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

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Abstract We cloned two β subunits of large-conductance calcium-activated potassium (BK) channels, hKCNMB3 (BKβ1) and hKCNMB4 (BKβ4). Profiling mRNA expression showed that hKCNMB3 expression is enriched in testis and hKCNMB4 expression is very prominent in brain. We coexpressed BK channel α (BKα) and BKβ4 subunits in vitro in CHO cells. We compared BKα/β4 mediated currents with those of smooth muscle BKα/β1 channels. BKβ4 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 BKβ1. BKα/β4 channels were not blocked by 100 nM charybdotoxin or iberiotoxin, and were activated by 17β-estradiol.

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 BKα subunits [1,2] and auxiliary BKβ subunits [3–6]. Association of BKβ with BKα subunits may confer an increased Ca^{2+} sensitivity, different kinetic properties as well as a different pharmacology to BK channels. BKα 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 BKα mRNA, the expression of BKβ mRNAs appears to be restricted to few specific tissues [3,5,6]. E.g., BKβ1 subunits are predominantly expressed in smooth muscle cells, whereas BKβ2 subunits were mainly detected in endocrine tissue. However, BKβ subunits, that matched the high expression levels of BKα 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 BKβ 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 BKα subunits and CNS-specific BKβ subunits. Possibly, the CNS BKβ subunits would be related in sequence and structure to the already known BKβ1 and BKβ2 subunits. Accordingly, a database search for EST clones potentially related to novel BKβ subunits resulted in the cloning of two novel human BKβ subunits (BKβ3, KCNMB3 and BKβ4, KCNMB4). We find that BKβ4 mRNA is highly expressed in human brain tissue and may contribute to BK channel diversity in brain. Comparably to BKβ1 [9–12], coexpression of BKα subunits with BKβ4 leads to slowed activation kinetics, increased Ca^{2+} sensitivity and an altered pharmacology of BK channels. So far, BK channel diversity has been attributed to alternative splicing of BKα 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 BKβ3 and BKβ4 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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2.2. Determination of tissue expression levels

For hybridization studies, the appropriate probe fragments were purified twice on agarose gels and labeled with ^{32}P-dATP using the Ambion StripEZ DNA Kit (AMS Biotech, Frankfurt, Germany). Human multiple tissue expression arrays (Clontech, Heidelberg, Germany) were hybridized according to the manufacturer's instructions and exposed for 1–14 days on a Fuji PhosphorImager (Raytest, Straubenhardt, Germany) and/or autoradiographed. Probe fragments were: KCNMB1, a 437 bp *Sma*I/*Bst*XI fragment of AI309617; KCNMB2, a 758 bp *Acc*I/*Nco*I fragment of AI299145; KCNMB3, a 320 bp PCR product (sense primer 5′-GGCTTCTCAGTCCTAATGTTC-3′; antisense primer 5′-GTCCAGAGCACTGTTGAGC-3′); KCNMB4, a 430 bp PCR product (sense primer 5′-CAACAGTACTGGAAAGATGAG-3′; antisense primer 5′-TACAGCAGATGGAATACAAGC-3′). For comparison, we probed the arrays with a 320 bp PCR product of the hKCNMA1 gene (sense primer 5′-GATGAGCAGCCGTCAACAC-3′; antisense primer 5′-GCATCACCAGGTTCCGGAG-3′).

2.3. Genomic localization of KCNMB4

The GenBank HTGS database was searched with the coding sequence of KCNMB4 as a probe. A human BAC clone RPC11–792c16 (Acc. No. AC011612) was identified, that covered three exons of KCNMB4 on three different contigs, enabling us to define the exon/intron structure of the gene. Electronic PCR of this clone at NCBI revealed STS markers on chromosome 12. One STS (A005A38, Acc. No. G20255) was located 570 bp downstream the KCNMB4 stop codon on an 8.1 kbp contig. Thus, the KCNMB4 gene was localized to 12q14.1–15.

