Leucine Zipper Domain Targets cAMP-dependent Protein Kinase to Mammalian BK Channels*

Received for publication, November 15, 2002, and in revised form, December 19, 2002
Published, JBC Papers in Press, December 30, 2002, DOI 10.1074/jbc.M211661200

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Large conductance, calcium- and voltage-activated potassium (BK) channels control excitability in many tissues and are regulated by several protein kinases and phosphatases that remain associated with the channels in cell-free patches of membrane. Here, we report the identification of a highly conserved, non-canonical, leucine zipper (LZ1) in the C terminus of mammalian BK channels that is required for cAMP-dependent protein kinase (PKA) to associate with the channel and regulate its activity. A synthetic polypeptide encompassing the central d position leucine residues in LZ1 blocks the regulation of recombinant mouse BK channels by endogenous PKA in HEK293 cells. In contrast, neither an alanine-substituted LZ1 peptide nor a peptide corresponding to another, more C-terminal putative leucine zipper, LZ2, had any effect on regulation of the channels by endogenous PKA. Mutagenesis of the central two LZ1 d position leucines to alanine in the BK channel also eliminated regulation by endogenous PKA in HEK293 cells without altering the channel sensitivity to activation by voltage or by exogenous purified PKA. Inclusion of the STREX splice insert in the BK channel protein, which switches channel regulation by PKA from stimulation to inhibition, did not alter the requirement for an intact LZ1. Although PKA does not bind directly to the channel protein in vitro, mutation of LZ1 abolished co-immunoprecipitation of PKA and the respective BK channel splice variant from HEK293 cells. Furthermore, a 127-amino acid fusion protein encompassing the functional LZ1 domain co-immunoprecipitates a PKA-signaling complex from rat brain. Thus LZ1 is required for the association and regulation of mammalian BK channels by PKA, and other putative leucine zippers in the BK channel protein may provide anchoring for other regulatory enzyme complexes.

Reversible protein phosphorylation represents a fundamental cellular regulatory mechanism to control the activity and function of plasma membrane ion channels (1). The co-ordination, specificity, and compartmentalization of ion channel regulation by reversible protein phosphorylation is facilitated by assembly with signaling complexes comprising cognate protein kinases and protein phosphatases. Assembly of ion channels with signaling complexes typically results from multiple protein-protein interactions mediated by distinct interaction domains (2) allowing signaling molecules to interact directly with an ion channel or indirectly as part of a higher order complex. Large conductance calcium- and voltage-activated potassium (BK) channels have been widely exploited as models of ion channel regulation by reversible protein phosphorylation; however, the molecular basis for kinase and phosphatase assembly with the BK channel is largely unknown (1, 3). BK channels play a central role in the regulation of cellular excitability because they are activated directly by both voltage and intracellular free calcium (4–6) and potently modulated by reversible protein phosphorylation (1). For example, they provide a dynamic link between electrical and chemical signaling events in cells, are major determinants of vascular smooth muscle tone (5, 7), and regulate action potential duration and frequency as well as neurotransmitter and hormone release in neurons and endocrine cells (6, 8). A single gene (KCNMA1) encodes for the pore-forming α-subunits of BK channels in all mammalian tissues (9). Phenotypic variation in native BK channels results from extensive alternative exon splicing of the α-subunit (6, 9, 10) as well as through interaction with regulatory β-subunits and accessory proteins (11–13). The BK channel α-subunit is a target for regulation by multiple protein kinases and protein phosphatases (3, 14–18), and several protein kinases have been reported to co-immunoprecipitate with mammalian BK channels (3, 16, 17). Although several consensuss phosphorylation sites have been identified by mutagenesis within the intracellular C-terminal domain, BK channel α-subunits do not contain previously identified protein-protein interaction domains. Thus, the molecular basis for protein kinase or phosphatase targeting to mammalian BK channels is essentially unknown. In Drosophila, the catalytic subunit, but not the holoenzyme, of cAMP-dependent protein kinase (PKA) binds directly to the intracellular C terminus (3, 19) of the channel. Although PKA co-immunoprecipitates with mammalian BK channels in a splice variant-independent manner, the mechanism of complex assembly is unknown (17).

Recently a structural motif, the leucine zipper (LZ), originally described in classes of DNA-binding proteins (20), has

* This work was supported by Biotechnology and Biological Sciences Research Council, Swindon, United Kingdom and Wellcome Trust grants (to M. J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Recipient of a Biotechnology and Biological Sciences Research Council, Swindon, United Kingdom Committee studentship.

