Protein Kinase A-Mediated Phosphorylation Contributes to Enhanced Contraction Observed in Mice That Overexpress β-Adrenergic Receptor Kinase-1

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ABSTRACT

Transgenic mice with cardiac specific overexpression of β-adrenergic receptor kinase-1 (βARK-1) exhibit reduced contractility in the presence of adrenergic stimulation. However, whether contractility is altered in the absence of exogenous agonist is not clear. Effects of βARK-1 overexpression on contraction were examined in mouse ventricular myocytes, studied at 37°C, in the absence of adrenergic stimulation. In myocytes voltage-clamped with microelectrodes (18–26 MΩ; 2.7 M KCl) to minimize intracellular dialysis, contractions were significantly larger in βARK-1 cells than in wild-type myocytes. In contrast, when cells were dialyzed with patch pipette solution (1–3 MΩ; 0 mM NaCl, 70 mM KCl, 70 mM potassium aspartate, 4 mM MgATP, 1 mM MgCl₂, 2.5 mM KH₂PO₄, 0.12 mM CaCl₂, 0.5 mM EGTA, and 10 mM HEPES), the extent of cell shortening was similar in wild-type and βARK-1 myocytes. Furthermore, when cells were dialyzed with solutions that contained phosphodiesterase-sensitive sodium-cAMP (50 μM), the extent of cell shortening was similar in wild-type and βARK-1 myocytes. However, when patch solutions were supplemented with phosphodiesterase-resistant 8-bromo-cAMP (50 μM), contractions were larger in βARK-1 than wild-type cells. This difference was eliminated by the protein kinase A inhibitor N-[2-[(4-bromocinnamylamino)ethyl]-5-isoquinoline (H89). Interestingly, Ca²⁺ current amplitudes and inactivation rates were similar in βARK-1 and wild-type cells in all experiments. These results suggest components of the adenylyl cyclase-protein kinase A pathway are sensitized by chronically increased βARK-1 activity, which may augment contractile function in the absence of exogenous agonist. Thus, changes in contractile function in myocytes from failing hearts may reflect, in part, effects of chronic up-regulation of βARK-1 on the cAMP-protein kinase A pathway.

Cardiac contraction is initiated by a rise in intracellular free Ca²⁺, derived primarily from internal stores in the sarcoplasmic reticulum (SR; Bers, 2001). Ca²⁺ release from the SR is triggered by excitation of the sarcolemma through a process called excitation-contraction (EC) coupling (Bers, 2001). In the healthy heart, Ca²⁺ cycling between intra- and extracellular compartments is tightly controlled. However, disruptions in Ca²⁺ cycling can impair cardiac contractile function in diseases such as congestive heart failure (Marks et al., 2002; Pieske et al., 2002). Alterations in the expression, function, or regulation of various components of EC coupling are thought to contribute to contractile dysfunction in heart failure (Striessnig, 1999). The sympathetic nervous system is activated in heart failure to compensate for diminished contractile function via activation of cardiac β-adrenergic receptors (βARs) (Keys and Koch, 2004). Stimulation of cardiac βARs activates adenylyl cyclase, which increases intracellular cAMP levels and leads to phosphorylation of protein targets via protein kinase A (Wallukat, 2002). However, chronic adrenergic stimulation results in phosphorylation and desensitization of βARs, which further impairs contractile function in the failing heart (Hausdorff et al., 1990; Post et al., 1999).

Desensitization of βARs is mediated by phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs) and second messengers. GRKs mediate homologous desensitization; thus, their kinase activity is dependent on agonist occupancy (Lohse et al., 1990). GRKs are a family of serine/threonine kinases (Penela et al., 2006). At present, seven isoforms have been identified, known as GRK1 through 7 (Penela et al., 2006). GRK2, also known as β-adrenergic...
receptor kinase-1 (βARK-1), is the predominant isoform expressed in the heart (Hausdorff et al., 1990). βARK-1 is expressed in both myocyte and nonmyocyte cardiac cells (Penela et al., 2006). Interestingly, βARK-1 expression and activity are increased in the failing heart (Ungerer et al., 1993). This up-regulation of βARK-1 increases phosphorylation of activated βARs, which leads to desensitization, and down-regulation of the receptors (Post et al., 1999). This dampens the βAR signaling cascade, which in turn alters Ca2+ cycling, and impairs cardiac contractile function (Rockman et al., 2002).

