

hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family

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Received 11 April 2000

Edited by Maurice Montal

Abstract We cloned two β subunits of large-conductance calcium-activated potassium (BK) channels, hKCNMB3 (BK β 1) and hKCNMB4 (BK β 4). Profiling mRNA expression showed that hKCNMB3 expression is enriched in testis and hKCNMB4 expression is very prominent in brain. We coexpressed BK channel α (BK α) and BK β 4 subunits *in vitro* in CHO cells. We compared BK α / β 4 mediated currents with those of smooth muscle BK α / β 1 channels. BK β 4 slowed activation kinetics more significantly, led to a steeper apparent calcium sensitivity, and shifted the voltage range of BK current activation to more negative potentials than BK β 1. BK α / β 4 channels were not blocked by 100 nM charybdotoxin or iberiotoxin, and were activated by 17 β -estradiol.

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Key words: Potassium channel; Calcium-activated; Accessory subunit; Expression pattern

1. Introduction

The large-conductance calcium-activated potassium (BK) channel is a member of the Shaker-related six transmembrane domain potassium channel superfamily that is sensitive to voltage and calcium. BK channels may occur as heterooligomeric complexes of poreforming BK α subunits [1,2] and auxiliary BK β subunits [3–6]. Association of BK β with BK α subunits may confer an increased Ca²⁺ sensitivity, different kinetic properties as well as a different pharmacology to BK channels. BK α mRNAs are nearly ubiquitously expressed in line with the detection of BK channels in many different tissues ranging from smooth muscle and kidney tubules to neurons in the central nervous system [7]. In contrast to BK α mRNA, the expression of BK β mRNAs appears to be restricted to few specific tissues [3,5,6]. E.g., BK β 1 subunits are predominantly expressed in smooth muscle cells, whereas BK β 2 subunits were mainly detected in endocrine tissue. However, BK β subunits, that matched the high expression levels of BK α mRNA in the central nervous system, had not been cloned.

Recently, it was shown that biochemically purified BK channels from brain are tightly associated with a small, as yet unidentified 25 kDa protein [8]. Most likely, the small associated protein represents a novel type of BK β subunit expressed in neuronal tissue. This observation suggested to us that like in smooth muscle cells, brain BK channels might consist of heteromultimeric assemblies containing BK α subunits and CNS-specific BK β subunits. Possibly, the CNS BK β subunits would be related in sequence and structure to the already known BK β 1 and BK β 2 subunits. Accordingly, a database search for EST clones potentially related to novel BK β subunits resulted in the cloning of two novel human BK β subunits (BK β 3, KCNMB3 and BK β 4, KCNMB4). We find that BK β 4 mRNA is highly expressed in human brain tissue and may contribute to BK channel diversity in brain. Comparably to BK β 1 [9–12], coexpression of BK α subunits with BK β 4 leads to slowed activation kinetics, increased Ca²⁺ sensitivity and an altered pharmacology of BK channels. So far, BK channel diversity has been attributed to alternative splicing of BK α subunit mRNA [13–17], to heteromeric assembly with other α -like subunits (slack) [18] and to various post-translational modification mechanisms [19–22]. When this manuscript was in preparation, comparable results were published for the cloning of BK β 3 and BK β 4 subunits and their expression in the *Xenopus* oocyte expression system [23].

2. Materials and methods

2.1. Identification and cloning of KCNMB subunits

The NCBI expressed sequence tag (EST) database was searched with the TBLASTN algorithm using the protein sequence of KCNMB1 as a query sequence. Three populations of EST sequences were identified and aligned using Lasergene DNASTar software (GATC, Konstanz, Germany). EST clones were obtained from Research Genetics (Huntsville, USA) and Resource Center of the German Human Genome Project (Berlin, Germany) respectively. EST AI309617 contained the complete open reading frame (ORF) of KCNMB1 [1]. EST clones AI299145 and AI301175 harbored the complete ORF of KCNMB2 [5]. EST clones AA761761 and AA236930 contained the incomplete ORF of KCNMB3. The KCNMB3 ORF was completed by RACE-PCR using the 5'-RACE system Version 2.0 (Life Technologies, Karlsruhe, Germany) and gene-specific primers. As template total RNA of human white matter was used. The sequence of the amplified product was used for the derivation of a primer to clone the putative 5'-end of the gene from human genomic DNA using Pfu Turbo Polymerase (Stratagene, Heidelberg, Germany). The derived KCNMB2 and KCNMB3 sequences were used as query sequences for further TBLASTN searches of the EST database. EST clone AA906027 contained an incomplete KCNMB4 open reading frame. It was completed by screening a commercial human cortex cDNA library (Clontech, Heidelberg, Germany) via PCR.

