

## 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  $\text{Ca}^{2+}$  channels via cAMP-dependent protein kinase (PKA) phosphorylation. However, regulation of the cloned L-type  $\text{Ca}^{2+}$  channel has been difficult to demonstrate conclusively. We stably transfected a human embryonic kidney (HEK-293) cell with the cardiac  $\alpha_1$  and  $\beta_2$  subunits, then examined PKA modulation of the  $\text{Ca}^{2+}$  current. Although forskolin did not increase basal  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel activity [1]. Despite the presence of direct G protein- $\text{Ca}^{2+}$  channel interactions [2], most of the  $\beta$ -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  $\text{Ca}^{2+}$  channel contains at least 3 subunits,  $\alpha_1$ ,  $\alpha_2\delta$ , and  $\beta_2$  [4–8]. Recent studies have demonstrated that PKA phosphorylates both  $\alpha_1$  and  $\beta_2$  [8,9]. Haase et al. [8] also showed that isoproterenol treatment in vivo led to the phosphorylation of  $\beta_2$ .

Regulation of the cloned cardiac  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels that are regulated by protein kinases A and C [10]. Furthermore, mammalian  $\beta$  subunits can stimulate these endogenous  $\text{Ca}^{2+}$  channels up to 10-fold [11]. In addition, both oocytes and Chinese hamster ovary (CHO) cells contain  $\alpha_2$  [12,13]. A second

complication is that  $\alpha_1$  does not form functional channels when expressed alone in mammalian cells, requiring co-expression with a  $\beta$  subunit [14,15].

We investigated the regulation of the cloned cardiac L-type  $\text{Ca}^{2+}$  channel using human embryonic kidney cells (HEK-293) stably transfected with the cardiac  $\alpha_1$  and  $\beta_2$  subunit. Untransfected cells express neither mRNA of  $\text{Ca}^{2+}$  channel subunits (X. Wei, unpublished observations) nor measurable  $\text{Ca}^{2+}$  currents. Transfected cells express large dihydropyridine-sensitive  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels, as observed in cardiac myocytes [3,16–18].

### 2. Materials and methods

#### 2.1. Sources

Similar results were obtained with *N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (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  $\alpha_1$  and rat  $\beta_2$  subunits has been described previously [6,19]. The cDNA insert encoding the  $\alpha_1$  subunit

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was subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA). The  $\beta_2$  cDNA insert was subcloned into the expression vector p91023(b) [20]. HEK-293 cells were transfected with 1  $\mu\text{g}$   $\alpha_1$  cDNA and 10  $\mu\text{g}$   $\beta_2$  cDNA using lipofectin (GIBCO, Grand Island, NY). Twenty-four hours after transfection, the cells were suspended in MEM  $\alpha$  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  $\alpha_1$  and  $\beta_2$  mRNA expression using polymerase chain reaction. Positive clones were analyzed for  $\alpha_1$  protein expression using dihydropyridine binding assays with the ligand, (+)[ $^3\text{H}$ ]PN200–110. Scatchard analysis of (+)[ $^3\text{H}$ ]PN200–110 binding to crude membrane preparations was performed as described previously [6]. Cell clone HCa $\alpha_1\beta_2$ -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  $\alpha$  medium supplemented with 0.4 g/l of G418, 10% horse serum, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Rabbit and ferret cardiac ventricular myocytes were prepared as described previously [21].

To minimize dialysis of the cell and  $\text{Ca}^{2+}$  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 M $\Omega$ . 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  $\mu\text{g}/\text{ml}$  nystatin, the internal pipette solution contained the following (in mM): 55 CsCl, 75 Cs $_2$ SO $_4$ , 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:  $Y = A_0 + A_1e^{-t/\tau_{\text{inact}}} + A_2e^{-t/\tau_{\text{inact}}}$ . 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  $\pm$  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  $\alpha_1$ [4,19] and  $\beta_2$  subunits [6], HCa $\alpha_1\beta_2$ -2. We recorded these currents using external solutions containing 10 mM of either Ca $^{2+}$  or

