Protein-protein interaction between cPLA2 and splice variants of α-subunit of BK channels

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Li J, Al-Khalili O, Ramosevac S, Eaton DC, Denson DD. Protein-protein interaction between cPLA2 and splice variants of α-subunit of BK channels. Am J Physiol Cell Physiol 298: C251–C262, 2010. First published November 25, 2009; doi:10.1152/ajpcell.00221.2009.— Altering the splice variant composition of large-conductance Ca2+-activated potassium (BK) channels can alter their activity and apparent sensitivity to Ca2+ and other regulators of activity. We hypothesized that differences in the responsiveness to arachidonic acid of GH3 and GH4 cells was due to a difference in two splice variants, one present in GH3 cells and the other in GH4 cells. The sequences of the two splice variants differ from one another in several ways, but the largest difference is the presence or absence of 27 amino acids in the COOH terminus of the BK α-subunit. Open probability of the variant containing the 27 amino acids is significantly increased by arachidonic acid, while the variant lacking the 27 amino acids is insensitive to arachidonic acid. In addition, sensitivity of BK channels to arachidonic acid depends on cytosolic phospholipase A2 (cPLA2). Here we used the Mammalian Matchmaker two-hybrid assay and two BK α-subunit constructs with [rSlo(27)] and without [rSlo(0)] the 27-amino acid motif to determine whether cPLA2 associates with one construct [rSlo(27)] and not the other. We hypothesized that differential association of cPLA2 might explain the differing responsiveness of the two constructs and GH3 and GH4 cells to arachidonic acid. We found that cPLA2 is strongly associated with the COOH terminus of rSlo(27) and only very weakly associated with rSlo(0). We also found that arachidonic acid has a lower affinity for rSlo(0) than for rSlo(27). We conclude that the lack of response of BK channels in GH4 cells to arachidonic acid, while the variant lacking the 27 amino acids is insensitive to arachidonic acid. It has been suggested that at least part of this difference in splice variant-dependent sensitivity to channel activators is due to differential association of accessory proteins with different BK channel splice variants that results in some splice variant channels forming specialized signaling complexes while channels formed from other splice variants do not (13). One specific case of such an accessory protein is the so-called BK β-subunit, which associates with the channel-forming α-subunit to alter its voltage and calcium dependence (15). Some β-subunits (β2 and β3) have also been suggested to alter the sensitivity to fatty acids like arachidonic acid.

Besides the β-subunits, there appear to be other accessory proteins. We have shown that cytosolic phospholipase A2 (cPLA2) is required for the optimal response of BK channels to intracellular Ca2+ and arachidonic acid in GH3 cells (7) and that cPLA2 is associated with BK channels since cPLA2 and BK channels can be coimmunoprecipitated and confocal microscopy shows that the two proteins colocalize at the plasma membrane (3). Most wild-type BK channels actually exist as heterotetramers comprised of four α- and four β-subunits. In a sense, the β-subunits can be viewed as accessory protein since four distinct β-subunits have been identified and characterized. Each β-subunit imparts different properties to the overall channel function depending on the anatomic location of the BK channel(s) (1).
As mentioned above, BK channels in GH3 cells are sensitive to arachidonic acid, while GH4 cells are not. GH3 and GH4 cells contain two different BK splice variants whose major difference is the presence in GH3 cells or absence in GH4 cells of a 27-amino acid domain near the COOH terminus of the BK α-subunit. Only GH3 cells containing the splice variant with the 27-amino acid domain are sensitive to arachidonic acid. The simplest explanation for this difference would be that the 27-amino acid region is, itself, the arachidonic acid binding site. However, our previous demonstration that arachidonic acid sensitivity appeared to require close association between cPLA2 and BK channels caused us to hypothesize that the 27-amino acid domain might actually be a site for cPLA2 interaction with the α-subunit of BK channels and that the differential cPLA2 interaction produced the differential sensitivity to arachidonic acid.

MATERIALS AND METHODS

Cell culture. GH3 and Chinese hamster ovary (CHO) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were grown at 37°C in 5% CO2 atmosphere. GH3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% heat-activated horse serum, 2.5% fetal bovine serum, 10 U of penicillin-streptomycin (Pen-Strep), and 2 mM glutamine. GH4C1 cells were originally obtained as a gift from Dr. Gerry Oxford (Univ. of North Carolina, Chapel Hill, NC) and were cultured identically to GH3 cells. Cells from passages 25–45 were used in the experiments described in this article. HEK-293 cells stably expressing the rSlO(27) or rSlO(0) subunit of BK channels (described below) were grown in DMEM supplemented with 10% fetal bovine serum, 10 U of Pen-Strep, and 2 mM glutamine. CHO cells were grown in F12K medium supplemented with 10% fetal bovine serum, 10 U of Pen-Strep, and 2 mM glutamine. CHO cells from passages 1–10 after receipt from ATCC and transfected HEK cells from passages 20–30 were used in the experiments described in this article. Cells for immunoprecipitation experiments were grown to confluence in T-75 culture flasks.

Antibodies and special reagents. Primary antibodies to BK-α channels (host: rabbit) and cPLA2 (host: mouse) were obtained from Chemicon International (Temecula, CA). A BK channel antibody designed specifically for immunoprecipitation was a generous gift from Dr. Irwin Levitan (University of Pennsylvania, Philadelphia, PA). Primary antibodies to BK channel β1- and β2-subunits (host: rabbit) were obtained from Millipore (formerly Chemicon International). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). A/G agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was used in the experiments described in this article. A/G-agarose was pelleted by centrifugation at 4,000 g for 10 min to precipitate unlysed cells. For immunoprecipitations, 1 ml of cell lysate and 10 μl of cPLA2 antibody (concent 1.0 mg/ml) were incubated overnight at 4°C. Thirty microliters of protein A/G PLUS-agarose was added and mixed at 4°C for 2 h with gentle agitation. A/G-agarose was pelleted by centrifugation at 4,000 g for 5 min at 4°C. The supernatant was carefully removed and saved for analysis by Western blot. The pellet was washed three times with 30 μl of Laemmlili sample buffer (Tris, pH 6.8, 1% SDS, 10% glycerol, and 0.025% bromophen blue). The resuspended sample, which also contained 20 mM DTT, was heated at 95°C for 5 min, and the pellet fraction was centrifuged at 4,000 g for 5 min before gel loading and Western blot analysis.

