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The role of constitutive PKA-mediated phosphorylation in the regulation of basal I_{Ca} in isolated rat cardiac myocytes

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1 Pharmacological inhibitors of protein kinase A (PKA) and protein phosphatases 1/2A were used to determine whether basal L-type Ca^{2+} current (I_{Ca}) observed in the absence of exogenous β-adrenergic receptor stimulation is sustained by PKA-mediated phosphorylation. Amphotericin B was used to record whole-cell I_{Ca} in the perforated patch-clamp configuration.

2 Calyculin A and isoprenaline (both $1\,\mu mol\,l^{-1}$) increased basal I_{Ca} ($P<0.05$), whereas H-89 inhibited I_{Ca} in a concentration-dependent manner with an IC_{50} $\sim 5\,\mu mol\,l^{-1}$. H-89 also inhibited the response to $1.0\,\mu mol\,l^{-1}$ isoprenaline, although relatively high concentrations ($30\,\mu mol\,l^{-1}$) were required to achieve complete suppression of the response.

3 Double-pulse protocols were used to study the effects of $10\,\mu mol\,l^{-1}$ H-89 on time-dependent recovery of I_{Ca} from voltage-dependent inactivation as well as the steady-state gating of I_{Ca}. $T_{0.5}$ (time for I_{Ca} to recover to 50% of the preinactivation amplitude) increased in the presence of H-89 ($P<0.05$) but was unaffected by calyculin A or isoprenaline.

4 Steady-state activation/inactivation properties of I_{Ca} were unaffected by $10\,\mu mol\,l^{-1}$ H-89 or $1\,\mu mol\,l^{-1}$ calyculin A, whereas isoprenaline caused a leftward shift in both curves so that $V_{0.5}$ for activation and inactivation became more negative.

5 Data show that basal I_{Ca} is regulated by cAMP-PKA-mediated phosphorylation in the absence of externally applied β-receptor agonists and that relatively high concentrations of H-89 are required to fully suppress the response to β-adrenergic receptor stimulation, thereby limiting the value of H-89 as a useful tool in dissecting signalling pathways in intact myocytes.
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Abbreviations: cAMP-PKA, cyclic AMP-dependent protein kinase A; EGTA, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic Acid; H-89, *N*-[2-*p*-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfonamide; I_{Ca}, L-type Ca^{2+} current; *I–V*, current–voltage; PP1/2A, protein phosphatase 1/2A; Ser, serine; $T_{0.5}$, time to recover to 50% of the preinactivation amplitude

Introduction

β-adrenergic receptor stimulation is well known to increase I_{Ca} in cardiac muscle (Mcdonald *et al*., 1994). This effect is mediated by cyclic AMP-dependent protein kinase A (cAMP-PKA)-dependent phosphorylation of Ser^{1928} on the α_1-subunit that constitutes the functional pore of the channel (De Jongh *et al*., 1996; Perets *et al*., 1996), although phosphorylation of the Ser^{478} and Ser^{479} on the β-subunit may also modulate the response (Bunemann *et al*., 1999). Increased phosphorylation of the α_1-subunit alters the gating properties of the channel to promote a higher open probability (Yue *et al*., 1990) that results in an increase in the macroscopic I_{Ca} across the whole cell.

While the role of cAMP-PKA-mediated phosphorylation in the response to catecholamines is undisputed (Kamp & Hell, 2000), it remains unclear as to whether cAMP-PKA also sustains the basal I_{Ca} observed in the absence of β-adrenergic receptor stimulation. This is important in the context of heart failure where downregulation of β-adrenergic receptors blunts the response to catecholamines so that other means of phosphorylating the channel become potentially more significant. Constitutive PKA-mediated phosphorylation, independent of β-adrenergic receptor stimulation, could under such circumstances play an important role in sustaining the I_{Ca} and thus contraction, as well as regulating other PKA-dependent phosphorylation targets. In addition, protein phosphatases are colocalised with the channel (Davare *et al*., 2000) and can also tonically regulate the phosphorylation status of the channel to modulate I_{Ca} (Herzig & Neumann, 2000; Dubell *et al*., 2002; Dubell & Rogers, 2004). Constitutive mechanisms that determine the basal levels of channel phosphorylation are therefore important determinants of I_{Ca} regulation.

Kinase and phosphatase inhibitors are a useful tool in elucidating the contribution of different mechanisms that regulate protein phosphorylation and thus cellular function. However, their usefulness depends largely on their selectivity and potency. H-89 (*N*-[2-*p*-bromo-cinnamylamino)ethyl]-5-isoquinolinesulfonamide) is of particular interest in this regard because it is described as being highly selective for PKA over

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the other kinases; its *in vitro* K_i is 50 nmol l^{-1} for PKA, 500 nmol l^{-1} for protein kinase G and 5–50 μmol l^{-1} for PKC and Ca^{2+}-calmodulin-dependent protein kinase (Chijiwa *et al*., 1990; Hidaka & Kobayashi, 1992). However, previous studies that have investigated the effects of H-89 have yielded some conflicting results: some have shown that H-89 decreased basal I_{Ca} (Hussain *et al*., 1999) and attenuated the response to isoprenaline (Mitarai *et al*., 2000), whereas others did not observe inhibition of basal I_{Ca} in cardiac myocytes (Yuan & Bers, 1995; Chen *et al*., 2002; Dubell & Rogers, 2004), concluding that constitutive PKA-mediated phosphorylation was not involved. Given these discrepancies, the present study examined the effects of H-89 in detail, alongside those of calyculin A (an inhibitor of protein phosphatases PP1/PP2A) to assess its potential usefulness in clarifying the role of constitutive PKA-mediated phosphorylation in regulating I_{Ca}.