2.4. Heterologous expression studies

KCNMB cDNAs were subcloned into pcDNA3 (Invitrogen, Groningen, The Netherlands) using standard cloning methods. CHO cells were transiently transfected with pcDNA3-hslo (GenBank™ Acc. No. U11058 [24]; a kind gift from Dr. F. Hofmann, University of Munich) with or without KCNMB pcDNA3 constructs using Lipofectamine™ (Life Technologies) as recommended by the manufacturer. An EGFP pcDNA3 construct was cotransfected to label transfected cells with EGFP-derived green fluorescence. Data for BKα and BKα/β1 in Fig. 4 were derived from HEK293 cell lines stably transfected with rat KCNMA and rat KCNMA/KCNMB1, respectively [25].

2.5. Electrophysiological measurements and analysis

Current measurements were performed 12–24 h following transfection, using either the whole-cell or the inside-out configuration of the patch-clamp technique. Patch pipettes were fabricated from borosilicate capillaries using a DMZ puller (Zeitz, Augsburg, Germany) and had resistances between 2 and 3 MΩ in recording solution. In whole-cell experiments the intracellular solution contained (in mM): 140 KCl, 1 EGTA, varied $CaCl_2$ and 10 HEPES (pH 7.2, KOH). Free Ca^{2+} concentrations were calculated using the program CALCIUM [26] as well as measured with the ratiometric Ca^{2+} indicators Fura-2 or BTC (Molecular Probes, Leiden, The Netherlands). During inside-out patch-clamp recordings the bath was perfused with the intracellular solution. The patch pipette was filled with the external solution (in mM): 135 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 5 HEPES (pH 7.4, NaOH). Single-channel recordings in the inside-out configuration were performed at isometric potassium concentrations using (in mM): 140 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 5 HEPES (pH 7.4, KOH) as pipette solution. All recordings were made at room temperature (20–22°C). Current traces were amplified using an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), sampled at 5 kHz and filtered at 2.9 kHz via a 4-pole low-pass bessel filter. The program package PULSE+PULSEFIT (HEKA Elektronik) was used for data acquisition and analysis. Leakage and capacitive currents were subtracted online using the P/4 subtraction method. Steady-state activation curves were derived from tail currents after stepping to the test potentials. Data were fit to a Boltzmann function

