Differential regulation of low and high voltage-activated calcium channels in neonatal rat myocytes following chronic PKA modulation

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Abbreviations: HVA, high-voltage activated; LVA, low-voltage activated; VDCC, voltage-dependent calcium channels; PKA, protein kinase A; 8Br-cAMP, 8bromo-cyclic AMP; DIV, days in vitro (culture)

The biophysical properties of cardiac voltage-dependent calcium channels (VDCC) can be modulated by protein kinases. In this study, we investigate whether long-term treatment with protein kinase A (PKA) modulators alter the VDCC activity in neonatal ventricular myocytes. Using whole-cell patch-clamp recordings, we found an increase in high-voltage activated (HVA) current density and a corresponding decrease in low-voltage activated (LVA) current density in neonatal rat ventricular myocytes up to 6 d in culture. Long-term exposure to 8Br-cAMP, a PKA stimulator, increased the HVA current density at 7 and 24 h. In contrast, H89, a PKA inhibitor, caused a biphasic change in the HVA current, with an initial reduction at 7 h-exposure, followed by an increase up to 4 d. In addition, H89 caused a sustained increase in LVA currents from 7 h to 4 d. These findings suggest that chronic exposure to H89 changes LVA and HVA calcium current activities in cardiac myocytes. PKA is a key target of β-adrenergic receptor activation, thus, our findings suggest long-term repeated use of β-adrenergic drugs may induce unexpected functional alteration of VDCCs.

Introduction

Voltage-dependent calcium channels (VDCCs) can be divided into two groups based on their activation properties—high-voltage activated (HVA) and low-voltage activated (LVA) channels.1 The channels are comprised of a pore-forming α subunit that can associate with, and be modulated by, auxiliary β, γ and αδ subunits.2-4 In the heart, HVA α subunits are encoded by two genes CACNAC and CACNAD, which express the Ca1.2 and Ca1.3 channels respectively; and LVA α subunits are encoded by two genes CACNAG and CACNAH, which express the Ca3.1 and Ca3.2 channels respectively.5-11 The expression of VDCCs in the heart is remodeled during pathological states such as cardiac hypertrophy,12-17 and cardiac ischemia.18

Calcium channels can be regulated by β-adrenergic receptor activation. G-protein coupled adrenergic stimulation elevates cAMP levels which activate PKA and MAPK signaling pathways19,20 leading to the phosphorylation of cAMP-response element binding protein (CREB) transcription factor.21 Long-term (2 h) stimulation with the β-adrenergic agonist isoproterenol results in an increase in the gene expression of Ca1.2 and the auxiliary subunits αδ and βα in myocytes, cultured in serum-containing media.22-24 PKA phosphorylation of the VDCCs also regulates channel activity. Upon β-adrenergic stimulation, PKA activation increases myocardial contractility (positive inotropy) via upregulation of the HVA Ca1.2 channel activity.25,26 In the adult heart, acute β-adrenergic receptor signaling stimulation of cAMP/PKA results in the phosphorylation of the Ca1.2 α subunit C-terminus, altering channel activity in the absence of changes in auxiliary subunits.27-30 Additionally, PKA may also modulate channel activity through its interaction with auxiliary subunits: phosphorylation of βα, the predominant VDCC β subunit in the heart, increases Ca1.2 channel activity even in absence of the αδ subunit C-terminus.31 PKA phosphorylation of the Ca1.3 channel also increases channel activity in the SA node.32,33 In recombinant-expression studies Ca3.2 channel activity is enhanced through forskolin stimulation of adenyl cyclase.34 Lastly, the coupling of LVA channels to both stimulatory and inhibitory G-proteins (G, and G, respectively) further increases the potential for differential modulation with the G, signaling cascade stimulating PKA-induced phosphorylation.35 Thus, PKA plays an important role in regulating cardiac VDCCs.

