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BK Channels – Focus on Polyamines, Ethanol/Acetaldehyde and Hydrogen Sulfide (H_2S)

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1. Introduction

Calcium (Ca^{2+})-activated potassium (K^+) channels are activated by the synergistic action of voltage as well as by Ca^{2+} which links these channels to cell metabolism. Because of their high level of functional diversity the channels are widely expressed in a remarkable amount of different cells from bacteria to men and found in a great variety of tissues such as sensory, muscle, vascular or the brain. The channels are among the most frequently studied K^+ channels giving rise to an impressive amount of knowledge about their structure and function. The idea of a Ca^{2+}-activated conductance was born in 1958 during studies on erythrocytes by Gardos (1958) who showed that metabolically deprived cells in the presence of internal Ca^{2+} augment the permeability of the cell plasma membrane to K^+ ions. The finding was further elaborated by direct injection of Ca^{2+} ions into mollusc neurons (Meech & Standen 1975; Gorman & Hermann 1979) which supported the idea of a Ca^{2+}- and voltage dependent membrane K^+ conductance and showed that it is also present in excitable cells. Up to present Ca^{2+}-activated K^+ conductances were and still are studied in great detail concerning their biophysical, physiological, pathophysiological, pharmacological, structural and functional properties (for early and recent reviews see Meech 1978; Hermann & Hartung 1983; Latorre et al.. 1989; Kaczorowski et al. 1996; Gribkoff, et al. 2001; Jiang et al., 2001; Weiger et al. 2002; Calderone 2002; Jiang et al., 2002) Maher & Kuchel 2003; Salkoff et al. 2006; Pluznick & Sansom 2006; Cui et al. 2009; Wu et al. 2010; Lee & Cui 2010; Grimm & Sansom 2010; Hill et al. 2010; Berkefeld et al. 2010; Cui 2010). In the first sections of this chapter after we briefly describe techniques to record BK channels we review some properties of BK channels which appeared important in the context of our further presentations.

Ethanol is produced by the cell metabolism and is generally known as one of the most ancient and most ubiquitous psychoactive drugs consumed by humans. There are myriads of publications on the effects of alcohol on body functions, behavior, social interactions or

cancer genesis. Research progressed rapidly in the field and scientists are vividly collecting data on the effects of alcohol and we experience growing understanding on the cellular level of some processes involved, however, many of its molecular mechanisms of action still remain elusive. We will review some aspects of the effects of ethanol as well as acetaldehyde - its first metabolite – on BK channels.

Polyamines (putrescine, spermidine and spermine), are simple molecules present in all eucaryotic cells. They have a wide array of functions from modulating ion channels, involvement in apoptosis and carcinogenicity and are required in cell proliferation and development. The Ca^{2+}-activated K^+ conductance was among the first to be reported being modulated by polyamines. We will briefly review the latest development in the field and cover the molecular mechanisms on polyamine interaction with BK channels.

Hydrogen sulfide (H_2S) is the third gasotransmitter discovered in brain next to nitric oxide and carbon monoxide. While H_2S is already well known to modulate ion channels, it was only recently discovered to also modulate BK channels. In the last section of this chapter we will briefly focus on this relatively new field in BK channel physiology.

2. Technical aspects of BK channel recordings

Due to their huge conductivity of 100 - 300 pikoSiemens (pS) BK channels are easily visible and discernible from other ion channels in single channel recordings. Since BK channels are well known to be asymmetric, i.e. drugs may act from the intracellular but not from the extracellular side, it is important to investigate BK channels in the inside out as well as in the outside out patch clamp mode. Choosing a model such as Chinese hamster ovarian (CHO) cells transfected with BK channels, inside out patches will allow to record macroscopic currents instead of single channels due to the huge number of channels expressed in a patch which add up to a macroscopic current. A good model for outside out single channel recordings are in our hands the GH3/GH4 cell lines from rat pituitary tumor cells. BK channels can be recorded in two different solution settings: a) a solution system which recalls the physiological situation with 3 - 5 milliMolar (mM) KCl in the extracellular bath and 100 - 145 mM KCl at the intracellular side, or b) in a more biophysical approach where a symmetric solution system with equal amounts of potassium (100 - 150 mM KCl) at either side of the membrane is used. The latter approach has been adopted by many researchers reported in the more recent literature. Since BK channels are Ca^{2+} sensitive a great deal of attention has to be paid to the Ca^{2+} concentration in the solution facing the intracellular side. Ca^{2+} has to be buffered and the resulting so called "free Ca^{2+} concentration" needs to be carefully adjusted according to the demands of the experiment. The Ca^{2+} concentration in a Ca^{2+} buffered solution reported as free Ca^{2+} contains only a fraction of the total Ca^{2+}. Depending on the buffer used the free Ca^{2+} concentration can be calculated using an online calculator (http://www.stanford.edu/~cpatton/webmaxcS.htm). Other Ca^{2+} buffering substances like magnesium or ATP have to be taken into account in these calculations. The best practice, however, is to finally measure the free Ca^{2+} concentration in the ready to use prepared solution with a Ca^{2+} sensitive electrode. At very low intracellular Ca^{2+} concentrations (below 1 microMolar (μM) free Ca^{2+}) and to remove potential other metal ion contaminants, solutions shall be passed over a Chelex 100 (BioRad) ion exchange column, prior to adding Ca^{2+} buffers and divalent ions (Erxleben et al. 2002). Low Ca^{2+} concentrations are in a range below 1 μM, while high Ca^{2+} concentrations for the BK channels are in a range of 10 - 100 μM free Ca^{2+}, depending on the type of BK Channel used.

The free Ca^{2+} concentration employed also determines the buffer to be used. BAPTA and EGTA are the best choice for low free Ca^{2+} concentration while HEDTA would be chosen for higher Ca^{2+} concentrations (Patton et al. 2004). It is good advice not to use these buffer systems at the edge of their buffer capacity since any additional Ca^{2+}, which may result for instance in whole cell recordings from Ca^{2+} influx by activation of Ca^{2+} channels, may not be buffered anymore and hence alter BK channel activity. Also higher concentrations of the Ca^{2+} buffer substance used like 10 mM are more favourable than low concentrations to make the system more stable. In addition small mistakes in balancing the salts for the solution or a sloppy adjustment of the pH can have serious consequences for the buffer range. Therefore great care has to be taken in the preparation of solutions and a freshly calibrated pH meter may help to adjust the free Ca^{2+} concentrations precisely. Ca^{2+} buffers can be of the fast type using BAPTA or of the slow type using EGTA. Fast buffers have the advantage that any input of additional Ca^{2+} from Ca^{2+} channels or a release of internal Ca^{2+} will not be sensed by the channel. Slow buffers like EGTA may be exceeded by the fast appearance of high amounts of Ca^{2+} but keep the overall Ca^{2+} concentration constant. For more information which Ca^{2+} buffer to use and how to calculate the free Ca^{2+} concentration see (Bers et al. 2010; Patton et al. 2004).

BK channels are located frequently in clusters in the cell membrane. This makes it sometimes almost impossible to obtain a patch with just a single channel. A way to work around this and to minimize the number of channels is to decrease the orifice of the tip of the patch electrode which increases the patch pipette resistance up to 5 - 6 MegaOhm. Indication that only one channel is in the patch, which is important for instance for kinetic analysis, can be obtained by increasing the Ca^{2+} concentration in the solution or by increasing the voltage to positive values and make sure that only one channel is observed. A good starting point to record single BK channels is to use a free Ca^{2+} concentration of 1 µM at a voltage of +30 mV. Submillimolar concentrations of tetraethylammonium (TEA) may be used as a low cost drug to block BK channels in initial experiments. To further specify the channel specific BK channel blockers such as iberiotoxin or paxilline shall be used.