Methods

Isolation of ventricular myocytes

Male Wistar rats (250–350 g) were killed by a blow to the head followed by cervical dislocation in accordance with the Home Office Guidelines (Schedule 1, Animals Scientific Procedures Act, 1986). The heart was removed and cleared of blood by retrograde perfusion for 2–3 min with HEPES-Tyrodes containing 0.75 mmol l^{-1} $CaCl_2$ (see *Solutions and drugs* for composition). This was followed by a 5 min perfusion with Ca^{2+}-free HEPES-Tyrode containing 0.1 mmol l^{-1} EGTA (ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid) and then with the enzyme solution containing 1.0 mg ml^{-1} collagenase (Type I; Worthington, Lakewood, Co, U.S.A.) and 0.05 mg ml^{-1} protease (Type XIV; Sigma, Poole, Dorset, U.K.) in low $CaCl_2$ (50.0 μmol l^{-1}) for 7 min. The left ventricle was removed, sliced into smaller chunks and agitated in the enzyme solution supplemented with 1.0% bovine serum albumin (Sigma, U.K.). Aliquots of myocytes were harvested at 5 min intervals by filtration of the digest through monofilament nylon cloth (Cadisch, U.K.) followed by gentle centrifugation of the filtrate (40 × *g* for 30 s). Pelleted myocytes were resuspended in enzyme-free isolation solution containing 0.75 mmol l^{-1} $CaCl_2$ and maintained at 4°C in a Petri dish until used. The process was repeated 2–3 times to obtain a total of 3–4 batches of myocytes from each heart. Only cells that showed clearly defined striations and produced rapid contractions in response to field stimulation were used in this study. Myocyte isolation was carried out at 36 ± 1°C.

Membrane currents

I_{Ca} was recorded in BAPTA AM (10 μmol l^{-1}; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)-loaded ventricular myocytes in the perforated patch-clamp configuration using amphotericin B (240 μg ml^{-1}) as the pore-forming agent. Voltage clamp was performed using the Axopatch 200 B amplifier (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) linked to a Digidata 1200B A/D interface. I_{Ca} was filtered at 5 kHz, acquired using Clampex 8.2 and analysed with Clampfit 8.2 (Molecular Devices Corporation). Glass micropipettes were fabricated from filamented borosilicate capillaries (GC150TF, Harvard, MA, U.S.A.) to give tip resistances ~1 MΩ when fire-polished and filled with a cesium-based pipette solution (see *Solutions* for composition). Current–voltage (*I–V*) curves for I_{Ca} were obtained by 5–10 mV steps from a holding potential of −40 to 60 mV. Conventional double-pulse protocols were used to obtain activation/inactivation curves: myocytes were depolarised from −40 to 60 mV for 400 ms in 5 mV increments, followed by a second test pulse to 0 mV. The currents obtained during the prepulse were corrected for the driving force and normalised to the peak current to obtain the activation curves, whereas I_{Ca} obtained during the second step (to 0 mV) was normalised and plotted against the prepulse potential to obtain the inactivation curves. A second series of double-pulse protocols was used to measure the time course of recovery from inactivation. Two successive pulses from –40 to 0 mV were separated by progressively increasing time intervals in 20 ms increments. I_{Ca} recorded during the second pulse were normalised and plotted against the interpulse interval. I_{Ca} were recorded at 35°C, unless stated otherwise.

Solutions

The composition of the physiological salt solution used during isolation procedure and storage of myocytes contained (mmol l^{-1}): NaCl, 130; KCl, 5.4; $MgCl_2$, 1.4; NaH_2PO_4, 0.4; HEPES, 10; glucose, 10; taurine, 20 and creatine, 10; and adjusted to pH 7.3 with 1 M NaOH. This was supplemented with either EGTA or Ca^{2+} as described above.

The external Tyrode solution used to superfuse cells during perforated patch-clamp experiments contained (mmol l^{-1}) NaCl, 140; KCl, 5.4; $MgCl_2$, 1; glucose, 10; HEPES, 10; $CaCl_2$, 1; and adjusted to pH 7.35 with 1 M NaOH. Patch pipette solution contained (mmol l^{-1}) Cs-glutamate, 120; KCl, 20; NaCl, 10; HEPES, 10, adjusted to pH 7.3 with 1 mol l^{-1} CsOH.

