Cloning and Functional Expression of Two Families of \( \beta \)-Subunits of the Large Conductance Calcium-activated \( K^+ \) Channel*

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We report here a characterization of two families of calcium-activated \( K^+ \) channel \( \beta \)-subunits, \( \beta_2 \) and \( \beta_3 \), which are encoded by distinct genes that map to 3q26.2–27. A single \( \beta_2 \) family member and four alternatively spliced variants of \( \beta_3 \) were investigated. These subunits have predicted molecular masses of 27.1–31.6 kDa, share \( \sim 30–44\% \) amino acid identity with \( \beta_1 \), and exhibit distinct but overlapping expression patterns. Coexpression of the \( \beta_2 \) or \( \beta_3 \)-c subunits with a BK \( \alpha \)-subunit altered the functional properties of the current expressed by the \( \alpha \)-subunit alone. The \( \beta_2 \) subunit rapidly and completely inactivated the current and shifted the voltage dependence for activation to more polarized membrane potentials. In contrast, coexpression of the \( \beta_3 \)-c subunits resulted in only partial inactivation of the current, and the \( \beta_3b \) subunit conferred an apparent inward rectification. Furthermore, unlike the \( \beta_1 \) and \( \beta_2 \) subunits, none of the \( \beta_3 \) subunits increased channel sensitivity to calcium or voltage. The tissue-specific expression of these \( \beta \)-subunits may allow for the assembly of a large number of distinct BK channels in vivo, contributing to the functional diversity of native BK currents.

Calcium-activated \( K^+ \) channels (\( K_{Ca} \))\(^1\) modulate cellular electrical excitability. These channels are gated by both cytoplasmic calcium and membrane potential and, therefore, provide feedback mechanisms to modulate \( Ca^{2+} \) influx. Activation of \( K_{Ca} \) channels hyperpolarizes cells, and the way in which this hyperpolarization regulates \( Ca^{2+} \) entry is dependent upon the nature of the influx pathway. For example, entry through voltage-gated calcium channels (e.g. in myocytes) may be decreased, due to voltage-dependent deactivation of the calcium channels (1–3). However, influx through voltage-independent channels (e.g. in endothelial cells) may be enhanced due to an increase in the driving force for \( Ca^{2+} \) (4, 5). Thus, the regulatory roles of \( K_{Ca} \) channels are context-dependent and may vary with cell type.

\( K_{Ca} \) currents have been recorded from a variety of tissues and have traditionally been classified into broad categories based on single channel conductance (e.g. large, intermediate, or small conductance). However, in addition to these differences in unitary current amplitude, distinct \( K_{Ca} \) currents may also vary in terms of their calcium- and voltage dependence, kinetics, or pharmacologic properties (3, 6, 7). Native \( K_{Ca} \) channels, therefore, comprise a large and functionally diverse family. In recent years, the structural basis of this diversity has been investigated. Significant progress in this regard has been made with large conductance (BK) \( K_{Ca} \) channels in particular, some of which have been both biochemically purified and cloned (8–12). BK channels have been purified from tracheal and aortic smooth muscles and demonstrated to be composed of two distinct types of subunits in those tissues: a large pore-forming \( \alpha \)-subunit and a smaller modulatory \( \beta \)-subunit (13). To date, only a single gene encoding a BK \( \alpha \)-subunit (\( KCNMA1 \)) has been found, although multiple variants are likely to be produced by alternative splicing (12). Two distinct genes encoding BK \( \beta \)-subunits have been identified: KCNM1, which encodes the \( \beta_1 \) subunit originally isolated from airway smooth muscle (11) has been localized to human chromosome 5q34 (14), and a recently isolated homologue encoding the \( \beta_2 \) subunit (15, 16). Although functional BK channels can be expressed from \( \alpha \)-subunits alone, coassembly with \( \beta \)-subunits can alter the biophysical and pharmacologic properties of the channel (15–19). However, the properties of some native BK currents are not well reproduced by combinations of currently known \( \alpha \) and \( \beta \)-subunits, suggesting the possibility that novel subunits of these channels may still exist.

