Functional Regulation of L-type Calcium Channels via Protein Kinase A-mediated Phosphorylation of the β2 Subunit*

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Moritz Bünnemann, Brian L. Gerhardstein, Tianyan Gao, and M. Marlene Hosey†
From the Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois 60611

Activation of protein kinase A (PKA) through the β-adrenergic receptor pathway is crucial for the positive regulation of cardiac L-type currents; however it is still unclear which phosphorylation events cause the robust regulation of channel function. In order to study whether or not the recently identified PKA phosphorylation sites on the β2 subunit are of functional significance, we coexpressed wild-type (WT) or mutant β2 subunits in tsA-201 cells together with an α1C subunit, α1CΔ1905, that lacked the C-terminal 265 amino acids, including the only identified PKA site at Ser-1928. This truncated α1C subunit was similar to the truncated α1C subunit isolated from cardiac tissue not only in size (∼190 kDa), but also with respect to its failure to serve as a PKA substrate. In cells transfected with the WT β2 subunit, voltage-activated Ba2+ currents were significantly increased when purified PKA was included in the patch pipette. Furthermore, mutations of Ser-478 and Ser-479 to Ala, but not Ser-459 to Ala, on the β2 subunit, completely abolished the PKA-induced increase of currents. The data indicate that the PKA-mediated stimulation of cardiac L-type Ca2+ currents may be at least partially caused by phosphorylation of the β2 subunit at Ser-478 and Ser-479.

It has been known for more than a decade that the cardiac L-type calcium channel is an important effector for positive modulation of cardiac contractility through signaling cascades initiated by activation of the β-adrenergic receptors (1). It is well accepted that activation of protein kinase A (PKA) through the βAR pathway is crucial for the positive regulation of cardiac L-type Ca2+ channels. The β2 subunits are composed of α1C, β2, and α2δ subunits (2, 3), and both the α1C and β2 subunits have been demonstrated to be direct targets of PKA-mediated phosphorylation (4–7). However, it has been difficult to elucidate how the phosphorylation of each of these subunits might contribute to functional regulation of the channels in intact cells and to assign specific roles of the multiple sites of phosphorylation to specific functional changes in channel properties. Studies in intact cardiac myocytes are extremely difficult due to the low abundance of channel proteins, thus studies in heterologous expression systems have the potential to define the roles of subunit phosphorylation in the regulation of the channels. However, a problem with this approach is that it has been difficult to reconstitute in heterologous expression systems the robust regulation of L-type channels that is observed in cardiac cells (8).

Cyclic AMP-dependent phosphorylation and functional regulation of the channels was facilitated in human embryonic kidney cells when the channels were coexpressed with the protein kinase A-anchoring proteins AKAP79 and AKAP15/18 (6, 9). While both the α1C and β2 subunits were phosphorylated when coexpressed with AKAP79, only phosphorylation of serine (Ser) 1928 in the pore-forming α1C subunit appeared to be functionally linked to channel regulation (6). However, compared with the robust PKA-mediated stimulation of native L-type currents (3–6-fold in many species), the effects of PKA in the heterologous expression systems in the presence of either AKAP were rather small (50% increase in peak currents) (6, 9).

It is not known whether or not phosphorylation of the β2 subunit plays a functional role in channel regulation. The goal of this study was to test whether or not PKA-mediated phosphorylation of the β2 subunit has functional consequences. The β2 subunit has been shown to undergo cAMP-dependent phosphorylation at multiple sites in vitro and in cardiac myocytes and intact hearts (12–14). While the rat β2a subunit has two consensus sequences at Thr-164 and Ser-591 that might serve as PKA sites, these sites are not phosphorylated by PKA (14). Rather, the actual sites of PKA-mediated phosphorylation on the β2a subunit are Ser-459, Ser-478, and Ser-479 (14). An additional goal of this study was to determine which, if any, of these sites might mediate functional changes in channel activity. In order to prevent contributions from the previously identified PKA phosphorylation site on the α1C subunit and to mimic conditions that may exist in native systems, we utilized a truncation mutant of the α1C subunit, α1CΔ1905, that lacked the C-terminal 265 amino acids, including the only identified PKA site at Ser-1928.

