Distinct stoichiometry of BK_{Ca} channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase

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Large conductance voltage- and calcium-activated potassium (BK_{Ca}) channels are important signaling molecules that are regulated by multiple protein kinases and protein phosphatases at multiple sites. The pore-forming α-subunits, derived from a single gene that undergoes extensive alternative pre-mRNA splicing, assemble as tetramers. Although consensus phosphorylation sites have been identified within the C-terminal domain of α-subunits, it is not known whether phosphorylation of all or single α-subunits within the tetramer is required for functional regulation of the channel. Here, we have exploited a strategy to study single-ion channels in which both the α-subunit splice-variant composition is defined and the number of consensus phosphorylation sites available within each tetramer is known. We have used this approach to demonstrate that cAMP-dependent protein kinase (PKA) phosphorylation of the conserved C-terminal PKA consensus site (S899) in all four α-subunits is required for channel activation. In contrast, inhibition of BK_{Ca} channel activity requires phosphorylation of only a single α-subunit at a splice insert (STREX)-specific PKA consensus site ($S4_{STREX}$). Thus, distinct modes of BK_{Ca} channel regulation by PKA phosphorylation exist: an ''all-or-nothing'' rule for activation and a ''single-subunit'' rule for inhibition. This essentially digital regulation has important implications for the combinatorial and conditional regulation of BK_{Ca} channels by reversible protein phosphorylation.

Large conductance voltage- and calcium-activated potassium (BK_{Ca}) channels are important regulators of cellular function in the endocrine, nervous, cardiovascular, and immune systems (1–6). BK_{Ca} channels are assembled as tetramers (7, 8) of pore-forming α-subunits encoded by a single gene (9) that undergoes extensive alternative splicing (10, 11). Distinct α-subunit splice-variant mRNAs may be expressed in the same cell, differentially expressed between tissues, or even neighboring cells (12, 13), and dynamic modification of splice-variant mRNA expression (14, 15) may result in altered BK_{Ca} channel phenotype and cellular regulation (5).

Similar to other tetrameric potassium channels BK_{Ca} channels are potently regulated by a variety of serine/threonine protein kinases (16), including cAMP-dependent protein kinase (PKA) (10, 17–20). The functional response of BK_{Ca} channels to PKA phosphorylation depends on the splice-variant α-subunit composition of the tetramer (10, 19). For example, PKA activates homotetramers of mammalian ZERO splice variants (10, 17, 19), whereas PKA inhibits homotetramers of STREX variants (10). This differential regulation of BK_{Ca} channels by PKA depends on functional consensus of PKA phosphorylation sites within the C terminus of the α-subunit. PKA activation of ZERO variants requires a functional conserved C-terminal PKA site (S899) (10, 17, 19), whereas PKA inhibition of STREX requires a functional PKA site ($S4_{STREX}$) within the STREX insert (10).

However, it is unknown how many α-subunits within the BK_{Ca} channel tetramer must be phosphorylated for functional regulation. An important step toward understanding how phosphorylation controls BK_{Ca} channel activity would be to determine the contribution of phosphorylating individual or multiple α-subunits within the tetramer. For example, do all four S899 sites within ZERO tetramers have to be phosphorylated for activation by PKA? Is phosphorylation of a single $S4_{STREX}$ site in STREX tetramers sufficient for PKA inhibition? Moreover, as the BK_{Ca} channel tetramerization domain is conserved between splice variants (8), does heterotetramerization of splice-variant α-subunits, such as ZERO and STREX, result in channels with a null or ''smoothed'' response to PKA phosphorylation or do mechanisms exist to allow either the activation or inhibition phenotype in response to PKA phosphorylation to be retained?

To address these fundamental issues we have exploited a strategy to determine the rules underlying phosphorylation of defined consensus sites across multiple α-subunits within BK_{Ca} channel tetramers in which the α-subunit stoichiometry is known *in situ*. By combining site-directed mutagenesis of predicted PKA consensus motifs of individual BK_{Ca} channel α-subunits with mutation (Y334V) of the external mouth of the channel pore, to modify the extracellular tetraethylammonium (TEA_o) sensitivity of assembled tetramers, we have been able to examine the PKA-dependent regulation of BK_{Ca} channel tetramers in which the stoichiometry of the consensus PKA sites within the tetramer is known. Using this strategy, we demonstrate that phosphorylation of S899 by PKA in all four α-subunits of the BK_{Ca} channel is required to increase channel activity, but that PKA phosphorylation within an alternatively spliced insert (STREX) of a single α-subunit is sufficient for channel inhibition. The structural basis of this effectively digital regulation and its physiological consequences for cellular excitability are highlighted.