3. Ca^{2+} activated K^+ channels

Ca^{2+}-activated K^+ channels channels are found in a great variety of excitable and non-excitable cells. The channels are broadly divided into three subfamilies mainly defined by their biophysical and pharmacological properties (Wei et al., 2005). In this chapter we will focus on the big (large or maxi conductance) K^+ channels (BK) which are also termed $K_{Ca}1.1$ or KCNM (gene name). The channels are also known as Slo1 channels - for "Slowpoke", a gene that was first cloned from the fruit fly *Drosophila* (Atkinson et al. 1991) and has since been cloned from a variety of organisms (Adelman et al. 1992; Salkoff et al., 2006). The channels are activated usually by both metal ions (Ca^{2+}/Mg^{2+}) and by membrane voltage synergistically, but can also be activated by either Ca^{2+}/Mg^{2+} or by voltage alone. In the absence of Ca^{2+} the channels require extremely large depolarization for activation (+100 to +200 mV). Some details of BK channels which bear relevance to the following section on ethanol/acetaldehyde, polyamines and H_2S are highlighted below.

3.1 BK channel properties

BK channels have a tetrameric structure with four independent alpha (α)-subunits containing the functional channel pore. The α-subunit subunit is a large protein of about

1,200 amino acids. Each BK channel α-subunit consists of a total of seven transmembrane segments with a unique S0 segment that precedes the usually six transmembrane segments (S1-S6). The total of seven segments (S0-S6) renders the N-terminus (amino terminal) at the extracellular side of the membrane (Meera et al., 1997). Multiple splice variants of the α-subunit have been identified resulting in a great variety of channel properties in various cell types (Fodor & Aldrich, 2009). The segments S1-S6 are conserved as in other voltage-dependent K^+ channels. BK channels consist of charged voltage sensing transmembrane segments (S1-S4) where charges appear to be functionally distributed (Ma et al. 2006; Aggarwal & MacKinnon 1996; Seoh et al., 1996). The S0 segment specific to BK channels appears to be involved in movements of the voltage sensor (Liu et al., 2008), and seems to be required for functional interaction of α-subunits and the accessory β-subunits as well as for insertion of the channels into the plasma membrane (Wallner et al. 1996; Morrow et al. 2006; Liu et al., 2008).

The pore forming segments (S5-S6) of each α-subunit have an amino acid sequence at the selectivity filter (glycine-tyrosine-glycine - GYG) which is also found in many other types of K^+ channels. The carboxyl (C) terminal tail comprises about two thirds of the α-subunit protein. In this region interactions take place with various channel modulating proteins including protein kinases and phosphatases (Wei et al., 1994; Schreiber & Salkoff 1997). It further includes a negatively charged Ca^{2+} binding region, the so called Ca^{2+} bowl (Wei et al., 1994; Schreiber & Salkoff 1997; Jiang et al. 2001) and a double negative charged region which is sensitive for Mg^{2+} as well as for Ca^{2+}, the so called RCK-domain (regulatory domain of K^+ conductance). In addition the biophysical functions of BK channels can be altered by interaction with auxiliary beta (β)-subunits. Tissue specificity is in part achieved by four different types of β-subunits (β1- β4) which associate with the α-subunit. β4 for instance is primarily expressed in the brain (Weiger et al., 2000) while the others are mainly found in the periphery (Torres et al. 2007). In addition to the β-subunits so called Slo binding proteins (Slob) have been identified which bind to and modulate Slo channels (Schopperle et al., 1998). Beside the complex pattern of channel gating by voltage, Ca^{2+} and β-subunits, other modulatory factors influence BK channel activity, like pH, the redox state or phosphoryation of the channel protein. Furthermore, gasotransmitters, like nitric oxide (NO) causing nitrosylation, carbon monoxide (CO) conveying carboxylation and H_2S imparting sulfuration may modulate channel activity (Wu & Wang 2005; Leffler et al., 2006; Kemp et al., 2009; Hou et al. 2009; Félétou 2009; Hu et al., 2011).

Through alternative splicing the pore forming α-subunit contains at its C-terminus a cysteine-rich 59-amino-acid insert between RCK1 and the Ca^{2+} bowl called stress-axis regulated exon (STREX). STREX exon expression is suppressed in hypophysectomized animals, whereas STREX exon expression is initiated by the stress-axis adrenocorticotropic hormone (Xie & McCobb 1998). Patch clamp recordings revealed that STREX causes BK channels to activate at more negative potentials and enhances activation and decreases deactivation which leads to increased repetitive firing of action potentials. STREX can be artificially induced by growing cells in phenol red which causes a significant increase in channel sensitivity to inhibition by oxidation but also to Ca^{2+} (Hall & Armstrong 2000). Coassembly of STREX/β1-subunits, however, could only be stimulated with a truncated N-terminus variation present which has physiological impact of channel regulation by Ca^{2+}, oxidation, and phosphorylation. β4-subunits together with the STREX insert alter BK channel biophysical properties in unexpected ways (Petrik & Brenner 2007). Individually β4 or the STREX insert promote channel opening by slowing deactivation at high Ca^{2+}.

BK channels have the largest single-channel conductance of all K^+ channels. The ideas why the conductance of these channels may be so large despite their high selectivity for K^+ can be summarized as followed: a) a negatively charged ring structure at the inner face of the channel which by electrostatic attraction of K^+ to the entrance approximately doubles the current amplitude (Brelidze et al. 2003; Nimigean et al. 2003; Zhang et al., 2006; Carvacho et al., 2008), b) a voluminous inner cavity with an excess of negatively charged amino acids near the selectivity filter which traps K^+ and facilitates their entrance into the selectivity filter (Brelidze & Magleby 2005; Li & Aldrich 2004), and c) a ring of four negative charges at the extracellular mouth of the channel (Haug et al., 2004), which pulls K^+ from the channel. The exact mechanism by which the high conductance of these channels is accomplished is still not fully understood in particular the contribution of the later two mechanisms to channel conductance have to be tested rigorously.

The dual modulation of BK channels by membrane voltage and by intracellular Ca^{2+} makes this channel to act as a molecular integrator of electrical events at the plasma membrane and intracellular signaling via Ca^{2+}. Since Ca^{2+} is involved in a multitude of cellular signaling processes this also provides a link to cell metabolism and gene activation. BK channels are widely distributed in brain and are often concentrated in neuronal cell bodies and nerve terminals (Knaus et al., 1996; Wanner et al., 1999). They facilitate membrane repolarization during action potential discharge and this way participate in the regulation of neurotransmitter release (Gho & Ganetzky 1992; Bielefeldt & Jackson 1994). BK channels play also a major role in relaxation of smooth muscles in the bladder, penis/clitoris or lung. The activity of BK channels therefore plays an essential role in controlling action potential discharge activity, hormone secretion or vasoconstriction (Weiger et al. 2002). The outward K^+ flux conducted by the BK channel moves the membrane potential in the hyperpolarizing direction suppressing activation of other voltage-dependent channels permeable to Ca^{2+}- or sodium. This provides a negative feedback for voltage-gated Ca^{2+} channels and hence prevents the accumulation of intracellular Ca^{2+}. Such a negative feedback system was already described for endogenous discharge activity in *Aplysia* pacemaker neurons (Gorman et al. 1981; Gorman et al. 1982).

