Pharmacological Investigations on Muscarinic and P2 Receptor Subtypes

Pharmacological Characterisation of the Stereoisomers of Glycopyrronium Bromide and their Tertiary Analogues

and

Evaluation of the Isolated Guinea-pig and Rat Ileal Longitudinal Smooth Muscle as Novel P2 Receptor Subtype Models

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Meiner Familie

True science teaches above all to doubt and
to be aware of one's ignorance.

Miguel de Unamuno

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1. INTRODUCTION

1.1. Muscarinic receptors

1.1.1. Historical background

The autonomic nervous system consists of various neural pathways associated with ganglionic synapses residing outside the central nervous system. In one part of the autonomic nervous system, the parasympathetic nervous system, acetylcholine (ACh) is the principal neurotransmitter.
Early in the past century, fundamental studies carried out by Hunt and Taveau (1906), Dale (1914) and Loewi (1921), provided the basis for the classical definition of muscarinic and nicotinic acetylcholine receptors: muscarinic receptors are selectively activated by muscarine and blocked by atropine, whereas effects at nicotinic receptors are mimicked by nicotine and abolished by d-tubocurarine. These receptors are members of two quite different gene superfamilies, and only share the property of being stimulated by the same endogenous ligand, ACh.

1.1.2. Subclassification and nomenclature

Whereas only a few years later the nicotinic receptors were further subdivided into a muscular and a ganglionic type, the muscarinic receptors were regarded as a homogeneous population for a long time. Historically, the first indications of a possible heterogeneity of muscarinic receptors were the cardioselective actions of the neuromuscular relaxant gallamine (Riker and Wescoe, 1951), antagonising effects induced by the muscarinic agonist methacholine. Gallamine did not affect the decrease of blood pressure caused by methacholine, but reduced its negative chronotropic effect. The reverse selectivity of 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; Barlow et al., 1976) further supported the view of different muscarinic receptor subtypes. The first functionally selective muscarinic agonist, 4-(3-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) was described by Roszkowski (1961). This quaternary ammonium compound was found to stimulate excitatory muscarinic receptors in sympathetic ganglia without affecting muscarinic receptors present in the heart or

jejunum. Since then, there was a growing interest in the subclassification of the muscarinic receptors.

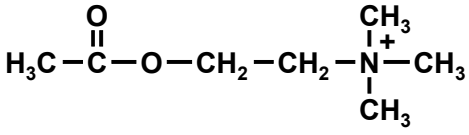
The first nomenclature system for the subdivision was introduced by Woodruff and Walker (1971), who proposed to denote muscarinic receptors stimulated by McN-A-343 as M_1 receptors, and those unaffected by this agonist as M_2 receptors. Subsequently, this classification was confirmed by investigations of many other groups (Goyal and Rattan, 1978; Mutschler and Lambrecht, 1984; Wess et al., 1987).

An important milestone in the classification of muscarinic receptors was the development of the muscarinic antagonist pirenzepine. Hammer et al. (1980) demonstrated in radioligand binding studies that pirenzepine displayed a higher affinity for muscarinic receptors expressed in neuronal tissues, e.g. cerebral cortex, hippocampus, sympathetic ganglia, compared to those expressed in peripheral organs, e.g. heart, smooth muscle, secretory glands, and certain brain stem areas. Soon afterwards, Hammer and Giacetti (1982) concluded from their observations made in functional and binding studies, that pirenzepine and McN-A-343 may recognise a common receptor subtype. The confirmation of these findings in subsequent studies led to the introduction of the first generally accepted nomenclature for muscarinic receptors (Hirschowitz et al., 1984) on the first international symposium ”Subtypes of Muscarinic Receptors” in 1983 in Boston/USA: muscarinic receptors with high affinity for pirenzepine were designated as M_1 receptors, and those with low affinity for pirenzepine were termed M_2 receptors. However, it had already been pointed out, that the M_2 receptors most likely represent a heterogeneous population.

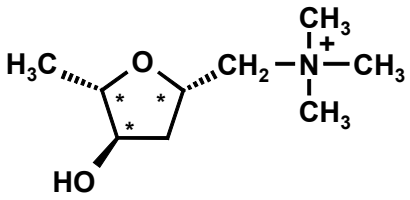
The following years were characterised by the development of selective muscarinic agonists and antagonists. The heterogeneity of M_2 receptors became substantially evident with the introduction of hexahydro-sila-difenidol (HHSiD) and its para-fluoro-analogue (p-F-HHSiD). In functional experiments as well as in radioligand binding studies these compounds exhibited an up to 70-fold higher affinity for smooth muscle and glandular muscarinic receptors than for muscarinic receptors in the heart (Mutschler and Lambrecht, 1984; Lambrecht et al., 1988, 1989a; Waelbroeck et al., 1991). Further support to the differentiation of muscarinic receptor subtypes was contributed by a number of structurally quite different compounds exhibiting higher affinities to M_2 than to M_3 receptors and, thus, displaying an inverse selectivity to the above mentioned compounds HHSiD and p-F-HHSiD, e.g. the alkaloid himbacine, the pirenzepine analogues AF-DX 116, AF-DX 384 and AQ-RA 741, and the polymethylene-tetraamines

methoctramine and mefurtramine (reviewed by Mutschler et al., 1995; Eglen and Watson, 1996). Based on the findings in functional and radioligand binding studies with the antagonists described above, Doods et al. (1987) suggested an extended nomenclature, which was presented on the ”Fourth Symposium on Subtypes of Muscarinic Receptors”: M_1, M_2 and M_3 receptors (Birdsall et al., 1989).
In the meantime, a fourth pharmacologically defined muscarinic receptor, the M_4 receptor, has been identified using radioligand and biochemical techniques in a variety of different tissues and cell lines. The confirmation in functional experiments was first shown by combining the data obtained with the relatively subtype-selective muscarinic antagonists. Therefore, a major step forward was the presentation of the isolated rabbit anococcygeus muscle as a robust test model for this receptor subtype by Gross et al. (1995, 1997b). Later, this group was able to confirm with this model data from binding studies with the novel M_4-selective muscarinic antagonist, PD102807 (Augelli-Szafran et al., 1997, 1998; Schwarz et al., 1997). This compound displayed in functional experiments the following selectivity profile: $M_4 > M_3$ (10-fold) > M_2 (3-fold) > M_1 (Gross et al., 1997a).

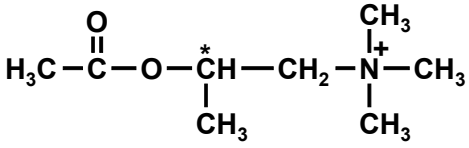
The chemical structure of some key muscarinic agonists and antagonists are given in Figs. 1.1. and 1.2., respectively.



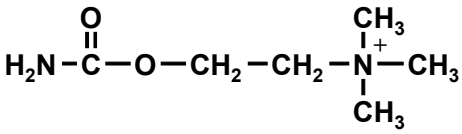
Acetylcholine



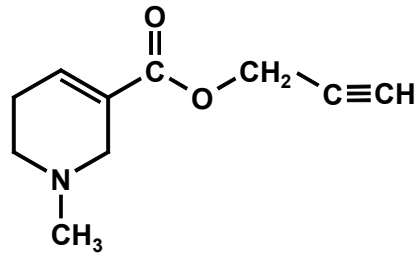
(+)-Muscarine



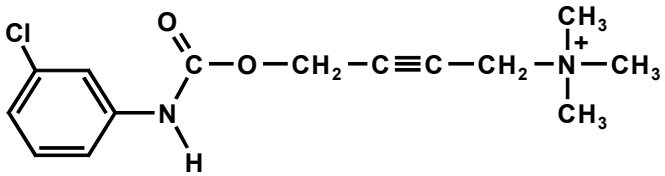
Methacholine



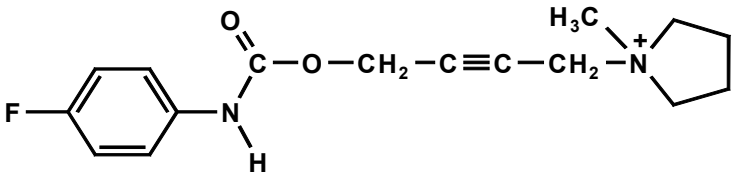
Carbachol



APE

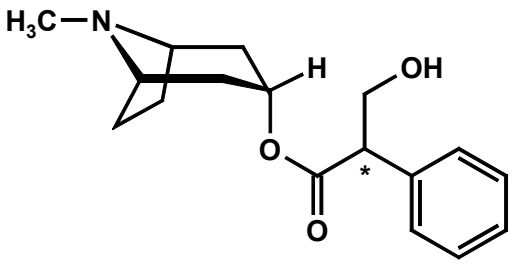


McN-A-343

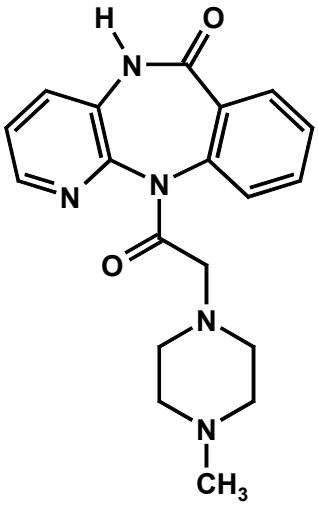


4-F-PyMcN^{+}

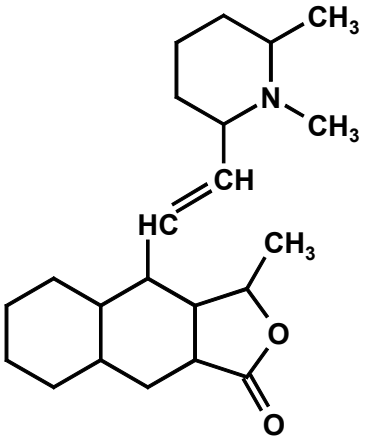
Fig. 1.1. *Chemical structure of some muscarinic key agonists. The asterisks denote the centres of chirality.*



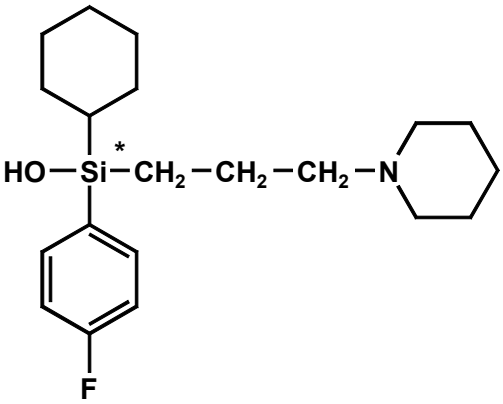
Atropine



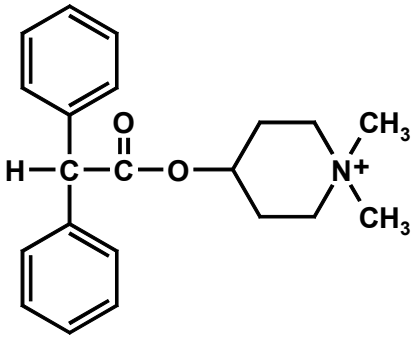
Pirenzepine



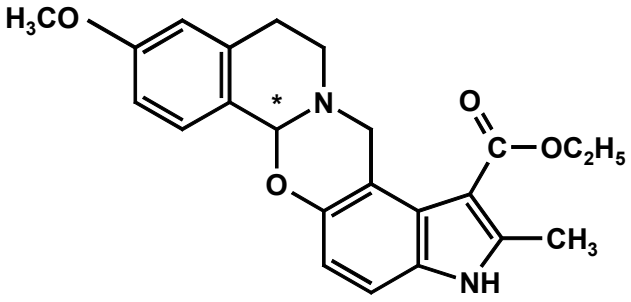
Himbacine



p-F-HHSiD



4-DAMP



PD 102807

Fig. 1.2. *Chemical structure of some muscarinic key antagonists. The asterisks denote the centres of chirality, except himbacine.*

A major aim of current research consequently lies in the identification of selective ligands for each of the muscarinic receptor subtypes. In terms of agonists, this aim has not really been achieved, because of the complexity of this field. Drug-receptor theory proposes that agonist potencies depend on several tissue factors, including receptor density, G protein density and the efficiency of the stimulus response coupling (Kenakin et al., 1992). All these factors may vary between tissues and cell lines with consequent variation in muscarinic agonist potency. This complication may be one reason why relatively little progress has been made in the area of muscarinic agonists with adequate subtype selectivity (Lambrecht et al., 1993; Angeli, 1995).

However, several advances have been made in the identification of selective antagonists. In the past few years a number of new muscarinic antagonists have been described, which will prove useful as both therapeutics with reduced side effects and tools to define muscarinic receptor subtypes operationally, because they show at least some selectivity for one of these subtypes:

- *M_1 receptor*: telenzepine (Eltze et al., 1985), guanylpirenzepine (Micheletti et al., 1990) and spirotramine (Melchiorre et al., 1995), MT7 (Jerusalinsky et al., 2000);
- *M_2 receptor:* tripitramine (Melchiorre et al., 1993), (S)-enantiomer of dimethindene (Pfaff et al., 1995; Pfaff, 1996);
- *M_3 receptor:* darifenacin (Wallis et al., 1995; Alexander and Peters, 1997), zamifenacin (Eglen, 1994; Wallis, 1995) and tiotropium bromide (Ba 679 BR), a muscarinic antagonist with kinetic selectivity for M_3 receptors (Barnes et al., 1995; Barnes, 2000; Norman et al., 2000);
- *M_4 receptor:* muscarinic toxin 3 (MT3), a peptide toxin isolated from green mamba venom (Jolkkonen et al., 1994; Olianas et al., 1999; Jerusalinsky et al., 2000), PD102807 (Augelli-Szafran et al., 1997, 1998; Gross et al., 1997a; Schwarz et al., 1997).

1.1.3. Cloning studies and structure

The use of recombinant receptors and polymerase chain reaction (PCR) confirmed the heterogeneity of muscarinic receptors. In 1986, the group of Numa was successful in cloning the first two muscarinic receptor genes from porcine cDNA libraries. These genes encoded the porcine cerebral and cardiac muscarinic receptors, termed m1 and m2 receptors, respectively (Kubo et al., 1986a, b). The sequence of the m2 receptor was subsequently reported by Peralta et al. (1987b).

The receptor genes for the muscarinic m3, m4 and m5 receptors have been cloned from rat cerebral cortex (Bonner et al., 1987, 1988). Finally, the last step was the cloning and sequencing of all five human muscarinic receptor subtypes (Peralta et al., 1987a; Bonner et al., 1988). When the gene products of the m1 - m5 receptors were expressed in *Xenopus* oocytes and stably transformed mammalian cell lines, e.g. COS-7 and CHO cells, functional muscarinic receptors were produced. Their antagonist binding properties were in good agreement with the corresponding pharmacologically defined M_1 - M_4 receptors (Buckley et al., 1989; Hulme et al., 1990; Dörje et al., 1991c; Richards, 1991; Bolden et al., 1992). However, no functional M_5 receptor has been characterised to date, although mRNA corresponding to the m5 gene as well as the respective receptor protein were detected at very low levels in rat brain (striatum, hippocampus, cerebellum and pons) by means of *in situ* hybridisation and immunoprecipitation (Vilaró et al., 1990; Yasuda et al., 1993). Recently, Reever et al. (1997) developed a novel pharmacological labelling strategy which permits for the first time a comparison of relative expression levels and autoradiographic localisation studies of the five muscarinic receptor subtypes in the brain. Their results suggest a potential physiological role for the M_5 receptor in modulating the actions of ACh in this tissue.

Based on the existing knowledge of the good homology between native and cloned muscarinic receptor subtypes, it is now recommended by the NC-IUPHAR that M_1, M_2, M_3, M_4 and M_5 terms should be used to describe both (Caulfield and Birdsall, 1998).

The size of the five human muscarinic receptor proteins ranges from 460 (M_1) to 590 (M_3) amino acids (Caulfield and Birdsall, 1998). They exhibit a substantial sequence homology, particularly within the seven putative transmembrane regions (TM I -TM VII). Variable regions include the third cytoplasmatic loop (i3) connecting TM V and TM VI as well as the extracellular located amino-(N-) and intracellular located carboxyl-(C-) terminal sequences (Hulme et al., 1990). In agreement with findings obtained with the photoreceptor protein bacteriorhodopsin, the ligand binding site of muscarinic receptors appears to be formed by the seven transmembrane domains, which enclose a well-conserved ligand binding pocket. Affinity labelling studies and mutagenesis experiments have demonstrated that an aspartic acid residue in the transmembrane region III appears to be essential in muscarinic ligand binding. Formation of an ionic bond

between the ammonium headgroup and the aspartic acid of TM III thus appears to be a general feature of ligand binding to muscarinic receptors. Moreover, Wess et al. (1991, 1992) have shown that two threonine and four tyrosine residues in the transmembrane regions III, V, VI and VII are probably involved in recognising the muscarinic ligands specifically. It has been proposed that the hydroxyl groups present in the side chains of these amino acids would form a hydrogen bond with the ester group of ACh. Studies on chimeric muscarinic receptors clearly indicate that sequences within the i3 loop dictate the G protein-coupling selectivity of the muscarinic receptor subtypes (Bonner, 1992; Wess, 1993; Blüml et al., 1994; Wess et al. 1995, 1997).

1.1.4. Signal transduction pathways

The muscarinic receptors are structurally classified as members of the superfamily of membrane-bound G protein-coupled receptors. In response to agonist stimulation, these heptahelical receptors activate heterotrimeric guanine-binding proteins (G proteins) by catalysing the exchange of GTP for GDP bound to the G_α subunit of the $G_\alpha G_{\beta\gamma}$ heterotrimer. The separated G protein subunits activate various second messenger systems directly or indirectly (Clapham, 1996).
While it was originally thought that effector regulation was exclusively accomplished via activated α subunits, there is now also increasing evidence that muscarinic receptors may act via $G_{\beta\gamma}$ subunits as well. Thus, it appears that activation of phospholipase C (PLC), adenylate cyclase (AC), cardiac K^+ channels as well as inhibition of neuronal Ca^{2+} channels can be produced by $G_{\beta\gamma}$ subunits. The physiological role of this route and its importance has yet to be established (Caulfield, 1993; Clapham and Neer, 1997).

On the basis of sequence homology and preferential G protein-coupling, muscarinic receptors can be divided into two functional classes: one consisting of M_1, M_3 and M_5 receptors and the other of M_2 and M_4 receptor subtypes.

Activation of the odd-numbered muscarinic receptor subtypes, M_1, M_3 and M_5, results in various biochemical effects, including stimulation of PLC, release of arachidonic acid, stimulation of protein kinase C, activation of intracellular calcium-dependent potassium and chloride currents. These responses are mediated by pertussis toxin (PTX)-insensitive G proteins of the $G_{q/11}$ family.

Activation of the even-numbered muscarinic M_2 and M_4 receptors is mainly coupled to inhibition of AC and stimulation of the inwardly rectifying potassium current. These responses are thought to be mediated by PTX-sensitive G proteins of the G_i/G_o class (Hulme et al., 1990; Jones, 1993). The signal transduction pathways of the M_1 to M_5 receptors are illustrated in Fig. 1.3.

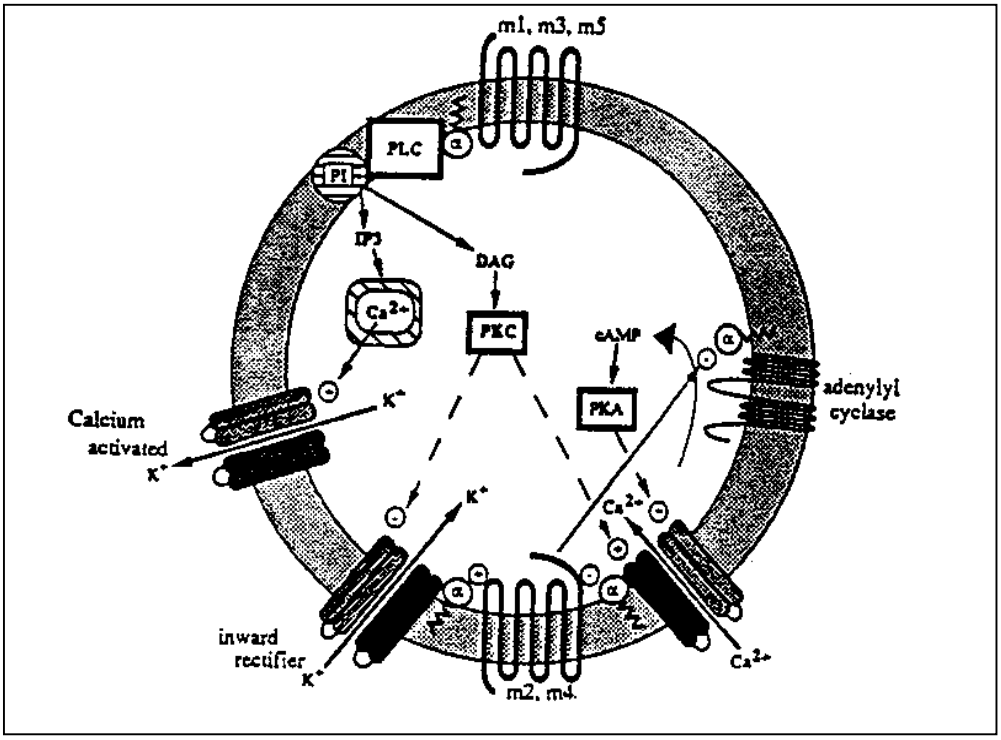


Fig. 1.3. *Summary of the signal transduction pathways of the five muscarinic receptor subtypes (taken from Jones, 1993). The solid lines represent known pathways and the dashed lines indicate suggested pathways.*

1.1.5. Localisation and biological function

The muscarinic receptors are widely expressed throughout the peripheral tissues and the central nervous system. The lack of muscarinic agonists and antagonists with pronounced subtype-selectivity has represented a major limitation in studying the physiological roles of the M_1 - M_5 receptors. However, the availability of the cloned muscarinic receptor genes, immunological methods and recent progress in gene knockout methodologies have provided the opportunity to examine the physiological functions of the individual muscarinic receptor subtype in an unambiguous fashion (Levey, 1993; Gomeza et al., 1999).

Cardiovascular system

The first demonstration that chemical transmission occurred between nerves and end organs was the classical experiment by Loewi (1921) on frog heart, showing that a substance, released by vagus nerve stimulation, was responsible for the cardiac slowing by the vagus. The substance was subsequently identified as ACh, and it is now accepted that both the reduction in rate and force of heart beat resulting from parasympathetic nerve action is due to an action of ACh on muscarinic M_2 receptors located on the heart cells (Caulfield, 1993). However, there is a fundamental difference in effector mechanisms activated by ACh in atria and ventricles. In both, stimulation of M_2 receptors, coupling to PTX-sensitive $G_{i/o}$ proteins leads to inhibition of AC and hence decreases intracellular cAMP. As a consequence, the force of contraction is decreased in an indirect manner via reduction of the L-type Ca^{2+} currents. In atrial myocytes ACh also opens inwardly rectifying potassium channels ($I_{K.ACh}$) through direct effects of G_α protein or $\beta\gamma$ subunits. This results in hyperpolarisation, slowing heart rate, abbreviation of the L-type Ca^{2+} currents, and reduction in the force of contraction in a direct way. In case of human ventricular myocardium activation of M_2 receptors has no direct negative inotropic effect, and it is still a matter of debate, whether ACh might stimulate $I_{K.ACh}$ (Brodde and Michel, 1999). In recent studies mRNA for M_1 receptors has been identified in rat and guinea-pig ventricular cardiomyocytes (reviewed by Brodde and Michel, 1999). Moreover, studies using molecular cloning and patch-clamp techniques as well as mRNA expression detection reported data showing the expression of mRNAs for M_3 and M_4 subtypes beside M_2 receptor subtype in the chick and canine heart (Shi et al., 1999). The role of these gene products and/or their presence has not yet been associated with any functional response.

Gastrointestinal tract

It is a well-recognised fact that parasympathetic nerve activity results in gut contraction, and that this effect is mediated by released ACh acting on muscarinic receptors. Muscarinic receptors are expressed abundantly in smooth muscle throughout the gastrointestinal tract in a manner that approximates a three-to-one mixture of the M_2 and M_3 subtypes (Ehlert et al., 1997, 1999). Muscarinic agonists elicited contractions through the M_3 receptor via stimulation of PLC causing IP_3 accumulation and calcium mobilisation. The M_2 receptor has been shown to cause an indirect contraction in the ileum by preventing the relaxant effects of forskolin and isoproterenol on histamine-induced contractions, when the M_3

receptor subtype was selectively blocked by 4-DAMP mustard (Thomas et al., 1993). Therefore, muscarinic agonists are known to have a dual effect on contraction, a direct M_3-mediated contraction and an indirect M_2-mediated inhibition of relaxation (Sawyer and Ehlert, 1998; Ostrom and Ehlert, 1999).

Respiratory tract

The respiratory tract receives efferent cholinergic parasympathetic innervation via the vagus nerve, stimulation of which produces rapid bronchoconstriction that is blocked by atropine (Richardson, 1979). Autoradiographic mapping studies in animals and human lung have demonstrated that muscarinic receptors are widely distributed, being identified on smooth muscle, submucosal glands, epithelium, blood vessels and parasympathetic nerves (Barnes, 1993; Pendry, 1993; White, 1995). In the respiratory tract, M_2 and M_3 receptor subtypes predominate, whereas M_1 receptors are the minor subtype. In rabbit lung, M_4 receptors are expressed on alveolar walls and smooth muscle (Lazareno et al., 1990; Dörje et al., 1991a; Mak et al., 1993; Vockert, 1996). M_1 receptors are thought to facilitate neurotransmission through parasympathetic ganglia and enhance cholinergic reflexes. M_2 receptors in the airway act as autoreceptors on postganglionic neurons, inhibiting ACh release, and may also act in smooth muscle to counteract the bronchodilatator actions of adrenergic stimulation. M_3 receptors appear to mediate smooth muscle constriction and secretion from submucosal glands (Barnes, 1993; Costello et al., 1998; Barnes, 2000). The localisation of the muscarinic receptors made it evident that cholinergic vagal stimulation provides the major bronchoconstrictor stimulus to the respiratory system and may contribute to airway narrowing in both chronic obstructive pulmonary disease (COPD) and chronic asthma.

Genitourinary tract

Activation of the parasympathetic system is the major pathway by which bladder contraction, and thus voiding, is achieved in man and primates. Binding and subtype-selective immunoprecipitation studies have demonstrated that the majority of muscarinic receptors in the urinary bladder is of the M_2 subtype, whereas on the other hand pharmacological studies using subtype-selective antagonists indicate that the M_3 receptor mediates smooth muscle contraction (Braverman and Ruggieri, 1999; Hegde and Eglen, 1999).

Central nervous system (CNS)

The brain is among the organs with the highest density of muscarinic receptors. M_1 - M_4 receptor proteins have been detected in nearly all parts of the brain. In the CNS, M_1 receptors are found in relatively high density in forebrain areas such as the hippocampus, cerebral cortex and striatum, and therefore play an important role in learning and memory processes. Muscarinic M_2 receptors are present in moderate abundance in forebrain areas, but are predominant in the brainstem, e.g. pons and cerebellum. These receptors are involved in the control of vegetative functions. The density of the M_3 receptor protein is very low in all parts of the brain and its function is still unknown. The M_4 receptor subtype is localised in high levels in the striatum, in moderate levels in the cerebral cortex, hippocampus and midbrain and with the lowest levels in hindbrain. Its localisation in the striatum has led to the proposal that this subtype is involved in the control of motor behaviour (Grimm et al., 1994b; Widzowski et al., 1997).

1.1.6. Therapeutic potential of selective muscarinic agonists and antagonists

Muscarinic antagonists, such as the belladonna alkaloids atropine and scopolamine, have long been used for treatment of a variety of human diseases, including disorders of the nervous system, e.g. Parkinson's disease and motion sickness, the digestive tract system, e.g. peptic ulcer and irritable bowel syndrome, and the respiratory system, e.g. COPD and asthma (Widzowski et al., 1997). However, while these compounds have modest clinical benefit, their utility is limited by the classical antimuscarinic side effects such as dry mouth, blurred vision, dizziness and tachycardia, because they are non-selective across the muscarinic receptor subtypes and many of the troublesome side effects result from interactions with certain receptor subtypes. Therefore, much research over the past years has focused on developing agonists and antagonists selective for one muscarinic receptor subtype.

Cardiovascular system

The peripherally acting muscarinic M_2 receptor antagonist, AF-DX 116 (Otenzepad) may be useful in the treatment of bradycardia (Schulte et al., 1991).

Gastrointestinal tract

In the digestive tract, cholinergic innervation reaches from the parasympathetic nervous system through the vagus nerve to various structures, including salivary glands, esophagus, stomach and gut. Preganglionic, vagal efferents innervate cholinergic postganglionic cells, such as the myenteric nerve plexus, which in turn innervates smooth muscle and glandular tissue, leading to smooth muscle contraction and secretory responses, respectively.

Clinical studies of M_1 antagonists have been ongoing since the 1980s and have generally demonstrated that these compounds are moderately effective in the acute treatment of duodenal and gastric ulcers. Pirenzepine, an antagonist with relatively high affinity for the muscarinic M_1 and modest affinity for M_2 - M_4 receptors, is approved for the treatment of peptic ulcer disease (reviewed by Hirschowitz et al., 1995). Structurally related compounds that had been in clinical development include telenzepine and nuvenzepine.

Respiratory system

Cholinergic mechanisms play an important role in the pathophysiology of diseases characterised by chronic airway obstruction, particularly in COPD and asthma (Barnes, 1986). The introduction of antimuscarinics as bronchodilatators has been hampered by the narrow safety margin of tertiary compounds, such as atropine. The new class of quaternary compounds promises better results.

Ipratropium bromide is a quaternised derivative of atropine, which is poorly absorbed into the systemic circulation when given by inhalation. Although non-selective between subtypes, the poor absorption following inhalation facilitates selective antagonism of airway muscarinic receptors (Eglen and Watson, 1996).

Tiotropium bromide (BA 679 BR) is a novel long-acting antimuscarinic agent, which is structurally related to ipratropium bromide. In *in vitro* receptor binding studies, tiotropium bromide showed kinetic receptor subtype selectivity with rapid dissociation from hm2 and slow dissociation rates from hm1 and hm3 (Disse et al., 1993). The prolonged duration of action after inhalation of tiotropium bromide could be confirmed in clinical studies with patients with COPD (Barnes et al., 1995; Disse et al., 1993, 1999; Maesen et al., 1995; Van Noord et al., 2000). Tiotropium bromide is in Phase III trials as a once-daily dry powder inhalation and is more effective than ipratropium bromide given three times daily (Barnes, 1999; Littner et al., 2000). The prolonged protection against cholinergic neural bronchoconstriction may also be useful in the control of nocturnal asthma (Barnes et al., 1995).

Genitourinary tract

Antimuscarinic agents remain the oldest and most widely prescribed drugs for the treatment of urge urinary incontinence.

Oxybutynin (Ditropan®) has been shown to be clinically effective in the treatment of detrusor instability and hyperreflexia. However, blockade of muscarinic receptors outside the bladder results in significant side-effects, e.g. dry mouth, that severely limit the utility of this compound (Butera and Argentieri, 1998).

Tolterodine (Detrusitol®, Detrol®) is a new muscarinic receptor antagonist, specifically developed for the treatment of bladder instability (Nilvebrant et al., 1997). Clinically it produces significantly less dry mouth than oxybutynin.

CNS

Much of the treatment research and development for Alzheimer's disease (AD) in recent years has been based on the cholinergic hypothesis of memory dysfunction and muscarinic regulation of amyloid metabolism.

The best-developed and most successful approach to cholinergic therapy is the acetylcholinesterase (AChE) inhibition. The AChE inhibitors currently available for the treatment of AD are tacrine, donezepil, rivastigmine and galantamine. Tacrine (Cognex®) was the first agent with Food and Drug Administration-approved labelling for use in the treatment of AD, but hepatotoxicity, cholinergic side effects, and a short half-life have limited the widespread use of tacrine. Donezepil (Aricept®) was the second drug available for the treatment of AD with advances of no hepatotoxicity, a long half-live (70 hours, which allows once-daily dosing), and a low incidence of peripheral side effects. Rivastigmine (Exelon®) also inhibits the butyrylcholinesterase, which could led to additional benefits in late-stage AD, but causes more gastrointestinal side effects at initiation of therapy (Jann, 2000). Galantamine (Reminyl®), a novel medicine for AD since march 2001, has a dual mechanism of action, combining allosteric modulation of nicotinic receptors with reversible, competitive inhibition of AChE (Scott and Goa, 2000). To date, cholinesterase inhibition has been the most effective approach to the treatment of AD. Several compounds have been tested or are in development, e.g. physostigmine, eptastigmine, velnacrine, metrifonate (Allain et al., 1997; Farlow and Evans, 1998; Francis et al., 1999; Hampel et al., 1999; Krall et al., 1999).

Another approach to the cholinergic treatment of AD is direct activation of the postsynaptically located M_1 receptors. WAL 2014 (talsaclidine) might be a promising candidate for cholinergic replacement therapy in AD (Ensinger et al., 1993). Xanomeline has been demonstrated as a potent but moderately selective

M_1 receptor agonist that might be another compound used in this respect (Shannon et al., 1994; Bymaster et al., 1998a; Christopoulos et al., 1999).
Furthermore, M_1 agonists may alter amyloid precursor protein (APP) processing in favour of the generation of neurotrophic APPs (soluble form of APP) and decreased production of amyloidogenic APP fragments (Roßner et al., 1998).
The selective M_2 receptor antagonist, BIBN 99, may be useful in the treatment of AD, since it could reverse the autoinhibitory control of ACh release (Doods et al., 1993; Doods, 1995).
The most exciting strategy is the search for a postsynaptic M_1 agonist coupled with a presynaptic autoinhibitory M_2 antagonist. Lu 25-109 was described as a combined M_1 agonist and M_2/M_3 antagonist, which effectively increases the soluble form of APP and may therefore be useful for both the replacement of ACh in AD as well as for the modulation of the time course of the disease via the interaction with APP procession pathways (Müller et al., 1998).

The poor results obtained in clinical studies with cholinergic drugs can be explained by the fact that the muscarinic agonists used so far possess very little selectivity for muscarinic receptor subtypes or, as in the case of esterase inhibitors, lack CNS selectivity. Due to the widespread involvement of muscarinic receptors in peripheral functions, unwanted side effects will occur, and consequently, these classical muscarinic agents possess a narrow therapeutic window (Eglen et al., 1999).

An additional CNS indication for muscarinic antagonists has been for idiopathic and drug-induced Parkinson's disease, particularly in the treatment of tremor and rigidity. Parkinson's disease is a hypokinetic movement disorder characterised by the loss of dopaminergic cells in the substantia nigra, with disruption of normal neural activity in basal ganglia circuits. Among other drugs like dopamine agonists, M_1/M_4 preferring muscarinic antagonists, e.g. trihexiphenidyl, procyclidine and biperiden are therapeutically used to restore the impaired balance between dopaminergic and cholinergic activity (Wess et al., 1990; Grimm et al., 1994b) The rational use of selective M_4 antagonists is based on the predominance of the M_4 subtype in the striatum relative to the M_1 subtype, although the functional role of the M_4 subtype remains unclear. Recently, Parke-Davis have discovered a new M_4 selective lead compound, PD102807 (Schwarz et al., 1997).

Furthermore, muscarinic receptors are abundant in pain pathways, including substantia gelatinosa of the dorsal horn of the spinal cord, thalamus and cortex, suggesting the possibility that muscarinic agonists could be acting at all levels of the CNS to modulate nociceptive information. Early studies have demonstrated that muscarinic agonists, e.g. arecoline and oxotremorine, as well as cholinesterase inhibitors, e.g. physostigmine and neostigmine, can produce antinociception in animals (Hartvig et al., 1989). However, the utility of the currently available non-selective muscarinic agonists is limited by their prominent parasympathomimetic side effects, and which muscarinic receptor subtype mediates antinociception is still debated. Recently, a novel compound, butylthio[2.2.2], (+)-(S)-3-(4-butylthio-1,2,5-thiadiazol-3-yl)-1-azabicyclo[2.2.2] octane, with mixed muscarinic receptor agonist (M_1) and antagonist (M_2 and M_3) activity was described (Shannon et al., 1997a, b; Swedberg et al., 1997). Butylthio[2.2.2] is currently undergoing clinical development as a novel analgesic (Shannon et al., 1997b).
Since it has been suggested that stimulation of the M_1 receptor subtype is not a requirement for antinociceptive effects (Sheardown et al., 1997), and, *in vivo*, the antinociceptive effect was blocked by pertussis toxin, the involvement of the muscarinic M_4 subtype in antinociception is under discussion (Ellis et al., 1999).

New insights in the involvement of the central cholinergic system in the pathophysiology of schizophrenia have been suggested by Bymaster et al. (1998b, 1999). They presented a new compound, PTAC, exhibiting partial agonist properties at the human M_2 and M_4 and antagonist properties at the human M_1, M_3 and M_5 muscarinic receptors, which does not produce tremor or salivation. Furthermore, PTAC shows functional dopamine antagonism despite its lack of affinity for dopamine receptors and exhibits a preclinical profile suggestive of antipsychotic efficacy.

1.2. P2 receptors

1.2.1. Historical background

For many years the focus of interest in purines and pyrimidines was the involvement of intracellular nucleotides in cell metabolism and the role of ATP as an energy source.
In 1929, the first concept of purines as extracellular signalling molecules was investigated in a pivotal study by Drury and Szent-Györgyi, demonstrating that adenosine and adenosine 5'-monophosphate (AMP), extracted from heart muscle, exert pronounced biological effects including bradycardia, coronary vasodilatation, decrease in systemic blood pressure, and inhibition of intestinal contraction. Since that time, the interest in extracellular nucleosides and nucleotides was constantly growing, and their pharmacological effects were studied in various tissues.
Nearly 50 years later, Burnstock proposed the existence of specific surface receptors for nucleosides (P1 receptors) and nucleotides (P2 receptors) mediating the physiological effects of adenosine and adenosine 5'-triphosphate (ATP; Burnstock, 1978). This first subdivision established the fundamental part of purine receptor classification and was based on the following four criteria: (i) comparison of the relative potencies of ATP, ADP, AMP, and adenosine; (ii) selective antagonism of the effect of adenosine, but not ATP, by methylxanthines; (iii) modulation of adenylate cyclase by adenosine, but not ATP; and (iv) induction of prostaglandin synthesis by ATP and ADP, but not adenosine.
Whereas adenosine acts on metabotropic A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 1994, 1997), it is now widely acknowledged that ATP and its analogues function as extracellular signalling molecules in the central, peripheral and enteric nervous system via cell surface metabotropic or ionotropic P2 receptors. The further development in the field of P2 receptors led to a more detailed classification according to their molecular structures, evidence of distinct effector systems, pharmacological profiles and tissue distribution.

1.2.2. Subclassification and nomenclature

As previously noted, based on studies on neurotransmission that was resistant to conventional adrenergic and cholinergic antagonists, Burnstock (1971) postulated that ATP, released at synaptic junctions, might mediate this "non-adrenergic, non-

cholinergic" (NANC) signalling. Nerves releasing ATP as putative neurotransmitter were termed "purinergic nerves", and the receptors that were activated by ATP or by its breakdown product, adenosine, were called "purinergic receptors".
Originally, P2 receptors were subclassified into P_{2X} and P_{2Y} purinoceptors on the basis of distinct pharmacological responses of various tissues to a series of analogues of ATP (Burnstock and Kennedy, 1985). The P_{2X} purinoceptors were shown to be most potently activated by α,β-methylene ATP (α,β-meATP) and β,γ-methylene ATP (β,γ-meATP), and were found to be localised in vas deferens, urinary bladder and vascular smooth muscle to mediate contraction. In contrast, P_{2Y} purinoceptors were mainly stimulated by 2-methylthio ATP (2-MeSATP), whereas α,β-meATP and β,γ-meATP were weak or inactive. P_{2Y} purinoceptors were shown to be present in guinea-pig taenia coli and on vascular endothelial cells causing relaxation. Also, repeated administration of α,β-meATP selectively desensitised the P_{2X} but not the P_{2Y} purinoceptors. After Burnstock and Kennedy's proposal, additional P2 purinoceptor subtypes have been described according to their different pharmacological profiles: P_{2T} purinoceptors, which were principally found in platelets and which prefer ADP as an agonist, whereas ATP is an antagonist; P_{2U} purinoceptors of the human neutrophils, at which ATP as well as UTP act as agonists, but α,β-meATP and 2-MeSATP have no effect; P_{2Z} purinoceptors, localised on cells of haemopoietic origin (e.g. macrophages, mast cells and lymphocytes), which are highly selective for the tetrabasic ATP^{4-} form and the unique response of which is characterised by the opening of non-selective membrane pores permeable to hydrophilic solutes as large as 9 kD (reviewed by Abbracchio and Burnstock, 1994; Fredholm et al., 1994; Boarder et al., 1995; Windscheif, 1996).
Besides the P_2 purinoceptor subtypes, there also appear to be receptors for adenine dinucleotide polyphosphates (Ap_xA), leading to the proposal of an additional receptor subtype, the P_{2D} purinoceptor (Abbracchio and Burnstock, 1994).

In 1994, Abbracchio and Burnstock outlined the basis for the currently accepted subclassification of P2 purinoceptors, namely the subdivision into a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors (Abbracchio and Burnstock, 1994).

Based on the rapid development in the field of the purinoceptors, the nomenclature has permanently been changed. The existence of receptors that are

structurally similar to receptors for ATP, but which strongly prefer, or are even selective for UTP or UDP, led to the suggestion that the family of receptors should be named 'P2 receptors' rather than 'P2 purinoceptors'. A P2 receptor is now defined as a receptor for purine or pyrimidine nucleotides, and its P2X/P2Y-classification is fully accepted and recommended by the IUPHAR Subcommittee for the Nomenclature and Classification of Purinoceptors (Fredholm et al., 1997).

Unambiguous delineation of P2 receptor subtypes has followed from the cloning and expression of functional proteins from nucleotide sequences encoding these receptors. To date, seven mammalian P2X receptors, $P2X_{1-7}$, and six P2Y receptor subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, have been cloned, pharmacologically characterised and accepted as valid members of the P2 receptor family (Ralevic and Burnstock, 1998; Burnstock and Williams, 2000).

1.2.3. Cloning studies and structure

1.2.3.1. Structural features of P2X receptors

The first two P2X receptor subunits were identified in rat vas deferens, $rP2X_1$, and PC12 cells, $rP2X_2$, by expression cloning. Subsequently, five additional P2X receptor clones were found in rat tissues either by screening of diverse libraries and/or PCR-techniques. The P2X proteins have 379 - 472 amino acids and are thought to have intracellular N- and C-termini, and two transmembrane domains separated by a large extracellular loop (Fig. 1.4.) (Brake et al., 1994; Valera et al., 1994; Newbolt et al., 1998). The amino acid identity among the different subunits (30 - 50 %) and the lack of homology with other ligand-gated ion channels indicate that they belong to a new family of membrane receptors (Soto et al., 1997). Despite the low amino acid identity between the members of the P2X family, some motifs are conserved in all subunits. The loop connecting the two transmembrane domains contains a number of conserved amino acids, largely glycines and lysines, which might be involved in the formation of the ATP binding site. Furthermore, there are ten cysteine residues conserved in the extracellular loop, suggesting a role in maintaining the tertiary structure of the protein, possibly by forming disulfide bridges. Additionally, a highly homologous hydrophobic segment (H5) preceding the second transmembrane domain is found in each P2X subunit. This region together with the second transmembrane domain is proposed to form

the ionic pore (reviewed by Barnard et al., 1997; Soto et al., 1997). In contrast to other ligand-gated channels, the putative transmembrane domains show a significant variability between the different P2X receptor subunits: one and two amino acids are conserved in transmembrane domain M1 and M2, respectively.
The subunit stoichiometry of the P2X receptors is still under investigation, but the involvement of three subunits or multiples of three was described by Nicke and colleagues (1998).