We identified two families of BK \( \beta \)-subunits. The first, which, to date, contains only a single member (\( \beta_2 \)), is identical to that recently identified in a lung carcinoid EST library (15, 16). The second, the \( \beta_3 \) family, comprises four distinct subunits (\( \beta_3a–d \)) that arise by alternative splicing of a single gene. Coexpression of the \( \beta_2 \) or \( \beta_3a, -b, \) or- \( c \) subunits with a BK \( \alpha \)-subunit alters the functional properties of the current from that of the \( \alpha \)-subunit expressed alone. However, unlike the \( \beta_2 \) subunit, which both inactivates the channel and increases its calcium and voltage sensitivity, the \( \beta_3 \) subunits do not increase the calcium or voltage sensitivity of the current. The differential expression of these novel \( \beta \)-subunits may underlie part of the large functional diversity observed in native BK currents.

EXPERIMENTAL PROCEDURES

Identification and Cloning of cDNAs Encoding BK \( \beta \)-Subunits—Sequence encoding the \( \beta_1 \) subunit (U61537) was used to search the GenBank\(^*\) data base for homologues using the BLASTN and TBLASTN algorithms of the GCG software package (Wisconsin Genetics Group). This search identified an EST (AA904191) that, when completely sequenced, was demonstrated to encode a full-length \( K_{Ca} \) \( \beta \)-subunit, \( \beta_2 \). A fragment of this cDNA containing the coding region and 105 bp of the 3'-UTR was amplified by PCR using gene-specific oligonucleo-
tide primers, cloned into pmPvpl+ (a modified version of pSP64T (20) containing an expanded polylinker), and confirmed by complete sequencing of both strands.

The open reading frame deduced amino acid sequence of β2 were then used to query the GenBank® database against GenBank® identifiers isolated amplified fragments by ESTs (AA195381, AA227991, AA236968, AA279911, AA761761, and AA934766) encoding partial sequences of novel putative BK β-subunits (β3). Commerically available cDNAs encoding these ESTs (AA195381, AA279911, and AA761761) were purchased from CLONTECH (a modified version of pSP64T (20)) containing the stability region common to all the β3 splice variants (nucleotides 1158–1450 of β3c). Hybridization was carried out overnight at 42 °C in 0.25 M NaPO4, 0.5 M NaCl, 1.0 m M EDTA, 7% SDS, and 1% bovine serum albumin.

Blots were washed twice in 5% SSC, 0.1% SDS at 42 °C for 30 min and twice in 1% SSC, 0.1% SDS at 42 °C for 30 min each and then exposed to x-ray film. Positives were scored as tissues exhibiting a specifically amplified cDNA fragment of the expected size that also hybridized to the cognate probe. Expression analysis was repeated once using a different lot of cDNA samples with consistent results.

In Situ Hybridization and Immunohistochemistry—In situ hybridization was used in a combination of bigenic probe derived from the sequenced cDNA β3. The antisense probe corresponded to nucleotides 907–857, and the control sense probe to nucleotides 825–875 of the β3c sequence. Probes were labeled at their 3' ends using the DIG oligonucleotide tailing kit (Roche Molecular Biochemicals) with biotin-16-DUTP (Roche) substituted for digoxigenin-DUTP. Formalin fixed human pancreas specimens (National Disease Research Interchange) were processed to paraffin, sectioned at 8 μm, and mounted on Superfrost plus slides (Fisher). In situ hybridization was carried out as described previously using 2 pmol of labeled probe/ml of hybridization buffer (24). The hybridization signal was amplified using the TSA Direct Red FISH tyramide reagent (NEN Life Science Products) according to manufacturer's directions.

Immunohistochemistry was performed sequentially following in situ hybridization. Sections demonstrating optimal β3 mRNA signal were incubated with either guinea pig anti-human insulin serum (Dako) or rabbit anti-human glucagon serum (Dako) for 1 h at room temperature.