Data analysis and statistics

All data are expressed as mean ± s.e.m. of *n* observations. Statistical comparisons were made (SPSS, vers. 11) using ANOVA and Student's *t*-test for independent samples. $P < 0.05$ was deemed statistically significant.

Results

Figure 1 shows representative tracings of I_{Ca} recorded upon depolarisation from −40 to 0 mV (A) and the *I–V* curves (B) to demonstrate that β-adrenergic receptor stimulation with 1.0 μmol l^{-1} isoprenaline significantly increased I_{Ca}. Control I_{Ca} at 0 mV was −1.23 ± 0.156 nA and increased by 181% to −3.45 ± 0.04 nA. Similarly, 1.0 μmol l^{-1} calyculin A also increased I_{Ca}, although not to the same extent as isoprenaline: I_{Ca} increased by 52% to 1.87 ± 0.17 nA ($P < 0.05$). These data confirm that basal I_{Ca} can be modulated not only by β-stimulation but also by inhibition of constitutively active endogenous protein phosphatases PP1/2A in the absence of external stimulation.

To investigate whether endogenous PKA also played a role in sustaining basal I_{Ca}, the PKA inhibitor H-89 was tested. Figure 2 shows original I_{Ca} recordings (A) in the absence and in the presence of a range of H-89 concentrations to show that

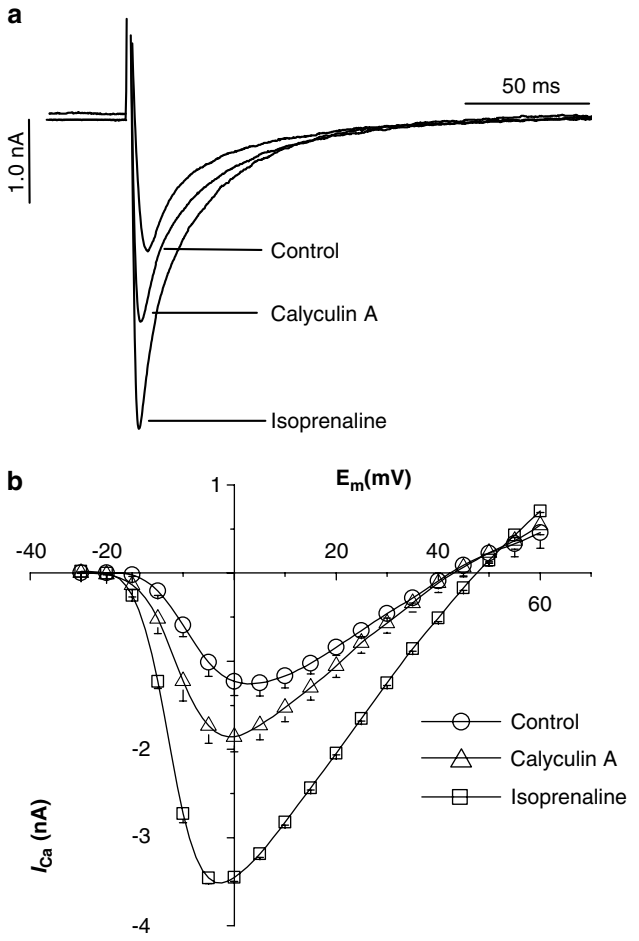

Figure 1 Effects of calyculin A and isoprenaline (both 1 μmol l^{-1}) on I_{Ca}. (a) Representative tracings of I_{Ca} elicited by square depolarising pulses from −40 to 0 mV in the absence and presence of calyculin A and isoprenaline. (b) *I–V* curves for I_{Ca} obtained in response to 5 mV step increments from −40 to 60 mV. Each data point is the mean ± s.e.m. of 8–10 different cells. Standard errors not visible are smaller than the symbols.

