Palmitoylation and Membrane Association of the Stress Axis Regulated Insert (STREX) Controls BK Channel Regulation by Protein Kinase C*§

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Background: Large-conductance potassium (BK) channels containing the stress axis regulated insert (STREX) are important regulators of cellular excitability.

Results: Membrane detachment of STREX by protein kinase A (PKA)-mediated phosphorylation or de-palmitoylation established channel inhibition by protein kinase C (PKC).

Conclusion: Membrane association of STREX regulates the access of PKC to its inhibitory phosphorylation site.

Significance: The interplay of phosphorylation by PKA, PKC, and palmitoylation determines BK-STREX channel activity.

Large-conductance, calcium- and voltage-gated potassium (BK) channels play an important role in cellular excitability by controlling membrane potential and calcium influx. The stress axis regulated exon (STREX) at splice site 2 inverts BK channel regulation by protein kinase A (PKA) from stimulatory to inhibitory. Here we show that palmitoylation of STREX controls BK channel regulation also by protein kinase C (PKC). In contrast to the 50% decrease of maximal channel activity by PKC in the insertless (ZERO) splice variant, STREX channels were completely resistant to PKC. STREX channel mutants in which Ser700, located between the two regulatory domains of K+-conductance (RCK) immediately downstream of the STREX insert, was replaced by the phosphomimetic amino acid glutamate (S700E) showed a ~50% decrease in maximal channel activity, whereas the S700A mutant retained its normal activity. BK channel inhibition by PKC, however, was effectively established when the palmitoylation-mediated membrane-anchor of the STREX insert was removed by either pharmacological inhibition of palmitoyl transferases or site-directed mutagenesis. These findings suggest that STREX confers a conformation on BK channels where PKC fails to phosphorylate and to inhibit channel activity. Importantly, PKA which inhibits channel activity by disassembling the STREX insert from the plasma membrane, allows PKC to further suppress the channel gating independent from voltage and calcium. Our results present an important example for the cross-talk between ion channel palmitoylation and phosphorylation in regulation of cellular excitability.

Large-conductance Ca2+- and voltage-activated potassium (BK) channels play an important role in the regulation of membrane excitability in cells of the neuronal, endocrine and vascular system. This is partly due to an unusually high single-channel conductance and the concerted activation by membrane depolarization and elevation of intracellular free calcium which enables BK channels to typically exert a negative feedback influence on cellular excitability by repolarizing cells and turning off voltage-gated Ca2+ channels. In some neuronal cells, however, BK channels are positive feedback regulators which by augmenting the afterhyperpolarization, facilitate recovery from inactivation of sodium channels and promote the discharge frequency of neurons (1). The importance of BK channels in regulation of vascular and non-vascular smooth muscle tone, determination of action potential duration and frequency, tuning of hearing frequencies in cochlear hair cells, the release of hormones and neurotransmitters has been impressively demonstrated in mice with targeted deletion of the pore-forming α-subunit of the BK channel (1–5).

Only one gene (KCNNMA1) codes for the pore-forming α-subunits of BK channels in all mammalian tissues (6, 7), but the functional properties of BK channels are exceptionally diverse, not only in different tissues but also in the same tissue or even in the same cell under different hormonal conditions (8–10). This complex phenotypic variation arises from many mechanisms encompassing transcriptional regulation (11),
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nuclear export of incompletely spliced intron-containing mRNAs (12), extensive alternative exon splicing of the α-subunit (7, 13–15), co-expression with different transmembrane modulatory β-subunits in a tissue-specific manner (16), and reversible protein phosphorylation (17). Several alternative splice sites and alternative exons have been identified in the KCNMA1 gene (18). The stress axis-regulated exon (STREX)

is by far the most well-characterized alternative exon in the KCNMA1 gene which encodes a 59-residue cysteine-rich insert at the C2 position of alternative splicing in the C terminus of mammalian BK channels (9). Intriguingly, both gonadal (sex) and adrenal (stress) steroids contribute to regulation of the BK channel properties as compared with the insertless ZERO splice variant (9). STREX enhances the Ca2+/voltage sensitivity, increases and decreases the rates of channel activation and deactivation, respectively, and by adding an additional phosphorylation site (Ser695), switches the channel from being activated by PKA to a state where BK channels are inhibited by PKA (22).