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$[G = G_{\max}/(1 + e^{(V-V_{1/2})/\text{slope}})]$ and then normalized to the maximum of the fit. EXCEL (Microsoft, Redmond, USA) and Igor Pro (WaveMetrics Inc., Lake Oswego, USA) were used for calculations and presentation of the data. Data are displayed as means $\pm$ S.E.M.

3. Results

3.1. Cloning of KCNMB3 and KCNMB4

A BLAST search in the NCBI-EST database identified several EST sequences which encoded novel BK β subunit sequences. Alignment with the known KCNMB1 [1,2] and KCNMB2 [5,6] sequences showed that the novel sequences represented KCNMB3 and, respectively, KCNMB4 in agree-

ment with recent reports [23,27]. The EST clones were used as probes to clone the complete open reading frames (ORFs) of KCNMB3 (BK β 3) and KCNMB4 (BK β 4). The two genes are localized to different chromosomal loci. The KCNMB3 sequence was previously mapped to a region of the human chromosome that is duplicated in the dup (3a) syndrome [27]. The KCNMB4 gene was localized to chromosome 12q14.1–12q15 by electronic PCR, which identified the STS marker A005A38 near the 3'-end of the last exon in the KCNMB4 ORF (see Section 2).

A sequence alignment of the BK β subunit family is shown in Fig. 1. Sequences between family members are only moderately conserved (21–43% identical residues). However, the

