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Functional characterisation of arteries and urinary bladder from mice with genetic ablation of the large conductance Ca^{2+} and voltage activated K^{+} (BK) channel

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Für Lavinia in Liebe

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1. Introduction

1.1. Large conductance Ca^{2+} and voltage activated K^+ channels

1.1.1. Ion channels, K^+ channels and Ca^{2+}-activated K^+ channels

Ion channels are ubiquitous specialized membrane proteins that form hydrophilic pores, through which ions move down their electrochemical gradients across the cellular membrane. The current carried by ions flowing through plasma membrane ion channels determines fundamental physiological phenomena. By channel gating, ion channels, which are dynamic proteins, can switch rapidly between their "open" and "closed" states, for allowing or, respectively, not allowing ion flow. There is an equilibrium between these different conformational states that determines the amount of current that flows across the membrane as a function of time. This equilibrium can be influenced by different factors, as the membrane voltage, the binding of extracellular neurotransmitters or intracellular messengers to the channel protein, or the covalent modification of the channel by protein phosphorylation (Hille, 2001).

Potassium channels were probably the first ion channels to evolve, most likely to participate (at least originally) in osmoregulation and cell volume control (Hille, 2001). They developed into a highly diverse and ubiquitous family of proteins. By selectively allowing K^+ ions to move across the cell membrane, they regulates multiple cellular functions, such as neuronal excitability, neurotransmitter release, hormone secretion, heart activity and smooth muscle tone (Weiger et al., 2002). In the last decade more then 200 genes encoding potassium channels have been described, in organisms ranging from bacteria to humans (with over 70 genes only in the human genome) (Calderone, 2002). One family, the Ca^{2+}-activated K^+ channels, open in response to increases in intracellular concentration of free Ca^{2+} ions. These channels are further subdivided into two principal groups: (i) the “BK” (big K^+) channels, characterized by an exceptionally large single channel conductance of 100-300 pS, and a unique dependence on both Ca^{2+} and voltage for activation; and (ii) the SK and IK channels which have a small (2-25pS) or intermediate (25-100pS)

conductance and essentially no voltage-dependence (Kohler et al., 1996; Ishii et al, 1997).

1.1.2. BK channels

Among the Ca^{2+}-activated K^+ channels BK channels are probably the most studied ones. A BK channel consists of a heterooctameric complex with four α-subunits and four β-subunits (Fig.1) (Orio et al., 1998). BK channels are widely expressed in both excitable and non-excitable cells. Electrophysiological experiments have shown that BK channels are particularly abundant in smooth muscles, where they are thought to set the pace of contractile activity. Although they are expressed to a lesser extent in neurons, it is thought that they play important roles in the regulation of transmitter release and spike shaping (Toro et al., 1998). At the mRNA level, both α- and β-subunits coexist in most tissues, although in brain the level of β-subunit mRNA is much lower than the level of α-subunit. In smooth muscles, both α- and β-subunit are equally expressed (Vogalis et al., 1996).

The pore forming α-subunits were first cloned from the fruit fly Drosophila (Atkinson et al., 1991). A Drosophila mutant called Slowpoke, whose behavioural phenotype is reflected in its name, was found to be defective in Ca^{2+} activated K^+ currents in nerve and muscle (Weiger et al., 2002). Therefore, the BK channels have been referred to as “Slo”. Recently, however, two other genes, Slo2 and Slo3, were shown to encode structurally similar channels (Schreiber et al., 1998; Yuan et al., 2000), determining the rename of Slo into Slo1. Nevertheless, the expression of Slo2 and Slo3 resulted in channels with different biophysical and pharmacological properties as the BK channel (Calderone, 2002). The BK channel α-subunit is encoded by only a single 250 kb gene, localized at 10q22.3 in the humane genome (Weiger et al., 2002). Initially, this was surprising because BK channels, which are ubiquitously distributed in mammalian organs, except in heart myocytes, liver cells and fibroblasts, have different properties within and among tissues, regarding mainly the Ca^{2+} sensitivity and macroscopic kinetics (Toro et al., 1998). The identification of multiple splice variants of the same gene and the interaction with different tissue-specific regulatory β-subunits explained the diversity of BK channels properties (Orio et al., 2002). Mammalian BK channel α-subunits have almost identical amino acid sequences among different species. The striking sequence conservation of the α-subunit may reflect a high evolutionary pressure to maintain an optimized function in

mammals (Toro et al., 1998). The primary sequence of the BK channels showed that they resemble the voltage-gated K^+ channel (K_v) superfamily (Jan & Jan, 1997).

Four different regulatory β-subunits are described. The β_1 subunit is coexpressed with the α subunit in smooth muscle cells, β_2 subunit is localized preferentially in chromaffin cells and brain, β_3 subunit was detected in testis, pancreas and spleen, and the β_4 subunit is expressed mainly in brain (Orio et al., 2002).

Topology of BK channel

The pore-forming α-subunits of the Slowpoke family of BK channels are large proteins (~1,200 amino acids) that resemble other voltage-dependent potassium channels in having six membrane-spanning domains (S1-S6), with a pore forming region between S5 and S6 (Weiger et al., 2002). The primary sequence of S1-S6 is homologous to the corresponding regions in K_v channels. In contrast to other members of the K_v superfamily, the BK channel has a seventh transmembrane segment (S0) that places the amino terminal outside the plasma membrane (Fig.1). This region is a critical determinant of β-subunit modulation (Wallner et al., 1996). As other voltage gated channels, the BK contains three critical charges at the S4 region that largely account for the voltage sensor gating current (Toro et al., 1998). Most notable is the extended carboxyl terminal tail domain (Fig.1), which is much larger than that of the K_v channels. The tail consists of four (S7-S10) intracellular hydrophobic domains and comprises about two-thirds of the α-subunit protein sequence. It includes a negatively charged region (the so-called Ca^{2+} bowl) that has been implicated in Ca^{2+} binding, and is the site of interaction with several channel modulatory proteins including protein kinases (Weiger et al., 2002). The auxiliary β-subunits are small proteins (~200 amino acids) with two membrane-spanning domains (TM1 and TM2) (Toro et al., 1998).

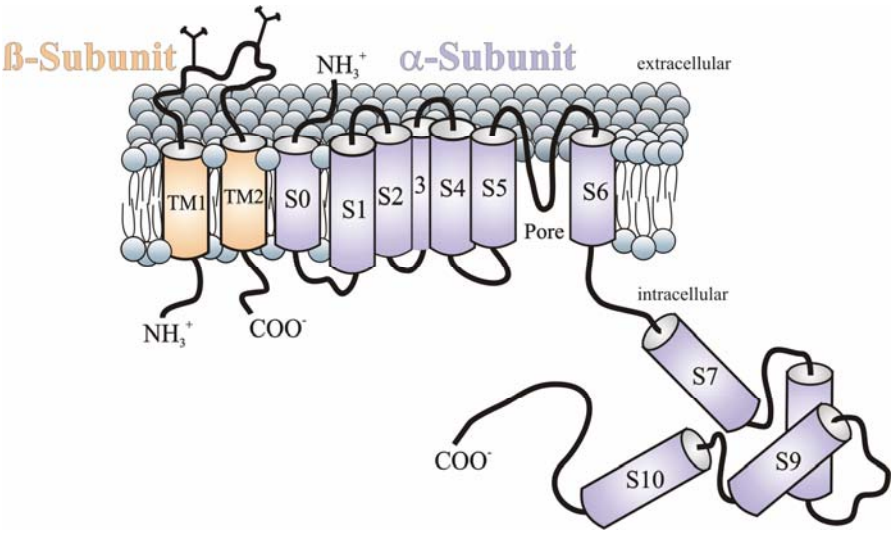


Fig.1 *Topology of the BK channel. The channel is a heterooctamer consisting of two subunits, the pore-forming α- and the regulatory β-subunit. The α-subunit has seven transmembrane segments (S0-S6) and four intracellular hydrophobic segments (S7-S10). The auxiliary β subunit consists of two transmembrane segments (TM1-TM2).*

Gating of BK channel

Under physiological conditions BK channels are activated by voltage and by the increase in free intracellular Ca^{2+}. Activation of the channel varies over a wide range of Ca^{2+} concentration from 10 nM to 10 µM (Marty, 1989; Latorre et al., 1989), and open probability changes in a voltage-dependent manner e-fold for every 10-15 mV of depolarization (Moczydlowski & Latorre, 1983; Blatz & Magleby, 1984). Under low- or zero-Ca^{2+} conditions the channel behaves like a purely voltage-dependent channel (Talukder & Aldrich, 2000; Horrigan & Aldrich, 1999). Thus, the voltage-sensing mechanism is independent of the binding of Ca^{2+} to the channel during the activation process, and Ca^{2+} is not absolutely required to activate BK channels. However, at Ca^{2+} concentrations above 100 nM, BK channels switches from a Ca^{2+}-independent to a Ca^{2+}-modulated conformational state. Therefore, the activation curve of BK channels is shifted to more negative voltages, allowing the channel to open at physiological potentials (Barrett et al. 1982; Cox et al., 1997).

In smooth muscle cells the primary role of the β_1-subunit is to increase the stability of the open state of BK channel (Cox & Aldrich, 2000). Only at free Ca^{2+} concentrations above 1 µM the β_1-subunit can upregulate the activity of the α-subunit in vascular muscle cells to produce a leftward shift of the open probability – voltage curve of the channel (Meera et al., 1996). The requirement for micromolar Ca^{2+}

suggests that colocalization or close vicinity of BK channel with Ca^{2+}-conducting channels of the plasma membrane or of internal stores are necessary for the channel to regulate cell excitability (Toro et al., 1998).

1.2. The role of BK channels in smooth muscles

BK channels are particularly abundant in smooth muscles, like the vasculature and the urinary bladder (Nelson et al., 1995; Toro et al., 1998; Heppner et al., 1997), were they control the membrane potential at rest and during depolarization (see below) and therefore the Ca^{2+} influx (Brayden & Nelson, 1992). However their functional importance and the regulation of their activity in different types of smooth muscles is still a matter of investigation.

1.2.1. Smooth muscle contraction

According to their contraction behavior smooth muscles are divided into “phasic” and “tonic” smooth muscle. In general, phasic smooth muscles have been defined as those muscles that maintain tone poorly, have relatively high shortening velocities, and are capable of displaying regenerative action potentials (Somlyo & Somlyo, 1994). They manifest a biphasic contractile response, i.e. an initial elevated phasic contraction, followed by a subsequent sustained tonic contraction (Fig.2). Tonic smooth muscles do not generally display action potentials or regenerative electrical activity under physiological conditions (Somlyo & Somlyo, 1994). They have slower shortening velocities, but more effectively maintain tone. The detrusor muscle belongs to the category of phasic smooth muscles while most of the vasculature, notably the aorta, is representative for the tonic smooth muscle (Horowitz et al., 1996).

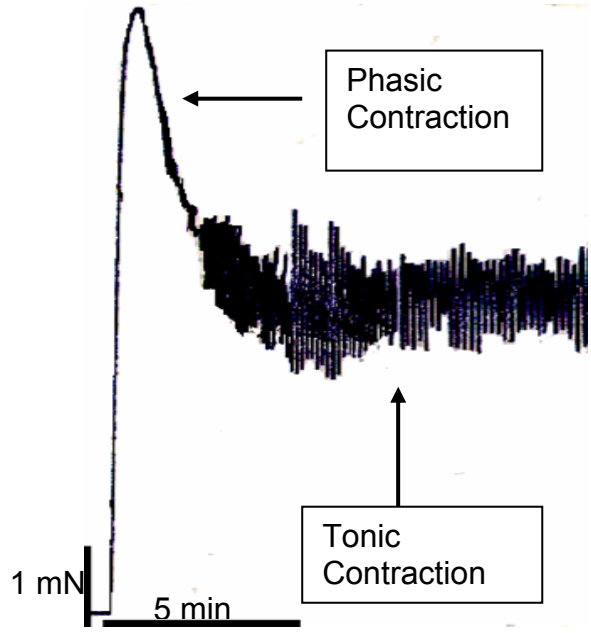


Fig.2 *Phasic and tonic component of contraction in "phasic" smooth muscles. Note also the rhythmical contractions occurring during the tonic contraction; (original trace of a carbachol-stimulated urinary bladder muscle strip).*

The main initiator of smooth muscle contraction is the rise in intracellular free Ca^{2+} (Filo et al., 1965). The increase in cytoplasmic Ca^{2+} is brought about by mobilization of intracellular Ca^{2+} stores, via inositol 1,4,5-triphosphate, and/or influx of Ca^{2+} from the extracellular fluid through voltage-dependent L-type Ca^{2+} channels (see Horowitz et al., 1996, for a review of smooth muscle contraction mechanisms). Increase of cytosolic Ca^{2+} promotes further release from intracellular stores by Ca^{2+}-induced Ca^{2+} release (Itoh et al., 1983, Hashitani et al., 2001). Free cytoplasmic Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complex associates with the catalytic subunit of the myosin light chain kinase (MLCK) and activates the enzyme to phosphorylate the regulatory myosin light chain (MLC_{20}), located on the myosin head. This phosphorylation is the main determinant of smooth muscle contraction (Somlyo & Somlyo, 1994). It allows the interaction between myosin heads and adjacent actin filaments, during which the myosin head hydrolyzes ATP (Somlyo & Somlyo, 1994). The ATP hydrolysis and subsequent dissociation of the tightly bound products (ADP and Pi) produce an ordered series of allosteric changes in the conformation of myosin that permits the sliding of the contractile filaments and therefore the muscle contraction.

Constrictors can also initiate contractions without changing the Ca^{2+} concentration (Somlyo & Somlyo, 1994). This process termed Ca^{2+} sensitization involves the phosphorylation of the regulatory myosin light chain (MLC_{20}), causing Ca^{2+}-independent contraction. The opposite process, namely Ca^{2+} desensitization, is induced by the dephosphorylation of MLC_{20} by the MLC phosphatase (MLCP). Thus,

the ratio between the activities of myosin light chain kinase (MLCK) and of MLC phosphatase is the major determinant of the Ca^{2+} sensitivity of myosin (Somlyo & Somlyo, 2003). The small GTPase RhoA and its effector, the Rho-associated kinase, is a major target for regulating Ca^{2+} sensitivity of smooth muscles myosin. Activated Rho kinase phosphorylates, and inactivates MLCP, preventing the dephosphorylation of myosin light chain (MLC_{20}), which leads to Ca^{2+}-independent smooth muscle contraction in the presence of basal or increased MLCK activity (Somlyo & Somlyo, 2003). A variety of agonists can induce Ca^{2+} sensitization by activating RhoA through G protein coupled receptors. Most $G\alpha_q$ coupled receptors activate both RhoA and the IP_3 pathway (Somlyo & Somlyo, 2003). Furthermore, agonists linked with $G\alpha_{12,13}$ can induce high force through Ca^{2+} sensitization via RhoA and without, or only minimal, activation of the IP_3 pathway (Nobe & Paul, 2001).

1.2.2. Smooth muscle relaxation; the role of cAMP and cGMP

The activity of the SERCA pump from the sarcoplasmic reticulum membrane, of the ATP dependent Ca^{2+} pumps from the cellular membrane and of the Na^+/Ca^{2+} exchanger determine the fall in intracellular free Ca^{2+} concentration (Horowitz, 1996), causing the inactivation of MLCK, which permits the dephosphorylation of the MLC_{20} by myosin light chain phosphatase (Somlyo & Somlyo, 1994). The subsequent inactivation of the actomyosin ATPase determines smooth muscle relaxation. Dephosphorylation of MLC_{20} by the MLC phosphatase (MLCP) also occurs independently from intracellular Ca^{2+} concentration by the so-called Ca^{2+} desensitization.

Two major mediators of smooth muscle relaxation are the cyclic nucleotides cAMP and cGMP. It seems that both nucleotides activate BK channels in several smooth muscles (see below). However, cAMP and cGMP also activate via their protein kinases (cAMP- and cGMP-protein kinase, respectively) (Walsh et al., 1995; Pfeifer et al., 1998; Carvajal et al., 2000) several other mechanisms, which determine smooth muscle relaxation by regulating the *intracellular Ca^{2+} concentration* and the *Ca^{2+} sensitivity* of the cells. Both nucleotides interact with Ca^{2+} release via the IP_3 pathway at multiple levels, summarized by Abdel-Latif (2001): (i) inhibition of the agonist-induced IP_3 production, probably by an inhibitory effect on PLC activity; (ii) stimulation of the conversion of IP_3 to IP_4; (iii) inhibition of agonist-induced intracellular Ca^{2+} mobilization, probably by a direct phosphorylation of the IP_3

receptor (Komalavilas & Lincoln, 1996). Cyclic GMP inhibits the IP_3 receptor in vivo also by phosphorylating the IP_3-receptor associated cGMP kinase substrate (IRAG) (Schlossmann et al., 2000). In addition, the blockade of Ca^{2+} channels activity (Ishikawa et al., 1993), and the stimulation of the Na^+/Ca^{2+} exchange pumps (Furukawa K, 1991) or of the SERCA pumps (Casteels, 1991) were also proposed as possible mechanisms by which the cyclic nucleotides reduce the intracellular Ca^{2+} concentration.

Ca^{2+} desensitization by RhoA phosphorylation via cGMP kinase (Somlyo & Somlyo, 2003) and subsequently inhibition of RhoA kinase activation also contributes significantly to relaxation mediated by cGMP and cAMP (Kawada et al., 1997; Sauzeau et al., 2000). A direct phosphorylation and activation of the myosin light chain phosphatase by cGMP kinase was also detected (Surks et al., 1999), as well as a cyclic GMP kinase-mediated phosphorylation of telokin, a protein that increases the MLC phosphatase activity (Wu et al., 1998; Komatsu et al., 2002). Cyclic AMP, but not cyclic GMP, phosphorylates myosin light chain kinase and inhibits thereby its activation by Ca^{2+}/calmodulin (Somlyo & Somlyo, 2003).

The cAMP and cGMP pathways in arteries and urinary bladder

Cyclic GMP and cyclic AMP are very important for the regulation of the activity of vessels and urinary bladder. In vascular smooth muscle cells the cGMP pathway is activated by the gas nitric oxide (NO). NO was recognized as an agent that is released from nitroglycerin and other vasodilating compounds and stimulates cyclic GMP production (Katsuki et al., 1977; Arnold et al., 1977). It is identical with the endothelium-derived relaxing factor, which decreases vascular tone (Furchgott & Zawadzki, l980; Ignarro et al., 1987; Palmer et al., 1987). In the vascular endothelial cells NO is produced from the amino acid L-arginine by Ca^{2+}/calmodulin activated nitric oxide synthase (NOS) and various cofactors (Boucher et al., 1999). It can act only locally because it has a short half-life (about 5-10 seconds) in the extracellular space (Ignarro et al., 1987) before it is converted to nitrates and nitrites by oxygen and water. It diffuses from the endothelial to the local smooth muscle cells and reacts with iron in the active site of the soluble guanylyl cyclase, stimulating it to produce cyclic GMP (Stone & Marletta, 1994; Ignarro et al, 1982).

NO appears to play an important role also in the relaxation of urinary bladder. Increasing evidences suggest that nitric oxide is the main mediator of the inhibitory

nonadrenergic noncholinergic effects which have been demonstrated in the low urinary tract of different species (Longhurst & Uvelius, 2001, Andersson & Persson, 1995; Burnett et al., 1995; Persson et al. 2000). Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are localized in nerves and urothelium of the detrusor (Burnett et al., 1997; Persson et al., 1993) and it was suggested that NO may be formed even in the bladder smooth muscles (James et al., 1993). It has been reported as well that intravesical administration of NO donors suppresses detrusor hyperactivity (Ozawa et al., 1999), whereas intravesical administration of oxyhemoglobin, an NO scavenger, stimulates bladder activity (Pandita et al., 2000). In addition, cGMP protein kinase, which mediates NO effects, is present in bladder smooth muscles (Persson et al. 2000). In cGMP kinase knock-out mice no bladder relaxation occurs in vitro after application of cGMP, and frequent voiding and nonvoiding bladder contractions were observed in vivo, indicating an unstable bladder (Persson et al. 2000).

Cyclic cAMP also plays a preeminent role in the relaxation of the urinary bladder. The detrusor contains a high density of β_1, β_2 and β_3 receptors, which mediate the adrenergic-induced relaxation of the bladder (Longhurst & Uvelius, 2001) by stimulating the adenylate cyclase and increasing the concentration of intracellular cyclic AMP. In addition, the decrease in cytoplasmic [cAMP] through the inhibition of adenylate cyclase contributes to the detrusor contraction, as shown in the case of the stimulation of muscarinic M_2 receptors (Longhurst & Uvelius, 2001; Matsui et al., 2003).

