High level production, characterization and structural analysis of neuronal calcium-activated potassium channels

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To Accursio, the nicest, sincere, vital, wonderful and lovely father that I could have wished for and to his infinite love for me.

To Alessandro, my great master of science and life and to his elegant mind.

To their love for life that I keep with me.

Muere lentamente
quien se transforma en esclavo del hábito,
repitiendo todos los días los mismos trayectos,
…y no le habla a quien no conoce.
Muere lentamente
quien evita una pasión,
quien prefiere el negro sobre blanco
y los puntos sobre las "íes" a un remolino de emociones,
justamente las que rescatan el brillo de los ojos,
sonrisas de los bostezos,
corazones a los tropiezos y sentimientos.
Muere lentamente
quien no voltea la mesa cuando está infeliz en el trabajo,
quien no arriesga lo cierto por lo incierto para ir detrás de un sueño,
quien no se permite por lo menos una vez en la vida,
huir de los consejos sensatos.
Muere lentamente
quien no viaja,
quien no lee,
quien no oye música,
quien no encuentra gracia en si mismo.
Muere lentamente
quien destruye su amor propio,
quien no se deja ayudar.
Muere lentamente,
quien pasa los días quejándose de su mala suerte
o de la lluvia incesante.
Muere lentamente,
quien abandona un proyecto antes de iniciarlo,
no preguntando de un asunto que desconoce o
no respondiendo cuando le indagan sobre algo que sabe…

Pablo Neruda

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Abstract

Calcium-activated potassium channels are fundamental regulators of neuron excitability. SK channels are activated by an intracellular increase of Ca^{++} (such as occurs during an action potential). They have a small single channel conductance (less than 20pS) and show no voltage dependence of activation.

To date, there are only a few examples of high-resolution structures of eukaryotic membrane proteins. All of them were purified from natural sources.

Since no abundant natural sources of eukaryotic K^+ channels are available we overexpressed rSK2 in order to produce the quantities necessary for structural analysis.

Unfortunately the *Pichia pastoris* expression system did not yield sufficient amount of pure protein, mainly because most of the protein was retained by in the ER and was only partially soluble.

Subsequently, two constructs were expressed: SK2-FCYENE (containing a specific sequence that promotes surface expression), and SK2-q-CaM a concatamer of SK2 and calmodulin. Although these proved an improvement in terms of solubilisation, little improvement was found in terms of amounts of purified material obtained.

For this reason we tested the Semliki Forest virus expression system, since the protein is expressed in a mammalian system where we hoped that it would be trafficked in the same way as *in vivo*.

Using this system it was possible to express rSK2 and solubilise it with several detergents and to achieve much better purification. However, the levels were still not sufficient for high-resolution structural studies, although sufficient for single particle electron microscopy analysis.

CHAPTER ONE

Introduction

1.1 The anatomy of the brain

The brain is the most complex part of the human body and the seat of intelligence, interpreter of senses, initiator of body movement, and controller of behaviour.

The brain is able to analyze information coming from peripheral receptors and to create perceptions, some of which are stored in memory.

Any cerebral process, either higher functions (learning, imagination, language) or simplest spinal reflexes, has the same cellular substrate: the neuron. The human brain contains as many as 10^{11} neurons. Although there are many different types of nerve cells, they all share common features. The functional versatility of neurons is based on a few principles that give rise to considerable complexity.

All sensations, movements, thoughts, memories, and feelings are the result of signals exchanged between neurons. Neurons consist of three parts: the cell body, the dendrites and the axon. The cell body, also known as the soma, includes the nucleus and perikaryon and is involved in the main metabolic functions of the cell; dendrites extend out from the cell body like the branches of a tree and receive messages from other nerve cells. Signals pass from the dendrites through the cell body and may travel away from the cell body down an axon to contact another neuron, a muscle cell, or cells in some other organ. The axon is the conduction component of the neuron, usually surrounded by many support cells that entwine the axon to form an insulating layer. This sheath is composed of multiple layers of myelin, which is chiefly lipid membrane and thus has a high electrical resistance. This causes the nerve signals to travel faster and further than in non-myelinated neurons. Axons may be very short, such as those that carry signals from

one cell in the cortex to another or may be very long, such as those that carry messages from the brain, down the spinal cord (Zigmond M.J. 1999; Kandel E.R. 2000).

1.2 The synapse

The structure through which a neuron interacts with another one or with other cell types is called the synapse and the space between the cells is called the synaptic cleft. The neuron sending out information is called the pre-synaptic cell and the cell receiving information is called the post-synaptic cell. When the signal reaches the end of an axon it induces the release of chemicals (neurotransmitters) into the synapse. Neurotransmitters are synthesized in the cell body and are transported to the terminal synaptic buttons of the axon where they are encapsulated into vesicles and stay close to the synaptic region. When an action potential occurs, an influx of calcium ions induces the vesicles to fuse with the pre-synaptic membrane and their contents are poured into the synaptic cleft. The neurotransmitters cross the synapse and bind to receptors on the post-synaptic cell. After binding to their ligands, the receptors trigger a cascade of events that end with the change of post-synaptic membrane potential. This event can result from either direct depolarization or hyperpolarization, or from activation of second messenger cascades that eventually lead to changes in firing rate.

Neurotransmitter receptors are transmembrane proteins with an extracellular site that binds the neurotransmitter and, after this binding, undergo some conformational change. In many cases, the receptor is an ion channel that can directly open or close upon ligand binding (direct gating). A second type of receptor is coupled to intracellular second messenger cascades that can also affect ion channels and consequently membrane potential. Each such receptor is coupled to its effector molecule by a guanosine nucleotide-binding proteins (G proteins). Typically, the effector is an enzyme that produces a second messenger, like cyclic adenosine monophosphate (cAMP), diacylglycerol, or an inositol polyphosphate. These second messengers activate a signal transduction pathway that involves either phosphorylation of different cellular proteins or mobilization of internal Ca^{2+} ions. The G proteins can also act by binding the ion channel directly.

Each neurotransmitter is produced through a specific biosynthetic pathway. However, three main classes can be described that share some enzymes or parts of metabolic pathways. The first class is made up of acetylcholine alone, the second class comprises biogenic amines, which derive from amino acid modifications; the third class is made up of amino acids *per se*.

There are also specific chains of enzymatic reactions that remove the transmitter from the synaptic cleft, either by destruction or by recycling it back to the producing neuron or, alternatively, to surrounding glial cells (astrocytes, in particular). This is important to stop unwanted signal transmission.

Neurotransmitters can act as either inhibitory or excitatory signals to the postsynaptic cell, by inducing hyperpolarization or depolarization of the plasma membrane. Some neurotransmitters can function both as inhibitors and exciters. This is because that while there are only a small number of neurotransmitters, there are a great variety of receptors on different types of cells.

Synaptic actions triggered by second messengers can cause ion channels, that are open at the resting potential, to close, thereby decreasing the conductance of the membrane. In addition, second messengers can alter the biochemical state of nerve cells, even by modulating gene expression and triggering a persistent change in function. For example, long lasting cellular modifications are extremely important in understanding memory formation. A single exposure to the transmitter can activate the cAMP second messenger system, and the cAMP-dependent protein kinase (PKA), that phosphorylates a K^+ channel, thus producing a change in synaptic potential that modifies neuronal excitability for a very short period of time. However, repeated exposure to the transmitter and the consequent activation of the same cascade (PKA-dependent), can also cause phosphorylation of one or more transcriptional activator proteins that regulate the expression of a gene encoding a protein able to modify the channel. This late event results in a more enduring alteration of the channel and in changes of neuronal excitability that last for days (Jacobson 1991; Siegel G.J. 1999; Zigmond M.J. 1999; Kandel E.R. 2000).

1.3 Ionic basis of the action potential

Every neuron (in fact every cell) has a specific distribution of electrical charges across its plasma membrane. The membrane potential results from a separation of positive and negative charges across the cell membrane. At the resting state, there is an excess of positive charges outside the cell and negative charges inside the cytoplasm. This distribution (resting potential: no signalling activity) is maintained because the lipid bilayer acts as a barrier to the diffusion of ions, and gives rise to an electric potential difference, which ranges from about 60 to 70 mV. Since, by convention, the potential outside the cell is arbitrarily defined as zero, and due to the high concentration of negative charges inside the membrane, the potential difference across the membrane is expressed as a negative value: Vr = -60 to -70 mV.

A reduction of the charge separation is called depolarization; conversely an increase in charge separation is called hyperpolarization. Transient current flow and therefore rapid changes in potential are triggered by ion channels, a class of integral membrane proteins. There are two types of ion channels in the membrane: gated and non-gated. Non-gated channels are always open: they are primarily important in maintaining the resting membrane potential. Gated channels, in contrast, open and close in response to specific electrical, mechanical, or chemical signals. Since ion channels recognize and select specific ions, the distribution of ionic species across the membrane depends on the particular distribution of ion channels in the cell membrane. Ion species are not distributed equally on the two sides of a nerve membrane: Na^+ and Cl^- are more concentrated outside the cell, while K^+ and organic anions are more concentrated inside (Hodgkin and Huxley 1952). There are two forces acting on a given ion species: the driving force of the chemical concentration gradient tends to move ions down the gradient (chemical potential) and the electrostatic force due to the charge separation across the membrane tends to move ions in a direction determined by its particular charge (Hille 1986). For example, chloride ions, which are more concentrated outside the cell, tend to move inward, down their concentration gradient through non-gated chloride channels. However, the relative excess of negative charge inside the cell favours chloride ion flow out of the cell. The reverse is true for the potassium ions. Na^+ is more

concentrated outside than inside and therefore tends to flow into the cell down its concentration gradient. Secondly, Na^+ is driven into the cell by the electrical potential difference across the membrane. Thus, at the resting membrane potential, the efflux of K^+ ions balances the movement of Na^+ ions into the cell (Hille 1970). Na^+-K^+ pumps prevent dissipation of ionic gradients, which extrudes Na^+ from the cell while taking in K^+. In addition, some cells also have chloride pumps that actively transport chloride ions toward the outside so that the ratio of extracellular to intracellular concentration of Cl^- is greater than the ratio that would result from passive diffusion alone. When a transient depolarizing potential occurs, some voltage-gated Na^+ channels open, and the increased Na^+ permeability allows Na^+ influx to exceed the K^+ efflux. Consequently, an influx of positive charge flows occurs; accumulation of positive charges inside the cell causes in turn further depolarization, opening of additional voltage-gated Na^+ channels and acceleration of depolarization. At the same time, K^+ efflux continues through the K^+ channels, and a slight diffusion of Cl^- into the cell also counteracts the depolarizing tendency of the Na^+ influx. The high number of voltage-gated Na^+ channels opened during the rising phase of the action potential creates permeability to Na^+ that is much higher than permeability to Cl^- and K^+. Two processes allow membrane re-polarization: i) inactivation of voltage-gated Na^+ channels and ii) delayed opening of voltage-gated K^+ channels. The combination of these two processes produces a net efflux of positive charge from the cell, which continues until the cell re-polarizes to its resting value. The action potential starts at the axon hillock where there is enrichment of voltage gated ion channels. Once the depolarization/repolarization wave reaches the axon, synaptic transmission takes place and another round of communication starts between the nerve cells (Hille 1992).

1.4 Ion Channels: Biophysical characteristics and ionic distribution and action potential

Ion channels often consist of several different subunits, where each subunit has a different function. Each subunit can be encoded by different genes. The functional diversity of ion channels is accomplished by combining different isoforms or by alternative splicing (Hille

1992; Jegla and Salkoff 1994). To date, there are more than 50 genes encoding for ion channels known (Hille 1992; Catterall 1994). Usually the voltage-gated channels have a main subunit, called the subunit, which carries out the function of the channel, and auxiliary subunits that modulate the physiological properties. In the brain, there are several different types of ion channels, but they all have some distinctive characteristics. Ion channels are usually glycoproteins that span the membrane several times. A common feature of channels is that they form multimeric assemblies, which create a pore inside the membrane where the ions can pass through. Generally, every channel is selective for a specific ion; in fact the flow of each ion depends on the electrostatic field inside the pore. The distribution within the same cell has great variability (Hille 1970; Hille 1992; Catterall 1994), usually the axon has larger density of sodium and potassium channels than the cell body, the myelinated fibers have a large number of Na^+ channels in the node of Ranvier, the vertebate neuromuscular junction has a high concentration of acetylcholine ligand-gated channels, while the Cl^- channel are very concentrated in muscle cells because they determine the resting membrane conductance (Marek and Davis 2003; Trimmer and Rhodes 2004). This distribution illustrates an elegant and complex system of protein localization (Futerman and Banker 1996; Weinberger et al. 1996; Hannan et al. 1998). Only a few ion channels are always open (called leak channels or non-gated channels), all the others have complex ways of opening and closing and are called gated channels. A conformational change of the channel may allow the opening or closing either passively or with energy expenditure. Sometimes channels change physical aspects inside the pore, thus leaving no space for ion flux, and sometimes the change involves a different distribution of charges inside the pore that constrain passage of charged particles. The open or closed state of a channel depends on signals coming from either inside or outside the cell. In voltage-gated channels, opening is controlled by the membrane potential. These channels have a high homology in their amino acid sequence called the S4 region. This sequence contains a set of positively charged amino acids (lysine and/or arginine) located in the transmembrane region of the protein. These charges act as the voltage sensor of the channel. Some gated channels need to be activated by specific small molecule ligands or by phosphorylation. The ligand-gated channels can be gated from the inside or from the outside of the cells. Gating from

outside is very important in chemical synaptic transmission and the molecules are usually neurotransmitters. Channels gated from the inside of the cell are generally controlled by second messengers, which are small signalling molecules inside the cell, for example cAMP and cGMP or internal calcium. These are called direct-gated receptors, or ionotropic receptors. One example of this is a potassium channel gated by the beta-gamma subunit of the G protein, which is a common signalling protein activated by certain membrane receptors (Hille 1992). Other channels can be activated or inactivated by protein phosphorylation.

1.5 Characteristic of potassium channels

Potassium channels display exceptional functional diversity. Many essential physiological processes are controlled by a cell's ability to rapidly and selectively alter its permeability to ions. Potassium channels are unique because the equilibrium potential for K^+ ions is very close to the resting membrane potential of the neuron. When the K^+ ions flow out of the cells, they cause hyperpolarization and consequently the membrane equilibrates to reach the resting potential; the same occurs when the ions flow into the cells, in this case there is depolarization but the effect is again to reach the resting potential. In the brain there are different families of K^+ channels that differ in their conductance and ion selectivity. The pore region of K^+ channels is highly conserved across the channel family (Jan and Jan 1990) but small changes in the amino acid residues can give functional diversity.

The first potassium channel gene was isolated in the 1987 from the fruit fly, *Drosophila melanogaster* and was called *Shaker* (Kamb et al. 1987). The channel was a voltage dependent K^+ channel and it was named after the observation of a mutant of this gene. The flies with this mutation strongly shook their legs in presence of ether. The region of the *shaker* mutation was found using cytological techniques on chromosome X and localized using the chromosome walking technique (Kamb et al. 1987; Papazian et al. 1987; Baumann et al. 1988; Schwarz et al. 1988). The shaker locus has a large open reading frame of 21 exons that can produce several transcripts by alternative splicing (Papazian et al. 1987; Tempel et al. 1987; Iverson et al. 1988). Only after the expression

of shaker in *Xenopus* oocytes and physiological studies, was it possible to confirm that the shaker protein was really a K^+ channel (Tempel et al. 1987; Iverson et al. 1988; Papazian et al. 1988; Tempel et al. 1988; Timpe et al. 1988). Using the shaker cDNA as a probe, it was possible to identify a K^+ channel from rat brain, RCK1 (Baumann et al. 1988) and RBK1 (Christie et al. 1989), and some inward rectifying channels (Kubo et al. 1993; Kubo et al. 1993). Subsequently, the RCK1 sequence was used to probe cDNA libraries and led to the isolation of several more neuronal voltage-dependent K^+ channel clones ((Stuhmer et al. 1988). At the same time, 3 more voltage-dependent K^+ channels (Shab, Shal and Shaw) were identified in *Drosophila* by cross-hybridisation with the shaker sequence (Covarrubias et al. 1991). As for shaker, equivalents of these channels were also found in mammalian cDNA libraries. Mammalian potassium channel subunits are encoded by multiple separate genes and not as in *Drosophila* by alternative splicing of a large transcriptional unit.

Molecular cloning and mutagenesis experiment has revealed that all potassium channels have the same pore constitution; all contain a conserved amino acid region, called the pore region, composed of the sequence GYG. The only exceptions are Kir6.2 and KCV which have GFG instead of GYG.

Whenever one of these amino acids are mutated, the channel is no longer able to discriminate between K^+ and Na^+ ions (Doyle et al. 1998).

1.5.1 Potassium channel families

Today, many potassium channels have been cloned and identified. Physiological and functional studies as well as and structural analysis show that they can be divided in different groups.

There are at least ten types of K^+ channel, each responding in different ways to different stimuli: voltage-sensitive [Ka, Kv, Kvr, Kvs and Ksr], known also as 6 TM (transmembrane) channels, Ca^{2+}-sensitive [BKCa, IKCa and SKCa] and receptor-coupled (KM and $KACh^+$ channels), that can have either 6 TM or 7 TM motifs. Inward rectified channels (Kir channels) that are 2 TM channels. The yeast Tok1 K^+ channel that has a 8 TM motifs. The TWIK-1 and -2, TREK-1, TRAAK, and TASK-1, all exhibit a duplicated 2 transmembrane unit and may therefore form a homodimeric

channel instead of a tetramer. A schematic model of potassium channel topology is shown in figure 1.

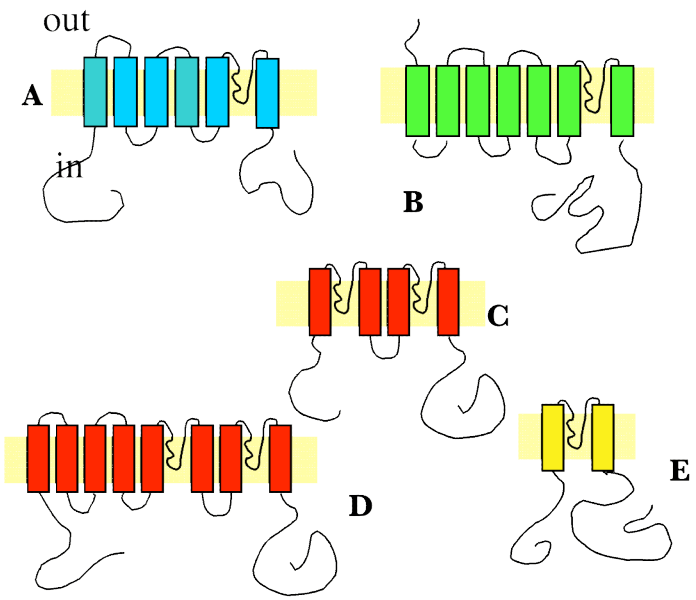

Figure 1: K^+ Channel Topologies. In this figure a shematic model of the secondary structure of different potassium channel families is shown, common feature is the pore region composed of two transmembrane segment and a re-enter loop. A) Typical six transmembranes motif of the voltage–gated potassium channels (Kv), like as well the small calcium activated potassium channel (SK). B) Seven transmembranes motif, that correspondes to the predicted topology of the large calcium activated potassium channel (BK). C) Four transmembrane unit with two pore regions of TWIK-1 and -2, TREK-1, TRAAK, and TASK-1 D) Eight transmenbrane domain of the Tok1 potassium channels, two pore regions are present. E) Two transmenbrane motif of, for example, the inward rectified potassium channel and the KcsA.

1.5.2 Regulation of Potassium Channel Assembly and Trafficking

Some potassium channels have trafficking motifs that control their movements between the endoplasmic reticulum (ER) and the Golgi apparatus, and between the plasma membrane, endosomes, and lysosomes. These motifs control the number and the subunit composition of channels on the plasma membrane (Ma and Jan 2002). For example, the

ATP-sensitive potassium channels have an ER retention/retrieval motif that guarantees the membrane insertion of only properly regulated octamers (Zerangue et al. 1999). Many voltage-gated potassium channels (Shaker family) have ER export motifs important in targeting these Kv1 channels to the axonal membrane. Motifs important for controlling Kv1.2 channel density on the axonal membrane of hippocampal neurons were identified. Mutations of the Kv1.1 channel gene increase the likelihood of action potential reflection from the motor nerve terminals and probably also the extent of action potential invasion of central neuronal axon branches, leading to behavioral abnormalities characteristic of episodic ataxia in patients and shaking phenotypes in mutant mice and flies (Ma et al. 2001; Ma and Jan 2002).

1.5.3 X-ray structure of potassium channels

The K^+ channel from *Streptomyces lividans* KcsA was the first whose structure was determined. This channel is one of the two transmembrane class, although the pore region is more homologous to mammalian voltage-dependent channels than inward rectifiers. The structure at 2 Å shows considerable detail in the pore region and reveals the mechanism of the selectivity filter (Doyle et al. 1998; Zhou et al. 2001) (figure 2). In KcsA the ion selectivity filter is 2.8 Å wide and 12 Å length. The potassium ions are attracted into the pore by the negative charge of a glutamic acid at the channel mouth, and then go into the chamber region, and finally the ions enter the selectivity filter by discarding water molecules and interacting with the carbonyl-oxygen atoms lining the pore. At the end the ions experience the field of a negative charged aspartic acid residues and it leaves the channel and enters into the extracellular space.

The potassium ion has a crystal radius of 1.33 Å, but in the cell, eight water molecules surround it, so the final radius is 4.2 Å.

The pore is so tight that the potassium ions have to lose their hydration shell to enter. The coordination by water-oxygen in the hydrate is replaced in the pore by coordination by the carbonyl-oxygen. The loss of energy by dehydration is compensated by interchange with the oxygen atoms of the protein.

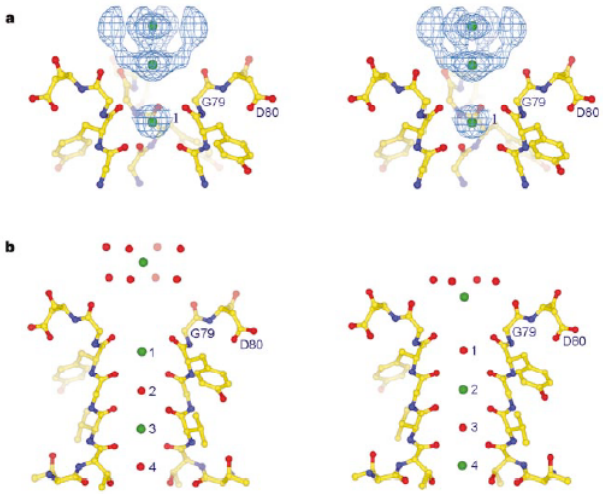

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Figure 2: Selectivity filter of the bacterical KcsA potassium channel. Potassium ion dehydration at the extracellular pore entryway. a) Stereo view of electron density at the extracellular pore entryway. Amino acids are shown in ball-and-stick representation and K^+ ions as green spheres. At position 1, the density for the K^+ ion is shown for reference. b) Interpretation of the two ion peaks outside the selectivity filter. When the configuration of ions (green spheres) and water (red spheres) inside the filter is K^+-water-K^+-water (left), an ion at the entryway is moved further away. When the configuration is water-K^+-water-K^+ (right), the ion outside the filter is drawn closer to the pore (From (Zhou et al. 2001).

Although the sodium ion is smaller than the potassium, it is unable to pass the selectivity filter in the presence of K^+. The selectivity appears to be conferred by the carbonyls in the pore, although it is not due to a size effect as the protein backbone probably fluctuates by up to 0.8 angstroms which is more than the difference between Nathe raddi of $^+$ and K^+ (Allen and Chung 2001; Chung et al. 2002; Chung and Kuyucak 2002; Berneche and Roux 2003; Allen et al. 2004).

1.5.4 Voltage-gated potassium channels

Voltage-gated potassium channels (Kv) can be classified into 9 families.

Usually, they have four identical domains, each consisting of about 300 amino acids and linkers of varying length connect them. Within the same family, different principal subunits can also assemble into functional heteromeric potassium channels. Each domain contains six putative transmembrane segments designated as S1- S6. S5 and S6 are always hydrophobic, while the other four segments are amphipathic. S1 and S3 have a net negative charge whears S4 is positively charged and is the segment that contains the voltage sensor, S2 has both positively and negatively charged regions.

Almost twenty years of electrophysiological, biochemical and mutagenesis studies have resulted in a common gating model. In this model, when the channel switches between the open and closed states, there are four charges per channel subunit that are translocated across the membrane. Gating appears to be due to a rotation of the four rigid helices surrounding the pore, S4 tilts in the membranes, exposing charged residues to solvent and causing torsion on S5 and S6, resulting in a open state.

However, recently, the Mackinnon group published the 3 Å resolution structure of the voltage-gated potassium channel from the thermophilic organism *Aeropyrum pernix* (Jiang et al. 2003) and a 1.9 Å resolution of the voltage sensor domain, that suggested a new and controversial gating model (Jiang et al. 2003).

The structure reveals a 4-fold symmetric tetramer with an antibody Fab fragment (required for crystalisation) bound to the S4 sequence at the outer perimeter of each subunit. This new structure shows several differences to the old model. The S3 helix, for example, is formed by two individual helices, S3a and S3b separated by a loop. The S3b helix and the amino terminal half of S4 are packed together forming a helix-turn-helix motif, the authors call this S3b-S4 unit the voltage sensor paddle. The position of the S4 helix is near the intracellular membrane surface, perpendicular to the pore axis. In the new model, it is the moving of the paddle across the membrane near to the protein-lipid interface that represents the gating charge movement (Figure 3).

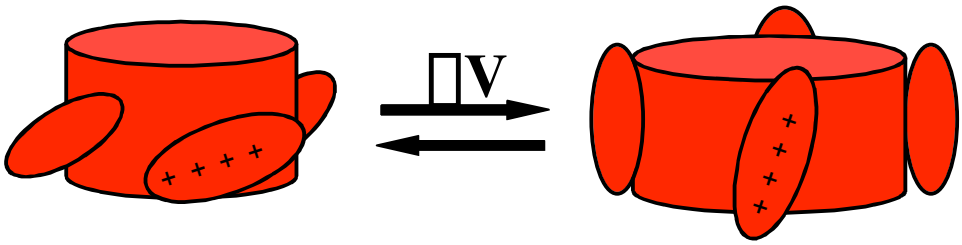


Figure 3: Proposed gating charge movements in the new model of the Kv channel activation. The gating charges (plus signs) in the S4 helices are supposed to move through the membrane from inside (bottom) to outside (top) by movements of the voltage-sensor paddles against the lipid membrane. This movement then lead to opening of a gate or gate and ion movement.

This structure is the center of a major debate, especially concerning the position of the S4 segment. In the structure the S4 segment is not parallel to the other helices, as predicted before, rather it is positioned at the periphery of the channel. During gating it does not move as piston, but instead moves through the lipid membrane, going from the intracellular side to the extracellular side of the membrane.

The new model means that the positively charged segment stays in a hydrophobic environment and during gating makes a 20 Å movement through the membrane.

One other big controversial feature is the position of the glycosylation site between the S1 and S2 segments that in the structure is found within in the membrane.

It has been suggested that the Fab fragment modifies the real structure of the channel (Miller 2003).

1.5.5 Calcium-activated potassium channels

Calcium-activated potassium channels are fundamental regulators of neuron excitability.

The activity of these channels is implicated in many physiological processes, including

neurosecretion and smooth muscle tone. Calcium activated potassium channel can be divided into three subfamilies: SK channels (small conductance calcium potassium channels), BK channels (large conductance calcium potassium channels) and IK channels (intermediate conductance calcium potassium channels).

1.5.5.1 Small calcium-activated potassium channels

Small calcium-activated potassium (SK) channels are characterized by a single channel conductance of less than 20 pS. They are activated by an intracellular increase of Ca^{2+} (such as occurs during an action potential) and show no voltage dependence of activation. SK channels subunits share an overall transmembrane topology with voltage gated potassium channels, exhibiting six transmembrane domains with amino and carboxyl termini residing within the cell. The only notable homology with other potassium channels is in the pore region, between the fifth and sixth domains. These channels are implicated in physiological processes like learning, memorization, regulation of circadian cycles and disruption of normal sleep patterns.

In the last decade, electrophysiological experiments in various systems have provided strong evidence that calcium channels, kinases and phosphatases must be in close proximity to calcium-activated potassium channels (Poolos and Johnston 1999).

Three members of the SK family of K^+ channels have been cloned (Kohler et al. 1996). All of them are voltage-insensitive and activated by submicromolar intracellular Ca^{2+}.

The channel shows a characteristic sensitivity to apamin which is useful indicator of the channel functionality. Toxins are an extremely valuable tool in studying ion channel structure, function and physiology. For example, the scorpion toxin charybdotoxin has been extensively used to achieve insights on the structure of the outer pore of the voltage-sensitive channels. Apamin is a bee venom neurotoxin polypeptide of 18 amino acids with sulphur bridges. The injection of sub lethal doses into mice causes extreme uncoordinated hypermotility and in a lethal dose it produces tonic convulsions and respiratory distress (Hugues et al. 1982).

Apamin specifically blocks SK2 and SK3 channels, whereas SK1 is apamin insensitive (Kohler et al. 1996). The use of this toxin in combination with in situ hybridization has

enabled the correlation of specific SK channel subunits with calcium-activated potassium currents in the central nervous system and has revealed the physiological function of SK-mediated currents in different neurons.

It has been found that Asp and Asn residues on each side of the SK pore domain are essential for apamin blocking, presumably due to electrostatic interactions with the basic apamin (Ishii et al. 1997).

Ca^{++} activated potassium channels have been detected by apamin binding experiments in rat brain synaptosomes (Habermann and Fischer 1979; Hugues et al. 1982), in primary neuronal cultures (Seagar et al. 1984), neuroblastoma (Hugues et al. 1982) and muscle cells (Hugues et al. 1982). In addition, scanning electron microscopy showed that there is a homogeneous distribution of the apamin binding protein/ SK channel on neuronal cell bodies (Schwarz and Passow 1983).

1.5.5.1.2 Biophysical characteristic of small calcium-activated potassium channels

In the central nervous system, the three SK channel isoforms (SK1, -2, -3) differentially distributed throughout the brain and selectively expressed in neuronal compartments, with elevated levels of expression in the regions where after hyperpolarization currents are common such as neocortex (SK1 and SK2), monoaminergic neurons (SK3), and in the hippocampus (SK1 and SK2) (Stocker and Pedarzani 2000).

In cultured hippocampal neurons using high-resolution immunofluorescence analysis, SK3 was localized in a punctate, synaptic pattern. This suggests SK3 may be a presynaptic channel in excitatory hippocampal synapses, and therefore involved in regulating neurotransmitter release (Obermair et al. 2003).

A single or a train of action potentials in neurones is followed by three types of afterhyperpolarisations (AHP): fast AHP, medium AHP and slow AHP. These AHPs contribute to the regulation of excitability and to the encoding function of neurons. The currents underlying the AHP are mediated by SK channels that can be divided into two groups depending on their kinetic and pharmacological properties (Sah 1996): medium AHP is sensitive to the bee venom toxin apamin and presents a relatively fast activation

and decay (Sah and McLachlan 1991); slow AHP is apamin insensitive, rises to peak and decays with time constants of several hundreds of milliseconds, and can be modulated by many neurotransmitters (Lancaster and Adams 1986; Storm 1990).

Activation of mI_{AHP} controls the firing frequency in tonically spiking neurons, whereas activation of sI_{AHP} leads to spike frequency adaptation (Sah 1996). While most excitable cells present an apamin-sensitive I_{AHP}, only a few nerve cells exhibit an apamin-insensitive sI_{AHP}, or both types of currents (Storm 1990; Sah 1996).

In many neurons of the central nervous system, action potentials are followed by a rise in intracellular Ca^{++} concentration that causes a prolonged after hyperpolarization of the membrane (Lancaster and Adams 1986; Constanti and Sim 1987; Schwindt et al. 1988; Storm 1990). Ca^{2+}-dependent changes in membrane K^+ permeability can also coordinate cell volume, metabolism and membrane transport.

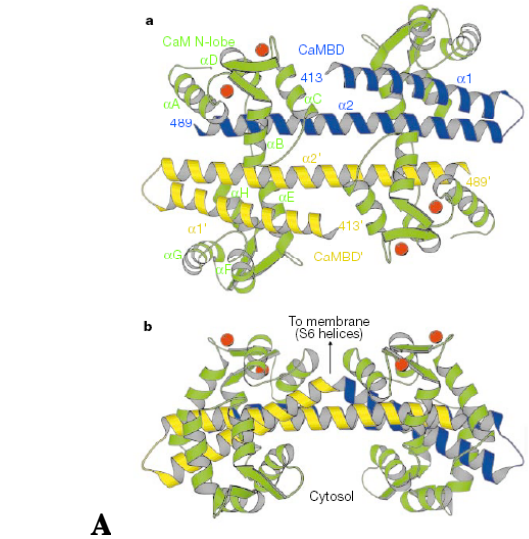
Interestingly, mice treated with apamin show memory potentiation because the blocking of SK channels leads to an increase in the excitability of hippocampal neurons and facilitates the induction of synaptic plasticity (Stackman et al. 2002).