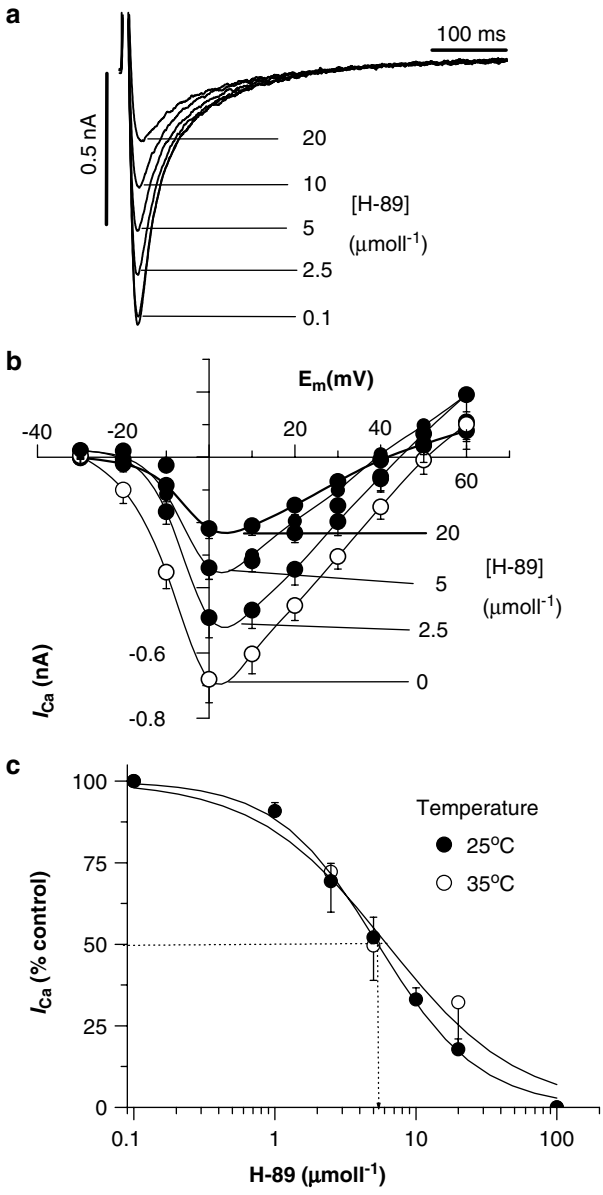

Figure 2 Effects of H-89 on I_{Ca} in isolated rat ventricular myocytes. (a) Representative original traces of I_{Ca} at differing concentrations of H-89. (b) *I–V* curves of I_{Ca} in the absence and presence of different concentrations of H-89. (c) Concentration–effect curve for H-89 at 35 and 25°C. Each data point is the mean ± s.e.m. from six to eight cells.

I_{Ca} is inhibited in a concentration-dependent manner. Figure 2b shows the effects of a range of H-89 concentrations on the *I–V* relations for I_{Ca} to illustrate that the inhibitory effect of H-89 was similar at all the potentials tested and did not alter the bell-shaped voltage dependence of the *I–V* curve or the reversal potential for the outward flow of Ca^{2+}. The IC_{50} was 5.4 μmol l^{-1} (Figure 2c; open symbols), a value similar to that described previously for the effects of H-89 on cell shortening in ferret ventricular myocytes (Hussain *et al*., 1999), as well as inhibition of tetanic Ca^{2+} transients in skeletal muscle (Blazev *et al*., 2001). Similar data were also obtained when I_{Ca} were recorded at room temperature, where the IC_{50} was 5.2 μmol l^{-1} (Figure 2c; closed symbols).

To establish whether the inhibitory effect of H-89 could be attributed to the inhibition of PKA, data in Figure 3 show the effects of 1 μmol l^{-1} isoprenaline in the presence of H-89. During these experiments, myocytes were first exposed to either 10 or 30 μmol l^{-1} H-89 until a steady-state level of I_{Ca} was achieved (typically 5–8 min). The solutions were then switched to H-89 plus isoprenaline. Figure 3a shows I_{Ca} tracings illustrating the effect of isoprenaline in the presence of 10 μmol l^{-1} H-89, a concentration that is almost double the IC_{50} value (see Figure 2). Although the response to isoprenaline was attenuated, it was not abolished: I_{Ca} increased by 93% in the presence of 10 μmol l^{-1} H-89 plus isoprenaline (Figure 3b). However, in the presence of 30 μmol l^{-1} H-89, the response to isoprenaline was almost completely blocked and I_{Ca} amplitude remained close to the values observed in the presence of 30 μmol l^{-1} H-89 alone (i.e. 23% of control; Figure 3d) and was not significantly different from this value. These data show that while 10 μmol l^{-1} H-89 did attenuate the effects of β-adrenergic receptor stimulation, relatively high concentrations (30 μmol l^{-1}) were required to fully suppress the isoprenaline-induced increase in I_{Ca}.