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been reported to play an important role in coordinating both the assembly of ion channels as well as their interaction with protein kinase and protein phosphatase signaling complexes (21–24). In several of these channels the catalytic subunit of PKA (PKAc) is targeted to the channel through a protein kinase A-anchoring protein (AKAP). The AKAP acts as an adapter protein by binding to both the LZ domain of the channel and the regulatory subunits of PKA. In this report we identify a putative LZ domain for BK channel assembly with a PKA signaling complex essential for the functional regulation of mammalian BK channels by PKA-dependent phosphorylation.

MATERIALS AND METHODS

Construction of BK Channel Splice Variant and Mutant cDNA Constructs—The cloning and sub-cloning of the mouse BK channel splice variants ZERO and STREX into the mammalian expression vector pcDNA3 or pcDNA3.1 (+) (Invitrogen) have been described previously (17, 25). A C-terminal hemagglutinin (HA) tag was introduced into each channel construct by replacing the normal stop codon. Amino acid numbering in the subsequent text and figures is in accordance with the amino acid sequence of the mouse mbr5 clone (accession number GI:347144; the start methionine being M, ELEH) for consistency. Alanine substitutions in the third and fourth LZ1 d position leucine residues (Fig. 1: amino acids Leu-530 and Leu-537) was performed by site-directed mutagenesis using a single mutagenic primer set with the QuikChange system according to the manufacturer (Stratagene, La Jolla, CA) to generate the HA-tagged LZ1 mutant channels ZERO(ISLO/hELEh) and STREX(ISLO/hELEh).

Thioredoxin fusion proteins were generated by PCR and subcloning fragments into the pBAD/Thio-TOPo vector (Invitrogen). Fusion proteins were constructed with an N-terminal His-patch (HP)-thioredoxin and C-terminal V5 and hexahistidine epitopes to facilitate purification and immunoprecipitation. All soluble fusion proteins were induced and purified from BL21 RIL Escherichia coli using standard methods. All immunoprecipitations using thioredoxin fusion proteins were performed with the intact HP-thioredoxin fragment, because cleavage of the thioredoxin fusion resulted in proteins that were largely insoluble.

The LZ1(ISLO/hELEh) thioredoxin fusion protein was designed to span the LZ1 domain from the end of the predicted regulator of potassium conductance domain (Estrada et al. (21)) and extending to amino acid Ile-616 (Fig. 5c). The ZERO(ISLO/hELEh) and STREX(ISLO/hELEh) thioredoxin fusion proteins were designed to span LZ2 and to include the mammalian STREX alternative site of splicing as well as the conserved PKA consensus site at S899 (Fig. 5c). The starting amino acid in the respective construct was Val-580 and terminating in amino acid Ala-984. ZERO(ISLO/hELEh) and STREX(ISLO/hELEh) are, thus, identical apart from the addition of 59 amino acids of the mouse STREX (25) insert in STREX(ISL0/hELEh) (Fig. 5c: amino acid numbering has been retained as for ZERO for consistency) and do not contain LZ1. All constructs were verified by DNA sequencing.

Concentrates—Leucine zipper (LZ) heptad repeat peptides were synthesized by Genemed Synthesis (South San Francisco, CA) and used at a final bath concentration of 25–80 μM. Concentrations of peptide <5 μM were largely ineffective (data not shown). The LZ1 peptide (IQKSCLAQGLSTMALNLAFS) corresponds to amino acids 523–539, spanning the 2nd, 3rd, and 4th d positions in the mouse 5-heptad repeat LZ1 motif (Fig. 1e) and was found that an N-terminal aspartic (D) residue was included to improve water solubility. The corresponding alanine substituted peptide (Ala-LZ1) was identical except the 2nd, 3rd, and 4th LZ1 heptad repeat d residues were replaced by alanine (DAASCLAQGASTMLNALSFS). The LZ2 peptide (DLRAVINVLCDMVCVLS) corresponds to conserved residues 816–834 within a putative C-terminal 4-heptad repeat LZ2 motif in the mouse BK channel (Fig. 1e).

HEK293 Cell Culture and Transfection—HEK293 cells were subcultured essentially as previously described (17, 25) except with one modification whereby cells were placed in serum-free (ITS, Invitrogen) medium 24 h before experiments. Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO2 at 37 °C. Cells were routinely passaged every 3–7 days using 0.25% trypsin in Hank’s buffered salt solution containing 0.1% EDTA. For immunoblotting studies cells were grown to 70–80% confluence in 75-cm² flasks. For electrophysiological assays cells were plated on glass coverslips in 6-well cluster dishes. Twenty-four hours before the experiment cells were washed, and medium was replaced with Dulbecco’s modified Eagle’s medium containing ITS serum replacement (Invitrogen). For transient transfections of BK channels HEK293 cells were seeded onto glass coverslips in 6-well cluster dishes at a density to allow cells reaching 40–60% confluence after 24 h. Cells were then transfected with 1 μg of the respective cDNA using LipofectAMINE (Invitrogen) in Dulbecco’s modified Eagle’s medium essentially as described previously. After 5 h medium was supplemented with 10% fetal calf serum, which was replaced after 24 h, and electrophysiological recordings made 24–72 h post-transfection. Stable cell lines were also created by selection and maintenance for Zeocin or Geneticin resistance using 0.2 mg ml⁻¹ Zeocin (Invitrogen) or 0.8 mg ml⁻¹ Geneticin (Invitrogen) as appropriate.