Materials and Methods

Channel Constructs and Expression. Cloned murine BK_{Ca} channel ZERO and STREX α-subunit splice variants with a C-terminal hemagglutinin epitope (−HA) tag in pcDNA3 (21) were transiently transfected into HEK293 cells by using Lipofectamine 2000 (Invitrogen) essentially as described (10, 21). The C-terminal −V5-epitope-tagged STREX channels were constructed in the pcDNA3.1-V5-His TOPO vector (Invitrogen). The PKA consensus phosphorylation site mutants and TEA pore mutants (Y334V) were created by QuikChange II site-directed mutagenesis kits (Stratagene), and mutation and sequence integrity were confirmed by sequencing. Coimmunoprecipitation of full-length epitope-

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Abbreviations: BK_{Ca}, large conductance voltage- and calcium-activated potassium; TEA_o, extracellular tetraethylammonium; IP, immunoprecipitate.

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tagged BK_{Ca} channels from HEK293 cells and Western blotting was performed essentially as described (21).

***In Vitro* PKA Phosphorylation Assays.** Immunoprecipitates (IPs) of −HA-tagged channels transiently expressed in HEK293 cells were subject to PKA-phosphorylation assay. In brief, IPs were incubated with 1 mM cold ATP, 10 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP (Amersham Pharmacia), and 40 units of PKA catalytic subunit (PKAc; Promega) in 2 mM $MgCl_2$/4 mM Tris·HCl (pH 7.4) in a 30-μl reaction. Additionally, 100 nM protein phosphatase inhibitor, okadaic acid, was added to inhibit endogenous serine/threonine protein phosphatase activity. Negative control reactions were performed either in the absence of PKAc or by pretreating PKAc with 10 μM H-89 10 min before and during the reaction; kemptide was used as a positive control for PKAc activity (data not shown). Reactions were incubated (30°C for 10 min), then quenched by being placed on ice and the addition of an equal volume of 2× gel-loading buffer (lithium dodecyl sulfate) before analysis by autoradiography.

Electrophysiology. Excised inside-out or outside-out patch-clamp recordings were performed at room temperature (22°C) with patches allowed to stabilize for at least 5–10 min before recording. All experiments were performed in equimolar potassium gradients with the intracellular face of the channel exposed to a solution containing 140 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM Hepes, 30 mM glucose, 1 mM ATP, and 1 or 5 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (pH 7.3) with free calcium buffered to 0.2 μM. The external face of the channel was exposed to a solution containing 140 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM Hepes, 30 mM glucose, and 0.1 mM $CaCl_2$ (pH 7.4). Patch pipette fabrication and solution application was performed as described (10, 21). Data were acquired on an Axopatch 200B patch-clamp amplifier, low-pass-filtered at 2 kHz, and digitized at 10 kHz by using pCLAMP 6 software (Axon Instruments, Foster City, CA). For patches containing single channels or fewer than three channels of the same α-subunit composition, open probability (P_o) was determined as described (10, 21) by using pCLAMP software or custom algorithms with IGOR PRO (WaveMetrics, Lake Oswego, OR). In some patches that contained more than one channel type, P_o was determined from Gaussian fits to the amplitude histograms.

Results

PKA Activation of ZERO Homotetramers Requires Phosphorylation of S899 in Each α-Subunit of the Tetramer. To address the rules that govern the outcome of multisubunit phosphorylation of BK_{Ca} channel tetramers by PKA, we studied single-ion channels comprising mixtures of BK_{Ca} channel splice variants with intact and mutated PKA consensus sites within the C terminus of each α-subunit (Fig. 1*A*). BK_{Ca} channels have an extracellular N terminus and an intracellular C terminus, thus, functional concatemers cannot be generated (22). The subunit stoichiometry of tetrameric channels was thus determined from the single-channel amplitude by introducing a point mutation Y334V in the subunit pore of one splice variant that reduces the sensitivity to TEA_o (Fig. 1*B*) (7, 23). In the symmetrical potassium gradients used, the IC_{50} for external blockade of wild-type ZERO BK_{Ca} channel homotetramers, by TEA_o was 0.11 ± 0.01 mM (n = 5), and for Y334V homotetramers, it was 80.5 ± 12.2 mM (n = 5). Thus, wild-type channels are completely blocked by 2 mM TEA_o whereas homotetramers of channels containing the TEA pore mutation (Y334V) are largely insensitive to 2 mM TEA_o (<10% inhibition of single-channel amplitude in the absence of TEA determined at +40 mV). The stoichiometry of mixtures of TEA-sensitive and TEA-insensitive α-subunits could thus be predicted from the single-channel amplitude in the presence of 2 mM TEA_o (Fig. 1 *C* and *D*) as described (7, 23).