Whole cell lysate immunoprecipitation procedure. Immunoprecipitation experiments were conducted based on the methods previously described for the precipitation of BK channel α- and β-subunits (12). One flask containing ~107 cells was lysed with RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 10% Triton X-100, and 1.0% sodium deoxycholate). The protease inhibitors described above were also added. After cell lysis (1 h at 4°C), the suspension was centrifuged at 4,000 g for 10 min to precipitate unlysed cells. For immunoprecipitations, 1 ml of cell lysate and 10 μl of cPLA2 antibody (concent 1.0 mg/ml) were incubated overnight at 4°C. Thirty microliters of protein A/G PLUS-agarose was added and mixed at 4°C for 2 h with gentle agitation. A/G-agarose was pelleted by centrifugation at 4,000 g for 5 min at 4°C. The supernatant was carefully removed and saved for analysis by Western blot. The pellet was washed three times with 30 μl of Laemmli sample buffer (Tris, pH 6.8, 0.1% SDS, 10% glycerol, and 0.025% bromophen blue). The resuspended sample, which also contained 20 mM DTT, was heated at 95°C for 5 min, and the pellet fraction was centrifuged at 4,000 g for 5 min before gel loading and Western blot analysis.

Western blot analysis. Western blot analysis was completed as previously described (7). Thirty microliters of the eluent and original supernatant was loaded in each lane on a 7.5% polyacrylamide gel. After electrophoresis (~1 h at 150 V), the proteins were transferred to nitrocellulose. The nitrocellulose blots were then probed with primary antibodies for BK-α channels (1:1,000). Blots were then washed and incubated with a secondary antibody (1:160,000). The secondary antibody used was goat anti-rabbit IgG, coupled to alkaline phosphatase (Kirkgaard and Perry Laboratories), and the antigen antibody complex was detected by the chemiluminescent detection system CDP-star (Tropix) and the Kodak 2000M camera system (Eastman Kodak, Rochester, NY).

Isolation of BK channel splice variants from GH3 and GH4 cells. Total RNA was prepared from GH3 and GH4 cells with an RNasey midi-kit (Qiagen) according to the manufacturer’s instructions. Five micrograms of total RNA and 50 μM oligo(dT)20 were used to make cDNA with the ThermoScript RT-PCR system (GIBCO BRL-Invitrogen). PCR primers specific for rat BK channel are as follows: TGGGTATGTAGGCTGAGACACAGACATC, AATTGTGTCATCGAGAAACG, and TGGAGGAGCCGAGGACGCAGCA for the α-subunit and CAGTGGGAAAGCCTGAGTCA for the β-subunit. The PCR products were run on a 0.8% gel and purified with a QIAquick Gel.
Extraction Kit (Qiagen). The products were cloned into PGEMTeasy (Promega), and the sequence of the insert was verified by sequencing the plasmid.

Quantitative PCR. Total RNA was prepared from GH3 and GH4 cells with an RNaseasy midi-kit (Qiagen) according to the manufacturer’s instructions. Five micrograms of total RNA and 50 μM oligo(dT)20 were used to make cDNA with the Thermoscript RT-PCR system (GIBCO BRL-Invitrogen). The following set of sense-antisense PCR primers was used: AGGGCCGTTGAGAGTGAGCAGGCA-ACCTAGGAGAGCTGTAGGGAGC; CTGACTTCTTCCAGGATGTCC-AGTCCGGACATGTATGCGACG; and AGGGCGGCAAA-GATTGCTCAT-ACCTAGGAGAGCTGTAGGGAGC.

The PCR products were run on a gel to confirm that there was a single product obtained for each primer set. The products were cloned into PGEMTeasy (Promega), and the sequence of the insert was verified by sequencing the plasmid. The plasmid was then used to generate a standard curve for real-time PCR using different known concentrations of the plasmid DNA dilutions (100 pg–0.01 pg). Real-time PCR was carried out with the iCycler PCR system (Bio-Rad) in 25-μl reactions on a 96-well plate. Reactions consisted of the following: double-distilled H2O, SYBR Green PCR buffer, MgCl2, dNTPs, AmpliTaqGold DNA polymerase (all from Applied Biosystems), PCR primers, and unknown cDNA template or plasmids. The reaction was cycled 40 times, and data were analyzed with the iCycler software and Microsoft Excel.

A melting curve program was run on each primer pair, and only one melting temperature was observed, suggesting one product for each PCR reaction.

Identification of BK channel β-subunits from GH3 and GH4 cells. Total RNA was prepared from GH3 and GH4 cells with an RNaseasy midi-kit (Qiagen) as described above. Five micrograms of total RNA and 50 μM oligo(dT)20 were used to make cDNA with the Thermoscript RT-PCR system (GIBCO BRL-Invitrogen). PCR primers specific for rat β1- and β2-subunits of BK channel are based on published sequences (accession nos. NM019273 for rSlo(27) and NM176861 for β2). The primers used were CTTGGTGGGCCCCTCTACACA, CAGAAAGCT-GGGCCAGAGG for the β1-subunit and TGAGGAGGACATGACCTCTGT, CTCTGGGTCAGAATAGCAGG for the β2-subunit.

The PCR products were run on a 1.5% gel and purified with a QIAquick Gel Extraction Kit (Qiagen). The products were cloned into PGEMTeasy (Promega) and sequence of the insert was verified by sequencing the plasmid. HEK cell cDNA and mouse brain cDNA were used as positive controls for β1 and β2, respectively. These results were independently confirmed by Western blot.

