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H-89 decreases the gain of excitation–contraction coupling and attenuates calcium sparks in the absence of beta-adrenergic stimulation

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ABSTRACT

This study used the selective protein kinase A (PKA) inhibitor H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) to determine the role of basal PKA activity in modulating cardiac excitation–contraction coupling in the absence of β-adrenergic stimulation. Basal intracellular cyclic AMP (cAMP) levels measured in isolated murine ventricular myocytes with an enzyme immunoassay were increased upon adenylyl cyclase activation (forskolin; 1 and 10 μM) or phosphodiesterase inhibition (3-isobutyl-1-methylxanthine, IBMX; 300 μM). Forskolin and IBMX also caused concentration-dependent increases in peak Ca^{2+} transients (fura-2) and cell shortening (edge-detector) measured simultaneously in field-stimulated myocytes (37 °C). Similar effects were seen upon application of dibutyryl cAMP. In voltage-clamped myocytes, H-89 (2 μM) decreased basal Ca^{2+} transients, contractions and underlying Ca^{2+} currents. H-89 also decreased diastolic Ca^{2+} and the gain of excitation–contraction coupling (Ca^{2+} release/Ca^{2+} current), especially at negative membrane potentials. This was independent of alterations in sarcoplasmic reticulum (SR) Ca^{2+} loading, as SR stores were unchanged by PKA inhibition. H-89 also decreased the frequency, amplitude and width of spontaneous Ca^{2+} sparks measured in quiescent myocytes (loaded with fluo-4), but increased time-to-peak. Thus, H-89 suppressed SR Ca^{2+} release by decreasing Ca^{2+} current and by reducing the gain of excitation–contraction coupling, in part by decreasing the size of individual Ca^{2+} release units. These data suggest that basal PKA activity enhances SR Ca^{2+} release in the absence of ß-adrenergic stimulation. This may depress contractile function in models such as aging, where the cAMP/PKA pathway is altered due to low basal cAMP levels.

1. Introduction

Upon activation of β-adrenoceptors in cardiomyocytes, adenylyl cyclase increases conversion of ATP into cyclic AMP (cAMP). cAMP activates protein kinase A (PKA), which is anchored to both Ca^{2+} channels and ryanodine receptors (Gray et al., 1997; Marx et al., 2000) and will phosphorylate components of the excitation–contraction coupling pathway to increase inotropy and lusitropy (Bers, 2002). Phosphorylation of L-type Ca^{2+} channels increases peak Ca^{2+} current (Kameyama et al., 1986; Mery et al., 1993). This triggers Ca^{2+} transients via Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors (Bers, 2002). Phosphorylation of troponin I decreases the affinity of troponin C for Ca^{2+}, causing Ca^{2+} to dissociate from the myofilaments more quickly and thus the rate of relaxation of the cardiac muscle increases (Zhang et al., 1995). Phosphorylation of phospholamban alleviates its inhibition of the SR Ca^{2+}-ATPase and increases SR Ca^{2+} uptake, which results in a faster decay of the Ca^{2+} transient (Li et al., 2000). Phosphorylation by PKA has also been shown to increase the open probability of ryanodine receptors, though this remains controversial (Kushnir and Marks, 2010).

N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) selectively and potently inhibits PKA from binding ATP, thus attenuating its enzymatic activity (Chijiwa et al., 1990; Hidaka and Kobayashi, 1992). H-89 has been shown to modulate components of excitation–contraction coupling in cardiomyocytes. In the presence of β-adrenoceptor agonists or adenylyl cyclase activators, H-89 attenuates the increase in inotropy and lusitropy that occurs (Bracken et al., 2006; Hussain et al., 1999; Yuan and Bers, 1995). Interestingly, H-89 has similar effects in the absence of β-adrenergic stimulation. This is likely due to basal intracellular cAMP levels that are tightly regulated by intrinsic adenylyl cyclase and phosphodiesterase activity, resulting in a

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degree of constitutive PKA activity (Chase et al., 2010; duBell et al., 1996; Iancu et al., 2008; Yan et al., 2011).