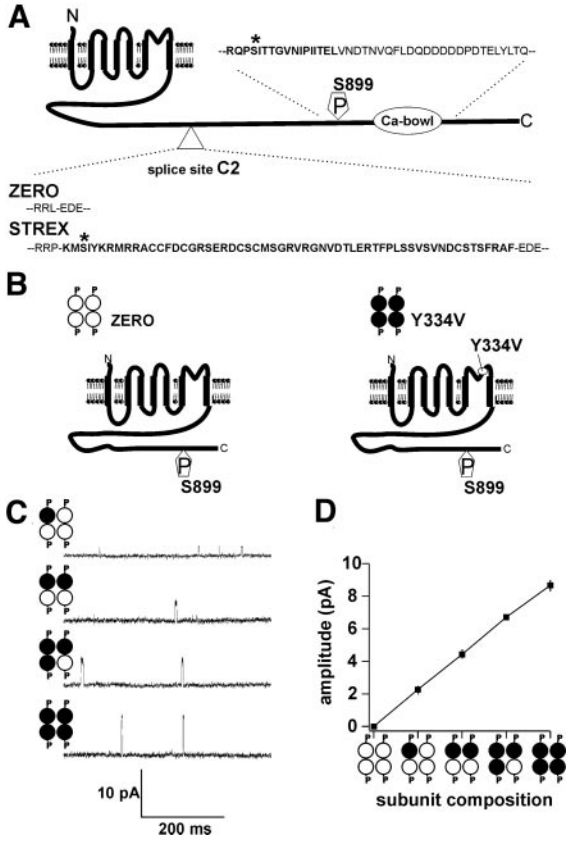

Fig. 1. Determining α-subunit composition of single BK_{Ca} channel tetramers through the TEA_o sensitivity of channels formed from mixtures of wild-type and TEA_o-binding site mutant α-subunits. (*A*) Schematic of alternative site of splicing C2, the conserved PKA consensus phosphorylation site (S899), and the calcium bowl in the C terminus of the BK_{Ca} channel α-subunit. The STREX α-subunit is identical with ZERO apart from an additional 59 aa at C2. The serine residue of the STREX-insert-specific ($S4_{STREX}$) and conserved (S899) PKA consensus motifs is indicated by ⋆. (*B*) Schematic of ZERO α-subunits (open circles) and the TEA_o-insensitive mutant (Y334V, filled circles). Representative single-channel traces (*C*) and summary plot of single-channel amplitude (*D*) of BK_{Ca} channels in the presence of 2 mM TEA_o, assembled as heterotetramers of ZERO and Y334V α-subunits. Recordings were at +40 mV in equimolar potassium gradients in the presence of 1 mM ATP and 0.2 μM free Ca^{2+} at the intracellular face of the patch and 2 mM TEA_o. Data are means ± SEM, n = 5–10 patches per group. Unless otherwise shown, error bars are within the symbol.

Previous site-directed mutagenesis studies have revealed a role for S899 (Figs. 1*A* and 2*A*) in PKA regulation of mammalian ZERO homotetramers (10, 17, 19), and *in vitro* phosphorylation studies demonstrate that the equivalent site in *Drosophila* (S942) is phosphorylated by PKA (20). To confirm biochemically that S899 is required for phosphorylation of full-length murine ZERO BK_{Ca} channels in HEK293 cells, homotetramers of ZERO channels immunoprecipitated from HEK293 cells were subjected to *in vitro* PKA phosphorylation (Fig. 2*B*). Full-length ZERO channels were phosphorylated by PKA *in vitro*, and this phosphorylation was completely abolished in the S899A mutant.

To investigate the number of α-subunits within the tetramer that must be phosphorylated by PKA for activation of homotetrameric ZERO channels, depending on the conserved S899 PKA consensus motif, we examined channels formed from mixtures of ZERO α-subunits containing the TEA_o mutation (Y334V, which retains the functional S899 PKA consensus site) coexpressed with TEA-sensitive ZERO subunits in which the S899 site has been mutated to alanine (S899A, Fig. 2*A*). The TEA_o sensitivity of S899A homotetramers was not significantly differ-