Full-drown and Immunoprecipitations—Immunoprecipitation (IP) of HA-tagged channels or PKA was performed using transient or stably expressing HEK293 cell lines essentially as previously described (17). Briefly, cells were solubilized in radioimmunoassay buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 0.2 mM NaCl, 10 mM EDTA, 20 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mg/ml bovine serum albumin, 1% (v/v) Triton X-100, and protease inhibitors (Roche Molecular Biochemicals). Insoluble material was removed by centrifugation (10,000 × g, 15 min, 4 °C), and the lysate was pre-clarified by incubation with 20 μl of 50% (v/v) protein G-Sepharose with agitation for 1 h at 4 °C. PKA- or HA-tagged channels were immunoprecipitated from the cleared lysate with the respective antibody (prebound to 40 μl of 50% (v/v) protein G-Sepharose) for 4 h at 4 °C. IP antibodies used were mouse anti-HA monoclonal antibody (clone: 12CA5, Roche Diagnostics) for HA-tagged channels, a sheep anti-PKAc polyclonal antibody (ab365 (29), a generous gift from Dr Roger A Clegg), or a rabbit anti-PKAc polyclonal antibody (Santa Cruz Biotechnology Inc.). The immuno- precipitates were washed 5 times with 1 ml of radioimmunoassay buffer before SDS-PAGE analysis.

For Western blot detection a rabbit anti-HA polyclonal (Y-11, Santa Cruz Biotechnology, Inc.) and the above sheep anti-PKAc polyclonal were used as described in figure legends (Figs. 4 and 5). IP of thioredoxin fusion proteins of the BK channel intracellular C terminus employed a mouse monoclonal anti-V5 antibody (Invitrogen). Detection was enhanced by chemiluminescence.

Patch Clamp 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 (17). The pipette solution (extracellular) contained 140 mM NaCl, 5 mM KCl, 0.1 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 or 5 mM BaCl₂, 30 mM glucose, 10 mM HEPES, 1 mM ATP, pH 7.3, with free calcium ([Ca²⁺]ᵢ) buffered to 0.2 μM unless indicated otherwise. For assays in which the catalytic subunit of PKA (PKAc, Promega, Madison, WI) was applied directly to patches, patches were exposed to the above intracellular bathing solution containing 0.1 mM dithiothreitol during control and PKA application to exclude effects due to dithiothreitol (30) present in purified PKA preparations. Data acquisition and voltage protocols were controlled by an Axopatch 200 A or B amplifier and pCLAMP6 software (Axon Instruments Inc., Foster City, CA). All recordings were filtered at 2 kHz and digitized at 10 kHz. After 40 min of channel activity was allowed to stabilize for at least 10 min (typically 10–15 min after excision), and stability plot experiments demonstrated that BK channel activity was stable for >1 h under the recording conditions used (data not shown) in the absence of channel modulators. Application of Ca²⁺ or other reagents to the intracellular face of patches was achieved by gravity-driven perfusion (10 volumes of the recording bath solution (bath volume, 0.5 ml) by gravity-driven perfusion at a flow rate of 1–2 ml/min) or direct application to the bath. Channel activity was determined during 30-s depolarizations to +40 mV.

Single-channel open probability (Pₒ) was derived either from single-channel analysis using pSTAT for patches with <4 channels or, in the case of patches with >4 channels, by an integrated dwell time al-
channel current amplitude from the peak intervals. After subtraction of the offset from the traces these were integrated over 0.5–80-s segments. The integral divided by integration time and single-channel current amplitude gives NP-

To determine mean percent (%) change in channel activity after a treatment in patches with low to moderate levels of channel expression, mean P, or N × P, was averaged from several minutes of recording at +40 mV immediately before and 10 min after the respective drug treatment. Mean change in activity was expressed as a percentage (% of the pretreatment control ± S.E. In the respective figure legends and text (see “Results”) a positive percentage (%) change in activity reflects activation, whereas a negative percentage (%) change reflects channel inhibition.

