually attributed to effects mediated via G protein-coupled P2Y receptors rather than ionotropic P2X receptors, as well as to the involvement of further transmitter substances/second messengers. Therefore, the effects of TTX, indomethacin and 8-SPT on CRC's to α,β-mATP were investigated. However, none of these compounds affected the α,β-mATP-induced relaxation of GPTC, thereby indicating that the agonist's site of action is most likely directly on P2 receptors present on the smooth muscle (Dudeck et al., 1995).

There is also controversy about the ADPβS-sensitive receptor being a species orthologue of the recombinant $P2Y_1$ receptor (Ralevic and Burnstock, 1998; Lambrecht et al., 1999).

Table 4.2. Comparison of antagonist potency estimates (pA_2 and [a]pIC_{50} values) obtained at turkey $P2Y_1$ receptors (Boyer et al., 1996a; J. L. Boyer and T. K. Harden, personal communication; Charlton et al., 1996; Camaioni et al., 1998), for ADPβS-induced relaxation mediated via taenia coli P2Y receptors (Lambrecht et al., 1996b; Bültmann et al., 1998; [b]pA_2 determined in one experiment for MRS 2179 30 μM, results not shown), and for ADPβS-induced contractions of guinea-pig ileal longitudinal smooth muscle (Czeche et al., 1998b; Lambrecht et al., 1999; G. Lambrecht, personal communication). n.d. = not determined.

Receptor	PPADS	Suramin	NF023	NF279	A3P5PS	MRS 2179
$tP2Y_1$	5.98	5.77	4.58[a]	4.63[a]	6.46	6.48[a]
P2Y (GPTC)	4.59	4.71	4.35	4.10	5.11	5.81[b]
P2Y (GPI)	6.20	5.68	4.74	5.32	n.d.	6.38[a]

Comparing antagonist potency estimates obtained at the recombinant turkey $P2Y_1$ with native P2Y receptors present in guinea-pig taenia coli and ileum, strikingly all compounds are roughly 10-fold less potent in taenia coli. The differences in potency for NF279 between $tP2Y_1$ and GPI P2Y receptors can be explained at least in part by differences in the experimental protocols applied, i.e. pre-incubation of antagonist in the tissue experiments and co-application of agonist and antagonist in the $tP2Y_1$ assays (J. L. Boyer and T. K. Harden, personal communication). However, this could not be accounted for the intraspecies differences observed for the two native P2Y receptors of guinea-pig (GPI and GPTC). On the one hand, antagonism of MRS 2179, NF023, NF279 and PPADS argue in favour of the $P2Y_1$ receptor subtype as inactivity of these antagonists was reported for $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (Lambrecht, 2000; Nandanan et al., 2000; von Kügelgen and Wetter, 2000). α,β-mATP-sensitive P2X receptors can be ruled out because no cross-desensitisation of relaxation of GPTC induced by ADPβS and α,β-mATP, respectively, was observed (Dudeck et al., 1995; North and Surprenant, 2000) and due to the weak antagonistic activity of NF023 (Soto et al., 1999). Moreover, ADPβS was shown to be inactive at recombinant $P2X_2$ receptors and only weakly active at the $P2X_4$ subtype (Bianchi et al., 1999). A contribution of these receptor subtypes can furthermore be excluded based on the antagonistic effect of MRS 2179 (Nandanan et al., 2000).

On the other hand, the potency observed for the antagonists is much lower in GPTC compared to the P2Y receptor present in the ileal longitudinal muscle (table 4.2.), the pharmacology of which is very similar to that of the recombinant $P2Y_1$ receptor (Czeche et al., 1998b; Lambrecht et al., 1999). However, the rank order of antagonist potencies for recombinant $tP2Y_1$ and taenia coli P2Y is very similar. On the basis of the remarkable impact of species differences with respect to the potency or activity of agonists and antagonists (see 1.1.2.2.1.1. and 1.1.2.2.2.1.), observed for example for species orthologues of $P2X_4$ and $P2Y_4$ receptors, it can be speculated that the taenia P2Y and the ileum P2Y might represent two isoforms of the guinea-pig $P2Y_1$ receptor. Taking into account, that the exchange of one amino acid renders PPADS-sensitivity to the PPADS-insensitive rat $P2X_4$ wild type receptor (Buell et al., 1996b), it is obvious, that very small variations of the primary structure of the receptor protein can have dramatic effects on its pharmacological characteristics. Nevertheless, the presence of a functional P2Y receptor subtype in GPTC that has not yet been cloned, cannot be excluded on the basis of these observations. Thus, this question will be resolved with the cloning of the P2 receptors from guinea-pig ileum and taenia coli. Unfortunately, except for the $P2X_2$ subtype, none of the other known recombinant P2 receptors have been cloned from guinea-pig tissue so far (Housley et al., 1999). Hence, the GPTC, although it is still one of the standard tissues to study native P2Y receptors, does not seem to be the ideal one, because of the problems discussed above. However, by the use of the metabolically stable agonist ADPβS, some of these problems can be circumvented, and even though the absolute potency determined for various antagonists in GPTC and GPI is clearly different, the rank order of antagonist potencies differs only marginally (table 4.2.).

In accordance with the screening assay applied for the study on native P2X receptors in RVD, inhibitory effects suramin analogues on CRC's to ADPβS were investigated at a single concentration of 10 μM. On the basis of the results obtained in GPTC, the fact that examination of a higher concentration of the compounds would have been more useful, has to be discussed. Unfortunately, most of the analogues as well as even the reference drugs suramin and NF023, did not show any significant inhibitory potency at the concentration investigated. Therefore, it is obvious, that a comparison of their potentially differing antagonistic effects on native P2Y receptors is impossible. On the other hand, the screening assays were not primarily designed to assess SAR's for suramin- and

NF023-derived large and small ureas, but to identify interesting compounds (displaying higher antagonistic potency and/or selectivity), worth performing a more detailed pharmacological characterisation with. Accordingly, to test equal concentrations of potential antagonists at both the native P2X and P2Y receptors allows to assess the selectivity of a certain analogue more exactly.

4.1.3. Comparison of the applicability of guinea-pig taenia coli and *Xenopus laevis* oocytes as ecto-nucleotidases bioassays

The exact identity of the ATP-degrading enzymes located on the cell surface of several smooth muscle preparations is still unknown. However, it is apparent from a number of studies, that enzymatic degradation of ATP (and other nucleotides) and some of its synthetic analogues may influence the pharmacological characterisation of P2 receptors, and lead to misinterpretations. On the one hand, the potency of agonists susceptible to enzymatic breakdown was underestimated (Humphrey et al., 1995; Kennedy and Leff, 1995) and thus, some of the P2 receptors originally characterised by rank orders of agonist potencies had to be redefined (cf. 1.1.). On the other hand, a phenomenon called "self-cancellation" occurred with antagonists such as suramin displaying dual enzyme inhibitory and P2 receptor antagonistic properties (Crack et al., 1994; Humphrey et al., 1995). Inhibition of ATP breakdown results in a potentiation of effects of ATP or other enzymatically unstable nucleotides and a leftward shift of the agonists' CRC, in contrast, P2 receptor blockade causes a rightward shift of the CRC and therefore suramin appeared to be rather inactive in certain preparations (Humphrey et al., 1995).

At present, there are two families of ecto-nucleotidases thought to play a predominant role in the inactivation of extracellular nucleotides. The members of the E-NTPDase family (syn. ecto-ATPases, ecto-apyrase, ecto-ATP-diphospho-hydrolase) are capable of hydrolysing nucleoside 5'-tri- (and) diphosphates generating the respective dephosphorylated mono- or dinucleotide and inorganic phosphate, and enzymes capable of hydrolysing ATP and ADP to AMP and either pyrophosphate or inorganic phosphate belonging to the E-NPP family (Zimmermann, 2000; Zimmermann et al., 2000; cf. 1.1.5.).

In GPTC, the presence of Ca^{2+}/Mg^{2+}-dependent nucleotide-degrading enzymes has been demonstrated (Ziyal, 1996). These enzymes were reported to display a broad substrate specificity metabolising ATP as well as other purine and pyrimidine nucleotides (Ziyal, 1996). In general, the coexistence of several nucleotide degrading enzymes in the same tissues is the rule rather than the exception (Zimmermann, 1999). For this reason, it is of some relevance when studying the impact of ecto-nucleotidases and enzyme inhibitors on the pharmacology of P2 receptor agonists and antagonists at native P2 receptors, to perform these studies in the same tissue. Unfortunately, the GPTC did not prove to be suitable for the investigation of the inhibitory activity of a larger number of compounds. In contrast to results obtained in a previous study (Ziyal, 1996), applying a similar experimental protocol the extent of inhibition of ATP degradation by suramin was considerably lower in the present study. Moreover, the inhibitory effect of suramin appeared to be limited. An explanation for these observations might be provided by results obtained in a recent study, analysing the inhibitory potency of suramin at recombinant NTPDase1 (CD39) and NTPDase2 (CD39L1). In this study, suramin at a concentration of 100 μM, was reported to be markedly more potent in inhibiting NTPDase2 than NTPDase1 (Heine et al., 1999). NTPDase1 was shown to hydrolyse ATP directly to AMP, without any detectable ADP in the medium. The hydrolysis of ADP, applied as substrate, to AMP revealed the same time course as that of hydrolysis directly from ATP to AMP. Expression of NTPDase2 alone led to the accumulation of ADP, which was only slowly further degraded to AMP by the enzyme. When both enzymes were co-expressed, AMP and ADP could be detected as hydrolysis products. AMP as a result of ATP degradation by both enzymes and of ADP degradation by NTPDase1 and ADP due to hydrolysis of ATP by NTPDase2. Assuming that both kinds of enzymatic activities are present in GPTC, hydrolysis of ATP by NTPDase2 leads to the formation of 1 P_i per molecule whereas breakdown of ATP by NTPDase1 generates 2 P_i . Under control conditions, both exogenously applied ATP as well as ADP, originating from the breakdown of ATP by NTPDase2 compete as substrates for NTPDase1, generating either 1 or 2 P_i per molecule ATP. In contrast, in the presence of suramin, ATP-degradation by NTPDase2 is largely inhibited, but ATP can be metabolised directly to AMP. Therefore, the possibility that inhibition of only one of a set of enzymes that might be present in GPTC, does not necessarily lead to a marked decrease in the amount of inorganic phosphate produced over a certain time course has to be taken into account.

However, the reason for the difference in the inhibitory potency of suramin in GPTC determined in the previous (Ziyal, 1996) and the present study is not clear.

The nucleotide-metabolising enzymes present on folliculated *Xenopus laevis* oocytes have been demonstrated to degrade all naturally occurring purine and pyrimidine nucleoside triphosphates as well as ADP and AMP (Ziganshin et al., 1995, 1996). In the absence of the divalent cations Ca^{2+} and Mg^{2+} P_i production was found to be decreased to 10 %, indicating the presence of Mg^{2+}- and/or Ca^{2+}-dependent enzymatic activity (Ziganshin et al., 1995) which is a general feature of ecto-nucleotidases. Under the experimental conditions of the present study the contribution of intracellular enzymes to the extent of ATP degradation can be excluded due to the following observations: neither had ouabain, as an inhibitor of the intracellular Na^+/K^+-ATPase, nor oligomycin, an inhibitor of mitochondrial (F-type) ATPases as well as sodium azide, a general inhibitor of intracellular ATPases which was also reported to have inhibitory effects on ecto-NTPDases, but only when applied at very high concentrations (Plesner, 1995; Zimmermann, 1996b), any effect on measured ATPases activity, i.e. generation of inorganic phosphate (Ziganshin et al., 1995). Although degradation of AMP indicates the presence of nucleoside monophosphate degrading ecto-5'-nucleotidase activity in *Xenopus* oocytes, this enzyme should not play any role when using ATP as substrate, because it is inhibited by micromolar concentrations of ATP and ADP (Zimmermann, 1996b). Furthermore, nucleotide-degrading activity by the non-specific alkaline phosphatases could be separated from ecto-nucleotidases activity because of their pH-optimum of about 8.5 (Fox, 1978; Zimmermann, 1996a). Additionally, inactivity of alkaline phosphatases was confirmed by the lack of generation of P_i from p-nitrophenyl phosphate which serves as a substrate for the enzyme (Ziganshin et al., 1995; see van der Ven and Hinds, 1996). The amount P_i detected should therefore be largely due to the degradation of ATP by enzymes belonging to the E-NTPDase family or the NPP family. The detection of P_i to assess the activity of ecto-nucleotidases does not allow to exactly characterise the enzymes involved in ATP-degradation, however, it represents a robust and convenient method to study effects of potential enzyme inhibitors. When using either ATP or ADP as substrate, slight differences were reported to occur in the extent of inhibition of certain compounds depending on the substrate analysed (Ziganshin et al., 1996), indicating that ATP and ADP might be degraded by separate enzymes.

