Supporting Information

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SI Materials and Methods

Phorbol-12-myristate-13-acetate (PMA), 4α-phorbol-12-myristate-13-acetate (4α-PMA), protein kinase C catalytic fragment (PKC), protein kinase C (19-31) pseudosubstrate inhibitor ($PKC_{19–31}$), protein kinase A catalytic subunit (PKA), protein phosphatase inhibitor 2 (PPI2), ocadaic acid, and Ro-31–8220 were obtained from Biomol. Carbamoylcholine chloride (CCh) was purchased from Sigma, IbTX from Alomone Laboratories, 8-(4-chlorophenylthio) guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP), Sp-5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphothioate (Sp-5,6-DCl-cBIMPS), and guanosine-3′,5′-cyclic monophosphate (cGMP) from BioLog. Protein kinase G Iα (PKG) was prepared as described (1). The PKG was consistently used in the presence of 10 μM cGMP. Drugs were either dissolved in physiological saline solution (PSS; see solutions) or in dimethyl sulfoxide (DMSO). The maximum 0.1% final concentration of DMSO in the bath solution did not affect BK currents. Solutions with IbTX contained 0.1% bovine albumin fraction V (Sigma). Collagenase type H (lot 014K8605), hyaluronidase type I-S, papain, and 1,4-dithio-D,L-threitol was purchased from Sigma.

Cell Culture and Transfection Procedure. HEK293 cells were cultured in minimum essential medium supplemented with Earle's salts medium (Biochrom) containing 10% FCS, 2 mM L-glutamine, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin at 37 °C, and 6% CO_2. For transfection, 10^5 cells were plated in a 35-mm dish and cultured for another 24 h. Thereafter, the HEK293 cells were transiently cotransfected with EGFP (Clontech), cloned into the pcDNA3 vector (Invitrogen), and the pcDNA3 plasmid containing either the BK channel α-subunit BK_A cloned from a bovine tracheal smooth muscle oligo dT-primed cDNA library (2), or the respective BK_A mutant. The GenBank accession number for the BK channel isoform is AAK54352.1 (protein) and AY033472 (mRNA; BK_A). Transfection of 1 μg of each cDNA per dish was achieved by calcium phosphate precipitation for 18 h at 35 °C and 3% CO_2. After the medium was exchanged several times with PSS (see below), the cells were transferred in a 35-mm dish to the stage of an inverted microscope (Zeiss Axiovert 200) for electrophysiological measurements. The transfection efficiency varied between 50% and 70% as judged by the expression of EGFP in transfected cells. Cells with similar intensity of EGFP fluorescence were used for the experiments.

Site-Directed Mutagenesis. Mutant channels were generated by extended overlap PCR or a one-step PCR-based mutation protocol, a modified version of the QuikChange protocol (Invitrogen). *PfuUltra* DNA Polymerase (Stratagene) was used in the extended overlap PCR with the pcDNA3/BK_A plasmid as template and the resulting amplicons were digested with suitable restriction enzymes and cloned back into the pcDNA3/ BK_A background (2). For the one-step PCR-based mutation protocol, the Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for amplification of the whole pcDNA3/BK_A plasmid. All mutations were confirmed by sequence analysis over the total length of amplification and the employed restriction sites.