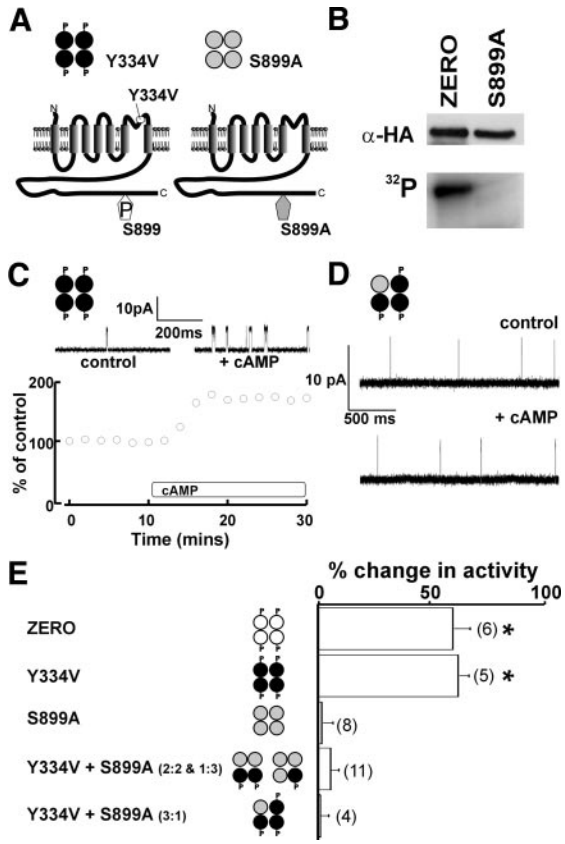

Fig. 2. PKA activation of ZERO homotetrameric BK_{Ca} channels requires phosphorylation of a C-terminal PKA consensus motif (S899) on all four α-subunits of the tetramer. (*A*) Schematic of TEA_o-insensitive Y334V α-subunits (filled circles) and the ZERO PKA consensus site mutant S899A (gray circles). (*B*) *In vitro* phosphorylation of homotetrameric ZERO BK_{Ca} channels and the S899A mutant. IPs were subjected to *in vitro* PKA phosphorylation in the presence of 100 nM okadaic acid and phosphorylation determined by ^{32}P autoradiography. Data are representative of three experiments. (*C*) Representative time-course plot of cAMP application to the intracellular face of patches containing homotetrameric Y334V α-subunits demonstrating channel activation by endogenous PKA. (*Inset*) Single-channel traces before (control) and after (+cAMP) application of cAMP. Recording conditions were as in Fig. 1*C*. The average P_o under control conditions was 0.06. (*D*) Representative single-channel records before (control) and after (+cAMP) application of cAMP to a patch containing a channel with a predicted 3:1 stoichiometry of Y334V to S899A α-subunits. (*E*) Summary of effect of cAMP on channel activity by using the various ZERO α-subunits expressed as a percentage change in activity from control. Pictograms illustrate predicted channel stoichiometry. Data from Y334V + S899A channels with (2:2) and (1:3) α-subunit stoichiometry are pooled. Data are means ± SEM. Number of patches is shown in parentheses. *, $P < 0.01$, ANOVA.

ent from ZERO channels (not shown). Activation of endogenous PKA in isolated inside-out patches with cAMP resulted in a robust activation of homotetrameric Y334V channels to an extent similar to that of wild-type ZERO channels (mean activation, 62.6 ± 4.5%, $n = 5$ vs. 60.3 ± 7.4, $n = 6$), demonstrating that the TEA mutation *per se* has no effect on channel activation by PKA (Fig. 2 *C* and *E*). The effect of cAMP was completely blocked by preincubating patches with the PKA inhibitor, PKI_{5-24} (10). In the presence of 0.45 μM PKI_{5-24}, the mean percentage change in channel activity after addition of cAMP to the intracellular face of the patch was −4.3 ± 6.7.8% ($n = 6$), confirming that the effect of cAMP is mediated by activation of *endogenous* PKA activity associated with the isolated patch. PKI_{5-24} alone applied after patch excision (as for cAMP in Fig. 2*C*) had no significant effect on channel activity, suggesting that "basal" PKA activity in isolated patches is low under our recording conditions (the mean percentage change in activity was −2.7 ± 4.1%, $n = 4$). Homotetrameric S899A channels were unresponsive to endogenous PKA (Fig. 2*E*), supporting the biochemical evidence (Fig. 2*B*) that S899 is required for channel activation by PKA. In channels containing a mixture of Y334V and S899A α-subunits no significant channel activation (mean change in activity, 5.3 ± 9.8%, $n = 11$) was observed upon application of cAMP (Fig. 2 *D* and *E*). Because cAMP failed to activate any channel in which fewer than four intact S899 sites are present, it is most unlikely that the lack of channel activation is due to the channel already being phosphorylated at all of its remaining S899 sites before cAMP application. The lack of PKA activation was observed even in channels that expressed a single S899A α-subunit (the mean change in activity in four patches was 1.6 ± 9.2%, Fig. 2 *D* and *E*). Altogether, these findings suggest that channel activation requires the coordinate phosphorylation of the S899 site on each of the four α-subunits of the tetramer and thus obeys an all-or-nothing rule.

PKA Phosphorylation of a Single α-Subunit at a Splice-Insert-Specific Consensus PKA Site Is Sufficient for Inhibition of STREX Homotetramer Channels. We sought to investigate whether phosphorylation of all four subunits within the tetramer is crucial for channels assembled from other BK_{Ca} channel α-subunit splice variants. The STREX variant is potently inhibited by endogenous PKA associated with the channel complex, and this regulation depends on a conserved serine residue ($S4_{STREX}$) within the 59-aa STREX insert (Figs. 1*A* and 3*A* and ref. 10). Full-length STREX channels were phosphorylated by PKA, and phosphorylation was abolished in the PKA consensus site double-mutant $S4_{STREX}$A-S899A (Fig. 3*B*). Mutation of either the $S4_{STREX}$ or S899 sites alone in STREX channels resulted in a reduced, but not abolished, phosphate incorporation, suggesting that either site is available for PKA phosphorylation in STREX homotetramer channels (Fig. 3*B*).