In contrast to results obtained in GPTC, suramin was shown to inhibit ecto-nucleotidases present on folliculated *Xenopus* oocytes in a dose-dependent manner, thereby confirming data from previous studies performed in oocytes (Ziganshin et al., 1995, 1996) as well as in smooth muscle preparations (Bültmann et al., 1996c; Ziyal, 1996), platelets (Beukers et al., 1995) and molecularly defined ecto-NTPDases (Heine et al., 1999). Furthermore, inhibition of ATP-degradation could also be demonstrated for the suramin-derived compound NF023 and the previously introduced ecto-nucleotidases inhibitor ARL 67156 (table 3.3.). Both compounds were described to display inhibitory potency on ATP-degrading enzymes present in smooth muscle preparations (Crack et al., 1995; Bültmann et al., 1996c; Ziyal, 1996), and for ARL 67156 ecto-nucleotidases-inhibiting activity has also been reported in blood cells (Crack et al., 1995). In accordance with results obtained in previous studies (Bültmann et al., 1996c; Ziyal, 1996), suramin was shown to be slightly more potent in inhibiting ATP-degradation than NF023. Consequently, the inhibitory potency of the suramin analogues was examined using the *Xenopus* oocytes assay, which in contrast to GPTC smooth muscle strips proved to be more suitable for the screening of a larger number of compounds.

4.1.4. Inhibitory activity of suramin-related large ureas and NF023-related small ureas at native P2 receptors and ecto-nucleotidases

Assessing and comparing the inhibitory potency of a series of suramin-related large ureas with the parent compounds suramin and NF037 (4'-des-methyl-suramin), respectively, as well as of a series of NF023-derived small ureas, provided some interesting antagonist SAR's:

4' substituents (figure 3.11., table 3.4.a.; figure 3.15., table 3.5.a.)

Compared to NF037, the insertion of an electron donating group (suramin) as well as an electronegative substituent (NF157) into the 4'-position of the benzamino residue led to a decrease in the inhibitory potency at native P2X receptors, whereas the activity on ecto-nucleotidases remained largely unchanged. At GPTC P2Y receptors no inhibitory activity could be detected. Surprisingly, the phenyl-substituted derivative NF198 displayed a slightly higher blocking potency at P2X

and P2Y receptors, indicating that an electron-enriched, larger substituent is well-tolerated in the 4'-position.

The 4''-methyl-substitution in the suramin and NF037 derivatives NF212 and NF213 generated compounds with reduced P2X receptor-blocking potencies and ecto-nucleotidases inhibitory activity.

Within the group of NF023-derived small ureas, insertion of electronegative substituents (NF156, NF255) also decreased the P2X receptor-blocking potency, which is almost abolished by substitution with electron donating groups (NF058, NF144). As assessed for the large ureas, the ecto-nucleotidases inhibitory activity remained rather unaffected. In contrast to NF198, the small urea analogue NF195 was slightly less potent than its parent compound NF023 in blocking P2X receptors, but more potent in blocking P2Y receptors.

In general, 4'-substituents regardless whether electronegative or electropositive lead to a decrease in P2X receptor-blocking potency. Accordingly, it has been reported, that rearrangement or increase in the number of methyl substituents, as in NF212 and NF213, decreased the inhibitory potency at P2X receptors in the pithed rat *in vivo*. In this study, NF023 has been reported to be at least as potent as suramin (Urbanek et al., 1990). Subsequent studies performed in ratVD and RVD revealed an even higher P2X receptor-blocking potency for NF023 compared to suramin (Bültmann et al., 1996c; Ziyal, 1996; see 1.1.4.2.). It was concluded from a comparison of suramin analogues, that an increase in molecular size enhances P2X affinity until a maximum is reached in NF023 and a further increase to suramin tends to reduce affinity (Bültmann et al., 1996c). Analysing a larger number of analogues, this conclusion cannot be confirmed regarding the results obtained in the present study. The P2X receptor-blocking potencies assessed for NF037 and NF023 in the screening assay as well as in ratVD (cf. chapter 3.2.) were almost similar, indicating that the reason for the reduced P2X potency of suramin might be more probably due to the 4'-methyl substitution than to the increase in molecular size.

Exchange of aminobenzoyl groups (figure 3.12., table 3.4.b.; figure 3.16., 3.5.b.)

In the large ureas-series two compounds have been analysed that display slightly lower P2X receptor-blocking potencies than the parent compound NF037. NF356, in which the aminobenzoyl groups are exchanged by aminofuranyl residues and the aminobenzimidazolyl derivative NF506.

Among the small ureas, the aminofuranyl analogue NF359 which is structurally closely related to NF550 shows a dramatic decrease in inhibitory potency at P2X and P2Y receptors.

In contrast, introduction of isomeric biphenyl residues generated compounds displaying increased P2X (NF139, NF192, NF136, NF201) and in part also increased P2Y receptor-blocking potencies (NF192, NF136) compared to the parent compound NF023. This group of derivatives can be described as intermediates between the large and small ureas. Interestingly, their P2 receptor inhibitory potencies differ depending on the spatial orientation of the aminonaphthalene sulphonic acid residues and the urea-bridge, in that the stretched form, the *para-para*-linked derivative NF130 is markedly less potent even than NF023, whereas the *para-meta*- and the *ortho-para*-linked compounds display increased potency at both P2 receptor subtypes. The inhibitory activity on ecto-nucleotidases also differs, albeit marginally, among the analogues, indicating that there might be possibilities to separate P2 receptor antagonistic properties from ecto-nucleotidases inhibition by structural variations within the group of suramin analogues. The increased antagonistic potencies displayed by some of the biphenyl isomers indicate that an increase in molecular size compared to NF023 is not necessarily accompanied by a decrease in P2 receptor-blocking potency.

The loss in potency found for NF359 together with the finding that in the group of large ureas the exchange of one pair of aminobenzoyl substituents with heteroaromatic systems is well-tolerated indicates, that at least one pair of aminobenzoyl moieties seems to be important for a certain P2 receptor-blocking potency.

7-aminonaphthalene di- and trisulphonic acid substituted 1'-4'-linked small ureas (figure 3.18., table 3.5.d.)

Compared to their parent compound NF278, no differences in P2 receptor-blocking and ecto-nucleotidases inhibitory potencies have been observed for the small ureas NF347 and NF291. All derivatives display very low potencies in blocking P2X receptors in ratVD. On the basis of these findings, the impact of the introduction of 7-aminonaphthalene instead of 8-aminonaphthalene trisulphonic acid residues cannot be clearly assessed.

Exchange of the central urea bridge (figure 3.14., table 3.4.d., figure 3.19., table 3.5.e.)

The introduction of a thiourea (NF513) instead of an urea bridge (NF037) in the large urea group led to decreased antagonistic potency at P2X receptors. Further variations analysed, the insertion of isophthalic and terephthalic acid (NF059, NF064) as well as piperazine (NF299) bridges also resulted in a loss of P2X receptor-blocking potency. Similar results were obtained in the small urea series, neither the phthalic acid analogues (NF344, NF343) nor the piperazine derivative (NF300) displayed considerable P2 receptor-blocking or ecto-nucleotidases inhibitory potencies, respectively. Therefore, the urea bridge seems to be the ideal link between the two symmetrical moieties.

Recent developments in the series of suramin analogues

Another series of suramin analogues has been introduced very recently (Braun et al., 2001; Lambrecht et al., 2002). With the aim of generating additional sites of ligand-receptor interaction the number of negatively charged structures within the suramin pharmacophore was increased by the introduction of four benzene disulphonic acid moieties instead of two naphthalene trisulphonic acid residues. Of these analogues, NF449 (figure 4.1.) displayed an outstanding antagonistic potency at $P2X_1$-like receptors present in ratVD (pIC_{50}'s of 7.15 and 6.31 using 1 or 10 μM α,β-mATP, respectively) as well as at recombinant $P2X_1$ receptors (*Xenopus* oocytes, pIC_{50} = 9.54). Antagonism of NF449 has been shown to be specific and selective for the $P2X_1$ receptor subtype as it was found to be inactive at α_{1A} adrenoceptors, histamine H_1 and muscarinic M_3 receptors and only a weak

antagonist at native and recombinant P2X$_3$ receptors as well as native P2Y$_1$-like and P2Y$_2$ receptors (Braun et al., 2001; Lambrecht et al., 2002).
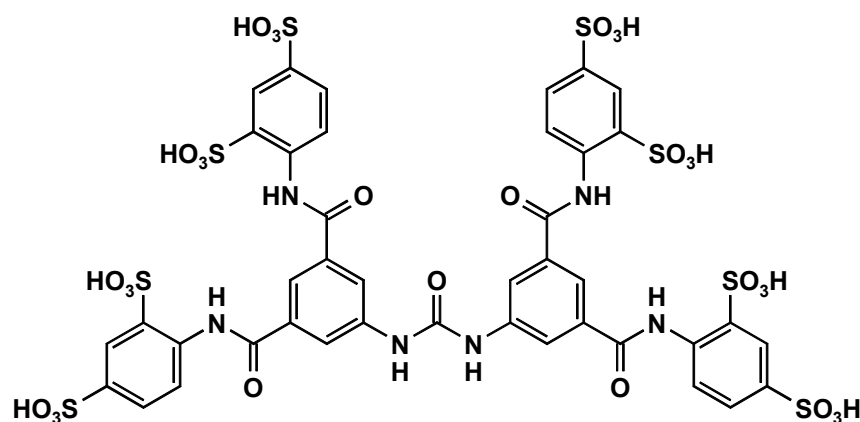
Fig. 4.1. *Chemical structure of NF449.*

4.2. Pharmacological characterisation of NF279 and its structural relatives

On the basis of the results obtained in the screening assays, small and large ureas varying in the substitution pattern of the benzoyl residues and especially one derivative, the suramin analogue NF279, were chosen to be subject of a more detailed pharmacological characterisation for two reasons: first, NF279 displayed the highest P2X receptor-blocking potency among all compounds analysed, being even markedly more potent than NF023; second, the fact that the relatively small structural variation from NF037 to NF279 resulted in such a remarkable increase in antagonistic potency argues in favour of a specific interaction between the compounds and the receptor protein, and third, a detailed pharmacological characterisation of analogues might provide some more interesting insights in SAR's of suramin-related compounds.

4.2.1. Inhibitory potency of small and large ureas III on native P2X receptors in rat vas deferens

For the analysis of the pharmacological properties of NF279 and its structural analogues (see figures 3.13., 3.17.) in terms of antagonistic potencies at native homomeric P2X$_1$ receptors, the rat vas deferens (ratVD) was chosen as model tissue. Contractions of ratVD elicited by α,β-mATP have been characterised in a

large number of studies as being mediated by a P2X receptor subtype which corresponds well in terms of rank orders of agonist as well as antagonist potencies with the P2X$_1$ homomer (Khakh et al., 1994, 1995b; Bültmann et al., 1999b; Ralevic and Burnstock, 1998; Bianchi et al., 1999; Lambrecht, 2000) which was originally cloned from the ratVD (Valera et al., 1994). Therefore, the use of the ratVD allowed a better comparison of the potency data obtained in the present study with potency estimates determined for other P2 receptor antagonists on recombinant rP2X$_1$ homomers and on its native counterpart in the ratVD. Heterogeneity of P2 receptors mediating contraction of the ratVD has been reported, based on the observation that in contrast to contraction evoked by α,β-mATP contractile responses to ATP were not totally blocked by suramin and iso-PPADS (Bültmann and Starke, 1994b). However, results obtained in a recent study confirmed earlier presumptions that the lack of effect of these antagonists might at least in part to be due to their dual activity: blocking P2 receptors and inhibiting ATP degradation by ecto-nucleotidases (Bültmann et al., 1999b), a phenomenon that has already been described for suramin and is referred to as "self-cancellation" (Crack et al., 1994). Nevertheless, the existence of additional subtypes of contraction-mediating P2 receptors cannot be totally ruled out by this explanation. Recently, UTP which has previously been shown to be inactive in pre-contracted ratVD (Khakh et al., 1994), was reported to elicit contraction of the tissue, when Evans blue was present to prevent enzymatic degradation of the nucleotide (Bültmann et al., 1999a). Contraction elicited by UTP was shown to be insensitive to desensitisation by α,β-mATP and persisted in the absence of extracellular calcium, indicating that contractile responses to uracil nucleotides were most likely to be mediated by a G protein-coupled P2Y receptor subtype. The fact that the UTP-induced contraction was inhibited by suramin together with a rank order of agonist potency of ATP $\geq$ UTP $>$ UDP = ADP led to the conclusion, that UTP might interact with P2Y$_2$ receptors in the ratVD preparation (Bültmann et al., 1999a). Based on the observation that UTP failed to desensitise ATP-induced contractions in the continuous presence of α,β-mATP the possibility of the presence of a third contraction-mediating P2 receptor cannot be ruled out (Bültmann et al., 1999a).

However, by the use of the P2X receptor-selective, non-degradable agonist α,β-mATP (cf. 1.1.3.1., 4.1.1., Khakh et al., 1995b; Lambrecht, 2000) contractile responses of ratVD seem to be almost exclusively mediated via P2 receptors

which closely resemble in their pharmacological properties those of the molecularly defined $P2X_1$ homomer (Khakh et al., 1994, 1995b; Valera et al., 1994; Trezise et al., 1995; Bianchi et al., 1999; see 4.3.).

