Supplemental Documentation

Results

Cloning and functional characterization of BK_{Ca} channel-forming (rslo cbv) subunits from rat cerebral artery smooth muscle cells

Smooth muscle cells were isolated from adult rat basilar and middle cerebral arteries as described elsewhere [1]. Following isolation, smooth muscle cells were individually collected using an enlarged patch-clamp micropipette; total RNA was obtained from ~100 cells. Following reverse transcription, 3'-RACE and PCR were conducted and amplicons were sequenced at the University of Tennessee Molecular Resource Center. Using restriction enzyme mapping and sequencing, two isoforms were screened (from ~18 clones) that contained the 3.6 kb insert attributable to rslo. After full sequencing, information corresponding to these rslo (termed cbv1 and cbv2) were deposited into Genbank (AY330293 and AY330294). After insertion into the pOX *Xenopus* expression vector, cbv1 and cbv2 cDNAs were linearized with NotI, and transcribed using T3 pol. cRNAs were injected as described for other slo [2]. After expression in *Xenopus* oocytes, rslo currents were acquired and analyzed as previously described for other slo channels in the same expression system [2, 3]. Cbv1 and cbv2 rslo currents were obtained from inside-out patches more than 5 minutes after patch excision from the cell. Pipette solution contained (in mM): Kgluconate 130, $CaCl_2$ 5.2, $MgCl_2$ 2.3, EGTA 5, HEDTA 1.6, HEPES 10 (free $[Ca^{2+}]_i$~11 μM). Bath solution contained (in mM): Kgluconate 130, $CaCl_2$ 3.84, $MgCl_2$ 1, EGTA 5, HEPES 10 (free $[Ca^{2+}]_i$~0.3 μM). Cbv1 and cbv2 rslo currents obtained from inside-out patches showed: 1) ohmic behavior between -60 and 40 mV, with slope conductances of 220±25 (cbv1) and 201±17 (cbv2) pS in symmetric 130 mM K^+, respectively. For both rslo channels, unitary currents displayed a Nernst shift of ~38 mV in $V_{i=0}$ when $[K^+]_i$ was changed

from 130 to 30 mM (Na^+ substituting for K^+). Together, these data indicate that cbv1 and cbv2 channels display high permeability and selectivity for K^+ over Na^+; 2) an elevation in channel steady-state activity (NP_o , calculated from all points amplitude histograms, as described in [4]) with more positive transmembrane potentials: 11±1 and 11±2 mV/e-fold change in NP_o for cbv1 and cbv2, respectively. Data are given as mean±SEM (n=3-4); 3) an increase in NP_o (× >10 times) with an elevation in $[Ca^{2+}]_i$ from subμM to μM (Supplemental Figure 1). Data from outside-out patches demonstrated that: 1) external TEA (1 mM) and iberiotoxin (100 nM) caused a flickery and a slow current block, respectively, almost totally abolishing rslo current (not shown), as described for native BK_{Ca} channels [5]; 2) external 17β estradiol (5-10 μM) increased currents (× ~5 times) only when rslo channels were coexpressed with β_1 subunits (Supplemental Figure 2), as previously reported for bslo channels [2].

In summary, full-length slo subunits were cloned from freshly isolated rat cerebral artery smooth muscle cells. Rslo expression led to single channel events that displayed all major characteristics of BK_{Ca} unitary currents. These data have been sent for communication at the Society for Neuroscience 2005 annual meeting (Liu, J, Liu, P, Crowley, JJ, Asuncion-Chin, MT, and Dopico, AM. "Cloning and functional characterization of BK_{Ca} channel-forming (rslo cbv) subunits from rat cerebral artery myocytes)".