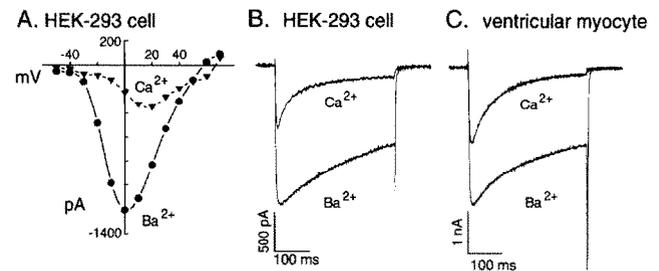


Fig. 1. Inward currents measured using 10 mM of either Ca $^{2+}$  or Ba $^{2+}$ . Panel A shows peak inward currents measured during 350 ms pulses to various test potentials from an HEK-293 cell transfected with the cardiac  $\alpha_1$  and  $\beta_2$  subunits. Current traces (average of 4 recorded 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).

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  $-662 \pm 60$  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 ( $\tau = 68 \pm 6$  ms,  $87 \pm 4\%$ ) in comparison to Ba $^{2+}$  ( $\tau = 330 \pm 41$  ms,  $39 \pm 3\%$ ,  $n = 4$ ,  $P < 0.01$ ). 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.

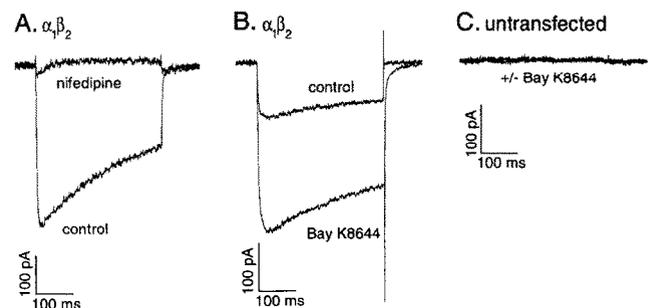


Fig. 2. Dihydropyridine sensitivity of currents measured in HEK-293 cells. The response to 10  $\mu\text{M}$  nifedipine (Panel A) or 1  $\mu\text{M}$  racemic BAY K8644 (Panel B) was measured in two different  $\alpha_1\beta_2$ -transfected HEK-293 cells. Panel C shows that no currents can be measured in an untransfected HEK-293 cell in either the absence or presence of 1  $\mu\text{M}$  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  $\text{Ca}^{2+}$  currents is their sensitivity to 'calcium antagonists', such as dihydropyridines. Fig. 2A shows that the dihydropyridine antagonist nifedipine ( $10 \mu\text{M}$ ) almost completely blocked the inward  $\text{Ba}^{2+}$  current. Fig. 2B shows that the dihydropyridine agonist, ( $\pm$ ) Bay K8644 ( $1 \mu\text{M}$ ), stimulates  $\text{Ba}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel activity 3- to 4-fold in ferret and rabbit cardiac myocytes [21,24]. In contrast, forskolin (2, 5, or  $10 \mu\text{M}$ ), in either the absence or presence of isobutylmethylxanthine (IBMX), did not stimulate  $\text{Ba}^{2+}$  currents recorded from transfected HEK-293 cells ( $n = 13$ ).

To test the possibility that the  $\text{Ca}^{2+}$  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  $\text{Ba}^{2+}$  currents. Analysis of the average dose-response relationship (Fig. 3A) revealed an  $\text{IC}_{50}$  of 76 nM. The maximum percent inhibition of peak currents was  $53 \pm 5\%$  ( $2 \mu\text{M}$  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,  $78 \pm 3\%$  ( $P < 0.01$ ). H-89 did not affect activation kinetics ( $\tau_{\text{act}}$  control,  $2.2 \pm 0.4$  ms; H-89,  $2.4 \pm 0.5$  ms), but did affect the inactivation rate ( $\tau_{\text{inact}}$  control,  $297 \pm 66$  ms; H-89,

