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Regulation of the cloned L-type cardiac calcium channel by cyclic-AMP-dependent protein kinase

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Abstract

Hormones can regulate cardiac L-type Ca^{2+} channels via cAMP-dependent protein kinase (PKA) phosphorylation. However, regulation of the cloned L-type Ca^{2+} channel has been difficult to demonstrate conclusively. We stably transfected a human embryonic kidney (HEK-293) cell with the cardiac α_1 and β_2 subunits, then examined PKA modulation of the Ca^{2+} current. Although forskolin did not increase basal Ca^{2+} current, the PKA inhibitors, H-89 and Rp-cAMPS, could inhibit basal current. We reversed H-89 inhibition with either forskolin or okadaic acid. We conclude that the channel was phosphorylated under basal conditions, and that inhibition of PKA allowed dephosphorylation. These studies demonstrate that reversible PKA regulation of cloned Ca^{2+} channels can be studied in HEK-293 cells.

Key words: Calcium channel; Recombinant DNA; Clone cell; Adenosine cyclic monophosphate; Protein kinase; Phosphoprotein phosphatase

1. Introduction

Regulation of cardiac contractility by catecholamines is due in part to the stimulation of L-type Ca^{2+} channel activity [1]. Despite the presence of direct G protein–Ca^{2+} channel interactions [2], most of the β-adrenergic regulation occurs via stimulation of adenylyl cyclase, which leads to increases in cellular cAMP and stimulation of cAMP-dependent protein kinase (PKA) activity [3].

Biochemical and molecular biological studies have shown that the cardiac L-type Ca^{2+} channel contains at least 3 subunits, α_1, $\alpha_2\delta$, and β_2 [4–8]. Recent studies have demonstrated that PKA phosphorylates both α_1 and β_2 [8,9]. Haase et al. [8] also showed that isoproterenol treatment in vivo led to the phosphorylation of β_2.

Regulation of the cloned cardiac Ca^{2+} channel has been difficult to demonstrate. This difficulty stems from the lack of an expression system that does not contain endogenous channels (or subunits). *Xenopus* oocytes contain endogenous Ca^{2+} channels that are regulated by protein kinases A and C [10]. Furthermore, mammalian β subunits can stimulate these endogenous Ca^{2+} channels up to 10-fold [11]. In addition, both oocytes and Chinese hamster ovary (CHO) cells contain α_2 [12,13]. A second complication is that α_1 does not form functional channels when expressed alone in mammalian cells, requiring co-expression with a β subunit [14,15].

We investigated the regulation of the cloned cardiac L-type Ca^{2+} channel using human embryonic kidney cells (HEK-293) stably transfected with the cardiac α_1 and β_2 subunit. Untransfected cells express neither mRNA of Ca^{2+} channel subunits (X. Wei, unpublished observations) nor measurable Ca^{2+} currents. Transfected cells express large dihydropyridine-sensitive Ca^{2+} currents. We show that PKA antagonists inhibit these currents, and that either forskolin or okadaic acid reverse this inhibition. We conclude that HEK-293 cells contain endogenous kinases and phosphatases that regulate cloned Ca^{2+} channels, as observed in cardiac cardiac myocytes [3,16–18].

2. Materials and methods

2.1. Sources

Similar results were obtained with *N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulf onami de (H-89) purchased from three suppliers (Calbiochem., La Jolla, CA; LC Laboratories, Woburn, MA; Seikagaku, Tokyo, Japan). The Rp diastereomer of adenosine cyclic 3',5'-phosphothiorate (Rp-cAMPS) and nifedipine were purchased from Calbiochem (La Jolla, CA). Okadaic acid (ammonium salt) was purchased from LC Laboratories (Woburn, MA). Nystatin and forskolin were purchased from Sigma (St. Louis, MO).