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Given that β-adrenergic blockers are commonly used in the treatment of myocardial arrhythmias and hypertension, it is fundamental to understand the long-term effects of PKA modulation on voltage-dependent calcium currents. To our knowledge, there is little known about the chronic effects of long-term exposure to PKA modulation on ventricular myocyte calcium channel function. In this study, we examined the long-term effect of PKA modulation on VDCCs in neonatal rat ventricular myocytes. Specifically, PKA activity was modulated by a cAMP analogue, 8Br-cAMP, and a PKA antagonist, H89, following exposure times ranging from 7 h to 4 d, and VDCC current activities were measured using patch-clamp recordings.

Results

Voltage-activated calcium currents in cultured neonatal myocytes. VDCCs undergo developmental changes as the contribution of the LVA currents to the voltage-dependent calcium influx is reduced, whereas the HVA currents increased during postnatal maturation or with time in culture.36,37 We first examined whole-cell (ruptured) calcium currents in neonatal rat ventricular myocytes following 2, 3 or 6 d in vitro (DIV) (Fig. 1A). Calcium current profiles (Fig. 1A1) and I–V curves (Fig. 1A2) were examined initially with a holding potential (V_h) of -100 mV. At 2 and 3 DIV, currents were activated at -40 mV and broad, dual-peak I–V curves were observed, indicating the presence of both LVA and HVA channels. The most notable change at 6 DIV was that the I–V curve shifted to the right (Fig. 1A2), and that maximal current activity was observed at higher voltages, indicative of the current component becoming predominately HVA channels. The maximal current density was 7.1 ± 0.7 pA/pF (n = 33) at 2 DIV and slightly increased to 8.1 ± 1.1 pA/pF (n = 10) at 6 DIV (Fig. 1A). The I–V profile at 3 DIV was similar to that seen at 2 DIV, suggesting that a shift from LVA to HVA dominance occurred between 3–6 d in culture. These findings are consistent with previous observations from myocytes grown in culture.37–40

To differentiate between HVA and LVA components, we took advantage of the biophysical and pharmacological properties of the channels. To record the LVA component, the HVA component was blocked using 10 μM nifedipine, a dihydropyridine L-type channel blocker that does not block T-type channels (Fig. 1C). Ventricular myocytes express both Ca_{1.2} and Ca_{1.3} channels. Previous reports showed 1 μM nifedipine blocked 90% of Ca_{1.2} and at least 50% of Ca_{1.3} currents carried by barium,41 and 10 μM nifedipine is sufficient to block all L-type channels.52,43 Thus, Ca current recorded in the presence of 10 μM nifedipine is mainly the DHP-insensitive currents, likely via the LVA channels.42,43 Consistent with these findings, a single LVA component I–V profile that activated at -40 mV and peaked at -20 mV was isolated in the presence of 10 μM nifedipine (Fig. 2A). This LVA current density increased slightly at 3 DIV, from 4.4 ± 0.6 pA/pF (n = 14) to 6.4 ± 0.8 pA/pF (n = 3), before significantly decreasing to 2.1 ± 0.3 pA/pF (n = 10; p < 0.05) at 6 DIV (Fig. 1C).

To measure the contribution of the HVA channels, the membrane potential was held at a holding potential (V_h) of -50 mV to inactivate the LVA channels, leaving the residual HVA current (Fig. 1B). The I–V curve obtained at the -50 mV holding potential showed that the current activated at -20 mV and peaked at +30 mV. In contrast to the LVA component (Fig. 1C), the HVA current density gradually increased over time in culture, from 3.9 ± 0.2 pA/pF (n = 4) at 2 DIV, to 5.4 ± 0.5 pA/pF (n = 11) at 3 DIV, and 7.4 ± 1.4 pA/pF (n = 12) at 6 DIV.

These time-dependent changes in current density are shown in Figure 1D. The maximal current densities of the HVA (V_h -50 mV) and LVA current (V_h -100 mV) at 10 μM nifedipine were compared with the total current recorded in the control condition (V_h -100 mV, Fig. 2C). The overall maximal current density did not significantly change with time in culture, whereas the LVA current was reduced significantly at DIV 6 (p < 0.05) while the HVA current increased.