RESULTS

Putative LZ Motifs in Mammalian BK Channels—The amino acid sequence of LZ motifs is typically characterized by a seven-residue (heptad) repeat (commonly denoted abcdedg, see Fig. 1, a and b) with positions a and d in each heptad repeat occupied by hydrophobic residues (20, 31). Leucine provides the most thermodynamically stable residue at position d (32). However, significant deviations in amino acid sequence from this “classical” leucine zipper motif may exist, for example, deletions or insertions of individual residues within the heptad repeat or the presence of polar residues at position a or d (22, 31, 32, 34).

Allowing for such variability, manual inspection of the C-terminal amino acid sequence of mammalian BK channels revealed several putative LZ motifs. A C-terminal LZ motif (LZ1, Fig. 1, a and b, between residues 513 and 548 of the murine BK channel variant mbr5 (accession number GI:347144 (9)) contains five heptad repeats downstream of residues that contribute to the proposed “fixed interface” of the BK channel regulator of K channel conductance (RCK, or tetramerization) domain (26–28). Although the second d residue of the five-heptad LZ1 repeat is glutamine (Q) and the fifth a residue is non-hydrophobic, the stability of “prototypical” LZ domains with a Glu (Q) residue at a single d position is, paradoxically, not significantly compromised compared with isoleucine or valine substitutions (32). Comparison of the amino acid sequence of mammalian BK channels with the structure of the RCK domain in calcium-activated potassium (MthK) channels from Methanobacterium thermoautotrophicum suggests that LZ1 forms the αG helix and also contributes to an extended linker region between the αG helix and βG strand (26–28). However, in MthK the extended linker is absent, and the mammalian LZ1 heptad repeats are not conserved in MthK or other prokaryotic RCK domains (Fig. 1a), suggesting LZ1 and the linker region play an additional role in mammalian BK channels.

A second putative four-heptad repeat LZ domain (LZ2: residues 816–843, Fig. 1a) is positioned between splice site 2 and the conserved PKA consensus site at serine residue Ser-899 (see Fig. 1a). At least two further three-heptad repeats may also be present in the C terminus.

To investigate whether LZ1 or LZ2 plays a functional role in targeting PKA to BK channels we examined the regulation of two distinct murine BK channel splice variants, STREX and ZERO, that are differentially regulated by PKA-dependent phosphorylation when expressed in HEK293 cells (17). Two functional strategies were exploited, first, by designing competitive peptide inhibitors of LZ1 and LZ2 interactions in an attempt to disrupt PKA regulation of STREX and ZERO channels, respectively, and second, by investigating PKA-dependent
regulation of STREX and ZERO channels in which candidate LZ1 motifs identified in the peptide inhibitor screen were disrupted by mutating the third and fourth position leucine residues (Leu-530 and Leu-537) to alanine.

LZ1 Is Required for PKA Regulation of the ZERO Splice Variant—Because the LZ1 domain is conserved in mammalian BK channels and distinct BK channel splice variants may be differentially regulated by PKA (17), we addressed whether the LZ1 motif was required for regulation of distinct splice variants by endogenous PKA. To assay for regulation of BK channel splice variants by endogenous PKA we applied cAMP to the intracellular face of isolated inside-out patches from HEK293 cells to activate PKA closely associated with the channel as previously reported (17). The mouse ZERO variant of BK is