1.5.5.1.3 SK2 channels and their interaction with calmodulin

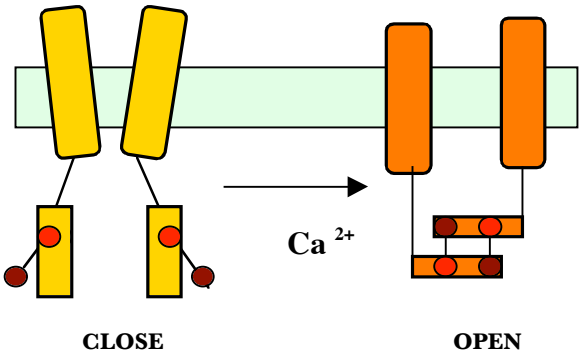
The SK2 channel has two subunits of 86 KDa (□) and a 30 KDa □) component, identified only by apamin cross-linking.

The SK protein alone is sufficient to produce Ca^{2+} sensitive channels and yet does not possess any known Ca^{2+} binding motif. This puzzle was resolved by the discovery that SK can bind calmodulin. The specific region of the channel that binds calmodulin is at the C-terminus (residues 463 to 497 in SK2).

Calmodulin is a small Ca^{2+}-binding protein, which acts as Ca^{2+} sensor in cells. It interacts with key components of numerous signaling pathways and together with kinases, phosphatases, cytoskeletal and ribosomal proteins plays a role as regulator in several cellular processes. It is a loop-helix-loop Ca^{2+} binding protein and when it binds Ca^{2+} it undergoes a remarkable conformational change.



A



B

Figure 4: A) Structure of the SK2/Ca2+/CaM complex. a) SK2 subunits (only the calmodulin binding domain) are in blue and yellow, CaM molecules are in green, and the Ca^{2+} ions are in red. Secondary structural elements, the CaM linker and the First and last observed residues in the CaMBD are labelled. b) View in a rotated by 90° showing the orientation of the complex relative to the membrane. Arrow indicates the positions of the first observed residue of each of the CaMBD monomers that are linked to the S6 pore helices (From (Schumacher et al. 2001).

B) Proposed chemo-mechanical gating model. Cutaway view showing only two of the four subunits of the channel, the S6 pore helices, the attached CaMBD and the linker (line) are shown. The CaM C-lobe and N-lobes are red and orange. Biochemical data demonstrates that the CaMBD/CaM complex is monomeric in the absence of Ca^{2+} and dimeric in presence. In this model, a rotary movement is the consequence of the formation of the dimeric complex that could drive a rotation between the S6 helices to open the gate.

The structure of the SK2 carboxyl terminal complexed with calmodulin has been solved by X-ray crystallography (Schumacher et al. 2001) (figure 4). Each SK carboxyl terminal links a calmodulin with or without the presence of Ca^{2+} in a 1:1 ratio. Activation of the channel occurs only when Ca^{2+} binds at least one of the N-terminal EF hands of CaM. Presumably, this interaction causes a conformational changes of the CaMs and therefore in SK2 leading to channel opening. Channel inactivation is the reverse process and occurs upon dissociation of Ca^{2+} from CaM. The model of the Ca^{2+} gating of SK channels has been described by a multiple-state model, where the rate-limiting transitions between closed stated are controlled by Ca^{2+} and strongly depend on Ca^{2+} concentration, while the open-state transition occurs rapidly and is not dependent on Ca^{2+}concentration (figure 4).

1.5.5.2 Large Conductance Calcium-Activated Potassium Channels

Large conductance voltage- and calcium-activated potassium channels (BK) participate in action potential repolarisation and generates the fast AHP. This means that in presynaptic terminals, they regulate the duration of the action potential and limit Ca^{2+} entry, thereby modulating neurotransmitter release.

In contrast to SK channels, BK channels have a large single channel conductance (greater than ~ 200 pS) (Latorre et al., 1989). Also unlike SK channels, BK is activated by the concerted influence of both membrane depolarisation and increases in Ca^{2+} concentration (Toro et al. 1998). They are thought to be feedback modulators of the activity of voltage-dependent Ca^{2+} channels with whom they coexist in both neurons and smooth muscle cells (Robitaille et al. 1993; Yazejian et al. 1997).

BK channels have been cloned and expressed from the slowpoke locus of *Drosophila* (Adelman et al., 1992). The first BK mammalian clone from mouse brain (mSlo) appeared in 1993 (Butler et al., 1993) and human analogues (hSlo) of these channels have subsequently been cloned from a variety of sources.

The BK channels contain two subunits: □ and □ (Garcia-Calvo et al. 1994); the □ subunit is characterized by seven membrane-spanning regions (S0-S6); the N-terminal extremity is extracellular, while the long C-terminal part is cytoplasmatic (Meera et al. 1997).

The region that binds the calcium, called the "calcium bowl" resides in the carboxyl terminal part. It contains many negatively charged residues, mostly aspartates (Vergara et al. 1998) and it differs significantly from the common calcium-binding motif EF-hand (Wei et al. 1996; Meera et al. 1997; Schreiber and Salkoff 1997).

In the carboxyl terminal part of the protein, is a domain which regulates the K^+ conductance. This so-called RCK domain appears to function by mechanically acting on the ion conduction pore by changing its conformation from the closed to the open state.

BK channels are sensitive to several toxins, for example charybdotoxin and iberiotoxin, which bind with high affinity to the external vestibule of the channel, and consequently block the K^+ flux by occluding the pore (Munujos et al. 1995; Koschak et al. 1997).

The α subunit has a proposed topology of spanning the membrane twice, with the amino- and carboxyl-termini residing inside the cell (Vergara et al. 1998).

Coexpression of the β subunit with the pore forming α subunit affects the apparent calcium sensitivity, by eliciting a negative shift in the activation voltage.

Also, the co-expression of the two subunits increases by 50-fold the affinity for charybdotoxin compared to the alpha subunit expressed alone (Hanner et al. 1998).

In additon, co-expression of the beta 1 subunit with the alpha subunit (mSlo) of BK channels increases the apparent Ca^{2+} sensitivity of the channel (Nimigean and Magleby 2000).

The beta1 subunit of large conductance Ca^{2+} and voltage-activated K^+ channels increases the open probability of BK channels and facilitate gating of BK channels by acting through the Ca^{2+} activating mechanisms (Qian and Magleby 2003), wheras the beta 2 subunit causes inactivation of BK channels. The inactivation effect has been shown to be mediated by the cytosolic amino terminal part of the beta 2 subunit (Xia et al. 2003).

1.5.5.2.1 X-ray structure of a bacterial calcium activated potassium channel

The structure of a calcium-activated potassium channel, called MthK, from *Methanobacterium thermoautotrophicum*, shows that functional potassium channel consists of four pore-forming subunits and four RCK dimers that create a gating ring inside the

cytoplasm (Jiang et al. 2002).

Two interfaces between the RCK domains maintain the structure of the ring. One interface is fixed, while the other one is flexible and forms a ligand binding pocket between adjacent domains. The Ca^{2+} ions are situated at the base of this cleft, between the two domains. In the presence of Ca^{2+}, this pocket undergoes a conformational change that cause a tilt of the rigid part, and consequently the diameter of the ring increases, causing a movement of the pore helices; this allows the opening of the pore (figure 5).

Further experiments have revealed that BK channels that lack the whole C terminus part are still able to retain the wild-type calcium sensitivity.

This means that the calcium bowl and the RCK domain do not make up the high affinity calcium binding sites in BK channels and another calcium binding site has to be present.

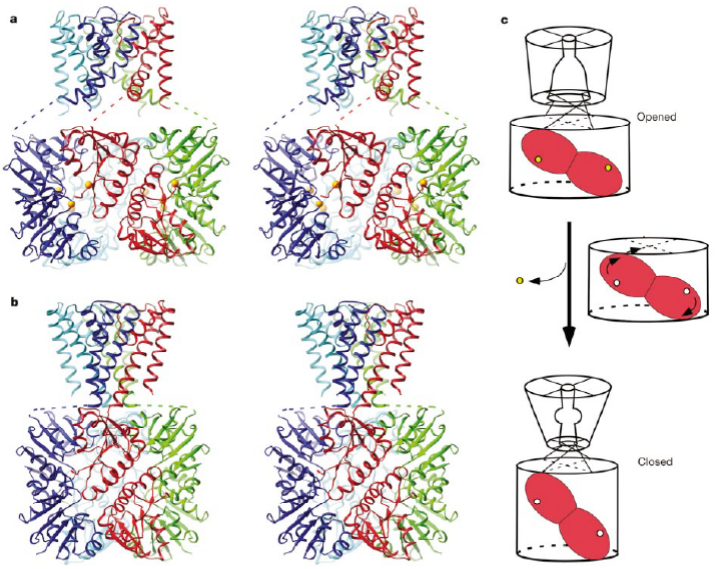


Figure 5: MthK structure and proposed mechanism of gating. A) Crystal structure of the MthK channel (opened conformation) with subdomains removed from the gating ring. RCK domains forming a fixed interface with each other are the same colour. Disordered linkers are shown as dashed lines and Ca^{2+} ions as yellow spheres. B) Hypothetical model of the MthK channel in its closed conformation. RCK domain pairs of the same colour were moved as rigid bodies. C) Proposed gating model. A single rigid unit is shown (red). Proposed direction of rotation and translation is shown from the arrows. Conformational changes in the gating ring that close the channel occur when Ca^{2+} ions (yellow circles) are released (From (Jiang et al. 2001).

1.5.5.2.2 Physiological and functional characteristics of BK channels

Physiological studies have shown the roles of BK channels in various tissues; they are involved in the regulation of smooth muscle contractility (Jaggar et al. 2000), endocrine and exocrine secretion (Ding et al. 1998; Xia et al. 1999), neuronal action potential duration and neurosecretion (Vergara et al. 1998). In neurons, they trigger the fast after-hyperpolarization that helps reset the membrane potential during an action potential (Stefani et al. 1997). BK channels are expressed in nerve terminals, where (after an action potential and consequently the rise of cytosolic calcium) they hyperpolarize the plasma membrane, thereby limiting the duration of voltage-dependent calcium influx and regulating synaptic transmission. Since they are important in regulating secretion, several studies have been done in order to identify proteins that regulate the properties of those channels.

BK channels are modulated by cyclic nucleotide-dependent protein kinases and by protein phosphatase 2A (Krause and Reinhart 1998; Tian et al. 1998). Most recently, two cytosolic proteins (called slob and slip1) have been shown to regulate BK channels probably by redistributing these channels within cells (Xia et al. 1998; Zhou et al. 2003; Jaramillo et al. 2004). In excitable cells, the transcriptional regulation of the single BK channel gene, *slo*, generates functional diversity (Tseng-Crank et al. 1994).

Many alternative transcripts of *slo* have been identified in both *Drosophila* and mammals (Adelman et al. 1992). These variants differ in their biophysical properties and in their expression patterns.

1.6 Heterologous Expression Systems

The production of a functional protein is directly related to the cellular machinery of the organism producing the protein. *E. coli* is a widely used organism for heterologous expression of proteins since its genome has been entirely mapped, it is easy to handle, grows rapidly, and allows simple recovery of the target protein. However, *E. coli* is a prokaryote and its lack of intracellular organelles, such as the endoplasmic reticulum and

the Golgi apparatus that are present in eukaryotes and that are responsible for post-translational modifications or glycosylation, often causes the production of non functional eukaryotic proteins since these modification cannot occur.

To overcome problems encountered with the use of *E. coli* to produce eukaryotic proteins, other organisms have been studied as suitable replacements. Mammalian, insect and yeast cells are the best substitute of *E.coli* system for the expression of eukaryotic proteins.

1.6.1 *Pichia Pastoris* expression System

Yeast cells combine simple genetic manipulation and rapid growth characteristics with the subcellular machinery for performing post-translational protein modification (Clare, 1991). Many foreign proteins have been successfully produced in the yeast *Saccharomyces cerevisiae*. But this system has several limitations. Generally the product yields are low because the stress caused by introduction of a foreign gene and proteolytic degradation is often encountered. Also, the use of inducible plasmid promoters to achieve a partial separation between the growth and protein production phase, has not been effective due to the instability of plasmids (Cregg 1987).

The methylotrophic *Pichia pastoris* has been developed to be an optimal host for the production of foreign proteins since its alcohol oxidase promoter was isolated and cloned (Buckholz and Gleeson 1991; de Hoop et al. 1991). The *Pichia pastoris* expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, which controls the gene that codes for the expression of alcohol oxidase, the enzyme which catalyzes the first step in the metabolism of methanol (de Hoop et al. 1991)

When grown on glucose or ethanol, alcohol oxidase is not detectable in the cells. However, when the yeast is grown on methanol, alcohol oxidase can be up to thirty-five percent of the total cellular protein. The control of the amount of alcohol oxidase is largely transcriptional (de Hoop et al. 1991; Shen et al. 1998). The key enzymes for methanol metabolism are compartmentalized in peroxisomes.

The AOX1 promoter has been characterized and incorporated into a series of *Pichia pastoris* expression vectors. The proteins produced in *Pichia pastoris* are usually folded correctly and the fermentation of genetically engineered *Pichia pastoris* provides an

excellent alternative to *E. coli* expression systems, since it is possible to achieve high yields of heterologous protein. Several proteins have been produced using this system, some examples are lysozyme, Kv potassium channels, mouse 5-hydroxytryptamine receptor and the human beta2-adrenergic receptor, human ET (B) endothelin receptor (Tschopp et al. 1987; Cregg et al. 1989; Weiss et al. 1998; Schiller et al. 2000; Parcej and Eckhardt-Strelau 2003).

The *Pichia pastoris* expression system offers many advantages when compared to other eukaryotic expression systems, since it does not have the endotoxin problem associated with bacteria or the viral contamination problem of proteins produced in animal cell culture. Furthermore, *Pichia pastoris* has a prolific growth rate especially when grown in fermentor.

1.6.2 Semliki Forest virus expression system

Semliki Forest virus (SFV) is an enveloped virus with a single-stranded positive sense RNA genome of the genus Alphavirus and the family Togaviridae. Semliki Forest virus, first isolated from mosquitoes in Uganda in 1944, has been used for many years as a model to study the molecular biology of RNA viruses. The virus has a broad host range, infecting most types of cells, and is relatively simple since it encodes only nine functional proteins of unique sequence. Different strains of SFV have been identified and infectious clones of SFV are now available. The RNA genome of SFV contains a 3'open reading frame (ORF) encoding the structural proteins, and a 5' ORF coding for the viral replicase. Transfection of cells with genomic viral RNA alone is enough to induce a productive replication cycle and budding of progeny virus particles. The viral RNA can also be produced by *in vitro* transcription, since the cDNA of the complete viral genome has been cloned into transcription vectors.

The expression system is based on an expression vector where the genes encoding the structural proteins are replaced by heterologous sequences. Transfection of cells with expression-vector derived RNA results in high level expression of the heterologous genes. A packaging system for production of recombinant SFV (rSFV) was previously developed using a helper vector, which encompasses the 3' ORF encoding the structural proteins.

The rSFV virions produced are able to infect cells and mediate biosynthesis of the desired protein/antigen but theoretically do not induce productive infection. Before virus budding, the spike protein p62 normally undergoes a proteolytic cleavage, which is necessary for subsequent virus infectivity. The p62 encoding sequence was modified in order that the cleavage can occur only *in vitro*. Virions therefore require protease activation in vitro to become infectious.

An important characteristic of Semliki Forest virus system is that the over-expressed proteins can undergo maturation, co- and post translational modifications that are fundamental feature of a functional eukaryotic protein.

This system has been applied for the expression of recombinant integral membrane proteins in several mammalian host cells. Numerous G protein-coupled receptors (GPCRs) such as the alpha (1B)-adrenergic receptor (Bjorklof et al. 2002) and alpha 2B - adrenergic receptor (Sen et al. 2003), several ion channels, for example the serotonin 5-HT3 receptor (Lundstrom et al. 1997; Hovius et al. 1998), and rat and human purinoreceptor P2x subtypes (Lundstrom et al. 1997), the histamine rH(2) receptor (Hoffmann et al. 2001), the glutamate receptors mGlu2 and mGlu3 (Schweitzer et al. 2000) and other types of transmembrane or membrane-associated proteins have been expressed at high levels.

The establishment of large-scale SFV technology allows the production of large quantities of recombinant proteins for biochemical and structure-function studies (Lundstrom 2003).

1.7 Aim of present study

In the last five years, several structural data were available to elucidate the function of the potassium channel. Despite this large amount of structural information, to date only few information are known about the eukaryotic potassium channels, so still many important aspects of these proteins need to be discovered.

In this project the attention was focalised in the study of one specific potassium channel family, the so-called: calcium-activated potassium channels.

Small calcium-activated potassium channels are implicated in many essential

physiological processes like learning, memorization, and regulation of circadian cycle and disruption of normal sleep pattern. Large calcium-activated potassium channels are involved in the regulation of the duration of the action potential and therefore in the modulation of neurotransmitter release.

In order to add to the understanding of the intriguing and mysterious world of the brain, it is important to obtain more information about these channels.

To date, there are no reports of purified SK2 channel from either natural or over-expressed sources and it has only been possible to get structural information about parts of the channel, in particular the X-ray structure of the carboxyl terminal part of the protein interacting with the calcium binding protein, calmodulin (Schumacher et al. 2001).

Large calcium-activated potassium channels are more abundant in the brain, in fact it has been possible to purify them from a native source, but the yield obtained is not sufficient for structural studies.

The aim of this project has been to express and characterise the small and large calcium-activated potassium channel from rat brain. Since in brain the expression level is low (especially SK2), it was necessary to over-express the channel.

To attempt this goal, the proteins were over-expressed employing two eukaryotic heterologous expression system, *Pichia pastoris* and Semliki Forest virus expression system. In both systems, the over-expressed protein can be subordinate to the subcellular machinery for post-translational protein modification.

Once the proteins are expressed in large amount, biochemical analysis can be performed in order to check the functionality, to purify them, to study their oligomeric state and to perform structural studies using electron microscopy and single particle image analysis.

CHAPTER TWO

Materials and methods

2.1 Materials

2.1.1 Chemicals, antibodies, detergents and lipids

Escherichia coli strain Top10F (Invitrogen) was used for sub-cloning and propagation of recombinant plasmids. The protease-deficient *Pichia pastoris* strain SMD1163 (*his4, pep4, prb*) (Invitrogen) was used routinely for the expression of recombinant SK2 and BK channels subunit genes. Enzymes for molecular biology were obtained from New England Biolabs or Stratagene. The following detergents were used: Thesit (Boehringer Mannheim or Simec trade AG), Tween 20 and Chaps (Calbiochem), DDM (Glycon). Anti-SK2 polyclonal antibody, reactive to residue 542-559, and anti BK policlonal antibody, reactive to residue 1098-1196 were obtained from Alomone. Anti-calmodulin monoclonal antibody was obtained from Upstate Biotechnology. Antibodies reactive with BK beta subunit protein were raised against the synthetic peptide, FWPTFLLTGGLLIIAS corresponding to residues 170-185 plus an additional N-terminal cysteine residue that was used for coupling to Ultralink Iodoacetyl resin (Pierce Chemical Co.). This affinity support was used to purify anti-BK-beta antibodies from crude sera using standard procedures (Harlow and Lane 1988). DNA sequencing was performed by MWG Biotech. All other reagents were obtained from Sigma Chemical Co. or Merck GmbH unless stated.

2.1.2 Solutions

Molecular biology buffers:

Agarose gel loading buffer 6× conc.	0.25% Bromphenolblue 40% Sucrose (w/v)
TAE buffer 50× conc. (agarose gel)	2M Tris pH 8,6 5.7 % acetic acid (v/v) 100 mM EDTA

Binding buffers:

Binding buffer 2× conc.	180 mM NaCl 10 mM KCl
Binding wash buffer	25 mM Imidazole pH 7,4 90 mM NaCl 5 mM KCl

Fermentation solutions:

Basal salts	68.1 mM $(NH4)_2SO_4$ 40%(w/v) Glycerol 5.4 mM $CaSO4 \cdot 2H_20$ 104.4 mM K_2SO_4 60.5 mM $MgSO_4 \cdot 7H_2O$

Na-hexametaphosphate 10× conc
filter sterile, keep at room temperature

PTM Trace salt	233.8 mM $FeSO_4 \cdot 7H_2O$ 0.82 mM Biotin 24.03 mM $CuSO4 \cdot 5\ H_2O$ 0.53 mM NaI 17.8 mM $MnSO_4 \cdot H_2O$ 8.3 mM $Na_2MoO_4 \cdot 2H_2O$ 0.32 mM H_3BO_3 2.1 mM $CoCl_2$ 146.7 mM $ZnCl_2$ 0.49% H_2SO_4

filter sterile, keep dark at room temperature

Breaking buffer (glassbeads)	50 mM Na-phosphate pH 7,4 1 mM EDTA 5% Glycerol (w/v) 0.1 mg/ml Trypsininhibitor 1 mM Benzamidine 0.1 mM Pefabloc
Breaking buffer (microfluidizer)	50 mM Tris/Hcl, pH 8.0 10% Glycerol (w/v)
SDS electrophoresis:	
Stacking buffer 2× conc.	180 mM Tris pH 6,8 0.2% SDS (w/v)
running buffer 10× conc.	250 mM Tris 192 mM Glycine 1% SDS (w/v)
SDS sample buffer 4× con.	250 mM Tris pH 6,8 6% SDS (w/v) 40% (v/v) Glycerol 0.02 % (w/v) Bromphenolblue
S&J anode buffer	0.2 M Tris/HCl, pH 8.9
S&J cathode buffer	0.1 M Tris 0.1 M Tricine 0.1% SDS (w/v)
S&J Buffer	0.3% SDS (w/v) 3 M Tris, pH 8.4 at RT
S&J sample buffer	50 mM Tris/HCl 12% glycerol (w/v) 4% SDS (w/v) 2% β-mercapto-ethanol, pH 6.8
Stain solution	45% Methanol (v/v) 10% Acetic Acid (v/v) 16 tablets /1 L (SERVA BLUE R)
Destain solution	7.4% Acetic acid (v/v)

	40% Methanol (w/v)
S&J staining solution	0.025% Serva Blue G (w/v) 10% Acetic acid (v/v)
Conditioner solution (silver staining)	0.1% $Na_2S_2O_3$ (w/v) 0.5% Glutaraldehyde (w/v) 30% Ethanol (v/v) 0.4 M Sodium acetate, pH 6.0
Silver staining solution	0.1% $AgNO_3$ (w/v) 0.0092% Formaldehyde (w/v)
Developer solution (silver staining)	2.5% Na_2CO_3 (w/v) 0.015% Formaldehyde (w/v)
Gel drying solution	5% Glycerol (w/v) 10% Ethanol (v/v)
Western blot:	
TBS (Tris buffer saline) 10 x conc.	200 mM Tris pH 7,5 1.5 M NaCl
Antibody solution	1% BSA (w/v) in 10× dil.TBS 0.05% Na-azid
Transferbuffer	25 mM Tris 192 mM Glycine
Blocking buffer	5% Milk powder (w/v) 0.05% Na-azide 10 % TBS 10 × conc
NTB (Nitro Blue Tetrazolium) stock	65.4 mg /ml DMF 50 % (w/v)
BCIP (5-Brom-4-chlor-3-indolylphosphat-p-toluidin) stock	50 mg /ml DMF 100 % (w/v)
Developing buffer	0.1 M Tris pH 9,5 0.1 M NaCl 5 mM $MgCl_2$ 50 µl NTB stock/10 ml buffer 33 µl BCIP stock/10 ml buffer

Stop solution	20 mM Na-EDTA
Protease inhibitors	1 µg/ml E64 0,7 µg/ml Pepstatin A 0,5 µg/ml Leupeptin
Solubilisation buffer	1.2 M NaCl 150 mM KCl 1 mM CaCl 40 mM hepes, pH7.0 5% Glycerol (w/v) + protease inhibitors + 4% detergent (w/v)
Washing buffer (Ni-NTA)	25 mM Imidazole pH 7.4 150 mM KCl 1 M NaCl 1mM CaCl 0.02 DDM or 0.01 digitonin (w/v) + protease inhibitors
Elution buffer (Ni-NTA)	300 mM Imidazole pH 7.4 50 mM KCl 150 mM NaCl 1mM CaCl 0.01% DDM (w/v) + protease inhibitors
Buffer S (strep II Chrom.):	20 mM Tris pH 8,2 50 mM KCl 1mM CaCl 0,02% NaN_3 (w/v) 0,1% DDM (w/v)
Washing buffer (ion exchange)	100 mM NaCl 50 mM KCl 1mM CaCl 40 mM Hepes, pH 7.0 5% Glycerol (v/v) 0.02% DDM (w/v)
Elution buffer (ion exchange)	1.2 M NaCl 150 mM KCl 1mM CaCl 40 mM Hepes, pH 7.0 5% Glycerol (v/v)

	0.02% DDM (w/v)
Gel filtration buffer	500 mM NaCl 50 mM KCl 1mM CaCl 20 mM Tris/HCl, pH 8.0 5% Glycerol (v/v) 0.02 DDM (w/v)

Bacterial culture medium:

LB (Luria broth) medium	10 gr. Peptone 10 gr. NaCl 5 gr, Yeast Extract

fill up to 1L with H_2O , autoclave
for Agar plates add15 gr. Agar /1L

SOC-medium	1.55 g Luria Broth 250 mM KCl 2 M $MgCl_2$ 1 M Glucose

fill up to 100 ml with H_2O, autoclave

Yeast culture medium:

MGY media	800 ml sterile H_2O 100 ml YNB (yeast nitrogen base)10× conc 100 ml Glycerol 10× conc 2 ml Biotin 500× conc
MM media	800 ml sterile H_2O 100 ml YNB 10× conc 100 ml Methanol 10× conc 2 ml Biotin 500× conc

Cell culture medium:

Phosphate buffered saline without calcium and magnesium (PBS)	2.68 mM KCl 1.47 mM KH_2PO_4 136.8 mM NaCl 8 mM $Na_2HPO_4.7H_2O$

Adjust to pH 7.0-7.2 with HCl and autoclave

Growth media	500 ml Dulbecco's MEM/nut mix F12 (HAM) 500 ml Iscove's modified Dulbecco's medium 100 ml foetal calf serum

2.1.3 Oligonucleotide primers

Oligonucleotide primers used to amplify the hBK alpha subunit cDNA by PCR:

hBK forward primer:

5'-AGATGCGCTCATCATCCCGGTGACCATGGA-3'

hBK reverse primer:

5'-GTTTCACACAGCGGCCGCGGATACACATAC-3'

Oligonucleotide primers used to amplify the rSK2 cDNA by PCR:

rSK2 forward primer:

5'-CAGCAGCTGCAGGTACAACGG-3'

rSK2 reverse primer

5'-CCGCCAGTGTGCTGGAATTCGCCC-3'

Oligonucleotide primers used to amplify rCaM cDNA by PCR:

Forward primer:

5'-TGATCCCCGTGCTCCGAATCCCCCGGGCCTCGCCA-3'

reverse primer:

5'-GAGAACGGGCATCGCCAGCGGCCCGGGGGGCCTTC-3'

SacII mutation on BK beta subunit

Forward primer:

5'-CTGCCACCGCGGTGAAGAAGCTGGTGATGGCCCAG-3'

reverse primer:

5'-GACGGTGGCGCCACTTCTTCGACCACTACCGGGTC-3'

SpeI mutation on pPIC3.5K-SK2 (to insert FCYENE oligo or Q oligo) by PCR:

forward primer:
5'-CCAACTTCATCTGAGACTAGTTAGAAGGGCGAATTCC-3'
reverse primer:
5'-GGAATTCGCCCTTCTAACTAGTCTCAGATGAAGTTGG-3'

StrepII oligos
Forward oligo:
5'-AATTCATGTGGTCTCACCCACAATTCGAGAAGGC-3'
revers oligo
5'-CTTCTCGAATTGTGGGTGAGACCACATG-3'

FCYENE oligo
Forward oligo:
5'-CTAGCTTCTGTTACGAGAACGAGTAGC-3'
reverse oligo:
5'-GGCCGCTACTCGTTCTCGTAACAGAAG-3'

Q oligo forward:
5'-GGCCGCTCCTAATGAATTCTGTTGCTGCTGTTGCTGTTGCTGTT
GCTGGC-3'
Q oligo reverse:
5'-CTAGGCCAGCAACAGCAACAGCAACAGCAGCAACAGAATTCATTA
GGAGC-3'

2.2 Methods

2.2.1 Molecular biological methods

Standard DNA manipulations and microbiological technique were performed as described in (Sambrook et al. 1989).

2.2.1.1 DNA cloning

Manufacture of expression constructs and *Pichia pastoris* transformation Polymerase chain reactions (PCR) employed either pfu I or the Taq Precision Plus system (Stratagene).

rSK2: the open reading frame encoding the rSK2 (small calcium activated potassium channel) protein was amplified from pCR2.1-SK2 by PCR (paragraph 2.1.3), introducing a 5' EcoRI restriction site. After gel purification and digestion with EcoRI, the PCR fragment was ligated into the similarly digested expression vector pNSHE 2.1 (prepared in the lab by Dr. D. Parcej based on pCR2.1 from Invitrogen) using EcoRI and EcoRV restriction sites.
pNSHE 2.1 contains an antibiotic resistance gene (ampicillin), a Kozak sequence, a 9-histidine tag, a step II-tag (Schmidt et al. 1996) and an enterokinase site-N terminal.
The antibiotic resistance gene allows the selection of positive clones in *E. Coli*, the Kozak sequence is used for increasing initiation of transcription, after subcloning the his tag and strep tag are used for purification and detection on a Western Blot, and the enterokinase site is recognized by the EK protease to selectively cleave the engineered tags.
pNSHE 2.1-SK2 was digested with BamHI and EcoRI and sub-cloned into a *Pichia Pastoris* expression vector, pPIC 3.5K (Invitrogen).

To insert SK2 into the Semliki forest virus expression vector pSFV2 gen, the open reading frame encoding the rSK2 (small calcium activated potassium channel) protein was cut from pPIC3.5K-SK2 with BamHI and NotI (paragraph 2.1.3) in order to ligate into the similarly digested expression vector pSFV2gen.

For the insertion of SK2 into the mammalian expression vector pcDNA3.1, SK2 fragment digested with BamHI and NotI from the construct pPIC3.5K-SK2, was ligated into similarly digested expression vector pcDNA3.1.

SK2-FCYENE: Oligonucleotides were designed to form a duplex containing the FCYENE coding sequence with SpeI and NotI compatible overhangs at the 5' and 3'

ends respectively. To insert FCYENE duplex into the pPIC 3.5K-SK2 construct, a SpeI restriction site was inserted at the 3' end of the SK2 coding sequence by mutagenic PCR. Subsequently the mutated SK2-pPIC3.5K construct was digested with SpeI and NotI to allow ligation of the FCYENE DNA.

For the insertion of SK2 into the mammalian expression vector pcDNA3.1, SK2 fragment digested with BamHI and NotI from the construct pPIC3.5K-SK2, was ligated into similarly digested expression vector pcDNA3.1.

SK2-q-calmodulin: SK2 channel was cloned in tandem with calmodulin, with a peptide linker containing 10 glutamine amino acids between the two proteins.
Oligonucleotides were designed to form a duplex containing 10 glutamine with SpeI and EcoRI compatible overhangs at the 5' and 3' ends respectively (paragraph 2.1.3). In order to insert into pPIC 3.5K-SK2 a SpeI restriction site was inserted at the 3' end of the SK2 coding sequence by mutagenic PCR.
The open reading frame encoding calmodulin was amplified from rat calmodulin cDNA (from ATCC) by PCR introducing a 5' EcoRI restriction site and a 3' NotI site.
After gel purification and digestion with EcoRI and NotI, the PCR fragment was ligated into the similarly digested mutated SK2-pPIC3.5K construct.

For the insertion of SK2 into the mammalian expression vector pcDNA3.1, SK2 fragment digested with BamHI and NotI from the construct pPIC3.5K-SK2, was ligated into similarly digested expression vector pcDNA3.1.

BK alpha subunit: the open reading frame encoding the human large calcium activated potassium channel □ subunit cDNA (generous gift from Dr. M. Wallner) was amplified from pcDNA3.1-hBK by PCR introducing a 3' NotI restriction site and a 3' EcoRV site. After gel purification and digestion with NotI, the PCR fragment was ligated into the expression vector pNSHE 2.1 or into another expression vector pNHE-□. (prepared in the lab by Dr. D. Parcej based on pCR2.1 from Invitrogen) digested with NotI and EcoRV restriction sites.

pNSHE 2.1-BK or pNHE-α-BK were digested with BamHI and NotI and sub-cloned into a *Pichia Pastoris* expression vector, pPIC 3.5K (Invitrogen).
Restriction analysis and DNA sequencing of the BK alpha subunit confirmed that the sequence of the two clones was correct.

