Alternative Splicing Switches Potassium Channel Sensitivity to Protein Phosphorylation*

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Lijun Tian‡, Rory R. Duncan‡, Martin S. L. Hammond‡, Lorraine S. Coghill‡§, Hua Wen§, Radda Rusinova§§, Alan G. Clarke***, Irwin B. Levitan††, and Michael J. Shipston‡‡‡

From the Department of Membrane Biology Group & Medical Research Council Membrane and Adapter Protein Co-operative Group, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom and the Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6074

Accelerated Publication

Alternative exon splicing and reversible protein phosphorylation of large conductance calcium-activated potassium (BK) channels represent fundamental control mechanisms for the regulation of cellular excitability. BK channels are encoded by a single gene that undergoes extensive, hormonally regulated exon splicing. In native tissues BK channels display considerable diversity and plasticity in their regulation by cAMP-dependent protein kinase (PKA). Differential regulation of alternatively spliced BK channels by PKA may provide a molecular basis for the diversity and plasticity of BK channel sensitivities to PKA. Here we demonstrate that PKA activates BK channels lacking splice inserts (ZERO) but inhibits channels expressing a 59-amino acid exon at splice site 2 (STREX-1). Channel activation is dependent upon a conserved C-terminal PKA consensus motif (S869), whereas inhibition is mediated via a STREX-1 exon-specific PKA consensus site. Thus, alternative splicing acts as a molecular switch to determine the sensitivity of potassium channels to protein phosphorylation.

Large conductance calcium- and voltage-activated potassium (BK) channels link intracellular chemical signaling events with the electrical properties of excitable cells in the endocrine, nervous, and vascular systems (1–3). BK channels are further potentially modulated by reversible protein phosphorylation (4–7). In native tissues BK channels display considerable diversity and plasticity in their regulation by reversible protein phosphorylation. For example, cAMP-dependent protein kinase (PKA) phosphorylation activates BK channels in smooth muscle cells and many neurons but inhibits channel activity in endocrine cells of the anterior pituitary (5, 7–11). Furthermore, the direction of channel regulation by PKA can be modified during challenges to homeostasis (9–11).

The pore-forming α-subunits of BK channels are derived from a single gene (Slo) that undergoes extensive alternative splicing to produce channels with distinct phenotypes (12–15). Importantly, alternative splicing of the α-subunit is dynamically regulated in adults, for example during stress or pregnancy (15, 16). Thus the diversity and plasticity of responses to PKA-dependent protein phosphorylation observed between BK channels in native tissues may result either from differential modulation of alternatively spliced BK channel α-subunits (12–15) or through their interaction with different signaling complexes and β-subunits (17–19).

To address whether BK channel alternative splice variants are differentially regulated by PKA-mediated protein phosphorylation, we have examined the regulation of three mouse (mslo) BK channel variants (20–22) expressed in HEK293 cells. BK channels are regulated by multiple protein kinase signaling pathways (5, 19, 23, 24). We have thus assayed the functional regulation of BK channel splice variants by directly activating PKA that remains closely associated with the channels in excised inside-out patches.

EXPERIMENTAL PROCEDURES

Molecular and Cell Biology—cDNAs encoding mouse BK channel variants were subcloned into the mammalian expression vector pcDNA3 or pcDNA3.1+ (Invitrogen BV, Leek, The Netherlands) and transfected into HEK293 cells using lipofectAMINE as previously described (22). The cloning and subcloning of the three splice variants have been described previously (ZERO and STREX-1 (22); and ZERO1/4C, also referred to as mB2 (21, 25)). ZERO lacks inserts at mammalian splice sites 1–5 and STREX-1 channels are identical except for a 59-amino acid cysteine-rich insert at mammalian splice site 2. ZERO1/4C contains inserts at splice sites 1 and 4 and has modified N and C termini compared with ZERO constructs. Site-directed mutagenesis was performed using QuikChange (Stratagene) and verified by DNA sequencing. Site-directed mutants displayed no significant change in single channel kinetics or conductance, although S869A channels displayed a significant shift in \( V_{0.5} \) (10–12 mV in the absence of intracellular ATP) compared with wild type ZERO channels. GST fusion proteins of the STREX-1 splice insert were constructed in pGEX-5x-1 by polymerase chain reaction and recombinant protein purified and assayed in a PKA phosphorylation reaction under standard conditions. Coimmunoprecipitation of channel variants with the catalytic subunit of PKA (PKAc) was performed essentially as described previously (24).