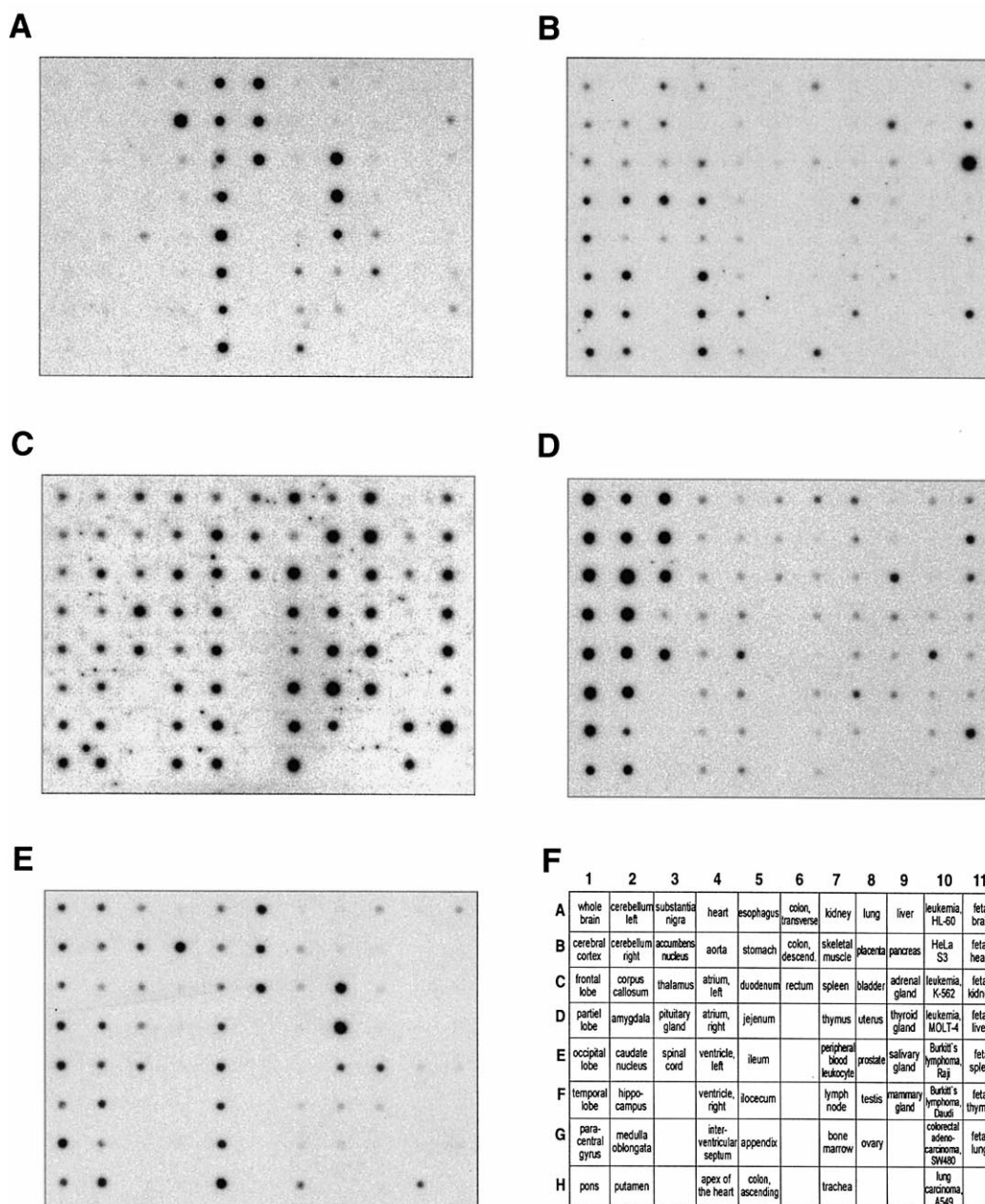


Fig. 2. Expression profiles of BK channel α and β subunits in human tissue. Multiple tissue expression arrays (MTETM, Clontech, Heidelberg, Germany) were hybridized with ³²P-labeled cDNA probes specific for BK channel β subunits KCNMB1 (A), KCNMB2 (B), KCNMB3 (C), KCNMB4 (D) and the BK channel α subunit KCNMA1 (E). Hybridization conditions and probes are described in Section 2. Autoradiographic exposure times were in A and D 2 days, B and E 9 days and in C 22 days. F: Arrangement of mRNA samples on multiple tissue expression arrays displayed in A–E.

structures show several motifs that have been conserved and may suggest that they are important for BK β subunit functions. The subunits have two homologous possibly membrane-spanning domains TM1 and TM2. Most likely, the amino- and carboxy-termini, which are not conserved, face the cytoplasm and the sequence between TM1 and TM2 with its consensus *N*-glycosylation sites represents an extracellular domain. Within this domain a characteristic pattern of four cysteine residues is recognized. By contrast, the sequence motif that has been correlated with the high-affinity charybdotoxin/iberiotoxin binding site of BK channels [28] has not been conserved and is absent in BK β 3 and BK β 4 (labeled with asterisks in Fig. 1).

3.2. Tissue distribution of BK β mRNA

We used commercial dot blots to compare the expression of BK β mRNAs in 76 different human tissues (Fig. 2). The results indicated that each BK β mRNA had a distinct pattern of expression. For comparison, we have included in this analysis BK α mRNA, which is expressed in every human tissue tested except heart atrium, ventricle and septum (Fig. 2E). Consistent with previous results [10,29] BK β 1 mRNA expression seems to be restricted to smooth muscle tissue, e.g. aorta, stomach, duodenum, ileum, colon, bladder and uterus (Fig. 2A). BK β 2 mRNA was strongly expressed in fetal kidney and to some extent in cardiac tissue, in pituitary gland, hippocampus and several other brain tissues (Fig. 2B). Of all four BK β subunits, BK β 3 mRNA seems to be least abundant. In case of the BK β 3 probe, relatively long exposure times were necessary to obtain hybridization signals of comparable intensity (Fig. 2C). By contrast, BK β 4 appears to be highly and specifically expressed in central nervous system tissue including spinal

cord, but not e.g. in pituitary gland and in non-neuronal tissue (Fig. 2D). The BK β 4 mRNA expression profile suggests that BK β 4 may represent a nervous system-specific β subunit of BK channels. Interestingly, biochemical purification of BK channels from brain indicated a BK β subunit of ~ 25 kDa molecular weight tightly associated with brain BK channel α subunits [8]. The derived size of BK β 4 protein (210 amino acid residues) is compatible with the notion that BK β 4 is a β subunit of neuronal BK channels. In multiple tissue Northern blots we have determined the transcript sizes of BK β 1–4 mRNAs (data not shown). In agreement with a previous report [23], BK β 4 hybridized strongly to a band of 1.9 kbp and to two minor bands of 3 kbp and 6.1 kbp in all brain tissues.