Animals and Cell Preparation. All experimental procedures were carried out according to the animal welfare guidelines of the University Medical Center Hamburg-Eppendorf. C57BL/6 mice of either sex were obtained from a colony bred and maintained at the animal house of the University Medical Center Hamburg-Eppendorf. Tracheae were dissected from mice killed by CO_2. After the connective tissue was removed, the tissue was cut with a sharp blade into pieces of ~1 mm side length and incubated under gentle agitation at 37 °C in Ca^{2+}-free PSS, including 0.7 mg mL^{-1} papain, 1 mg mL^{-1} 1,4-dithio-D,L-threitol, and 1 mg mL^{-1} fat-free BSA. The tissue pieces were transferred 30 min later into PSS solution containing 50 μM Ca^{2+}, 1 mg mL^{-1} collagenase, 1 mg mL^{-1} hyaluronidase, and 1 mg mL^{-1} albumin, and digested for another 8–10 min at 37 °C. Single cells were released by gentle trituration of the digested tissue and stored in PSS at room temperature. After isolation, >50% of the cells were relaxed, and only these cells were used for the electrophysiological studies. The experiments were conducted within 6 h of cell isolation. A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1 mL) mounted on the stage of an inverted microscope (Zeiss Axiovert 200). The myocytes were allowed to adhere to the bottom of the chamber for 5–10 min and were then superfused at 2–3 mL min^{-1} with PSS at room temperature until the beginning of the experiment.

Electrophysiology. Standard patch-clamp recording techniques were used to measure currents in the whole-cell, outside-out, or inside-out patch-clamp configuration (3). Patch electrodes were fabricated from borosilicate glass capillaries (MTW 150F; World Precision Instruments, Inc.) and filled with prefiltered solutions of different composition (see below). Currents were recorded at room temperature with an EPC-7 amplifier (HEKA Elektronik), connected via a 16-bit A/D interface to a Pentium IBM clone computer. The signals were low-pass filtered (1 kHz) before 5 kHz digitization. Data acquisition and analysis were performed with an ISO-3 multitasking patch-clamp program (MFK M. Friedrich). Pipette resistance ranged from 2 to 3 MΩ in whole-cell, 2.5–3 MΩ for macroscopic current recording, and 8–9 MΩ for single-channel recording in the excised-patch experiments. NP_o was derived from single-channel analysis for patches with 1–4 channels open. The open and closed level of a channel was determined from an all-point histogram. Channel open state was defined as 50% of single-channel level. NP_o was calculated by the equation:

$$NPo = \left(\sum_{j=1}^{N} t_j j\right) \Big/ T,$$

where *P*o is the single-channel open-state probability, T is the duration of the measurement, t_j is the time spent with j = 1,2.. N channels open, and *N* is the maximal number of simultaneous channel openings seen in the patch. NP_o calculations were based on 15-s segments of single-channel recordings. The NP_o averages of 12–20 segments (3-5 min), obtained immediately before and after the addition of a compound, were used for statistical analysis.

Open- and closed-time histograms were obtained from records with only one active channel. Estimation of the time constants was done according to Sigworth and Sine (4) by the maximum-likelihood method.

A holding potential of −10 mV was used in whole-cell patch-clamp experiments and in inside-out patches when macroscopic currents were recorded. For the determination of NP_o, the holding potential was +40 mV.

After establishment of the whole-cell, inside-out, or outside-out configurations, the cells or patches were superfused for 5–10 min with control solutions until the currents or channel activity became stable. If the current or channel activity was not stable

after 10 min, the recording was terminated. For experiments with protein kinases, see below.

Solutions. For whole-cell experiments, the bath was superfused with PSS containing (in mM) 127 NaCl, 5.9 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, and 10 Hepes adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM) 126 KCl, 6 NaCl, 1.2 $MgCl_2$, 5 EGTA, 11 glucose, 3 dipotassium ATP, 0.1 Na_3GTP, and 10 Hepes adjusted to pH 7.4 with KOH. The free Ca^{2+} concentration was adjusted to 0.3 μM by adding the appropriate amount of $CaCl_2$ as described previously (5). Free Ca^{2+} was checked by fura-2 fluorescence. For macroscopic current recordings in inside-out patches from HEK293 cells, the extracellular (pipette) solution is PSS. The bath solution (cytosolic surface of the patch) contained (in mM) 126 KCl, 6 NaCl, 1.2 $MgCl_2$, 5 EGTA, 11 glucose, 3 dipotassium ATP, 0.1 Na_3GTP, and 10 Hepes adjusted to pH 7.4 with KOH. The free Ca^{2+} concentration was adjusted to 1 μM. For single-channel recordings in HEK293 and TSMC cells, the extracellular (pipette) solution is (in mM):132 KCl, 6 NaCl, 1.2 $MgCl_2$, 5 EGTA, 11 glucose, 10 Hepes, pH 7.4 adjusted with KOH, and the bath solution (cytosolic surface of the patch) contained 126 KCl, 6 NaCl, 1.2 $MgCl_2$, 5 EGTA, 11 glucose, 3 dipotassium ATP, 0.1 Na_3GTP, and 10 Hepes, pH 7.4 adjusted with KOH. The free Ca^{2+} concentration was 0.3 μM. For outside-out experiments, the same solutions as for single-channel recordings in inside-out experiments were used, except that the pipette and bath solutions were reversed. The free Ca^{2+} concentration in the pipette was 0.3 μM.