BK-α splice variant constructs. A full-length clone of rSlo encoding rSlo(27) was used as the template for constructing cDNA of a specific sequence variant, rSlo(0). rSlo(0) cDNA was constructed by replacing a 1,040-bp DNA fragment of rSlo NheI/Xmal digest (2,783 ± 3,822) with the corresponding 959-bp DNA fragment of mSlo NheI/Xmal digest (2,317 ± 3,275). These regions of rSlo and mSlo cDNA encode identical amino acid sequences, except that rSlo(0) lacks 81 bp, resulting in a deletion of a 27-amino acid sequence. The deletion of the 81-bp section in the rSlo(0) construct was confirmed by subsequent DNA sequencing. The individual rSlo variants were subcloned into pcDNA 3.1 (+) (Invitrogen) for expression in HEK-293 cells as described above.

cPLA2 activity assay. Cells from two confluent T-75 flask were washed twice with cold PBS (4°C), and the PBS was removed and replaced with 1.5 ml of 250 mM sucrose buffer. Cells were removed from the flask by scraping. Cells were then lysed by sonication at 4°C. After sonication, the supernatants were centrifuged at 160,000 g for 1 h. The cytosolic supernatant was removed, and the membrane fraction was resuspended in 0.5 ml of sucrose buffer. Protein determinations were made on the cytosol and membrane fractions as described above. PLA2 activity was assayed by the hydrolysis of dipalmitoyl-[2-14C]palmitoyl-1-α-phosphatidylcholine to [14C]palmitic acid, as described previously (7, 21, 22). Each reaction mixture contained 20 μg of protein for cytosolic measurements and 10 μg of protein for membrane measurements.

Mammalian two-hybrid screen. For two-hybrid experiments, cells were plated in 35-mm culture dishes and allowed to grow to ~90% confluence before transfection. A full-length clone for cPLA2 was inserted into the Matchmaker BD plasmid, and three different α-subunit clones were inserted into the Matchmaker AD plasmid. The first of these constructs contained amino acids 1-594 from the NH2 terminus, which are identical for both rSlo(27) and rSlo(0). The second construct contained 456 amino acids from the COOH terminus of rSlo(0) (lacking the 27-amino acid domain), and the third contained 491 amino acids from the COOH terminus of rSlo(27) (containing the 27-amino acid domain) in addition, to determine the possible influence of the BK channel β-subunit on any cPLA2-BK protein interaction, a full-length construct for the BK channel β-subunit (β1) was tested for association with the three α-subunit constructs in the presence and absence of cPLA2. The Matchmaker Mammalian Two-Hybrid Kit was obtained from Clontech Laboratories (Palo Alto, CA), and the plasmids provided were used for further cloning. The pM plasmid was used to produce one NH2-terminal DNA binding domain fusion protein encoding plasmids [the same for both rSlo(0) and rSlo(27)] and two COOH-terminal DNA binding domain fusion protein encoding plasmids [1 for rSlo(0) and 1 for rSlo(27)]. The pVP16 plasmid was used to produce a full-length cPLA2-VP16 or a full-length BK-fusion protein encoding plasmid. PCR products for rSlo(0), the NH2 terminus of rSlo(0), and rSlo(27) domains were ligated into pM with BamHI at the 5’ end and XbaI at the 3’ end. cPLA2 was ligated into pVP16 with EcoRI at the 5’ end and XbaI at the 3’ end. BK-β was ligated into pVP16 with EcoRI and HindIII. Two-hybrid assays were performed by transfecting and harvesting CHO cells according to the manufacturer’s instructions. Typical transfection mixtures contained a total of 22 μg of DNA with 2 μg of PG5CAT plasmid, 10 μg of pM or pM-rSlo, and 10 μg of pVP16 or pVP16-cPLA2. Cells were harvested 48 h after transfection, and six replicate experiments were analyzed for the presence of chloramphenicol acetyltransferase (CAT) with a commercially available UV assay (FAST CAT, Molecular Probes). Blots were visualized and intensities measured with a Kodak 2000MM camera system (Eastman Kodak). Measurement areas for each CAT-containing product were defined with intensity minima set at 25% above background.

Arachidonic acid binding assay. We took advantage of the fact that our anti-BK-α antibody immunoprecipitated rSlo(0) and rSlo(27) constructs equally well. We bound anti-BK-α to A/G beads. We then lysed ~107 cells from two flasks, one transfected with rSlo(0) and the other with rSlo(27), in a gentle lysis buffer (mM: 150 NaCl, 50 HEPES, 1 EGTA, 1 EDTA, 100 mM sodium fluoride, and 10 tetrasodium pyrophosphate, with 100 μM sodium orthovanadate; 10% glycerol, 1% TX-100, pH 7.4, 300 mM Na3-p-nitrophenylphosphate, 1 mM PMSF, 1 mM Pefablock, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) for 30 min at 4°C. After cell lysis (1 h at 4°C), the suspension was centrifuged at 4,000 g for 10 min to precipitate unlysed cells. Thirty microliters of protein A/G PLUS-agarose beads was added to the supernatants and mixed at 4°C for 2 h with gentle agitation to remove any nonspecifically binding proteins and any nonsolubilized membrane fragments. A/G-agarose was pelleted by centrifugation at 4,000 g for 5 min at 4°C. The supernatants were carefully removed and added to the anti-BK-α A/G beads. The equivalence of the immunoprecipitation of rSlo(0) and rSlo(27) was confirmed by performing Western blots on a small amount of the two sets of beads. Since under these conditions there might be significant nonspecific binding of arachidonic acid to the A/G beads and antibody plus protein complexes, we also prepared beads with anti-GAPDH antibody and GAPDH protein. All of the A/G beads containing antibodies and protein were centrifuged, and the supernatant was removed. We then prepared arachidonic acid by evaporating 100 μl of EtOH containing 10 μCi of [3H]arachidonic acid to dryness under dry N2. We dissolved the arachidonic acid in 1 ml of PBS containing 1% BSA and...
determined the specific activity. Two hundred fifty microliters of the arachidonic acid in PBS was added to each set of beads [rSlo(27), rSlo(0), and GAPDH (control)] and shaken at room temperature for 15 min, after which the beads were centrifuged and four 50-µl samples of supernatant were removed from each set and counted. The remainder of the supernatant was discarded and replaced with 1 ml of fresh cold PBS with 1% BSA. This procedure was repeated at 15 min, 30 min, 1 h, 2 h, and 3 h. After the supernatant was removed from the last wash, the beads were resuspended in 100 µl of fresh PBS with 1% BSA and four 25-µl samples were counted to determine the arachidonic acid remaining on the beads. The rates and half-lives of unbinding were determined for each condition by calculating the number of counts remaining on the beads at each of the sample times and on the beads at the end of the 3-h period.