Bound antibody was detected with fluorescein isothiocyanate-conjugated donkey anti-guinea-pig IgG (Jackson Immunoresearch; 15 μg/ml in phosphate-buffered saline) or donkey anti-rabbit IgG (Jackson Immunoresearch; 15 μg/ml in phosphate-buffered saline), respectively. Sections were counterstained with 4',6-diamino-2-phenylindole, and images were obtained and processed using a Nikon E1000 microscope, Micromax CCD camera (Princeton Instruments), and Metamorph imaging program (Universal Imaging).

Electrophysiology—The cDNA encoding the BK α-subunit was a kind gift from Ligia Toro (identical in sequence to U11058 with one exception—identification of the conservative R1112K mutation (25)). Plasmids encoding channel subunit cDNAs were linearized with appropriate restriction enzymes and cRNA synthesized by standard procedures (26, 27). cRNAs were injected into Xenopus oocytes using 1.5 ng of α-subunit RNA/oocyte ± β-subunit RNA at equimolar concentration, 5-fold, or 10-fold molar excess. The molar ratio of the β/α RNAs in coinjection experiments was varied from 1 to 10 in attempts to maximize stoichiometric assembly of the two subunits. Preliminary comparisons of the magnitudes of functional effects induced by β-subunits demonstrated saturation of effects at <5-fold molar excess of β RNA. Therefore, all subsequent studies were done with β-subunit RNA in 10-fold molar excess over α. Oocytes were maintained at 18 °C in ND-96 (28), and macroscopic K+- currents were recorded from inside-out patches 3–14 days following injection. Recordings were performed in symmetrical potassium; the standard pipette and bath solutions contained 116 mM potassium gluconate, 4 mM potassium chloride, and 10 mM HEPES, pH 7.2. CaCl2 was added to the bath solution to yield a final concentration of free ionized calcium of 30 μM, taking the stability constant for calcium gluconate (15.9 s−1) into account (29). Currents were recorded using an EPC-7 amplifier (HEKA) and pClamp software (Axon Instruments). Data were sampled at 2 kHz using an 8-pole Bessel filter (Frequency Devices) and sampled at 5 kHz, but some records, included in group data, were filtered at 1 kHz and sampled at 3 kHz.

Currents were elicited in the presence of 30 μM bath Ca2+ using a voltage command consisting of a holding potential of −80 mV, followed by a 200-ms prepulse to −160 mV, and finally 500-msec step depolarizat...
tions from –80 to +80 mV in 10-mV increments. Tail currents were recorded for 120 ms after returning to the –80 mV holding potential. The hyperpolarizing prepulse was needed to remove steady state inactivation at the holding potential when an inactivating β-subunit was coexpressed. Peak currents were measured, transformed to macroscopic conductances, and plotted as a function of test potential to assess changes in the voltage dependence of activation at 30 μM Ca²⁺. Where appropriate, Boltzmann equations were fit to these data to estimate midpoints of activation. Maximal inactivation parameters, fractional non-inactivating current, and inactivation rates were measured from current traces acquired in 30 μM Ca²⁺ at +80 mV. These values were used to calculate saturation of β-subunit effects after coinjection of β- and α-subunit cRNAs at increasing ratios. Inactivation rates were determined from the exponential fits to the data. Fractional non-inactivating current was calculated as steady-state current/peak current, and fractional inactivating current was estimated as peak current minus steady-state current divided by peak current. Values are presented as mean ± S.E.