![Diagram showing representative single channel traces and corresponding diary plots of channel activity from isolated inside-out patches from HEK293 cells expressing wild type HA-tagged mouse ZERO channels (ZERO) (a) or the alanine-substituted leucine zipper (LZ1) mutant channels (ZERO<sub>L530A:L537A</sub>) before (control) and 10 min after (+cAMP) application of 1 mM cAMP to the intracellular face of the patch (b). NP<sub>o</sub>, number of functional channels × open probability of channel). c, summary of the effect of cAMP application to the intracellular face of patches from ZERO channels in the absence (ZERO, n = 8) or presence of 80 μM corresponding leucine zipper-competing peptides (LZ1, n = 6; Ala-LZ1, n = 4; LZ2, n = 4) as well as for the ZERO<sub>L530A:L537A</sub> channels (n = 8). All data are expressed as the percentage of change to pretreatment BK channel activity measured at +40 mV in the presence of 0.2 μM [Ca<sup>2+</sup>]<sub>i</sub> and 2 mM Mg-ATP as described under “Materials and Methods.” Mean ± S.E. **, p < 0.01 compared with ZERO group.
activated by PKA closely associated with the channel and dependent upon a C-terminal serine residue (Ser-899) conserved in all mammalian BK channel splice variants (17, 25). C-terminal HA-tagged ZERO channels were stimulated upon application of cAMP to the intracellular face of inside-out patches from HEK293 cells only in the presence of Mg-ATP (48.6 ± 8.0%, n = 8, Fig. 2) in a similar fashion to untagged channels (17). cAMP activation of ZERO channel activity was not observed in the presence of 25–80 μM LZ1 competing peptide (the mean activation was 57.9 ± 3.6%, n = 8, Fig. 2). LZ1-competing peptide also blocked cAMP stimulation of the untagged channels (the mean change in activity was −1.9 ± 3.6%, n = 3). However, cAMP regulation of ZERO channels was unaffected by the alanine-substituted LZ1-competing peptide (the mean activation was 56.8 ± 14.6%, n = 4) or LZ2-competing peptide (the mean activation was 57.9 ± 8.9%, n = 4). Although the effect of the LZ1 peptide is specific, relatively high concentrations of peptide are required. LZ1 peptide concentrations <5 μM were largely ineffective (not shown). Thus, to confirm the requirement for a LZ1 domain in ZERO channel regulation by endogenous PKA, we mutated the third and fourth d position LZ1 leucine residues to alanine (ZERO<sub>L530A/L537A</sub>). This mutation had no significant effect on the half-maximal voltage required for channel activation (V<sub>0.5</sub>) under the assay conditions; V<sub>0.5</sub> was −47 ± 8 mV (n = 4) (ZERO) and 52 ± 5 mV (n = 3) (ZERO<sub>L530A/L537A</sub>). Importantly, ZERO<sub>L530A/L537A</sub> channels were unaffected by application of cAMP to their intracellular face (mean change in activity was 2.5 ± 5.6%, n = 6) (Fig. 2b).

To address whether mutation of the LZ1 motif prevents transduction of the effect of PKA-mediated phosphorylation of the mutant channel rather than preventing interaction of PKA with the channel, we applied the purified catalytic subunit of PKA (PKAc) to the intracellular face of patches containing ZERO<sub>L530A/L537A</sub> channels. Application of PKAc resulted in a 49.3 ± 5.2% (n = 3) activation of ZERO<sub>L530A/L537A</sub> channels, similar to that upon activation of endogenous PKAc with cAMP. Taken together these data suggest that the LZ1 motif is a common site required for targeting of PKA to murine ZERO variant BK channels.

**LZ1 Is Required for PKA-dependent Inhibition of the STREX Splice Variant**—To examine whether the conserved LZ1 domain is also required for regulation of other BK channel splice variants by endogenous PKA, we examined the regulation of the mouse STREX variant expressed in HEK293 cells. STREX channels are identical to ZERO apart from inclusion of a 59-amino acid insert (17, 25). The mouse STREX splice variant is potently inhibited by PKA closely associated with the channel complex and dependent upon a conserved serine residue within the STREX insert (17, 25). STREX channels expressing a C-terminal HA tag were similarly inhibited (mean % change in activity was −56.1 ± 11.2%, n = 6) by endogenous PKA upon application of cAMP to the intracellular face of excised inside-out patches from HEK293 in the presence of Mg-ATP (Fig. 3). No significant cAMP inhibition of STREX channel activity was observed in the presence of 25–80 μM LZ1 1-competing peptide (mean % change in activity was 5.8 ± 8.9%, n = 10, Fig. 3). A similar LZ1 peptide block of PKA inhibition of the untagged channel was also observed (mean % change in activity was 9.6 ± 3.9%, n = 3). However, robust PKA-mediated inhibition of STREX was observed in the presence of 80 μM alanine-substituted LZ1 peptide (Ala-LZ1; mean inhibition −61.5 ± 6.2%, n = 7) or 80 μM alanine peptide corresponding to the more C-terminal putative LZ2 domain (mean % change in activity was −61.6 ± 9.3%, n = 6). To further address the requirement for a functional LZ1 domain for PKA action, the third and fourth d position LZ1 leucine residues (Leu-530 and Leu-537) in the HA-tagged STREX channel were mutated to alanine and expressed in HEK293 cells. Although the half-maximal voltage for activation of STREX channels is shifted to more negative potentials than for ZERO channels (25), the LZ1 mutant STREX channels (STREX<sub>L530A/L537A</sub>) expressed in HEK293 cells displayed no significant shift in their half-maximal activation voltage compared with wild type STREX channels under the assay conditions used. In the presence of 0.2 μM free calcium and Mg-ATP, the respective V<sub>0.5</sub> was 47 ± 8 mV (STREX) and 52 ± 5 mV (STREX<sub>L530A/L537A</sub>). Importantly, application of cAMP to the intracellular face of patches expressing STREX<sub>L530A/L537A</sub> had no significant effect on channel activity (mean change in channel activity was 14.6 ± 6.9%, n = 6).