There is a vast body of evidence to show that BK channels are also modulated by a antagonistic cycle of protein kinases/phosphatases as well as by G-proteins (Toro et al. 1990; Reinhart et al., 1991; Chung et al., 1991; Wei et al., 1994; Bielefeldt & Jackson 1994; Schreiber & Salkoff 1997; Schubert & Nelson 2001; Zhou et al., 2010; Tian et al., 2004; Xia et al., 1998). Channels remain functionally associated to kinase/phosphatase and G-proteins even after isolation and reconstitution into lipid bilayer membranes. Furthermore, BK channels are directly activated by internal GTP or GTPγS (a non-hydrolysable GTP analogue) in the presence of Mg^{2+}, characteristic for a G-protein mediated mechanism (Toro et al. 1990). Modulation of channels by kinases/phosphatases is involved in physiological processes such as transmitter release, hormone secretion or muscle contraction (Levitan 1994; Schubert & Nelson 2001; Newton & Messing 2006; Dai et al. 2009). In many cases BK channels and kinases/phosphatases are arranged in "nano-domains", and are constitutively attached to the channel proteins. The kinases themselves are regulated by substrate availability (ATP, GTP, phosphoinositoldiphosphat (PIP_2), by spatial factors (closeness of kinase to the channel within the membrane, association to the channel via specific binding sites) or by hydrolysis via phosphodiesterases.

The activity of BK channels is modulated by the redox state of critical cysteine sulfhydryl groups of the channel protein or an associated regulatory protein involving free thiols and disulfides (DiChiara & Reinhart 1997; Wang et al., 1997; Gong et al., 2000; Tang et al., 2001). Cysteine residues known for their responsibility of redox modulation are usually located at the cytoplasmic side of the channel. Under reducing conditions the channel activity is augmented as shown in different cell types (DiChiara & Reinhart 1997; Gong et al., 2000; Wang et al., 1997), whereas inclusion of the STREX insert makes the channels extremely sensitive to inhibition by oxidation (Erxleben et al., 2002).

BK channel activity is also influenced by their lipid surrounding. This has been studied by insertion of the channels into artificial lipid bilayer membranes. For example the probability of channel opening (Po) was significantly greater in phosphatidylethanolamine (PE) compared to phosphatidylserine (PS) at the same Ca^{2+} concentration and voltage (Moczydlowski et al., 1985). Also bilayer thickness and specific lipids such as sphingomyelin, which cluster in micro-domains have been identified as a critical factors that modulate BK channel conductance (reviewed in Yuan et al. 2004). Beside lipids cholesterol is a major component of cell membranes in animals. BK channels are generally inhibited by accessory cholesterol in native as well as in reconstituted cell membranes by shortening mean open and extending mean closed times. Depletion of membrane cholesterol results in an increase of channel open probability (Bolotina et al., 1989; Chang et al., 1995b; Crowley et al. 2003; Lin et al., 2006; Bukiya et al., 2008).

4. BK channels - and ethanol/acetaldehyde

Ethanol ($CH3\text{-}CH_2OH$) is a product of cell metabolism and can affect all living organisms from bacteria to men where it has a multitude of effects at the cellular level. For almost a century it was generally accepted that many of the pharmacological actions of ethanol result from nonspecific interactions with cellular membranes causing a „disordering" (fluidizing) effect. This was thought to alter membrane ionic conductances based on the „lipid theory of alcohol action" by Meyer and Overton (in Lynch 2008). Later, it was found that physiological concentrations of ethanol produced rather small disordering membrane effects and Franks & Lieb (1987) pointed out that a change in temperature of less than 1°C is sufficient to mimic the effects of anesthetics on lipid bilayers. During the last decades it became clear that ethanol directly acts on proteins such as receptors and ion channels located in the plasma membrane or at intracellular signalling molecules. Experimental evidence revealed that some effects of ethanol are due to specific actions including most ligand-gated ion channels, such as glutamate-, γ-aminobutyric acid- (GABA) (Lobo & Harris 2008), dopamine- (Di Chiara & Imperato 1986), 5-hydroxytryptamine-, or acetylcholine-, opioid-, (Di Chiara et al. 1996; Herz 1997; Gianoulakis 2009), adenosine-, ATP- (Asatryan et al., 2011; Ostrovskaya et al., 2011), or TRP receptors (Benedikt et al., 2007), as well as voltage-gated ion channels, such as K^+, Na^+, and in particular Ca^{2+} channels (Gonzales & Hoffman 1991; Crews et al., 1996b; Dopico et al. 1996; Jakab et al. 1997; Horishita & Harris 2008; Dopico & Lovinger 2009; Kerschbaum & Hermann 1997). Ethanol was also found to interact with signal-transduction mechanisms including G-proteins and protein kinases (Messing et al. 1991; Lahnsteiner & Hermann 1995; Newton & Ron 2007; Martin 2010; Kelm et al. 2011).

Ca^{2+} activated K^+ channels are among those channels being directly modulated by ethanol (in Dopico et al., 1999; Brodie et al., 2007; Mulholland et al., 2009; Dopico & Lovinger 2009; Treistman & Martin 2009; Martin et al., 2010). Activation of K^+ channels drives the membrane potential in hyperpolarizing direction which led to the speculation that these channels may be involved in the sedative action of ethanol (Nicoll & Madison, 1982). However, many of the early studies on the ethanol effects used very high ethanol concentrations far above the lethal dose in humans. For instance extracellular application of 500 - 2500 mM ethanol to cat trigeminal neurons caused a short burst of action potentials which was followed by hyperpolarization. This was interpreted as an ethanol-induced Ca^{2+} inward current that activated a Ca^{2+}-dependent electrogenic K^+-pump (Baranyi & Chase 1984). Studies at more relevant pharmacological concentrations showed that 20 mM ethanol (this equals the legal blood concentration in many countries) enhances the Ca^{2+}-dependent after-hyperpolarization, but not the Ca^{2+}-independent after-hyperpolarization in rat hippocampus CA1 cells (Carlen et al., 1982). Similar findings were reported in other studies for hippocampus CA3 neurons, granule cells and cerebellar Purkinje cells (Niesen et al., 1988). Initial evidence of an increase in a Ca^{2+} activated K^+ conductance by ethanol came from experiments on identified mollusc (*Helix*) neurons (Madsen & Edeson 1990). First studies showing the involvement of Ca^{2+} activated K^+ channels as a target of ethanol were presented in parallel by Dopico et al., (1996) and by Jakab et al., (1997). Ethanol augmented BK channel activity of isolated neuro-hypophyseal synaptic nerve terminals (Dopico et al. 1996) and increased BK channel open probability of rat pituitary tumor cells (Jakab et al. 1997). The increase in channel activity was considered as a result of modification of channel gating induced by ethanol acting on the channel protein or at some signalling mediator. The reduction of neuropeptide release (vasopressin, oxytocin) by ethanol from neuro-hypophyseal terminals was explained by inhibition of voltage-dependent Ca^{2+} channels (Wang et al., 1991) and it was speculated that the decrease in circulating vasopressin levels is involved in the generation of diuresis, a frequently observed phenomenon after alcohol ingestion.