Recently, we have demonstrated that protein kinase C markedly inhibited the activity of the insertless bovine BK-ZERO channel isoform. This effect was due to phosphorylation of serine 695 (Ser695), which is one of several putative PKC-dependent phosphorylation sites, located between the two regulatory domains of K+ conductance (RCK) (23). We also found that phosphorylation of Ser695 was not only dependent on the preceding phosphorylation of Ser1151, another PKC consensus motif at the C-terminal end, but also prevented the stimulatory effects of PKG and PKA. Here we analyzed the effect of PKC on BK channels expressing the STREX insert which is located immediately upstream of the critical PKC-dependent phosphorylation site (Ser695 in BK-ZERO). Surprisingly, we found that BK channels containing the STREX insert were completely resistant to PKC due to the membrane association of the C terminus via palmitoylation of conserved cysteine residues within STREX. Thus, abolition of palmitoylation or disassembling STREX from the plasma membrane by PKA-mediated phosphorylation resulted in channels which were strongly inhibited by PKC. This so far unrecognized divergent regulation of ZERO- and STREX-BK channels by protein kinase C is likely of physiological importance for cellular excitability as the proportion of STREX-BK channels varies under hormonal influence.

EXPERIMENTAL PROCEDURES

Phorbol-12-myristate-13-acetate (PMA), 4α-phorbol-12-myristate-13-acetate (4α-PMA), protein kinase C catalytic fragment (PKC), protein kinase C 19–31 pseudosubstrate inhibitor (PKC19–31), protein kinase A catalytic subunit (PKA), were obtained from Biomol (Hamburg, Germany). 2-bromopalmitate was purchased from Sigma (Taufkirchen, Germany), iberiotoxin (IbTX) from Alomone Laboratories (Jerusalem, Israel), guanosine-3’,5’-cyclic monophosphate (cGMP) from BioLog (Bremen, Germany). Protein kinase G ια (PKG) was prepared as described (24) and was consistently used in the presence of 10 μM cGMP. Drugs were either dissolved in physiological saline solution (PSS; see solutions) or in dimethyl sulfoxide (DMSO). The maximum 0.1% final concentration of DMSO in the bath solution did not affect BK currents. Solutions with IbTX contained 0.1% bovine albumin fraction V (Sigma).

Cell Culture and Transfection Procedure—HEK293 cells were cultured in minimum essential medium supplemented with Earle’s salts medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum, 2 mM L-glutamine, 100 units ml−1 penicillin, 100 μg ml−1 streptomycin at 37 °C and 6% CO2. For transfection, 105 cells were plated in a 35-mm dish and cultured for another 24 h. Thereafter, the HEK293 cells were transiently co-transfected with EGFP (Clontech, Heidelberg, Germany), cloned into the pcDNA3 vector (Invitrogen, Karlsruhe, Germany), and the pcDNA3 plasmid containing the murine BK channel α subunit either with or without STREX exon (BK-STREX or BK-ZERO channel), or the respective BK mutant. Mouse BK-STREX used in this study is composed of the BK channel with the accession number L16912 (6) starting with MDALII and containing the STREX insert from the BK channel AF156674 (25) instead of IYF from L16912 at STREX splice site C2. The position of amino acids important for phosphorylation and palmitoylation in BK-STREX is as follows: The inhibitory (within the STREX insert) and the stimulatory PKA phosphorylation site is KMSQ941Y and QPS927IT, respectively, the palmitoylation site is RAC645C646FD (located within STREX), the PKC phosphorylation sites are TLS700PK and PKS1150RE, and the PKG phosphorylation site is KSS1139SV. Mouse BK-ZERO corresponds to the BK channel with the accession number L16912 (6) starting with MDALII but without the IYF insert from L16912 at the STREX splice site C2. Transfection of 1 μg of each cDNA was achieved by calcium phosphate precipitation for 18 h at 35 °C and 3% CO2. After washing, the cells were cultivated for another 24–48 h at 37 °C and 6% CO2. After the medium was exchanged several times with PSS (see below), the cells were transferred in a 35-mm dish to the stage of an inverted microscope (Zeiss Axiovert 200) for electrophysiological measurements. The transfection efficiency varied between 50 and 70% as judged by the expression of EGFP in transfected cells. Cells with similar intensity of EGFP fluorescence were used for the experiments.

GH3/B6 cells, a kind gift from Dr. C. Bauer (University Medical Center Hamburg-Eppendorf, Germany) were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham medium (Sigma) supplemented with 15% horse serum (Invitrogen), 2.5% fetal calf serum (Biochrom), and 2 mM L-glutamine (Biochrom). Cells were maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO2. The medium was changed every 2–3 days, and cells were passaged once a week. Patch clamp- or PCR studies were performed 3–5 days after passaging.