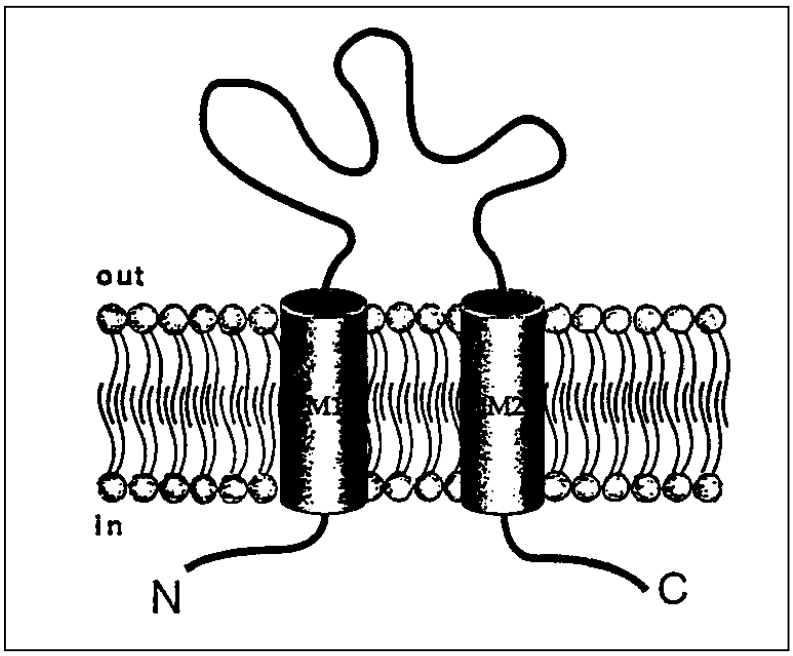


Fig. 1.4. *Schematic model of the putative topology of P2X receptors (taken from Soto et al., 1997).*

1.2.3.2. Cloned P2X receptors

$P2X_1$ receptor

The $P2X_1$ was the first recombinant P2X receptor to be identified by expression cloning from rat vas deferens (Valera et al., 1994). Recently, species orthologues were isolated from mouse and human urinary bladder (Valera et al., 1995). When the $P2X_1$ receptor was expressed in *Xenopus* oocytes or in HEK 293 cells large inward currents to ATP, α,β-meATP, and 2-MeSATP were observed (Valera et al., 1994, 1995). The rank order of potency of the different P2 agonists are: 2-MeSATP $\geq$ ATP > α,β-meATP >> ADP. The currents are characterised by a rapid desensitisation (within hundreds of milliseconds). The P2 antagonists, suramin and PPADS, readily antagonise the agonist-evoked responses. When human $P2X_1$ receptors were stably expressed in 1321N1 human astrocytoma cells, 2'- and 3'-O-(4-benzoylbenzoyl) ATP (BzATP), which has been widely reported as a $P2X_7$-selective agonist, was shown to be the most potent agonist (pEC_{50} = 8.74; Bianchi et al., 1999).

$P2X_2$ receptor

The cDNA was originally isolated by Brake et al. (1994) from a library of PC12 rat pheochromocytoma cells and displays only 41 % amino acid homology with the rat vas deferens $P2X_1$ receptor. Agonists, such as ATP $\geq$ 2-MeSATP $\geq$ ATPγS, are usually 20 - 30 times less potent at this receptor than at the $P2X_1$ receptor subtype. α,β-meATP has no agonist activity. During prolonged application of agonist this channel shows very little desensitisation (Brake and Julius, 1996; Bianchi et al., 1999). Suramin and PPADS are, as it was found for the $P2X_1$ receptor, potent antagonists. Recently, Lynch et al. (1999) reported on the cloning and characterisation of a novel human P2X receptor gene with structural and functional properties identifying it as a homologue of the rat $P2X_2$ subtype.

$P2X_3$ receptor

The $P2X_3$ receptor was cloned from a dorsal root ganglion cDNA library, which encoded a 397-amino-acid-protein, having 41 - 46 % identity with other P2X receptors (Chen et al., 1995a; Lewis et al., 1995). In terms of agonist selectivity, rate of desensitisation and antagonism, this recombinant receptor has similar pharmacological properties to that of the $P2X_1$ subtype. The following order of activity was found for the $P2X_3$ receptor obtained from rat and human: 2-MeSATP

> ATP > α,β-meATP (Garcia-Guzman et al., 1997b). Expression of the $P2X_3$ receptor in HEK293 cells has shown the following properties: (i) ATP and α,β-meATP evoked rapidly desensitising currents, (ii) currents induced by ATP (30 μM) were maximally inhibited by 100 μM suramin and 30 μM PPADS, (iii) inhibition by PPADS developed rapidly (< 30 s) and could be washed out within 2 min, unlike the very slow kinetics of block observed with $P2X_1$ and $P2X_2$ receptors.

$P2X_4$ receptor

The isolation of this cDNA from rat hippocampus was first reported by Bo et al. (1995). cDNAs encoding the $P2X_4$ subunit were also isolated from rat cervical superior ganglia (Buell et al., 1996) and, more recently, from human brain (Garcia-Guzman et al., 1997a). The P2 agonists showed the following rank order of potency: ATP ≥ 2-MeSATP > ATPγS >> ADP > α,β-meATP (weak or inactive) (reviewed by Barnard et al., 1997). The rat and human homologues of the $P2X_4$ receptor appear to differ in their sensitivity to suramin and PPADS, where the human $P2X_4$ receptor is weakly sensitive and the rat $P2X_4$ is relatively insensitive to these putative inhibitors (Bo et al., 1995; Garcia-Guzman et al., 1997a). A lysine residue present in the $P2X_1$ and $P2X_2$ receptors, but absent in the $P2X_4$ receptor, is critical for the binding of antagonists but not agonists (Buell et al., 1996).

$P2X_5$ receptor

The cDNA was isolated from a library prepared from rat coeliac ganglia (Collo et al., 1996). The predicted protein has 417 amino acids. The $P2X_5$ receptor was characterised by rapid activation by ATP > 2-MeSATP > ADP, minimal desensitisation and lack of effect of α,β-meATP. The currents were inhibited readily by suramin (1 - 30 μM) and PPADS (1 - 30 μM). The inhibition by PPADS developed slowly and was only partially reversible.

$P2X_6$ receptor

The $P2X_6$ receptor cDNA was isolated from a superior cervical ganglion cDNA library (Collo et al., 1996). The predicted protein has 379 amino acids. Currents evoked by ATP in cells expressing $P2X_6$ receptors showed only little desensitisation. Rank order of potency of P2 agonists are: ATP ≥ 2-MeATP > ATPγS >> ADP. α,β-meATP has no effect. ATP-induced currents were unaffected by suramin (1 - 30 μM) or PPADS (1- 30 μM), when the antagonists were present in the bath for up to 15 min. Longer applications (30 - 60 min) resulted in partial inhibition (Collo et al., 1996).

Currently it is under discussion, that homomeric $P2X_6$ channels were not readily expressed (summarised by Khakh et al., 2001).

$P2X_7$ receptor

The $P2X_7$ receptor, cloned from rat brain, has a pharmacological profile typical of the receptor previously termed P_{2Z} (Surprenant et al., 1996). Brief application of agonist in the presence of divalent cations opens the $P2X_7$ channel, permeable only to small cations like the other known P2X receptors. Agonist application after removal of the extracellular divalent cations produces a much larger, sustained inward current and leads to lysis of the cells, because of the formation of large membrane pores (Surprenant et al., 1996). A special structural feature of the $P2X_7$ receptor is a significantly longer intracellular C-terminal (240 amino acids), of which at least 117 are crucial for the induction of the non-selective pore. The $P2X_7$ shows the following agonist pharmacology: 2'- and 3'-O-(4-benzoylbenzoyl) ATP (BzATP) >> ATP > 2-MeSATP > ATPγS >> ADP, suggesting that ATP^{4-} is the active form of ATP. α,β-meATP was found to be inactive. The evoked current desensitised very slowly (> 10 s) and was relatively insensitive to suramin and moderately sensitive to PPADS (reviewed by Barnard et al., 1997). Recently, Bianchi et al. (1999) demonstrated that the human $P2X_7$, when stably expressed in 1321N1 human astrocytoma cells, mediates submaximal responses to ATP as compared to BzATP, whereas all other tested nucleotide ligands, including 2-MeSATP, ATPγS and ADP, were functionally inactive as human $P2X_7$ agonists.

Based on agonist efficacy and desensitisation characteristics the P2X receptors can be grouped into three classes (reviewed by Burnstock and Williams, 2000):

(1) $P2X_1$ and $P2X_3$:
high affinity for ATP (EC_{50} = 1 µM) and α,β-meATP, rapidly activated and desensitised;

(2) $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$:
lower affinity for ATP (EC_{50} = 10 µM) and α,β-meATP-insensitive, slow desensitisation and sustained depolarising currents;

(3) $P2X_7$:
very low affinity to ATP (EC_{50} = 300 - 400 µM), little or no desensitisation, can also function as a non-selective ion pore.

1.2.3.3. Co-assembly of distinct P2X receptor subunits

The phenotypic differences among the expressed receptors, particularly with respect to the actions of α,β-meATP, the rate of desensitisation, and the effectiveness of antagonists do not always correspond to those observed in native tissues, suggesting that the native channels might be heteropolymers or that they contain additional subunits not yet cloned. Only the $P2X_7$ receptor is unique in that its subunits do not co-immunoprecipitate with any others (North and Surprenant, 2000).

Heteropolymerisation of P2X subunits has been first demonstrated for $P2X_2$ and $P2X_3$ receptor subunits heterologously expressed in HEK293 cells (Lewis et al., 1995). These receptors, when expressed as homo-oligomers, have clearly different properties: the $P2X_2$ channel being insensitive to α,β-meATP and desensitising very slowly, and the $P2X_3$ channel being sensitive to α,β-meATP and desensitising rapidly. Coexpression of $P2X_2$ and $P2X_3$ subunits in mammalian cells produced a channel with mixed characteristics (α,β-meATP-sensitive and little desensitisation).

Torres and colleagues reported on the coexpression of $P2X_1$ and $P2X_5$ receptor subunits revealing a novel ATP gated ion channel (Torres et al., 1998), and furthermore, they presented results about the co-assembly for several subunits of the P2X family. Only the $P2X_7$ subunits preferentially form homo-oligomeric rather than hetero-oligomeric assemblies (Torres et al., 1999).

The P2X heteromeric receptor, containing central $P2X_4$ and $P2X_6$ subunits, was also described by Lê et al. (1998). Coexpression in *Xenopus* oocytes led to the generation of this novel pharmacological phenotype of ionotropic P2 receptors, $P2X_{4+6}$, which is activated by low-micromolar α,β-meATP concentrations (EC_{50} = 12 µM) and is blocked by suramin and reactive blue 2 (RB-2), contrary to the fact, that RB-2 at low concentrations can potentiate actions at homomeric $P2X_4$ receptors. Furthermore, studies by Lê and co-workers supported the existence of hetero-oligomeric $P2X_{1+5}$ channels, demonstrating that, when expressed in *Xenopus* oocytes, these channels were characterised by slow desensitisation as well as sensitivity to α,β-meATP (EC_{50} = 1.1 µM) and trinitrophenyl ATP (TNP-ATP; IC_{50} = 64 nM; Lê et al., 1999).

However, not all known recombinant subunits can form heteromeric receptors, as suggested by observation from a co-immunoprecipitation study where protein-protein interactions could be detected neither for $P2X_1$/$P2X_4$, $P2X_2$/$P2X_4$,

$P2X_3/P2X_4$, $P2X_3/P2X_6$, nor for any combination containing $P2X_7$ subunits (Torres et al., 1999).
It is important to elucidate if heteromultimerisation is a common occurrence in the native setting, especially, if the hetero-oligomeric nature of such combinations may be masked by the ability of a single subunit type to dominate the phenotype of the complex. If such dominance is exhibited, it could be misleading, as it might affect only the obvious biophysical phenotype but not any underlying developmental and/or regulatory mechanisms, and would thus prevent a more complete understanding of the physiological significance of ATP transmission via that hetero-oligomer.

1.2.3.4. Properties of P2Y receptors

The molecular structure and membrane topology of P2Y receptors is shown in Fig. 1.5.

$P2Y_1$ receptor

A cDNA encoding a protein of 362 residues was originally cloned from late-embryonic chick brain and confirmed to be a P2Y-type receptor, by oocyte expression (Webb et al., 1993). In $P2Y_1$-transfected cells, agonist application led to the formation of IP_3 (Simon et al., 1995). ATP, ADP, and many of their substituted derivatives were found to be agonists at this receptor, with 2-MeSATP as the most potent agonist, whereas pyrimidine based molecules and α,β-meATP were inactive. This was hence assigned as a new subtype of the pharmacologically defined P2Y receptors, $P2Y_1$. The P2Y antagonists suramin, RB-2 and PPADS are strongly inhibitory at the $P2Y_1$ receptors (Simon et al., 1995; Charlton et al., 1996b). The cloned chick receptor, called $cP2Y_1$, was the first member of a proposed extended family of metabotropic P2 receptors (Abbracchio and Burnstock, 1994). Subsequently, by cross-hybridisation and PCR amplification based upon this clone, $P2Y_1$ has been cloned from a variety of species, e.g. turkey, bovine, mouse, rat and human. Notably, the relative potency of ATP and ADP differs widely between recombinant and endogenous $P2Y_1$ receptors (summarised by Fredholm et al., 1996; Barnard et al., 1997; Ralevic and Burnstock, 1998).

$P2Y_2$ receptor

In 1993, Lustig and co-workers isolated the mouse $P2Y_2$ receptor cDNA using a functional expression cloning strategy (Lustig et al., 1993). UTP and ATP, with approximately equal potency, as well as Ap_4A were full agonists at this receptor subtype, whereas 2-MeSATP was inactive. Agonist effects were antagonised by suramin, whereas PPADS was ineffective (Charlton et al., 1996b). The $P2Y_2$ receptor has shown an agonist profile consistent with that of the P_{2U} receptor in mammalian tissues, and since its discovery, it has been widely held that the recombinant $P2Y_2$ receptor corresponds to the P_{2U} receptor (King et al., 1998).

$P2Y_4$ receptor

The $P2Y_4$ was cloned from human genomic DNA and from placental and pancreas RNA (see Barnard et al., 1997). Analysis of the nucleotide efficacy at this receptor subtype originally indicated that UTP and UDP acted as full agonists, whereas ATP and ADP appeared to be either weak partial agonists or inert. Other groups have observed that UTP was a full agonist, UDP was inactive, and ATP acted as a full agonist with lower potency than UTP (for review see Barnard et al., 1997; King et al., 1998). Recent studies, monitoring the release of intracellular Ca^{2+} evoked by extracellular nucleotides, indicate that the human $P2Y_4$ has a restricted nucleotide selectivity for UTP, GTP and ITP, whereas the rat $P2Y_4$ was additionally activated by ATP, Ap_4A, CTP and XTP (Kennedy et al., 2000). In view of its agonist profile the rat $P2Y_4$ receptor expressed in *Xenopus* oocytes did not match the profile for the human $P2Y_4$ but, instead, was closer to the profile of rat $P2Y_2$ (Bogdanov et al., 1998). ATP was shown to act as a competitive antagonist at the human $P2Y_4$ receptor. $P2Y_4$ receptors have been reported to be insensitive to suramin, whereas the effect of PPADS remains unclear, as it has been found to be either inactive or active (Charlton et al., 1996a; Communi et al., 1996a).

$P2Y_6$ receptor

The $P2Y_6$ receptor was originally cloned from rat aorta cells (Chang et al., 1995), and subsequently, a human sequence has been isolated from placenta and spleen (Communi et al., 1996b). In several studies, UDP and UTP were found to be the most potent agonists, whereas 2-MeSATP and ATP were less active (Barnard et al., 1997). This receptor is blocked by PPADS and suramin (reviewed by Von Kügelgen and Wetter, 2000).

$P2Y_{11}$ receptor

Sequences encoding the $P2Y_{11}$ receptor have been isolated from human placenta cDNA (Communi et al., 1997). In terms of protein structure, the second and third extracellular loops are significantly longer than those of the other P2Y receptors. The recombinant human $P2Y_{11}$ receptor stably expressed in 1321N1 astrocytoma cells for measuring IP_3 and CHO-K1 cells for measuring cAMP has been shown to couple to both pathways, a unique feature among the P2Y receptor family (Communi et al., 1997). The rank order of potency of the tested nucleotides was almost identical for both pathways: ATP > 2-MeSATP >>> ADP. UTP and UDP were inactive. The ATP analogue AR-C67085, a potent inhibitor of ADP-induced platelet aggregation, was the most potent agonist. Suramin behaved as a competitive antagonist with a pA_2 value of 6.09, and PPADS was inactive (Communi et al., 1999).

$P2Y_{12}$ receptor

This ADP-sensitive receptor (formerly designated as P_{2T} or $P2Y_{ADP}$), which plays an important role in blood platelet aggregation, has long been waiting for its molecular biological characterisation. Latest reports from Hollopeter and colleagues (2001) provided evidence that this receptor is now cloned from a cDNA library from rat as well as from human platelets. On the basis of measuring PTX-sensitive G_i-linked responses through a sensitive electrophysiological assay they could clearly identify the long-sought ADP receptor on platelets as $P2Y_{12}$ receptor subtype. 2-MeSADP was shown to be the most potent agonist tested, displaying two orders of magnitude higher potency than ADP (with half-maximal responses at 0.9 nM and 300 nM, respectively). UDP and adenosine failed to have agonist effects in *Xenopus* oocytes expressing the rat or human $P2Y_{12}$. The $P2Y_1$-selective antagonist A3P5P had no inhibitory effect in this assay. Northern blot analysis demonstrated that the $P2Y_{12}$ receptor has a restricted expression pattern and is abundantly present in platelets and to a smaller extend in the brain.

The deduced protein sequences of cloned P2Y receptors share approximately 25 % identity with other members of the G protein-coupled receptor family and about 40 % identity with one another, indicating that they form a distinct branch of the G protein-coupled receptor superfamily (Brake and Julius, 1996).

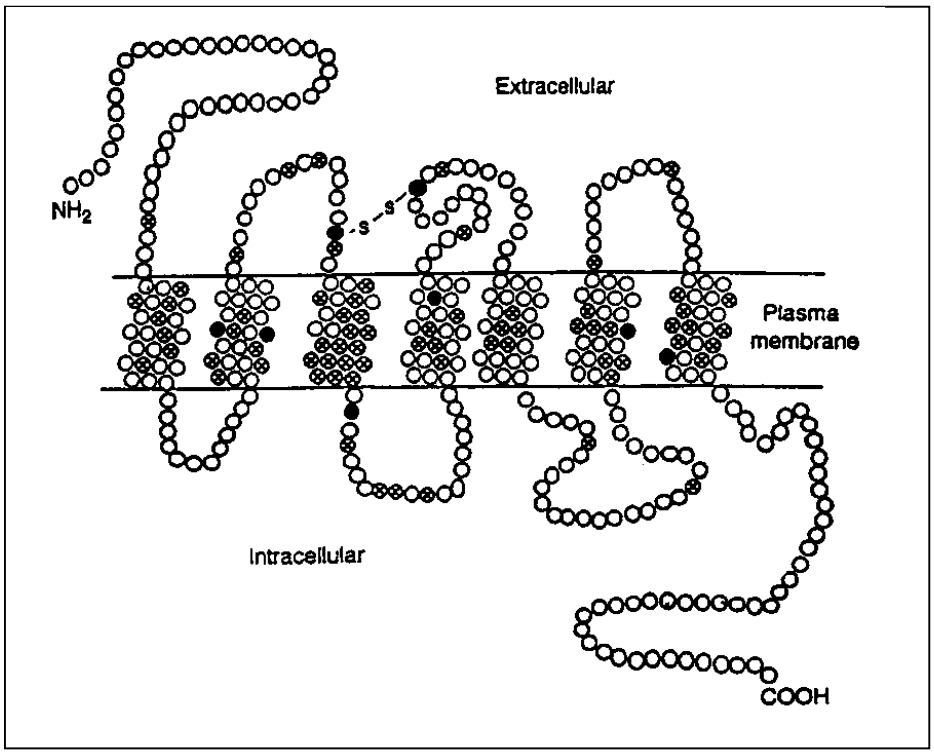


Fig. 1.5. *Molecular structure of P2Y receptors (modified from Burnstock, 1996).*

1.2.3.5. Further P2Y receptors

$P2Y_3$ receptor

The cDNA has been cloned from chick brain (Webb et al., 1996). This receptor shows a preference for UDP, and to a lesser extent for UTP and ADP. Because no mammalian homologue has yet been identified, it is not included as a distinct subtype within the P2Y receptor family by the IUPHAR nomenclature committee. It has been suggested that the p2y3 may be the chick homologue of the mammalian $P2Y_6$ receptor, but this has not yet been confirmed (for review see Ralevic and Burnstock, 1998; Von Kügelgen and Wetter, 2000).

The cloned G protein-coupled $p2y_5$, $p2y_9$, and $p2y_{10}$ receptors have now been shown unequivocally not belonging to the P2Y receptor family. The receptor initially termed $p2y_7$ was found to be a leukotriene B_4 receptor. There are also some doubts about the inclusion of the turkey tp2y receptor as well as the $p2y_8$ receptor expressed in the neural plate of *Xenopus laevis*, as mammalian homologues have not been identified (summarised by Ralevic and Burnstock,

1998). However, they seem to be similar to the rat $P2Y_4$ receptor (Von Kügelgen and Wetter, 2000).

The originally termed P_{2D} ($P2Y_{ApnA}$ or P_4) receptor, which has high affinity for diadenosine polyphosphates, has not yet been cloned and thus is now designated as the dinucleotide receptor (Pintor and Miras-Portugal, 2000). This receptor might be a subtype of the P2Y receptor family because it seems to couple to G proteins (Ralevic and Burnstock, 1998).

1.2.4. Agonists

According to the widespread occurrence of P2 receptors, they have a broad ligand specificity, recognising ATP, ADP, UTP, and UDP as well as the diadenosine polyphosphates. However, most of the agonist ligands used for pharmacological characterisation of P2 receptor subtypes are structural variations of the parent purine or pyrimidine nucleotide pharmacophore at the ring system, the ribose moiety, or the polyphosphate side-chain (Fig. 1.6.). Structure-activity relationships (SAR) efforts have predominantly focused on improved ligands potency, enzymatic stability and/or receptor subtype-selectivity (reviewed by Jacobson and Suzuki, 1996; Ralevic and Burnstock, 1998).

The stable analogue of ATP, α,β-meATP, which is more resistant than ATP to hydrolysis by ecto-nucleotidases present on cell membranes, was first shown to produce a selective desensitisation of P2 receptors in guinea-pig bladder and vas deferens. Since then, α,β-meATP has been the most widely used compound in the investigation of P2X receptor-mediated responses in many tissues, because it is effective especially at recombinant $P2X_1$, $P2X_3$, heteromeric $P2X_{2+3}$ and $P2X_{1+5}$ receptors as well as at their endogenous counterparts, and generally inactive at P2Y receptors (see review Dalziel and Westfall, 1994; Ralevic and Burnstock, 1998; Lambrecht, 2000).
Another stable and also useful purine analogue is adenosine 5'-O-(2-thiodiphosphate) (ADPβS), as it is an agonist at some P2Y receptors, e.g. $P2Y_1$, but weak or inactive at P2X receptors (Ralevic and Burnstock, 1998).

The use of other P2 receptor agonists, e.g. 2-MeSATP, is limited in whole tissue studies by their insufficient stability in the presence of ecto-nucleotidases.

1.2.5. Antagonists

Functional subtype-identification of P2 receptors in native tissues is based on the use of agonists and subtype-preferring antagonists. However, most antagonists are highly limited in terms of their kinetics of antagonism, receptor-affinity, selectivity and P2 receptor-specificity (Lambrecht et al., 1999).
In the following, such P2 antagonists are described, which were extensively tested at recombinant and/or native P2 receptors, and which were used for P2 receptor characterisation in the present study (see chapter 4.2.) (Fig. 1.7.).

Suramin, the symmetrical urea of 8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid (Fig. 1.7.), a polysulfonated aromatic antitrypanocidal drug, is an effective antagonist of P2 receptors, and might be a useful tool in discriminating between P1 and P2 receptor-mediated responses. The antagonist effect of suramin was first shown in the mouse vas deferens (Dunn and Blakeley, 1988). Subsequent studies have investigated the P2 antagonism of suramin in a variety of tissues, such as mouse vas deferens, rat vas deferens, pithed rat, guinea-pig urinary bladder and taenia coli, and rabbit ear artery (for review see Dalziel and Westfall, 1994; Ralevic and Burnstock, 1998). Unfortunately, the compound also interacts with glutamate, nicotinic, 5-hydroxytryptamine and GABA receptors, ecto-nucleotidases, various protein kinases, and G protein subunits (Bhagwat and Williams, 1997; Ralevic and Burnstock, 1998; Lambrecht, 2000), indicating that suramin possesses a spectrum of biological activities that might limit its usefulness as a P2 receptor antagonist. Simplification of the structure of suramin has resulted in NF023.

The symmetrical 3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulfonic acid (NF023; Fig. 1.7.) was found to be selective for native $P2X_1$ receptors (pA_2 = 5.5 - 5.7) antagonising effects at these receptors in rat, hamster and rabbit tissues (Bültmann et al., 1996; Lambrecht, 1996; Ziyal et al., 1996, 1997). The effect of NF023 studied on voltage-clamped *Xenopus* oocytes heterologously expressing homomultimeric $P2X_1$ - $P2X_4$ as well as heteromultimeric $P2X_{2+3}$ receptors confirmed the subtype-selectivity for the $P2X_1$ receptor (Soto et al., 1999). Like the parent compound suramin, NF023 inhibited ecto-nucleotidases activity, but its $P2X_1$-selectivity is dominating (Lambrecht, 2000). In order to increase the potency of NF023 at the $P2X_1$ receptor and, hence, to alter its functional selectivity in favour of the $P2X_1$ subtype, further structural variations have been examined.

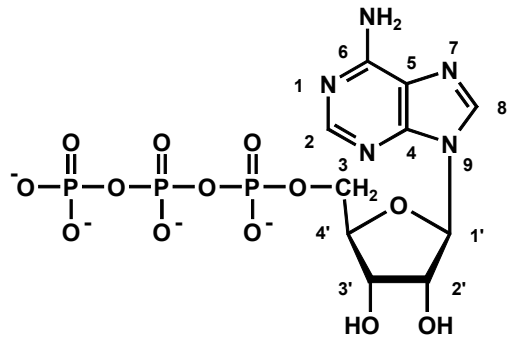
The suramin-related compound NF279 (8, 8'-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(1,3,5-naphthalene-trisulfonic acid); Fig. 1.7.) is a novel and highly potent antagonist blocking P2X receptor-mediated responses in rat vas deferens (pIC_{50} = 5.71), and having only little effects at P2Y receptors in guinea-pig taenia coli (pA_2 = 4.10; Damer et al., 1998). At cloned P2X receptors expressed in *Xenopus* oocytes ($P2X_1$ - $P2X_4$, $P2X_7$), the compound was wholly selective for $P2X_1$ receptors (Lambrecht, 2000). NF279 was found to be weak at recombinant $P2Y_1$ (pIC_{50} = 4.6) and inactive (up to 100 µM) at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes (Boyer and Harden, personal communication). Thus, NF279 is the compound with the highest $P2X_1$ vs. P2Y receptor and ecto-nucleotidase selectivity presently available (Lambrecht et al., 1999; Lambrecht, 2000).

A diazo derivative of the coenzyme pyridoxal phosphate, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Fig. 1.7.), is another widely used P2 receptor antagonist, which is not selective for one of the P2 receptor subtypes (Lambrecht et al., 1992; Lambrecht, 1996). It exhibits pA_2 values of 6 - 6.7 at endogenous $P2X_1$-like receptors in a variety of smooth muscle preparations (Ralevic and Burnstock, 1998). At recombinant $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_5$ receptors PPADS had antagonistic effects with IC_{50} values of 1 - 2.6 µM, whereas rat $P2X_4$ and $P2X_6$ receptors were unaffected (Collo et al., 1996). Within the P2Y receptor family, PPADS generally antagonises endogenous $P2Y_1$-like and recombinant $P2Y_1$ receptors coupled to PLC but not P2Y receptors coupled to inhibition of AC. PPADS has been reported to block $P2Y_1$-like receptors in rat duodenum (pA_2 = 5.1), guinea-pig taenia coli (pA_2 = 5.3; Windscheif et al., 1995), and rat mesenterial arterial bed (pA_2 = 6.0; Ralevic and Burnstock, 1996), but was inactive at those in smooth muscle of rabbit mesenteric artery and endothelium of rabbit aorta (Ziganshin et al., 1994b). PPADS also blocks $P2Y_2$-like receptors in astrocytes from the dorsal horn of the spinal cord, but not those on rat mesenteric arterial endothelium or on cultured bovine aortic endothelial cells. Furthermore, it antagonises responses to UTP at recombinant $P2Y_4$ receptors, and at high concentrations PPADS blocks $P2_{ADP}$ receptor-mediated platelet aggregation and inhibits ecto-nucleotidase activity (reviewed by Ralevic and Burnstock, 1998).

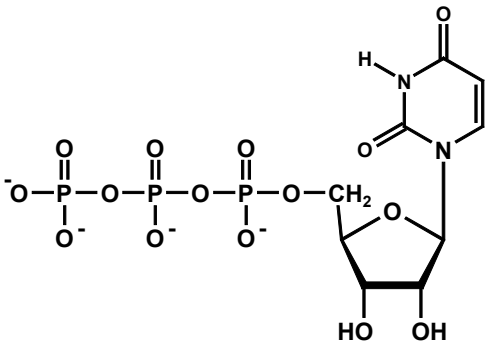
In order to increase the selectivity of PPADS in favour of the $P2X_1$ subtype a series of 6-naphthylazo analogues was synthesised and pharmacologically characterised by Lambrecht and co-workers (1999). The most promising

compound, pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS; Fig. 1.7.), has shown high affinity at $P2X_1$ in rat vas deferens (pK_B = 7.43) and in inhibition of inward currents to 1 μM ATP in *Xenopus laevis* oocytes expressing homomeric cloned rat $P2X_1$ receptors (pIC_{50} = 7.84). In contrast, its antagonistic properties at the P2Y receptor in guinea-pig taenia coli (pA_2 = 4.84) and at the $P2Y_1$ subtype in guinea-pig ileum (pA_2 = 6.13) were clearly lower. Thus, PPNDS is a novel P2 antagonist with high affinity at and selectivity for the $P2X_1$ receptor subtype.

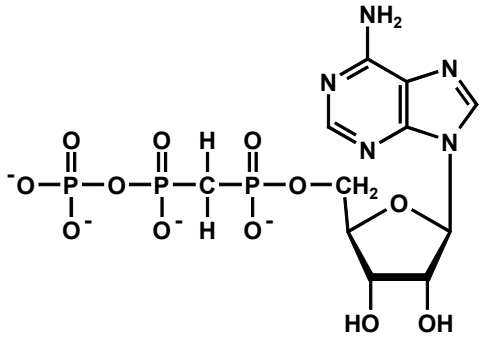
Recently, Lambrecht and co-workers (2000) introduced an interesting antagonist, SB9 (Fig. 1.7.), resulting from extensive SAR studies with heterodimers, which potently antagonises $P2Y_1$ receptor-mediated responses. SB9, 6-[(4,6,8-trisulfo-1-naphthyl)iminocarbonyl-1,3-(4-methylphenylene)iminocarbonyl-1,3-phenylene-azo]-pyridoxal-5'-phosphate, is a bivalent ligand consisting of pyridoxal-5'-phosphate and the monomer of suramin linked through a diazo group as spacer. It has been found that the compound exerts a remarkable selectivity for $P2Y_1$-like receptors in GPI (pA_2 = 6.98 ± 0.07) with an about 10-fold lower antagonistic potency at $P2X_1$-like receptors in rat vas deferens (pA_2 = 6.05 ± 0.13). In the ecto-nucleotidase assay, SB9 has shown a 447-fold lower inhibitory potency. Thus, heteromeric bivalent ligands might be useful P2 antagonists and especially SB9 could be used as lead compound for further investigations.



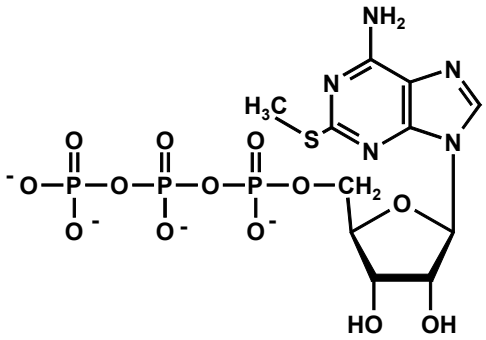
ATP



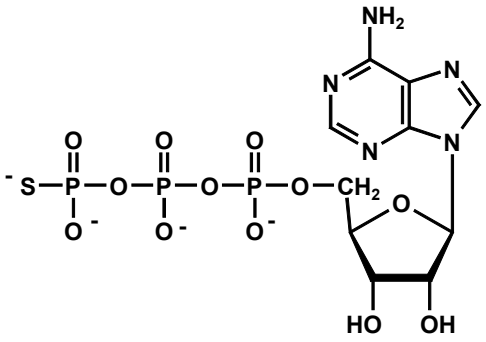
UTP



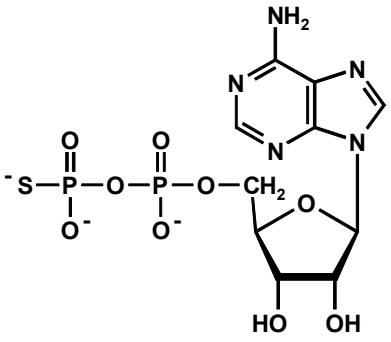
α,β-meATP



2-MeSATP

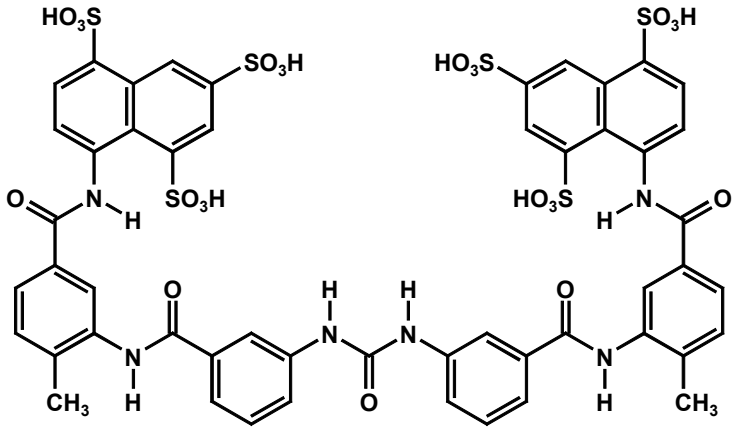


ATPγS

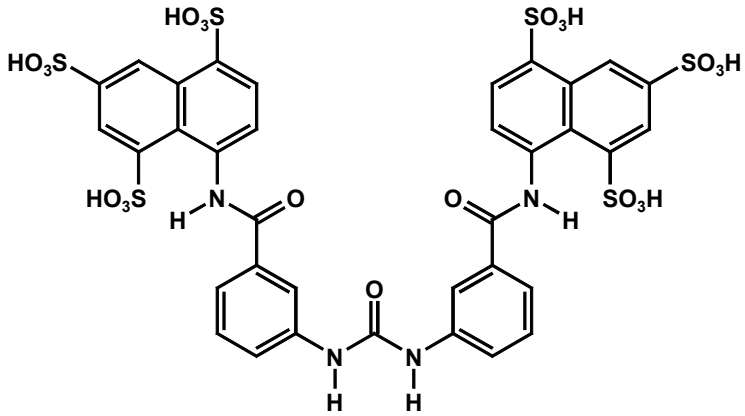


ADPβS

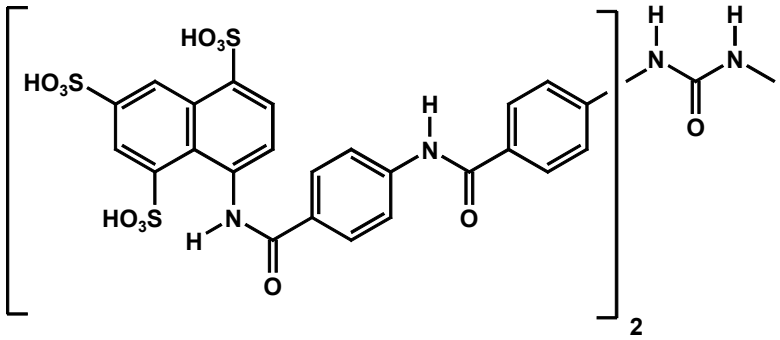
Fig. 1.6. *Chemical structure of some P2 receptor agonists.*



Suramin

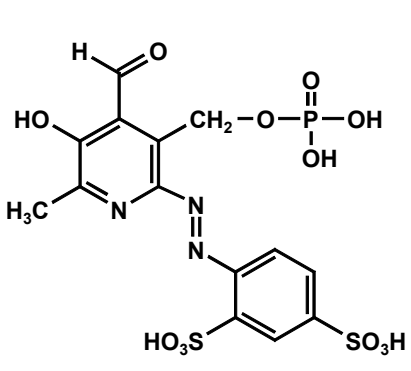


NF023

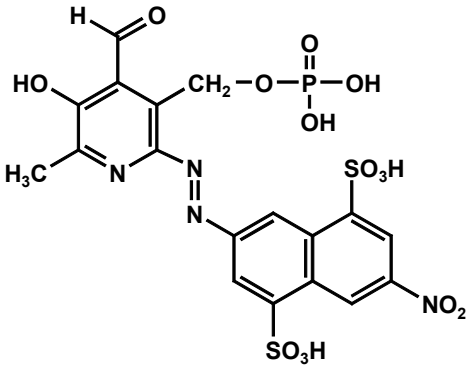


NF279

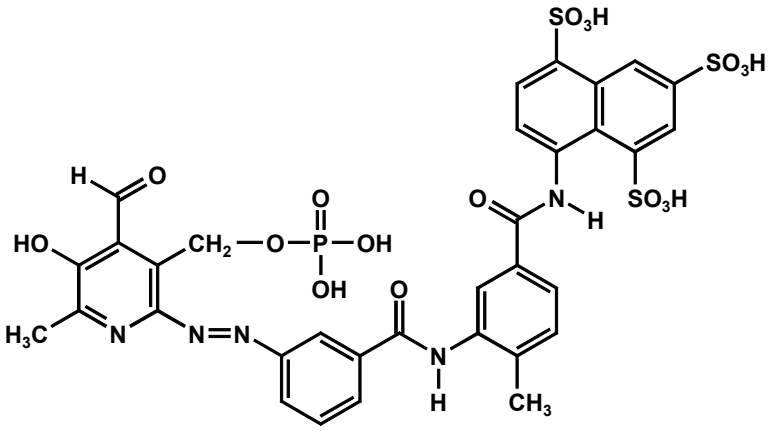
Fig. 1.7. *(legend, see page 35)*



PPADS



PPNDS



SB9

Fig. 1.7. *Chemical structure of some P2 receptor antagonists.*

1.2.6. Agonist breakdown

As already emphasised, the problems associated with the breakdown of ATP and its hydrolysable analogues have profound implications for the pharmacology of P2 receptors. It is evident that many tissues have cell-surface enzymes that are capable of dephosphorylating ATP sequentially to ADP, AMP and adenosine (Ziganshin et al., 1994a). The catalytic site of this ectonucleotidases faces the extracellular medium, and for its catalytic activity the presence of divalent cations

such as Ca^{2+} or Mg^{2+} is necessary. The expression of the individual enzymes can vary between tissues, cells, and developmental stages. A key function of these enzymes presumably is the inactivation of nucleotides or nucleosides that have been released as signalling substances or during pathological events and, subsequent, purine salvage (for review see Zimmermann, 1996, 1999). The simplistic nomenclature used so far in the literature, e.g. ecto-ATPase, ecto-ADPase, has to be revised after the molecular and functional characterisation of several novel enzyme families with overlapping substrate specificities and tissue distribution. The following enzymes are mainly involved in the degradation of nucleotides (see Zimmermann, 2000).

Ecto-nucleoside 5'-triphosphate diphosphohydrolase (E-NTPDase) family

Members of this family can hydrolyse nucleoside 5'-triphosphates (NTP) and nucleoside 5'-diphosphates (NDP) albeit with varying preference for an individual type of nucleotide. According to their presumptive membrane topography they can be separated into two groups: (1) NTPDase1 to 4, which are predicted to have a transmembrane domain at the N- and C-terminus; (2) NTPDase5 and putative 6; but only NTPDase5 has been expressed and characterised (lack of C-terminus, whereas N-terminal hydrophobic leader sequence is cleaved, resulting in a soluble and secreted form of the enzyme). The enzymes of the E-NTPDase family differ regarding their preference for NTP and NDP.

- NTPDase1 (Ecto-ATPDase): NTP $\rightarrow$ NMP + 2 Pi; NDP $\rightarrow$ NMP + Pi
- NTPDase2 (Ecto-ATPase): NTP $\rightarrow$ NDP + Pi; (NDP $\rightarrow$ NMP + Pi)
- NTPDase3 (Ecto-ATPDase): NTP $\rightarrow$ NDP + Pi; NDP $\rightarrow$ NMP + Pi
- NTPDase4 (UDPase): UDP $\rightarrow$ UMP + Pi; UTP $\rightarrow$ UDP + Pi

- NTPDase5: NDP $\rightarrow$ NMP + Pi

Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family

The members of this family are characterised by a single transmembrane domain, an intracellular N- and an extracellular C-terminus. The enzymes (NPP1 to NPP3) possess a surprisingly broad substrate specificity - alkaline phosphodiesterase as well as nucleotide pyrophosphatase activity. That means that these enzymes are capable of hydrolysing:

- NTP → NMP + PPi (ATP → AMP + PPi)
- ADP → AMP + Pi
- AMP → adenosine + Pi

Alkaline phosphatases

These enzymes are non-specific ecto-phosphomonoesterases that are releasing inorganic phosphate from a variety of organic compounds including NTP, NDP and NMP. They may also hydrolyse PPi.

- NTP → NDP + Pi
- NDP → NMP + Pi
- NMP → nucleoside + Pi
- PPi → 2 Pi

Ecto-5'-nucleotidase

This enzyme is responsible for the formation of extracellular adenosine from released adenine nucleotides.