RESULTS

Arachidonic acid differentially affects BK channels from GH3 and GH4 cells. We have shown that BK channels in GH3 cells are directly activated by arachidonic acid, and yet other investigators, using a subclone of GH3 cells (GH4C1) have shown that the BK channels in these cells are not sensitive to arachidonic acid (although they are sensitive to arachidonic acid metabolites) (5, 7, 8). Figure 1 shows that in GH3 cells arachidonic acid produced a significant increase in probability (P_o) from 0.2 ± 0.05 to 0.57 ± 0.13 (P < 0.001) but in GH4 cells arachidonic acid had no effect on P_o (untreated P_o was 0.16 ± 0.09, and after treatment with arachidonic acid P_o was 0.16 ± 0.1). We hypothesized that the difference between the two cell types was that they contained two different splice variants, one sensitive to arachidonic acid and the other not.

**Fig. 1.** Arachidonic acid differentially affects splice variants in the α-subunit of large-conductance Ca^{2+}-activated potassium (BK) channels from GH3 and GH4 cells. Experiments were conducted with inside-out patches depolarized to +20 mV and exposed to 0.1 µM Ca^{2+}. Open probability (P_o) values for individual patches are plotted along with means ± SD, and significant changes in P_o are indicated by an asterisk. One micromolar arachidonic acid caused P_o to increase from 0.2 ± 0.05 to 0.57 ± 0.13 (P < 0.0001) in GH3 cells (n = 8) (A), while P_o in GH4 cells was unaffected by arachidonic acid (0.16 ± 0.09 vs. 0.16 ± 0.10) (n = 7) (B). C and D: representative single-channel records from BK channels in GH3 (C) and GH4 (D) cells. The “o” and “c” denote the open and closed state of the channel(s), respectively.

GH3 and GH4 cells contain different BK channel α-subunit splice variants. Using PCR with primers described in MATERIALS AND METHODS, we isolated β-subunits and the COOH-terminal cytosolic domains of α-subunits.

Examination of the amplified fragments of the α-subunits revealed two different predominant splice variants from the two cell types. Quantitative PCR confirmed the difference in the levels of mRNA for the two splice variants in the two different cell types (Fig. 2). For example, in GH3 cells there is 86 ± 12 times as much of an α-subunit splice variant previously identified in rat brain and rat adrenal chromaffin cells, rMBKα1 (accession nos.: nucleotide AF135265, protein AAD34786), as that of an α-subunit splice variant that has not been previously described in rat but is homologous to a mouse variant, muBKα1 (accession nos.: nucleotide L16912, protein AAA39746). In contrast, in GH4 cells there is 549 ± 80 times as much muBKα1 as rMBKα1. The GH3 splice variant has one small (4 amino acids) and one large (27 amino acids) insertion and one (3 amino acids) deletion not present in the GH4 variant, and the two sequences vary at their COOH-terminal end (Fig. 3).

Identification of BK channel β-subunits from GH3 and GH4 cells. BK β-subunits were identical in both types of cells and were found to be identical to the sequence for the β1-subunit (accession no. AF020712). To determine whether GH3 or GH4 cells also contained the β2-subunit, total RNA was used to prepare cDNA from GH3 and GH4 cells. PCR primers specific for rat β_1 and β_2-subunits of BK channel were based on published sequences (accession nos. NM019273 for β_1 and
NM176861 for β2). Amplification with these primers produced bands of the appropriate size for the β1-subunit from GH3 and GH4 cells but no detectable product for the β2-subunit (although the primers were able to amplify the β2-subunit from rat brain cDNA). Observable PCR products were isolated and cloned, after which the sequence of the PCR products was determined. The sequence of the 2 splice variants that differed between GH3 and GH4 cells. Relative amounts of the 2 splice variants in GH3 (A) and GH4 (B) cells are shown (note logarithmic scale). In GH3 cells there is 86 ± 12 times (mean ± SD; n = 3) as much of a rat splice variant, mBKa1.1, as that of a splice variant previously only observed in mouse, mBKa1.2. In contrast, in GH4 cells there is 549 ± 80 times (mean ± SD; n = 3) as mBKa1.1 as mBKa1.2. The larger difference in the GH4 cells may reflect the fact that they are a clonal line derived from GH3 cells.

Constructs to examine function of GH3 and GH4 splice variants. Fortuitously, α-subunit splice variants similar to those in GH3 and GH4 cells have been isolated from rat brain to examine BK channel Ca\(^{2+}\) sensitivity (10). In this work, Park and colleagues examined the role of the large 27-amino acid difference between the GH3 and GH4 splice variants by modifying the mouse Slo sequence to produce two constructs that differed only in the presence or absence of the 27-amino acid region near the COOH terminus that we hypothesized was the arachidonic acid or PLA2 binding site. Figure 5 is a schematic illustrating the differences between the two splice variants. The two variants were designated rSlo(0) (no putative binding site) and rSlo(27) (full 27-amino acid putative binding site). Using excised patches from HEK cells transiently transfected with rSlo(27), we found that arachidonic acid treatment resulted in a significant increase in \(P_o\) from 0.07 ± 0.03 to 0.19 ± 0.06 (\(P < 0.005\)). On the other hand, arachidonic acid treatment of excised patches from HEK cells transiently transfected with rSlo(0) had no significant effect on \(P_o\) (0.05 ± 0.03 vs. 0.05 ± 0.03). These data are summarized in Fig. 6, and support our hypothesis that the 27-amino acid region in the COOH terminus of the α-subunit is necessary for arachidonic acid sensitivity.