To directly examine whether each STREX α-subunit in the tetramer must be phosphorylated at the STREX-insert-specific PKA site, $S4_{STREX}$, we generated STREX channels with (STREX-Y334V) or without (STREX) the TEA_o mutation and STREX channels in which the $S4_{STREX}$ site is mutated to alanine ($S4_{STREX}$A, Fig. 3*A*). In all three constructs, the conserved C-terminal S899 site is intact, and phosphorylation assays indicate it is available for phosphorylation (Fig. 3*B*). Both STREX and STREX-Y334V homotetramers were inhibited to the same extent by activation of endogenous PKA by cAMP in isolated inside-out patches (mean inhibition, −56.5 ± 6.8%, $n = 12$, and −60.6 ± 10.3%, $n = 5$, respectively), demonstrating that the TEA_o mutation had no effect on the ability of endogenous PKA to inhibit the channel (Fig. 3 *C* and *E*). The effect of cAMP was completely prevented upon prior application of the PKA inhibitor peptide PKI_{5-24} to the patch (the mean percentage change in channel activity was 1.8 ± 4.5%, $n = 8$). PKI_{5-24} alone had no significant effect on channel activity (not shown). Furthermore, the TEA_o sensitivity of STREX and STREX-Y334V was not significantly different from ZERO or Y334V channels, respectively. (The IC_{50} for TEA_o inhibition of STREX was 0.19 ± 0.09 mM, and for STREX-Y334V it was 83.5 ± 10.5 mM.) Homotetrameric $S4_{STREX}$A channels are activated under the same conditions depending on a functional S899 site (10) (Fig. 3*E*). However, channels formed from mixtures of STREX-Y334V and $S4_{STREX}$A α-subunits were inhibited by endogenous PKA to the same extent as homotetramers of STREX or STREX-Y334V subunits (Fig. 3 *D* and *E*). Similar inhibition of STREX-Y334V and $S4_{STREX}$A heterotetramers was observed irrespective of the presence of 1, 2, or 3 $S4_{STREX}$A α-subunits in the heterotetramer, suggesting that even a single $S4_{STREX}$ site is sufficient to exert inhibition in channels containing four STREX inserts. [In all

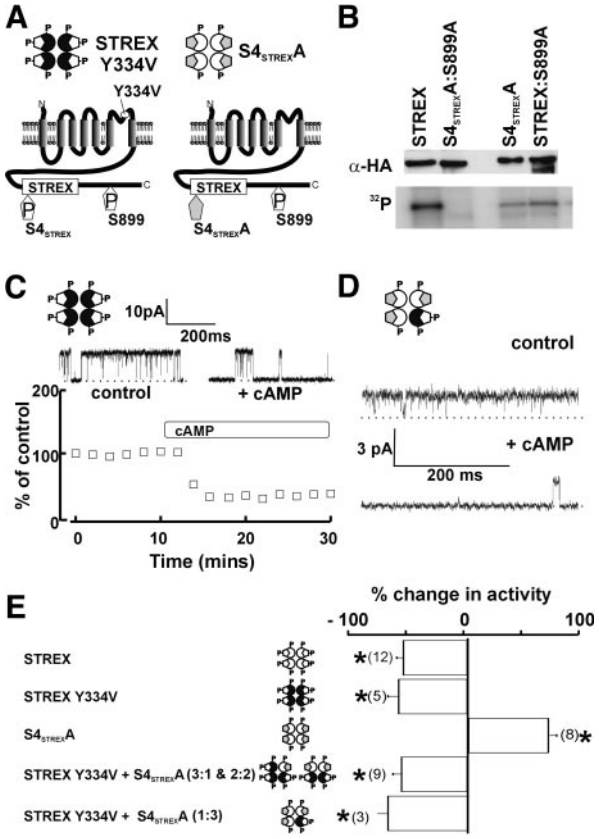

Fig. 3. PKA inhibition of STREX homotetrameric BK_{Ca} channels requires a functional PKA consensus motif ($S4_{STREX}$) on a single STREX α-subunit within the tetramer. (*A*) Schematic of the TEA_o-insensitive STREX α-subunit (STREX-Y334V, filled circles with open pentagon) and STREX α-subunit in which the STREX-insert-specific PKA consensus motif is mutated to alanine ($S4_{STREX}A$, open circle with shaded pentagon). (*B*) *In vitro* phosphorylation of homotetrameric STREX BK_{Ca} channels and the respective PKA consensus site mutants, $S4_{STREX}$A:S899A, STREX:S899A, and $S4_{STREX}$A. IPs were subjected to *in vitro* PKA phosphorylation as in Fig. 2*B*. Data are representative of three experiments. (*C*) Representative time-course plot of cAMP application to the intracellular face of patches containing homotetrameric STREX-Y334V α-subunits demonstrating inhibition of channel activity by endogenous PKA. (*Inset*) Single-channel traces before (control) and after (+cAMP) application of cAMP. Recording conditions were as in Fig. 1*C*. The average P_o under control conditions was 0.58. (*D*) Representative single-channel records before (control) and after (+cAMP) application of cAMP to a patch containing a heterotetrameric channel with a predicted 1:3 stoichiometry of STREX-Y334V to $S4_{STREX}$A α-subunits. (*E*) Summary of effect of cAMP on channel activity by using the various STREX α-subunit mutations expressed as a percentage change in activity from control. Pictograms illustrate predicted channel stoichiometry in each group. Data for STREX-Y334V + $S4_{STREX}$A heterotetramers with (3:1) and (2:2) stoichiometry are pooled. Data are means ± SEM. Number of patches is shown in parentheses in histogram. *, $P < 0.01$, ANOVA.