**Generation of Antiserum and Immunoprecipitation—** Rabbit antipeptide antisera was raised (Genosys) against the amino-terminal 15 amino acids of β3b, and initially characterized by immunoprecipitation of in vitro translated β-subunits. Proteins were translated in a rabbit reticulocyte lysate (Promega), in the presence of translation grade [35S]Met (NEN Life Science Products) and canine microsomal membranes (Promega), according to the manufacturers’ recommended protocol. Immunoprecipitations were then carried out as described previously (30) using 1% Triton X-100 to solubilize the proteins and a 1/100 dilution of either preserum or antiserum. Proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

To investigate the synthesis of β-subunits in Xenopus oocytes, cells were injected with cRNAs encoding β3b or β3d and incubated overnight at room temperature in 1 ml ND-96 supplemented with 0.5 mM of ICN Trans-[35S]cysteine (1175 Ci/mmol) to metabolically label newly synthesized proteins. Solubilization and immunoprecipitation (from batches of 24–30 oocytes) was carried out as described previously (31), using 1% Triton X-100 as the detergent and a 1/100 dilution of preserum or antiserum. Immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

**RESULTS**

**Identification and Cloning of Five BK β-Subunits—** A combination of data base searching and classical molecular biological techniques resulted in the identification of two families of β-subunits, β2 and β3. The initial data base searches were carried out using the amino acid and nucleotide sequences of the BK β-subunit purified from airway and vascular smooth muscle (β1) (11) as the query and identified an EST encoding a full-length homologue (β2). Subsequent searches with the β2 sequence then identified several ESTs encoding parts of another putative β-subunit (β3). None of these latter cDNAs encoded a full-length protein, so 5′-RACE was performed to complete the coding sequences. Because many of these ESTs were derived from a tonsil preparation enriched for B cells, cDNA from spleen (an available tissue that is also rich in B cells) was used as the template. A novel family of 4 β-subunits (β3a–d) was cloned from the products of that reaction. The PCR reactions were repeated several times using two different spleen cDNA preparations with consistent results.

Although differing in primary sequence, the overall structure of the β2 and β3 subunits is similar to that of β1, containing two hydrophobic, putative transmembrane domains (Fig. 1). The deduced amino acid sequence of the β2 subunit is 235 residues in length, predicting a 27.1-kDa protein that shares 44% pairwise amino acid identity with β1. The β3 family consists of four related subunits (Fig. 1), ranging in length from 257 to 279 amino acids (28.1–31.6 kDa) that are approximately 32% and 41% identical to β1 and β2, respectively. The β3a–d subunits vary only in their cytoplasmic, amino-terminal sequence and share 256 carboxyl-terminal amino acids. A single nucleotide polymorphism was identified within this conserved domain of the β3 family. The nucleotide (at position 1350 of the β3c cDNA sequence) was found to be either G or A (11 G and 9 A in 20 independent cDNAs), resulting in either a Ser or an Asn in the encoded protein (position 161 in the β3c amino acid sequence), and this single nucleotide polymorphism was found in cDNAs encoding each of the β3 splice variants.

Alignment of the deduced amino acid sequences of BK β-subunits (Fig. 1) demonstrates that sequence conservation between families is strongest within the two putative transmembrane domains and, to a lesser extent, in the connecting
extracellular loop. The unique amino-terminal sequences of the β2 and β3 variants are longer than that of β1 and contain consensus sequences for phosphorylation by several different protein kinases, including some that are found in all of the subunits (e.g., protein kinase A sites) and some that are restricted to particular family members (e.g., tyrosine kinase sites in β3). Each of the β-subunits contains potential N-linked glycosylation sites within the extracellular loop, but the number is subunit-dependent, ranging from 1 to 3. The carboxyl-terminal domain of the β3 subunit is also longer than that found in either β1 or β2, and contains a leucine zipper motif that starts in the second transmembrane domain.