Previous work has shown that inhibition of PKA with H-89 reduces peak Ca^{2+} transients in isolated ventricular myocytes (Chase et al., 2010; Hussain et al., 1999). It is possible that H-89 inhibits SR Ca^{2+} release by inhibiting tonic phosphorylation of Ca^{2+} channels in ventricular myocytes (Bracken et al., 2006; Chase et al., 2010; Crump et al., 2006; Hussain et al., 1999; Mitarai et al., 2000). In contrast, some studies report that H-89 has no effect on basal Ca^{2+} current (duBell and Rogers, 2004; Yuan and Bers, 1995), which suggests that H-89 inhibits SR Ca^{2+} release by effects on other components of the excitation–contraction coupling pathway. However, the effect of H-89 on the relationship between Ca^{2+} current, SR Ca^{2+} release and contraction has not been previously investigated. The objective of this study was to determine the role of basal PKA activity in modulating cardiac excitation–contraction coupling. In these studies, Ca^{2+} currents, Ca^{2+} transients and contractions were simultaneously recorded in isolated murine ventricular myocytes in the absence and presence of H-89. Effects of H-89 on SR Ca^{2+} content, excitation–contraction coupling gain and unitary Ca^{2+} release (Ca^{2+} sparks) were also evaluated.

2. Materials and methods

2.1. Isolation of ventricular myocytes

Experiments were conducted in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (CCAC, Ottawa, ON: Vol. 1, 2nd ed., 1993; Vol. 2, 1984) and were approved by the Dalhousie University Committee on Laboratory Animals. C57BL/6 female mice (7.8 ± 0.3 mos) were obtained from Charles River Laboratories (St. Constant, QC). Ventricular myocytes were isolated via enzymatic dissociation as previously described (Fares et al., 2012). Briefly, mice were anaesthetized with sodium pentobarbital (200 mg/kg, i.p.) and 100 U of heparin. Hearts were cannulated in situ through the aorta, excised, and perfused retrogradely at 2 ml/min for 10 min with 37 °C oxygenated (100% O_2) Ca^{2+}-free buffer solution containing (in mM): 105 NaCl, 5 KCl, 25 HEPES, 0.33 NaH_2PO_4, 1 $MgCl_2$, 20 glucose, 3 Na-pyruvate and 1 lactic acid (pH 7.4). The heart was then perfused with solution of the same composition plus 50 μM Ca^{2+}, collagenase (8 mg/30 ml, Worthington Type I, 250 U/mg), dispase II (3.5 mg/30 ml, Roche) and trypsin (0.5 mg/30 ml, Sigma) for 8–10 min. The ventricles were removed from the atria, minced, and stored at room temperature in modified Kraftbrühe (KB) buffer containing (in mM): 50 L-glutamic acid, 30 KCl, 30 KH_2PO_4, 20 taurine, 10 HEPES, 10 glucose, 3 $MgSO_4$ and 0.5 EGTA (pH 7.4). The tissue was gently agitated to dissociate individual myocytes and the supernatant was filtered with a 225 μm polyethylene filter (Spectra/Mesh). Quiescent rod-shaped myocytes with clear striations were used in experiments.