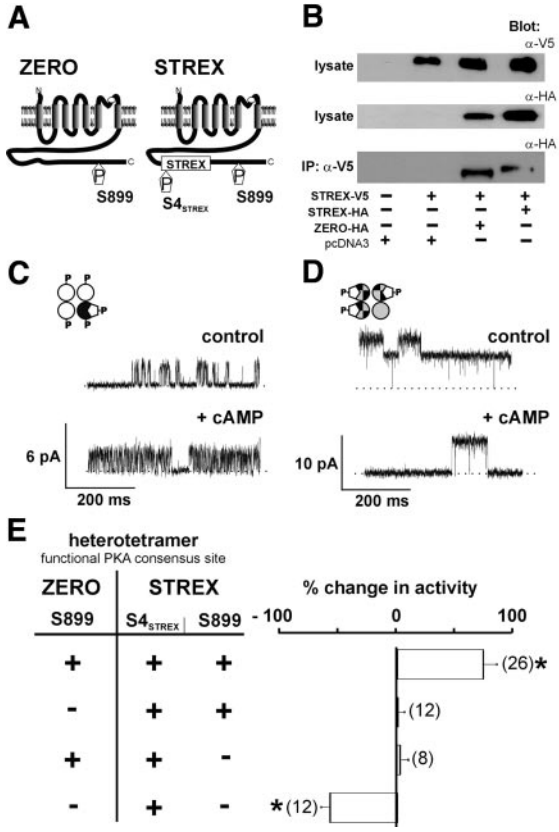

Fig. 4. PKA regulation of ZERO/STREX heterotetramer channels is conditional upon the stoichiometry of α-subunits containing functional C-terminal PKA consensus phosphorylation motifs (S899). (*A*) Schematic of the PKA-consensus sites within the ZERO and STREX α-subunit splice variants. (*B*) Biochemical confirmation of heterotetramerization by co-IP of epitope (−HA or −V5) tagged ZERO and STREX α-subunits. Cell lysates and IPs were subjected to SDS/PAGE and Western blotting. Data are representative of four experiments. (*C*) Representative single-channel records before (control) and after (+cAMP) application of cAMP to a patch containing a heterotetrameric channel with a predicted 3:1 stoichiometry of ZERO to STREX-Y334V α-subunits. (*D*) Representative single-channel records before (control) and after (+cAMP) application of cAMP to a patch containing a heterotetrameric channel with a predicted 1:3 stoichiometry of S899A to STREX-Y334V-S899A (checkerboard circle with open pentagon) α-subunits. In this patch, a smaller channel can be observed with a predicted 3:1 stoichiometry that is also inhibited upon cAMP application. (*E*) Summary of effect of cAMP on BK_{Ca} channels assembled from ZERO and STREX splice-variant α-subunits in which the respective consensus PKA sites are intact (+) or mutated to alanine (−). Data are expressed as a percentage change in activity from control. Channel stoichiometry was determined from the single-channel sensitivity to TEA_o with the Y334V mutation in either the respective ZERO or STREX α-subunit as indicated in *Results*. Data for heterotetramers in each group include data from channels predicted to contain one, two, or three of the respective TEA-insensitive α-subunits. Data are means ± SEM. Number of patches is shown in parentheses. *, $P < 0.01$, ANOVA.

three patches containing channels with predicted 1:3 STREX-Y334V/$S4_{STREX}$A α-subunit stoichiometry, channel activity was significantly inhibited; mean inhibition was $-67.5 \pm 9.3\%$, not significantly different from STREX-Y334V homotetramers alone (Fig. 3 *D* and *E*); $P > 0.05$, Mann–Whitney *U* test).] This finding suggests that, under our recording conditions, the $S4_{STREX}$ site is constitutively dephosphorylated before cAMP application. In addition, these data, in conjunction with the phosphorylation assays in Fig. 3*B*, suggest that a single $S4_{STREX}$ site in a tetramer composed of four STREX α-subunits may override four functional S899 sites. Thus, in STREX homotetramers, phosphorylation of a single $S4_{STREX}$ site is sufficient for channel inhibition and thus obeys a single-subunit rule.