- NMP → nucleoside + Pi

The situation is more complicated since many of the enzymes have shown tissue colocalisation. Finally, there are many other surface-located converting enzymes, e.g. ecto-nucleoside diphosphokinase that may interconvert ATP and UDP to ADP and UTP, the presence of which can result in an underestimation of ligand potency. These misinterpretations have led to an intensive search for compounds that inhibit ecto-nucleotidases and preferably have no or only a small effect on P2 receptors.
The only selective ecto-nucleotidase inhibitor that has just a small antagonistic effect on P2X receptors is the ATP analogue 6-N,N-diethyl-D-β,γ-dibromomethylene ATP (ARL 67156, formerly FPL 67156) (Kennedy et al., 1996).
When purified ecto-enzymes were used, the analysis of structure-activity requirements of the enzyme suggested that substitutions at the purine ring had virtually no effect on removal of the terminal phosphate from the ATP analogues, and compounds, such as 2-MeSATP and 2-ClATP, are good substrates of ecto-nucleotidases. However, methylene isoesters of ATP, e.g. α,β-meATP and β,γ-

meATP, or compounds with substitutions on the terminal phosphate of the nucleotide, ADPβS and ADPγS, proved to be resistant to degradation (for review see Zimmermann, 1996).
Furthermore, the ability of P2 receptor antagonists to inhibit some ecto-nucleotidases and thereby protect ATP and other nucleotidases from degradation may complicate their use (Chen et al., 1996; Lambrecht et al., 1999).

1.2.7. Signal transduction mechanisms

P2X receptors can modulate cell function by gating Na^+, K^+ and Ca^{2+} permeability resulting in an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and depolarisation (see Surprenant, 1996). The direct flux of extracellular Ca^{2+} through the channel constitutes a significant source of the increased $[Ca^{2+}]_i$. On the other hand, membrane depolarisation leads to the secondary activation of voltage-dependent Ca^{2+} channels, which probably make the primary contribution to Ca^{2+} influx and to the increase of $[Ca^{2+}]_i$. Because this transduction mechanism does not depend on the production and diffusion of second messengers within the cytosol or cell membrane, the response time is very rapid, and appropriately plays an important role in fast neuronal signalling and regulation of muscle contractility (Ralevic and Burnstock, 1998).

P2Y receptors belong to the seven transmembrane domain superfamily of receptors, which may couple, via heterotrimeric G proteins, to ion channels as well as to enzymes. In the following the substantial second messenger pathways for P2Y receptors are described briefly.

Phospholipase C (PLC)

The main signal transduction pathway of all cloned and functionally defined P2Y receptors is activation of PLC via PTX-sensitive $G_{i/o}$ and -insensitive $G_{q/11}$ proteins, resulting in increased levels of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The consequence of IP_3 formation and entailed Ca^{2+} mobilisation is the stimulation of a variety of signalling pathways, e.g. activation of phosphokinase C (PKC), phospholipase A_2 (PLA_2), Ca^{2+}-dependent K^+ channels, nitric oxide synthase (NOS) and subsequent endothelium-derived relaxing factor (EDRF) formation. DAG leads to activation of PKC, which in turn may stimulate PLC, phospholipase D (PLD), mitogen-activated protein kinase (MAPK) pathway

and voltage-dependent Ca^{2+} channels (reviewed by Barnard et al., 1997; Ralevic and Burnstock, 1998).

Adenylate cyclase (AC)

The regulation of cAMP levels by P2Y receptors is a result of direct inhibition of the AC via PTX-sensitive G_i proteins. This pathway has been observed for $P2Y_{11}$ (Communi et al., 1997) and $P2Y_{12}$ (Hollopeter et al., 2001) receptor subtypes.

Ion channels

P2Y receptors can regulate plasmalemma ion channels indirectly, as a result of second messenger production, or directly via either α or $\beta\gamma$ subunits. The receptor stimulation results in a rise of intracellular Ca^{2+} by mobilisation of intracellular stores, leading to the sequential opening of two types of Ca^{2+}-sensitive channels: transient depolarising Cl^- (for example described for P2Y receptors in airway epithelia) and hyperpolarizing K^+ channels (Boarder and Hourani, 1998).

Phospholipase A_2 (PLA_2)

In endothelial cells, activation of Ca^{2+}-sensitive PLA_2 results in an increased production of prostacyclin (reviewed by Ralevic and Burnstock, 1998).

Tyrosine kinase - MAPK cascades

MAPK are key elements of signal transduction pathways involved in cell growth. These cytoplasmatic enzymes transduce signals from both receptor tyrosine kinases and G protein-coupled receptors to the nucleus, resulting in the activation and/or induction of transcription factors, thereby leading to gene expression and cell growth. The tyrosine kinase and p42-p44 MAPK cascades are now recognised as a part of response to activation of P2Y receptors. Three routes for the regulation of MAPK by P2Y receptors are conceivable: activation of the tyrosine kinase - MAPK cascades directly by $\beta\gamma$ subunits, activation following PLC stimulation, and by an autocrine mechanism involving production of a growth factor, which then acts on a tyrosine kinase receptor (Neary, 1996; Boarder and Hourani, 1998). As with the $P2Y_1$ receptor, protein tyrosine phosphorylation and MAPK activation seem to be the major route for $P2Y_2$ receptor-mediated prostacyclin production in endothelial cells.

1.2.8. Localisation and physiological roles

In view of the ubiquitously occurrence of purines and pyrimidines and the widespread distribution of P2 receptors (for review see Ralevic and Burnstock, 1998), only a few examples have been selected and will be focused in the following.

Nervous system

Since Burnstock's first proposal of purinergic nerves, a large body of evidence has been accumulated and unambiguously demonstrated that ATP, released upon stimulation and action on P2 receptors, is a neurotransmitter in the efferent autonomic nervous system and peripheral sensory nerves (Chen et al., 1995b).

In 1976, Burnstock introduced the concept of co-transmission. It appears that ATP is a principal transmitter and that it has been retained as a co-transmitter with other neurotransmitters, such as noradrenaline and ACh, in many different nerve types, albeit in proportions that vary between locations and species. So it was found, that in sympathetic nerves ATP co-exists and is co-released with noradrenaline and neuropeptide Y, in some parasympathetic nerves with ACh, in some sensory-motor nerves with calcitonin gene-related peptide (CGRP) and substance P, and in NANC inhibitory nerves together with NO and vasoactive intestinal peptide (VIP; for review see Burnstock, 1996).

Additionally, ATP co-released with noradrenaline or ACh, also plays a feedback role in modulation of transmitter release via presynaptic P2 receptors in the autonomic nervous system. The facilitation on noradrenergic and/or cholinergic transmission has been reported in the rabbit ear artery, GPI, saphenous artery and rat sympathetic neurons (for review see Chen et al., 1995b).

Glia cells, as a constitutional and functional part of the nervous system, play an important role in the modulation of synaptic efficacy. Astrocytes, oligodendrocytes and microglial cells are all direct targets for extracellular nucleotides via various P2 receptor subtypes, which mediate a variety of biological processes (Chen et al., 1995b; Abbracchio and Burnstock, 1998; Inoue, 1998).

Cardiovascular system

The importance of ATP as an extracellular signalling molecule was first appreciated in the cardiovascular tissue. ATP, acting at distinct P2 receptor subtypes located on endothelial and smooth muscle cells, affects vascular tone and blood flow by causing either contraction or dilatation.

Vasoconstriction is mediated through the stimulation of P2X receptors present on vascular smooth muscle cells. Synaptic release of ATP from sympathetic perivascular neurons activates excitatory P2X receptors to produce the NANC component of vasoconstriction that remains after blockade of adrenergic and cholinergic receptors. The purinergic nature of this response was demonstrated by its attenuation following application of α,β-meATP, which desensitises these receptors (reviewed by Brake and Julius, 1996). In the rat vascular system mRNAs for $P2X_1$, $P2X_2$ and $P2X_4$ receptor subtypes were found by *in situ* hybridisation and reverse transcriptase PCR (Nori et al., 1997).

P2Y receptors are also involved in the regulation of vascular tone. ATP, released from endothelial cells during hypoxia, activates endothelial $P2Y_1$, $P2Y_2$ and probably $P2Y_4$ receptors resulting in activation of endothelial NOS and release of EDRF, thereby in potent vasodilatation. Adenosine, produced following breakdown of ATP, contributes to the later component of vasodilatation by direct action of P1 receptors on vascular smooth muscle (reviewed by Abbracchio and Burnstock, 1998).

Within the cardiovascular system, ATP and ADP also function as paracrine regulators of platelet aggregation. Platelets aggregating at sites of damage in arterial walls release high concentrations of ATP and ADP as constituents of their dense granules. ADP recruits additional platelets through activation of $P2Y_{12}$ (Hollopeter et al., 2001) receptors on their surface. It has been shown that ADP stimulates platelets acting on three distinct P2 receptors, $P2X_1$ (Sun et al., 1998), $P2Y_1$ and $P2Y_{12}$, leading to increase of intracellular Ca^{2+}, rapid Ca^{2+} influx, activation of PLC and inhibition of AC, shape change of the platelets, exposure of glycoprotein IIb/IIIa complex as binding sites for cross-linking of platelets by fibrinogen, amplification of the response by release of a range of proaggregatory mediators including thromboxane A_2, 5-hydroxytryptamine and ADP, and sustained aggregation (Kunapuli, 1998; Ingall et al., 1999; Brass, 2001).

Immune system

Extracellular ATP is known to act on a variety of cells within the immune system. The $P2X_7$ receptor is thought to be involved in this action of ATP, since it was found in functional studies that $P2X_7$ receptor distribution is generally limited to cells of hematopoietic origin including mast cells, macrophages and lymphocytes (reviewed by Abbracchio and Burnstock, 1998; Ralevic and Burnstock, 1998).

ATP acting via $P2X_7$ receptors has been implicated as an initiator of programmed cell death, i.e. apoptosis. For example, exposure of mouse macrophages to extracellular ATP leads to nuclear DNA fragmentation and release of processed interleukin-1β, two responses that are associated with apoptotic death in some cell types (reviewed by Brake and Julius, 1996).

1.2.9. Therapeutic targets

Cardiovascular system

In the cardiovascular system the most immediate target for therapeutic intervention is in the control of platelet function. The central role of ADP as an aggregating agent, not only in the physiological processes of haemostasis but also in the development and extension of arterial thrombosis, has been long established and makes ADP receptors of potential clinical importance. Platelet activation by ADP leads to rapid calcium entry and mobilisation of intracellular calcium stores, inhibition of AC, shape change from discoid to spherical form, and aggregation.

The antithrombotic drugs ticlopidine and clopidogrel are thought to act by reducing the number of $P2Y_{12}$ receptors on platelets, thereby reducing the responsiveness of platelets to ADP. Both compounds are inactive *in vitro* and must be metabolised in the liver to thiol derivatives in order to acquire their anti-aggregatory properties in this way that they modify a cysteine residue on the receptor (Hollopeter et al., 2001).

A more direct mechanism to prevent platelet aggregation is the ATP analogue APL66096, a platelet ADP receptor antagonist, which has shown promising results in experimental models of thrombosis (for review see Boarder and Hourani, 1998; Hechler et al., 1998). Recently, Ingall and co-workers reported on two ATP analogues, which structurally differ from ATP in modifications of the polyphosphate side chain to prevent breakdown and substitution of the adenine

moiety to enhance affinity and selectivity for *$P2Y_{ADP}$* ($P2Y_{12}$) receptors. The compounds, termed AR-C67085MX and AR-C69931MX, exhibited IC_{50} values of 2.5 nM and 0.4 nM, respectively, against ADP-induced aggregation of human platelets (Ingall et al., 1999). Antagonists of the $P2Y_{12}$ receptor represent a major step forward in the treatment of thrombotic disease (reviewed by Cusack and Hourani, 2000).

Respiratory tract

Cystic fibrosis (CF), a hereditary disease, is recognised as a result of mutations in a gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) that results in a defect in cAMP-stimulated chloride transport. Subsequently, mucus may become altered in its rheological properties and bacterial adhesivity, leading to inflammation and then mucus hypersecretion. ATP and UTP have been proposed as therapeutic agents acting on P2 receptors resulting in intracellular calcium mobilisation through PLC. This increase in calcium leads to an activation of a chloride channel distinct from CFTR, which is localised on respiratory epithelia and which is not defective in cystic fibrosis (Boarder et al., 1995; Merten et al., 1998). UTP is currently in phase II clinical trials as a treatment for CF (Bennett et al., 1996; Olivier et al., 1996). Disadvantages of ATP are the bronchoconstrictor and cardiac depressant effects of adenosine formed from the hydrolysis of ATP (Bhagwat and Williams, 1997).

Sensory neurons

ATP has long been known to induce nociceptive responses following peripheral administration. These effects are due to direct activation of peripheral nerve terminals or indirectly through actions on inflammatory cells. ATP, originating from platelets, mast cells, postganglionic sympathetic nerve terminals or from sensory afferents, may be released into extracellular spaces as a consequence of tissue injury. Which P2 receptor subtype could mediate the nociceptive effect of ATP is still unknown, but there is strong evidence that homomeric $P2X_3$ or heteromeric $P2X_{2+3}$ receptors are involved (Cook et al., 1997; Ding et al., 2000; Burnstock and Williams, 2000; Hamilton and McMahon, 2000).

Endocrinal system

ATP also has important endocrine functions acting on P2Y receptors, probably $P2Y_1$ receptors, of pancreatic ß cells. Insulin secretion induced by

physiological agents such as ACh and glucose was potentiated by ATP and ADP. The stable analogue ADPßS was approximately 100-fold more potent than ATP itself in increasing insulin secretion in isolated perfused rat pancreas. *In vivo* experiments in rats and dogs showed that after oral administration of ADPßS the insulin secretion was stimulated and glucose tolerance was improved (Hillaire-Buys et al., 1993). Studies with ADPßS in streptozotocin-diabetic rats further supported the hypothesis that P2Y receptors of the pancreatic ß cells may be suitable target for the development of new orally-active antidiabetic drugs (for review see Abbracchio and Burnstock, 1998). Recently, Petit and colleagues provided evidence that on pancreatic ß cells, additionally to the P2Y, P2X receptors are localised, which transiently stimulate insulin release at low non-stimulating glucose concentration and slightly affect the potassium conductance of the membrane (Petit et al., 1998).

Tumor growth

P2 receptor agonists such as ATP, ADP, AMP, α,β-meATP, β,γ-meATP and ATPγS were claimed as antineoplastic agents (for review see Fischer, 1999). These compounds may be useful for the treating of any hormone independent cancer, including prostate, ovarian, breast and endometrial cancer. The claim is based on the expression of P2 receptors on these cancer cells, and stimulation of these receptors inhibits the growth of the cells. This new approach for the treatment of neoplasm is of special interest since the metastatic disease cannot be controlled by a hormonal therapy.

In conclusion, P2 receptors may be promising clinical targets and P2 agonists or antagonists helpful for the treatment of various diseases. Nonetheless, the use of knockout mice will be a beneficial way to clarify the participation of P2 receptor subtypes in physiological as well as in pathophysiological processes (Burnstock and Williams, 2000).

2. AIM OF THE THESIS

2.1. Investigations of the isomers of glycopyrronium bromide and their tertiary analogues at muscarinic receptors

As mentioned above, muscarinic receptors are composed of a family of four functionally characterised subtypes, which can be distinguished pharmacologically and structurally. The physiological role of each subtype in the central and peripheral nervous system remains to be clarified due, in part, to a lack of agonists and antagonists with adequate subtype selectivity. In this regard, the development of subtype selective muscarinic ligands could provide both improved tools for receptor classification and novel therapeutics with reduced cholinergic side effects. Indeed, several selective muscarinic receptor ligands are now in advanced clinical evaluation (Eglen and Watson, 1996).

Selective blockade of muscarinic M_3 receptor may be therapeutically useful in the treatment of respiratory disorders, such as COPD. Vagal stimulation induces brochoconstriction and mucus secrection, by activation of muscarinic M_3 receptors located on smooth muscle, vascular endothelium, submucosal cells and neural elements (Barnes, 1993). The evidence that quaternary atropine derivatives such as ipratropium bromide (Fig. 2.1.) produce significant bronchodilation has recently been stimulated interest in antimuscarinics as bronchodilators. These highly polar ammonium compounds are poorly absorbed across mucosal surfaces, and thus systemic effects are unlikely with drug inhalation.

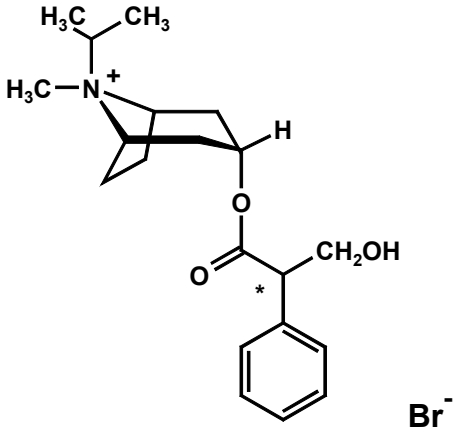
Instead of subtype-selective M_3 receptor antagonists, some new therapeutic approaches exploit differences in receptor kinetics. Tiotropium bromide (BA 679 BR; Fig. 2.1.) is a novel antagonist, developed as a long-acting antimuscarinic bronchodilator for the treatment of patients with COPD. It inhibits exogenous ACh-induced bronchospasm three times more potently than ipratropium bromide. Tiotropium bromide binds non-selectively to cloned human M_1 - M_3 receptors but dissociates more rapidly from M_2 and M_1 than from M_3 receptors, thus achieving M_3 subtype selectivity through a kinetic mechanism (Disse et al., 1993; Maesen et al., 1993; Haddad et al., 1994).

2. AIM OF THE THESIS

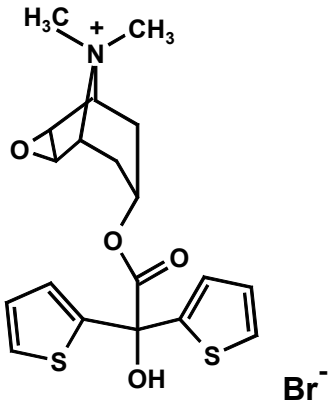
Glycopyrronium bromide (glycopyrrolate; Fig. 2.1.) is a quaternary compound with antimuscarinic properties similar to those of atropine, but with minimal cardiovascular, ocular and CNS effects. It has two chiral carbon atoms resulting in four stereoisomers. Glycopyrrolate (Robinul®) is one of the two diastereomeric forms of glycopyrronium bromide and represented by the (R/S)-(S/R) pair (Demian and Gripshover, 1990). The safety of glycopyrrolate has been established by nearly 20 years of clinical use, orally to control gastric acidity and parenterally as an antisialogogue, and in the reversal of neuromuscular blockade as a substitute of atropine (Mirakhur and Dundee, 1981; Johnson et al., 1984; Ali-Melkkilä et al., 1993).

The impetus for the present study came from the interesting findings by Fuder and Meincke (1993), who analysed the antimuscarinic properties of glycopyrronium bromide *in vitro* at rabbit vas deferens (RVD; M_1), paced rat left atria (M_2), and guinea-pig ileum (GPI; M_3). Glycopyrronium bromide blocked with very high affinity (>11, apparent -log pK_B) the muscarinic M_1 receptor, and it showed intermediate affinity (10.31) at M_3 and lower affinity (9.09) at M_2 receptors. With exception of M_1 receptors, the antagonism appeared to be of simple competitive type. At RVD the slope of the Schild regression line was lower than unity at higher concentrations (1 nM) and steepened when the lowest concentration (0.1 nM) was taken into account. Thus, a virtual apparent -log K_B value of 11.41 ± 0.14 was estimated from dose ratios of all antagonist concentrations assuming a slope of unity. Testing low antagonist concentrations comparatively close to the apparent K_B resulted, probably, in incomplete drug equilibrium between organ bath and tissue even after an equilibration time of 3 to 5 h.

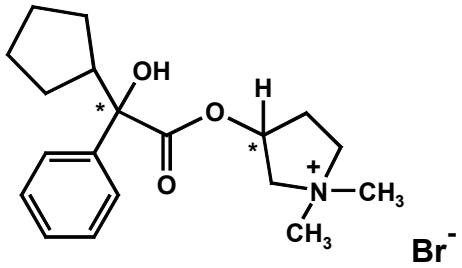
In light of these results, the selectivity profile of the individual stereoisomers of glycopyrronium bromide at the muscarinic receptor subtypes would be interesting to investigate. Hence, the aim of the present investigation was to determine the affinities of the quaternary isomers of glycopyrronium bromide as well as of the corresponding tertiary analogues and to evaluate the stereochemical demands for the binding to the muscarinic receptor subtypes.



Ipratropium bromide



Tiotropium bromide



Glycopyrronium bromide

Fig. 2.1. *Chemical structure of the muscarinic antagonists used or in development for the treatment of COPD. The asterisks denote the centres of chirality.*

2.2. Evaluation of the guinea-pig ileal longitudinal smooth muscle with respect to the distribution of P2 receptors

ATP plays a widespread role in cell signalling, acting at surface receptors called P2 receptors and promoting a diverse array of physiological functions. Classification based on rank order of agonist potency generated an initial subdivision into P2X and P2Y receptors, which are now known to be ion channels and seven transmembrane G protein-coupled receptors, respectively. Recently, successful cloning strategies have had a major impact on this area of pharmacology, with the cloning of seven P2X and six P2Y subtypes (see chapter 1.2.3.). The integration of this molecular information into an understanding of the cellular and tissue functions of these receptors is very important, especially since a heterogenous population of P2 receptors is very often found in native tissues.

The guinea-pig ileal longitudinal smooth muscle (GPI) preparation is established as a robust *in vitro* test system for different receptor classes, e.g. muscarinic, 5-hydroxytryptamine and histamine receptors. Recently, in several studies P2 receptors were found to be located in this tissue. Using α,β-meATP as agonist contraction of the ileal longitudinal muscle segments could be shown which may be due to its action at P2X receptors. Since the evoked contractile response was inhibited by tetrodotoxin and atropine, α,β-meATP seems to act, probably, on P2X-like receptors situated on cholinergic nerves and, subsequently, to release acetylcholine causing an atropine-sensitive contraction of the GPI. Moreover, it is proposed the involvement of an endogenous P2 ligand, possibly ATP, acting as a positive modulator of ACh release or stimulating a further P2 receptor located at the postsynaptic site, since its contractile effect was atropine- and TTX-insensitive (Kennedy and Humphrey, 1994; Barthó et al., 1997; Matsuo et al., 1997; Sawyer et al., 2000).

Accordingly, the present study was addressed to elucidate the pre- and postsynaptically located receptor subtypes mediating the responses to α,β-meATP and other P2 receptor agonists in guinea-pig ileal longitudinal smooth muscle as well as their sensitivity to various commonly used P2 antagonists (Fig. 2.2.).

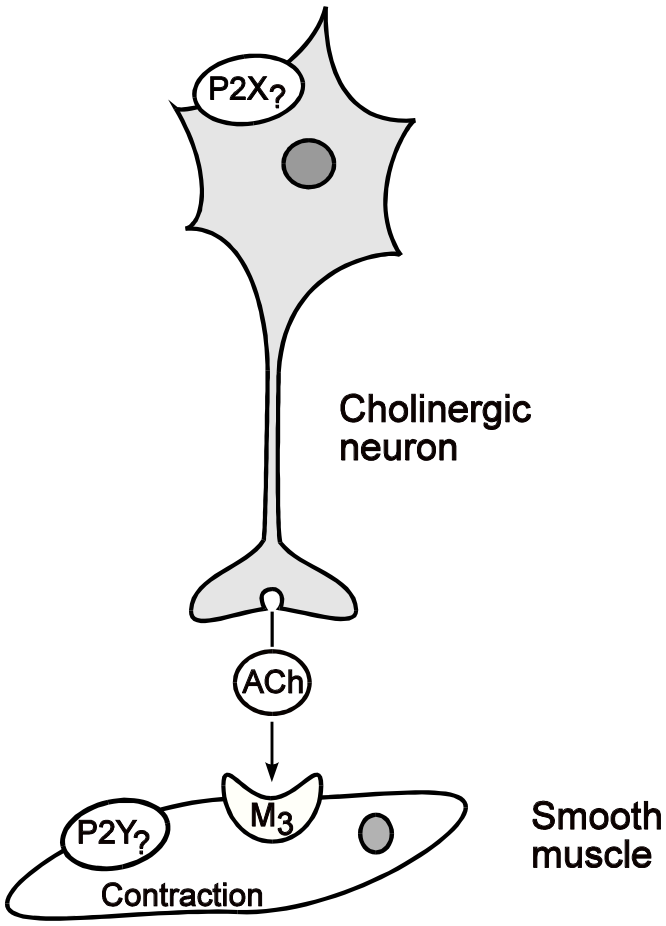


Fig. 2.2. *Funtional heterogeneity of P2 receptors mediating contraction in guinea-pig ileal longitudinal smooth muscle.*

2.3. Characterisation of functional P2 receptor subtypes mediating contraction in the rat ileal longitudinal smooth muscle

The identification and characterization of rat P2 receptors in native tissues are of special interest because one of the main current challenges is to relate the cloned rat P2X and P2Y receptor subtypes (see chapter 1.2.3.) to the diverse physiological responses caused by nucleotides.

Previous studies examined the effect of ATP on myenteric plexus preparation of rat ileum. Using myocytes isolated from the longitudinal muscle layer of rat ileum, Pacaud et al. (1996) postulated a PPADS-sensitive P2 receptor with properties similar to the $P2Y_1$ subtype stimulation of which led to a rise in intracellular Ca^{2+}, resulting from coupling to PLC. Moreover, Blottière et al. (1996) suggested that these myocytes do not possess functional P2X receptors, because α,β-meATP had no effect, but that 2-MeSATP and UTP gave hints for the existence of P2Y or P2U receptor subtypes. Both agonists induced a rise in $[Ca^{2+}]_i$, probably via an all-or-nothing mobilisation of Ca^{2+} from intracellular stores.
Furthermore, the coexistence of ATP and NO was reported in myenteric neurons of the rat ileum (Belai and Burnstock, 1994). In experimens with electrical field stimulation, Smits and Lefebvre (1996) have found that ATP, NO and putative other neurotransmitters caused relaxation in the precontracted rat ileal longitudinal smooth muscle (RI). Because of the complex pattern resulting from EFS which in non-adrenergic, non-cholinergic conditions in precontracted RI consists of a primary (nitrergic) contraction, a primary relaxation, an off-relaxation (also nitrergic in nature) and a rebound contraction, it has been very difficult to determine the P2 receptors that were involved.

Thus, the present study was undertaken to characterise the P2 receptor subtypes subserving contraction of RI in order to define similarities and differences to native P2 receptors found functionally in GPI.

3. MATERIALS AND METHODS

3.1. Drugs

3.1.1. Commercially available drugs

- Adenosine 3',5'-diphosphate (A3P5P; Sigma, Deisenhofen, Germany)
- Adenosine 5'-O-(2-thiodiphosphate) dilithium salt (ADPßS; Sigma, Deisenhofen, Germany)
- Adenosine 5'-O-(3-thiotriphosphate) dilithium salt (ATPγS; Sigma, Deisenhofen, Germany)
- Atropine sulfate (Merck, Darmstadt, Germany)
- 2-Chloro-N^6-cyclopentyladenosine (CCPA; RBI, Natick, USA)
- 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; RBI, Natick, USA)
- Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany)
- Glycopyrronium bromide (Brenner Efeka - Wyeth, Münster, Germany)
- Hexamethonium chloride (Sigma, Deisenhofen, Germany)
- Histamine dihydrochloride (Sigma, Deisenhofen, Germany)
- α,ß-Methyleneadenosine 5'-diphosphate dilithium salt (α,ß-meADP; Sigma, Deisenhofen, Germany)
- α,ß-Methyleneadenosine 5'-triphosphate dilithium salt (α,ß-meATP; Sigma, Deisenhofen, Germany)
- 2-Methylthioadenosine 5'-triphosphate tetrasodium salt (2-MeSATP; RBI, Natick, USA)
- Naloxone (Sigma, Deisenhofen, Germany)
- N^G-Nitro-L-arginine (L-NOARG; Sigma, Deisenhofen, Germany)
- Pentobarbital sodium (Nembutal®; Sanofi, Hannover, Germany)
- Physostigmine (Merck, Darmstadt, Germany)
- Procaine hydrochloride (Sigma, Deisenhofen, Germany)
- Suramin (Bayer, Leverkusen, Germany)
- Tetrodotoxin (TTX; Sigma, Deisenhofen, Germany)
- Yohimbine hydrochloride (Sigma, Deisenhofen, Germany)

3.1.2. Gifts

- MRS2179 (K. A. Jacobson; National Institutes of Health, Bethesda, USA)
- p-F-HHSiD (Prof. Dr. R. Tacke, University of Würzburg, Germany)
- Himbacine hydrochloride (Dr. W. C. Taylor, University of Sidney, Australia)
- NF023, NF031, NF279 (Prof. Dr. P. Nickel, Bonn, Germany)
- PPADS (Dr. H. G. Bäumert, University of Frankfurt, Germany)
- Pirenzepine hydrochloride (Dr. Karl Thomae GmbH, Biberach, Germany)

3.1.3. Syntheses

Arecaidine propargyl ester (APE) and 4-(4-fluorophenylcarbamoyloxy)-2-butynyl-N-methyl-pyrrolidinium tosylate (4-F-PyMcN$^+$) were synthesised in our laboratories by Dr. U. Moser and Mrs. U. Hermanni.
The four isomers of glycopyrronium bromide and their four tertiary analogues were synthesised by M. Elgert, University of Frankfurt, Germany.

3.2. Preparation of buffer and stock solutions

Bath fluids and buffers were freshly prepared for each experiment from commercially available chemicals of analytical grade purity. Their compositions are listed in the table below (Tab. 3.1.).

Drugs were dissolved and diluted in 0.9 % (w/v) aqueous NaCl solution prior to use in the experiments. Stock solutions of CCPA and DPCPX were made up in dimethyl sulfoxide. Control experiments assured that the final concentration of dimethyl sulfoxide present after dilution in the bath fluid had no pharmacological effect itself.

Tab. 3.1. *Compositions (in mM) of the bath fluids used in the pharmacological experiments*

	Tyrode solution	Krebs solution
NaCl	137.0	118.0
KCl	2.7	4.7
$CaCl_2$	1.8	1.0
$MgCl_2$	1.05	-
$MgSO_4$	-	0.6
$NaHCO_3$	11.9	25.0
NaH_2PO_4	0.42	-
KH_2PO_4	-	1.2
(+)-Glucose	5.6	11.1

3.3. Animals

All laboratory animals were housed in an air-conditioned room at a temperature of 23°C and a relative atmospheric humidity of 50 % in a light-dark cycle of 12 hours each.

Male New Zealand white rabbits (2.5 - 3.0 kg body weight) were purchased from Koch, Edingen-Neckarhausen, Germany.

Male guinea-pigs (250 - 350 g body weight) and male Sprague Dawley rats (200 - 300 g body weight) were generously provided by Hoechst AG, Frankfurt/Main, Germany.

All animals were fed a standard diet (rabbits/guinea-pigs: Altromin® 3022, rats: Altromin® 1324, Altromin GmbH, Lage, Germany) with free access to food and tap water.

3.4. Methods

3.4.1. Rabbit vas deferens (RVD)

Experiments on RVD were carried out according to the method described by Eltze (1988), Eltze et al. (1988) and Dörje et al. (1991b). Briefly, new Zealand white rabbits were killed by i.v. injection of 120 mg/kg of pentobarbital sodium (Nembutal®) into the ear vein, locally anaesthetised by a procaine solution (10 %, w/v). The abdomen was opened and the vasa deferentia were excised. After clearing of connective tissue the vasa deferentia were divided into six segments of approximately 1.0 cm length. Each segment was fixed vertically by means of platinum ring electrodes (Fleck, Mainz, Germany) and a cotton thread, which was connected to a force displacement transducer, in 6 ml water-jacketed organ baths containing Krebs buffer (for its composition see Tab. 3.1.). Additionally, 1 µM yohimbine was added to the buffer to block α_2-adrenoceptors.
The bath fluid was maintained at 31°C and was continuously bubbled with carbogen (95 % O_2, 5 % CO_2). The tension of the preparation was set at 375 mg and they were left to equilibrate for 30 min before the continuous field stimulation (0.05 Hz, 0.5 ms, 40 V; HSE Stimulator, II/215, Hugo Sachs Elektronik, March-Huchstetten, Germany) was started. The contractions were measured isometrically via a force displacement transducer (TF6V5) connected to a DMS DC amplifier (both from Fleck, Mainz, Germany), and were registered on a pen recorder (Knauer, Berlin, Germany).

Antagonist studies: The neurogenic contractions were concentration-dependently inhibited by the M_1 receptor agonist p-F-PyMcN$^+$ (Lambrecht et al., 1993; Moser et al. 1993; Grimm et al., 1994a). Antagonist affinities were determined by constructing cumulative concentration-response curves of the agonist in the absence and presence of the antagonist. The equilibration time was dependent on the used concentration of the antagonist, but it was at least 1 hour. EC_{50} values of p-F-PyMcN$^+$ for the control (absence of antagonist) and the shifted concentration-response curve (presence of antagonist) were determined graphically for calculation of the dose ratio from which the pA_2 value was assessed.

3.4.2. Guinea-pig atria (GPA)

Male guinea-pigs were killed by cervical dislocation. After exsanguination of the animals the left atria were isolated and transferred into Tyrode solution (pH = 7.4; T = 32°C, for its composition see Tab. 3.1.) gassed with carbogen. The organs were divided into two segments and fixed vertically by a cotton thread and platinum electrodes connected to a force displacement transducer (TF6V5) in 6 ml water-jacketed organ baths containing Tyrode solution. Atria were paced electrically with supramaximal rectangular impulses (2 Hz, 3 ms, 5 V). The force of the resulting contractions was measured using a Hellige amplifier and recorder (Hellige, Freiburg, Germany). After 10 min equilibration time with half-maximum tension, the resting tension was raised to 250 mg and kept throughout. After another 20 min equilibration period, the negative inotropic responses to the cumulative addition of the muscarinic agonist APE were registered as changes in isometric tension.

Antagonist studies: Antagonist affinities were determined by constructing concentration-response curves to APE as described for RVD.

3.4.3. Guinea-pig ileum (GPI)

3.4.3.1. Preparation for experiments with exogenously applied muscarinic agonist

Male guinea-pigs were killed by cervical dislocation and exsanguinated. The distal part of the ileum was dissected at about 5 cm from the ileocaecum. Strips of ileal longitudinal smooth muscle (1 - 1.5 cm) were prepared according to the technique described by Paton and Zar (1968), mounted in 6 ml water-jacketed organ baths and loaded with a tension of 500 mg. Tyrode solution (pH = 7.4; T = 32°C, for its composition see Tab. 3.1.) aerated with carbogen served as bath fluid. Mechanical responses of ileal longitudinal muscle strips to APE were measured as isotonic contractions via an isotonic force displacement transducer (TF6V5 iso) and registered with a DMS DC amplifier and a Knauer recorder.

Antagonist studies: Antagonist affinities were determined in essentially the same manner as described for RVD.

3.4.3.2. Preparation for experiments with endogenous acetylcholine as agonist

The prepared ileal longitudinal smooth muscle strips were fixed by means of two platinum electrodes, transferred in 6 ml water-jacketed tissue baths containing Krebs solution (pH = 7.4; T = 37°C, for its composition see Tab. 3.1.) gassed with carbogen and connected to a force displacement transducer (TF6V5). The tissue was excited with supramaximal single rectangular impulses (0.1 Hz, 0.5 ms, 60 V). Neurogenic twitch contractions were measured isometrically using a DMS DC amplifier, and were registered on a Knauer pen recorder.

Antagonist studies: After a stabilisation period (1 - 2 h) the inhibition of neurogenic twitch contractions was measured by cumulative application of increasing concentrations of muscarinic antagonists to obtain pIC_{50} values. The inhibition of the contractile responses was calculated as percentage of control contractions.

3.4.3.3. Preparation for experiments with exogenously applied P2 agonists

The GPI was prepared and fixed as described in 3.4.3.1. and incubated, under a resting tension of 1 g, in 6 ml tissue baths containing modified Krebs solution (1.3 mM Ca^{2+}; Tab. 3.1.) aerated with carbogen (pH = 7.4; T = 37°C). Mechanical responses of the muscle strips to the P2 agonists were measured isometrically via a force displacement transducer (TF6V5) and registered with a DMS DC amplifier and a Knauer recorder.

Antagonist studies: First, the muscle strips were contracted by administration of 100 µM α,β-meATP or ADPβS, respectively, to increase the sensitivity of the tissue. After a rest of 1 hour, contractions were obtained to single doses (every 15 min) of exogenous α,β-meATP (Krebs solution supplemented with 70 nM physostigmine) or ADPßS (Krebs solution containing 300 nM atropine). Two concentration-response curves were constructed on each preparation, the first in the absence and the second in the presence of the antagonist (60-min exposure). One ileal strip from each animal was used as a control to monitor time-dependent variation in responses to the respective agonist. Generally, all contractile responses from a single tissue were normalised and expressed as percentage of

those evoked by the highest agonist concentration used in the control curve. Concentration-effect curves were fitted to the data in the form $\log_{10}$ concentration of the agonist vs. normalised effects by logistic, non-linear regression analysis (Fig. P Software Corporation, Durham, NC, USA).

3.4.4. Rat ileum (RI)

Male Sprague Dawley rats (body weight 250 - 350 g) were killed by asphyxiation, exsanguinated, and the ileum (distal 10 cm discarded) was removed. Strips of ileal longitudinal smooth muscle (1 - 1.5 cm) were prepared and mounted as described for GPI (see 3.4.3.1.). The plexus-containing smooth muscle strips of the rats were equilibrated in modified Krebs solution (1.3 mM Ca^{2+}; Tab. 3.1.) in 6 ml organ baths under a resting tension of 1 g. Mechanical muscular activity to the P2 agonists was recorded isometrically by a force displacement transducer (TF6V5) and registered with a DMS DC amplifier and a Knauer recorder.

Antagonist studies: As described above, muscle strips were contracted by administration of 100 µM of the respective P2 receptor agonist. After a stabilisation period (1 - 2 h), the contractions were obtained to single doses (every 15 min) of the exogenously applied agonist. Two concentration-response curves were constructed on each preparation, the first in the absence and the second in the presence of the antagonist (60-min exposure).
To obtain pIC_{50} values, the inhibition of contractions to one concentration of the P2 agonist was measured by application of increasing concentrations of the antagonist. The inhibition of the contractile responses was calculated as percentage of control contractions.

3.5. Evaluation of data

Antagonist affinities were obtained by constructing concentration-response curves for agonists in the absence and presence of increasing antagonist concentrations (log intervals 0.5 or 1.0). The corresponding EC_{50} values were determined graphically from semilogarithmic plots (effects vs. log concentration). Control experiments were carried out to assure that equilibrium conditions were achieved within the incubation period. If possible, four concentrations of the antagonist were

tested and tissues of at least two animals were used. Each concentration of antagonist was investigated in at least two independent experiments. Apparent affinities of antagonists (pA_2 values) were obtained from Arunlakshana-Schild plots (Arunlakshana and Schild, 1959). Slopes were determined by linear regression of the experimental data to the following equation using the method of least squares:

$$\log (DR - 1) = m\ pA_x - \log K_D$$

DR = dose ratio of EC_{50} values of the agonist in the presence and absence of the antagonist
m = slope of regression line
pA_x = -log [antagonist] (M)
K_D = antagonist dissociation constant

If the slope of the regression line was found not significantly different from -1.00 ($P > 0.05$), the antagonism was regarded as purely competitive. In this case, the pA_2 value was calculated as the x-intercept of a regression line by fitting to the data the best straight line with a slope of unity (m = -1), the latter called ‘constrained plot’ (McKay, 1978; Tallarida et al., 1979; Tallarida and Murray, 1986).

When it was impossible to investigate more than two concentrations (or even only one concentration) of an antagonist, the pA_2 value was calculated from the individual dose ratios according to the following equation (Tallarida et al., 1979):

$$pA_2 = \log (DR - 1) + pA_x$$

3.6. Statistics

Data are presented as means ± SEM (n) with n indicating the number of individual experiments. Differences between mean values were tested for statistical significance by Student’s t-test; $P < 0.05$ was accepted as being significant. Linear regression analysis was carried out using least-squares fit. Calculations are based on the personal computer program ”Pharm/PCS-Version 4.0” (Pharmacological Calculation System, Tallarida and Murray, 1986).

4. RESULTS

4.1. Functional *in vitro* experiments with the isomers of glycopyrronium bromide and their corresponding tertiary analogues at muscarinic receptors

4.1.1. General considerations

Neurogenic twitches elicited by electrical field stimulation of the rabbit vas deferens were concentration-dependently inhibited by the muscarinic agonist 4-F-PyMcN$^+$ yielding a pD_2 value of 6.62 (± 0.05; n = 66). At guinea-pig atria and ileum, arecaidine propargyl ester caused negative inotropic effects in atria with a pD_2 value of 7.99 (± 0.02; n = 86), and contractions of the ileal longitudinal smooth muscle with a pD_2 of 7.63 (± 0.11; n = 15), respectively. All effects served as standards to assess antagonist potencies of the compound to be examined.

4.1.2. Affinity profile of the parent compound glycopyrronium bromide

Glycopyrronium bromide, 1,1-dimethyl-3-(2-cyclopentyl-2-hydroxy-phenylacetyl-oxy)-pyrrolidinium bromide (Fig. 2.1.), is a quaternary ammonium compound with two chiral carbon atoms in its molecular structure. The parent compound is commercially available as a mixture of (R/S)-(S/R) stereoisomers (Demian and Gripshover, 1990). Fuder and Meincke (1993) described this compound as an ultrapotent M_1-selective muscarinic receptor antagonist *in vitro*. Therefore, at first the antimuscarinic potency of the glycopyrrolate was determined functionally at muscarinic M_1 (RVD), M_2 (GPA) and M_3 (GPI) receptors.