BK beta subunit: the human large calcium activated potassium channel β subunit cDNA (generous gift from Dr. M. Wallner) was amplified from pcDNA3.1-BKβ by PCR introducing a 5' EcoRI restriction site and a 3' NotI site. After gel purification and digestion with NotI and EcoRI, the PCR fragment was ligated into the *Pichia pastoris* vector pPICZB. Oligonucleotides were designed to form a duplex containing the a strep II coding sequence with SacII and NotI compatible overhangs at the 5' and 3' ends respectively.
To insert the duplex into the pPICZb-BKβ construct, a SacII restriction site was inserted at the 5' end of the BKβ coding sequence by mutagenic PCR. Subsequently the mutated pPICZB-BKβ construct was digested with SacII and EcoRI to allow ligation of the strep II duplex.

2.2.1.2 Transformation of *E.coli* Strain Top 10' by plasmid DNA.

40 µl electro competent Top 10' *E.coli* cells were incubated for 1 min on ice with the DNA construct, then transfered into a 0.2 cm cuvette and put to the Gene Pulser (Bio Rad). After the electro shock (time constant should be 4.5-4.6), 1 ml SOC-medium was added in the cuvette and resuspended, pour to the falcon tube.
The Gene Pulser was set at 25 F, 2.5 kV, Pulser Controller at 200 Ohm.
The cells were then incubated 1 hr in a incubator at 37°C. The cell were then plated on LB-Amp Plates, incubate overnight at 37°C. Not for pPICZB.

2.2.1.3 Transformation of *Pichia pastoris* cells

Pichia pastoris cells were transformed by electroporation with 15–20 µg of Pme I (for the pPIC3.5K constructs) or of NruI (for pPICZB construct) linearized vectors, using

conditions recommended by the manufacturer. The Gene Pulser was set at 25 F, 1.5 kV, Pulser Controller at 400 Ohm. After initial selection for His^+ transformants, multicopy integrants were selected by their resistance to increasing concentrations of G418. Clones resistant to 0.2–1.0 mg/ml of G418 were selected.

After BstX1 linearization, pPICZB-BK was used to transform *Pichia pastoris* SMD1163 cells or cells already expressing pPIC3.5K-BK construct. Colonies were selected from plates with different concentrations of G418 or zeocin and grown in MGY and then induced with MM, at 30°C in a flask to test foreign protein production.

2.2.1.4 Preparation of mRNA *in vitro*

DNA was linearised for the *in vitro* transcription of RNA. 10-20 g of plasmid DNA is cut with NruI (for pSFV2 plasmid) and with SpeI (for the helper vector) restriction enzymes at 37°C for 1-3 hr in total volume of 50-100 l.

Complete digestion was confirmed by loading on a 0.8% agarose gel an aliquot of the digestion together with the uncut control.

DNA was purified on Microspin™ S-200HR (Amersham Pharmacia Biotech) and DNA was resuspended in H_2O at 0.5 g/ l.

The following reaction mixtures was prepared at room temperature:

Mix
- 5 l (2.5mg) linearized DNA
- 5 l SP6 buffer
- 5 l 10 mM $m^7G(5')ppp(5')G$
- 5 l 50mM DTT
- 5 l rNTP mix
- 20 l Rnase free water
- 1.5 l (50U/ l) Rnase inhibitor
- 3.5 l 20U/ l SP6 RNA polymerase

Incubated at 37°C for 60 min and then immediately used for electroporation.

2.2.1.5 Electroporation of RNA into BHK-21 cells

Recently passaged (less than 3 days) BHK (baby hamster kidney fibroblasts) cells grown in 175 cm^2 tissue culture flasks to 80% confluency were used.

Growth medium was aspirated and cells washed with PBS and then detached with 5 ml of trypsin/EDTA. Cells were resuspended thoroughly to remove clumps and 25 ml medium added, transferred to a 50 ml falcon tube and centrifuged for 5 min at 1500rpm (450g) at 4°C. Medium was then removed and the cells resuspended in 30 ml of PBS and centrifuged again for 5 min. The cells pellet was resuspended in 2 ml of PBS (~ $2 \cdot 10^7$ cells/ml) and immediately used for electroporation.

Electroporation of cells in one 0.2 cm cuvette results in 10ml virus stock

The mRNA was briefly centrifuged down and 0.4 ml of cell suspension was transferred to 0.2 cm gene pulser cuvette, together with a mix of 25 µl of Helper RNA and 50 µl of RNA, then the cuvette was inserted into the holder and pulse twice at room temperature with following setting for the BioRad Gene PulserII.

BioRad Gene PulserII setting:

Cuvette	0.2 cm
Pulse controller setting	"high range"
	" Infinity"
Capacitance rotary switch	" high capacitance"
Voltage	360 V
Capacitance	75µF
Resistance obtained	10Ω
Expected time constant	0.7-0.8 sec

The electroporated cells were transferred into a flask with 10 ml of medium and then put in a 5% CO_2 incubator at 37°C for 24 h.

Recombinant virus stocks were harvested after 24 hr of incubation.The medium was filtered through a 0.22 µM filter unit (Millex-GP Millipore) to remove cell debris and possible contaminants from the *in vitro* transcription reactions and stored at –80°C.

2.2.1.6 Transfection of mammalian cells using Lipofectamine 2000

COS 7 (African green monkey kidney) cells (generous gift from Dr.G.O'Sullivan) were grown on a 175 ml flask and cultured at 37°C until they were confluent and then they were transfected with pcDNA3.1-SK2, pcDNA3.1-SK2-FCYENE or pcDNA3.1-SK2-q-cam DNA constructs using the cation lipid mixture Lipofectamine 2000 (from invitrogen), according with the manufacturer's instruction.

2.2.2 Protein production

2.2.2.1 Small scale production in *Pichia pastoris*

Pichia pastoris SMD1163 cells carring the foreign gene were grown overnight in MGY medium (0.34% (w/v) yeast nitrogen base, to an absorbance at 600 nm (A_{600}) of 2–6. After pelleting at 1500g for ten min, the cells were resuspended to an A_{600} of 1.0 in MM and grown for 24–48 hr at 30 °C. Additional methanol was added after 24 hr to a final concentration of 0.5% (v/v), in order to maintain inducing conditions. Then the cells were centrifuged and the pellets stored at –80 °C or used immediately for further studies.

2.2.2.2 Large scale production in *Pichia pastoris*

Fermentation was conducted in a benchtop fermentor (INFORS AG) with autoclavable vessels of 5 L working volume. *P. pastoris* fermentations were run as multi-stage fed-batch processes with oxygen supplementation. Pure oxygen was supplied automatically to meet the dissolved oxygen requirements for high-density cell growth.

A frozen vial of 1 ml *P. pastoris* SMD 1163 cells carring a foreign gene was inoculated into a 1 L shake flask with 350 ml MGY. The culture was incubated at 30°C, 240rpm, for 14-20 hr in an environmental incubator shaker. The entire 350 mL volume of inoculum was transferred to the fermentor vessel containing 3.5 l of basal salts medium (see media components, plus 4.4 mL/L trace metal solution. The temperature was controlled at 30°C. The dissolved oxygen was set at 30% and pH is at 5.0. Ammonium hydroxide

solution was used as the base solution to adjust the pH. After ~20 hr of batch culture, the glycerol fed-batch process was initiated. After the growth phase (800 ml of glycerol) , four hours carbon-source starvation period was established before the culture was switched to the production phase.

The production phase (methanol feeding) was divided into three stages: 6 hr induction, 12 hr in a high-feed-rate stage and 6 hr in a low-feed-rate stage (700-800 ml of methanol).

2.2.2.3 Small scale production in BHK cells

Recombinant virus was activated by adding 25 µl of α-chymotrypsin/ml of virus suspension at room temperature for 20 min and then the reaction was stopped by addition of 25 µl aprotinin/ml of virus suspension.

BHK cells were grown 175 ml flasks and cultured at 37°C until they were confluent and then infected by aseptically adding 3-5 ml of the activated virus directly to flask culture.

The flask was then put back in the incubator for the desired time.

The cells were usually harvested between 16-32 hr by centrifugation for 5 minutes at 2000rpm at 4 °C and then the pellet washed twice with PBS.

2.2.2.4 Large scale production in BHK cells

Cells were grown until the cell density was ~ $7.5 \cdot 10^5$ cells/ml in spinner flasks and cultured at 37°C with a stirring speed of 95-100rpm and then infected by aseptically adding 30 ml/l of activated virus directly to the spinner flask culture. Cells were usually harvested between 16-48 hr by centrifugation for 15 min at 2000rpm at 4 °C and then the pellet washed twice with PBS.

2.2.3 Membrane extraction

2.2.3.1 Small scale membrane preparation with Glass beads

Small shake-flask culture volumes (up to 400 ml), cells were suspended in 0.05 M sodium phosphate (pH 7.4), 1 mM EDTA, 5% (w/v) glycerol, 0.1 mg/ml of soybean trypsin inhibitor, 1 mM benzamidine and 0.1 mM Pefabloc SC to an A_{600} of 50–100.
An equal volume of ice-cold, acid-washed glass beads (0.25–0.5 mm diameter) was added and the cells were broken by vortex mixing for 10 min (30 sec bursts separated by 30 sec cooling on ice). Glass beads, unbroken cells and other cell debris were removed by centrifugation at 1500g for ten minutes and the pellet washed with an equal volume of buffer and recentrifuged. The combined supernatants were then centrifuged at 50,000g for 30 min. The crude membrane pellet was resuspended in ice-cold water and the protein content determined using the DC protein assay (BioRad).

2.2.3.2 Large scale preparation of membranes using a Microfluidiser

Cells grown in a fermenter (300–400g of packed pellet after 24 hr induction with methanol) were suspended to 25–35% wet weight in 50 mM Tris–HCl (pH 8.2) and protease inhibitors as described above. The cells were then passed five times through a microfluidiser model M-110L (Microfluidics Corp., Newton, MA) equipped with a 110 M interaction chamber and cooling coil, which were immersed in water at 4°C. After centrifugation at 1500 g for twenty minutes, the pellet was passed again through the microfluidiser and the supernatants combined. Glycerol, polyethylene glycol (average molecular mass 4000) and NaCl were added to final concentrations of 10% (w/v), 10% (w/v) and 0.1 M, respectively in order to precipitate the crude membrane fraction. After incubation on ice for 15 min, the mixture was centrifuged at 12,000g for 20 min. Then the pellet was washed once by resuspending in water, centrifuged for one hour at 30,000g and again resuspended in ice-cold water. Protein concentration was determined as described above.

2.2.3.3 Preparation of synaptosomal plasma membrane

12 brains (frozen from Harlan Labs) were resuspended in 90 ml 0.32 M sucrose buffer (25mM Hepes/KOH, pH 7.0, plus protease inhibitors) and homogenised with 2x6 strokes of a glass-perspex homogeniser.
Then the material was centrifuged at 4000rpm for 10 min in a 45 Ti rotor. supernatant S1 was collected. Pellet was resuspend in 60 ml sucrose solution with gentle shaking. Then spin again and supernatant was collected as above. Centrifuged pooled supernatants at 15000rpm for 20 min in 45 Ti rotor. Pellet was resuspend in 120 ml of resuspension buffer and left 30 minutes at 4^{o}C. Afterwards the pellet was loaded on a sucrose gradient and centrifuged at 25000rpm for 80 min in SW 25 rotor. Finally the synaptosomal fraction was collected and frozen at -70^{o}C.

2.2.4 Polyacrylamide gel analysis

SDS-PAGE analysis were performed using the discontinuous method (Laemmli 1970), while blue native electrophoresis using the Schagger and von Jagow method (Schagger and von Jagow 1987).
For discontinuous electrophoresis, 10% polyacrylamide gels were used. The samples were mixed in a 1:1 ratio with sample buffer (paragraph 2.1.2); the electrophoresis was conducted at a current of 15 mA/gel.

For blue native electrophoresis, 13-16% gradient gel was prepared. The samples were mixed in a 1:1 ratio with the S&J sample buffer (paragraph 2.1.2). The anode buffer chamber was loaded with S&J anode buffer whereas the cathode chamber with S&J cathode buffer. The gels were run at 30 V for 1 hour and then to 20 mA/gel.

2.2.4.1 Staining of SDS-PAGE gels

For Coomassie blue staining the gel was incubated for 30-60 min with stain solution (paragraph 2.1.2) under continuous shaking. Then the gel was washed with destain solution until the background was clear.

For blue native staining, proteins were fixed for 30 min by incubation in 10% acetic acid (v/v) and 50% methanol (v/v). The gel was then stained for 2 hours in S&J staining solution (paragraph 2.1.2) and destained in 10% acetic acid (v/v) until the background became clear.

For silver stain, proteins in the gel were fixed for 20 min in 10% acetic acid (v/v) and 30% methanol (v/v). The gel was then incubated for 20 minutes in conditioner solution and washed 3 times for 10 min. After the washing, the gel was incubated for 20 min with the silver staining solution. After washing again 3 times for 10 min, the gel was incubated with the developer solution, until the protein bands were visible. Then the developing reaction was stopped by replacing the developer solution with 5% acetic acid solution (v/v).

2.2.5 Western blotting

PVDF was wetted by incubation in 100% methanol for 2-10 min and rinsed thoroughly with water followed by transfer buffer (paragraph 2.1.2). Three pieces of Whatman 3 MM filter paper were soaked in transfer buffer and placed on the bottom electrode of the Bio Rad semi-dry blotter, then in order, the PVDF, the gel (after briefly rinse with transfer buffer) and other 3 pieces of soaked filter paper were placed one on top of the others.

After the transfer, the PVDF was incubated for 1hour in blocking buffer and afterwards washed 10 minutes with TBS. The primary antibody, in antibody dilution buffer (paragraph 2.1.2), was added and incubated overnight at 4^{o}C. The following day, the PVDF was washed 3 times for 10 minutes with TBS + 25% blocking buffer (paragraph

2.1.2), and then the secondary antibody conjugated with alkaline phosphatase in antibody dilution buffer was added and incubated for 1hour and 30 minutes. The PVDF was again washed 3 times for 10 min with TBS + 25% blocking buffer and once with TBS.
The PVDF was incubated for 5 min in developing buffer (paragraph 2.1.2) and then in developing solution (50 μl NBT + 33 μl BCIP per 10 ml developing buffer) until the protein bands were visible. Then, the PDVF was immediately transferred onto stop solution.

2.2.6 Solubilisation

Membranes (20 mg/ml) were resuspended in 2x solubilisation buffer (in presence of protease inhibitor) to a final concentration of 10 mg/ml. After the addition of detergent (1-5%), the solution was stirred or shaken at 4°C for the desired time. Then the insoluble materials were sedimented at 160,000g for 40 min at 4°C. Soluble and insoluble materials were subjected to protein concentration determination and SDS-PAGE analysis.

2.2.7 Purification of solubilized membrane proteins

2.2.7.1 Affinity Chromatography

2.2.7.1.1 Immobilised metal affinity chromatography

The principle of this chromatography is the affinity between histidine, cysteine or tryptophane residues to transition metals, as for example Ni^{+2} or Co^{+2} (Porath et al, 1975). The proteins after binding the resin can be eluted either increasing imidazole concentration or by decreasing the pH.
Ni-NTA resin (from Qiagen) was packed either into disposable polystyrene column (2, 5, or 10 ml) or into XK16 column (Pharmacia Biotech). After column equilibration, the sample was loaded with a flow rate of 0.5 ml/min. The column was then washed with 3 column volumes of washing buffer (paragraph 2.1.2) and the protein eluted either with a linear gradient of imidazole (from 50 mM to 300 mM) or directly with 300 mM of imidazole. Fractions were collected for further analysis during the loading, washing and elution steps. For a gradient elution Äkta system (Amersham) was used.

2.2.7.1.2 Affinity chromatography on Streptactin column

Streptactin-agarose chromatography takes advantage of the strong interaction between the 8 amino acids (WSHPQFEK) of the tag and Streptactin engineered streptavidin (IBA).

Streptactin-agarose resin (from IBA) was packed into a disposable polystyrene 2 ml column. After column equilibration in buffer S, the sample was loaded at a flow rate of 0.25 ml/min. The column was then washed with 3 column volumes of buffer S and the protein eluted with buffer S + 2.5 mM desthiobiotin.

Fractions were collected for further analysis during the loading, washing and elution steps.

2.2.7.2 Ion Exchange Chromatography

In the ion exchange chromatography, the proteins bind the ion exchange matrix at low salt concentration and they are then eluted by increasing the salt concentration or changing the pH of the buffer.

Before performing ion exchange chromatography, the salt concentration of the sample was decreased during the Ni-NTA washing and elution (paragraph 2.1.2).

SP resin (from Pharmacia Biotech) was packed either into disposable polystyrene column (2, 5, or 10 ml) or into XK16 column (Pharmacia Biotech). After column equilibration, the sample was loaded with a flow rate of 0.5 ml/min. The column was then washed with 3 column volumes of washing buffer (paragraph 2.1.2) and the protein eluted either with a linear gradient of NaCl (from 200 mM to 1.2 M) or by a step of with 300, 500, 800 mM and 1.2 M of NaCl.

Fractions were collected for further analysis during the loading, washing and elution steps. For a gradient elution Äkta system (Amersham) was used.

2.2.7.3 Size exclusion chromatography

In the gel filtration chromatography the proteins are separated by their differences in size and shape. The proteins do not bind to the matrix and for this reason it is considered a mild chromatography, even if membrane proteins can endure strong delipidating effect that can seriously affect their functionality.

Gel filtration chromatography was performed on a Superose 6 column (Amersham) using SMART system (Amersham) for analytical analysis.

50 µl of sample (either from Ni-NTA elution fraction or ion exchange elution fraction) was applied and the flow rate was of 50 µl/min.

The fractions were collected for SDS-PAGE analysis and the chromatogram was recorded.

2.2.8 Immunofluorescence analysis

For immunofluorescence experiments, cells were grown on 24 wells plate on 12-15 mm cover glasses. After a given time of foreign protein expression, the cells were washed twice in PBS and the fixed for 10 min in 4 % paraformaldehyde (w/v) in PBS, then washed 3 times in PBS and incubated for 5 minutes at room temperature with 0.02% triton x-100 (w/v). After washing again 3 times, the cover glass was incubated for 30 min with 2% BSA or goat serum (w/v) and then incubated for 1 hr with the primary antibody diluted in PBS+2% BSA or goat serum (w/v). After the incubation the cover glass was washed other 3 times in PBS, the excess of buffer dried with filter paper and then again incubated for 1 hr with the secondary antibody conjugated with a fluorochrome in PBS+2% BSA or goat serum (w/v). Afterwards the cover glass was again washed 3 times in PBS and one time in water, the excess of water dried with filter paper and finally the coverglass was placed on a glass, up side down on top of a drop of water mounting medium. The glasses are then left for few hours at 4°C before being observed in the fluorescence microscope.

2.2.9 Immunogold electron microscopy

Pichia pastoris cells over-expressing different constructs were harvested at different induction times and fixed for 3hr with 4% paraformaldehydein (w/v), 0.1 M sodium cacodylate buffer, pH7.2 containing 0.5 M sorbitol. After incubation the fixative was washed with sodium cacodylate buffer supplemented with deacrising concentration of sorbitol. Afterwards the sample was treated for 10 min with 1% sodium metaperiodate (w/v), then water and finally incubated overnight with 0.05 M NH_4Cl.

For resin embedding, the cells were enclosed in agar, then dehydrated with ethanol and finally infiltrated with LR white resin (London resin company Ltd., Reading, England). After the polymerization (55^{o}C, 25hr), thin sections were cut with Ultracut microtome (Reichert, Vienna, Austria) and collected on Formvar (Serva Feinbiochemica GmbH, Heidelberg, FRG) coated nickel grids (Plano GmbH, Wetzlar, FRG).

For immunogold labeling, the grids were sequentially incubated with saturated sodium metaperiodate (10min), water (3 times, 10min), PBS + 2% glycine (w/v) (10min), PBS + 1% BSA (w/v), 0.2% tween 20 (w/v), 0.1% triton x-100 (w/v) (10min), and PBS + 0.1% BSA (w/v), 0.05% tween 20 (w/v) (2 times, 10min).

Primary antibodies were incubated for 3 hr and after washing, a secondary gold-coupled antibody was applied for 1 hr. After washing the sections were fixed with 1% glutarardialdehyde in PBS (10min), washed again and then double contrasted with uranyl acetate and lead citrate. Grids were analysed in the electron microscope EM208S (Philips).

2.2.10 Measurement of ^{125}I-apamin binding to SK2 channels

These experiments were performed as described (Seagar et al. 1984; Seagar Mj Fau - Granier et al. 1984; Seagar et al. 1986; Seagar Mj Fau - Labbe-Jullie et al. 1986; Seagar et al. 1987; Seagar Mj Fau - Marqueze et al. 1987).

Membranes prepared either from *Pichia pastoris* cells expressing SK2, SK2-FCYENE, SK2-q-CaM (24 hours induction), or from mammalian cell lines (BHK expressing SK2 or CHO expressing SK2 or SK2-FCYENE) were tested for functional studies.

Membranes (60 μg) were incubated with 50 pM [^{125}I]-apamin in 500 μl of incubation buffer on ice. After 1 hr incubation, a 450 μl aliquot was applied to a GF/B glass-fiber disc (Whitman) soaked previously in 0.3% (w/v) polyethelynimine (Bruns et al. 1983), which was then washed rapidly with two 5 ml portions of ice-cold washing buffer.
Bound ligand was quantified by determining the γ-radiation level of the filter. Non displaceable binding was assessed by including a 100-fold excess of unlabelled toxin. For determination of binding parameters, various concentrations of [^{125}I]-apamin were included in the reaction with or without 100-fold molar excess of unlabelled apamin for determination of non-specific binding. In competition experiments, membranes were incubated with 50 pM [^{125}I]-apamin, together with increasing concentrations of unlabelled apamin or gallamine. Data were treated as detailed elsewhere (Seagar et al. 1987; Parcej and Dolly 1989).

2.2.11 Electron microscopy of single particle

Purified SK2 channel was diluted to 0.05 mg/ml and a drop of 6 μl was applied to 400 mesh copper grids previously coated with a thin carbon film.
The specimen was stained with 2% ammonium molybdate (w/v). Micrographs were recorded under low dose conditions on a Philips CM120 electron microscope (FEI) equipped with a LaB_6 cathode at an accelerating voltage of 100 kV and a nominal magnification of 60000x. The defocus values were approximately 1 μm.
Micrographs were recorded on Kodak SO-163 film and developed in full strength D-19 developer for 12 minutes at 20 °C. Negatives were initially visually checked for the distribution of particles and quality of staining. Then suitable micrographs were inspected on an optical diffractometer and those suffering from astigmatism or drift were discarded. Selected negatives were scanned on a Zeiss SCAI flat bed scanner (Zeiss, Germany) with 7 μm raster size. Images were converted to SPIDER format and reduced 3 times by binning to a final pixel size corresponding to 0.36 nm on the sample scale.
Image processing was carried out using SPIDER and WEB (Frank et al. 1996) and classification was performed using XMIPP (Marabini and Carazo 1994). All alignments

were performed using a simultaneous translational/rotational alignment algorithm, based on correlation of Radon transforms (Radermacher et al. 2001).

CHAPTER THREE

Results and Discussion

3.1 Expression of a small calcium-activated potassium channel in *Pichia pastoris*.

3.1.1 Cloning and expression

DNA encoding the rat small calcium-activated potassium channel (rSK2) was cloned from a rat brain cDNA using the PCR method and employing oligonucleotide primers corresponding to the published sequence (accession number: P70604) (Kohler et al. 1996). After subcloning into an intermediate vector containing tags and other sequences, it was inserted into the *Pichia pastoris* expression vector pPIC3.5K. The final construct (Figure 6) contains a Kozak sequence around the start codon to enhance transcription, followed by a Strep II and a nonohistidine tag for affinity purification. An enterokinase site is also present in order to cleave the engineered tags if desired. Additional features of the expression vector include fragments of the *P. Pastoris* AOX gene which promotes insertion into the host DNA at the AOX locus, the Histidinol (*His4*) gene and a gene encoding resistance to G418. The latter two are used in a two-step selection procedure for *Pichia* transformants. The correct sequence of the construct was verified by restriction analysis and DNA sequencing using primers complementary to the AOX gene fragments flanking the insert.

P. pastoris SMD1163 cells were transformed by electroporation after linearisation of the plasmid in the 5'AOX region. This leads to integration of the gene into the host genome such that the insert is under the control of the strong AOX promoter. SMD1163 cells were chosen initially because they lack 3 endogenous proteases, which may aid

production of full-length protein. After selection on medium lacking histidine (selecting transformants containing the *HIS4* gene), the positive clones were selected again on plates containing G418. By titrating the G418 concentration, it is possible to select clones containing multiple expression cassettes (Figure 6)

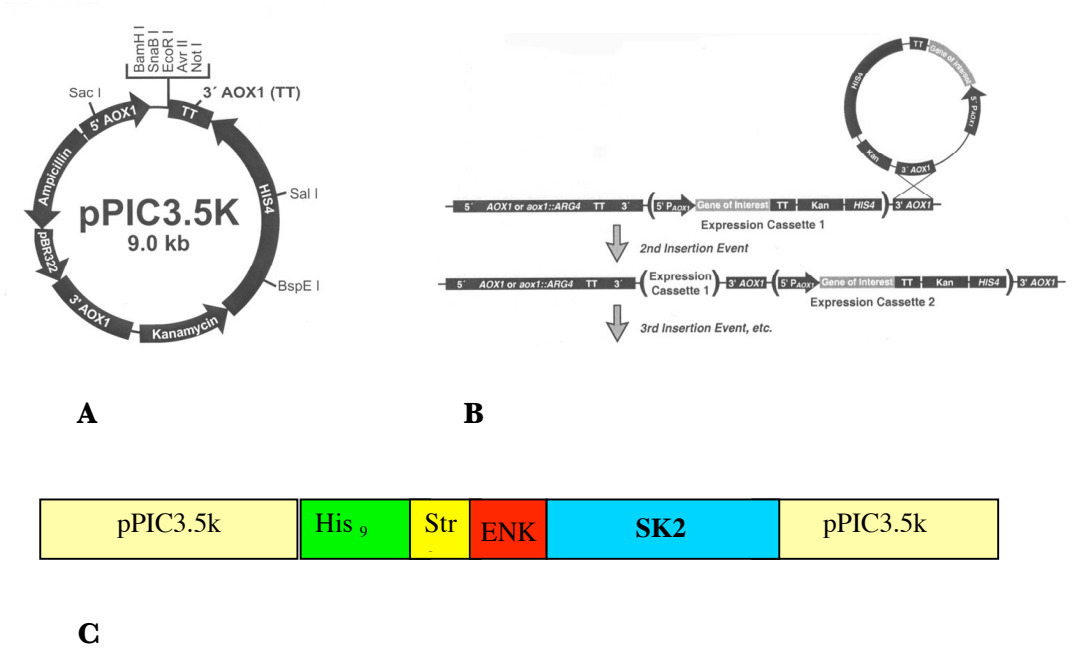


Figure 6: A) *Pichia pastoris* expression vector, pPIC 3.5K and schematic representation of cloning strategy. B) Schematic representation of constructed expression vector pPIC 3.5K: His_9: nine histidine tag; Str: strept II tag; ENK: enterokinase recognition sequence; SK2: SK2 coding sequence.

Several colonies were tested for expression. Twenty-four hours after induction in methanol medium, the cells were harvested and membranes were prepared.

Two antibodies were used for immunological detection on Western blots. One, anti-SK2, specifically recognises an epitope at the C-terminus of rSK2 while the second, anti-His tag, will label the His tag at the protein N-terminus.

Both antibodies identified a protein band at ~65 K after induction (Figure 7). The identical pattern observed for the antibodies indicates that the full length protein is produced and that no marked degradation has taken place. The mass of 65K is close to the expected value of 64KDa calculated from the amino acid sequence. In addition to this

major band, a higher molecular mass band was also detected, again with both antibodies. Because of its high mass, an accurate Mr could not be determined, however, it is possible that it represents a stable tetrameric form of the channel, or else is a result of aggregation during sample preparation.

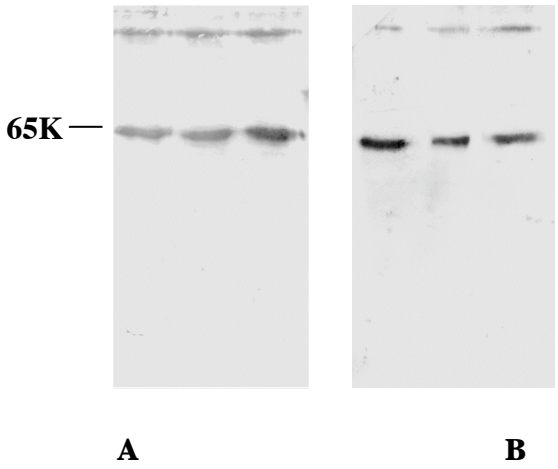


Figure 7: Western blot of *Pichia pastoris* membranes over-expressing the SK2 channel (30 □g of membranes). Blots were probed with anti-SK2 polyclonal antibody (A) or anti his-tag monoclonal antibody (B) and then visualized with alkaline-phosphatase-coupled secondary antibody.

The amount of protein produced by cells from colonies grown on different concentrations of G418, did not show noticeable differences on Western blot analysis. This indicated that the gene dose has no or little effect on levels of protein production, a situation observed for Kv1.2 K^+ channel (Parcej and Eckhardt-Strelau 2003).

A time course of induction showed that the expression level was already maximal after 6 hours. Levels seen at 12 and 24 hours were similar, though after extended periods (eg. 48 h) of induction less protein was detectable (Figure 8). This may be due to protein degradation, down regulation of the expression and/or cell death.

In addition to SMD1163, GS115 and SMD1168 *Pichia pastoris* strains were also tested. GS115 contains the full complement of endogenous protease while SMD1168 has deletions for the *pep4*$^-$ that is deficient in protease A, that it is required for the proteolytic activation of a number of proteases, including carboxypeptidase Y. However, no significant variation, in expression or in stability were observed.

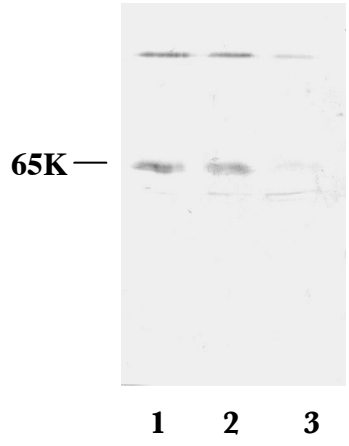


Figure 8: Time course of expression of SK2 expressed in SMD1163 *Pichia pastoris* cells. Blot was probed with anti-SK2 polyclonal antibody. Lane 1) Sk2 expression at 12 hour induction; lane 2) 24 hours; lane 3) 48 hours.

3.1.2 Localisation of expressed SK2 channel

In order to establish the cell localization of expressed SK2 channel, cells were induced, fixed and thin sections prepared for immuno-electron microscopy. After labelling with anti-SK2 antibody, a secondary gold-coupled antibody was applied. The electron microscopic images confirmed the presence of the protein in the cells. However, instead of being found in the plasma membrane, like SK2 channels in neuronal cells, it was mainly localized on the surface of internal membrane structures identified as the endoplasmic reticulum (Figure 9). This is not unprecedented for membrane proteins which are over-expressed in *P. pastoris* (Weiss et al. 1998; Maeda et al. 2000; Grunewald et al. 2004) and it has also been observed for several K^+ channels expressed in other systems. For example, Kv1.2 expressed in CHO cells fails to reach the cell surface unless it is co-expressed with the Kv subunit (Trimmer 1998), while the separate components of the I_{KATP} channel, sulphonyl urea receptor and Kir6.2 are retained in the endoplasmic reticulum unless expressed together (Lorenz et al. 1998). Retention in the endoplasmic reticulum does not necessary indicate that the produced protein is mis-folded. Endoplasmic reticulum located Kv1.2 for example appears to bind specific ligands in a

normal way (Shamotienko et al. 1999). Several explanations for the abnormal localisation seen here may be advanced. Firstly, it may be that the protein is indeed misfolded. However, the incorrect localisation may also be caused by overloading of the cell machinery due to excessive amounts of protein produced. Also conceivable is that, like Kv1.2 or the I_{KATP} channels, an additional subunit is required for proper trafficking. Finally, the protein may be toxic for the cell and consequently be retained in the endoplasmic reticulum.

3.1.3 Binding of specific ligands to *P. Pastoris* expressed SK2 channels

It is often found that proteins over-expressed in heterologous systems are misfolded and consequently inactive, this is a particular problem for eukaryotic membrane proteins (Klaiber et al. 1990; Tate et al. 2003).

For this reason, it was important to test the functionality of the SK2 proteins expressed in *Pichia pastoris* cells, and to address this question, binding experiments using radiolabeled apamin were performed.

Apamin, as already mentioned, is an octadecapeptide from bee venom with neurotoxic activity and has been further demonstrated to be a specific blocker of calcium activated potassium channels (SK2 and SK3).

It has been shown (Labbe-Jullie et al. 1991) that three specific residues, Arg-13, Arg-14 and Gln-17, are fundamental for the interaction between apamin and the channel.