4.1 Ethanol - BK channels – and cellular signalling

Ethanol/drugs and cellular signaling is covered extensively in several reviews (McIntire 2010; Ron & Messing 2011; Newton & Messing 2006; Harris et al. 2008; Chao & Nestler 2004; Newton & Ron 2007; Hoffman & Tabakoff 1990). In GH3 pituitary tumor cells the ethanol-induced potentiation of channel activity was prevented in the presence of PKC inhibitors and phosphatase inhibitors augmented the effect whereas blockade of phospholipase C was not able to prevent BK channel activation (Jakab et al. 1997). Taken together the experiments suggested a PKC-mediated phosphorylation and stimulation of the channels. PKC involvement in acute and chronic ethanol action has been summarized by Stubbs & Slater (1999) and Brodie et al., (2007). Using transgenic mice two PKC isoenzymes have been identified that mediate opposing behavioural effects of ethanol (Newton & Ron 2007). Deletion of PKCγ produced mice with high ethanol drinking phenotype requiring a high level of ethanol to reach intoxication - maybe similar to humans at risk to acquire alcoholism. On the other hand, deletion of PKCε produced animals with a low ethanol intake which were more sensitive to acute effects of ethanol - perhaps modelling humans with a low risk of developing alcoholism. The authors conclude that drugs interfering with different PKC isoforms may be beneficial in treating alcoholism. Ethanol has also been reported in cultured hippocampal neurons to transiently elevate intracellular Ca^{2+} by a Ca^{2+}-

induced Ca^{2+} release mechanism from internal stores by involvement of PKC activation (Mironov & Hermann 1996). Concomitant Ca^{2+} elevations in the cell soma as well as in dendrites were observed which appears important considering the effects of ethanol in the modulation of synaptic BK channels (Dopico et al. 1996). Ethanol activation of PKC was mimicked by application of the actin depolymerising drugs cytochalasin B and D suggesting that in intact cells cytoskeleton rearrangements may also contribute to Ca^{2+} liberation from internal pools (Mironov & Hermann 1996). This notion of an interaction of ion channels and the actin cytoskeleton is in concert with findings of BK channels in lipid rafts where they co-localize with the actin cytoskeleton (Brainard et al., 2005). Disruption or stabilization of actin increased or decreased BK channel activity, respectively. A similar finding of an ethanol increased elevation of intracellular Ca^{2+} was reported for GH4/C1 pituitary tumor cells which appeared to result from Ca^{2+} influx as well as liberation of Ca^{2+} from internal stores (Sato et al., 1990; Jakab et al., 2006). The ethanol initiated increase of internal Ca^{2+}, therefore, may be an additional factor to the activation of BK channels. Activation of BK channels is known to also derive from stretch activation of the cell membrane (Gasull et al., 2003; Kawakubo et al., 1999). Ethanol has been found to induce cell swelling even under isoosmotic conditions evoking transmitter and hormone secretion (Jakab et al., 2006). However, BK channels were reported to be stretch activated but insensitive to cell volume changes (Grunnet et al., 2002; Hammami et al., 2009) which makes it more likely that Ca^{2+} influx induced by ethanol activates BK channels but not cell swelling.

Experiments with cloned BK channels from mouse brain (*mslo* α-subunits) expressed in oocytes suggested that auxiliary subunits were not required for the action of ethanol (Dopico et al. 1998). Ethanol reversibly increased *mslo* activity in excised patches with a potency (EC_{50} = 24 mM) similar to native channels. Using this system it was concluded that the ethanol effect is unlikely to be mediated by second-messengers or G-proteins favouring a direct interaction of ethanol with the α-subunit of BK channels. Since BK channel activation by an increase of intracellular Ca^{2+} was reduced it was hypothesized that ethanol and intracellular Ca^{2+} act as agonists (Dopico et al. 1998). In further experiments BK channels were incorporated into artificial lipid bilayers to avoid complexities as from native cell membranes such as cytoplasmic constituents or complex membrane lipid composition. Even under these minimum conditions ethanol increased the activity of BK channels with a decrease of mean closed time or increase of mean open time, whereas channel conductance was not affected (Chu et al., 1998; Crowley et al.2005).

Recently the site of ethanol action at the BK channel protein has been targeted. A single mutation of threonine to valine (T107V) in the non-conserved S0-S1 linker loop has been identified to modify bovine BK channel (*bslo*) responses to acute ethanol exposure (Liu et al., 2006). Ethanol increased *bslo* T107V channel activity caused by augmenting frequency of channel openings. In addition, incremental phosphorylation at T107 by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) progressively increased channel activity which depending on the state of phosphorylation was gradually inhibited by ethanol. Therefore, phosphorylation at T107 is considered as a "molecular dimmer switch" that via post-translational protein modification imposes tolerance to BK channels. It still remains to be seen where and how exactly ethanol impacts the channel structure to exert its effect and how tolerance is achieved. In intact cells the situation may be more complicated again since channels may be in different phosphorylated/dephorphorylated states and ethanol may

also affect intracellular signalling systems. BK channels have been found to cluster into nano-domains including α-, β-subunits with Ca^{2+}/Mg^{2+}-binding sites and attachments of slob protein(s), as well as kinases and phosphatases. Isolation of channels and insertion into lipid bilayers therefore does not preclude the possibility that other constituents of the channel also respond to ethanol or to second messenger mediated interaction.

4.2 Ethanol – and membrane lipids

Although modern studies have produced a large amount of experimental evidence that ethanol directly affects proteins the lipid theory is not obviated by those findings. Indeed the lipid environment is an important modulator of channel properties. Ethanol action on channels is influenced by the composition of the native cell membrane which may differ in different cell types. The lipid composition and changes in the lipids environment by ethanol which interacts with lipids may modulate channel activity. Prolonged exposure to ethanol alters the lipid composition of membranes (Taraschi et al., 1991). Recent studies show that the lipid environment impacts BK channel function and is involved in causing acute tolerance to ethanol. BK channels reconstituted into lipid bilayers exhibit increased open probability by ethanol similar to native channels but the baseline characteristics of the channels differed depending on the lipid composition (Chu et al., 1998). BK channel activity induced by ethanol was dependent on the size and shape of the phospholipids independent of their charges (Crowley et al. 2005). Altering the thickness of the bilayer into which BK channels from HEK cells (human embryonic kidney cells) were inserted changed the ethanol response from potentiation in thin bilayers to inhibition in thick bilayers which correlated with mean closed time of the channels (Yuan et al., 2008). As mechanism for the biphasic channel modulation was proposed that forces of lateral stress within the lipid bilayer combine with hydrophobic mismatch to the channel gating spring structure (Yuan et al., 2007). It appears conceivable therefore that molecules such as cholesterol or alcohol inserted into the membrane bilayer may change its thickness and affect gating of BK channels. In fact elevation of membrane cholesterol decreased channel open probability (Bregestovski et al. 1989; Bolotina et al., 1989) and antagonized the potentiating effect of ethanol on BK channels (Crowley et al. 2003). Depletion of cholesterol resulted in activation of BK channels, an increase of BK current density and reduced firing of action potentials (Lam et al., 2004; Lin et al., 2006). Furthermore, the effect of ethanol as well as cholesterol was greatly reduced in the absence of phosphatidylserine in the bilayer membrane stressing the complexity of lipid impact on BK channel activity. This is of special interest since brain cholesterol in mice (Chin et al. 1978) or cerebellar granula cells is elevated after exposure to alcohol (Omodeo-Salé et al., 1995). Ethanol also reduced the asymmetric distribution of cholesterol between the cytofacial (higher cholesterol) and exofacial leaflet of the lipid bilayer (Wood et al., 1990). Cholesterol by itself concentration dependently moved BK channels into the closed state (Chang et al., 1995a) and hence appears to override the augmenting effect of ethanol. Furthermore, basal channel activity and its potentiation by ethanol in bilayers containing phosphatidylcholine are not as forceful as in those containing phosphatidylserine (PS). In natural membranes PS is abundant in the inner leaflet of the cell membrane and serves as an anchor for membrane-associated signalling molecules that regulate ion channel activity. PS is involved in Ca^{2+}-dependent PKC translocation to the cell membrane being a well-known modulator for both basal BK channel activity (Schubert & Nelson, 2001) as well as for ethanol potentiation of BK channels (Jakab et al., 1997). It is conceivable therefore that the

presence of PS in cell membranes is specifically required for ethanol to modulate BK channel function given the links that exist between this phospholipid and signalling molecules (Crowley et al., 2005).