To address whether the action of cAMP is dependent upon targeting of endogenous PKAc with the channel via a “typical” protein kinase A-anchoring protein, as for several other types of ion channel (21–23), we examined cAMP regulation in the presence of the AKAP-competing peptide, ht31 (33). In parallel experiments, cAMP-dependent inhibition of STREX channel activity in the presence of ht31 peptide was not significantly different from that with cAMP alone (mean % inhibition was −57.2 ± 2.2%, n = 4). Taken together these data suggest that LZ1 is required to target endogenous PKAc to BK channel splice variants and that targeting or regulation is not dependent upon a typical protein kinase A-anchoring protein.

**PKAc Docking with Mouse BK Channel Variants Mediated via a LZ1 Complex**—The catalytic subunit of PKA (PKAc) has previously been shown to co-immunoprecipitate with both the ZERO and STREX BK channel splice variants (17). Because PKA regulation of both ZERO and STREX splice variants was dependent upon a functional LZ1 motif we addressed whether alanine substitution of the third and fourth d position LZ1 leucine residues prevented co-immunoprecipitation of PKAc with either splice variant subunit. PKAc co-immunoprecipitated with STREX or ZERO channels upon pull-down of the HA epitope-tagged channels expressed in HEK293 cells (Fig. 4a) as previously reported for the untagged splice variants (17). However, PKAc did not co-IP with either alanine-substituted LZ1 BK channel splice variant (Fig. 4a). Furthermore, association of PKAc with either STREX or ZERO channels did not require interactions mediated via PKA regulatory subunits because an antibody that IPs PKAc that is dissociated from its regulatory subunits (29) resulted in robust co-IP of the respective BK channel variant (Fig. 4b). Similar co-IP was observed with anti-PKAc antibodies that IP the holoenzyme (not shown). Furthermore, co-IP was not dependent upon channels being phosphorylated at the putative PKA consensus sites because PKAc co-immunoprecipitated with the STREX channel in which both the STREX (STREX Ser-4) and conserved (Ser-899) PKA consensus serine residues were mutated to alanine (STREX<sub>S4A/S899A</sub> construct, Fig. 4b). This suggests that LZ1 is required for targeting of PKAc to the channel and that targeting is independent of substrate recognition.

Because the LZ1 motif is immediately downstream of the core RCK domain (also referred to as the tetramerization domain) (26–28) mutation of this site may disrupt the C-terminal conformation of the channel, thus preventing association of a PKAc signaling complex with other domains in the channel. Indeed, in Drosophila PKAc binds directly with the C terminus of the channel at a region close to the Drosophila equivalent (Ser-942) of the conserved mammalian PKA consensus site at mammalian serine residues Ser-899 (3, 19). To directly test whether the LZ1 domain in mouse BK channels is required as
FIG. 3. LZ1 is required for PKA inhibition of STREX channels. Representative single channel traces and corresponding diary plots of channel activity from isolated inside-out patches from HEK293 cells expressing wild type HA-tagged mouse STREX channels (STREX) (a) or the alanine-substituted leucine zipper (LZ1) mutant channels (STREXL530A/L537A) before (control) and 10 min after (+ cAMP) application of 1 mM cAMP to the intracellular face of the patch (b). NP₀, (number of functional channels × open probability of channel). c, summary of the effect of cAMP application to the intracellular face of patches from STREX channels in the absence (STREX, n = 6) or presence of 25–80 μM corresponding leucine zipper competing peptides (LZ1, n = 10; Ala-LZ1, n = 7; LZ2, n = 8) as well as for the STREXL530A/L537A channels (n = 6). All data are expressed as the percentage of change to pretreatment BK channel activity measured at +40 mV in the presence of 0.2 mM [Ca²⁺], and 2 mM Mg-ATP as described under “Materials and Methods.” Mean ± S.E. **, p < 0.01 compared with STREX group.
Zipping PKA to BK Channels

DISCUSSION

Increasing evidence suggests that LZ domains play an important role in both the assembly of ion channel signaling complexes as well as ion channel assembly per se (21–24). Allowing for known variations in the amino acid sequence of classical leucine zipper motifs, manual inspection of the intracellular C-terminal domain of mammalian BK channels revealed at least four possible LZ domains with LZ1 and LZ2 (Fig. 1a), displaying the highest criteria for functional LZ domains.