Site-directed Mutagenesis—Mutant channels were generated either by extended overlap polymerase chain reaction, or by a
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BACKGROUND—We have shown earlier that phosphorylation of Ser695 in the extracellular (pipette) surface of the patch was the same as the pipette solution in whole cell recordings. The free Ca\(^{2+}\) concentration was adjusted to 1 \(\mu M\). The storage buffers for purified protein kinases were: catalytic fragment of PKC (PKCc): 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 15 mM DTT, 10% glycerol, 20 mM Tris, pH 7.5; catalytic subunit of PKA (PKA\(_c\)): 50 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 50% glycerol, 20 mM Tris, pH 7.5; PKG I\(_a\): 10 mM TES, 1.25 mM DTT, 0.25 \(\mu\)g ml\(^{-1}\) leupeptin, 0.05% NaN\(_3\), 50% glycerol. When the effect of protein kinases was investigated in inside-out patches, appropriate amounts of storage buffers were diluted into the bath solution. To avoid significant effects of storage buffers on channel activity, patches were first superfused with storage buffer containing solutions alone until channel activity was stable (usually within 5 to 10 min), then superfusion with the same solution containing the respective protein kinase was started.

RESULTS—STREX Insert Impedes BK Channel Regulation by PKC—The PKC-dependent regulation of BK channels was investigated with the murine pore-forming BK \(\alpha\)-subunit (Kcnam1) containing the 59 amino acid STREX exon (22, 30). When inside-out membrane patches obtained from transfected HEK293 cells were superfused for at least 5 min at the cytosolic side with 20 nM of PKCc, no significant change of macroscopic currents was observed at all voltages tested (−120 to +100 mV; Fig. 1 A). This finding is in contrast to the distinct current decrease obtained with PKCc under otherwise identical experimental conditions from the insertless (ZERO) splice variant of the murine BK \(\alpha\)-subunit (Fig. 1B). As reported before the STREX variant exhibited an increased channel activity compared with ZERO (31). The half-maximal activating voltage (\(V_{1/2}\)) was 24.2 ± 2.1 mV and 46.2 ± 1.1 mV for STREX and ZERO, respectively. Although the application of 20 nM PKCc in the same patches did not significantly change \(V_{1/2}\) of the ZERO channels 46.7 ± 3.2 mV in the presence of PKCc, the macroscopic currents were decreased at all voltages tested. At +80 mV, PKCc reduced mean membrane conductance (\(G_m\)) from 21.9 ± 2.8 to 10.7 ± 1.5 nS, i.e. by 51.5 ± 4.9% (Fig. 1B). Cyclic nucleotide-dependent protein kinases showed the well-known stimulatory effect of PKG and inhibitory effect of PKA on BK-STREX channel currents. PKG (300 nM) enhanced BK currents and shifted the \(V_{1/2}\) to the left from 25.6 ± 1.9 (control) to 0.4 ± 4.1 mV (Fig. 1C). In contrast, PKA (300 nM) shifted the \(V_{1/2}\) to the right from 24.2 ± 2.1 (control) to 56.5 ± 5.3 mV (Fig. 1D).

We have shown earlier that phosphorylation of Ser\(^{695}\) in the ZERO splice variant of the bovine BK \(\alpha\)-subunit is responsible for the PKC-dependent channel inhibition (23). To investigate whether the STREX insert prevents PKC to phosphorylate Ser\(^{700}\), which corresponds to Ser\(^{695}\) in the bovine ZERO variant and is located immediately downstream of the STREX...
exon, we constructed mutants in which Ser\textsuperscript{700} was replaced by either the phosphomimetic amino acid glutamate (S700E) or by alanine (S700A). Introducing a negative charge at position 700 (S700E), resulted in channels with a strongly reduced membrane conductance compared with wild-type BK-STREX channels. At 20 mV, the mean membrane conductance was 6.5 ± 0.6 nS (\textit{Ctrl} in Fig. 2A), which is 43% of the conductance measured in the wild-type BK channel at the same potential (Wt: 15.0 ± 1.5 nS in Fig. 2A). No significant change in membrane conductance was observed by superfusion with 20 nM PKCc for 5 min (6.1 ± 0.7 nS). Interestingly, exposure of the intracellular face of the patch to 300 nM PKG, failed to influence \( G_m \) in patches carrying the S700E mutant channel, whereas PKAc decreased \( G_m \) from 6.1 ± 0.9 to 1.9 ± 0.5 nS (Fig. 2A). The basal \( G_m \) of STREX channels with the mutation of Ser\textsuperscript{700} to alanine, was similar to the wild type and not altered by PKCc (Fig. 2B). PKG enhanced the mean \( G_m \) at 20 mV from 18.6 ± 2.6 to 29.6 ± 2.7 nS, whereas PKAc decreased the mean membrane conductance from 16.7 ± 1.2 to 7.1 ± 1.4 nS. Altogether, these findings suggest that the STREX insert prevents BK channel phosphorylation at position Ser\textsuperscript{700} by PKC, and thus, in contrast to the insertless ZERO splice variant, no decrease in membrane conductance can occur. A phosphomimetic negative charge in Ser\textsuperscript{700}, however, reduces the channel conductance to a similar extent as PKC phosphorylation does in the ZERO variant and abolishes the enhancing effect of PKG on channel activity. The inhibitory effect of PKA, mediated via phosphorylation of Ser\textsuperscript{636} within the STREX insert, is apparently independent from the charge at the Ser\textsuperscript{700} position.