1.2.3. Regulation of BK channels in smooth muscles

By hyperpolarising the membrane and consequently closing a number of channels, including L-type Ca^{2+} channels, BK channels determine the excitability and contractility of smooth muscles (Brayden & Nelson, 1992). BK channels are activated by depolarization of the membrane potential and by increased concentrations of intracellular Ca^{2+} (see above), but their activity can also be modulated by second messengers such as cAMP and cGMP. Indeed, both cGMP kinase (Robertson et al., 1993; Stockand & Sansom 1996) and cAMP kinase (Kume et al., 1989; Kume et al., 1994) phosphorylate BK channels and contribute therefore to smooth muscles relaxation (Nelson & Quayle, 1995). However, the responses of BK channels to

hormones and drugs that activate either the cAMP or the cGMP-signalling pathway vary from tissue to tissue and are still investigated (Zhou et al., 2001).

BK channels are active not only in depolarized and contracting smooth muscles but also at rest (Toro et al., 1998). This appeared initially surprising taking into consideration the high amount of Ca^{2+} needed for the activation of BK channels (see above). However the discovery of the so-called "Ca^{2+} sparks" in a variety of smooth muscle cells and intact tissues (Nelson et al., 1995, ZhuGe et al., 1998; Sanders, 2001; Ohi, 2001) revealed the mechanism underlying BK channels activation at resting membrane potential. Ca^{2+} sparks are highly localized, transient increases in Ca^{2+} concentration [up to 10 μM close to the releasing site of a Ca^{2+} spark (Perez et al., 1999)], which arise from the opening of one or more ryanodine receptors (RyRs) of the sarcoplasmic reticulum. The proximity of RyR in the SR to the plasma membrane (the space between SR and the cellular membrane is about 12-20 nm) creates significant transient elevations of Ca^{2+} near the plasma membrane. Thereby, Ca^{2+} sparks can activate Ca^{2+}-dependent ion conductances of the membrane, to determine transient changes in transmembrane ionic currents. The hyperpolarizing spontaneous transient outward K^+ currents (STOCs) and the depolarizing spontaneous transient inward Cl^- currents (STICs) in smooth muscles appear to be activated by locally Ca^{2+} increases (Gordienko et al., 1999). STOCs are up to 250 pA large and about 100 ms lasting currents, which were first identified in smooth muscle cells by Benham & Bolton in 1986. The characteristics of the currents suggested from the beginning that they are determined by the opening of up to hundred Ca^{2+}-activated K^+ channels, in response to sudden discharge of Ca^{2+} from internal stores (Benham & Bolton, 1986; see also Bolton & Imaizumi**,** 1996; Plüger et al., 2000).

Several evidences exist for the link between Ca^{2+} sparks and the BK channels-carried STOCs. Simultaneous optical and electrical measurements on arterial myocytes indicate that virtually all Ca^{2+} sparks activate STOCs (Perez et al., 1999); depletion of intracellular Ca^{2+} stores abolishes STOCs (Benham & Bolton 1986, Ganitkevich & Isenberg, 1990); in pressurized cerebral arteries, ryanodine (by blocking Ca^{2+} release from the sarcoplasmic reticulum) and thapsigargin (by unloading Ca^{2+} stores) inhibited Ca^{2+} sparks and depolarized the membrane potential by about 10mV, which is similar to the depolarization induced by BK blockers (Knot et al. 1998); smooth muscle cells from cerebral arteries of mice lacking the auxiliary

β1 subunits of the BK channels, which increases the Ca^{2+} sensitivity of the channel, generated Ca^{2+} sparks of normal amplitude and frequency, but STOCs frequencies were largely reduced at physiological membrane potential (Plüger et al., 2000). However there are also some disparities between sparks and STOCs, and some evidences suggest that STOCs are not only carried by BK channels (see chapter 4.1.).

In smooth muscles Ca^{2+} sparks occur at rest but also during depolarization and contraction (Jaggar et al., 1988 a). In arteries and urinary bladder depolarization increases sparks amplitude and frequency, an effect which depends on Ca^{2+} influx via L-type Ca^{2+} channels (Jaggar et al., 1988 a; Herrera et al., 2001). It was proposed that in depolarized smooth muscles the increased cytoplasmic and/or sarcoplasmic (Nelson & Nelson, 1990; Gregoire et al., 1993) Ca^{2+} activates ryanodine receptor channels, resulting in Ca^{2+} spark with higher amplitude and frequency. Ca^{2+} sparks activate Ca^{2+}-activated K^+ channels to cause STOCs, which hyperpolarize the membrane potential, close voltage-dependent Ca^{2+} channels, and hence decrease global Ca^{2+} (Jaggar et al., 1998 b) (Fig. 3).

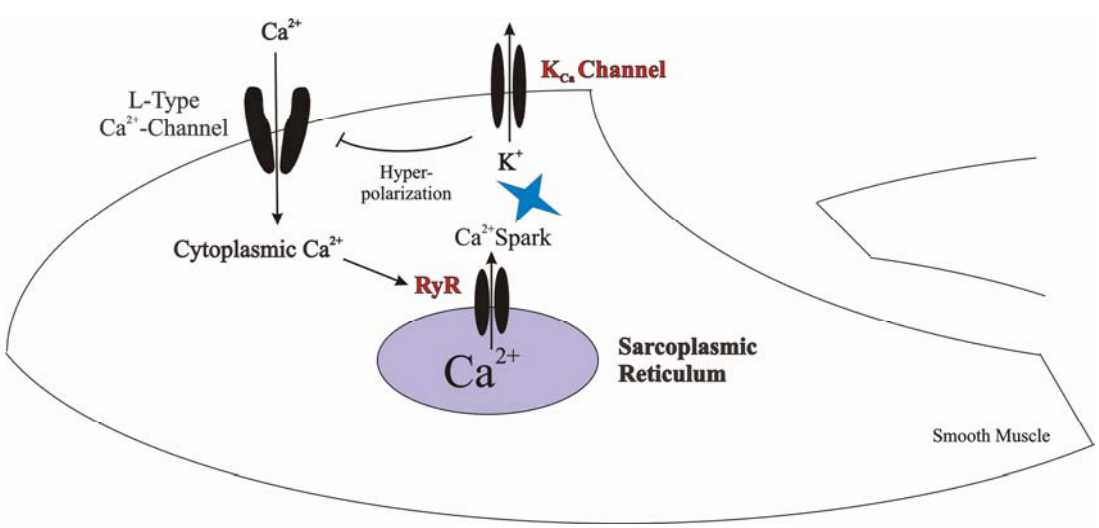


Fig.3 *Activation of Ca^{2+}-regulated K^+ channels by Ca^{2+} sparks (according to Jaggar et al., 1998 b). For details see text.*

1.3. Aim of this study

In excitable smooth muscle cells, BK channels are thought to regulate the membrane potential, and thereby the intracellular Ca^{2+} concentration and contractility. However, the exact physiological role of BK channels in many smooth muscle tissues is still unclear. Mice with genetic deletion of BK channels are an excellent model for delineating channel-dependent physiological processes. This study examines the effect of BK channel deficiency on the functions of arteries and urinary bladder of $BK^{-/-}$ mice. The following specific aspects have been evaluated:

1. It is known that in vascular smooth muscle cells α_1 adrenergic receptor agonists cause both Ca^{2+} release from the sarcoplasmatic reticulum and Ca^{2+} influx via voltage dependent Ca^{2+} channels (Miriel et al., 1999; Furutani et al. 2002, Moosmang et al., 2003). To examine whether BK channel deficiency affects the Ca^{2+} influx, phenylephrine-induced Ca^{2+} transients were measured by fluorescence microscopy in isolated aortic cells and compared between wt and $BK^{-/-}$ mice.

2. Suppression of Ca^{2+} transients is the major mechanism of the NO/cGMP/cGMP kinase-induced vascular relaxation (Pfeifer et al. 1998). BK channels are targets of the cGMP protein kinase phosphorylation in vivo (Alioua et al., 1998), which increases the open probability of the channels (Furukawa et al. 1996). To test whether the BK channels activation plays a role in the cGMP effect on intracellular Ca^{2+}, phenyleprine-induced Ca^{2+} transients were monitored in aortic cells from wt and $BK^{-/-}$ mice preincubated with membrane permeant derivates of cGMP.

3. It was further reinvestigated whether BK channels carry the Ca^{2+} sparks-induced spontaneous transient outward currents (STOCs). In addition, Ca^{2+} spark parameters were evaluated by confocal microscopy in $BK^{-/-}$ and wt cerebral artery cells, in order to evaluate the influence of BK channel deficiency on Ca^{2+} sparks.

4. In the urinary bladder, a major aim was to elucidate the role of BK channels for the contractility of the detrusor. The urinary bladder manifests a complex pattern of contraction allowing proper micturation. The carbachol-, the depolarization- and the electrical field stimulation-induced contractions, as well as the rhythmical contractility of the bladder, were analyzed in wt and $BK^{-/-}$ mice.

5. A further objective was to study whether cAMP and cGMP relax urinary bladder via BK channels activation. Therefore, the relaxation determined by

membrane permeant derivates of cyclic nucleotides was compared between wild type and $BK^{-/-}$ urinary bladder.

2. Methods

All the experiments described in this thesis were performed by me; the STOCs measurements were performed together with Kirill Essine, from the group of Dr. M. Gollasch, Helios Franz-Volhard-Klinik, MDC für Molekulare Medizin, Humboldt Universität, Wildbergstr. 50, Berlin.

2.1. Ca^{2+} transients measurements

Mice were killed by asphyxiation with CO_2. The thoracic and abdominal aorta was removed and placed in *isolation and bath solution* (see below) to be cleaned from the surrounding tissues. After cleaning the aorta was opened longitudinally, cut into two segments and washed in *Ca^{2+} free isolation and bath solution*. Afterwards the aortic segments were digested in Ca^{2+}-free *enzyme solution 1* at 37°C for 30 min under continuous shaking (25 stroke/min). They underwent also a second digestion in Ca^{2+}-free *enzyme solution 2* at 37°C for 10 min under continuous shaking (25 stroke/min). Following enzyme treatment the tissue was washed with could *isolation and bath solution*, and individual cells were freed from the tissue by passing tissue bundles through the tip of a fire-polished Pasteur pipette. Cells were kept on ice in an Eppendorf 1ml tube in *isolation and bath solution* for 15 min to recover. Dispersed cells were then loaded in the same Eppendorf 1 ml tube with 2,5 µM Fura 2-AM for 45-60 min in the dark at room temperature. Excess buffer containing Fura 2-AM was removed by centrifugation at 1200 rpm for 3 minutes. The pellet of cells was placed on ice and, after 10-15 min, 300 µl of *isolation and bath solution* were added slowly over 2-3 min. For Ca^{2+} measurements a suspension of vascular smooth muscle cells (10µl) was gently aspirated from the surface of the cell pellet and spread on a glass coverslip (Iversen & Arendshorst, 1999). To improve cell adhesion to the slide a drop of Poly-L-Lysine solution 0,01% was placed on the glass coverslip, allowed to remain for 20 minutes and then aspirated. During measurements the cells were continuously superfused with *isolation and bath solution* at 36°C and a flow rate of 2-4 ml/min. Ca^{2+} measurements were performed using the dual-wavelength microfluorescence technique (see below). Two Ca^{2+} transients were elicited consecutively by application of the α_1-agonist phenylephrine for 1 minute, with a 15 min interval in between to allow the refilling of intracellular Ca^{2+} stores. In some experiments the cells were

treated with 8-Br-cGMP for 5-7 min before eliciting the second transient. In these experiments 8-Br-cGMP was maintained in the bath also during the stimulation with phenylephrine. Ca^{2+} transients were analyzed using the Microcal Origin 6.0 software, (Microcal Software, Inc., Northampton, USA).

Chemicals

Fura-2 AM was obtained from Molecular Probes, Eugene, USA. All other chemicals were obtained from Sigma, Deisenhofen, Germany.

Solutions

Isolation and bath solution

	mM
NaCl	127
KCl	5,9
$CaCl_2 \times 2H_2O$	2,4
$MgCl_2 \times 6H_2O$	1,2
Glucose	11,8
HEPES	10 pH=7,4

Ca^{2+} free isolation and bath solution

	mM
NaCl	127
KCl	5,9
$MgCl_2 \times 6H_2O$	1,2
Glucose	11,8
HEPES	10 pH=7,4

Ca^{2+} Solution

	mM
$CaCl_2 \times 2H_2O$	50

Enzyme Solution 1

	mg/ml Ca^{2+}-free isolation and bath solution
Albumin	1
Papain	1
DTT (Dithioerythritol)	1

Enzyme Solution 2

	mg/ml Ca^{2+}-free isolation and bath solution
Albumin	1
Collagenase	1
Hyaluronidase	1
+ Ca^{2+} Solution	1µl/ml Ca^{2+}-free isolation and bath solution

Fura 2-AM

1 mg Fura-2 AM was dissolved in 400 µl DMSO, aliquots were kept at -20°C.

Dual wavelength microfluorescence

The free cytosolic Ca^{2+} concentration was measured by using the fluorescent Ca^{2+} chelator fura-2. Fura-2 is a dual wavelength ratiometric Ca^{2+} dyes (Grynkiewicz et al., 1985). Ratiometric indicators exhibit not only intensity changes with changing [Ca^{2+}] but the Ca^{2+}-free and Ca^{2+}-bound forms of the indicator actually have distinct spectra, the maxima in which are located at different wavelengths (the spectra show wavelength shifts). Significant shifts are observed in the excitation but not in the emission spectra of Fura-2 (Fig.4). The maximal absorption for the Ca^{2+} free compound is at about 360-380 nm, while the maximal absorption for the Ca^{2+} bound form is at about 340 nm. The emission maximum is at about 510 nm, and is Ca^{2+} independent. Because the Ca^{2+} free and the Ca^{2+}-bound forms of ratiometric indicators are characterized by spectral peaks at different wavelengths, intensity measurements can be made at two different excitation wavelengths and a ratio can be obtained (Grynkiewicz et al., 1985). Obtaining of a ratio minimizes the effect of many artefacts that are unrelated to changes in Ca^{2+}, such as changes in the cell thickness, loss of indicator from a cell by leakage, differences in concentration of the Ca^{2+} dye in cells.

In the experiments performed in this study isolated cells loaded with Fura-2 were exposed alternatively to light at 340 nm and 380 nm, and the emitted fluorescent signal at 510 nm was measured. The light source was a xenon lamp, the alternative stimulation was performed using a computer controlled filter wheel, and the emitted light was measured by a high resolution digital photo camera and was acquired and stored in a computer. Since Fura-2 cannot pass the cellular membrane the cells were loaded with Fura-2 AM (Acetoxymethylester) at a final concentration of 2,5 µM. By intracellular esterases the Ca^{2+} chelator Fura-2 was released, and remained in the cells because of its hydrophilic character.

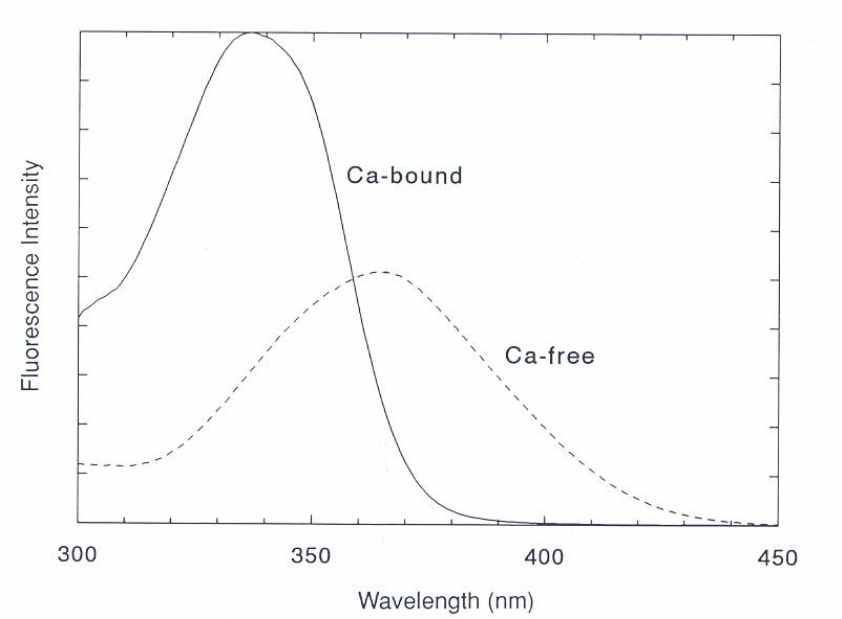


Fig.4 *Excitation spectra of Ca^{2+}-bound and Ca^{2+}-free form of Fura-2 (measured at emission wavelength of 505 nm) (Kao J. et al., 1994).*

Equipment

Microscope	Axiovert S 100, Zeiss, Stuttgart, Germany
Objective	PlanNeofluar40x, Zeiss, Stuttgart, Germany
Camera	SPOT, Diagnostic Instruments, inc., Sterling Heights, USA
Light source	XBO75, Zeiss, Stuttgart, Germany
Filter wheel	99A042, LEP Ltd., New York, USA
Acquisition Software	MetaFluor 4.6r9, Universal Imaging Corporation, Downingtown, USA

2.2. Ca^{2+} sparks measurements

Mice were killed by cervical dislocation. The brain was removed and placed into ice cold oxygenated (95% O_2 / 5% CO_2) *physiological salt solution (PSS)* (see below). Cerebral basilar arteries were carefully dissected in ice-cold *PSS* and cleaned of surrounding connective tissue. Arteries were then washed with low Ca^{2+} *enzyme solution* and then digested with *papain enzyme solution* and *collagenase enzyme solution* as follows. The *papain enzyme solution* was first activated for 10 minutes at 36°C and thereafter the cerebral arteries have been placed in and incubated for 15 minutes at 36°C under continuous shaking (20 stroke/min) and then transferred into the *collagenase enzyme solution*, which was activated before for 6 minutes at 36°C. The collagenase digestion lasted 6 minutes at 36°C and under continuous shaking (20 stroke/min). The enzyme digestion was stopped by placing the artery for 5 minutes in ice cold low Ca^{2+} *enzyme solution* contained in an Eppendorf 1 ml tube. The digested tissue was triturated with a fire polished glass Pasteur pipette to yield single smooth muscle cells.

Dispersed cells were loaded in an Eppendorf 1 ml tube with the Ca^{2+} indicator Fluo 3 by incubation in *enzyme solution* containing 10 µM Fluo3-AM (Acetoxymethylester) and Pluronic F-127 0,05% for 30 min in the dark at room temperature. Excess buffer containing Fluo 3-AM was removed by centrifugation at 1200 rpm for 3 minutes. The cells were re suspended in 100 µl of *bath solution* kept on ice and used within the same day. For Ca^{2+} measurements a suspension of vascular smooth muscle cells (10µl) was gently aspirated from the surface of the cell pellet and spread on a glass coverslip. Measurements have been performed in the *bath solution*.

Ca^{2+} sparks were measured by line scanning microscopy. A line was chosen near the cellular membrane. This line was scanned every 2 ms, for a period of 10 s. Each cell was scanned for a maximal duration of 30 s. A two dimensional image was obtained from plotting light intensity of the scanned line on the ordinate versus time on the abscissa. The baseline fluorescence (F_0) was determined by averaging line-scan images without occurring sparks.

Sparks were analyzed off-line by “Spark analysis”, program written by Kirill Essine in C++ and running under the Windows operating system. Amplitudes of Ca^{2+} sparks were measured as local fractional fluorescence increases (F/F_O). The threshold of

sparks detection was set as fluorescence increases greater than 1.2. Rise time was measured from 10 to 90 % of peak. Decay time was measured at 50% of peak. Spark width was calculated at 5% of peak. Frequency of sparks was calculated for the lines with sparking sites as: number of sparks/scan time.

Chemicals

Fluo 3-AM was obtained from Molecular Probes, Eugene, USA.

All other chemicals were obtained from Sigma, Deisenhofen, Germany.