ZERO/STREX Heterotetramers Are Activated by PKA Through Phosphorylation of All Four Subunits at S899. Because BK_{Ca} channel activation depends on the presence of a functional S899 site in each α-subunit, whereas channel inhibition only requires phosphorylation of a single $S4_{STREX}$ site, we sought to address the functional consequence of channel regulation by PKA in heterotetramers formed from ZERO and STREX α-subunits in which multiple PKA phosphorylation sites are available (Fig. 4*A*). For example, does STREX act as a dominant α-subunit to confer inhibition in all heterotetramers, as might be predicted from the single-subunit rule in STREX homotetramers? Does

regulation depend on a titration of α-subunit stoichiometry? Or, alternatively, is the activation by PKA through phosphorylation of all four α-subunits at the conserved S899 site the default phenotype of heterotetramers?

Coexpression of epitope-tagged ZERO and STREX α-subunits in HEK293 cells resulted in robust coimmunoprecipitation of the respective channel α-subunit, confirming biochemically that heterotetramers may exist *in vivo* (Fig. 4*B*). Furthermore, coexpression of Y334V α-subunits with STREX α-subunits (or ZERO with STREX-Y334V α-subunits) resulted in functional expression of heterotetramers as determined by single-channel TEA_o sensitivity (Fig. 4 *C* and *D*). However, in contrast to the dominant effect of PKA inhibition determined by the $S4_{STREX}$ site in STREX homotetramers, in heterotetrameric channels containing ZERO and STREX α-subunits, channels were always activated by endogenous PKA (Fig. 4 *C* and *E*). For ZERO/STREX heterotetramers in which all S899 and all $S4_{STREX}$ sites are available for phosphorylation, mean activation was 72.3 ± 10.0% ($n = 26$) (Fig. 4*E*). Activation was independent of whether the TEA_o mutation for determination of subunit stoichiometry was in the ZERO or STREX α-subunit (for ZERO + STREX-Y334V, activation was 64.6 ± 15.0%, $n = 12$; for Y334V + STREX, mean activation was 79.4 ± 15.5%, $n = 14$) or the number of respective TEA-mutant subunits in the tetramer (not shown). These heterotetrameric channels contain fewer than four α-subunits expressing the STREX insert (and, thus, fewer than four $S4_{STREX}$ sites), but they contain four α-subunits each with a functional S899 site. Thus, to examine whether this dominant activation requires functional S899 sites in each of the four α-subunits, as would be predicted from the all-or-nothing rule of ZERO homotetramers, we coexpressed STREX-Y334V α-subunits with S899A α-subunits. Under these conditions (i.e., functional conserved S899 sites are only present in STREX subunits), no significant change of channel activity was observed (% change in activity was −1.7 ± 6.8%, $n = 12$; Fig. 4*C*). Furthermore, if intact S899 sites were in the ZERO but not STREX α-subunit of the tetramer, no significant effect of PKA was observed (Fig. 4*E*). In heterotetramers of ZERO α-subunits and STREX α-subunits that contain both the TEA_o mutation and the S899A mutation (STREX-Y334V-S899A α-subunits), no significant effect of endogenous PKA was observed (mean change in activity, 3.9 ± 6.3%, $n = 8$). These data are consistent with the hypothesis that the default mode of PKA regulation of ZERO/STREX heterotetramers is activation-dependent on phosphorylation of all four α-subunits at S899 by the all-or-nothing rule.

However, channels formed from ZERO and STREX splice variants in which the functional S899 site is mutated to alanine in all α-subunits of the tetramer (mixtures of S899A + STREX-Y334V-S899A) resulted in channels that are *inhibited* by endogenous PKA (Fig. 4 *D* and *E*; mean inhibition, −58.8 ± 8.3%, $n = 12$), not significantly different from STREX, STREX-Y334V (Fig. 3*E*), or STREX-Y334V-S899A homotetramers. (Mean inhibition for STREX-Y334V-S899A homotetramers was −55.9 ± 5.9% $n = 6$.) This finding suggests that in heterotetrameric channels, (*i*) the inhibitory effect of a phosphorylated $S4_{STREX}$ site is conditional upon the phosphorylation status of the S899 sites within the tetramer, i.e., the PKA-inhibited channel phenotype observed in STREX homotetramers is only manifest in ZERO/STREX heterotetramers if all four S899 sites are dephosphorylated, and (*ii*) under these conditions, the single-subunit rule for PKA inhibition through phosphorylation of $S4_{STREX}$ applies.

Discussion

We examined the regulation of tetrameric BK_{Ca} channels by cAMP-dependent protein kinase (PKA) phosphorylation to determine the rules of α-subunit phosphorylation within the tetramer that governs functional regulation of the channel by PKA. By exploiting a strategy to allow determination of tetramer α-subunit composition with known numbers of PKA consensus motifs within each α-subunit *in situ*, we demonstrate that the α-subunit composition of the tetramer determines the stoichiometry of tetramer phosphorylation required for PKA regulation.