**Prevention of BK Channel Inhibition by PKC Depends on Palmitoylation of STREX**—To examine whether palmitoylation, and thus association of the STREX domain with the plasma membrane (31), prevents PKC from inhibiting BK channel gating, we abolished STREX palmitoylation and membrane localization by 1) pharmacological inhibition of palmitoyl transferases, 2) site-directed mutagenesis of palmitoylation sites, and 3) detachment of STREX from the cell membrane by PKA phosphorylation. In accordance with published data on the role of STREX palmitoylation (31) pre-treatment of transfected HEK293 cells with the palmitoyl transferase inhibitor 2-bromopalmitate (100 \( \mu \)M 2-BP) for 24 h, resulted in the expression of BK-STREX channels with reduced activity (supplemental Fig. S1). Most important, these channels were sensi-
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FIGURE 3. Prevention of STREX palmitoylation established PKC-dependent channel inhibition. Bars represent conductances at +40 mV from inside-out membrane patches obtained from HEK293 cells expressing STREX (A), the S700A- (B) or the C645A/C646A (C) mutant channel. A and B, HEK293 cells were treated for 24 h with 100 µM 2-BP before patches were obtained. A, inhibition of palmitoylation by 2-BP established PKC-dependent inhibition of STREX and abolished PKA-dependent inhibition. The enhancing effect of PKG was inhibited in the presence of PKC. B, in the S700A STREX mutant treated with 2-BP, PKC failed to inhibit $G_m$, in the palmitoylation-deficient double mutant C645A/C646A PKC inhibited $G_m$, and the inhibitory effect of PKA was abolished. As in A, the enhancing effect of PKG was blocked in the presence of PKC. The upper parts of A–C show representative macroscopic current recordings with the interventions as indicated. Bars in the lower parts represent means ± S.E. The numbers within bars represent the number of membrane patches. The intracellular (bath) Ca²⁺ concentration was 1 μM. **, $p<0.01$; ns, not significant.

It has been demonstrated that the palmitoylation of cysteine Cys⁶⁴⁵ and Cys⁶⁴⁶ within the STREX insert is required for membrane attachment of the STREX C terminus at the plasma membrane (31). We therefore studied the STREX palmitoylation-deficient double mutant C⁶⁴⁵A/C⁶⁴⁶A in inside-out membrane patches from transfected HEK293 cells (Fig. 3C). This mutant showed similar characteristics as the wild type STREX channel treated with 2-BP. At 40 mV, 20 nM PKC, decreased $G_m$ by 57.0 ± 6.9% and additional application of PKG had no further effect. In the absence of PKC, PKG enhanced $G_m$ from 15.8 ± 3.0 (control) to 25.7 ± 4.4 nS, whereas PKA had lost its inhibitory action (15.5 ± 1.8 nS). Original current records from the C645A/C646A double mutant are also shown in Fig. 3C. To investigate whether BK channels become sensitive toward PKC when the STREX domain is detached from the plasma membrane by PCA-mediated phosphorylation, we superfused inside-out patches with 300 nM PKA first. When the decrease of $G_m$ was stable for at least 1 min, the additional application of 20 nM PKC, induced a further decline of current amplitude (Fig. 4A). At 60 mV, PKC reduced $G_m$ from 16.9 ± 1.4 (PKA) to 9.2 ± 1.0 nS (PKA plus PKC). Additional application of 5 μM of the PKC pseudosubstrate inhibitor peptide PKC₁₉₋₃₁ reversed the PKC effect completely. When we applied PKG in the presence of PKA plus PKC, (Fig. 4B), no change in channel activity was observed. In conclusion, the inhibitory effect of PKC via phosphorylation of Ser⁷⁰⁰ requires that the STREX insert is not membrane associated via palmitoylation.