Other Related Sequences—Data base searches with the nucleotide sequences encoding β2 and β3a–d resulted in the identification of two additional DNAs that are homologous to different regions of the β3 gene. The first, homologous to the β3b domain, is a cDNA (U696609 and AJ223075) encoding a transcription factor, GCF2 (32, 33). Although transcribed from an upstream promoter, this cDNA is ~95% identical to exon 1b and part of the 5′-UTR of the β3 gene (Fig. 2A). However, since exon 1b encodes only the 5′-UTR and the initiating methionine of the β3b subunit, there is no homology between GCF2 and any of the BK β-subunits at the amino acid level. The second sequence, a fragment of chromosome 22q11.2 (AP000365 and AP000547), is homologous to the conserved core region of the β3 variants. Sequence analysis revealed >95% nucleotide identity between this region of chromosome 22 and exons 3, 4, and the intervening intron of the β3 gene (Fig. 2). However, there is no further homology to any of the other introns or exons of the β3 gene in the additional >100 kilobase pairs of chromosome 22 sequence available in AP000365 or AP000547.

Alternative Splicing Generates the BK β3 Family—Analysis of the structure of the gene encoding β3a–d demonstrated that this family of subunits arises from alternative splicing of a single gene. A search of the high throughput genomic sequence data base revealed a single entry (AC007823) that contains the unique (5′) sequences of the β3a, β3b, β3c, and β3d cDNAs. All of these sequences, as well as the first 191 bp of the conserved core domain, are present on a 37-kilobase pair fragment of human genomic DNA in the following order: β3a-β3b-β3c/β3d-core (1′–191) (Fig. 2). The sequences unique to the β3a and β3b subunits are present on distinct exons (1a and 1b), whereas the unique β3c and β3d sequences arise by differential splicing of a third exon (1c/1d). The first 191 bp of the β3 conserved core domain are encoded on another exon (2) that is contiguous with exon 1c.

Since no additional β3 genomic sequence was yet present in the data base, a human genomic DNA library was screened to isolate the remaining part of the gene. Four BACs were isolated in two screens, and each was analyzed by a combination of PCR and direct sequencing to determine which parts of the β3 gene they encode. In this way, a contig of two overlapping BACs (B766 and B767) was demonstrated to contain all of the coding sequence of the β3 gene. Detailed characterization of B767 demonstrated that two additional exons (3 and 4) encode the β3 core sequence (and some 3′-UTR) that was not present in the genomic data base (Fig. 2). Thus, the β3 subunits arise by alternative splicing of a gene containing 6 exons, 3 of which (1a–c/d) encode sequence unique to each of the splice variants. The other 3 exons (2–4) encode the carboxyl-terminal sequence common to all members of this family (Fig. 2).

Chromosomal Mapping—The chromosomal locations of the β2, β3, and GCF2 genes were mapped by radiation hybrid analysis. PCR reactions were carried out against DNAs isolated from G3 and G4 radiation hybrid panels using subunit- and splice variant-specific primers. Due to the high degree of sequence homology, primers used to amplify the unique β3b domain also amplified a fragment of GCF2. However, β3b and GCF2 amplification products were distinguished by their differential susceptibility to digestion by either BsrBI (which specifically cleaves the β3b fragment) or PstI (which cleaves only the GCF2 fragment). Therefore, fragments amplified from the β2, β3a–c/d, and GCF2 exons could each be identified unambiguously. These experiments mapped the β2 gene to human chromosome 3q26.2–27.1 by FISH. Results of a typical FISH experiment, demonstrating hybridization of BAC (B766) DNA to the distal long arm of chromosome 3 (in green). Cohybridization of a chromosome 3-specific marker resulted in the labeling of the centromere (in pink).
linkage to D2S331 (LOD > 19), confirming that the GCF2 and β3 proteins are encoded by distinct genes. These data also demonstrate that the β3 gene is distinct from the homologous fragment located on chromosome 22q11.2.

**Fig. 4. Tissue-specific expression of β2 and β3α–d transcripts.** Expression in different tissues was analyzed by RT-PCR using primers that distinguish the individual subunits (see "Experimental Procedures"). Products were detected by Southern analysis. The positive control (from CLONTECH) used to clone each of the β3 variants and serves as an additional positive control. Sizes of the products are indicated in bp.