When control membranes prepared from *Pichia pastoris* cells transformed with empty pPIC3.5K vector and induced with methanol were tested, no specific apamin binding was observed. In contrast, apamin bound with high affinity to membranes from cells expressing SK2 after 24 hr of induction. The total number of binding sites was 100 (+/- 20) fmol/mg (Figure 24). This compares with the 10 fmol/mg for rat brain membranes measured in parallel. It is likely that the amount of active channel observed in *P. pastoris* is actually more than 10-fold higher than in the brain, since in brain not only SK2 but also SK3 channels are present and bind apamin (Stocker and Pedarzani 2000), consequently part of the specific binding observed in the synaptosomal membrane could be attributed to SK3 or something else. In addition, the level of expression of the three

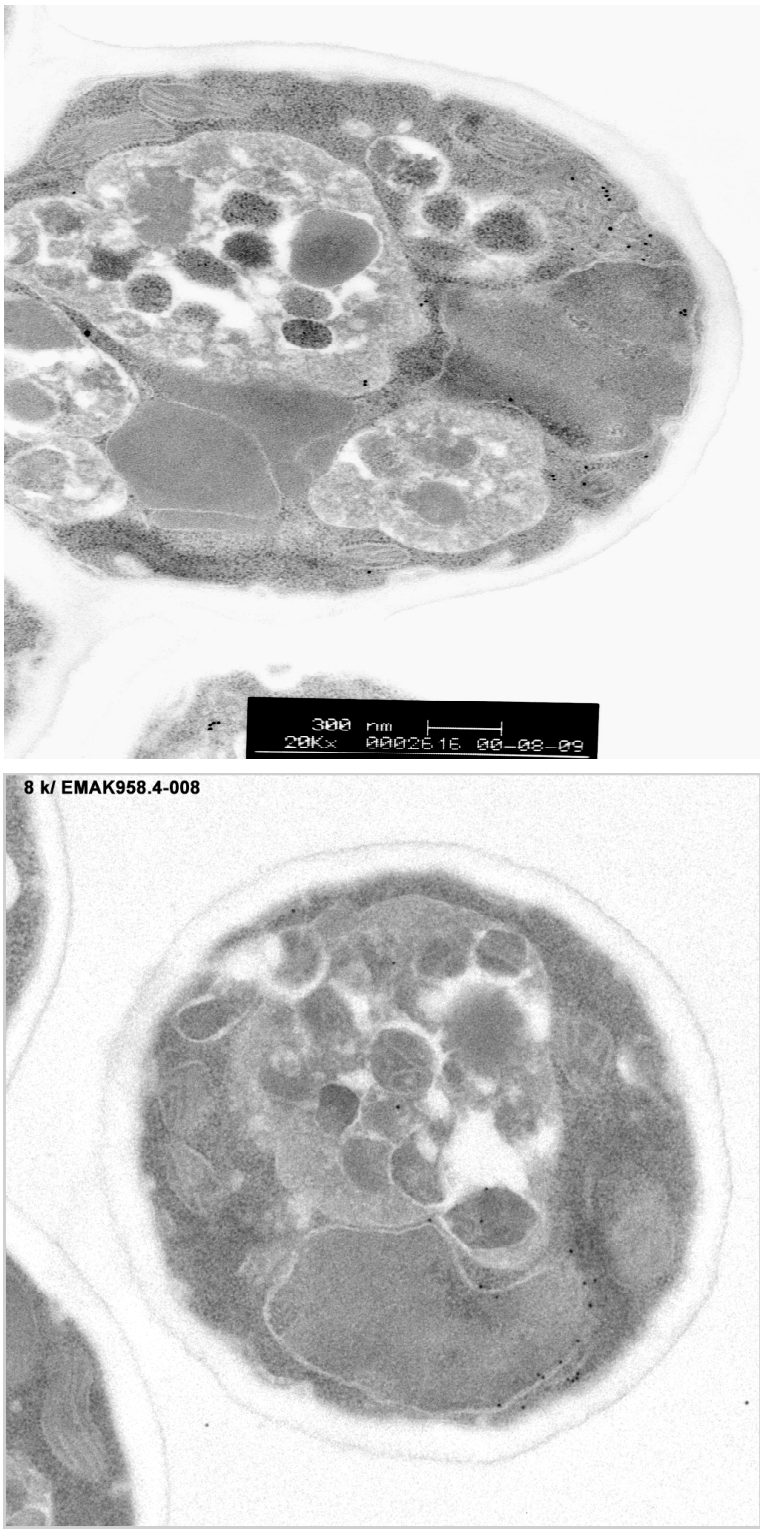


Figure 9: Indirect post embedding imunogold labelling of *Pichia pastoris* cells expressing SK2 after 24 h induction. The primary polyclonal anti-SK2 antibody was visualised with a gold coupled (10nm) anti rabbit antibody. Speciment preparation and electron microscopy were performed by Dr.Winfried Haase, MPI of Biophysics, Frankfurt/M.

channels has a differential distribution (Stocker and Pedarzani 2000) in the central nervous system and heterotetramerization between different SK channels also occurs (Benton et al. 2003; Monaghan et al. 2004) which makes direct comparison difficult. Furthermore, Western blots indicate that the levels of SK2 expressed in *P. pastoris* are probably substantially more than 10-fold higher than the brain membrane levels.

Our native brain synaptosomal binding data are in agreement with other previous native brain binding data. In fact it has been reported that in rat brain synaptosomal, the specific I^{125}-apamin Binding Capacity (B_{max}) is 12.5 fmol/mg (Hugues et al. 1982) and in primary neuron cultures is 3-8 fmol/mg (Seagar et al. 1984).

The apamin-binding receptors present in the rat heart have a B_{max} of 24 fmol/mg, while in the cultured rat heart cells is 2.8 fmol/mg.

Only membranes extracted from ileum and liver of Guinea pig show a high specific I^{125}-apamin binding capacity that is 42 fmol/mg in the ileum membranes and 43 fmol/mg in the liver membranes (Marqueze et al. 1987).

3.1.4 Solubilisation of SK2 channels from *P. Pastoris* membranes.

In order to purify the expressed SK2 protein it was first necessary to obtain them in a soluble state.

Initial trials used sodium cholate since this was the only detergent found to solubilise native apamin receptor/SK channels with retention of I^{125}-apamin binding activity, although other detergents were able to solubilise the apamin receptor but with less stability (Seagar et al. 1987).

The efficiency of the solubilisation conditions was estimated by Western blot analysis using either the anti SK2 or anti His-tag antibodies, comparing the soluble fraction with membranes before extraction and with the insoluble pellets.

Unfortunately, no SK2 channel could be solubilised with sodium cholate (Figure 5), so a detergent screen was performed.

First, nonionic detertergents, Triton X-100, Triton X-114, n-dodecyl-β-D-maltoside (DDM), n-octyl-β-D-glucoside (OG), Tween 20, APO12, Thesit and zwitterionic detergents as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS),

Lauryl dimethylamine oxide (LDAO), Zwittiger L-12 and 14 were tested since they do not usually denature proteins (Helenius and Simons 1975; Hjelmeland 1990). In addition the ionic detergent taurocholic acid was tested. Diheptanoylphosphatidylcholine (DHPC), a short-chain phosphatidylcholine that act as a mild detergent, was also tested especially because it has been reported that the protein conformation and activity is usually preserved when this detergent is used (Kessi et al. 1994).

For example, Ca^{+2}-ATPase from sarcoplasmic reticulum was solubilised with DHPC in higher yield and better specific activity than with $C_{12}E_8$ or cholate (Shivanna and Rowe 1997). Ammonium perfluorooctanoate (APFO), a fluorinate surfactant, was also tried since it has been reported to be able to solubilise membrane proteins (for example the photosystem II and the equine herpes virus (EHV) envelope protein), and is particularly good in increasing the yield of solubilisation and as well to solubilise proteins that are usually difficult to extract from the membrane (Shepherd and Holzenburg 1995)

None of the detergents above was able to solubilise the channel with the protein remaining in the high-speed pellet after the extraction (Figure 10).

Further tests with various concentrations of these detergents, different pH, salt concentration, and glycerol addition did not bring any improvement.

However, digitonin was able to extract the channel (Figure 11). Digitonin is a saponin extracted from the plant *Digitalis purpurea* and useful for receptor solubilization (Niznik et al. 1986; Rauh et al. 1986; Nunnari et al. 1987).

Frequently applied as haemolysis reagent and for permeabilization of certain types of cells like blood platelets, hepatocytes, yeast or tumor cells, it is also used for the estimation of cholesterol and appears to selectively solubilise cholesterol-containing membranes.

Unfortunately, using the standard conditions, the amount of SK2 channel extracted with digitonin was not high. In order to try to improve the efficiency of solubilisation, the effects of detergent concentration and buffer components were tested.

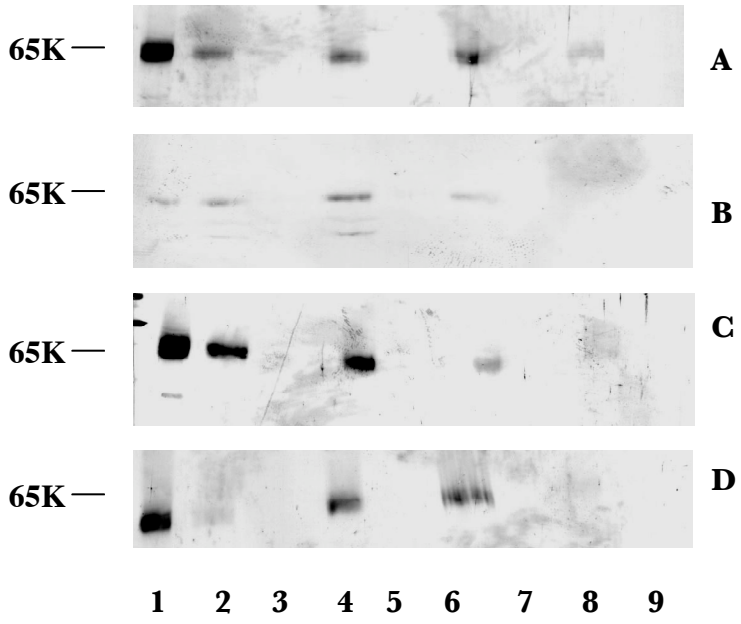


Figure 10: Solubilisation of *Pichia pastoris* membranes over-expressing SK2.
Membranes (10 mg/ml) were incubated for 1h at 4 °C with various detergents at 4% (w/v), in presence of 100 mM KCl, 600 mM NaCl, 5% glycerol, 40 mM hepes, pH 7.0. Immunoblot analysis were performed with anti SK2 antibody.
A: Lane1) Membranes (30 µg); solubilisation mixture before, lane 2) and after extraction with Triton X-100, lane 3); solubilisation mixture before, lane 4) and after extraction with cholic acid, lane 5); solubilisation mixture before, lane 6) and after extraction with thesit, lane 7); solubilisation mixture before, lane 8) and after extraction with OG, lane 9).
B: Lane1) Membranes (30 µg); solubilisation mixture before, lane 2) and after extraction with tauric acid, lane 3); solubilisation mixture before, lane 4) and after extraction with Z-12, lane 5); solubilisation mixture before, lane 6) and after extraction with Z-14, lane 7); solubilisation mixture before, lane 8) and after extraction with C12E8, lane 9).
C: Lane1) Membranes (30 µg); solubilisation mixture before, lane 2) and after extraction with DDM, lane 3); solubilisation mixture before, lane 4) and after extraction with CHAPS, lane 5); solubilisation mixture before, lane 6) and after extraction with LDAO, lane 7); solubilisation mixture before, lane 8) and after extraction with Tween 20, lane 9).
D: Lane1) Membranes (30 µg); solubilisation mixture before, lane 2) and afteextraction withr Triton X-114, lane 3); solubilisation mixture before, lane 4) and after extraction with APFO, lane 5); solubilisation mixture before, lane 6) and after extraction with DHPC, lane 7); solubilisation mixture before, lane 8) and after extraction with APO-12, lane 9).

As expected, increasing the detergent concentration had a positive effect, in terms of material extracted, with a maximal effect at 4% of digitonin. Further experiments were therefore conducted using digitonin at 4%.

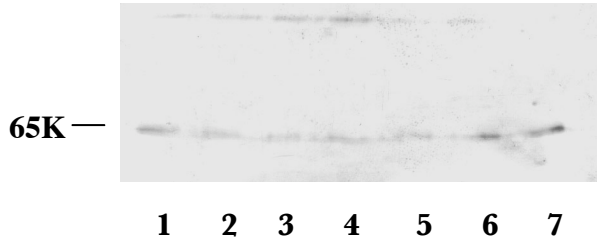


Figure 11: Effects of salt concentration on solubilisation of *Pichia pastoris* membranes over-expressing SK2. Membranes were incubated for 1h at 4 °C with 4% digitonin, in presence of 100 mM KCl, 5% glycerol, 40 mM hepes, pH 7.0 and different concentration of NaCl. Immunoblot analysis with anti SK2. Lane1) membranes (30 □g); lane 2) Extract, 0 mM NaCl; lane 3) Extract, 100 mM NaCl; lane 4) Extract, 300 mM NaCl; lane 5) Extract, 500 mM NaCl; lane 6) Extract, 600 mM NaCl; lane 7) Extract, 1.2 M NaCl.

The time of solubilisation had small but noticeable effects with over-night incubation increasing extraction slightly. However due to the tendency of digitonin to precipitate after several hours at 4°C, shorter incubation times (2-3 hours), were chosen.

In contrast, buffer composition, especially the total salt concentration had a strong effect on the channel solubilisation, as well on the solubility of digitonin at 4°C.

Increasing the concentration from 0 mM to 600 mM gave a considerable improvement in receptor solubilisation, whereas from 600 mM to 1.2 M only a slight improvement was seen (Figure 11). However the higher salt concentration had a much larger beneficial effect on digitonin solubily with the detergent remaining in solution for 48 hours at 4°C.

Inclusion of glycerol in the solubilisation mixture was found to have no significant effect, but since the glycerol could be useful to stabilize the channel, 5% (w/v) was added to the buffer.

The final optimised solubilisation buffer was: 1.2 M NaCl, 150 mM KCl, 5% glycerol, 40 mM hepes, pH 7.0 and 4% digitonin.

However even under this best condition, the extraction as detected on Western blots was poor compared to the amount of SK2 channel observed in the membrane.

3.1.5 Large scale protein production

Although digitonin does not give high channel solubility and has the added disadvantages of been poorly soluble at 4°C, it was decided to scale-up the production of the channel in

order to try to purify it. For this purpose, the *Pichia pastoris* cells carrying the SK2 gene construct were grown in a 5l fermentor.

Protein production in *Pichia* can improve by using the fermentor. The fermentor facilitates the increase of dissolved oxygen levels by increasing agitation, airflow, and by supplementing the air stream with pure oxygen. It can be run in fed-batch mode, meaning that fresh media or growth limiting nutrients can be pumped into the vessel, whenever necessary. Moreover, it is possible to control methanol flow rates, so only the optimal amount of methanol for induction of expression and protein synthesis is added so avoiding an overload of potentially toxic compound.

Under optimised grow this conditions 300 g/L of cells expressing SK2 channel was achieved with the best level of expression was reached after 24 hours of induction

In the case of SK2, the specific expression level in the fermenter was worse than that seen in flask culture (Figure 12). However, this disadvantage is outweighed by the vastly superior cell density possible (flask culture gave a maximum of 10g/l).

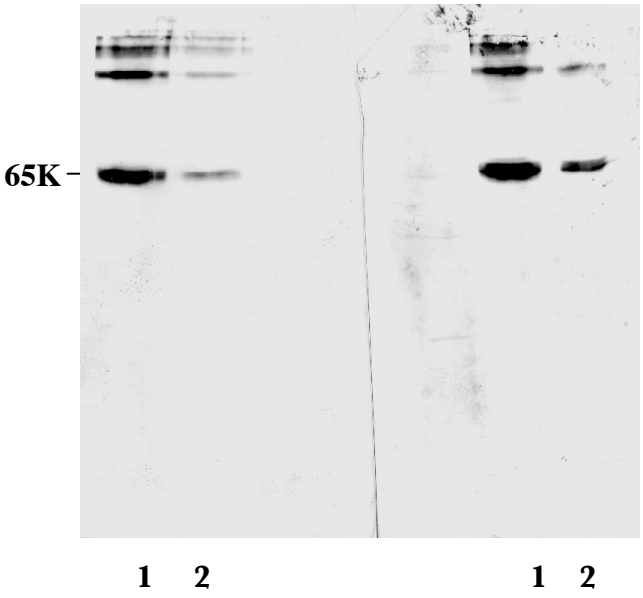


Figure 12: Large scale production of SK2. Immunoblot analysis with anti His-tag (A) and anti SK2 (B) antibodies; Lane 1) membranes after 24h induction in flask; lane 2) membranes after 24h induction in the fermentor.

3.1.6 Purification of SK2

3.1.6.1 Immobilised metal affinity chromatography (IMAC)

Large-scale membrane production was achieved by using a microfluidizer to break the cells. The membranes were then solubilised for 2-3 hours with 4% digitonin, in the optimised buffer described above except with the addition of 25 mM imidazole.

After protein extraction, the soluble material was loaded on a Ni-NTA column previously equilibrated with the solubilisation buffer plus 0.01% of digitonin (w/v). After washing with washing buffer (paragraph 2.1.2), bound material was eluted with 300 mM imidazole solution. However, although SK2 was present in the eluate as determined by immunoblotting (Figure 13), a Coomassie stained gel showed the presence of many other proteins beside rSK2.

Although disappointing, this is not unprecedented since often it is not possible to achieve a good level of purification for proteins of eukaryotic origin using IMAC due to the high intrinsic content of these proteins. In the case of rSK2, this is made worse by the relatively low expression level.

The addition of higher concentration of imidazole in the solubilisation buffer often overcomes this problem since it suppresses the unspecific binding of other proteins.

Unfortunately, this could not be exploited here because in the presence of just 50 mM imidazole, the majority of the SK2 did not bind the column.

However, the IMAC column offered the possibility of detergent exchange, which circumvents the digitonin precipitation problem. This was performed using DDM and Triton X-100 where the recovery of the protein was the same as when only digitonin was used. For this reason, DDM was used for further purification steps.

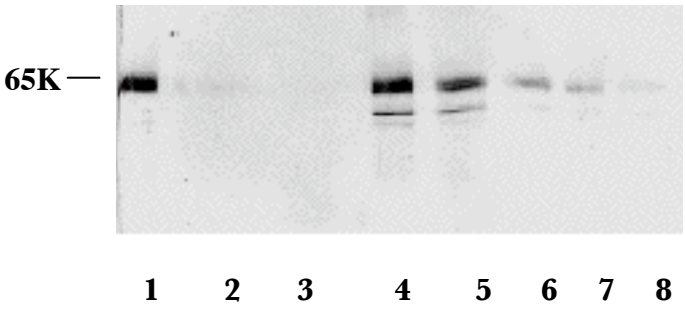


Figure 13: Ni-NTA chromatography. Imunoblot analysis with anti His-tag. Lane 1) 30 µg of membranes; lane 2) proteins extracted with 4% digitonin; lane 3) flow-through of Ni-NTA column; lanes 4-8) eluted fractions.

3.1.6.2 Affinity chromatography on Streptactin column

In anticipation of a further purification being required, the SK2 construct also contains a strep II tag, which enables affinity chromatography on streptactin-agarose (Schmidt, Köpke et al. 1996). This procedure is very effective for many soluble proteins and has been used successfully for some membrane proteins (Parcej and Eckhardt-Strelau 2003). Unfortunately the SK2 channel did not bind to the column, with all the SK2 in the Ni-NTA eluate recovered in the flow-through (Figure 14).

There was no improvement even when the Ni-NTA eluate was dialysed to remove salt and imidazole, which may interfere with the binding of the strep II tag to streptactin.

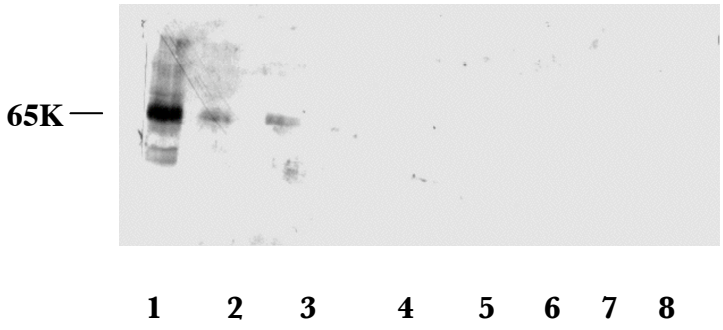


Figure 14: Affinity chromtography on streptactin column. Imunoblot analysis with anti SK2. Lane 1) 30 µg of membranes; lane 2 Ni-pool; lane 3) flow-through; lanes 4-8) eluted fraction.

3.1.6.3 Ion exchange chromatography

Since the theoretical isoelectric point (pI) of SK2 is 9.5 it was decided to test cation exchange chromatography for purification.

To allow binding of the protein to the cation exchange resin, the high salt concentration, normally used in the Ni-NTA protocol, was reduced during washing and elution steps to 100 mM NaCl, 50 mM KCl, 5% glycerol and 40 mM hepes, pH 7.0 plus imidazole.

The eluted fractions containing SK2 were applied to an SP resin equilibrated with 100 mM NaCl, 50 mM KCl, 5% glycerol, 40 mM hepes, pH 7.0, and 0.02% DDM.

Then, after washing, the column was eluted using a salt gradient up to 1 M NaCl.

The channel bound strongly to the resin and started to be eluted only after more than 400 mM NaCl was applied (Figure 15); this of course, was an extremely important feature for the purification because most of the contaminant proteins were eluted at lower salt concentration.

The purity of the channel appeared to be better after cation exchange although there were still a few contaminants present. However, it was not very efficient in terms of sample concentration.

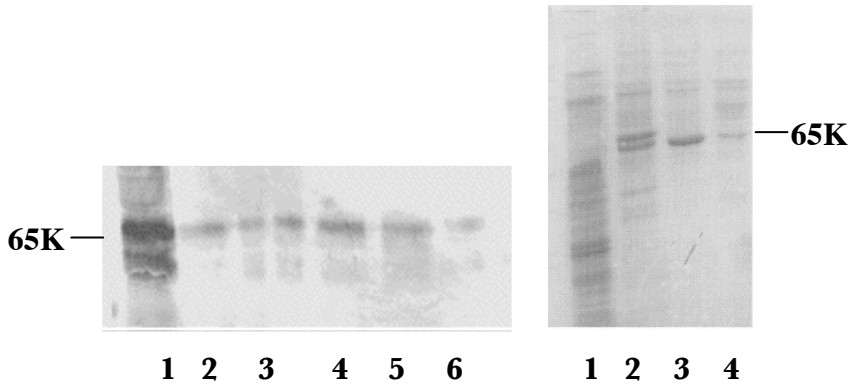


Figure 15: Ion exchange chromatography on streptactin column. A) Imunoblot analysis with anti SK2 antibody. Lane 1) 30 μg of membranes; lane 2) Ni-pool; lanes 3-8) eluted fraction. B) Blue Coomassie stain 10% SDS gel of SK2 purification. Lane 1) membranes (30 μg); 2) Ni pool; 3) SP flow through; 4) SP pool.

3.1.6.4 Size exclusion chromatography

Gel filtration chromatography was used to verify the oligomeric state of the channel rather than as a purification step.

SK2 is expected to be a homotetramer like other potassium channels (MacKinnon 1991), and its predicted molecular weight is about 280 Kda. However, the channel will bind a considerable and unknown amount of detergent, which will increase the Stokes radius of the channel particle and lead to an elution volume considerably smaller than that expected for just the protein tetramer. Despite these uncertainties, an elution volume of 1.5 ml on a Superose 6 column combined to a SMART system would indicate a higher oligomeric state (likely a tetramer). The elution volume of 1.5 ml is that seen for Kv1.2 tetramer under the same conditions.

By western blot analysis it was possible to recognize that channel did indeed elute after 1.5 ml, the first peak after the void volume (Figure 16).

The other peaks present in the profile are probably contaminant proteins present in the sample, since even after the ion exchange chromatography the sample is still not totally pure.

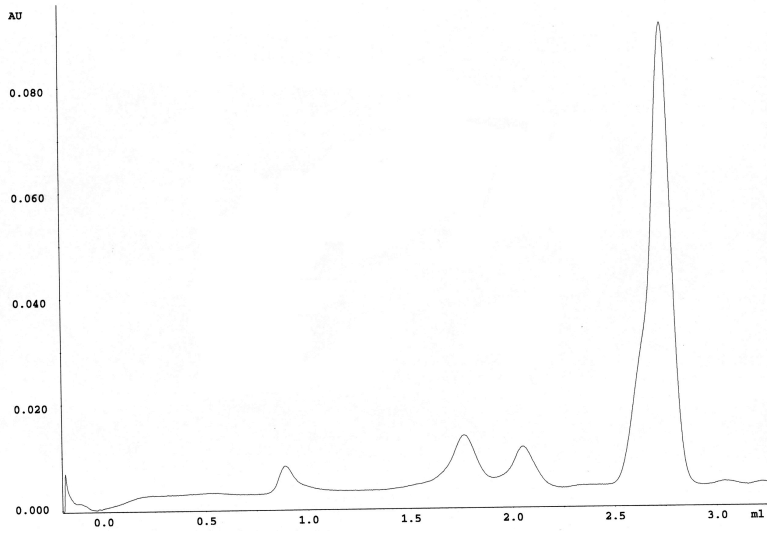


Figure 16: Chromatogram of gel filtration analysis of SP eluted fraction on a SMART/Superose 6 column.

3.2 Expression of small calcium activated potassium channel-FCYENE in *Pichia pastoris* SMD1163 cells.

Because the yield of purified wild-type SK2 was small, it was decided to test another construct in order to try to obtain the quantity of material needed for further structural studies. The major difficulty when trying to isolate the wild-type channel was the inability to extract it from the membranes with any detergent except digitonin, which itself was not very effective. One possible explanation could be that the channel is retained in the endoplasmic reticulum in an unfolded and therefore insoluble state. This could also be the reason why it appeared that the level of apamin binding was less than would be expected by the relative intensity of the protein observed on Western blots. This would in fact suggest that only the channels that are able to overcome the ER quality control system (Ellgaard et al. 1999) and these then subsequently migrate to the Golgi and plasma membrane are solubilisible.

Several examples of amino sequences have been identified in proteins, including K^+ channels, which are required for the proteins to move from the ER to the Golgi (and consequently to the plasma membrane) only when the sequences are masked in a correctly folded and assembled protein (Zerangue et al. 1999). Another sequence that acts to promote surface expression has been identified in the mammalian inward rectifier channel, Kir 2.1 (Ma et al. 2001).

This latter sequence is composed of 7 amino acids (FCYENE) of which all except the cysteine at position 2 appear to be essential for exit from the ER. However, the sequence has no apparent effect on folding, gating or assembly of the channel. Importantly, unlike the ER retention signals, the effect of FYCENE is not position dependent.

Further studies have shown (Stockklausner et al. 2001) that the FCYENE sequence represents a recognition signal for the recruitment and the incorporation of the Kir channel into COP II-coated transport vesicles (Klumperman 2000). Importantly, when this sequence is inserted into the carboxyl terminal of other types of K^+ channel (e.g. Kv1.2) it was able to induce efficient translocation to the plasma membrane (Ma et al. 2001; Stockklausner et al. 2001; Zhang and Harris-Warrick 2004). The aforementioned studies were conducted in mammalian cell expression systems. However, the FCYENE

sequence was shown to have a similar effect on *Saccharomyces cerevisae* expressed inward rectifier channels (Stockklausner et al. 2001). It was therefore, decided to test the effect of this sequence on SK2 expression in *Pichia pastoris*.

3.2.1 Cloning and expression of SK2-FCYENE in *Pichia pastoris* cells

Oligonucleotides containing the FCYENE coding sequence were inserted at the carboxyl terminal part of the SK2 channel.

After screening and verification by DNA sequencing analysis of the rSK2 gene region the plasmid was linearized and transformed into *Pichia pastoris* SMD1163 cells by electroporation as for the wild type channel.

As previously, the production of the SK2-FCYENE mutant protein was tested by immuno-blotting after 24h of induction.

The SK2-FCYENE expression was assessed on immunoblots using anti-SK2 and anti His-tag antibodies. As for the wild-type construct, a band at ~65K was detected (Figure 17). Also, as both antibodies labelled identically, it is apparent that no significant proteolytic degradation occurred.

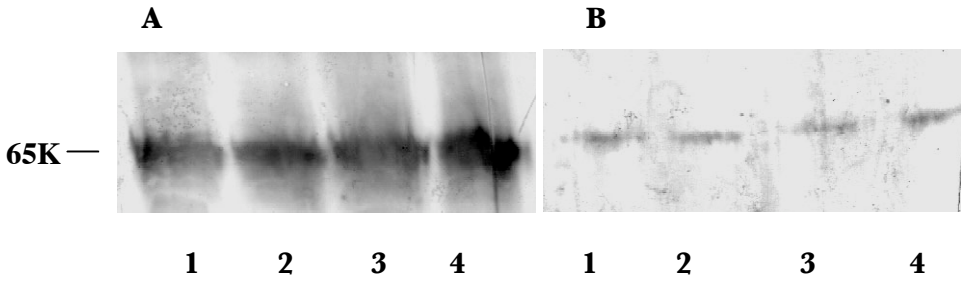


Figure 17: Western blot of *Pichia pastoris* membranes, from four different colonies, over-expressing SK2-FCYENE channel (30 μg of membranes). Blots were probed with anti-SK2 polyclonal antibody (A) or anti his-tag monoclonal antibody (B) and then visualised with alkaline-phosphatase-coupled secondary antibody.

3.2.2 Binding of ^{125}I-apamin to membranes of SK2-FCYENE expressing cells

Saturable ^{125}I-apamin binding was observed in membranes of the cell expressing the mutant construct. The binding was mono-phasic with a total number of binding sites of 110 (+/- 15) fmol/mg (Figure 20). These values are similar to that observed for the expressed wild-type SK2, indicating that the FCYENE tag had no effect on total expression levels of binding-competent channels.

3.2.3 Solubilisation of SK2-FCYENE

The efficacy of channel solubilisation by detergents was assessed by western blot analysis using either the anti SK2 or anti His-tag antibody as for wild-type SK2.

For the reasons mentioned above (paragraph 3.1.4) sodium cholate was the first detergent tested, using either the published buffer conditions or the buffer found optimal for extraction with digitonin (1.2 M NaCl, 200 mM KCl, 5% glycerol, 40mM hepes, pH7.0). Unlike the wild-type channels, some of the SK2-FCYENE mutant protein could be solubilised by this detergent albeit with low yield.

Further, when digitonin, Triton X-100, DDM, and octyl glucoside were tested, all were found to solubilise the channels, although to different extents. This is in striking contrast to the situation seen with the wild type where only digitonin showed significant levels of extraction. Although once again digitonin was among the more efficacious detergents for SK2-FCYENE solubilisation, it was apparent that dodecyl maltoside was equally effective (Figure 18). In addition, the fraction extracted from the membrane appeared to be higher with these two detergents compared with the amount of wild-type SK2 extractable by digitonin. This enhancement of solubilisation for the FCYENE containing channel may result from improved folding of the protein or by relocation to a membrane whose lipid composition is more soluble in dodecyl maltoside.

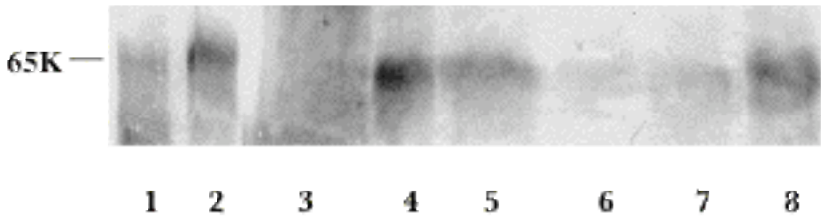


Figure 18: Western blot analysis of SK2-FCYENE solubilisation. Lane1) SK2 membranes; lane 2) SK2-FCYENE membranes; solubilisation mixture before, lane 3) and after extraction with 4% digitonin, lane 4); solubilisation mixture before, lane 5) and after extraction with 4% cholic acid, Lane 6); solubilisation mixture before, lane 7) and after extraction with 4% DDM, Lane 8). Blots were probed with anti-SK2 polyclonal antibody and then visualised with alkaline-phosphatase-coupled secondary antibody.

3.2.4 Localisation of SK2-FCYENE mutant channel

The localisation of the SK2-FCYENE protein produced in *Pichia pastoris* was investigated by immunogold labelling analysis (Figure 19) as before. In contrast to the wild-type, the channel was no longer found solely in ER membranes. Rather, it appeared concentrated in vesicle clumps. The identity of these clumps is not clear.

In the cell, lipid rafts play an important functional role in the specific distribution of different proteins in micro domain of plasma membrane depending on lipid composition and concentration (Brown DA 2000). It is possible that this is the way to control the distribution of proteins inside the cell, and segregation into a specific membrane platform is used to render a protein insoluble and unfunctional (Brown DA 2000; Bock et al. 2003). In this case, it is also possible that the segregation into specific vesicles make the channel easier to solubilise due to the lipid composition of these vesicles.