In this paper we have identified, using both competing peptide and mutagenesis studies, a leucine zipper motif present in BK channels that is required for the targeting and functional regulation of mammalian BK channels by PKA. Using a combination of functional and biochemical assays we demonstrate that LZ1, but not LZ2, is required for BK channel assembly with a PKA signaling complex. First, short competing peptides designed to disrupt specific LZ domain interactions blocked PKA-dependent regulation of both STREX and ZERO BK channel variants. In these assays we stimulated PKA activity intimately associated with the channel by applying cAMP to the intracellular face of isolated inside-out patches. As previously reported (17) the effects of cAMP in this system are dependent upon the presence of Mg-ATP, and the actions of cAMP are completely abolished by the PKA inhibitor peptide PKI(5–24).

Although STREX channels are inhibited whereas ZERO channels are activated by PKA closely associated with the channel (17), the short LZ1-competing peptides effectively blocked PKA-dependent regulation of either splice variant. In contrast, alanine-substituted LZ1-competing peptides or peptides directed against LZ2 were not able to block PKA-dependent regulation of the respective BK channel splice variant. Secondly, disruption of the LZ1 domain in STREX or ZERO by mutating the third and fourth position leucine amino acids to alanine (Leu-530 and Leu-537) prevented channel regulation by endogenous PKA. A functional LZ1 is not required for the transduction of the effect of PKA-dependent phosphorylation because application of purified PKAc to patches containing the LZ1 mutant channels was still able to regulate channel activity. This supports the hypothesis that PKA-dependent phosphorylation rather than PKA binding to the channel complex per se is responsible for PKA regulation of mammalian BK channels.

Third, although PKAc co-immunoprecipitates with both wild type STREX and ZERO channels, pull-down of PKAc was not evident with either splice variant when the respective LZ1 motif was mutated. Finally, although PKAc does not directly interact with a fusion protein incorporating LZ1, a PKAc signaling complex can be isolated from native brain tissue using a fusion protein that contains an intact LZ1 domain but not an intact LZ2 domain. Although we cannot exclude that LZ1 is required for correct conformation of a binding site within the 127-amino acid LZ1 fusion protein rather than acting as the direct PKAc signaling complex interaction motif, our data strongly support the hypothesis that LZ1 functions as a conserved motif for directing assembly of a PKAc signaling complex with mammalian BK channels.

The adapter protein linking PKAc with the BK channel has not been identified; however, three independent lines of evidence suggest the putative adapter protein is not a typical AKAP that targets PKAc to several other ion channels (21–23). First, in electrophysiological assays concentrations of ht31 peptide (33) that disrupt PKA/ARAP interactions at other ion

Fig. 4. PKAc co-immunoprecipitation with BK channel splice variants requires a functional LZ1. a, representative Western blots of co-IPs from HEK293 cells expressing HA-tagged STREX or ZERO channels and their respective LZ1 mutants (STREX L530A/L537A and ZERO L530A/L537A). IPs were performed using a mouse monoclonal anti-HA antibody, and immunoprecipitates were probed with either rabbit anti-HA antibody or sheep anti-PKAc antibody. b, representative Western blots of co-IPs of PKAc with HA-tagged BK channel variants using a sheep anti-PKAc antibody to IP PKAc that is not associated with its regulatory subunits (see "Materials and Methods"). Cell lysates were probed with the same anti-PKAc antibody, and channels were detected in the IPs using a rabbit anti-HA antibody. The STREX579-984 construct is identical to STREX except that the STREX insert (S4) and conserved (S899) conserved PKA phosphorylation serine residues are mutated to alanine. Detection was by enhanced chemiluminescence with purified PKAc run as a positive control for PKAc blots. Apparent molecular masses in SDS-PAGE gels were: PKAc, ~45 kDa; HA-tagged BK channels, ~128 kDa.

a targeting motif for a PKAc signaling complex we generated soluble thioredoxin C-terminal fusion proteins encompassing the LZ1 domain (LZ11489-616 construct, Fig. 5a) as well as fusion proteins encompassing LZ2, the STREX alternative site of splicing, and the conserved PKA consensus motif at Ser-899 (ZERO L530A/L537A) and STREX L530A/L537A constructs respectively, Fig. 5a). All assays were performed with the thioredoxin fusions because cleaved proteins were largely insoluble.