Differential Expression of the β2 and β3α–d Subunits—Since the high degree of nucleotide identity among 1) GCF2 and β3b, 2) β3c and β3d, and 3) β3 core and chromosome 22q11.2 precluded unambiguous Northern analysis for expression of those subunits, tissue distribution was studied by RT-PCR. Specificity was ensured in several ways. For example, primers were designed to span at least one intron, thereby allowing differentiation of products amplified from mRNA and contaminating genomic DNA (or incompletely spliced RNA). In addition, since the β3 core has no homology to GCF2 cDNA, the use of an antisense primer annealing in that domain allowed β3b-specific amplification. Similarly, sense primers were designed to anneal within the 5′ unique regions of each of the β3 splice variants to prevent amplification of transcripts from chromosome 22. The β3c and β3d products were distinguished based on their 101-bp size difference, and the β3b distribution was independently confirmed using a sense primer that anneals in the region not present in β3d.

This RT-PCR analysis demonstrated that the BK β-subunits exhibit distinct patterns of expression (Fig. 4) and that the β2 and β3α subunits are more restricted in their tissue distribution than are the β3b–d variants. Although precise quantitation of expression is not possible using the data in Fig. 4 suggest that expression of β2 is strongest in kidney and pancreas with weaker expression (some requiring prolonged exposures of the blot) in ovary, testes, and small intestine. β3α is expressed in placenta, pancreas, kidney, and heart, whereas β3b–d are widely distributed.

The abundance of β3b mRNA in pancreas prompted further sublocalization studies. In situ hybridization analysis demonstrated colocalization of β3c with insulin, and not with glucagon, indicating that expression of this subunit in the pancreas is limited to β cells (Fig. 5). Similar results were obtained using either an antisense oligonucleotide probe specific for β3c (data in Fig. 5) or a 600-nucleotide antisense riboprobe that did not distinguish between β3c and β3d (data not shown).

**Functional Effects of the β2 and β3 Subunits**—To examine the effects of these β-subunits on the function of K<sub>Ca</sub> channels, cRNA encoding a BK α-subunit (h-slo) (25) was injected into *Xenopus* oocytes with or without each β-subunit cRNA. Coinjection of the β2 transcript had significant effects on the biophysical properties of the current (Fig. 6). For example, whereas cells expressing the α-subunit alone exhibited noninactivating currents, coexpression of the β2 subunit resulted in rapid (τ = 51 ± 6 ms at +80 mV, 30 μM Ca<sup>2+</sup>, n = 7) and complete inactivation. Furthermore, the β2 subunit shifted the voltage dependence for activation of the channel by approximately −60 mV (Table 1).

Three of the four β3 splice variants also altered the properties of the K<sub>Ca</sub> channel, but their effects differed from those induced by β2 and, in fact, also varied between splice variants. For example, coexpression of the β3a, -b, and -c subunits resulted in partial inactivation (Fig. 6) of the current. Thus, although the time constants for inactivation of β3a and β3c currents were similar to that induced by β2 (τ = 45 ± 15 ms for β3a (n = 6), and 60 ± 6 ms for β3c (n = 9)), and voltage-independent under the conditions tested (data not shown), the fractional inactivation during a 500-ms pulse to +80 mV (30 μM Ca<sup>2+</sup>) was only 0.76 ± 0.03 (n = 6) and 0.61 ± 0.03 (n = 10) in cells coexpressing β3a or β3c, respectively, compared with 0.97 ± 0.02 (n = 7) in cells expressing β2. The β3b subunit conferred a small component of extremely fast inactivation that could be resolved in only a fraction of the patches (τ = 1.5 ± 0.2 ms, n = 3 out of 7; Fig. 6, inset). The β3d subunit did not induce detectable inactivation.

Although the measurable time-dependent inactivation conferred by β3b was small and rapid, a comparison of the ratio of steady state currents at +80 mV to peak tail current at −80 mV revealed an apparent rectification consistent with very rapid inactivation that was established within 1–2 ms. In cells expressing the α-subunit alone, this ratio was 0.97 ± 0.03 (n = 11), demonstrating equivalent current magnitudes at +80 and −80 mV, consistent with a linear, ohmic conductance. In contrast, in cells coexpressing α and β3b, this ratio was 0.46 ± 0.05 (n = 7), indicating an apparent inward rectification conferred by the β3b subunit. This apparent rectification may be the result of an extremely rapid inactivation, as has been previously described for currents expressed from h-erg (34).

