Differential regulation of Ca\textsubscript{V}1.2 channels by cAMP-dependent protein kinase bound to A-kinase anchoring proteins 15 and 79/150

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The Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 voltage-gated calcium channels initiate excitation-contraction coupling in skeletal and cardiac myocytes, excitation-transcription coupling in neurons, and many other cellular processes. Up-regulation of their activity by the β-adrenergic–PKA signaling pathway increases these physiological responses. PKA up-regulation of Ca\textsubscript{V}1.2 activity can be reconstituted in a transfected cell system expressing Ca\textsubscript{V}1.2Δ1800 truncated at the in vivo proteolytic processing site, the distal C-terminal domain (DCT; Ca\textsubscript{V}1.2[1801–2122]), the auxiliary α2ß and β subunits of Ca\textsubscript{V}1.2 channels, and A-kinase anchoring protein-15 (AKAP15), which binds to a site in the DCT. AKAP79/150 binds to the same site in the DCT as AKAP15. Here we report that AKAP79 is ineffective in supporting up-regulation of Ca\textsubscript{V}1.2 channel activity by PKA, even though it binds to the same site in the DCT and inhibits the up-regulation of Ca\textsubscript{V}1.2 channel activity supported by AKAP15. Mutation of the calcineurin-binding site in AKAP79 (AKAP79ΔPIX) allows it to support PKA-dependent up-regulation of Ca\textsubscript{V}1.2 channel activity, suggesting that calcineurin bound to AKAP79 rapidly dephosphorylates Ca\textsubscript{V}1.2 channels, thereby preventing their regulation by PKA. Both AKAP15 and AKAP79ΔPIX exert their regulatory effects on Ca\textsubscript{V}1.2 channels in transfected cells by interaction with the modified leucine zipper motif in the DCT. Our results introduce an unexpected mode of differential regulation by AKAPs, in which binding of different AKAPs at a single site can competitively confer differential regulatory effects on the target protein by their association with different signaling proteins.

INTRODUCTION

Voltage-gated Ca\textsuperscript{2+} (Ca\textsubscript{V}) channels initiate excitation-contraction coupling in muscle cells, excitation-transcription coupling in neurons, and many other physiological events (Reuter, 1979; Catterall, 1991; Bers, 2002; West et al., 2002). In skeletal and cardiac muscle, up-regulation of the activity of Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 channels increases contractile force in response to activation of the β-adrenergic signaling pathway in the fight or flight response (Reuter, 1983; Tsien et al., 1986; Catterall, 2000). In neurons, activation of the dopamine and β-adrenergic signaling pathways increases Ca\textsubscript{V}1.2 channel activity and modulates gene transcription and synaptic plasticity (Lovingier, 2010; Gerfen and Surmeier, 2011; Qian et al., 2012). β-Adrenergic receptors activate adenyl cyclase, increase cAMP, activate cAMP-dependent protein kinase (PKA), and phosphorylate Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 channels (Reuter, 1983; Tsien et al., 1986; Catterall, 2000). Targeting PKA to specific subcellular compartments or substrates by binding to A-kinase anchoring proteins (AKAPs) exerts spatiotemporal control over these regulatory processes (Wong and Scott, 2004).

Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 channels form autoinhibitory signaling complexes, which are essential for regulation of their activity by the PKA pathway (Hulme et al., 2004; Catterall, 2010). Their pore-forming α\textsubscript{1} subunits are proteolytically processed in vivo near the center of their large intracellular C-terminal domains (De Jongh et al., 1989, 1991, 1996; Hell et al., 1996). The membrane-anchored AKAP15/18\textsuperscript{1} (Gray et al., 1997, 1998; Fraser et al., 1998) binds to the distal C-terminal domain (DCT; Ca\textsubscript{V}1.2[1801–2122]) of these channels via a modified leucine zipper motif (Hulme et al., 2002, 2003). The DCT and AKAP binding are required for regulation of Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 channels by PKA in skeletal and cardiac myocytes (Gray et al., 1998; Hulme et al., 2002, 2003; Ganesan et al., 2006; Fu et al., 2011). Moreover, the proteolytically cleaved DCT binds to the remainder of the Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.2 channels by interaction with a site in the proximal C-terminal domain (Hulme et al., 2005, 2006) and is a potent autoinhibitor of the activity.