As is true for endogenous BK-α subunits, coexpressing β-subunits with either of the rSlo constructs does increase \(P_o\). The addition of the β1- or the β2-subunit increased the control \(P_o\) (0.05 ± 0.03) of the rSlo(0) channels to a level near those from GH4 cells [0.09 ± 0.05 (\(n = 4\)) for β1 and 0.13 ± 0.03 (\(n = 4\)) for β2]. However, the addition of arachidonic acid to cells expressing α-subunits and either the β1- or the β2-subunit did not result in a significant increase in activity \([0.18 ± 0.05 (n = 4); P = 0.125]\) for β2 and 0.11 ± 0.05 (\(n = 4\); \(P = 0.092\)) for β1], implying that the β-subunits were not responsible for producing arachidonic acid sensitivity.

Addition of accessory proteins is required to activate BK channel α-subunits. While some of the differences in the splice variants found in GH3 and GH4 cells were suggestive of fatty acid binding sites, the 27-amino acid deletion was not obviously a fatty acid binding site. We have previously demonstrated (4, 7) that maximum activation of endogenous BK channels in GH3 cells requires functional cPLA2. We further confirmed that cPLA2 could activate α-subunits as strongly as BK β-subunits. For these experiments, we used excised patches depolarized to +20 mV with four different cytosolic Ca\(^{2+}\) concentrations: 0.1, 1, 10, and 100 μM. BK channels in GH3 cells were compared with HEK cells stably expressing an α-subunit almost identical to the major splice variant in GH3 cells (muBKα) alone or with HEK cells expressing the mBKa α subunit and transiently expressing either cPLA2 or the β1-subunit of BK channels or both (Fig. 7) (clones of both subunits were generously provided by Owen McManus at Merck Research Labs (Rahway, NJ), and the cPLA2 clone was a generous gift from John Turk at Washington University, St. Louis, MO). The coexpression of either cPLA2 or the β1-subunit increased the ability of Ca\(^{2+}\) to activate the channel above that of the α-subunit alone. The magnitudes of the increase for the coexpression of the α- and β1-subunits are virtually identical to those reported by McManus et al. (15).

cPLA2 and BK channel α-subunit coimmunoprecipitate from GH3 and GH4 cells. As we have previously reported (7), any manipulation that prevents activation or expression of cPLA2 shifts the Ca\(^{2+}\) and voltage activation relationships for BK channels to the right (in the absence of cPLA2 full activation requires higher voltages or higher intracellular Ca\(^{2+}\)). This implies that cPLA2 and BK channel proteins must be either in extremely close proximity or actually physically associated. We have subsequently shown that cPLA2 and BK channels colocalize at the surface membrane of both GH3 and GH4 cells (3). However, the difference in arachidonic acid sensitivity of GH3 and GH4 suggests some difference in the cPLA2 activation of specific BK channel α-subunit splice variants. There are several possible explanations for the difference: there could be a difference in the cellular expression of cPLA2, a difference in the activity of cPLA2, or a difference in the association of...
cPLA2 and BK channels. Western blotting (Fig. 8B) shows that cPLA2 protein is equally expressed in both GH3 and GH4 cells, and Fig. 8A shows that there is no significant difference in cPLA2 enzymatic activity (cPLA2 activities for GH3 and GH4 cells were 67.3 ± 7.4 and 62.4 ± 6.9 pmol palmitic acid hydrolyzed-min⁻¹-mg protein⁻¹, respectively). However, when we immunoprecipitated BK channel α-subunits with an antibody (generously supplied by Dr. Irwin Levitan) and then probed the immunoprecipitate for cPLA2, cPLA2 coimmunoprecipitated with the α-subunit of BK channels from both GH3 and GH4 cells, but the amount that immunoprecipitated was much greater in the GH3 cells than in the GH4 cells (Fig. 8C). These data suggest that the BK channel α-subunits in both GH3 and GH4 cells can associate with cPLA2 but that the association is not as strong in GH4 cells.

Differential binding of cPLA2 to BK channel α-subunits from GH3 and GH4 cells in mammalian two-hybrid assay. Coimmunoprecipitation implies an interaction between cPLA2 and some site on the BK channel protein. However, it was unclear how the differences in association were related to the observation that BK channels in the two cell types respond differently to arachidonic acid. Therefore, we decided to examine more specific interactions between cPLA2 and the two different splice variants in the arachidonic acid-sensitive cells (GH3) and the arachidonic acid-insensitive cells (GH4). As mentioned above, we were aided in this investigation since these two splice variants had been previously isolated by us and others (10). The two constructs were identical to the complete mouse Slo gene except that the two constructs differed only in the presence [rslo(27)] or absence [rslo(0)] of the 27-amino acid domain near the COOH terminus.

We used these two constructs to test the hypotheses that cPLA2 and BK channels are physically associated and that the association depends on the presence of the 27-amino acid domain in rslo(27) near the COOH terminus. We also thought that the differences in the association of cPLA2 with the two α-subunit splice variants could explain the differential effects of arachidonic acid. rslo(27) exhibits an extremely high homology to the major α-subunit of BK channels found in GH3 cells, while rslo(0) exhibits an extremely high homology to the major α-subunit found in GH4 cells. To test for specific interaction and to identify the region of BK channels that interacts with cPLA2, we used the Mammalian Matchmaker (Clontech) two-hybrid assay.