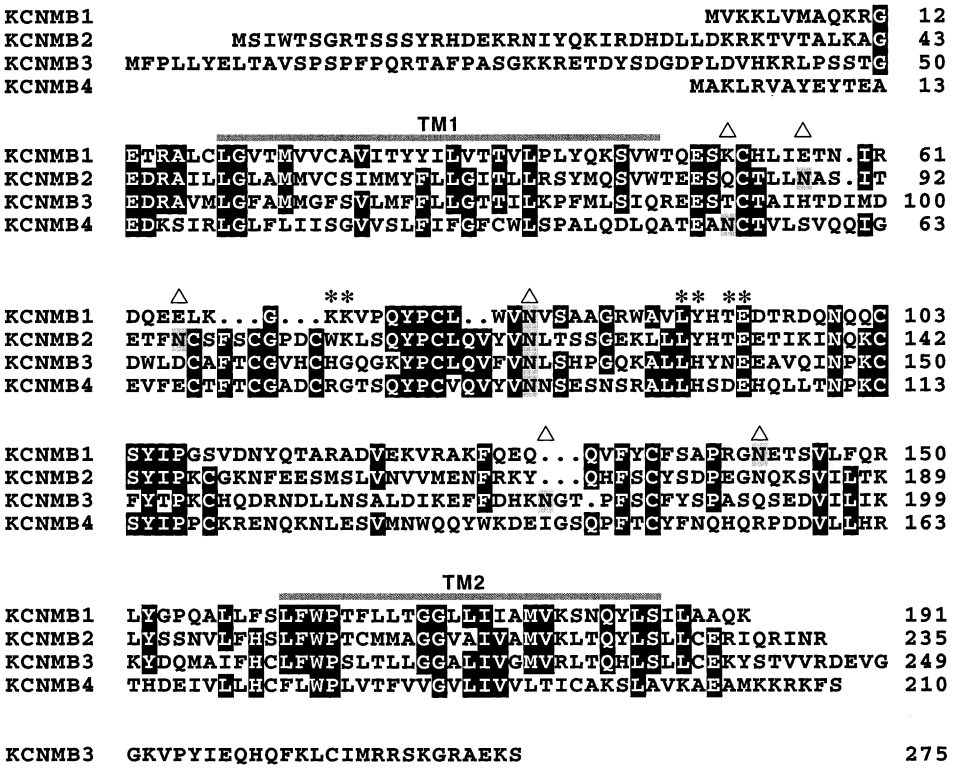

Fig. 1. Sequence alignment of human BK channel β subunit family. Sequence comparison of open reading frames derived from human BK channel β subunit genes KCNMB1 to KCNMB4. Sequences were aligned using the GCG software alignment program (Genetics Computer Group, Madison, WI, USA). Aligned residues are boxed in black for three or more identical amino acid residues. Numbers at right refer to last amino acid residue in each row. One-letter code denotes amino acids. Dots indicate gaps in the sequence alignment. Putative transmembrane-spanning segments TM1 and TM2 are overlined. Δ = consensus *N*-glycosylation sites, respective asparagine residues are boxed in gray. Note, only one consensus glycosylation site is conserved in all four BK channel β subunits. Amino acid residues in the KCNMB1 protein sequence that have been implicated in high-affinity binding of ChTX to BK channels [33] are labeled with an asterisk.

$[G = G_{max}/(1 + e^{(V-V_{1/2})/slope})]$ 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 ± 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 BKβ 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 agreement with recent reports [23,27]. The EST clones were used as probes to clone the complete open reading frames (ORFs) of KCNMB3 (BKβ3) and KCNMB4 (BKβ4). 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 BKβ subunit family is shown in Fig. 1. Sequences between family members are only moderately conserved (21–43% identical residues). However, the

