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cAMP-Dependent Regulation of Cardiac L-Type Ca^{2+} Channels Requires Membrane Targeting of PKA and Phosphorylation of Channel Subunits

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Summary

The cardiac L-type Ca^{2+} channel is a textbook example of an ion channel regulated by protein phosphorylation; however, the molecular events that underlie its regulation remain unknown. Here, we report that in transiently transfected HEK293 cells expressing L-type channels, elevations in cAMP resulted in phosphorylation of the α_{1C} and β_{2a} channel subunits and increases in channel activity. Channel phosphorylation and regulation were facilitated by submembrane targeting of protein kinase A (PKA), through association with an A-kinase anchoring protein called AKAP79. In transfected cells expressing a mutant AKAP79 that is unable to bind PKA, phosphorylation of the α_{1C} subunit and regulation of channel activity were not observed. Furthermore, we have demonstrated that the association of an AKAP with PKA was required for β-adrenergic receptor-mediated regulation of L-type channels in native cardiac myocytes, illustrating that the events observed in the heterologous expression system reflect those occurring in the native system. Mutation of Ser1928 to alanine in the C-terminus of the α_{1C} subunit resulted in a complete loss of cAMP-mediated phosphorylation and a loss of channel regulation. Thus, the PKA-mediated regulation of L-type Ca^{2+} channels is critically dependent on a functional AKAP and phosphorylation of the α_{1C} subunit at Ser1928.

Introduction

The regulation of ion channels by second messenger-mediated protein phosphorylation is widely recognized as an important physiological control mechanism. The voltage-activated Ca^{2+} channels in cardiac cells were among the first ion channels discovered to be regulated by protein phosphorylation. Many electrophysiological studies demonstrated that cardiac L-type channel currents are markedly increased by β-adrenergic receptor (β-AR) agonists, and studies performed over the course of 20 years have strongly implicated that regulation occurs through a cAMP- and PKA-dependent pathway that results in the phosphorylation of the channels or as yet unidentified regulatory proteins (reviewed by McDonald et al., 1994; Hosey et al., 1996). Despite the extensive study of this pathway, the specific in vivo phosphorylation events underlying channel regulation have not been identified.

L-type Ca^{2+} channels are multisubunit complexes, composed minimally of pore-forming α_1 subunits along with accessory β and $\alpha_2\delta$ subunits. Multiple isoforms of L-type Ca^{2+} channels exist and differ in the type of α_1 and β subunits (De Waard et al., 1996; Hosey et al., 1996). Studies at the mRNA and protein level suggest that cardiac L-type channels contain the α_{1C} and the β_{2a} subunit isoforms (Perez-Reyes et al., 1992; Yoshida et al., 1992; Witcher et al., 1995; Gao et al., 1997). Similar forms of this channel are expressed in brain and smooth muscle, as well as in nonexcitable cells such as fibroblasts (De Waard et al., 1996). Biochemical studies of native cardiac Ca^{2+} channels have been hampered by the fact that the channels are rare membrane proteins. Several heterologous expression systems have been utilized to study the regulation of cardiac L-type Ca^{2+} channels; however, these studies have met with unexpected, and unexplained, difficulties. Most studies of expressed cardiac L-type channels were unable to reproduce the expected increase in Ca^{2+} channel currents upon activation of the PKA pathway (Perez-Reyes et al., 1994; Singer-Lahat et al., 1994; Zong et al., 1995; Perets et al., 1996). However, in other studies, the expected activation of the expressed channels was observed after increases in cAMP (Yoshida et al., 1992; Yatani et al., 1995). Thus, no clear picture has emerged, and the molecular events underlying regulation of cardiac L-type Ca^{2+} channels have not been identified.

Recent biochemical studies have shown that the α_{1C} subunit is a substrate for PKA in vitro (De Jongh et al., 1996; Puri et al., 1997), and Ser1928 has been identified as a potential PKA target (De Jongh et al., 1996; Mitterdorfer et al., 1996; Perets et al., 1996). However, the lack of a functional assay has hampered progress, and so far, no direct functional correlation exists between the in vitro phosphorylation of the α_{1C} subunit and PKA-mediated regulation of the channel. Moreover, the β_{2a} subunit has also been identified as a PKA substrate in vitro (Puri et al., 1997), but its role in the regulation of Ca^{2+} channel function upon phosphorylation has not been explored. Thus, although channel subunits can serve as substrates for phosphorylation in vitro, the inconsistencies observed in channel regulation between cardiac myocytes and heterologous expression systems suggest that important factors involved in mediating the regulation of Ca^{2+} channels by PKA are missing from the expression systems.

Several investigations have suggested that subcellular anchoring of PKA through association with A-kinase anchoring proteins (AKAPs) is an efficient means to control the spatiotemporal resolution of cAMP-responsive phosphorylation events (Faux and Scott, 1996). More recently, two multifunctional anchoring proteins that simultaneously bind more than one signaling enzyme have been cloned (Coghlan et al., 1995a, 1995b; Faux and Scott, 1996). For example, AKAP79 can bind PKA, protein kinase C, and phosphatase 2B (calcineurin) and can tether the enzymes to cell membranes (Coghlan et al., 1995a, 1995b; Klauck et al., 1996). It has been suggested that targeting kinases to particular intracellular sites by AKAPs may increase the local concentration of the kinase and facilitate phosphorylation reactions by localizing kinases in close proximity to their substrates (McCartney et al., 1995a). For example, anchoring of PKA has been shown to be necessary for the regulation of AMPA receptor/channels by PKA in cultured hippocampus neurons (Rosenmund et al., 1994). In addition, an AKAP has been implicated in the voltage-dependent facilitation of the skeletal isoform of L-type Ca^{2+} channels expressed in skeletal myotubes and in transfected tsA-201 cells using electrophysiological approaches (Johnson et al., 1994, 1997). In this study, we investigated the role of AKAPs in facilitating the phosphorylation and regulation of cardiac Ca^{2+} channels upon PKA activation.

Results

AKAP79 Is Required for Phosphorylation of Ca^{2+} Channel Subunits by PKA in Transfected HEK293 Cells

We reasoned that localization of PKA at the cell periphery in close proximity to the Ca^{2+} channel may be necessary to reproduce the known regulation of the cardiac L-type channels in heterologous expression systems. To test the hypothesis that AKAPs may be involved in mediating phosphorylation of the channels by PKA in vivo, we used HEK293 cell lines stably expressing wild-type (AKAP79wt) or mutant AKAP79 (which is unable to bind PKA). Studies with these cells were prompted after we were unable to detect an increase in phosphorylation of the α_{1C} subunit of the Ca^{2+} channels in vivo upon activation of PKA in numerous attempts in naive HEK cells, even though we were able to measure receptor- or forskolin-mediated increases in cAMP levels and activation of PKA.

In untransfected HEK cells, very little AKAP79 was detected by immunofluorescent staining, and staining of the RII-regulatory subunit of PKA was diffuse and largely cytoplasmic (Figure 1A, top row). In contrast, in the stable AKAP79wt cells, marked staining of AKAP79 was observed at the plasma membrane, and RII staining at the plasma membrane was intense and coincident with AKAP79 staining (Figure 1A, bottom row). The RII binding domain of AKAPs is a conserved element of secondary structure that is predicted to form an amphipathic α helix. Substitution of a proline at position 396 for alanine has been shown to disrupt the helical secondary structure and abolish the binding of the RII subunit to AKAP79 (Carr et al., 1992). A cell line stably expressing AKAP79-A396P (which we refer to as AKAP79pro) was created. Although both the AKAP79wt and AKAP79pro proteins were expressed in the appropriate stable cell lines (Figure 1B) and localized to the cell periphery (data not shown), AKAP79pro did not bind to the RII subunit in overlay assays, whereas RII binding to the AKAP79wt protein was readily visualized (Figure 1B). Thus, the AKAP79pro cells express a nonfunctional PKA binding protein, which serves as a negative control for these studies.