A full-length clone for cPLA2 was inserted into the Matchmaker BD plasmid, and three different α-subunit clones were inserted into the Matchmaker AD plasmid. The first of these constructs contained amino acids 1-594 from the NH2 terminus, which are identical for rslo(27) and rslo(0). The second construct contained 456 amino acids from the COOH terminus of rslo(0) (lacking the 27-amino acid domain), and the third contained 491 amino acids from the COOH terminus of rslo(27) (containing the 27-amino acid domain). These constructs and the structure of the BK α-subunit are shown schematically in Fig. 5. In addition, to determine the possible influence of the BK channel β-subunit on any cPLA2-rslo-interaction, a full-length construct for the BK channel β-subunit (β1) was tested for association with the three α-subunit constructs in the presence and absence of cPLA2. cPLA2 most strongly associated with the COOH terminus of rslo(27) (Fig. 9A). cPLA2 is also associated with the NH2 terminus of the α-subunit [the region that both rslo(0) and rslo(27) have in common], but the binding to the COOH terminus of rslo(27) is 3.6 ± 1.1 fold stronger. cPLA2 is only weakly associated with the COOH terminus of rslo(0), to the extent that this association was not even detectable in some experiments. In the experiments in which association was weak but detectable, the binding of rslo(27) was 11 ± 3.3-fold greater than that of rslo(0). In our hands, the negative controls were below the level of detection for four of the six experiments, clearly demonstrating that for a detectable CAT product to be formed, a physical association between the proteins contained in both the binding and activation domains is required. Statistical comparisons, therefore, were limited to the fold differences between binding to the NH2 terminus and binding to the two COOH termini (Fig. 9B).
and to differences in the raw intensity data for binding to the NH2 terminus and to the two COOH termini. Since the data were normally distributed, individual fold changes between the NH2 terminus and the COOH termini of rSlo(0) and rSlo(27) were compared with a Student’s t-test. This analysis showed that the association between cPLA2 and rSlo(27) was significantly greater ($P < 0.02$) than between cPLA2 and rSlo(0). In all experiments, a paired t-test showed that the association was significantly greater for the COOH terminus of rSlo(27) compared with the NH2 terminus ($P < 0.03$) and significantly weaker for the COOH terminus of rSlo(0) compared with the NH2 terminus ($P < 0.01$). Somewhat surprisingly, experiments with the β-subunit showed a physical association with all of the rSlo constructs (Fig. 9C).

cPLA2 binding prevents BK β-subunit from binding to rSlo(27). We anticipated that the β-subunit would preferentially associate with the NH2 terminus since the NH2 terminus contained the membrane spanning domains that conventional wisdom suggests are the regions with which the β-subunits interact (17). However, the association of cPLA2 with rSlo(27) is so strong that this association prevents association of the β-subunit with rSlo(27). On the other hand, the presence of cPLA2 had no apparent effect on the association between the β-subunit and either the NH2 terminus of rSlo or the COOH terminus of rSlo(0) (Fig. 9C).

rSlo(27) has a higher affinity for arachidonic acid than rSlo(0). If rSlo(27) is sensitive to arachidonic acid and rSlo(0) is not, then we would anticipate that rSlo(27) should interact more strongly with arachidonic acid than rSlo(0). Since the presumptive arachidonic acid binding site is intracellular and arachidonic acid is moderately hydrophobic, most whole cell methods and single-channel methods were unsuitable for examining arachidonic acid affinity to BK channels. However, we took advantage of the fact that our BK-α antibody immunoprecipitated rSlo(0) and rSlo(27) constructs equally well. We bound anti-BK-α to A/G beads and then immunoprecipitated rSlo(0) or rSlo(27) from transfected CHO cells. We used the immobilized proteins as targets for [3H]arachidonic acid binding. Since under these conditions there might be significant nonspecific binding of arachidonic acid to the A/G beads and antibody plus protein complexes, we also prepared beads with anti-GAPDH antibody and GAPDH protein. The beads were repeatedly washed, and the rate of release of arachidonic acid from the three different sets of beads was determined. The half-lives of arachidonic acid binding for binding of arachidonic acid to rSlo(27), rSlo(0), and GAPDH (control) were 0.89 ± 0.15, 0.55 ± 0.09, and 0.52 ± 0.11 h, respectively (means ± SE, $n = 5$) (Fig. 10). The unbinding half-life for rSlo(27) was significantly longer ($P < 0.05$) than for either rSlo(0) or GAPDH, while the unbinding half-life for rSlo(0) was not significantly different from that for the GAPDH control (ANOVA followed by a Holm-Sidak multiple comparison test). The implication is that rSlo(27), similar to the BK channel variant in GH3 cells, binds arachidonic acid more strongly than rSlo(0), similar to the variant in GH4 cells. Since the only difference between rSlo(27) and rSlo(0) is the 27-amino acid insertion in rSlo(27), the arachidonic acid binding appears to require this region.

DISCUSSION

Arachidonic acid activates BK channels in GH3 cells but does not activate BK channels in GH4 cells. Arachidonic acid is a critical precursor in several cellular signaling pathways (19) and regulates several types of ion channels within the CNS (11, 16). Arachidonic acid production is primarily controlled by regulating the activity of the membrane-associated phospholipase A$_2$ (cPLA$_2$), an enzyme that hydrolyzes membrane lipids to produce arachidonic acid (18, 20). Once produced, arachidonic acid can itself act on ion channels or can be further metabolized to produce additional signaling molecules. BK channels can be very sensitive to cPLA$_2$ expression and arachidonic acid (6, 8, 29). Arachidonic acid applied to the cytosolic surface of patches formed from GH3 cells strongly activates BK channels, but not all BK channels are so sensitive to arachidonic acid. Arachidonic acid does not activate BK channels in GH4 cells (Fig. 1). We suspected that the difference was because the two different cell types express different splice variants, one of which is sensitive to arachidonic acid and the other insensitive. In fact, when we examined the major splice variants in the two cell types we found two alternative splice variants. We simultaneously confirmed that both cell types expressed the same β-subunit, β$_2$. The variants differ at sites that have been previously described for mammalian BK channels (27). The GH3 splice variant differs from the GH4 variant in having one large 27-amino acid insertion, one
smaller 4-amino acid insertion, and one 3-amino acid deletion (Fig. 3), and the two sequences vary at their COOH-terminal end. The large 27-amino acid insertion is adjacent to the Ca\(^{2+}\) "bowl" and has been previously described as altering the Ca\(^{2+}\) sensitivity of the channel (10). The insertion has some positive charge coupled with hydrophobic residues that could be a binding site for anionic lipids like arachidonic acid or a hydrophobic protein (like PLA\(_2\)). Thus it is possible that the