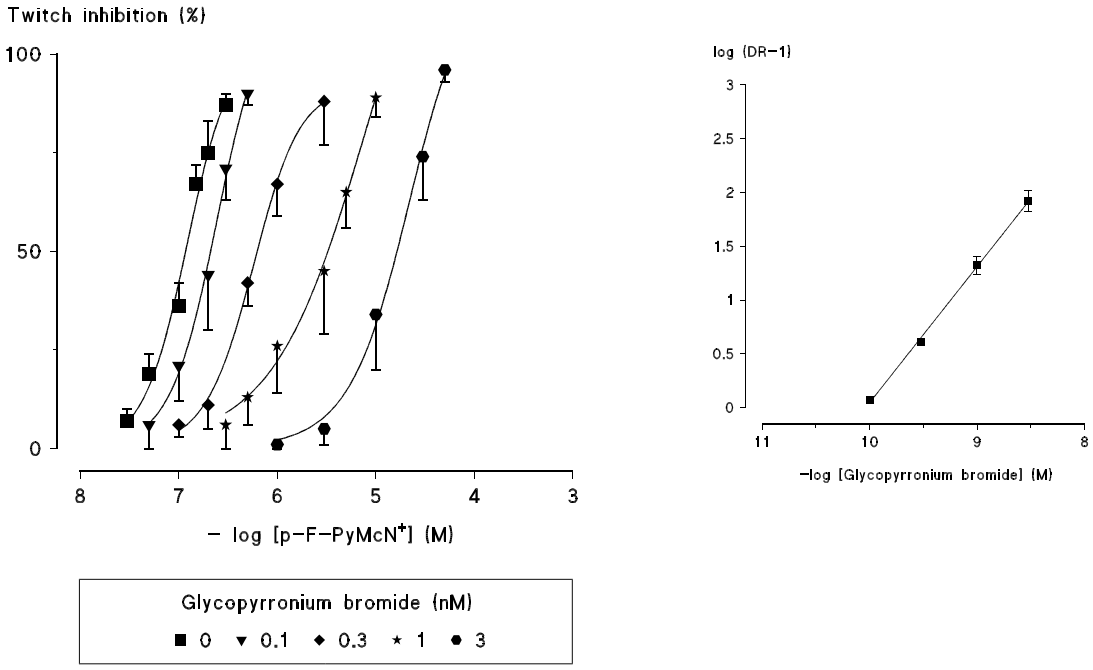


Fig. 4.1. *Concentration-response curves of p-F-PyMcN$^+$ in the absence and in the presence of glycopyrronium bromide in RVD (left panel; n = 13), and the corresponding Schild plot (right panel) with slope of 1.29 ± 0.06. Error bars falling within the area covered by a symbol are not shown.*

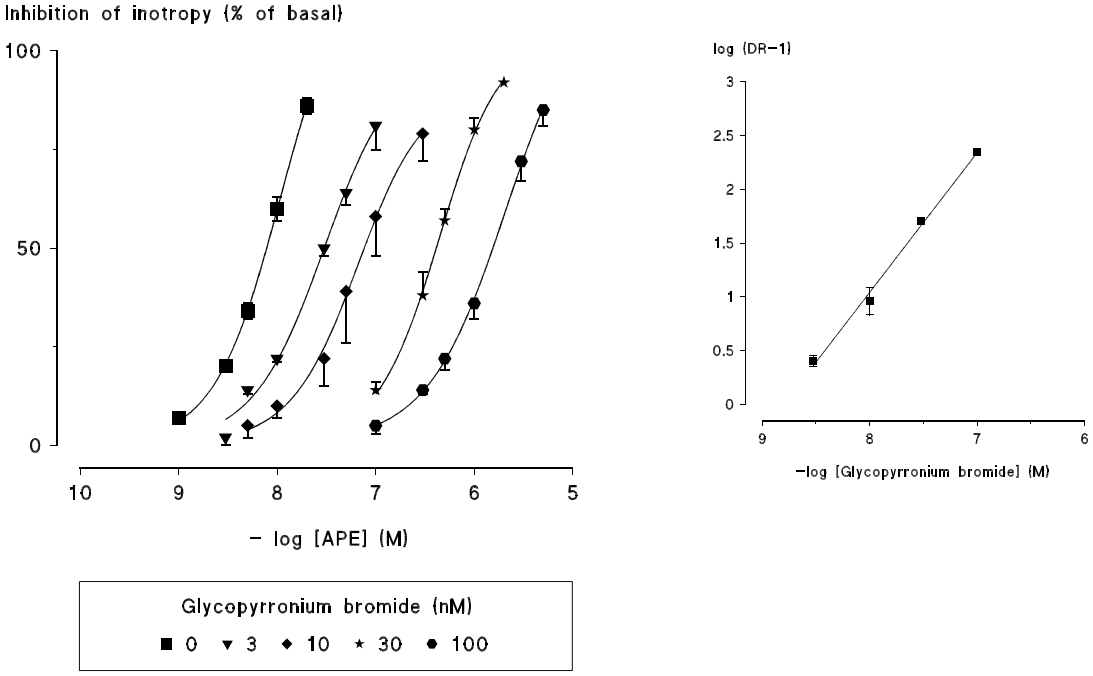


Fig. 4.2. *(legend, see page 61)*

Fig. 4.2. *Concentration-response curves of APE in the absence and in the presence of glycopyrronium bromide in GPA (left panel; n = 12), and the corresponding Schild plot (right panel) with slope of 1.30 ± 0.06. Error bars falling within the area covered by a symbol are not shown.*

At M_1 (Fig. 4.1.) and M_2 (Fig. 4.2.) receptors glycopyrronium bromide produced parallel shifts of the agonist concentration-response curve to higher concentrations with pA_2 values of 10.24 ± 0.06 (n = 13) for the M_1 and a pA_2 value of 9.11 ± 0.06 (n = 12) for the M_2 receptor subtype. In contrast to the above mentioned results the compound exhibited only a slight M_1 receptor selectivity (13-fold) over the M_2 subtype.

At the M_3 receptor subtype a very slow dissociation of the antagonist was observed in the functional experiments and the agonist could not reach the maximum of the control curve in presence of the antagonist. Thus, a pIC_{50} value was determined at electrically stimulated GPI to give a value of the antimuscarinic potency. The pIC_{50} value resulting from inhibition of neurogenic twitch contractions was 8.89 ± 0.06 (Fig. 4.3.).

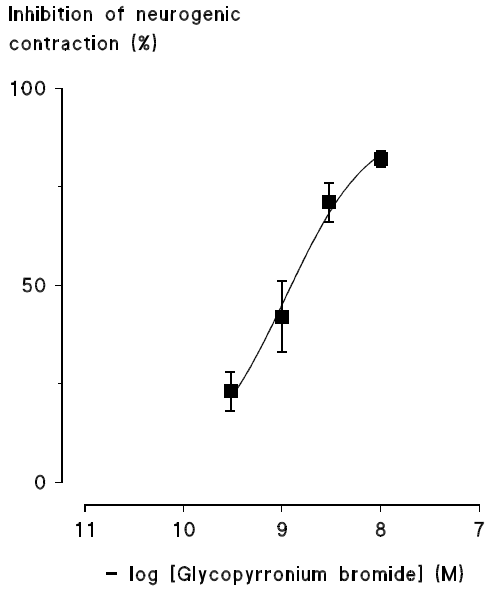


Fig. 4.3. *Inhibition curve of glycopyrronium bromide to contractions elicited by electrical field stimulation in GPI. Data are presented as mean values ± S.E.M. (n = 8). Error bars falling within the area covered by a symbol are not shown.*

The potency estimates of glycopyrronium bromide at M_1, M_2 and M_3 receptors are summarised in Fig. 4.4.

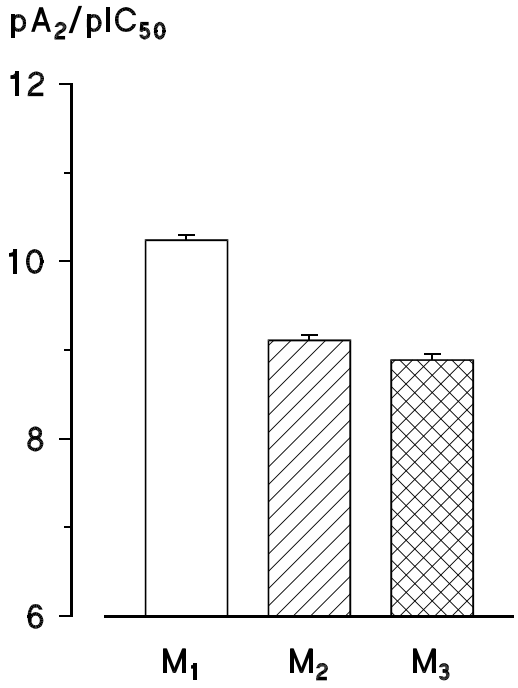


Fig. 4.4. *Antagonist potency of glycopyrronium bromide (pA_2: M_1 and M_2; pIC_{50}: M_3) determined in functional studies at muscarinic receptor subtypes. Data are shown as mean values ± S.E.M. from 8 - 13 experiments.*

4.1.3. Affinity profile of the quaternary stereoisomers of glycopyrronium bromide

The two centres of chirality of the parent compound, glycopyrronium bromide, result in the existence of four stereoisomers whose absolute configurations are shown in Fig. 4.5. (R = CH_3). To get detailed information about the influence of the steric arrangement it was of special interest to examine the antagonistic properties of these stereoisomers. Consequently, in the following experiments the four isomers of glycopyrronium bromide, designated **1** (3S/2'S), **2** (3S/2'R), **3** (3R/2'S) and **4** (3R/2'R), were tested in three functional muscarinic M_1, M_2 and M_3 receptor models.

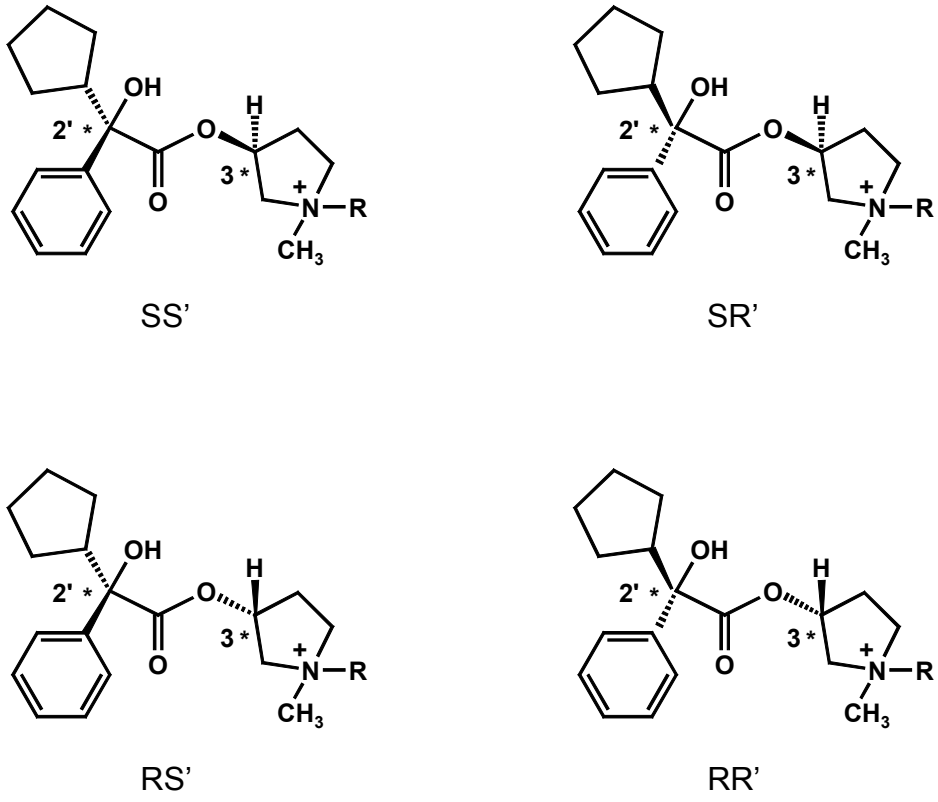


Fig. 4.5. *Chemical structure of the tertiary (R = H) and quaternary (R = CH_3) stereoisomers of glycopyrronium bromide. The asterisks denote the centres of chirality.*

In RVD and GPA the quaternary stereoisomers shifted the dose-response curves of 4-F-PyMcN$^+$ and APE, respectively, to the right. The Schild plots have shown that the rightward shifts were only parallel for the (3S/2'S)-configured isomer with slopes not significantly different from unity in both models.

The antagonistic properties (pA_2 values for M_1 and M_2; pIC_{50} values for M_3) are listed in Tab. 4.1.

Tab. 4.1. *Antimuscarinic potencies (pA_2/pIC_{50}) and slopes of Schild plots (in parenthesis) of the quaternary compounds **1**, **2**, **3** and **4** at muscarinic receptor subtypes examined in functional studies in RVD (M_1), GPA (M_2) and GPI (M_3). Data are presented as means ± S.E.M. (n = 3 - 13).*

compound	configuration	M_1 pA_2	M_2 pA_2	M_3 pIC_{50}
1	3S / 2'S	**8.22** ± 0.04 (1.01 ± 0.06)	**7.92** ± 0.03 (1.02 ± 0.05)	**6.82** ± 0.06
2	3S / 2'R	**10.27** ± 0.14	**9.39** ± 0.10 (1.52 ± 0.10)	**9.39** ± 0.09
3	3R / 2'S	**9.53** ± 0.10	**8.69** ± 0.03 (1.34 ± 0.08)	**8.57** ± 0.08
4	3R / 2'R	**10.30** ± 0.07	**9.43** ± 0.11 (1.59 ± 0.14)	**8.76** ± 0.06

In case of compounds **2** and **4** only one concentration (0.1 nM; n = 3; for compound **2** see Fig. 4.6.) and of compound **3** two concentrations (1 and 3 nM; n = 8) were included in the calculation at the M_1 receptor, since at higher concentrations pseudoirreversible antagonism was observed. However, the values for compounds **2** and **4** are probably underestimated because of the long time needed to reach equilibrium at M_1 receptor.

Figs. 4.7. and 4.8. show examples for the concentration-response curves that are observed in RVD and GPA, respectively.

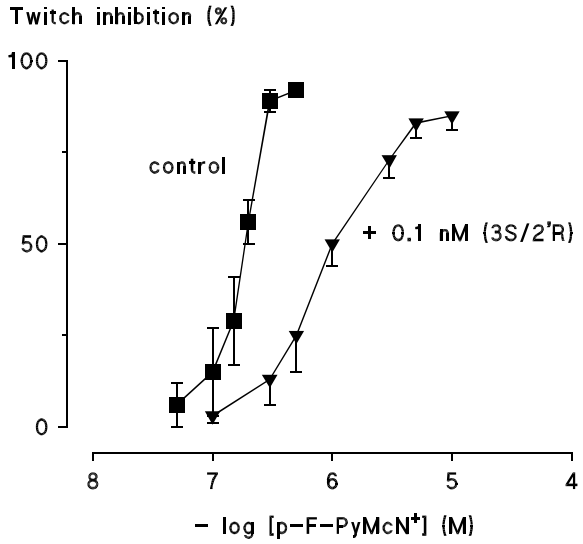


Fig. 4.6. *Concentration-response curves of p-F-PyMcN$^+$ in the absence and in the presence of 0.1 nM of the quaternary (3S/2'R)-configured stereoisomer of glycopyrronium bromide in RVD (n = 3).*

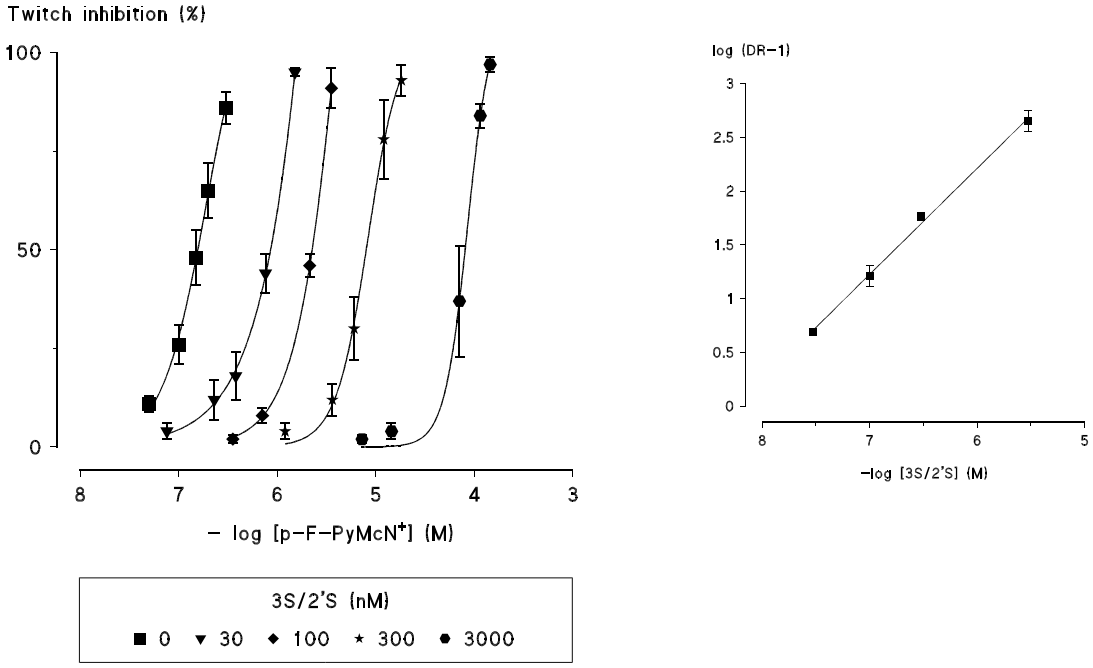


Fig. 4.7. *Concentration-response curves of p-F-PyMcN$^+$ in the absence and in the presence of the quaternary (3S/2'S)-configured stereoisomer of glycopyrronium bromide in RVD (left panel; n = 13), and the corresponding Schild plot (right panel) with slope of 1.01 ± 0.06 not significantly different from unity (P > 0.05). Error bars falling within the area covered by a symbol are not shown.*

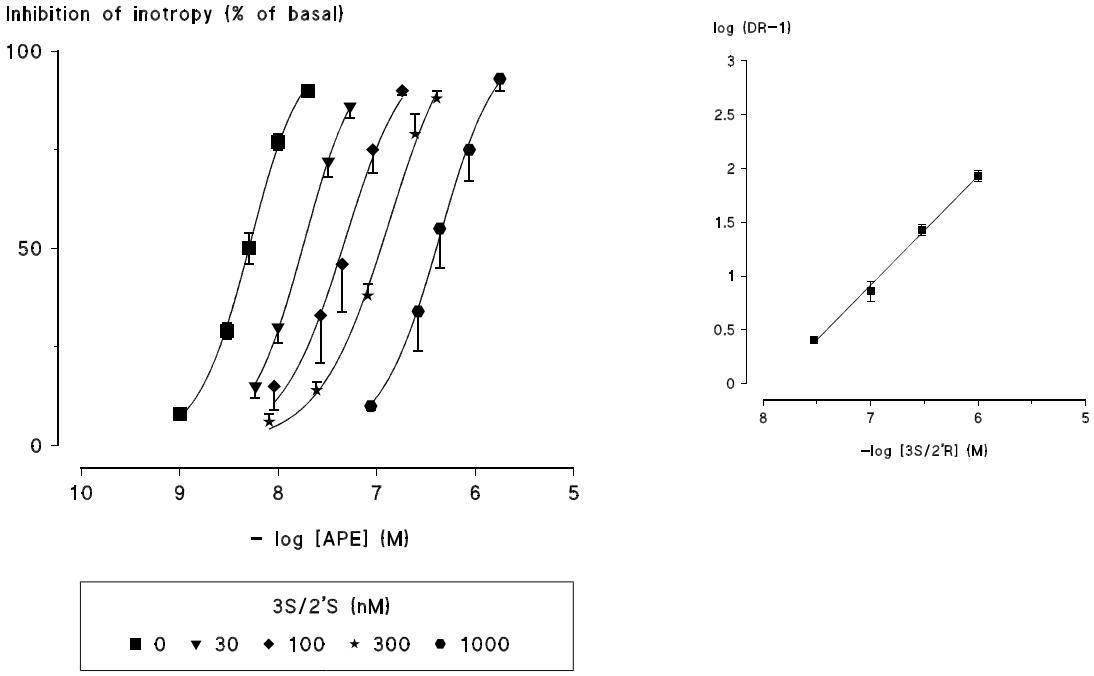


Fig. 4.8. *Concentration-response curves of APE in the absence and in the presence of the quaternary (3S/2'S)-configured stereoisomer of glycopyrronium bromide in GPA (left panel; n = 12), and the corresponding Schild plot (right panel) with slope of 1.02 ± 0.05 not significantly different from unity (P > 0.05). Error bars falling within the area covered by a symbol are not shown.*

In the concentration range tested, the quaternary amines (**1** - **4**) proved to be surmountable antagonists in the functional studies at muscarinic receptors in rabbit vas deferens (M_1) and guinea-pig atria (M_2).

However, because all isomers behaved as pseudoirreversible antagonists in the M_3 assay, the inhibition of neurogenic twitch contractions in GPI was measured by increasing concentrations of the antagonist, to obtain pIC_{50} values. The pIC_{50} values, resulting from the inhibition curves of the respective isomers to contractions elicited by electrical field stimulation (Fig. 4.9. A - D), are listed in table 4.1.

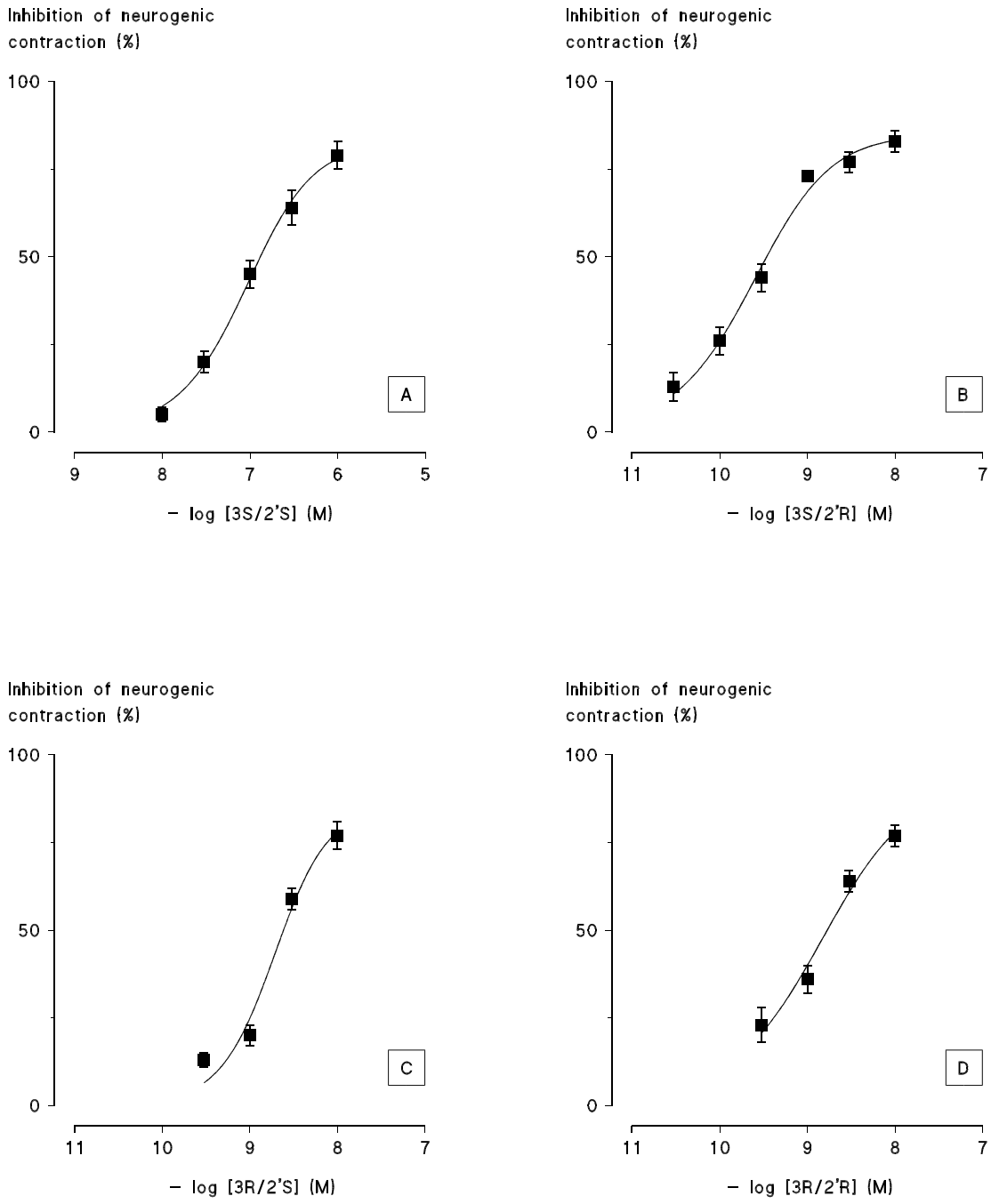


Fig. 4.9. *Inhibition curves of the quaternary stereoisomers (3S/2'R) [A], (3S/2'R) [B], (3R/2'S) [C] and (3R/2'R) [D] of glycopyrronium bromide to contractions elicited by electrical field stimulation in GPI. Data are presented as mean values ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown.*

The results show that the affinity/potency and muscarinic subtype selectivity of the tested compounds depend greatly on their absolute configuration. All isomers displayed in functional experiments a slight preference for M_1 over M_2 [2-fold for **1** (3S/2S') to 8-fold for **2** (3S/2R')]. Finally, the diastereomers **2** and **4**, with (R)-configuration in the glycolic acid part, were the most potent and the isomer **1** with (3S/2'S)-configuration the least potent among the four quaternary stereoisomers at the three muscarinic receptor subtypes.

4.1.4. Affinity profile of the tertiary stereoisomeric analogues of glycopyrronium bromide

Besides the quaternary compounds, also antimuscarinic potencies of the corresponding four tertiary analogues (Fig. 4.5., R = H), 1-methyl-3-(2-cyclopentyl-2-hydroxy-phenylacetyloxy)-pyrrolidium hydrogentartrate, were tested functionally in the same models at muscarinic M_1 - M_3 receptors. The compounds were designated **5** (3S/2'S), **6** (3S/2'R), **7** (3R/2'S) and **8** (3R/2'R). With exception of compound **5** (pA_2 = 7.61 ± 0.05 with a slope of the Schild plot of 1.19 ± 0.07; Fig. 4.10 A), all isomers behaved as pseudoirreversible antagonists in GPI, and thus, pIC_{50} values were determined to examine their antimuscarinic potencies. As an example, the inhibition curve of the (3S/2'S)-configured tertiary compound to contractions elicited by electrical field stimulation in GPI is illustrated in Fig. 4.10 B.

In Tab. 4.2, affinity/potency estimates are presented. The pA_2 values resulted from the rightward shift of the agonist curves in the presence of increasing concentrations of the antagonist in the RVD (M_1) and GPA (M_2), respectively. To determine the pIC_{50} values the following concentrations of the respective isomer were used: **5** (0.03 - 3 µM), **6** (0.3 - 10 nM), **7** (1 - 10 nM) and **8** (0.1 - 3 nM). The pIC_{50} values resulted from inhibition curves of the stereoisomers to contractions elicited by electrical field stimulation in GPI.

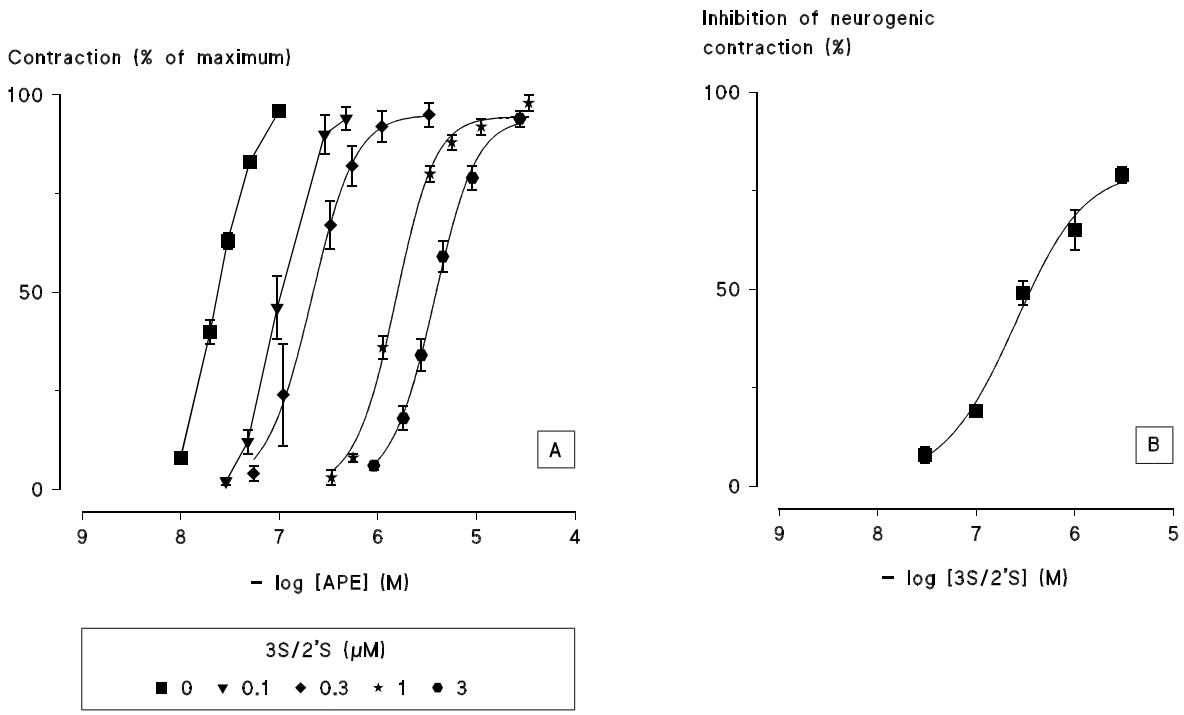


Fig. 4.10. *[A] Concentration-response curves of APE in the absence and in the presence of the tertiary (3S/2'S)-configured stereoisomer of glycopyrronium bromide in GPI (n = 16), and [B] inhibition curve of this compound to contractions elicited by electrical field stimulation in GPI. Data are presented as mean values ± S.E.M. (n = 3). Error bars falling within the area covered by a symbol are not shown.*

Tab. 4.2. *Antimuscarinic potencies (pA_2/pIC_{50}) and slopes of Schild plots (in parenthesis) of the tertiary amines **5**, **6**, **7** and **8** at muscarinic receptor subtypes examined in functional studies in RVD (M_1), GPA (M_2) and GPI (M_3). Data are presented as means ± S.E.M. (n = 9 - 13).*

compound	**configuration**	**M_1** pA_2	**M_2** pA_2	**M_3** pIC_{50}
5	3S / 2'S	**7.70** ± 0.07 (0.80 ± 0.09)	**7.52** ± 0.03 (1.02 ± 0.05)	**6.35** ± 0.08
6	3S / 2'R	**9.98** ± 0.04 (0.92 ± 0.09)	**9.03** ± 0.04 (1.18 ± 0.06)	**8.80** ± 0.02
7	3R / 2'S	**8.97** ± 0.07 (0.98 ± 0.15)	**8.71** ± 0.04 (1.12 ± 0.06)	**8.47** ± 0.02
8	3R / 2'R	**10.00** ± 0.12 (0.70 ± 0.23)	**9.45** ± 0.02 (1.02 ±0.03)	**9.05** ± 0.03

As with the quaternary analogues **1** - **4** (Tab. 4.1.), the highest antimuscarinic potency was observed for the tertiary diastereomers **6** and **8** possessing the (R)-configuration in the acid part of the molecules (Tab. 4.2.). As far as M_1 and M_2 receptors are concerned, compound **6** (3S/2'R) displayed the greatest subtype-selectivity (9-fold, based on affinity data).

4.2. P2 receptor heterogeneity in the guinea-pig ileal longitudinal smooth muscle preparation

4.2.1. Experiments at neuronal P2 receptors

4.2.1.1. Preliminary experiments with α,β-meATP

Based on observations by Kennedy and Humphrey (1994) the metabolically stable and $P2X_1$- and $P2X_3$-selective agonist α,β-meATP was used in the following experiments to elucidate the exact nature of the P2X-like receptors expressed in myenteric neurons of GPI.

Single doses of α,β-meATP (10 μM) elicited biphasic contractile responses consisting of a rapid (phasic) response followed by a more sustained (tonic) response. In the present study, only the phasic contractions, which desensitised rapidly (Fig. 4.11.), were examined.

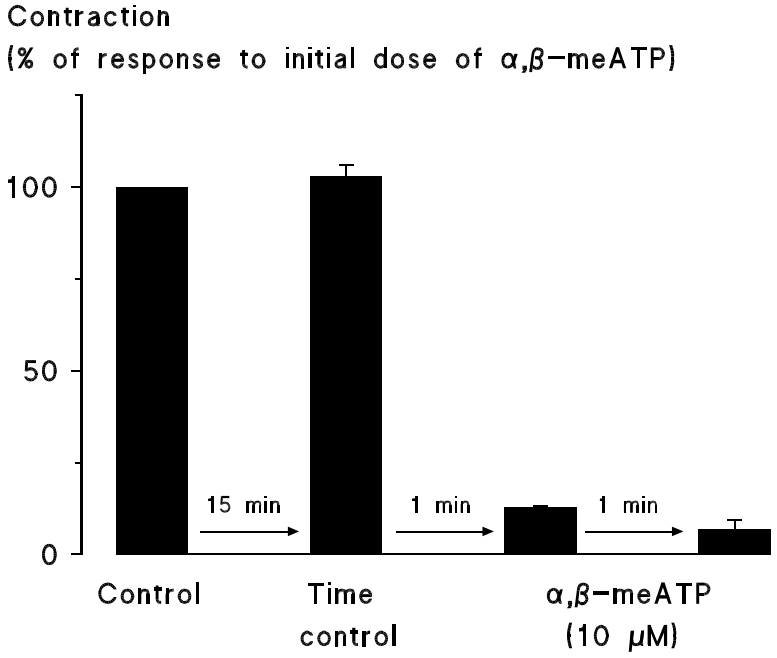


Fig. 4.11. *Desensitisation of α,β-meATP (10 μM)-induced contractions of the GPI. Responses are expressed as % of response to initial dose of α,β-meATP administered. Control responses to α,β-meATP amounted to 1201 ± 122 mg tension (n = 3).*

Since the contractile effects of α,β-meATP in GPI are mediated by acetylcholine, released from cholinergic neurons (Kennedy and Humphrey, 1994), and since these cholinergic neurons are endowed with prejunctional adenosine (Moody and Burnstock, 1982) and opiate receptors (Vizi et al., 1981), control experiments were carried out to characterise the action of α,β-meATP in our experimental design. Therefore, in a first run of experiments the influence of 2-chloro-N^6-cyclopentyladenosine (CCPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), naloxone and physostigmine on the contractions elicited by 10 or 30 µM α,β-meATP, respectively, was investigated.

CCPA: The A_1-selective agonist CCPA (Lohse et al., 1988) concentration-dependently reduced contractions (1000 nM CCPA: reduction to 4 % of control without CCPA) elicited by 10 µM α,β-meATP (Fig. 4.12.), consistent with the existence of a prejunctional adenosine A_1 receptor.

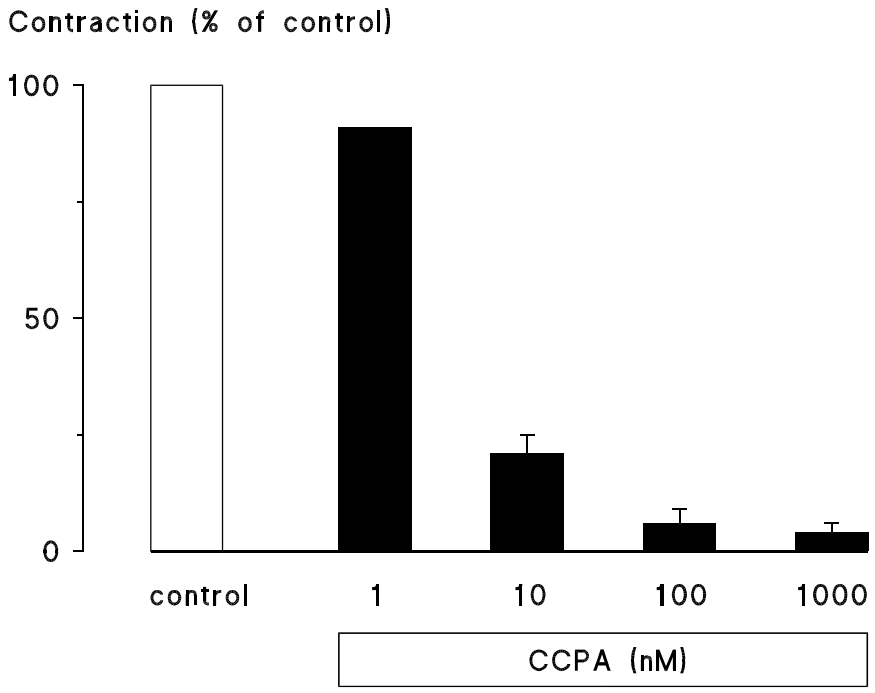


Fig. 4.12. *Effect of increasing concentrations of CCPA on the contractile response of the GPI produced by α,β-meATP (10 µM). Responses are expressed as percentage of control responses (1114 ± 103 mg tension generated) without CCPA (n = 3 - 5).*

DPCPX: Additionally, three concentrations of the A_1 receptor antagonist DPCPX (Lohse et al., 1987) (30, 100 and 300 nM) were tested against 30 µM α,β-meATP (n = 1 - 2). However, contractions elicited by α,β-meATP remained unaffected in the presence of DPCPX.

Naloxone: To exclude the influence of prejunctional opiate receptors, naloxone (30 - 1000 nM) was tested against 30 µM α,β-meATP (n = 2). Again, there was no difference between α,β-meATP potency in the absence and in the presence of naloxone.

Physostigmine: As an inhibitor of the enzyme acetylcholinesterase physostigmine (30 and 100 nM) increased the contractile effect to α,β-meATP (30 µM). At higher concentrations (300 and 1000 nM) it also acted as an antimuscarinic agent (Fig. 4.13.). Consequently, all subsequent experiments were carried out in the continuous presence of 70 nM physostigmine.

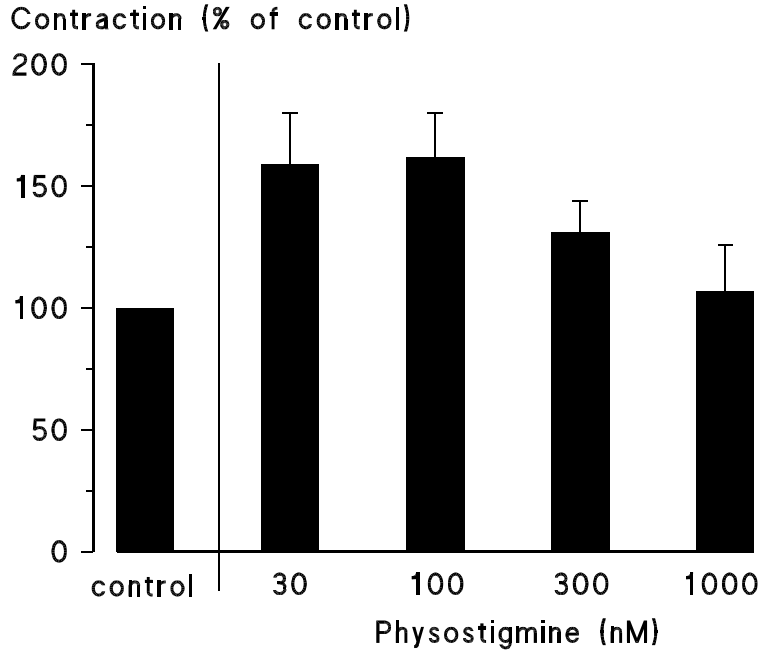


Fig. 4.13. *Effect of increasing concentrations of physostigmine on the contractile response of the GPI produced by α,β-meATP (30 µM). Responses are expressed as percentage of control responses (848 ± 79 mg tension generated) without physostigmine (n = 3 - 4).*

In order to confirm the neuronal localisation of the P2X-like receptor and the release of ACh via this receptor, the Na^+ channel blocking agent tetrodotoxin (TTX) as well as the non-selective muscarinic antagonist atropine were tested. As illustrated in Fig. 4.14., the responses to 10 µM α,β-meATP were totally abolished by TTX (1 µM) and greatly reduced by atropine (300 nM), respectively.

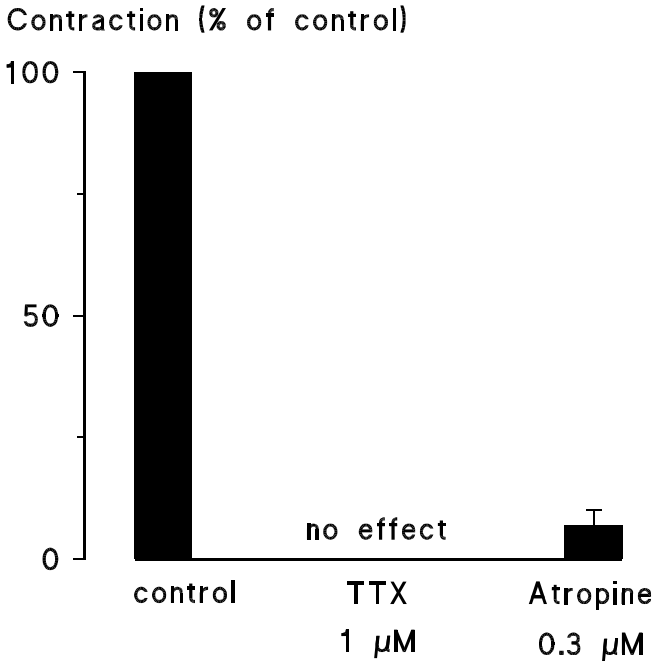


Fig. 4.14. *Influence of TTX (1 µM) and atropine (0.3 µM) on contractions elicited by α,β-meATP (10 µM) in GPI. Responses are expressed as percentage of control responses (1728 ± 222 mg tension generated) without TTX or atropine (n = 4).*

4.2.1.2. Antagonism by suramin, PPADS and NF023

α,β-meATP (0.1 - 30 µM; EC_{50} = 0.79 ± 0.09 µM; 1401 ± 87 mg tension generated in control experiments) produced concentration-dependent contractions in GPI. To avoid desensitisation, the agonist was added at regular intervals of 15 min. Time controls revealed that the dose-response curves remained stable during the course of the experiments. Unless stated otherwise, the Krebs buffer (1.3 Ca^{2+}) was supplemented with 70 nM physostigmine to prevent breakdown of endogenously released ACh.

Suramin (10 - 100 μM) antagonised contractile responses to α,β-meATP in a roughly parallel manner, with a pA_2 value of 5.10 ± 0.07. Fig. 4.15. demonstrates the dose-response curves of suramin to α,β-meATP (left panel) as well as the resulted Schild plot, the slope of which (0.94 ± 0.09) was not significant different from unity (right panel).