\textsuperscript{1}Nomenclature of AKAPs. The common nomenclature for AKAPs (Wong and Scott, 2004) is used, in which the protein names are defined from apparent molecular masses observed in SDS-PAGE. AKAP15 (Gray et al., 1997, 1998) is also designated AKAP18 (Fraser et al., 1998) and AKAP7 (Wong and Scott, 2004), but AKAP15 is used here to refer to all work on this protein. AKAP79 in human is the homologue of AKAP150 in mouse, which has an insertion of a large additional domain (Wong and Scott, 2004). AKAP79/150 is also designated AKAP5 (Wong and Scott, 2004). AKAP79 was used in the experiments described in this study, and for simplicity, AKAP79 is used as the name for this protein when referring to research in the literature, whether it was carried out with the mouse or human protein.
of CaV1.2 channels when coexpressed in nonmuscle cells (Hulme et al., 2006). Activation of protein phosphorylation by PKA increases ion conductance activity by relieving the autoinhibitory effect of the DCT (Fuller et al., 2010).

Regulation of CaV1.2 channels by PKA has been reconstituted by coexpression of the components of this autoinhibitory signaling complex in transfected cells (Fuller et al., 2010). CaV1.2Δ1800 truncated at the site of in vivo proteolytic processing (Emrick et al., 2010) and the DCT composed of CaV1.2[1801–2122] interact with each other when expressed as separate proteins, and the DCT markedly inhibits CaV1.2 channel activity (Hulme et al., 2006; Fuller et al., 2010). Coexpression of these two components of the α1 subunit as separate proteins together with the auxiliary α2β and β subunits of CaV1.2 channels and AKAP15 yields an autoinhibited CaV1.2 signaling complex whose activity can be increased three- to fourfold by activation of adenyl cyclase in transfected cells (Fuller et al., 2010). Normal regulation of basal activity requires phosphorylation of Ser1700 and Thr1704, located at the interface between the DCT and the proximal C-terminal domain, and up-regulation of CaV1.2 channel activity requires PKA phosphorylation of Ser1700 (Fuller et al., 2010). Cardiac myocytes from mice in which these sites are mutated to Ala have reduced basal L-type Ca\(^{2+}\) current and impaired up-regulation by β-adrenergic agonists, confirming the crucial role of this regulatory mechanism in vivo (Fu et al., 2013).

AKAP15 also binds to CaV1.2 channels in brain neurons (Marshall et al., 2011), and AKAP79/150\(^{1}\) binds to CaV1.2 channels in both brain and cardiac muscle (Gao et al., 1997; Hall et al., 2007). Both of these AKAPs are involved in regulation of gene expression in response to activation of CaV1.2 channels in neurons (Oliveria et al., 2007; Marshall et al., 2011). The effects of AKAP79 on gene transcription in neurons are mediated by the Ca\(^{2+}\)-regulated phosphoprotein phosphatase calcineurin, which binds directly to AKAP79 (Oliveria et al., 2007). Like AKAP15, AKAP79 binds to CaV1.2 channels via the modified leucine zipper motif in the DCT (Oliveria et al., 2007). Therefore, it is of great interest to explore PKA regulation of CaV1.2 channels mediated via AKAP79 compared with AKAP15 in our reconstituted regulatory system. Here we report strikingly different regulatory properties of these two AKAPs, which depend on binding of the phosphoprotein phosphatase calcineurin by AKAP79. Our results introduce an unexpected mode of differential regulation by AKAPs, in which different AKAPs can compete for binding at a single site and confer differential regulatory effects on the target protein by association with different signaling proteins.

**MATERIALS AND METHODS**

**cDNA constructs**

Constructs used in this study include rabbit α1.2a, rat β2b, rabbit α281, AKAP15, AKAP79, PKA-Cα, and PKA-RIIα in pcDNA3 (Fuller et al., 2010). CaV1.2 leucine zipper motif triple mutant (I2073A, F2080A, I2087A) was constructed using PCR overlap extension. Construction of AKAP\(_{12M}\) was previously described (Hulme et al., 2002). AKAP79PIX was provided by J.D. Scott (University of Washington, Seattle, WA). The mutant sequence, orientation, and reading frame of all constructs were confirmed by DNA sequencing.

