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Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels

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Abstract

Large conductance calcium- and voltage-gated potassium (BK) channels are important regulators of physiological homeostasis and their function is potently modulated by protein kinase A (PKA) phosphorylation. PKA regulates the channel through phosphorylation of residues within the intracellular C terminus of the pore-forming α-subunits. However, the molecular mechanism(s) by which phosphorylation of the α-subunit effects changes in channel activity are unknown. Inhibition of BK channels by PKA depends on phosphorylation of only a single α-subunit in the channel tetramer containing an alternatively spliced insert (STREX) suggesting that phosphorylation results in major conformational rearrangements of the C terminus. Here, we define the mechanism of PKA inhibition of BK channels and demonstrate that this regulation is conditional on the palmitoylation status of the channel. We show that the cytosolic C terminus of the STREX BK channel uniquely interacts with the plasma membrane via palmitoylation of evolutionarily conserved cysteine residues in the STREX insert. PKA phosphorylation of the serine residue immediately upstream of the conserved palmitoylated cysteine residues within STREX dissociates the C terminus from the plasma membrane, inhibiting STREX channel activity. Abolition of STREX palmitoylation by site-directed mutagenesis or pharmacological inhibition of palmitoyltransferases prevents PKA-mediated inhibition of BK channels. Thus, palmitoylation gates BK channel regulation by PKA phosphorylation. Palmitoylation and phosphorylation are both dynamically regulated; thus, cross-talk between these 2 major posttranslational signaling cascades provides a mechanism for conditional regulation of BK channels. Interplay of these distinct signaling cascades has important implications for the dynamic regulation of BK channels and physiological homeostasis.

Large conductance calcium- and voltage-gated potassium (BK) channels are potently regulated by protein phosphorylation (1) and are important determinants of neuronal, cardiovascular, endocrine, and epithelial function where channel dysfunction may lead to major disorders such as hypertension (2, 3), ataxia (4), epilepsy (5, 6), and incontinence (7). BK channels are potently regulated by phosphorylation, and several putative phosphorylation motifs on the pore-forming α-subunit have been identified (8–12). However, as for other potassium channels, the molecular basis through which phosphorylation of the α-subunit effects changes in BK channel activity is essentially unknown.

BK channel pore-forming α-subunits are encoded by a single gene, KCNMA1 (13), and native BK channels show functional heterogeneity in their response to protein kinase A (PKA)-mediated phosphorylation. This diversity results, in large part, from the extensive alternative pre-mRNA splicing of the pore-forming α-subunits (10, 12). Previous studies have demonstrated that PKA phosphorylation of a conserved C-terminal phosphorylation motif, RQRPSOCP, results in BK channel activation (9, 10, 14). Inclusion of the stress regulated exon (STREX) (15) in the intracellular C terminus generates an additional PKA consensus motif (serine residue 3 of the STREX insert, S3) that results in channel inhibition by PKA (10, 14). PKA inhibition of STREX follows a single-subunit rule, whereby only 1 α-subunit within the BK channel tetramer is required to be phosphorylated by PKA on S3 for inhibition to be conferred (14). Thus, phosphorylation of a single STREX α-subunit probably induces major conformational rearrangements in the BK channel C terminus to mediate channel inhibition.

The STREX insert is cysteine-rich (6 of 58 aa) that, when included into the BK channel α-subunit C terminus, generates a cysteine-rich domain (CRD) in the intracellular linker between the 2 regulator of conductance (RCK) domains (Fig. 1A). In many proteins, including other voltage- and ligand-gated ion channels (16–21), cysteine residues are common targets for protein palmitoylation, the covalent attachment of a palmitate lipid to a cysteine residue via a thioester bond. Palmitoylation can exert diverse effects on protein function including allowing cytosolic protein domains to anchor to the plasma membrane (22–24).

We thus hypothesized that palmitoylation of the STREX insert might target the C-terminal domain of the BK channel to the plasma membrane independently of the N-terminal transmembrane domains. Furthermore, this suggested a mechanism by which phosphorylation of a single STREX subunit could result in channel inhibition—through regulation of STREX domain interaction with the plasma membrane. To test these hypotheses, we exploited an integrated imaging, electrophysiological, and biochemical approach. We demonstrate that palmitoylation of BK channels results from dissociation of the STREX domain from the plasma membrane. Importantly, palmitoylation of STREX provides a conditional gate for regulation of BK channel activity by PKA phosphorylation.