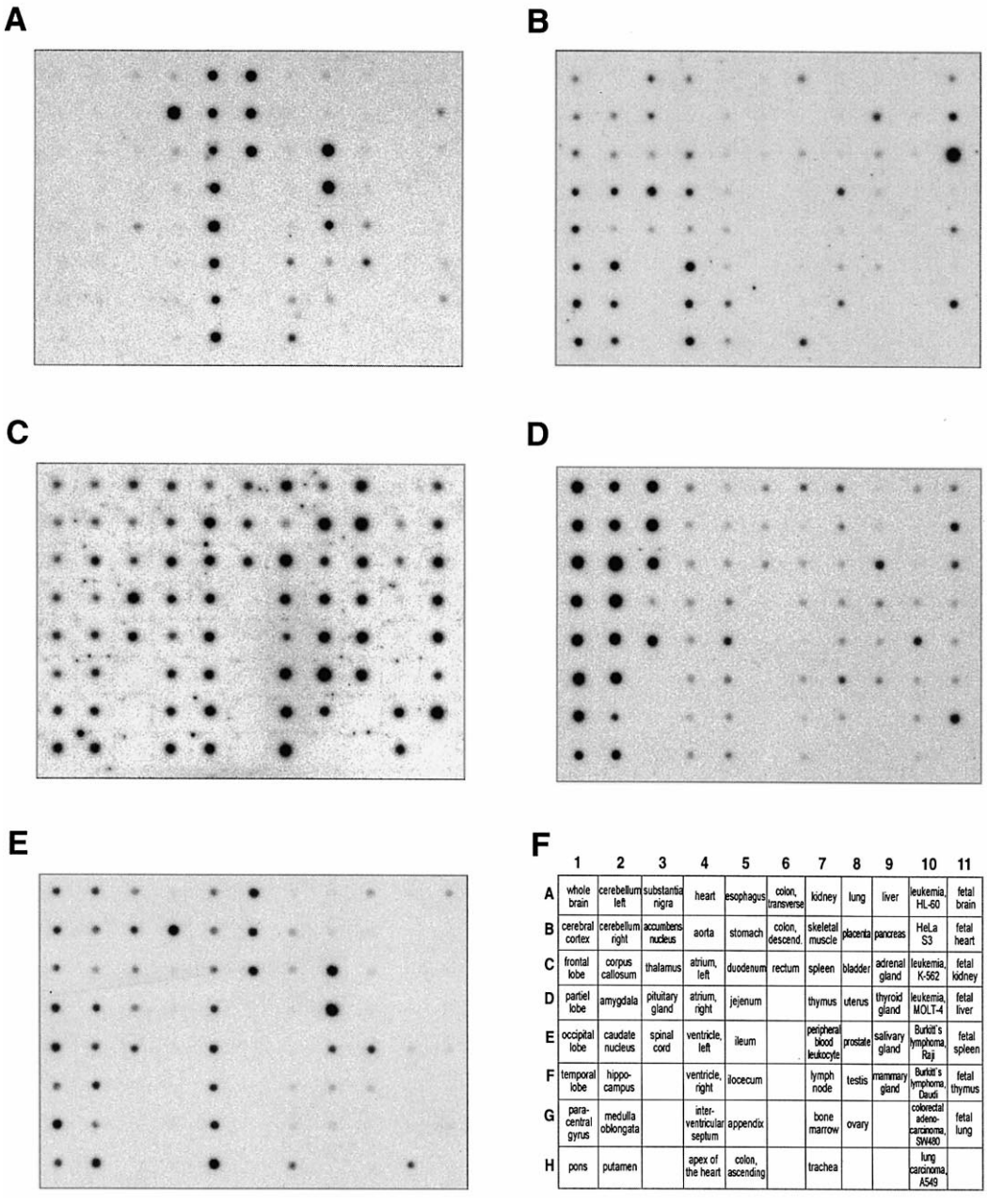

Fig. 2. Expression profiles of BK channel α and β subunits in human tissue. Multiple tissue expression arrays (MTE™, Clontech, Heidelberg, Germany) were hybridized with ^{32}P-labeled cDNA probes specific for BK channel β subunits KCNMB1 (A), KCNMB2 (B), KCNMB3 (C), KCNMB4 (D) and the BK channel α subunit 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 BKβ 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 BKβ3 and BKβ4 (labeled with asterisks in Fig. 1).

3.2. Tissue distribution of BKβ mRNA

We used commercial dot blots to compare the expression of BKβ mRNAs in 76 different human tissues (Fig. 2). The results indicated that each BKβ mRNA had a distinct pattern of expression. For comparison, we have included in this analysis BKα mRNA, which is expressed in every human tissue tested except heart atrium, ventricle and septum (Fig. 2E). Consistent with previous results [10,29] BKβ1 mRNA expression seems to be restricted to smooth muscle tissue, e.g. aorta, stomach, duodenum, ileum, colon, bladder and uterus (Fig. 2A). BKβ2 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 BKβ subunits, BKβ3 mRNA seems to be least abundant. In case of the BKβ3 probe, relatively long exposure times were necessary to obtain hybridization signals of comparable intensity (Fig. 2C). By contrast, BKβ4 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 BKβ4 mRNA expression profile suggests that BKβ4 may represent a nervous system-specific β subunit of BK channels. Interestingly, biochemical purification of BK channels from brain indicated a BKβ subunit of ~25 kDa molecular weight tightly associated with brain BK channel α subunits [8]. The derived size of BKβ4 protein (210 amino acid residues) is compatible with the notion that BKβ4 is a β subunit of neuronal BK channels. In multiple tissue Northern blots we have determined the transcript sizes of BKβ1–4 mRNAs (data not shown). In agreement with a previous report [23], BKβ4 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 BKα with BKβ4 subunits