The current densities of voltage-gated calcium channels change with prolonged PKA modulation. Having established the recording protocols, we next examined whether chronic PKA modulation alters the total calcium channel current profile, and that of the LVA (recorded at V_h -100 mV in presence of 10 μM nifedipine) and/or HVA channels (recorded at V_h -50 mV). Acute exposure (up to 2 h) to the β-adrenergic agonist isoproterenol was shown to enhance the gene expression of Ca_{1.2} and the α, βδ and βα auxiliary subunits and was prevented by pretreatment with either the β-adrenergic blocker propranolol or the PKA inhibitor H89.22,23 In this study, we examined whether long-term exposure of neonatal ventricular myocytes to either PKA stimulation (Fig. 2) or inhibition (Fig. 3) alters the functional properties of LVA and/or HVA channels. To keep the culture condition consistent, the compounds were added into the culture medium at 2 DIV, and the effects of compounds on the current activities were observed at either 7 h (2 DIV), 24 h (3 DIV) or 96 h (6 DIV) following treatment.

To induce PKA stimulation, 1 μM 8Br-cAMP, a cAMP analogue, was added to the culture. Figure 2 shows the current profiles (Fig. 2A1–C1) and I–V curves (Fig. 2A2–C2) of the total current, and the HVA and LVA current components at different DIVs. Similar to that seen in control conditions (Fig. 1), both HVA and LVA components contributed equally to the total current at 2 DIV (Fig. 2A), whereas the LVA component was reduced at 6 DIV only (Fig. 2C). The HVA component, however, significantly increased at both 3 DIV (Fig. 2B) and 6 DIV (Fig. 2C). A summary of the time-dependent changes in current densities resulting from 8Br-cAMP treatment is shown in Figure 2D. A modest increase in current density of the HVA channel component was observed between 7 h and 6 DIV (24 h at 8.9 ± 0.9 pA/pF, n = 13, DIV 3) after 8Br-cAMP exposure. In contrast, the current density of the nifedipine-insensitive component (LVA channels) remained relatively stable between 7 h and 6 DIV. An increase in the total calcium current density (11.4 ± 1.1, n = 12) was observed after 4 d of chronic exposure of the 8Br-cAMP (6 DIV). This was due primarily to a significant increase in the HVA component (10 ± 1.0 pA/pF; n = 10), even though the LVA component was reduced (2.5 ± 0.5 pA/pF; n = 16), relative to the 24 h treatment regimen (3 DIV).
Figure 1. Time-dependent changes in calcium current properties in cell culture. Whole-cell voltage-clamp recordings were carried out from neonatal rat ventricular myocytes at various days in culture. (A) Total currents were recorded from a holding potential of -100 mV. (B) HVA currents were determined by inactivating LVA currents by setting the holding potential at -50 mV. (C) LVA currents were determined by blocking DHP-sensitive currents with 10 μM nifedipine and recording currents from a holding potential of -100 mV. Typical representative current traces are presented in (A1–C1) and current-voltage (I–V) curves are in (A2–C2). (I–V) curves were determined from the peak current of the traces, normalized to the cell capacitance. DIV (days in vitro) indicates the number of days cells were in culture. (D) Time-dependent changes in peak current densities of total, HVA and LVA currents for myocytes grown in culture over 2, 3 and 6 d. I_{max} was determined from the from peak of the (I–V) density plot. *represent statistical difference relative to 7 or 24 h nifedipine treatment (p < 0.05).