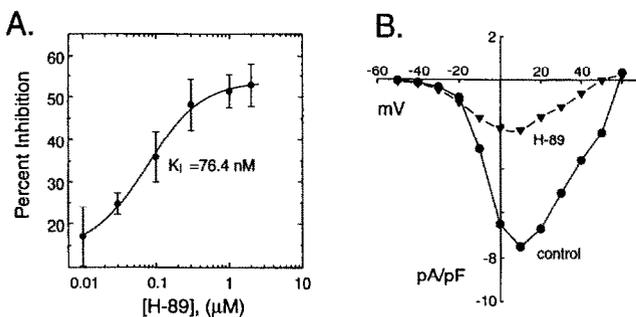


Fig. 3.  $\text{Ba}^{2+}$  currents in transfected HEK-293 cells were inhibited by H-89. Panel A shows the percent inhibition of currents measured with varying concentrations of H-89. Peak inward currents were measured during test pulses to  $+10 \text{ mV}$  every 30 s. Each data point represents the average of 3–10 cells (mean  $\pm$  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 ( $\bullet$ ) or presence of  $1 \mu\text{M}$  H-89 ( $\blacktriangledown$ ).

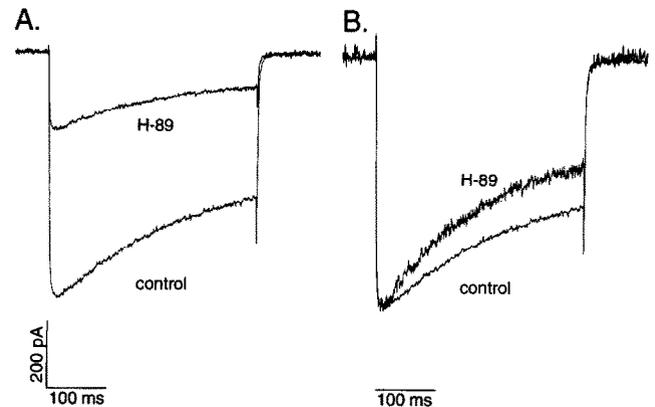


Fig. 4. H-89 affects the inactivation rate. Currents were recorded during test pulses to  $10 \text{ mV}$  in the absence or presence of  $1 \mu\text{M}$  H-89. Four traces were averaged. The currents in Panel A were rescaled and plotted together in Panel B.

$169 \pm 32 \text{ ms}$ ,  $n = 5$ ,  $P < 0.01$ ). 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 ( $A_o$ ) by 76% (control,  $174 \pm 47 \text{ pA}$ ; H-89,  $42 \pm 15 \text{ pA}$ ,  $n = 5$ ,  $P < 0.05$ ). 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  $-10 \text{ mV}$ ), and similar results were obtained using test potentials between 0 and  $+40 \text{ mV}$  (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  $\text{Ca}^{2+}$  [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  $5 \mu\text{M}$  Rp-cAMPS led to a rapid inhibition of the  $\text{Ba}^{2+}$  currents. This concentration inhibited currents  $54 \pm 3\%$  ( $n = 4$ ), 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 ( $5 \mu\text{M}$ ) plus IBMX ( $20 \mu\text{M}$ ) dramatically stimulated the  $\text{Ba}^{2+}$  currents, 3.6-fold ( $\pm 0.6$ ,  $n = 4$ ). The time course of one such experiment is shown in Fig. 5A, which plots the change in peak currents measured during  $+10 \text{ mV}$  test pulses. Adding H-89 with forskolin blocked this stimulation ( $n = 4$ ), then there was no stimulation of  $\text{Ba}^{2+}$  current (Fig. 5B). However, subsequent addition of racemic Bay K8644 stimulated the peak currents 4.0-fold ( $\pm 1.0$ ,  $n = 4$ ).