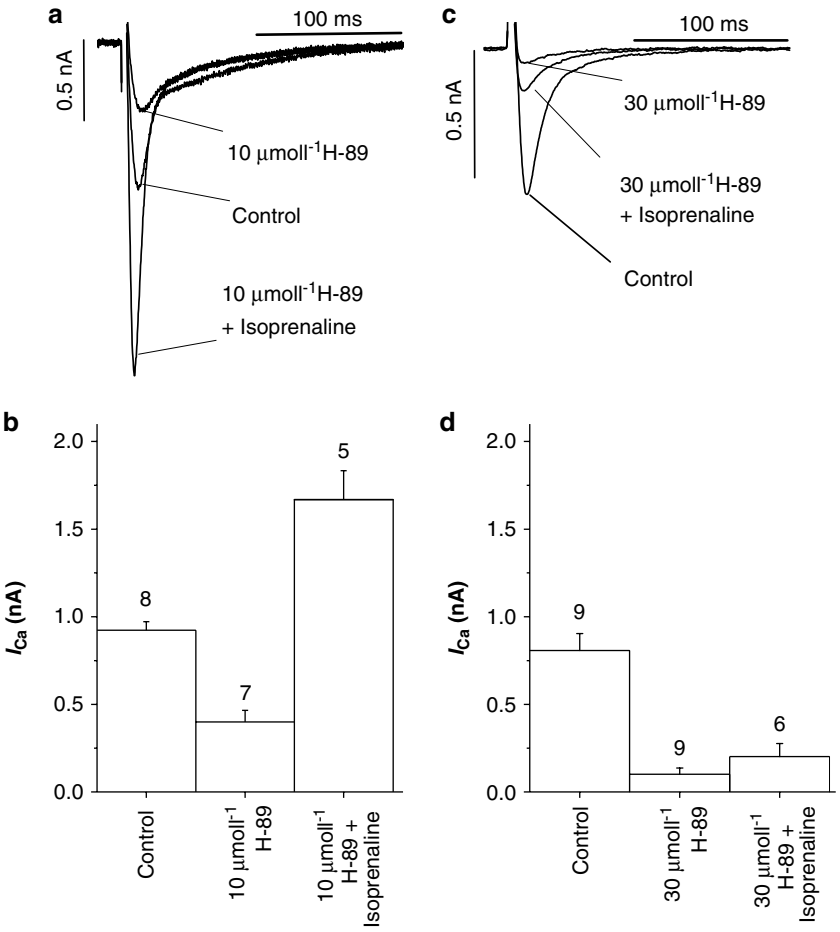

Figure 3 Effects of H-89 on the response to isoprenaline. The response to isoprenaline was determined following equilibration of myocytes with either 10 (a and b) or 30 μmol l^{-1} (c and d) H-89. The number above each bar is the number of separate myocytes tested. All the bars shown were significantly different from one another, except the effects of 30 μmol l^{-1} H-89 vs 30 μmol l^{-1} H-89 plus isoprenaline.

To gain further insights into mechanisms by which H-89 might act on the L-type Ca^{2+} channels, double-pulse protocols were used to investigate the effects of H-89, calyculin A and isoprenaline on time-dependent recovery of I_{Ca} from voltage-dependent inactivation. Original tracings in Figure 4a illustrate that under control conditions, I_{Ca} amplitude during the second test-pulse was small when the interpulse interval was short (e.g. 20 ms for the first pulse) and that I_{Ca} increased as the rest period was progressively lengthened such that at long interpulse intervals I_{Ca} recovered to the same amplitude as the I_{Ca} observed during the prepulse. A similar recovery of I_{Ca} from voltage-dependent inactivation was observed in the presence of calyculin A but not in the presence of H-89. This is shown quantitatively in Figure 4b and c, where I_{Ca} amplitude determined during the second test pulse was normalised to that in the pre–pulse and plotted against time before fitting with the Boltzmann function to determine $T_{0.5}$ (the time taken for I_{Ca} to recover to 50% of the I_{Ca} amplitude observed during the pre–pulse). Mean ($\pm$s.e.m.) $\%I_{Ca}$ recovered is shown in Figure 4b alongside the effects of calyculin A, isoprenaline and H-89. $T_{0.5}$ values are shown in Figure 4c to illustrate that the time course of recovery from voltage-dependent inactivation was significantly slowed in the presence of H-89 ($P<0.05$), but was not significantly different in the presence of calyculin A or isoprenaline (both 1 μmol l^{-1}).

To investigate the effects of the three compounds on channel availability, a second series of double-pulse protocols were performed to obtain the steady-state activation and inactivation curves for I_{Ca}. In these experiments, a 400 ms pulse to potentials between -40 and 60 mV was followed by a second pulse to 0 mV. I_{Ca} obtained at each potential were converted to conductance (g) using the following equation: $g = I_{Ca}/(E_m - E_{rev})$ to account for potential-dependent differences in the driving force for Ca^{2+} entry. The conductance was then normalised to the maximal value to generate the activation curves, which were then fitted with the Boltzmann function to obtain $E_{0.5}$ (V_m at which half-maximal conductance is obtained). In order to generate the inactivation curves, I_{Ca} amplitudes during the second step to 0 mV were first converted to conductance (as above) and then plotted against the prepulse potential before fitting with the Boltzmann equation to obtain $E_{0.5}$ for inactivation. Figure 5a shows I_{Ca} during the second step to 0 mV after the pre-test potential was varied between -40 to 60 mV in 5 mV increments. Maximal I_{Ca} during the second step were obtained when the pre-test potential did not activate I_{Ca} during the first few steps (e.g. -30 mV). I_{Ca} then progressively declined as the prepulse voltage was stepped to more positive