Results and Discussion

STREX Insert Is a Membrane-Anchoring Domain of the Cytosolic C Terminus of the BK Channel. To address whether the cysteine-rich STREX domain is a membrane-anchoring module we developed an imaging assay to screen the ability of the STREX insert, and its cognate cysteine residues, to anchor the STREX C terminus to the plasma membrane in the absence of the N-terminal transmembrane domains of the α-subunit. We generated fluorescent-GFP fusion constructs of the entire BK channel C terminus (Fig. 1B) as well as constructs that encompass the CRD as fluorescent fusions with...
flanking mCer- and/or YFP fusion proteins to mimic the STREX linker region between the RCK domains (Fig. 1C).

Expression of STREX C-terminal (Fig. 1B) or CRD constructs (Fig. 1C) resulted in robust plasma membrane expression of the fusion proteins in HEK293 cells in the absence of full-length BK channels or transmembrane segments. Identical results were also obtained in cells that endogenously express STREX variant channels including murine anterior pituitary corticotrope (AtT20) cells, rat pheochromocytoma PC12 cells, and human insulinoma INS-1 cells (data not shown). In contrast, in all these systems, expression of a STREX construct (STREX*) in which the transmembrane segment of a C-terminal, or CRD, fusion protein that lacks the STREX insert was HA-tagged with an epitope (Fig. 1B, Lower Right). Thus, a STREX subunit within a heteromeric assembly is sufficient to localize the BK channel C terminus at the plasma membrane. These data demonstrate that STREX acts as a membrane-targeting/anchoring domain and that the key plasma membrane localization motifs must reside within the CRD domain.

To determine whether the cysteine residues within the CRD control membrane localization of the STREX C terminus, we mutated cysteine residues to alanine in both the C-terminal and CRD STREX constructs. Mutation of residue C13 to alanine almost abolished membrane targeting in both fusion proteins (Fig. 1D), and mutation of its upstream vicinal cysteine residue (C12) significantly reduced membrane localization. The double-mutant C12:13A abolished membrane targeting of both fusion proteins (Fig. 1D). In contrast, mutation of any of the other cysteine residues within the CRD had no significant effect on membrane localization (Fig. 1D).

Palmitoylation of STREX Is Required for Membrane Targeting of the C Terminus. Using the CSS-palm palmitoylation algorithm (25), we predicted that of the 6 cysteine residues within the STREX insert, only cysteine residues C12 and C13 are conserved cysteine residues in the STREX insert predicted to be palmitoylated by the CSS-palm algorithm (shaded) and the PKA phosphorylation site serine S3 (indicated by the asterisk). Cysteine residues are numbered in the CRD as follows: STREX residues numbered from the first STREX residue (K) and upstream cysteines labeled by letters, (B and C). Expression of STREX C-terminal (Fig. 1C) or CRD constructs resulted in robust plasma membrane expression of the fusion proteins in HEK293 cells in the absence of full-length BK channels or transmembrane segments. Identical results were also obtained in cells that endogenously express STREX variant channels including murine anterior pituitary corticotrope (AtT20) cells, rat pheochromocytoma PC12 cells, and human insulinoma INS-1 cells (data not shown). In contrast, in all these systems, expression of a STREX construct (STREX*) in which the transmembrane segment of a C-terminal, or CRD, fusion protein that lacks the STREX insert was HA-tagged with an epitope (Fig. 1B, Lower Right). Thus, a STREX subunit within a heteromeric assembly is sufficient to localize the BK channel C terminus at the plasma membrane. These data demonstrate that STREX acts as a membrane-targeting/anchoring domain and that the key plasma membrane localization motifs must reside within the CRD domain.
PKA Phosphorylation Dissociates the STREX C Terminus from the Plasma Membrane. PKA inhibition of STREX channels results from phosphorylation of serine residue 3 within the STREX insert (10, 14) that is just upstream of the site of palmitoylation (C12:13). We reasoned that phosphorylation of S3, which would introduce a negative charge into an otherwise basic region immediately upstream of C12:13, might result in destabilization of the STREX domain with the plasma membrane leading to significant structural rearrangements and thus channel inhibition. We took 3 approaches to test this idea. First, we generated the STREX PKA phosphomimetic S3E by mutating STREX S3 to glutamic acid. The S3E mutation abolished STREX C-terminal or CRD construct localization at the plasma membrane (Fig. 3 A and C). The serine-to-aspartate mutation (S3D, data not shown) also significantly reduced membrane expression, suggesting that both the S3E and S3D mutants largely mimic PKA phosphorylation of S3. In contrast, phosphomimetic mutation of S899 in the downstream C terminus, which is the major site for PKA activation (9, 10, 14), had no significant effect on STREX C terminus association with the plasma membrane (Fig. 3 A and C). However, the phosphomimetic mutations do not allow us to distinguish between a plasma membrane trafficking defect and a reduced association of the C-terminal domains once at the plasma membrane. Thus, in the second series of experiments, we acutely treated HEK293 cells, expressing the wild-type STREX C-terminal or CRD constructs at the plasma membrane, with the membrane-permeable cAMP analogue 8-CP(cAMP) to establish whether constructs resident at the plasma membrane could be dissociated by PKA phosphorylation. Pretreatment of cells with 0.1 mM SCPT-cAMP, in the presence or absence of 10 nM okadaic acid, significantly reduced membrane association of the STREX constructs (Fig. 3 B). Importantly, cAMP-induced dissociation was abolished by pretreatment with the cell-permeable PKA inhibitor H89. In contrast, activation of protein kinase G (PKG) or C (PKC) had no effect on STREX localization at the plasma membrane (Fig. 3 D). In addition, neither acute (<3% O2), hypoxia (Fig. 3 D), which also regulates BK channels through the STREX insert (28), nor ionomycin-induced calcium elevation or KCl-induced depolarization (Fig. S1) had any significant effect on the plasma membrane localization of STREX. These data support a model in which PKA phosphorylation of STREX results in dissociation of the STREX domain from the plasma membrane.