PKC-dependent Inhibition in Cells Co-expressing STREX and ZERO BK Channels—Many native cells express both, the ZERO and the STREX splice variant of the BK channel. Moreover, as the BK channel tetramerization domain is conserved in splice-variant α-subunits (32), heterotetramerization of ZERO and STREX α-subunits may occur in these cells with an unpredictable response to PKC. To investigate the PKC-dependent channel gating in cells co-expressing ZERO and STREX α-subunits, we used a cloned rat somatomammotrope pituitary cell line (GH3/B6) endogenously expressing both variants and HEK293 cells co-transfected with both isoforms. In GH3/B6 cells, PCR products without (ZERO) and with the 174 bp STREX exon could be detected (Fig. 5A). When we elicited whole-cell outward currents ($I_{out}$) in GH3/B6 cells, the PKC activator phorbol 12-myristate 13 acetate (PMA, 100 nM) decreased current densities at a potential of +80 mV from 227.6 ± 18.1 (control) to...
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FIGURE 4. Removal of STREX membrane localization by PKA re-established PKC-dependent channel inhibition. A and B, conductance-voltage relationships obtained from inside-out membrane patches of HEK293 cells expressing the STREX BK channels. Control patches (Ctr) were first superfused with 300 nm PKA, and when the decrease of \( G_m \) was stable for at least 1 min, 20 \( \mu \)M PKC, (PKC) was additionally applied. The further decline of \( G_m \) induced by PKC was completely reversed by the PKC pseudosubstrate inhibitor peptide \( \text{PKC}_{19-31} \) (5 \( \mu \)M; A), or remained unchanged when the patch was additionally superfused with 300 nm PKG (B). Means \( \pm \) S.E. when exceeding the size of the symbol are shown. n, number of patches. The intracellular (bath) Ca\(^{2+}\) concentration was 1 \( \mu \)M.

\[ 160.8 \pm 17.5 \text{ pA pF}^{-1}, \text{i.e. by} 35.3 \pm 5.8\% \]

The inactive phorbol ester analog 4\( \alpha \)-PMA (100 nm) had no significant effect on \( I_{\text{out}} \) (203.0 \( \pm \) 16.3 pA pF\(^{-1}\); Fig. 5B). Addition of the specific BK channel-blocking peptide ibiotoxin (IbTX, 300 nm) revealed that under our experimental conditions more than 90% of \( I_{\text{out}} \) was conducted by BK channels. To inhibit plasma membrane attachment of the STREX domain via palmitoylation, we treated GH3/B6 cells for 24 h with 100 \( \mu \)M 2-BP. In these cells, 100 nm PMA reduced \( I_{\text{out}} \) from 171.4 \( \pm \) 22.3 (control) to 82.7 \( \pm \) 13.6 pA pF\(^{-1}\) which is a significantly larger decrease (by 48.0 \( \pm \) 2.8%; \( p < 0.05 \)) than that in cells not treated with 2-BP (compare Fig. 5, B and C). Again, 4\( \alpha \)-PMA was ineffective, and 300 nm IbTX reduced \( I_{\text{out}} \) to less than 10% of the control current. In a second series of experiments, we co-transfected HEK293 cells with the same amount of plasmid DNA encoding for the ZERO and the STREX variant of the BK channel, respectively. To examine the PKC effect in these cells, inside-out patches were obtained and the cytosolic side was superfused with 20 nm PKC. The inhibitory effect of PKC, could be detected at potentials from -120 to +100 mV. At 80 mV, PKC decreased \( G_m \) from 35.1 \( \pm \) 2.6 to 25.4 \( \pm \) 2.1 nS, \text{i.e. by} 25.3 \( \pm \) 4.8% (Fig. 5D). As observed in the GH3/B6 cells, the prevention of the membrane-anchoring of the STREX domain by 2-BP treatment, resulted in an enhanced sensitivity of the BK channels toward PKC. At +80 mV, 20 nm PKC decreased \( G_m \) from 38.3 \( \pm \) 3.5 to 19.1 \( \pm \) 1.9 nS, \text{i.e. by} 49.3 \( \pm \) 2.2% (Fig. 5E). This decrease is significantly \( (p < 0.01) \) larger than the reduction induced by PKC in the absence of 2-BP (compare Fig. 5, D and E). As the dependence of the observed inhibitory effect of PKC on 2-BP treatment in HEK293 cells largely resembles the situation in GH3/B6 cells, the data demonstrate that both BK channel isoforms co-expressed in GH3/B6 cells are incorporated into the cell membrane and are functionally active.