3.3. Expression of BK α with BK β 4 subunits

Since our hybridization results indicated that BK β 4 was most likely a prominent BK β subunit of neuronal BK channels, we have concentrated on the coexpression of BK α subunits with BK β 4 subunits in comparison to that with BK β 1 subunits. For this purpose, BK β 1 and BK β 4 cDNAs were cloned into pcDNA3 expression vectors (pcDNA3 KCNMB1; pcDNA3 KCNMB4). They were cotransfected into CHO cells with pcDNA3-hslo. 12–24 h post-transfection, we recorded Ca²⁺-activated outward currents from the transfected cells. Macroscopic currents were characterized using both whole-cell and inside-out patch-clamp configurations.

Representative current traces mediated by BK channels with or without β subunits are shown in Fig. 3. They were evoked at voltage pulses ranging from -80 mV to $+100$ mV in $0.8 \mu\text{M}$ internal Ca²⁺. In the absence of BK β subunits, BK α subunits (Fig. 3A,D) typically gave rise to relative rapidly activating biphasic outward currents ($\tau_{\text{fast}} = 1.2 \pm 0.2$ ms and

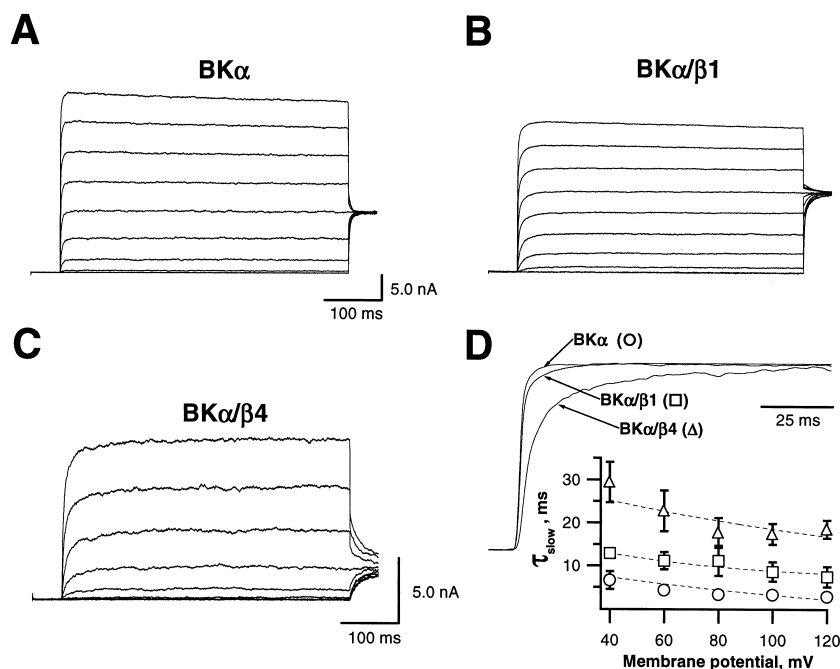


Fig. 3. Currents mediated by BK channels in the absence or presence of β subunits KCNMB1 and KCNMB4 in transfected tissue culture cells. A–C: Macroscopic currents were elicited in whole-cell patch-clamp experiments by stepping the membrane voltage from a holding potential of -80 mV to depolarized test potentials in $+20$ mV increments up to $+100$ mV. Cells were buffered to $0.8 \mu\text{M}$ Ca²⁺. Duration of test potentials was 500 ms. Tail currents were measured at $+40$ mV subsequent to the test potentials. A–C are labeled above the current traces for the subunits expressed. D: Voltage dependence of activation kinetics of BK α , BK α/β 1, BK α/β 4 mediated currents ($n = 3$ –5). For comparison, current traces elicited with a $+80$ mV test pulse are normalized to the maximum amplitude.