Detailed Materials and Methods

Thin layer chromatography

Native heme-binding pocket peptide (DAKEVKRAFFYCKACHDDITDPK, BKPP) and double mutant (C to S and H to R) peptide (DAKEVKRAFFYCKASRDDITDPK, BKMP) were dissolved in water (stock solution: 1 mM). Peptides (10μl) were applied 2 cm from the bottom edge of 5 x 20 cm silica gel 60 plates and allowed to dry. Deoxygenated Krebs (dKrebs) was prepared by vigorously diffusing N_2 through Krebs solution in a parafilm-sealed Erlenmeyer flask. Fe^{2+}-heme (500 μM) solution was prepared by dissolving Fe^{3+}-hemin in basic (pH 8) dKrebs and reducing it with sodium dithionate (SDT, added to a final concentration of 2 mM). Exposure of porphyrins to light and air was minimized. Heme (10μl, 1 mM) was applied 3 cm from the bottom edge (1 cm above the peptide) and the plate was immediately placed in a gas controlled, environmental chamber development tank. The chromatography solvent was ethanol/isopropanol/PBS (15/15/70). Solvent migration time was 2hr in atmospheres of either 100% N_2, 100% CO, or 10% CO and 90% N_2 (data for 100% and 10% CO were the same and thus, combined as CO). Under these conditions heme does not move but the peptide migrates into the heme spot. If the peptide binds to the heme, its migration will be either stopped or slowed. Plates were dried in room air and peptides stained with naphthol blue black (500 mg dissolved in methanol: acetic aid: water (45: 10:45), 3 min), rinsed with water (3 min), and destained with methanol:acetic acid:water (90:2:8)). Line scans were obtained and intensity quantified using a digital camera, digital imaging and densitometry with NIH Image software.

Spectrophotometry

Carbon monoxide was prepared as a saturated solution (1 mM) by vigorously bubbling distilled water with 100 % CO for 2 hours under a headspace gas of 100 % CO. CO concentrations in the solutions were measured by GC-MS. Under reducing conditions (0.5mM SDT and anoxic Krebs), CO (as saturated solution in water, 1mM) was added to heme to make final concentrations of 20, 40, and 100 µM. The stock solution of BKPP peptide (1 mM), corresponding to the heme-binding pocket of the BK channel, was prepared in water and stored at –20˚C. For experimentation, BKPP peptide was dissolved in dKrebs to final concentrations of 20-100 µM. All solutions were protected from air and light, and the spectra were taken immediately after mixing the solutions in a parafilm-sealed optical cuvette with the head space purged with N_2. To investigate interactions between heme and CO under reducing conditions, the absorption spectrum of heme solution was recorded first, then CO was added to make a final concentration of 20-100 µM in the cuvette, and spectra were recorded again. To investigate the effects of BKPP on heme - CO interactions under reducing conditions, BKPP was dissolved in dKrebs to make final concentrations of 20-40 µM and was added to the heme solution a) in the absence of CO, b) immediately before CO, and c) immediately after CO, and the absorption spectra of heme solutions were recorded. To investigate interactions among hemin, CO, and BKPP under non-reducing conditions, the above experimental protocol was repeated in the absence of SDT. Scans were performed using an Ultraspec 2100 UV/visible spectrophotometer (Amersham, Piscataway, NJ) and analyzed using Biochrom Data Capture spreadsheet interface software. Each experiment was repeated at least 5 times.

Mass Spectrometry

A Micromass Qtof2 mass spectrometer with a nano-electrospray ionization probe and MassLynx software (version 3.5) was used for mass spectrometry (MS). For MS, collision energies of 5 or 8V were used and the instrument scanned at 100 to 2000 m/z for all solution ions. The voltages were kept very low to enable peak visualization of weakly bound ion complexes. Spray needle voltage was 1.8 kV, cone voltage was 5V. Solutions were sprayed in 25 mM ammonium bicarbonate, 12 to 25 % acetonitrile. Syringe infusion rate through a 30 µm orifice spray needle was 0.4 µl/min. In all cases, stable protein or peptide charge states were formed, and the protein was not denatured. For MSMS, progressively increasing voltages were used while fixing on one precursor m/z and scanning the fragments. MSMS plots demonstrated affinity of the heme for peptide or protein.

Hemin solutions were made in small amounts of 0.2N ammonium hydroxide, with final dilution to1mg/ml and 70% acetonitrile. Oxygen was kept from solutions by N_2 purging. Dithiothreitol (DTT) was used at 2 to 5 times hemin on a molar basis to reduce the hemin to heme. Myoglobin was made to 500µM in 50mM ammonium bicarbonate. SDT at a 1.3-fold higher concentration than myoglobin was used to reduce the integral heme iron, followed by desalting on a Sephadex G-25 column. Amounts of protein/peptide electrosprayed varied from 20 to 150 µM.