Fig. 6. Arachidonic acid differentially affects splice variants rSlo(27) and rSlo(0) isolated from rat brain. P\(_o\), values for individual patches are plotted along with means ± SD, and significant changes in P\(_o\) are indicated by an asterisk. When the \(\alpha\)-subunit [rSlo(27)], similar to the splice variant from GH3 cells, was transfected into HEK-293 cells, arachidonic acid treatment significantly increased P\(_o\), from 0.07 ± 0.03 to 0.19 ± 0.06 (P < 0.005) (n = 6) (A). On the other hand, when the \(\alpha\)-subunit [rSlo(0)], similar to the splice variant from GH4 cells, was transfected into HEK-293 cells, arachidonic acid treatment had no significant effect on P\(_o\), (0.05 ± 0.03 to 0.05 ± 0.03) (n = 8) (B). C and D: representative single-channel records from BK channels in HEK-293 cells expressing rSlo(27) (C) and rSlo(0) (D). The "o" and "c" denote the open and closed state of the channel(s), respectively. These data suggest that the effect of arachidonic acid requires the presence of the 27-amino acid sequence contained in rSlo(27), since when it is absent, either in rSlo(0) or in GH4 cells, arachidonic acid has no effect on channel P\(_o\).
27-amino acid region could contain both PLA2 and arachidonic acid binding sites. However, one of the insertions (SRKR) or the variation at the COOH terminus (RK) and the adjacent amino acids have a concentration of positive charge coupled with hydrophobic residues that could also contribute to arachidonic acid binding.

Arachidonic acid sensitivity of BK channels could arise either because arachidonic acid failed to interact with the channel or because cPLA2 failed to interact and, therefore, was incapable of delivering high concentrations of arachidonic acid in close proximity to the channel. We have shown that any manipulation that prevents activation or expression of PLA2 in GH3 cells, the Ca2+ sensitivity of BK channels derived from stable HEK cell line expressing only the muBKα was greatly diminished. Coexpression with the β1-subunit, cPLA2, or both the β1-subunit and cPLA2 resulted in a sequential marked increase in Ca2+ responsiveness of the channels.

On the other hand, BK channels in GH4 cells are not sensitive to arachidonic acid. Originally we thought that it was possible that GH4 cells do not have cPLA2 or that cPLA2 and BK channels do not associate as they do in GH3 cells. This explanation was quickly eliminated because Western blotting showed that both GH3 and GH4 cells had equal amounts of cPLA2 and that the enzyme was equally active (Fig. 8). However, unlike the result in GH3 cells, the α-subunit of BK channels in GH4 cells was only poorly communoprecipitated with cPLA2. These results suggest that cPLA2 does not associate with the BK channel splice variant in GH4 cells as well as it does with the BK channel splice variant in GH3 cells.

We took advantage of the fact that the same splice variants as those in GH3 and GH4 cells had been isolated from rat brain to examine BK channel Ca2+ sensitivity (10). For these studies, parts of the COOH termini of the two splice variants were used to replace the COOH terminus of mSlo, resulting in two constructs whose only difference was the presence [in rSlo(27)] or absence [in rSlo(0)] of the 27-amino acid region near the COOH terminus that we hypothesized was the arachidonic acid or PLA2 binding site. Using excised patches from HEK-293 cells transiently transfected with rSlo(27) or rSlo(0), we found that arachidonic acid treatment resulted in a significant increase in \( P_o \) in the former but not the latter, implicating the 27-amino acid region in arachidonic acid sensitivity.

rSlo(27) binds both cPLA2 and arachidonic acid much more strongly than rSlo(0). Because of the differential sensitivity to arachidonic acid and the difference in communoprecipitation, we used a direct binding assay to examine cPLA2 interaction with rSlo(27) and rSlo(0). The mammalian two-hybrid assay that we used can provide quantitative measures of the binding and rules out the possibility of indirect interaction that might occur in communoprecipitation experiments. Using this assay,

![Fig. 7. Coexpression of either cytosolic phospholipase A2 (cPLA2) or the β1-subunit with muBKα results in an increase in Ca2+ sensitivity. Groups of 6-8 cells from each treatment were studied at each Ca2+ concentration. For these experiments, inside-out patches depolarized to +20 mV were used. Compared with wild-type GH3 cells, the Ca2+ sensitivity of BK channels derived from stable HEK cell line expressing only the muBKα was greatly diminished. Coexpression with the β1-subunit, cPLA2, or both the β1-subunit and cPLA2 resulted in a sequential marked increase in Ca2+ responsiveness of the channels.](Image 393x193 to 465x221)

![Fig. 8. cPLA2 is equally expressed in both GH3 and GH4 cells but associates more strongly with the α-subunit of BK channels from GH3 cells. A: cPLA2 activities for GH3 and GH4 cells were not different (67.3 ± 7.4 and 62.4 ± 6.9 pmol palmitic acid·min·mg protein·
1), respectively, by assay of PLA2 function in whole cell lysates from GH3 and GH4 cells as described previously (21, 22). B: typical Western blot in which whole cell lysates from both GH3 and GH4 cells were probed with a commercially available antibody (Millipore) to cPLA2. This blot shows that cPLA2 is expressed to the same extent in both cell lines (as expected from A). C: we communoprecipitated BK channel α-subunits, resolved the communoprecipitate on SDS gels, and probed with a commercially available antibody (Millipore) to cPLA2. cPLA2 was associated with BK channels in both GH3 and GH4 cells but was much more strongly associated in GH3 cells. These data suggest that BK channel α-subunits in both GH3 and GH4 cells are associated with cPLA2, albeit to different extents.](Image 393x259 to 465x290)
we found a strong association of cPLA$_2$ with the COOH terminal end of rSlo(27) but not with rSlo(0).