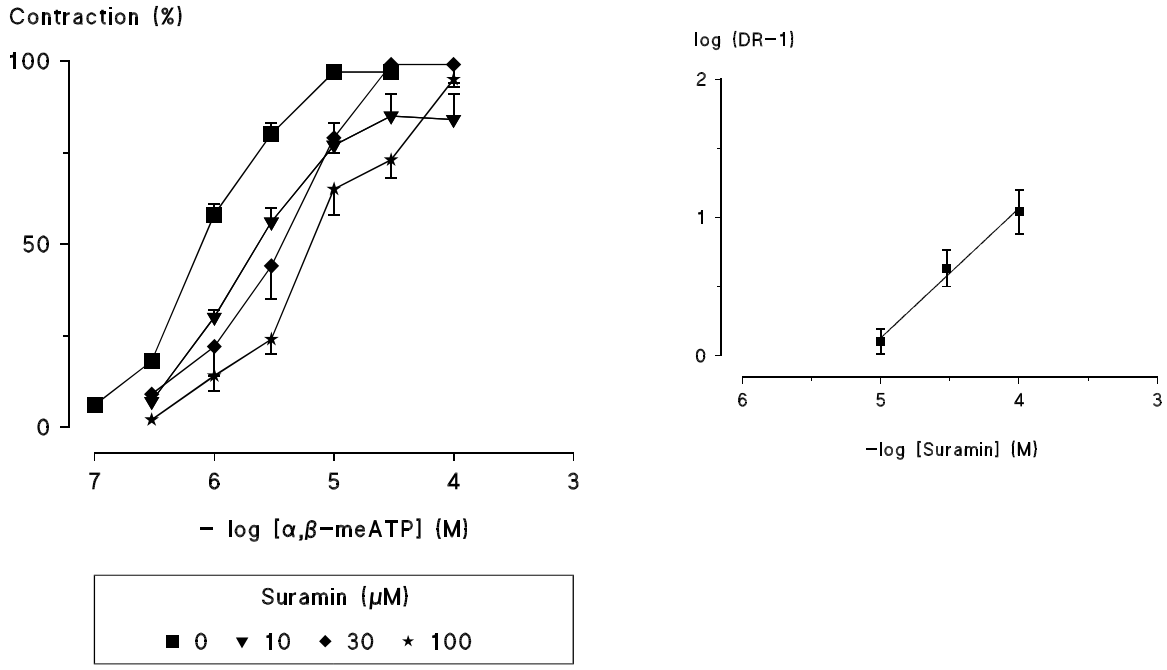


Fig. 4.15. *Concentration-response curves of α,β-meATP in the absence and in the presence of suramin in the GPI, and the corresponding Schild plot with slope not significantly different from unity (P > 0.05). The graphs show mean data ± S.E.M (n = 4). Error bars falling within the area covered by a symbol are not shown.*

PPADS (0.3 - 100 μM) antagonised contractile responses to α,β-meATP in a pseudoirreversible fashion. The calculated apparent pK_B of PPADS, derived from a double reciprocal regression (Kenakin, 1993), was 6.17 ± 0.09 (Fig. 4.16.).

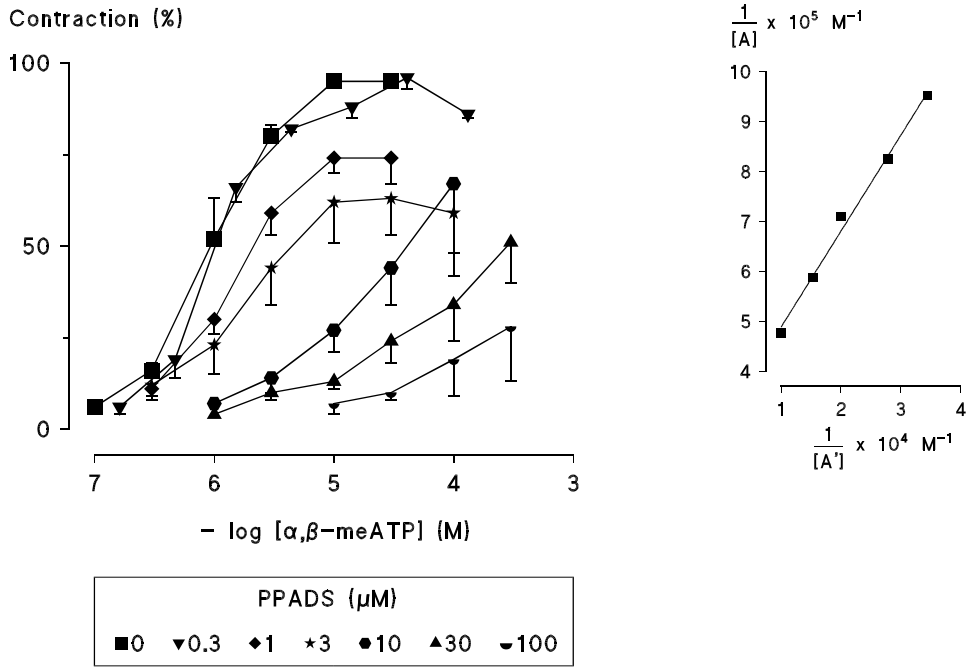


Fig. 4.16. *Effect of PPADS on the mechanical response of the GPI produced by α,β-meATP. The graphs show mean data ± S.E.M. (n = 3 - 4). Error bars falling within the area covered by a symbol are not shown. The right panel shows the double reciprocal plot: antagonism by PPADS of α,β-meATP-induced contractions of the GPI. [A] and [A'] denote the equieffective agonist concentrations in the absence and in the presence of PPADS (10 μM).*

Increasing concentrations of NF023 (30 - 300 μM) produced rightward shifts of the concentration-effect curve for α,β-meATP, and Schild transformation of these data revealed that NF023 is a competitive P2X receptor antagonist with a pA_2 value of 4.56 ± 0.07 and a slope of the Schild regression line of 0.94 ± 0.01, which is not significantly different from unity (Fig. 4.17.).

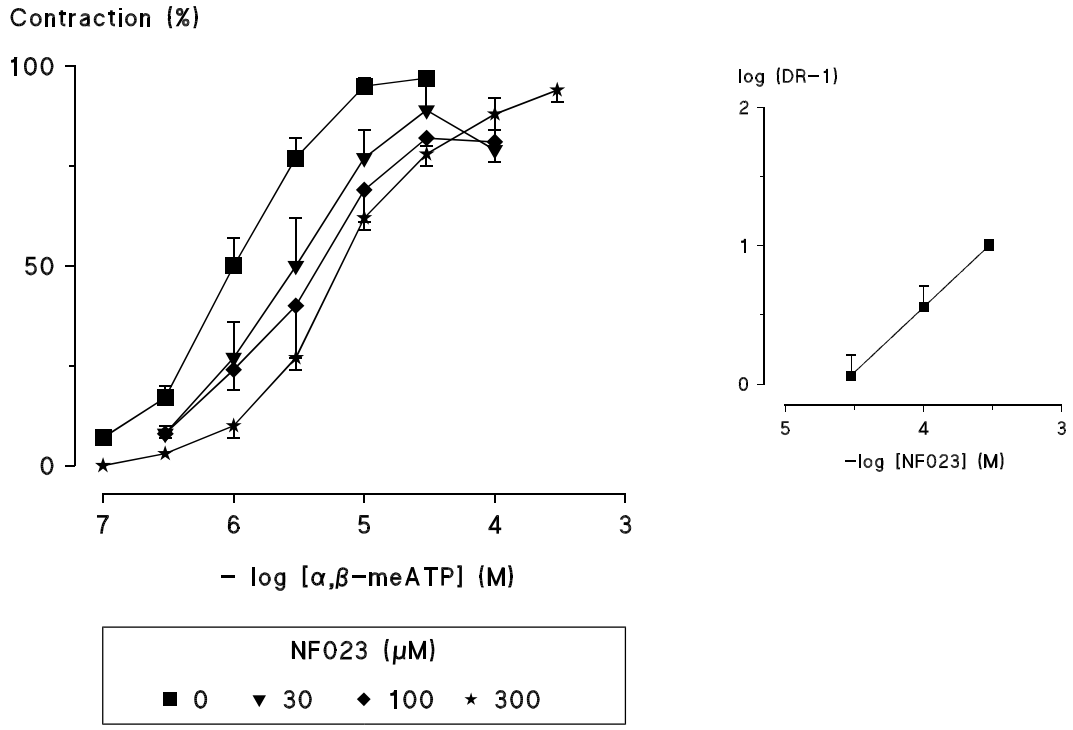


Fig. 4.17. *Concentration-response curves of α,β-meATP in the absence and in the presence of NF023 in the GPI (left panel), and the corresponding Schild plot (right panel) with slope not significantly different from unity (P > 0.05). The graphs show mean data ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown.*

4.2.1.3. Antagonism by NF279

The antagonist properties of NF279 were very interesting due to its specific $P2X_1$ receptor selectivity (Lambrecht, 2000). The concentration-response curves for NF279 against α,β-meATP are shown in Fig. 4.18.

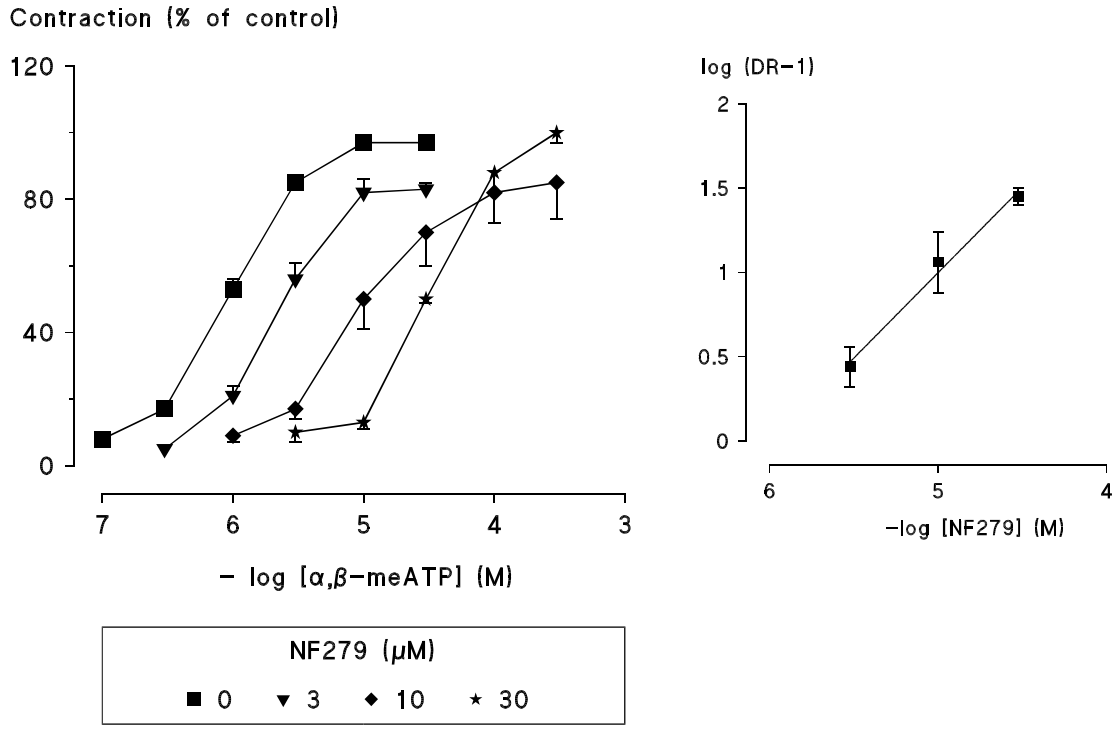


Fig. 4.18. *Concentration-response curves of α,β-meATP in the absence and in the presence of NF279 in the GPI (left panel), and the corresponding Schild plot (right panel) with slope not significantly different from unity (P > 0.05). The graphs show mean data ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown.*

NF279 (3 - 30 μM; Fig. 4.18.) produced concentration-related rightward displacements of the concentration-response curve to α,β-meATP in a parallel fashion. A pA_2 value of 5.95 ± 0.08 could be determined. The corresponding Schild plot was linear, and the slope of the regression line (1.01 ± 0.10) was not significantly different from unity (P > 0.05), indicating a competitive nature for this antagonistic effect.

4.2.1.4. Investigations on the postjunctional muscarinic receptor subtype responsible for contraction of GPI

Previous studies have shown, that the neuronal P2X-like receptor, which has been investigated, acts after its stimulation through facilitation of ACh release causing a contraction of the GPI. In the following set of experiments the postjunctional muscarinic receptor subtype responsible for ACh-mediated contractions to α,β-meATP or contractions elicited by electrical field stimulation of GPI was characterised by means of atropine and the subtype-preferring muscarinic antagonists pirenzepine, himbacine and p-F-HHSiD. It is worth mentioning that PPADS (100 µM), suramin (100 µM), NF023 (100 µM) and NF279 (100 µM) failed to affect contractions eliciting by single doses (100 nM) of the muscarinic agonist arecaidine propargyl ester (APE) at GPI. Hence, a direct interaction of the P2 antagonists employed with postjunctionally located muscarinic receptors can be excluded (Lambrecht, 2000 and unpublished results).

As previously shown, contractions elicited by single doses of α,β-meATP were greatly reduced by atropine (300 nM; cf. 4.2.1.1.), indicating that these responses are indirect-muscarinic, ACh-mediated and caused by stimulation of soma-dendritic P2X-like receptors on cholinergic neurones. To examine the exact nature of the postjunctionally localised muscarinic receptor, the antagonistic effect of atropine (0.003 - 0.1 µM), p-F-HHSiD (0.01 - 3 µM), himbacine (0.3 - 30 µM) and pirenzepine (0.1 - 30 µM) was determined to α,β-meATP (3 µM)-induced contractions. All these muscarinic antagonists concentration-dependently decreased contractile responses of the GPI to almost 100 % (Fig. 4.19.).

Electrical field stimulation (EFS; 0.1 Hz) of the GPI elicited neurogenic (TTX-sensitive, 1 µM) and monophasic rapid twitch contractions. These responses were muscarinic (ACh-mediated) in nature, since they were (nearly) abolished by muscarinic antagonists atropine (0.3 - 30 nM), p-F-HHSiD (1 - 1000 nM), himbacine (10 - 1000 nM) and pirenzepine (30 - 3000 nM; Fig. 4.19.).

Although the antagonists used (Fig. 1.2.) were less potent against α,β-meATP than against EFS, the same antagonist profile could be noticed for both methods: atropine > p-F-HHSiD (7- to 10-fold) > himbacine (5- to 8-fold) > pirenzepine (1- to 2-fold) (Tab. 4.3.).

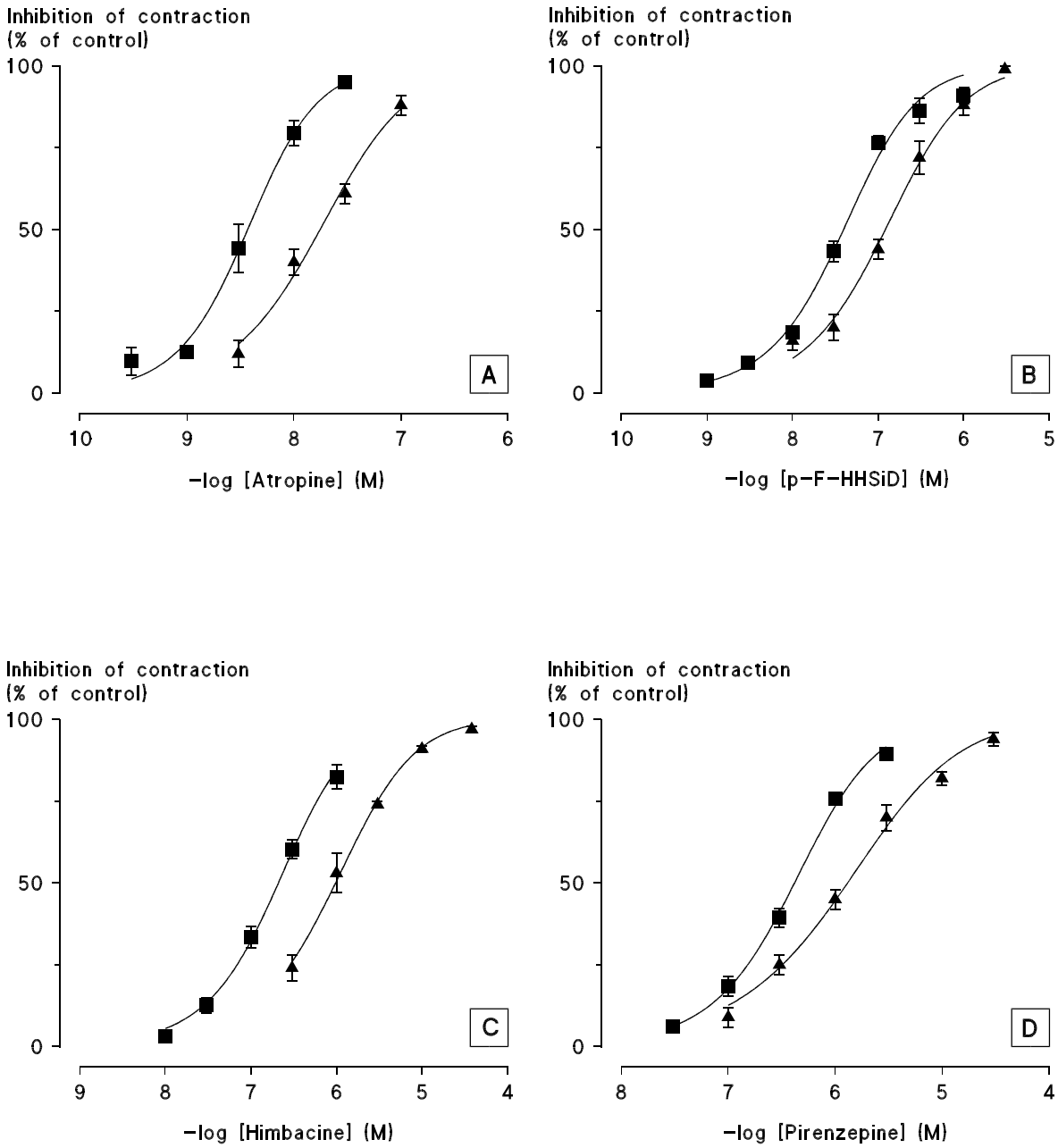


Fig. 4.19. *Concentration-inhibition curves of atropine (**A**), p-F-HHSiD (**B**), himbacine (**C**) and pirenzepine (**D**) in GPI on contractions elicited by EFS (0.1 Hz; ■) or α,β-meATP (3 μM; ▲). The graphs show mean data ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown.*

Tab. 4.3. *Potencies (pA_2 and pIC_{50} values) of muscarinic antagonists to inhibit cholinergically mediated responses in the GPI. The pIC_{50} values are given as mean values ± S.E.M. from 4 - 6 experiments.*

Muscarinic antagonist	pA_2 **M_3**	pIC_{50} **α,β-meATP**	pIC_{50} **EFS**
Atropine	**8.99**[2)]	**7.76** ± 0.05	**8.44** ± 0.04
p-F-HHSiD	**7.84**[1)]	**6.93** ± 0.04	**7.42** ± 0.04
Himbacine	**7.10**[2)]	**6.01** ± 0.02	**6.69** ± 0.02
Pirenzepine	**6.88**[1)]	**5.88** ± 0.04	**6.39** ± 0.02

[1)] Lambrecht et al., 1988; [2)] Eltze et al., 1993.

Table 4.3. summarises the pA_2 values derived from Schild plots at M_3 receptors in GPI and the pIC_{50} values obtained either with α,β-meATP (3 µM) or EFS (0.1 Hz) in GPI for the four muscarinic key antagonists. The excellent correlations, r = 0.993 for pIC_{50} (EFS) vs. pIC_{50} (α,β-meATP) and r = 0.992 for pA_2 (M_3) vs. pIC_{50} (α,β-meATP), are shown graphically in Fig. 4.20. A and B.

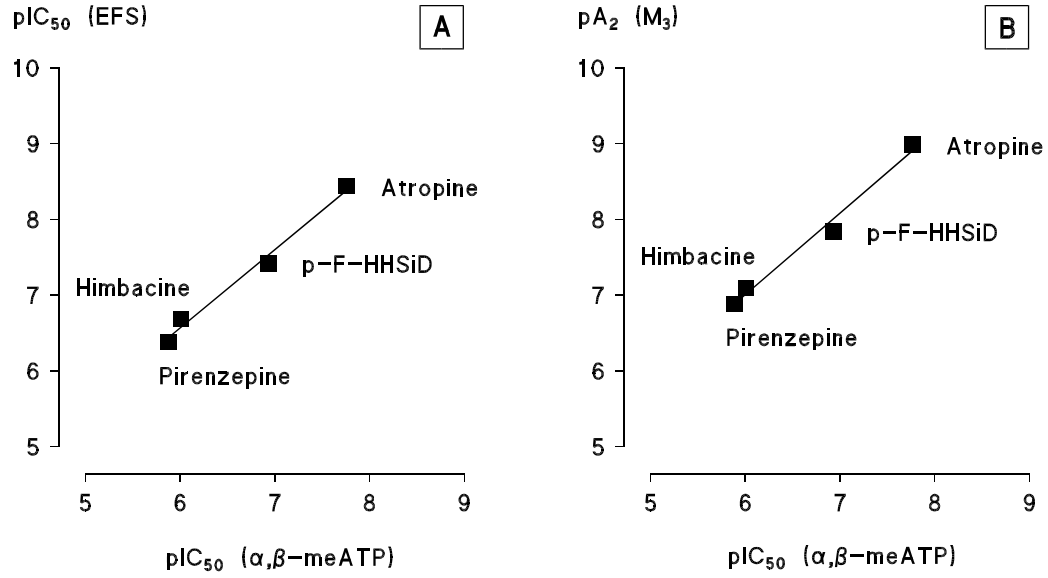


Fig. 4.20. *Correlation plots for the pIC_{50} values (using α,β-meATP as agonist) obtained with muscarinic receptor antagonists in GPI and (**A**) pIC_{50} values for these compounds towards neurogenic (EFS-induced) contractions or (**B**), respectively, affinity estimates (pA_2) towards M_3 receptors identified by classical Schild analysis. The correlation coefficients of 0.993 (A) and 0.992 (B) regard the best fitting line through the data points.*

4.2.2. Experiments at postjunctionally localised P2 receptors

4.2.2.1. Potency profile of P2 agonists

The three nucleotide agonists ADPßS (0.3 - 100 µM), α,β-meADP (0.3 - 100 µM) and 2-MeSATP (0.3 - 100 µM) were investigated for their contractile activity in the GPI in order to establish a rank order of potency (Fig. 4.21.).

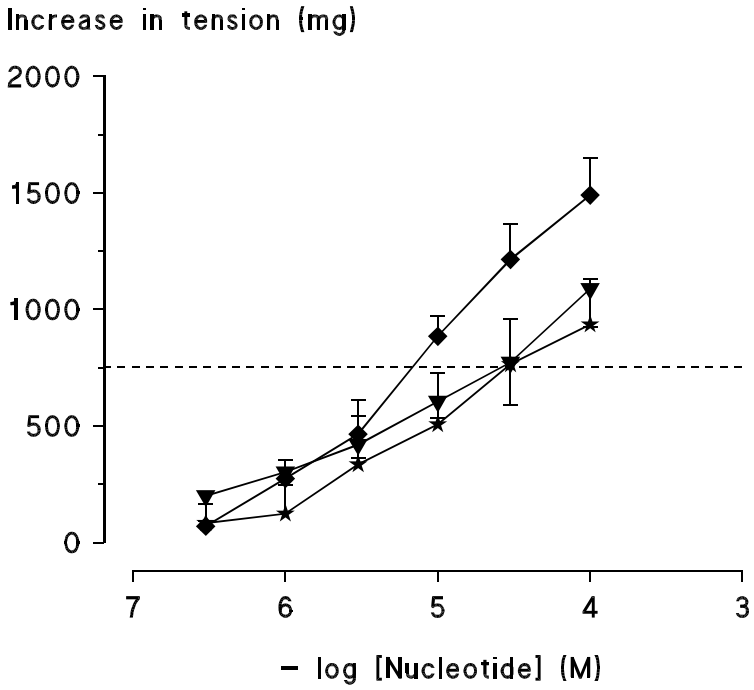


Fig. 4.21. *Concentration-response curves of nucleotides (◆ ADPßS; ▼ 2-MeSATP; ★ α,β-meADP) eliciting contractions in the GPI. Contractions were measured as increase in tension. Mean values (± S.E.M.) consist of 3 - 8 individual determinations. Error bars falling within the area covered by a symbol are not shown.*

The nucleotides produced biphasic contractile responses consisting of a phasic contraction, followed by a tonic response. In the present study, only the phasic contractions were examined (Fig. 4.21.). The following rank order of potency based on the generation of 750 mg tension (pEC_{750} value) was obtained (Tab. 4.4.): ADPßS > 2-MeSATP ≥ α,β-meADP.

Tab. 4.4. *Potency of the P2 agonists ADPßS, 2-MeSATP and α,β-meADP based on generation of 750 mg tension (pEC_{750} values ± S.E.M.) in GPI.*

ADPßS	2-MeSATP	α,β-meADP
5.18 ± 0.04	4.67 ± 0.03	4.46 ± 0.07
(n = 8)	(n = 4)	(n = 3)

Although a maximum response was not reached by any of the agonists, ADPßS clearly was the most efficacious compound and therefore used for the following experiments.

4.2.2.2. Effects of atropine, TTX and CCPA

Exogenously administered ADPßS (10 µM) produced contractions of GPI, which were partially reduced by TTX (1 µM; 32 ± 3 %), atropine (0.3 µM; 45 ± 1 %) or CCPA (1 µM; 24 ± 3 %; Fig. 4.22.).

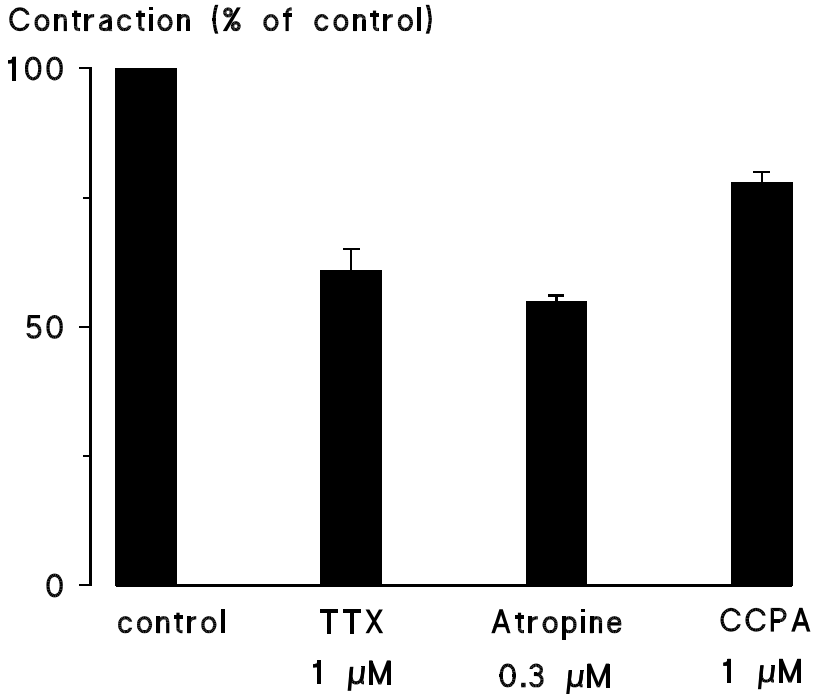


Fig. 4.22. *Influence of TTX (1 µM), atropine (0.3 µM) and CCPA (1 µM) on contractions of the GPI elicited by ADPßS (10 µM). Responses are expressed as percentage of control responses (1066 ± 112 mg tension generated) without TTX, atropine or CCPA (n = 3 - 4).*

Higher concentrations of atropine (1 and 3 µM; n = 4) did not enhance the inhibition to contractions produced by ADPßS.

Contractions produced by 2-MeSATP (10 µM) in GPI were also decreased either by TTX (1 µM; 49 ± 6 %; n = 3) or atropine (0.3 µM; 50 ± 3 %; n = 5). Similar

observations were made with 100 µM α,β-meADP, where contractions were depressed by 1 µM TTX (80 ± 8 %; n = 4) and 0.3 µM atropine (62 ± 7 %; n = 4). These results imply that ADPßS (as well as 2-MeSATP and α,β-meADP) acts at different P2 receptors located at two sites in GPI: (1) the neuronal α,β-meATP-sensitive receptor (α,β-meATP >> ADPßS), and (2) a postjunctional α,β-meATP-insensitive receptor (ADPßS >>> α,β-meATP). Thus, to isolate the stimulatory action of ADPßS at the postjunctional P2 receptors pharmacologically, atropine (0.3 µM) was present throughout the experiments with P2 antagonists.

4.2.2.3. Effects of N^G-nitro-L-arginine and hexamethonium on contractions elicited by ADPßS

In the following experiments the influence of N^G-nitro-L-arginine (L-NOARG) and hexamethonium on contractions elicited by ADPßS was investigated.
The nicotine receptor antagonist hexamethonium (30 and 100 µM) and the NO-synthase inhibitor L-NOARG (30 and 300 µM) failed to modify the effects of ADPßS, suggesting that nicotine receptors as well as NO may not be involved in the action of ADPßS.

4.2.2.4. Antagonism by suramin, PPADS and NF023

Single doses of ADPßS (0.3 - 300 µM; EC_{50} = 0.70 ± 0.04 µM; 1232 ± 59 mg tension generated in control experiments; n = 33) produced concentration-dependent contractions in GPI (Krebs 1.3 mM Ca^{2+}; containing 0.3 µM atropine) (Figs. 4.23. - 4.25.). No desensitisation to these responses could be observed by addition of single doses of 10 µM ADPßS given every minute without wash-out phase.

Suramin (10 - 100 μM; Fig. 4.23.) shifted the concentration-response curves of ADPßS in a dose-dependent manner to the right. The regression line of the corresponding Schild plot had a slope of 0.94 ± 0.05 (not significantly different from unity), and a pA_2 value of 5.68 ± 0.24 could be estimated.

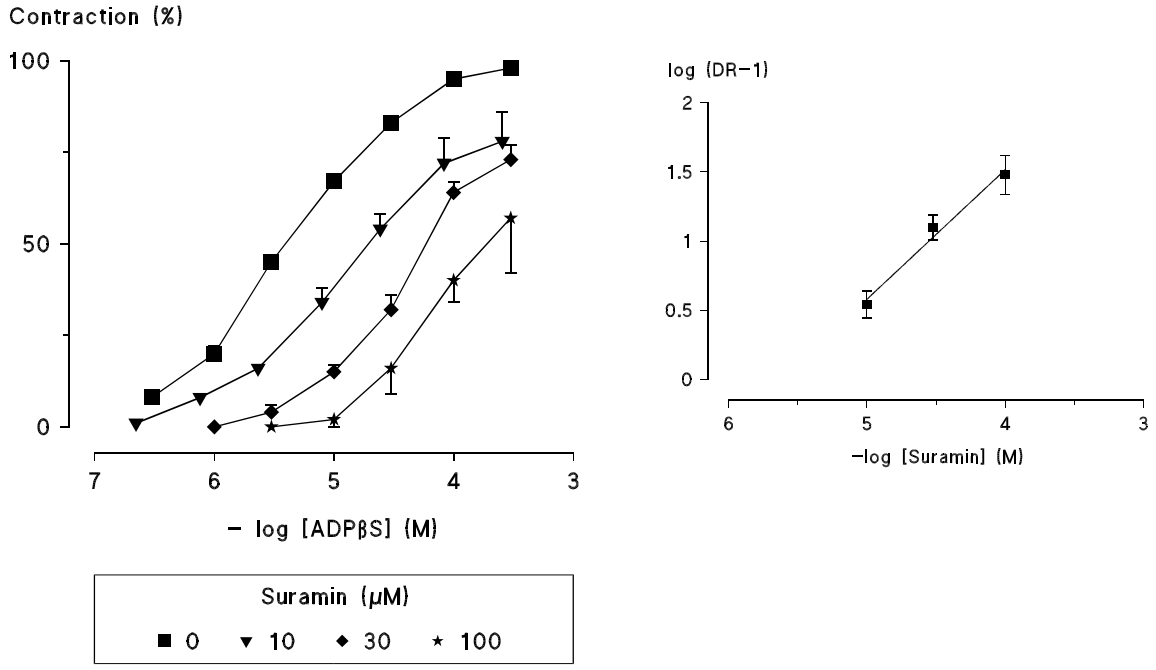


Fig. 4.23. *Concentration-response curves of ADPßS in the absence and in the presence of suramin in the GPI (left panel), and the corresponding Schild plot (right panel) with slope not significantly different from unity (P > 0.05). The graphs show mean data ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown.*

PPADS (1 - 10 µM; Fig. 4.24.) antagonised contractile responses to ADPßS in a pseudoirreversible manner. Analysis of these data by a double reciprocal plot yielded a pK_B estimate of 6.20 ± 0.05.

The effects of PPADS were reversed to the maximum of the control curve after a wash-out period of 15 - 30 min.

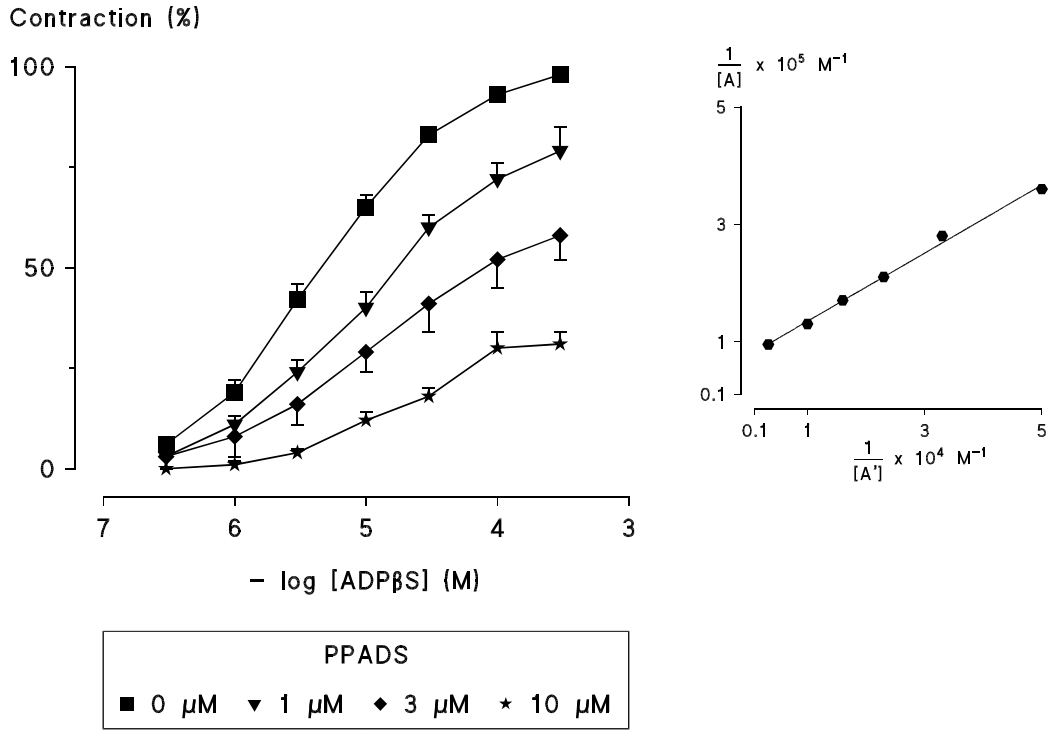


Fig. 4.24. *Effect of PPADS on the mechanical response of the GPI produced by ADPßS (left panel). The graphs show mean data ± S.E.M. (n = 4). Error bars falling within the area covered by a symbol are not shown. The right panel shows the double reciprocal plot: antagonism by PPADS of ADPßS-induced contractions of the GPI. [A] and [A'] denote the equieffective agonist concentrations in the absence and in the presence of PPADS (3 µM).*

NF023 (100 - 1000 µM; Fig. 4.25.) caused reversible and concentration-related antagonism of responses to ADPßS. The corresponding Schild plot was linear with a slope not significantly different from unity (1.01 ± 0.06), and a pA_2 value of 4.74 ± 0.03 could be determined.

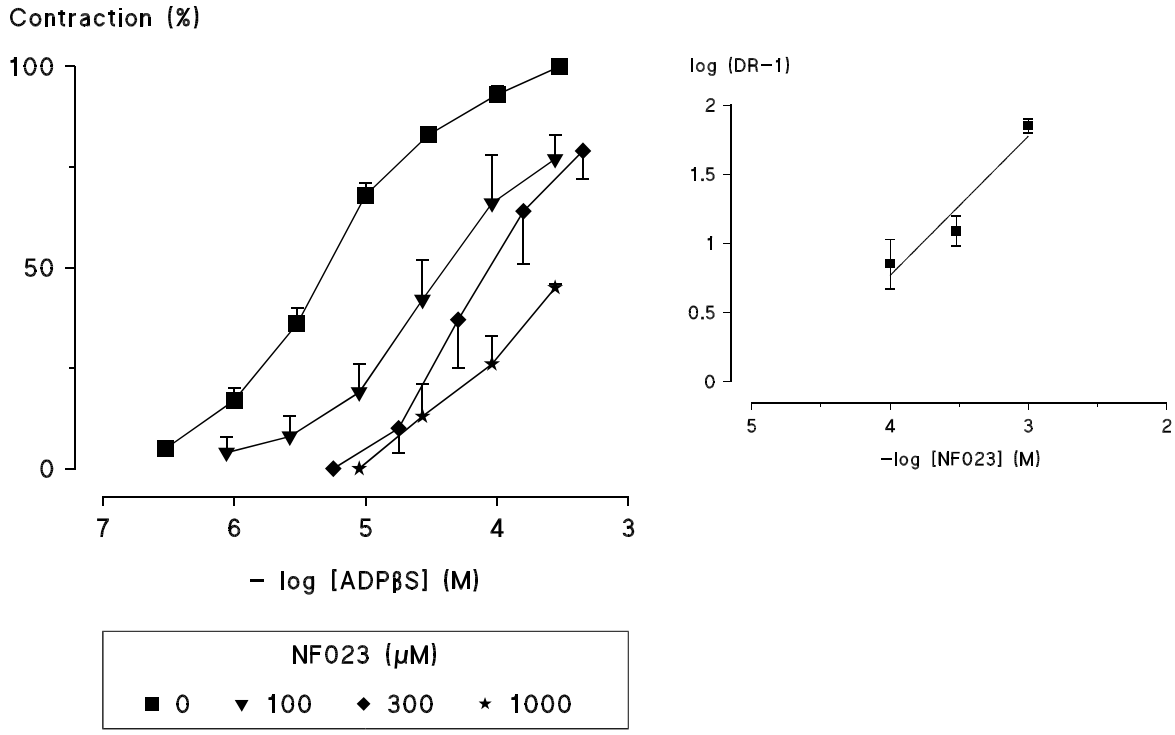


Fig. 4.25. *Concentration-response curves of ADPßS in the absence and in the presence of NF023 in the GPI (n = 3; left panel), and the corresponding Schild plot (right panel) with slope not significantly different from unity (P > 0.05). Error bars falling within the area covered by a symbol are not shown.*

4.2.2.5. Antagonism by NF279

The antagonism of NF279 (30 µM) to ADPβS was determined only for one concentration.

NF279 shifted the concentration-response curve of ADPβS to the right in a parallel fashion with a resulting pA_2 value of 5.11 ± 0.04 (n = 6; Fig. 4.26).

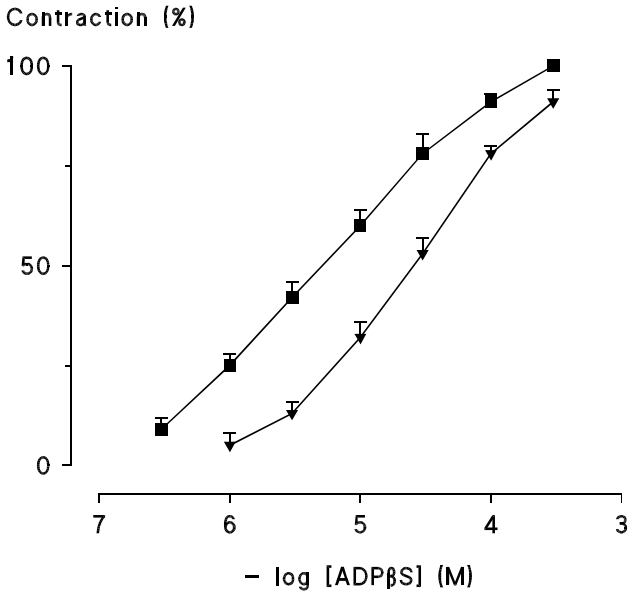


Fig. 4.26. *Concentration-response curves for the contractile responses to ADPßS in the absence (ν) and in the presence of 30 µM NF279 (τ) in GPI. Data are shown as means ± S.E.M. (n = 6). Error bars falling within the area covered by a symbol are not shown.*

4.2.2.6. Antagonism by A3P5P and MRS2179

Progress in the P2 receptor field has been slowed by the lack of availability of receptor subtype-selective antagonists. Thus, the observation by Boyer et al. (1996) that adenosine 3',5'-bisphosphate (A3P5P; Fig. 4.27.) acted as competitive antagonist with slight partial agonist activity at $P2Y_1$ receptors in turkey erythrocyte membranes was of considerable interest. The effect of this bisphosphate ATP analogue was selective for the PLC-coupled $P2Y_1$ receptor, since neither agonist nor antagonist activities were observed on recombinant $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.

In the present study, A3P5P (100 µM; Fig. 4.27.) shifted the concentration-response curve to ADPßS to the right in a roughly parallel manner with depression of the maximum of the control response curve. The resulting pA_2 value was 4.45 ± 0.16 (n = 6). No agonist activity of A3P5P itself could be observed.

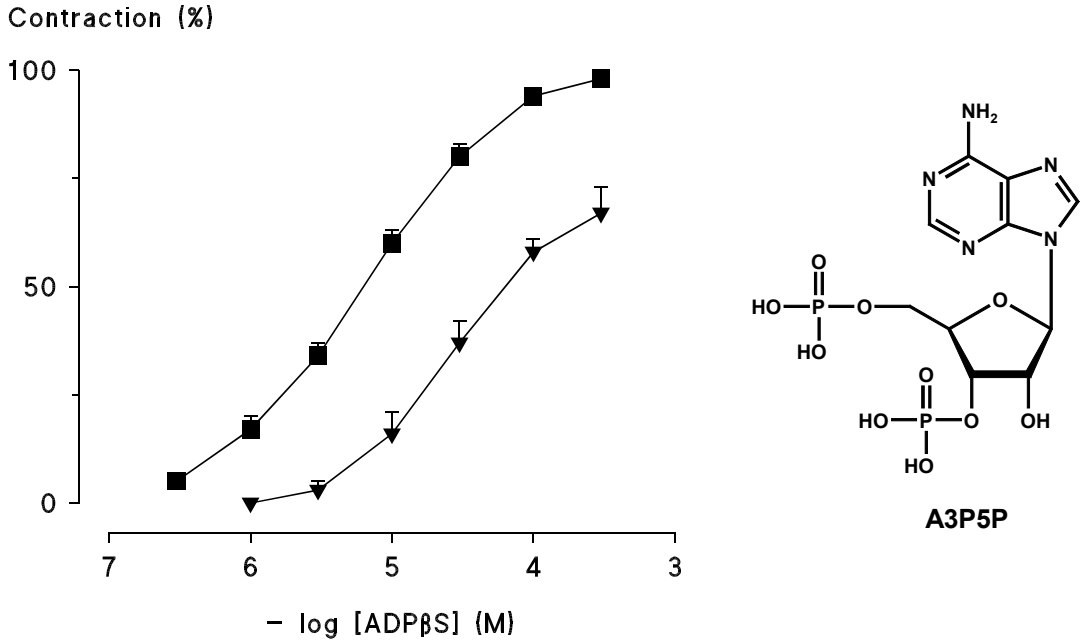


Fig. 4.27. *Concentration-response curves for the contractile responses to ADPßS in the absence (ν) and in the presence of 100 μM A3P5P (τ) in GPI. Data are shown as means ± S.E.M. (n = 6). Error bars falling within the area covered by a symbol are not shown.*

Another $P2Y_1$-selective antagonist, 2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate (MRS2179; Fig. 4.28.), was described by Camaioni et al. (1998). The potency of this antagonist was measured in turkey erythrocyte membranes with a K_i value of approximately 100 nM.