Antagonistic potencies determined for the reference drugs suramin and NF023 (tables 3.6. and 3.7.) were very similar to those obtained for the antagonists in previous studies comparing the apparent pA_2 values calculated from the pIC_{50} values with affinity estimates derived from Schild plots (Khakh et al., 1994; Bültmann et al., 1996c). The potency estimates for small and large ureas derived from concentration-inhibition curves largely confirmed the results obtained in the screening study performed on RVD (figure 4.2.). Among the large ureas the 1'-4',1''-4''-linked 1,3,5-trisulphonated naphthylurea NF279 displayed the highest $P2X_1$-like receptor-blocking potency being about one order of magnitude more potent than the 1'-3',1''-3''-linked parent compounds suramin and NF037. The $P2X_1$-like receptor-blocking potencies of the 1'-4',1''-3''-linked structural intermediates NF280 and NF032 were decreased, even in comparison to suramin and NF037. In contrast to the 1'-3',1''-3'' and the 1'-4',1''-3'' analogues, an impact of the substitution pattern of the naphthyl moieties' sulphonic acid residues became obvious comparing the potency of NF279 and NF031: a shift of one sulphonic acid residue from the 5- to the 6-position in the naphthyl ring led to a marked loss in antagonistic potency at ratVD $P2X_1$-like receptors.

The 1'-4'-linked small ureas NF278 and NF013 being only poor $P2X_1$-like receptor antagonists, displayed the opposite to the effect observed in the group of large ureas. Comparable to the impact of the same structural variation in the large urea series, a change in the substitution pattern of the naphthalene sulphonic acid residues of the 1'-3' linked derivatives (NF023: 1,3,5; NF550: 1,3,6) did not alter the $P2X_1$-like receptor-blocking potency.

Fig. 4.2. *(see page 158) Selectivity profile of suramin-related large ureas (A.) and NF023-derived small ureas (B.) on native contraction-mediating $P2X_1$-like receptors present in rat vas deferens (ratVD) and $P2Y_1$-like receptors mediating contraction of guinea-pig ileal longitudinal smooth muscle preparation (GPI) (cf. 3.2.). The $P2X_1$-like receptor-blocking potencies of NF278 and NF013 have been examined at a single concentration of the respective antagonist, apparent pA_2 values may therefore be even lower than indicated by the broken line.*

A.

pA$_2$ value

B.

pA$_2$ value
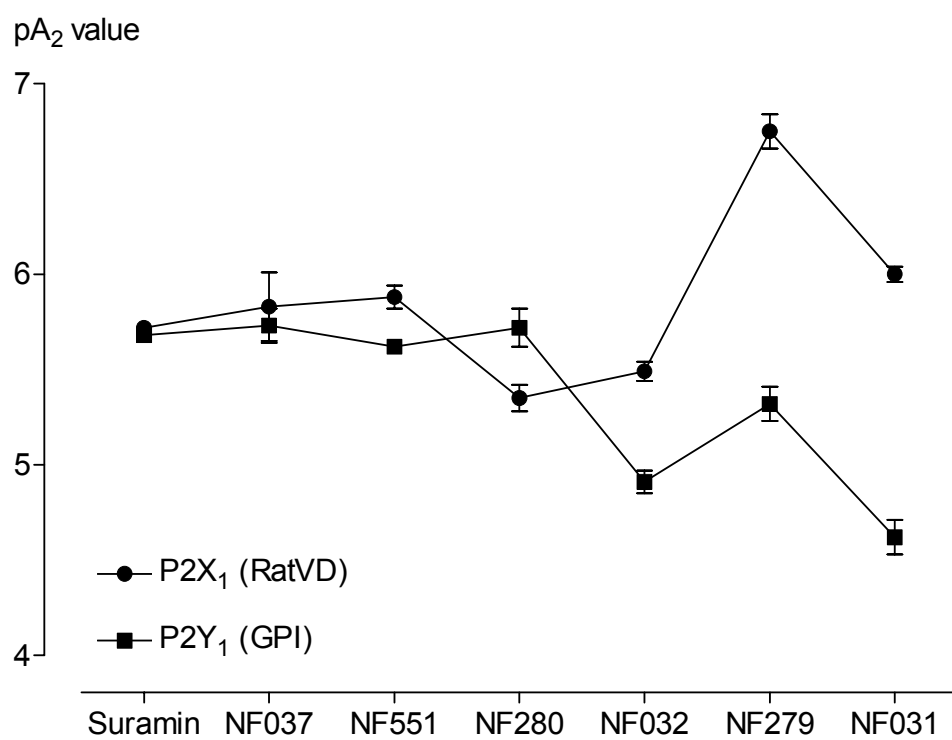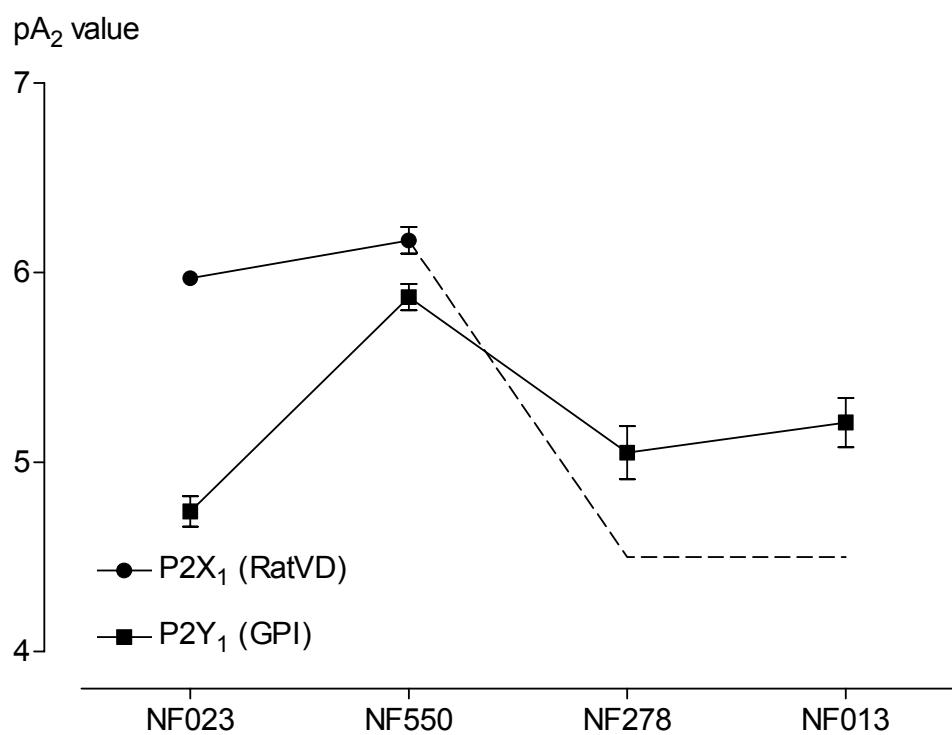
4.2.2. Inhibitory potency of small and large ureas III on native P2Y receptors in guinea-pig ileum and taenia coli

Suramin competitively antagonised P2Y receptor-mediated relaxation of GPTC evidenced by a Schild plot whose slope was not significantly different from unity (figure 3.4.). The pA_2 value of 4.45 derived from the constrained plot is slightly lower than affinity estimates determined for suramin in GPTC in previous studies (Bültmann et al., 1996a; Ziyal, 1996). Using ADPβS as agonist in GPTC, Bültmann and colleagues (1996a) reported pA_2 values of suramin to decrease when higher concentrations of the antagonist were examined. Although not different from unity, a slope of < 1 obtained for suramin and its desmethyl-derivative NF037 for the regression according to Arunlakshana and Schild (1959) in the present study indicate that there is a trend to lower pA_2 values, thereby confirming those previous observations. Similar to results described for the analysis of the antagonistic effects of NF023 at GPTC P2Y receptors, the pA_2 values obtained in the present study decreased markedly with increasing concentrations (cf. 3.1.2.2.; Bültmann et al., 1996c). Accordingly, limited antagonism was also observed for the small urea NF550 as CRC's to ADPβS in the presence of increasing concentrations of the analogue were almost superimposable. The reason for this deviation from competitive antagonism is not clear. One possible explanation, the presence of a heterogeneous receptor population in GPTC, has been proposed previously (Dudeck et al., 1995; Bültmann et al., 1996a; Lambrecht et al., 1996b; see 4.1.2.). Due to the fact that no cross-desensitisation of relaxation induced by either α,β-mATP or ADPβS has been reported, a heterogeneity of receptors mediating relaxation induced by ADPβS has to be anticipated. This might also reflect the differences observed comparing the potencies of antagonists in GPTC with those obtained for GPI P2Y receptors (table 4.2., 4.3.).

In GPI, ADPβS was shown to act at two sites: a P2 receptor located on cholinergic neurons, the stimulation of which causes indirect (acetylcholine-mediated) contractions via postjunctional muscarinic M_3 receptors (Czeche et al. 1998a), recently characterised as $P2X_3$ receptor subtype based on its sensitivity to α,β-mATP and the relative potencies of several antagonists (Lambrecht et al., 2000a); and a postjunctional P2 receptor on the ileal longitudinal smooth muscle (Kennedy and Humphrey, 1994; Czeche et al., 1998b). In the presence of

atropine, α,β-mATP was shown to be inactive and therefore contractile responses to ADPβS proved to be mediated exclusively via the postjunctional P2 receptor, which exactly resembles in its pharmacological properties the molecularly defined P2Y$_1$ receptor subtype (Czeche et al., 1998b; Lambrecht et al., 1999).

Table 4.3. Comparison of potency estimates (pA$_2$ values) obtained for large and small ureas inhibiting relaxation of guinea-pig taenia coli (GPTC) induced by ADPβS or blocking ADPβS-evoked contractions of guinea-pig ileal longitudinal smooth muscle (GPI). In GPTC, pA$_2$ values determined for NF023 and NF550 decreased testing increasing concentrations of the antagonists, therefore the concentration of antagonist for which the respective pA$_2$ value was calculated is given in parentheses.

Large ureas	Suramin	NF037	NF551	NF280	NF032	NF279	NF031
P2Y (GPTC)	4.45	4.86	4.60	4.87	3.95	4.10	3.30
P2Y (GPI)	5.68	5.73	5.62	5.72	4.91	5.32	4.56
Small ureas	NF023		NF550		NF278		NF013
P2Y (GPTC)	4.40 (100 μM)		5.43 (10 μM)		4.27		4.53
	4.07 (300 μM)		4.78 (30 μM)				
P2Y (GPI)	4.74		5.87		5.05		5.21

The large ureas NF037 and NF551 were equipotent to their parent compound suramin in inhibiting ADPβS-induced relaxation of GPTC as well as contraction of GPI elicited by ADPβS, indicating that the 4'-methyl residues are not a prerequisite for binding to the P2Y receptors present in these preparations. Similar results were obtained in a binding study performed on turkey erythrocyte membrane P2Y receptors comparing the affinity of suramin and NF037 (van Rhee et al., 1994). In contrast to the ratVD P2X$_1$-like receptors the 1'-4',1"-4"-linkage in NF279 and NF031 led to a loss in antagonistic potencies at GPI P2Y$_1$-like receptors, whereas the 1'-4',1"-3"-linked compound NF280 was almost equipotent to the 1'-3',1"-3"-linked derivatives suramin, NF037 and NF551. The substitution pattern of the naphthalene sulphonic acid residues affected P2Y$_1$-like receptor-blocking potency not only in the 1'-4',1"-4"-linked compounds (comparable to the effect of this structural variation at ratVD P2X$_1$-like receptors), the 5- to 6-shift of one

sulphonic acid residue also resulted in a decreased antagonistic potency of the 1'-4', 1"-3"-linked analogue NF032.

Comparing the $P2Y_1$-like receptor-blocking potencies of the small ureas in GPI (table 4.3.), where in contrast to GPTC the Schild plot for NF023 was linear with a slope (1.01) not significantly different from unity (Czeche et al., 1998b; Lambrecht et al., 1999), NF550 being more potent than NF023, its 1'-4'-linked analogue NF013 and NF278, a 1,3,6-substitution of the naphthalene sulphonic acid residues seems to be in favour of $P2Y_1$-like receptor affinity. As assessed for $P2X_1$-like receptors, 1'-4'-linkage generates compounds displaying reduced antagonistic potency at $P2Y_1$-like receptors.

4.2.3. Inhibitory potency of small and large ureas III on ATP breakdown (*Xenopus laevis* oocytes)

The inhibitory activity of large and small ureas on ATP breakdown by ecto-nucleo-tidases (ectoN) present in *Xenopus laevis* oocytes was almost similar ranging from 18 to 65 % inhibition in the presence of 300 μM of the respective analogue. The assessment of structure-activity relationships for ectoN inhibition is difficult to establish because of all compounds being only poor inhibitors of ATP degradation. In general, inhibitory potency on ectoN seems to increase in parallel to the antagonistic potency of the derivatives at either $P2X_1$-like or $P2Y_1$-like receptors (see table 4.4.). In the group of small ureas, inhibitory potencies on ectoN seem to correlate well with the $P2Y_1$-like receptor-blocking potencies whereas in the large urea series, inhibition of ATP degradation increases with increasing antagonistic potencies at $P2X_1$-like receptors.