Electrophysiology

Smooth muscle cells were isolated from resistance-size (~150 µm) rat cerebral arteries as previously described [6]. Cells were used within 8 hours of isolation. Cos-1 cells were transfected with cloned rat cerebral artery smooth muscle channels (cbv1) or cbv1 in which CKACH was

mutated to CKASR between amino acids 612 and 616, as in the BKPP and BKMP peptides. Channel currents were measured from inside-out membrane patches using an Axopatch 200B patch-clamp amplifier and a Digidata 1322A. Bath solution contained (in mM): 140 KCl, 5.2 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 1.6 HEDTA, and 10 HEPES (pH 7.2), and pipette solution contained (in mM): 140 KCl, 2 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES (pH 7.4). The free [Ca^{2+}] bathing the intracellular membrane surface was 10 µM, as confirmed with a Ca^{2+}-sensitive and reference electrode (Corning) CO was prepared as a saturated, deoxygenated solution (1 mM) and diluted as required. Low O_2 solutions were prepared from anoxic bath solution that was made by purging with 100 % N_2. Following preparation, bath solutions were placed in a gas impermeant container and continually perfused through the patch-clamp chamber at a rate of 4 ml min^{-1}. O_2 pressure in the patch-clamp chamber was monitored using a PO_2 electrode (Extech Instruments) and confirmed that gas exchange with room air was minimal at this perfusion rate. Dilution of heme to a 100 nM concentration in bath solution was accompanied by addition of 2 µM dithionite. Dithionite (2 µM) did not alter BK_{Ca} channel activity (data not shown). K_{Ca} channel open probability (P_o) was calculated from the following equation: $P_o = NP_o/N$, where N is the total number of channels in the patch (determined by application of 100 µM free Ca^{2+} at +40 mV). Where appropriate, data were fit with a Hill equation: $P_o=P_{max}$ $[Hemin]^n/(K_d^n + Hemin]^n)$, where P_o is the open probability, K_d is the dissociation constant, n is the Hill coefficient, and P_{max} is maximal P_o.

cDNA synthesis from cerebral artery smooth muscle cells

Individual smooth muscle cells were isolated from rat basilar and middle-cerebral arteries using enzymes, as previously described [1]. Smooth muscle cells were identified under an inverted

microscope using Hoffmann optics and individually aspirated into glass micropipettes (≤10 μm diameter). Smooth muscle cells were released into Eppendorf tubes containing ~50 μl of PicoPure extraction buffer (Arcturus), and stored at -80ºC. Total RNA from ~100 smooth muscle cells was isolated using the PicoPure RNA Isolation Kit (Arcturus).

First strand cDNA was synthesized from the total RNA using an oligo(dT)-primed 20 μl reaction by SuperscriptII Reverse transcriptase (Invitrogen) under standard conditions (final concentrations: 8 mM Magnesium acetate, 50 mM Tris-HCl, 75 mM KCl, 500 μM each dNTP, 25μM oligo(dT) primer and 7.5 U SuperscriptII Reverse transcriptase).

PCR amplification of slo1 conserved fragment and 3'-RACE

Amplification of rat cerebral artery smooth muscle cell slo1 (KCNMA1) fragments was performed using the following PCR primer pairs: forward, 5'-gca agt gat gcc aaa gaa gtt a-3' and reverse, 5'-atc tgt cca ttc cag gag gt-3'. PCR amplification was performed on an AMPLITRON II-Thermocycler (Barnstead Thermolyne) using 2 μl of the first strand cDNA in a final volume of 50 μl containing 2 U Platinum TaqDNA polymerase (Invitrogen), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 40 mM KCl, 16 mM Tris-HCl, and a primer concentration of 200 mM each. The cycling protocol for the PCR reaction was: 94 ºC for 2 min, [94 ºC for 30 s, 52 ºC for 45 s and 72 ºC for 50s] x 25 cycles, and 72ºC for 2 min. Amplicons were automatically sequenced at the University of Tennessee Health Science Center Molecular Resource Center. Based on these rat KCNMA1 fragment sequences, we designed two primers: GSP1, cca gtc tgt ctc att cct ccc ac, and GSP2, ctg tcc act cca tcc cgt cca to conduct 3'RACE. Target 3' cDNA ends were amplified by two rounds of PCR under optimal conditions using the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen).