In addition, in separate experiments in which the rSlo constructs were bound to agarose beads, we showed that arachidonic acid bound to rSlo(27) much more strongly than rSlo(0) (Fig. 10). In these experiments, if the nonspecific binding is subtracted from the binding for the two BK constructs, rSlo(27) binds arachidonic acid at least 12-fold more strongly than rSlo(0). This value is interesting since the COOH terminus of rSlo(27) binds cPLA$_2$ 11.3-fold better than rSlo(0) and implies that the binding of cPLA$_2$ may be necessary for the binding of arachidonic acid.

The simple interpretation of these results would be that both cPLA$_2$ and arachidonic acid bind to the 27-amino acid insertion in rSlo(27). However, there is an alternative possibility. Since, in the arachidonic acid binding experiments, both of the rSlo constructs were immunoprecipitated from cells transfected with one or the other of the constructs, it is possible that accessory proteins might be coimmunoprecipitated with the rSlo proteins. One candidate for such coimmunoprecipitation,
at least for rSlo(27), is cPLA₂, since we know it binds strongly to rSlo(27). In this scenario cPLA₂ would act as an accessory protein for BK channels in a manner analogous to the better-known β-subunits. In fact, Fig. 7 shows that cPLA₂ cotransfected with a mouse α-subunit can increase the activity of the channel as much or more than cotransfection with the β₁-subunit. The activity of the wild-type channel obtained from cells that contained rat BKα1, the β₁-subunit, and cPLA₂ is approximately the same as cells transfected with the α-subunit and cPLA₂ alone. This is consistent with our observation that cPLA₂ is capable of displacing the β₁-subunit from the COOH-terminal end of rSlo(27). In the presence of β₁ or cPLA₂ alone, either accessory protein binds to the α-subunit and increases its responsiveness to voltage and Ca²⁺. In the presence of both (as in the native GH3 cell), cPLA₂ preferentially binds and the activation is similar to cPLA₂ alone (Fig. 7).

In addition to arachidonic acid, cPLA₂ and other lipases can produce other cis unsaturated fatty acids in addition to arachidonic acid that increase BK channel activity (5). Physical association of cPLA₂ with the BK channel would allow synthesis and efficient delivery of arachidonic acid directly to its binding site on the BK channel protein.

Role of BK channel accessory proteins. Our results imply that an association between the α-subunit and accessory proteins can result in changes that shift the Ca²⁺ or voltage response so that lower Ca²⁺ concentrations and lower voltages activate the channel. While the β-subunit is one example of a molecule that increases Ca²⁺ sensitivity, cPLA₂ is apparently another. We conclude that the lack of response of BK channels in GH4 cells to arachidonic acid is explained, in part, by the poor binding of cPLA₂ to the COOH terminus of the α-subunit [rSlo(0)].

Our results contrast with those of Sun et al. (25), who suggested that all arachidonic acid sensitivity of BK channels was conferred by β-subunits and that the α-subunit by itself did not respond to arachidonic acid. We, of course, would agree that some α-subunit isoforms (like the one present in GH4 cells) are not sensitive to arachidonic acid. They do not explicitly mention which human Slo isoform they expressed in Xenopus oocytes, but we presume that it is homologous to rSlo(0). They also used 30 times the amount of arachidonic acid that we use in our work. However, the interesting issue is whether a β-subunit could restore sensitivity to arachidonic acid. We originally examined the effect of the β₁-subunit, which does not. They examined the β₂-, β₃-, and β₄-subunits but did not examine β₁. Like β₁, β₄ does not produce arachidonic acid sensitivity. On the other hand, they suggested that the β₂-subunit strongly, and the β₃-subunit to a lesser extent, confer arachidonic acid sensitivity to channels. If their conclusion that the β₂-subunit was solely responsible for the arachidonic acid sensitivity was correct, then we should be able to convert rSlo(0) channels, which are unresponsive to arachidonic acid, to channels that are responsive to arachidonic acid simply by cotransfecting cells expressing rSlo(0) with the β₂-subunit. In fact, our results show that, as expected, cotransfecting cells with rSlo(0) and either the β₁- or the β₂-subunit increased the control P₀ of the rSlo(0) channels to a level near channels in native GH4 cells (in which α and β are presumably always present). However, the addition of arachidonic acid to these channels did not result in a significant increase in channel activity. Therefore, β-subunits, by themselves, do not seem to induce sensitivity to arachidonic acid. These results are consistent with our hypothesis that the arachidonic acid sensitivity is predominantly dependent on the presence or absence of a 27-amino acid sequence residing on the BK channel α-subunit. One interesting aspect of the study of Sun et al. (25) is their use of ETYA as a lipoxigenase inhibitor. While this agent does produce inhibition, it also acts as a nonmetabolizable arachidonic acid analog, which did not activate channels. This suggests that some of the effect produced by the β-subunits were produced by metabolites. This will be an interesting area for future investigation.

The results presented here reinforce the importance of arachidonic acid as a signaling molecule in the CNS and specific cells. They also show that the expression of different splice variants can have profound effects on the responses of cells to environmental humoral agents. We further suggest that the constructs we have used are an interesting model in which to study the effects of arachidonic acid and possibly its metabolites. Our results also add to the rapidly growing body of evidence that suggests that the pore-forming subunits of many ion channels exist in the membrane as one of the components of a regulatory protein complex along with one or several signaling molecules that contribute to the modulation of the channel.

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DISCLOSURES

The authors are not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

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