Table 4.4.   (see page 162) Antagonistic potencies (pA_2 values) of small and large ureas on P2X receptors in rat vas deferens (ratVD) and P2Y receptors in guinea-pig ileal longitudinal smooth muscle (GPI) in comparison to the inhibitory potency (%) of the analogues (300 μM) on ATP breakdown by ecto-nucleotidases (ectoN) present in folliculated *Xenopus laevis* oocytes.

Large ureas	Suramin	NF037	NF551	NF280	NF032	NF279	NF031
P2X (ratVD)	5.72	5.83	5.88	5.35	5.49	6.75	6.00
P2Y (GPI)	5.68	5.73	5.62	5.72	4.91	5.32	4.56
ectoN	41.2	57.0	49.6	43.1	28.6	66.4	52.8
Small ureas	NF023		NF550		NF278		NF013
P2X (ratVD)	5.97		6.17		< 5		< 5
P2Y (GPI)	4.74		5.87		5.05		5.21
ectoN	28.2		43.3		26.1		18.3

4.2.4. Inhibitory properties of NF279

Of the antagonists displaying considerable potency and selectivity for a certain P2 receptor subtype, the large urea NF279, which was shown to be significantly more potent in blocking ratVD $P2X_1$-like receptors (six-fold) than NF023 (Lambrecht et al., 1996a; Soto et al., 1999), was further analysed in terms of mechanism of P2 receptor antagonism as well as P2 receptor subtype selectivity and specificity of its antagonistic effects.

4.2.4.1. P2X receptor antagonism of NF279

4.2.4.1.1. Antagonism of NF279 at native P2X receptors

In order to elucidate the mechanism of antagonism by NF279 on $P2X_1$-like receptors in ratVD, CRC's to α,β-mATP were determined in the absence and presence of NF279. Increasing concentrations of NF279 shifted the CRC's progressively to the right, indicating concentration-dependent antagonism, and simultaneously increased the slope in a non-concentration-dependent manner (see figure 3.22.). An increase of the slope or maximum response of the α,β-mATP CRC's has been previously described in ratVD for suramin, NF023 and congeners (Bültmann et al., 1996c) as well as for the histochemical dyes Evans blue, Trypan blue and RB2 (Bültmann and Starke, 1993; Bültmann et al., 1994; Tuluc et al., 1998), and additionally in other tissues containing P2X receptors (Trezise et al., 1994; Ziyal, 1996; see Bültmann et al., 1996c). The increase in the

maximum response seems to be a common property of reversibly acting P2X receptor antagonists, the underlying mechanism, however, is not known. Originally, it was anticipated that it might be due to α,β-mATP acting on a low affinity P2 receptor which is antagonised by low concentrations of suramin (Blakeley et al., 1991; Mallard et al., 1992). This possibility seems to be rather unlikely for several reasons: first, the increase in the maximal response seems to be independent of the concentration and the antagonist examined; second, TTX failed to inhibit contraction of ratVD induced by α,β-mATP (cf. 3.3.1.), thereby indicating that the agonist acts exclusively postsynaptically and, third, this potentiating effect of antagonists has been observed analysing P2X receptor-mediated effects in different tissues as well as in vasa deferentia of different species. An alternative explanation, that inhibition of ecto-nucleotidases by the antagonists might account for the increase in maximum seems not conceivable, as well, when using the enzymatically stable agonist α,β-mATP. However, it has been reported recently that α,β-mATP itself inhibits ecto-nucleotidases present in bovine pulmonary endothelial cells with an IC_{50} of about 30 μM (Chen and Lin, 1997). Considering the ability of almost all antagonists to inhibit ecto-nucleotidases (although for most of the antagonists the concentration required for enzyme inhibition is higher than the concentration necessary for P2 receptor antagonism) it is possible that rapid breakdown of ATP which can be released during contraction of the smooth muscle (Westfall et al., 1996) is prevented and therefore endogenous ATP can contribute to the contraction induced by exogenously applied α,β-mATP.

A further deviation from competitive antagonism observed for NF279 was the increase in mean pA_2 estimates calculated from the shift of the CRC's in the presence of increasing NF279 concentrations (see table 3.8.). It may result when the lower NF279 concentrations have not reached equilibrium (Leff et al., 1990). However, there was no difference in affinity estimates determined for the lowest NF279 concentration examined after 60- and 120-min pre-incubation, respectively. In contrast, NF279 was found to act as a purely competitive antagonist ($pA_2 = 5.95$) on P2X$_3$-like receptors modulating ACh-release in GPI longitudinal smooth muscle preparation, indicated by a Schild plot, the slope of which was not significantly different from unity (Lambrecht et al., 1999; Lambrecht et al., 2000a).

4.2.4.1.2. Antagonism of NF279 at recombinant P2X receptor subtypes

Electrophysiological studies with the aim to examine the P2X receptor subtype selectivity of NF279 revealed remarkable differences in the antagonistic potencies determined for NF279 either pre-incubated or co-applicated at the rapidly desensitising homomeric P2X$_1$- and P2X$_3$ receptors expressed in *Xenopus laevis* oocytes (Rettinger et al., 2000). When pre-incubated for 10 s NF279 was about 100-fold more potent in blocking current responses from rP2X$_1$ receptors (pIC$_{50}$ = 7.72) than from rP2X$_3$ receptors (pIC$_{50}$ = 5.79) (Damer et al., 1998; Rettinger et al., 2000). The inhibitory potency determined for NF279 on hP2X$_1$ receptors (pIC$_{50}$ = 7.30) was almost similar to its potency on rP2X$_1$, the slight variance may be due to differences in the protein structure or differences in the experimental protocols (i.e. the solution exchange times) applied, respectively, (Klapperstück et al., 2000; Rettinger et al., 2000), although, for NF023 similar pIC$_{50}$ values (hP2X$_1$: 6.68; rP2X$_1$: 6.62) have been found for the human and the rat homologue (Soto et al., 1999). In contrast to NF279, pre-incubation in comparison to co-application had no significant influence on the inhibitory potency of NF023 on the current induced by ATP (Soto et al., 1999). An explanation of this differential action of the two antagonists might come from the comparison of the time course and reversibility of their inhibitory actions determined for NF023 (3 μM) in RVD (Ziyal, 1996) and NF279 (1 μM) in ratVD (figure 3.23.). The times of on-set as well as off-set of NF279's antagonism turned out to be markedly longer than those of NF023, being 180 min for NF279 compared to 50 min for NF023 as well as 90 min (NF279) compared to 50 min (NF023) for the wash-out of the respective compound.

At rP2X$_1$, increasing concentrations of NF279 caused both a shift to the right of the ATP CRC and an increasing depression of the maximum current. Since the dissociation of NF279 from the receptor is slow compared to the time required to reach the maximal response after application of ATP, no equilibrium is reached during the occurrence of the peak current on which the analysis is based (Rettinger et al., 2000). Whether the antagonism of NF279 can be considered competitive can neither be confirmed nor rejected on the basis of the results obtained in that study, although the competitive nature of antagonism by the structurally related compound NF023 favours this interpretation (Soto et al., 1999). Further experiments are needed to clarify this issue.

Furthermore, NF279 was shown to concentration-dependently decelerate both, activation and desensitisation of the $hP2X_1$ receptor (Klapperstück et al., 2000). This effect has so far not been reported for NF023. Recently, desensitisation of ratVD $P2X_1$ receptors has been shown to be due to internalisation of the agonist-activated receptor. Subsequently, rapid recycling of these receptors to the cell-surface is followed by recovery of the receptor from an inactivated state which was described to be the rate-limiting step in desensitisation and recovery of $P2X_1$ receptors (Ennion and Evans, 2001). Since desensitisation/internalisation is dependent on receptor activation, the slowly dissociating antagonist NF279 competing with ATP may therefore cause deceleration of activation as well as desensitisation of $P2X_1$ receptors (see Klapperstück et al., 2000).

At the almost non-desensitising $rP2X_2$ receptor antagonism of NF279 was demonstrated to be competitive, and a mean pK_B value of 6.44 was calculated when the slope of the regression according to Arunlakshana and Schild (1959) was constrained to unity (Rettinger et al., 2000).

The $hP2X_4$ receptor was less sensitive to inhibition than rat $P2X_1$, $P2X_2$ and $P2X_3$ receptor subtypes (41 % inhibition at 300 μM NF279) (Rettinger et al., 2000). Antagonistic potency at $hP2X_7$ receptors measured at an activating ATP concentration of 1 mM yielded a pIC_{50} value of 4.5 (Klapperstück et al., 2000). Therefore, the P2X receptor selectivity profile of NF279 ($P2X_1 > P2X_2 > P2X_3 \geq P2X_7 >> P2X_4$) is unique and different from those of the few P2 receptor antagonists which have been examined in terms of P2X receptor subtype selectivity.

4.2.4.2. Antagonism of NF279 at native and recombinant P2Y receptors

In GPI longitudinal smooth muscle preparation increasing concentrations of NF279 shifted the concentration-contraction curves obtained to single doses of ADPβS progressively to the right. A pA_2 estimate of 5.32 derived from Schild analysis was slightly higher than that found for NF023 (4.74) and in turn moderately lower than the antagonistic affinity determined for suramin on GPI $P2Y_1$-like receptors (Lambrecht, 2000). This rank order of potency was largely confirmed when the antagonistic potencies of the compounds were examined at recombinant $P2Y_1$ receptors. NF279 (pIC_{50} = 4.6) was found to be equipotent to

NF023 (pIC_{50} = 4.6), and both compounds were inactive at recombinant $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (J.L. Boyer and T.K. Harden, personal communication).

4.2.4.3. Inhibitory potency of NF279 on ATP breakdown

Like suramin and NF023, NF279 inhibits ecto-nucleotidases activity in *Xenopus laevis* oocytes at very high concentrations (100 - 300 μM).

4.2.4.4. P2 receptor specificity of NF279 and NF031

At a concentration of 100 μM NF279 did not interact with α_{1A} receptors in ratVD, adenosine A_1, histamine H_1 and muscarinic M_3 receptors in GPI and adenosine A_{2B} as well as neuronal nicotinic receptors in GPTC (table 3.12.). Thus, similar to NF023 (Lambrecht et al., 1996a) but in contrast to suramin (1.1.4.) the antagonism of NF279 appears to be specific for P2 receptors.

4.3. Regional variation of purinergic responses in rat vas deferens

There are several reports addressing the regional variation of adrenergic and purinergic responses in vasa deferentia of different species with respect to the prostatic and epididymal origin of the segment. In general, segments from the prostatic end of the muscle exhibit primarily a rapid, phasic, purinergic response whilst segments from the epididymal end show mainly a slower, tonic, adrenergic contraction (Sneddon and Machaly, 1992). Moreover, heterogeneity of P2 receptors mediating contraction of the ratVD has been postulated, based on potency profiles of both agonists and antagonists (Bültmann and Starke, 1994b, 2001; Bültmann et al., 1999a). However, whether the P2 receptor subtypes are distributed symmetrically along the length of the vas deferens and as a consequence, prostatic as well as epididymal segments are equally suitable for the study of $P2X_1$ receptor-mediated responses, has not been examined so far.

4.3.1. Comparison of agonist potencies in prostatic and epididymal segments of the rat vas deferens

By the use of agonists with different selectivity profiles slight differences were observed comparing their potencies in the two ends of the ratVD. α,β-mATP was the most potent agonist in prostatic as well as epididymal segments, the potency estimates determined in the present study (pEC_{1000} values) being very similar to potency estimates (EC_{50}'s) reported previously (Khakh et al., 1995b). A rank order of agonist potency of α,β-mATP $>$ L-β,γ-mATP $\geq$ ATPγS $>$ ADPβS was observed in both portions of the ratVD. However, α,β-mATP displayed a slightly higher potency in prostatic segments and the slope of the agonist's CRC was markedly decreased in epididymal segments. In contrast, potency of the $P2X_1$ receptor-selective agonist L-β,γ-mATP (Trezise et al., 1995; Rae et al., 1998) differed only marginally among the segments and the CRC's were almost superimposable. As already described for α,β-mATP, the potency estimates for L-β,γ-mATP correspond well with an EC_{50} that has been determined in a previous study for the agonist in prostatic segments of ratVD (Trezise et al., 1995).