Basal cAMP levels in myocytes may be sufficient to activate PKA, thus the effects of additional stimulation by 8Br-cAMP may be stunted. To test this hypothesis, we examined calcium current profiles in the presence of the PKA inhibitor, H89 (1 μM, Fig. 3). To test whether the effect of H89 was influenced by the solvent, the currents were also recorded in the presence of...
Interestingly, the HVA component (2.0 ± 0.4 pA/pF, n = 8, -50 mV) was smaller relative to the LVA current (7.6 ± 0.8 pA/pF, n = 12; -100 mV, 10 μM nifedipine) following 7 h of H89 treatment (Fig. 3A and 2 DIV), whereas there were no differences between DMSO (0.01%) alone. The current profiles of the total, the HVA and LVA current components that were recorded in the presence of H89 at different DIVs are shown in Figure 3A1–C1, and the mean I–V curves are shown in Figure 3A2–C2, respectively.
control and 8Br-cAMP treated LVA channels at this time point (Figs. 1A and 2A). The HVA component increased significantly following 24 h-treatment (3 DIV, Fig. 3B) and after 4 d (6 DIV, Fig. 3C). The LVA component became reduced with the time in culture (Fig. 1), however, increased and remained consistent during H89 treatment (7 h: 7.6 ± 0.8 pA/pF, n = 30; 24 h: 7.8 ± 1.9, n = 9; and 4 d: 8.0 ± 0.9, n = 9) (Fig. 3D). The current profiles in DMSO treated cells (Fig. 3E) were similar to that seen in the untreated, control condition (Fig. 1D), indicating the solvent did not affect the current properties.

Figure 3. Effects of the PKA inhibitor, H89, on calcium currents in neonatal rat ventricular myocytes. Currents were recorded from a holding potential of -100 mV (total current, A), -50 mV (HVA currents, B), or -100 mV in the presence of 10 μM nifedipine (LVA currents, C). Typical representative current traces are presented in (A1–C1), and (I–V) curves are in (A2–C2). Time-dependent changes in current densities of neonatal rat ventricular myocytes treated with 1 μM H89 (D) or 0.01% DMSO (E). Drug treatment started at 2 DIV, and recordings were carried out at 7, 24 or 96 h post-treatment, equivalent to 2 DIV, 3 DIV and 6 DIV. *represents statistical difference relative to other treatments (p < 0.05).
For a better comparison, all the data were replotted to illustrate the chronic effects of 8Br-cAMP or H89 on the maximal current density of the total current (Fig. 4A), HVA (Fig. 4B) or LVA (Fig. 4C) current components. At 7 h post-treatment, the HVA current component was significantly increased by 8Br-cAMP, and reduced by H89, suggesting functional VDCC channels are regulated through a PKA-dependent mechanism. The LVA current was not affected by 8Br-cAMP, but was significantly increased by H89. The HVA current in H89 was lower relative to the solvent control (4.7 ± 0.4 pA/pF, n = 18) after 7 h of treatment, and increased significantly by 24 h to 9.2 ± 1.4 pA/pF (n = 11; DMSO control: 6.4 ± 0.6 pA/pF, n = 13), a level which was comparable to the 8Br-cAMP 24 h-exposure (8.9 ± 0.9 pA/pF, n = 5) (Fig. 4B). Continued H89 treatment led to an additional significant increase in HVA current, reaching 13.6 ± 1.7 pA/pF (n = 10) by 4 d, which was also significantly larger relative to the DMSO control (8.2 ± 1.3 pA/pF, n = 14). The LVA component (-100 mV, 10 μM nifedipine) remained unchanged in the first 3 DIV, and declined at 6 DIV for all treatments except H89, where it significantly increased compared to its DMSO-vehicle control (7 h: 4.7 ± 0.8 pA/pF, n = 18; 24 h: 5.6 ± 0.6 pA/pF, n = 4; 4 d: 3.0 ± 0.4 pA/pF, n = 11) (Fig. 4C). Taken together, our results show that long-term exposure to H89 increases total calcium current densities by enhancing both HVA and LVA current components.

Discussion

This study demonstrated chronic effects of 8-Br-cAMP and H89 on peak calcium current activity in neonatal rat ventricular myocytes. Myocytes showed a general remodeling of functional VDCCs over time in culture, with an increased HVA current component (currents elicited from Vh = -50) and a decreased LVA current component (nifedipine-insensitive currents). Chronic treatment with the cAMP analogue, 8Br-cAMP, appeared to hasten the HVA current changes, with the increase in peak current density reaching saturation between 1- to 4 d-treatment. In contrast to the stimulation of PKA with 8Br-cAMP, long-term exposure to H89 significantly increased both HVA and LVA calcium currents.