4.3 BK channels – ethanol and behaviour

BK channels play a pivotal role in behavioural responses to ethanol. Ethanol applied to the nematode *Caenorhabditis elegans* at human intoxicating concentrations dose-dependently and reversibly cause impairment of locomotion and egg-laying (Davies et al., 2003). Using BK channel knock outs the *slo-1* mutants were highly resistant to ethanol in behavioural assays. Behaviour of *slo-1* gain-of-function mutants again resembled those of ethanol-intoxicated animals as they show behavioural responses like in-coordination and a loss of social inhibition. Selective expression revealed that only *sol-1* in neurons but not in muscle rescued ethanol sensitivity. Investigation of excised BK channels showed that channel open probability was increased by ethanol as shown in previous single BK channel studies (Dopico et al. 1996; Jakab et al. 1997). In a molecular model for ethanol intoxication increased BK channel activity increases action potential repolarization and/or causes membrane hyperpolarization which shuts down Ca^{2+} channels and reduces transmitter release at synaptic terminals (Crowder, 2004). The experiments clearly demonstrate that mutation of a single gene affects ethanol sensitivity, although this is most probably not the only mechanism involved and it remains interesting to further monitor extensions of these findings to higher animals or to humans. Martin, et al. (2008) recently examined the generation of action potentials in brain spiny neurons using whole cell patch clamp recordings. They found that the number of action potentials evoked by current injection was increased in β4-subunit knockout mice compared to wild type under the influence of ethanol. However, the role of BK channels on the membrane resting potential was not investigated.

4.4 BK channels – and ethanol tolerance

Tolerance is generally defined as reduction or loss of response to a drug over time or after repeated exposure which may involve ion channels, receptors and/or gene expression (Chandler et al. 1998; Chao & Nestler 2004; Atkinson 2009; Treistman & Martin 2009). Tolerance in the nervous system is associated with down-regulation of excitatory receptors, such as NMDA-, nicotinic acetylcholine receptors or voltage dependent Ca^{2+} channels. It is also accompanied with up-regulation of inhibitory channels such as $GABA_A$, glycine or serotonin receptors (Harris et al.2008). Different types of tolerance may be categorized into: a) **acute tolerance** – which is a time-dependent type of tolerance that occurs during drug exposure in a time frame of seconds to minutes, b) **rapid tolerance** – occurs after a single usually high dosage drug experience, and c) **chronic tolerance**, which takes place after prolonged, repeated, identical, low dose drug exposures in a time frame of hours, days or weeks (Berger et al. 2004; Treistman & Martin 2009; McIntire 2010; Cowmeadow et al. 2005). Eventually drug tolerance may lead to increased consumption and addiction defined as compulsive drug-seeking and drug-taking behaviour (Chao & Nestler 2004).

In the early studies using excised single BK channel recordings from GH3 cells it was found that the potentiating effect after ethanol exposure rapidly declined. Within minutes both, mean open time and open probability of channels returned to control values (Jakab et al. 1997). In contrast, BK channel activity from synaptic terminals after application of ethanol

remained elevated over minutes (Dopico et al. 1996). BK channels of rat hypothalamic-neurohypophysial terminals also become rapidly tolerant to ethanol including two components: decreased ethanol potentiation (short term within minutes) and decreased channel density (long term >24 hours) (Pietrzykowski et al., 2004). These two types of tolerance appear to reflect different mechanisms: a) decreased BK potentiation by ethanol and, b) down-regulation of BK channels and reduction of channel clustering associated with internalization of channels as suggested from immunolabeling. In the *Drosophila* nervous system a null mutation of the slowpoke gene completely eliminated rapid tolerance to ethanol (Cowmeadow et al. 2005). Ethanol increased slowpoke expression in the nervous system coincident with the induction of ethanol tolerance (Cowmeadow et al., 2006). Since an increase of slowpoke expression is also caused by cold, by CO_2 sedation (Ghezzi et al., 2010) or by heat-shock promoters (Cowmeadow et al., 2006) it was suggested that this is a more common mechanism for acquisition of tolerance. Interestingly the *Drosophila* slowpoke gene appears to contain a binding site for CREB (cyclic-AMP response element binding protein) which has been implicated in learning and memory and hence may also be involved in the ethanol response (Cowmeadow et al., 2006) and possibly in the memory deficits after excessive alcohol intake. Further experimentation into the molecular mechanism of tolerance using single channel recoding revealed that only after expression of the somatic BK α-subunit together with the brain specific β4-subunit ethanol dose-dependently increased the open probability of channels and decreased the duration of action potentials whereas BK α-subunit together with the ß1-subunit expressed in dendrites was insensitive to ethanol (Martin et al., 2004; Martin et al., 2008)

Human BK channels (*hslo*) are also potentiated by alcohol being dependent on the presence of auxiliary β-subunits (Feinberg-Zadek & Treistman 2007). BK channel activity containing only the α-subunit were substantially increased by ethanol, together with the β4-subunit the channel mean open time was also increased but to a lesser extent and channel activity was unaffected in the presence of β1-subunit. After prolonged ethanol exposure (24 h) down regulation of the BK current containing only *hslo* or *hslo*+β4 was observed - but not with β1 (Feinberg-Zadek, et al. 2008). Moreover, neuronal BK channels from wild-type mice expressing α- and β4-subunits show little tolerance whereas BK channels from β4 knockout (KO) mice also exhibit acute tolerance to ethanol. Studies at the behavioural level revealed that β4-KO mice drink more compared to wild-type companions (Martin et al., 2008). The authors point out that because subunit expression - in particular β4 - differs between many cells types, i.e. in neurons and even in neuronal compartments this could determine variations in individual alcohol responses such as tolerance which may lead to abuse and alcoholism.

Ethanol, via an epigenetic mechanism involving microRNA, induces alternative splicing and mediates rapid reorganization of BK α-isoforms (Pietrzykowski et al., 2008). This leads to destruction of a subset of BK α-subunits but persistence of ethanol-insensitive, mainly STREX BK channels. Acute molecular tolerance to ethanol was found to be a function of exposure time and once initiated tolerance persists in the absence of the drug (Velázquez-Marrero et al., 2011). During prolonged ethanol exposure (6 hours, but not at 1 or 3 hours) mRNA levels of the ethanol-insensitive STREX isoform were increased and transition to the biophysical properties of BK-STREX channels occurred.

Chronic tolerance to alcohol is observed in rats that have been maintained on an ethanol-containing diet for 3 to 4 weeks (Knott et al., 2002). On the cellular level it was found that

long-term ethanol exposure leads to a compensatory change in the expression of two channels acting as functional dyads: L-type Ca^{2+} channels current density increased, whereas BK current decreased but BK channels also became less sensitive to ethanol.