Since our hybridization results indicated that BKβ4 was most likely a prominent BKβ subunit of neuronal BK channels, we have concentrated on the coexpression of BKα subunits with BKβ4 subunits in comparison to that with BKβ1 subunits. For this purpose, BKβ1 and BKβ4 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 Ca^{2+}-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 μM internal Ca^{2+}. In the absence of BKβ subunits, BKα subunits (Fig. 3A,D) typically gave rise to relative rapidly activating biphasic outward currents ($\tau_{fast} = 1.2 \pm 0.2$ ms and

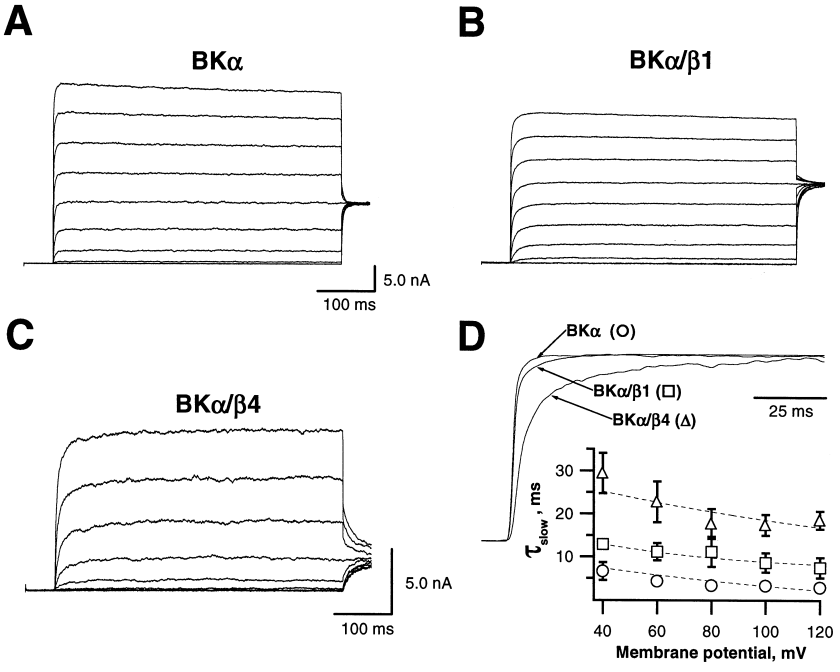

Fig. 3. Currents mediated by BK channels in the absence or presence of β subunits KCNMB1 and KCNMB4 in transfected tissue culture cells. A–C: Macroscopic currents were elicited in whole-cell patch-clamp experiments by stepping the membrane voltage from a holding potential of −80 mV to depolarized test potentials in +20 mV increments up to +100 mV. Cells were buffered to 0.8 μM Ca^{2+}. 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 BKα, BKα/β1, BKα/β4 mediated currents ($n = 3$–5). For comparison, current traces elicited with a +80 mV test pulse are normalized to the maximum amplitude.