Temporal changes. The temporal alterations in the observed calcium channel current density during culture supports previous work showing an increase in the DHP-sensitive HVA current over time and a reduction in the LVA current component observed from a variety of ventricular myocyte models. In adult guinea pig myocytes there is a decrease in the ratio of ICaT/ICaL over time.40 In contrast to the stimulation of PKA with 8Br-cAMP, long-term exposure to H89 significantly increased both HVA and LVA calcium currents.

*indicates statistical difference over time (p < 0.05); #indicates statistical difference compared to control treatment (p < 0.05).

For a better comparison, all the data were replotted to illustrate the chronic effects of 8Br-cAMP or H89 on the maximal current density of the total current (Fig. 4A), HVA (Fig. 4B) or LVA (Fig. 4C) current components. At 7 h post-treatment, the HVA current component was significantly increased by 8Br-cAMP, and reduced by H89, suggesting functional VDCC channels are regulated through a PKA-dependent mechanism. The LVA current was not affected by 8Br-cAMP, but was significantly increased by H89. The HVA current in H89 was lower relative to the solvent control (4.7 ± 0.4 pA/pF, n = 18) after 7 h of treatment, and increased significantly by 24 h to 9.2 ± 1.4 pA/pF (n = 11; DMSO control: 6.4 ± 0.6 pA/pF, n = 13), a level which was comparable to the 8Br-cAMP 24 h-exposure (8.9 ± 0.9 pA/pF, n = 5) (Fig. 4B). Continued H89 treatment led to an additional significant increase in HVA current, reaching 13.6 ± 1.7 pA/pF (n = 10) by 4 d, which was also significantly larger relative to the DMSO control (8.2 ± 1.3 pA/pF, n = 14). The LVA component (-100 mV, 10 μM nifedipine) remained unchanged in the first 3 DIV, and declined at 6 DIV for all treatments except H89, where it significantly increased compared to its DMSO-vehicle control (7 h: 4.7 ± 0.8 pA/pF, n = 18; 24 h: 5.6 ± 0.6 pA/pF, n = 4; 4 d: 3.0 ± 0.4 pA/pF, n = 11) (Fig. 4C). Taken together, our results show that long-term exposure to H89 increases total calcium current densities by enhancing both HVA and LVA current components.
density. Although the degree of Ca\textsubscript{1,3} channel contribution to the HVA currents in ventricles is still to be resolved conclusively, Ca\textsubscript{1,3} channels have been implicated in early ventricular myocyte development.\cite{42,43} The sensitivities of HVA channels to dihydropyridines are dependent on channel subtype,\cite{42,43} thus we used a higher concentration of nifedipine (10 μM) to block >90% of Ca\textsubscript{1,2}/Ca\textsubscript{1,3} HVA channel components. At this concentration, LVA channels are not significantly affected.\cite{50} Our observed changes in both current density and \( V_{1/2} \) activation reflect the changes in contribution between LVA to HVA channels during culture and development from 2–6 d in vitro. The time-dependent reduction in LVA currents observed in control culture conditions was prevented by chronic treatment of H89. More critically, the activation of such a pathway raises concerns regarding how long-term β-adrenergic therapy may alter intracellular signaling cascades that activate this pathway.

**PKA modulation.** The focus of this current study was to test the role of long-term PKA modulation on the functional activity of VDCC in neonatal rat ventricular myocytes. PKA modulation of the HVA calcium current density in myocytes is a well-documented phenomenon, however, most work has focused on acute effects of PKA modulation.\cite{27,28,29,31} The activity of recombinantly expressed Ca\textsubscript{3,2} channels appears to be enhanced following 30 min application of forskolin.\cite{31} The facilitation of LVA currents was suggested to be under modulation by both stimulatory and inhibitory G-proteins (G\textsubscript{s} and G\textsubscript{i}, respectively), the former rents was suggested to be under modulation by both stimulatory and inhibitory G-proteins (Gs and Gi, respectively), the former

Furthermore, CaMKII activity increases during heart failure when myocyte protein expression has been shown to revert to “fetal” like characteristics, suggesting that the LVA component may be targeted in this scenario.