In this study, the antagonistic property of MRS2179 (0.1 - 30 μM; Fig. 4.28.) against 10 μM ADPβS was determined. In this way, a pIC_{50} value of 5.37 ± 0.04 (n = 4) could be estimated.

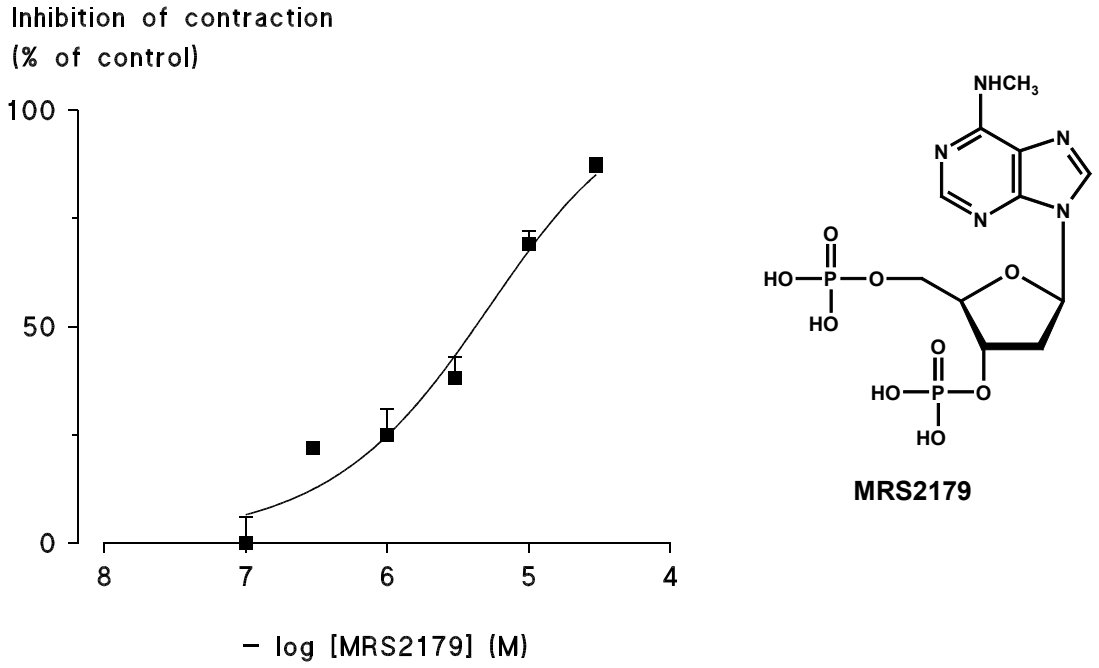


Fig. 4.28. *Inhibition curve of MRS2179 to 10 µM ADPßS in GPI. Data are means ± S.E.M. from 4 experiments. Error bars falling within the area covered by a symbol are not shown.*

4.3. Experiments with the longitudinal smooth muscle of the rat ileum

4.3.1. Experiments with P2 agonists

In a first set of experiments the four nucleotide agonists ADPßS, α,β-meADP, α,β-meATP and ATPγS were investigated for their contractile activity in the rat ileal longitudinal smooth muscle (RI) in a concentration range from 0.3 to 300 µM in order to establish a rank order of potency.

All agonists elicited concentration-dependent contractions of the RI. The highest efficacy was observed with the P2X-selective agonist α,β-meATP, which showed a relatively steep concentration-response curve. The curves for the other three nucleotides appeared to be parallel with ADPßS being more potent than α,β-meADP, which in turn was more potent than ATPγS (Fig. 4.29.).

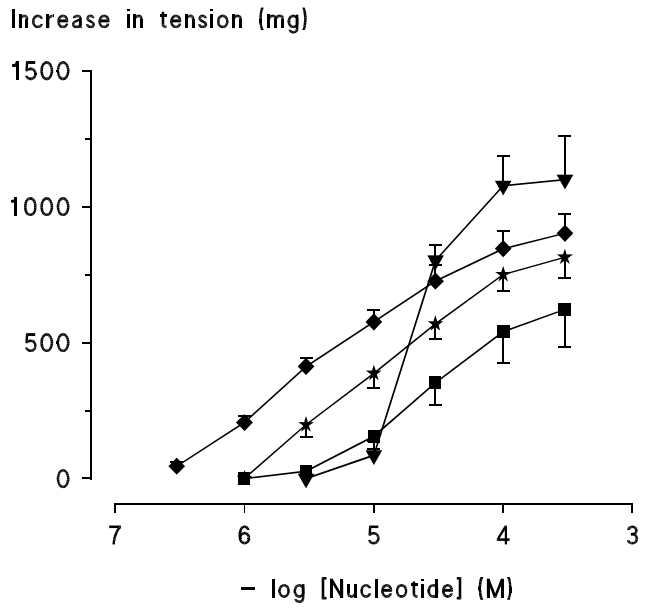


Fig. 4.29. *Concentration-response curves of nucleotides (◆ ADPßS; ▼ α,β-meATP; ★ α,β-meADP; ■ ATPγS) eliciting contractions in the RI. Contractions were measured as increase in tension. Mean values (± S.E.M.) consist of 4 - 14 individual determinations. Error bars falling within the area covered by a symbol are not shown.*

α,β-meATP: After cumulative addition (3 times) of 30 µM α,β-meATP every minute without washing-out period no desensitisation of the receptor could be noticed.

ADPßS: The contractile effect of ADPßS could not be increased neither through a priming with 100 µM ADPßS before starting the dose-response curve nor through extension of the time between the next addition of the agonist.
After cumulative addition (3 times) of 10 µM ADPßS every minute without washing-out period no desensitisation of the receptor could be noticed.

4.3.2. Effects of TTX and atropine on responses to α,β-meATP

TTX: Contractions elicited by 30 µM α,β-meATP were reduced by 1 µM TTX to 34 ± 2 % (n = 5; 961 ± 46 mg tension generated) (Fig. 4.30.).

Atropine: The contractile response to 100 µM α,β-meATP after addition of 0.3 µM atropine amounted to 44 ± 8 % (n = 4; 585 ± 133 mg tension generated) of control response without atropine (Fig. 4.30.).

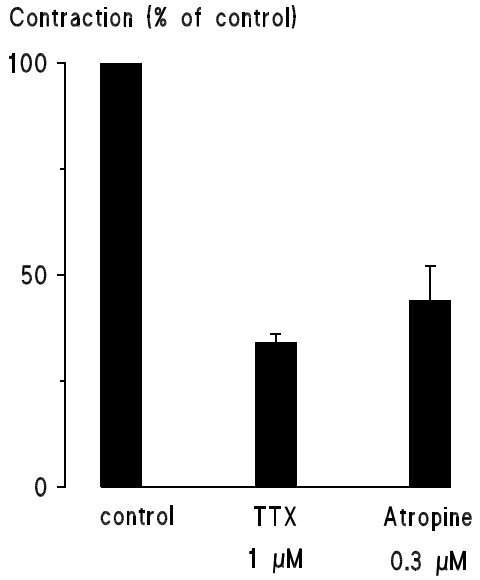


Fig. 4.30. *Influence of TTX (1 µM) and atropine (0.3 µM) on contractions elicited by α,β-meATP (30 µM in case of TTX or 100 µM in case of atropine) in RI. Responses are expressed as percentage of control responses without TTX or atropine (n = 4 - 5).*

4.3.3. Effects of TTX and atropine on responses to ADPßS

No influence of either 1 µM TTX (n = 6) or 0.3 µM atropine (n = 4) on contractions elicited by 10 µM ADPßS was observed, indicating that the agonist acts at P2 receptors, probably P2Y receptors, located on the postsynaptic site.

4.3.4. Effects of NF023, suramin and PPADS on responses to ADPßS

In the following experiments, ADPßS (0.3 - 300 µM) was used as agonist and the influence of NF023, suramin and PPADS was investigated.

ADPβS (EC_{50} = 5.10 ± 0.03 µM; 1550 ± 81 mg tension generated in control experiments; n = 36) produced concentration-dependent contractions in RI. The agonist was added in single doses every 15 min. Time controls revealed that the dose-response curves remained stable during the course of experiments. The three antagonists shifted the concentration-response curve of the agonist to the right in a roughly parallel fashion. However, they exhibited only a weak antagonism with pA_2 values of 4.34 ± 0.25 for NF023 (n = 3; Fig. 4.31.), 4.60 ± 0.10 for suramin (n = 3 - 4; Fig. 4.32.) and 4.79 ± 0.16 for PPADS (n = 3; Fig. 4.33.).

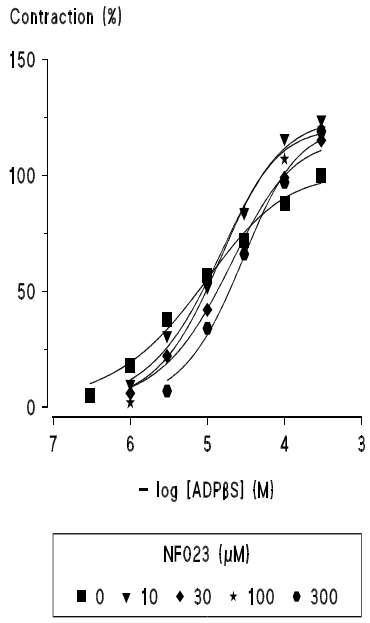


Fig. 4.31. *Concentration-response curves of ADPßS in the absence and in the presence of NF023 (10 - 300 µM) in RI. Data are shown as mean values of 3 experiments.*

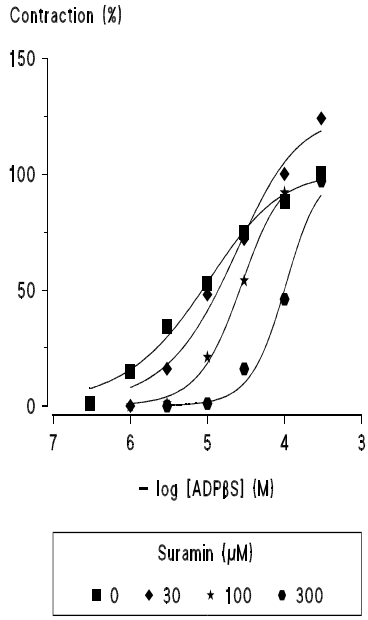


Fig. 4.32. *Concentration-response curves of ADPßS in the absence and in the presence of suramin (30 - 300 μM) in RI. Data are shown as mean values of 3-4 experiments.*

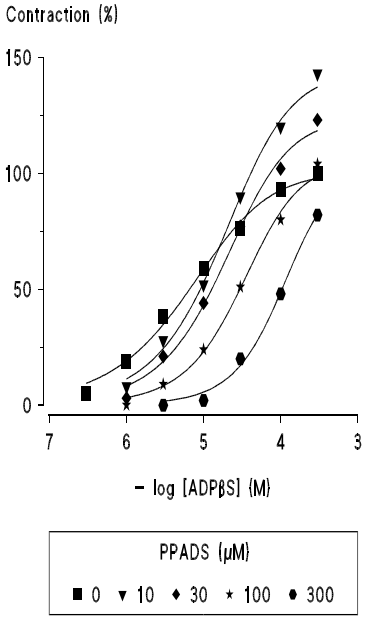


Fig. 4.33. *Concentration-response curves of ADPßS in the absence and in the presence of PPADS (10 - 300 μM) in RI. Data are shown as mean values of 3 experiments.*

Remarkably, the antagonist curves shown in Figs. 4.31. - 4.33. were always steeper than the corresponding control curve and in some cases the maximum of the antagonist curves was higher than that of the agonist control curve. The pA_2 values were calculated from the shift that was observed at 50 % of the respective control curve.

4.3.5. Effects of NF279 and A3P5P on responses to ADPßS

For these two selective antagonists, pIC_{50} values to 10 µM ADPßS were determined.

NF279, a wholly $P2X_1$-selective antagonist, showed only a weak antagonism against ADPβS in RI (Fig. 4.34.; pIC_{50} = 3.92 ± 0.04, n = 4).

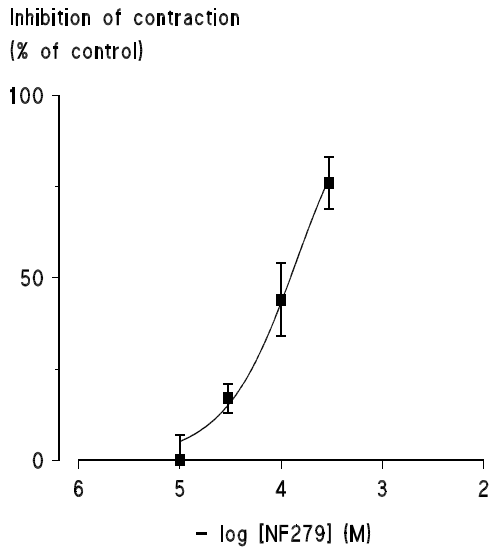


Fig. 4.34. *Inhibition curve of NF279 to 10 µM ADPßS in RI. Data are means ± S.E.M. from 3 - 4 experiments.*

A3P5P, described as a $P2Y_1$-selective antagonist, had no influence on contractions elicited by ADPßS (n = 4) up to a concentration of 100 µM.

5. DISCUSSION

5.1. Functional *in vitro* experiments with glycopyrronium bromide, its four stereoisomers and their corresponding tertiary analogues at muscarinic receptors

5.1.1. Affinity profile of glycopyrronium bromide

As mentioned before, glycopyrronium bromide is a synthetic quaternary ammonium compound with two asymmetrical carbon atoms. One of the racemates (R/S-S/R) was shown to exhibit antimuscarinic properties in functional as well as in radioligand binding experiments (Lau and Szilagyi, 1992; Gomez et al., 1995). Basic findings about the affinity and subtype selectivity of glycopyrronium bromide at muscarinic M_1 - M_3 receptors were demonstrated by Fuder and Meincke (1993). They described that this compound blocks effects elicited by muscarinic agonists at rabbit vas deferens (M_1), paced rat left atria (M_2) and guinea-pig ileum (M_3) with very high potency (chapter 2.1.). Glycopyrrolate was about 10- or 100-fold more potent at the M_1 receptor than in blocking M_3 or M_2 receptors, respectively.

In the present study, the affinity profile of glycopyrronium bromide was determined at RVD (M_1), GPA (M_2) and GPI (M_3) as reference for subsequent experiments. The affinity of the parent compound at M_1 and M_2 receptors pointed to a slight preference for the M_1 receptor subtype (13-fold over M_2; Fig. 4.4.). At the M_3 receptor subtype a pIC_{50} value was determined because of the very slow dissociation of the antagonist from the receptor observed in the functional experiments (chapter 4.1.2.). So, a direct comparison with pA_2 values was not possible.
The results obtained in radioligand binding studies with recombinant human muscarinic receptor subtypes stably expressed in CHO-K1 cells showed the following antagonistic profile: $M_3 = M_4 = M_1 \geq M_2$ with K_i values in the lower nanomolar range (Kreutzmann, personal communication). The pK_i value at the M_1 receptor was only 4-fold lower than the functionally determined pA_2 value, and in case of the M_3 affinity there was a difference by a factor of 6. Nevertheless, if one can speculate that the pIC_{50} at the M_3 receptor of GPI is lower than the respective

pA_2 value, the same rank order of antagonist potency could be assumed for functionally obtained data.

5.1.2. Affinity profile of the four stereoisomers of glycopyrronium bromide

Since glycopyrronium bromide contains two chiral centres in its molecule the compound consists of the following four stereoisomers: **1** (3S/2'S), **2** (3S/2'R), **3** (3R/2'S), and **4** (3R/2'R) (Fig. 4.5.).

First of all, the data obtained in functional experiments (Tab. 4.1.) were confirmed in radioligand binding studies at recombinant M_1, M_3 and M_4 receptors stably expressed in CHO cells as well as at native M_2 receptors present in rat heart (Fig. 5.1.). [^{3}H]N-methylscopolamine was used as radioligand and its binding to muscarinic receptors was competitively inhibited by compounds **1 - 4** (Czeche et al., 1997).

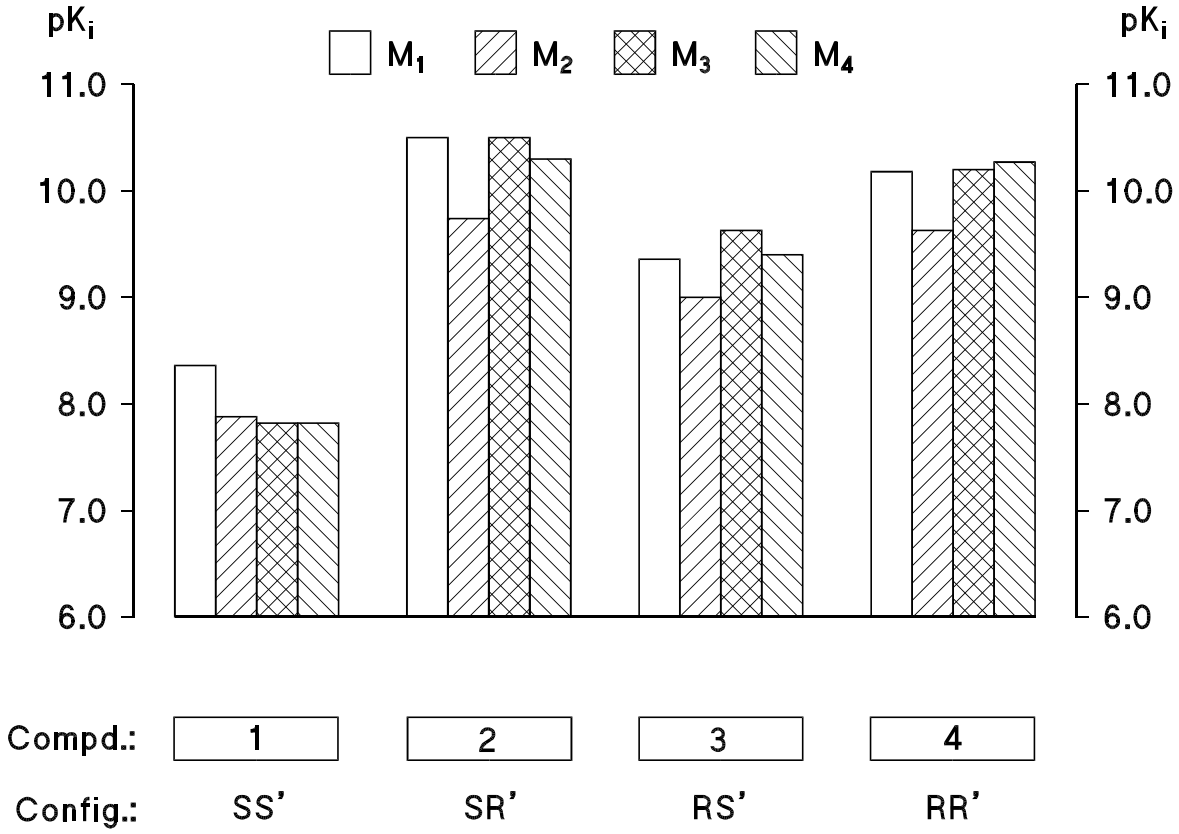


Fig. 5.1. *(legend, see page 100)*

Fig. 5.1. *Antagonist affinities of the quaternary stereoisomers of glycopyrronium bromide determined in radioligand binding studies at recombinant M_1, M_3 and M_4 receptors stably expressed in CHO cells and at native M_2 receptors present in rat heart.*

The affinities of the quaternary compounds **1 - 4** determined in functional experiments at M_1 and M_2 receptor subtypes were in good agreement with data examined in radioligand binding studies (Fig. 5.2.; M_1: slope = 0.94, r = 0.99; M_2: slope = 1.19, r = 0.99). Although the regression lines at the M_1 and M_2 receptor subtypes have not been represented for reasons of clearness, the location of the data points around the solid line (y = x) proves the excellent correlation. Data at M_3 receptor (pK_i vs. pIC_{50}) are best correlated by the dotted line (slope = 1.08, r = 0.99). A comparison of pK_i values obtained at M_3 receptors with pIC_{50} values obtained in GPI showed that the pIC_{50} values are consistently lower (up to 32-fold) than the pK_i values due to the experimental design using endogenous acetylcholine as agonist in functional studies.

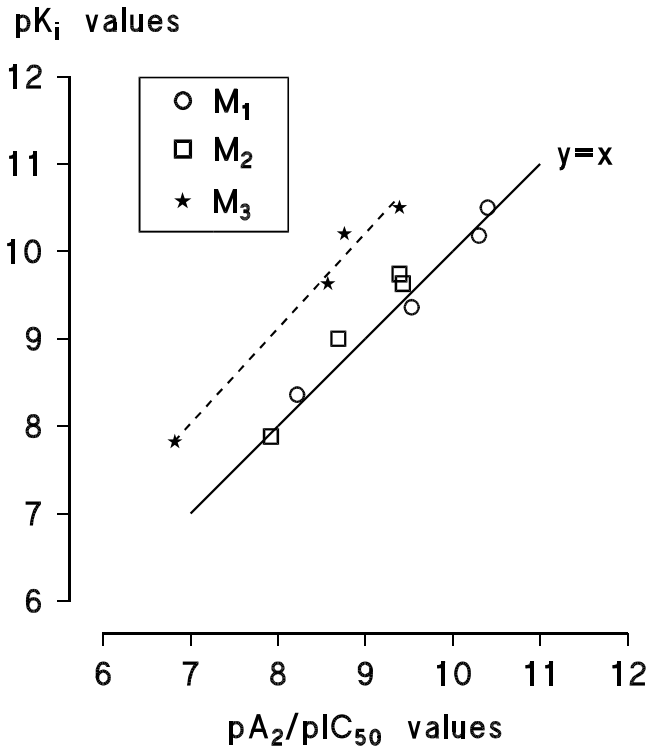


Fig. 5.2. *Comparison of pK_i and pA_2/pIC_{50} values for the quaternary isomers of glycopyrronium bromide obtained in binding and functional studies, respectively, at muscarinic receptor subtypes. The solid line represents the 1:1 relationship.*

Taken together, it can be concluded that the quaternary compounds **1 - 4** behaved as surmountable competitive antagonists in functional studies at muscarinic receptors in RVD (M_1 receptors) and GPA (M_2 receptors) as well as in radioligand competition experiments at M_1 - M_4 receptors. Because of the very slow dissociation from the receptor, which could be observed, but unfortunately has still not been quantified, during the experimental study at M_3 receptor subtype in GPI, it was impossible to determine pA_2 values. However, because of the long time needed to get an equilibrium at the M_3 receptor in the binding assay too, Waelbroeck has determined the dissociation half-lifes ($t_{1/2}$) of the four stereoisomers at this receptor subtype (personal communication). The following $t_{1/2}$ (min) could be observed: 1 [**1** (3S/2'S)] << 70 [**2** (3S/2'R)] < 90 [**3** (3R/2'S)] < 120 [**4** (3R/2'R)]. Consequently, the determination of pIC_{50} values in the electrical stimulated GPI was used as an alternative method. The benefit arised from this design was the evaluation of the antagonistic properties of the compounds, but disadvantageous was that the release of endogenous ACh could not be quantified. If compared the rank order of $t_{1/2}$ with the rank order of the antagonistic potency of the compounds, no direct correlation could be estimated.

Taken the data from binding studies into account, the quaternary stereoisomers were almost non-selective at the four muscarinic receptor subtypes or had only a slight preference (up to 6-fold for compound **2**) for M_1, M_3 and M_4 over M_2 receptors.

5.1.3. Affinity profile of the tertiary stereoisomeric analogues of glycopyrronium bromide

In analogy to the quaternary stereoisomers, the tertiary derivatives of glycopyrronium bromide exist in the corresponding steric forms: **5** (3S/2'S), **6** (3S/2'R), **7** (3R/2'S), and **8** (3R/2'R).

A comparison with data obtained in the binding assays has been undertaken as well. The data determined in radioligand competition studies (Czeche et al., 1997; Fig. 5.3.) at M_1 - M_4 receptor subtypes showed that all tertiary amines exhibit a slight selectivity (up to 13-fold for compound **6**) for M_1, M_3 and M_4 over M_2 receptors.

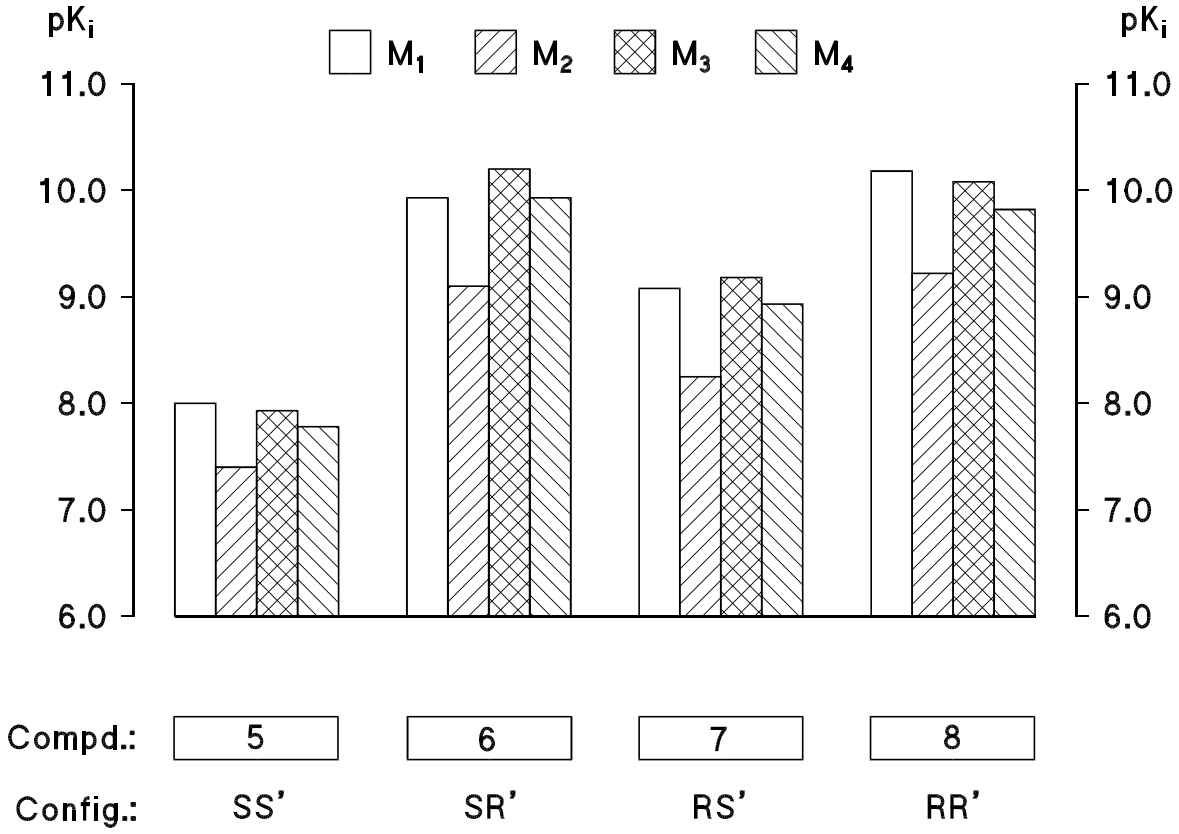


Fig. 5.3. *Antagonist affinities of the tertiary stereoisomeric analogues of glycopyrronium bromide determined in radioligand binding studies at recombinant M_1, M_3 and M_4 receptors stably expressed in CHO cells and at native M_2 receptors present in rat heart.*

There is also an excellent correlation between functional and binding data at M_1 and M_2 receptors (Fig. 5.4.: M_1: slope = 0.90, r = 0.99; M_2: slope = 0.99, r = 0.97), which is proved by the small straggling of data points around the solid line (y = x). Data at M_3 receptor (pK_i vs. pIC_{50}) are best correlated by the dotted line (slope = 0.88, r = 0.96). As already mentioned for the quaternary derivatives, the regression line of the pIC_{50} values was displaced to the left in a roughly parallel fashion. The factor of the shift was up to 27 due to the experimental design considering the use of endogenous acetylcholine of unknown concentration.

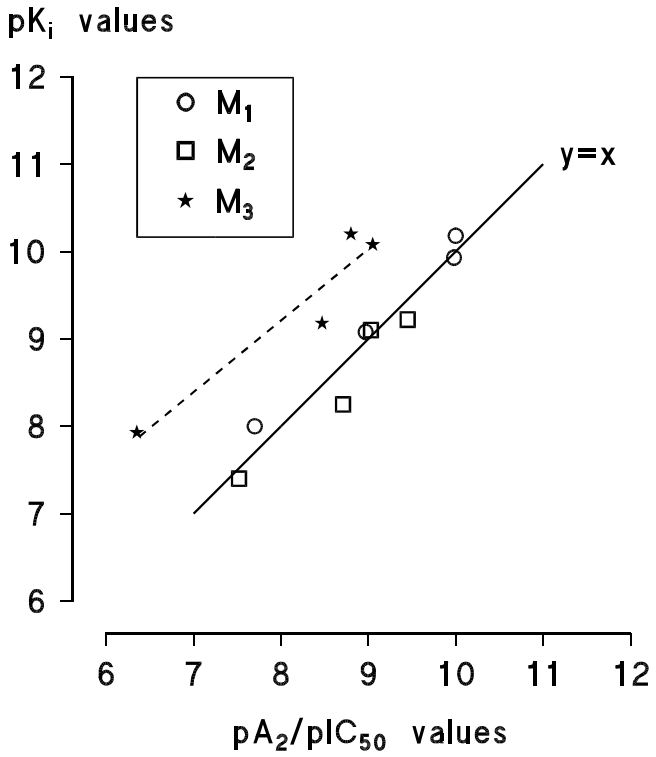


Fig. 5.4. *Comparison of pK_i and pA_2/pIC_{50} values for the tertiary stereoisomeric analogues of glycopyrronium bromide obtained in binding and functional studies, respectively, at muscarinic receptor subtypes. The solid line represents the 1:1 relationship.*

As their quaternary analogues, compounds **5 - 8** behaved as surmountable competitive antagonists in functional studies at muscarinic receptors in RVD (M_1 receptors) and GPA (M_2 receptors), and in radioligand binding experiments at M_1 - M_4 receptors. The problems occurring with inclusion of pIC_{50} values in the comparison are already discussed in chapter 5.1.2. Again, dissociation half-lifes were determined only at the M_3 receptor subtype in radioligand binding studies (Waelbroeck, personal communication), and were not quantified in the respective functional model at GPI. The following rank order of $t_{1/2}$ (min) resulted: 20 [**5** (3S/2'S)] < 50 [**6** (3S/2'R)] = 50 [**7** (3R/2'S)] < 60 [**8** (3R/2'R)], and compared with the rank order of $t_{1/2}$ values of the quaternary isomers, the differences between the stereoisomers were less.

5.2. Stereoselectivity and eudismic analysis of the isomers of glycopyrronium bromide

Differences between the biological activity of optical isomers have been of continuing interest. The study of chiral compounds can help to understand how chemical modifications could change the binding affinity for different receptor subtypes. In this context, stereoselectivity has been proven to be a useful tool for structure-activity relationship studies and in understanding how the structure of a given molecule contributes to the pharmacological receptor-mediated effects. The analysis of dependence of stereoselectivity on affinity was considerably simplified by developing a method called eudismic analysis (Lehmann et al., 1976; Testa, 1990; Casy, 1993). To describe this model the following terminology was introduced: In a series of congeneric chiral pairs the more potent isomer in each is designated eutomer and the less potent distomer regardless of their absolute configuration (Lehmann, 1986; Testa, 1990). For any given pair the potency/affinity ratio is called eudismic ratio (= stereoselectivity), and its logarithm, eudismic index.

Since glycopyrronium bromide possesses two centres of chirality the stereoisomers can be separated into enantiomers and epimers. Enantiomers are characterised by the fact that they have identical physical and chemical properties in achiral environments. Epimers are so-called diastereomers in which only one of the two (or more) chiral centres is inverted (Lehmann, 1990). Contrary to the enantiomers, there is no mirror-image relationship and therefore they differ in their physico-chemical properties. Thus, enantiomers of chiral drugs and chiral diastereomers are equal in that they are composed of the same number and kinds of atoms bonded in the same fashion, but differ in the way in which the atoms within the molecule are oriented in space (Lambrecht and Mutschler, 1986). In case of glycopyrronium bromide and its tertiary analogues diastereomers with the same configuration in the **pyrrolidinyl part** [(**3R**/2'R) vs. (**3R**/2'S) and (**3S**/2'R) vs. (**3S**/2'S)] as well as diastereomers with the same configuration in the **acid part** [(3S/**2'R**) vs. (3R/**2'R**) and (3R/**2'S**) vs. (3S/**2'S**)] exist besides the enantiomeric pairs.

The influence of the configuration is portrayed as stereoselectivity ratio for the enantiomers (enantioselectivity) in Figs. 5.5. (quaternary compounds) and 5.7.

(tertiary compounds) and for the diastereomers (diastereoselectivity) in Figs. 5.6. (quaternary compounds) and 5.8. (tertiary compounds).

It is noteworthy that meanwhile the quaternary and tertiary stereoisomers of glycopyrronium bromide have been synthesised in an alternative way (Walter, personal communication) and furthermore, binding data have been determined in our laboratory, with CHO cells stably expressing muscarinic M_1 to M_5 receptor subtypes, by Kreutzmann (personal communication). The data obtained with these 'new' isomers are in good agreement with data using the 'older' isomers presented here (see chapter 4.1.3., Tab. 4.1. and chapter 4.1.4., Tab. 4.2. as well as chapter 5.1.2., Fig. 5.1. and chapter 5.1.3., Fig. 5.3.). An exception has been found for the quaternary compound **3** (3R/2'S) and in analogy for the corresponding tertiary compound **7**, with (R)-configuration in the pyrrolidinyl part and (S)-configuration in the acid part of the molecule, too. These differences are a result of impurity of these compounds with the respective (3R/2'R) isomer. And, as the (3R/2'R)-configured substances exhibited a very high antagonistic potency at the muscarinic receptor subtypes the potency of the (3R/2'S)-configured substances was markedly increased. In consequence, for the interpretation of the eudismic ratios the binding data from Kreutzmann for compounds **3** (M_1 = 8.10 ± 0.13; M_2 = 8.11 ± 0.05; M_3 = 7.94 ± 0.06) and **7** (M_1 = 8.22 ± 0.10; M_2 = 7.97 ± 0.05; M_3 = 8.01 ± 0.12) were included. The stereochemical purity of both compounds has been checked by analytical methods (Lambrecht, personal communication).

Quaternary compounds

For almost all quaternary compounds the stereoselectivity was least pronounced at M_2, middle at M_1 and highest at M_3 receptors. The low ratios at M_2 receptors (24 to 72) go in keeping with the lower affinities found for the respective eutomers in binding studies (Kreutzmann, personal communication) as well as the higher M_1 (70 to 74) and M_3 (87 to 110) ratios in nearly the same range correspond well with the compounds' preference for these subtypes.

Exceptions are the extremely lower selectivity ratios of 1 and 2 at M_1 to M_3 subtypes for the diastereomers with the same configuration, either (R) or (S), in the glycolic acid part of the molecule (Fig. 5.6., right panel). As these low ratios are a result of similar affinities of the respective eutomers vs. distomers, one can postulate, that the steric arrangement of the acid part is of paramount importance. On the one hand, when it is (R)-configured, high affinities were obtained, on the other hand, when it was (S)-configured the affinities were likewise lower at the

individual muscarinic receptor subtypes. Thus, the influence of the steric alignment in the pyrrolidinyl part was of minor importance.

However, regarding the individual muscarinic receptor subtypes no stringent configurational demands could be concluded for one subtype, although quantitative differences exist. Therefore, in case of the quaternary isomers of glycopyrronium bromide and its tertiary analogues, which are discussed below, the stereoselectivity ratios failed to be helpful as an additional parameter to characterise muscarinic receptor subtypes (Waelbroeck et al., 1991, 1996).

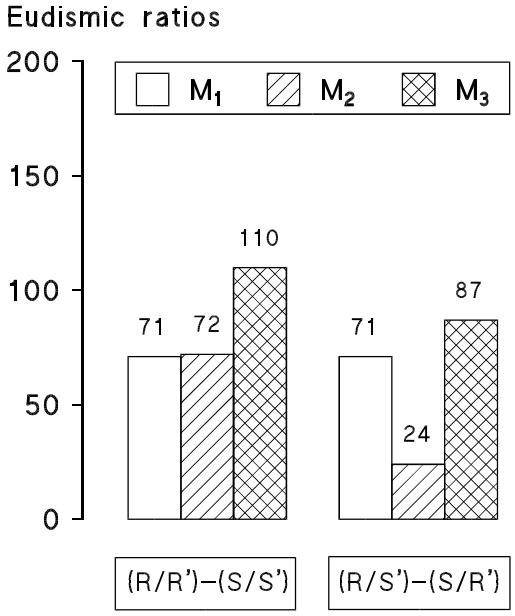


Fig. 5.5. *Eudismic ratios for the quaternary enantiomers:* ***4*** *(3R/2'R) vs.* ***1*** *(3S/2'S) and* ***3*** *(3R/2'S) vs.* ***2*** *(3S/2'R) at the muscarinic receptor subtypes in RVD, GPA and GPI.*

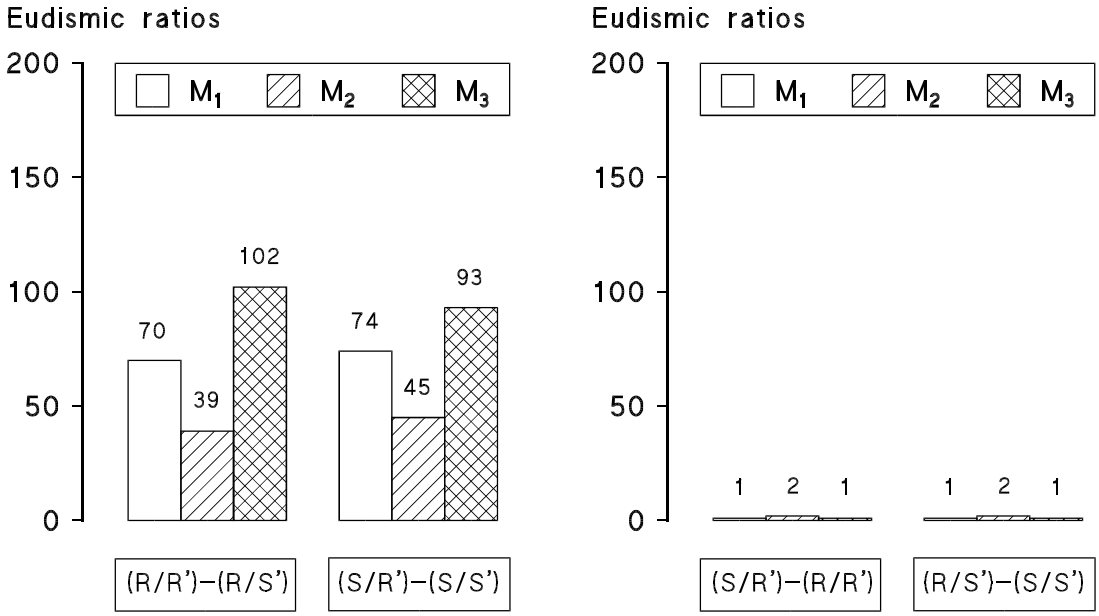


Fig. 5.6. *Eudismic ratios for the quaternary diastereomers with the same configuration in the pyrrolidinyl part,* ***4*** *(3R/2'R) vs.* ***3*** *(3R/2'S) and* ***2*** *(3S/2'R) vs.* ***1*** *(3S/2'S) (left panel), and with the same configuration in the acid part,* ***2*** *(3S/2'R) vs.* ***4*** *(3R/2'R) and* ***3*** *(3R/2'S) vs.* ***1*** *(3S/2'S) (right panel), respectively, at native or recombinant muscarinic receptor subtypes M_1 - M_3.*

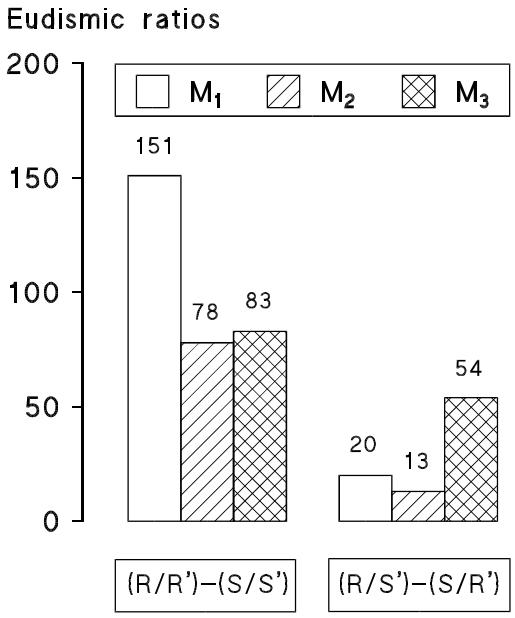


Fig. 5.7. *Eudismic ratios for the tertiary enantiomers:* ***8*** *(3R/2'R) vs.* ***5*** *(3S/2'S) and* ***7*** *(3R/2'S) vs.* ***6*** *(3S/2'R) at the muscarinic receptor subtypes in RVD, GPA and GPI.*

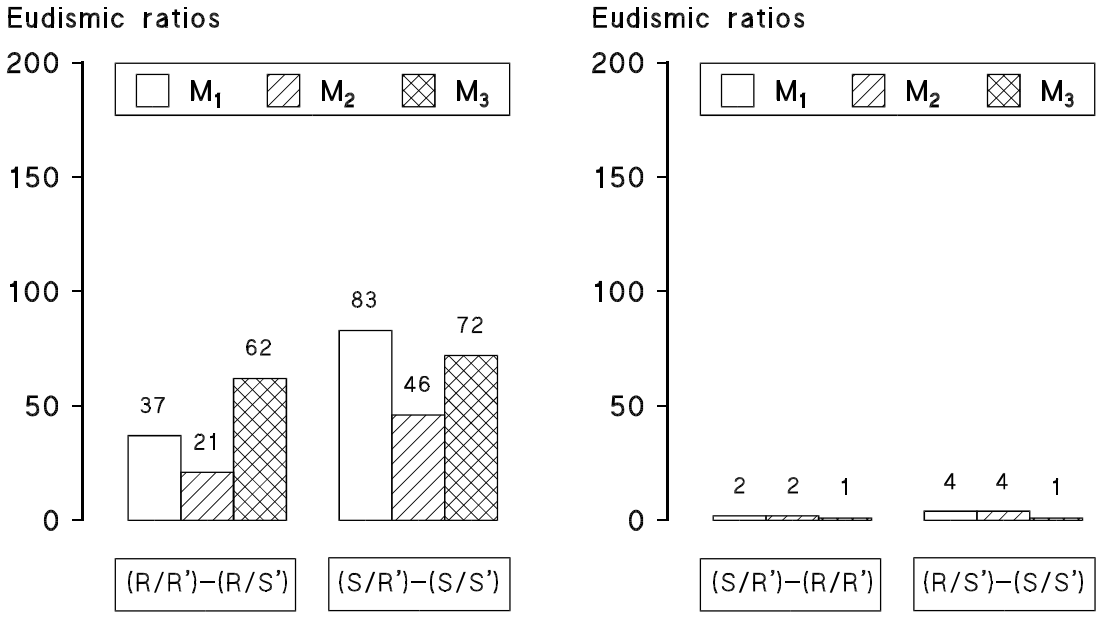


Fig. 5.8. *Eudismic ratios for the tertiary diastereomers with the same configuration in the pyrrolidinyl part,* ***8*** *(3R/2'R) vs.* ***7*** *(3R/2'S) and* ***6*** *(3S/2'R) vs.* ***5*** *(3S/2'S) (left panel), and with the same configuration in the acid part,* ***6*** *(3S/2'R) vs.* ***8*** *(3R/2'R) and* ***7*** *(3R/2'S) vs.* ***5*** *(3S/2'S) (right panel), respectively, at native or recombinant muscarinic receptor subtypes M_1 - M_3.*

Tertiary compounds

The calculated stereoselectivity ratios of the tertiary stereoisomers show similarities to that of the quaternary compounds, with a wide range of differences in affinity for the individual isomers at the receptor subtypes. The lowest ratios were found for the M_2 subtype (13 to 78) that is in a good agreement with the fact that almost all compounds were least potent at this receptor in the binding assay (Kreutzmann, personal communication). A selectivity or preference for either the M_1 or M_3 subtype could not been noted, so relatively wide ranges (20 to 151 for M_1 and 54 to 83 for M_3) resulted.