In vitro pull-down assays using the LZ11489-616 ZERO L579-984 or STREX L579-984 fusion proteins as bait failed to pull down recombinant purified PKAc catalytic subunit (Fig. 5, b and c). However, using solubilized whole rat brain lysate (Fig. 5, b and c) or HEK293 cell lysate (not shown) resulted in specific pull down of PKAc with the LZ11489-616 fusion protein (Fig. 5b) but not with either the ZERO L579-984 or STREX L579-984 fusions or thioredoxin alone (Fig. 5c). To confirm that a functional LZ1 domain is required for PKAc complex assembly with the LZ11489-616 fusion protein we mutated the third and fourth position LZ1 leucine residues (Leu-530 and Leu-537) within the LZ11489-616 fusion protein to alanine. This construct was expressed at equivalent levels as the wild type fusion protein; however, mLZ11489-616 failed to pull down PKAc from brain lysates. Taken together these data suggest that a functional LZ1 domain is required for assembly of a PKAc signaling complex, dependent upon an as yet unidentified adapter protein(s), with mammalian BK channels.
channels (21–23) had no significant effect on channel regulation by cAMP. Second, co-IP of PKAc with the channel was observed both with anti-PKAc antibodies that IP holoenzyme as well as with antibodies that IP PKAc subunits not associated with their regulatory subunits. Finally, PKAc pull-down was observed in the presence of saturating concentrations of cAMP that would dissociate PKAc from its regulatory subunits.

In contrast to the requirement for an adapter protein to target PKAc with mammalian BK channels reported here PKAc binds directly to the C terminus of Drosophila BK channels (3, 19). BK channels from Caenorhabditis elegans and Drosophila, in contrast to mammalian BK channels, display amino acid substitutions at the 3rd and 5th d positions with phenylalanine and arginine residues, respectively. Thus, whether PKAc can also be targeted to dSlo via the putative LZ1 domain in addition to direct PKA binding to more C-terminal domains in dSlo remains to be elucidated.

The mammalian LZ1 domain is located after residues important for the proposed fixed interface (resulting from helices αD and αE in the crystal structure of the calcium-activated potassium channel (MthK) from M. thermoautotrophicum) as well as for salt bridge formation in the RCK (or tetramerization) domain of mammalian BK channels (26–28). Comparison of the mammalian BK channel sequence with residues in the RCK domain of MthK channels suggests that LZ1 would contribute to the αG helix as well as the extended linker region between the αG helix and βG strand. Thus, LZ1 would be predicted to contribute to the RCK variable subdomain that protrudes from the gating ring (26). The αG helix and variable subdomain in MthK together contribute to a flexible interface between RCK domains (26). However, both the mammalian LZ1 heptad repeats and extended linker is absent in MthK and other prokaryotic RCK domains, suggesting LZ1 plays an important additional role in mammalian BK channels (26–28). Because the LZ1 motif is highly conserved in vertebrates and is not disrupted by known sites of alternative splicing in mammals (6), LZ1 may thus contribute to both a flexible interface as well as a domain required for targeting of a PKAc signaling complex with the channel. Whether LZ1 is the actual interaction interface or is required to target the PKAc signaling complex to downstream regions within the 127-amino acid LZ1489–616 fusion protein (i.e. that would be predicted to form the majority of the variable flexible domain) remains to be elucidated. Importantly, LZ1-competing peptides or mutagenesis of the third and fourth d position LZ1 leucine residues to alanine had no significant effect on the half-maximal voltage required for activation of STREX or ZERO channels at free calcium concentrations of <1 μM used in this study. Recently, mutagenesis of methionine Met-540 to isoleucine, which lies within the last heptad repeat of the LZ1 motif, was shown to reduce murine BK channel calcium sensitivity at calcium concentrations of 10 μM and above (35). Because the BK channel RCK domain contributes to calcium sensitivity (35, 36), the M540I mutation may disrupt the LZ1 motif and modify intrinsic calcium bind-
izing sites within the RCK or other C-terminal region of the channel. Alternatively the M540I mutation may displace an extrinsic calcium sensor that interacts with the RCK in a LZ1-dependent manner.

Because the PKAc signaling complex can interact with a fusion protein encompassing the LZ1 domain but not incorporating residues within the core RCK domain essential for tetramerization (26–28), our data would suggest that each subunit or subunit dimer of the BK channel tetramer may interact with its own PKAc signaling complex. Furthermore, the interaction of LZ1 with the PKAc signaling complex is not promiscuous as PKAc failed to co-immunoprecipitate with fusion proteins spanning other putative LZ domains, including LZ2. Whether, other potential BK channel LZ motifs play an important role in BK channel targeting with other signaling proteins or channel assembly per se remains to be elucidated.

Acknowledgments—We are grateful to Dr. Roger Clegg (Hannah Research Institute) for the generous gift of a mouse BK channel clone containing a C-terminal HA epitope. We thank members of the Membrane Biology Group for useful discussions.

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J. Biol. Chem. 2003, 278:8669-8677.
doi: 10.1074/jbc.M211661200 originally published online December 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M211661200

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