In the absence of PKA activation, compensation or upregulation of other signaling cascades may become more prominent in cardiac function. For instance, in the heart when PKA interaction is disrupted by interfering with AKAPs in vivo, there is an upregulation of a cTN1 truncation in order to maintain inotropy in the absence of PKA-linked β adrenergic signaling to its downstream effectors.\cite{57} Moreover, temporal changes in calcium current properties of newborn rabbit ventricular myocytes in culture following β-adrenergic stimulation are neither PKA nor CaMKII dependent.\cite{58} A recent work shows that physiological stressors, such as hypoxia can alter neonatal rat ventricular myocytes LVA calcium component via a reduction in Ca\textsubscript{3,1} gene expression by HIF-1α (hypoxia induced factor 1α).\cite{46} Since 8Br-cAMP and H89 target cAMP/PKA, which can regulate gene expression via CREB or CaMKII,\cite{19,21,27,29,31} the effect of the drugs may be explained by differential regulation of gene expression of the channel subunits. Whether the effects of H89 on VDCCs are likely mediated by inhibiting PKA to phosphorylate its target substrates, H89 at the concentration used in our studies (1 μM) may also inhibit other signaling pathways, such as S6K1, MSK1 and ROCK-II.\cite{60} Whether and how H89 inhibiting these kinases are involved in functional calcium expression remain to be investigated in future.

The relative density of T- and L-type channel expression reduces during cardiac maturation, such that T-type current activity is downregulated,\cite{61,62} whereas L-type current activity is upregulated.\cite{61,62,63} T-type current increases in ventricles in adult cardiac hypertrophy,\cite{14} sudden death\cite{65} and heart failure.\cite{66} Thus, T-type channel re-expression may involve cardiac dysfunction, premature and arrhythmogenic death. Ca\textsubscript{3,2} \( \gamma ^{\text{a}} \), but not Ca\textsubscript{3,1} \( \gamma ^{\text{a}} \), mice fail to develop a hypertrophic phenotype in response to either pressure overload or Ang II,\cite{66} indicating that T-type channels play a role in hypertrophic remodelling. Although L-type current increases in hypertrophic myocytes,\cite{12,13,17} it reduces in severe hypertrophy and heart failure.\cite{12} These altered Ca\textsuperscript{2+} signals may activate abnormal signal transduction pathways leading to an imbalance in gene regulation, and subsequently to disease progression. β-adrenoceptor activation induces cAMP-dependent gene regulation,\cite{79,80,68,69} through PKA and mitogen activated protein kinase (MAPK) signalling cascades.\cite{21} Signalling pathways involved in cardiomyocyte hypertrophic stimuli include MAPK, Gp130/Stat3, CaMKs and calcineurin-dependent signalling pathways.\cite{71,72} In this study, we showed that chronic PKA modulation differentially regulated LVA- and HVA calcium currents. Whether this modulation may be one of the critical processes resemble that occur in the remodeling of hypertrophic myocytes or other cardiac conditions remains to be further investigated.

This study highlights the putative regulatory mechanisms of functional VDCC expression. The ability for changes in functional expression in neonatal rat ventricular myocytes during PKA modulation raises an important question, particularly in light of overwhelming chronic use of β adrenergic antagonists in the prevention and treatment of heart failure. Most studies to date have examined acute effects of β-adrenergic signaling on VDCC.
expression. The rationale for using β-blockers is based on clinical evidence and theories of their mechanism for protection including the reduction in PKA induced hyperphosphorylation of RyR associated with heart failure and the inhibition of βARK induced downregulation. However, the potential compensatory effects on signaling cascades must continue to be examined. In addition, VDCCs can be chronically regulated in a PKA-independent/H89 sensitive manner. Future studies should be carried out to determine the mechanisms underlying the chronic regulation of functional VDCCs mediated by H89 and cAMP agonists chronic regulation of functional VDCCs. Understanding whether repeated use of β-adrenergic drugs alters calcium current expression will assist our current therapeutic strategy for the treatment of cardiovascular disorders.