Palmitoylation Gates BK Channel Regulation by PKA. If this model were correct, we would predict that PKA inhibition of STREX BK channels would be lost in channels that are not palmitoylated. To test this hypothesis, we examined the regulation of wild-type STREX channels and the STREX palmitoylation-deficient mutant C12:13A in electrophysiological assays. STREX channels are robustly inhibited by endogenous PKA closely associated with the channel upon exposure of the intracellular face of the patch to cAMP (Fig. 3 E and G). The effect of cAMP is abolished by the PKA inhibitor PKI24, but not by inhibitors of PKG, PKC, or CaMKII (Fig. S2) and is mimicked by application of exogenous catalytic subunit of PKA (PKAc) (Fig. 3 G). Although full-length C12:13A channels are robustly expressed at the plasma membrane (Fig. 3 F and H), suggesting the full-length C12:13A mutant is not itself trafficking deficient, cAMP or PKAc fail to inhibit this mutant. Furthermore, the inhibitory effect of cAMP on wild-type STREX channels was abolished in cells pretreated overnight with the inhibitor of palmitoylation, 2-BP (Fig. 3 G). The C12:13A mutation, and inhibition of STREX channel palmitoylation by 2-BP, which both dissociate the C terminus from the plasma membrane (Fig. 2 C), resulted in a right shift in the half-maximal voltage for activation (Fig. S3 A). Reduced basal activity per se is not responsible for the loss of cAMP-mediated inhibition because cAMP potently inhibited STREX channels recorded under conditions to match the shift in V0.5 resulting from the C12:13A

24). Importantly, the STREX CRD construct is robustly palmitoylated, and 3H-palmitate incorporation was essentially abolished in the C12:13A mutant, suggesting that these residues are the major sites of palmitoylation within the CRD (Fig. 2 A). As an alternative biochemical approach to confirm endogenous palmitoylation of STREX channels on cysteine residues, we also exploited a cysteine-accessibility assay (26) after treatment of STREX channels with 1 M neutral hydroxylamine to cleave endogenous palmitate thioester bonds to cysteine residues. Accessible cysteines were probed by using biotin-BMCC (Upper) with total protein probed by α-HA (Lower). (C) Summary imaging data of STREX and C12:13A C terminus (W) or CRD (N) as in Figs. 1 B-D and the effect of 24-h pretreatment of cells with the palmitoyltransferase inhibitor 2-bromopalmitate (2-BP, 100 μM) or the myristoylation inhibitor 2-hydroxymyristate (2-HM, 0.1–1 mM). Data are means ± SEM, N > 14, n > 950 for each construct/treatment. *, P < 0.01 compared with respective STREX construct (ANOVA with Student–Neuman–Keuls post hoc test).
mutation (e.g., at −20 mV in 0.1 μM free calcium, inhibition was 67 ± 7% of control, n = 12).

