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Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels

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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 palmitoyl transferases 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.

KCNA1 | acylation | protein kinase A | maxi-K


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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 C-terminal, or CRD, fusion proteins that lack the STREX insert (ZERO constructs), but that are otherwise identical to the STREX constructs, did not localize to the membrane (Fig. 1C). Thus, a STREX subunit within a heteromeric assembly is sufficient to localize the BK channel C terminus at the plasma membrane. These STREX cysteine residues are highly conserved in vertebrates (Fig. 1D). Using the CSS-palm palmitoylation algorithm (25), we predicted that 4 of the 6 cysteine residues within 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) Schematic of C-terminal GFP fusion (B) and CRD domain (C) fused between CFP and YFP constructs and representative single confocal section images of STREX; STREX cysteine mutants C12:13A and C23:25A, and the ZERO variant (STREX insert excluded) expression in HEK293 cells. In B Lower Right, the C-terminal ZERO–GFP fusion construct was co-transfected with a modified C-terminal STREX construct (STREX*) in which the −GFP tag of STREX was replaced with an −HA epitope. (Scale bars: 5 μm.) (D) Summary bar chart of the respective C-terminal (■) or CRD (□) construct localization at the plasma membrane expressed as a percentage of the respective STREX expression. Data are means ± SEM, N > 12, n > 350 for each construct. ** P < 0.01 compared with respective STREX construct (ANOVA with Student–Neuman–Keuls post hoc test).

Fig. 1. STREX targets BK channel C terminus to the plasma membrane. (A) Schematic illustrating the topology of the BK channel pore forming α-subunit. The STREX insert is located in the linker between the 2 predicted regulator of K⁺ conductance (RCK) domains in the intracellular C terminus. Inclusion of STREX generates a CRD encompassing the heme-binding domain (hb) and STREX. Sequence alignment indicates evolutionarily 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) Schematic of C-terminal GFP fusion (B) and CRD domain (C) fused between CFP and YFP constructs and representative single confocal section images of STREX; STREX cysteine mutants C12:13A and C23:25A, and the ZERO variant (STREX insert excluded) expression in HEK293 cells. In B Lower Right, the C-terminal ZERO–GFP fusion construct was co-transfected with a modified C-terminal STREX construct (STREX*) in which the −GFP tag of STREX was replaced with an −HA epitope. (Scale bars: 5 μm.) (D) Summary bar chart of the respective C-terminal (■) or CRD (□) construct localization at the plasma membrane expressed as a percentage of the respective STREX expression. Data are means ± SEM, N > 12, n > 350 for each construct. ** P < 0.01 compared with respective STREX construct (ANOVA with Student–Neuman–Keuls post hoc test).
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-CPT-cAMP to establish whether constructs resident at the plasma membrane could be dissociated by PKA phosphorylation. Pretreatment of cells with 0.1 mM 8-CPT-cAMP, in the presence or absence of 10 nM okadaic acid, significantly reduced membrane association of the STREX constructs (Fig. 3). 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. 3D). In addition, neither acute (<3% O2), hypoxia (Fig. 3D), 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 PKI6-24, but not by inhibitors of PKG, PKC, or CaMKII (Fig. S2) and is mimicked by application of exogenous catalytic subunit of PKA (PKAc) (Fig. 3G). 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. 3G). The C12:13A mutation, and inhibition of STREX channel palmitoylation by 2-BP, which both dissociate the C terminus from the plasma membrane (Fig. 2C), resulted in a right shift in the half-maximal voltage for activation (Fig. S3A). 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 V005 resulting from the C12:13A

Fig. 2. Palmitoylation of STREX required for membrane localization. (A) Representative fluorographs (Upper) and Western blots (Lower) of full-length STREX-HA and ZERO-HA channels (Left) and the wild-type STREX and mutant C12:13A CRD-YFP constructs (Right) expressed in HEK293 cells. Constructs were labeled with H-palmitate for 4 h and the respective constructs immunoprecipitated (IP) by using α-HA or α-GFP magnetic microbeads respectively and detected by fluorography. (B) Representative blots from a cysteine-accessibility assay after IP and 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 (WT) or CRD (C) as in Fig. 1B–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).

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. 2A). 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 (ha). Ha cleaves the thioester bond between 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 (WT) or CRD (C) as in Fig. 1B–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).
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 (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-CTP-cAMP (0.1 mM, 10–30 min) on STREX C-terminal (14) or CRD (13) 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 (~3% O2), PKG activation with the cell-permeable cGMP analogue 8-CTP-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 (14) or C12:13A (13) 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 (~3% 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).
Palmitoylation is a post-translational modification that plays a crucial role in the regulation of ion channel function. It involves the covalent attachment of palmitic acid to cysteine residues on the extracellular domains of ion channels. This process is carried out by palmitoyltransferases, which transfer palmitate from palmitoyl-CoA to the thiol group of cysteine residues. The resulting palmitoylated channels are then targeted to the plasma membrane, where they function as ion channels.

Channels are 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).

In the nervous system, PKA is a key modulator of ion channel function. It regulates the activity of BK channels, among others, by phosphorylating them. The phosphorylation of BK channels increases their sensitivity to activation by voltage, calcium, or nucleotides, and mediates their desensitization. PKG, another cGMP-dependent protein kinase, also regulates BK channel activity, but its effects are less well understood.

The functional significance of protein palmitoylation in the nervous system is not fully understood. However, it is thought to play a role in the regulation of channel trafficking, stability, and function. For example, palmitoylation may be involved in the regulation of BK channel activity in the basal forebrain, where it is thought to modulate the excitability of neurons.

In conclusion, protein palmitoylation is a dynamic and versatile mechanism that regulates ion channel function in the nervous system. Further studies are needed to fully understand the role of this process in the regulation of ion channel activity and to explore its potential therapeutic applications in neurological disorders.
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 precleared with protein-G beads (Sigma), and incubated overnight at 4 °C with mouse monoclonal α-HA antibody. Immunopurified channels 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. HEK293 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, 10 mM glucose, and 100 mM Mg-ATP, 30 mM glucose, 1 mM ATP (pH 7.4) with or without 0.002 tetrodotoxin. The patch pipette (intracellular) contained 140 mM KCl, 2 mM MgCl₂, 10 mM Heps, 30 mM glucose, 1 mM BAFTA, and 1 mM ATP (pH 7.4) with intracellular free calcium ([Ca²⁺]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 PI4 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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