Ethanol and other drugs such as benzyl alcohol, a common sedative, induces neural expression of the *slo* gene and the production of rapid tolerance (Cowmeadow et al. 2005; Ghezzi et al., 2004). The drugs increased expression of the *slo* gene, enhanced neuronal excitation by reducing the refractory period between action potentials and augmented seizure susceptibility (Ghezzi et al., 2010). Mutant BK channels exhibiting increased activity were found in humans to cause increased excitability due to rapid repolarization of action potentials (Du et al., 2005). This condition can lead to epilepsy and paroxysmal movement disorders and alcohol appears to be responsible for initiation of dyskinesia in these individuals. The molecular pathway that mediates the upregulation of *slo* transcription in *Drosophila* using benzyl alcohol has been linked to a CREB transcription factor. Down regulation of a CREB repressor isoform releases other CREB activator isoforms which after phosphorylation bind to CRE (cyclic AMP response element) within the *slo* promoter region and induces acetylation of histones (Wang et al., 2007). This eventually stimulates specific promoters to increase the expression of BK channels. Increased BK availability is suggested to enhance neural discharge activity by shorting action potentials. Reduced Ca^{2+} influx via voltage activated channels gives rise to sedation and development of rapid tolerance (Wang et al., 2009). If this mechanism also applies to ethanol remains to be investigated. Tolerance to alcohols may also include changes in membrane lipid composition (Yuan et al., 2007).

4.5 Ethanol – blocks BK channels

Although in most cases ethanol is found to increase BK channels activity it has also been reported to act as suppressant. Rat aortic myocyte BK channels expressed in *Xenopus* oocytes are in majority inhibited by 30 - 200 mM ethanol. Coexpression of the ß1-subunit together with the α-subunit in this tissue failed to influence ethanol action on *bslo* channels. The inhibition of BK channels in rat aortic myocytes may contribute to the direct contraction of aortic smooth muscle produced by acute alcohol exposure (Dopico, 2003). In supraoptic neuronal cell bodies ethanol failed to increase BK channel activity but increased nerve terminal BK channels (Dopico et al., 1999). Moreover, BK channels from vascular tissue are also blocked by ethanol (Walters et al. 2000; Liu et al., 2003). The reason for this difference is not clear but may include expression of different channel isoforms, different auxiliary proteins (β-subunits) or different lipid composition around the channels.

4.6 Ethanol – and transmitter/hormone secretion

Ethanol influences the duration of action potentials by facilitating their repolarization and their after-hyperpolarization (Gruss et al., 2001). This negative feedback on cell excitation closes Ca^{2+} channels, shortens the duration of Ca^{2+} entering the cells and decreases the Ca^{2+} triggered release of hormones or neurotransmitters (reviewed in Dopico et al., 1999).Ethanol also directly acts on Ca^{2+} channels. At low concentrations (10 mM - ca. 0.5 per mille) ethanol has been found to reduce vasopressin release from nerve terminals isolated from rat neurohypophysis by inhibition of the Ca^{2+} current which explains the reduction in plasma vasopression levels (Wang et al., 1991). In hippocampal CA1 neurons ethanol at extremely low concentration (0.01 per mille) enhanced, but at higher concentrations (5 per mille)

decreased, synaptic transmission by activation of a G-protein/protein kinase C signalling pathway (Lahnsteiner & Hermann 1995). Voltage dependent Ca^{2+} currents were also suppressed by ethanol in invertebrate preparations (Camacho-Nasi & Treistman 1987; Oyama et al. 1986) by activation of a G-protein/protein kinase transduction pathway resulting in decreased action potential duration (Kerschbaum & Hermann 1997).

Despite the wealth of knowledge about alcohol interaction with receptors, ion channels, enzymes and signaling molecules questions about its main target(s) and its binding site(s) at these proteins still remain. It is thought that the most likely target sites of ethanol are amphipathic pockets in membrane proteins like K^+ channels of the inward rectifier type (Harris, et al. 2008; Howard, et al. 2011). Alcohol binding sites have been identified in the crystal structure of "alcohol dehydrogenase (ADH)" (Ramaswamy et al., 1996; Rosell et al., 2003) and for LUSH, an odorant binding protein from *Drosophila* (Kruse et al., 2003). This may help to develop further ideas on how the ethanol binding site may look like in other proteins. However, little is known if ethanol directly binds to these proteins or if accessory ethanol-binding proteins that target the functional protein are effective. Furthermore, it remains to be determined to which extent and how ethanol interferes with the lipid phase of the membrane or the lipid-protein interaction.

5. BK channels – and acetaldehyde

Acetaldehyde (ACA) is the primary metabolite of ethanol oxidation and in numerous studies a role for it in the action of ethanol on the brain has been proposed. Indeed evidence is accumulating that ACA is responsible for some of the effects that so far have been attributed to ethanol (reviewed in Hunt 1996; Quertemont et al. 2005; Correa et al., 2011). On basis that ACA has been generally considered as an aversive, treatment for alcoholics with disulfiram (Antabus, an inhibitor of ACA metabolism) has been established and used clinically. However, it was also noticed that ACA has central reinforcing effects (Melis et al., 2007; Quertemont & Tambour 2004; Rodd-Henricks et al., 2002; Quertemont & De Witte 2001). The metabolism and regulation of ACA particularly in blood or liver occurs via activities of alcohol dehydrogenase (ADH), cyctochrome P450, catalase and aldehyde dehydrogenase. The blood concentrations of ACA after ethanol consumption was found extremely low (<0.5 μM) (Eriksson & Fukunaga 1993; Eriksson 2007) and together with the activity of the blood-brain barrier it appeared unlikely to penetrate the brain in any pharmacological relevant amounts. However, ACA can be produced within the brain from ethanol through catalase and/or cytochrome P-4502E1 which makes it more likely that biologically significant concentrations at least in some brain areas can be achieved (Karahanian et al., 2011; Correa et al., 2011; Deng & Deitrich 2008; Quertemont et al. 2005). There is also evidence that ACA may mediate tolerance and dependence. Nevertheless, the actual ACA concentrations in the brain after ethanol consumption and its rapid oxidation remain to be determined. Most clear cut studies on the modulation of neurotransmission by acetaldehyde/alcohol have been performed on the dopaminergic system (reviewed in Correa et al., 2011). ACA appears to modulate dopaminergic function particularly in the mesolimbic pathway which indicates relevance to motivational behaviour. Studies of the action of ACA on the cellular level, on single channels or on electrical activity are scarce. In smooth muscle cells it was reported that ACA inhibits voltage-dependent Ca^{2+} currents (Morales et al., 1997). Furthermore, in vitro ACA was found to enhance firing of action

potentials of dopaminergic neurons in the ventral tegmental area by reduction of the A-type K^+ current and activation of a hyperpolarization-activated inward current (Melis et al., 2007). The stimulating properties were prevented by blockade of local catalase.