Ser\(^{1156}\) as the Master Switch for STREX BK Channel Regulation by Protein Kinases—Phosphorylation and dephosphorylation of Ser\(^{1156}\), located close to the C-terminal end of the bovine ZERO channel, plays a key role in the complex regulation of BK channels by PKC, PKG, and PKA (23). To examine the role of Ser\(^{1156}\) in STREX channels which corresponds to Ser\(^{1151}\) in the bovine ZERO isoform, we constructed mutants in which Ser\(^{1156}\) was replaced either by the phosphomimetic amino acid aspartate (S1156D) or by alanine (S1156A). In inside-out patches from HEK293 cells expressing the S1156D mutant, we found a channel-behavior identical to that of the non-mutated STREX channel shown in Fig. 1. Application of 20 nm PKC, to the intracellular face of the patch was ineffective with respect to \( G_m \) (Fig. 6A), whereas PKG enhanced (by 117.0 \( \pm \) 35% at 20 mV), and PKA inhibited \( G_m \) (by 68.8 \( \pm \) 11.5%). In the S1156A mutant, neither PKC nor PKG was effective, whereas PKA retained its inhibitory action on \( G_m \) (decrease by 61.6 \( \pm \) 8.1%; Fig. 6B). 2-BP treatment (100 \( \mu \)M, 24 h), resulted in STREX S\(^{1156}\)-D mutant channels in which superfusion of inside-out patches with PKC, reduced \( G_m \), by 52.1 \( \pm \) 2.5%. PKG enhanced \( G_m \) by 73.7 \( \pm \) 9.6% and PKA was ineffective (Fig. 6C). In the STREX S\(^{1156}\)-A mutant treated with 2-BP, BK channel conductance remained unchanged in the presence of PKC, and PKG, but PKA, surprisingly, enhanced \( G_m \) by 101.0 \( \pm \) 37%; Fig. 6D). Taken together, the data indicate that phosphorylation of Ser\(^{1156}\) of STREX BK channels is the precondition for PKG to enhance and for PKC to inhibit the activity of the membrane-detached channel. The inhibitory effect of PKA is independent from Ser\(^{1156}\), but depends on the attachment of the STREX domain to the cell membrane. When the STREX insert is detached from the plasma membrane, PKA enhances channel activity if Ser\(^{1156}\) is dephosphorylated, a regulation which has been described before for the bovine ZERO variant not phosphorylated at Ser\(^{1151}\) (23).

DISCUSSION

In the present study we examined the PKC-dependent regulation of BK channels from mouse brain expressing the 59-amino acid insert at splice site 2 (STREX) of the C-terminal domain of the channel pore-forming \( \alpha \) subunit (22, 30). The STREX exon is widely expressed in excitable cells with a predominant distribution in neuroendocrine cells (9, 25), including pancreatic islets and adrenal chromaffin cells (15, 33) and it confers a higher \( \text{Ca}^{2+} /\text{voltage} \) sensitivity on the channel compared with the ZERO splice variant which contains no inserts at the alternative splice sites (9, 13, 25). This well-known functional difference between the two channel isoforms is illustrated by the respective control current-voltage-relationship shown in Fig. 1, A and B. In addition, STREX speeds and slows rates of channel activation and deactivation, respectively, effects which may have profound effects on the intrinsic firing properties of excitable cells (9, 15, 34). Phorbol esters as activators of PKC have been shown to inhibit BK channel activity in arterial- (35, 36) and tracheal smooth muscle cells (23) that mainly express the BK channel ZERO isoform. In inside-out patches from HEK293 cells transiently transfected with the pore-forming BK channel \( \alpha \)-subunit cloned from bovine trachea, it was shown that PKC decreased BK channel open probability, \( N_P /\alpha \), by shortening channel open time and prolonging the closed state of the channel (23). PKC neither affected single-channel conductance, nor the voltage dependence or the calcium sensitivity of the channel. Surprisingly, we found that the
catalytically active fragment of PKC, when applied directly to the intracellular side of inside-out patches expressing the STREX channel, was completely ineffective (Fig. 7, A and B).