Solutions

Physiological Salt Solution (PSS)

	mM
NaCl	119
KCl	4,7
KH_2PO_4	1,2
$NaHCO_3$	25
$CaCl_2$	2,5
Mg_2SO_4	1,2
Glucose	11,1
EDTA	0,026

Aerated with 95% O_2 and 5% CO_2
to obtain pH 7,4

Enzyme Solution

	mM	
NaCl	55	
KCl	5,6	
Na Glutamat	80	
$MgCl_2$	2	
Glucose	10	
HEPES	10	pH=7,4

Ca^{2+} Solution

	mM
$CaCl_2x2H_2O$	50

Papain Enzyme Solution

	mg/ml Enzyme Solution
Bovine Serum Albumin	1
Papain	0,5
DTT (Dithioerythritol)	1

Collagenase Enzyme Solution

	mg/ml Enzyme Solution
Bovine Serum Albumin	1
Collagenase	1
(Collagenase type F / Collagenase type H = 30% / 70%)	
+ Ca^{2+} Solution	2µl/1ml Enzyme Solution

Bath Solution

	mM	
NaCl	134	
KCl	6	
$MgCl_2$	1	
$CaCl_2$	2	
Glucose	10	
HEPES	10	pH=7,4

Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) used in conjunction with Ca^{2+}-sensing fluorescent indicators dyes provide a great improvement in horizontal and vertical resolution over conventional fluorescent microscopy, giving one the possibility to image Ca^{2+} within the context of detailed intracellular structure. In LSCM the laser beam is scanned across the specimen and emitted light is collected through a pinhole aperture (Fig.5). The pinhole provides the necessary mechanism for the rejection of out-of-focus light, but also necessitates the detection and collection of signal from only one point of the specimen at time.

In the experiments performed in this study a krypton/argon laser was used as a light source. The cells were exposed to a 488 nm wavelength light, and the emitted fluorescent signal was measured at wavelength longer than 515 nm. The measurements depth of the recording was estimated to be about 5 µm. As a Ca^{2+} dye Fluo-3 was used, which is a single wavelength Ca^{2+} indicator. For single wavelength indicators changes in Ca^{2+} bring about changes in the intensity of their fluorescence emission spectra, whereas the spectral maximum remains essentially unchanged. The absorption maximum for fluo-3 is at about 505 nm for Ca^{2+} free and Ca^{2+} bound form (Fig.6), and its emission maximum is at 526 nm, also for both forms.

The Ca^{2+} free form of Fluo-3 is more than 40 times less bright than the Ca^{2+}-bound form (Fig.6). Since Fluo-3 can not pass the cellular membrane the cells were loaded with Fluo-3 AM (Acetoxymethylester). Through intracellular esterases the Ca^{2+} chelator Fluo-3 was released, and remained in the cells because of its hydrophilic character. Because of the low solubility of Fluo-3 AM in aqueous media, Pluronic F-127, a mild nonionic surfactant which disperses the AM esters, was added to the cell containing solution together with Fluo-3 AM.

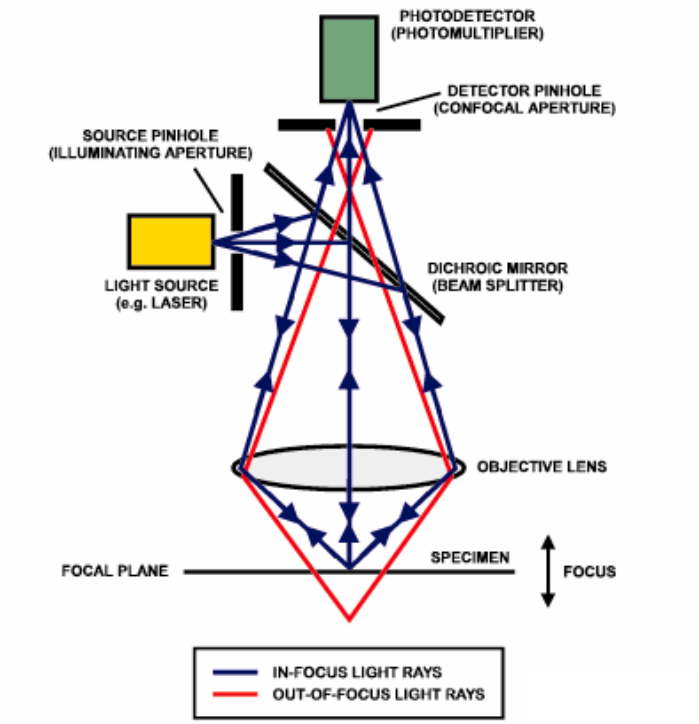


Fig.5 *In LSCM, a laser light beam is deviated by the dichroic mirror and focused to a small spot by an objective lens onto a fluorescent specimen. The emitted fluorescent light passes through the dichroic mirror in the direction of the photodetector (photomultiplier). A confocal aperture (pinhole) is placed in front of the photodetector, such that the fluorescent light from points on the specimen that are not within the focal plane (the so called out-of-focus light) where the laser beam was focused will be largely obstructed by the pinhole. In this way, out-of-focus information (both above and below the focal plane) is greatly reduced. The spot that is focused on the centre of the pinhole is often referred to as the "confocal spot" (Ladic, 1995).*

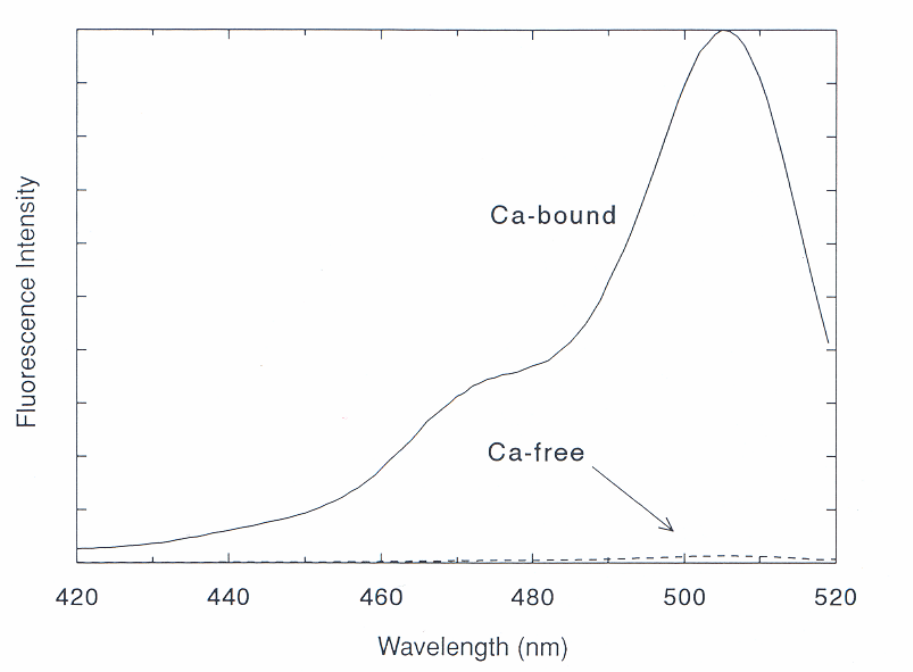


Fig.6 *Excitation spectra of fluo-3. The Ca^{2+}-free form is more than 40 times less bright than the Ca^{2+}-free form (measured at emission wavelength of 525 nm) (Kao J. et al., 1994).*

Equipment

Microscope	BioRad (Munich, Germany) laser scanning confocal microscope attached to a Nikon Diaphot microscope (Melville, NY, USA)
Objective	Nikon 60x oil immersion objective with the numerical aperture of 1.2
Acquisition Software	Lasersharp 2000, BioRad, Munich, Germany

2.3. STOCs measurements

STOCs measurements were performed together with Kirill Essine. Cerebral arteries smooth muscle cells were isolated as described above for sparks measurements. From isolated cells STOCs were electrophysiological measured in the perforated patch mode (Gollasch et al., 1996), at room temperature (20-24°C). The holding potential was set at -60 mV while depolarizing test potentials were applied in 10 mV steps from -50 to 0 mV. STOCs analysis was performed off-line using “STOCs Analysis” program written by Kirill Essine in C++ and running under the Windows operating system.

Solutions

Bath Solution

	mM
NaCl	134
KCl	6
$MgCl_2$	1
$CaCl_2$	2
Glucose	10
HEPES	10 pH=7,4

Pipette Solution

	mM
KCl	30
K aspartate	110
NaCl	10
$MgCl_2$	1
EGTA	0,05
HEPES	10 pH=7,2
Amphotericin	200 µg/ml

Equipment

Amplifier	Axopatch 200B, Axon Instruments, Foster City, CA, USA
Series interface	CED 1401, Cambridge Electronic Design Limited, Cambridge, UK
Software	CED Voltage Clamp Software Version 6.08, Cambridge Electronic Design Limited, Cambridge, UK

2.4. Urinary Bladder Contractility Experiments

Mice were killed by asphyxiation with CO_2. The entire urinary bladder was removed and pinned to the bottom of a Sylgard-coated Petri dish containing ice-cold *dissection solution* (see below). After removing the surrounding adipose and connective tissue the bladder was cut open with a longitudinal incision beginning from the urethral orifice. Four muscle strips (1-3 mm long) were cut free from the bladder wall. Each muscle strip was bound to two small hooks and was placed in thermostatically controlled (37 °C) tissue baths (10 ml volume), containing *physiological salt solution*. One end of the strip was attached to a stationary metal hook while the other end was connected to a force-displacement transducer (FSG-01/20, Experimetria Ltd., Force range: ±20g; Displacement: ±500µm/g) for tension recording (Fig. 7). The force generation was recorded on a computer-based acquisition system and on a chart recorder. The strips were suspended under a 3 mN tension. There was a 60-90 min equilibration period. During this period the bath solution was changed every 5-10 min.

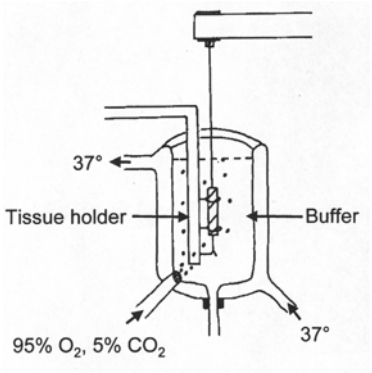


Fig.7 *Diagram of double-walled glass organ bath system for measuring responses of bladder strips. The bladder strip is suspended on silk ligatures between the lower hook and the force displacement transducer (Longhurst & Uvelius, 2001).*

Dose-response contractions measurements were performed by applying increasing concentrations of carbachol (10 nM, 100 nm, 1 µM, 10 µM and 100 µM) and KCl (20 mM, 40 mM, 60mM, 80 mM, and 100 mM) to the baths containing bladder muscle strips with intact urothelium. The interval between applications was of about 10 minutes to allow the muscles to develop a constant contraction before applying a higher concentration of carbachol or KCl, respectively. Both compounds were added cumulatively. Electrical field stimulation (EFS) of the bladder strips was performed as follows. Increasing frequencies of EFS were applied (0.5 Hz, 1 Hz, 2

Hz, 4 Hz, 8 Hz, 12 Hz, 16 Hz, 20 Hz, 25 Hz, 30 Hz, 35 Hz, 40 Hz, 50 Hz) for a period of 1 minute with a period of 2 minutes in between for allowing the muscles to recover. The voltage of the electrical field was 10 V and the duration of the pulse was 5 ms.

For monitoring the effect of cyclic nucleotides on bladder contraction cGMP and cAMP analogs were applied cumulatively during the constant component of carbachol-induced contraction. Briefly, 15 minutes after stimulation of the bladder strips with 10 µM carbachol, increasing concentrations (1 µM, 10 µM, 100 µM) of 8-(4-Chlorophenylthio)-guanosine 3',5' cyclic monophosphate (8-pCPT-cGMP), and 5,6-Dichloro-1-b-D-ribofuranosylbenzimidazol-3',5'-cyclic monophosphorothioate (cBIMPS), respectively, were added to the bath. The interval between applications was 15 min to obtain a constant effect before applying a higher concentration of 8-pCPT-cGMP or cBIMPS, respectively. In the case of EFS two consecutives dose response measurements were performed. 15 minutes before the second application of the same EFS protocol 100 µM 8-pCPT-cGMP was added to the bath. 8-pCPT-cGMP was maintained in the bath until the end of the experiment.

Carbachol-induced rhythmical contractions were also analyzed in this study. The amplitude of rhythmical contractions was normalized to the amplitude of the initial phasic contraction induced by the application of 10µM carbachol (see Fig. 13). Frequency of the rhythmical contractions was assessed over a period of 3 minutes.

Recorded data were analyzed with the FlexPro 5.0 (Weisang GmbH & Co. KG, St. Ingbert, Germany) and the Microcal Origin (Microcal Software, Inc., Northampton, USA) software.

Solutions

Dissection Solution

	mM	
NaCl	55	
KCl	6	
Na Glutamat	80	
MgCl2	2	
Glucose	10	
HEPES	10	pH=7,4

Physiological Salt Solution

	mM
NaCl	119
KCl	4,7
NaHCO3	24
KH2PO4	1,2
CaCl2	2,5
MgSO4	1,2
Glucose	11

Aerated with 95% O2 and 5% CO2 to obtain pH 7,4

Chemicals

All chemicals were obtained from Sigma, Deisenhofen, Germany.

Equipment

Isolated Tissue Bath System	TSZ-04, Experimetria Ltd., Budapest, Hungary
Analog/Digital Converter	DT 3010 Series, Data Translation GmbH, Bietigheim Bissingen, Germany

2.5. Statistics

Measured values were expressed as mean ± S.E.M. for n, the number of different animals used in the study. When specified, n was the number of cells or strips used. Statistical significance was tested using paired t-test, and probabilities of less than 5% different from control were considered significant. Statistical analysis was performed using the Microsoft Excel (Microsoft Corporation, Redmond, USA) software.

3. Results

3.1. Characterization of the vascular phenotype of $BK^{-/-}$ mice

3.1.1. Ca^{2+} transients in aortic cells

Phenylephrine (an α_1 adrenergic receptor agonist) elicits Ca^{2+} transients in aortic cells due to Ca^{2+} release from the sarcoplasmatic reticulum (via the PLC IP_3 pathway) and to Ca^{2+} influx via Ca^{2+} channels (due to membrane depolarization) (Furutani et al. 2002, Moosmang et al., 2003). The effect of BK channel deficiency on phenylephrine-induced Ca^{2+} transients was tested by fluorescent microscopy in isolated aortic cells loaded with Fura 2-AM. Ca^{2+} transients were elicited by application of 10μM phenylephrine for 1 minute. The area under curve (AUC) of the fluorescence ratio due to Ca^{2+} transients was similar in wt and $BK^{-/-}$ cells [3.8±0.3 arbitrary units in wt (n=109 cells) versus 4.0±0.3 arbitrary units in $BK^{-/-}$ (n=81 cells), P=n.s.] (Fig.8). Thus, BK channel deficiency did not affect the rise in cytosolic free Ca^{2+} during the α_1 adrenergic stimulation. In addition, the fluorescent ratio determined by the Ca^{2+} concentration at rest was compared between the phenylephrine-reactive wt and $BK^{-/-}$ cells. There was no difference between wt and $BK^{-/-}$cells [0.3±0.01 A.U. in wt (n=102 cells) versus 0.3±0.01 A.U. in $BK^{-/-}$ (n=93 cells), P=n.s.] (Fig.8). Thus, it seems that BK channel deficiency does not affect the basal Ca^{2+} concentration in vascular cells.

The effect of the BK channel activation by cGMP/cGMP kinase (Furukawa et al., 1996; Alioua et al., 1998; Fukao et al., 1999) on phenylephrine-induced Ca^{2+} transients was also determined in this study. Two consecutive Ca^{2+} transients were elicited by application of 10 μM phenylephrine for 1 minute, with a 15 min interval in between to allow the refilling of intracellular Ca^{2+} stores. The AUC of the fluorescent ratio of the second transient was expressed as percent of the AUC of the first Ca^{2+} transient, and was compared between wt and $BK^{-/-}$. The normalized AUC was similar in $BK^{-/-}$ and wt aortic cells in the absence of 8-Br-cGMP [103.2±7% (n=40 cells) and

86.9±7.6% (n=53 cells), respectively; n.s.) (Fig.9). However, preincubation for 7-8 minutes with 8-Br-cGMP determined an increased suppression of the second Ca^{2+} transient in wt compared to $BK^{-/-}$ cells [37.8±7.2% (n=63 cells) (see also Pfeifer et al., 1998) and 72.2±10.9%, (n=44 cells), respectively; P=0.007]. This suggests that the BK channels activation is important for the cGMP-induced suppression of Ca^{2+} transients in vascular muscle cells.

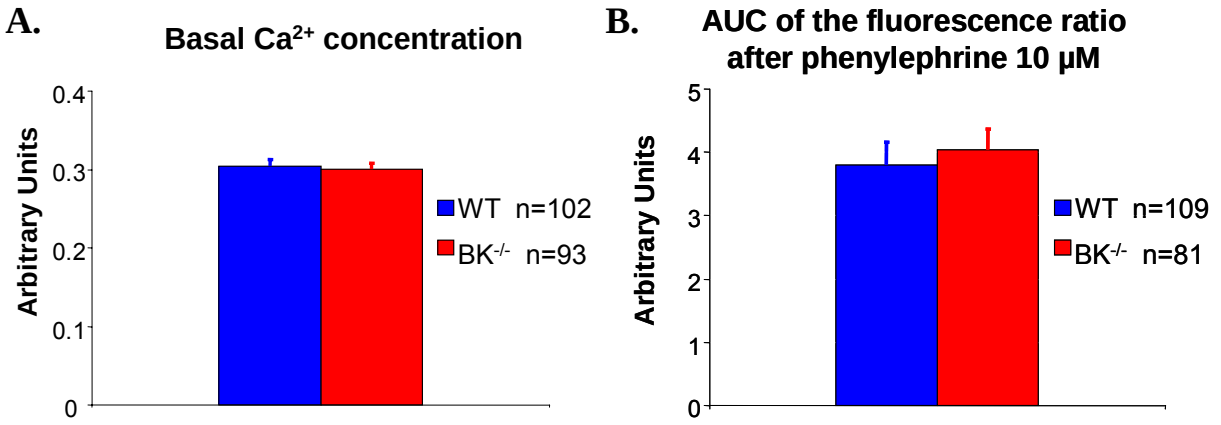


Fig.8 *Statistics of basal Ca^{2+} concentration (A) and α_1-agonist-induced Ca^{2+} transients (B) in isolated vascular smooth muscle cells from aorta; n represents the number of cells used for the statistics (Ca^{2+} transients were quantified as area under curve of the fluorescent ratio).*

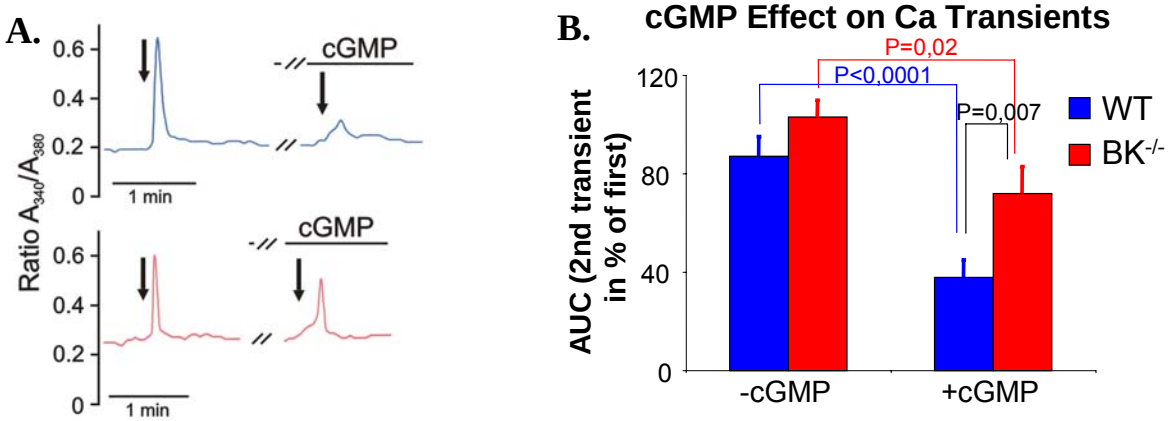


Fig.9 *Representative traces (A) of Ca^{2+} transients from wt and $BK^{-/-}$ aortic muscle cells preincubated with 8-Br-cGMP for 7 min. Consecutive Ca^{2+} transients, elicited by phenyleprine in wt (blue traces) and $BK^{-/-}$ (red traces) cells, are presented. Arrows mark the start of phenyleprine application. In (B) the AUC of the fluorescent ratio of the second transient is presented as percent of the AUC of the corresponding first Ca^{2+} transient, in the absence (left columns) and in the presence of 8-Br-cGMP (right columns); n=53 wt cells and n=40 $BK^{-/-}$ cells in the absence of 8-Br-cGMP; n=63 wt cells and n=44 $BK^{-/-}$ cells in the presence of 8-Br-cGMP.*

3.1.2. Ca^{2+} sparks and STOCs in cerebral arterial cells

Ca^{2+} sparks and STOCs were measured in muscle cells from cerebral arteries of $BK^{-/-}$ and wt mice. STOCs were absent in $BK^{-/-}$ vascular cells, even at depolarized membrane potential that promotes the activation of the Ca^{2+} dependent BK channels (Fig.10) (work was done together with Kirill Essine). The frequency of STOCs was 1.1±0.2 STOCs/s in wt cells at a holding potential of -40 mV.