To investigate the role of βARK-1 in the modulation of cardiac contractility, a number of studies have examined the effects of βARK-1 overexpression on cardiac function in a transgenic mouse model (Koch et al., 1995; Rockman et al., 1996; Chen et al., 1998). Overexpression of βARK-1 in these mice is driven by the α myosin heavy chain promoter (Koch et al., 1995). This leads to a 3- to 5-fold overexpression of βARK-1 in cardiac myocytes, which is similar to the levels seen in heart failure (Koch et al., 1995). However, βARK-1 is overexpressed only in myocytes in this model and may therefore not reflect the overall role of βARK-1 overexpression in heart failure (Penela et al., 2006). In vivo studies have shown that isoproterenol-stimulated increases in heart rate and contractility are significantly reduced in βARK-1 mice compared with wild-type controls (Koch et al., 1995; Rockman et al., 1996; Chen et al., 1998). In addition, isoproterenol-stimulated adenyl cyclase activity is reduced in sarcolemmal membranes from βARK-1 mice compared with wild-type animals (Koch et al., 1995). Interestingly, adenyl cyclase activity is also reduced in sarcolemmal membranes from βARK-1 mice in the absence of isoproterenol (Koch et al., 1995). This suggests that chronic overexpression of βARK-1 may alter the adenyl cyclase-protein kinase A pathway in the heart, and thereby alter cardiac function even in the absence of agonist. However, little is known about the effects of βARK-1 overexpression on intrinsic myocyte function.

We hypothesized that alterations in the cAMP-protein kinase A pathway in βARK-1 ventricular myocytes alter cardiac contractile function in the absence of exogenous βAR agonist. The objectives of this study were to 1) compare the extent of cell shortening and magnitude of L-type Ca2+ current (Ica,L) in myocytes from βARK-1 and wild-type mice when cells were voltage-clamped with high-resistance microelectrodes to minimize intracellular dialysis with pipette solutions; 2) determine whether the extent of cell shortening and magnitude of Ica,L differed between βARK-1 and wild-type myocytes when cells were dialedyzed with patch pipette solutions in the absence and presence of various analogs of cAMP; and 3) determine whether differences between βARK-1 and wild-type myocytes were mediated by protein kinase A. All experiments were conducted in regularly paced ventricular myocytes, at physiological temperature (37°C), in the absence of exogenous βAR stimulation.

### Materials and Methods

#### Experimental Animals

Experiments were performed on cardiac ventricular myocytes isolated from approximately 6-month-old male and female βARK-1 mice and wild-type littermates. The age ranges used were similar in the wild-type and βARK-1 groups, as shown in Table 1. In addition, there was no significant difference in weight between the wild-type and βARK-1 mice (Table 1). The initial breeding pair were made up of female wild-type (B6SJLF1/J) and male βARK-1 (B6SJL-TgN[ARK12]1Wjk) mice obtained from The Jackson Laboratory (Bar Harbor, ME). The breeding colony was maintained by breeding βARK-1 animals with wild-type littermates. βARK-1 mice are hemizygous for the transgene. Therefore, transgenic mice were identified by genotyping with a protocol provided by The Jackson Laboratory (www.jax.org/jaxmice/micetech). All experiments respected the guidelines stated in the Canadian Council on Animal Care (1980, 1984) and were approved by the Dalhousie University Committee on Animal Care.