In our laboratory we have investigated some of the effects of ACA on single BK channels from GH cells (Handlechner et al., 2008; Handlechner et al., 2011). Given the fact that the simultaneous presence of ACA and ethanol reflects the physiological situation in the brain after alcohol consumption we assumed that both molecules may either act synergistically or antagonistically. Hence we started to investigate the BK channel response to ethanol in the presence of ACA. Extracellular ethanol increased BK channel open probability as reported previously (Jakab et al. 1997). In the presence of intracellular ACA the ethanol related increment of BK channel activity was inhibited in a dose dependent manner. BK channel amplitudes were not affected but mean channel open time and open probability were significantly reduced. In contrast, extracellular ACA had no effect on ethanol induced channel activity. Our results reveal that ACA interferes with BK channel activity blunting the effect of ethanol. The action of ACA on the channel can be considered as direct and not through some metabolic product or adduct, activation of transmitters/hormones or gene expression since we use cell free recordings, ACA is always in excess and the effect is acute.

Our findings may have consequences for the pharmacological/toxicological effects of ACA/ethanol on the electrical activity of cells, on nervous function and animal behaviour. From our findings we may speculate that ACA counteracts the effect of ethanol and may potentiate tolerance to ethanol. In any case, in the context of ethanol actions ACA effects have to be considered carefully. Further investigation shall be concerned with the dependence of the ACA-mediated effect at variable concentrations of free internal Ca^{2+}, possible ACA interference with intracellular signaling cascades, i.e. the phosphorylation or redox state of the BK channels or interference with the brain specific β4 subunit in the action of EtOH/ACA on BK channel properties.

6. BK channels – modulation by hydrogen sulfide (H_2S)

H_2S is a colorless gas and well know because of its peculiar odor of rotten eggs. It also is an extremely toxic gas and inhaled in higher concentrations causes coma and eventually death (Reiffenstein et al. 1992; Beauchamp et al., 1984). H_2S is produced endogenously in many living cells from the amino acid L-cysteine. Three synthetic pathways in various organs have been described such as in vascular system, liver, kidneys and the brain (Shibuya et al., 2009; Ishigami et al., 2009; Stipanuk & Beck 1982; Łowicka & Bełtowski 2007). After its generation H_2S diffuses either immediately in the surrounding milieu or is bound to and stored in proteins until it is released by an adequate stimulus. H_2S – similar to the other gasotransmitters NO or CO – is water and lipid soluble and therefore also easily passes membranes. The physiology, pathophysiology, pharmacology of H_2S particularly in the vascular system and brain has been reviewed in an impressive amount of recent publications (Wang 2011; Kimura 2011; Hu et al., 2011; Bucci & Cirino 2011; Wang 2010; Tan et al. 2010; Gadalla & Snyder 2010; Mustafa et al., 2009; Mancardi et al., 2009; Qu et al., 2008; Li & Moore 2008; Li et al., 2011; Łowicka & Bełtowski 2007; Szabó 2007; Wallace 2007; Wallace 2010; Lloyd 2006; Wang 2002; Boehning and Snyder 2003; Caliendo et al., 2010).

Besides many other cellular targets H_2S also acts on ion channels. In neurons an increase of the cytosolic Ca^{2+}-concentration by H_2S appears to be caused by activation of Ca^{2+} entry

through L-type Ca^{2+}-channels (García-Bereguiaín et al., 2008). Modulation of pain processing by H_2S appears to involve activation of T-type Ca^{2+} channels responsible for its pro-nociceptive effect, whereas analgesia is due to activation of K_{ATP} channels (Distrutti 2011). In peripheral tissue, however, H_2S reduces T-type Ca^{2+} channel activity leading to hyperalgesia (Kawabata et al., 2007). T-type calcium channels are also involved in pain processing of spinal nociceptive neurons (Maeda et al., 2009), in colon (Matsunami et al., 2009) and in pancreas (Nishimura et al., 2009). H_2S decreased the mechanical contraction of rat cardiomyocytes through inhibition of L-type calcium channels (Sun et al., 2008). One of the most well-known actions of H_2S is the activation of ATP-sensitive K^+ channels by which H_2S causes vasorelaxation (Zhao & Wang 2002; Tang et al., 2005; Zhao et al., 2001; Jiang et al., 2010; Liang et al., 2011; Liu et al., 2011), inhibits insulin secretion (Yang et al., 2005; Wu et al., 2009), or protects primary cortical neurons from oxidative stress (Kimura & Kimura 2004). However, the universal applicability of a K_{ATP} dependent action has been questioned (Kubo et al., 2007; Szabó 2007). In the gastrointestinal tract (human jejunum smooth muscle) H_2S activates sodium channels in a partially redox dependent manner (Strege et al., 2011). In contrast to other gasotransmittes H_2S appears not to act on the intracellular signaling pathway guanylyl cyclase (Garthwaite 2010). The interaction of H_2S with ion channels has been reviewed by Tang et al. (2010).

We choose GH3 cells since they are widely used as model cells to investigate BK channel activity in natural settings (Sitdikova et al. 2010). Sodium hydrosulfide (NaHS) was used as H_2S donor since it can be readily handled and quantified. Our experiments showed that H_2S dose-dependently increased single channel open probability (P_{open}) (Sitdikova et al. 2010). In our cell free, single channel recordings where Ca^{2+} is kept constant the increase of BK channel activity indicates that H_2S does not act via elevation of the Ca^{2+} concentration. The fast onset of the H_2S effect after application within seconds, but also the rapid decrease after washout of the drug, further suggests a direct effect at the channel protein. A half maximal effective concentration of 90 µM NaHS indicates that H_2S induces BK channel activation in a physiological relevant concentration range. To study the effect of H_2S on BK channel sensitivity to intracellular Ca^{2+} we used a range of Ca^{2+} concentrations at a constant membrane potential. The experiments show that there was no difference in H_2S effects on BK channel activity at different cytoplasmic Ca^{2+} concentrations. Hence H_2S appears not to interfere at the Ca^{2+}binding sites of the channel. Also ß4 subunits appear to be an unlikely target of our BK channels since iberiotoxin rapidly blocked the current indicating that BK channels in GH3 cells are not accompanied by ß4-subunits.

Redox modification is among the recognized mechanisms for cellular effects of H_2S including NMDA receptors (Kimura & Kimura 2004; Kabil & Banerjee 2010), K_{ATP} channels Zhao et al., 2001; Yang et al., 2005) or T-type Ca^{2+}-channels (Kawabata et al., 2007). We hypothesized that the increase of BK channel P_{open} may be mediated by redox modulation of cysteine residues. In our experiments the effect of NaHS was prevented when the reducing agent DTT was applied to the pipette solution accessing the cytoplasmic side of the channel. If channels were in the oxidized state by application of thimerosal, P_{open} was further increased by NaHS compared to the already increased thimerosal control.

In contrast to our findings a recent report indicates that BK channels expressed in HEK293 cells were inhibited by H_2S and activated by CO (Telezhkin et al., 2009; Telezhkin et al., 2010). In carotid body chemoreceptors, which are important to maintain oxygen homeostasis

by regulating ventilation, H_2S caused an excitation of these cells by blocking BK channels which appear to play a crucial role in oxygen sensing (Li et al., 2010). In other preparations, however, H_2S causes dilatation and hyperpolarization of vascular smooth muscle (Jackson-Weaver et al., 2011) and activates BK channels in cultured endothelial cell (Zuidema et al., 2010). These differences in the response to H_2S are unclear but might be due to different tissues containing different BK channel splice variants or may be due to a different phosphorylation or redox state of the channels.

BK channels mediate or modulate many physiological functions as well as patho-physiological conditions. Future studies will have to show how H_2S or H_2S related substances may be involved and may contribute to those conditions. Techniques to determine H_2S even at low concentrations (in the micro- to nanomolar range) in biological preparations which are available now will help to facilitate the investigation of H_2S in biology and medicine (Doeller et al., 2005; Peng et al., 2011). In pharmacology the development of new drugs modulating H_2S signaling might be rewarding in the treatment of diseases like high blood pressure, pain therapy or erectile dysfunction.