MATERIALS AND METHODS

Cell Culture—tsA-201 cells (large T antigen-transformed human embryonic kidney cells) were maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units of penicillin and streptomycin (Life Technologies, Inc.) in an humidified atmosphere containing 5% CO2. TsA cells were transiently cotransfected with the truncated α1CΔ1905 subunit (rabbit) and either the wild-type (WT) rat β2a subunit or the S459A or S478A/S479A β2a mutants. Each construct was in the pCR3 vector and 3 μg of each was used per 10-cm plate, along with the CD8 reporter vector, pH3-CD8.
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(0.5 µg/10 cm plate) (15), using the Effectene transfection kit following the manufacturer's recommended protocol (Qiagen). Cells were replated 24–48 h following transfection on 3-cm plates that were previously coated with collagen (Sigma). Transfected cells were visualized by using the CDS receptor as a reporter gene and marking transfected cells with anti-CDS antibody coated Dynabeads (Dynal) (15).

In Vitro Phosphorylation of Calcium Channel Subunits by PKA—The wild-type α1c or the truncation mutant α1cΔ1905 subunits were coexpressed with the WT β2 subunit in tsA-201 cells. Whole cell lysates were prepared from the transfected cells using lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, and protease inhibitors (11)). The channel subunits were immunoprecipitated from the cell lysates using an α1c subunit-specific antibody, Card I, coupled to Ultra-Link protein G (Pierce). Native α1c subunits from rabbit heart were assayd in the phosphorylation experiments as well. To isolate the native α1c subunits, crude membranes from frozen rabbit heart were prepared. For immunoprecipitation, membranes were solubilized in lysis buffer and incubated with the Card I antibody coupled to protein G (11). The immunoprecipitated channel subunits from transfected tsA cells and rabbit heart were phosphorylated with the purified catalytic subunit of PKA following the procedures described previously (4). The phosphorylated channel proteins were separated by SDS-polyacrylamide gel electrophoresis followed by phosphorimaging and immunoblot analysis. Detection of the α1c subunits on the immunoblot was with biotinylated Card I.

**Solutions**—For the measurement of Ba2+ currents through L-type calcium channels, the external solution consisted of 10 mM BaCl2, 105 mM NaCl, 25 mM CsCl, and 10 mM Hepes, pH 7.4. The pipette solution was composed of 100 mM cesium aspartate, 40 mM CsCl, 1 mM MgCl2, 2 mM Mg-ATP, 0.5 mM GTP, 5 mM EGTA, and 5 mM Hepes, pH 7.4. When present, PKA was included in the pipette at a final concentration of 20 nM. Current measurement—Ba2+ currents through L-type calcium channels were measured in the whole cell configuration of the patch clamp technique using fire-polished borosilicate glass pipettes (GF-150–10, nA) Currents were measured in the whole cell configuration of the patch clamp amplifier (Axopatch 200, Axon Instruments). Signals were analog-filtered using a low pass Bessel filter (1–3 kHz corner frequency). Data were digitally stored using an IBM-compatible PC equipped with a hardware/software package (ISO2 by MFK) for voltage control, data acquisition, and data evaluation.

After establishing the whole cell configuration, cells were clamped at −90 mV and voltage pulses (test pulses) (100-ms duration) to +10 mV were applied every 10 s in order to activate L-type calcium channels. After reaching a steady state of the current amplitude (define as current amplitude that did not change in three subsequent test pulses), which took 3–5 min, the current-voltage relationship (I-V curve) was measured by varying the potential of the test pulses from −80 to +50 mV. Capacitative currents due to recharging the cell membrane were compensated. Currents were normalized to capacitance.

The activity of protein kinase A was purified to homogeneity from bovine heart (17). In all experiments in which the effect of PKA was studied, control experiments (without PKA) were performed in parallel in order to minimize variations in current amplitudes due to differences from transfection to transfection. The summarized data were pooled from at least three different transfections, unless otherwise noted and are expressed as mean ± S.E.

**RESULTS**

Can PKA regulate channels lacking a phosphorylatable α1c subunit? The first question we addressed was whether or not PKA can regulate cardiac L-type calcium channels comprised of a wild-type β2 subunit and a truncated α1c subunit that lacks Ser-1928, the site that was previously shown to be phosphorylated both in vitro (4, 5) and in intact cells (6). The truncated α1c subunit was isolated in these studies because its deletion results in the loss of its C terminal domain downstream of residue 1905 (α1cΔ1905). This mutant α1cΔ1905 subunit had a similar molecular mass when analyzed by SDS-polyacrylamide gel electrophoresis as the truncated α1c subunit isolated from cardiac tissue (Fig. 1). Importantly, α1cΔ1905 was not a substrate for PKA (Fig. 1), confirming that Ser-1928 is the sole site phosphorylated by PKA in the α1c subunit (4, 6).

Representative voltage-dependent Ba2+ currents through channels consisting of α1cΔ1905 and WT β2 subunits were elicited in response to depolarization to different test potentials. The current voltage relationship exhibited the typical properties of Ba2+ currents through L-type Ca channels (Fig. 2).