To examine whether expression of AKAP79 influenced the phosphorylation of channel subunits, AKAP79wt or AKAP79pro cells were transiently transfected with Ca^{2+} channel α_{1C} and β_{2a} subunits and labeled with $^{32}P_i$. Cells were treated with various agents, or with the vehicle DMSO as a control, and channel subunits were isolated by immunoprecipitation (see Experimental Procedures). The first observation from these experiments was that both the α_{1C} and the β_{2a} subunits were basally phosphorylated in the AKAP79wt and AKAP79pro cells (as well as in naive HEK cells; data not shown; Figure 2A). The degree of basal phosphorylation was quite high for both subunits; we estimated the stoichiometry of basal phosphorylation for the α_{1C} and β_{2a} subunits to be 3.2 ± 0.4 ($n = 4$) and 1.9 ± 0.3 ($n = 4$) mol phosphate/mol protein, respectively.

When AKAP79 cells transfected with channel subunits were treated with forskolin (FSK) alone, no increase in phosphorylation of the α_{1C} subunit over basal level was observed in either cell line (Figure 2). However, an increase in phosphorylation of the β_{2a} subunit was detected (Figure 2). Since the α_{1C} subunit is a substrate for PKA in vitro (De Jongh et al., 1996; Puri et al., 1997), we asked whether the lack of PKA-induced phosphorylation of the α_{1C} subunit in intact cells might in part be due to rapid dephosphorylation of the channels. AKAP79 can bind and localize phosphatase 2B together with PKA (Klauck et al., 1996), and this could increase local phosphatase activity at the membranes. Accordingly, we tested the effects of okadaic acid (OA), which inhibits phosphatases 1 and 2A, and FK506, which inhibits phosphatase 2B, on channel phosphorylation by PKA. Treatments with OA alone (Figure 2) or FK506 alone (data not shown) did not increase the phosphorylation of either channel subunit in the AKAP79wt cells, whereas treatments with OA or FK506 plus FSK increased the phosphorylation of both the α_{1C} and the β_{2a} subunits up to 2-fold in AKAP79wt cells (Figure 2). The increases were highly significant since they were due to stoichiometric phosphorylation of the subunits upon PKA activation; the net increases over basal levels for the α_{1C} and the β_{2a} subunits was determined to be 1.4 ± 0.1 ($n = 4$) and 1.1 ± 0.1 ($n = 4$) mol phosphate/mol protein, respectively. The requirement for phosphatase inhibitors to detect channel phosphorylation by PKA suggested that phosphatase activity may be relatively high in these cells, and that phosphorylation and dephosphorylation of the channel subunits are fast dynamic processes.

Another important finding was that the presence of the wild-type AKAP79 was necessary for the phosphorylation of channel α_{1C} subunits by PKA in intact cells. The results from 3–5 independent in vivo phosphorylation

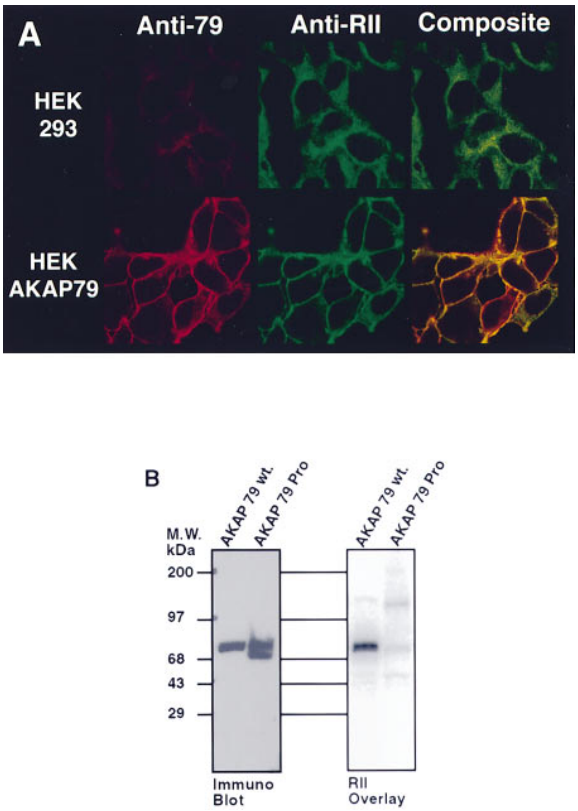

Figure 1. Characterization of Stable AKAP79wt and AKAP79pro Cell Lines

(A) Redistribution of PKA-RII in cells overexpressing AKAP79. Stable AKAP79wt (bottom row) and untransfected HEK293 cells (top row) were costained with the anti-RII antibody and the anti-AKAP79 mAb. The staining patterns of both proteins were detected by double immunofluorescence microscopy. RII staining is shown in green and AKAP79 staining is shown in red. In AKAP79wt cells, colocalization of RII and AKAP79 at the cell periphery is inferred by the areas of yellow present in the composite image.

(B) Expression and RII binding properties of AKAP79wt and AKAP79-pro in the stable cells. Proteins in total cell extracts were fractionated by SDS-PAGE and analyzed by either immunoblotting with anti-AKAP79 mAb or overlay blotting with ^{32}P-labeled RII subunits.

experiments, in both AKAP79 cell lines, were averaged and summarized (Table 1; Figures 2B and 2C). The relative phosphorylation levels of either the α_{1C} or the β_{2a} subunits under control conditions (basal phosphorylation) were expressed as 100%, and the extent of phosphorylation of all of the drug-treated groups were compared to the control. As described above, both the α_{1C} and the β_{2a} subunits were substrates of PKA when FSK plus phosphatase inhibitors were used in AKAP79wt cells. However, the phosphorylation of the α_{1C} subunit did not increase upon PKA activation in AKAP79pro cells, even in the presence of the phosphatase inhibitors (Figure 2B). Similar negative effects were observed in naive HEK cells (data not shown). These results suggested that a functional AKAP79 was required for the PKA-mediated phosphorylation of the α_{1C} subunits. Somewhat surprisingly, PKA-mediated phosphorylation of the β_{2a} subunits was observed in either AKAP79wt or AKAP79pro cells, and the extent of stimulated phosphorylation was similar in both cell lines (Figure 2C). These results, and the ability to detect phosphorylation of the β_{2a} subunit with FSK alone, suggested that phosphorylation of the β_{2a} subunit occurs more readily than phosphorylation of the α_{1C} subunit in the HEK cells. Although it has been shown that the β subunit is membrane localized (Chien et al., 1995), it is very hydrophilic and possesses no transmembrane segments. Thus, it is possible that the sites of phosphorylation in the β subunit are more cytoplasmically oriented and accessible to cytoplasmically localized PKA in the absence of AKAP79. In addition, phosphorylation sites of the β subunit may be less sensitive to phosphatases, making it easier to detect phosphorylation of the β subunit.

AKAP79 Is Required for PKA-Mediated Regulation of L-Type Ca^{2+} Channel Currents

To investigate the regulation of the Ca^{2+} channels by PKA, the α_{1C} and the β_{2a} subunits were transfected into either AKAP79wt or AKAP79pro cells for electrophysiological studies. As channel currents had not been measured in either the wild-type or the mutant AKAP79 cells previously, we first performed whole-cell patch-clamp experiments to ensure that the expressed channels possessed the expected electrophysiological properties

Table 1. Effects of Different PKA Stimulators on Phosphorylation of Ca^{2+} Channel α_{1C} and β_{2a} Subunits in AKAP79wt or AKAP79pro Cells

	α_{1C}				β_{2a}			
	AKAP79wt		AKAP79pro		AKAP79wt		AKAP79pro	
Treatment	*X* ± *SEM*	*N*	*X* ± *SEM*	*N*	*X* ± *SEM*	*N*	*X* ± *SEM*	*N*
Control	100	5	100	3	100	5	100	3
FSK	96 ± 6	4	99 ± 1	3	161 ± 11	5	120 ± 11	3
OA	115 ± 23	5	123 ± 16	3	122 ± 26	5	132 ± 16	3
OA + FSK	191 ± 19	5	117 ± 11	3	192 ± 21	5	187 ± 23	3
FK506 ± FSK	167 ± 33	5	133 ± 34	3	185 ± 29	5	158 ± 6	3

The phosphorylation levels of Ca^{2+} channel subunits were determined by normalizing the amount of phosphate incorporated to the amount of protein, as described in the Experimental Procedures. The data summarized in this table were obtained as described in Figure 2. The phosphorylation of the channel subunits under control conditions (i.e., basal phosphorylation) in both AKAP79 cells was expressed as 100%. The increases in phosphorylation upon PKA activation were compared to those of the controls. The values are means ± SEM, and the number of experiments is indicated as N.