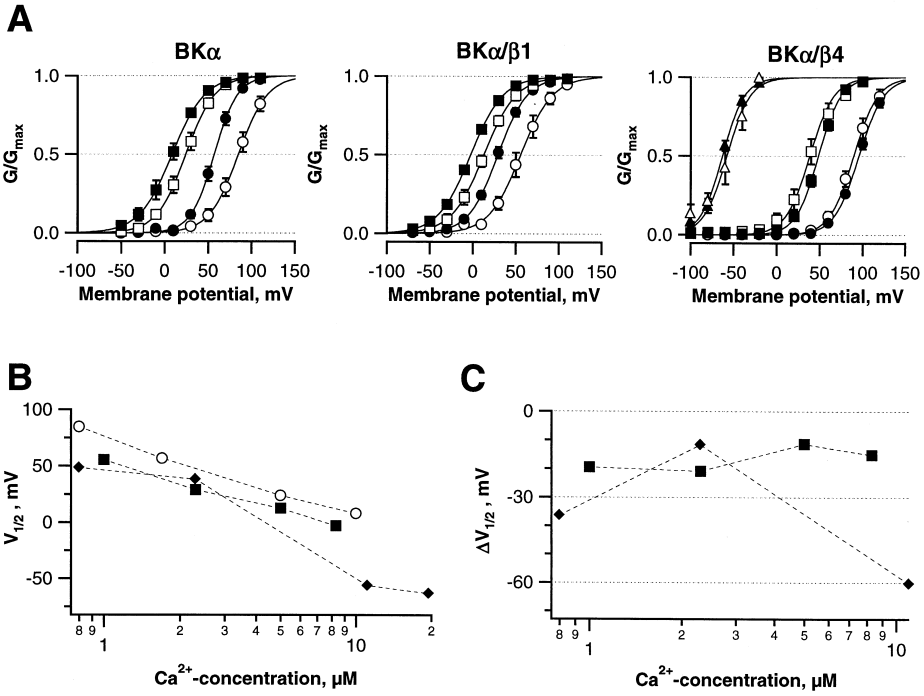

Fig. 4. KCNMB4 confers increased Ca^{2+} sensitivity to BK channels. A: Plot of conductance versus voltage relation of BKα subunits alone or coexpressed with BKβ1 or BKβ4 subunits. Normalized conductance was measured for each test potential from current amplitude taken 2 ms after repolarization to tail potentials. Data are displayed as the mean ± S.E.M. ($n = 3$–7). Recordings were made from inside-out patches bathed in solutions buffered to various calcium concentrations (in μM): for BKα, 0.8 (○), 1.7 (●), 5.0 (□), 10.0 (■); for BKα/β1, 1.0 (○), 2.3 (●), 5.0 (□), 8.3 (■); and for BKα/β4, 0.02 (○), 0.35 (●), 0.8 (□), 2.3 (■), 11.0 (△), 19.5 (▲). Solid curves show fits of data to a Boltzmann function ($G = G_{max}\,[I/(1 + e^{-(V - V_{1/2})zF/RT})]$) and then normalized to the maximum of the fit. B: Plot of $V_{1/2}$ versus log calcium concentration. BKα (○) expressed alone; BKα expressed in the presence of BKβ1 (■) and of BKβ4 (♦), respectively. C: Calcium dependence of $\Delta V_{1/2}$ ($V_{1/2}$ BKα minus $V_{1/2}$ BKα/β1 or BKα/β4). Values at comparable Ca^{2+} concentrations for BKα 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 ± 0.2 mV (Fig. 4A,B). In agreement with previous results [9–12,23], coexpression of BKα with BKβ1 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 BKβ1 (Fig. 3B,D). The slow activation time constant (Fig. 3D) at +40 mV for BKα/β1 was 12.9 ± 0.9 ms ($n = 2$). Coexpression of BKβ4 with BKα subunits affected the activation kinetics of BK currents more pronouncedly ($\tau_{slow} = 29.4 \pm 8.8$ ms at +40 mV, $n = 5$) than BKβ1 (Fig. 3C,D). Like BKβ1, BKβ4 shifted the voltage range of BK current activation to more negative potentials (Fig. 4A,B). In contrast to BKβ1, 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, BKβ4 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 ± 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 BKα/β4 channels similar to BKα/β1 [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 BKβ1 [12], BKβ4 did not significantly influence the single-channel conductance of BK channels.