**Cell culture and transfection**

Human embryonic kidney tsA201 cells were cultured in DMEM/Ham’s F12 supplemented with 10% FBS and 100 U/ml penicillin and streptomycin. Cells were grown to ~70% confluence in 10% CO\(_{2}\) and transiently transfected with cDNAs encoding α1.2a truncated at Ala1800 (CaV1.2Δ1800), β2b, and α281 subunits at a 1:1 molar ratio using the FuGENE 6 method (Roche). Wild-type or mutant DCT constructs composed of CaV1.2[1801–2271] were transfected with CaV1.2Δ1800 using a molar ratio of 0.75:1 (DCT/CaV1.2Δ1800). In addition, cDNA encoding eGFP in the pcDNA3 vector was added at a molar ratio of 0.1:1 to each transfection mixture as an indicator of transfection efficiency.

**Electrophysiology**

24 h after transfection, cells were plated at low density, and recordings were made 38–48 h after transfection using the whole-cell configuration of the patch clamp technique. Patch pipettes (1.5–2 MΩ) were pulled from micropipette glass (VWR Scientific) and fire-polished. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments Inc.) and sampled at 5 kHz after anti-alias filtering at 2 kHz. Data acquisition and command potentials were controlled by either pCLAMP or HEKA Pulse software, and data were stored for later offline analysis. Voltage protocols were delivered at 10-s intervals, and leak and capacitive transients were subtracted using a P/4 protocol. Approximately 80% of series resistance was compensated with the patch clamp circuitry. The extracellular bath solution contained (mM) 150 Tris, 10 glucose, 1 MgCl\(_2\), and 10 BaCl\(_2\) (adjusted to pH 7.4 with CH\(_3\)SO\(_4\)). The intracellular solution contained (mM) 135 CsCl, 10 EGTA, 1 MgCl\(_2\), 4 MgATP, and 10 HEPES (pH 7.3, adjusted with CsOH).

**Analysis of electrophysiological recordings of CaV1.2 channels**

Current-voltage relationships from peak inward Ba\(^{2+}\) currents were normalized to gating charge (Q) to correct for variation in protein abundance. Gating currents result from the voltage-driven movement of gating charges as conformational changes occur preceding channel opening and are independent of channel unitary conductance and open probability (P\(_{o}\)). Gating charge movement was measured as the integral of the gating current transient at the reversal potential of the ionic current (Fig. S1). The reversal potential was determined by applying a series of test pulses at 10-s intervals from the holding potential of −80 mV to potentials between 60 and 80 mV in 2-mV increments. The ionic current that flows upon repolarization (the tail current) gives a functional readout proportional to the number of open channels, the single channel conductance, and channel P\(_{o}\) at the end of the depolarizing step. By comparing the ionic and gating currents we determined the efficiency of coupling of the charge movement of the voltage sensors to the subsequent opening of the pore by calculating the ratio of tail current to gating charge (tail current [nA]/integrated gating charge [pC]). Tail currents were recorded after repolarization to −50 mV after each test pulse. All data are expressed as means ± SEM of n cells. Bar graphs are presented for coupling ratio data in the figures, and scatter plots containing all of the individual cell values are presented for representative experiments in Fig. S2. Statistical significance was tested with Student’s t test for pairwise analysis and ANOVA followed by Dunnett’s test for comparison of multiple conditions.
RESULTS

Differential regulation of Ca$_{1.2}$ channels by association with AKAP15 versus AKAP79

Neurons, myocytes, and other excitable cells express a broad array of AKAPs, which are involved in many aspects of cell signaling (Logue and Scott, 2010). AKAP15 and AKAP79 are both expressed in nerve and muscle cells and interact with a common regulatory site on Ca$_{1.2}$ channels in those cell types (Gray et al., 1997, 1998; Hulme et al., 2003, 2006; Hall et al., 2007; Oliveria et al., 2007; Marshall et al., 2011). We used reconstitution of Ca$_{1.2}$ regulation in transfected cells to compare the effects of these two AKAPs on regulation of Ca$_{1.2}$ channels via the PKA pathway, and we recorded barium currents ($I_{Ba}$) conducted by Ca$_{1.2}$ channels to minimize activation of Ca$^{2+}$-dependent regulatory processes. Human embryonic kidney tsA-201 cells were cotransfected with Ca$_{1.2}\Delta 1800$, DCT, and Ca$^{2+}$ channel auxiliary subunits, plus either AKAP15 or AKAP79 (Fig. 1).