It is not clear if, in this specific case, this segregation has a functional role or if it is simply a non-specific reaction from *Pichia* cells to the over-expression of this particular construct. However, as before little labelling is observed in the plasma membrane. It appears that the FCYENE sequence is able to promote exit from the ER but the channels are not translocated further.

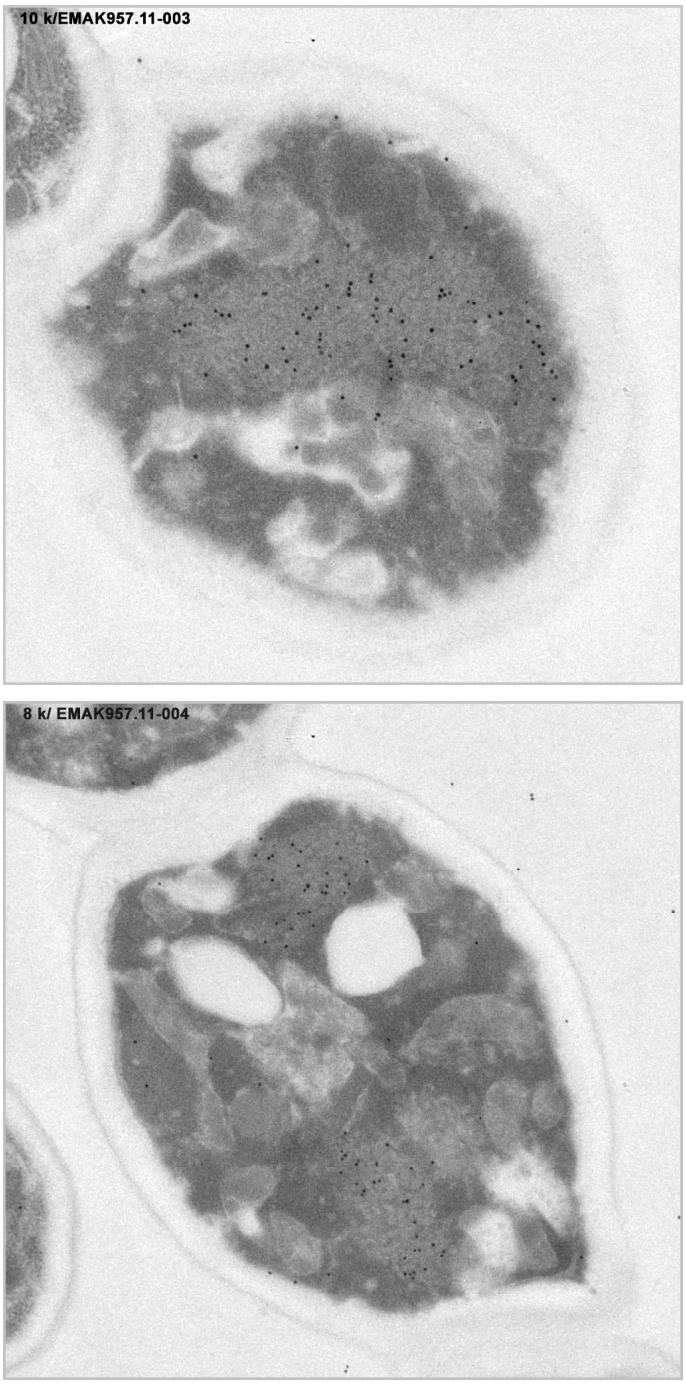


Figure 19: Indirect post embedding immunogold labelling of *Pichia pastoris* cells expressing SK2-FCYENE after 24 hr induction, The primary policlonal anti-SK2 antibody was visulised with a gold coupled (10nm) anti rabbit antibody. Speciment and electron microscopy were performed by Dr. Winfried Haase, MPI of Biophysics, Frankfurt/M.

3.2.5 Purification

For purification, membranes were soulubilised in DDM to circumvent the problems caused by digiton in marginal solubility at 4°C. However, as was the case for wild-type SK2 protein, immobilised metal affinity chromatography on Ni-NTA was not sufficient to obtain pure channel protein.

In addition, streptactin-agarose chromatography was ineffective, with all the material loaded appearing in the flow-through of the column.

Ion exchange chromatography on SP resin was then performed following the same conditions described for the wild-type channel (Figure 21).

Again, similar to the wild type, this step significantly improved the purity of the channel. As for the wild-type construct, a few contaminants are still present in the protein stained gel.

The FCYENE sequence clearly has a strong effect of the amount of SK2 channel that could be solubilised from the *Pichia* membranes. It is tempting to speculate that this was due to the apparent ER retention observed for wild-type SK2 channels being at least partially overcome. This is supported by the different localisation observed by immuno electron microscopy. However, it is clear that although most of the channel has escaped from the ER, it fails to reach the plasma membrane, instead being found in vesicle clumps. It is possible that movement through a subsequent step in the pathway to the plasma membrane requires other, as yet unidentified, determinants.

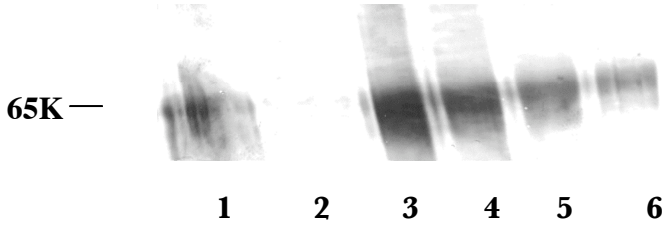


Figure 20: Western blot analysis of SK2-FCYENE purification on Ni-NTA column. Lane 1) Membranes; 2) flow through; lanes 3, 4, 5, 6, 7, 8) eluted fractions. Blots were probed with anti-SK2 polyclonal antibody and then visualised with alkaline-phosphatase-coupled secondary antibody

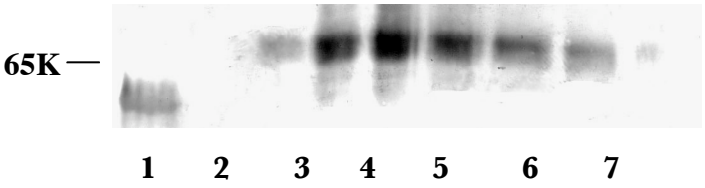


Figure 21: Western blot analysis of Ion-exchange chromatography on SP resin. Lane 1) flow trhough; lanes 2, 3, 4, 5, 6, 7) eluted fractions. Blots were probed with anti-SK2 polyclonal antibody and then visualised with alkaline-phosphatase-coupled secondary antibody

3.3 Co-expression of SK2 and calmodulin in *Pichia pastoris* SMD1163 cells.

Calmodulin is a small Ca^{2+}-binding protein, which acts as a sensor of Ca^{2+} levels in the cells. It functions as a regulator of cellular processes by interacting with major components of numerous signaling pathways involving various kinases, phosphatases, cytoskeletal and ribosomal proteins. CaM regulates many important cellular processes such as smooth muscle contraction. Calmodulin is a rather ubiquitous calcium-sensing protein belonging to a class of loop-helix-loop cation binding proteins of similar structure and function. Calmodulin undergoes a dramatic conformational change upon Ca^{2+} binding which defines its regulatory properties. It can assume at least three different conformations due to its flexible linker between the two globular domains that link Ca^{+2} and partner proteins.

Calmodulin is well known to play important roles in the regulation of several proteins such as CaM-dependent protein kinases, receptors and other channels.

Calmodulin links to the N-methyl-$_D$-aspartate (NMDA) receptor mediating the Ca^{+2}-dependent inactivation process (Ehlers et al. 1996; Zhang et al. 1998). In neurons, binding of calmodulin to B50/GAP 43 protein (also known as neuromodulin) is involved in neurite outgrowth, cone growth function and movement (Verkade et al. 1997).

Calmodulin is tethered constitutively to KCNQ 2/3, voltage-dependent potassium channels which are evolutionarily distinct from other Kv channels, and its binding is essential for channel functionality (Wen and Levitan 2002). Human ether à go-go (EAG), another voltage dependent potassium channel also interacts with calmodulin but in a Ca^{+2} dependent manner which leads to inhibition (Schonherr et al. 2000).

Calmodulin has also been shown to bind the L-type Ca^{+2} channel *via* the consensus 'IQ' calmodulin binding motif, located in the carboxyl terminal part of the channel subunit. In this case the binding of calmodulin regulates both inactivation and facilitation (Zuhlke et al. 1999). In 2001, it was shown by Adelman *et al.* that the calcium sensitivity of SK channels was conferred by interaction with calmodulin (Xia et al. 1998).

The subsequent crystal structure of the SK2 C-terminal and calmodulin illustrated the tight interaction of the proteins and led to speculation as to how binding of calcium could trigger channel opening. Importantly, this and other studies showed that calmodulin is always bound to the channel, even in the absence of calcium (Schumacher et al. 2001). In the case of SK channels, it has been reported that the binding between calmodulin and SK2 is not only essential for the gating of the channel, but also plays an important role in the trafficking of the channel from internal compartments to the plasma membrane. This was shown when the amino acids in SK essential for the tight interaction between the two proteins were mutated. These mutant channels then lost the ability to reach the plasma membrane, being retained in internal organelles, probably the ER (Lee et al. 2003). It is interesting to note that while the amino acid sequence of calmodulin among vertebrates is invariant, the yeast protein shows a number of differences, particularly at the C-terminus (Figure 22). Importantly, these changes alter the affinity for calcium, with loop four unable to bind calcium at all. It is therefore possible that the abnormal localisation of expressed SK2 could have been due to a missing or faulty association with the native *Pichia pastoris* calmodulin.

Interspecies Sequence Alignment

```
CALM_HUMAN     1 -ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG
CALM_YEAST     1 MSSNLTEEQIAEFKEAFALFDKDNNGSISSSELATVMRSLGLSPSEAEVNDLMNEIDVDG
consensus      1 .  .************* *****  *.*.. **.*******  *.***..*..**.*

CALM_HUMAN    60 NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE
CALM_YEAST    61 NHQIEFSEFLALMSRQLKSNDSEQELLEAFKVFDKNGDGLISAAELKHVLTSIGEKLTDA
consensus     61 *  *.* *** .* * .*  *** *. ***.**** * * ******.**.* .*****

CALM_HUMAN   120 EVDEMIREADIDGDGQVNYEEFVQMMTAK
CALM_YEAST   121 EVDDMLREVS-DGSGEINIQQFAALLSK-
consensus    121 ***.*.**  .** * .*   *  ....
```

Figure 22: Sequence alignment between the human and the *Saccaromices cerevisiae* calmodulin.

3.3.1 Cloning and expression

The SK2 channel sequence was cloned in tandem with calmodulin, with a peptide linker containing 10 glutamines added between the two proteins.

The glutamine linker was inserted in order to give to the two proteins the room to fold correctly. Glutamine was chosen, as it is a neutral but polar amino acid, which has the propensity to form an extended conformation in solution when in a peptide.

The length of the linker was chosen by examining PDB file of the structure of the carboxyl terminal part of SK2 complexed with calmodulin (Schumacher et al. 2001). The distance in Å between the last amino acid of SK2 and calmodulin was found to be 15 Å, which corresponds to about 10 glutamine residues. The tandem cloning of two different proteins or different subunits of the same protein is a good co-expression strategy because it allows a predefined stoichiometry.

Once the tandem construct (called pPIC3.5K-SK2-q-CaM) was obtained and the accuracy of the sequence ensured by sequencing, the plasmid was inserted in the *Pichia pastoris* genome as described for the wild type construct (paragraph 3.1.1). As previously, the production of the SK2-q-CaM tandem protein was tested by Western blot after 24 h of induction.

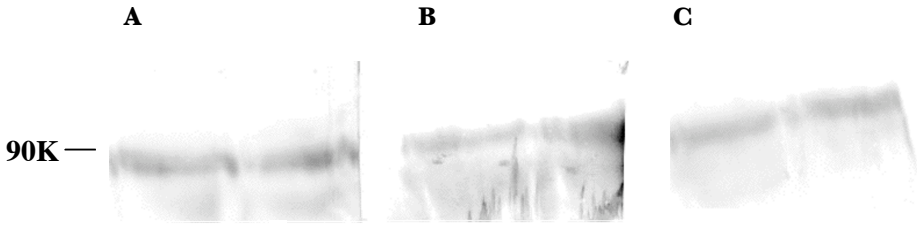


Figure 23: Western blot of *Pichia pastoris* membranes over-expressing SK2-q-calmodulin construct (30 □g of membranes). Blots were probed with anti-SK2 polyclonal antibody (A) or anti his-tag monoclonal antibody (B) or anti calmodulin (C) and then visualized with alkaline-phosphatase-coupled secondary antibodies.

The use of anti-SK2, anti-His and anti-Calmodulin antibodies confirmed the expression of the protein and that no degradation or partial cleavage of the proteins has occurred; all the three antibodies detected a band at about 90 K (Figure 23), as expected for the tandem linked protein. Like the other constructs, expression was maximal at 24h or earlier.

3.3.2 Specific I^{125}-apamin binding to *Pichia pastoris* membrane expressing SK2-q-Calmodulin tandem construct and localization studies

Saturable I^{125}-apamin binding was detected in membranes of *Pichia* cells expressing the tandem construct. The total number of binding sites varied from 110 fmol/mg to a maximum of 400 fmol/mg, which is considerably higher than the apamin binding sites observed for the expressed wild-type SK2, and the FCYENE mutant (Figure 24). It is not clear if this is due to a higher level of total channel protein expressed or an increase in the proportion of active channels in the population.

Interestingly, binding experiments on *Pichia pastoris* membranes expressing the wild type channel showed no difference, in term of total number of binding sites, when calmodulin was included in the in the binding buffer (data not shown). This would suggest that any enhancement of binding due to calmodulin it is linked with an improvement of folding and/or assembly.

Indirect post-embedding immunogold labelling of *Pichia pastoris* cells carrying the SK2-q-Cam tandem construct shows the presence of the calmodulin dramatically changes the distribution of the channel in the cell.

Unlike the other constructs discussed above, some of the SK2-q-CaM chimera is found on the plasma membrane. In addition labelling is observed in membrane layers (possibly Golgi derived) in proximity of the plasma membrane and also in the ER (Figure 25).

Previously it has been shown by electron microscopy that *Pichia pastoris* cells contain stacked Golgi organelles (Rossanese et al. 1999) instead of the individual cisternae distributed throughout the cytoplasm that are found in *Saccharomyces cerevisiae* cells. These Golgi stacks are found either near the nucleus or adjacent to the peripheral ER elements that are below the plasma membrane. This distribution is similar to the membrane stacks

seen here.

A typical *Pichia pastoris* cell contains two to six transitional ER sites, each of which is adjacent to a Golgi stack (Rossanese et al. 1999); the new cis-Golgi cisternae are generated by the fusion of membranes derived from the ER.

From the electron microscope images it appears that the channel is being transported from the ER *via* the Golgi apparatus and further to the plasma membrane.

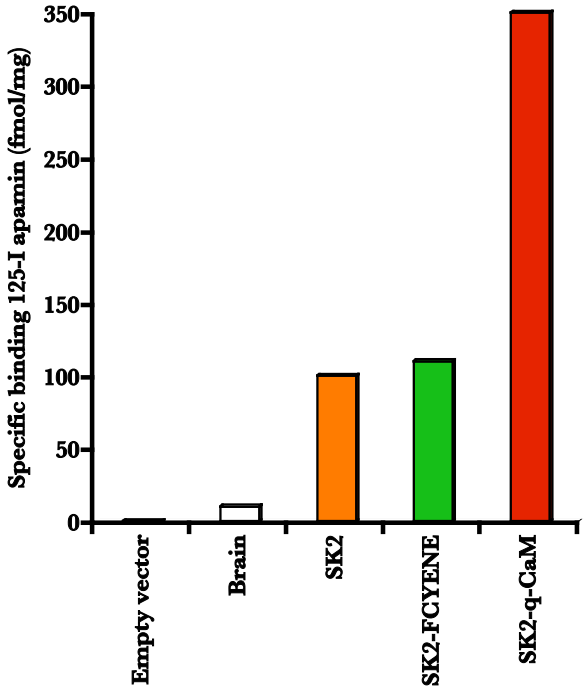


Figure 24: Binding of ^{125}I-apamin to Pichia membranes expressing different constructs. Crude cell membranes were prepared from *Pichia pastoris* cells and ^{125}I-apamin binding measured as described in material and methods. Specific binding of 50 pM labeled apamin to SMD1163 cells expressing SK2, SK2fcyene and SK2qCaM. Parallel binding to rat synaptosomal membranes and to SMD1163 cells expressing empty pPIC 3.5K vector are shown.

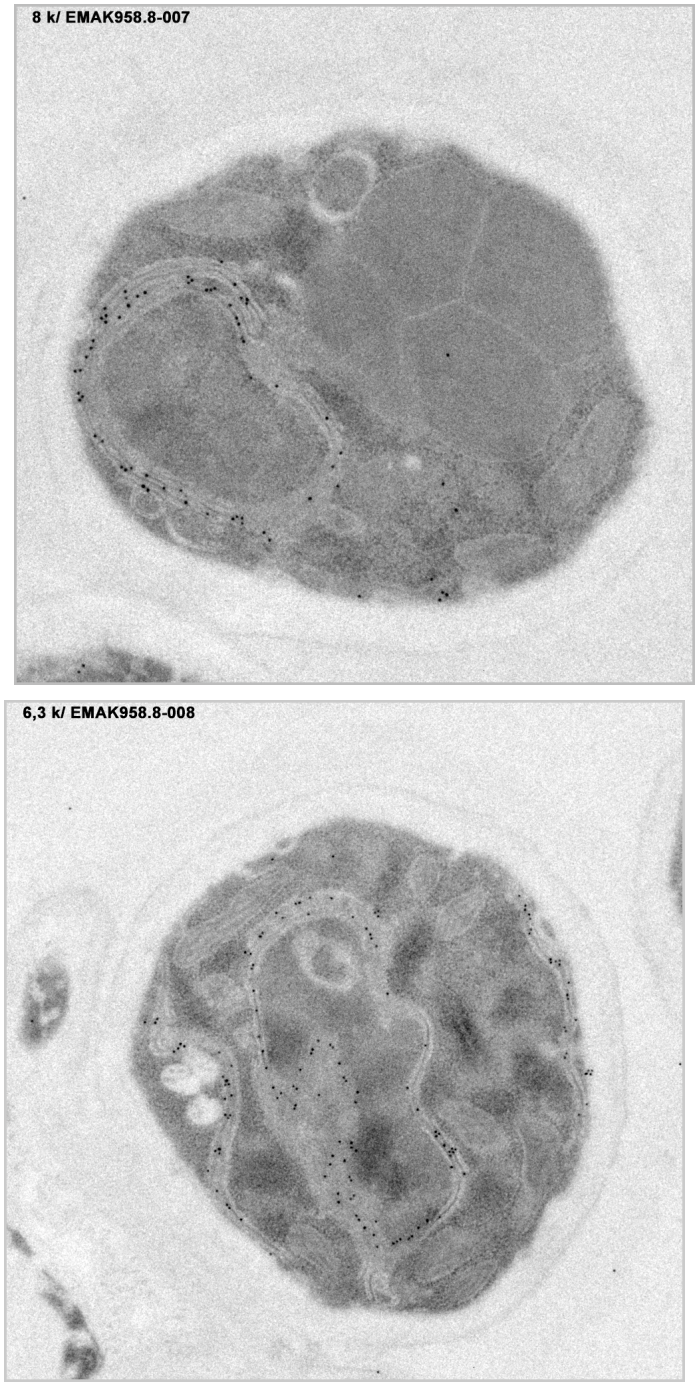


Figure 25: Indirect post embedding immunogold labelling of Pichia pastoris cells expressing SK2-q-CaM after 24 h induction. The primary polyclonal anti-SK2 antibody was visualised with a gold coupled (10nm) anti rabbit antibody. Speciment preparation and electron microscopy were performed by Dr.Winfried Haase, MPI of Biophysics, Frankfurt/M.

3.3.3 Solubilisation of the tandem linked SK2-q-calmodulin

Once again detergent channel solubilisation was determined by Western blot analysis using either anti SK2, anti His-tag or anti-calmodulin antibodies.

Detergent screening was performed and a number of different detergents were able to solubilise the SK2-q-CaM chimera. The most effective ones were (as for the FCYENE construct) digitonin and DDM. However, a high level of soluble channels was recovered also after extraction with CHAPS, Triton X-100, octyl glucoside and cholic acid (Figure 26). Importantly, in all cases the efficiency of extraction was much higher for the tandem-linked construct than for the wild-type and even FCYENE mutant channel.

These findings are in agreement with the binding and electro microscopy experiments and confirm that calmodulin is important for the proper assembly of the channel.

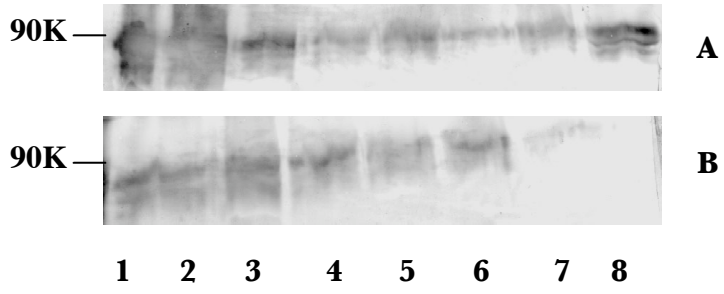


Figure 26: Solubilisation of *Pichia pastoris* membranes over-expressing SK2-q-CaM.
Membranes (10 mg/ml) were incubated for 1h at 4 °C with various detergent at 4% (w/v), in presence of 100 mM KCl, 600 mM NaCl, 5% glycerol, 40 mM hepes, pH 7.0. Immunoblot analysis were performed with anti SK2 antibody.
Blot A: Lane1) Membranes (30 □g); solubilisation mixture before, lane 2) and after extraction with Triton X-100, lane 3); solubilisation mixture before, lane 4) and after extraction with cholic acid, lane 5); solubilisation mixture before, lane 6) and after extraction with thesit, lane 7); solubilisation mixture before, lane 8) and after extraction with OG, lane 9).
Blot B: Lane1) Membranes (30 □g); solubilisation mixture before, lane 2) and after extraction with tauric acid, lane 3); solubilisation mixture before, lane 4) and after exraction with Z-12, lane 5); solubilisation mixture before, lane 6) and after extraction with Z-14, lane 7); solubilisation mixture before, lane 8) and after extraction with C12E8,lane 9).

3.3.4 Large scale production and purification of SK2-q-calmodulin tandem construct

In order to obtain enough *Pichia pastoris* cells carrying the SK2-q-CaM tandem construct, a large scale production in a 5l fermentor was performed.

The same parameters used for the SK2 production were employed and also for this construct, high cell density was achieved, with the best level of expression being reached after 24 hours of induction.

Membranes from fermentation cells were solubilised in DDM and the soluble material subjected to Ni-NTA chromatography. However, as already experienced, immobilised metal affinity chromatography was not sufficient by itself to purify the channels. In fact the purity was lower compared with the wild type channel and SK2-FCYENE mutant while the purified fraction was less concentrated because it was eluted in a broader peak.

Since the presence of calmodulin may have caused the strep II tag to be exposed in a different way to the wild type, streptactin-agarose chromatography was attempted again. Unfortunately it was once again ineffective, with little or no binding to the column. Ion exchange chromatography on SP resin was then performed following the same conditions described for the wild-type channel.

Unfortunately, ion exchange chromatography was also less efficient in terms of purity and concentration compared to the wild-type and FCYENE constructs. This it was quite disappointing and unexpected, as previously this purification step had substantially improved the purity of the channel. Presumably, the relatively low efficiency of the Ni-NTA step was due to alteration in the availability of the His tag due to the association with calmodulin, although the calmodulin binding site is at the C-terminal of SK2. Likewise, the cation exchange procedure would be expected to be different due to the calmodulin binding. The calculated pI for the SK2-Calmodulin tandem is 6.6.

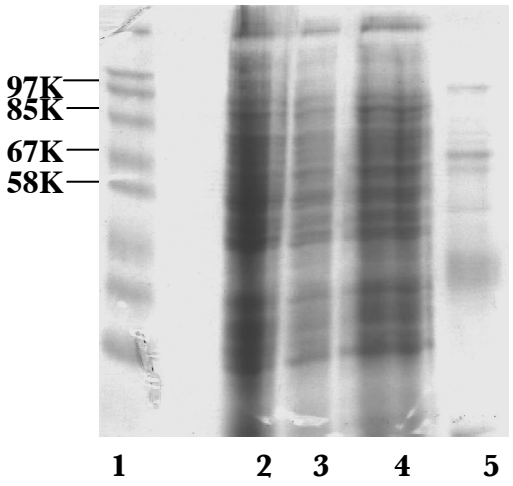


Figure 27: Detergent pre-extraction experiment. Membranes (10 mg/ml) expressing SK2-q-CaM construct were extracted first with 4% thesit, then the pellet was resuspended and solubilised again with 4% DDM, the soluble material was then loaded on a immobilised metal affinity chromatography (Ni-NTA column) and on ion exchange chromatography (SP). Lane 1) Markers; Lane 2) membranes (30 □g); lane3) pellet extracted in 4% DDM (30 □g); lane 4) membranes extracted in 4% thesit (30 □g); lane 5) SP eluted fraction.

3.3.4.1 Detergent pre-extraction experiments

A pre-extraction of membranes using Thesit, a detergent unable to solubilise SK2-q-CaM concatamer protein, was attempted in order to try to remove contaminating proteins before the first chromatography step. After pre-extraction, the insoluble pellet was solubilised in DDM before immobilised metal chromatography on Ni-NTA and cation exchange chromatography as previously described.

However, this purification technique only partially improved the purity of the sample (Figure 27).

3.3.4.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation was also tried as alternative purification step.

After extraction of membranes in dodecyl maltoside, the solubilised material was incubated with increasing concentration of ammonium sulphate (from 20% to 90%

saturation) and each fraction examined on Western blots to determine the location of SK2-q-CaM and Coomassie blue stained gel to examine the total protein content. The channel was found in almost every fraction, rendering this method useless.

3.4 Over-expression and characterisation of small calcium activated potassium channel (SK2) using Semliki Forest virus expression system

It was decided to attempt expression of SK2 channel in mammalian cell lines using Semliki Forest virus mediated transfection because this system has been shown to produce high yields of certain other recombinant proteins with native post-translational modifications and trafficking (Lundstrom et al. 1997; Lundstrom 2003). For example, the mouse serotonin 5-HT3 receptor (Hovius et al. 1998) was produced using using Semliki Forest virus expression system and several milligrams of pure protein were obtained.

3.4.1 Cloning of SK2 channel DNA into the Semliki Forest virus vector pSFV2gen.

The SK2 gene was excised from the construct pPIC3.5K-SK2 using BamHI and NotI restriction enzymes and ligated with the vector (pSF2gen) specific for Semliki Forest virus infection (Figure 28). The insert thus retains both the Kozac consensus sequence and the N-terminal His and Strep II affinity tags as verified by DNA sequencing.
The mammalian cell line BHK (Baby Hamster Kidney cells) were transformed by electroporation with RNA transcribed *in vitro* from the SK2-pSF2gen and helper vectors. Virus particles were subsequently harvested for protein production trials.

3.4.2 SK2 channel production and localization.

Virus particles were activated with chymotrypsin and used to infected BHK cells either adherent to culture dishes or in suspension.

The cells were harvested at different times after infection to test the expression level and no differences were found from 20 hours to 36 hours of infection (data not shown). By 48 hours cells started to die due to the virus infection.

The presence of SK2 channel was verified by Western Blot and immunofluorescence analysis. Immunoblotting, using either anti SK2 or anti His-tag antibodies, detected a band around 65 K, which is the same length as SK2 expressed in *Pichia pastoris* cells (Figure 28). In control cells expressing empty pSFV2gen or pSFVgen containing green fluorescent protein (GFP), no signal was detected.

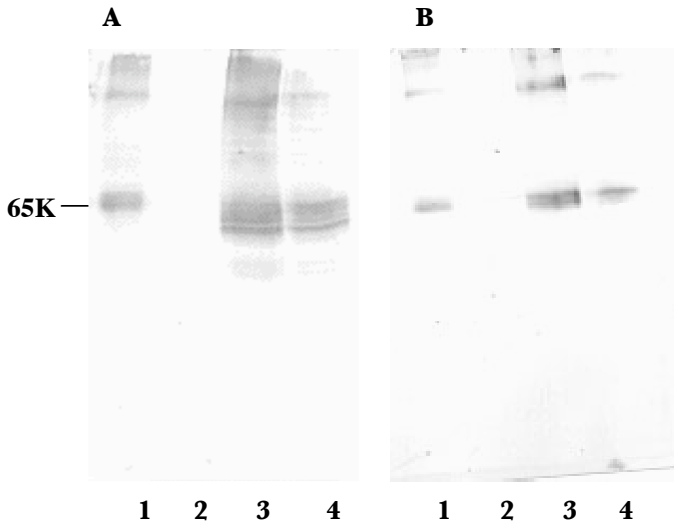


Figure 28: Expression of SK2 in BHK after infection with Semiliki forest virus compared with the expressiom in *Pichia pastoris* cells. Lane 1: SK2 expressed in *Pichia pastoris* cells (30 □g membranes); lane 2: control, BHK cells infected with empty vector (30 □g membranes), lane 3-4: SK2 expressed in BHK cells (30 □g membranes). Imunoblot analysis with (A) anti SK2 and anti His-tag (B) antibodies.

Interestingly, both antibodies recognized in the SK2 expressing cells a multiple band, the anti-SK2 antibody recognized also a third band a bit below the multiple band, that could be a partial N-terminal degradation of SK2. The presence of this multiple band suggests differential post-translational modification. However, the SK2 sequence does not contain

a consensus sequence for N-glycosylation. It is possible that the different mobilities of the two forms may be a result of phosphorylation which is know to affect migration of Kv1.2 channel on SDS-PAGE (Parcej and Eckhardt-Strelau 2003).

However, other modifications such as tyrosine sulphation or lipid modification cannot be ruled out.

Immunofluorescence light microscope images also show the presence of SK2 in BHK cells expressing pSFVgen-SK2 construct (Figure 29C), while no fluorescent signal was seen in negative control cells infected with viruses comprised of empty pSFVgen vector (Figure 29 D). Positive control cells expressing pSFV2gen-GFP construct were also tested to check if Semliki Forest virus expression system was working well (Figure 29A-B). In addition post-embedding immunogold electron microscopy of BHK cells carrying the pSFV2 -SK2 construct was also performed.

Images show strong labelling of the channel in the cells. The majority of SK2 channels was present in the ER membranes but some were also localized to the plasma membrane (Figure 30), showing that the protein is successfully translocated.

3.4.3 Specific apamin binding in BHK cells expressing SK2

The functionality of SK2 proteins expressed in BHK cells was again demonstrated by binding experiment using radiolabelled apamin. Membranes (60 □g), extracted from BHK cells expressing SK2 after 24 hours of viral infection, were incubated with ^{125}I-apamin and in parallel the same amount of membranes were incubated with the radioligand and an excess of unlabeled ligand to determine the non-specific binding. As a control, membrane extracted from BHK cells expressing GFP after 24 hours of infection were also tested.

Saturation binding data were found to fit to a single-site binding model with B_{max} and K_D of 120-400 fmol/mg and 20-60 pM respectively.