PKA activation of BK_{Ca} channels depends on all four α-subunits being phosphorylated at the conserved C-terminal PKA consensus site, S899. This four-subunit all-or-nothing rule applies for channels assembled as homotetramers of the ZERO α-subunit splice variant, which contains a single PKA consensus site (S899), and for heterotetrameric channels assembled from ZERO and STREX α-subunits.

In contrast, PKA-dependent inhibition of BK_{Ca} channel activity requires only a single α-subunit to be phosphorylated at a splice-insert-specific (STREX) PKA consensus motif ($S4_{STREX}$) and thus follows a single-subunit rule. STREX α-subunits contain two PKA consensus motifs: the conserved C-terminal S899 site and the STREX-insert-specific site, $S4_{STREX}$. In channels assembled as STREX homotetramers, phosphorylation of a single $S4_{STREX}$ site can override the effect of phosphorylating the S899 sites within the STREX α-subunits. Although the single-subunit rule for PKA inhibition can apply to ZERO/STREX heterotetramers, inhibition mediated by PKA phosphorylation of a $S4_{STREX}$ site is conditional upon all four S899 sites within the tetramer being dephosphorylated. The rules of phosphorylation regulation are distinct from those of BK_{Ca} channel tetramer regulation by intracellular free Ca^{2+} through the C-terminal "calcium bowl"; each α-subunit calcium bowl provides a stepwise contribution to the cooperative activation and apparent Ca^{2+} affinity of BK_{Ca} channel tetramers (23). These inter- and intra-subunit phosphorylation rules have important implications for the coordinated regulation of BK_{Ca} channels by reversible protein phosphorylation.

Because PKA-dependent activation follows the four-subunit all-or-nothing phosphorylation rule, but PKA-dependent inhibition follows the single-subunit rule, a functional consequence of our data is that PKA-dependent activation and inhibition may be subject to differential combinatorial control by protein phosphatases. For example, for PKA-dependent activation dephosphorylation of any one S899 site within the tetramer would prevent PKA activation and could be accomplished by distinct serine/threonine protein phosphatases targeting separate α-subunits, i.e., multiple phosphatases may converge at a single BK_{Ca} tetramer to regulate PKA activation. Conversely, antagonism of PKA inhibition would require all available $S4_{STREX}$ PKA consensus sites to be dephosphorylated. Although BK_{Ca} channels are potently regulated by dephosphorylation by multiple protein phosphatases (18, 24, 25), how or if these enzymes are targeted to BK_{Ca} channel α-subunits is currently unknown.

Our data also suggest an additional level of conditional regulation by reversible protein phosphorylation (19, 25, 26). In ZERO/STREX heterotetramers, PKA inhibition is conditional upon the phosphorylation status of the S899 sites in the tetramer, and this effect can be exerted across α-subunits within the heterotetramer (i.e., the phosphorylated S899 site does not need to reside on the same subunit as the $S4_{STREX}$ site). Although it is unknown whether differential, site-specific dephosphorylation/phosphorylation can occur *in vivo*, our data imply that cross talk between α-subunits within the tetramer is an important determinant of BK_{Ca} channel regulation by phosphorylation.

These data suggest that alternative pre-mRNA splicing and posttranslational PKA phosphorylation of BK_{Ca} channel α-subunits act in concert to enable two distinct BK_{Ca} behaviors to be expressed from tetramers assembled from distinct BK_{Ca} channel α-subunit splice variants. The default action of BK_{Ca}

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channels is to facilitate cell repolarization, and this function can be enhanced through PKA activation of the tetramer by means of the four-subunit all-or-nothing rule (ZERO or default heterotetramer phenotype). In contrast, the single-subunit PKA-inhibition rule allows a subset of BK_{Ca} channels to facilitate depolarization upon PKA phosphorylation (STREX homotetramers or ZERO/STREX heterotetramers in which all S899 sites are dephosphorylated). Considerable phenotypic diversity in the regulation of native BK_{Ca} channels by PKA-dependent phosphorylation has been reported. In many neurons and smooth muscle cells, PKA activates BK_{Ca} channels (13, 16, 27–29), whereas in endocrine cells of the anterior pituitary, BK_{Ca} channels are inhibited (6, 24, 30). Whether such physiological diversity results from homo- and/or heterotetramer assembly of α-subunit splice variants in native tissues and/or involves accessory subunits remains to be determined.

Posttranslational protein phosphorylation acting in concert with changes in channel α-subunit composition, governed by transcription or alternative pre-mRNA splicing, likely represents a generic mechanism to regulate potassium ion channel phenotype and function. Analysis of the stoichiometry of BK_{Ca} channel tetramer α-subunit phosphorylation by other kinase-signaling pathways, with the approach outlined in this study, combined with determination of the effects of phosphorylation on voltage and calcium sensitivity, should provide important insights into the structural basis for the coordinated multifactorial regulation of BK_{Ca} channels.

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