Cells transfected with Ca$_{1.2}\Delta 1800$ without DCT conducted high levels of $I_{Ba}$ upon depolarization in whole-cell voltage clamp (Fig. 1, A [top] and B). In contrast, cells transfected with Ca$_{1.2}\Delta 1800$ + DCT had much lower levels of $I_{Ba}$, reflecting the autoinhibitory effect of the DCT (Fig. 1, A [top] and B). The autoinhibitory effect of the DCT was also observed in measurements of the coupling ratio of ion channel opening to gating charge movement, which was calculated from measurements of gating charge movement at the reversal potential and tail currents immediately after repolarization (Fig. 1, A [bottom] and C). This measurement is unaffected by the efficiency of transfection and expression of the Ca$_{1.2}$ channels because it is a ratio of ionic current to gating charge for channels at the cell surface. The coupling ratio ($I_{Tail}/Q_{Gating}$) was ~45 nA/pC for Ca$_{1.2}\Delta 1800$ alone and 15 nA/pC for Ca$_{1.2}\Delta 1800$ + DCT (Fig. 1 C), as we have observed in previous work (Fuller et al., 2010).

We used 5 μM forskolin to activate adenyl cyclase and the PKA signaling pathway. Activation of adenyl cyclase with forskolin gave a substantial increase in $I_{Ba}$ and coupling ratio for Ca$_{1.2}\Delta 1800$ + DCT coexpressed with an optimal level of AKAP15 (Fig. 1, A–C). We have shown previously that this increase results from phosphorylation of Ser1700 in Ca$_{1.2}\Delta 1800$ by PKA (Fuller et al., 2010). In contrast, forskolin treatment of Ca$_{1.2}\Delta 1800$ + DCT coexpressed with AKAP79 over a wide range of cDNA ratios did not result in an increase of either $I_{Ba}$ or coupling ratio (Fig. 1, A–C), even though AKAP79 is known to bind to the AKAP-binding site in the DCT of Ca$_{1.2}$ channels (Oliveria et al., 2007). To assure that PKA was not limiting in these experiments, we overexpressed PKA as described previously (Fuller et al., 2010) and conducted a similar series of experiments (Fig. 1, D and E). Forskolin had no effect in the absence of any AKAP or in the presence of AKAP79, in contrast to the substantial increase in $I_{Ba}$ and coupling ratio in the presence of AKAP15. These results reveal striking differential regulation of Ca$_{1.2}$ channels via the PKA pathway dependent on their association with AKAP15 versus AKAP79.

Functional competition of AKAP15 and AKAP79 in regulation of Ca$_{1.2}$ channels

If AKAP15 and AKAP79 both interact with the AKAP-binding domain in the DCT of Ca$_{1.2}$ channels, they should compete with each other for binding to that regulatory site and PKA regulation via AKAP15 should be inhibited by coexpression of AKAP79. To examine this point, we expressed Ca$_{1.2}\Delta 1800$ + DCT with AKAP15 + AKAP79 and measured regulation by activation of adenyl cyclase with forskolin (Fig. 2). The results show that AKAP79 does indeed inhibit PKA regulation of Ca$_{1.2}\Delta 1800$ + DCT coexpressed with AKAP15, as measured by the amplitude of $I_{Ba}$ and the coupling ratio (Fig. 2). One potential mechanism of competitive interaction between AKAP15 and AKAP79 would be competitive binding of PKA by AKAP79, which could potentially deplete the cellular pool of PKA. To rule out this possibility, we overexpressed PKA in the presence of the two AKAPs (Fig. 2 C). Even under these conditions, expression of AKAP79 substantially reduced PKA regulation of Ca$_{1.2}\Delta 1800$ + DCT via AKAP15 (Fig. 2 C). Together, these results demonstrate competitive regulation of Ca$_{1.2}$ channels by AKAP15 and AKAP79, dependent on their binding to the AKAP-binding domain in the DCT.