2.2. cAMP assay

Myocytes were isolated as described above and ~10 ml aliquots of KB supernatant were centrifuged until a pellet was formed (~70 min, 18 g). The pellet was resuspended in HEPES buffer containing (in mM): 145 NaCl, 10 glucose, 10 HEPES, 4 KCl, 1 $CaCl_2$, and 1 $MgCl_2$ (pH 7.4). A hemocytometer was used to determine myocyte density, and cells were added to 96-well plates at a density of ~1000 cells/well. Cells were incubated at room temperature for one hour, followed by a ten min treatment with one of the following: DMSO solvent control (0.1%), forskolin (1 or 10 μM), or 3-isobutyl-1-methylxanthine (IBMX; 300 μM). Cells were then incubated in a solution of dodecyltrimethylammonium bromide (0.25%) for 10 min to rupture cellular membranes. The cell lysates were stored at −20 °C for a maximum of 14 day until the cAMP assay was performed. Samples were thawed and acetylated to increase assay sensitivity. Intracellular cAMP levels in the cell lysates were determined using an Amersham™ cAMP Biotrak™ Enzymeimmunoassay (EIA) System (GE Healthcare Life Sciences, Baie d'Urfe, QC). A plate reader (450 nm, ELx800, BioTek Instruments, Winooski, VT) was used to measure sample absorbances and cAMP concentrations were calculated from a standard curve (2 to 128 fmol cAMP) fit with a nonlinear regression (R^2=0.99). The protein content of each sample was determined using a detergent-compatible Lowry assay kit (BioRad, Mississauga, ON) and cAMP concentrations were normalized to the amount of protein in the sample.

2.3. Electrophysiology

Isolated myocytes were incubated with the Ca^{2+} sensitive fluorescent dye fura-2 acetoxymethyl (AM) ester (5 μM; Invitrogen, Burlington, ON) for 20 min in darkness. Aliquots of cell suspension were placed in a custom-made glass-bottomed chamber mounted on the stage of an inverted microscope (Nikon Eclipse, TE200, Nikon Canada, Mississauga, ON). Cells were superfused at 3 ml/min (37 °C) with HEPES buffer containing (mM): 145 NaCl, 10 glucose, 10 HEPES, 4 KCl, 1 $CaCl_2$, and 1 $MgCl_2$ (pH 7.4). In field-stimulation experiments, a pair of platinum electrodes were placed in the bath and positioned on either side of the microscope field of view. Bipolar pulses (3 ms, 4 Hz) were generated by a stimulus isolation unit (Model # SIU-102; Warner Instruments, Hamden, CT) and controlled by pClamp 8.1 software (Molecular Devices, Sunnyvale, CA). Ca^{2+} transients and contractions were measured simultaneously. In voltage clamp experiments, the HEPES buffer also contained 4-aminopyridine (4 mM) to inhibit transient outward K^+ current, and lidocaine (0.3 mM) to inhibit Na^+ current. Na^+ current was also inactivated by a pre-pulse to −40 mV prior to test pulses. Ca^{2+} transients, contractions and Ca^{2+} current, were measured simultaneously. All experiments were conducted at 37 °C.

Simultaneous recordings of whole cell fluorescence and cell shortening were made by splitting the microscope light between a CCD camera (model TM-640, Pulnix America) and a photomultiplier tube (Photon Technologies International (PTI), Birmingham, NJ) with a dichroic cube (Chroma Technology Corp., Rockingham, VT). Camera images were displayed on a television monitor and unloaded cell shortening was measured with a video edge-detector (Crescent Electronics, Sandy, UT). Fura-2 was alternately excited with 340 and 380 nm light and fluorescence emission was measured at 510 nm (5 ms sampling interval) with a DeltaRam fluorescence system and Felix v1.4 software (PTI). Intracellular Ca^{2+} concentrations were obtained with an in vitro calibration curve, as previously described (Fares et al., 2012; O'Brien et al., 2008). For IBMX, forskolin and dibutyryl cAMP concentration-response curves, recordings were made following at least a 3 min exposure to each drug concentration (as highlighted in the results section). No further inotropic effects were seen with longer incubation times.

Membrane potentials and currents were recorded by impaling cells with high-resistance microelectrodes (18–28 MΩ) filled with filtered 2.7 M KCl. Discontinuous single electrode voltage clamp was performed with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA; 5–6 Hz) and protocols were generated with ClampEx v8.2 software (Molecular Devices). Trains of five 50 ms conditioning pulses from −80 to 0 mV (2 Hz) were delivered to cells, followed by repolarization to −40 mV for 450 ms. Ca^{2+} currents and transients were recorded simultaneously