Materials and Methods

Animals and cell culture. Neonatal rat ventricular myocytes were isolated from 1-d-old Sprague Dawley pups as previously described in reference 74. Rats were sacrificed following the University of Toronto animal guidelines, and the hearts were placed in ice cold Hanks balanced salt solution (HBSS). The apex of the ventricle from the lower 1/3 of the heart was separated from the rest of the heart and cut into smaller pieces with surgical scissors. The pieces were incubated in 1 mg/mL trypsin and 10 μg/mL gentamycin in HBSS and gently rocked at 4°C for 3 h. After the incubation, the solution was replaced with 5 mL of digestion buffer (1 mg/mL trypsin, 0.75 mg/mL collagenase and 10 μg/mL gentamycin in HBSS), and gently stirred at room temperature for 10 min. The digest was collected in 5 mL heat-inactivated FBS. The digestion process was repeated six times. The cells were then centrifuged at 283 g for 5 min and resuspended in serum-containing media (10% FBS, 1% penicillin-streptomycin, 1:1 DMEM:F12). The cells were pre-plated in Primaria dishes (BD Falcon #353802) for 1 h to attach non-cardiac cells. The density of non-attached cells was counted using a hemocytometer and plated at a density of 2 x 10⁶ cells/dish for patch-clamp recording. Twenty-four hours later (1 d in vitro, DIV 1), the media was changed to serum-free media (1% Insulin-Transferrin-Selenium, 1% penicillin-streptomycin, 1:1 DMEM:F12, Invitrogen).

Drugs and treatments. 8Br-cAMP was dissolved in water and H89 in DMSO at a concentration of 10 mM. Approximately 36 h after initial plating of the myocytes in serum-free media, cells were treated to a final concentration of 1 μM of 8Br-cAMP or H89 (2DIV). Media and drug treatments were changed daily, and cells were examined following 7 h, 24 h or 4 d after exposure to the drugs, on 2, 3 or 6 DIVs. Water and 0.01% DMSO were used as vehicle controls. Nifedipine was used at a final concentration of 10 μM. All drugs were purchased from Sigma; DMSO was purchased from Fisher Scientific.

Patch-clamp recordings. Whole-cell calcium current (ruptured) was recorded using patch electrode with voltage-clamp protocols, as described previously with some modifications. Bath solution comprised of (in mM) 20 CaCl₂, 85 CsCl, 40 TEA-Cl, 1 MgCl₂, 10 HEPES, 10 glucose (pH 7.3 adjusted with CsOH); pipettes (2–5 MΩ) were filled with (in mM) 140 Cs-methanesulfonate, 4 MgCl₂, 2 MgATP, 9 EGTA, 9 HEPES (pH 7.2 adjusted with CsOH). The membrane potential was held at -100 mV for 1–2 min after whole-cell rupture to allow for sufficient equilibration before recording for 2–5 min. Total current was measured on a MultiClamp7A amplifier (Molecular Devices) by stepping from -100 mV to increasingly depolarized potentials (from -80 mV to +80 mV), on a 4 pole Bessel filter (1 kHz, -3dB) and sampled at 2 kHz. The high-voltage activated component was subsequently measured by holding the membrane at -50 mV to inactivate the low-voltage activated component. The low-voltage activated component was recorded independently in the presence of the 10 μM nifedipine, which inhibits HVA channel activation. Current densities were determined by normalizing against the cell capacitance. Currents were fit with a modified Boltzmann function.

Statistics. All electrophysiology data were analyzed using Clampfit (Molecular Devices). Statistical analysis was carried out using Sigma Stat 3.0 software (SPSS Inc.). The significance of differences between mean values from each experimental group were tested using a Student’s t-test for two groups and one way analysis of variance (ANOVA) for multiple comparisons followed by Tukey post-hoc test. Differences were considered significant if p < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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