Differential regulation requires calcineurin association with AKAP79

One potential mechanism that could contribute to differential PKA regulation via AKAP15 and AKAP79 is the ability of AKAP79 to bind other signaling molecules and bring them into close association with Ca$_{1.2}$ channels (Logue and Scott, 2010). In particular, AKAP79 binds the Ca$^{2+}$-regulated phosphoprotein phosphatase calcineurin (Coghlan et al., 1995; Oliveria et al., 2007), which could dephosphorylate Ca$_{1.2}$ channels and reduce their up-regulation by PKA. Calcineurin binds to the PXIXIT motif on AKAP79 (Dell’Acqua et al., 2002; Oliveria et al., 2007). Therefore, we examined regulation of Ca$_{1.2}\Delta 1800$ + DCT coexpressed with AKAP79 with the PXIXIT site deleted (AKAP79ΔPIX). Under these conditions, forskolin treatment increased the activity of Ca$_{1.2}\Delta 1800$ + DCT coexpressed with AKAP79ΔPIX as...
effectively as Ca\(_{\text{v}1.2}\Delta1800 + \text{DCT}\) coexpressed with AKAP15, when measured as the amplitude of I\(_{\text{Ba}}\) or the coupling ratio (Fig. 3, A–C). Overexpression of PKA further increased I\(_{\text{Ba}}\) and coupling ratio for Ca\(_{\text{v}1.2}\Delta1800 + \text{DCT}\) coexpressed with either AKAP79\(\Delta\text{PIX}\) or AKAP15 (Fig. 3, D and E). Under experimental conditions similar to ours, Oliveria et al. (2007) found that competing peptide inhibitors of the binding of calcineurin to Ca\(_{\text{v}1.2}\) channels gave similar results as the \(\Delta\text{PIX}\) mutation, indicating that the effects of this mutation are caused by inhibition of calcineurin binding and not by a more global conformational change in AKAP79. Moreover,
our experiments show a gain of function effect of AKAP79ΔPIX, fully restoring its ability to support PKA regulation at the same level as AKAP15 (Fig. 3 E). Complete restoration of the activity of AKAP79 to the equivalent of AKAP15 would not be expected for a mutation-induced, nonspecific conformational change in AKAP79. Therefore, our results implicate binding of calcineurin to AKAP79 as the primary reason for its differential regulation of CaV1.2 channels via the PKA pathway.

Although calcineurin is strongly Ca2+ regulated, it has a significant basal activity (Stewart et al., 1982; Perrino et al., 1992; Stemmer and Klee, 1994). In our experiments, we measure Ba2+ currents in low extracellular Ca2+ and we chelate intracellular Ca2+ with EGTA; therefore, it is likely that the basal activity of calcineurin in the presence of entering Ba2+ is sufficient to oppose PKA regulation in this experimental system. Consistent with this conclusion, we found that treatment with 5 µM cyclosporin A, which blocks up-regulation of calcineurin activity by Ca2+ without affecting basal activity (Fruman et al., 1992), did not significantly increase Ba2+ currents (P = 0.33). The mechanism of this effect of basal calcineurin activity on PKA regulation of CaV1.2 channels is considered further in the Discussion.