Electrophysiology—All experiments were performed in the inside-out configuration of the patch clamp technique at room temperature (20–24 °C) using physiological potassium gradients essentially as described previously (29). The pipette solution (extracellular) contained (in mM): 140 NaCl, 5 KCl, 0.1 CaCl₂, 2 MgCl₂, 20 glucose, 10 HEPES, pH 7.4. The bath solution (intracellular) contained (in mM): 140 KCl, 5 NaCl, 2 MgCl₂, 1 or 5 BAPTA, 30 glucose, 10 HEPES, 1 mM ATP, pH 7.3, with free calcium [Ca\(^{2+}\)] buffered to 0.2 μM, unless indicated otherwise.

one S-transferase; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid.

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‡‡ To whom all correspondence should be addressed: Membrane Biology Group, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom and the Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6074.

The abbreviations used are: BK, large conductance calcium-activated potassium; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N′,N′″-tetraacetic acid.
RESULTS AND DISCUSSION

Five major alternative splice sites and a conserved weak PKA consensus motif (RQPS869) have been identified in the C-terminal region of mammalian BK channels (14, 20–22, 26, 27) (Fig. 1a).

Application of cAMP to the intracellular face of excised inside-out patches containing channels that lack inserts at any splice site (ZERO construct channels (Fig. 1a) (20, 22)) resulted in significant (p < 0.01) activation of mean channel activity in 10 out of 16 patches (Fig. 1, b and c). The mean percentage activation in response to cAMP was 128.6 ± 45.2%. cAMP-mediated inhibition of channel activity was never observed in patches containing ZERO channels. This activation was dependent upon closely associated cAMP-dependent protein kinase activity as cAMP had no effect in any patch in the absence of ATP (mean percent change in activity was: 2.9 ± 15.6%, n = 4) or in the presence of the specific PKA inhibitor peptide PKI5–24 (−9.3 ± 8.2%, n = 6).

In contrast, under identical recording conditions, application of cAMP to the intracellular face of patches containing channels expressing the 59-amino acid cysteine-rich exon at splice site 2 (STREX-1, Fig. 1a) (22, 26) resulted in significant inhibition of channel activity in eight out of nine patches (Fig. 1, b and c). Mean percent change in activity: −56.5 ± 6.8%, cAMP had no significant effect in the absence of ATP (18.6 ± 11.9%, n = 4) or in the presence of PKI5–24 (7.8 ± 11.8%, n = 6). The STREX-1 exon is widely expressed in neuroendocrine tissues and confers enhanced apparent calcium sensitivity on native BK channels compared with the ZERO variant that is highly expressed in brain (22, 26). The inhibitory effect of cAMP was not a result of the enhanced single channel Po observed in STREX-1 compared with ZERO channels under identical recording conditions as cAMP also inhibited STREX-1 channels when recording conditions were adjusted to give a Po similar to that of ZERO (not shown).

The data suggest that an endogenous cAMP-dependent protein kinase A activity (PKA) remains closely associated with STREX-1 or ZERO channels in excised patches from HEK293 cells. Indeed, the catalytic subunit of PKA (PKAc) binds directly to the C-terminal tail of dSlo (24) and coimmunoprecipitates with both mouse ZERO and STREX-1 channel splice variants expressed in HEK293 cells (Fig. 2a). This interaction is specific as PKAc did not coimmunoprecipitate with KCNQ3 potassium channel subunits under the same conditions.2 It is interesting that, in the case of one splice variant of dSlo, PKA regulatory subunit competes with the channel for PKAc binding, and hence the holoenzyme does not bind to the channel.3

In contrast to the robust regulation of ZERO or STREX-1 channels, cAMP had no significant effect on channels containing inserts at splice sites 1 and 4 with different N and C termini (Fig. 1c; ZERO1/4AC variant (21), mean percent change in activity was 7.8 ± 5.2%, n = 8). As PKAc also coimmunopre-

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2 H. Wen and I. B. Levitan, unpublished data.