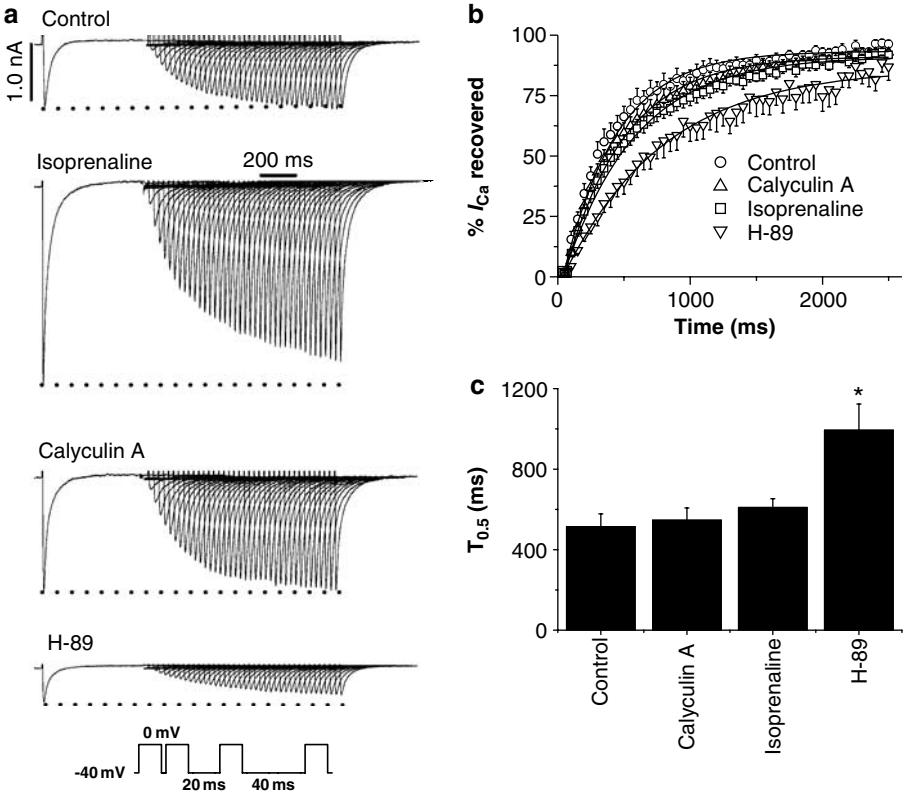

Figure 4 Effects of H-89, calyculin A and isoprenaline on recovery of I_{Ca} from voltage-dependent inactivation. (a) The inset in the bottom part of the figure shows the double-pulse protocol during which myocytes were depolarised from −40 to 0 mV with a progressively increasing interpulse interval (20 ms increments). The other panels in (a) show representative tracings illustrating the recovery of I_{Ca} in control conditions and in the presence of calyculin A (1 μmol l^{-1}), isoprenaline (1 μmol l^{-1}) and H-89 (10 μmol l^{-1}). (b) Mean (±s.e.m.) time course of I_{Ca} recovery fitted with the Boltzmann equation. (c) Mean (±s.e.m.) $T_{0.5}$ under control conditions ($n=6$) and in the presence of calyculin A ($n=6$), isoprenaline ($n=4$) and H-89 ($n=9$). *$P<0.05$.

values. Figure 5b shows the inactivation curves alongside the $E_{0.5}$ (Figure 5d) in control and in the presence of calyculin A, isoprenaline and H-89. Similarly, Figure 5c shows the activation curves and $E_{0.5}$ for activation of I_{Ca} (Figure 5e). Only isoprenaline significantly decreased $E_{0.5}$ for activation and inactivation by shifting the curves to more negative V_m values. Although calyculin A and H-89 both decreased $E_{0.5}$ for both activation and inactivation, these effects did not reach statistical significance (Figure 5d and e).

Discussion

A range of protein kinase and phosphatase inhibitors has previously been used to investigate the identity of the protein kinase(s) and phosphatases that sustain the basal I_{Ca} (e.g. Hartzell *et al*., 1995; Yuan & Bers, 1995; Hirayama & Hartzell, 1997; Dubell *et al*., 2002; Dubell & Rogers, 2004). Such tools can be an invaluable adjunct to other techniques, such as biochemical measurements of phosphorylation, in deciphering the cellular mechanisms of function and even the interactions between different pathways. However, for this to be possible, it is necessary to establish the selectivity and potency of the compounds used in the setting in which they are tested. In the present study, we evaluated the PKA inhibitor H-89 to assess the role of PKA-mediated phosphorylation in maintaining basal I_{Ca} in the absence of β-adrenergic receptor stimulation, as well as its role in the response to such stimulation.

The main findings were that H-89 produced a concentration-dependent inhibition of I_{Ca} with an IC_{50} of ~5.0 μmol l^{-1}, irrespective of the experimental temperature. H-89 also blocked the response to β-adrenergic receptor stimulation with 1 μmol l^{-1} isoprenaline, although concentrations as high as 30 μmol l^{-1} were required to achieve this. Secondly, basal I_{Ca} could be increased by inhibition of PP1/PP2A by calyculin A, although the response was smaller than that to isoprenaline. The overall interpretation of these data seems fairly straightforward–that basal I_{Ca} is regulated by constitutive PKA-mediated phosphorylation, which also appears to be modulated by protein phosphatases PP1/PP2A. However, as noted in the introduction, some previous studies were unable to demonstrate such inhibition of basal I_{Ca} with H-89, leading to the conclusion that PKA-mediated phosphorylation was unlikely to be involved. Data from the present study (Figure 3) can explain this apparent discrepancy.