Full-length slo1 amplification and sequence analysis

After comparing the sequence of slo1 3' cDNA ends of rat cerebral artery smooth muscle cells, another two primers for first round PCR were designed: 5 out, tcc tcc tct tcc tcc tcg tcc tcg g, and 3 out, acg tca cca ttt atg cag ttt gtc ag. For the second round of PCR, primers were: 5 in, gtc cac gag ccc aag atg gat gcg c, and 3 in, cct ggg aat caa cat tca tct tca act tc. Target cDNAs were amplified by nested PCR in optimal conditions according to the following protocol: first round PCR was conducted in a 0.5-ml microcentrifuge tube containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 300 nM primer 5out, 300 nM primer 3out, 200 µM of each dNTP, 2 µl cDNA, and 1.5 units of DNA polymerase (Expand High Fidelity PCR system, Roche) for a total volume of 50 µl. The cycling protocol for PCR was: 94 °C for 1 min; denaturation, 94°C for 30 s; annealing of primers, 61 °C for 30 s with a decrease of 0.3 °C/cycle; primer extension, 72°C for 1 min x 10 cycles; denaturation, 94°C for 30 s; annealing of primers, 58 °C for 30 s; extension, 8 s/cycle; primer extension, 72 °C for 1 min x 15 cycles, followed by final extension, 72 °C for 5 min. Second round PCR was conducted following a similar protocol.

A band of ~3.6 kb was amplified, rescued and ligated to the pCR-XL-Topo vector (Invitrogen). Using restriction enzyme mapping and sequencing analysis, we screened two slo1 isoforms (from ~18 clones) that contained the 3.6 kb insert. After full sequencing, information corresponding to the two isoforms of rat cerebral artery smooth muscle cell slo1 (termed cbv1 and cbv2) was deposited into Genbank (AY330293 and AY330294).

Cbv1 insertion for mammalian expression and mutagenesis

PCR primers were designed starting from the second Met: forward, cac caa gat gga tgc gct cat cat ccc, and reverse, tct gta aac cat ttc ttt ttt ctg ttt gtc gcg. A Kozak sequence was introduced at the 5' end to improve expression in a mammalian expression system. Amplified PCR fragments were then directly ligated to the pcDNA3/V5/His-TOPO vector (Invitrogen).

Mutagenesis of cbv1 was conducted using the Quickchange system (Stratagene) in order to eliminate the heme binding motif (CKACH) by introducing a double mutation (CKASR). Oligo sequences used for mutagenesis were: forward, gca ttt ttt tac tgc aag gcc tct cgt gat gac gtc ac, and reverse, gtg acg tca tca cga gag gcc ttg cag taa aaa aat gc. The fidelity of the desired mutations and the absence of unwanted mutations were verified by sequencing.

Cell culture and transfection

Cos1 cells were maintained in DMEM (Cellgro) supplemented with 10 % FBS (Gibco) and 1 % penicillin/streptomycin. pCDNA3encoding cbv1 and pEGFP-C3 were co-transfected into cos1 cells using FuGENE 6 (Roche). Cells were cultured at 37 ^{o}C in a 95 % air / 5 % CO_2 atmosphere and used between 24 and 96 hours following transfection. eGFP was excited at 450-490 nm and emitted light between 500 and 550 nm was observed. Transfected cells were positively identified due to their green fluorescence.

Statistics

Summary data are expressed as mean ± standard error of the mean. ANOVA, paired or unpaired Students *t* , Student-Newman-Keuls, and/or Wilcoxon matched-pairs signed-ranks test were

performed according to experimental design and data distribution (Kolmogorov and Smirnov distance).