Palmitoylation appears to specifically gate PKA-mediated inhibition of STREX channels because the palmitoylation status of STREX does not alter its intrinsic hypoxia sensitivity (28) (Fig. 3G and H) or the regulation by PKG-dependent phosphorylation (Fig. S3b). Moreover, inhibition of palmitoylation by 2-BP does not modulate PKA-dependent activation of the ZERO splice variant (Fig. S3c) that depends on phosphorylation of a PKA consensus motif (S899) outwith the STREX insert.

Furthermore, the model proposed for palmitoylation-dependent gating of PKA inhibition of STREX would be expected to adhere to a “same-subunit” rule based on the previous demonstration that only a single subunit of STREX needs to be phosphorylated at S3 for channel inhibition (14). The same-subunit model would predict that PKA inhibition, as a result of phosphorylation of STREX at S3, would occur only if the same subunit is also palmitoylated at C12:13. By using a TEA-pore mutation strategy (Y334V) to determine channel subunit stoichiometry (14), cotransfection of subunits that could be palmitoylated but not phosphorylated (constructs with S3A mutation) together with subunits that could be phosphorylated but not palmitoylated (constructs with C12:13A mutation) revealed that cAMP was unable to inhibit channel activity (mean change in activity was 4 ± 10%, n = 12). In contrast, introduction of even a single subunit that could be both phosphorylated and palmitoylated (i.e., a wild-type STREX subunit) with subunits that could be palmitoylated but not phosphorylated (S3A constructs) resulted in robust channel inhibition by cAMP (inhibition was 71 ± 6%, n = 8). These data demonstrate that palmitoylation of the same subunit in which the channel is phosphorylated is required for PKA-mediated inhibition.

To examine whether palmitoylation gates native STREX channel regulation, we examined the regulation of BK channels in mouse anterior pituitary corticotrope (AtT20) cells. STREX variant channels are robustly expressed in this system and are potently inhibited by cAMP-dependent protein phosphorylation (29). CAMP potently inhibited the outward paxilline-sensitive (BK) current in these cells (Fig. 4). Pretreatment of AtT20 cells with 2-BP abolished cAMP-mediated inhibition of the BK current in the whole-cell configuration (Fig. 4). Similar data were obtained in perforated-patch recordings: 8-CPT-cAMP-mediated inhibition of the paxilline-sensitive BK current in 2-BP-treated cells was only 7 ± 8% (n = 4) of that observed by 8-CPT-cAMP in vehicle-treated controls. BK channels are remarkable in the range of physiological processes they control, their functional heterogeneity as a result of alternative splicing of the single gene encoding the pore-forming α-subunits, and their extensive regulation by reversible protein phosphorylation. Our data reveal the molecular basis for PKA-mediated inhibition of BK channels through the regulation of STREX domain interaction with the plasma membrane. Palmitoylation of the STREX domain uniquely allows the large intracellular C terminus of the STREX splice variant to associate with the plasma membrane and, importantly, gates the regulation of STREX channels by PKA. Critically, this regulation depends on the site of palmitoylation being adjacent to the site of phosphorylation in the same subunit polypeptide. Importantly, STREX channel activation by PKG-mediated phosphorylation was not affected by palmitoylation status. Furthermore, PKA-activation of ZERO