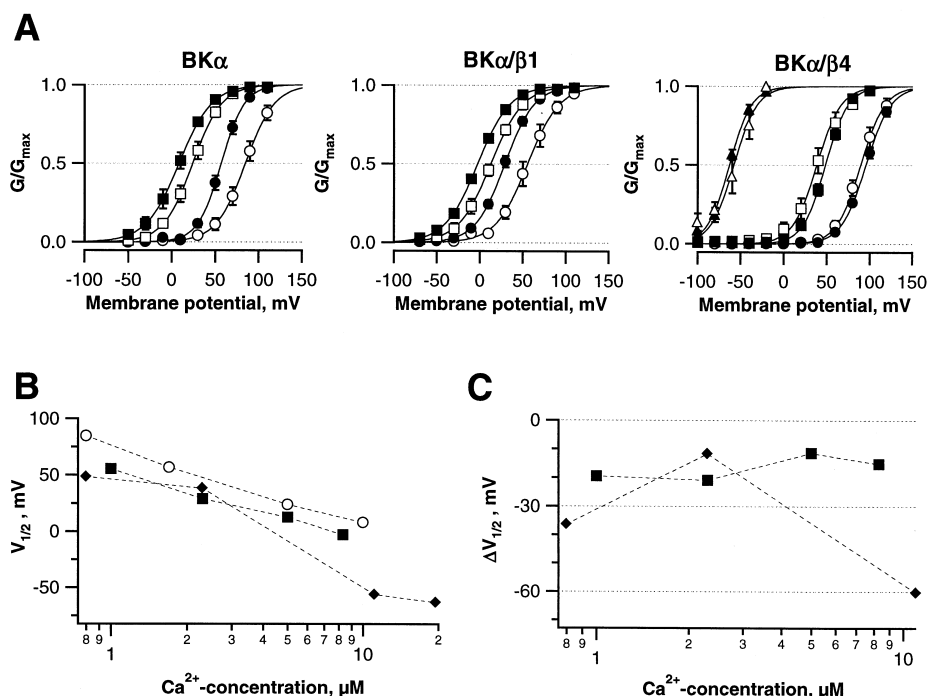


Fig. 4. KCNMB4 confers increased Ca^{2+} sensitivity to BK channels. A: Plot of conductance versus voltage relation of BK α subunits alone or coexpressed with BK $\beta 1$ or BK $\beta 4$ subunits. Normalized conductance was measured for each test potential from current amplitude taken 2 ms after repolarization to tail potentials. Data are displayed as the mean $\pm$ S.E.M. ($n=3-7$). Recordings were made from inside-out patches bathed in solutions buffered to various calcium concentrations (in μM): for BK α , 0.8 ($\circ$), 1.7 ($\bullet$), 5.0 ($\square$), 10.0 ($\blacksquare$); for BK $\alpha/\beta 1$, 1.0 ($\circ$), 2.3 ($\bullet$), 5.0 ($\square$), 8.3 ($\blacksquare$); and for BK $\alpha/\beta 4$, 0.02 ($\circ$), 0.35 ($\bullet$), 0.8 ($\square$), 2.3 ($\blacksquare$), 11.0 ($\triangle$), 19.5 ($\blacktriangle$). Solid curves show fits of data to a Boltzmann function ($G = G_{max} [1/(1 + e^{-(V-V_{1/2})zF/RT})]$) and then normalized to the maximum of the fit. B: Plot of $V_{1/2}$ versus log calcium concentration. BK α ($\circ$) expressed alone; BK α expressed in the presence of BK $\beta 1$ ($\blacksquare$) and of BK $\beta 4$ ($\blacklozenge$), respectively. C: Calcium dependence of $\Delta V_{1/2}$ ($V_{1/2}$ BK α minus $V_{1/2}$ BK $\alpha/\beta 1$ or BK $\alpha/\beta 4$). Values at comparable Ca^{2+} concentrations for BK α were extrapolated from B. Labels as in B.

$\tau_{slow} = 6.6 \pm 2.1$ ms at +40 mV, $n=3$) [5,9–13,23]. BK currents were activated at 0.8 μM internal Ca^{2+} by depolarizing test potentials above 0 mV. The conductance/voltage relation had a midpoint ($V_{1/2}$) at $+84.7 \pm 0.2$ mV (Fig. 4A,B). In agreement with previous results [9–12,23], coexpression of BK α with BK $\beta 1$ subunits (Fig. 3B) conferred to BK channels an apparent increased sensitivity to intracellular calcium (Fig. 4A,B). At comparable intracellular Ca^{2+} concentrations, BK currents activated at more hyperpolarized membrane potentials giving rise to a negative shift ($\Delta V_{1/2}$) in the midpoint of the conductance/voltage relations by ~ -15 to ~ -30 mV (Fig. 4C). Also, activation of BK channels is slowed in the presence of BK $\beta 1$ (Fig. 3B,D). The slow activation time constant (Fig. 3D) at +40 mV for BK $\alpha/\beta 1$ was 12.9 ± 0.9 ms ($n=2$). Coexpression of BK $\beta 4$ with BK α subunits affected the activation kinetics of BK currents more pronouncedly ($\tau_{slow} = 29.4 \pm 8.8$ ms at +40 mV, $n=5$) than BK $\beta 1$ (Fig. 3C,D). Like BK $\beta 1$, BK $\beta 4$ shifted the voltage range of BK current activation to more negative potentials (Fig. 4A,B). In contrast to BK $\beta 1$, however, increasing intracellular calcium concentrations from 0.8 to 11 μM dramatically enlarged the shift in the midpoint of the conductance/voltage relations from ~ -20 mV to ~ -60 mV (Fig. 4C). Thus, BK $\beta 4$ conferred a significantly steeper Ca^{2+} dependence onto the apparent conductance/voltage relation of BK currents (Fig. 4B,C).