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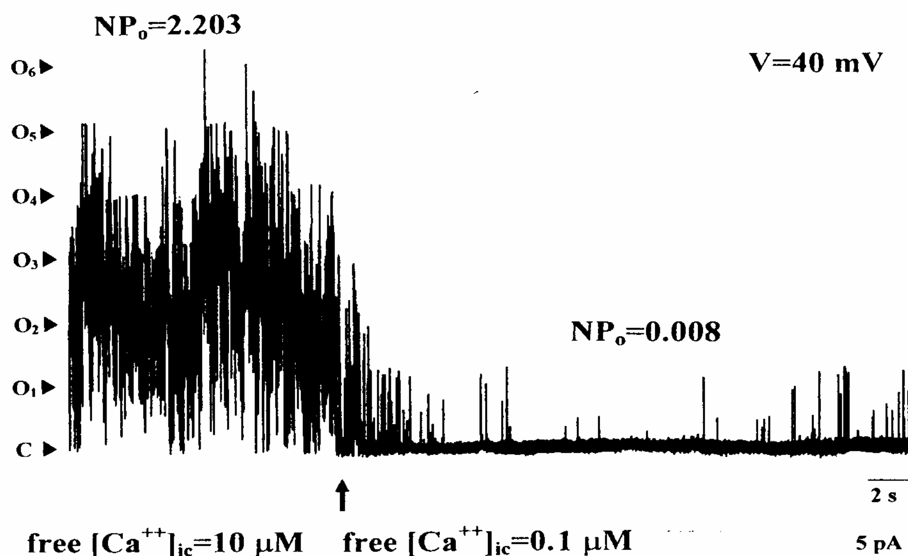
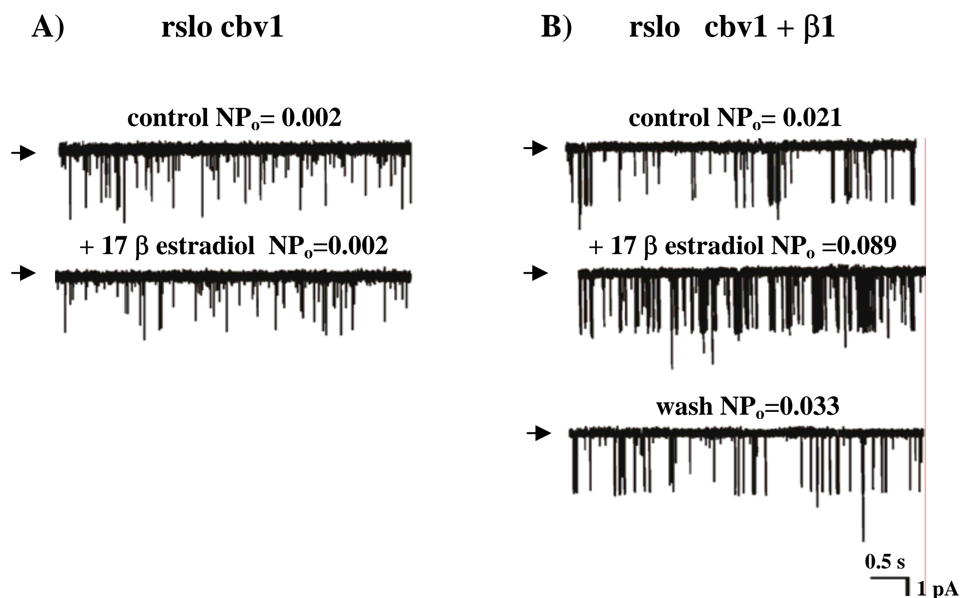


Online Figure 1. Ca^{2+}-depdendent rslo cbv2 channel steady-state activity (NP_o) in an inside-out patch. Similar results were obtained with rslo cbv1 channels. Channel openings are shown as upward deflections. Arrowheads indicate unitary current levels: C: closed state (baseline), O_1-O_6: first to sixth level of openings. The arrow at the bottom indicates the time at which $[Ca^{2+}]_i$ was changed. Records were obtained at +40 mV in symmetric 130 mM K^+. The bath solution contained (mM): 130 Kgluconate, 4.6 $CaCl_2$, 2.5 $MgCl_2$, 5 EGTA, 1.6 HEDTA, 10 HEPES. The pipette solution contained (mM): 130 Kgluconate, 5.2 $CaCl_2$, 2.3 $MgCl_2$, 5 EGTA, 1.6 HEDTA, and 10 HEPES.



Online Figure 2. Bath application of 17β-estradiol (10 µM) fails to regulate homomeric rslo cbv1 channels (A), but reversibly increases β_1+rslo cbv1 channel steady-state activity (NP_o) (B). Similar results were obtained when 17β-estradiol action was evaluated on β_1+rslo cbv2 vs. homomeric rslo cbv2 channels. Channel NP_o was continuously recorded during voltage-clamp conditions (V=-20 mV) and $[Ca^{2+}]_i \approx 100$ nM. Channel openings are shown as downward deflections, and arrows indicate the zero current level. The estradiol concentration is maximal for β_1+hslo channel activation [7]. Pipette solution contained (mM): 130 Kgluconate, 4.6 $CaCl_2$, 2.5 $MgCl_2$, 5 EGTA, 1.6 HEDTA, 10 HEPES. Bath solution contained (mM): 130 Kgluconate, 5.2 $CaCl_2$, 2.3 $MgCl_2$, 5 EGTA, 1.6 HEDTA, 10 HEPES.