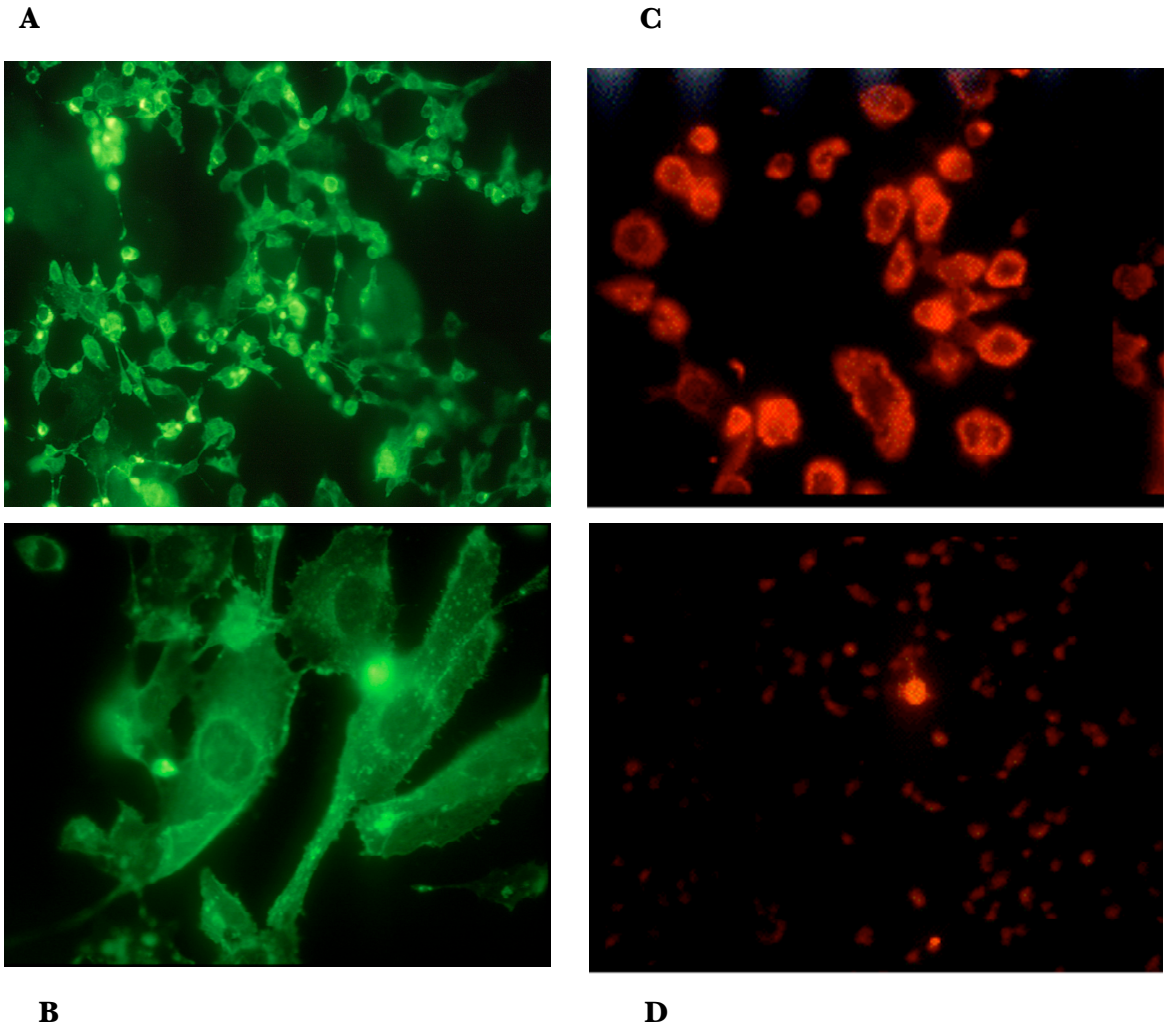


Figure 29: Immunofluorescent images of BHK cells. A-B) Immunofluorescent micrographs of BHK cells expressing pSFV2-GFP after 24 hours of Semliki Forest virus infection. BHK cells expressing pSFV2-SK2 (C) or pSFV2 alone (D) after 24 hours of Semliki Forest virus infection. Primary antibody: anti SK2; secondary antibody: anti rabbit conjugated with rhodamin

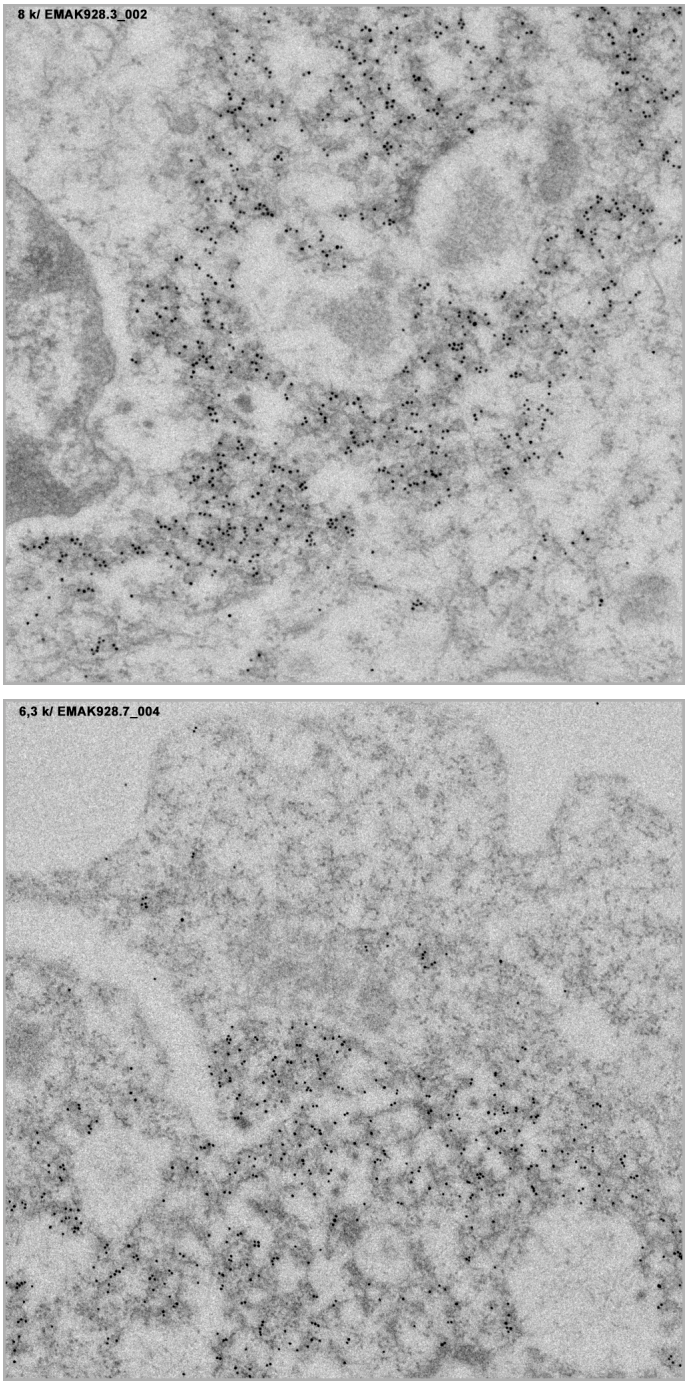


Figure 30: Indirect post embedding imunogold labelling of BHK cells expressing SK2 after 24 h induction. The primary polyclonal anti-SK2 antibody was visualised with a gold coupled (10nm) anti rabbit antibody. Speciment preparation and electron microscopy were performed by Dr.Winfried Haase, MPI of Biophysics, Frankfurt/M.

These experiments (Figure 32) show that the apamin links to the channel with high affinity ($K_{D=}$ 20-60 pM). These values are comparable with those observed in native membranes (Seagar et al. 1984; Marqueze et al. 1987; Seagar et al. 1987; Marqueze et al. 1989; Wadsworth et al. 1994; Wadsworth et al. 1997), and similar to the levels of binding (90-120 fmol/mg) observed for *P. pastoris* expressed SK2. Furthermore competitive displacement experiments using an increasing amount of unlabelled apamin (Figure 33) or of the neuromuscular blocker gallamine (Figure 34) also exhibit similar affinity to that seen in native brain membranes.

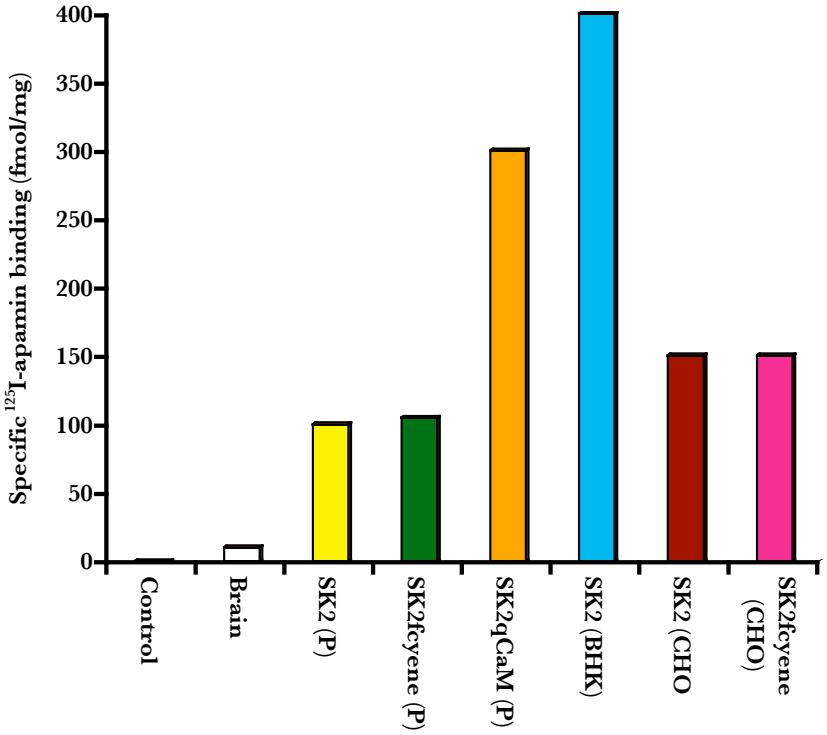


Figure 31: Schematic comparison of ^{125}I-apamin binding to various membranes. Crude cell membranes were prepared from *Pichia pastoris* cells and ^{125}I-apamin binding measured as described in material and methods. Specific binding of 50 pM labelled apamin to SMD1163 cells expressing SK2, SK2fcyene, SK2qCaM, SK2 in BHK cells using Semliki Forest virus system, SK2 in CHO cells using lipofectamine trasfection and SK2-FCYENE in CHO cells using lipofectamine transfection. Parallel binding to rat synaptosomal membranes and to SMD1163 cells expressing empty pPIC 3.5K vector are shown.

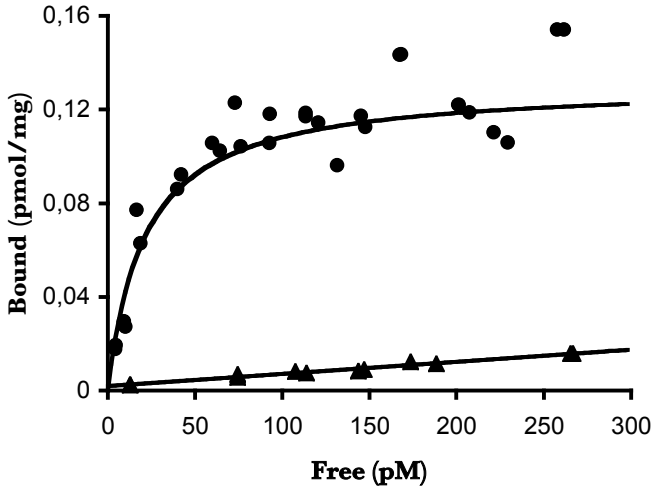


Figure 32: Scatchard analysis of binding to membranes from SK2-producing BHK cells. Data were fit to a single-site binding model using the non-linear curve fitting program GRAFIT. (●) Saturable binding (▲) total binding.

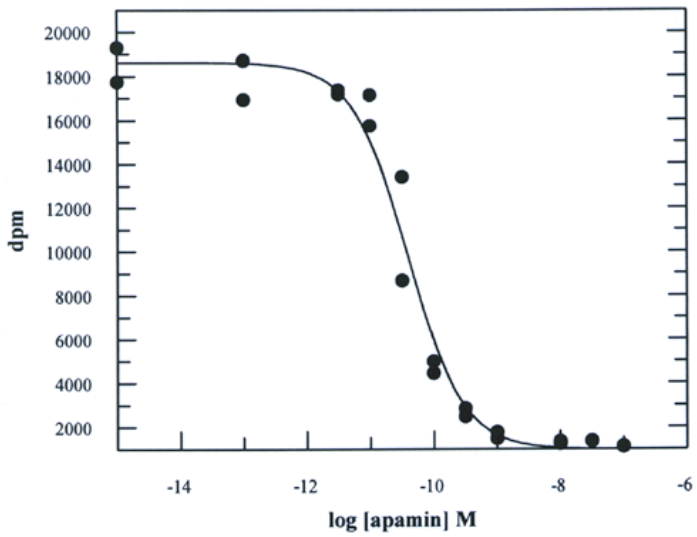


Figure 33: Competition for binding between ^{125}I-apamin and unlabellad apamin. BHK cell membranes expressing SK2 were incubated with 50 pM of ^{125}I-apamin in presence of various concentration of unlabelled apamin.

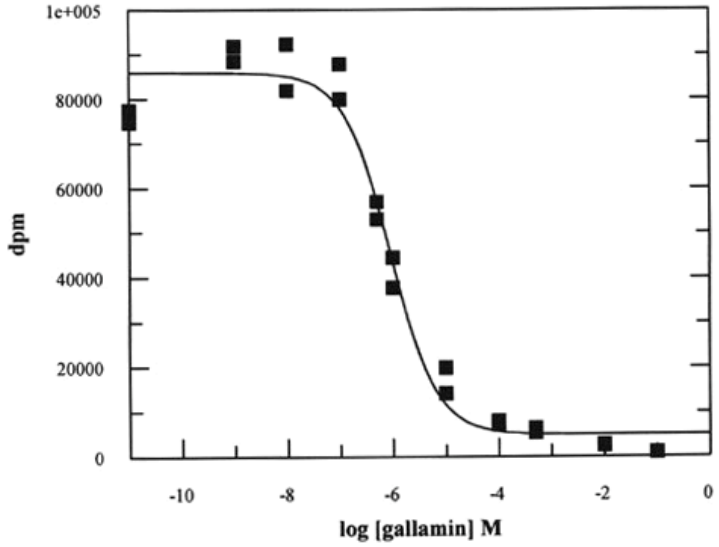


Figure 34: Inhibition of ^{125}I-apamin binding by gallamine. BHK cell membranes expressing SK2 were incubated with 50 pM of ^{125}I-apamin in presence of various concentrations of gallamine.

3.4.4 Solubilisation of SK2 channel from BHK membranes.

Digitonin, Cholic acid, Triton X-100, DDM and octyl glucoside were tested and all were found to solubilise the SK2 channel from BHK cell membranes. The yield of soluble channels was comparable to the level of extraction obtained after solubilisation of membrane expressing pPIC3.5K-SK2-q-Cam construct and much better than the levels seen for SK2-pPIC3.5K constructs in *P. pastoris* cells (data not shown).

It is possible that the channel expressed in mammalian cells the channel is more easily extracted due to the different lipid composition of membrane microdomains in *Pichia pastoris* and in BHK membranes. Another possible explanation for the better solubilisation and specific binding could be that in BHK cells the channel is able to assemble with the calmodulin available in the cell and therefore fold correctly.

3.4.5 Purification of SK2 channel over-expressed in BHK cells.

Membranes prepared from cells harvested after 24 hours of infection were solubilised in DDM and the soluble material loaded onto a Ni-NTA column and the eluate applied to a SP ion exchange chromatography.

After ion exchange chromatography (Figure 35) the channel appears pure on a Coomassie or silver stained gel. Furthermore it was possible to achieve a protein concentration of 0.1-0.2 mg/ml (Bio-Rad protein assay).

Despite the relatively low amounts of protein recovered, this represents a big step forward in the characterization of small calcium activated potassium channel. To date there are no other reports of purified SK2 channel from either natural or overexpressed sources.

Of note is that on the Coomassie or silver stained gels no band corresponding to calmodulin was observed. This was confirmed by immunoblotting using anti-calmodulin antibody on BHK membranes, which confirmed the absence of calmodulin.

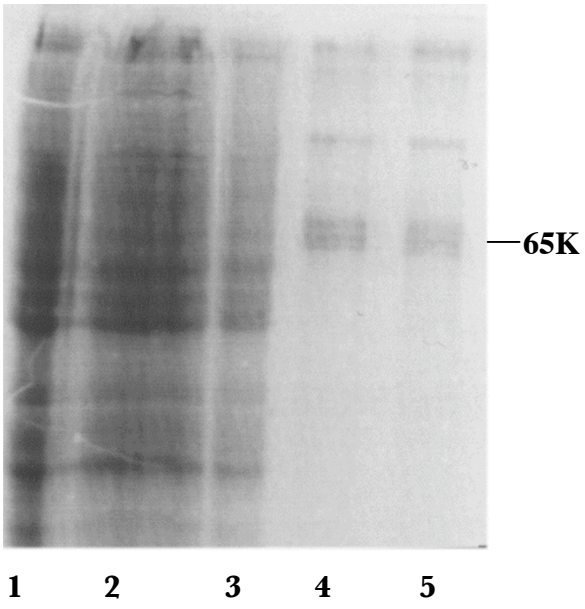


Figure 35: Purification of SK2 channel over-expressed in BHK cell by Ni-NTA and ion exchange chromatography. Blue Coomassie stain 10% SDS. Lane 1) membranes (30 μg); lane 2) membrane proteins extracted with 4% DDM; lane 3) Ni pool; lane 4-5) SP pool.

3.4.5.1 Size exclusion chromatography

Gel filtration chromatography was employed to verify the oligomeric state of the channel. As before channel in the tetrameric state should elute from a Superose 6 column at about 1.5 ml (Figure 36).

By western blot analysis it was possible to recognize that the channel eluted in the expected position, the first peak after the void volume.

This is similar to the profile recorded for the native SK2 channel produced in *Pichia pastoris* (Figure 16).

The other peaks were also present when the buffer alone was loaded and are presumably due to mixed detergent-lipid micelles.

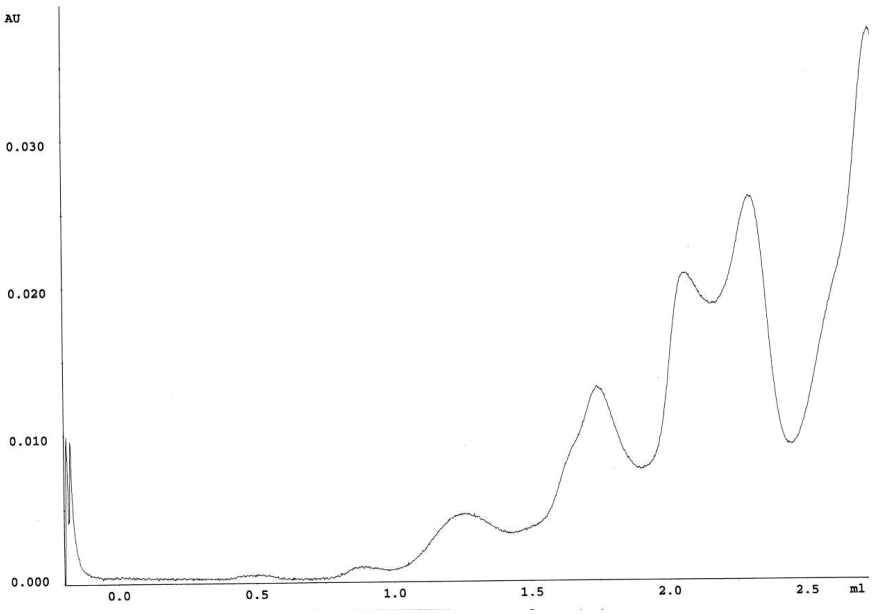


Figure 36: Gel filtration analysis of SP eluted fraction on a SMART/Superose 6 column. The arrow indicates at which volume is eluted SK2.

3.4.6 Blue native gel electrophoresis

To further determine the oligomeric state of purified SK2 blue native PAGE (BN-PAGE) was performed (Figure 37).

The gel showed that the channel migrates at a relative molecular mass of around 500 K. The identity of the stained bands was confirmed by immunoblot analysis. The upper band is probably due to some aggregation or artefacts of the electrophoresis methods. The smear under the 500 K band could be due to DDM since the sample was concentrated before the loading.

The expected tetrameric size of SK2 without calmodulin in a BN-PAGE is 280KDa.

It has been demonstrated that in BN-PAGE membrane proteins run with a molecular weight that is increased by a factor of 1.8 (Heuberger et al. 2002). This factor takes in account the mass of bound dye and detergent, compared to the soluble standards. Since the molecular weight of the tetrameric form of SK2 is 280 KDa, multiplying for the factor would give an expected value of 500K**,** which is close to that observed. This means that the expressed SK2 channel assembles in a correct oligomeric state.

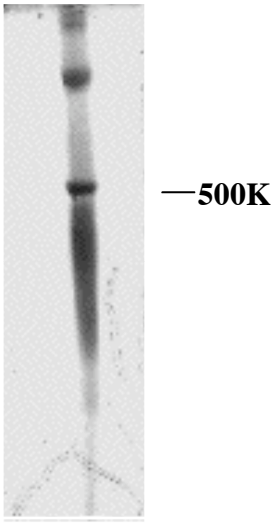


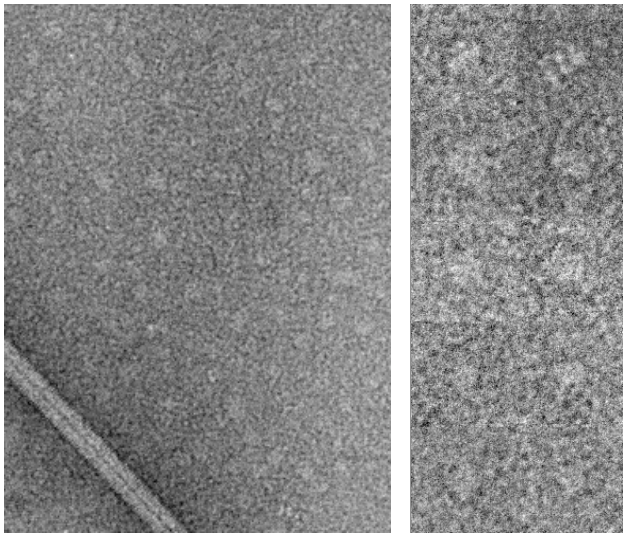
Figure 37: Blue native gel (BN-PAGE). SP pool containing SK2 protein was loaded on a gradient gel to analyse the oligomeric state of the purified preotein. 1ml fraction was concentrated to 50 µl using centricon tube (10.000 cut off) and 20 µl were loaded on the gel.

3.4.7 Electron microscopy of purified SK2 channel.

SK2 purified channel was analyzed as single particles in deep negative stain (ammonium molybdate) by electron microscopy. The micrographs (Figure 38A) showed the particles to be uniformly distributed, with few small aggregates.

A data set of 1200 selected images of SK2 particles was analysed. After a reference-free alignment a first reference was obtained, which was used for aligning the whole data set. For classifying the data a neuronal network technique (Marabini and Carazo 1994) was used.

Four node images were selected from this second analysis and used as starting references for multireference alignment of the full data set. The multireference alignment was iterated, recalculating the reference images after each step, by averaging the corresponding aligned particle images. Figure 39A shows the four class averages obtained: class 1 corresponded to 27.9%, class 2 to 36.8%, class 3 to 21.8%, and class 4 to 13.9% of the number of particles.



A **B**

Figure 38: A) Electron micrograph of SK2 channel negatively stained with 2% ammonium molybdate. B) Gallery of single particles.

Even if these are only preliminary studies, it is possible to observe that the class 1 average of particles resembles a tetramer and it may be a top view of the channel. Class 4 is smaller than the others and may represent disassembled channels or a side view. Class 2 is unusual in that it appears to possess an extra mass in addition to the expected four subunits. It may be a slightly tilted version of the top view or it may result from two channels in close proximity. Interpretation is difficult because these images represent projection of an unknown 3D structure, which will vary depending on the orientation of the particles on the carbon film. Notably, the width of the four-fold class of approximately 70 Å, this value is in agreement with that seen for the top view of Kv1.2 (Parcej and Eckhardt-Strelau 2003) which possesses four subunits of similar size to SK2. For understanding the nature of these projection averages and the relationship between them, 3D reconstruction is required.

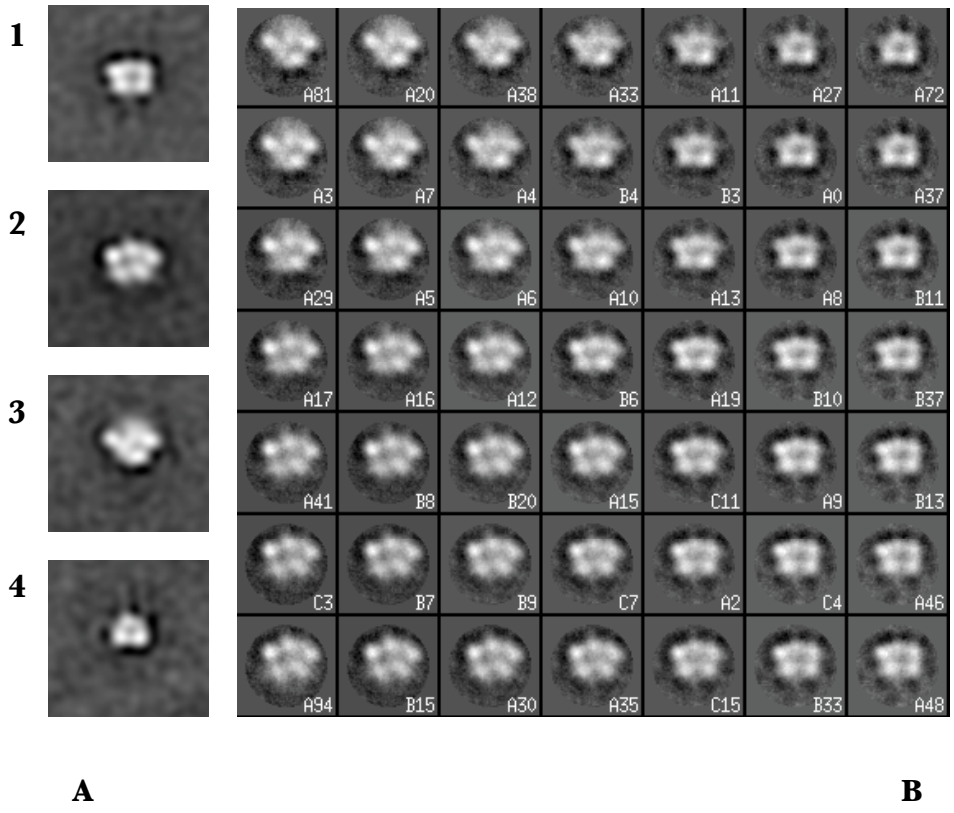


Figure 39: Image analysis of SK2 channel. A) Self-organizing map from a neural network analysis of single particles. The numbers in the label of each square indicate the number of images that contribute to this node in the map. B) Class averages obtained after several rounds of multireference alignment.

3.5 Over-expression and characterisation of a large calcium-activated potassium channel (BK) in *Pichia pastoris*

3.5.1 Cloning and expression of BK alpha subunit in *Pichia pastoris* cells

In order to obtain enough protein for functional and structural studies, the human large calcium-activated potassium channel α subunit (BK) cDNA (generous gift from Dr. M. Wallner) was subcloned either into the vector pNSHE 2.1 (paragraph 2.2.1.1) or into pNHE-α.

The pNHE-α contains the ampicillin resistance gene, Kozak sequence, 9-histidine tag, and enterokinase site-N terminal (but not strepII sequence), but unlike pNSHE contains in addition the *Saccharomyces cerevisae* alpha factor signal.

The alpha factor signal sequence is an insertion and secretion signal that may help the protein to reach the cell surface and has been shown to improve expression of the 5HT receptor in *Pichia.* Unlike SK2, BK is predicted to have 7 transmembrane helices with the N-terminal extracellular. Both the clones pNSHE 2.1-BK and pNHE-α-BK were excised as BamHI, EcorI fragments from the vectors and sub-cloned into the *Pichia Pastoris* expression vector, pPIC 3.5K (Figure 6). Restriction analysis and DNA sequencing of the BK insert region confirmed that the sequence of the two clones was correct. The plasmid was then linearized and transformed into competent *Pichia Pastoris* cells, SMD1163, by electroporation.

The production of the BK and BK alpha-factor protein was tested by immuno-blotting after 24h of induction. For immunological detection two different antibodies were used: anti-BK antibody that recognises the carboxyl terminal part of the protein and an anti his-tag, which reacts with the N-terminal His tag.

BK channels lacking the alpha factor signal sequence were identified in induced cells as a band of ~ 110 K. The identical patterns observed for the antibodies, which recognise opposite ends of the sequence shows that the protein has not undergone degradation (Figure 40). A time course of induction showed no difference in expression level after 6, 12 and 24 hours.

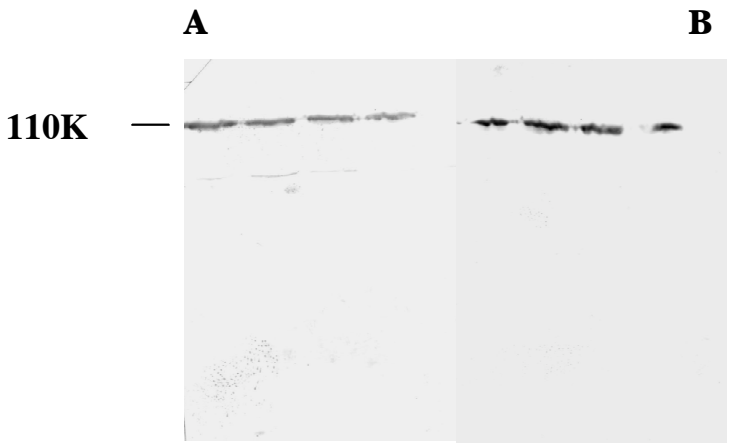


Figure 40: Western blot of *Pichia pastoris* membranes over-expressing BK-□ (30 □g of protein). Expression of BK Blots were probed with anti-BK polyclonal antibody (A) and with anti his-tag monoclonal antibody (B) and then visualised with alkaline-phosphatase-coupled secondary anti body.

In contrast BK channels expressed with the alpha-factor sequence at the N-terminus were partly degraded as evidenced by differential labelling by the two antibodies. This suggests a cleavage at the N terminus part of the protein (Figure 41), probably because the presence of the alpha factor causes a mis-localization of the channel.

Harvesting the cells after 6 or 12 hours and addition of several protease inhibitors during membrane preparation did not improve the situation.

Consequently, all further experiments were carried out only with the over-expressed pPIC3.5K-BK construct.

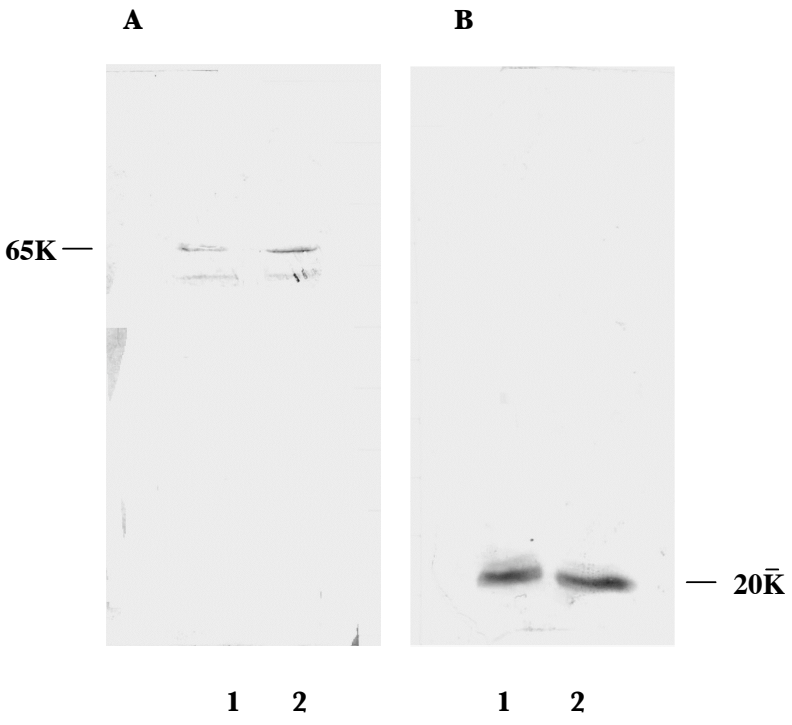


Figure 41: Western blot of *Pichia pastoris* membranes over-expressing BK □/□ channel (30 □g of protein). Expression of BK □/□ Blots were probed with anti streptactin polyclonal antibody (A) and with anti his-tag monoclonal antibody (B) and then visualised with alkaline-phosphatase-coupled secondary anti body.

3.5.2 Localisation of BK in Pichia *pastoris* cells

In order to establish the cell localization of expressed BK channel, immunogold stained electron microscopic analysis was performed.

The electron microscopic images showed the presence of the protein in the cells in large vesicles probably lipid clumps (Figure 42).

Such clumps are not normally present in *Pichia pastoris* cells under physiological conditions and may be due to a cell stress caused by the channel over-expression.

As already mentioned above, this could be a way to control the distribution of proteins inside the cell, with segregation into these clumps used to make insoluble and non-functional a protein (Brown DA 2000; Bock et al. 2003).