In order to test the effects of PKA on these currents, the purified catalytic subunit of PKA was added to the patch pipette at a final concentration of 20 nM. This resulted in an approximately two-fold increase (from −47.5 ± 11.4 pA/pF to −116.2 ± 26.2 pA/pF at 0 mV test potential, n = 10–11) in current amplitude of the Ba2+ current generated by the channels formed by the α1cΔ1905 and the WT β2 subunits (Fig. 2, A and B). These results demonstrated that PKA could indeed cause increases in currents generated from channels lacking a phosphorylatable α1c subunit. In addition, the 2-fold increase in peak current amplitude resembled that seen in native cardiac myocytes. On the other hand, no apparent hyperpolarizing shift in the current-voltage (I-V) curve of the Ba2+ current was observed. This latter effect is routinely observed in native cardiac myocytes.

In order to test whether or not the PKA-mediated increase in Ba2+ currents was due to phosphorylation of the β2a subunit, we expressed the α1cΔ1905 subunit with mutant β2a subunits that lacked the identified PKA phosphorylation sites (14). These mutants contained point mutations of serines to alanines either at position 459 or 478/479 (14). Voltage-dependent Ba2+ currents in cells expressing α1cΔ1905 and mutant β2a S459A were indistinguishable from currents obtained in cells expressing the WT β2a subunit (Fig. 3), indicating that this mutation did not alter the basic functional properties of the regulatory β2a subunit. The addition of the catalytic subunit of PKA to the patch pipette caused a significant increase in Ba2+ currents compared with the controls (−138.1 ± 37.7 pA/pF versus −28.2 ± 7.0 pA/pF at 0 mV, n = 6–7) in cells expressing α1cΔ1905 and the mutant β2a S459A (Fig. 3, A and B). This effect was comparable with that observed with the WT β2a subunit (compare Fig. 2 with Fig. 3). In addition and in contrast to the results obtained with the WT β2a subunit, a significant (p < 0.05) shift of the voltage that caused half-maximal activation of calcium channels from −7.8 ± 2.4 mV to −14.4 ± 0.6 mV (n = 6–7) was observed in response to PKA by analysis of steady state activation curves (Boltzmann fit).

The channels formed by α1cΔ1905 and β2a S478A/S479A also produced currents that were similar in current density and voltage dependence to those obtained with the WT β subunit in the absence of PKA (Fig. 4 compared with Fig. 2), indicating that these point mutations did not lead to gross misfolding of...
the $\beta_2$ subunit protein. However, in marked contrast to what was observed with the WT and S459A mutant $\beta_2a$ subunits, the addition of PKA to the pipette did not augment currents obtained with $\alpha_{1C}$D1905 and the S478A/S479A $\beta_2a$ subunit ($259.1 \pm 612.1 \text{ pA/pF}$ versus $263.5 \pm 612.4 \text{ pA/pF}$ at $0 \text{ mV}$, $n = 9$). We have previously demonstrated that Ser-478 and Ser-479 are key residues for phosphorylation by PKA and that mutation of these two serines to alanines causes a 75% reduction in the PKA-mediated phosphorylation of the $\beta_2$ subunit. Taken together with the fact that the $\alpha_{1C}$D1905 subunit was not a substrate for PKA, the functional effects of the PKA-mediated regulation seen in the studies reported here are likely to occur through phosphorylation of the $\beta_2a$ subunit. These results demonstrated the importance of phosphorylation of the $\beta_2a$ subunit to the regulation of the cardiac calcium channel and identified the functionally important residues in the $\beta_2$ subunit that are important for channel regulation.

**DISCUSSION**

The regulation of the cardiac L-type calcium channel by activation of PKA has been extremely well characterized through electrophysiological studies (1); however the underlying phosphorylation reactions have not been resolved completely. In particular, the substrates for PKA that are responsible for the stimulation of the calcium current in intact cardiac myocytes are unknown. The results shown here, together with those in recent companion studies (6, 7, 14), give new insights into this process and demonstrate that the regulation of the calcium channel may occur through more than one process. While early studies encountered difficulties in obtaining PKA-mediated stimulation of the cardiac L-type channel in various heterologous expression systems (8), we now have learned of two scenarios that will allow for expression of the PKA effects. In studies with full-length $\alpha_{1C}$ and $\beta_2a$ subunits, cAMP-dependent effects can be observed only if the channels are co-expressed with an AKAP (6). In this scenario, the cAMP-dependent effects were attributed to phosphorylation of Ser-1928 in the C terminus of the $\alpha_{1C}$ subunit, as mutation of this site alone led to a loss of the PKA effect (6). In addition, the PKA-mediated phos-