2.2. Generation of a stably-transfected HEK-293 cell

The cloning of the rabbit cardiac α_1 and rat β_2 subunits has been described previously [6,19]. The cDNA insert encoding the α_1 subunit

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was subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA). The β_2 cDNA insert was subcloned into the expression vector p91023(b) [20]. HEK-293 cells were transfected with 1 μg α_1 cDNA and 10 μg β_2 cDNA using lipofectin (GIBCO, Grand Island, NY). Twenty-four hours after transfection, the cells were suspended in MEM α medium supplemented with 0.4 g/l of G418 and 10% fetal bovine serum. Single colonies were isolated using cloning cylinders, then plated in 24 well plates. Total RNA was prepared from half the cells of each clone and assayed for α_1 and β_2 mRNA expression using polymerase chain reaction. Positive clones were analyzed for α_1 protein expression using dihydropyridine binding assays with the ligand, (+)[^{3}H]PN200–110. Scatchard analysis of (+)[^{3}H]PN200–110 binding to crude membrane preparations was performed as described previously [6]. Cell clone HC$a\alpha_1\beta_2$-2, which expressed 250 fmol of binding sites per mg of membrane protein, was selected for further study.

2.3. Electrophysiological analysis

HEK-293 cells were plated on polylysine-coated cover slips and cultured 3–5 days before electrophysiological studies. Cells were grown in MEM α medium supplemented with 0.4 g/l of G418, 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rabbit and ferret cardiac ventricular myocytes were prepared as described previously [21].

To minimize dialysis of the cell and Ca^{2+} channel rundown, we used the nystatin perforated patch technique [22]. Whole-cell currents were recorded from nystatin patches using an Axopatch 200 amplifier and pCLAMP software (Axon Instruments, Foster City, CA). Data were digitized at 2 kHz and filtered at 1 kHz or off-line. Capacitive transients were subtracted using a P/4 subpulse routine. All experiments were performed at room temperature. Pipettes were fabricated from TW-150–6 capillary tubing (World Precision Instruments, Sarasota, FL), using a Model P-97 Flaming-Brown pipette puller (Sutter Instruments, Novato, CA). Pipettes were typically 0.8–1.5 MΩ. After gigaseal formation, incorporation of nystatin and formation of the voltage clamp was monitored using the capacitive transient induced by a 5 mV test pulse. In addition to 60 μg/ml nystatin, the internal pipette solution contained the following (in mM): 55 CsCl, 75 Cs_2SO_4, 10 $MgCl_2$, 0.1 EGTA, 10 HEPES, pH adjusted to 7.2 with CsOH. The external Tyrodes solution was the following (in mM): 140 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 HEPES, pH 7.4. Voltage-clamp control was usually achieved in 5 min, then the external solution was replaced with the following (in mM): 10 $BaCl_2$ (or 10 $CaCl_2$, as noted), 140 tetraethylammonium chloride, 5 CsCl, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH adjusted to 7.4 with tetraethylammonium hydroxide. The holding potential was −80 mV.

Peak currents and exponential fits were calculated using the pCLAMP software program clampfit (Axon Instruments, Foster City, CA). Current kinetics were calculated by fitting the data with the following equation: $Y = A_0 + A_1 e^{-t/\tau act} + A_2 e^{-t/\tau inact}$. Cell capacitance was measured by integrating the charging current during a 10 mV hyperpolarizing pulse followed by a 15 mV depolarizing pulse. Pooled data are expressed as mean ± S.E. Statistical significance was analyzed using Student's *t*-test for paired data.

All test compounds were diluted in external solution, then perfused into the bath at a rate of 3–5 ml/min. The bath was continuously perfused throughout the experiment. Despite the use of nystatin patches, there was still rundown of the Ba^{2+} currents. To minimize the contribution of this rundown all drug responses were measured 3–5 min after application.