Cross-desensitisation studies yielded two interesting observations: first, although in prostatic portions the α,β-mATP-induced contraction was nearly abolished by desensitisation using L-β,γ-mATP, in epididymal segments one third of the response remained. Second, contraction elicited by ADPβS in prostatic segments was markedly decreased after desensitisation using α,β-mATP, which was not the case for the ADPβS-induced contraction in epididymal segments. Moreover, especially in the epididymal portions tachyphylaxis rather than desensitisation was observed for contraction elicited by ADPβS, indicating that the agonist might act at one of the non- or slowly-desensitising P2 receptor subtypes. A further interesting finding of the present study was the difference in the time course of desensitisation induced by α,β-mATP. Desensitisation appeared to be faster in prostatic than in epididymal portions of the ratVD. This might be in favour of α,β-mATP also acting on a P2 receptor subtype different from the rapidly desensitising $P2X_1$ receptor.

The fact that TTX did not alter the responses elicited by α,β-mATP and ADPβS neither in prostatic nor in epididymal segments, respectively, argues against an involvement of presynaptic receptors.

Accordingly, based on the cross reactivity of L-β,γ-mATP and α,β-mATP as well as of α,β-mATP and ADPβS, in prostatic segments of ratVD, P2X$_1$ receptors can be regarded at least as the predominating P2 receptor subtype mediating contraction induced by all of the three agonists applied (Trezise et al., 1995; Rae et al., 1998). In contrast, contractions elicited by α,β-mATP and ADPβS in epididymal segments seem to be mediated via (a) distinct, moderately or slowly-desensitising P2 receptor subtype(s) present on the postsynaptical site in addition to the P2X$_1$ receptor subtype. Whether this additional site of action is the same for both agonists was the subject of a more detailed analysis, applying P2 receptor antagonists.

In general, homomeric P2X$_3$ as well as heteromeric P2X$_2$P2X$_3$ and P2X$_1$P2X$_5$ receptors are possible candidates for the receptor subtype sensitive to α,β-mATP, regarding the selectivity profile of the agonist (Lambrecht, 2000; North and Surprenant, 2000). Whereas ADPβS has been reported to act as an agonist at the G protein-coupled P2Y$_1$, P2Y$_6$, P2Y$_{11}$ and P2Y$_{12}$ receptors as well as P2X$_1$, P2X$_3$ and P2X$_4$ receptors (Filtz et al., 1994; Chang et al., 1995; Bianchi et al., 1999; Communi et al., 1999; Hollopeter et al., 2001; Jin et al., 2001), indicating overlapping activities to exist.

4.3.2. P2 receptor subtype(s) mediating α,β-mATP-induced contraction of epididymal segments of the rat vas deferens

Assessing and comparing the inhibitory potencies of several P2 receptor antagonists on α,β-mATP-induced contraction of prostatic and epididymal segments of the ratVD, in certain cases marked differences were observed. The highest ratios (IC$_{50}$[epididymal]/IC$_{50}$[prostatic]) have been obtained for PPADS, NF279, and NF037, whereas the non-selective antagonist suramin displayed almost similar potencies in epididymal and prostatic ends. In general, pIC$_{50}$ values determined for the antagonists in prostatic ends were higher than those found in epididymal segments although, regarding the differences in the agonistic potency of α,β-mATP with respect to the anatomical origin of the tissue, the opposite should have been the case: IC$_{50}$ values depend on the concentration of agonist applied in the way that the higher potency/concentration of agonist the higher the concentration of antagonist required to block the agonist-induced effect.

Therefore, pIC_{50} values derived from epididymal segments would have been expected to be slightly higher than those derived from prostatic segments.

As could be anticipated from the comparison of the antagonistic potencies, the differences increased when affinities of NF279 (independent of the agonist concentration), determined in prostatic and epididymal portions, were compared. pA_2 values assessed for NF279 (10 μM) were one order of magnitude higher in prostatic than in epididymal segments of the ratVD.

The pA_2 values determined for NF279 (3 μM) in the two segments of ratVD correspond well with the apparent pA_2 estimates calculated from the pIC_{50} values of the antagonist (tables 3.16. and 3.17.). Therefore, the modification of the relationship introduced by Cheng and Prussoff (1973) and applied in order to generate potency estimates for NF279, which are not depending on the potency of the respective agonist, yielded acceptable results.

The fact, that only minor differences have been observed for the antagonistic effect of the $P2X_1$ receptor-selective compound NF279 in prostatic and epididymal segments of the ratVD when the $P2X_1$ receptor-selective agonist L-β,γ-mATP was applied to induce contractions, confirms the presence of functional $P2X_1$-like receptors in both ends of the tissue. Whereas the potency differences observed using the non-selective agonist ATPγS as well as the P2X receptor-selective agonist α,β-mATP are in favour of the presence of additional P2 receptor subtype(s), at least in epididymal segments. Although these findings confirm the results obtained in the agonist studies, due to the absence of antagonists displaying selectivity for any of the other P2X receptor subtypes sensitive to α,β-mATP, the identity of the agonists additional site of action cannot be clearly delineated. However, it might be further confined regarding the P2 receptor subtypes' mRNA and proteins detected in ratVD so far.

P2 receptor subtypes present in ratVD

Based on reverse transcription-polymerase chain reaction (RT-PCR) analysis, not only the mRNA encoding for the $P2X_1$ receptor subtype but also $P2X_2$, $P2X_3$ and $P2X_4$ receptor mRNA's have been detected in both segments of ratVD (C. Büttner and G. Schmalzing, personal communication). Almost recently, the availability of

antibodies to unique peptide sequences of the seven P2X receptor subtypes enabled a immunohistochemical screening for the presence of P2X receptor subtype proteins to be performed in ratVD tissue (Lee et al., 2000). In addition to P2X$_1$ receptors, for which strong immunostaining has been observed in the smooth muscle membranes (Lee et al., 2000; Mulryan et al., 2000), a more diffuse staining pattern was seen with P2X$_2$ receptor antibodies also in the smooth muscle cells. P2X$_3$ immunoreactivity has been reported in nerve fibre bundles outside the muscle proper, in the adventitial tissue. However, no differences in staining pattern between prostatic and epididymal ends of the tissue have been observed (Lee et al., 2000).

A further study revealed a more discrete distribution pattern of P2X receptor subtypes in ratVD. Immunohistochemical analysis localised the presence of P2X$_1$ receptor subunit proteins to the outer longitudinal and circular muscle layers, whereas P2X$_2$ immunolabelling was particularly concentrated within the lamina propria (Burton et al., 2000) (see figure 4.3.). Two alternatively spliced isoforms of the P2X$_2$ receptor subunit mRNA were detected applying RT-PCR (Burton et al., 2000). One corresponding in sequence to the original clone (P2X$_{2(a)}$), the other being identical with the P2X$_{2(b)}$ isoform encoding a protein with a truncated C-terminal domain (Brake et al., 1994; Brändle et al., 1997). P2X$_{2(b)}$ receptor subunits have been reported to exhibit a degree of desensitisation not found when P2X$_{2(a)}$ receptors were expressed to form homomeric ATP-gated ion channels (Brake et al., 1994; Simon et al., 1997).

The cells expressing the P2X$_2$ receptor isoforms were identified as interstitial cells of Cajal (ICC). Those cells, localised in the enteric plexuses and between gastrointestinal smooth muscle fibres, have been described to co-ordinate contraction via a reticular network of processes which synapse with enteric neurons and gastrointestinal smooth muscle cells, serving as the pacemaker cells for the basal electrical rhythm essential for the orthograde lumenal transport in the gut (Hagger et al., 1997; Horowitz et al., 1999). Therefore, P2X$_2$ receptors present on ICC in ratVD may be involved in the intrinsic regulation of smooth muscle contraction (Burton et al., 2000).
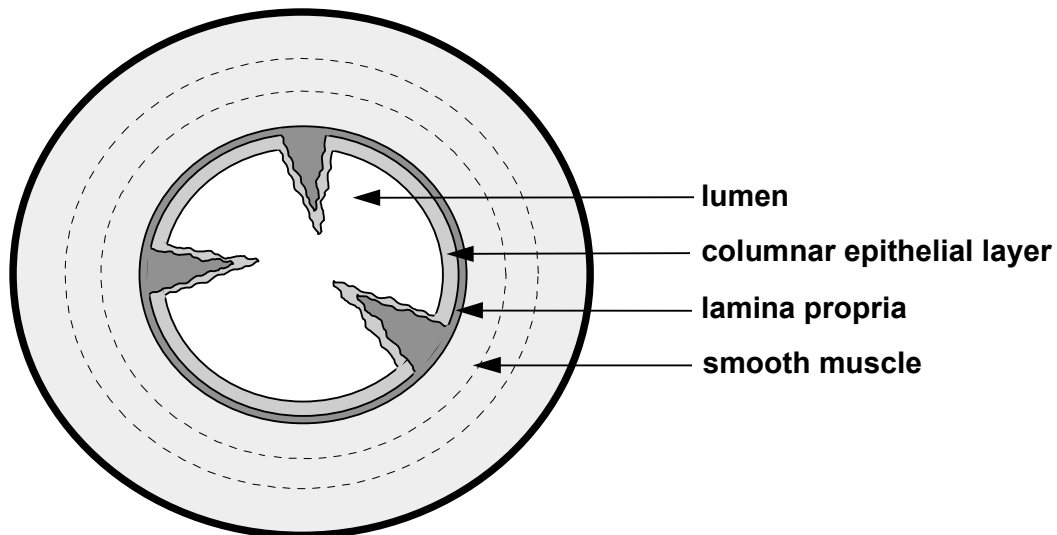
Fig. 4.3. *Schematic structure of a cross-section of the rat vas deferens.*

In contrast, in mouse vas deferens, the presence of $P2X_{1-6}$ receptor proteins has been detected by use of subtype-specific antibodies (Barden et al., 1999). Interestingly, $P2X_1$ receptor clusters of two size categories were found, larger ones occurring under most sympathetic varicosities and small-sized clusters being present throughout the smooth muscle. From the distribution pattern of the other P2X receptor subtypes it was concluded that heteropolymerisation of subtypes seems very unlikely at the varicosities in mouse vas deferens, as these usually do not possess subtypes other than the $P2X_1$, and $P2X_2$ to $P2X_6$ receptor subtypes mainly occur as small size clusters throughout the muscle. It was suggested, that the large $P2X_1$ clusters are responsible for the fast junction potential component and the large number of small P2X receptor clusters of different subtypes might provide the input for the slow junction potential component that has been previously observed in the mouse vas deferens smooth muscle (Barden et al., 1999). Whether these findings are of relevance for the ratVD needs to be established.

Studies using P2Y receptor subtype-specific antibodies have not been performed in ratVD so far. The only P2Y receptor subtype reported to be present in vas deferens tissue, based on RT-PCR mRNA detection, was the rat $P2Y_4$ receptor (Webb et al., 1998).

These findings and the results obtained in the present study, support the view that in epididymal segments contraction elicited by α,β-mATP is not exclusively

mediated via $P2X_1$ receptors. Based on the selectivity profile of α,β-mATP, an involvement of P2Y receptor subtypes as well as homomeric $P2X_2$, $P2X_4$, $P2X_6$, and $P2X_7$ receptors can be excluded (Lambrecht, 2000; Bianchi et al., 1999). So far, besides the $P2X_1$ subtype, $P2X_2$ and $P2X_3$ receptor proteins have been detected in ratVD (Lee et al., 2000). Although mRNA encoding for the $P2X_4$ has been reported to be present in ratVD (Bo et al., 1995; C. Büttner and G. Schmalzing, personal communication), no immunostaining was observed using $P2X_4$ receptor-specific antibodies (Lee et al., 2000). Considering all these facts, the unidentified α,β-mATP-sensitive P2 receptor present in epididymal segments of the ratVD should belong to the P2X receptor subfamily, the most probable subtypes are $P2X_3$, as well as $P2X_2$ and/or $P2X_3$ contributing to a heteromeric assembly of two or probably more P2X receptor subunits (cf. Torres et al., 1999).

4.3.3. P2 receptor subtype(s) mediating ADPβS-induced contraction of epididymal segments of the rat vas deferens

In order to determine the P2 receptor subtype(s) involved in mediating contraction of epididymal segments of the ratVD elicited by ADPβS and whether these are probably similar to the additional site of action of α,β-mATP, the effects of the non-selective antagonist suramin, the $P2X_1$ receptor-selective suramin analogues NF023 and NF279 (Lambrecht, 2000) as well as the $P2Y_1$-selective ligand MRS 2179 (see von Kügelgen and Wetter, 2000; cf. 1.1.3.2.) were examined.

MRS 2179 (10 and 100 μM) did not alter the CRC obtained to cumulatively applied ADPβS (figure 3.32.), indicating that the contraction is most probably not mediated via $P2Y_1$ receptors.

The non-selective P2 receptor antagonist suramin shifted the ADPβS CRC to the right, as did NF023 and NF279 (figure 3.32.). The rightward shift was accompanied by an increase of the contractile responses induced by ADPβS. Particularly in the presence of NF279, of which four different concentrations have been examined, contractions were augmented by lower concentrations of the antagonist (1 and 3 μM) in a dose-dependent manner. The simultaneous action of ADPβS at two different P2 receptor subtypes, one mediating relaxation, the other mediating contraction - NF279 being more potent in antagonising the relaxation-mediating receptor subtype - might be a possible explanation for these

observations. In fact, the presence of relaxation-mediating P2 receptors has been postulated in rat and mouse vas deferens (Boland et al., 1992; Mallard et al., 1992). However, in ratVD pre-contracted with high potassium, ADPβS as well as α,β-mATP have been reported to induce solely contraction whereas contractile responses elicited by ATP as well as 2-MeSATP were followed by relaxation (Bültmann and Starke, 2001).