Tail current decay during hyperpolarizing steps was markedly slower in channels containing β3a subunits (Fig. 6). For
FIG. 5. **BK β3c is expressed in pancreatic β-cells.** In situ hybridization experiments were performed with labeled sense and antisense oligonucleotide probes specific for the β3c splice variant. Panel A demonstrates specific hybridization of the antisense probe to the islets of Langerhans (red), whereas no hybridization was observed using the control, sense primer (panel B; scale bars = 20 μm). Higher magnification of staining for β3c within islet cells (panel C; scale bar = 5 μm). Immunohistochemistry was used to demonstrate colocalization of β3 and insulin. Panel D shows in situ hybridization of the β3c probe (green; scale bar = 20 μm), panel E the immunohistochemical localization of insulin in the same section (blue; scale bar = 20 μm), and panel F shows a superposition (double exposure) of panels D and E, demonstrating coexpression of β3c and insulin. Panel G shows in situ hybridization of the β3c probe (green; scale bar = 20 μm), panel H the immunohistochemical localization of glucagon in the same section (blue; scale bar = 20 μm), and panel I shows a superposition of G and H, demonstrating the lack of colocalization of these two markers. Taken together, the data demonstrate expression of β3c mRNA in pancreatic β-cells and not in pancreatic α-cells. Cells were counterstained with 4',6-diamino-2-phenylindole.

example, two time constants were required to fit α + β3a tail currents at −80 mV (1.3 ± 0.06 ms and 72 ± 16 ms, n = 6). In contrast, when α was either expressed alone, or with other β3 subunits, tail currents could be fit with a single time constant that approximates the fast component observed with β3a: 1.3 ± 0.18 ms for α alone (n = 11), 2.3 ± 0.65 ms for α + β3b (n = 7), 1.0 ± 0.05 ms for α + β3c (n = 9), and 1.1 ± 0.22 ms for α + β3d (n = 8). Tail currents in cells expressing β2-containing channels were too small for analysis.

The effects of the β3 splice variants on the Ca2+ and voltage dependence of the channel were also distinct from that of β2. As noted above, at a given Ca2+ concentration, the β2 subunit, like β1, induced a large shift to more polarized potentials in the voltage dependence for activation of the channel. None of the β3 splice variants, however, induced a similar phenotype (Table I). Coexpression of the β3b or β3d subunits caused no significant change in voltage dependence for activation (V1/2 = −2 ± 4 mV, −11 ± 9 mV, and −3 ± 6 mV for α alone, α plus β3b, and α plus β3d, respectively, at 30 μM Ca2+). Even more dissimilar to the β1 and β2 phenotype, coexpression of the β3a and β3c subunits resulted in shifts in channel activation to more depolarized potentials (V1/2 = +28 ± 5 mV and +15 ± 0.3 mV for β3a and β3c, respectively, at 30 μM Ca2+). Thus, the biophysical properties of this KCa channel α-subunit were modified in diverse ways by different β-subunits.

As demonstrated above, functional effects of the β2 and β3a, and β3c subunits are readily apparent when they are coexpressed with a BK β-subunit, while the effects of the β3b subunit are more subtle. To examine expression of β3b in oocytes, we have generated an antisera that is capable of immunoprecipitating each of the β3 splice variants from in vitro translations. Using this antiserum, we have been able to immunoprecipitate β3b from oocytes injected with β3b cRNA (data not shown.) Thus, the small functional effects noted upon coexpression of the β3b subunit are supported by the synthesis of that protein in those cells. Despite successful in vitro translation and immunoprecipitation of the β3d subunit, we have neither been able to demonstrate functional effects nor immunoprecipitation of this subunit from oocytes injected with encoding cRNA (data not shown).