Previous studies, rather cautiously, used relatively low concentrations (1 μmol l^{-1}) of H-89, presumably to avoid nonspecific effects on other kinases (Yuan & Bers, 1994; Dubell & Rogers, 2004). Such low concentrations were probably selected on the basis that the *in vitro* K_i for H-89 is 50 nmol l^{-1}. However, concentration–effect curves in the present study clearly show that the IC_{50} for H-89 in rat ventricular myocytes is ~5 μmol l^{-1} and that 1 μmol l^{-1}

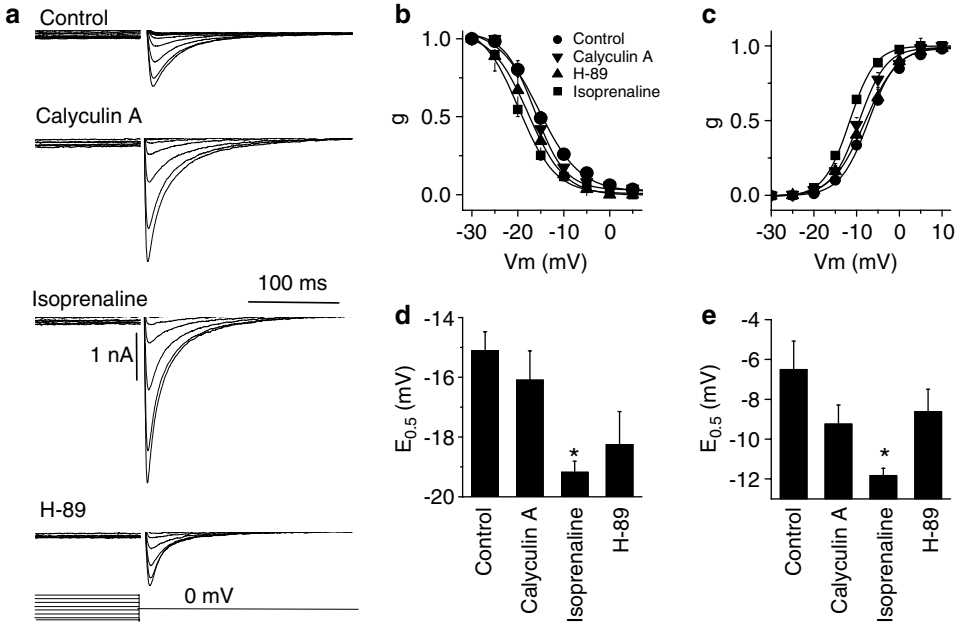

Figure 5 Effects of cayculin A, isoprenaline and H-89 on voltage-dependent activation and inactivation of I_{Ca}. Myocytes were stimulated using a double-pulse protocol where the voltage of the first pulse was increased in 5 mV step increments (400 ms) from −40 to 60 mV followed by a second step to 0 mV after 2 ms. I_{Ca} recorded were converted to conductance (see text). (a) I_{Ca} during the second pulse to 0 mV in control conditions and in the presence of 1 μmol l^{-1} calyculin A, 1 μmol l^{-1} isoprenaline and 10 μmol l^{-1} H-89. In each case, the largest amplitude I_{Ca} was recorded during the first step in the protocol. (b) Steady-state inactivation curves. (c) Steady-state activation curves. (d) Mean (±s.e.m.) voltage at half-maximal conductance for inactivation. (e) Mean (±s.e.m.) voltage for half-maximal conductance for activation. Both activation and inactivation curves were fitted with the Boltzmann function. Mean data are from 6–8 separate myocytes. *$P<0.05$.

produces only a small effect (<10% inhibition). It seems reasonable, therefore, to suggest that the low concentrations used in some of the previous studies were simply too low to produce any significant inhibition of I_{Ca}. It is difficult, however, to explain why 10 μmol l^{-1} H-89 was ineffective in ferret ventricular myocytes (Yuan & Bers, 1995), in which we have previously shown that the IC_{50} for inhibiting cell shortening was also ~5.0 μmol l^{-1} (Hussain *et al*., 1999). Furthermore, similar concentrations of H-89 have also been shown to be effective in inhibiting Ca^{2+} transients during tetanic contractions in skeletal muscle (Blazev *et al*., 2001), and even lower H-89 concentrations (K_i 76.4 nmol l^{-1}) could inhibit I_{Ca} recorded from cloned channels in HEK-293 cells (Perez-Reyes *et al*., 1994). One possible reason for these apparent discrepancies could be that the *in vitro* K_i determined using purified kinases (Chijiwa *et al*., 1990) is not directly applicable to intact cardiac myocytes, where higher concentrations are required, perhaps due to inadequate equilibration even after a steady state is reached. Differences in the K_i values could also arise if different channel types are studied for example, cloned vs native Ca^{2+} channels (Perez-Reyes *et al*., 1994). It is therefore essential that the effective concentrations be determined empirically in the test system under study.