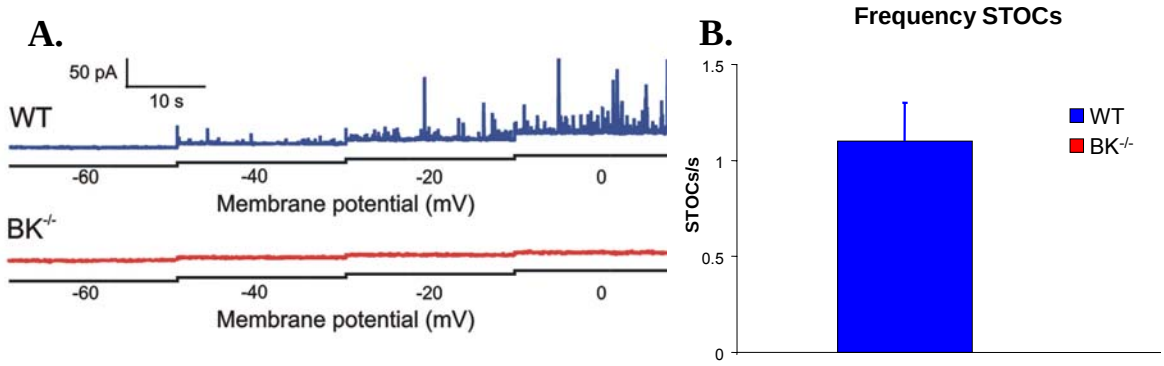


Fig.10 *(A) Original traces from STOCs measurements at different membrane potentials in cerebral arterial cells; (B) STOCs frequency at -40 mV; n=9 wt cells from 4 wt mice and n=12 $BK^{-/-}$ cells from 3 $BK^{-/-}$ mice.*

Sparks were detected in wt and $BK^{-/-}$ vascular cells, thus the absence of the STOCs in $BK^{-/-}$ cells is not due to the absence of the Ca^{2+} sparks. The spatiotemporal characteristics of sparks were similar in wild type (n=29 sparks, from 5 cells) and $BK^{-/-}$ cells (n=41 sparks from 11 cells) (for comparison see Perez et al. 1999; Pluger et al. 2000). Sparks amplitude was 2.1±0.2 arbitrary units in wt versus 1.9±0.1 arbitrary units in $BK^{-/-}$ cells (n.s.); the half time of decay of sparks was 52±5 ms in wt and 52±4 ms in $BK^{-/-}$ cells (n.s.); sparks width was 2.4±0.2 μm in wt and 2.1±0.1 μm in $BK^{-/-}$ cells, (n.s.), and the frequency of sparks was 0.7±0.2 sparks/s in wt and 0.7±0.2 sparks/s in $BK^{-/-}$ cells, (n.s.) (Fig.11). Thus, the BK channel deficiency does not affect Ca^{2+} sparks characteristics in smooth muscle cells from cerebral arteries.

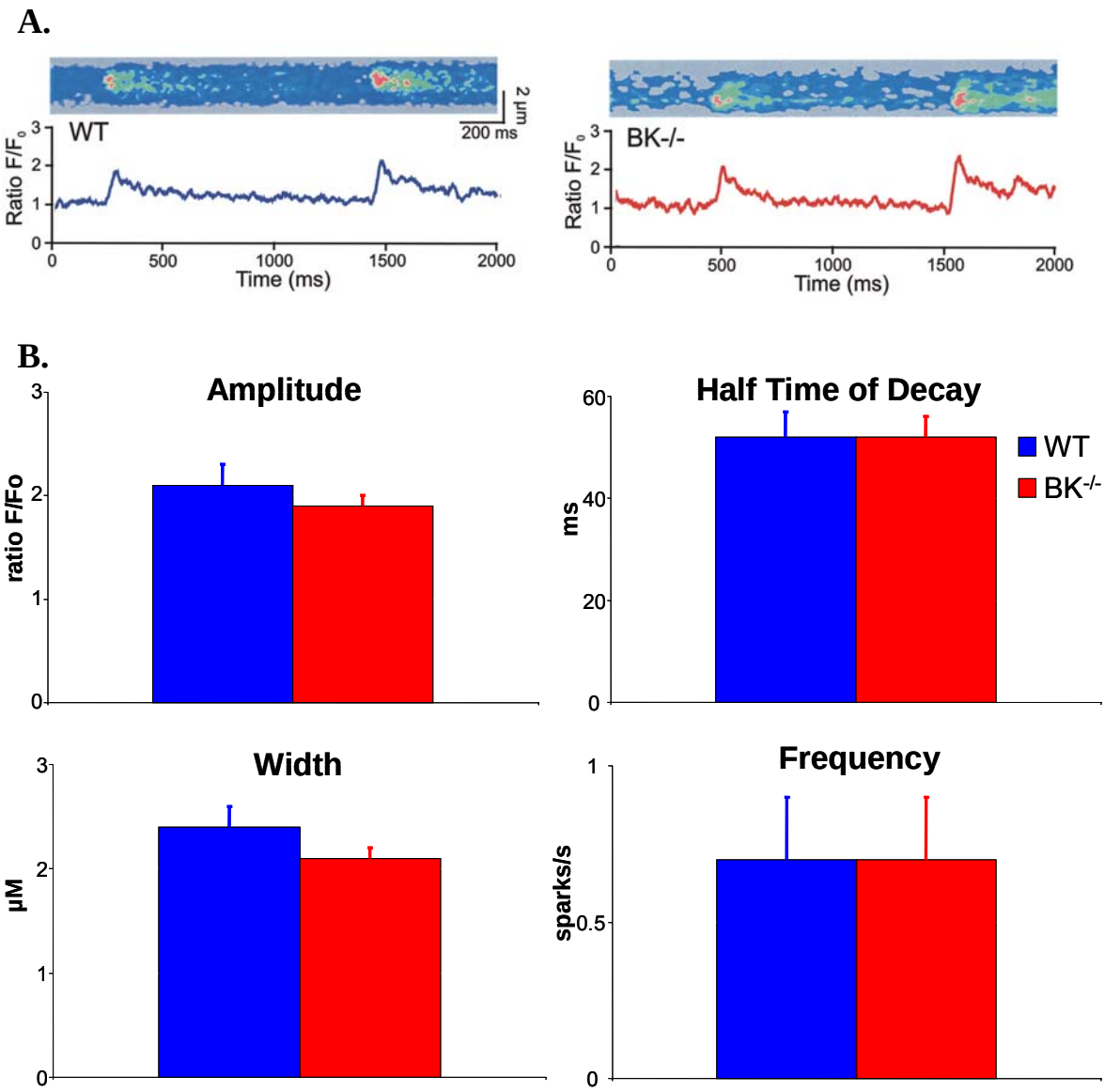


Fig.11 *Confocal line scans of fluo-3-loaded wt and BK$^{-/-}$ cerebral arterial cells, and time-course of corresponding Ca^{2+} sparks (A). Statistics of spark parameters are presented in (B). Spark amplitudes were measured as local fractional fluorescence increases (F/F_0; F_0 is the baseline). Spark duration was measured at half-maximal amplitude. Spark width was calculated at 5% of peak. Frequency of sparks was calculated for scan lines with sparking sites as: number of sparks/scan time; n=29 sparks detected in 5 wild type cells and n=41 sparks detected in 11 BK$^{-/-}$ cells.*

3.2. Characterization of the urinary bladder function

3.2.1 Carbachol-induced phasic and tonic contractions in wild type and $BK^{-/-}$ detrusor

Concentrations of 10 nM, 100 nm, 1 μM, 10 μM and 100 μM carbachol were applied cumulatively to the baths containing urinary bladder muscle strips with intact urothelium from wt and $BK^{-/-}$ mice. Both the phasic and the tonic components (see pages 4-5) of detrusor contractions were analyzed. Maximal phasic and tonic contractions were obtained at carbachol 10 μM for wt and for $BK^{-/-}$ detrusor (Fig.12). The phasic component of the carbachol-induced contraction was not affected by the BK channel deficiency. At 1 μM carbachol the phasic contraction represented 43.4±1.6% and 45.8±0.6% (n.s.), of the 10 μM carbachol-induced phasic contraction in wt (n=7 mice) and in $BK^{-/-}$ (n=7 mice), respectively (Fig.12). There was no phasic component of contraction at carbachol concentration below 1 μM. The tonic response of the detrusor was more pronounced in $BK^{-/-}$ than in wt mice at sub maximal concentration of carbachol: 14.1±3.7% in $BK^{-/-}$ bladder strips (n=7 mice) versus 2.9±0.9% in wt strips (n=7 mice) (P=0.01), and 84.6±5.8% in $BK^{-/-}$ bladder strips(n=7 mice) versus 60.9±2.9% in wt strips (n=7 mice) (P=0.003) (tonic contractions were normalized for each strip to the tonic contraction induced by 10 μM carbachol; the presented values represent the means of the normalized amplitudes) (Fig.12).

In experiments performed on different mice the phasic force developed after the application of 10 μM carbachol was normalized to the weight of the bladder strips. The normalized force was similar in wt and $BK^{-/-}$ bladder strips: 2.8±0.7 mN/mg in wt (n=7 strips) and 3.2±0.8 mN/mg in $BK^{-/-}$ mice (n= 8 strips) (n. s.). Thus, following muscarinic stimulation the BK deficient bladder develops similar force per mass unit as the normal bladder.

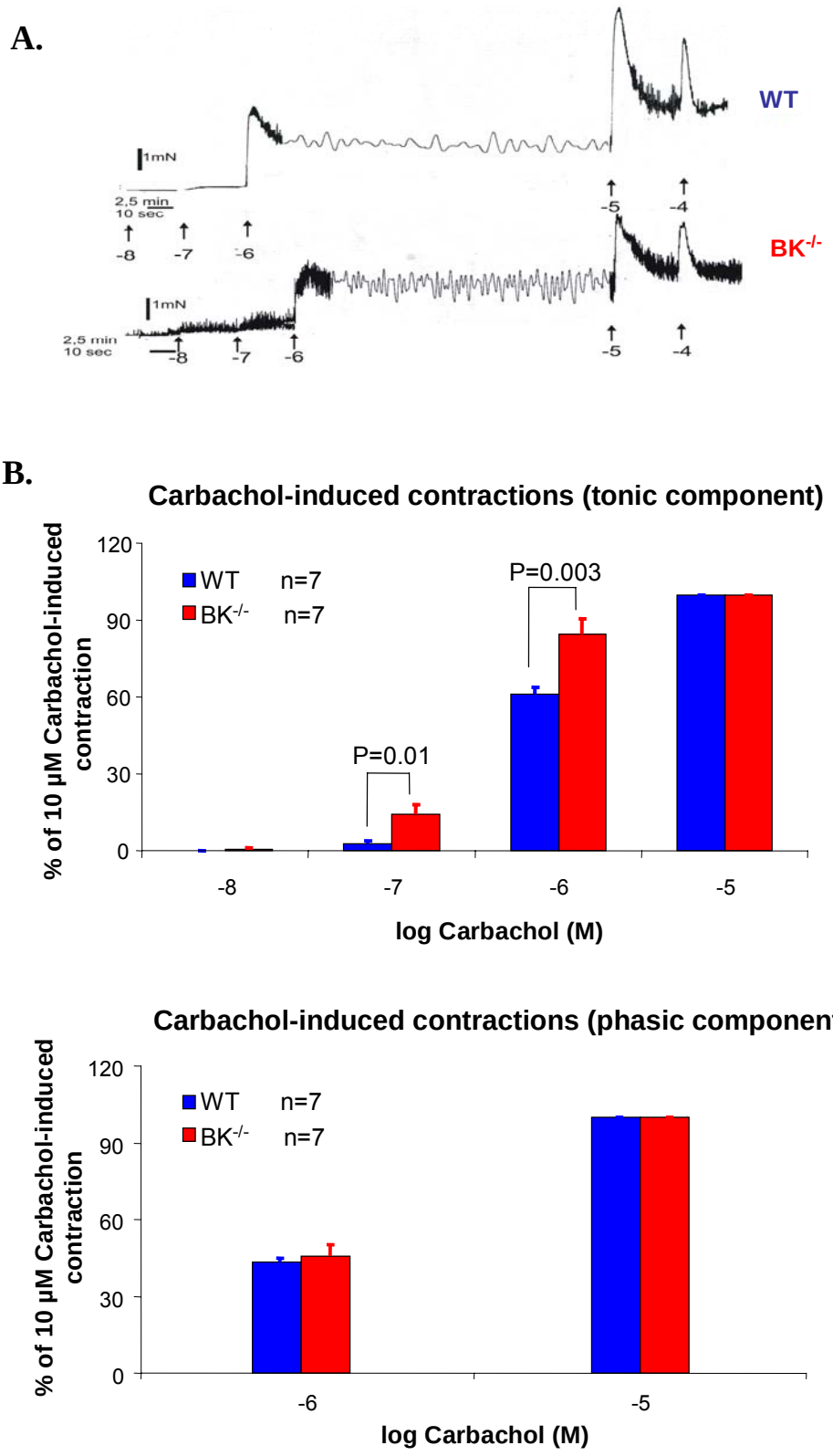
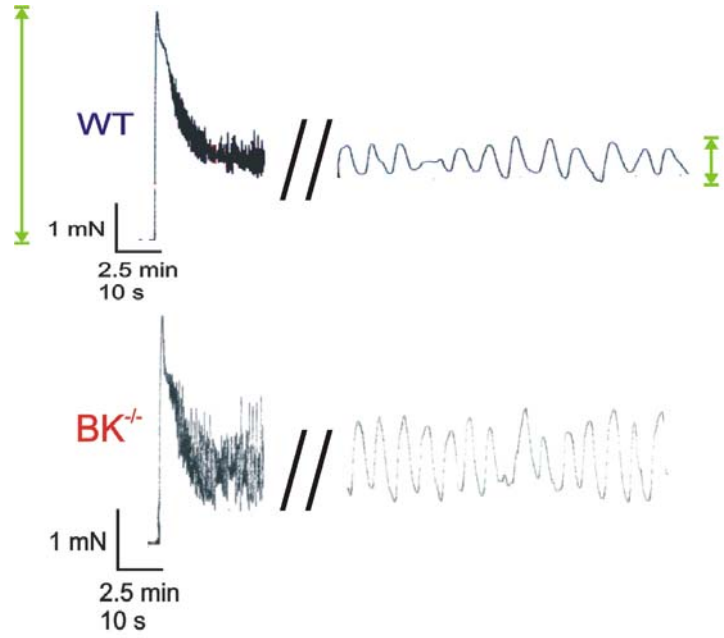


Fig.12 *(A) Representative traces of carbachol-induced contractions in wt and BK$^{-/-}$ bladder strips; arrows mark the application of increasing concentrations of carbachol; numbers represent logarithm of carbachol concentrations. (B) Statistics of the phasic and tonic component of carbachol-induced contractions. The amplitudes of the phasic and tonic components have been normalized to the amplitude of 10 µM carbachol-induced phasic and, respectively, tonic contraction; n is the number of different mice used for the statistic.*

3.2.2 Characterization of carbachol-induced rhythmical contractions of the detrusor

The urinary bladder muscle strips generate spontaneous and agonist-induced rhythmical contractions (Brading, 1992; Hashitani et al., 2001). During this study spontaneous mechanical activity could be observe only in $BK^{-/-}$ bladder strips. Unlike guinea pig urinary bladder, the mouse normal bladder often does not exhibit spontaneous rhythmical contractions (Petkov et al. 2001). However, rhythmical contractions occurred often during the carbachol-induced tonic contractions, both in wt and in $BK^{-/-}$ strips (Fig.13). The tonic contractions determined by carbachol are different in muscle strips from wt and from $BK^{-/-}$ mice at submaximal agonist concentrations (see above). Therefore, for analyzing the rhythmical contractions the maximal 10 μM carbachol concentration was applied to wt and $BK^{-/-}$ bladder strips with intact urothelium. The amplitudes of rhythmical contractions were normalized to the amplitude of the initial phasic component of contraction (see Fig.13). The normalized mean amplitude of the rhythmic contractions was more then twice more pronounced in $BK^{-/-}$ than in wt [26.1±2.2% of the phasic component of carbachol-induced contraction in $BK^{-/-}$ (n=8 mice), versus 12.1±1.6% in wild type (n=9 mice), (P=0.0001)] (Fig.13). Also the frequency of the carbachol-induced rhythmic contractions was significantly increased in $BK^{-/-}$ versus wt bladder strips [11.2±0.5 contractions per minute in $BK^{-/-}$ (n=8 mice), versus 9.1±0.5 contractions per minute in wild type (n=9 mice), (P=0.007)] (Fig.13). These data suggest that BK channels play an important role in the regulation of rhythmic contractions in mice bladder.

A.



B.

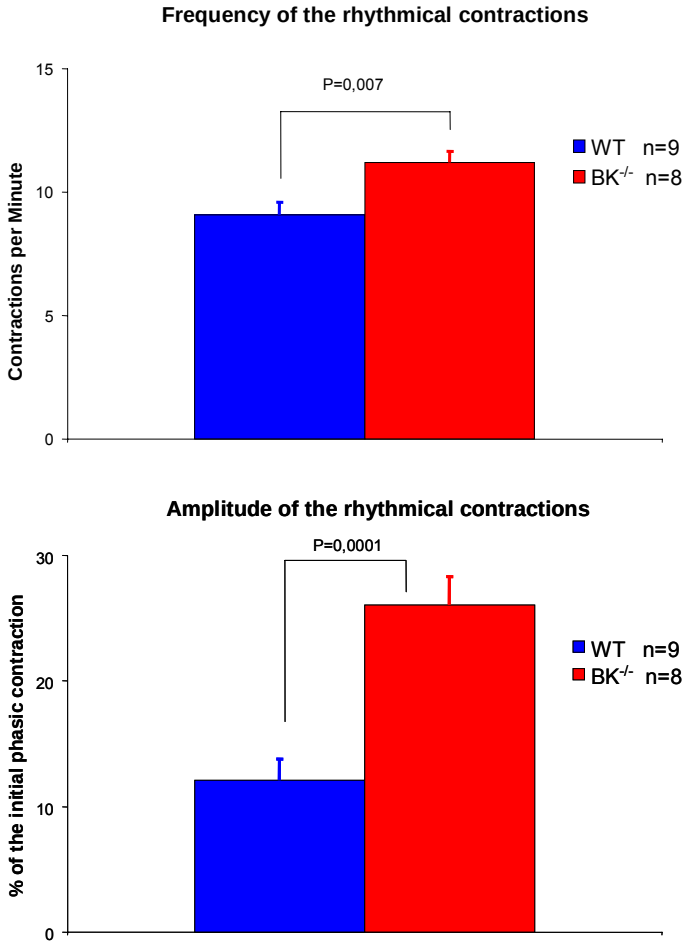


Fig.13 *Original traces (A) and statistics (B) of the frequency and the amplitude of rhythmical detrusor contractions after stimulation with 10 μM carbachol. The amplitude of rhythmical contractions (green bar right) was normalized to the amplitude of the initial phasic component of the 10 μM carbachol-induced contraction (green bar left). Frequency of the rhythmical contractions was assessed over a period of 3 minutes; n is the number of different animals used for the statistic.*

3.2.3 KCl-induced contractions in wild type and $BK^{-/-}$ detrusor

Detrusor muscle strips with intact urothelium were depolarized by KCl solutions of different concentrations (20 mM, 40 mM, 60mM, 80 mM, and 100 mM) but with similar osmolarity. Both the phasic and the tonic components (see pages 4-5) of detrusor contractions were analyzed. The maximal phasic contraction was obtained at similar concentration in wt and $BK^{-/-}$ mice (60 mM KCl) (Fig.14). At submaximal KCl concentrations the phasic response was more pronounced in $BK^{-/-}$ (n=5 mice) than in wt (n=6 mice) muscle strips: 7.3±2% in $BK^{-/-}$ strips versus 1.2±0.8% in wt strips (P=0.01) at 20 mM KCl, and 49±6.8% in $BK^{-/-}$ strips versus 21.2±3.9 % in wt strips (P=0.005) at 40 mM KCl (phasic contractions were normalized for each strip to the phasic contraction induced by 60 mM KCl; the presented values represent the means of the normalized amplitudes) (Fig.14). There was, however, no difference in the tonic component of the KCl induced contractions between wt (n=6 mice) and $BK^{-/-}$ (n=5 mice) strips. At 20 mM KCl the amplitude of the tonic contraction was 3.4±2.8% in wt and 11.2±3.5% in $BK^{-/-}$ mice (n. s.); at 40 mM the amplitudes were 23.1±4.8% in wt versus 27.3±4.5% in $BK^{-/-}$ strips (n. s.), and at 60 mM KCl the amplitudes were 85.1±4.2% in wt versus 74.6±3.8% in $BK^{-/-}$ bladders (n. s.) (tonic contractions were normalized for each strip to the tonic contraction induced by 80 mM KCl; the presented values represent the means of the normalized amplitudes).