Due to the lack of desensitisation of ADPβS-induced contractions as well as the potentiation rather than inhibition of these responses by lower concentrations of NF279, the P2X$_1$ receptor is very unlikely to be the predominant site of action of ADPβS in epididymal segments of the ratVD. Furthermore, NF279, at a concentration of 10 μM, has been demonstrated to effectively antagonise P2X$_1$-like receptors in prostatic segments of the ratVD (cf. 3.2.1.3.1.), whereas this concentration causes only a slight shift to the right of the ADPβS CRC in epididymal segments. However, desensitisation of a small fraction of the ADPβS-induced contraction in epididymal segments, as well as marked desensitisation observed in prostatic segments using α,β-mATP as desensitising agent, indicates that part of the contraction elicited by ADPβS is most probably mediated via P2X$_1$ receptors.

ADPβS has been previously shown to elicit contraction of vasa deferentia of guinea-pig and rabbit (Bailey and Hourani, 1995; Lambrecht et al., 1996b). In rabbit vas deferens, suramin as well as NF023 and PPADS have been reported to antagonise contractile responses elicited by ADPβS, although their potencies were low, especially the potency estimate obtained for NF023, when compared to pA$_2$ values obtained using α,β-mATP as agonist (Lambrecht et al., 1996b).

Based on the fact that ADPβS-induced contraction is inhibited by the P2X$_1$ receptor-selective antagonists NF023 and NF279 (Lambrecht, 2000), and P2Y$_1$ receptors - the only P2Y receptor subtypes at which both antagonists have been reported to display, albeit low, activity - can be excluded, an involvement of P2X receptor subtype(s) in mediating ADPβS-induced effects has to be anticipated. However, the exact identity of these P2 receptor subtype(s) remains to be established.

5. Summary

In the early 70s, adenosine-5'-triphosphate (ATP), which has long been known as an intracellular source of energy, has been postulated to act extracellularly as a signalling molecule, i.e. as neuro-/cotransmitter. However, it lasted several years until the "purinergic nerves hypothesis" introduced by G. Burnstock and co-workers as well as the existence of so-called purinergic receptors (P2 receptors) mediating responses induced by ATP was finally accepted. At present, there is no doubt about ATP and other nucleotides such as adenosine-5'-diphosphate as well as uridine-5'-tri- and -diphosphate acting as signalling substances in the peripheral and central nervous system.

The P2 receptors can be subdivided into two major groups, the P2X receptor family of ligand-gated cation channels and the P2Y receptor family of G protein-coupled nucleotide receptors. To date seven mammalian P2X receptor subunits (P2X$_{1-7}$) and six P2Y receptors (P2Y$_{1,2,4,6,11,12}$) have been cloned, characterised pharmacologically and accepted as valid members of the P2 receptor family.

The neurotransmitting activity of nucleotides is rapidly terminated by enzymatic cleavage of the phosphate chain by a group of membrane-bound enzymes (sometimes also present in soluble forms) generally referred to as ecto-nucleotidases. The final degradation product of ATP, adenosine, which itself has been characterised as a neurotransmitter/neuromodulator elicits its mainly inhibitory effects acting at P1 receptors (A$_1$, A$_{2A}$, A$_{2B}$, A$_3$).

One of the most exciting challenges in the rapidly expanding P2 receptor field is to relate the cloned P2 receptor subtypes to the diverse physiological responses mediated by native receptors. The identification of potent and selective P2 receptor ligands (agonists and antagonists) will aid in defining their physiological/ pharmacological function. Unfortunately, most of the P2 receptor antagonists evaluated so far are limited in their usefulness by their irreversibility of antagonism or lack of potency, subtype-selectivity and P2 receptor specificity. The property of many of these antagonists to either inhibit ecto-nucleotidases or to be susceptible to enzymatic breakdown, further complicates their use. Therefore, a high affinity P2 receptor antagonist would be a valuable pharmacological tool to improve radioligand binding assays, which in turn are critical to the use of high throughput screening assays to identify novel pharmacophores and - finally - new therapeutic agents.

Suramin (Germanin®), which is in use for the treatment of trypanosomiasis and onchocerciasis, has been reported to be a non-selective antagonist at recombinant and native P2 receptor subtypes. Particularly one analogue, the small urea NF023, displayed considerable potency and selectivity in competitively blocking P2X receptors present in vascular and non-vascular preparations. This was confirmed in studies at recombinant P2X receptor subtypes.

Therefore, a pharmacological evaluation of compounds related to suramin and NF023 was performed to gain further insights in structure-activity relationships with the aim to identify novel P2 receptor antagonists.

Development of screening assays for the investigation of P2 receptor and ecto-nucleotidases inhibitory activities of suramin analogues

To investigate the inhibitory activity of a larger number of suramin analogues screening assays were developed. The contraction-mediating P2 receptor subtype present in rabbit vas deferens (RVD) has been previously characterised as a member of the P2X receptor subfamily. However, several findings indicated that contraction elicited by electrical field stimulation, resulting in the release of the naturally occurring non-selective ligand ATP, might be mediated by a heterogeneous P2 receptor population. Taking these investigations into account, the inhibitory activity of suramin analogues was determined on contractions of the RVD elicited by a single dose of the exogenously applied P2X receptor selective agonist α,β-methylene ATP (α,β-mATP).

Furthermore, the inhibitory activity of the derivatives was investigated in guinea-pig taenia coli (GPTC) which is one of the prototype tissues for the study of native P2Y receptors. Originally, the P2 receptor subtype mediating relaxation of the carbachol-pre-contracted GPTC has been assumed to be identical to the recombinant $P2Y_1$ receptor. However, several studies indicated that relaxation of GPTC is mediated by at least two different P2 receptor subtypes. Therefore, the original classification has been questioned.

Consequently, in addition to GPTC P2Y receptors, the detailed pharmacological evaluation of selected derivatives was performed at $P2Y_1$-like receptors mediating contraction of the guinea-pig ileal longitudinal smooth muscle.

Inhibitory activities of the small and large ureas on ATP breakdown were determined on ecto-nucleotidases of folliculated *Xenopus laevis* oocytes. ATP-degrading activity in the presence of oocytes has been characterised in previous studies to be due to the extracellular enzymes present in the follicle epithelium.

Structure-activity-relationships of suramin analogues

The inhibitory activities of suramin- and NF023-related compounds determined in the screening assays provided some interesting insights in structure-depending activity (NF023, suramin and 4'-desmethyl-suramin [NF037] served as reference drugs):

- 4'-Substituents either electropositive or electronegative lead to a decrease in inhibitory potency of small and large urea derivatives at P2X receptors.
- Introduction of an electron-enriched 4'-phenyl group enhances the P2X as well as P2Y receptor-blocking potency of large ureas, whereas the small urea analogue displays reduced antagonistic activity at P2X receptors and an increase in P2Y receptor inhibitory activity.

Based on these observations, the reason for the reduced antagonistic activity of suramin at P2X receptors when compared to NF023 seems to be more probably due to the 4'-methyl substitution than - as originally anticipated - to the increase in molecular size.

- Introduction of isomeric biphenyl instead of aminobenzoyl residues generates NF023 analogues displaying increased P2X and in some cases also P2Y receptor-blocking potencies.
- An exchange of the urea bridge resulted in all cases - thiourea, phthalic acid, piperazine - in a loss in antagonistic activity at the P2X receptor subtype.

The detailed pharmacological evaluation of suramin and NF023 analogues varying in the substitution pattern of the aminonaphthalene sulphonic acid residues as well as in the linkage of the aminobenzoyl moiety confirmed preliminary results obtained in the screening assays and enabled to suggest further structure-activity relationships.

Investigations of the P2X receptor-blocking potency of these compounds were performed in prostatic segments of the rat instead of the rabbit vas deferens. This

tissue was chosen for two reasons: first, the rat vas deferens (ratVD) has been widely used to study native $P2X_1$-like receptors, and second, the presence of $P2X_1$ receptor subtypes in ratVD has been unambiguously proved by expression cloning. Therefore the use of the ratVD facilitates the comparison of results obtained from other groups at native $P2X_1$-like receptors.

Among these analogues, the 1'-4',1"-4"-linked large ureas NF279 and NF031 displayed high antagonistic potency and considerable selectivity for ratVD $P2X_1$-like receptors, whereas the antagonistic potency determined for the 1'-4',1"-3"-linked structural intermediates NF280 and NF032 was markedly decreased. Introduction of 1,3,6- instead of 1,3,5-aminonaphthalene trisulphonic acid residues resulted in a substantial decrease of antagonistic potency of 1'-4',1"-4"-linked large ureas without affecting the P2X receptor selectivity. In contrast, the same structural variation in the group of small ureas led to a loss in P2X receptor selectivity due to an increase in P2Y receptor-blocking potency.

Regarding the inhibitory effect of the suramin derivatives on ecto-nucleotidases in folliculated *Xenopus laevis* oocytes, a general conclusion can be drawn: almost all analogues were shown to inhibit ecto-nucleotidases at relatively high concentrations in a similar concentration range. Therefore, the higher the P2 receptor antagonistic potency of a certain analogue, the higher the P2 receptor vs. ecto-nucleotidases selectivity.

Specificity and P2 receptor subtype selectivity of NF279

In prostatic segments of the ratVD, increasing concentrations produced a progressive rightward shift, and independent of the concentration an increase in the maximum as well as in the slope of the α,β-mATP concentration-contraction curve. Due to an increase of the resulting pA_2 values (6.07 [1 μM], 6.41 [3 μM], 7.06 [10 μM]), the antagonism of NF279 at $P2X_1$-like receptors cannot be regarded as purely competitive. However, the progressive rightward shift in the presence of increasing concentrations of NF279 clearly indicates competitive interactions between NF279 and α,β-mATP. Investigation of the antagonistic activity of NF279 on recombinant P2X receptor subtypes might provide a possible explanation, as kinetics of NF279 and ATP interacting with $P2X_1$ receptors were found to be markedly different. Equilibration with and dissociation from the receptor of NF279 was shown to be considerably slow. Therefore, at native

$P2X_1$-like as well as recombinant $P2X_1$ receptor subtypes, a competitive interaction of NF279 could neither be confirmed nor rejected. On the other hand, NF279 displayed competitive antagonism at native $P2Y_1$-like and $P2X_3$-like receptors of GPI. The $P2X_1$ receptor-selectivity determined for NF279 in studies at native receptors was confirmed and extended, analysing the pharmacological profile on recombinant P2X receptor subtypes. The resulting rank order of antagonistic potency $P2X_1 > P2X_2 \geq P2X_3 \geq P2X_7 >> P2X_4$ as well as the $P2X_1$ receptor-blocking potency is unique among the metabolically stable P2 receptor antagonists described so far.

NF279 was shown to be without influence on α_{1A} receptor-induced contraction in ratVD, adenosine A_1, histamine H_1 and muscarinic M_3 receptor mediated effects in GPI, adenosine A_{2B} and neuronal nicotinic receptors in GPTC. Thus, the antagonism of NF279 appears to be highly specific for P2 receptors.

Heterogeneity of P2 receptors mediating contraction of prostatic and epididymal segments of the rat vas deferens

Comparing the inhibitory potency of NF279 and other P2 receptor antagonists on contractions of ratVD elicited by α,β-mATP, differences were observed with regard to the anatomical origin - either prostatic or epididymal - of the segment. Investigation of contractions of prostatic and epididymal segments induced by agonists and blocked by antagonists with different selectivity profiles revealed a differential distribution of P2 receptor subtypes along the length of the tissue. In prostatic segments, contraction was shown to be mediated almost exclusively via $P2X_1$-like receptors, whereas in epididymal segments in addition to the $P2X_1$-like receptor, at least one further P2 receptor subtype is involved in the contractile response. Therefore, studies on $P2X_1$ receptor-mediated effects on both segments of the ratVD might lead to misinterpretations. Unfortunately, the additional P2 receptor subtype(s) present in epididymal segments could not be identified by use of the pharmacological tools presently available. However, based on the results obtained in the present study and regarding the P2 receptor proteins detected in ratVD so far, the unknown receptor most probably belongs to the P2X receptor family and may be the result of an heteromeric assembly of different P2X receptor subunits, generating a novel pharmacological phenotype.