7. BK channels – and polyamines

The polyamines putrescine, spermidine and spermine are hydrocarbon molecules with two, three or four positively charged amino groups under physiological conditions. Polyamines are metabolized from the decarboxylation products of ornithine and S-adenosyl-methionine in nearly all eukaryotic cells. They are multifunctional molecules which are inevitable for development or cell proliferation and modulate a number of cellular targets, like DNA, RNA or signaling proteins, but are also involved in pathological mechanisms, like cancer (Igarashi and Kashiwagi 2010; Bachrach, 2005). In addition to the above mentioned functions polyamines play a major role in modulating a number of ion channels. In the potassium channel family they act as modulators of the inward rectifiers K_{ir}, the BK, the TASK (two-pore-domain potassium channels), the KCNQ and the delayed rectifier channels (reviewed in Weiger & Hermann 2009). Furthermore, AMPA and NMDA receptors as well as Ca^{2+} and sodium channels are modulated by polyamines (Huang & Moczydlowski 2001; Williams, 1997). The ideas to test polyamines on ion channels was initially reported using mollusk neurons (Drouin & Hermann 1994; Drouin & Hermann 1990) and pituitary tumor GH cells (Weiger & Hermann 1994). Drouin & Hermann described a blocking action of polyamines on BK currents using whole cell two electrode voltage clamp experiments in *Aplysia californica* neurons on a K^+ channel which is pharmacologically similar to BK channels. They found spermine injected into the cell to have a dual action: immediately after injection the Ca^{2+} activated current was blocked, whereas after a prolonged time the current was increased. As explanation for these phenomena it was suggested that after prolonged Ca^{2+} injection the Ca^{2+} buffer capacity of the cells was exhausted or/and during the time course of the experiments the channels became more sensitive to Ca^{2+} which overcame the blocking effect caused by spermine. When they applied spermine in high concentrations up to 10 mM to the extracellular side of the cells they observed no or only a minor reduction (10%) of the current after prolonged application (10-15 min). The interpretation given for this result was that spermine possibly entered the cells by a polyamine transporter and acted at the intracellular face of the channels. To overcome the limitations of whole cells experiments Weiger & Hermann (1994) used a cell free patch system investigating single BK channel

activity. They confirmed the blocking action of polyamines which acted in a voltage depended manner on the channel when applied to the intracellular face of the membrane but had no effect when applied extracellularly. The effect of polyamines on BK channel was dual: firstly, by a so called fast blocking mechanism the current amplitude was apparently reduced (caused by limitations of the recording system) and secondly, the open probability of the channel was decreased. The order of effectiveness of the various polyamines tested was: spermine > spermidine> putrescine. At high Ca^{2+} concentrations applied to the intracellular side polyamines were ineffective on single channel kinetics while the reduction of the amplitude remained. The stoichiometry of the channel block by spermine was 1:1, the reduction of the open probability had a 2:1 relationship. These data were in agreement with the whole cell recordings in *Aplysia* and suggested two interactions sites of BK channels with polyamines: namely the channel pore where the polyamine does not bind firmly but rather slips in and out at high frequency (flickery block, causing the reduced amplitude) as well as the Ca^{2+} sensor of the BK channel. The question why polyamines are not effective when applied to the outside the channel was probed with a series of diamines which differed in length up to 1,12 diaminododecane (Weiger et al. 1998). Diamine molecules are similar to polyamines in carrying a positively charged amino group at each end which is separated by a variable length CH-chain. Only 1,12-diaminododecane was found to act as a blocker from the extracellular face of the channel while diamines with a shorter chain length were ineffective. In silico molecular modeling revealed that 1, 12-diaminododecane and spermine although they have the same length the latter is more flexible and is completely hydrated. 1,12-diaminodocane has only small water caps at its ends, positioned over the charged amino groups separated by a long hydrophobic segment. It was hypothesized that spermine, putrescine or spermidine as well as the shorter diamines are not able to block the channel from the extracellular side due to energetic reasons which prevents to strip of the water shell in order to interact with the channel pore.

BK channels of rabbit pulmonary smooth muscle in contrast to other cells exhibit strong rectification (Snetkov et al., 1996). This was attributed to the presence of spermine and spermidine but not putrescine in the cytoplasm. Blocking polyamine synthesis with the ornithine decarboxylase inhibitor DFMO (difluoromethylornithine) released BK channel rectification supporting the notion of a rectifying action imposed by polyamines. Similar data were reported for BK channels in myocytes from the saphenous branch of the rat femoral artery (Catacuzzeno et al., 2000). These discoveries remind to the mechanism of current rectification caused by polyamines at inward rectifier channels (K_{ir}) (Fakler et al., 1995).

A more detailed molecular explanation of how polyamines block BK channels was presented by Zhang et al., 2006. They found the ring of 8 negative charges at the inner channel mouth to be responsible for the attraction of polyamines to the channel pore. Mutation of these charges to neutral amino acids reduced the blocking effect of polyamines 90-fold and reduced rectification. In another experiment they removed the polyamine block by a simple competition of positive charges at the negative ring at the channel entrance by applying 3 M KCl. Thus under physiological condition polyamines are attracted to channel by the ring of negative charges as well as the negative charges in the channel's pore driving them into the ion conduction pathway to block the channel when positive voltage is applied.

A study in humans suggests that BK channel block by polyamines may be a reason for the development of the overactive bladder syndrome (Li et al., 2009). In people with the syndrome high levels of polyamines were found in biopsies of the urothelium in parallel with a reduced or blocked BK channel activity. By preventing polyamine synthesis in these cells in vitro, BK channel activity could be restored to normal. This result opens a new window of opportunity for a possible future treatment of the disease.

While the majority of reports indicate a block of BK channels by polyamines, they were found to be ineffective in blocking the channel in retinal Müller glia cells (Biedermann et al., 1998). This result may be explained by the rather low concentration of polyamines used in these experiments or by a different, less sensitive splice variant of the channel being expressed in these cells. In summary polyamines appear to modulate BK channels by interacting with the channel pore from the inside of the cell membrane while they are not effective from the outside. They may either cause a block or rectification of the BK current.

8. Synopsis

BK channels are important integrators of cellular signals and hence are involved in a huge diversity of cellular actions and serve in initiating many cellular pathways. Here we summarized the action of ethanol/acetaldehyde, polyamines and hydrogen sulfide on BK channels – only a few of many modulators. Interestingly all these agents appear to interfere with quite different targets at the channel indicating its enormous plasticity. Although there is a vast array of input sites which modulate the channels its output is rather simple - once activated it hyperpolarizes the membrane potential. Since these channels use a combined mechanism of activation by voltage and intracellular Ca^{2+} concentration any of these signals and their minute manipulation by external factors is integrated by the channels imposing far-reaching effects for physiology, pathophysiology or pharmacology. These features makes BK channels so unique and warrants further interesting research in the future to discover even more interactions of this channel with its environment and its further modulatory action on the biology of cells.

9. Abbreviations

ACA = acetaldehyde; BK = maxi calcium-activated potassium channel; H_2S = hydrogen sulfide; EtOH = ethanol; STREX = stress-axis regulated exon; PS = phosphatiylserine; CREB = cyclic AMP response element-binding protein; P_{open} = open probability

10. References

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