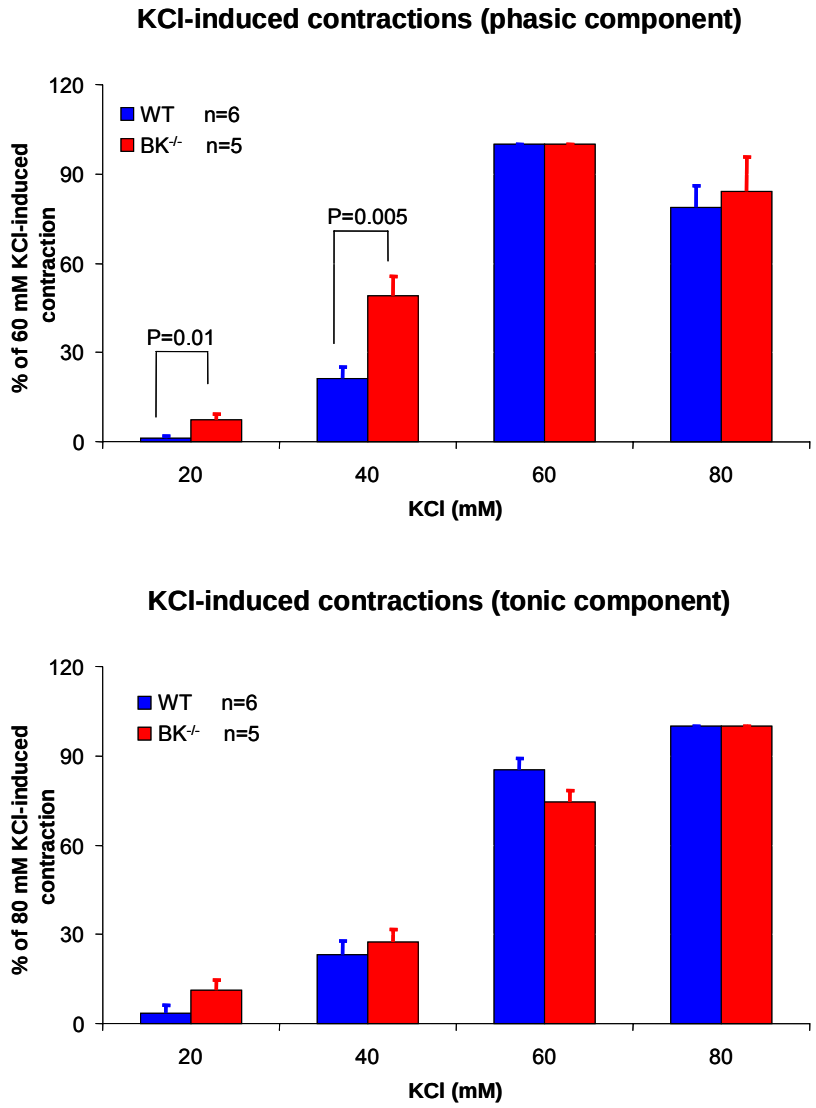


Fig.14 *Statistics of the phasic and tonic components of bladder strips contractions following KCl-stimulation. Increasing concentrations of KCl have been applied by replacing the bath solution. The amplitudes of the phasic and tonic components have been normalized to the amplitude of the 60 mM KCl-induced phasic contraction and, respectively, 80 mM KCl-induced tonic contraction; n is the number of different mice used in the study.*

3.2.4 Electrical field stimulation-induced contraction in wild type and $BK^{-/-}$ detrusor and its modulation by cGMP

Electrical field stimulation (EFS) elicits bladder contraction by releasing neurotransmitters from nerve endings in the bladder body (Longhurst & Uvelius, 2001). EFS with increasing frequencies were applied to wt and $BK^{-/-}$ detrusor strips

with intact urothelium, and the initial phasic component of contraction was analyzed. For each trace the amplitudes of the initial phasic contractions at different EFS frequency were normalized to the amplitude obtained at 30 Hz. Normalized contractions were more pronounced in BK channels deficient strips compared to wt strips at frequencies between 2-20 Hz, and the maximal contraction was obtained at lower EFS frequency in $BK^{-/-}$ as in wt bladder strips (Fig. 15; Table 1). When the maximal phasic contraction of every strip was normalized to the mass of the strip a significant difference was obtained between the $BK^{-/-}$ and the wt bladders: 2.7±0.5 mN/mg in $BK^{-/-}$ strips (n=12 strips) versus 1.3±0.4 mN/mg in wt strips (n=8 strips) (P=0.049). Thus, following electrical field stimulation the BK channels deficient bladder can develop a higher force per mass unit as the wt bladder.

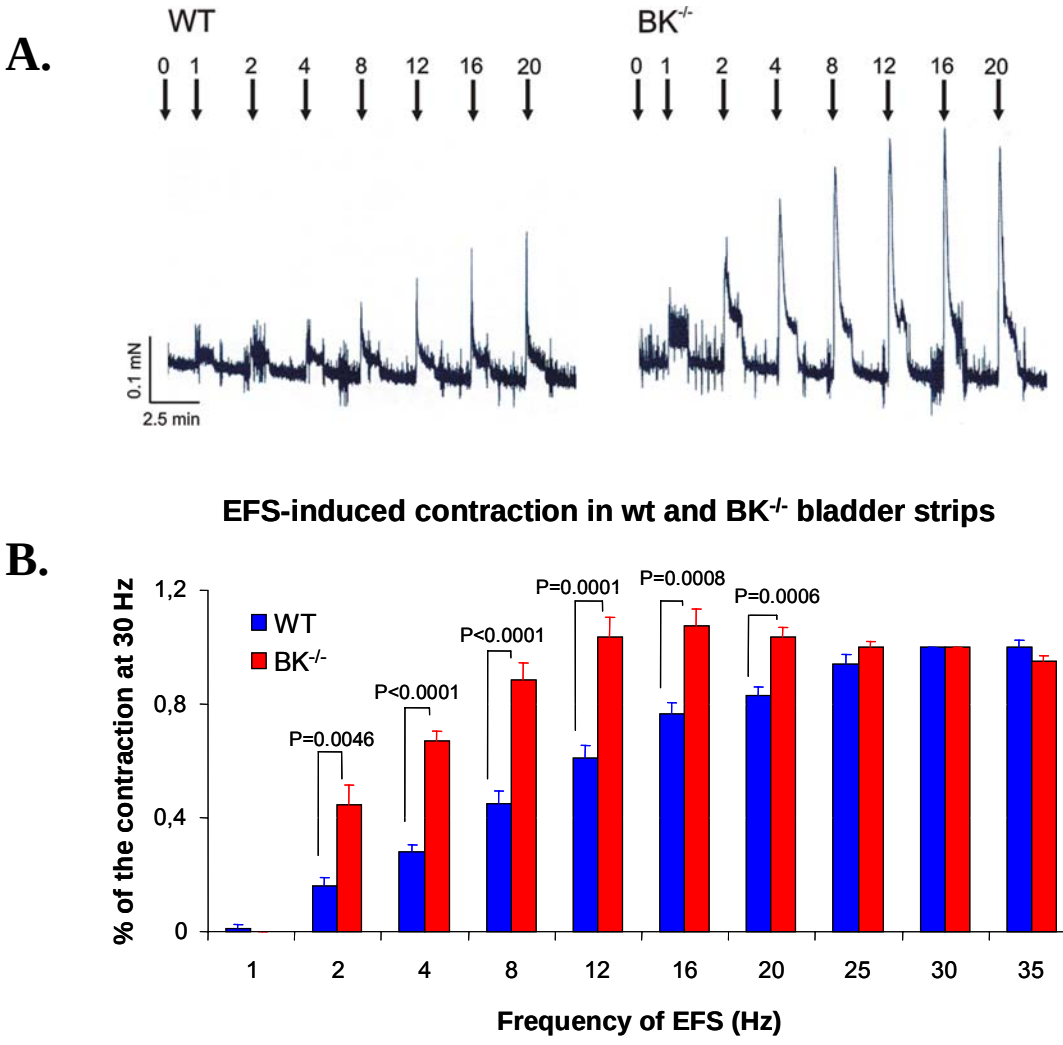


Fig. 15 *Electrical field stimulation (EFS) induced contractions in wt and $BK^{-/-}$ bladder strips. In (A) representative traces are presented; arrows mark the application of EFS of different frequencies. In (B) the statistical analysis is presented; the amplitude of the initial phasic contractions was normalized to the amplitude obtained at 30 Hz; n=9 strips from 3 wt mice and n=12 strips from 3 $BK^{-/-}$ mice. The means of the normalized values of contractions are presented in Table 1.*

Table 1. *Means of the normalized values (see Fig. 15) of EFS-induced contractions in wt and BK$^{-/-}$ bladder strips; values are expressed as percent.*

	EFS Frequency										
	0.5	**1**	**2**	**4**	**8**	**12**	**16**	**20**	**25**	**30**	**35**
WT	0	0.01± 0.01	0.16± 0.03	0.28± 0.03	0.45± 0.04	0.61± 0.04	0.77± 0.04	0.83± 0.03	0.94± 0.03	1	1± 0.02
BK$^{-/-}$	0	0	0.44± 0.07	0.67± 0.04	0.88± 0.06	1.03± 0.07	1.07± 0.06	1.03± 0.04	1± 0.02	1	0.95± 0.02
P		n.s.	0.005	$<10^{-4}$	$<10^{-4}$	$2*10^{-4}$	$8*10^{-4}$	$6*10^{-4}$	n.s.		n.s.

To ensure that the response to EFS was neurogenic and not myogenic in origin, the same experiment was repeated in the presence of tetrodotoxin. Tetrodotoxin inhibited 80-90% of the initial contractility in wt as well as in BK$^{-/-}$ strips, showing that the main part of the EFS-evoked response is nerve mediated. The EFS-induced contraction in the presence of tetrodotoxin was similar in wt and in BK$^{-/-}$ bladder strips (Table 2).

Table 2. *Means of the normalized amplitudes of EFS-induced contractions in the presence of tetrodotoxin; for every strip contractions amplitudes were normalized to the amplitude of the contraction at 30 Hz in the absence of tetrodotoxin; values are expressed as percent.*

	EFS Frequency										
	0.5	**1**	**2**	**4**	**8**	**12**	**16**	**20**	**25**	**30**	**35**
WT	0	0.02 ±0.02	0.03± 0.02	0.03± 0.02	0.04± 0.03	0.07± 0.04	0.09± 0.04	0.13± 0.05	0.16± 0.07	0.17± 0.05	0.18± 0.06
BK$^{-/-}$	0	0	0	0	0	0	0.02± 0.01	0.05± 0.06	0.06± 0.02	0.07± 0.02	0.09± 0.03
P		n.s.	n.s.	n.s.	n.s.	0.043	n.s.	n.s	n.s.	n.s.	n.s.

In order to asses the effect of cGMP on EFS-induced contractions consecutive dose-response measurements were performed, first in the absence and afterwards in the presence of 100 µM 8-pCPT-cGMP (see also page 23). In BK$^{-/-}$ bladder strips cGMP determined the inhibition of EFS-induced contractions by about 50%

compared to the contractions induced by EFS in the absence of cGMP (Table 3, Fig. 16). In wt strips EFS-induced contractions were unaffected by cGMP.

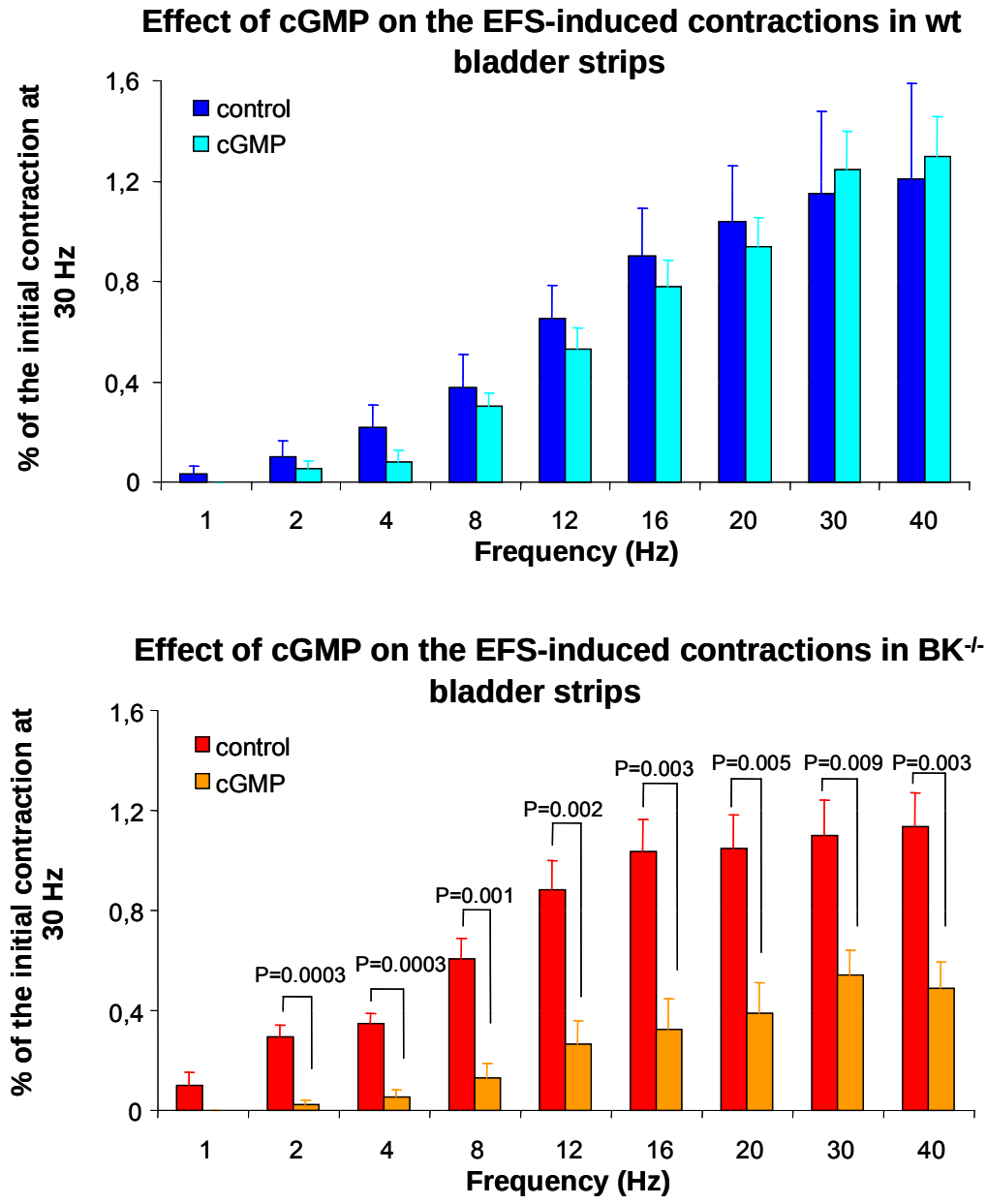


Fig 16. *The effect of cGMP on EFS-induced contractions in wt and BK$^{-/-}$ bladder strips. Only the values of the second dose-response measurements are presented; for every strip the contractions were normalized to the amplitude of the contraction at 30 Hz from the first dose-response measurements. Control strips represent wt or BK$^{-/-}$ bladder strips to which no 8-pCPT-cGMP was applied; n=5 wt strips from 3 mice to which cGMP was applied, n=4 wt control strips from 2 mice, n=6 BK$^{-/-}$ strips from 3 mice to which cGMP was applied and n=6 BK$^{-/-}$ control strips from 3 mice. The means of the normalized values of contractions are presented in Table3.*

Table 3. *Means of the normalized values (see Fig. 15) of EFS-induced contractions during the second dose-response measurements in wt and BK$^{-/-}$ bladder strips in the presence or in the absence of cGMP*

	EFS Frequency (Hz)								
	1	**2**	**4**	**8**	**12**	**16**	**20**	**30**	**40**
WT Control	0.03± 0.03	0.1± 0.06	0.22± 0.09	0.37± 0.13	0.65± 0.14	0.9± 0.19	1.04± 0.22	1.15± 0.33	1.21± 0.38
WT cGMP	0	0.05± 0.03	0.08± 0.05	0.3± 0.05	0.53± 0.08	0.78± 0.11	0.94± 0.12	1.25± 0.15	1.3± 0.16
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BK$^{-/-}$ Control	0.1± 0.05	0.3± 0.05	0.34± 0.05	0.6± 0.09	0.88± 0.11	1.03± 0.13	1.05± 0.13	1.1± 0.14	1.14± 0.13
BK$^{-/-}$ cGMP	0	0.02± 0.02	0.05± 0.03	0.13± 0.06	0.26± 0.1	0.33± 0.12	0.39± 0.12	0.54± 0.1	0.49± 0.11
P	n.s.	0.0003	0.0003	0.001	0.002	0.003	0.005	0.009	0.003

3.2.5 The effect of cGMP and cAMP on precontracted bladder strips

To test the effect of cGMP on the urinary bladder, detrusor muscle strips with intact urothelium from wt and BK$^{-/-}$ mice were firstly stimulated with 10μM carbachol, a concentration that elicits maximal phasic and tonic responses. After the muscles reached a steady-state tonic contraction, concentrations of 1 μM, 10 μM and 100 μM 8-pCPT-cGMP, a soluble analog of cGMP, were applied to the bath. The amplitude of the tonic contraction before the application of the lowest concentration of the cGMP analog was considered 100% contraction and the difference between 100% contraction and the percent contraction after the application of 8-pCPT-cGMP gave the percent of relaxation. Control experiments were also performed with bladder strips from different wt and BK$^{-/-}$ mice in order to asses the rundown of the tonic contraction. In these experiments the relaxation was determined at the same time points as in the cGMP experiments.