The storage buffers for purified protein kinases were PKC: 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 15 mM DTT, 10% glycerol, 20 mM Tris (pH 7.5); PKA (catalytic subunit): 50 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 50% glycerol, 20 mM Tris (pH 7.5); PKG Iα: 10 mM TES, 1.25 mM DTT, 0.25 μg mL^{-1} leupeptine, 0.05% NaN_3, 50% glycerol. When the effect of protein kinases was investigated in inside-out patches, appropriate amounts of storage buffers were diluted into the bath solution. To avoid significant effects of storage buffers on channel activity, patches were first superfused with storage buffer containing solutions alone until channel activity was stable (usually within 5–10 min), then superfusion with the same solution containing the respective protein kinase was started.

Statistical Analyses. SigmaPlot for windows (Jandel Scientific, version 11) was used for statistical analyses. Significance was determined by paired or unpaired *t* test or by one-way ANOVA. When a significant effect was detected with ANOVA, Student's *t* test was used for pairwise comparisons. The effects were deemed significant when a $P < 0.05$ was obtained. Results are expressed as means ± SE where applicable.

1. Ruth P, et al. (1997) Identification of the amino acid sequences responsible for high affinity activation of cGMP kinase Iα. *J Biol Chem* 272:10522–10528.
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4. Sigworth FJ, Sine SM (1987) Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys J* 52:1047–1054.
5. Zhou X-B, Ruth P, Schlossmann J, Hofmann F, Korth M (1996) Protein phosphatase 2A is essential for the activation of Ca^{2+}-activated K^+ currents by cGMP-dependent protein kinase in tracheal smooth muscle and Chinese hamster ovary cells. *J Biol Chem* 271: 19760–19767.