3.4. Pharmacology of BKα/β4 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 β1 subunits [24,33]. IC_{50} values for block of BKα/β1 channels by ChTX or IbTX are in

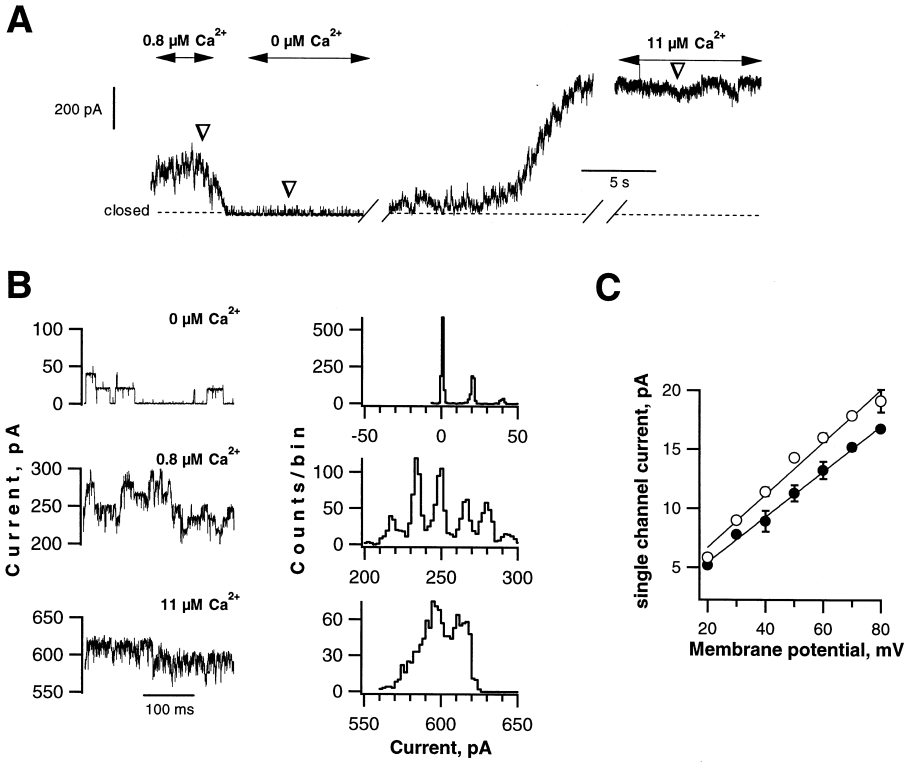

Fig. 5. Single-channel characteristics of BKα coexpressed with BKβ4. A: Calcium dependence of single-channel activity, recorded in the inside-out configuration at +80 mV. A bath superfusion was used to adjust the calcium concentration. Arrows indicate the completed bath exchange. B: Single-channel events of BKα/β4 (*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 BKα/β4 at 0 μM Ca^{2+} (○) and 0.8 μM 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 BKα/β1 channels ($n = 3$). Native BK channels in smooth muscle tissue have a similar scorpion toxin sensitivity consistent with the presence of BKα/β1 channels in this tissue [1,2,34]. BKα/β4 mediated outward currents were measured at 1 μM intracellular calcium and at test potentials ranging from −80 mV to +120 mV. We found that BKα/β4 channels are not blocked by 100 nM ChTX or IbTX, respectively ($n = 3$; data not shown). The high expression level of BKβ4 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 17β-estradiol acts as a BK channel opener on BKα/β1 [37]. Apparently, 17β-estradiol binds to an extracellular site on BKα/β1 channels that depends on the presence of BKβ1 subunits. Here, we show that 17β-estradiol may also activate BKα/β4 channels. They were recorded from outside-out patches bathed in 0 Ca^{2+} solution (Fig. 6). In the absence of Ca^{2+} and 17β-estradiol the patches showed very little single-channel activity, but as soon as 17β-estradiol was applied to the bath, single-channel activity markedly increased and correlated to the concentration of 17β-estradiol added to the bath. 10 μM estradiol activated BK channel to a similar degree like 1 μM intracellular calcium.