**FIG. 2.** PKA-mediated regulation of L-type calcium currents. **A,** the voltage dependence of Ba$^{2+}$ currents through L-type calcium channels in cells cotransfected with the truncated $\alpha_{1C}$D1905 and the WT $\beta_2a$ were measured. Representative current traces obtained from a cell in response to test potentials of $-80$, $-40$, $-20$, $-10$, $0$, $10$, $20$, $30$, $50 \text{ mV}$, with or without PKA (20 nM) in the (intracellular) pipette solution, are shown. **B,** current-voltage relationships (I-V curves) for the peak $I_{Ba}$ obtained from 10–11 cells from four independent transfections in the presence (filled circles) or absence (open circles) of PKA (20 nM) in the pipette solutions are shown.

**FIG. 3.** PKA-mediated stimulation of Ba$^{2+}$ currents through channels lacking phosphorylation site Ser-459 on the $\beta_2a$ subunit. **A,** representative current traces in response to different test potentials (see Fig. 1) obtained from cells expressing S459A $\beta_2a$ subunits with the $\alpha_{1C}$D1905 subunit with or without 20 nM PKA in the pipette (as indicated). **B,** summarized data for the voltage dependence of peak $I_{Ba}$ obtained from similar experiments as described in A ($n = 6–7$).

**FIG. 4.** Lack of PKA-mediated stimulation of L-type calcium channels in cells expressing S478A/S479A $\beta_2a$ subunits. Experiments were performed as described for Fig. 1 using the S478A/S479A $\beta_2a$ subunits instead of $\beta_2a$ WT subunits. **A,** representative barium currents obtained in response to different test potentials with or without 20 nM PKA in the internal solution (as indicated). **B,** summarized data for the I-V curves for voltage-activated $I_{Ba}$ measured in the absence or presence of PKA in the internal solution ($n = 9$).
Phosphorylation of Ser-1928 in the α1C subunit was AKAP-dependent, while phosphorylation of the β2a subunit was not (6). Thus, even though the β2a was phosphorylated at multiple sites when it was coexpressed with the full-length α1C subunit in the presence or absence of an AKAP, there did not appear to be a functional consequence of the phosphorylation of the β2a subunit (6). Interestingly, in this scenario, the increases in peak current were small, but a significant hyperpolarizing shift in the current-voltage relationship was observed (6). In the second scenario reported here, in studies with a C-terminally truncated, nonphosphorylatable α1C subunit, we have demonstrated the functional importance of the phosphorylation of two adjacent sites at Ser-478 and Ser-479 in the β2 subunit for regulation of the channel in response to PKA. In this second scenario, the increase in peak current was more substantial than observed in the first scenario, but a small hyperpolarizing shift in the current-voltage relationship was only observed in the context of the β2a S459A mutant. These effects did not require the expression of an AKAP, in agreement with the previous observation that the β2a subunit could undergo PKA-dependent phosphorylation whether or not it was co-expressed with an AKAP. These results contribute new aspects to mechanisms of regulation of the cardiac L-type channel, in particular that the β subunit may be directly involved in the regulatory process. Further studies are necessary to define whether one or both, or even other, events contribute to the PKA-mediated regulation of the channels in intact myocytes. Since neither scenario alone exhibits both the large increases in peak current amplitude and the hyperpolarizing shift in the current-voltage relationship that are observed in native cardiac myocytes, it is possible that both scenarios contribute to current regulation in the heart.

Key to understanding exactly what modes of regulation exist in cardiac myocytes is to elucidate the status and role of the α1C C terminus. It is possible that the truncation of the α1C subunit is necessary to allow for expression of the functional consequences of PKA-mediated phosphorylation of the β2 subunit and that regulation proceeds through a different mechanism when the full-length α1C subunit is the major form present. Yet another mechanism of regulation of the channel may be possible if the α1C subunit is cleaved in intact cells and the C-terminal fragment remains functionally associated with the "body" of the channel. This latter possibility is suggested by the observations that ~85–90% of the α1C subunit appears to be truncated at the C terminus when biochemically isolated from native tissues, yet immunofluorescent studies suggest that the C terminus is present in cardiac myocytes in stoichiometric amounts and co-localized with the α1C and β2 subunits (11). Potentially the truncation of the C terminus may allow new conformations of the channel to exist and alter the functional consequences of the phosphorylation of both the α1C and β2 subunits. Future studies will address these potentially complex mechanisms of channel regulation and further probe the types of regulation that occur in native systems.

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