Fig. 3. PKA phosphorylation of STREX dissociates STREX from plasma membrane. (A) Representative single confocal sections from HEK293 cells expressing wild-type STREX C-terminal constructs and the corresponding STREX and C-terminal PKA phosphorylation site phosphomimetic constructs S3E and S899E. (Scale bars: 5 μm.) (B) Effect of cell-permeable cAMP analogue 8-CPT-cAMP (0.1 mM, 10–30 min) on STREX C terminus (CRD) or CRD membrane localization in the presence or absence of 10 nM okadaic acid or the PKA inhibitor H89 (1 μM). (C) Summary of S3E and S899E construct expression at the plasma membrane (na, S899 site not present in CRD construct). (D) Effect of acute hypoxia (1% O2) or PKG activation with the cell-permeable cGMP analogue 8-CPT-cGMP (0.1 mM in the presence of 10 nM okadaic acid) or PKA activation with the phorbol ester PMA (100 nM in the presence of 10 nM okadaic acid) on construct localization at the plasma membrane. Data are means ± SEM, N > 4, n > 350. (E and F) Representative single-channel traces and diary plots of single-channel mean open probability (Po) from isolated inside-out patches of HEK293 cells expressing full-length STREX (E) or C12:13A (F) channels before and 10 min after exposure to cAMP. Single channels were assayed in physiological K+ gradients exposed to 0.2 μM free calcium and 2 mM Mg-ATP. (G and H) Inhibition of STREX (G) or C12:13A (H) channel Po by cAMP (0.1–1.0 mM) in the presence or absence of the PKA inhibitor PKI5-24 (0.45 μM) or 24-h cell pretreatment with 100 μM 2-BP; application of catalytic subunit of PKAc (300 nM) or exposure to acute hypoxia (1% O2). Data are means ± SEM, n = 5–14 for each treatment. **, P < 0.01 compared with respective control (ANOVA with Student–Neuman–Keuls post hoc test).
channels was not affected by inhibitors of protein palmitoylation. PKA and PKG activation of BK channels is independent of the STREX insert and is thought to be mediated by consensus phosphorylation sites in the more distal C terminus (8–10, 12, 14). Importantly, these sites are not juxtaposed to predicted sites of palmitoylation, further supporting our model in which the cross-talk between PKA inhibition and palmitoylation status of STREX depends on both PKA phosphorylation and palmitoylation being in close proximity on the same subunit in the tetramer, thus obeying the single-subunit rule of PKA inhibition (14).

To what extent may such cross-talk between these major, dynamically regulated, lipid and phosphorylation signaling pathways occur in other ion channels? Protein phosphorylation is a fundamental mechanism to control ion channel function, and increasing evidence suggests that multiple ion channels are also regulated by palmitoylation (16–21). We would predict that phosphorylation–palmitoylation cross-talk is likely to occur when sites of palmitoylation are flanked by sites of serine/threonine phosphorylation. In support of this, our pilot sequence analysis reveals (M.J.S. and I.C.M.R., unpublished work) that in several ion channels reported to be of much broader significance to conditionally regulate a functional mechanism to control ion channel function, and increasing evidence suggests that multiple ion channels are also regulated by palmitoylation (16–21). We would predict that phosphorylation–palmitoylation cross-talk is likely to occur when sites of palmitoylation are flanked by sites of serine/threonine phosphorylation. In support of this, our pilot sequence analysis reveals (M.J.S. and I.C.M.R., unpublished work) that in several ion channels reported to be palmitoylated, the sites of palmitoylation are flanked by sites of serine/threonine phosphorylation. In particular, the palmitoylation inhibitor 2-bromopalmitate (2-BP; Sigma) was made as a fresh 100 mM stock in DMEM and applied at a final concentration of 100 μM overnight. For the myristoyl and palmitoyl C-terminal insert double-labeled construct, fresh stock solutions were made either complexed to BSA, or dissolved in DMSO as for 2-BP, and applied at 0.1–1 mM final concentration in each well. For acute imaging assays, cells were incubated for 1 h in DMEM containing 15 mM Hepes and 0.25% BSA (pH 7.4) at 37 °C before incubation for 10–30 min with fresh medium containing the respective drug treatment before rapid fixing. For acute hypoxia, medium was continuously bubbled with nitrogen gas (O2 level are <3%, equivalent to ~25 mM Hg) and during fixation.

Materials and Methods

Channel Constructs. The generation of full-length murine ZERO and STREX channel epitope-tagged as well as TEA-pore mutant (Y334V) constructs have been described (14, 31). C-terminal–GFP constructs, the respective CRD constructs, and site-directed mutants were generated and sequence-verified as described in SI Methods.

Cell Lines, Transfection, and Treatments. HEK293 cells and mouse anterior pituitary corticotrope AtT20 D16:16. HEK293 cells and mouse anterior pituitary corticotrope AtT20 D16:16 (passage 18–32) were subcultured essentially as described (10, 14, 29). Cells were transiently transfected by using Lipofectamine 2000 (Invitrogen) or Fugene-HD.