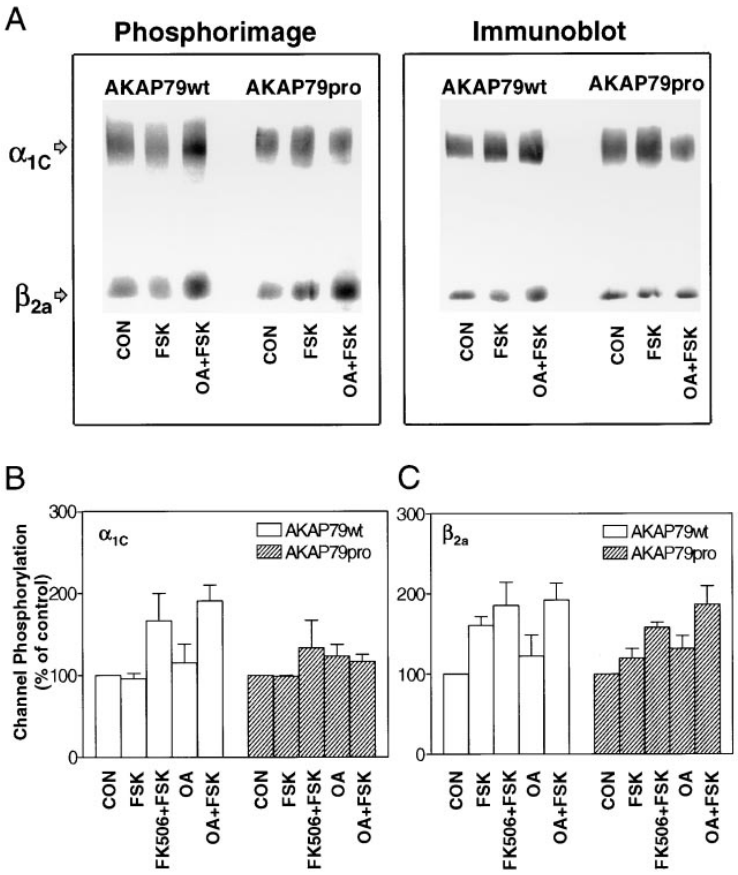

Figure 2. Phosphorylation and Immunoprecipitation of L-Type Ca^{2+} Channel α_{1C} and β_{2a} Subunits in Stable AKAP79wt or AKAP79pro Cells

Stable AKAP79 cells transiently transfected with the α_{1C} and β_{2a} subunits were labeled with ^{32}P and subsequently incubated with indicated agents. The Ca^{2+} channel subunits were solubilized and immunoprecipitated with the Card I antibody and separated by SDS-PAGE, followed by phosphorimage and immunoblot analysis. (A) shows a representative phosphorimage and immunoblot. (B) and (C) show averaged results from 4–5 independent experiments. The data from the phosphorimage analysis were normalized to the amount of protein loaded per lane, as assessed from the densitometric analysis of the immunoblot. The basal phosphorylation of both channel subunits in control conditions was expressed as 100%, and the increases in phosphorylation were compared to the basal phosphorylation in control cells.

(Figure 3). Transient transfection of the α_{1C} and β_{2a} subunits resulted in the expression of inward currents carried by 40 mM Ba^{2+} (I_{Ba}) in both AKAP79wt or AKAP79pro cell lines. There were no variations in current density or voltage-dependent properties in Ca^{2+} channel currents expressed in both types of cells, suggesting that the basal Ca^{2+} channel activity was not affected by transfection with either the wild-type or the mutant AKAP79 protein. The average expressed current (data pooled from both types of cells) was 516 $\pm$ 47 pA (range, 70–2000 pA; n = 65) for all cells studied.

An example of the currents recorded from a transfected and an untransfected AKAP79wt cell are shown in Figure 3. Cells transfected with the α_{1C} and β_{2a} subunits showed marked voltage-activated inward I_{Ba}, which inactivated slowly during depolarizing steps (Figure 3A). In contrast, untransfected cells did not demonstrate any measurable inward current at any potential (Figure 3B). A representative current–voltage (I–V) relationship obtained from transfected and untransfected AKAP79wt cells is also shown (Figure 3C). In transfected cells, I_{Ba} activated at potentials around -30 mV, peaked near 20 mV, and reversed around 60 mV. The voltage dependence of the current activation and inactivation of I_{Ba} was also characterized (Figure 3D). The absence of any endogenous voltage-activated inward currents in untransfected HEK cells agrees with previous studies (Perez-Reyes et al., 1994; Perez-Garcia et al., 1995).

The currents expressed in both AKAP79wt and AKAP79pro cells displayed a sensitivity to Ca^{2+} channel agonists and antagonists similar to those observed in native cardiac L-type channels (data not shown). The currents carried by Ca^{2+} (I_{Ca}) inactivated more rapidly during depolarization, and the peak I_{Ca} was much smaller compared to Ba^{2+} as the charge carrier in these cells (data not shown). Since we could record inward currents with higher resolution, Ba^{2+} was used as the charge carrier throughout the rest of this study.

We tested whether Ca^{2+} channel currents expressed in either AKAP79wt or AKAP79pro cells are responsive to agents that increase cAMP. Although the number of cells responsive to FSK was similar for AKAP79pro and AKAP79wt cells ($\sim$50%), the increase in peak I_{Ba} by FSK was greater in AKAP79wt than in AKAP79pro cells (Figure 4A). The data shown are from all cells tested (responsive cells plus cells with an unclear response). Addition of FSK increased the amplitude of I_{Ba} by 1.14 $\pm$ 0.05-fold (n = 10) in AKAP79pro cells and by 1.32 $\pm$ 0.08-fold (n = 15) in AKAP79wt cells.

To examine the possibility that the failure to observe cAMP-dependent phosphorylation effects was caused by endogenous protein phosphatase activity, we repeated these experiments in the presence of OA. Cells were preincubated with OA (100–200 nM) for 1–2 hr, and currents were measured in the presence of OA (2 μM) in the internal solution. Neither preincubation nor internal application of OA significantly affected the maximal conductance of I_{Ba} by itself. However, when OA was used, the number of cells responsive to external application of FSK was significantly increased in both cell lines. In addition, OA significantly augmented the effects of FSK in AKAP79wt cells but had no effect in AKAP79pro cells