AKAP15 and AKAP79ΔPIX require the modified leucine zipper motif in the DCT

AKAP15 binds to skeletal muscle CaV1.1 channels and cardiac CaV1.2 channels via a modified leucine zipper interaction between a heptad repeat of hydrophobic residues in the AKAP-binding domain in the DCT and a similar heptad repeat of two Leu residues in AKAP15, and this modified leucine zipper interaction is required for PKA regulation of CaV1.2 channels in skeletal and cardiac myocytes (Hulme et al., 2002, 2003). To confirm that this leucine zipper interaction is also required for PKA regulation of CaV1.2Δ1800 + DCT in our reconstituted regulatory system in transfected tsA-201 cells, we tested the regulatory effects of AKAP15ΔLZM, in which the two Leu residues in heptad repeat in AKAP15 are mutated to Ala (Fig. 4, A–C). Our results show that AKAP15ΔLZM is completely ineffective in supporting PKA regulation of CaV1.2Δ1800 + DCT, as measured by increased I\textsubscript{Ba} or increased coupling ratio (Fig. 4, A–C). In complementary experiments, we substituted Ala for the three hydrophobic residues in the heptad repeat that forms the AKAP-binding domain in the DCT of CaV1.2 channels to create the triple mutant DCT\textsubscript{LZM}. Coexpression of CaV1.2Δ1800 + DCT\textsubscript{LZM} with AKAP15 also resulted in loss of regulation via the PKA pathway (Fig. 4, D–F). These results confirm that this leucine zipper interaction is also required for PKA regulation of CaV1.2Δ1800 + DCT in our reconstituted system requires interaction of the leucine zipper motif in AKAP15 with the complementary modified leucine zipper motif in the DCT of CaV1.2 channels.

Although AKAP79 is known to interact with CaV1.2 channels via the modified leucine zipper motif in the DCT (Hall et al., 2007; Oliveria et al., 2007), the role of this interaction in up-regulation of ion conductance activity of CaV1.2 channels has not been directly tested. Our

Figure 2. AKAP15 and AKAP79 compete for binding to the DCT of CaV1.2 channels. (A) Representative I\textsubscript{Ba} through CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels coexpressed with cDNA ratios of 0.003:1 WT AKAP15 and/or 0.01:1 AKAP79 in the presence of 5 µM forskolin (Fsk) elicited by a test pulse to 10 mV from a holding potential of −80 mV. (B) Mean current-voltage relationships for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels coexpressed with 0.003:1 WT AKAP15 and/or 0.01:1 AKAP79 and 5 µM forskolin. Error bars are SEM. (C) Coupling efficiency (nA/pC) for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels coexpressed with 0.003:1 WT AKAP15 and/or 0.01:1 AKAP79 and 5 µM forskolin. Error bars are SEM. (D) Mean coupling efficiency (nA/pC) for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels coexpressed with 0.003:1 WT AKAP15 and/or 0.01:1 AKAP79 and 5 µM forskolin. Error bars are SEM.
ability to reconstitute PKA regulation of CaV1.2 channels by coexpression with AKAP79ΔPIX in transfected cells now allows a direct test of its site of interaction using the DCTLZM mutant. We found that CaV1.2Δ1800 + DCTLZM coexpressed with AKAP79ΔPIX was not up-regulated by activation of adenylyl cyclase with forskolin (Fig. 4, G and H). These results show directly that the regulatory effects of AKAP79ΔPIX, as well as those of AKAP15, require interaction with the modified leucine zipper motif in the DCT of CaV1.2 channels.

D I S C U S S I O N

Reconstitution of regulation of CaV1.2 channels by the PKA signaling pathway

The results presented here further establish the relevance of our reconstitution system for studies of regulation of CaV1.2 channels by the PKA signaling pathway. Coexpression of different members of the autoinhibitory signaling complex formed by CaV1.2 channels allows their differential regulatory properties to be directly determined and compared with the properties of CaV1.2 channels expressed with AKAP15. Using this approach, we have found that AKAP79 confers strikingly different regulation from AKAP15. These results imply that differential expression and localization of AKAP15 and AKAP79 can lead to differential regulation of CaV1.2 channels in different cell types and subcellular compartments. Our results highlight the importance of binding of the Ca2+-regulated phosphoprotein phosphatase calcineurin in determining the regulatory properties of AKAP79. A previous study has demonstrated its role in regulation of basal activity of CaV1.2 channels and in regulation of gene expression via the PKA signaling pathway.