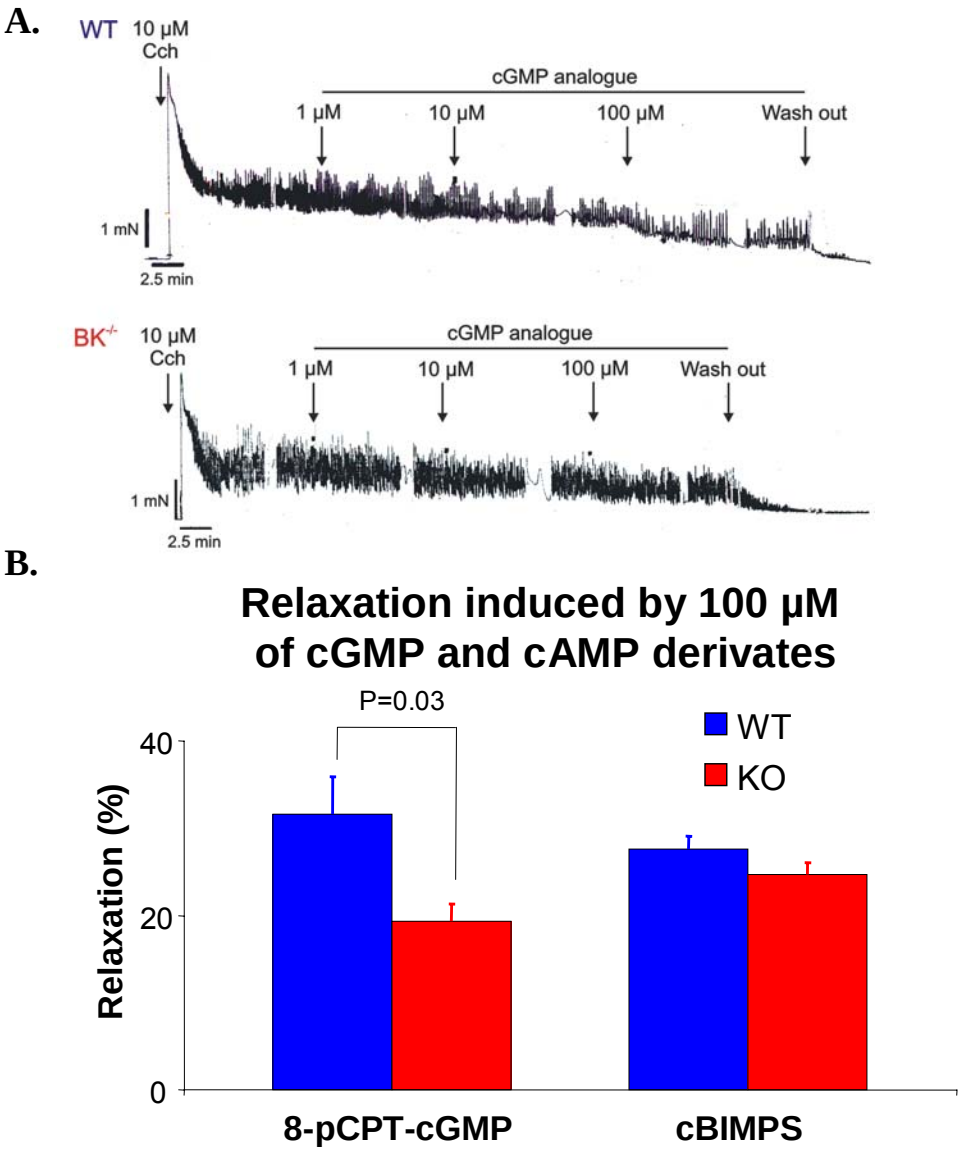


Fig.17 *Representative traces (A) and statistics (B) of the cGMP and cAMP effects on wt and BK$^{-/-}$ detrusor muscle strips. Cyclic GMP and cAMP analogs were applied cumulatively during the tonic component of carbachol-induced contraction. The percent of relaxation was calculated as the difference between 100% contraction and the percent contraction after the application of 8-pCPT-cGMP or cBIMPS. Because 1 μM and 10 μM of 8-Br-cGMP and cBIMPS determined no relaxation, only the effects induced by 100 μM 8-Br-cGMP and by 100 μM cBIMPS are presented in the diagrams. The amplitude of the tonic contraction before the application of 100 μM cGMP or 100μM cAMP derivates was considered 100% contraction; n=6 wt mice and n=5 BK$^{-/-}$ mice used in the cGMP experiments, and n=4 wt mice and n=5 BK$^{-/-}$ mice used in the cAMP experiments.*

The relaxation after the application of 1 μM and 10 μM cGMP analog was similar in wt (n=6 mice) (13.3±1.8% and 28.7±4.1%, respectively) and BK$^{-/-}$ bladder strips (n= 5 mice) (16.8±1.2% and 30.2±3%, respectively), but was also similar with the "relaxation" (rundown) in control wt strips (n=5 mice) (16.3±3.9% and 25.5±4.6%,

respectively) and control $BK^{-/-}$ strips (n=5 mice) (15.9±3.4% and 24.9±6.6%, respectively). This suggests that low concentrations of cGMP have no effect on the tonic contraction. A significant difference between the control and the cGMP experiments was obtained only at concentrations of 100 µM 8-pCPT-cGMP and only for wt bladder strips: 51.5±3.7% in wt (n=6 mice) versus 30.4±3.6% in wt control (n=5 mice) (P=0.003), but 43.5±3% in $BK^{-/-}$ (n= 5 mice) and 30.5±6.5% in $BK^{-/-}$ control (n=5 mice) (n. s.). However, the relaxation in wt was not significant different from the relaxation in $BK^{-/-}$ bladder strips. Nevertheless, when the amplitude of the tonic contraction before the application of 100 µM 8-pCPT-cGMP was considered as 100% contraction (and thus the rundown effect during the application of low concentrations of cGMP agonists was neglected), a significant difference between the relaxation in wt and $BK^{-/-}$ strips was obtained: 31.6±4.3% in wt (n=6 mice) and only 19.3±2% in $BK^{-/-}$ (n=5 mice) (P=0.03). The rundown effect during the application of 100 µM 8-pCPT-cGMP, as assessed in control experiments, was significantly different from the relaxation induced by the cGMP derivate: 5.9±3.6% in wt and 7.6±1.6% in $BK^{-/-}$ bladder strips. Thus, when the rundown of the tonic contraction during the application of low cGMP concentrations was not neglected, the relatively high rundown masked the significant but relatively small difference in relaxation between wt and $BK^{-/-}$ strips. These data suggest that the cGMP-induced relaxation of the carbachol-precontracted detrusor smooth muscle is mediated in part by BK channels.

The importance of BK channels for the cAMP/cAMP kinase-induced relaxation of the detrusor was investigated as well. Similar experiments as before were performed by using increasing concentrations (1 µM, 10 µM and 100 µM) of the soluble analog of cyclic AMP, cBIMPS. As in the cGMP experiments only 100 µM concentrations of the cAMP analog determined increased relaxation compared to control experiments, which were performed on bladder strips from the same mice as those used in the cAMP experiments. When as 100% contraction was considered the amplitude of the tonic contraction before the application of the lowest concentration of the cAMP analog, 100 µM cBIMPS determined a 49.5±2.8% relaxation in wt strips (n=4 mice) while in wt control (n=5 mice) only a 30.4±3.6% relaxation (P=0.005) was measured. In $BK^{-/-}$ strips (n=5 mice) 100 µM cBIMPS induced a 53±1.9% relaxation which was significant different from the 30.5±6.5% relaxation measured in $BK^{-/-}$ control strips (n=5 mice) (P=0.01). There was no difference between the relaxations induced by 100 µM cBIMPS in wt and $BK^{-/-}$ bladder strips. Also when the amplitude of

the tonic contraction before the application of 100 μM cBIMPS was considered as 100% contraction (in order to eliminate the rundown effect during the application of low cBIMPS concentrations) no significant difference between the relaxation of wt and $BK^{-/-}$ detrusor was obtained: 27.6±1.5% in wt (n=4 mice) and 24.7±1.3% in $BK^{-/-}$ (n=5 mice) bladder strips. These values were significant higher than the rundown of the tonic contraction during the application of 100 μM cBIMPS, as determined in control experiments (5.9±3.6% in wt and 7.6±1.6% in $BK^{-/-}$ bladder strips). Therefore, BK channels appear to be not important for the cAMP-induced relaxation of the carbachol-precontracted detrusor smooth muscles.

3.2.6 The effect of cGMP and cAMP on the rhythmical contractions of the detrusor

The analogs of cGMP and cAMP, 8-pCPT-cGMP and cBIMPS, respectively, had similar effects on the rhythmical contractions of bladder strips. Both of them decreased the mean amplitude of the carbachol-induced rhythmical contractions in $BK^{-/-}$ mice to a similar value as in wt, without affecting it in wt (Fig.18). 100 μM 8-pCPT-cGMP reduced the mean normalized amplitude of rhythmical contractions from 25.9±1.4% to 13±1.2% in $BK^{-/-}$ strips (n=5 strips; P=0.0002), while the amplitude in wt remained unaffected (11.9±1.8% before and 14±2.9% after 8-pCPT-cGMP application, respectively; n=7 strips, n.s.). In $BK^{-/-}$ bladder strips 100 μM cBIMPS decreased the normalized amplitude of rhythmical contractions from 27.4±3.5% to 12.75±1.2% (n=9 strips; P=0.001). In wt detrusor the amplitude was not significantly affected by the cAMP analog (11.5±2.1% before and 7.5±2.4% after cBIMPS application, respectively; n=6 strips, n.s.).

The frequency of rhythmical contractions was reduced by cyclic nucleotides analogs only in wt strips. 100 μM 8-pCPT-cGMP decreased the contraction frequency in wt bladders from 9.5±0.7 to 5.7±0.8 contractions per minute (n=6 strips; P=0.006). 100 μM cBIMPS had a similar effect: it reduced the frequency of bladder contraction in wt from 9.4±0.5 to 7.06±0.9 contractions per minute (n=6 strips, P=0.046). In $BK^{-/-}$ detrusor the frequency of rhythmical contractions was not significantly affected by the nucleotides analogs [11.4±0.6 contractions per minute before and 13.3±1 contractions per minute after 8-pCPT-cGMP application (n=5

strips; n.s.), and 10.9±0.7 contractions per minute before and 11.5±0.9 contractions per minute after cBIMPS application (n=9 strips, n.s.)] (Fig.19).

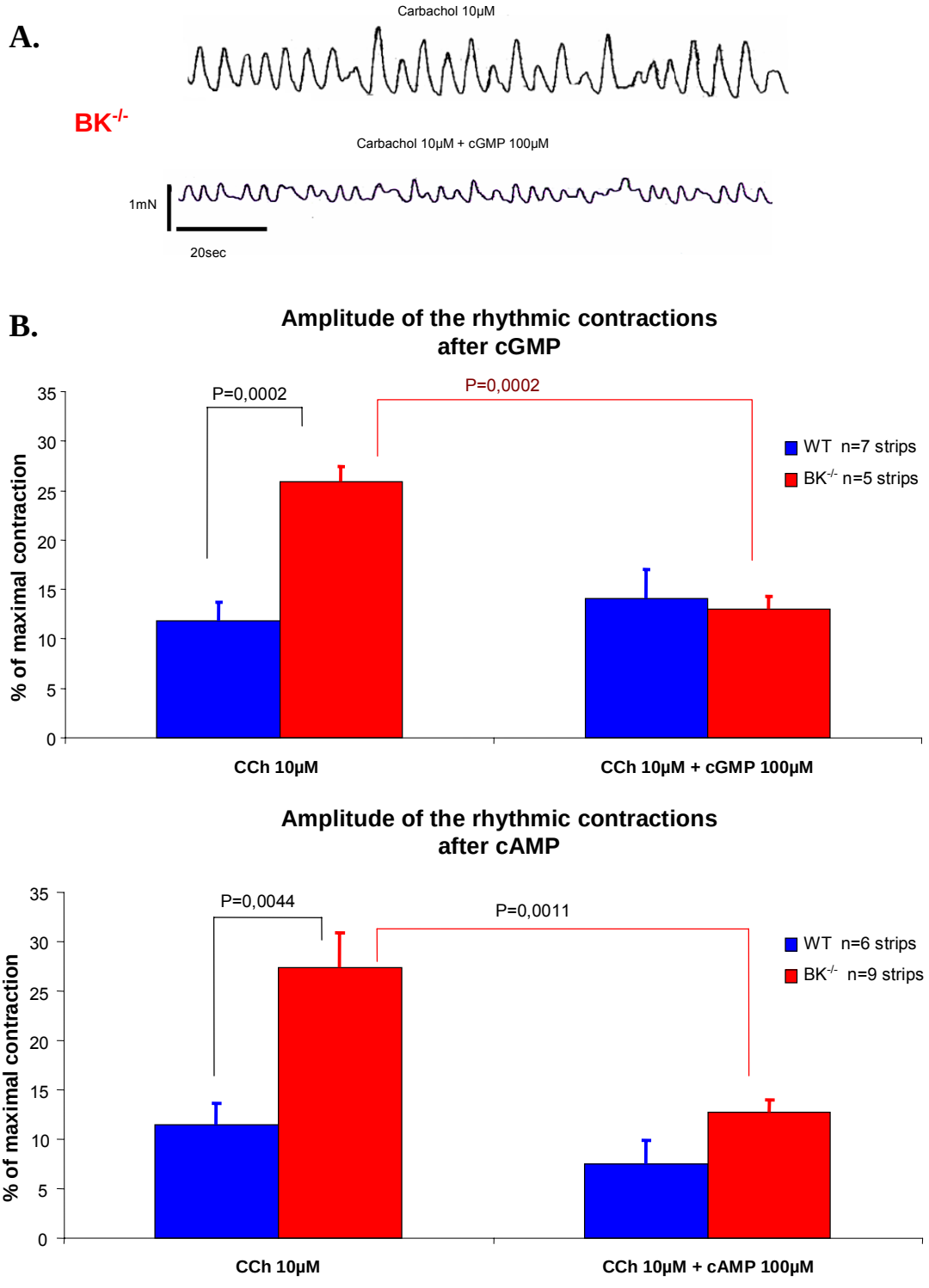


Fig.18 *Representative traces for BK$^{-/-}$ strips (A) and statistics (B) of the cGMP and cAMP effects on carbachol-induced rhythmical contractions* ***amplitude*** *in wt and BK$^{-/-}$ detrusor muscle strips. The amplitude of rhythmical contractions was normalized to the amplitude of the initial phasic component of the 10 μM carbachol-induced contraction as in figure 17; n is the number of strips used for the statistic.*

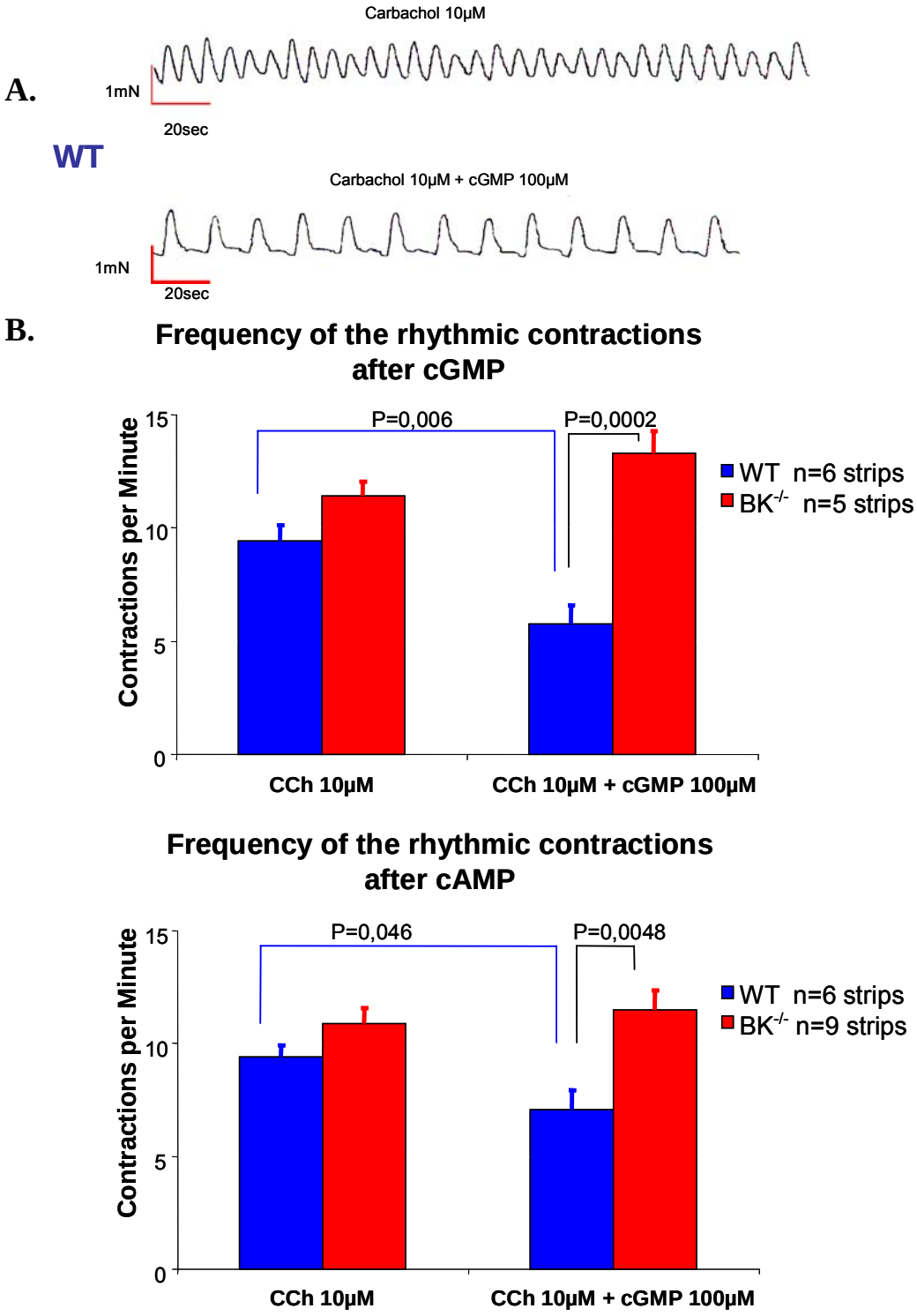


Fig.19 *Representative traces for wt strips (A) and statistics (B) of the cGMP and cAMP effects on carbachol-induced rhythmical contraction* ***frequency*** *in wt and BK$^{-/-}$ detrusor muscle strips. The frequency of rhythmical contractions was assessed over a period of 3 minutes; n is the number of strips used for the statistic.*

4. Discussion

4.1. The role and activation mechanisms of BK channels in vascular smooth muscle cells

The tone of vascular smooth muscle, especially from small arteries, controls the resistance to blood flow, and thus the blood pressure. The intracellular free Ca^{2+} concentration determines the tone of smooth muscles (Filo et al., 1965). Therefore, mechanisms that control the Ca^{2+} homeostasis in vascular cells play an important role in the regulation of blood pressure. BK channels are postulated to be essential for maintaining arterial tone (Brayden & Nelson, 1992; Jaggar et al., 1998 b; Plüger et al., 2000). *In vitro* evidence suggests that BK channels limit Ca^{2+} entry by hyperpolarizing the cells and closing voltage-dependent Ca^{2+} channels (Brayden & Nelson, 1992). However, the importance and the exact regulatory mechanisms of BK channels in vascular smooth muscle cells remain yet unclear. In this study, the role of BK channels in the regulation of Ca^{2+} homeostasis of vascular muscles was examined. The results show that BK channels are effectors of the NO/cGMP signaling cascade in vascular cells. It was also indubitably confirmed that BK channels carry the Ca^{2+} sparks-induced STOCs to determine a steady hyperpolarization of the cellular membrane. The lack of these mechanisms causes probably the enhanced systemic blood pressure of $BK^{-/-}$ mice (Sausbier et al., 2003).

Ca^{2+} measurements performed in this study show that there are no differences between wild type and $BK^{-/-}$ aortic cells regarding the basal Ca^{2+} concentration (Fig. 8), although BK channels deficient cells are more depolarized than control cells (Sausbier et al, 2003). Therefore it appears that in resting vascular smooth muscles the Ca^{2+} homeostasis is not affected by the lack of BK channels. Further, the effect of BK channel deficiency on cytoplasmic [Ca^{2+}] during agonist-induced smooth muscle contraction was analyzed. α_1 adrenergic receptor agonists cause both Ca^{2+} release from the sarcoplasmatic reticulum (over the phospholipase C - IP_3 pathway) and Ca^{2+} influx via Ca^{2+} channels (due to membrane depolarization) (Miriel et al., 1999; Furutani et al. 2002, Moosmang et al., 2003). One hypothesis tested in this study affirmed that the absence of BK channels will prolong the membrane depolarisation

and will increase the Ca^{2+} amount entering the cells during the α_1 agonist stimulation. There were, however, no differences between the phenylephrine-induced [Ca^{2+}] transients of wt and $BK^{-/-}$ mice, suggesting that BK channels play normally a minor role in this process. It is known that BK channels can be activated by global or local ("sparks") increase in Ca^{2+}, and that sparks and STOCs frequencies and amplitudes increase during depolarization (Jaggar et al., 1988 a; see Fig. 10 for the effect of depolarization on STOCs). However, Ca^{2+} sparks-activated STOCs are annihilated by agents that empty the cytoplasmic Ca^{2+} stores (Benham & Bolton, 1986; McCarron et al., 2002; Ohi et al., 2002), such as phenyleprine, suggesting that during α_1 agonist stimulation BK channels probably cannot be activated by Ca^{2+} sparks. This might be one possible explanation for the similar phenylephrine-induced [Ca^{2+}] transients in wt and $BK^{-/-}$ vascular muscle cells.