6. Zusammenfassung

Zu Beginn der 70er Jahre wurde für Adenosin-5'-triphosphat (ATP), das bereits lange als innerhalb der Zelle vorkommender Energieträger bekannt war, eine extrazelluläre Wirkung als Neurotransmitter postuliert. Es dauerte allerdings noch eine Reihe von Jahren, bis die von der Arbeitsgruppe um G. Burnstock aufgestellte Hypothese der "purinergen Nerven" und die damit verbundene Existenz von sogenannten P2-Rezeptoren (früher purinerge Rezeptoren) allgemein anerkannt wurde. Heute besteht kein Zweifel mehr, dass ATP alle der unten genannten Anforderungen an eine als endogener Überträgerstoff innerhalb des vegetativen und zentralen Nervensystems fungierende Substanz erfüllt:

- Synthese und Speicherung des entsprechenden Überträgerstoffes,
- Freisetzung einer definierten Menge an Transmitter bei einem einlaufenden Aktionspotential,
- Rezeptoren für die Reaktion mit dem Transmitter sowie
- die rasche Inaktivierung des freigesetzten Neurotransmitters.

Die Familie der P2-Rezeptoren gliedert sich in zwei Unterfamilien, von denen jeweils weitere Subtypen existieren. P2X-Rezeptoren sind Ligand-gesteuerte Kationenkanäle, die P2Y-Rezeptoren gehören zur großen Familie der G-Protein-gekoppelten Rezeptoren. Mittlerweile sind sieben P2X-Rezeptor-Subtypen ($P2X_{1-7}$) sowie sechs P2Y-Rezeptor-Subtypen ($P2Y_{1,2,4,6,11,12}$) molekularbiologisch und pharmakologisch charakterisiert und von der IUPHAR (International Union of Pharmacology) als Mitglieder der P2-Rezeptor-Familie anerkannt. Neben ATP interagieren auch andere Nukleotide wie z. B. Adenosin-5'-diphosphat sowie Uridin-5'-tri- und -diphosphat als physiologische Liganden mit einigen Subtypen der P2Y-Rezeptor-Familie.

Die Inaktivierung der freigesetzten Nukleotide erfolgt durch enzymatische Abspaltung der Phosphat-Gruppen (Dephosphorylierung) durch größtenteils membranständige, zum Teil aber auch lösliche Enzyme, die häufig unter dem Begriff "Ekto-Nukleotidasen" zusammengefasst werden. Endprodukt dieses extrazellulär verlaufenden Abbaus ist Adenosin, das ebenfalls ein Neuro-modulator/-transmitter ist, und dessen meist inhibitorischen Effekte über P1-Rezeptoren (A_1, A_{2A}, A_{2B}, A_3) vermittelt werden.

Eine der größten Herausforderungen auf dem immer noch vergleichsweise "jungen" Forschungsgebiet der Nukleotid-Rezeptoren ist die Verknüpfung der auf molekularbiologischer Ebene gewonnenen Erkenntnisse mit erwiesenermaßen

durch native P2-Rezeptoren vermittelten physiologischen Effekten. Für die Charakterisierung der nativen Rezeptoren sind Antagonisten, die die Wirkung des physiologischen Liganden an seinen Rezeptoren möglichst potent, spezifisch und möglichst selektiv für einen bestimmten Rezeptor-Subtyp blockieren - sogenannte "pharmakologische Werkzeuge" - von großer Bedeutung. Diese könnten z.B. auch als Radioliganden in Bindungsstudien eingesetzt werden, um so ein breiteres Screening zur Identifizierung neuer Leitstrukturen und - letztendlich - neuer Arzneimittel zu ermöglichen. Neben der oft mangelnden Wirkstärke, Subtypselektivität und Reversibilität des inhibitorischen Effektes einer Vielzahl der bisher eingesetzten Antagonisten ist ihre Wechselwirkung mit Nukleotid-abbauenden Enzymen, vor allem für Untersuchungen an nativen P2-Rezeptoren, ein erheblicher Nachteil.

Der in der Therapie der Schlafkrankheit sowie der durch Filarien ausgelösten Onchozerkose eingesetzte Wirkstoff Suramin (Germanin®) erwies sich in pharmakologischen Untersuchungen an nativen und klonierten P2-Rezeptor-Subtypen als unspezifisch wirkender, nicht-selektiver, zum Teil auch nicht-kompetitiver Antagonist. Aus einer kleineren Anzahl untersuchter Suramin-Derivate zeigte NF023 eine im Vergleich zur Muttersubstanz erhöhte inhibitorische Wirksamkeit an vaskulären und nicht-vaskulären P2X-Rezeptoren (P2X$_1$-ähnliche Rezeptoren) sowie eine stark verminderte antagonistische Potenz an glattmusklär vorkommenden P2Y-Rezeptoren (P2Y$_1$-ähnliche Rezeptoren). Nachfolgende Untersuchungen an klonierten P2-Rezeptor-Subtypen bestätigten diese Ergebnisse, und die Substanz ist bis heute einer der wenigen kompetitiven, P2X$_1$-Rezeptor-selektiven Antagonisten.

Die Gruppe der Suramin-Derivate bot daher die Möglichkeit: (1) möglicherweise weitere interessante P2-Rezeptor-Antagonisten mit neuen pharmakologischen Profilen zu identifizieren und (2) durch Untersuchung struktureller Modifikationen, Struktur-Wirkungs-Analysen innerhalb dieser Substanzgruppe durchzuführen.

Entwicklung von Methoden zur Charakterisierung der antagonistischen Wirkstärke von Suramin-Derivaten an nativen P2-Rezeptor-Subtypen und Ekto-Nukleotidasen

Im Rahmen dieser Arbeit wurden zunächst basierend auf bereits bestehenden pharmakologischen Modellen Methoden entwickelt, die die Untersuchung einer

Vielzahl von Substanzen, die zum Teil nur in geringer Menge verfügbar waren, an glattmuskulären P2X- und P2Y-Rezeptoren sowie den Nukleotid-abbauenden Ekto-Nukleotidasen ermöglichen.

Der postsynaptische kontraktionsvermittelnde P2-Rezeptor-Subtyp im Samenleiter des Kaninchens war bereits in früheren Arbeiten als P2X-Rezeptor (P2X$_1$-ähnlich) charakterisiert worden. Aus diesen Arbeiten ergaben sich jedoch Hinweise, dass die durch elektrische Feldstimulation - durch Freisetzung des endogenen Liganden ATP - ausgelösten Kontraktionen nicht ausschließlich durch diesen P2-Rezeptor-Subtyp vermittelt werden. Daher wurde der inhibitorische Effekt der Derivate des Suramin auf durch exogene Zugabe einer Einzeldosis des P2X-Rezeptor-selektiven Agonisten α,β-Methylen-ATP ausgelöste Kontraktionen des Gewebes bestimmt.
Suramin und NF023 dienten hier, wie auch für die anderen verwendeten pharmakologischen Modelle, als Vergleichssubstanzen.

Für die Bestimmung der antagonistischen Wirkstärken der Suramin-Derivate an glattmuskulären P2Y-Rezeptoren erwies sich die Taenia coli des Meerschweinchens, eines der Prototyp-Gewebe für diesen Rezeptor-Subtyp, als brauchbar. Hier wurde der inhibitorische Effekt der Substanzen auf durch den P2-Rezeptor-Agonisten ADPβS ausgelöste Relaxationen des durch Carbachol vorkontrahierten Präparates (in Form von Dosis-Wirkungs-Kurven) durch Auswertung des Ausmaßes der Rechtsverschiebung der Dosis-Wirkungs-Kurven in Gegenwart des potentiellen Antagonisten (pA$_2$-Wert) bestimmt. Inzwischen ist die frühere Charakterisierung des relaxationsvermittelnden P2Y-Rezeptors als P2Y$_1$-Rezeptor-Subtyp umstritten. Aus diesem Grund wurde für die detaillierte pharmakologische Untersuchung ausgewählter Substanzen die Longitudinal-muskulatur des Ileum des Meerschweinchens, in der durch ADPβS ausgelöste Kontraktionen erwiesenermaßen durch P2Y$_1$-ähnliche Rezeptoren vermittelt werden, als zusätzliches pharmakologisches Modell verwendet.

Da sich die Taenia coli des Meerschweinchens im Rahmen der vorliegenden Untersuchungen aufgrund der geringen, nicht konzentrations-abhängigen inhibitorischen Wirkung der Referenzsubstanz Suramin als unbrauchbar für die Untersuchung einer potentiellen Hemmung von Nukleotid-abbauenden Enzymen durch die Derivate des Suramins erwies, wurden diese an Oozyten des Krallenfrosches *Xenopus laevis* durchgeführt. Die ATP-abbauenden Enzyme im

Follikelepithel der Oozyten wurden bereits in vorhergehenden Arbeiten als zur Gruppe der Ekto-Nukleotidasen gehörig charakterisiert.

Struktur-Wirkungsanalyse von Suramin-Derivaten

Die im Rahmen des Screenings bestimmten inhibitorischen Effekte einer einzelnen Konzentration (10 μM) der von Suramin abgeleiteten sogenannten "großen Harnstoffe" sowie der von NF023 abgeleiteten "kleinen Harnstoffe", läßt nur erste Rückschlüsse auf einen möglichen Zusammenhang zwischen Struktur und Wirkung zu. In erster Linie wurde dieses breit angelegte Screening durchgeführt, um neue, in ihrer Wirkstärke bzw. ihrem pharmakologischen Profil den Muttersubstanzen überlegene P2-Rezeptorliganden zu identifizieren. Dennoch ließen sich diese vorläufigen Befunde in eingehenderen pharmakologischen Untersuchungen ausgewählter Verbindungen bestätigen. Aus der Vielzahl der untersuchten Strukturvariationen sollen im folgenden nur einige besonders bemerkenswerte Befunde erläutert werden (als Vergleichssubstanz diente hier - neben den bereits erwähnten Muttersubstanzen Suramin und NF023 - das 4'-Desmethyl-Derivat des Suramin NF037):

- 4'-Substitution mit Elektronen-ziehenden wie auch Elektronen-schiebenden Substituenten führen sowohl in der Gruppe der großen als auch der kleinen Harnstoffe zu einer verminderten inhibitorischen Aktivität am P2X-Rezeptor.
- Die Einführung eines großvolumigen, Elektronen-reichen Phenylrestes in die 4'-Position wird in beiden Gruppen, bezogen auf die inhibitorische Aktivität an den untersuchten P2-Rezeptor-Subtypen, besser toleriert.

Somit lässt sich die im Vergleich zu Suramin erhöhte antagonistische Potenz von NF023 am P2X$_1$-Rezeptor bzw. die verminderte inhibitorische Wirkung an P2Y$_1$-ähnlichen Rezeptoren eher auf den fehlenden 4'-Methyl-Substituenten und nicht, wie ursprünglich angenommen, auf das Fehlen einer aromatischen Teilstruktur zurückführen.

- Wird das Phenyl-Fragment in der Gruppe der kleinen Harnstoffe durch eine Biphenyl-Struktur ersetzt, so konnte, in Abhängigkeit von der Verknüpfung dieser Biphenyl-Struktur mit der Harnstoffbrücke und den beiden Naphthalin-tri-sulfonsäure-Resten, eine Zunahme der inhibitorischen Wirksamkeit der entsprechenden Derivate an beiden P2-Rezeptor-Subtypen festgestellt werden.

- Der Austausch der Harnstoffbrücke gegen andere Strukturelemente - z.B. Thioharnstoff, Phthalsäure, Piperazin - hat in allen untersuchten Fällen eine Abnahme der inhibitorischen Aktivität zur Folge.

Detaillierte pharmakologische Untersuchungen von Suramin- (bzw. 4'-Desmethyl-Suramin) und NF023-analogen Verbindungen mit 1,3,5- sowie 1,3,6-Naphthalintrisulfonat-Substituenten, die sich außerdem in der Verknüpfung (1'-4',1"-3" und 1'-4',1"-4" [4'-Desmethylsuramin] bzw. 1'-4' [NF023]) der Amino-benzoyl-Teilstrukturen unterscheiden, ermöglichten weitere Aussagen zu Struktur-Wirkungs-Beziehungen.

Zur Bestimmung der antagonistischen Potenz der o. g. Substanzen an P2X-Rezeptoren wurden an Stelle des Samenleiters des Kaninchens prostatische Segmente des Samenleiters der Ratte verwendet. Gründe für den Wechsel der Spezies waren (1) die weit verbreitete Verwendung dieses Präparates für Untersuchungen an nativen $P2X_1$-ähnlichen Rezeptoren und (2) der eindeutige Nachweis der Anwesenheit des $P2X_1$-Rezeptor-Subtyps, der erstmals durch Expressionsklonierung aus diesem Gewebe isoliert wurde. Auf diese Weise ließen sich Ergebnisse der vorliegenden Arbeit besser mit Untersuchungen anderer Arbeitsgruppen an nativen $P2X_1$-ähnlichen Rezeptoren, vor allem aber rekombinanten $P2X_1$-Rezeptoren, vergleichen.