The second aspect to the inhibitory action of H-89 on I_{Ca} was the concentration required to inhibit the response to β-adrenergic receptor stimulation. A previous study showed that the response to 0.1 μmol l^{-1} isoprenaline could be blocked by 10 μmol l^{-1} H-89 (Mitarai *et al*., 2000). In the present study, 10 μmol l^{-1} H-89 attenuated the response to 1 μmol l^{-1} isoprenaline, but a much higher concentration (30 μmol l^{-1}) was required to produce complete inhibition. The most likely explanation for this observation is that a much greater concentration of the free catalytic subunit of PKA becomes available in the presence of 1.0 μmol l^{-1} isoprenaline. Consequently, much higher concentrations of H-89 are required for effective inhibition. This explanation is plausible given that the proposed mechanism of action of H-89 is thought to involve competitive binding at the ATP binding site on the catalytic subunit rather than interactions with the cAMP binding site on the regulatory subunit. [ATP] in myocytes is normally in the millimolar range and therefore not a limiting factor (Elliott *et al*., 1989). This mechanism of action has been postulated from binding studies performed to assess the degree of competition between binding of ATP and H-89 to PKA (Hidaka & Kobayashi, 1992).

L-type Ca^{2+} channels are subject to Ca^{2+}-dependent inactivation as well as voltage-dependent inactivation, which has several components (Hadley & Lederer, 1991; Findlay, 2002a). Ca^{2+}-dependent inactivation occurs as Ca^{2+} enters the cell across the channel pore, as well as due to the Ca^{2+} released from the sarcoplasmic reticulum. Voltage-dependent inactivation, although not particularly well characterised in molecular terms, is likely to involve conformational changes in the channel complex to impede or obstruct the flow of Ca^{2+} across the channel pore. The inactivation mechanism(s) may also be modulated by the ancillary subunits as well as the phosphorylation status of any number of these components (Mitarai *et al*., 2000; Findlay, 2002b; 2004).

β-Adrenergic stimulation is well known to alter the steady-state activation and inactivation of L-type Ca^{2+} channels and the $E_{0.5}$ for activation and inactivation of I_{Ca} is typically shifted to the left (Chen *et al*., 2002). This implies that the channel is

more susceptible to activation and inactivation when phosphorylated, and that lower voltages would be required to activate or inactivate the channel. A logical extension of this argument is that dephosphorylation, for example with H-89, may be expected to have the opposite effect and shift the activation/inactivation curves to the right. Data in Figure 5 show that while the expected leftward shift in the activation/inactivation curves was observed in response to isoprenaline, H-89 and calyculin A had no significant effect in either direction, suggesting that the voltage sensor was unlikely to be affected. In fact, although the effects of H-89 were not significant, the general trend was for the $E_{0.5}$ values to be decreased in the presence of H-89 (i.e. in the same direction as in response to isoprenaline). Similar data were also reported by Yuan & Bers (1994; 1995), where the $E_{0.5}$ values were significantly shifted in the negative direction. These data suggest that the effects of H-89 may be more complex than simple inhibition of PKA.

In addition, in paired-pulse experiments performed to examine whether there was an effect on the overall time course of recovery of I_{Ca} from inactivation, H-89 ($10\,\mu mol\,l^{-1}$) was found to slow the time-dependent recovery of I_{Ca} from inactivation (Figure 4), whereas isoprenaline and calyculin A had no significant effect. Whether this effect of H-89 was due to decreased PKA-mediated phosphorylation is difficult to say given that isoprenaline and calyculin did not have the opposite effect. It is interesting to note, however, that this effect of H-89 on recovery from inactivation was previously observed in studies where I_{Ca} amplitude was largely unaffected (Yuan & Bers, 1994; 1995). The fact that the two effects can occur independently suggests that different mechanisms could be involved simultaneously. Inhibition of I_{Ca} could, for instance, occur in response to the effects of H-89 on PKA-mediated phosphorylation, whereas the slowed recovery from inactivation could occur owing to nonspecific actions on other targets. That H-89 has the potential to produce additional non-PKA-related effects is supported by several lines of evidence. First, we have previously shown that H-89 could inhibit SR Ca^{2+} uptake in SR vesicles independently of the effect on phosphorylation of phospholamban, probably due to a direct effect on the SR Ca^{2+} ATPase (Hussain *et al*., 1999). Secondly, in the accompanying paper, we show that H-89 can also inhibit K^+ currents that are not sensitive to modulation by β-adrenergic receptor stimulation. It is therefore necessary to consider the concentrations of H-89 used carefully before implicating the involvement or exclusion of PKA-mediated phosphorylation.

Given that basal PKA-mediated phosphorylation does appear to be involved in sustaining I_{Ca}, it is of interest to know the mechanisms responsible for PKA activity that exists in the absence of β-adrenergic receptor stimulation, that is, is there a basal level of activation of β-adrenergic receptors even in the absence of catecholamines? or are there separate constitutive mechanism(s) responsible for the tonic levels of PKA-mediated phosphorylation? These questions could be important in the context of heart failure, where alterations in basal phosphorylation of L-type Ca^{2+} channels, perhaps due to enhanced phosphatase activity (Chen *et al*., 2002; Neumann, 2002), could contribute to the pathogenesis of the disease.

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