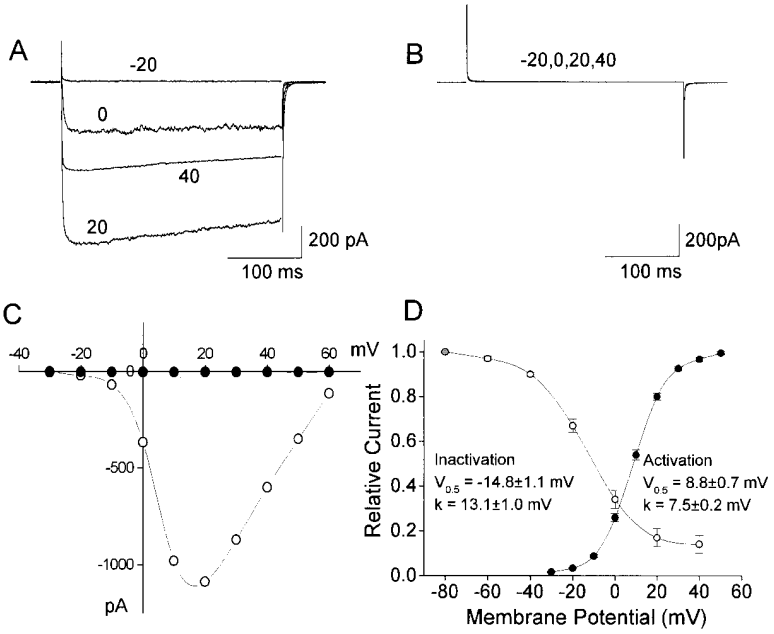

Figure 3. Properties of Expressed Ca^{2+} Channels

Whole-cell currents recorded from an AKAP79wt cell transfected with the α_{1C} and β_{2a} subunits (A) and from an untransfected AKAP79wt cell (B). Traces show currents recorded from a holding potential of −80 mV to the indicated test potentials. The current–voltage curves obtained from the transfected cell (open circles) and the untransfected cell (closed circles) are shown in (C). The voltage dependence of activation (closed circles) and inactivation (open circles) of the Ca^{2+} channel current are shown in (D). The voltage dependence of the current activation was determined by measuring the peak current elicited by depolarizing pulses to various potentials. Conductance was then calculated by dividing the peak current by the driving force. The apparent reversal potential was obtained by extrapolating the peak I–V plot through the zero current axis. These normalized values were then fit to a Boltzmann equation (Equation 1) to obtain the midpotential ($V_{0.5}$) and the slope factor (k). The voltage-dependent channel inactivation was determined by applying 5 s depolarizing pulses to different potentials and then measuring the peak current evoked in response to a subsequent test pulse to 10 mV; the gap between the two pulses was 15 ms. The prepulse-inactivation curves were fitted to the Boltzmann equation (Equation 2). Since no variations in the voltage dependence of activation and inactivation were observed in Ca^{2+} channel currents recorded from AKAP79wt or AKAP79pro cells, data were pooled and plotted. For activation kinetics, the average $V_{0.5}$ and k were 9.0 ± 0.8 mV and 7.7 ± 0.2 mV in AKAP79wt cells (n = 43) and 8.4 ± 1.5 mV and 7.1 ± 0.4 mV for AKAP79pro cells (n = 22). For inactivation kinetics, the average $V_{0.5}$ and k were −14.6 ± 2.0 mV and 13.4 ± 1.9 mV in AKAP79wt cells (n = 5) and −15.0 ± 1.3 mV and 12.7 ± 0.7 mV for AKAP79pro cells (n = 5).

(Figure 4A; 1.54 ± 0.09-fold in AKAP79wt cells and 1.14 ± 0.03-fold in AKAP79pro cells). These results agree with biochemical data presented earlier and suggest that phosphatase activity may contribute to the reduced responses to cAMP-dependent stimulation of the expressed Ca^{2+} channel currents in HEK cells. In the remaining studies, OA was therefore present to enhance such phosphorylation-dependent regulation of channel activity.

In addition to FSK, a membrane permeable cAMP analog, 8-Br-cAMP (50 μM), stimulated I_{Ba} gradually and reached a new steady-state level about 2–3 min after application. Figure 4B compares the effects of FSK and 8-Br-cAMP on peak I_{Ba} in AKAP79wt and AKAP79pro cells. Both FSK and 8-Br-cAMP were much more effective in AKAP79wt than in AKAP79pro cells. Extracellular perfusion with 8-Br-cAMP increased the amplitude of I_{Ba} by 1.35 ± 0.07-fold (n = 12) in AKAP79wt cells but only by 1.11 ± 0.05-fold in AKAP79pro cells (n = 6, $p < 0.05$). These results indicate that the presence of a functional AKAP79 protein is critical for cAMP-dependent modulation of Ca^{2+} channel currents expressed in HEK cells.

An example of the time course of the effect of FSK recorded in AKAP79wt cells is shown in Figure 5. The peak I_{Ba} was increased following the application of FSK plus OA and reached a new steady-state level about 2–3 min after application (Figure 5A). The peak I–V relationships ± FSK (Figure 5C) illustrated that FSK increased I_{Ba} at all potentials measured; however, the I–V relation in the presence of FSK was not simply a scaled-up version of the control I–V. This skewing was related to FSK-induced negative shifts (~5 mV) of the I–V relation.

Taken together, the biochemical and electrophysiological results clearly indicated that submembrane tethering of PKA through association with AKAP79 is a key factor that facilitates PKA-mediated phosphorylation and regulation of heterologously expressed cardiac L-type channels. Phosphorylation of the α_{1C} subunit seems to be critical for channel regulation, as conditions that did not allow for α_{1C} subunit phosphorylation also did not result in channel activation. In contrast, PKA anchoring had no significant effects on cAMP-dependent phosphorylation of the β_{2a} subunits. Phosphorylation of the β_2 subunit alone does not appear sufficient to activate currents, or may only allow the small increases in current seen under any of these conditions. Thus, PKA-mediated modulation of these Ca^{2+} channels is, most likely, largely due to phosphorylation of the α_{1C} subunits.

The Association of AKAP and PKA Is Required for β-AR-Mediated Regulation of L-type Ca^{2+} Channel Currents in Native Cardiac Myocytes

It is well established that activation of β-adrenergic receptors by isoproterenol (Iso) stimulates L-type Ca^{2+} channel currents through a cAMP- and PKA-dependent pathway in native cardiac myocytes (McDonald et al., 1994). To investigate the potential role of AKAP in regulating native cardiac Ca^{2+} channels, the effects of an anchoring inhibitory peptide, Ht 31, were tested on the enhancement of I_{Ca} by Iso in isolated murine cardiac myocytes. Ht 31 is a 24 amino acid peptide containing the minimal region required for RII subunit binding (Carr et al., 1992). Ht 31 can block the interaction between

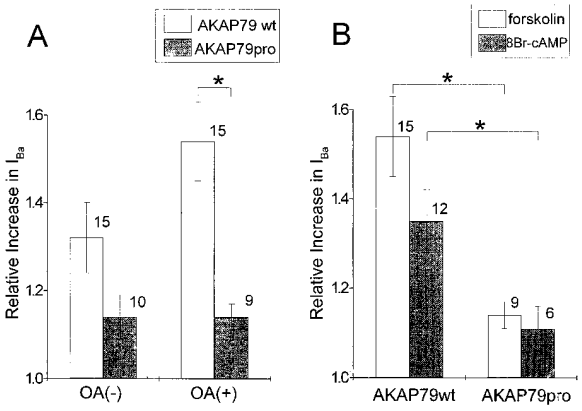

Figure 4. AKAP79 Is Required for PKA-Mediated Regulation of Expressed Ca^{2+} Channel α_{1C} and β_{2a} Subunits

(A) shows the mean effects of FSK (5 μM) on I_{Ba} recorded from AKAP79wt and AKAP79pro cells expressing the α_{1C} and β_{2a} subunits in the absence or presence of okadaic acid (OA). Numbers correspond to the total number of cells tested, including responsive and nonresponsive cells. The number of responsive (R) cells and those with unclear (U) effects were: for FSK alone, AKAP79wt, 9 (R), 6 (U); for FSK alone, AKAP79pro, 6 (R), 4 (U); for FSK plus OA, AKAP79wt, 14 (R), 1 (U); for FSK plus OA, AKAP79pro, 7 (R), 2 (U). In (B), the effects of FSK or 8-Br-cAMP on I_{Ba} in the presence of OA are compared. Numbers correspond to the total number of cells tested: for 8-Br-cAMP, AKAP79wt, 11(R), 1 (U); for 8-BR-cAMP, AKAP79pro, 7 (R), 2 (U). Values are mean ± SE. Significance between groups is indicated (*$p < 0.05$).

AKAPs and RII subunits, thus displacing PKA from its anchoring sites in intact cells to uncouple cAMP-responsive events (Carr et al., 1992; Rosenmund et al., 1994). Depolarization to 0 mV from a holding potential of −50 mV elicited an L-type Ca^{2+} channel current in cardiac myocytes that was completely blocked by 10 mM cadmium (data not shown). Perfusion of Iso (100 nM) increased the amplitude of I_{Ca} 2-fold (Figure 6A). However, when Ht 31 (100 μM) was included in the intracellular solution, the potentiation of the current by Iso was substantially reduced, without affecting the level of basal Ca^{2+} channel current (Figure 6C). Similar inhibition of Iso effects was obtained with a specific inhibitor of the catalytic subunit of PKA, PKI (5–24) amide (10 μM; Figure 6B). The effect of the Ht 31 peptide developed more slowly (5–10 min) than that of PKI (which occurred within 5 min), consistent with the concept that the Ht 31 peptide competes with AKAPs and leads to a gradual displacement of PKA from its anchoring sites (Rosenmund et al., 1994). As a negative control, an inactive peptide, Ht 31P, was tested under similar conditions. Ht 31P is identical to Ht 31, except that the isoleucine residue at position 10 is substituted with a proline, resulting in an inactive peptide that cannot interrupt the interaction between AKAP and PKA (Rosenmund et al., 1994). Intracellular application of Ht 31P (100 μM) did not affect the enhancement of I_{Ca} by Iso (Figure 6D). The effects of Iso on I_{Ca} with different intracellular solutions are summarized in Figure 6E. Under control conditions analyzed 10 min after establishing the whole-cell configuration, the mean Iso-promoted increase in I_{Ca} was 1.77 ± 0.14-fold ($n = 8$). This augmentation was significantly blocked by the AKAP inhibitory peptide Ht 31 (100 μM) to 1.17 ± 0.05 ($n = 15$) or by PKI (10 μM) to 1.10 ± 0.07 ($n = 8$). In contrast, the inactive form of the inhibitor, Ht 31P (100 μM) had no effect compared to control conditions (1.72 ± 0.10; $n = 12$). These results indicated that Iso-induced augmentation of Ca^{2+} channel currents in cardiac myocytes was specifically blocked by dissociating PKA from the AKAP anchoring sites with the inhibitory peptide Ht 31, and they strongly suggested that AKAPs played a critical role in the β-AR-mediated activation of L-type Ca^{2+} channels in native cardiac myocytes. These results complement those discussed earlier with the heterologous expression system and add further support to the conclusion that AKAP tethering of PKA is a key factor in the cAMP-mediated regulation of cardiac L-type Ca^{2+} channels.