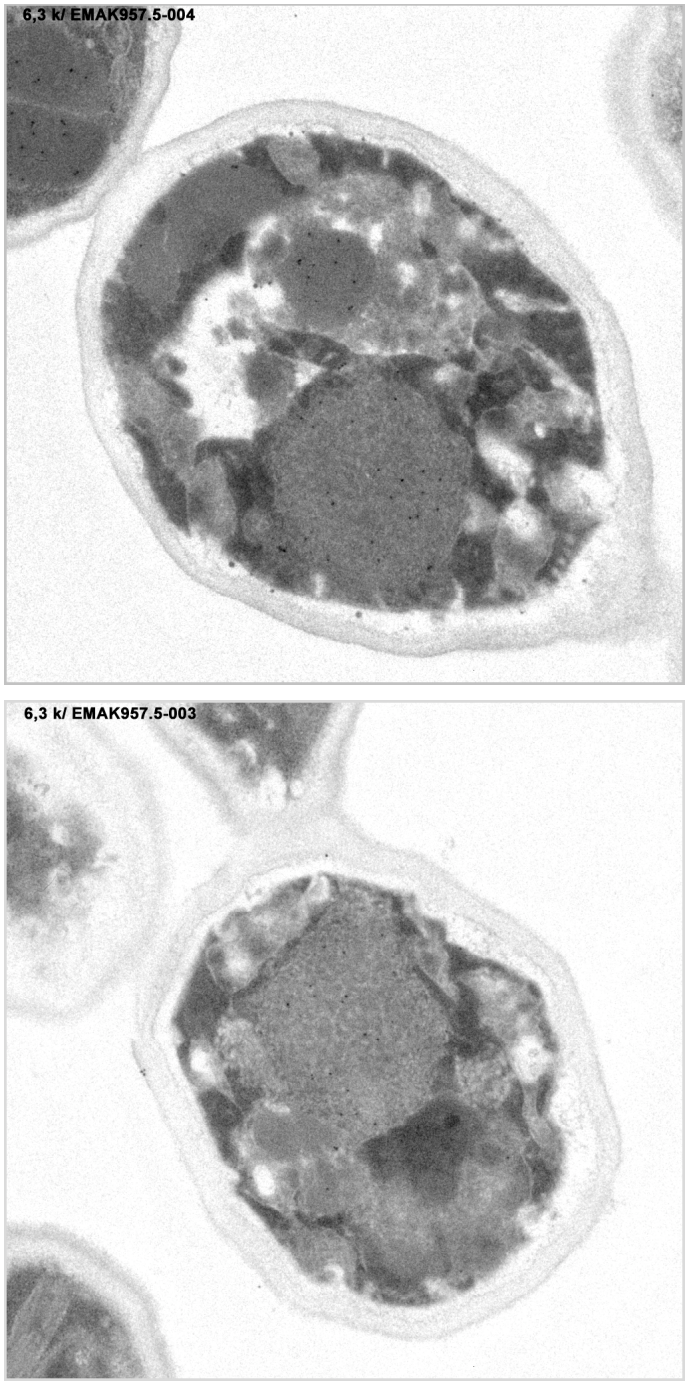


Figure 42: Indirect post embedding imunogold labelling of *Pichia pastoris* cells expressing BK channel alpha subunit after 24 h induction. The primary polyclonal anti-BK antibody was visualised with a gold coupled (10nm) anti rabbit antibody. Speciment preparation and electron microscopy were performed by Dr.Winfried Haase, MPI of Biophysics, Frankfurt/M.

3.5.3 Solubilisation of BK channel

Unlike the SK2 channel, BK channels are expressed in relatively high amounts in several tissue especially muscle. For this reason it was possible to purify and characterize the channel from native sources (Garciacalvo et al. 1994; Giangiacomo et al. 1995) although the yield of pure channel was insufficient for structural analyses.

Several detergents have been reported to be able to solubilise the BK channel but only digitonin and CHAPS were able to extract the protein in active form (Garciacalvo et al. 1994).

Therefore, different concentrations of digitonin and CHAPS (from 2% to 10%) were tested using the buffer conditions described in this previous work.

Western blot analysis was employed to estimate the efficiency of solubilisation.

Unexpectedly, BK channel could not be solubilised with either digitonin or CHAPS.

Detergent screening was performed and none of the following detergents were able to solubilised the protein: cholic acid, Triton X-100, Triton X-114, Tween 20, DDM, OG, APFO, DHPC, Thesit, Taurocholic acid, APO12, LDAO, and Zwittiger L-12 and L-14.

In all cases, the protein was found in the pellet after the centrifugation (an example is shown in Figure 43).

No improvement was achieved by varying the detergent concentrations or by using different salt or buffer conditions.

It is possible that the channel cannot be solubilised by any detergents because, as shown in Figure 42 , it is stacked into clumps and therefore in an insoluble form.

To try to overcome this problem we decided to express together the alpha and the beta subunit of the BK channel, following the idea that the presence of the beta subunit could allow the right assembly and localization of the channel.

As reported in the chapter 1, in the brain but also in other tissues, the presence of the beta subunit is essential for the functionality of the channel.

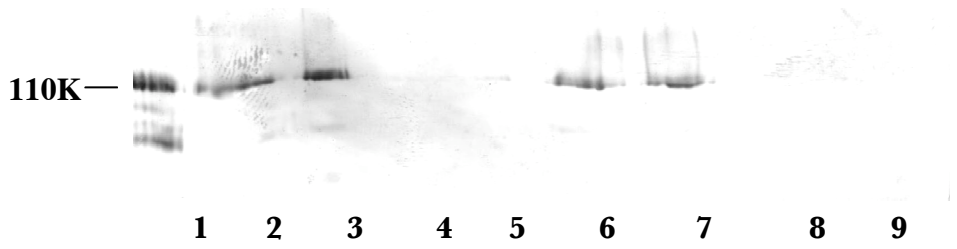


Figure 43: Solubilisation of *Pichia pastoris* membranes over-expressing BK alpha subunit.
Membranes (10mg/ml) were incubated for 1h at 4 °C with various detergents at 4% (w/v), in presence of 100 mM KCl, 600 mM NaCl, 5% glycerol, 40 mM hepes, pH 7.0. Immunoblot analysis was performed using anti BK antibody.
Lane1) Membranes (30 μg); lane 2) soluble mixture before extraction with digitonin; lane 3) soluble mixture before extraction with CHAPS; lane 4) soluble mixture after extraction with digitonin; lane 5) soluble mixture after extraction with CHAPS; lane 6) soluble mixture before extraction with DDM; lane 7) soluble mixture before extraction with Triton X-100; lane 8) soluble mixture after extraction with DDM; lane 9) soluble mixture after extraction with Triton X-100.

3.5.4 Cloning of the BK beta subunit and co-expression with the BK channel into *Pichia pastoris* cells.

The human large calcium-activated potassium channel β subunit cDNA (generous gift from Dr. M. Wallner) was subcloned into the *Pichia pastoris* vector pPICZB. A strep II sequence was subsequently inserted to be in frame at the N-terminal part of the protein. Restriction analysis and DNA sequencing of the beta subunit were employed to verify the correctness of the sequence.
The plasmid was then linearized and transformed into competent *Pichia Pastoris* SMD1163 cells already expressing pPIC 3.5K-BK.
Production of the BKβ and BKβ proteins was tested after 24h of induction by immuno-blotting using anti his-tag antibody to label the BK subunit and anti strepII-tag antibody for BKβ (Figure 44), often a double band was detected around 30K.
When the anti-strepII and the anti- BKβ antibodies (raised against a syntethic peptide

corresponding to residues 170-185) were tested against control membranes, they were able to detect a band around 30K. This is similar to the expected size of BKα, so it is no possible to establish if the BKα gene really was expressed.
At this time no commercially BKα antibodies were available, and it is known that anti-BKα antibodies raised against synthetic peptides usually react aspecifically (LaTorre R., personal communication).

3.5.5 Solubilisation of membranes from cells transfected with BK/BKα channels

For the reason mentioned above, digitonin and CHAPS were the first two detergents employed to solubilise the channel but as before no extraction could be observed.
Again detergent screening was performed and all the detergents described above were tested. None was able to extract the channel from the membrane (Figure 45).As already pointed out, it is not easy to comment on these results since it is not clear if BKα is actually produced. Interesting, however is the observation the neither the BK or the 30K bands are solubilisable under any conditions.
It has been demonstrates that slob, a multi-functional channel-binding proteins interacts with BK channels in *Drosophila* incrising their activity (Schopperle et al. 1998). It could be therefore possible that slob has a regulatory function and that its present it is important for a correct functionality of the channels and that this protein is not expressed in *Pichia pastoris* cells.

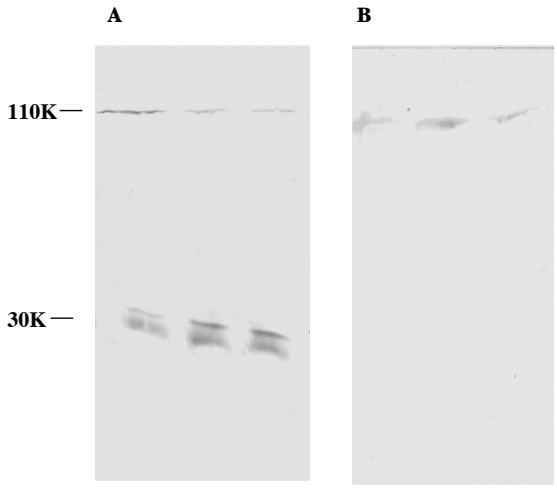


Figure 44: Western blot of *Pichia pastoris* membranes over-expressing BK □/□ channel (30 □g of protein). Expression of BK □/□ Blots were probed with anti streptactin polyclonal antibody (A) and with anti his-tag monoclonal antibody (B) and then visualised with alkaline-phosphatase-coupled secondary anti body.

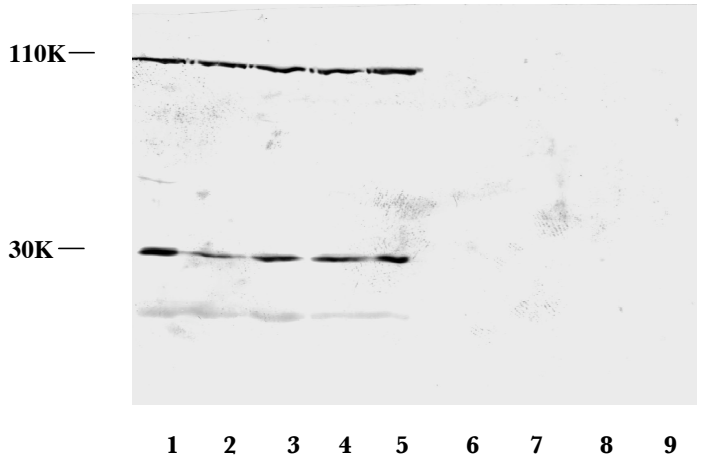


Figure 45: Solubilisation of *Pichia pastoris* membranes over-expressing Bk □/□ subunits.
Membranes (10mg/ml) were incubated for 1h at 4 °C with various detergents at 4% (w/v), in presence of 100 mM KCl, 600 mM NaCl, 5% glycerol, 40 mM hepes, pH 7.0. Immunoblot analysis was performed using anti streptactin antibody.
Lane1) Membranes (30 □g); lane 2) soluble mixture before extraction with digitonin ; lane 3) soluble mixture before extraction with CHAPS; lane 4) soluble mixture before extraction with DDM; lane 5) soluble mixture before extraction with TRITON X-100; lane 6) soluble mixture after extraction with digitonin; lane 7) soluble mixture after extraction with CHAPS; lane 8) soluble mixture after extraction with DDM; lane 9) soluble mixture after extraction with Triton X-100.

CHAPTER FOUR

Conclusions and Outlook

Potassium channels are present in both bacterial and eukaryotic cells and are related members of a single protein family, all of which contain a highly conserved sequence of amino acids called the selectivity filter. They conduct K^+ ions across the cell membranes and they are involved in several cellular processes as electrical impulse, cell volume regulation and hormone secretion.

During evolution, the function of potassium channels probably developed from basic cellular tasks as osmotic regulation and energy production to more specialized and complex function, such as, for example, the control of the electrical impulse in excitable cells, that in the nervous system is the base of higher function as learning, imagination, language, perception and consciousness.

Since the 1953, when Hodgink and Huxley described the action potential propagation in the squid giant axon (Hodgkin and Huxley 1952), amazing discoveries were done in the field of ion channels. Potassium permeability was for the first time proposed by Hodgkin and Keynes after their subtle isotopic K^+ flux-ratio experiment (Hodgkin and Keynes 1955), these researchers suggested that it could be possible that: "ions cross the membrane along a chain of negative charges or through narrow tubes or channel…in which they are constrained to move in single file [with] several ions in the channel at any moment" (Hille 1970). After almost fifty years of biophysical, biochemical, mutagenesis and structural studies, their prediction was definitively confirmed when the structure of the bacterial potassium channel KcsA was solved (Doyle et al. 1998; Zhou et al. 2001).

The aim of my project was to characterize two channels that belong to the calcium-activated potassium channel subfamily. In particular, the small calcium activated potassium channel (SK2) and the large calcium-activated potassium channel (BK).

SK2 from rat was cloned into *Pichia pastoris* expression system. Expression of a large amount of full length protein was obtained. Localization studies revealed that the SK2 channel was mostly localized on the surface of the endoplasmic reticulum or of other internal membrane structures, rather than in the plasma membrane, where it resides in neuronal cells.

Nevertheless, binding experiments using radiolabelled apamin confirmed that at least part of the expressed channel was functional.

The solubilisation of the channel was particularly hard: among the several detergents tested, only digitonin was able to solubilise the channel. Moreover the yield was poor compared to the amount of SK2 channel estimated in the membrane.

After several chromatographic methods, the channel was only partially purified and the yield was low. Gel filtration analysis showed that SK2 channel has, as expected, a homotetrameric form, like the other potassium channels (MacKinnon 1991).

The lumen of the endoplasmic reticulum provides a dynamic and efficient environment for the folding of proteins destined for secretion and for a variety of cellular compartments and membranes. Usually, the folding process begins on the nascent chains and is completed minutes or hours later during assembly of oligomers. It is assisted by molecular chaperones, some of which are unique to the ER. Quality control and selective degradation systems ensure only conformationally mature proteins are transported from the ER (Helenius et al. 1992).

For example, studies on ATP-sensitive K^+ channel trafficking reveal an essential quality control function for a trafficking motif present in each of the alpha (Kir6.1/2) and beta (SUR1) subunits. This motif for endoplasmic reticulum (ER) retention/retrieval is required at multiple stages of assembly of these channels to restrict surface expression to fully assembled and correctly regulated octameric channels. (Zerangue et al. 1999).

Often proteins, including K^+ channels, require amino acid sequences which are essential for the proteins to move from the ER to the Golgi (Zerangue et al. 1999). One of these sequences that acts to promote surface expression has been identified in the mammalian inward rectifier channel, Kir 2.1 (Ma et al. 2001).

To overcome the ER retention problem of SK2 channel expressed in *Pichia pastoris* cells, this sequence containing a 7 amino acids (FCYENE) tag, was inserted in the carboxyl terminal part of SK2.

It was therefore decided to test the effect of this sequence on SK2 expression in *Pichia pastoris*. Also in this case, a full length protein was produced.

Moreover, binding experiment revealed that total number of binding sites is similar to that observed when expressing wild-type SK2, thus indicating that the FCYENE tag did not improve the expression levels of binding-competent channels.

Digitonin, Triton X-100, n-dodecyl-β-D-maltoside, and octyl glucoside allowed the solubilisation of the SK2-FCYENE mutant protein. This demonstrates that the FCYENE tag has a remarkable effect on the amount of SK2 channel that could be solubilised from *Pichia* membranes.

In contrast to the wild type, the SK2-FCYENE mutant protein exhibited a different distribution inside the yeast cells: it indeed concentrated in vesicle clumps and not only in ER membranes.

However, again, a faint labelling is observed at the plasma membrane. Although the FCYENE sequence is able to promote exit from the ER, it is not able to drive translocation of the channel to the plasma membrane.

Despite the higher amount of soluble starting material, notable improvement was not observed in terms of purified channel.

Calcium sensitivity of SK channels is conferred by interaction with calmodulin (Xia et al. 1998; Schumacher et al. 2001).

The binding between calmodulin and SK2 is not only essential for the gating, but also for the correct trafficking of the channel (Lee et al. 2003).

It is not unusual that a protein needs to interact with specific proteins in order to assemble properly or to be transported outside the ER. Also protein interaction may be required for proper subcellular localization into the plasma membrane. As already reported in Chapter 3, calmodulin is a fundamental partner of many proteins and it is extremely important in several cellular processes. In the nervous system for example, it interacts with several receptors and channels.

Beside calmodulin, many other proteins were discovered that play important roles for the potassium channel assembly, folding and localization.

For example, calnexin is part of an ER chaperone system that monitors and promotes the proper folding and assembly of glycosylated membrane proteins. Calnexin has been found to interact transiently with wild-type Shaker protein in the ER and glycosylation of Shaker protein is necessary for association with calnexin (Nagaya et al. 1999). A novel molecular chaperone, KChAP, transiently interacts with the Kv2.1 amino terminus increasing the total number of Kv2.1 protein and the function of the channel in *Xenopus* oocytes. It is also a chaperon for Kv1.3 and Kv4.3 (Kuryshev et al. 1999; Kuryshev et al. 2000).

Proper ion channel function often requires specific combinations of pore-forming alpha and regulatory beta subunits. The interaction between the alpha and beta subunits of the voltage-gated potassium is important for correct folding, trafficking and the differences in functionality.

In brain, for example, Kv beta 2, associates with the Kv1.2 alpha subunit early in channel biosynthesis and Kv beta 2 has multiple chaperone-like effects on associated Kv1.2 including promotion of co-translational N-linked glycosylation of the nascent Kv1.2 polypeptide, increased stability of Kv beta 2/Kv1.2 complexes, and increased efficiency of cell surface expression of Kv1.2 (Shi et al. 1996).

As the presence of auxiliary proteins is very often fundamental for the correct maturation and plasma membrane localization of a channel, SK2 channel was cloned in tandem with calmodulin, with a peptide linker containing 10 glutamines connecting the two proteins. Large amounts of expressed tandem protein were achieved in *Pichia pastoris* cells. Interestingly, binding experiments showed that the total number of binding sites is considerably higher than the expressed wild-type SK2, and the FCYENE mutant.

Moreover the presence of calmodulin dramatically modifies the distribution of the channel in the cell, with some of the SK2-q-CaM chimera localized on the plasma membrane. It is important to point out that efficiency of detergent extraction was higher than for the other two constructs. Unfortunately, despite the enormous solubilisation improvement, channel purification did not improve.

SK2 channel was also expressed in mammalian cell lines using *Semliki Forest Virus*.
The channel reached a good expression level after 24h virus infection.
The channel was integrated into plasma membrane, although the majority was found in the ER membranes. Saturation binding data were found to fit to a single-site binding model, the apamin links to the channel with high affinity. The values obtained together with the ones derived from competitive displacement experiments are comparable with measurements done on native brain membranes (Marqueze, Seagar et al. 1989; Wadsworth, Doorty et al. 1994; Wadsworth, Torelli et al. 1997).
Solubilisation of the channel was successful, with several detergents able to extract the channel, of which the best one was DDM.
The crucial finding was that pure channel was obtained. This represents a significant advancement for the characterization of small calcium-activated potassium channels. To date there are no other reports of purified SK2 channel from either natural or recombinant sources.
Gel filtration chromatography and blue native PAGE showed that the pure channel has a tetrameric form, as expected.
Preliminary electron microscopy single particle image analysis showed that one class average of particles resembles a tetramer and presents a possible top view of the channel. The width of the four-fold class is approximately 70 Å, this value is comparable with the top view of Kv1.2 (Parcej and Eckhardt-Strelau, 2003) which possesses four subunits of similar size to SK2.
In the next future, more work on single particle image analysis will be done, aimed at a better understanding of these projection averages and 3D reconstruction. It will also be interesting to perform single particle experiments on SK2 bound to calmodulin, at this aim co-expression of SK2 and calmodulin or expression of the concatameric form, SK2-q-CaM, in mammalian cells will be tried.
More studies on the behavior of the channel inside the cells, concerning its biogenesis, folding, trafficking and its interaction with other cellular partners have to be done in order to fully understand this class of channels.

Also the human large calcium-activated potassium channel alpha subunit cDNA was expressed in *Pichia pastoris* cells in order to obtain high yield of protein for structural studies. The full length protein was expressed in large amount. Electron microscopic images revealed the presence of the protein in the cells in large aggregate vesicles. Despite the vast number of detergents tried, BK channel could not be solubilised with any of them. Even when the beta subunit of the protein was cloned together with the alpha subunit, no improvements were obtained.

It has been shown that BK channels in *Drosophila* interact with slob, a new class of multi-functional channel-binding protein, and their interaction leads to an increase in channel activity (Schopperle et al. 1998). Furthermore, co-immunoprecipitation experiments from *Drosophila* heads and transfected cells showed that the protein 14-3-3 interacts with BK via slob. All three proteins are co-localized presynaptically at *Drosophila* neuromuscular junctions. Two serine residues in slob are required for 14-3-3 binding, and the binding is dynamically regulated in Drosophila by calcium/calmodulin-dependent kinase II phosphorylation. 14-3-3 co-expression dramatically alters BK channel properties when slob is present. (Zhou et al. 1999).

In the future it will be important to try also expression of BK channel in mammalian cells using Semliki Forest virus system, to see whether it is possible to solubilize the channel. Furthermore co-expression of BK channel together with the beta subunit and/or slob protein could give more information about the behavior of this channel inside the cell.

Even if today several structural data of different potassium channels are available, still little is known about the structural characteristics and their role in eukaryotic cell biology. Furthermore little information regarding biogenesis, assembly, post-modifications, trafficking and cell surface expression is available.

Many experiments have revealed that as early as in biogenesis, the channels undergo several processes and modifications, all highly controlled and regulated by for example, specific amino acid motifs of channel itself, or by the ER machinery as translocon and chaperons, or auxiliary subunits, lipid interaction, segregation into specific vesicles and selective localization along the plasma membrane.

Understanding structure-function relationship in the eukaryotic context is therefore a fascinating project.

Concluding remarks

At the beginning of last century, science has revealed us new theories that revolutionized the physics and the old reductionistic theory, thanks to the scientific and intellectual work of a genial group of physicists, as Planck, Einstein, Heisenberg, Bohr, De Broglie, Schrödinger, Pauli and Dirac. New horizons were opened and what at that time was considered as paradoxal by these scientists that were observing the new events, are today asserted theories of modern physics. These new theories have shown us the unity of the universe and that it is not possible to observe and study physical phenomenon as a single entity, but only if we consider them within unified system made of interconnections.

In the same way, each organism, from the simplest one to the more complex, is made by an ensemble of biological elements that are interconnected and that endure the influence of the immediate environment (as for example, protein-protein, lipid-protein, cell-cell, cell-extracellular matrix, temperature and pH), namely the nature that surround them. Thus, every time we try to study a part or an aspect of a biological system, as for instance a protein, we have to deal with the arduous task to try to recreate *in vitro,* the physico-chemical and environmental conditions that are present *in vivo.* It is of course evident that the more complex a biological system is, as for example the nervous system, the more intricate it is to reproduce these conditions and to study it. A better knowledge of biological systems in their complexity will allow us to understand better this fabulous world that surround us.

"The world thus appears as a complicated tissue of events in which connections of different kinds alternate or combine and thereby determine the texture of the whole" (Heisenberg 1962).

Bibliography

Adelman, J. P., K. Z. Shen, et al. (1992). "Calcium-activated potassium channels expressed from cloned complementary DNAs." Neuron **9**(2): 209-16.

Allen, T. W. and S. H. Chung (2001). "Brownian dynamics study of an open-state KcsA potassium channel." Biochim Biophys Acta **1515**(2): 83-91.

Allen, T. W., O. S. Andersen, et al. (2004). "Energetics of ion conduction through the gramicidin channel." Proc Natl Acad Sci U S A **101**(1): 117-22.

Baumann, A., A. Grupe, et al. (1988). "Structure of the voltage-dependent potassium channel is highly conserved from Drosophila to vertebrate central nervous systems." Embo J **7**(8): 2457-63.

Benton, D. C., A. S. Monaghan, et al. (2003). "Small conductance Ca2+-activated K+ channels formed by the expression of rat SK1 and SK2 genes in HEK 293 cells." J Physiol **553**(Pt 1): 13-9.

Berneche, S. and B. Roux (2003). "A microscopic view of ion conduction through the K+ channel." Proceedings of the National Academy of Sciences of the United States of America **100**(15): 8644-8.

Bjorklof, K., K. Lundstrom, et al. (2002). "Co- and posttranslational modification of the alpha(1B)-adrenergic receptor: effects on receptor expression and function." Biochemistry **41**(13): 4281-91.

Bock, J., I. Szabo, et al. (2003). "Ceramide inhibits the potassium channel Kv1.3 by the formation of membrane platforms." Biochemical & Biophysical Research Communications **305**(4): 890-7.

Brown DA, L. E. (2000). "Structure and function of sphingolipid- and cholesterol-rich membrane rafts." The Journal of Biological Chemistry **275**: 17221-17224.

Bruns, R. F., K. Lawson-Wendling, et al. (1983). "A rapid filtration assay for soluble receptors using polyethylenimine-treated filters." Analytical Biochemistry **132**(1): 74-81.

Buckholz, R. G. and M. A. Gleeson (1991). "Yeast systems for the commercial production of heterologous proteins." Biotechnology (N Y) **9**(11): 1067-72.

Catterall, W. A. (1994). "Molecular properties of a superfamily of plasma-membrane cation channels." Curr Opin Cell Biol **6**(4): 607-15.

Christie, M. J., J. P. Adelman, et al. (1989). "Expression of a cloned rat brain potassium channel in Xenopus oocytes." Science **244**(4901): 221-4.

Chung, S. H. and S. Kuyucak (2002). "Ion channels: recent progress and prospects." Eur Biophys J **31**(4): 283-93.

Chung, S. H., T. W. Allen, et al. (2002). "Modeling diverse range of potassium channels with Brownian dynamics." Biophys J **83**(1): 263-77.

Constanti, A. and J. A. Sim (1987). "Calcium-dependent potassium conductance in guinea-pig olfactory cortex neurones in vitro." J Physiol **387**: 173-94.

Corry, B., M. Hoyles, et al. (2002). "Reservoir boundaries in Brownian dynamics simulations of ion channels." Biophys J **82**(4): 1975-84.

Covarrubias, M., A. A. Wei, et al. (1991). "Shaker, Shal, Shab, and Shaw express independent K+ current systems." Neuron **7**(5): 763-73.

Cregg, J. M., K. R. Madden, et al. (1989). "Functional characterization of the two alcohol oxidase genes from the yeast Pichia pastoris." Mol Cell Biol **9**(3): 1316-23.

de Hoop, M. J., J. Cregg, et al. (1991). "Overexpression of alcohol oxidase in Pichia pastoris." FEBS Letters **291**(2): 299-302.

Ding, J. P., Z. W. Li, et al. (1998). "Inactivating BK channels in rat chromaffin cells may arise from heteromultimeric assembly of distinct inactivation-competent and noninactivating subunits." Biophys J **74**(1): 268-89.

Doyle, D. A., J. M. Cabral, et al. (1998). "The structure of the potassium channel - molecular-basis of K^+ conduction and selectivity." Science **280**(N5360): 69-77.

Doyle, D. A., J. Morais Cabral, et al. (1998). "The structure of the potassium channel: molecular basis of K+ conduction and selectivity." Science **280**(5360): 69-77.

Ehlers, M. D., S. Zhang, et al. (1996). "Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit." Cell **84**(5): 745-55.

Ellgaard, L., M. Molinari, et al. (1999). "Setting the standards: quality control in the secretory pathway." Science **286**(5446): 1882-8.

Frank, J., M. Radermacher, et al. (1996). "SPIDER and WEB: processing and

visualization of images in 3D electron microscopy and related fields." Journal of Structural Biology **116**(1): 190-199.

Futerman, A. H. and G. A. Banker (1996). "The economics of neurite outgrowth--the addition of new membrane to growing axons." Trends Neurosci **19**(4): 144-9.

Garcia-Calvo, M., H. G. Knaus, et al. (1994). "Purification and reconstitution of the high-conductance, calcium-activated potassium channel from tracheal smooth muscle." J Biol Chem **269**(1): 676-82.

Garciacalvo, M., H. G. Knaus, et al. (1994). "Purification and Reconstitution Of the High-Conductance, Calcium- Activated Potassium Channel From Tracheal Smooth-Muscle." Journal Of Biological Chemistry **269**(1): 676-682.

Giangiacomo, K. M., M. Garcia-Calvo, et al. (1995). "Functional reconstitution of the large-conductance, calcium-activated potassium channel purified from bovine aortic smooth muscle." Biochemistry **34**(48): 15849-62.

Gouaux, E. (1998). "Single potassium ion seeks open channel for transmembrane travels: tales from the KcsA structure." Structure **6**(10): 1221-6.

Grunewald, S., W. Haase, et al. (2004). "Production of the human D2S receptor in the methylotrophic yeast P. pastoris." Receptors Channels **10**(1): 37-50.

Habermann, E. and K. Fischer (1979). "Apamin, a centrally acting neurotoxic peptide: binding and actions." Adv Cytopharmacol **3**: 387-94.

Hannan, A. J., P. Gunning, et al. (1998). "Structural compartments within neurons: developmentally regulated organization of microfilament isoform mRNA and protein." Mol Cell Neurosci **11**(5-6): 289-304.

Hanner, M., R. Viannajorge, et al. (1998). "The beta-subunit of the high-conductance calcium-activated potassium channel - identification of residues involved in charybdotoxin binding." Journal of Biological Chemistry **273**(N26): 16289-16296.

Harlow, E. and D. Lane (1988). Antibodies: A Laboratory Manual, Cold Spring Harbor Press.

Heisenberg, W. (1962). Physics and Philosophy. New York, Harper & Row.

Helenius, A. and K. Simons (1975). "Solubilization of membranes by detergents." Biochim Biophys Acta **415**(1): 29-79.

Helenius, A., T. Marquardt, et al. (1992). "The endoplasmic reticulum as a protein-folding compartment

Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum." Trends Cell Biol **2**(8): 227-31.

Heuberger, E. H., L. M. Veenhoff, et al. (2002). "Oligomeric state of membrane transport proteins analyzed with blue native electrophoresis and analytical ultracentrifugation." J Mol Biol **317**(4): 591-600.

Hille, B. (1970). "Ionic channels in nerve membranes." Prog Biophys Mol Biol **21**: 1-32.

Hille, B. (1986). "Ionic channels: molecular pores of excitable membranes." Harvey Lect **82**: 47-69.

Hille, B. (1992). Ionic Channels in Excitable Membranes. Sunderland, MA., Sinauer Associates.

Hjelmeland, L. M. (1990). "Solubilization of native membrane proteins." Methods Enzymol **182**: 253-64.

Hodgkin, A. L. and A. F. Huxley (1952). "The components of membrane conductance in the giant axon of Loligo." J Physiol **116**(4): 473-96.

Hodgkin, A. L. and A. F. Huxley (1952). "Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo." J Physiol **116**(4): 449-72.

Hodgkin, A. L. and R. D. Keynes (1955). "The potassium permeability of a giant nerve fibre." J Physiol **128**(1): 61-88.

Hoffmann, M., D. Verzijl, et al. (2001). "Recombinant Semliki Forest virus for over-expression and pharmacological characterisation of the histamine H(2) receptor in mammalian cells." Eur J Pharmacol **427**(2): 105-14.

Hovius, R., A. P. Tairi, et al. (1998). "Characterization of a mouse serotonin 5-HT3 receptor purified from mammalian cells." J Neurochem **70**(2): 824-34.

Hugues, M., D. Duval, et al. (1982). "Preparation of a pure monoiodo derivative of the bee venom neurotoxin apamin and its binding properties to rat brain synaptosomes." J Biol Chem **257**(6): 2762-9.

Hugues, M., G. Romey, et al. (1982). "Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: voltage-clamp and

biochemical characterization of the toxin receptor." Proc Natl Acad Sci U S A **79**(4): 1308-12.

Hugues, M., H. Schmid, et al. (1982). "The Ca2+-dependent slow K+ conductance in cultured rat muscle cells: characterization with apamin." Embo J **1**(9): 1039-42.

Ishii, T. M., J. Maylie, et al. (1997). "Determinants of apamin and d-tubocurarine block in sk potassium channels." Journal of Biological Chemistry **272**(N37): 23195-23200.

Iverson, L. E., M. A. Tanouye, et al. (1988). "A-type potassium channels expressed from Shaker locus cDNA." Proc Natl Acad Sci U S A **85**(15): 5723-7.

Jacobson, M. (1991). Developmental Neurobiology. New York and London, Plenum Press.

Jaggar, J. H., V. A. Porter, et al. (2000). "Calcium sparks in smooth muscle." Am J Physiol Cell Physiol **278**(2): C235-56.

Jan, L. Y. and Y. N. Jan (1990). "A superfamily of ion channels." Nature **345**(6277): 672.

Jaramillo, A. M., X. Zheng, et al. (2004). "Pattern of distribution and cycling of SLOB, Slowpoke channel binding protein, in Drosophila." BMC Neurosci **5**(1): 3.

Jegla, T. and L. Salkoff (1994). "Molecular evolution of K+ channels in primitive eukaryotes." Soc Gen Physiol Ser **49**: 213-22.

Jiang, Y., A. Pico, et al. (2001). "Structure of the RCK domain from the E. coli K+ channel and demonstration of its presence in the human BK channel." Neuron **29**(3): 593-601.

Jiang, Y., A. Lee, et al. (2002). "Crystal structure and mechanism of a calcium-gated potassium channel." Nature **417**(6888): 515-22.

Jiang, Y., A. Lee, et al. (2003). "X-ray structure of a voltage-dependent K+ channel." Nature **423**: 33-41.

Jiang, Y., V. Ruta, et al. (2003). "The principle of gating charge movement in a voltage-dependent K+ channel." Nature **423**(6935): 42-8.

Kamb, A., L. E. Iverson, et al. (1987). "Molecular characterization of Shaker, a Drosophila gene that encodes a potassium channel." Cell **50**(3): 405-13.