The conductance of single BK channels has been shown to be ~ 200 pS in various in vitro expression systems [30]. Exposure of BK channels to intracellular Ca^{2+} dramatically increased the open probability of the channels without affecting single-channel conductance [30–32]. We recorded from inside-

out patches BK channel activity $\pm Ca^{2+}$ (Fig. 5). Fig. 5A shows a representative patch recording, where the inside-out patch was first bathed in buffer containing 0.8 μM Ca^{2+} , then in Ca^{2+} -free bathing solution, followed by an exposure to 11 μM Ca^{2+} . As expected for BK channels, channel activity is very sensitive and proportional to the Ca^{2+} concentration added to the cytoplasmic side of the BK channels. At free Ca^{2+} a sporadic, mostly single opening of BK channels was seen (Fig. 5B). At 0.8 μM Ca^{2+} multiple BK channel openings were seen and at 11 μM BK channel openings were so frequent that single-channel events could no longer be discerned. The data clearly show that increasing Ca^{2+} in the bath profoundly increased the open probability of BK $\alpha/\beta 4$ channels similar to BK $\alpha/\beta 1$ [31,32]. Single-channel current/voltage relations were obtained from inside-out patch recordings at 0 and 0.8 μM Ca^{2+} , respectively, such as shown in Fig. 5A,B. The results showed (Fig. 5C) that the slope of the current/voltage relations was linear between +20 mV and +80 mV. The single-channel conductance derived from the slope at 0 and at 0.8 μM Ca^{2+} was 223 ± 6 pS ($n=2$) and 193 ± 14 pS ($n=2$), respectively. Like BK $\beta 1$ [12], BK $\beta 4$ did not significantly influence the single-channel conductance of BK channels.

3.4. Pharmacology of BK $\alpha/\beta 4$ channels

BK channels are sensitive to block by nM concentrations of scorpion toxins like charybdotoxin (ChTX) and iberiotoxin (IbTX) [24,33]. The sensitivity to scorpion toxin block is increased when BK channels contain $\beta 1$ subunits [24,33]. IC_{50} values for block of BK $\alpha/\beta 1$ channels by ChTX or IbTX are in