In analogy to the quaternary isomers, clearly lower eudismic ratios (1 to 4) were observed at M_1 - M_3 receptors for the diastereoisomeric pairs with the same configuration in the acid part. Thus, also within the series of tertiary compounds the configuration of the acid part is of greatest relevance.

As far as the absolute configuration of the quaternary stereoisomers of glycopyrronium bromide and their tertiary analogues are concerned, the rank order of affinity for M_1 - M_4 receptors is (calculated from binding and functional data): [RR'] ≅ (1- to 2-fold) [SR'] > (13- to 87-fold) [RS'] ≅ (1- to 4-fold) [SS']. This implies, as previously mentioned, that the steric arrangement in the glycolic acid centre of the molecules, (R)-configuration, is of paramount importance for high affinity. In the case of compounds with (S)-configuration in the acid part, affinity for muscarinic receptors is also governed by the configuration in the pyrrolidinyl moiety with (R) > (S). Similar results were reported by Noronha-Blob et al. (1992) with the four optical isomers of 3-quinuclidinyl atrolactate (QNA). The rank order of potency of the QNA analogues was the same at M_1, M_2 and M_3 receptors: (RR) > (RS) > (SR) > (SS) with (RR)-QNA being 182 to 308-fold more potent than (SS)-QNA. These findings suggest that (R)-isomers conform more stringently to the stereochemical demands imposed by muscarinic receptor subtypes than do (S)-isomers. However, comparing a series of structurally non-related chiral muscarinic antagonists the priority rules of the Cahn-Ingold-Prelog nomenclature has to be taken into account (Waelbroeck et al., 1996).

Related to the structure of the quaternary stereoisomers of glycopyrronium bromide and their tertiary analogues are the enantiomers of 3-benziloyloxy-N-methylpyrrolidine and their methiodides that have been investigated by Wehrle (1997). The exchange glycolic acid/ benzilic acid resulted in a loss of the chiral carbon atom in the acid part of the molecule with the consequence that only

enantiomers exist with either (R)- or (S)-configuration in the pyrrolidinyl part. As determined by Wehrle, the quaternary enantiomers of the benzilate analogues of glycopyrronium bromide displayed higher affinities at the muscarinic receptors than the corresponding derivatives. And also contrary to the results of the present study was that these compounds showed no stereoselectivity. And although the affinities for muscarinic receptors were very high, up to the subnanomolar range, for the quaternary (R)-configured benzilate, a really subtype-selectivity as well as kinetic-selectivity could not be observed. However, a slight preference for muscarinic M_1 and M_3 receptor subtypes (up to 4-fold) over M_2 has been found.

5.3. Therapeutical implications of the isomers of glycopyrronium bromide

Parasympathetic nerves provide the dominant neuronal bronchoconstrictor control of human airway smooth muscles. Cholinergic neuronal tone is the major reversible component in COPD and, through the circadian variation of vagal tone, also contributes to nocturnal asthma. The duration of action of the present available antimuscarinic drugs as well as the unwanted side effects are insufficient to provide convenient maintenance therapy for the patients. Furthermore it was shown that prejunctional muscarinic M_2 receptors on postganglionic cholinergic nerves in airways inhibit ACh release. Non-selective antagonists, such as ipratropium bromide, therefore increase the release of ACh via blockade of M_2 receptors (Barnes, 2000). Consequently, there is a clinical need to develop more selective antimuscarinic agents that have a longer duration of action than present drugs.

In radioligand binding studies with CHO-K1 cells, the (3R/2'R)-configured quaternary isomer of glycopyrronium bromide showed at the M_3 receptor a dissociation half-live of 120 min (Waelbroeck, personal communication). The compound exhibits in functional experiments at muscarinic receptors pA_2 values of 10.30 (M_1; RVD), 9.43 (M_2; GPA) and a pIC_{50} value of 8.76 (M_3; GPI) and in the binding assay pK_i values of 10.18 (M_1), 9.63 (M_2), 10.20 (M_3) and 10.27 (M_4). Thus, in comparison with the (SS')-, (SR')- and (RS')-configured quaternary isomers this compound was potent at the M_3 receptor and had a long duration of action. These results suggest a 'kinetic receptor subtype selectivity' for this stereoisomer, although the kinetic parameters at M_1, M_2 and M_4 receptor have yet to be quantified.

Similar results were reported by Disse et al. (1993) with Ba 679 BR (Fig. 2.1.). It has been demonstrated that Ba 679 BR binds with high affinity but non-selectively to cloned human M_1 - M_3 receptors and dissociates more rapidly from M_2 ($t_{1/2}$ = 3.6 h) than from M_1 ($t_{1/2}$ = 14.6 h) and M_3 ($t_{1/2}$ = 34.7 h) receptors, thus achieving subtype selectivity through a kinetic mechanism. Studies by Takahashi et al. (1994) at human bronchi confirmed the high potency (IC_{50} = 0.24 nM), slow onset (43.5 min) and long duration of action (> 300 min) of Ba 679 BR, suggesting that it may be a useful drug to provide convenient therapy for patients with COPD.

Johnson et al. (1984) reported on the effect of glycopyrrolate, the mixture of (SR')- and (RS')-configured stereoisomers, in asthma precipitated by exercise and cold air inhalation. Glycopyrrolate was as effective as atropine in producing brochodilation in this *in vivo* study. However, its long duration of action and the few side effects with inhalation may enhance its clinical utility. Walker et al. (1987) supported these results by studying the effectiveness, optimal dose and duration of action of glycopyrrolate in patients with nonexercise-asthma. In view of these findings it can be expected that the quaternary (RR')-configured isomer of glycopyrronium bromide may be a promising new drug for patients with COPD.

5.4. P2 receptor heterogeneity in the guinea-pig ileal longitudinal smooth muscle preparation

5.4.1. Experiments at neuronal P2 receptors

5.4.1.1. Preliminary experiments with α,β-meATP

In the present study, P2 receptors expressed in myenteric neurons of the GPI were stimulated with single doses of the stable ATP analogue α,β-meATP, a putative P2X-selective agonist (Lambrecht, 2000). These contractions were biphasic, consisting of a rapid, i.e. phasic, response followed by a more sustained, i.e. tonic, contraction. To get comparable results in the following experiments with α,β-meATP only the phasic, rapidly desensitising contractions were evaluated.

To examine, whether the response to α,β-meATP is based exclusively on P2X-like receptors, mediating ACh release, CCPA, DPCPX, and naloxone were tested in this model. The A_1-selective agonist CCPA (0.001 - 1 µM, Fig. 4.12.) concentration-dependently reduced the contractions elicited by α,β-meATP (10 µM), indicating the existence of prejunctional A_1 receptors in the GPI. Addition of the A_1-selective receptor antagonist DPCPX (0.03 - 0.3 µM) has no effect on the contractile responses evoked by α,β-meATP, indicating that there was no endogenous tone at the prejunctional A_1 receptors under the present experimental conditions. Thus, these results are consistent with earlier studies suggesting the existence of adenosine receptors on cholinergic nerve terminals in the GPI (Moody and Burnstock, 1982), which later were characterised as A_1 receptor subtype (Nitahara et al., 1995). It seems likely that stimulation of prejunctional adenosine receptors causes modulation, i.e. inhibition, of ACh release (Somogyi and Vizi, 1988). Nitahara et al. (1995) confirmed these findings by investigating the presynaptic receptor-mediated modulation of evoked release of [^{3}H]ACh and consequent contraction of GPI. On one hand, adenosine and N^6-cyclopentyladenosine (A_1 receptor agonist) inhibited both the release of ACh in a concentration-dependent manner, on the other hand, 8-phenyltheophylline and DPCPX antagonised these inhibitory effects. In consequence, for the following experiments the metabolically stable P2X-selective agonist α,β-meATP was used for P2X receptor subtype characterisation, because the influence of possible degradation products, such as adenosine, could be circumvented.

Furthermore, it has been found that cholinergic neurons in GPI are endowed with opiate receptors stimulation of which might influence contractile responses (Somogyi and Vizi, 1988; Katsoulis et al., 1992). In the present study, α,β-meATP-induced contractions remained unaffected by naloxone. Thus, there was no tone at prejunctional opiate receptors by endogenous opiates in the present experiments.

The next step was to confirm that really ACh, as already mentioned, is also released when the presynaptic P2X-like receptor has been stimulated. The administration of physostigmine, an inhibitor of the acetylcholinesterase, led to an increase of the contractile responses to 30 µM α,β-meATP at concentrations of 30 and 100 nM (Fig. 4.13.). This expected observation was due to the prevented breakdown of ACh and a resulting increased quantity of this endogenous agonist in the synaptic cleft. The maximum effect of 30 µM α,β-meATP was reached with 100 nM physostigmine, whereas at higher concentrations (300 and 1000 nM physostigmine) no benefit was observed. It can be concluded that physostigmine at higher concentrations also shows antimuscarinic properties. Thus, for the characterisation of the P2X-like receptor, which can modulate the release of ACh, and to reinforce the contractile response to ACh by preventing its breakdown, all further experiments with P2 antagonists were done in the presence of 70 nM physostigmine.

5.4.1.2. Effects of TTX and atropine

A helpful tool for the determination that the receptor's location is at the neuronal site is the sodium channel blocker TTX. In the present study, 1 µM TTX completely abolished responses to 10 µM α,β-meATP (Fig. 4.14.), confirming the neuronal nature of this P2X receptor.
The blocking effect of the non-selective antagonist atropine (300 nM; Fig. 4.14.) following administration of 10 µM α,β-meATP has confirmed the fact that ACh is released via stimulation of P2X receptors, and additionally, the observed contractions of the GPI are a result of binding of ACh to postjunctional muscarinic receptors. These interesting findings will be discussed in detail in chapter 5.4.1.5.

To summarise these results (5.4.1.1. and 5.4.1.2.) it can be concluded that α,β-meATP acts on P2 receptors, presumably P2X-like receptors, which are

expressed on the cell bodies of myenteric neurones in GPI. The stimulation of these receptors resulted in the release of endogenous ACh and a subsequent contraction of the muscle via muscarinic receptors.

5.4.1.3. Antagonism by P2 antagonists

The pharmacological characterisation of P2 receptor subtypes requires a battery of different antagonists, since there is still a lack of highly selective compounds for the individual P2 receptor subtypes. Suramin, PPADS, NF023 and NF279 belong to the most popularly and best examined antagonists at P2 receptors in native tissues as well as at recombinant receptor subtypes (Lewis et al., 1995; Ziyal et al., 1996, 1997; Soto et al., 1997, 1999; Lê et al., 1998; Ralevic and Burnstock, 1998; Bianchi et al., 1999; Lambrecht et al., 1999; Lambrecht, 2000). Despite the non-selective behaviour of suramin and PPADS for a single receptor subtype, comparison with the affinity estimates determined in various earlier studies at native and recombinant receptors can give hints to the receptor subtype to be investigated.

It has already been reported that the P2 agonists α,β-meATP, 2-MeSATP and ATP led to contraction of GPI by acting at two different P2 receptors. Responses to α,β-meATP, but not to 2-MeSATP or ATP, were antagonised by TTX and atropine, suggesting that α,β-meATP activates P2 receptors on cholinergic nerve terminals, whilst the other two compounds act on smooth muscle P2 receptors (Kennedy and Humphrey, 1994). Matsuo et al. (1997) confirmed these findings and described that α,β-meATP produced contractions of ileal longitudinal smooth muscle segments. The contractile response was inhibited by suramin, TTX and atropine indicating that this effect results mainly from stimulation of, probably, P2X receptors on cholinergic nerves. Further evidence for the involvement of P2 receptors in the cholinergic contraction of the GPI was given by Barthó et al. (1997). They found that PPADS and suramin strongly inhibited the ACh-mediated contractile effect of α,β-meATP, but contractions evoked by exogenous ACh remained to be unaffected by both P2 antagonists. Additionally, cholinergic contractions due to electrical field stimulation (EFS) were inhibited by PPADS and suramin in a non-additive manner, suggesting that an endogenous ligand that stimulates prejunctional P2 receptors, possibly ATP, is involved in the contractile effect of EFS as a positive modulator of ACh release.

In the present study, α,β-meATP (0.1 - 30 μM) elicited concentration-dependent contractile responses. Suramin (10 - 100 μM; Fig. 4.15.), NF023 (30 - 300 μM; Fig. 4.17.) and NF279 (3 - 30 μM; Fig. 4.18.) shifted the concentration-response curve of α,β-meATP to the right in a parallel manner. The corresponding Schild plots of these compounds were linear, and the slopes of their regression lines did not differ from unity.

In the presence of PPADS (0.3 - 100 μM; Fig. 4.16.) concentration-response curves of α,β-meATP were displaced to the right in a roughly parallel fashion with reduction of the maximum response. This might be due to a pseudoirreversible or non-competitive antagonism of PPADS (Windscheif, 1995).

To summarise the antagonistic properties of the four compounds mentioned above, it could be concluded that they, with exception of PPADS, behaved as competitive antagonists at the neuronal P2X-like receptor. On the basis of the affinity estimates (pA_2 values) the following antagonist profile was obtained: PPADS > NF279 > suramin > NF023. The pA_2 values are presented in decreased order graphically in Fig. 5.9.

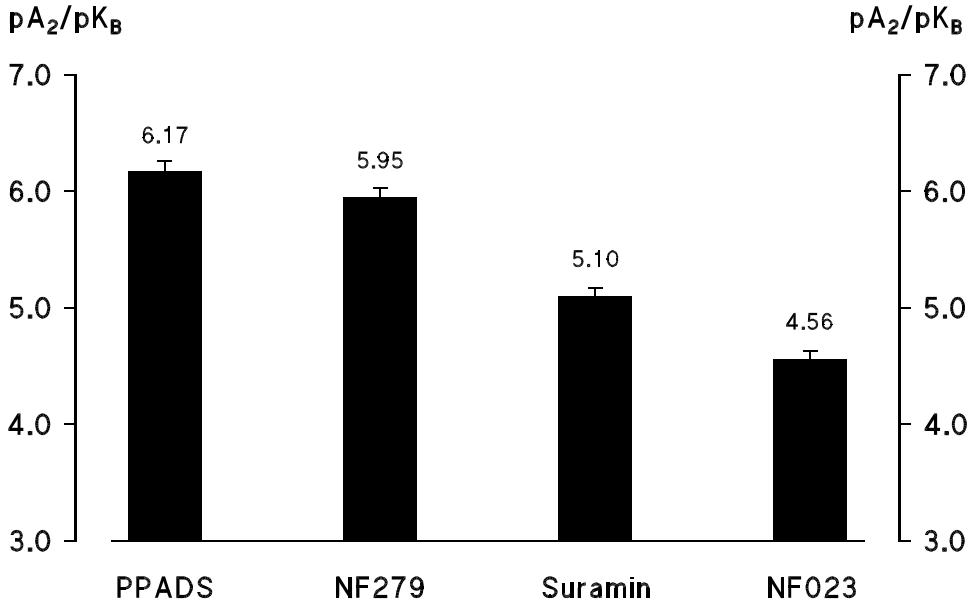


Fig. 5.9. *Affinity estimates (pK_B value for PPADS; pA_2 values for NF279, suramin and NF023) of P2 receptor antagonists in GPI using α,β-meATP as agonist (n = 4 for each antagonist).*

As already pointed out, the comparison between functional affinity estimates and potencies reported at recombinant receptors could be a suitable method for subtype identification by means of a battery of antagonists. Especially suramin, PPADS, NF023 and NF279 have been widely used in functional studies at native and cloned P2 receptors of various species. Their antagonistic properties at recombinant rat and human P2 receptor subtypes are illustrated in Figs. 5.10. to 5.12.

• Comparison of pA_2 values functionally determined at P2X-like receptors in GPI with pIC_{50} values determined at recombinant human and rat P2X receptors for PPADS and suramin:

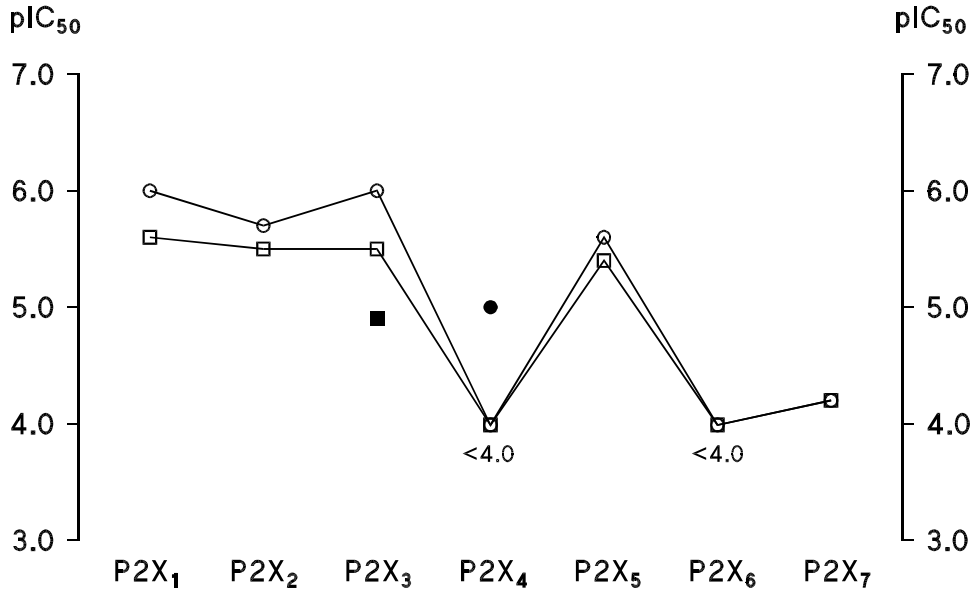


Fig. 5.10. *Selectivity profiles of PPADS (○, ●) and suramin (□, ■) at recombinant rat (opened symbols) and human (filled symbols) P2X receptor subtypes. Potency estimates (pIC_{50} values) were determined as inhibition of ATP-induced inward currents. Data taken from Soto et al., 1997; Ralevic and Burnstock, 1998.*

The pA_2 value of 6.17 determined in functional experiments for PPADS seems to correlate best with potencies (pIC_{50} values) found at recombinant rat $P2X_1$ (6.0), rat $P2X_2$ (5.7) and/or rat $P2X_3$ (6.0; Fig. 5.10.). However, the $P2X_2$ receptor

subtype can be excluded, because it belongs to the α,β-meATP-insensitive receptors, which show slow desensitisation or sustained depolarising currents (reviewed by MacKenzie et al., 1999; Burnstock and Williams, 2000), whereas, as already mentioned (see 4.2.1.1.), in GPI α,β-meATP produced rapidly desensitising contractile responses.

The functionally determined pA_2 value (5.1) of suramin is very similar to the pIC_{50} value (4.9) obtained at recombinant human $P2X_3$ receptors. However, taken the pIC_{50} value of 5.5 determined at recombinant rat $P2X_3$ into account, there is a slight difference by a factor of 2.5. Besides, also the pIC_{50} values of the rat $P2X_1$ (5.6), rat $P2X_2$ (5.5) and rat $P2X_5$ (5.4) are nearby the pA_2 value found in GPI, but the latter two subtypes can be excluded because of their α,β-meATP-insensitivity.

• Comparison of pA_2 values functionally determined at P2X-like receptors in GPI with pA_2 values determined at recombinant human P2Y receptors for PPADS and suramin:

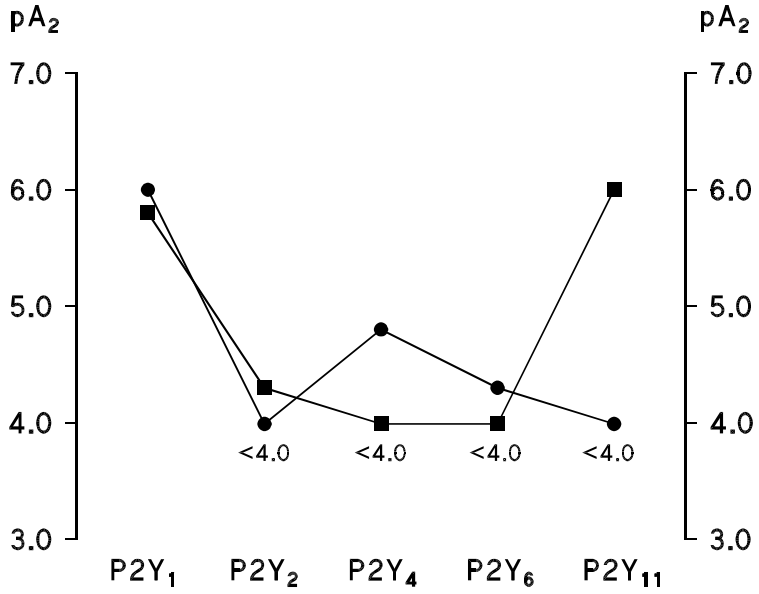


Fig. 5.11. *Selectivity profiles of PPADS (●) and suramin (■) at recombinant human P2Y receptor subtypes. Affinity estimates (pA_2 values) were determined as inhibition of inositol phosphate accumulation induced by nucleotides. Data taken from Soto et al., 1997; Ralevic and Burnstock, 1998; Communi et al., 1999.*

Although the effect of α,β-meATP strongly indicated the presence of a neuronal P2X-like receptor subtype, a comparison with affinities at P2Y is still necessary to argument against them. A good agreement to the functional pA_2 value of PPADS has only shown the pA_2 value (6.0) at recombinant human $P2Y_1$ receptors. Neither the affinity estimates of PPADS at any further recombinant human P2Y receptor subtype nor the pA_2 values for suramin at these receptors were nearly in the range of the pharmacologically determined affinity estimates for both antagonists in GPI. Apart from these facts, the 'agonist-selectivity' of α,β-meATP for P2X receptors led to the conclusion that P2Y receptors can be excluded, although this ligand has also been described as a weak P2Y agonist and the relationship between its function and selectivity for both P2 classes deserves careful attention (Fredholm et al., 1994).

• *Comparison of pA_2 values functionally determined at P2X-like receptors in GPI with pIC_{50} values determined at recombinant P2X and P2Y receptors for NF023 and NF279:*

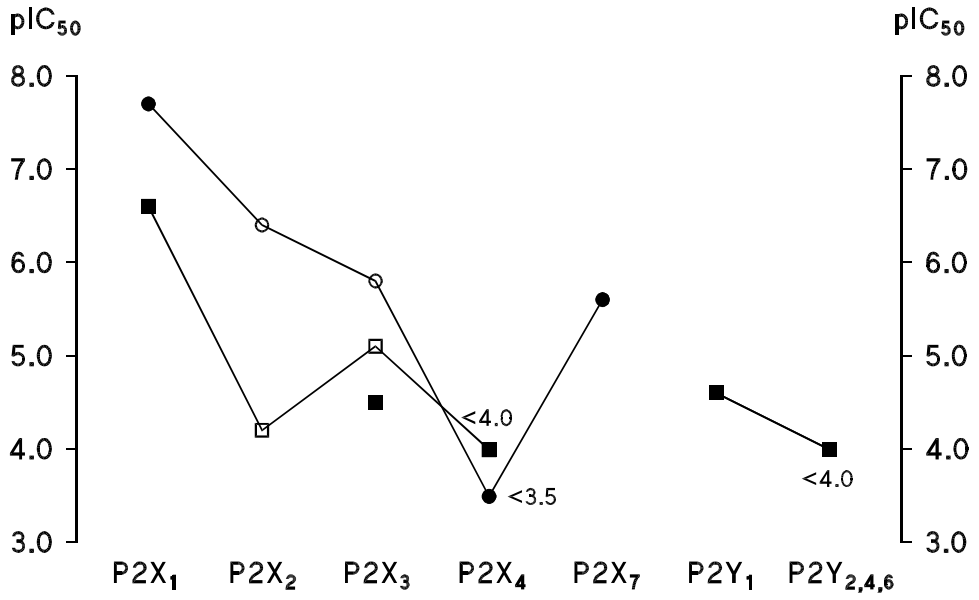


Fig. 5.12. *(legend, see page 119)*

Fig. 5.12. *Selectivity profiles of NF023 (□, ■) and NF279 (○, ●) at recombinant rat (opened symbols) and human (filled symbols) P2X as well as at human (filled symbols) P2Y receptor subtypes. At $P2X_1$ and $P2X_4$ receptors opened symbols are covered by the filled symbols, i.e. the affinity properties were the same for these rat and human subtypes. Both compounds have also the same potency at the human $P2Y_1$ and $P2Y_{2,4,6}$ receptors (symbols are covered). Potency estimates for P2X receptors were determined as inhibition of ATP-induced inward currents, and for P2Y receptors as inhibition of inositol phosphate accumulation induced by nucleotides. Data are taken from Soto et al., 1999; Klapperstück et al., 2000; Rettinger et al., 2000; Boyer, J.L. and Harden, T.K., personal communication.*

Compared to the functionally found pA_2 values of NF023 (4.56) and NF279 (5.95), the pIC_{50} values that have been found for both antagonists at the rat recombinant $P2X_3$ receptor subtype correlated best (Fig. 5.12.). At the $P2X_1$ receptor subtype, which was considered at first (cf. antagonistic profile of PPADS at recombinant rat P2X subtypes), the potency of NF023 is higher by a factor of 110 and this of NF279 by a factor of 56 compared with the pA_2 values determined in GPI.

In conclusion, PPADS, NF279, suramin and NF023 competitively antagonised α,β-meATP-induced contractions in GPI with potency decreasing in that order (Fig. 5.9.). The best correlation of pA_2 values from functional experiments and potencies reported at recombinant P2X receptor subtypes for these four antagonists was found at the $P2X_3$ receptor.

Another aspect seems to be important: human and rat P2X receptor subtypes have shown different functional phenotypes with regard to agonist responsiveness, especially to α,β-meATP, antagonist sensitivity, particularly to PPADS and suramin, and desensitisation (North and Barnard, 1997). $P2X_1$ and $P2X_3$ are the only two subtypes at which α,β-meATP is an effective agonist with EC_{50} around 1 µM. Moreover, $P2X_1$ and $P2X_3$ desensitise within milliseconds, whereas the other P2X subtypes needs a few seconds for desensitisation. In view of the antagonists PPADS and suramin the situation is not so simple. Only rat $P2X_4$ receptors are rather insensitive to both antagonists. For further potency estimates see Figs. 5.10. and 5.11.

Eventually, the question arises, which subtypes are responsible for contraction of GPI since P2X receptors have been functionally characterised by whole-cell patch clamp method in neurons isolated from guinea-pig myenteric plexus resulting in the identification of two types of cells: The major proportion of neurons (92 %) responded to ATP with slowly desensitising inward currents, which were antagonised by PPADS. α,β-meATP was a rather poor agonist in these cells. In a minority of neurons (8 %), ATP and α,β-meATP activated with similar potency rapidly desensitising inward currents. Thus, it could be speculated that a heterogeneous P2X receptor population may exist: It was proposed that the greater proportion may bear $P2X_2$-like receptors, whereas the smaller proportion of myenteric neurons may be of $P2X_1$ or $P2X_3$ receptor subtype (Zhou and Galligan, 1996; Nörenberg and Illes, 2000). In view of these results it can be assumed that in the present study with α,β-meATP the minority of neurons of guinea-pig myenteric plexus neurons has been activated, but further P2X receptor subtypes, homomeric or heteromeric, may be present, and it would be interesting to investigate this suggestion.

5.4.1.4. Concluding remarks

Upon comparing the above characteristics of each subtype with the pharmacological profile obtained in the present study, the data suggest again that neuronal P2X receptors of the GPI that can be stimulated by α,β-meATP are of $P2X_3$ subtype. Hence, one should be cautious in extrapolating pharmacological data, based largely on findings in guinea-pig, to other species including human and rat. But unfortunately, with exception of the $P2X_2$ receptor subtype, no other P2X receptor has been cloned from guinea-pig tissue so far (Housley et al., 1999). It can also not be excluded that this subtype is a heteromer. Thus, to answer these questions and to establish the GPI as a robust and unequivocal model for studying $P2X_3$ receptors, the receptor has to be cloned from this tissue.

5.4.1.5. Investigations on the muscarinic receptor subtype responsible for contraction of GPI

In this set of experiments the effect of α,β-meATP in GPI has been compared with that of electrical field stimulation based on the assumption that with both methods

endogenous ACh would be released, which in turn, leads to stimulation of postjunctional muscarinic receptors. Additionally, the muscarinic receptor subtype responsible for the resulted contraction of the ileal smooth muscle has been investigated by means of some muscarinic key antagonists.

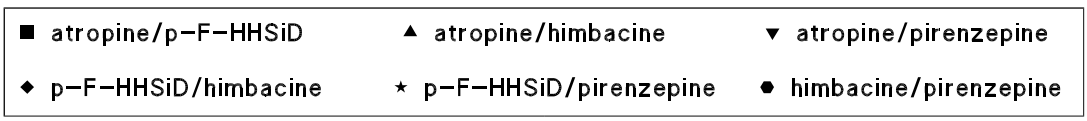
It is important to note, that the P2 antagonists, used so far in this study, at a concentration of 100 µM failed to affect contractions elicited by single doses of the muscarinic agonist arecaidine propargyl ester (APE; 100 nM). Accordingly, a direct interaction of PPADS, suramin, NF023 and NF279 with postjunctionally located muscarinic receptors can be excluded at their highest concentration used.

Hints to the muscarinic nature of the postjunctionally localised cholinergic receptor were already given in chapter 4.2.1.1.: the non-selective muscarinic antagonist atropine in a concentration of 300 nM strongly suppressed contractile responses to 3 µM α,β-meATP. For further investigations of the antagonistic properties a series of different muscarinic antagonists were employed, since there is still a shortage of highly-selective compounds. Atropine (0.003 - 0.1 µM), the 'M_3-selective' antagonist p-F-HHSiD (0.01 - 3 µM), the 'M_2-selective' antagonist himbacine (0.3 - 30 µM), and the 'M_1-selective' antagonist pirenzepine (0.1 - 30 µM) antagonised contractions to 3 µM α,β-meATP to almost 100 % (Fig. 4.19. A - D). The following antagonist profile in GPI was found: atropine > p-F-HHSiD > himbacine > pirenzepine (Tab. 4.3.). The low pIC_{50} value of pirenzepine (5.88) excludes the existence of M_1 receptors. In addition, himbacine exhibited a low affinity with pIC_{50} value of 6.01 and therefore it can be excluded that M_2 or M_4 receptors are involved. p-F-HHSiD displayed a pIC_{50} value of 6.93 and thus can exclude the participation of M_2 receptors, but is in good agreement with the existence of M_3 receptors. The highest pIC_{50} value was found for atropine (7.76), which was also in best accordance with the occurrence of M_3 receptors. It is important to note that most potency estimates of antagonists are documented as pA_2 values in literature. The pIC_{50} values obtained in the present study remained constantly by a factor of 10 lower than the respective pA_2 values (Tab. 4.3.).

As outlined before, activation of the soma-dendritic P2X receptor modulates release of endogenous ACh accompanied by contractile responses in GPI. Another standard method to liberate endogenous ACh is the electrical field stimulation of this tissue with 0.1 Hz. As before, the muscarinic antagonists were tested in the following concentrations: atropine (0.03 - 30 nM), p-F-HHSiD (1 -

1000 nM), himbacine (10 - 1000 nM), and pirenzepine (30 - 3000 nM). The antagonist profile (pIC_{50} values are given in parenthesis) was identical to that of GPI stimulated with α,β-meATP: atropine (8.44) > p-F-HHSiD (7.42) > himbacine (6.69) > pirenzepine (6.39). However, the inhibition curves of the electrically stimulated GPI were shifted to the left in a parallel manner, indicating that more ACh was released. The excellent correlation with r = 0.993 between both methods is illustrated in Fig. 4.20. A.

Previous reports (Eglen et al., 1996) have clearly identified the muscarinic receptor located at the postsynaptic site of GPI and responsible for contraction of the smooth muscle as M_3 subtype. Meanwhile, the GPI is a standard model for characterisation of new muscarinic antagonists and their structure-activity relationships in a functional way. To evaluate, whether the way of indirect ACh release after stimulation of the neuronal $P2X_3$-like receptor might be used for investigations in the muscarinic field, the exact nature, i.e. subtype, of this postsynaptically located muscarinic receptor has been determined. For that purpose, differences of pIC_{50} values of the above mentioned four muscarinic antagonists examined with α,β-meATP were compared to the corresponding differences in pK_i values obtained from radioligand binding studies at native M_1 - M_4 receptor subtypes. The graphical correlation is given in Fig. 5.13. Hence it becomes evident that only the regression line for the M_3 receptor subtype with a slope of 1.03 ± 0.16 provides a convincing correlation: M_1: r = 0.35; M_2: r = 0.15; M_3: r = 0.96; M_4: r = 0.54. To confirm this finding, pIC_{50} values obtained with α,β-meATP in GPI were compared to pA_2 values identified functionally at M_3 receptors in GPI (Fig. 4.20. B). The noticeable excellent correlation (r = 0.992) again led to the conclusion that activation of soma-dendritic $P2X_3$-like receptors in GPI causes an indirect ACh-mediated contraction via postjunctional M_3 receptors.



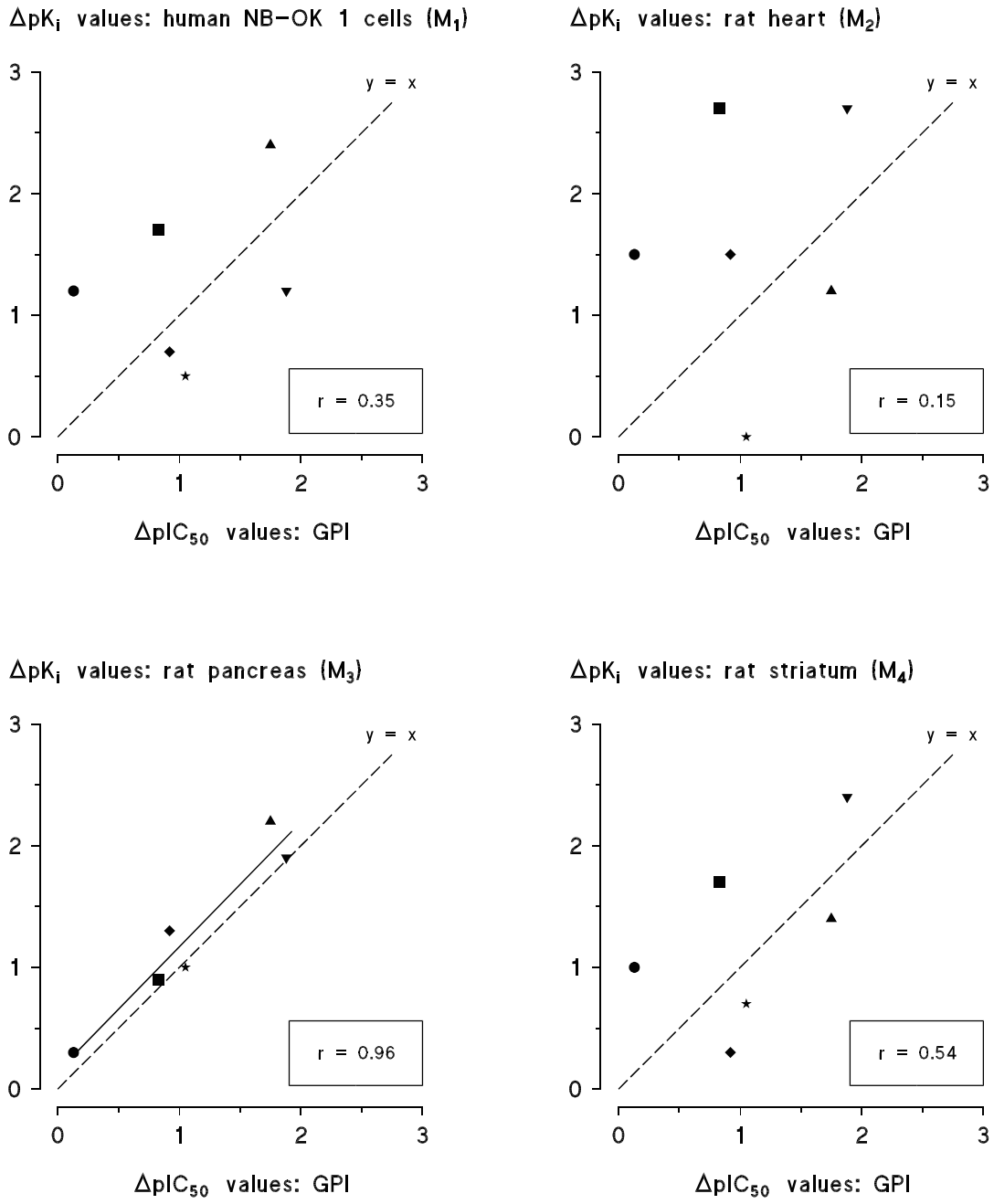


Fig. 5.13. *Comparison of differences (Δ) of pIC_{50} values for atropine, p-F-HHSiD, himbacine and pirenzepine, respectively, inhibiting α,β-meATP-induced contractions at postsynaptically located muscarinic receptors in GPI (present study) with differences (Δ) in binding affinities (pK_i values) determined in radioligand binding assays (data from Waelbroeck et al., 1989, 1990, 1991, and personal communication) at native M_1 to M_4 receptors (Pfaff et al., 1995). The dotted lines represent the 1:1 relationship.*

5.4.2. Experiments at postjunctionally localised P2 receptors

5.4.2.1. Potency profile of P2 agonists

In the field of P2 receptors a first idea of the receptor subtype to be characterised may be given through rank order of different agonists. ADPβS, α,β-meADP and 2-MeSATP in concentrations from 0.3 to 100 µM produced concentration-dependent contractions in GPI. As none of them reached a really maximum response comparable pEC_{750} values based on the development of 750 mg tension were uniformly determined, because for ADPβS it represents the presumable halfmaximal effect in the concentration range used. In consequence, the following affinity profile could be established: ADPβS > 2-MeSATP > α,β-meADP, with ADPβS as the most potent agonist indicating the presence of postjunctional P2Y receptors.

5.4.2.2. Effects of atropine, TTX and CCPA

The contractile responses of the aforementioned agonists were partially reduced by either TTX (1 µM), atropine (0.3 µM) or CCPA (1 µM). These observations (see Fig. 4.22.) imply that these three P2 agonists stimulated not only the P2 receptors on smooth muscle but also P2 receptors, probably $P2X_3$, located in myenteric neurons and characterised in chapter 4.2.1. The inhibitory effect of the A_1-selective agonist CCPA could be explained by its modulatory effect on ACh release following stimulation of soma-dendritic P2X receptors with P2 agonists (Somogyi and Vizi, 1988). Upon these findings it can be concluded that there are two different P2 receptors in GPI: a neuronal α,β-meATP-sensitive receptor with α,β-meATP >> ADPβS; and a postjunctional α,β-meATP-insensitive with ADPβS >>> α,β-meATP. Again, these results are in good agreement with previous studies (Kennedy and Humphrey, 1994; Matsuo et al., 1997). For the characterisation of the postjunctional P2 receptor ADPβS was used as agonist, since it was the most effective one, and atropine was present throughout the experiments with the P2 antagonists to prevent the influence of the neuronal $P2X_3$ receptor, which mediates ACh release and, subsequently, a contractile response via postjunctional muscarinic M_3 receptors.

5.4.2.3. Antagonism by P2 antagonists

Single doses of ADPβS (0.3 - 300 µM; 1232 ± 59 mg tension generated in control experiments) produced concentration-dependent contractions in GPI.

The commonly used antagonists PPADS, suramin, NF279 and NF023 antagonised contractions elicited by ADPβS with potency decreasing in that order (Fig. 5.14.).

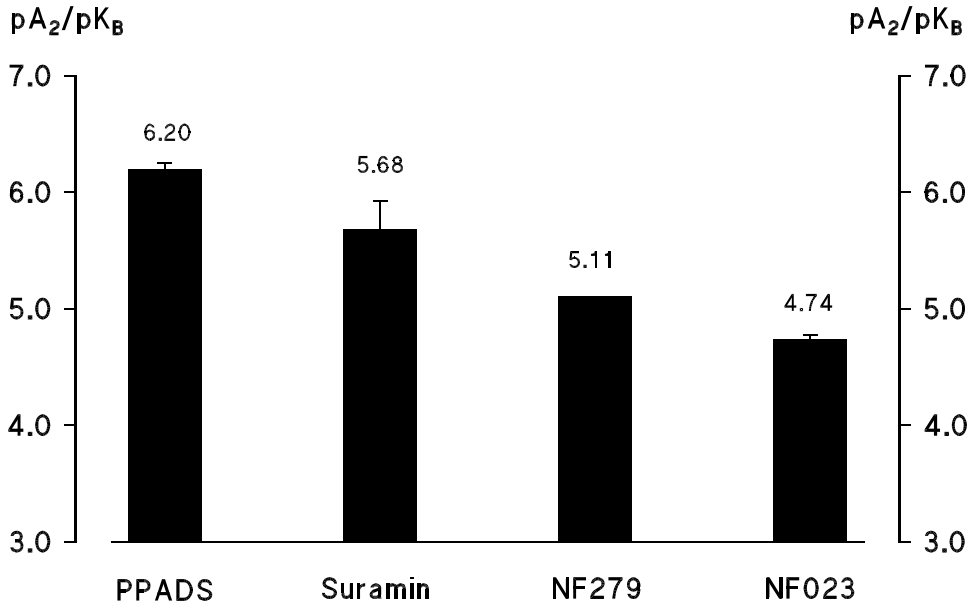


Fig. 5.14. *Affinity estimates (pA_2 values for NF279, suramin and NF023; pK_B value for PPADS) of P2 receptor antagonists in GPI using ADPβS as agonist (n = 3 - 6).*

First, strong evidence for the involvement of P2Y receptors was given since the GPI under the present experimental design, e.g. with atropine in the bath solution to exclude the described effect of $P2X_3$-like receptors located postjunctionally, was sensitive to the P2Y receptor agonist ADPβS. Second, if compared the functional affinity estimates with those obtained at recombinant rat and human P2Y receptors (Fig. 5.11.) the following can be summed up: the high potencies of PPADS and suramin correlate best with affinities found for both compounds at human $P2Y_1$ receptor subtype. At $P2Y_{11}$, suramin has also shown high affinity,

but there is a great discrepancy to the pA_2 value of PPADS that is less than 4.0. At all other P2Y subtypes the pA_2 values were clearly lower than observed in the present study from functional experiments in GPI. The pA_2 values of NF023 and NF279 at recombinant $P2Y_1$ receptors also correlate best with pA_2 values functionally determined in GPI.