3. Results

3.1. Electrophysiological characterization of transfected HEK-293 cells

In contrast to untransfected HEK-293 cells (see Fig. 2C), we measured large inward currents from the cell line transfected with the cardiac α_1[4,19] and β_2 subunits [6], HC$a\alpha_1\beta_2$-2. We recorded these currents using external solutions containing 10 mM of either Ca^{2+} or Ba^{2+}, and tetraethylammonium chloride to block outward K^+ currents. Fig. 1A shows peak current voltage relationships of a single cell in either Ca^{2+} or Ba^{2+}. Typically the inward Ba^{2+} currents had a voltage threshold of −40 mV, peaked around +10 mV, and reversed around +60 mV (internal pipette solution contained Cs). In comparison, the peak of the Ca^{2+} current–voltage relationship was shifted approximately 15 mV to depolarizing potentials. Similar shifts have been noted before and attributed to changes in surface potential [23]. The mean peak Ba^{2+} currents were −662 ± 60 pA (n = 20, 10 mV test potential), since the average capacitance was 81 pF this corresponds to a current density of −8.2 pA/pF. Individual current traces recorded during test pulses to +15 mV (from the cell in Panel A) are shown in Fig. 1B. Currents inactivated faster and more completely when Ca^{2+} was the charge carrier (τ = 68 ± 6 ms, 87 ± 4%) in comparison to Ba^{2+} (τ = 330 ± 41 ms, 39 ± 3%, n = 4, $P < 0.01$). We obtained similar results in rabbit or ferret cardiac myocytes (Fig. 1C). Since we could measure inward currents more reliably with Ba^{2+}, we used it throughout the rest of this study.

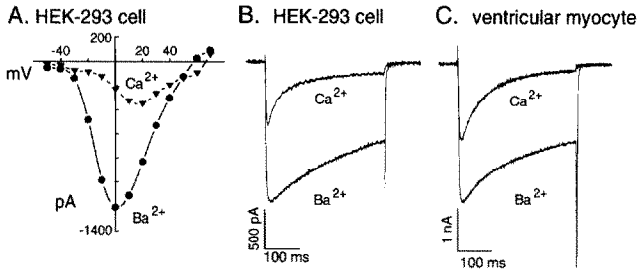

Fig. 1. Inward currents measured using 10 mM of either Ca^{2+} or Ba^{2+}. Panel A shows peak inward currents measured during 350 ms pulses to various test potentials from an HEK-293 cell transfected with the cardiac α_1 and β_2 subunits. Current traces (average of 4 recorded during test pulses to +15 mV) are shown in panel B. Panel C shows individual current traces from a ferret cardiac ventricular myocyte recorded in either 1 mM Ca^{2+} or Ba^{2+} (test potential 0 mV).

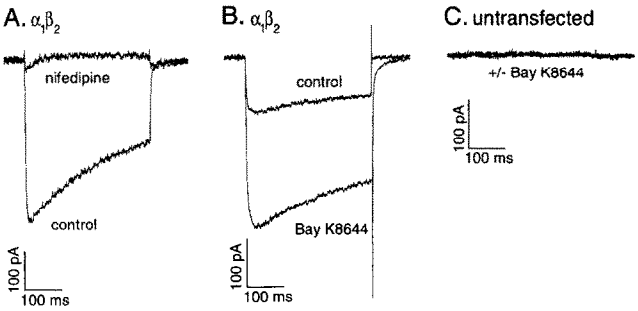

Fig. 2. Dihydropyridine sensitivity of currents measured in HEK-293 cells. The response to 10 μM nifedipine (Panel A) or 1 μM racemic BAY K8644 (Panel B) was measured in two different $\alpha_1\beta_2$-transfected HEK-293 cells. Panel C shows that no currents can be measured in an untransfected HEK-293 cell in either the absence of presence of 1 μM racemic BAY K8644. The currents elicited by 4 depolarizing pulses to +10 mV from a holding potential of −80 mV were averaged.

An identifying feature of L-type Ca^{2+} currents is their sensitivity to 'calcium antagonists', such as dihydropyridines. Fig. 2A shows that the dihydropyridine antagonist nifedipine (10 μM) almost completely blocked the inward Ba^{2+} current. Fig. 2B shows that the dihydropyridine agonist, (±) Bay K8644 (1 μM), stimulates Ba^{2+} currents. In contrast, we did not observe inward currents in untransfected HEK-293 cells, even in the presence of Bay K8644 (Fig. 2C).