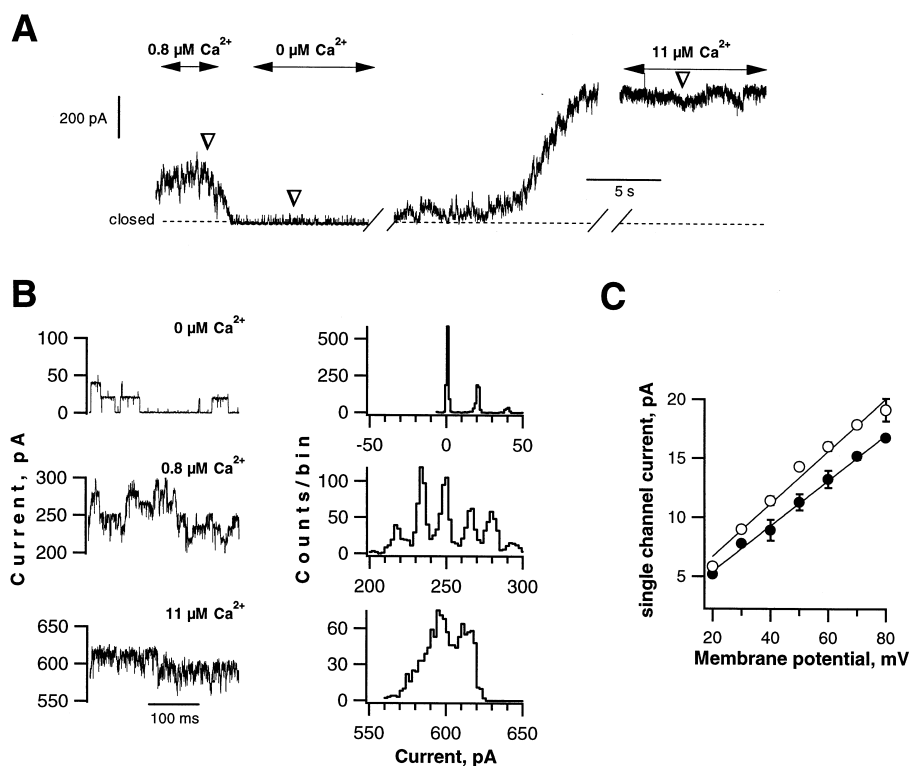


Fig. 5. Single-channel characteristics of BK α coexpressed with BK β 4. A: Calcium dependence of single-channel activity, recorded in the inside-out configuration at +80 mV. A bath superfusion was used to adjust the calcium concentration. Arrows indicate the completed bath exchange. B: Single-channel events of BK α / β 4 (left) were extracted from the record shown in A, at times indicated by the triangles. Corresponding amplitude histograms of the events (right). C: Single-channel conductance of BK α / β 4 at 0 μ M Ca $^{2+}$ (○) and 0.8 μ M Ca $^{2+}$ (●), respectively. Single-channel currents were measured by amplitude histogram analysis of two patches for each condition.

the 10 nM range. Typically, bath application of 100 nM toxin resulted in a complete block of BK α / β 1 channels ($n=3$). Native BK channels in smooth muscle tissue have a similar scorpion toxin sensitivity consistent with the presence of BK α / β 1 channels in this tissue [1,2,34]. BK α / β 4 mediated outward currents were measured at 1 μ M intracellular calcium and at test potentials ranging from -80 mV to $+120$ mV. We found that BK α / β 4 channels are not blocked by 100 nM ChTX or IbTX, respectively ($n=3$; data not shown). The high expression level of BK β 4 subunits in neuronal tissue is consistent with the observation that neuronal BK channels may not be sensitive to ChTX or IbTX [35,36]. Recently, it has been reported that 17 β -estradiol acts as a BK channel opener on BK α / β 1 [37]. Apparently, 17 β -estradiol binds to an extracellular site on BK α / β 1 channels that depends on the presence of BK β 1 subunits. Here, we show that 17 β -estradiol may also activate BK α / β 4 channels. They were recorded from outside-out patches bathed in 0 Ca $^{2+}$ solution (Fig. 6). In the absence of Ca $^{2+}$ and 17 β -estradiol the patches showed very little single-channel activity, but as soon as 17 β -estradiol was applied to the bath, single-channel activity markedly increased and correlated to the concentration of 17 β -estradiol added to the bath. 10 μ M estradiol activated BK channel to a similar degree like 1 μ M intracellular calcium.

4. Discussion

The biochemical purification of BK channels from tracheal smooth muscle showed that they are coassembled from BK α and BK β 1 subunits [2]. The subsequent cloning of BK α and

BK β 1 cDNAs [10,38] demonstrated that BK α mRNA is expressed in many different mammalian tissues, whereas BK β 1 mRNA expression appeared to be restricted mainly to smooth muscle tissue [29,38]. Our mRNA expression profiling of human BK α and BK β 1 mRNAs is in agreement with the previous reports. It shows that human BK β 1 mRNA expression occurs predominantly in tissue of smooth muscle origin, whereas BK α mRNA was detectable in almost all the 70 mRNA samples investigated. The discrepancy between BK α and BK β 1 mRNA expression profiles suggested that possibly the tissues and cells, which do not express BK β 1 subunits, may in fact express related, but distinct BK β subunits. Recent findings [5,23] and our results show that BK β subunits repre-

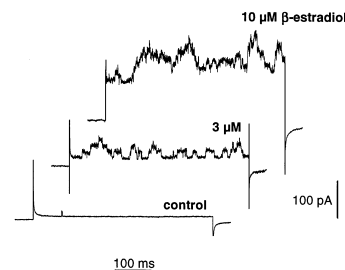


Fig. 6. Agonistic effect of 17 β -estradiol on BK α / β 4 mediated currents. Depicted are current traces from the same outside-out patch superfused successively with external solution alone (control) or containing 3 and 10 μ M 17 β -estradiol, respectively. The patch was held at -80 mV and currents were recorded after depolarizing voltage steps of 500 ms duration to $+80$ mV. Internal solution was practically free of calcium.