Using the same protocol as in Fig. 5A, we found that okadaic acid ( $1 \mu\text{M}$ ) also stimulated H-89-inhibited currents, 2-fold ( $\pm 0.2$ ,  $n = 4$ ). This stimulation was partially

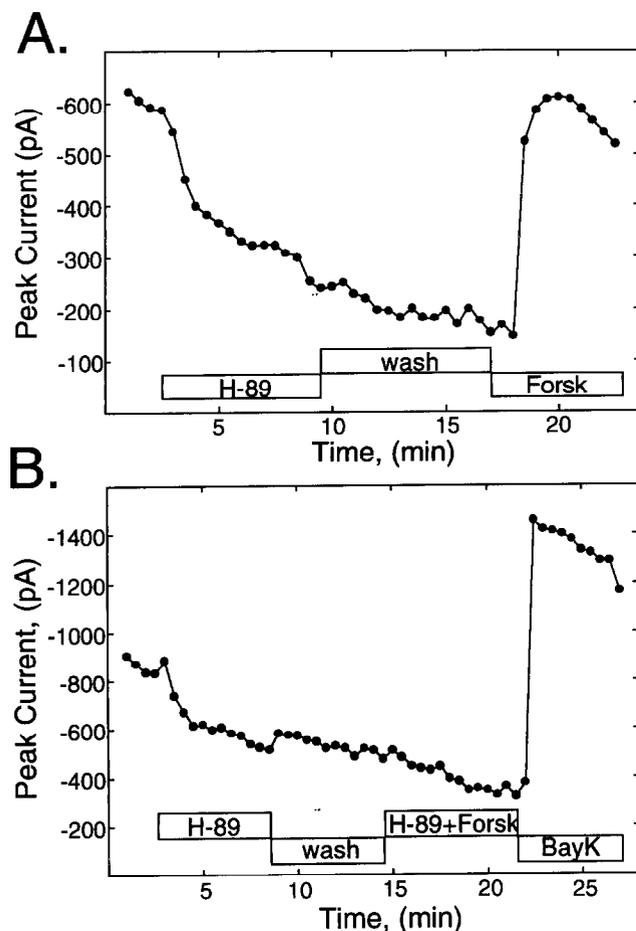


Fig. 5. (A) Forskolin reverses the inhibition caused by H-89. Peak currents were measured during test pulses to +10 mV from a holding potential of  $-80$  mV. Addition of  $1 \mu\text{M}$  H-89, wash, and  $5 \mu\text{M}$  forskolin plus  $20 \mu\text{M}$  IBMX are indicated by the boxes. (B) H-89 blocks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

blocked if H-89 was added with okadaic acid ( $1.2\text{-fold} \pm 0.1$ ,  $n = 6$ ).

#### 4. Discussion

We found that cotransfection of the cardiac  $\alpha_1$  with the cardiac  $\beta_2$  led to the expression of functional  $\text{Ca}^{2+}$  channels in HEK-293 cells. We showed that these channels have  $\text{Ca}^{2+}$  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  $\beta$  (skeletal muscle  $\beta_1$ ) 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  $\text{IC}_{50}$  observed in the present experiments ( $76 \text{ nM}$ ) is similar to that observed in pheochromocytoma cells ( $48 \text{ nM}$ ) [25]. H-89 does not block the  $\text{Ca}^{2+}$  channel directly, since Bay K8644 could still stimulate activity. Further evidence is that H-89 does not block  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel expressed in HEK-293 cells. Similar effects of forskolin [18], okadaic acid [17], and Rp-cAMPS [3] have been reported for L-type  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  currents, but could reverse the stimulation by forskolin [24]. Thus, the PKA-mediated stimulation of cloned L-type  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels can be studied. The advantages of these cells are that they do not have endogenous  $\text{Ca}^{2+}$  channels, they do not express  $\text{Ca}^{2+}$  channel subunits, and they contain kinases and phosphatases that modulate the activity of transfected  $\text{Ca}^{2+}$  channels.

Recent evidence suggests there may be two modulatory phosphorylation sites [27]. One site may be on  $\alpha_1$  [9,28] and the other on  $\beta_2$  [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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