**DISCUSSION**

We report here the characterization of 2 families of BK β-subunits. The first, β2, contains only a single member and is identical to that recently described by two other groups (15, 16). The second β3a–d, consists of four related proteins, which arise by alternative splicing of a distinct gene. The sequence of one (β3c) of these four splice variants was also recently reported (35). Although similar in general structure to the known β1 subunit, each of these novel β-subunits exhibits properties that distinguish it from the others. For example, the pattern of expression for each of these subunits is distinct, though several subunits are often present within a single tissue. In addition, the various channel subunits contain distinct sites for regulation by diverse kinases, raising the possibility of subunit-specific modulation by various second messengers in vivo. Finally, coexpression of these β-subunits with a BK α-subunit demonstrated that most alter the properties of the channel and do so in subtype- and splice variant-specific ways. For example, coexpression of the β1 subunit has dramatic effects on the voltage and Ca2+ dependence of the channel (shifting V1/2 to significantly more negative potentials at a given Ca2+ concentration (18). Although similar to β1 in terms of its effects on voltage dependence, β2 also significantly alters the inactivation prop-
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properties of the BK current in these studies. Although formally possible, we think this subunit is not a cloning artifact since it was independently isolated several times and detected in several tissues. There are several other possible explanations for the lack of observed β3d functional effects. As discussed above, we have not yet been able to biochemically demonstrate synthesis of β3d in oocytes injected with its cRNA, despite successful in vitro translation and immunoprecipitation of the same lot of cRNA. We cannot be certain whether this negative result represents a failure of the antibodies to detect β3d synthesized in oocytes or whether β3d is inefficiently translated in oocytes. The lack of functional effects of β3d may, therefore, reflect a lack of expression of this subunit in Xenopus oocytes, inactivity of the protein in this particular expression system, or its inability to assemble with the particular splice variant of the BK α-subunit used in these studies, or it may have effects beyond those we have examined. It is also possible that some of these β-subunits may have effects on protein processing or on other types of KCa channels (e.g. small or intermediate conductance channels) in lieu of or in addition to effects on BK channels, and further work will be required to distinguish these possibilities.

This work has increased the total number of KCa, β-subunits currently known to eight, from five families: the original β1 subunit purified and cloned from smooth muscle (11), the β2 (15, 16) and β3a–d variants reported here, and two additional subunits, Y21839 (36) and C06 (37) (β4 and putative β5). Y21839 is another protein that shares 25–31% identity to human β1–β3 and represents a novel family (β4) of this rapidly expanding class of channel subunits. The Y21839 sequence was recently released in a patent data base (the GENSEQ data base), and functional characterization has recently been reported (36). C06 is an avian homologue, most closely related to β1, that has functional properties similar to those reported for other β1 species variants (37, 38). However, the low degree of sequence conservation between CO6 and β1 (47% pairwise amino acid identity with human β1 compared with ~80% identity between other known β1 species variants) suggests that CO6 might indeed represent a novel family of β-subunits, β5. Therefore, although only one gene encoding a BK α-subunit has been identified, the large number of genes encoding distinct β-subunits and their splice variants allows generation of functional diversity in vivo through coassembly of different α-subunit splice variants and different modulatory β-subunits. Because the functional channel is thought to be composed of four α and up to four β-subunits (9), diversity may be further enhanced by coassembly of multiple, functionally distinct splice variants of these β-subunits into the same KCa channel.

The mapping of the β2 and β3 genes to chromosome 3q26.2–27.1 makes them potential candidate genes in several diseases that also have been mapped to this region. These include cerebral cavernous malformations-3 (3q25.2–27), myelodysplasia syndrome (3q26), Cornelia de Lange syndrome (3q25.3), and autosomal dominant optic atrophy (3q28–29) (39–43). Further work will be required to elucidate the role, if any, of these BK channel subunits in the etiology of these pathophysiologic states.

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