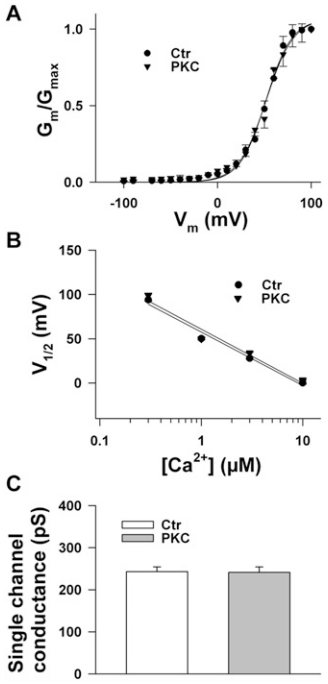

Fig. S1. PKC has no influence on the voltage- and calcium-dependent activation of BK_A channels and on single-channel conductance. (*A*) Normalized conductance-voltage relationships before (Ctr) and in the presence of 30 nM PKC_c (PKC). Means ± SEM of eight inside-out patches obtained from HEK293 cells expressing BK_A channels are shown. The half-maximally activating voltage ($V_{1/2}$) was 51.6 ± 4.6 mV before and 50.9 ± 3.7 mV in the presence of PKC. (*B*) Ca^{2+} does not affect the voltage required for half-maximal channel activation. The $V_{1/2}$ values were calculated from normalized conductance-voltage relationships obtained from inside-out membrane patches superfused sequentially at the cytosolic side with solutions containing 0.3, 1, 3, and 10 μM Ca^{2+}. Means ± SEM of nine patches before (Ctr) and of eight patches after application of 30 nM PKC_c (PKC) are shown. Error bars are within the symbols. (*C*) Single-channel conductance was not affected by PKC. Means ± SEM of 13 inside-out membrane patches before (Ctr; 243 ± 12 pS) and after the application of 30 nM PKC_c (PKC; 241 ± 13 pS). Holding potential +40 mV. The intracellular (bath) Ca^{2+} concentration was 1 μM in *A* and 0.3 μM in *C*.

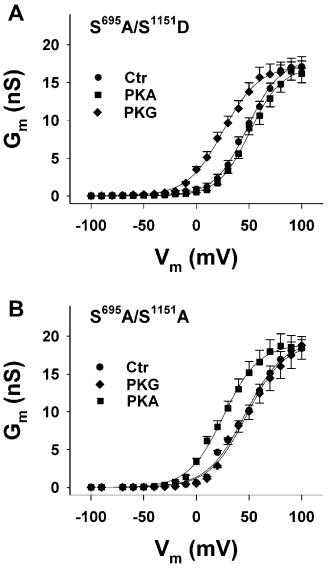

Fig. S2. The double mutants $S^{695}A/S^{1151}D$ and $S^{695}A/S^{1151}A$ of the BK channel retain their specific activation by PKG and PKA, respectively. Inside-out patches were superfused with either 300 nM PKG or PKA. Conductance-voltage relations were obtained by 300 ms depolarizing pulses every 5 s in 10-mV increments from −100 to +100 mV. Holding potential was −10 mV. Means ± SEM are shown. (*A*) PKG-dependent activation of the mutant channel $S^{695}A/S^{1151}D$. The respective $V_{1/2}$ values (in mV) were 48.4 ± 5.0 (Ctr; n = 13), 50.9 ± 5.7 (PKA; n = 7), and 26.4 ± 3.7 mV (PKG; n = 6). (*B*) PKA-dependent activation of the mutant channel $S^{695}A/S^{1151}A$. The respective $V_{1/2}$ values (in mV) were 46.5 ± 4.6 (Ctr; n = 16), 47.7 ± 5.2 (PKG; n = 8), and 25.8 ± 2.8 (PKA; n = 8). The intracellular (bath) Ca^{2+} concentration was 1 μM.

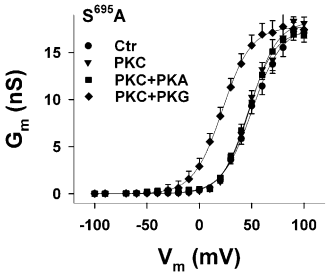

Fig. S3. Mutation of Ser^{695} to alanine (S^{695}A) has no influence on the PKG-dependent activation of BK channels in the presence of PKC. Inside-out patches were superfused with 30 nM PKC$_c$ first, and then additionally with either 300 nM PKG or PKA. Conductance-voltage relations were obtained by 300-ms depolarizing pulses every 5 s in 10-mV increments from −100 to +100 mV. Holding potential was −10 mV. Means ± SEM are shown. The respective $V_{1/2}$ values were 50.3 ± 4.8 (Ctr; n = 16), 48.7 ± 5.0 (PKC; n = 16), 46.8 ± 5.1 (PKC + PKA; n = 8), and 22.1 ± 3.0 mV (PKC + PKG; n = 8). The intracellular (bath) Ca^{2+} concentration was 1 μM.