Ser1928 of the α_{1C} Subunits Is Important in Mediating PKA-Induced Phosphorylation and Regulation of the Ca^{2+} Channels

Based on previous studies, in which a C-terminal truncated α_{1C} subunit purified from heart was found not to be a substrate of PKA in vitro (Chang and Hosey, 1988; Yoshida et al., 1992; De Jongh et al., 1996), we predicted that the PKA phosphorylation site(s) would be in the C-terminus of the α_{1C} subunits. A schematic figure shows the approximate locations of these putative PKA sites in the α_{1C} subunits (Figure 7A). We first tested a triple α_{1C} mutant, α_{1C}TriM (S1575A, T1626A, S1627I), in in vivo phosphorylation experiments. Both basal and PKA-stimulated phosphorylation of the α_{1C}TriM subunits were

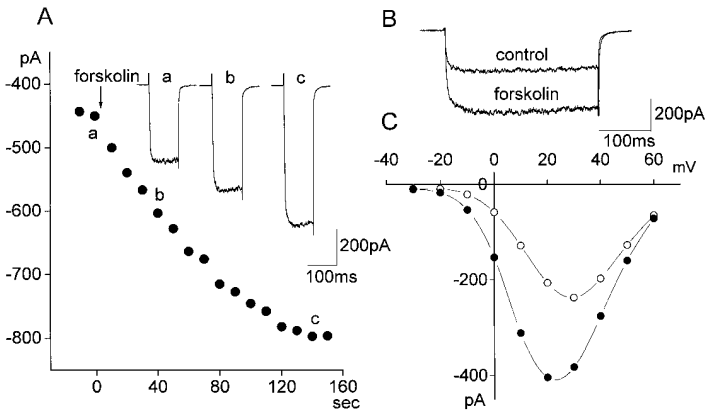

Figure 5. Time Course of Peak I_{Ba} during Superfusion with Forskolin (5 μM) in AKAP79wt Cell Expressing Ca^{2+} Channel α_{1C} and β_{2a} Subunits

(A) shows the time course of peak I_{Ba} during perfusion with FSK (in the presence of OA). Pulses to 10 mV were applied every 10 s from a holding potential of −80 mV. Insert shows the current traces indicated by a–c.

(B) shows the effects of FSK on voltage dependence of I_{Ba}. The currents recorded at 10 mV before (control trace) and 2 min after the application of FSK are shown. The holding potential was −80 mV.

(C) shows current–voltage relationships before (open circles) and after (closed circles) FSK.

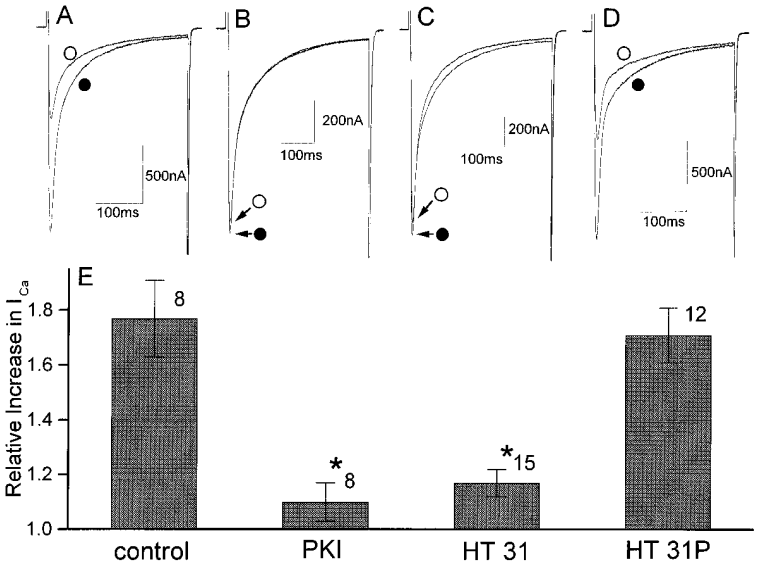

Figure 6. Effects of PKA Anchoring on Modulation of Isoproterenol (100 nM) Stimulated Ca^{2+} Channel Currents in Cardiac Myocytes

Current traces were recorded from a holding potential of −50 mV to 0 mV and were superimposed, before (open circles) and after (closed circles) superfusion with Iso. Iso was added 10 min after breaking into the cell with a pipette containing (A) control solution or (B) in the presence of 10 μM PKI (5–24), (C) 100 μM Ht 31, or (D) 100 μM Ht 31P. Note the differences in scaling used for the records shown. (E) shows the mean increases of I_{Ca} by Iso measured under the indicated conditions. Numbers correspond to the number of cells. Values are mean ± SE. Significance between groups is indicated (*$p < 0.05$).

the same as phosphorylation of the wild-type α_{1C} subunits (data not shown). To further narrow down the region containing potential PKA sites, we used a C-terminal truncation mutant of the α_{1C} subunit, α_{1C}del3′1773, generated by engineering a stop codon at residue 1773 (the full-length α_{1C} protein contains 2171 amino acid residues). This α_{1C} truncation mutant forms functional Ca^{2+} channels and conducts larger currents compared to the wild-type channel (Wei et al., 1994). We tested whether this truncated α_{1C} subunit could be phosphorylated by PKA in vivo (all the different mutant α_{1C} subunits were cotransfected with the β_{2a} subunits). The α_{1C}del3′1773 mutant subunit completely lost basal and PKA-stimulated phosphorylation (data not shown). Taken together, these results indicated that all basal and PKA-stimulated phosphorylation sites are at the C-terminus of α_{1C} subunits after residue 1773.

Previous studies have suggested that Ser1928 of the α_{1C} subunits might play a role in PKA-mediated regulation of cardiac Ca^{2+} channels (De Jongh et al., 1996; Mitterdorfer et al., 1996; Perets et al., 1996). In the present study, we transfected the AKAP79wt cells with either α_{1C}/β_{2a} or α_{1C}S1928A/β_{2a} subunits and performed in vivo phosphorylation experiments. The basal phosphorylation of the α_{1C}S1928A subunit was similar to that of wild-type α_{1C}; however, phosphorylation of the α_{1C}S1928A mutant did not increase following PKA activation (Figure 7B). This observation was further confirmed by studying the time course of phosphorylation in intact cells (Figure 7D). The α_{1C} and the β_{2a} (when complexed with wild-type α_{1C}) subunits became rapidly phosphorylated by PKA to their maximal levels within 2–5 min (Figure 7D). In contrast, the phosphorylation of the α_{1C}S1928A subunits remained the same as basal even after 10 min of treatment (Figure 7D). These results indicated that Ser1928 of the α_{1C} subunit is a key residue in mediating phosphorylation of the channel by PKA in vivo.