Nevertheless, the pIC_{50} values obtained for the standard antagonists in this study at P2X receptors one should into account. In case of PPADS, only the pIC_{50} values at recombinant rat $P2X_1$ (6.0) and $P2X_3$ (6.0) and for suramin at $P2X_1$ (5.6), $P2X_2$ (5.5) and $P2X_3$ (5.5) receptors could be taken into consideration (cf. Fig. 5.10.). However, because of their rapid desensitisation the $P2X_1$ and $P2X_3$ receptors can be excluded. Regarding the pIC_{50} values for NF023 (4.2) and NF279 (6.4) at $P2X_2$ receptors it seems rather doubtful whether this subtype is involved.

5.4.2.4. Antagonism by A3P5P and MRS2179

Although progress has been made in identifying adenine nucleotide analogues that exhibit selectivities among P2 receptors, the availability of antagonists with subtype-selectivity is still limited. In consequence, a step forward were structure-activity relationships (SAR) from adenine nucleotides with phosphate substitution in 2'- and/or 3'-positions. From this series, A3P5P was the most promising one. In the adenine nucleotide-promoted inositol lipid hydrolysis response of turkey erythrocyte membranes A3P5P caused a parallel rightward shift of the 2-MeSATP concentration-effect curve, and a calculated pK_B of 5.66 ± 0.21 was determined from Schild regression with slope not significantly different from unity. Similar antagonistic activities were observed in 1321N1 human astrocytoma cells stably expressing the cloned $P2Y_1$ receptor. The calculated pK_B value of 6.05 ± 0.01 was essentially the same as that obtained in the turkey erythrocyte membrane preparation, but in contrast, no partial agonist activity could be noticed. Additionally, A3P5P was neither agonist nor antagonist at P2Y receptor of C6 rat glioma cells, human $P2Y_2$ and $P2Y_4$, or rat $P2Y_6$ receptors stably expressed in 1321N1 astrocytoma cells (Boyer et al., 1996). Recent reports from Hollopeter et al. (2001) have shown, that A3P5P failed to exhibit inhibitory effects on signals evoked by ADP on recombinant rat or human $P2Y_{12}$ receptors in concentrations up to 300 µM.

In the study presented here, the functionally determined pA_2 value resulting from only one concentration (100 µM) of A3P5P was 4.45 ± 0.16. The difference to the above mentioned antagonist potencies amounted to a factor of 16 ($P2Y_1$ in turkey erythrocytes) to 40 ($P2Y_1$ stably expressed in astrocytoma cells). However, the selectivity of A3P5P observed exclusive for $P2Y_1$ receptors gives strong evidence for this subtype in the smooth muscle of guinea-pig.
The chemical structure of A3P5P could raise the question to a potential degradation by enzymes described in chapter 1.2.6. Possibly from these enzymes the ecto-5'-nucleotidase may split of the terminal phosphate group resulting in adenosine 3'-phosphate. However, Park et al. (1998) have examined the degradation of adenosine 3'-phosphate 5'-phosphosulfate in rat vas deferens. It was rapidly broken down to A3P5P, but no further degradation of A3P5P was observed. In view of these results one could assume that A3P5P is also a stable compound under the experimental conditions of the present study in GPI.

Extension of SAR culminated in a new series of deoxyadenosine bisphosphates with substitutions in the adenine base, ribose moiety and phosphate groups. Among them, the most interesting compound was MRS2179 (Camaioni et al., 1998; Nandanan et al., 1999). MRS2179 behaved as competitive antagonist at the turkey $P2Y_1$ receptor with a pK_B value of 102 nM, and was inactive at $P2Y_2$, $P2Y_4$ and $P2Y_6$ subtypes as well as at the adenylate cyclase-linked P2Y receptor in C6 glioma cells and canine $P2Y_{11}$ receptor (Nandanan et al., 1999, 2000). The pK_B value of 6.99 ± 0.13 found by Boyer et al. (1998) for MRS2179 at human $P2Y_1$ receptors expressed in 1321N1 human astrocytoma cells provides further evidence for its $P2Y_1$ selectivity since no antagonistic effect of this compound has been observed at human $P2Y_2$, human $P2Y_4$ or rat $P2Y_6$ receptors. The presence of an N^6-methyl group and the absence of a 2'-hydroxyl group both enhanced affinity and decreased agonist efficacy, thus resulting in a pure antagonist at both turkey and human $P2Y_1$ receptors. In the present study, MRS2179 yielded a pIC_{50} value of 5.37 ± 0.04, and in view of its selectivity, this result is in a good agreement with the previous findings indicating that the P2 receptor located on the postsynapic site of the GPI is a $P2Y_1$ subtype. Although this bisphosphate derivative was helpful to characterise the P2Y receptor subtype on smooth muscle, it should be noted that MRS2179 also inhibited $P2X_1$ receptor-mediated responses and that its affinity at the human $P2Y_{11}$ is not known (Von Kügelgen and Wetter, 2000), whereas its affinity to the newly cloned $P2Y_{12}$ receptor to date can be excluded (Savi et al., 2001).

5.4.3. Conclusion

Upon comparing the functionally determined antagonist properties of the aforementioned antagonists with data from recombinant P2 receptors, the postjunctional P2Y receptor in GPI mediating contractile responses is certainly of $P2Y_1$ subtype. The cloning of P2 receptors from the GPI might be helpful for further investigations.

In summary, the guinea-pig ileal longitudinal smooth muscle preparation is an excellent model in which $P2X_3$ and $P2Y_1$ receptor selectivity of antagonists can be determined concomitantly with different agonists. Thus, it is a unique system in which $P2X_3$-like receptors seem to be located on the soma-dendritic region and $P2Y_1$ receptors on the postsynaptic site (Fig. 5.15.).

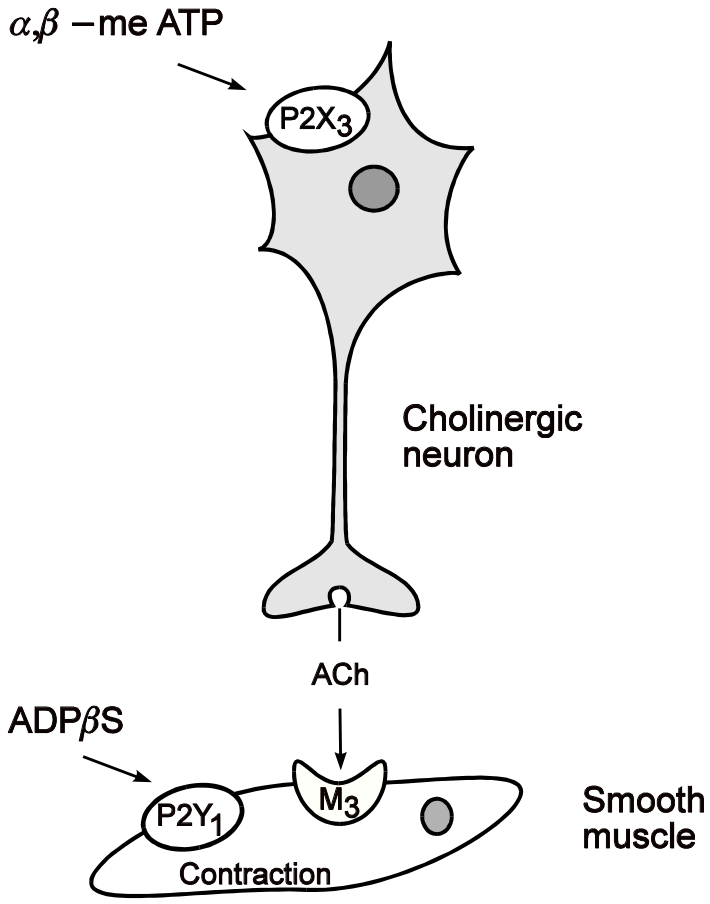


Fig. 5.15. *Functional heterogeneity of P2 receptors mediating contraction in GPI.*

5.5. Experiments at the longitudinal smooth muscle of rat ileum

The present study was undertaken to characterise the P2 receptors in RI to establish a further possible model for studying P2 receptor subtypes, and to compare the situation of P2 receptors in RI with that of GPI.

5.5.1. Experiments with P2 agonists

First of all, the potency of commonly used P2 agonists were examined. α,β-meATP, ADPβS, α,β-meADP and ATPγS elicited concentration-dependent contractile responses in RI with efficacy/potency decreasing in that order.

Since α,β-meATP and ADPβS were the most efficacy/potent agonists they were used for further investigations.
α,β-meATP has shown no desensitisation after cumulative addition. This observation led to the conclusion that the α,β-meATP-sensitive, but rapidly desensitising P2X receptor subtypes, i.e. $P2X_1$ and $P2X_3$ receptors, can be excluded. On the other hand, $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors show slow desensitisation, but are rather insensitive to α,β-meATP. Therefore, one can speculate, that the P2 receptor subtype could be a hetero-oligomeric channel, e.g. $P2X_{2+3}$ or $P2X_{1+5}$ (Lewis et al., 1995; Lê et al., 1999). Another explanation could be that in RI α,β-meATP mainly stimulates P2Y receptors.
Administration of 1 μM TTX reduced contractions elicited by 30 μM α,β-meATP to nearly 40 % of control (Fig. 4.29.) indicating that the agonist acts at the neuronal as well as at the postsynaptic site. Addition of 0.3 μM atropine led to a decrease of contraction evoked by 100 μM α,β-meATP to about 45 % of control (Fig. 4.29.). Thus, it can be assumed that, as it was observed at GPI, contractions of RI are partly indirectly ACh-mediated.

In case of ADPβS, cumulative addition also failed to result in desensitisation. But contrary to α,β-meATP, contractions elicited by ADPβS remained unaffected by administration of either 1 μM TTX or 0.3 μM atropine indicating that this agonist acts only directly at the smooth muscle of the rat ileum. ADPβS was therefore used to determine the potency of antagonists in RI.

5.5.2. Effects of P2 antagonists on responses to ADPβS

The commonly used P2 antagonists PPADS (10 - 300 µM; Fig. 4.32.), suramin (30 - 300 µM; Fig. 4.31.) and NF023 (10 - 300 µM; Fig. 4.30.) produced rightward displacements of the concentration-effect curve of ADPβS in a roughly parallel manner with potency decreasing in that order. It was characteristic for all these antagonists that the control curve was somewhat flatter and the antagonist curves showed a higher maximum than the control curve. A reason for this fact could be that by the antagonist a relaxation-mediating P2 receptor was blocked.

5.5.3. Conclusion

Little information is given in the literature concerning the localisation of P2 receptor subtypes in the smooth muscle of the rat ileum (cf. chapter 2.3), and in the study presented here, the presence of P2 receptors could be proved unequivocally, but it was impossible with the functional methods to characterise the involved subtypes exactly. Besides the lack of really subtype-selective agonists and antagonist for P2 receptors, there are many further caveats within the field of P2 receptors that might complicate the receptor characterisation: first, the existence of hitherto unknown P2 receptor subunits can not be excluded or a single subunit may predominate the phenotype of a heteromeric complex. A further complication may arise from alternative splicing. Second, the coexistence of ionotropic P2X together with metabotropic P2Y receptors could also influence the neuronal response.
In summary, the situation concerning the localisation and response of P2 receptors in RI is quite different from that observed in GPI.

6. SUMMARY

The interest in receptors for acetylcholine hands back into the passed century as nicotinic and muscarinic receptors as well as their subtypes could to be discovered and characterised. Due to the abundance of muscarinic receptors within the central and peripheral nervous system as well as in innervated organs they are still of large interest as targets for certain clinical indications.
Particularly within the area of the chronical obstructive pulmonary disease (COPD) bronchodilators are the first line approach to treatment. Although the use of muscarinic receptor antagonists as ipratropium bromide, either alone or in combination with a short-acting β_2 receptor agonist, is recommended, their use is hampered by unwanted side effects. Besides M_3 receptors human airways contain also M_2 and its blockade results in an increased ACh release and is therefore undesirable. Thus, the development of antagonists with M_3-selectivity and/or extended duration of action would be a large progress for the treatment of patients with COPD or even asthma.

Investigations on the stereoisomers of glycopyrronium bromide and their four corresponding tertiary analogues

In the present study both the four pure stereoisomers of glycopyrronium bromide and the corresponding tertiary analogues were investigated. To date, the diastereoisomeric pair (RS/SR) of this compound (launched as Robinul®) is predominantly used therapeutically as antisialagogue for premedication under anaesthesia or as antispasmodic.

The molecular structure of glycopyrronium bromide indicates two centres of chirality, which result in four stereoisomers. The pharmacological investigation of both the quaternary and corresponding tertiary isomers in functional standard models, rabbit vas deferens for M_1 receptors, guinea-pig left atria for M_2 receptors and the guinea-pig ileal longitudinal smooth muscle for M_3 receptors, has shown that all compounds behaved as potent antagonists at the respective muscarinic receptor subtypes. However, their receptor selectivity was relatively small, whereby generally the lowest affinity was observed at the M_2 receptors. Among the stereoisomers, the (R/R')- and (S/R')-configured compounds exhibited the highest, the (S/S')-configured isomers the lowest antagonistic properties. These

results could be confirmed in radioligand binding studies. Remarkably, at M_3 receptors an extremely slow dissociation of the substances has been determined. With a dissociation half-life of 120 min, determined in the binding assay and an affinity in the subnanomolar range, the quaternary (R/R')-configured stereoisomer of glycopyrronium bromide might be a suitable alternative to the present treatment of COPD: the long dissociation half-life would permit a once-daily administration which may enhance patients' compliance, the high affinity enables a small dosage and the so-called 'kinetic selectivity' as well as the quaternary structure may result in a minimisation of unwanted side effects. Due to these advantages the substance was patented and put to the pharmaceutical industry for resuming investigations at the disposal.

Evaluation of the guinea-pig ileal longitudinal smooth muscle preparation with respect to P2 receptor heterogeneity

Due to the pioneering work, which was carried out at the end of the 70's by Burnstock and co-workers, the picture of ATP as an energy source of the cell has been changed to ATP as a neurotransmitter of ubiquitous occurrence with appropriate target structures, the P2 receptors. In the meantime it is generally recognised that one can differentiate between metabotropic P2Y and ionotropic P2X receptors. Cloning techniques have validated the original receptor classification and have gone on to unveil an exciting range of different receptor subtypes. Until today, seven subtypes of P2X ($P2X_{1-7}$) receptors and six P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$) receptor subtypes have been cloned, pharmacologically characterised and accepted as true representatives of the P2 receptor family.

In this rapidly expanding field, one of the main current challenges is to relate the cloned P2 receptors to the diverse physiological responses mediated by native receptors. Since the guinea-pig ileal longitudinal smooth muscle is a well-known preparation, for instance for studying muscarinic receptors, the goal of the present study was to evaluate this model with respect to P2 receptor subtype distribution.
Besides agonists, which indicate a preference for ionotropic (α,β-meATP) or metabotropic (ADPβS) P2 receptors, a number of well-examined antagonists with partially high affinity for a receptor subtype were used for the functional investigations.

6. SUMMARY

The neuronal localisation of P2X receptors could be demonstrated. The almost complete inhibition of the contraction to α,β-meATP after application of atropine or TTX, indicating an indirect, ACh-mediated effect. Due to these observations in all further experiments with P2 antagonists 70 µM physostigmine were added to the bath solution.

The antagonists suramin, NF023 and NF279 behaved as competitive antagonists against α,β-meATP, while PPADS was found to be a pseudoirreversible antagonist. A comparison of functionally determined pA_2 values with potencies obtained at recombinant rat and human P2 receptors let to the conclusion that the $P2X_3$ receptor subtype causes a contraction of the ileal smooth muscle via an indirect mechanism of ACh release. Since P2X subunits can assemble to form heteromeric besides homomeric functionally active channels the present soma-dendritic P2X receptor can also be a heteromer, at which the $P2X_3$ predominates the native phenotype. As long as no unequivocal identification of this receptor has been reported on molecular level, one should use the designation $P2X_3$-like receptor.

The criteria of antagonist affinities were used to verify that the muscarinic receptor present in GPI which could be indirectly stimulated via neuronal localised $P2X_3$-like receptors causing ACh release were the same as previously characterised in GPI in a direct way with APE or electrical field stimulation of the tissue.
These correlations demonstrated unequivocally that the postjunctional muscarinic receptors of GPI, stimulated through ACh, which was released after activation of soma-dendritical $P2X_3$-like receptors, are equivalent to native or recombinant M_3 receptors.

Moreover, addition of ADPβS elicited contractions in GPI, which could not be abolished by either TTX or atropine. These observations led to the idea that there are postsynaptically localised P2Y receptors. In order to prevent the influence of the neuronal $P2X_3$ receptors, all experiments were done in the presence of 0.3 µM atropine.
Again, suramin, NF023 and NF279 behaved as competitive antagonists against ADPβS , while PPADS caused a rightward shift of the control curve with maximum depression. A comparison of the antagonist potencies with described potency values obtained at recombinant P2 receptors permitted the conclusion that the P2Y receptor located postsynaptically in the GPI showed characteristics of the

$P2Y_1$ receptor subtype. The $P2Y_1$-selective bisphosphates A3P5P and MRS2179, although in the present study possessing lower pIC_{50} values than described in the literature, confirmed this conclusion.

With the identification of the neuronal $P2X_3$ receptor and the postsynaptic $P2Y_1$ receptor, the GPI is a unique functional pharmacological model to date, in which both P2 receptor subtypes can be pharmacologically isolated by the use of the respective agonist, α,β-meATP or ADPβS.

Characterisation of P2 receptors in the rat ileal longitudinal smooth muscle

One, in comparison to the GPI, completely different situation was found to be in the RI. The tested P2 agonists ADPβS, α,β-meATP, α,β-meADP and ATPγS produced contractions of the tissue, whereby α,β-meATP proved to be the most efficacious agonist. However, in contrast to the GPI, a repeated addition of α,β-meATP led not to a desensitisation of the receptor. The inhibition of the contraction after addition of TTX and atropine suggested the presence of both pre- and postsynaptically located P2X receptors.
Contractile responses to ADPβS failed to be affected by the addition of TTX or atropine. Also suramin, NF023 and PPADS proved to be very weak antagonists at this preparation. Remarkably, in each case the antagonist curves were steeper and had a higher maximum effect than the agonist control curves. Thus, one can only speculate that the antagonists have blocked a relaxation-mediating receptor and in consequence only the effect of the contraction-mediating receptor remained.

Although tissues of the rat are frequently used as functional models in experimental pharmacology, in face of the lack of subtye selective agonists and antagonists in the present study it was not possible to characterise the P2 receptor subtypes responsible for contraction of RI. Moreover, a comparison with GPI shows that differences might exist when using the same tissue of two different species.

7. ZUSAMMENFASSUNG

Die Beschäftigung mit Muskarinrezeptoren reicht bis in das vergangene Jahrhundert zurück als, in Folge der verschiedenen Wirkungen des Neurotransmitters Acetylcholin, einerseits Nikotin- und andererseits Muskarinrezeptoren sowie deren Subtypen entdeckt und charakterisiert werden konnten. Aufgrund der weiten Verbreitung von Muskarinrezeptoren innerhalb des zentralen und peripheren Nervensystems sowie in entsprechend innervierten Organen sind diese nach wie vor als Target für bestimmte klinische Indikationen von großem Interesse.
Besonders im Bereich der chronisch obstruktiven Atemwegserkrankungen (COPD) sind Bronchodilatoren Mittel der Wahl. Obwohl die derzeitige Behandlungsstrategie im wesentlichen auf dem Einsatz des unselektiven muskarinischen Antagonisten Ipratropiumbromid, allein oder in Kombination mit einem kurzwirksamen β_2-Sympathomimetikum, beruht, ist ihr Einsatz aufgrund der dabei auftretenden unerwünschten Nebenwirkungen limitiert. Neben M_3-Rezeptoren findet man in der menschlichen Lunge auch präsynaptische M_2-Rezeptoren, deren Blockade zu einem Anstieg der Acetylcholinfreisetzung führt. Demzufolge würde die Entwicklung eines hochselektiven und/oder langwirksamen muskarinischen M_3-Rezeptorantagonisten einen großen Fortschritt für die Behandlung von Patienten mit COPD und auch Asthma bedeuten.

Untersuchung der Stereoisomere des Glycopyrroniumbromids und der entsprechenden tertiären Analoga

Im Rahmen der vorliegenden Arbeit wurden sowohl die vier reinen Stereoisomere des Glycopyrroniumbromids als auch die entsprechenden vier tertiären Analoga untersucht. Bislang wird das Diastereomerengemisch (RS/SR), das als Robinul® im Handel ist, vorwiegend als Antisialagogum in der Prämedikation der Narkose oder als Spasmolytikum therapeutisch eingesetzt.

Die molekulare Struktur des Glycopyrroniumbromids weist zwei Chiralitätszentren auf, woraus sich vier stereoisomere Verbindungen ergeben. Die pharmakologische Untersuchung sowohl der quartären als auch der korrespondierenden tertiären Isomere in den funktionellen Standardmodellen,

Kaninchen-Vas-deferens für M_1-Rezeptoren, linker Vorhof des Meerschweinchenherzens für M_2-Rezeptoren und die Längsmuskulatur des Meerschweinchenileum für M_3-Rezeptoren, ergab, dass sich alle Verbindungen an den untersuchten Muskarinrezeptorsubtypen als potente Antagonisten verhielten. Ihre Rezeptorselektivität war jedoch relativ gering, wobei im Allgemeinen die niedrigste Affinität zum M_2-Rezeptor beobachtet wurde. Innerhalb der Stereoisomeren zeigten die (R/R')- und (S/R')-konfigurierten Verbindungen den stärksten, die (S/S')-konfigurierten Isomere hingegen den geringsten antagonistischen Effekt. Diese Ergebnisse konnten durch Radioligand-Bindungsstudien bestätigt werden. Bemerkenswert war jedoch, dass insbesondere am M_3-Rezeptor eine extrem langsame Dissoziation der Substanzen vom Rezeptor festgestellt wurde. Verbunden mit der hohen Affinität und der in Bindungsstudien ermittelten Dissoziationshalbwertszeit von 120 min könnte das quartäre (R/R')-konfigurierte Stereoisomer des Glycopyrroniumbromids eine geeignete Alternative zur Behandlung der COPD darstellen: Die lange Halbwertszeit sollte eine Einmalgabe pro Tag erlauben und somit die Patientencompliance erhöhen, die hohe Affinität eine geringe Dosierung ermöglichen und die 'kinetische Selektivität' sowie die quartäre Struktur könnten zur Minimierung unerwünschter Nebenwirkungen führen. Aufgrund dieser Vorteile wurde die Substanz patentiert und der pharmazeutischen Industrie für weiterführende Untersuchungen zur Verfügung gestellt.

Untersuchungen an der Längsmuskulatur des Meerschweinchenileum in Hinblick auf die Verteilung von P2-Rezeptoren

Aufgrund der Pionierarbeit, die Ende der 70er Jahre von Burnstock und seinen Mitarbeitern geleistet wurde, wandelte sich das Bild von ATP als einer Energiequelle der Zelle zu einem Neurotransmitter ubiquitären Vorkommens mit entsprechenden Zielstrukturen, den P2-Rezeptoren. Inzwischen ist allgemein anerkannt, dass zwischen metabotropen P2Y-Rezeptoren und ionotropen P2X-Rezeptoren unterschieden werden kann. Mit Hilfe von Klonierungstechniken konnte diese Klassifizierung validiert und außerdem eine Vielzahl unterschiedlicher Subtypen identifiziert werden. Bis heute wurden sieben P2X- ($P2X_{1-7}$) und sechs P2Y- Rezeptorsubtypen ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$) kloniert und pharmakologisch charakterisiert. Sie gelten unumstritten als Vertreter der P2-Rezeptorfamilie.

Heute besteht eine der größten Herausforderungen auf diesem sich explosionsartig expandierendem Gebiet darin, die geklonten P2-Rezeptoren mit den verschiedenen physiologischen Antworten, die durch native P2-Rezeptoren vermittelt werden, in Einklang zu bringen. Da die Längsmuskulatur des Meerschweinchenileum ein bekanntes Modell, z.B. für Untersuchungen an Muskarinrezeptoren, darstellt, war das Ziel der vorliegenden Arbeit, dieses Modell in Bezug auf die Verteilung von P2-Rezeptoren hin zu untersuchen.
Neben Agonisten, die eine Präferenz für entweder ionotrope (α,β-meATP) oder metabotrope (ADPβS) P2-Rezeptoren aufweisen, wurden im wesentlichen eine Reihe gut untersuchter Antagonisten mit zum Teil hoher Affinität für einen Rezeptorsubtyp für die funktionellen Untersuchungen verwendet.

Die neuronale Lokalisation des P2X-Rezeptors konnte durch die komplette Aufhebung der durch α,β-meATP-vermittelten Kontraktionen nach Zugabe von TTX charakterisiert werden. Die ebenfalls fast vollständige Hemmung der Kontraktion nach Einsatz von Atropin wies auf einen indirekten, durch Acetylcholin vermittelten Effekt hin. Aufgrund dieser Beobachtungen wurden sämtliche Versuche mit P2-Antagonisten unter Zusatz von 70 µM Physostigmin in der Nährlösung durchgeführt.
Die eingesetzten Antagonisten Suramin, NF023 und NF279 erwiesen sich als kompetitive Antagonisten, während PPADS neben der Rechtsverschiebung einen Maximumabfall der Agonistenkurven bewirkte. Ein Vergleich der funktionell ermittelten pA_2-Werte mit den Wirkstärken an rekombinanten P2-Rezeptoren von Ratte und Mensch lässt vermuten, dass es sich hierbei um einen $P2X_3$-Rezeptorsubtyp handelt, der über die Freisetzung von Acetylcholin eine Kontraktion der glatten Muskulatur über einen indirekten Mechanismus auslöst. Da sich P2X-Rezeptoruntereinheiten neben homomeren auch zu heteromeren, funktionell aktiven Kanälen vereinen können, könnte es sich bei dem vorliegenden soma-dendritischen P2X-Rezeptor aber auch um ein Heteromer handeln, bei dem der $P2X_3$-Rezeptor den Phänotyp bestimmt. Solange noch keine eindeutige Identifizierung dieses Rezeptors speziesspezifisch auf molekularer Ebene erfolgt ist, sollte man deshalb die Bezeichnung $P2X_3$(-ähnlicher)-Rezeptor verwenden.

Es konnte gezeigt werden, dass eine Stimulation des präsynaptischen $P2X_3$(-ähnlichen)-Rezeptors zur Ausschüttung von Acetylcholin führt, das wiederum postsynaptisch einen kontraktionsvermittelnden Muskarinrezeptor aktiviert. Um zu

beweisen, dass es sich dabei um denselben Muskarinrezeptorsubtyp handelt, der bereits auf direktem Weg durch APE oder mittels EFS als M_3-Rezeptor charakterisiert werden konnte, wurden die Affinitäten muskarinischer Antagonisten als Kriterien herangezogen. Die erhaltenen Korrelationen wiesen eindeutig darauf hin, dass dieser postsynaptisch im GPI lokalisierte Muskarinrezeptor dem nativen und rekombinanten, kontraktionsvermittelnden M_3-Rezeptorsubtyp entspricht.

Durch Zugabe von ADPβS konnten im GPI Kontraktionen ausgelöst werden, die allerdings mit TTX und Atropin nur zum Teil gehemmt wurden. Diese Beobachtungen führten zu der Erkenntnis, dass postsynaptisch P2Y-Rezeptoren lokalisiert sind. Ihre Subtypcharakterisierung erfolgte unter Zusatz von 0.3 µM Atropin in der Nährlösung, um den Einfluss der neuronalen $P2X_3$-Rezeptoren zu unterbinden. Suramin, NF023 und NF279 zeigten wiederum einen kompetitiven Antagonismus gegen ADPβS, während PPADS auch hier eine Rechtsverschiebung mit Maximumdepression der Agonistenkurve hervorrief. Ein erneuter Vergleich mit beschriebenen Affinitätswerten von rekombinanten P2-Rezeptoren ließ den Schluss zu, dass der im GPI postsynaptisch gefundene P2Y-Rezeptor Eigenschaften des $P2Y_1$-Rezeptorsubtyps aufweist. Eine Bestätigung dafür gaben außerdem die $P2Y_1$-selektiven Bisphosphate A3P5P und MRS2179, obgleich sie geringere pIC_{50}-Werte aufzeigten als in der Literatur beschrieben.

Mit der Charakterisierung des neuronalen $P2X_3$-Rezeptors und des postsynaptischen $P2Y_1$-Rezeptors ist das GPI ein bisher einzigartiges funktionelles pharmakologisches Modell, in dem beide P2-Rezeptorsubtypen durch Einsatz des jeweiligen Agonisten, α,β-meATP oder ADPβS, pharmakologisch isoliert werden können.

Charakterisierung von P2-Rezeptoren in der Längsmuskulatur des Rattenileum

Eine im Vergleich zum GPI gänzlich andere Situation zeigte sich im RI. Die getesteten P2-Agonisten ADPβS, α,β-meATP, α,β-meADP und ATPγS erzeugten Kontraktionen im untersuchten Gewebe, wobei sich α,β-meATP als effektivster Agonist erwies. Im Unterschied zum GPI führte eine wiederholte Gabe von α,β-meATP allerdings nicht zu einer Desensibilisierung des Rezeptors. Die durch Zugabe von TTX und Atropin erreichte Kontraktionshemmung lässt auf das

Vorhandensein von sowohl prä- als auch postsynaptischen P2X-Rezeptoren schließen. Eine Trennung der durch diese Rezeptoren hervorgerufenen Effekte war im funktionellen Experiment jedoch nicht durchführbar.
Keinerlei Effekt zeigte allerdings der Zusatz von TTX und Atropin auf die durch ADPβS ausgelösten Kontraktionen. Suramin, NF023 und PPADS erwiesen sich als sehr schwache Antagonisten an diesem Präparat. Auffällig war hingegen, dass die durch den Antagonisten verschobenen Kurven jeweils steiler und im Maximum höher waren als die Kontroll-Agonistenkurve. Man kann also hier nur spekulieren, dass durch die Antagonisten zunächst ein relaxationsvermittelnder Rezeptor geblockt wurde und in Folge nur noch der Effekt des kontraktionsvermittelnden Rezeptors sichtbar war.

Obwohl Gewebe der Ratte häufig als funktionelle Modelle in der experimentellen Pharmakologie eingesetzt werden, konnten auf Grund fehlender subtypselektiver Agonisten und Antagonisten die kontraktionsvermittelnden P2-Rezeptorsubtypen im RI nicht identifiziert werden. Des weiteren zeigt ein Vergleich mit dem GPI, dass bedeutende Unterschiede bei der Verwendung gleichen Gewebes zweier verschiedener Spezies existieren können, die bei der vergleichenden Betrachtung von Affinitätswerten von Agonisten und Antagonisten zur Charakterisierung von Rezeptorsubtypen beachtet werden müssen.

8. GLOSSARY OF ABBREVATIONS

A	AC	Adenylate cyclase
	ACh	Acetylcholine
	AChE	Acetylcholinesterase
	AD	Alzheimer's disease
	ADP	Adenosine 5'-diphosphate
	ADPßS	Adenosine 5'-O-(2-thiodiphosphate)
	AF-DX 116	11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one
	AF-DX 384	5,11-Dihydro-11-[[[2-[2-(dipropylamino)methyl)-1-piperidinyl]ethyl]amino]-carbonyl]-6H-pyrido-(2,3-b)(1,4)-benzodiazepine-6-one
	AMP	Adenosine 5'-monophosphate
	APE	Arecaidine propargyl ester; 1,2,5,6-tetrahydro-1-methyl-3-[(2-propynyloxy)carbonyl]pyridinium hydrobromide
	APP	Amyloid precursor protein
	A3P5P	Adenosine 3',5'-diphosphate
	APPs	Soluble form of APP
	Ap_xA	Adenine dinucleotide polyphosphates
	AQ-RA 741	11-({4-[4-(Diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3b)(1,4)benzodiazepine-6-one
	AR-C67085MX	2-Propylthio-β,γ-dichloromethylene-D-ATP
	AR-C69931MX	N^6-(2-Methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-D-ATP
	ARL66096	2-Propylthio-β,γ-difluoromethylene-D-ATP
	ARL 67156	6,N,N-Diethyl-D-β,γ-dibromomethylene ATP (formerly FPL 67156)
	ATP	Adenosine 5'-triphosphate
	ATPγS	Adenosine 5'-O-(3-thiotriphosphate)
B	BIBN 99	5,11-Dihydro-8-chloro-11-([4-{3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl}-1-piperidinyl]-acetyl)-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one
	BzATP	2'-and 3'-O-(4-Benzoyl-benzoyl)adenosine 5'-triphosphate
C	cAMP	Cyclic adenosine 3',5'-monophosphate
	cDNA	Complementary desoxyribonucleic acid
	CCPA	2-Chloro-N^6-cyclopentyladenosine
	CF	Cystic fibrosis
	CGRP	Calcitonin gene-related peptide
	CHO	Chinese hamster ovary
	CNS	Central nervous system
	COPD	Chronic obstructive pulmonary disease
	CTP	Cytosine 5'-triphosphate
	CTRF	Cystic fibrosis transmembrane conductance regulator
D	DAG	Diacylglycerol
	4-DAMP	4-Diphenylacetoxy-N-methylpiperidine methiodide

8. GLOSSARY OF ABBREVATIONS

	DMSO	Dimethyl sulfoxide
	DNA	Desoxyribonucleic acid
	DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
	DR	Dose ratio
E	EC_{50}	Concentration of agonist producing 50 % of the maximum effect
	EDRF	Endothelium-derived relaxing factor
	EFS	Electrical field stimulation
F	4-F-PyMcN^{+}	4-(4-Fluorophenylcarbamoyloxy)-2-butynyl-N-methyl-pyrrolidinium tosylate
G	G_i	Inhibitory G protein
	G_o	G protein of yet unknown function
	G_q	Phospholipase C stimulating G protein
	GABA	γ-Aminobutyric acid
	GDP	Guanosine 5'-diphosphate
	GPA	Guinea-pig atria
	GPI	Guinea-pig ileal longitudinal smooth muscle
	G protein	Guanine nucleotide binding regulatory protein
	GTP	Guanosine 5'-triphosphate
H	h	Hour(s)
	HEK	Human embryonic kindey
	HHSiD	Hexahydro-sila-difenidol
	Hz	Hertz
I	IC_{50}	Concentration of agonist/antagonist producing halfmaximal effect
	IP_3	Inositol 1,4,5-trisphosphate
	ITP	Inositol 5'-triphosphate
K	K_D	Dissociation constant
	kD	Kilodalton
L	L-NOARG	N^G-Nitro-L-arginine
	Lu 25-109	5-(2-Ethyl-2H-tetrazol-5-yl)-1-methyl-1,2,3,6-tetrahydropyridine maleate
M	m	Slope of regression line
	M	Molar
	MAPK	Mitogen-activated protein kinase
	McN-A-343	4-(3-Chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride
	α,ß-meADP	α,ß-Methyleneadenosine 5'-diphosphate
	α,ß-meATP	α,ß-Methyleneadenosine 5'-triphosphate
	β,γ-meATP	β,γ-Mehthyleneadenosine 5'-triphosphate
	2-MeSADP	2-Methylthioadenosine 5'-diphosphate
	2-MeSATP	2-Methylthioadenosine 5'-triphosphate
	min	Minutes

8. GLOSSARY OF ABBREVATIONS

	mRNA	Messenger ribonucleic acid
	MRS2179	N^6-Methyl 2'-deoxyadenosine 3',5'-bisphosphate
N	n	Number of individual experiments
	NANC	Non-adrenergic non-cholinergic
	NB-OK 1	Human neuroblastoma cell line
	NC-IUPHAR	International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification
	NDP	Nucleoside 5'-diphosphate
	NF023	Symmetrical 3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulfonic acid
	NF279	8, 8'-(Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis(1,3,5-naphthalenetrisulfonic acid)
	NG 108-15	Neuroblastoma x glioma hybrid cell line
	NMP	Nucleoside 5'-monophosphate
	NO	Nitric oxide
	NOS	Nitric oxide synthase
	NTP	Nucleoside 5'-triphosphate
P	P	Probability level
	pA_2	Negative logarithm of the antagonist concentration shifting the agonist concentration-response curve twofold to the right
	pA_x	Negative logarithm of the antagonist concentration used
	PC12	Pheochromocytoma cells
	PCR	Polymerase chain reaction
	pD_2	Negative logarithm of the agonist concentration producing 50 % of the maximum effect
	p-F-HHSiD	(±)-para-Fluoro-hexahydro-sila-difenidol
	Pi	Inorganic phosphate
	pH	Negative logarithm of the concentration of hydrogen ions
	pIC_{50}	Negative logarithm of the agonist/antagonist concentration producing halfmaximal inhibition
	PKA	Protein kinase A
	PKC	Protein kinase C
	pK_i	Negative logarithm of the equilibrium dissociation konstant of inhibitor
	PLA_2	Phospholipase A_2
	PLD	Phospholipase D
	PLC	Phospholipase C
	PPADS	Pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid
	PPi	Inorganic pyrophosphate
	PPNDS	Pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate)
	PTAC	6-(3-Propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo-[3.2.1]-octane
	PTX	Pertussis toxin
Q	QNA	3-(1-Azabicyclo[2.2.2]octyl)2-hydroxy-2-phenylpropionate
R	r	Correlation coefficient
	RB-2	Reactive blue 2

	RI	Rat ileal longitudinal smooth muscle
	RNA	Ribonucleic acid
	RVD	Rabbit vas deferens
S	s	Second(s)
	SAR	Structure-activity relationship
	SB9	6-[(4,6,8-Trisulfo-1-naphthyl)iminocarbonyl-1,3-(4-methylphenylene)iminocarbonyl-1,3-phenylene-azo]-pyridoxal-5'-phosphate
	S.E.M.	Standard error of the mean
T	TM	Transmembrane domain
	TTX	Tetrodotoxin
	$t_{1/2}$	Dissociation half-life
U	UDP	Uridine 5'-diphosphate
	UTP	Uridine 5'-triphosphate
V	V	Volt
	VIP	Vasoactive intestinal polypeptide
	vs.	Versus
W	w/v	Weight per volume
X	XTP	Xanthine 5'-triphosphate

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10. ACTIVE PARTICIPATION IN CONGRESSES AND SYMPOSIA

November 1996
Seventh International Symposium on Subtypes of Muscarinic Receptors, Vienna, Virginia, USA

Czeche, S., Elgert, M., Noe, C., Waelbroeck, M., Mutschler, E. and Lambrecht, G. (1996). Antimuscarinic properties of the stereoisomers of glycopyrronium bromide. *Life Sci.* **60**, 1167.

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Deutsche Gesellschaft für Pharmakologie und Toxikologie, 38th Spring Meeting, Mainz, Germany

Czeche, S., Elgert, M., Noe, C., Waelbroeck, M., Mutschler, E. and Lambrecht, G. (1997). Stereoselective interaction of glycopyrronium bromide with four muscarinic receptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355** (Suppl.), R24.

March 1997
Deutsche Pharmazeutische Gesellschaft, 9th Spring Meeting for the New Generation of Academics, Würzburg, Germany

Czeche, S., Elgert, M., Noe, C., Waelbroeck, M., Mutschler, E. and Lambrecht, G. (1997). Stereoselektive Wechselwirkung von Glycopyrroniumbromid mit vier Muskarinrezeptor-Subtypen. Book of abstracts.

February 1998
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Czeche, S., Niebel, B., Bäumert, H.G., Mutschler, E. and Lambrecht, G. (1998). Neuronal P2X-like receptors mediate cholinergic contraction via postjunctional

muscarinic M_3-receptors in guinea-pig ileal longitudinal smooth muscle. *Pharmazie* **53** (Suppl. 1), 11.

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May 1998

Sixth International Symposium on Adenosine and Adenine Nucleotides, Ferrara, Italy

Czeche, S., Niebel, B., Mutschler, E., Nickel, P. and Lambrecht, G. (1998). P2-receptor heterogeneity in the guinea-pig ileal longitudinal smooth muscle preparation. *Drug Dev. Res.* **43**, 52.

August 1998

Eighth International Symposium on Subtypes of Muscarinic Receptors, Danvers, Massachusetts, USA

Czeche, S., Niebel, B., Mutschler, E. and Lambrecht, G. (1998). Facilitation of acetylcholine release from myenteric neurones by α,ß-methylene ATP causes a contraction of the guinea-pig ileum via postjunctional M_3 receptors. *Life Sci.* **64**, 592.

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Damer, S., Niebel, B., Czeche, S., Nickel, P., Ardanuy, U., Schmalzing, G., Rettinger, J., Mutschler, E. and Lambrecht, G. (1998). NF279: A novel potent and selective antagonist of P2X receptor-mediated responses. *Eur. J. Pharmacol.* **350**, R5-R6.

Lambrecht, G., Damer, S., Niebel, B., Czeche, S., Nickel, P., Rettinger, J., Schmalzing, G. and Mutschler, E. (1999). Novel ligands for P2 receptor subtypes in innervated tissues. *Prog. Brain Res.* **120**, 107-117.

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12. CURRICULUM VITAE

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22. Februar 1968	geboren in Gotha/Thüringen
1974 - 1976	Polytechnische Oberschule Otto-Grotewohl, Gotha
1976 - 1984	Polytechnische Oberschule mit Erweitertem Russisch-unterricht Anna-Seghers, Gotha
1984 - 1986	Erweiterte Oberschule Arnoldi, Gotha
1986 - 1988	Sertürner-Apotheke in Gotha
09/1986 - 08/1987	Ausbildung zur Apothekenfacharbeiterin
05/1988 - 08/1989	Facharbeiter für Galenische Forschung im VEB Arzneimittelwerk Dresden
09/1989 - 09/1993	Studium der Pharmazie an der Martin-Luther-Universität Halle/Saale
09/1993 - 04/1994	Diplomarbeit am Pharmakologischen Institut der Martin-Luther-Universität Halle/Saale
05/1994 - 09/1994	Pharmaziepraktikantin in der Steintor-Apotheke in Halle/Saale
11/1994	Approbation als Apothekerin
seit 12/1994	Anfertigung der vorliegenden Dissertation am Pharmakologischen Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Frankfurt/Main
seit 01. Mai 1999	Referentin bei der ABDA - Bundesvereinigung Deutscher Apothekerverbände, Abt. Apotheker/Apotheke, Bereich Aus- und Fortbildung, Eschborn/Taunus
10.10.1999	Geburt von Lea