3.2. Regulation of the expressed L-type currents

Agents that either increase protein kinase activity, or decrease phosphatase activity, stimulate L-type Ca^{2+} channel activity in a variety of cells [1]. We have previously used forskolin, which by stimulating cAMP formation leads to activation of PKA, to stimulate Ca^{2+} channel activity 3- to 4-fold in ferret and rabbit cardiac myocytes [21,24]. In contrast, forskolin (2, 5, or 10 μM), in either the absense or presence of isobutylmethylxanthine (IBMX), did not stimulate Ba^{2+} currents recorded from transfected HEK-293 cells (n = 13).

To test the possibility that the Ca^{2+} channel was phosphorylated under basal conditions, we measured the effect of the selective PKA inhibitor, H-89 [25]. Addition of H-89 to the external solution led to a rapid inhibition of the Ba^{2+} currents. Analysis of the average dose–response relationship (Fig. 3A) revealed an IC_{50} of 76 nM. The maximum percent inhibition of peak currents was 53 ± 5% (2 μM H-89, n = 6). Current–voltage analysis shows that inhibition occurs at most test potentials (Fig. 3B).

H-89 had a more pronounced inhibition of the current remaining at the end of the 350 ms test pulse, 78 ± 3% ($P < 0.01$). H-89 did not affect activation kinetics (τ_{act} control, 2.2 ± 0.4 ms; H-89, 2.4 ± 0.5 ms), but did affect the inactivation rate (τ_{inact} control, 297 ± 66 ms; H-89,

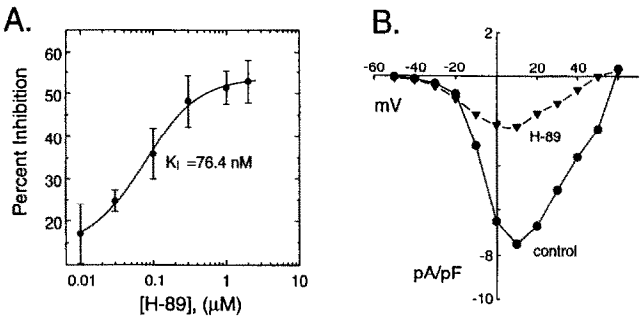

Fig. 3. Ba^{2+} currents in transfected HEK-293 cells were inhibited by H-89. Panel A shows the percent inhibition of currents measured with varying concentrations of H-89. Peak inward currents were measured during test pulses to +10 mV every 30 s. Each data point represents the average of 3–10 cells (mean ± S.E.). The results were obtained from a total of 40 cells. The average data were fit using the Hill equation, and used to plot the smooth line. Panel B shows the average (n = 3) peak current–voltage relationship obtained in either the absence (●) or presence of 1 μM H-89 (▼).

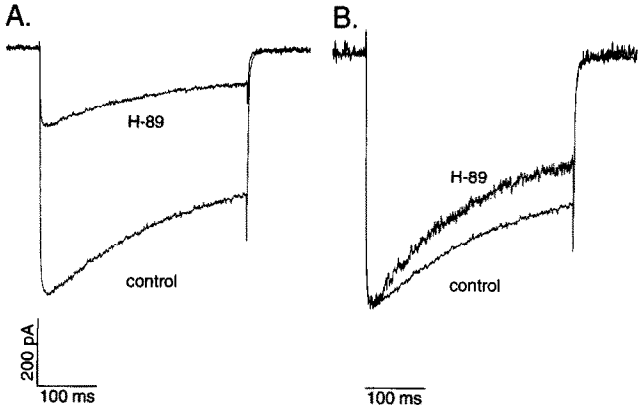

Fig. 4. H-89 affects the inactivation rate. Currents were recorded during test pulses to 10 mV in the absence or presence of 1 μM H-89. Four traces were averaged. The currents in Panel A were rescaled and plotted together in Panel B.