Phosphorylation of the β_{2a} subunits when complexed with α_{1C}S1928A was very intriguing. The basal phosphorylation of the β_{2a} subunits was increased (up to 2-fold) when coexpressed with the α_{1C}S1928A mutant (compared to when complexed with wild-type α_{1C}), and no further augmentation was obtained upon PKA activation (Figures 7C and 7D). Since the α_{1C} and β_{2a} subunits form tightly associated complexes (De Waard et al., 1995), the mutation at Ser1928 of the α_{1C} subunit might alter the interaction between the α_{1C} and β_{2a} subunits and make the β_{2a} subunit more accessible to kinases under basal conditions. The resulting increase of basal phosphorylation of the β_{2a} subunits might preclude any further PKA-induced phosphorylation.

The effect of the Ser1928 mutation on PKA-mediated regulation of the Ca^{2+} channel was assessed by parallel electrophysiological studies. The AKAP79wt cells were transfected with α_{1C}S1928A/β_{2a} subunits, and whole-cell currents were recorded (Figure 8A). The current–voltage relationship of the mutant channel was similar to that of the wild-type channel (compare Figures 3C and 8B). Cells were treated with OA and FSK, and I_{Ba} currents were recorded over a 2 min time course. In contrast to the wild-type channel, the peak currents of the α_{1C}S1928A mutant channel were not increased upon PKA activation (Figure 8C). The results obtained from the indicated number of cells were averaged and summarized in Figure 8D. The peak current of the α_{1C}S1928A mutant channel could not be stimulated by FSK or 8-Br-cAMP treatment, indicating that phosphorylation of Ser1928 is required for PKA-mediated regulation of cardiac Ca^{2+} channels.

Discussion

The regulation of cardiac L-type Ca^{2+} channels by the β-adrenergic receptor-mediated signaling pathway has been extensively studied electrophysiologically in cardiac myocytes (McDonald et al., 1994). However, biochemical studies have been hindered by the rareness of the channel proteins in native tissues. Several heterologous expression systems have been used to attempt to reveal the molecular events underlying channel regulation, but most of them failed to reproduce the receptor- and PKA-mediated regulation of the channel. Recently, several groups have shown that the α_{1C} subunit of cardiac L-type channels is a substrate of PKA in vitro (De

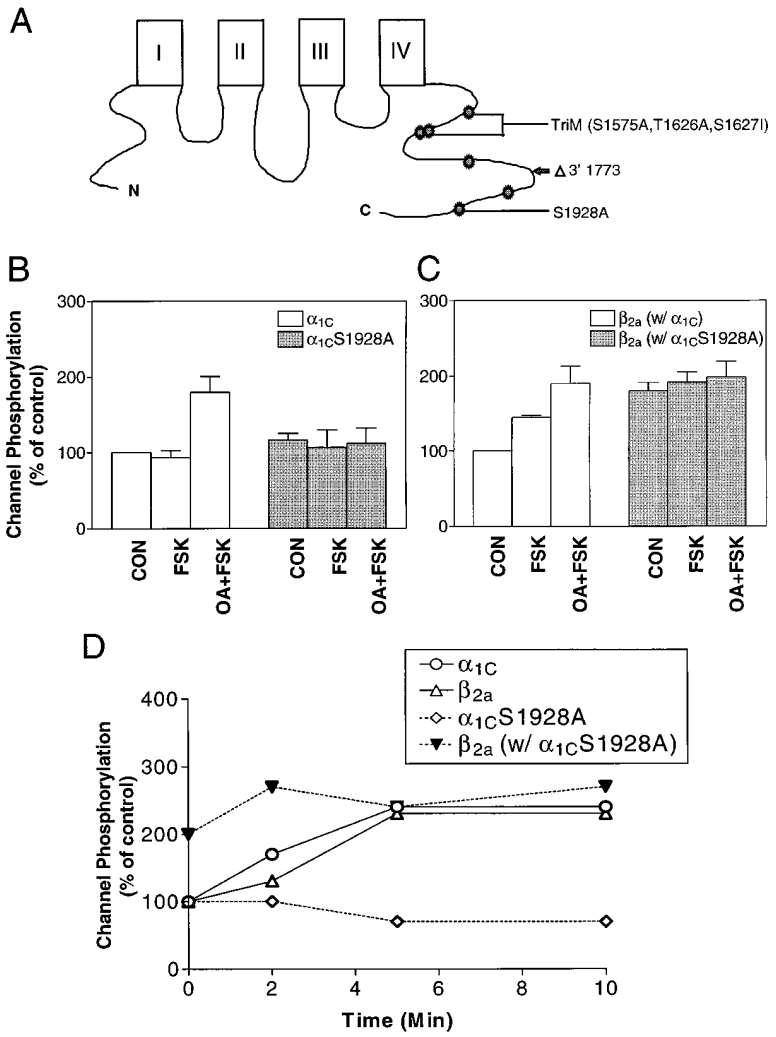

Figure 7. Effects of PKA Activation on Phosphorylation of α_{1C}S1928A and β_{2a} Subunits in AKAP79wt Cells

(A) shows a schematic representation of the α_{1C} subunit and the PKA site mutants tested in this study. Stable AKAP79wt cells were transfected with either α_{1C}/β_{2a} or α_{1C}S1928A/β_{2a} subunits. Cells were labeled with ^{32}P and treated with indicated drugs. The phosphorylation of channel subunits was analyzed as described in Figure 2. The basal phosphorylation of the α_{1C} and the β_{2a} subunit in control cells was expressed as 100%. The phosphorylation levels of the α_{1C}S1928A mutant and the β_{2a} (when complexed with α_{1C}S1928A) were all compared to the control α_{1C}/β_{2a} cells. Results from 3–5 independent experiments were quantified and are shown in (B) and (C). (D) shows the time course of phosphorylation of α_{1C}/β_{2a} and α_{1C}S1928A/β_{2a} in AKAP79wt cells. Cells were stimulated for 2, 5, or 10 min with 15 μM FSK after preincubation with 100 nM OA and 1 mM IBMX for 10–15 min. Phosphorylation of each channel subunit at the different time points was quantified as in Figure 2.

Jongh et al., 1996; Puri et al., 1997), but the correlation between channel phosphorylation and underlying functional changes was not identified due to lack of a suitable functional assay. In the present studies, we combined biochemical and electrophysiological approaches to demonstrate that both the α_{1C} and β_{2a} subunits are substrates of PKA in intact cells, and a functional AKAP79 is required for PKA-mediated phosphorylation of the Ca^{2+} channel α_{1C} subunit and the modulation of channel function. In addition, we demonstrated that the association between AKAP and PKA is critical for the β-AR-mediated activation of L-type Ca^{2+} channels in native cardiac myocytes. Furthermore, a site for PKA-induced phosphorylation in vivo and regulation of the channel has been mapped to Ser1928 of the α_{1C} subunit using site-directed mutants.

The requirement for PKA anchoring to enable Ca^{2+} channel phosphorylation was well demonstrated by comparing the results obtained from the AKAP79wt and AKAP79pro cells. The only difference between the wild-type and the mutant AKAP79 proteins is their ability to bind RII subunits of PKA (Carr et al., 1992; Figure 1B). However, the α_{1C} subunits were phosphorylated in response to PKA activation in AKAP79wt cells but not in AKAP79pro cells, and the Ca^{2+} currents from expressed channels were significantly stimulated by PKA in AKAP79wt cells but not in AKAP79pro cells. The extent of stimulation of the currents in the AKAP79wt cells was smaller than previously observed in some atrial and ventricular myocytes, but similar to the extent of stimulation we observed in the native murine cardiac myocytes. The parental HEK293 cells express a low level of endogenous AKAP79, and the RII and C subunits of PKA were mainly found in cytosolic fractions (Ndubuka et al., 1993; Figure 1A). Others (Zong et al., 1995) have observed a lack of PKA-mediated modulation of the channel in native HEK cells, and we were unable to detect PKA-induced phosphorylation of the α_{1C} subunit in native HEK cells. These results are likely due to the fact that the levels of the kinases and AKAPs at the plasma membrane is insufficient to allow for PKA-mediated phosphorylation of the α_{1C} subunit. Anchoring of PKA to cell membranes by AKAPs not only increases the local concentration of the enzyme, but may also help to determine the specificity of phosphorylation events by providing a linkage between the kinase and its substrates. It has been suggested that the action of serine/