The STREX insert contains an additional PKA consensus motif located at Ser636 in the mouse channel (Fig. 7 C). Phosphorylation of this serine has been shown to cause inhibition of channel activity by producing a \( \sim 20 \) mV rightward shift of the voltage-dependent activation curve (Fig. 1 D; Ref. 22). In contrast, PKC reduced the maximal BK channel activity in the mouse ZERO mutant without affecting the apparent voltage sensitivity (Fig. 1 B). These findings indicate that both PKA and PKC are principally able to inhibit BK channels but by different mechanisms. It has been shown before that inhibition of the bovine ZERO-BK channel by PKC depends on phosphorylation of Ser695 located within the linker between the two regulators of K conductance RCK1 and RCK2 (23). Ser695 corresponds to Ser700 in the STREX isoform and when we replaced Ser700 by glutamate (S700E) to mimic the negative charge induced by PKC-dependent phosphorylation, \( G_m \) was markedly reduced, resembling the effect of PKC in the ZERO channel. Obviously, the inhibitory effect of the negative charge at the Glu700 position is not dependent on membrane attachment of the STREX insert as it similarly occurred in cells treated with and without 2-BP. Furthermore the S700E mutant did not respond to either PKC or PKG (Figs. 2 A and 7 D). PKG usually increases BK channel activity not only in the ZERO (26) but also in the STREX...
Palmitoylation Prevents STREX-BK Channel Regulation by PKC

FIGURE 6. Phosphorylation of serine 1156 determines the sensitivity of STREX channels to protein kinases. A–D, bars represent conductances at +20 mV from inside-out membrane patches obtained from HEK293 cells expressing either the phosphomimetic S1156D (A and C) or the phospho-resistant S1156A STREX mutant channels (B and D). Cells in A and C were treated with the palmitoyl transpherase inhibitor 2-BP (100 μM). Phosphorylation of Ser1156 is a precondition for PKG-dependent STREX activation (A and B) and for PKC-dependent STREX inhibition which, however, occurs only when STREX is detached from the plasma membrane (+2-BP in C and D). The PKA-induced inhibition of STREX is independent of Ser1156 (A and B) but relies on the membrane-attached STREX insert (C). In the S1156A mutant that mimics the dephosphorylated channel at this position, PKA enhances Gm when STREX is detached from the membrane (+2-BP in D). PKC was applied at 20 mM and PKG and PKA, at 300 mM, respectively. Means ± S.E. are shown. Numbers within bars represent the number of membrane patches. The intracellular (bath) Ca2+ concentration was 1 μM, *p < 0.05, **p < 0.01, ***p < 0.001.

isoform (Fig. 1C) by phosphorylation of a serine residue within an optimal consensus sequence close to the C-terminal end of the BK channel (Ser1139 in STREX; (26, 37)). This stimulatory effect was abolished when Ser695 in bovine ZERO was either phosphorylated by PKC or replaced by a phosphomimetic amino acid (23). Since PKC treatment did not inhibit the STREX channel (Figs. 1A and 7B), our findings in the S700E mutant strongly indicate that the STREX insert induces a conformation of the C terminus of the BK channel in which the access of PKC to Ser700 is hindered. This interpretation is further supported by the data in which we interfered with the membrane attachment of the STREX insert. A palmitoylated cysteine-rich domain (CRD) (15) targets the large intracellular C terminus of the STREX channel to the plasma membrane (31). After treatment with the largely irreversible palmitoyl transferase inhibitor 2-BP (38) or mutation of the two critical cysteines for palmitoylation to alanine (C645A/C646A; Ref. 31), the STREX channels became sensitive to PKC, i.e. PKC markedly decreased Gm at all potentials. In addition, PKA lost its inhibitory action and PKG failed to enhance channel activity in the presence of PKC (Figs. 3 and 7D). As PKC did not inhibit Gm in the S700A mutant treated with 2-BP (Fig. 3B), Ser700 would appear to be accessible to phosphorylation by PKC only when the anchoring of the C terminus in the cell membrane was prevented. Interestingly, palmitoylation impairs PKC-dependent phosphorylation not only in STREX channels but also in some ligand-gated ion channels. A palmitoylation resistant mutant of the GluR6 subunit of the kainate receptor was a better substrate for PKC-dependent phosphorylation than the wild-type GluR6 receptor (39). Depalmitoylation of a specific cysteine residue of the GluR1 subunit of the AMPA receptor facilitated phosphorylation by PKC, which in turn enhanced the interaction with binding partners promoting the GluR1 insertion in the plasma membrane (40). All these C-terminal PKC consensus sites are near the palmitoylation sites and therefore it is attractive to speculate that palmitoylation, determining the accessibility of PKC to its target sites, is a mechanism to regulate these ion channels. Protein palmitoylation is a reversible process depending on a variety of protein palmitoyl transferases and thioesterases (41, 42). Thus it is likely that both palmitoylated and depalmitoylated BK channels may exist in the same cell. With regard to the STREX channel the complete resistance to PKC in our electrophysiological assay, however, indicates that the membrane-attached C terminus is the predominant form. Therefore, the question arises, whether a physiological mechanism exists that disassembles the C terminus of STREX channels from the membrane to allow PKC-induced inhibition. PKA-dependent phosphorylation of Ser1156 immediately upstream of the conserved palmitoylated cysteine residues within the STREX insert dissociates the C terminus from the plasma membrane and thereby inhibits the channel activity (31). When we used PKAc to remove the C terminus from the membrane, we found that the additional application of PKCc further reduced the channel activity, which contrary to PKA resulted in a decrease of maximal channel gating that was insurmountable by voltage or [Ca2+]i (Fig. 4 and Fig. 7D). Such an effect could be of physiological relevance in particular in endocrine cells of the anterior pituitary gland that express the STREX variant such as somatomamotropes and corticotrrophs. In both these cell types cellular excitability and hormone secretion is regulated by neuropeptides that activate both the PKA- and PKC-dependent signaling cascades resulting in inhibition of endogenous BK channels (43–45). When we investigated cells which co-express STREX and ZERO BK channels such as GH3/B6 cells, a clonal rat somatomamotrope pituitary cell line, we found that the maximal PKC-dependent channel inhibition was significantly amplified in cells treated with 2-BP. In HEK293 cells co-transfected with equal amounts of ZERO- and STREX cDNA, the maximal PKC-dependent channel inhibition was only half compared with cells exclusively expressing the ZERO variant. These results indicate that co-expressed ZERO and STREX channels are incorporated in the plasma membrane and are functionally active. The sensitivity to PKC can be regulated in these cells by mechanisms that increase or decrease the expression of either channel isoform or lead to the detachment of the STREX C terminus from the plasma membrane such as PKA-mediated phosphorylation or depalmitoylation.