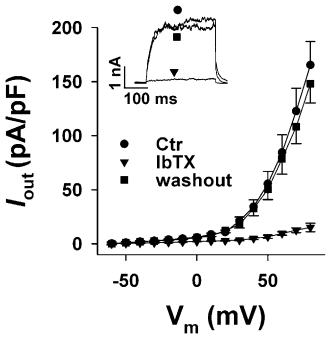

Fig. S4. More than 90% of whole-cell I_{out} in TSMCs is conducted through BK channels. Current-voltage relations of seven TSMCs (obtained from three mice) before (Ctr, 165.3 ± 21.7 pA pF^{-1} at 80 mV), 2–3 min after the addition of 300 nM iberiotoxin (IbTX, 15.2 ± 3.8 pA pF^{-1} at 80 mV), and after washout of the toxin (148.1 ± 17.8 pA pF^{-1} at 80 mV) are shown. Holding potential −10 mV. Means ± SEM. (*Inset*) Representative I_{out} recordings elicited by 300-ms pulses at +80 mV. The Ca^{2+} concentration in the pipette was 0.3 μM.

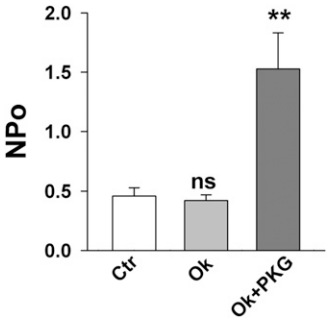

Fig. S5. Inhibition of protein phosphatase 2A failed to inhibit BK channel activity and to abolish PKG-dependent activation. Inside-out patches from TSMCs were superfused at the cytosolic side with 3 nM ocadaic acid first (ok), and then additionally with 300 nM PKG. Means ± SEM of six patches from three mice at +40 mV are shown. $^{**}P < 0.01$ vs. control (Ctr); ns, not significant. The intracellular (bath) Ca^{2+} concentration was 0.3 μM.

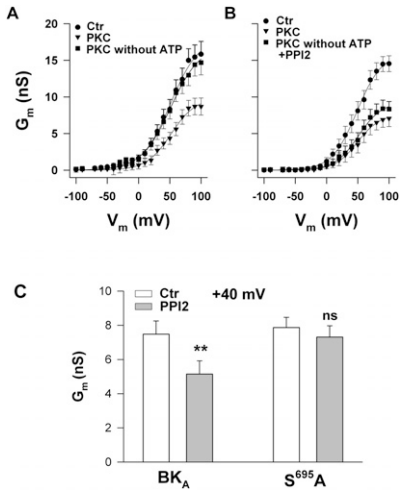

Fig. S6. Protein phosphatase 1 is constantly dephosphorylating Ser^{695} in BK channels expressed in HEK293 cells. (*A* and *B*) Conductance-voltage relationships obtained from inside-out membrane patches of HEK293 cells expressing BK_A channels. Means ± SEM are shown. (*A*) The curves were obtained before (Ctr), 5 min after applying 30 nM PKC_c to the cytosolic side of the patch (PKC), and 6 min after changing to an ATP-free bath solution with PKC still present. Note that the ATP-free solution completely reversed the inhibitory PKC effect ($n = 6$). (*B*) Same experimental protocol as in *A*, except that the ATP-free solution contained 30 nM PKC_c plus 20 nM of the protein phosphatase 1 inhibitor peptide PPI2. Note that the inhibition of PP1 prevented the reversal of the inhibitory PKC effect ($n = 6$). (*C*) Application of 20 nM PPI2 to the cytosolic side of inside-out membrane patches obtained from HEK293 cells expressing BK_A channels resulted in a significant decrease of membrane conductance (G_m) from 7.5 ± 0.8 to 5.0 ± 0.7 nS ($n = 8$). In the BK channel $S^{695}A$ mutant, however, PPI2 failed to affect G_m ($n = 7$). The bars represent means ± SEM at 40 mV. The intracellular (bath) Ca^{2+} concentration was 1 μM. $^{**}P < 0.01$ vs. Ctr; ns, not significant.