4. Discussion

The biochemical purification of BK channels from tracheal smooth muscle showed that they are coassembled from BKα and BKβ1 subunits [2]. The subsequent cloning of BKα and BKβ1 cDNAs [10,38] demonstrated that BKα mRNA is expressed in many different mammalian tissues, whereas BKβ1 mRNA expression appeared to be restricted mainly to smooth muscle tissue [29,38]. Our mRNA expression profiling of human BKα and BKβ1 mRNAs is in agreement with the previous reports. It shows that human BKβ1 mRNA expression occurs predominantly in tissue of smooth muscle origin, whereas BKα mRNA was detectable in almost all the 70 mRNA samples investigated. The discrepancy between BKα and BKβ1 mRNA expression profiles suggested that possibly the tissues and cells, which do not express BKβ1 subunits, may in fact express related, but distinct BKβ subunits. Recent findings [5,23] and our results show that BKβ subunits repre-

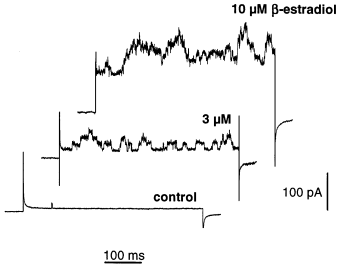

Fig. 6. Agonistic effect of 17β-estradiol on BKα/β4 mediated currents. Depicted are current traces from the same outside-out patch superfused successively with external solution alone (control) or containing 3 and 10 μM 17β-estradiol, respectively. The patch was held at −80 mV and currents were recorded 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 BKα channels [5,23]. Here we show that BKβ4 is a BK channel subunit, which is prominently expressed in human brain. Therefore, BKα/β4 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, BKα/β4 channels could not be blocked by ChTX or IbTX concentrations that typically blocked BKα/β1 channels [35,36]. These results support the notion that BKα/β4 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 BKβ1 (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 BKβ4 protein sequence. It remains to be shown whether these mutations are indeed responsible for the absence of a high toxin sensitivity of BKα/β4 channels. At low calcium concentrations, the open probability of BKα/β4 channels was significantly increased by extracellular 17β-estradiol like BKα/β1 [37]. Apparently, the extracellular 17β-estradiol binding site has been conserved between BKα/β1 and BKα/β4 channels. The results suggest that 17β-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 17β-estradiol application and how steroid hormone induced opening of neuronal BK channels may affect neuronal excitability.

It has been shown that BKβ1 subunits increase the open probability of BK channels [39]. The BKβ4 subunits seem to have a similar effect on BK channels. However, the influence of BKβ1 and BKβ4 subunits on BK channels appeared not uniform. BKβ4 slowed the gating kinetics of BK channels more profoundly than BKβ1. While this manuscript was in preparation, a recent report [23] showed that in the *Xenopus* oocyte expression system 1 to 10 μM Ca^{2+} concentrations shifted $V_{1/2}$ of BKα/β4 current activation to more positive potentials and Ca^{2+} concentrations of <50 μM to negative potentials. In contrast, the expression of BKα/β4 channels in CHO cells produced BK currents, whose $V_{1/2}$ values were dramatically shifted to negative potentials by 1 to 20 μM Ca^{2+} concentrations. For example, 10 μM Ca^{2+} shifted BKα/β4 currents by $+7.5\pm4.9$ 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 BKα/β4 currents were shifted by ~20 to -60 mV when the Ca^{2+} concentration was raised from 1 to 11 μM. This may indicate that BKβ4 confers an exquisite Ca^{2+} sensitivity onto BK channels in a physiologically important Ca^{2+} concentration range. At 10 μM Ca^{2+} BKα/β4 channels may operate in a more negative voltage range than BKα/β1 channels and, thereby, significantly contribute to attenuating neuronal excitability. Note, at very low calcium concentrations (0.02 μM) we apparently observed a less negative shift in the conductance/voltage relation of BKα/β4 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 Ca^{2+} sensitivity in pharmacology have been attributed to alternatively spliced BKα subunits [13–17] and/or posttranslational modifications [19–21]. Recent reports [5,6,23] and our results show that heteromultimerization of BKα subunits with different BKβ subunits may also make a significant contribution to BK channel diversity. The analysis of the tissue expression of BKβ1 to BKβ4 mRNAs suggests that specific BKα/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.

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