One physiological relevant mechanism for the activation of BK channels in vascular smooth muscle cells might be the NO/cGMP/cGK (cGMP kinase I) pathway. NO, as endothelium-derived relaxing factor (Ignarro et al., 1987, Palmer et al., 1987), plays an important role in vascular relaxation. It increases the cyclic GMP concentration via guanylyl cyclase in the cells (Vaandrager & de Jonge 1996) and activates therefore the cGMP-dependent protein kinase (Pfeifer et al., 1998). BK channels are targets of the cGMP protein kinase phosphorilation in vivo (Alioua et al., 1998), which increases the open probability of the channels (Furukawa et al. 1996). Application of 8-Br-cGMP reduces the phenylephrine-induced Ca^{2+} transients almost twice more pronounced in wt as in $BK^{-/-}$ vascular cells (see Fig. 9), showing that BK channels are implicated in the cGMP-mediated inhibition of Ca^{2+} transients. However, it seems that BK channels are not alone responsible for the suppression of Ca^{2+} transients by cyclic GMP. The inhibition of Ca^{2+} transients in $BK^{-/-}$ cells too suggests that there is at least a second target for the cyclic nucleotide in vascular smooth muscles, which can determine this effect. In the literature it is described that cGMP decreases IP_3-stimulated elevation in intracellular Ca^{2+} by direct phosphorylation of the IP_3-receptor (Komalavilas & Lincoln, 1996) or by the phosphorylation of IP_3-receptor-associated cGMP kinase substrat (IRAG) (Schlossmann et al., 2000). Thus, the reduction of Ca^{2+} transients in $BK^{-/-}$ aortic cells by cGMP is probably caused by the inhibition of the phenylephrine-induced Ca^{2+} release from intracellular stores.

In conclusion, it seems that, the NO/cGMP/cGK pathway regulates both Ca^{2+} release and Ca^{2+} influx in vascular smooth muscle cells. Figure 18 shows a scheme

of the probable mechanisms leading to the suppression of Ca^{2+} transients in vascular smooth muscle cells by cyclic GMP.

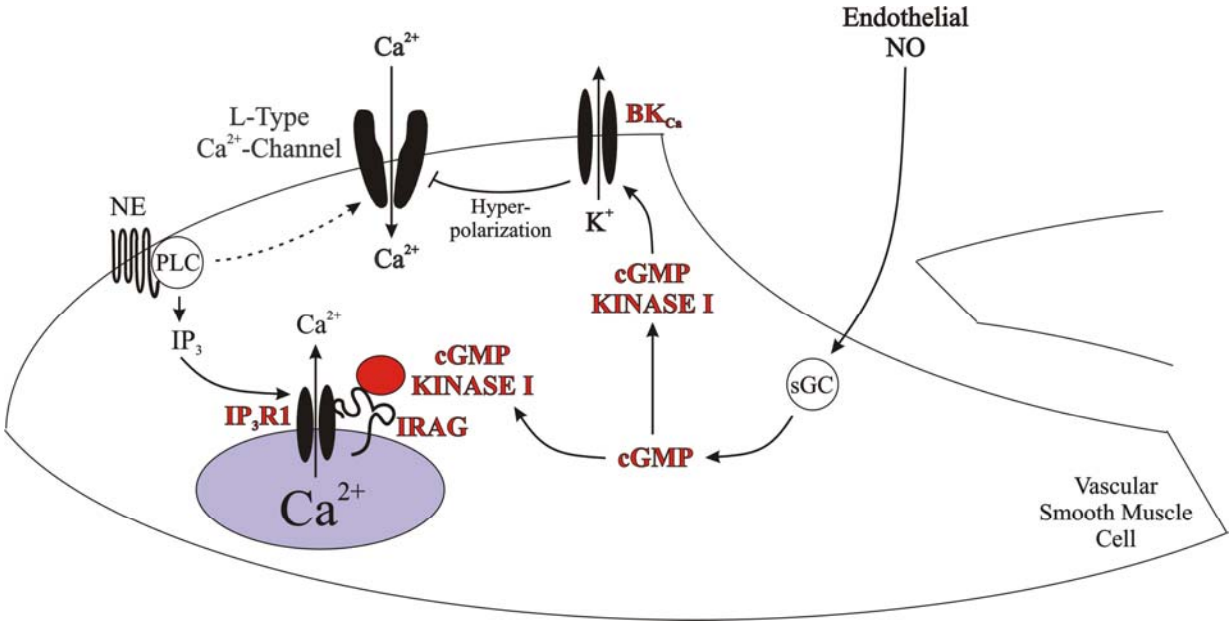


Fig.20 *Suggested mechanisms for the suppression of Ca^{2+} transients in aortic smooth muscle cells by cGMP; NE=norepinephrine, PLC=phospholipase C, IP_3=inositol triphosphate, IP_3R=IP_3 receptor, IRAG=IP_3-receptor-associated cGMP kinase substrate, BK_{Ca}=big conductance Ca^{2+} activated K^+ channel, sGC=soluble guanylyl cyclase; arrows=stimulatory effect, blunt arrows=inhibitory effect, dotted line=indirect effect.*

BK channels are believed to carry spontaneous transient outward currents (STOCs) in smooth muscle cells after being activated by Ca^{2+} sparks, i.e. highly localized, transient increases in Ca^{2+} concentration occurring near the plasma membrane (Benham & Bolton, 1986; Nelson et al., 1995) (see pages 9-10). However, the exact relationship between Ca^{2+} sparks, BK channels and STOCs is yet unclear. Disparities exist, for example, regarding the peak Ca^{2+} concentration of a Ca^{2+} spark measured with fluo3 (up to 0.5 µM) and the Ca^{2+} requirement for an increase in BK channel activity (local Ca^{2+} should reach levels of 10-100 µM) (Jaggar et al., 2000). Also, in vascular muscle cells, approximately 40% of the STOCs appear without detectable sparks (Perez et al., 1999), and the sparkless STOCs have smaller amplitude (Perez et al., 1999). Kong et al. (2000) and Bayguinov et al. (2000) described in colonic myocytes small-amplitude STOCs (Kong et al.) with resistance to the BK-channels blockers but sensitive to the small-conductance Ca^{2+} sensitive K^+ channels (SK)-inhibitor apamine. Since the Ca^{2+} concentration needed for the

activation of the SK channels is 10-100 nM, much lower than for BK channels, it is conceivable that SK channels might contribute to the generation of (sparkless) STOCs in vascular muscle cells.

STOCs were, however, completely absent in $BK^{-/-}$ muscle cells from cerebral arteries even at depolarized membrane potentials that promote Ca^{2+} influx via voltage-gated Ca^{2+} channels. Since Ca^{2+} sparks have been detected in both wt as in $BK^{-/-}$ cells the absence of STOCs cannot be attributed to the absence of Ca^{2+} sparks. Therefore, these results show clearly that STOCs are carried only by BK channels, at least in vascular smooth muscle cells from cerebral arteries.

By carrying STOCs, BK channels inhibit the myogenic tone in arteries (Nelson et al., 1995; Jaggar et al., 1998 b) and therefore limit the intra-arterial blood pressure. Myogenic tone represents the vascular graded constriction in response to increased intravascular pressure (Bayliss, 1902). It is developed in small resistance arteries, is independent of nervous stimulation and it is caused by graded membrane depolarization of the smooth muscle cells and activation of voltage-dependent Ca^{2+} channels (Harder, 1984; Brayden & Nelson, 1992; Meininger & Davis, 1992). Figure 21 provides a scheme of the contribution of BK channels-carried STOCs to the regulation of the myogenic tone.

It is established that RyR channels are activated by increases in cytoplasmic and SR Ca^{2+} concentration (Nelson & Nelson, 1990; Herrmann-Frank et al., 1991; Gregoire et al., 1993) (see also Fig. 21). It was reported (Jaggar et al., 1998 a) that in intact preparation of cerebral arteries, membrane depolarisation increases via Ca^{2+} influx the frequency and amplitude of Ca^{2+} sparks. However, in the experiments presented in this thesis amplitude, half time of decay, duration and frequency of Ca^{2+} sparks were similar in wt and $BK^{-/-}$ isolated muscle cells from cerebral arteries (Fig. 11), although $BK^{-/-}$ cells from the aorta were about 10 mV more depolarized than wild type cells (Sausbier et al., 2003). Nevertheless, as already shown, the Ca^{2+} homeostasis is not affected by the lack of BK channels in vascular smooth muscles at rest. Thus, at resting membrane potential the BK channel deficiency does not affect Ca^{2+} sparks characteristics, probably because it does not alter the cytoplasmic $[Ca^{2+}]$.

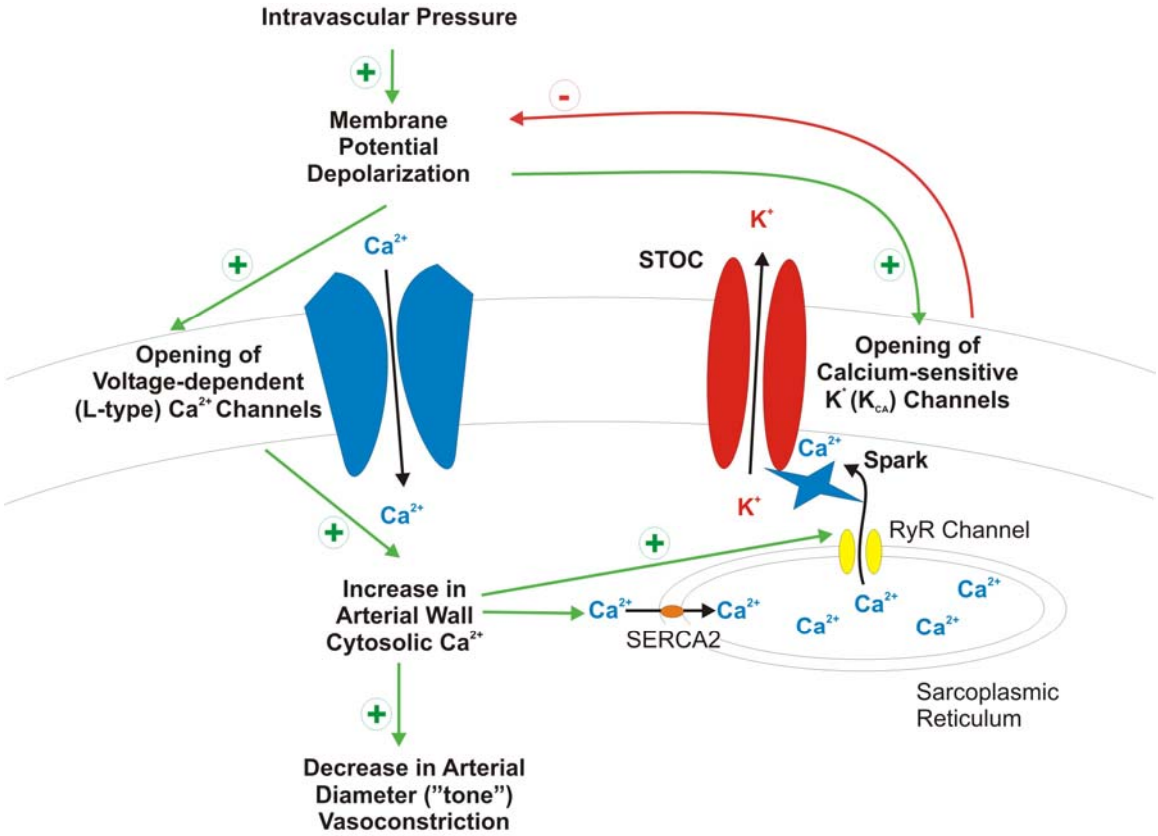


Fig.21 *Myogenic tone development and its regulation by BK channel-carried STOCs. The intravascular pressure causes membrane depolarization and the opening of voltage activated Ca^{2+} channels. The increase in cytoplasmic and sarcoplasmic Ca^{2+} stimulates ryanodine channels to release Ca^{2+} sparks. Ca^{2+} sparks determine transient outward currents by the opening of several BK channels.*

4.2. The role of BK channels in the urinary bladder

Dysfunctions regarding the activity of the bladder can determine clinical relevant symptoms, such as urinary incontinence, and therefore it is important to understand the mechanisms that control the detrusor activity. Large-conductance Ca^{2+} activated K^+ channels are present in the urinary bladder smooth muscles (Klöckner & Isenberg, 1985; Hashitani & Brading, 2003). As activated by both voltage and Ca^{2+}, BK channels are uniquely suited to serve as a Ca^{2+}-voltage signal integrator in the modulation of membrane potential and excitability (Petkov et al., 2001; Heppner et al., 1997). By controlling the membrane potential, BK channels control also the Ca^{2+} influx into the cells through voltage-gated Ca^{2+} channels, and, thus, the contraction and relaxation of the detrusor. The experiments presented in this thesis show that BK channels regulate agonist-induced, depolarization-induced and electrical field stimulation-induced contractions of the bladder, and are important for the control of spontaneous and agonist-induced bladder rhythmical contractility. Ca^{2+}-activated K^+ channels also mediate and regulate the effects of cyclic AMP and cyclic GMP on rhythmical contractions and on EFS-induced contractions of the detrusor. The results obtained in vitro can explain the increased micturition frequency and the elevated intravesical pressure of $BK^{-/-}$ mice detected in vivo (Sausbier et al., 2004). Thus, it appears that BK channels play a critical role in urinary bladder, by controlling its excitability and contractility.

Phasic carbachol-induced contractions (see pages 4-5) of the detrusor were not different between wild type and $BK^{-/-}$ (Fig. 12). However, the data about the tonic component of carbachol-induced contraction in wt and $BK^{-/-}$ muscle strips suggest a significant contribution of BK channels as a negative feedback mechanism to the tonic force development. Carbachol determines detrusor contraction mainly by binding to the M_3 receptors, which activates the inositol 1,4,5-triphosphate pathway (Matsui et al., 2000; Stengel et al., 2000; Longhurst & Uvelius, 2001). The phasic component of the muscarinic agonist-induced contraction is attributed to an IP_3-evoked release of Ca^{2+} from the sarcoplasmic reticulum, while the tonic component is ascribed to Ca^{2+} influx as a result of membrane depolarization (Baron et al., 1984; Somlyo et al., 1985; Kobayashi et al., 1989, Fry & Wu, 1998; McCarron et al., 2002). Therefore, the phasic component of contractions cannot be significantly regulated by

BK channels. However, during the tonic phase of contraction the lack of BK channels produces probably an increased depolarization in $BK^{-/-}$ compared to wild type bladder smooth muscles at similar muscarinic stimulus. This depolarization determines probably a higher Ca^{2+} influx and an increased tonic force development in $BK^{-/-}$ bladder strips.

Carbachol, by activating the IP_3 pathway, can also determine the depletion of intracellular Ca^{2+} stores, abolishing therefore Ca^{2+} sparks and consequently the BK channels carried STOCs (Benham & Bolton, 1986; McCarron et al., 2002; Wu et al., 2002). This effect would reduce the contribution of BK channels to the regulation of the membrane potential. As far I know STOCs inhibition was demonstrated only in studies where the Ca^{2+} releasing agonist was applied for a short period of time, which would correspond probably to the initial phasic contraction (McCarron et al., 2002; Wu et al., 2002). In the continuous presence of carbachol a partial refilling of Ca^{2+} stores might occur, in order to ensure the constancy of the contractile response. If so, during the tonic component of the carbachol-induced contraction the sarcoplasmic reticulum is possible to refill with Ca^{2+}, which would lead to the reapparition of Ca^{2+} sparks and therefore to an increased contribution of BK channels to the regulation of membrane potential and contractility of detrusor smooth muscles. Electrophysiological studies are needed in order to confirm or infirm this theory.

KCl stimulates smooth muscle to contract by depolarizing the membrane potential and facilitating Ca^{2+} influx (Hashitani & Brading, 2003; Trujillo et al., 2000). Application of increased extracellular K^+ to the bladder causes a biphasic contractile response (see pages 4-5) and a similar variation of intracellular free Ca^{2+} (Trujillo et al., 2000; Löfgren et al., 2003). In the experiments described in this study the phasic component of the KCl-induced contraction was significant higher in $BK^{-/-}$ versus wild type bladder strips, at submaximal KCl concentrations (Fig. 14), underlining the role of the BK channel as a negative feedback to membrane depolarization and Ca^{2+} influx. Although one might expect the maximal “phasic” force development to be obtained at lower KCl concentrations in BK channel deficient muscles, it was measured at the same KCl concentration in wt and $BK^{-/-}$ bladders (60mM). It is possible that the KCl steps that have been used in these experiments were too large for an accurate determination of the concentration that elicits maximal contraction.

Tonic KCl-induced contractions of the detrusor, however, were similar in wild type and $BK^{-/-}$ strips (Fig. 14). This was unexpected because there are no reasons to believe that the tonic contraction is caused by other factors than membrane depolarization. A possible explanation is provided below. During the KCl-induced contraction the cytoplasmic [Ca^{2+}] decreases from a peak, corresponding probably to the initial phasic contraction, to a steady state concentration, which might correspond to the tonic component of contraction (see Löfgren et al., 2003, and Trujillo et al., 2000, for Ca^{2+} measurements in phasic smooth muscles depolarized by KCl). It is well known that L-type Ca^{2+} channels inactivates in a [Ca^{2+}] and voltage dependent manner (Chad et al., 1984; Kalman et al., 1988, Hashitani and Brading, 2003). So, the decline in force and cytoplasmic [Ca^{2+}] during the tonic phase of contraction might be determined by the inactivation of Ca^{2+} channels, which might be similar in wt and $BK^{-/-}$ detrusor. Thus, it is possible that the number and/or opening time of Ca^{2+} channels during tonic contraction rather depends on the inactivation of Ca^{2+} channels than on the closing of the same channels via hyperpolarizing outward currents.

Increased extracellular [K^+] determines also a decrease in the driving force for K^+, which might reduce the currents flowing through K^+ channels. However, the significant difference between $BK^{-/-}$ and wt bladder strips regarding the phasic component of contractions, suggests that if the BK channels are open an outward K^+ current can flow through them also at high extracellular [K^+].

Electrical field stimulation (EFS) elicited a contractile response which was mediated mainly by neurotransmitter release from nerve terminals in the bladder body, since the application of tetrodotoxin caused its inhibition at all at low frequencies or by about 80-90% at high frequencies. The initial phasic contractions were more accentuated in BK channel deficient strips compared to control strips, and the maximal contraction was obtained at lower frequencies in $BK^{-/-}$ than in wt bladder strips. Also the maximal contraction per mass unit was higher in $BK^{-/-}$ detrusor. These results suggest an increased excitability and contractility of the $BK^{-/-}$ urinary bladder. Since the EFS-induced contraction in the presence of tetrodotoxin was absent at submaximal frequencies (lower than 16 Hz) in $BK^{-/-}$ strips and since the EFS-induced contractility in the presence of tetrodotoxin is similar in wt and $BK^{-/-}$ strips, the BK deficiency does not increase the sensitivity of the bladder muscles to the direct effect of EFS.

In other experiments of this study carbachol-induced initial phasic contraction were similar in wt and $BK^{-/-}$ strips (Fig. 12). This suggests that either EFS determines an increased release of acetylcholine in $BK^{-/-}$ detrusor, or that acetylcholine is not the only neurotransmitter that mediates contraction in mice bladder. Indeed, although in human bladder the nerve-mediated activity is exclusively cholinergic (Sibley, 1984), in small mammals non-adrenergic non-cholinergic (NANC) contractions predominate, and cholinergic contractions can be detected only when trains of stimuli are applied at high frequency (Sibley, 1984; Brading & Mostwin, 1989; Longhurst & Uvelius, 2001). ATP was demonstrated to be the major nonadrenergic, noncholinergic transmitter released from the bladder body after stimulation of the pelvic nerves and in many species responses to low frequency electrical field stimulation (0.5-8 Hz) results primarily from ATP release (Longhurst et al., 1984; Theobald, 1992; Tammela et al., 1994; Longhurst & Uvelius, 2001). In addition, in small mammals the initial phasic contraction determined by EFS is attributed mainly to ATP release from purinergic nerves (Sneddon & McLees, 1992; Longhurst & Uvelius, 2001). The contractile responses of bladder strips to ATP are mediated by $P2X_1$ receptors (Palea et al, 1994., 1995; McMurray et al., 1998), which are cation-selective channels that show little selectivity for sodium over potassium, and manifest a relatively high permeability to calcium (Evans et al., 1996). Activation of the purinergic receptors in the urinary bladder of small mammals initiate so called “excitatory junction potentials”, which open L-type Ca^{2+} channels and therefore trigger action potentials, transient increases in Ca^{2+} and contractions (Brading & Mostwin, 1989; Hashitani & Suzuki, 1995; Hashitani et al., 2000). In $BK^{-/-}$ mice the BK channel deficiency increases probably the open time and/or the number of opened L-type Ca^{2+} channels to cause more elevated phasic EFS-induced contractions than in wt bladder strips. Thus, the negative feedback via BK channels appears to be very important in the regulation of nerve-mediated bladder contractions.