3 Y. Zhou and I. B. Levitan, unpublished data.
The presence of the STREX-1 exon may expose or modify the functional effect of PKA-mediated phosphorylation. Precipitates subjected to Western blotting with anti-PKAc antibodies were subjected to immunoprecipitation using anti-mSlo and immunoprecipitates subjected to channel expression. Lysates by Western blotting using an anti-mSlo antibody that recognizes all transiently expressed in HEK293 cells, and cell lysates were analyzed the STREX exon.

noprecipitates with mSlo splice variants and phosphorylates a cent change was the site-directed STREX-1 variant (STREX-S869A, mean percent change activity was 3.2 ± 5.8%, n = 6). Importantly, the inhibitory effect of cAMP on S4STREX channels is dependent upon the conserved C-terminal serine residue at position 869 (Ser869) has been reported as the target for PKA-mediated activation of the hSlo ZERO variant expressed in Xenopus oocytes (27). Furthermore, the equivalent site in dSlo (Ser843) is phosphorylated by PKA (24).

Mutation of serine 869 to alanine (S869A) completely abolishes cAMP-mediated activation of the ZERO variant (S869A construct, mean percent change activity was 3.2 ± 1.5%, n = 6) but had no significant effect on cAMP-mediated inhibition of the site-directed STREX-1 variant (STREX-S869A, mean percent change was −58.1 ± 12.1%, n = 11) in excised patches (Fig. 3, a and b). Importantly, the inhibitory effect of cAMP on S4STREX on cAMP-mediated regulation of ZERO and STREX-1 variants. Data are expressed as the mean percent change in channel activity ± S.E. after activation of endogenous PKA with 1 mM cAMP for each construct shown on the left. cAMP was applied in the presence (+cAMP) of ATP or after pretreatment with 0.45 μM of the specific PKA inhibitor PKI5–24 in the presence of ATP. A positive percent change in activity indicates activation, a negative percent change indicates inhibition.

Splicing switches potassium channel sensitivity to PKA.

**Fig. 2.** Catalytic subunit of protein kinase A (PKAc) commumnoprecipitates with mSlo splice variants and phosphorylates the STREX exon. a, PKAc and the respective splice variant were transiently expressed in HEK293 cells, and cell lysates were analyzed by Western blotting using an anti-mSlo antibody that recognizes all three variants (top panel) to normalize for channel expression. Lysates were subjected to immunoprecipitation with anti-mSlo and immunoprecipitates subjected to Western blotting with anti-PKAc antibodies (lower panel). b, autoradiograph of 32P-phosphoproteins analyzed by SDS-polyacrylamide gel electrophoresis from *in vitro* PKA phosphorylation assays of the STREX-1 insert (as a GST fusion protein, GST-STREX-1) and the corresponding site-directed mutant GST-S4STREX-A.

**Fig. 3.** Ser869 and S4STREX are required for cAMP-mediated activation and inhibition of ZERO and STREX-1 respectively. a, representative single channel recordings and diary plots of mean single channel open probability (P0) of S4STREX-A and S869A channels in inside-out patches from HEK293 cells before (Control) and 10 min after application of 1 mM cAMP to the intracellular face of the patch as in Fig. 1. b, summary of the effect of site-directed mutation of Ser869 and S4STREX on cAMP-mediated regulation of ZERO and STREX-1 variants.活性 of the properties and regulation of potassium ion channels, including multiple gene families, heteromultimerization of sub-
units, association with regulatory or accessory subunits, and post-translational modifications such as reversible protein phosphorylation. As BK channels are derived from a single gene alternative, exon splicing plays a major role in determining the functional diversity of BK channels.

Our data reveal that alternative exon splicing of BK channels also provides a fundamental context-sensitive mechanism for switching BK channel sensitivity to protein phosphorylation that has significant implications for both the long and short term control of cellular excitability. As BK channel splice variant expression can be dynamically regulated, for example during stress or pregnancy (15, 16), the functional consequence of PKA-mediated regulation of BK channel activity may be switched in the long term by altering the splice variant expressed (e.g. from ZERO to STREX-1). In contrast, short term switching of BK channel sensitivity to PKA could be achieved by specific dephosphorylation of residues important for PKA regulation within a splice insert. For example, in STREX-1 containing channels that are normally inhibited by PKA, dephosphorylation of the PKA site specifically within the STREX-1 insert would allow PKA-dependent activation of the channel through conserved sites in the C terminus (as for S4STREXA construct). Further diversity and plasticity may be achieved through splice variant heteromultimerization and/or association with regulatory subunits (17–19).

In conclusion, alternative exon splicing acts as a powerful long and short term molecular switch to define the sensitivity of BK channels to protein phosphorylation and provides a fundamental mechanism for specifying the diversity, plasticity, and functional role of such modular coincidence detectors of excitable cells.

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