In analogy to ZERO BK channels (23), we found that besides Ser700 a second PKC-dependent phosphorylation site, Ser1156
at the C-terminal end, was critical for STREX channel regulation by PKC, PKG, and PKA (see Fig. 7). When we introduced a negative charge by replacing Ser<sup>1156</sup> by aspartate (S1156D) to mimic PKC-dependent phosphorylation at this site, PKCc was ineffective as in the wild type channel, whereas after detachment of the C terminus from the membrane by prior inhibition of palmitoyl transferases, STREX was effectively inhibited by PKCc. This inhibitory effect was lost in the S1156A mutant, indicating that phosphorylation of Ser<sup>700</sup> is conditional, depending on the preceding phosphorylation of Ser<sup>1156</sup> which is the default setting in our electrophysiological assay. Activation of STREX channels by PKG was also dependent on phosphorylation of Ser<sup>1156</sup> and was not dependent on the membrane association of the STREX domain. As outlined above, the inhibitory effect of PKA was dependent on the association of the STREX domain with the plasma membrane but independent of Ser<sup>1156</sup>. Interestingly, when Ser<sup>1156</sup> was dephosphorylated as in the mutant S1156A and the STREX C terminus dissociated from the cell membrane by 2-BP, PKA<sub>c</sub> enhanced BK channel activity (Fig. 7E). This activating PKA effect is dependent on phosphorylation of Ser<sup>927</sup> which is a second PKA-dependent phosphorylation site located in the C terminus downstream of the STREX insert (22, 26).

In summary, we show in this study that STREX BK channels are insensitive to PKC due to the membrane attachment of its C terminus to the plasma membrane which in turn most likely poses a steric hindrance for PKC to phosphorylate the critical Ser<sup>700</sup>. Abolition of STREX palmitoylation or membrane localization resulted in STREX channels that were inhibited by PKC similar to the insertless ZERO channel. Disassembling the C terminus from the cell membrane via PKA-mediated phosphorylation enables PKC to further inhibit BK channel activity suspending the negative feedback regulation on voltage-dependent Ca<sup>2+</sup> influx. Such an effect may be important in neuroendocrine cells where PKA- and PKC-dependent regulations of BK channel activity synergize in hormone secretion. As highlighted in Fig. 7, the activity of BK channels can thus obviously be modulated by a concert of regulatory mechanisms including palmitoylation and phosphorylation at various sites. In individual cells and specialized tissues this complex regula-
tion offers adaptive mechanisms to achieve fine tuning of cellular excitability.

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