169 ± 32 ms, n = 5, $P < 0.01$). Consistent with the effect of H-89 on current amplitude at the end of the test pulse, H-89 inhibited the extrapolated steady-state current (A_0) by 76% (control, 174 ± 47 pA; H-89, 42 ± 15 pA, n = 5, $P < 0.05$). Current traces from a representative cell are shown in Fig. 4. This effect on inactivation kinetics is not due to errors in the voltage clamp since the observed inactivation rate was only weakly voltage-dependent (although current decay was only observed during pulses above −10 mV), and similar results were obtained using test potentials between 0 and +40 mV (control, 380 to 275 ms; H-89, 193 to 105 ms). Previous studies in cardiac myocytes showed that isoproterenol stimulation slows the inactivation of current through L-type Ca^{2+} [21].

To confirm that H-89 inhibited the currents by acting on PKA, we also studied the effect of the PKA antagonist, Rp-cAMPS [26]. Addition of 5 μM Rp-cAMPS led to a rapid inhibition of the Ba^{2+} currents. This concentration inhibited currents 54 ± 3% (n = 4), similar to the maximal H-89 inhibition. If these effects are due to dephosphorylation of the channel, then agents that increase cAMP formation or inhibit phosphatase activity should restore activity.

The effect of saturating concentrations of H-89 showed little recovery upon washout (Fig. 5). However, subsequent addition of forskolin (5 μM) plus IBMX (20 μM) dramatically stimulated the Ba^{2+} currents, 3.6-fold (± 0.6, n = 4). The time course of one such experiment is shown in Fig. 5A, which plots the change in peak currents measured during +10 mV test pulses. Adding H-89 with forskolin blocked this stimulation (n = 4), then there was no stimulation of Ba^{2+} current (Fig. 5B). However, subsequent addition of racemic Bay K8644 stimulated the peak currents 4.0-fold (± 1.0, n = 4).

Using the same protocol as in Fig. 5A, we found that okadaic acid (1 μM) also stimulated H-89-inhibited currents, 2-fold (± 0.2, n = 4). This stimulation was partially