sent a family of at least four related subunits, which have distinct expression patterns in human (mammalian) tissue and also confer distinct gating properties to BK α channels [5,23]. Here we show that BK β 4 is a BK channel subunit, which is prominently expressed in human brain. Therefore, BK α / β 4 type BK channels may resemble BK channels measured in neuronal tissue. It has been shown that neuronal BK channels are not blocked by IbTX and ChTX in contrast to smooth muscle BK channels [35,36]. Indeed, BK α / β 4 channels could not be blocked by ChTX or IbTX concentrations that typically blocked BK α / β 1 channels [35,36]. These results support the notion that BK α / β 4 channels correlate with channels as they occur in native neuronal tissue. It has been shown that certain amino acid residues in the extracellular loop sequence of BK β 1 (see Fig. 1) are involved in the generation of the high-affinity ChTX binding site of BK channels [33]. Some of the amino acid residues are mutated in the derived BK β 4 protein sequence. It remains to be shown whether these mutations are indeed responsible for the absence of a high toxin sensitivity of BK α / β 4 channels. At low calcium concentrations, the open probability of BK α / β 4 channels was significantly increased by extracellular 17 β -estradiol like BK α / β 1 [37]. Apparently, the extracellular 17 β -estradiol binding site has been conserved between BK α / β 1 and BK α / β 4 channels. The results suggest that 17 β -estradiol may not only open smooth muscle BK channels, but also neuronal BK channels. It will be interesting to see whether BK channel activity in neurons can be modulated by 17 β -estradiol application and how steroid hormone induced opening of neuronal BK channels may affect neuronal excitability.

It has been shown that BK β 1 subunits increase the open probability of BK channels [39]. The BK β 4 subunits seem to have a similar effect on BK channels. However, the influence of BK β 1 and BK β 4 subunits on BK channels appeared not uniform. BK β 4 slowed the gating kinetics of BK channels more profoundly than BK β 1. While this manuscript was in preparation, a recent report [23] showed that in the *Xenopus* oocyte expression system 1 to 10 μ M Ca²⁺ concentrations shifted $V_{1/2}$ of BK α / β 4 current activation to more positive potentials and Ca²⁺ concentrations of <50 μ M to negative potentials. In contrast, the expression of BK α / β 4 channels in CHO cells produced BK currents, whose $V_{1/2}$ values were dramatically shifted to negative potentials by 1 to 20 μ M Ca²⁺ concentrations. For example, 10 μ M Ca²⁺ shifted BK α / β 4 currents by $+7.5 \pm 4.9$ mV to $V_{1/2} \approx +50$ mV in the *Xenopus* oocyte expression system [23] and by -60 mV to $V_{1/2} = -56$ mV in the CHO expression system. The reasons for this discrepancy are not clear. Our data show that BK α / β 4 currents were shifted by ~ 20 to -60 mV when the Ca²⁺ concentration was raised from 1 to 11 μ M. This may indicate that BK β 4 confers an exquisite Ca²⁺ sensitivity onto BK channels in a physiologically important Ca²⁺ concentration range. At 10 μ M Ca²⁺ BK α / β 4 channels may operate in a more negative voltage range than BK α / β 1 channels and, thereby, significantly contribute to attenuating neuronal excitability. Note, at very low calcium concentrations (0.02 μ M) we apparently observed a less negative shift in the conductance/voltage relation of BK α / β 4 currents. For experimental reasons, we did not go to test potentials above +120 mV and, therefore, could not determine maximum conductance values at very low calcium concentrations. Instead, the values were extrapolated from the fit of the data to a Boltzmann function.

Therefore, the $V_{1/2}$ values at very low calcium concentrations may be an underestimate.

Previously, much of the BK channel diversity in gating kinetics, apparent voltage dependence and Ca²⁺ sensitivity in pharmacology have been attributed to alternatively spliced BK α subunits [13–17] and/or posttranslational modifications [19–21]. Recent reports [5,6,23] and our results show that heteromultimerization of BK α subunits with different BK β subunits may also make a significant contribution to BK channel diversity. The analysis of the tissue expression of BK β 1 to BK β 4 mRNAs suggests that specific BK α / β combinations are expressed in different tissues and have distinct expression patterns. In conclusion, our results indicate that smooth muscle and neuronal BK channels may have different subunit compositions.

Acknowledgements: O.P. thanks the European Union for generous support.

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