Urinary bladder muscle strips generate spontaneous rhythmical contractions, which are the basis for the rhythmical contractility that occurs during the tonic phase of the carbachol-induced bladder contraction (Petkov et al., 2001). Action potentials determined by the opening of Ca^{2+} channels underlie, single or as bursts, the rhythmical contractions of the detrusor (Hashitani et al., 2001), which appear to be myogenic in nature (Liu et al., 1998; Herrera et al., 2000). Simultaneous

measurements of spontaneous actions potentials, intracellular Ca^{2+} and contractions could show a one to one relationship between action potentials or action potentials bursts, Ca^{2+} transients and mechanical activity of small bladder muscle strips (Hashitani et al, 2004). However, in large bladder strips preparations, as used in the experiments presented in this study, significant differences between the frequency of spontaneous action potentials and contractions have been reported (Herrera et al., 2000; Hashitani et al, 2001). These differences occurs because in the normal bladder the spontaneous contractions develop often locally and do not spread readily throughout the tissue (Hashitani et al., 2000), as a result of the low electrical coupling between detrusor smooth muscles (Bramich & Brading, 1996; Hashitani et al., 2001). Thus, stimulatory factors are needed for an integrated contraction over the entire muscle in larger bladder strips. This is probably also the explanation for the fact that in the experiments performed for this study spontaneous rhythmical contractions did not occur in unstimulated muscle strips from wild type mice, but they were present after carbachol addition to the bath. Therefore, the rhythmical contractility was analyzed only after carbachol stimulation. Nevertheless, unstimulated mechanical activity could be observed in muscle strips from $BK^{-/-}$ mice, suggesting an increased excitability.

The amplitude of carbachol-induced rhythmical contractions was more than doubled in $BK^{-/-}$ mice compared to control mice (Fig. 18). In addition, the frequency of rhythmical contractions was also significantly increased in BK knockout versus wt bladder muscles (Fig. 19). These data suggest an important role for the BK channels in the regulation of rhythmical contractions in the bladder, supporting and partially clarifying previous reports from the literature. Hashitani & Brading (2003 a) reported that blockers of BK channels, such as charybdotoxin and iberiotoxin, increased the amplitude of detrusor spontaneous rhythmical contractions more than twice, and also enhanced the amplitude and the duration of action potentials. In addition, afterhyperpolarizations which regulate the frequency of action potentials were abolished (Hashitani & Brading, 2003 a). Similar results have been obtained with bladder muscle strips from mice lacking the β1-subunit of the BK channel, which determine a higher Ca^{2+}/voltage sensitivity of the channel. The amplitude of the rhythmical contractions induced by 20 mM KCl or 1 μM carbachol was twice to four times higher in preparations from $BK\beta1^{-/-}$ versus control mice (Petkov et al., 2001).

There have been, however, contradictory reports considering the role of BK channels in the modulation of action potentials and rhythmical contractions frequency. In the experiments of Hashitani & Brading (2003 a), iberiotoxin or charybdotoxin determined an initial increase in the frequency of spontaneous action potentials, followed, during prolonged application, by a decrease to lower values as in controls. In accordance with this, BK channel blockers initially slightly depolarized the membrane potential, but eventually the membrane potential became slightly more negative than its original value (Hashitani & Brading, 2003 a). The spontaneous rhythmical contractions had a similar comportment; their frequency initially increased but then decreased below the control values when the BK channels blockers were applied over a long period (Hashitani & Brading, 2003 a). Herrera et al. (2000) described also a significant reduction of the spontaneous contractions frequency after iberiotoxin application. In the experiments of Petkov et al. (2001), 20mM KCl- and 1μM carbachol-induced rhythmical contractility had a lower frequency in bladder muscle strips from BKβ1$^{-/-}$ versus control mice, but a similar one as in iberiotoxin-treated wt muscle strips. On the other hand, Heppner et al. (1997) report an increment of action potential frequency and duration, and membrane depolarization after iberiotoxin treatment of bladder muscle strips, and Imai et al. (2001) describe a significant increase in the frequency of contractions after iberiotoxin treatment. These conflicting results may confirm the limitation of the chemical blockade of channels in obtaining an accurate image of the roles played by channels in biological processes.

The experiments presented in this thesis, which were performed on mice with genetic deletion of the BK channel, show however that BK channels have an inhibitory role on both the amplitude and the frequency of rhythmical contractions. The increased amplitude of carbachol-induced rhythmical contractions in BK$^{-/-}$ strips can be explained by an enhanced Ca^{2+} influx into the cells, because of the impaired L-type Ca^{2+} channels inactivation. This is probably reflected by an elevated amplitude and longer duration of single action potentials, but also by a possible increased number of electrical events occurring during a single burst of action potentials. The increased frequencies of the carbachol-induced rhythmical contractions indicate an enhanced excitability of the BK$^{-/-}$ detrusor, and are concordant with the described (Heppner et al., 1997) membrane depolarization and the elevation of action potential frequency after iberiotoxin treatment. It is worth to mention here that measurements performed on isolated bladder smooth muscle at rest could also show a significant

depolarization of BK channel deficient versus control cells (Sausbier et al., 2004), suggesting a role for BK channels in the regulation of the resting membrane potential. Following carbachol stimulation, the BK channel deficiency determines probably an enhanced depolarization of the membrane and a shortening of the interval between burst or single action potentials, to induce a higher frequency of rhythmical contractions. The present data underline therefore that BK channels are involved in the regulation of the membrane potential during and between action potentials. It seems that large conductance Ca^{2+}-activated K^+ channels not only regulate the amplitude and the duration of action potentials but also the occurrence of electrical events.

It is important to know the role of rhythmical contractility in the bladder functions, for a better understanding of the implications of the presented results. When integrated over the entire wall of the bladder via neuronal inhibitory or stimulatory input, the rhythmical contractions are proposed to maintain a level of tone that can be relaxed during filling, to allow the urine storage without an increased intraluminal pressure, or, respectively, can be synchronized to achieve voiding (Brading, 1997; Stevens et al. 1999). However, in some pathological conditions, such as 'idiopathic detrusor instability' (Brading & Turner, 1994; Brading, 1997) or 'bladder partial outlet obstruction' (Bing et al., 2002; Su et al., 2003), increased spontaneous contractions, caused partially by myogenic mechanisms (Park, 2000), determines an involuntary rise in intravesical pressure, which results in urinary incontinence. Thus, the enhanced rhythmical contractions amplitude and frequency in $BK^{-/-}$ bladders measured in vitro might probably explain the elevated bladder pressure and the increased micturition frequency of $BK^{-/-}$ mice (Sausbier et al., 2004). It seems that BK channels deficient mice manifest detrusor instability and urinary incontinence, which appears to be determined, at least partially, by a myogenic mechanism. This results show that changes in the status of smooth muscle may cause bladder overactivity and underline the importance of the detrusor in the generation of the instable bladder. If enhancement of the muscle excitability, as a result of BK channel deficiency, can determine urinary incontinence, it is conceivable that an opposite process, namely a decrease in the excitability by the stimulation of BK channels, can be a valuable alternative for the treatment of this symptom.

The activation of the cGMP and cAMP pathways determines smooth muscle relaxation (see chapter 1.2.2.) and both pathways are physiologically active in the

urinary bladder (for details see pages 7-8). The experiments presented in this thesis (Fig. 17) confirm the relaxing effects of cGMP and cAMP derivates on the bladder of normal mice. However, even high concentrations (100 µM) of the cAMP and cGMP analogs produced a relatively small relaxation of only 25-30% of the tonic contraction. The similar relaxation produced by cGMP and cAMP on wt detrusor strips might suggest an interaction between the two pathways (see also the similar effects on the rhythmical contractions, and see Bonnevier et al, 2003, and White et al., 2000, for examples of cross-activation of the two pathways in both directions). In BK channel deficient mice the relaxing effect of cGMP on the urinary bladder was reduced compared to wt, suggesting a role for BK channels in the cGMP-induced relaxation of the detrusor. Since cBIMPS had a similar effect on wt and $BK^{-/-}$ bladder strips, it seems that BK channels are not involved in the relaxation of the carbachol-induced tonic contraction via cAMP. Nevertheless this does not exclude an activation of the BK channels by the cAMP pathway but only a functional importance of this stimulation for the tonic contraction, at least in our experimental conditions (see the different effects of cBIMPS on rhythmical contractions in wt and $BK^{-/-}$ bladder, which are discussed below).

The effect of the cyclic nucleotides on the rhythmical activity of the bladder, which is superimposed over the tonic phase of the carbachol-induced contraction, suggests that BK channels in the detrusor are targets of both the cAMP and the cGMP pathway. Both agents had surprising and interesting effects on the frequency and on the amplitude of carbachol-induced rhythmical contractions in wild type and $BK^{-/-}$ mice. 100 µM 8pCPT-cGMP and 100 µM cBIMPS determined a reduction of the carbachol-induced rhythmical contraction frequency in wt muscle strips, without affecting it in $BK^{-/-}$ strips (Fig. 19). These data suggest that the cGMP- and cAMP-induced inhibition of contractions frequency in control mice is due to the activation of BK channels. The presented results are consistent with the report about the isoproterenol-induced reduction of the frequency of spontaneous action potentials and associated Ca^{2+} transients in detrusor smooth muscles of guinea pig (Nakahira et al., 2001) and are in accordance with the described activation of BK channels in bladder smooth muscle following the addition of cAMP increasing agents (Kobayashi et al., 2000). Hashitani et al. (2004) also registered a reduction of the frequency of spontaneous action potentials, Ca^{2+} transients and corresponding contractions in

guinea pig detrusor muscle strips after forskolin and 8Br-cGMP application. Thus, cyclic nucleotides can regulate the frequency of spontaneous and stimulated rhythmical contractions in the bladder and this occurs, at least partially, by activating the BK channels.

Neither cAMP nor cGMP affected the amplitude of the carbachol-induced rhythmical contractions in wild-type mice bladder strips. However, Hashitani et al. (2004) reported that forskolin and 8Br-cGMP suppress spontaneous contractions in isolated guinea-pig bladder muscle strips, and proposed the inhibition of the Rho kinase-induced Ca^{2+} sensitization (see pages 6 and 7) as an explanation for the cGMP and cAMP effects on the rhythmical contractions (see also Sauzeau et al, 2000; Somlyo & Somlyo, 2003). Therefore, it might appear somehow surprisingly that in the experiments presented in this thesis the amplitude of the carbachol-induced contractions was not affected by cyclic nucleotides in wt detrusor strips. Nevertheless, carbachol can antagonize, via a G_q-mediated stimulation of Rho kinase (Fukumoto et al., 2001; Chikumi et al., 2002; Wibberley et al., 2003) the reported cAMP- and cGMP-induced inhibition of Ca^{2+}-senzitivity and probably also the subsequent inhibition of the rhythmical contractility. On the other hand, cyclic nucleotides can also exert their relaxing effect on smooth muscles in a number of other ways, involving among others also the reduction of free cytoplasmic Ca^{2+} (Hashitani et al., 2004). However, although they caused a relaxation of the carbachol-induced tonic contraction and a reduction in the frequency of the rhythmical contractility, the two cyclic nucleotides have not decrease the amplitude of rhythmical contractions in wt bladders.

In $BK^{-/-}$ bladder strips application of cGMP and cAMP analogs reduces the amplitude of carbachol-induced rhythmical contractions to the same level as in wt. These results suggest that BK channel deficiency activates or up regulates cGMP and cAMP sensitive mechanisms in the detrusor, which are responsible for the elevated contractions. These mechanisms seem to be not active or do not have the same functional importance in wild type bladders. Some theories that may explain these results are provided below:

1. Ca^{2+} sensitization and the overexpressed Rho kinase apparently determine the increased spontaneous contractions registered in an animal model of urinary incontinence, namely the bladder outflow obstruction (Su et al, 2003; Bing et al., 2003). It has been reported as well that membrane depolarization activates Rho-

kinase (Mita et al., 2002; Sakurada et al, 2003). Since $BK^{-/-}$ detrusor smooth muscles are more depolarized than wild type muscles (Sausbier et al, 2004), an increased activation of the Rho kinase pathway and a stronger dependency of the rhythmical contractions on Ca^{2+} sensitization may be the case in these smooth muscle cells. Therefore, cAMP and cGMP might decrease the elevated Ca^{2+} sensitivity (see page 7; see also Kawada et al., 1997; Wu et al., 1998; Surks et al., 1999; Sauzeau et al., 2000; Komatsu et al., 2002; Somlyo & Somlyo, 2003; Hashitani et al., 2004) in $BK^{-/-}$ bladder muscles and lower the amplitude of rhythmical contractions to the same level as in wild type.

2. An increased Ca^{2+} influx is possible to occur in $BK^{-/-}$ detrusor during the action potentials, as a result of the impaired repolarization. The elevated Ca^{2+} entrance may determine Ca^{2+}-induced Ca^{2+} release (CICR) from some distinct Ca^{2+} pools that are active only in $BK^{-/-}$ bladder muscles, and that are sensitive to cAMP and cGMP. The inhibition of these stores by the cyclic nucleotides in BK channels deficient strips may cause the reduction in the rhythmical contractions amplitude. Participation of CICR to the generation of Ca^{2+} transients following actions potentials has been demonstrated in the detrusor (Imazumi et al, 1998; Hashitani et al, 2001; Ohi et al, 2001), and functional and morphological distinct Ca^{2+} stores have been as well described in many cells, inclusive smooth muscles (Golovina and Blaustein, 1997; Flynn et al., 2001).

3. Blockers of the IP_3 receptors, but also blockers of the phospholipase C have been shown to inhibit the spontaneous mechanical activity of the bladder (Imai et al., 2002; Tanaka et al., 2003; Hashitani et al., 2004; see also Tanaka et al., 1994; Valle-Rodriguez et al., 2003). Therefore, it is all the more conceivable that the IP_3 pathway plays an important role in the carbachol-induced rhythmical contractions (see also Hashitani et al., 2000, discussion). On the other hand, membrane depolarization, by activating the phospholipase C, increases the Ca^{2+}-releasing effect of agonists via IP_3 (Somlyo and Somlyo, 2003). It is established as well that elevated cytoplasmic Ca^{2+} activates the IP_3 receptors (McCarron et al., 2004)**.** Thus, because of the increased depolarization and Ca^{2+} influx, the contribution of the IP_3 pathway to the generation of rhythmical contractions is probably more important in $BK^{-/-}$ than in wild type bladder. Cyclic GMP kinase and cyclic AMP kinase can inhibit the IP_3 receptor by a direct phosphorylation (Komalavilas and Lincoln, 1996) and, in case of cGMP kinase, by the phosphorylation of the IP_3 receptor associated cGK substrate (IRAG)

(Schlossmann et al., 2000). In conclusion, the cyclic nucleotides-mediated reduction of rhythmical contractions amplitude in $BK^{-/-}$ bladder strips may relay, at least partially, on the inhibition of the IP_3 pathway.

Similar as in the case of rhythmical contractions, cGMP determined the inhibition of the initial phasic component of EFS-induced contraction only in $BK^{-/-}$ bladder strips without affecting it in wt strips (Fig. 16). These results confirm the fact that the BK channel deficiency activates some cGMP-sensitive mechanisms in the detrusor. The mainly determinant of the initial phasic contraction induced by electrical field stimulation is the release of ATP (Sneddon & McLees, 1992), which causes membrane depolarization, Ca^{2+} influx via L-type Ca^{2+} channels, but also Ca^{2+} release from intracellular stores probably through Ca^{2+}-induced Ca^{2+} release (Hashitani et al., 2000). Also a cholinergic contribution to the initial component of contraction can not be excluded at least at high EFS frequency (Longhurst & Uvelius, 2001). Thus, similar mechanisms as proposed for rhythmical contractions can also account for the inhibitory effect of cGMP on the phasic component of EFS-induced contraction in $BK^{-/-}$ bladder. The reduction of Ca^{2+} sensitivity, which can be more pronounced in $BK^{-/-}$ bladder, the inactivation of possible cGMP sensitive Ca^{2+} stores, which open only in the BK channel deficient detrusor, the inhibition of the IP_3 pathway, which can be more active in smooth muscles lacking BK channels, and/or some other mechanisms can explain the cGMP effects. However, further studies are necessary for confirming or infirming these theories.

5. Abstract

BK channels respond to elevations in intracellular calcium and membrane depolarization, and inhibit the excitability of smooth muscles. This study addressed the role of BK channels in arteries and urinary bladder by using mice with inactivated Slo1 gene. BK channel deficiency did not affected α1 agonist-induced Ca^{2+} transients in aortic cells, suggesting that BK channels normally play a minor role in this process. However, activation of BK channels by cGMP in wt cells determined a more pronounced inhibition of α1 agonist-induced Ca^{2+} transients as in $BK^{-/-}$ cells. It appears that the NO/cGMP signaling pathway regulates via BK channels the influx of Ca^{2+} into vascular cells. The fact that cGMP determines suppression of Ca^{2+} transients in $BK^{-/-}$ vascular cells too suggests that the cGMP effect is also mediated by BK channel-independent mechanisms. In $BK^{-/-}$ cells from cerebral arteries STOCs were completely absent even at depolarized membrane potentials, indicating that only BK channels carry STOCs in this tissue. Since Ca^{2+} sparks parameters were not affected by the BK channel deficiency a relationship between STOCs and Ca^{2+} sparks is unlikely to exist. Summarizing the vascular experiments, BK channels participate in at least two mechanisms involved in vascular smooth muscle relaxation: (1) they are effectors of the NO/cGMP signaling cascade, and (2) they carry Ca^{2+} sparks-induced STOCs. The lack of these mechanisms causes probably the enhanced systemic blood pressure of $BK^{-/-}$ mice (Sausbier et al., 2003).

In the urinary bladder, muscarinic submaximal stimulation of muscle strips determined increased tonic contractions in $BK^{-/-}$ versus wild type mice. The phasic component of the KCl-induced contraction was also significantly higher in $BK^{-/-}$ detrusor strips at submaximal KCl concentration. The electrical field stimulation-induced nerve-mediated phasic contraction of the bladder was, as well, more accentuated in BK channels deficient as in control strips, and the maximal contraction was obtained at a lower frequency in $BK^{-/-}$ as in wt bladder. Also the maximal force developed per mass unit after EFS was higher in $BK^{-/-}$ than in wt bladders. In addition, the amplitudes and frequencies of carbachol-induced rhythmical contractions were increased in BK channel deficient bladder muscles, and spontaneous rhythmical contractility occurred only in strips from $BK^{-/-}$ mice. All these results suggest enhanced excitability and contractility of the $BK^{-/-}$ detrusor. BK

channel deficiency did not affect the cAMP-induced relaxation of the carbachol-precontracted detrusor, but the relaxation induced by cGMP was more pronounced in wt than in $BK^{-/-}$ bladder strips, suggesting that the relaxing effect of cGMP is mediated in part by BK channels. Preincubation with cGMP determined the reduction by about a half of the amplitude of the initial phasic component of EFS-induced contraction in $BK^{-/-}$ bladder strips, but did not affect the contractions of wt bladder. In addition, both cAMP and cGMP reduced the amplitudes of carbachol-induced rhythmical contractions only in $BK^{-/-}$ muscles and to the same level as in wt, indicating that BK channel deficiency activates or upregulates cGMP and cAMP sensitive mechanisms in the detrusor, which are responsible for the higher rhythmical contractions. The cyclic nucleotides inhibited also the frequency of the rhythmical contractility, but only in wt muscle strips, suggesting that this effect is mediated by the activation of BK channels. Thus, it appears that BK channels have a prominent role in urinary bladder. The results obtained in vitro can probably explain the elevated bladder pressure and the enhanced micturition frequency of BK deficient mice (Sausbier et al., 2004).

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