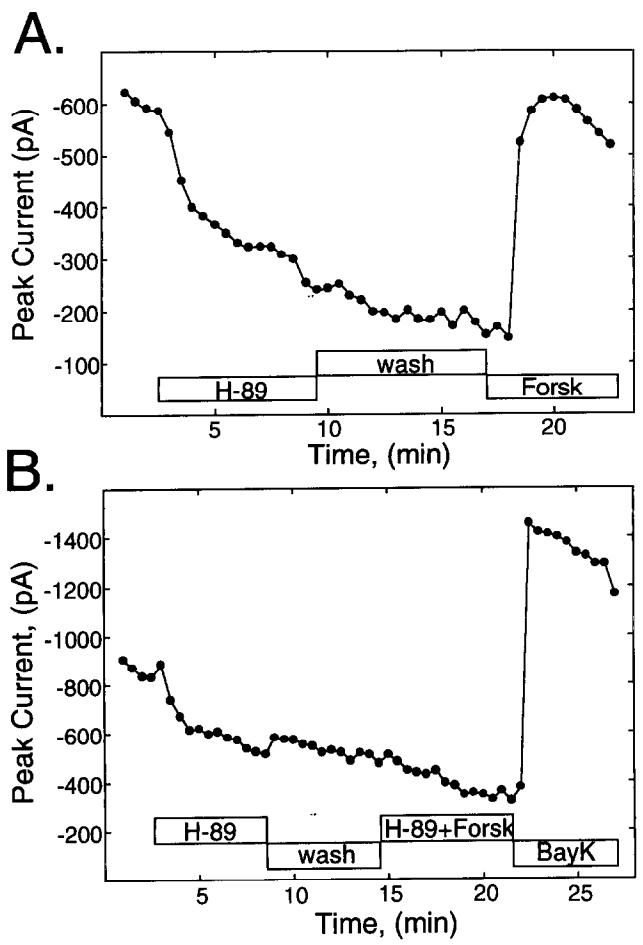

Fig. 5. (A) Forskolin reverses the inhibition caused by H-89. Peak currents were measured during test pulses to +10 mV from a holding potential of −80 mV. Addition of 1 μM H-89, wash, and 5 μM forskolin plus 20 μM IBMX are indicated by the boxes. (B) H-89 blocks the ability of forskolin to stimulate currents. The cell was treated sequentially with the following (additions indicated by boxes): 1 μM H-89; control external solution (wash); 5 μM forskolin, 20 μM IBMX plus 1 μM H-89; and finally 1 μM racemic Bay K8644.

blocked if H-89 was added with okadaic acid (1.2-fold ± 0.1, $n = 6$).

4. Discussion

We found that cotransfection of the cardiac α_1 with the cardiac β_2 led to the expression of functional Ca^{2+} channels in HEK-293 cells. We showed that these channels have Ca^{2+} dependent inactivation and pharmacological responses similar to those observed in native cardiac myocytes. Similar results were found using either CHO cells or mouse L cells, where coexpression with a β (skeletal muscle β_1) was shown to be required for functional channel activity [14,15].

We demonstrated that H-89 was a potent inhibitor of channel activity. Previous studies have shown that this isoquinolinesulfonamide derivative is highly selective for PKA [25]. In fact, the IC_{50} observed in the present experiments (76 nM) is similar to that observed in pheochromocytoma cells (48 nM) [25]. H-89 does not block the Ca^{2+} channel directly, since Bay K8644 could still stimulate activity. Further evidence is that H-89 does not block Ca^{2+} currents in rabbit cardiac myocytes [24]. Rp-cAMPS also inhibited basal activity. Thus, we conclude that the channel is phosphorylated in the basal state and that both H-89 and Rp-cAMPS inhibit PKA, allowing dephosphorylation and deactivation of the channel.

Basal phosphorylation also explains why forskolin did not directly stimulate currents. In contrast, forskolin dramatically stimulated currents after H-89 treatment. We suggest that this is due to forskolin's ability to stimulate adenylyl cyclase activity directly, leading to increases in cAMP, followed by re-phosphorylation of the channel. Evidence that PKA mediates forskolin stimulation was provided by the ability of H-89 to block this stimulation. The ability of okadaic acid to stimulate currents after H-89 treatment provides evidence for the involvement of a protein phosphatase.

We have shown how agents that modulate the activity of PKA and protein phosphatases modulate the activity of the cloned cardiac Ca^{2+} channel expressed in HEK-293 cells. Similar effects of forskolin [18], okadaic acid [17], and Rp-cAMPS [3] have been reported for L-type Ca^{2+} channels in cardiac myocytes. The magnitude of the forskolin stimulation of H-89-inhibited currents is nearly identical to the isoproterenol stimulation observed intact cardiac myocytes [1]. In parallel experiments with rabbit and ferret cardiac myocytes, we found that H-89 had little effect on basal Ca^{2+} currents, but could reverse the stimulation by forskolin [24]. Thus, the PKA-mediated stimulation of cloned L-type Ca^{2+} channel activity in HEK-293 cells is similar to that observed in cardiac myocytes. The only major difference is that the expressed channel is phosphorylated under basal conditions.

We conclude that HEK-293 cells provide a useful expression system where phosphorylation of cloned Ca^{2+} channels can be studied. The advantages of these cells are that they do not have endogenous Ca^{2+} channels, they do not express Ca^{2+} channel subunits, and they contain kinases and phosphatases that modulate the activity of transfected Ca^{2+} channels.

Recent evidence suggests there may be two modulatory phosphorylation sites [27]. One site may be on α_1 [9,28] and the other on β_2 [8]. Although our studies do not address this issue, we do show that HEK-293 cells provide an expression system where this can be studied. Future studies will determine if phosphorylation alters the single channel properties of the cloned channel as observed in cardiac myocytes [27,29].

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