Kandel E.R., S. J. H., Jessell T.M. (2000). Principles of Neural Science, McGrow-Hill,

Health Professions Division.

Kessi, J., J. C. Poiree, et al. (1994). "Short-chain phosphatidylcholines as superior detergents in solubilizing membrane proteins and preserving biological activity." Biochemistry **33**(35): 10825-36.

Klaiber, K., N. Williams, et al. (1990). "Functional expression of Shaker K+ channels in a baculovirus-infected insect cell line." Neuron **5**(2): 221-6.

Klumperman, J. (2000). "Transport between ER and Golgi." Curr Opin Cell Biol **12**(4): 445-9.

Kohler, M., B. Hirschberg, et al. (1996). "Small-conductance, calcium-activated potassium channels from mammalian brain." Science **273**(5282): 1709-14.

Kohler, M., B. Hirschberg, et al. (1996). "Small-conductance, calcium-activated potassium channels from mammalian brain [see comments]. Comment in: Science 1996 Sep 20;273(5282):1677." Science **273**(5282): 1709-14.

Koschak, A., R. O. Koch, et al. (1997). "[I-125]Iberiotoxin-D19Y/Y36F, the first selective, high specific activity radioligand for high-conductance calcium-activated potassium channels." Biochemistry **36**(7): 1943-1952.

Krause, J. D. and P. H. Reinhart (1998). "Ca2+ activation properties of hslo ca2+-activated k+ channels." Biophysical Journal **74**(N2): A 210-210.

Kubo, Y., E. Reuveny, et al. (1993). "Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel." Nature **364**(6440): 802-6.

Kubo, Y., T. J. Baldwin, et al. (1993). "Primary structure and functional expression of a mouse inward rectifier potassium channel." Nature **362**(6416): 127-33.

Kuryshev, Y. A., B. A. Wible, et al. (1999). "K+ channel associated protein (KChAP) modulates expression of specific Kv channels." Biophysical Journal **76**(1): A79-A79.

Kuryshev, Y. A., T. I. Gudz, et al. (2000). "KChAP as a chaperone for specific K(+) channels." Am J Physiol Cell Physiol **278**(5): C931-41.

Labbe-Jullie, C., C. Granier, et al. (1991). "Binding and toxicity of apamin. Characterization of the active site." European Journal of Biochemistry **196**(3): 639-45.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head

of bacteriophage T4." Nature **227**(259): 680-5.

Lancaster, B. and P. R. Adams (1986). "Calcium-dependent current generating the afterhyperpolarization of hippocampal neurons." J Neurophysiol **55**(6): 1268-82.

Lee, W. S., T. J. Ngo-Anh, et al. (2003). "Small conductance Ca2+-activated K+ channels and calmodulin: cell surface expression and gating." J Biol Chem **278**(28): 25940-6.

Lorenz, E., A. E. Alekseev, et al. (1998). "Evidence for direct physical association between a k+ channel (kir6.2) and an atp-binding cassette protein (sur1) which affects cellular-distribution and kinetic-behavior of an atp-sensitive k+ channel." Molecular and Cellular Biology **18**(N3): 1652-1659.

Lundstrom, K., A. Michel, et al. (1997). "Expression of ligand-gated ion channels with the Semliki Forest virus expression system." J Recept Signal Transduct Res **17**(1-3): 115-26.

Lundstrom, K. (2003). "Semliki Forest virus vectors for rapid and high-level expression of integral membrane proteins." Biochim Biophys Acta **1610**(1): 90-6.

Ma, D., N. Zerangue, et al. (2001). "Role of ER export signals in controlling surface potassium channel numbers." Science **291**(5502): 316-9.

Ma, D. and L. Y. Jan (2002). "ER transport signals and trafficking of potassium channels and receptors." Curr Opin Neurobiol **12**(3): 287-92.

Maeda, Y., R. Kuroki, et al. (2000). "High-level secretion of biologically active recombinant human macrophage inflammatory protein-1alpha by the methylotrophic yeast Pichia pastoris." Protein Expr Purif **18**(1): 56-63.

Marabini, R. and J. M. Carazo (1994). "Pattern recognition and classification of images of biological macromolecules using artificial neural networks." Biophys J **66**(6): 1804-14.

Marek, K. W. and G. W. Davis (2003). "Controlling the active properties of excitable cells." Curr Opin Neurobiol **13**(5): 607-11.

Marqueze, B., M. J. Seagar, et al. (1987). "Photoaffinity labeling of the K+-channel-associated apamin-binding molecule in smooth muscle, liver and heart membranes." Eur J Biochem **169**(2): 295-8.

Marqueze, B., M. J. Seagar, et al. (1989). "Characterization of apamin-binding protein

associated with a Ca2+ -activated K+ channel." J Protein Chem **8**(3): 425-7.

Meera, P., M. Wallner, et al. (1997). "Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus." Proceedings of the National Academy of Sciences of the United States of America **94**(25): 14066-71.

Miller, G. (2003). "Neuroscience. The puzzling portrait of a pore." Science **300**(5628): 2020-2.

Monaghan, A. S., D. C. Benton, et al. (2004). "The SK3 subunit of small conductance Ca2+-activated K+ channels interacts with both SK1 and SK2 subunits in a heterologous expression system." J Biol Chem **279**(2): 1003-9.

Munujos, P., H. G. Knaus, et al. (1995). "Cross-linking of charybdotoxin to high-conductance calcium-activated potassium channels: identification of the covalently modified toxin residue." Biochemistry **34**(34): 10771-6.

Nagaya, N., C. T. Schulteis, et al. (1999). "Calnexin associates with shaker K+ channel protein but is not involved in quality control of subunit folding or assembly." Receptors & Channels **6**(4): 229-239.

Nimigean, C. M. and K. L. Magleby (2000). "Functional coupling of the beta(1) subunit to the large conductance Ca(2+)-activated K(+) channel in the absence of Ca(2+). Increased Ca(2+) sensitivity from a Ca(2+)-independent mechanism." Journal of General Physiology **115**(6): 719-36.

Niznik, H. B., N. Y. Otsuka, et al. (1986). "Dopamine D1 receptors characterized with [3H]SCH 23390. Solubilization of a guanine nucleotide-sensitive form of the receptor." J Biol Chem **261**(18): 8397-406.

Nunnari, J. M., M. G. Repaske, et al. (1987). "Regulation of porcine brain alpha 2-adrenergic receptors by Na+,H+ and inhibitors of Na+/H+ exchange." J Biol Chem **262**(25): 12387-92.

Obermair, G. J., W. A. Kaufmann, et al. (2003). "The small conductance Ca2+-activated K+ channel SK3 is localized in nerve terminals of excitatory synapses of cultured mouse hippocampal neurons." Eur J Neurosci **17**(4): 721-31.

Papazian, D. M., T. L. Schwarz, et al. (1987). "Cloning of genomic and complementary

DNA from Shaker, a putative potassium channel gene from Drosophila." Science **237**(4816): 749-53.

Papazian, D. M., T. L. Schwarz, et al. (1988). "Ion channels in *Drosophila*." Annual Review of Physiology **50**: 379-94.

Parcej, D. N. and J. O. Dolly (1989). "Dendrotoxin acceptor from bovine synaptic plasma-membranes - binding- properties, purification and subunit composition of a putative constituent of certain voltage-activated K^+ channels." Biochemical Journal **257**(3): 899-903.

Parcej, D. N. and L. Eckhardt-Strelau (2003). "Structural characterisation of neuronal voltage-sensitive K+ channels heterologously expressed in Pichia pastoris." J Mol Biol **333**(1): 103-16.

Poolos, N. P. and D. Johnston (1999). "Calcium-activated potassium conductances contribute to action potential repolarization at the soma but not the dendrites of hippocampal CA1 pyramidal neurons." J Neurosci **19**(13): 5205-12.

Qian, X. and K. L. Magleby (2003). "Beta1 subunits facilitate gating of BK channels by acting through the Ca2+, but not the Mg2+, activating mechanisms." Proceedings of the National Academy of Sciences of the United States of America **100**(17): 10061-6.

Radermacher, M., T. Ruiz, et al. (2001). "The structure of the V1-ATPase determined by three-dimensional electron microscopy of single particles." Journal of Structural Biology **135**: 26-37.

Rauh, J. J., M. P. Lambert, et al. (1986). "Glycoprotein properties of muscarinic acetylcholine receptors from bovine cerebral cortex." J Neurochem **46**(1): 23-32.

Robitaille, R., M. L. Garcia, et al. (1993). "Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release." Neuron **11**(4): 645-55.

Rossanese, O. W., J. Soderholm, et al. (1999). "Golgi structure correlates with transitional endoplasmic reticulum organization in Pichia pastoris and Saccharomyces cerevisiae." J Cell Biol **145**(1): 69-81.

Sah, P. and E. M. McLachlan (1991). "Ca(2+)-activated K+ currents underlying the afterhyperpolarization in guinea pig vagal neurons: a role for Ca(2+)-activated

Ca2+ release." Neuron **7**(2): 257-64.

Sah, P. (1996). "Ca(2+)-activated K+ currents in neurones: types, physiological roles and modulation." Trends Neurosci **19**(4): 150-4.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press.

Schagger, H. and G. von Jagow (1987). "Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa." Analytical Biochemistry **166**(2): 368-79.

Schiller, H., W. Haase, et al. (2000). "The human ET(B) endothelin receptor heterologously produced in the methylotrophic yeast Pichia pastoris shows high-affinity binding and induction of stacked membranes." Receptors Channels **7**(2): 93-107.

Schmidt, T. G. M., J. Köpke, et al. (1996). "Molecular interaction between the *Strep* tag affinity peptide and its cognate target streptavidin." Journal of Molecular Biology **225**: 753-776.

Schonherr, R., K. Lober, et al. (2000). "Inhibition of human ether a go-go potassium channels by Ca(2+)/calmodulin." Embo J **19**(13): 3263-71.

Schopperle, W. M., M. H. Holmqvist, et al. (1998). "Slob, a novel protein that interacts with the slowpoke calcium-dependent potassium channel." Neuron **20**(N3): 565-573.

Schreiber, M. and L. Salkoff (1997). "A novel calcium-sensing domain in the bk channel." Biophysical Journal **73**(N3): 1355-1363.

Schumacher, M. A., A. F. Rivard, et al. (2001). "Structure of the gating domain of a Ca2+-activated K+ channel complexed with Ca2+/calmodulin." Nature **410**(6832): 1120-4.

Schwarz, W. and H. Passow (1983). "Ca2+-activated K+ channels in erythrocytes and excitable cells." Annu Rev Physiol **45**: 359-74.

Schwarz, T. L., B. L. Tempel, et al. (1988). "Multiple potassium-channel components are produced by alternative splicing at the Shaker locus in Drosophila [published erratum appears in Nature 1988 Apr 21;332(6166):740]." Nature **331**(6152): 137-42.

Schweitzer, C., C. Kratzeisen, et al. (2000). "Characterization of [(3)H]-LY354740 binding to rat mGlu2 and mGlu3 receptors expressed in CHO cells using semliki forest virus vectors." Neuropharmacology **39**(10): 1700-6.

Schwindt, P. C., W. J. Spain, et al. (1988). "Multiple potassium conductances and their functions in neurons from cat sensorimotor cortex in vitro." J Neurophysiol **59**(2): 424-49.

Seagar, M. J., C. Granier, et al. (1984). "Interactions of the neurotoxin apamin with a Ca2+-activated K+ channel in primary neuronal cultures." J Biol Chem **259**(3): 1491-5.

Seagar, M. J., C. Labbe-Jullie, et al. (1986). "Molecular structure of rat brain apamin receptor: differential photoaffinity labeling of putative K+ channel subunits and target size analysis." Biochemistry **25**(14): 4051-7.

Seagar, M. J., B. Marqueze, et al. (1987). "Solubilization of the apamin receptor associated with a calcium-activated potassium channel from rat brain." J Neurosci **7**(2): 565-70.

Seagar Mj Fau - Granier, C., F. Granier C Fau - Couraud, et al. (1984). "Interactions of the neurotoxin apamin with a Ca2+-activated K+ channel in primary neuronal cultures." J Biol Chem **259**(3): 1491-5.

Seagar Mj Fau - Labbe-Jullie, C., C. Labbe-Jullie C Fau - Granier, et al. (1986). "Molecular structure of rat brain apamin receptor: differential photoaffinity labeling of putative K+ channel subunits and target size analysis." Biochemistry **25**(14): 4051-7.

Seagar Mj Fau - Marqueze, B., F. Marqueze B Fau - Couraud, et al. (1987). "Solubilization of the apamin receptor associated with a calcium-activated potassium channel from rat brain." J Neurosci **7**(2): 565-70.

Sen, S., V. P. Jaakola, et al. (2003). "Functional expression and direct visualization of the human alpha 2B -adrenergic receptor and alpha 2B -AR-green fluorescent fusion protein in mammalian cell using Semliki Forest virus vectors." Protein Expr Purif **32**(2): 265-75.

Shamotienko, O., S. Akhtar, et al. (1999). "Recreation of neuronal Kv1 channel oligomers by expression in mammalian cells using Semliki Forest virus."

Biochemistry **38**(51): 16766-16776.

Shen, S., G. Sulter, et al. (1998). "A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast Pichia pastoris." Gene **216**(1): 93-102.

Shepherd, F. H. and A. Holzenburg (1995). "The potential of fluorinated surfactants in membrane biochemistry." Anal Biochem **224**(1): 21-7.

Shi, G., K. Nakahira, et al. (1996). "Beta subunits promote K+ channel surface expression through effects early in biosynthesis." Neuron **16**(4): 843-52.

Shivanna, B. D. and E. S. Rowe (1997). "Preservation of the native structure and function of Ca2+-ATPase from sarcoplasmic reticulum: solubilization and reconstitution by new short-chain phospholipid detergent 1,2-diheptanoyl-sn-phosphatidylcholine." Biochem J **325 (Pt 2)**: 533-42.

Siegel G.J., A. B. W., Albers R.W., Fisher S.K., Uhler M.D (1999). Basic Neurochemistry. Philadelphia, Baltimora, New York, London, Lippincott Williams Wilkins.

Stackman, R. W., R. S. Hammond, et al. (2002). "Small conductance Ca2+-activated K+ channels modulate synaptic plasticity and memory encoding." J Neurosci **22**(23): 10163-71.

Stefani, E., M. Ottolia, et al. (1997). "Voltage-controlled gating in a large conductance Ca2+-sensitive K+channel (hslo)." Proceedings of the National Academy of Sciences of the United States of America **94**(10): 5427-31.

Stocker, M. and P. Pedarzani (2000). "Differential distribution of three Ca(2+)-activated K(+) channel subunits, SK1, SK2, and SK3, in the adult rat central nervous system." Mol Cell Neurosci **15**(5): 476-93.

Stockklausner, C., J. Ludwig, et al. (2001). "A sequence motif responsible for ER export and surface expression of Kir2.0 inward rectifier K(+) channels." FEBS Lett **493**(2-3): 129-33.

Storm, J. F. (1990). "Potassium currents in hippocampal pyramidal cells." Prog Brain Res **83**: 161-87.

Stühmer, W., M. Stocker, et al. (1988). "Potassium channels expressed from rat brain cDNA have delayed rectifier properties." FEBS Lett **242**(1): 199-206.

Tate, C. G., J. Haase, et al. (2003). "Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter." Biochimica et Biophysica Acta **1**: 141-53.

Tempel, B. L., D. M. Papazian, et al. (1987). "Sequence of a probable potassium channel component encoded at Shaker locus of Drosophila." Science **237**(4816): 770-5.

Tempel, B. L., Y. N. Jan, et al. (1988). "Cloning of a probable potassium channel gene from mouse brain." Nature **332**(6167): 837-9.

Tian, L., H. G. Knaus, et al. (1998). "Glucocorticoid regulation of calcium-activated potassium channels mediated by serine/threonine protein phosphatase." J Biol Chem **273**(22): 13531-6.

Timpe, L. C., T. L. Schwarz, et al. (1988). "Expression of functional potassium channels from Shaker cDNA in Xenopus oocytes." Nature **331**(6152): 143-5.

Toro, L., M. Wallner, et al. (1998). "Maxi-k-ca, a unique member of the voltage-gated k channel superfamily." News in Physiological Sciences **13**(JUN): 112-117.

Trimmer, J. S. (1998). "Analysis of K+ channel biosynthesis and assembly in transfected mammalian cells." Methods in Enzymology **293**: 32-49.

Trimmer, J. S. and K. J. Rhodes (2004). "Localization of Voltage-Gated Ion Channels IN Mammalian Brain." Annu Rev Physiol **66**: 477-519.

Tschopp, J. F., P. F. Brust, et al. (1987). "Expression of the lacZ gene from two methanol-regulated promoters in Pichia pastoris." Nucleic Acids Res **15**(9): 3859-76.

Tseng-Crank, J., C. D. Foster, et al. (1994). "Cloning, expression, and distribution of functionally distinct Ca(2+)-activated K+ channel isoforms from human brain." Neuron **13**(6): 1315-30.

Vergara, C., R. Latorre, et al. (1998). "Calcium-activated potassium channels." Current Opinion in Neurobiology **8**(N3): 321-329.

Verkade, P., L. H. Schrama, et al. (1997). "Ultrastructural co-localization of calmodulin and B-50/growth-associated protein-43 at the plasma membrane of proximal unmyelinated axon shafts studied in the model of the regenerating rat sciatic nerve." Neuroscience **79**(4): 1207-18.

Wadsworth, J. D., K. B. Doorty, et al. (1994). "Comparable 30-kDa apamin binding polypeptides may fulfill equivalent roles within putative subtypes of small conductance Ca(2+)-activated K+ channels." J Biol Chem **269**(27): 18053-61.

Wadsworth, J. D., S. Torelli, et al. (1997). "Structural diversity among subtypes of small-conductance Ca2+-activated potassium channels." Arch Biochem Biophys **346**(1): 151-60.

Wei, A., T. Jegla, et al. (1996). "Eight potassium channel families revealed by the C. elegans genome project." Neuropharmacology **35**(7): 805-29.

Weinberger, R., G. Schevzov, et al. (1996). "The molecular composition of neuronal microfilaments is spatially and temporally regulated." J Neurosci **16**(1): 238-52.

Weiss, H. M., W. Haase, et al. (1998). "Comparative biochemical and pharmacological characterization of the mouse 5HT5A 5-hydroxytryptamine receptor and the human beta2-adrenergic receptor produced in the methylotrophic yeast Pichia pastoris." Biochem J **330 (Pt 3)**: 1137-47.

Wen, H. and I. B. Levitan (2002). "Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels." J Neurosci **22**(18): 7991-8001.

Xia, X. M., B. Fakler, et al. (1998). "Mechanism of calcium gating in small-conductance calcium-activated potassium channels." Nature **395**(6701): 503-7.

Xia, X., B. Hirschberg, et al. (1998). "dSLo interacting protein 1, a novel protein that interacts with large-conductance calcium-activated potassium channels." J Neurosci **18**(7): 2360-9.

Xia, X. M., J. P. Ding, et al. (1999). "Molecular basis for the inactivation of Ca2+- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells." J Neurosci **19**(13): 5255-64.

Xia, X. M., J. P. Ding, et al. (2003). "Inactivation of BK channels by the NH2 terminus of the beta2 auxiliary subunit: an essential role of a terminal peptide segment of three hydrophobic residues." Journal of General Physiology **121**(2): 125-48.

Yazejian, B., D. A. DiGregorio, et al. (1997). "Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured Xenopus nerve-muscle synapses." J Neurosci **17**(9): 2990-3001.

Zerangue, N., B. Schwappach, et al. (1999). "A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels." Neuron **22**(3): 537-48.

Zhang, S., M. D. Ehlers, et al. (1998). "Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors." Neuron **21**(2): 443-53.

Zhang, Y. and R. M. Harris-Warrick (2004). "An ER export signal accelerates the surface expression of shal potassium channels in pyloric neurons of the lobster stomatogastric ganglion." Pflugers Arch **447**(4): 401-4.

Zhou, Y., W. M. Schopperle, et al. (1999). "A dynamically regulated 14-3-3, Slob, and Slowpoke potassium channel complex in Drosophila presynaptic nerve terminals." Neuron **22**(4): 809-18.

Zhou, Y., J. H. Morais-Cabral, et al. (2001). "Chemistry of ion coordination and hydration revealed by a K+ channel-Fab complex at 2.0 A resolution." Nature **414**(6859): 43-8.

Zhou, Y., H. Fei, et al. (2003). "An interaction domain in Slob necessary for its binding to the slowpoke calcium-dependent potassium channel." Neuropharmacology **45**(6): 714-9.

Zigmond M.J., B. F. E., Landis S.C., Roberts J.L., Squire L.L. (1999). Fundamental Neuroscience. San Diego, London, Boston, Academic Press.

Zuhlke, R. D., G. S. Pitt, et al. (1999). "Calmodulin supports both inactivation and facilitation of L-type calcium channels." Nature **399**(6732): 159-62.

Zusammenfassung in deutscher Sprache

Viele essentiellen physiologischen Prozesse sind durch die Fähigkeit der Zelle kontrolliert, schnell und selektiv ihre Durchlässigkeit für Ionen zu verändern. Kaliumkanäle vermitteln als reguliertes Porenprotein den selektiven Transport von Kaliumionen durch die Zellmembran. Sie sind für physiologische Prozesse wie Neurosekretion und Tonus der glatten Muskulatur von Bedeutung. Kaliumkanäle sind einzigartig unter den Ionenkanälen, weil das Gleichgewichtspotential für Kaliumionen sehr nahe dem Ruhemembranpotential des Neurons ist, während sich das Ruhepotential anderer Ionenkanäle meist stark von dem der umgebenden Membran unterscheidet. Im Gehirn kommen verschiedene Kaliumkanäle vor, die sich in ihrer Leitfähigkeit und Selektivität unterscheiden. Eine Familie stellen die Calcium-aktivierten Kaliumkanäle dar, welche wiederum in drei Unterfamilien aufgeteilt werden können: SK-Kanäle (geringe Leitfähigkeit), BK-Kanäle (hohe Leitfähigkeit) und IK-Kanäle (mittlere Leitfähigkeit). Sie werden durch ein Ansteigen der intrazellulären Ca^{2+}-Konzentration, wie sie während eines Aktionspotentials auftritt, aktiviert und zeigen keine Spannungsabhängigkeit der Aktivierung. SK-Kanäle sind durch eine Leitfähigkeit des einfachen Kanals von weniger als 20 pS gekennzeichnet.

Die Untereinheiten der SK-Kanäle teilen ihre gesamt-Transmembrantopologie mit spannungsabhängigen Kaliumkanälen. Sie verfügen über sechs Transmembrandomainen, und Amino- und Carboxyterminus liegen im Zellinneren. Die einzige erwähnenswerte Homologie mit anderen Kaliumkanälen besteht in der Porenregion zwischen der fünften und sechsten Transmembrandomaine. SK-Kanäle sind in physiologische Prozesse wie Lernen, Erinnerungsvermögen, Regulation des Tagesrhytmus und Unterbrechung des normalen Schlafmusters involviert. Drei Vertreter der SK-Familie der Kaliumkanäle wurden kloniert (Kohler et al. 1996). Sie alle agieren spannungsunabhängig und werden durch submikromolare intrazelluläre Ca^{2+}-Konzentrationen aktiviert. Die Proteine zeigen eine charakteristische Empfindlichkeit gegenüber Apamin, welche ein nützlicher Anzeiger für die Funktion

des Kanals ist.

Der SK2-Kanal besitzt zwei Untereinheiten, eine von 86 kDa (□) und eine weitere von 30 kDa (□), die bisher nur durch Apamin-Quervernetzungsstudien identifiziert wurde. Die klonierte □-Untereinheit des SK2-Kanals besitzt keine signifikante Sequenzhomologie zu anderen bisher klonierten Kaliumkanälen mit Ausnahme eines 12-Aminosäure-Abschnittes innerhalb der mutmaßlichen Porendomäne. Die Sensitivität gegenüber Calcium wird durch Calmodulin hervorgerufen, welches an die Aminosäuren 463 bis 497 bindet. Calmodulin ist ein kleines Ca^{2+}-bindendes Protein, das in der Zelle als Ca^{2+}-Sensor fungiert.

Das SK2-Protein der Ratte (rSK2) wurde in *Pichia pastoris*-Zellen überexprimiert, um das Protein zu reinigen und genügend Material für strukturelle Studien zu erhalten. Dazu wurde die rSK2-cDNA in das *Pichia Pastoris*-Expressionssystem kloniert. Eine Expression großer Mengen des Vollängenproteins wurde erreicht. Elektronenmikroskopische Lokalisationsstudien wie Immunogold-Markierung und Gefrierbruch-Analyse zeigten, daß der SK2-Kanal hauptsächlich an der Oberfläche des Endoplasmatischen Retikulums oder andersartigen internen Membranstrukturen lokalisiert ist. Dies steht im Gegensatz zu neuronalen Zellen, in denen das Protein eher in der Plasmamembran zu finden ist. Bindungsstudien mit radioaktiv markiertem Apamin bestätigten, daß zumindest ein Teil des exprimierten Kanalproteins funktionell war. Die Gesamtzahl an Bindungsstellen war zehnfach höher als in Membranen aus Rattenhirnen, die parallel für die Messungen eingesetzt wurden.

Die Solubilisierung des Kanalproteins mit Detergentien war besonders schwierig zu erreichen. Von 15 getesteten Detergentien konnte das Kanalprotein nur mit Digitonin aus der Membran gelöst werden. Zudem war der Anteil an gelöstem SK2-Protein im Vergleich zur geschätzten in der Membran befindlichen Menge niedrig. Verschiedene chromatographische Verfahren wurden zur Reinigung des Kanals eingesetzt, aber das Protein wurde nur teilweise gereinigt und die Ausbeute war gering. Eine Gelfiltrationsanalyse zeigte, daß der gereinigte SK2-Kanal wie andere Kaliumkanäle als

Homotetramer vorlag (MacKinnon 1991). Um einen verbesserten Einbau in die Plasmamembran zu erreichen, wurde versucht, ein weiteres SK2-Konstrukt zu exprimieren. Dieses verfügte über eine zusätzliche sechs Aminosäuren umfassende Sequenz (FCYENE), welche sich als bedeutsam für die Membranlokalisation anderer Kaliumkanäle herausgestellt hatte (Ma et al. 2001). Die Sequenz war an den Carboxyterminus angefügt. Der Effekt dieser Sequenz auf die SK2-Expression in *Pichia pastoris* wurde untersucht.

Mit dem SK2-FCYENE-Konstrukt wurde ebenfalls ein Vollängenprotein erhalten. Bindungsstudien brachten B_{max}-Werte hervor, welche denen für exprimiertes wildtyp-SK2-Protein glichen. Dies zeigte, daß die FCYENE-Sequenz keinen Effekt auf den gesamt-Expressionslevel bindungskompetenter Kanäle hatte. Digitonin, TritonX-100, n-Dodecyl-β-D-maltosid (DDM) und Octyl-glycosid erlaubten eine Solubilisierung des SK2-FCYENE-Proteins. Dies zeigte, daß die FCYENE-Sequenz einen bemerkenswerten Effekt auf die Löslichkeit des SK2-Proteins hatte. Im Gegensatz zum Wildtyp zeigte das SK2-FCYENE-Protein auch eine veränderte Verteilung innerhalb der Hefezellen. Es lag nicht nur im Endoplasmatischen Retikulum, sondern auch konzentriert in Vesikeln vor. Trotzdem waren wie zuvor nur geringe Mengen an Protein in der Plasmamembran zu finden. Offenbar kann die FCYENE-Sequenz zwar den Austritt aus dem Endoplasmatischen Retikulum fördern, aber die Kanäle werden nicht in die Plasmamembran translokiert. Trotz der erhöhten Menge an löslichem Ausgangsmaterial wurde die Menge an gereinigtem Kanalprotein nicht nennenswert erhöht.

Die Calcium-Sensitivität der SK-Kanäle wird durch eine Wechselwirkung mit Calmodulin hervorgerufen (Xia et al. 1998; Schumacher et al. 2001). Die Bindung zwischen Calmodulin und SK2-Protein ist nicht nur für das Öffnen, sondern auch für den Transport des Kanals essentiell (Lee et al. 2003). Deshalb wurde das SK2-Protein *in tandem* mit Calmodulin kloniert (SK2-q-CaM), wobei ein Verbindungsstück aus zehn Glutaminresten die beiden Proteine verband. In *Pichia pastoris*-Zellen wurden große Mengen des Proteins erhalten.

Interessanterweise zeigten Bindungsexperimente, daß die Gesamtzahl an Bindungsstellen erheblich höher war als für exprimiertes wildtyp- und SK2-FCYENE-Protein. Es ist nicht geklärt, ob dies durch einen erhöhten Wert an gesamtem exprimierten Kanalprotein oder durch ein Ansteigen des Anteils an aktiven Kanälen in der Population verursacht wurde. Das Calmodulin veränderte dramatisch die Verteilung des Kanalproteins in der Zelle. Dies zeigte die Immunogold-Markierung des SK2-q-CaM-Proteins in den *Pichia pastoris*-Zellen. Einige der SK2-q-CaM-Chimären waren in der Plasmamembran lokalisiert. Ferner war die Effektivität der Solubilisierung mit Detergenz weitaus höher als für das wildtyp- und das SK2-FCYENE-Protein. Trotz der enormen Verbesserung der Löslichkeit war die Ausbeute an SK2-q-CaM-Protein geringer als für die beiden anderen Konstrukte, was vermutlich auf eine veränderte Verfügbarkeit der His-Sequenz durch die Wechselwirkung mit Calmodulin zurückzuführen war.

Das SK2-Protein wurde zudem in der Säugerzelllinie BHK (Baby Hamster Kidney) unter Verwendung des *Semliki Forest Virus*-Systems exprimiert. Das Protein erreichte 24 h nach Virusinfektion einen hohen Expressionslevel. Eine Immunogold-Markierung bestätigte, daß das SK-Protein in die Plasmamembran eingebaut wurde, wenngleich ein Großteil des Proteins in den Membranen des Endoplasmatischen Retikulums verblieb. Apamin band mit hoher Affinität an das Kanalprotein, wobei die Daten der Sättigungskurven auf eine einzelne Bindungstelle hinwiesen. Diese Werte und Daten aus kompetitiven Verdrängungs-Experimenten waren mit jenen Werten, die mit nativen Hirnmembranen gemessen wurden, vergleichbar (Marqueze, Seagar et al. 1989; Wadsworth, Doorty et al. 1994; Wadsworth, Torelli et al. 1997). Das Kanalprotein ließ sich erfolgreich mit verschiedenen Detergentien, vor allem mit DDM, aus der Membran lösen. Mittels Ionenaustausch-Chromatographie konnten 0,1-0,2 mg/ml reines SK-Protein erhalten werden. Gelfiltrations-Chromatographie und Blau-Nativ Gelelektrophorese (BN-PAGE) zeigten, daß das reine Kanalprotein wie erwartet als Tetramer vorlag.

Auch die cDNA der α-Untereinheit des humanen BK-Kanals wurde in *Pichia pastoris* exprimiert, um eine hohe Proteinausbeute für strukturelle Untersuchungen zu erhalten. Das Vollängenprotein wurde in großer Menge produziert. Elektronenmikroskopische Aufnahmen zeigten das Protein in den Zellen in großen Vesikeln lokalisiert. Obwohl eine große Anzahl von Detergentien ausprobiert wurde, vermochte keines das BK-Protein zu lösen. Selbst durch eine Co-Expression von α- und β-Untereinheit konnte keine Verbesserung erzielt werden, und auch in diesem Falle konnte keines von verschiedenen getesteten Detergentien das Kanalprotein lösen.

Bis heute gibt es keine weiteren Berichte von gereinigtem SK2-Protein aus natürlicher oder rekombinanter Quelle. Trotz der relativ geringen Proteinausbeute ist die gelungene Reinigung des Proteins daher eine zentrale Erkenntnis für die Charakterisierung von SK-Kanälen.

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Publications

Cappello F, Bellafiore M, Palma A, Marciano V, Licata L, Cannino G, Gentile C, Zummo G, Farina F, Bucchieri F.
Protective role of the complement regulatory protein human CD-55 in cardiac xenograft: a descriptive study and a revision of the literature.
Histol Histopathol. 2002 Oct;17(4):1085-9

Cestelli A, Catania C, D'Agostino S, Di Liegro I, Licata L, Schiera G, Pitarresi GL, Savettieri G, De Caro V, Giandalia G, Giannola LI.
Functional feature of a novel model of blood brain barrier: studies on permeation of test compounds. J Control Release. 2001 Sep 11;76(1-2):139-47

Savettieri G, Di Liegro I, Catania C, Licata L, Pitarresi GL, D'Agostino S, Schiera G, De Caro V, Giandalia G, Giannola LI, Cestelli A.
Neurons and ECM regulate occludin localization in brain endothelial cells.
Neuroreport. 2000 Apr 7;11(5):1081-4

Savettieri G, Licata L, Catania C, Raneri R, Di Liegro I, Cestelli A.
Synergistic effects of laminin and thyroid hormones on neuron polarity in culture.
Neuroreport. 1999 Apr 26;10(6):1269-7