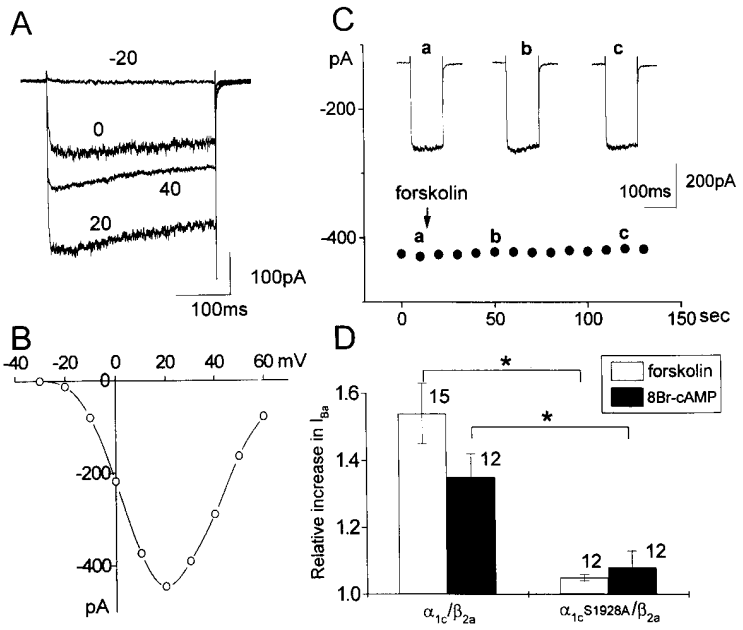

Figure 8. Whole-cell Currents and Modulation of I_{Ba} in AKAP79wt Cells Expressing the α_{1C}S1928A and β_{2a} Subunits by cAMP-Dependent Phosphorylation

(A) shows whole-cell currents recorded from a holding potential of −80 mV to the indicated test potentials. The corresponding current–voltage curve is shown in (B). (C) shows the time course of peak I_{Ba} during superfusion with FSK (5 μM). Pulses to 10 mV were applied every 10 s from a holding potential of −80 mV. Inset shows the current traces indicated by a–c. (D) shows mean effects of FSK (5 μM) and 8-Br-cAMP (50 μM) on I_{Ba} recorded from AKAP79wt expressing α_{1C}/β_{2a} (reproduced from data in Figure 4 for comparison) or α_{1C}S1928A/β_{2a}. Numbers correspond to the number of cells. Values are mean ± SE. Significance between groups is indicated (*$p < 0.05$).

threonine kinases (i.e., PKA and PKC) can be well regulated and restricted through protein–protein interactions by using anchoring proteins as common mediators (Faux and Scott, 1996).

A muscle-specific isoform of the AKAPs, AKAP100, has been cloned recently (McCartney et al., 1995b). AKAP100 has a similar RII subunit binding domain as all known AKAPs and is expressed exclusively in cardiac and skeletal muscle (McCartney et al., 1995b). In the present study, we demonstrated that application of the inhibitory peptide Ht 31 was able to block the Iso-induced augmentation of L-type Ca^{2+} currents, while the nonfunctional peptide Ht 31P had no effect in native cardiac myocytes. These data strongly suggested that the association between an AKAP and PKA is required for β-AR-mediated regulation of the channels in heart cells. Interestingly, a very recent study suggested that an AKAP may be closely associated with the skeletal muscle Ca^{2+} channel (Gray et al., 1997).

In the present study, we demonstrated that the PKA-mediated regulation of the cardiac L-type Ca^{2+} channel can only be detected when the phosphorylation of the α_{1C} subunits is stimulated by PKA. A lack of the PKA-mediated phosphorylation of the α_{1C} subunits, as in the AKAP79pro cells, leads to loss of stimulation of Ca^{2+} currents by PKA in intact cells. This finding provides direct and compelling evidence that the phosphorylation of the α_{1C} subunit is necessary for the PKA-mediated regulation of channel function. Moreover, an important site of phosphorylation on the α_{1C} subunit responsible for the regulation was identified as Ser1928 using site-directed mutants. That this site might be important was first suggested by indirect results obtained in oocytes (Perets et al., 1996). It also has been shown that the native α_{1C} subunits isolated from heart are substrates of PKA in vitro, and one site of phosphorylation was identified as Ser1928 using a phosphoserine sensitive antibody (De Jongh et al., 1996). A chimeric channel with the C-terminus of the cardiac L-type channel was expressed in yeast and was also phosphorylated by PKA in vitro at Ser1928 (Mitterdorfer et al., 1996). In this study, we were able to provide direct evidence that Ser1928 of the α_{1C} subunit is required for PKA-mediated phosphorylation of the α_{1C} subunits and the augmentation of channel peak currents in intact cells. All the mutant α_{1C} subunits tested here bound dihydropyridines in a manner similar to that of the wild-type α_{1C} subunit (data not shown), suggesting that these mutations do not disrupt the basic channel structure and drug binding properties.

The effect of phosphorylation of the β_{2a} subunit is less clear at this point. The β_{2a} subunit has been shown to be an excellent substrate of PKA both in vitro (Puri et al., 1997) and in vivo (Figure 2). However, loss of PKA-mediated regulation of the Ca^{2+} channels in AKAP79pro cells was not accompanied by loss of phosphorylation of the β_{2a} subunit, suggesting that PKA-induced phosphorylation of the β_{2a} subunit is not sufficient to significantly modulate channel function. In addition, these data demonstrated that the phosphorylation of the β subunit did not require the AKAP, suggesting a more cytoplasmic orientation of the phosphorylation site and its accessibility to cytoplasmic PKA. On the other hand, the Ca^{2+} channels formed by the α_{1C}S1928A and β_{2a} subunits were not regulated by PKA, and neither channel subunit was phosphorylated upon PKA activation. In addition, the fact that the basal phosphorylation of the β_{2a} subunits was increased when coexpressed with α_{1C}S1928A suggests that the phosphorylation state of the β_{2a} subunits is influenced by complexing with different α_{1C} subunits. Further studies are required to assess these intriguing results and the effect of subunit phosphorylation in modulating channel functions.

In summary, we have shown that membrane tethering of PKA by AKAP79 is required for PKA-mediated phosphorylation of the L-type Ca^{2+} channel α_{1C} subunits and

for PKA-induced augmentation of Ca^{2+} currents in HEK293 cells. In addition, we have demonstrated that the interaction between AKAP and PKA is important for β-AR-mediated regulation of Ca^{2+} channels in native cardiac myocytes. A key site of PKA-mediated phosphorylation of the α_{1C} subunits has been identified as the C-terminal residue Ser1928; phosphorylation of this residue is necessary for regulation of the channel by PKA. The in vivo phosphorylation of the β_{2a} subunit has been demonstrated, and the functional effects of this subunit will be determined by future studies.

Experimental Procedures

Materials

The expression construct pRSα_{1C}del3′1773 was a gift from Drs. Roman E. Shirokov and Eduardo Rios (Rush University). FK506 was a gift from Dr. Neil A. Clipstone (Northwestern University). Okadaic acid (OA) was purchased from Gibco/BRL. Forskolin was obtained from Calbiochem. ^{35}S[methionine] and $^{32}P_i$[orthophosphate acid] were purchased from Amersham. All other reagents were from standard sources.

Antibodies

Ca^{2+} channel antibodies, including the α_{1C} subunit-specific Card C and Card I and the β subunit-directed anti-β_2 and -β_{gen} antibodies were prepared and characterized previously (Chien et al., 1995, 1996). Specific anti-RII rabbit polyclonal antibody, which was generated against murine RII subunits, and which cross-reacts with human RII subunits, was provided by Dr. J. Goldenring (Medical College of Georgia). Anti-AKAP79 monoclonal antibody (mAb) was obtained from ICOS Corporation.

Preparation of Expression Vectors

The pSP72α_{1C}S1928A and pSP72α_{1C}TriM constructs (Perets et al., 1996) were first digested with *Kpn*I and blunt-ended with Klenow; subsequently, the α_{1C} mutant sequences were excised from the pSP72 vector using *Xho*I. The mutant cDNAs were then cloned into *Sal*I/*Sma*I sites of the pCMV5 polylinker. Other expression vectors used in this study were prepared as described previously (Chien et al., 1995, 1996).