Die 1'-4',1"-4" verknüpften Verbindungen NF279 und NF031 waren die potentesten Antagonisten innerhalb der o. g. Gruppen und zeigten darüber hinaus eine beachtliche Selektivität für den $P2X_1$-ähnlichen Rezeptor im Samenleiter der Ratte. Im Vergleich zu den Muttersubstanzen war eine reduzierte antagonistische Potenz der 1'-4', 1"-3"-verknüpften strukturellen Zwischenstufen NF280 und NF032 an $P2X_1$-ähnlichen, für NF032 auch an $P2Y_1$-ähnlichen Rezeptoren zu verzeichnen. Ein Einfluss des Substitutionsmusters der Naphthalintrisulfon-säure-Reste konnte, was die antagonistische Wirkstärke an $P2X_1$-ähnlichen Rezeptoren angeht, nur für die beiden 1'-4',1"-4" verknüpften großen Harnstoffe NF279 und NF031 festgestellt werden. Hier zeigte sich jedoch, dass die Verschiebung eines Sulfonat-Restes zu einem deutlichen Verlust der antagonistischen Potenz an beiden P2-Rezeptor-Subtypen führt.

Auch in der Gruppe der kleinen Harnstoffe konnte ein - wenn auch etwas anderer - Einfluss dieser Strukturvariation gezeigt werden. Die Verschiebung eines

Sulfonat-Substituenten von der 5- auf die 6-Position des Naphthalin-Ringsystems steigerte die antagonistische Wirkstärke an $P2Y_1$-ähnlichen Rezeptoren um mehr als eine Zehnerpotenz, während die inhibitorische Aktivität von NF550 an $P2X_1$-ähnlichen Rezeptoren im Vergleich zu NF023 unverändert blieb.

Bezogen auf die inhibitorische Aktivität der Suramin- und NF023-Derivate an Ekto-Nukleotidasen läßt sich folgendes feststellen: Generell zeigen die Substanzen gegenüber den Nukleotid-abbauenden Enzyme im Follikelepithel der Oozyten des Krallenfrosches eine relativ ähnliche inhibitorische Wirkstärke. Das läßt den Schluss zu, dass mit steigender antagonistischer Potenz der Suramin-Derivate an einem oder mehreren P2-Rezeptor-Subtypen die Selektivität ihrer antagonistischen Wirkung bezogen auf die als Nebeneffekt auftretende Hemmung der Ekto-Nukleotidasen zunimmt.

Untersuchungen der P2-Rezeptor-Subtyp-Selektivität und Spezifität von NF279

An prostatischen Segmenten des Samenleiters der Ratte führte die Inkubation steigender Konzentrationen des Antagonisten NF279 zu einer zunehmenden Rechtsverschiebung, und zusätzlich zu einer Konzentrations-unabhängigen Steigerung des Maximums sowie zu einer Versteilung der Konzen-trations-Wirkungs-Kurve von α,β-Methylen-ATP. Darüber hinaus war für steigende Konzentrationen des Antagonisten eine Zunahme der resultierenden pA_2-Werte zu verzeichnen (pA_2-Werte: 6.07 [1 μM], 6.41 [3 μM], 7.06 [10 μM]), so daß eine Regression nach Arunlakshana und Schild (1959) eine Gerade mit einer Steigung > 1 (signifikant von 1 verschieden) ergeben hätte. NF279 kann somit nicht als "kompetitiver Antagonist" im strengen Sinne bezeichnet werden. Die zunehmende Rechtsverschiebung in Gegenwart steigender Konzentrationen des Antagonisten ist allerdings ein eindeutiger Hinweis auf eine kompetitive Wechselwirkung von α,β-Methylen-ATP und NF279 am $P2X_1$-ähnlichen Rezeptor des Samenleiters der Ratte. Untersuchungen zur antagonistischen Wirkung von NF279 an rekombinanten $P2X_1$-Rezeptor-Subtypen ergaben Hinweise, dass die oben beschriebenen nicht-kompetitiven Wechselwirkungen möglicherweise durch Unterschiede in der Kinetik von Agonist (ATP) und NF279 zustande kommen. Dennoch konnte auch im Rahmen dieser Untersuchungen nicht abschließend geklärt werden, ob es sich bei NF279 um einen rein kompetitiven $P2X_1$-Rezeptor-selektiven Antagonisten handelt. Sowohl an den $P2Y_1$-ähnlichen wie auch an den

$P2X_3$-ähnlichen Rezeptoren der Longitudinal-Muskulatur des Ileum des Meerschweinchens erwies sich NF279 als rein kompetitiver Antagonist (pA_2-Werte von 5.32 bzw. 5.95). Bezogen auf native P2-Rezeptor-Subtypen ergibt sich damit das folgende pharmakologische Profil: P2X (Samenleiter der Ratte) > P2X (Ileum des Meerschweinchens) > P2Y (Ileum des Meerschweinchens). Das aus Untersuchungen an rekombinanten P2-Rezeptor-Subtypen resultierende, bisher einzigartige, pharmakologische Profil - $P2X_1$ > $P2X_2 \geq P2X_3 \geq P2X_7 \gg P2X_4$ - unterstreicht die Bedeutung von NF279.

NF279 (100 μM) zeigte keinerlei Einfluss auf α_{1A}-Rezeptor-vermittelte Kontraktionen im Samenleiter der Ratte, Adenosin A_1-Rezeptor- und Nicotin-Rezeptor-vermittelte sowie A_{2B}-Rezeptor-, Histamin H_1- und Muskarin M_3-Rezeptor-vermittelte Effekte in der Taenia coli bzw. in der Longitudinal-Muskulatur des Ileum des Meerschweinchens. Der Antagonismus von NF279 erwies sich somit als hoch spezifisch für P2-Rezeptor-Subtypen.

Untersuchungen zur Heterogenität P2-Rezeptor-vermittelter Effekte in prostatischen und epididymalen Segmenten des Samenleiters der Ratte

Aus dem Vergleich der antagonistischen Potenz von NF279 und einigen anderen P2-Rezeptor-Antagonisten an prostatischen und epididymalen Segmenten des Samenleiters der Ratte ergaben sich Hinweise auf eine asymmetrische Verteilung kontraktionsvermittelnder P2 Rezeptoren innerhalb des Gewebes. Weitergehende Untersuchungen zeigten, dass die Kontraktion prostatischer Segmente - wie ursprünglich für den gesamten Samenleiter angenommen - nahezu ausschließlich durch $P2X_1$-ähnliche Rezeptoren vermittelt wird, während in epididymalen Segmenten α,β-Methylen-ATP und ADPβS mindestens einen weiteren P2-Rezeptor-Subtyp stimulieren. Die Verwendung beider Segmente für die Untersuchung potentieller Antagonisten kann somit, zumindest für Substanzen, deren pharmakologisches Profil dem des NF279 ähnelt, zu Fehleinschätzungen führen. Leider ist es mit den vorhanden pharmakologischen Werkzeugen nicht gelungen, diese(n) weiteren Subtyp(en) eindeutig zu charakterisieren. Basierend auf den vorliegenden Ergebnissen und auf Untersuchungen mit Subtyp-spezifischen Antikörpern kann nach dem heutigen Kenntnisstand davon ausgegangen werden, dass es sich hierbei um einen P2X-Rezeptor-Subtyp handeln muss, möglicherweise auch einen durch Assemblierung unterschiedlicher P2X-Rezeptor-Untereinheiten bisher noch nicht identifizierten Phänotyp.

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8. Glossary of Abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
ADP	Adenosine-5'-diphosphate
ADPβS	Adenosine 5'-O-(2-thiodiphosphate)
ADPγS	Adenosine 5'-O-(3-thiotriphosphate)
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine-5'-monophosphate
AMP-PNP	β,γ-imidoadenosine-5'-triphosphate
ANAPP$_3$	Arylazidoaminopropionyl-ATP
Ap$_n$A	Adenine dinucleotides ("n" indicating the number of phosphates between the two adenosine moities)
A2P5P	Adenosine-2'-phosphate-5' phosphate
A3P5P	Adenosine-3'-phosphate-5' phosphate
A3P5PS	Adenosine-3'-phosphate-5'-phosphosulfate
ARC-66096MX	2-Propylthio-D-β,γ-difluoromethylene ATP
AR-C67085MX	2-propylthio-D-β,γ-dichloromethylene ATP
AR-C69931MX	2-(3,3,3-trifluoropropylthio)-N^6-methylthioethyl-D-β,γ-dichloromethylene ATP
ARL 67156	6-N,N-diethyl-D-β,γ-dibromomethylene ATP
ATP	Adenosine-5'-triphosphate
ATPγS	Adenosine-5'-O-3-thiotriphosphate
Bst101	N-[3-(N-(1,3,5-Trisulpho-8-naphthyl)carbamoyl)-phenyl]-N'-[3-(N-(1-naphthyl)-carbamoyl)phenyl]-urea
BzATP	2',3'-O-(4-benzoylbenzoyl) ATP
CHO cells	Chinese hamster ovary cells
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
2-Cl-Ado	2-Chloroadenosine
CMP	Cytidine-5'-monophosphate
CNS	Central nervous system
CRC	Concentration-response curve

CIC	Concentration-inhibution curve
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonate
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
DR	Dose ratio
EC_{50}	Molar concentration producing 50 % of the individual maximal response
EFS	Electrical field stimulation
GPI	Guinea-pig ileal longitudinal smooth muscle
GPTC	Guinea-pig taenia coli
G protein	Guanyl nucleotide binding protein
HEK293 cells	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
ICC	Interstitial cells of Cajal
INS-316	UTP
INS-365	Diuridine-tetraphosphate
IP_3	Inositol 1,4,5-triphosphate
Ip_5I	Diinosine pentaphosphate
iso-PPADS	Pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid
IUPHAR	International Union of Pharmacology
KN-04	N-[1-[N-methyl-p-(5-isoquinolinesulfonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinolinesulfon-amide
KN-62	1-[N,O-bis(5-isoquinolinesufonyl)-N-methyl-L-tyro-syl]-4-phenylpiperazine
α,β-mADP	α,β-Methylene-ADP
α,β-mATP	α,β-Methylene-ATP
β,γ-mATP	β,γ-Methylene ATP
2-MeSADP	2-Methylthio-ADP
2-MeSATP	2-Methylthio-ATP
min	Minute(s)
MRS 2179	N^6-Methyl-2'deoxy-adenosine.3',5'- biphosphate
MRS 2220	Cyclic pyridoxine-$\alpha^{4,5}$-monophosphate-6-phenyl-azo-2',5'-disulfonate
ms	Millisecond(s)

NA	Noradrenaline
NANC	Non-adrenergic, non-cholinergic
NBTG	S-p-Nitrobenzyl-6-thioguanosine
n.d.	Not determined
NDPK	Nucleoside diphosphokinase
NECA	5'-N-Ethylcarboxamidoadenosine
NF018	8-(3-(3-Nitrobenzamido)benzamido)-naphthalene-1,3,5-trisulphonic acid
NF019	8-(3-(3-Aminobenzamido)benzamido)-naphtha-lene-1,3,5-trisulphonic acid
NF023	8,8'-(Carbonylbis(imino-3,1-phenylenecarbonyl-imino))bis(1,3,5-naphthalenetrisulphonic acid)
NF105	8-(2-Methyl-3-(3-aminobenzamido)-benzamido)-naphthalene-1,3,5-trisulphonic acid
NIDDM	Non-insulin-dependent diabetes mellitus
oxoATP	2',3'-dialdehyde-ATP
PAGE	Polyacrylamide gel electrophoresis
PC	Pheochromocytoma
PCR	Polymerase chain reaction
PDNP	Phosphodiesterase/nucleotide pyrophosphatase
P_i	Inorganic phosphate
PLC	Phosholipase C
PP_i	Pyrophosphate
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
PPNDS	Pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate)
pA_2	Negative logarithm of the antagonist concentration shifting the agonist-concentration-response curve two-fold to the right
pA_2*	Apparent pA_2 values
RatVD	Rat vas deferens
RB2	Reactive blue 2
RT-PCR	Reverse transcription-polymerase chain reaction
RVD	Rabbit vas deferens
s	Second(s)

SAR	Structure-activity relationship
SB9	6-[(4,6,8-trisulpho-1-naphthyl)iminocarbonyl-1,3-(4-methylphenylene)-iminocarbonyl-1,3-phenylene-azo]-pyridoxal-5'-phosphate
SCG	Superior cervical ganglia
8-SPT	8-(p-Sulfophenyl) theophylline
τ	Time constant
TM	Transmembrane region
TNP	Trinitrophenyl
TNP-ATP	2',3'-O-(2',4',6')-Trinitrophenyl-ATP
UDP	Uridine-5'-diphosphate
UMP	Uridine-5'-monophosphate
UTP/INS-316	Uridine-5'-triphosphate
w/v	Weight per volume
XAMR0721	8-(3,5-Dinitro-phenylenecarbonylimino)-1,3,5-naphthalenetrisulphonic acid

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10. Curriculum Vitae

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5. September 1991	1. Abschnitt der Pharmazeutischen Prüfung
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November 1993 - April 1994	Pharmaziepraktikum, St. Hubertus-Apotheke, Berlin
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Dezember 1994 - November 1999	Wissenschaftliche Mitarbeiterin am Pharmakologischen Institut für Naturwissenschaftler
Januar 2000 - August 2000	Apothekerin, Steglitz-Apotheke, Berlin
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