Cell treatments: The palmitoylation inhibitor 2-bromopalmitate (2-BP; Sigma) was made as a fresh 100 mM stock in DMEM and applied at a final concentration of 100 μM overnight. The myristoyl and palmitoyl C-terminal insert double-labeled construct, fresh stock solutions were made either complexed to BSA, or dissolved in DMSO as for 2-BP, and applied at 0.1–1 mM final concentration in each well. For acute imaging assays, cells were incubated for 1 h in DMEM containing 15 mM Hepes and 0.25% BSA (pH 7.4) at 37 °C before incubation for 10–30 min with fresh medium containing the respective drug treatment before rapid fixing. For acute hypoxia, medium was continuously bubbled with nitrogen gas (O2 level are <3%, equivalent to ~25 mM Hg) and during fixation.

Imaging Assays. Cells were fixed with 4% paraformaldehyde, mounted in Mowiol, and analyzed under epifluorescence by using an inverted Nikon Eclipse 2000 microscope with a 100× oil objective lens. Confocal images were acquired on a Zeiss LSM510 laser scanning microscope with a 63× oil Plan Apochromat (N.A. = 1.4) objective lens, in multitracking mode to minimize channel cross-talk. Membrane expression was quantified by using the LSM browser or Velocity software as described in SI Methods. Data are shown as mean ± SEM for N independent experiments where n = minimum total number of cells analyzed across experiments for each construct.

Palmitoylation Prediction and Palmitoylation Assays. CSS-palm prediction. We exploited the published CSS-palm palmitoylation algorithm (25) to predict cysteine residues within the entire coding sequence of the murine STREX BK channel isoform as well as the STREX insert alone (see Table S1). Sequences were analyzed with both the published CSS-palm v1.0 algorithm as well as the recently refined CSS-palm v2.0 web interface. In both cases palmitoylation prediction was initially set to the highest cutoff in both algorithms.

**3H palmitic acid incorporation.** HEK293 cells were transiently transfected in 6-well cluster dishes (~3 × 105 cells per well) with full-length STREX-HA, ZERO-HA subunit as well as the STREX insert alone (see Table S1). Sequences were recorded by using the CSS-palm v2.0 web interface. In both cases palmitoylation prediction was initially set to the highest cutoff in both algorithms.
mM NaCl, 50 mM Hepes (pH 7.5), 1.5 mM MgCl₂, 1 mM EDTA, and 1% Triton-X-100 containing 25–50 mM N-ethylmaleimide (NEM) to block reactive cysteines. Cell lysates were spun, supernatants precluded with protein-G beads (Sigma), and incubated overnight at 4 °C with mouse monoclonal α-HA antibody. Immunopurified complexes were rapidly washed 3 times in lysis buffer without NEM and treated with 1 M ha (+ha) (pH 7.4) for 1 h or 1 M Tris-HCl (pH 7.4) as a control (−ha). After washing, beads were exposed to the sulfhydryl-specific biotinylating reagent biotin-BMCC (10 μM, Pierce) for 2 h at room temperature. Labeled proteins were run on SDS-PAGE, transferred to PVDF membrane, and probed with streptavidin-conjugated horseradish peroxidase (HPR) and detected by ECL.

Electrophysiological Assays. HXK293 cells. Single-channel current recordings were performed in the inside-out configuration of the patch-clamp technique, at room temperature (20–24 °C). The pipette solution (extracellular) contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, 10 mM Hepes (pH 7.4). The bath solution (intracellular) contained 140 mM KCl, 5 mM NaCl, 2 mM MgCl₂, 1 mM BAPTA, 30 mM glucose, 10 mM Hepes, and 20 mM glucose (pH 7.4) with or without 0.002 tetrodotoxin. The patch pipette (intracellular) contained 140 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 30 mM glucose, 1 mM BAPTA, and 1 mM ATP (pH 7.4) with intracellular free calcium ([Ca^{2+}]_i) buffered to 200 nM. Perforated-patch recordings were conducted by using amphotericin in the patch pipette. Cells were voltage clamped at −50 mV and depolarized to the respective potentials for 100 ms with leak subtraction applied by using a P/4 protocol and series resistance compensation of −50%. Steady-state outward current was determined 90 ms into the pulse and was stable for ~30 min under these conditions.

Statistical Analysis. All data are presented as means ± SEM with N = number of independent experiments and n = number of individual cells analyzed in imaging assays. Data were analyzed by ANOVA with post hoc Student–Neuman–Keuls test with significance set at P < 0.01.

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