Cell Culture and Transfection

The stable HEK293 cell lines, AKAP79wt and AKAP79pro, were generated as described previously (Klauck et al., 1996). Cells were maintained in DMEM (Gibco/BRL) containing 10% fetal bovine serum (Atlanta Biologicals), 500 μg/ml G418 (Gibco/BRL), and 1% penicillin/streptomycin at 37°C in 5% CO_2. Transient expression of various Ca^{2+} channel subunits in AKAP79wt or AKAP79pro cells was carried out using the calcium phosphate precipitation method (Chien et al., 1995).

Immunofluorescence Microscopy

HEK293 and AKAP79 cells were seeded on acid-washed glass coverslips in growth media 48 hr prior to the day of experiments. Cells were washed twice in PBS, fixed in 3.7% formaldehyde/PBS, and permeabilized with acetone for 1 min. The fixed and permeabilized cells were then washed with PBS and blocked in labeling buffer (0.1% BSA/PBS) for 30 min. Cells were colabeled with anti-AKAP79 mAb (1:250) and anti-RII antibody (1:2000) and visualized using anti-rabbit-FTIC and anti-mouse-Texas Red antibodies (Molecular Probes). The stained coverslips were mounted on slides using a Molecular Probes Slow Fade Kit and were viewed with a laser-scanning confocal microscope (Leitz). A single representative focal plane through the approximate center of the analyzed cells is shown in Figure 1A.

Immunoblotting and ^{32}P-RII Overlay Blotting of AKAP Proteins

Stable AKAP79 cells were washed twice in PBS and lysed in lysis buffer (1% Triton X-100, 25 mM Tris [pH 8.0], 140 mM NaCl, 10 mM EDTA, 2 μg/ml leupeptin/pepstatin, 1 mM benzamadine, and 1 mM AEBSF), followed by centrifugation (40,000 × g, 15 min). Proteins (50 μg) in the cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters. The anti-AKAP79 mAb (1:1000) and secondary anti-mouse horseradish peroxidase (HRP) (Amersham) were used for immunoblotting, and detection was with enhanced chemiluminescence (ECL; Pierce). ^{32}P-labeled RII subunits were used for overlay blotting as described previously (Carr and Scott, 1992).

In Vivo Phosphorylation and Immunoprecipitation

Approximately 40 hr posttransfection, transiently transfected AKAP79 cells were labeled for 4 hr with 0.1–0.25 mCi/ml $^{32}P_i$ in phosphate-free DMEM (without serum). The cells were then treated at room temperature with either (a) 15 μM FSK + 1 mM IBMX for 10 min; (b) 100 nM OA for 10–15 min; (c) 100 nM OA for 10 min, then with FSK and IBMX as in (a); or (d) 10 ng/ml FK506 during $^{32}P_i$ labeling, then with FSK and IBMX as in (a). Control cells were treated with DMSO only. After washing with ice cold PBS, the cells were harvested in phosphate buffer (PB; 20 mM Na_2HPO_4 [pH 7.4], 20 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, and protease inhibitors; Chien et al., 1995), and crude membranes were prepared as described (Pals-Rylaarsdam et al., 1995). The membranes were solubilized using solubilization buffer (0.1% SDS, 1% Triton X-100, and 500 mM NaCl in PB containing protease inhibitors [pH 7.4]). Solubilized proteins were immunoprecipitated overnight using Card I and/or the β_{gen} antibody coupled to protein G-Sepharose. Since the interaction between the α_{1C} and the β_{2a} subunits is almost irreversible (De Waard et al., 1995), the α_{1C} and β_{2a} subunits coprecipitated by using either Card I (as shown in Figure 2A) or the β_{gen} antibody alone (data not shown) in the presence of a low concentration of SDS. The immunoprecipitates were washed 8–10 times alternately in solubilization buffer and wash buffer (1% Triton X-100 and 250 mM NaCl in PB [pH 7.4]) and subsequently separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting and phosphorimaging (Pals-Rylaarsdam et al., 1995).

Analysis of Phosphorylated Ca^{2+} Channel Subunits

For detection of the immunoprecipitated α_{1C} and the β_{2a} subunits, the nitrocellulose filters were blotted with Card C and the anti-β_2 antibody. ECL and HRP were used as the detection method. In order to calculate the stoichiometry of phosphorylation of the channel subunits, it was necessary to calculate both the quantity of channel protein in each immunoprecipitate and the amount and specific activity of ^{32}P incorporated. As the amount of protein in each immunoprecipitate varied to some degree, the amount of protein was assessed using a densitometric analysis to compare the immunoreactivity of the precipitated proteins to a linear immunoreactivity standard curve, generated by using known amounts of channel subunits (Pals-Rylaarsdam et al., 1995). The standard curves were constructed as described (Puri et al., 1997). The phosphorylation levels of the α_{1C} and β_{2a} subunits were assessed by phosphorimaging and ^{32}P standards. The specific activity of ATP in the cells was determined as described (Richardson and Hosey, 1992). In order to quantitate the amount of ^{32}P in each immunoprecipitate and to take into account the variations in the amount of protein in each immunoprecipitate, we expressed the results as the amount of ^{32}P incorporated/unit protein. Since both channel subunits were basally phosphorylated to a significant degree, we compared all increases in phosphorylation to the basal level of phosphorylation of each subunit in control cells, which we expressed as 100% in each experiment.

Electrophysiology

For electrophysiological measurements, transfected cells were seeded onto glass coverslips 24 hr after transfection and incubated in culture medium for 1–4 days. Whole-cell currents were recorded by patch-clamp techniques as previously described (Yatani et al., 1994). The external solution contained 40 mM $BaCl_2$ or $CaCl_2$, 1 mM $MgCl_2$, 80 mM tetraethyl ammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, and 10 mM HEPES (pH 7.3). The control pipette solution contained 130 mM CsCl, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM GTP, 5 mM EGTA, amd 5 mM HEPES (pH 7.3). The patch pipettes had

resistances of 2–4 MΩ. Currents elicited in response to depolarizing steps were recorded from a holding potential of −80 mV. Cell capacitance was measured using voltage ramps of 0.8 V/s from a holding potential of −50 mV. The average cell capacity was 21.6 ± 0.7 pF (n = 65). Current density was expressed as pA/pF by dividing peak inward current by cell density. Experimental data are shown as raw currents, without capacity or leak-current subtraction. The amplitude of Ca^{2+} channel currents was measured by subtracting leak currents using P/4 protocol or subtracting currents with and without cadmium (100 μM).

The conductance–voltage relations were determined using an interactive nonlinear regression fitting procedure to the Boltzmann equation (Equation 1): $G/G_{max} = 1/\{1 + \exp[(V^{0.5} - V_m)/k]\}$ where V_m is the membrane potential, $V_{0.5}$ is the midpotential and k is the slope factor, and $G_{max} = I_{max}/(V_m - E_{rev})$. Similarly, voltage-dependent inactivation was determined using the Boltzmann equation (Equation 2): $I/I_{max} = 1/\{1 + \exp[(V_m - V_{0.5})/k]\}$.

Single ventricular myocytes were prepared from adult mouse hearts as described previously (Masaki et al., 1997). Ca^{2+} channel currents were isolated from other membrane currents by placing cells in an external solution containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 135 mM tetraethyl ammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, and 10 mM HEPES (pH 7.3). The patch pipettes had a resistance of 2 MΩ or less and contained 100 mM Cs aspartate, 20 mM CsCl, 1 mM $MgCl_2$, 2 mM MgATP, 0.5 mM GTP, 5 mM EGTA, and 5 mM HEPES (pH 7.3). Depolarizing pulses were applied every 10 s from a holding potential of −50 mV. PKI (5–24) amide, Ht 31, and Ht 31P peptides (Rosenmund et al., 1994) were dissolved in DMSO as 10 mM stock solution and were added to the patch pipette solution as noted. The final concentration of DMSO was less than 1%, and this concentration had no effect on Ca^{2+} channel currents (data not shown).

Solution changes were made using a modified Y-tube technique (Yamamoto et al., 1996). To avoid accumulation of test substances in the bulk solution, the chamber was continuously perfused at 5 ml/min. To minimize the contribution of run up or run down of channel activity, the effects of drugs were determined 2–3 min after obtaining the whole-cell patch-clamp configuration. All experiments were done at room temperature (20°C–21°C). Comparisons between conditions were evaluated using the Student's t test, with significance imparted at the $p < 0.05$ level.

Acknowledgments

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