Figure 3. PKA-dependent regulation of CaV1.2Δ1800 channels via AKAP15 and AKAP79ΔPIX. (A) Representative I_{Ba} through CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels coexpressed with cDNA ratios of either 0.003:1 AKAP15 or 0.01:1 AKAP79ΔPIX in tsA-201 cells in the absence or presence of 5 µM forskolin elicited by a test pulse to 10 mV from a holding potential of −80 mV. (B) Mean current-voltage relationships for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels with 0.003:1 AKAP15 or 0.01:1 AKAP79ΔPIX and 5 µM forskolin (Fsk). Dashed black line indicates zero current level. (C) Coupling efficiency (nA/pC) for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels with AKAP15 or AKAP79ΔPIX and 5 µM forskolin. Dashed black line indicates mean coupling ratio for unstimulated CaV1.2Δ1800 + DCT. Values and ±SEM are indicated. **, P < 0.01; and *, P < 0.05 versus control. Significance was determined by ANOVA followed by Dunnett’s post-test. (D) Mean current-voltage relationships determined as in B. Fits to current-voltage relationships showed that there was no significant difference in the voltage dependence of activation (P > 0.7). (B and D) Mean current-voltage relationships showed that there was no significant difference in the voltage dependence of activation (P > 0.7). (B and D) Error bars are SEM. (E) Coupling efficiency (nA/pC) for CaV1.2Δ1800 and CaV1.2Δ1800 + DCT channels with PKA Ca catalytic subunit plus PKA RIα regulatory subunit, AKAP15, AKAP79ΔPIX, and 5 µM forskolin. Dashed black line indicates mean coupling ratio for unstimulated CaV1.2Δ1800 + DCT. ***, P < 0.001; and *, P < 0.05 versus control without forskolin. Significance was determined by Student’s t test. The control results for AKAP15 + DCT are values from the dataset published in Fuller et al. (2010). The experiments presented here overlapped in time with those previously published experiments.
Differential regulation of Ca\textsubscript{V}1.2 channels by PKA bound to different AKAPs

Most cells express several different AKAPs, which have been shown to participate in many different cell signaling pathways (Logue and Scott, 2010). Thus, differential regulation of distinct signaling pathways by different AKAPs is well established. Our results with Ca\textsubscript{V}1.2 channels add a new perspective on the potential molecular mechanisms for differential regulation by AKAPs by entry of Ca\textsuperscript{2+} through Ca\textsubscript{V}1.2 channels (Oliveria et al., 2007). Our results provide direct evidence for an important role for calcineurin bound to AKAP79 in opposing up-regulation of Ca\textsubscript{V}1.2 channel function by the PKA pathway. A recent study shows that AKAP79 also binds phosphoprotein phosphatase-1 (Le et al., 2011), which may contribute additional modes of differential regulation of Ca\textsubscript{V}1.2 channels and other PKA signaling targets.
revealing that multiple AKAPs can bind at a single regulatory site on their common target protein and have differential effects on the same cell signaling pathway that are mediated by their associations with different regulatory proteins. AKAP15 and AKAP79 have previously been shown to interact with the same, short modified leucine zipper motif, which is required for their binding and support of PKA regulation (Hulme et al., 2002, 2003; Oliveria et al., 2007). However, it was unknown whether interaction with this common site would cause functional competition between the two proteins. We found that AKAP15 and AKAP79 do indeed compete functionally when coexpressed with Cav1.2 channels in the autoinhibitory signaling complex. Moreover, this functional competition is caused by the ability of AKAP79 to bind calcineurin. Because different AKAPs bind many different kinases, phosphoprotein phosphatases, and other signaling proteins (Logue and Scott, 2010), this form of functional competition and differential regulation of target proteins by different AKAPs acting at a common binding site would provide a broad range of regulatory options controlled by expression and localization of AKAPs. These findings add an additional layer of flexibility and complexity to cell signaling pathways in which AKAPs organize multiple regulatory proteins.

Our results demonstrating functional competition among AKAPs interacting with the same site on Cav1.2 channels take on additional significance in light of recent mouse genetic studies of AKAP regulation of the heart (Jones et al., 2012). Deletion of both AKAP15 and AKAP79 in mice is not sufficient to prevent β-adrenergic up-regulation of Cav1.2 channel activity by isoproterenol in ventricular myocytes (Jones et al., 2012). Because β-adrenergic stimulation in ventricular myocytes requires AKAP anchoring at the AKAP-binding domain on Cav1.2 channels (Hulme et al., 2003), these mouse genetic results imply that one or more additional AKAPs besides AKAP15 and AKAP79 can mediate β-adrenergic stimulation of Cav1.2 channels in ventricular myocytes through interaction with the same site. Thus, functional competition for regulation of Cav1.2 channels by AKAPs likely extends to at least one more, yet-unidentified AKAP. The levels of expression and affinity and the different regulatory properties of these AKAPs will determine which one is dominant in regulating Cav1.2 channels and therefore will define the overall pattern of regulation of channel activity. Changes in expression of these AKAPs in different physiological and/or pathophysiological states may be important determinants of the activity of Cav1.2 channels.

Regulation of Cav1.2 channels by calcineurin bound to AKAP79

Differential regulation of Cav1.2 channels by AKAPs might reflect altered interactions between the channel and AKAP or differential interactions of the bound AKAP with other signaling proteins. Our results with AKAP79 show that its binding of calcineurin is responsible for its inability to support PKA regulation of Cav1.2 channels. Surprisingly, the basal phosphatase activity of calcineurin is sufficient for this regulatory effect. Our measurements are made using Ba2+ as the permeant ion, and intracellular Ca2+ is strongly buffered with EGTA in the recording pipette. Therefore, it is unlikely that calcineurin is substantially up-regulated by Ca2+ binding in our experiments. Calcineurin has a significant basal activity, which is ~0.25–1% of its maximal activity when fully activated by Ca2+ and calmodulin in biochemical assays in solution (Stewart et al., 1982; Perrino et al., 1992; Stemmer and Klee, 1994). The rate of dephosphorylation of phosphoprotein substrates depends on their local concentration in the vicinity of the phosphatase, and tethering of calcineurin directly to the DCT of Cav1.2 channels would increase the local concentration of its substrate site at Ser1700-P by hundreds or thousands of fold. Evidently, the effect of proximity afforded by binding to AKAP79 allows effective dephosphorylation of the Cav1.2 channel by the basal activity of calcineurin at a rate that is comparable with or greater than the rate of phosphorylation by PKA, preventing accumulation of phosphorylated Cav1.2 channels and resulting in functional competition between AKAP15 and AKAP79 at their common binding site. In a cardiac myocyte, increases of cAMP near Cav1.2 channels may be faster and larger because of localized signaling; therefore, activation of calcineurin by Ca2+ entering through Cav1.2 channels may be required to return the activity of these channels to the basal level in vivo. These considerations further emphasize the importance of a signaling complex for regulation of Cav1.2 channels in vivo.

Regulation of Cav1.2 channels in different tissues

Cav1.2 channels conduct L-type Ca2+ currents in several different cell types. In skeletal and cardiac myocytes, up-regulation of Cav1.2 channel activity in response to activation of the β-adrenergic signaling pathway contributes to the increase in contractile force during the fight or flight response (Reuter, 1983; Tsien et al., 1986; Catterall, 1991). In brain neurons, Cav1.2 channels are involved in synaptic plasticity on the postsynaptic side of the synapse, and up-regulation of their activity by the β-adrenergic and dopaminergic signaling pathways enhances synaptic transmission (Davare et al., 2001; Young and Yang, 2004; Hall et al., 2007). In endocrine cells, Cav1.2 channels mediate Ca2+ entry that triggers secretion of hormones, and regulation by the PKA signaling pathway is an important regulator of hormone release (Baldelli et al., 2004; Yang and Berggren, 2006). AKAP15 and AKAP79 can regulate Cav1.2 channels in skeletal and cardiac myocytes (Hulme et al., 2002, 2003) and brain neurons (Hall et al., 2007; Marshall et al., 2011), and AKAPs are also implicated in control of hormone
secretion (Lester et al., 2001; Yang and Berggren, 2006). Our results presented here imply that competitive binding of AKAP15, AKAP79, and potentially other AKAPs at the AKAP-binding domain on Ca_{v}1.2 channels can transform the regulatory responses of Ca_{v}1.2 channels to the PKA signaling pathway and potentially to other intracellular signaling pathways and thereby can fine-tune the regulation of muscle contraction, synaptic transmission, and hormone secretion.

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