#### Cell Isolation

Myocytes were anesthetized with an intraperitoneal injection of pentobarbital sodium (200–300 mg/kg), coinjected with heparin (100 U) to inhibit blood coagulation. The thoracic cavity was opened with a parasternal incision. The aorta was incised, cannulated in situ, and perfused at 2.2 ml/min for 10 min with oxygenated, Ca2+-free solution of the following composition: 130 mM NaCl, 5 mM KCl, 25 mM HEPES, 0.33 mM Na2HPO4, 1 mM MgCl2, 20 mM glucose, 3 mM sodium pyruvate, and 1 mM lactic acid (pH 7.4 with NaOH). The solution was maintained at 37°C with a heated circulating water bath. Then, the hearts were perfused with the Ca2+-free solution supplemented with 50 μM Ca2+, 24 mg/30 ml of collagenase (type I; Worthington, Biochemicals, Freehold, NJ), 10 mg/30 ml of neutral protease dispase II, and 1 mg/ml trypsin. After approximately 10 min, the ventricles were excised, minced, and stored at room temperature until use in a high K+ solution containing 80 mM KOH, 50 mM glutamic acid, 30 mM KCl, 30 mM KH2PO4, 20 mM taurine, 10 mM HEPES, 10 mM glucose, 3 mM MgSO4, and 0.5 mM EGTA (pH 7.4 with KOH).

#### Data Acquisition

Isolated myocytes were placed on the stage of an inverted microscope and allowed to settle for approximately 10 min. Then, the cells were superfused at 3 ml/min with a standard buffer solution of the following composition: 145 mM NaCl, 10 mM glucose, 10 mM HEPES, 1 mM MgCl2, and 1 mM MgCl2 (pH 7.4 with NaOH). This solution was supplemented with 0.3 mM lidocaine and 4 mM 4-aminoipyridine to block Na+ and K+ currents, respectively. All experiments were conducted at 37°C.

In experiments designed to minimize intracellular dialysis with the electrode filling solutions, cells were voltage-clamped with high-resistance microelectrodes (18–26 MΩ; filled with 2.7 M KCl). In other experiments, cells were voltage-clamped with patch pipettes (1–3 MΩ) filled with 0 mM NaCl, 70 mM KCl, 70 mM potassium aspartate, 4 mM MgATP, 1 mM MgCl2, 2.5 mM KH2PO4, 0.12 mM CaCl2, 0.5 mM EGTA, and 10 mM HEPES (pH 7.2). In some experiments, this pipette filling solution was supplemented with either 50 μM 8-bromo-cAMP or 50 μM sodium-cAMP. In all cases, discontinuous single-electrode voltage clamp (5–8 kHz) was conducted with pCLAMP software, version 8.0 (Molecular Devices, Sunnyvale, CA) and an Axoclamp-2B amplifier (Molecular Devices). Unloaded cell shortening was recorded with a video edge detector (Crescent Electronics, Sandy, UT) and video camera (model TM-640; Pulnix America, Inc., Sunnyvale, CA) operating at 120 Hz. In some experiments, the protein kinase inhibitor H89 (5 μM) was added to the extracellular buffer. In these experiments, the control extracellular solution contained the same concentration of DMSO as used in the H89 studies as a solvent control.

Several voltage-clamp protocols were used. In all cases, voltage-
clamp test steps were preceded by five 200-ms conditioning pulses from the holding potential of −80 to 0 mV to provide a consistent history of activation. Cells were then repolarized to −40 mV for 500 ms. In some experiments, complete contraction-voltage and current-voltage relations were elicited by a series of 250-ms depolarizing steps from −40 mV to potentials between −30 and +60 mV, in increments of 10 mV. However, a single 250-ms test step from −40 to 0 mV was used to elicit peak inward ICa-L, and contraction in experiments with HS9. A single test step was used in these experiments because preliminary experiments showed that the concentration of DMSO used to dissolve the HS9 caused contractions to run down during lengthy voltage-clamp protocols.

Data Analysis. All analyses were performed with pCLAMP software. The extent of cell shortening was measured as the difference between the peak contraction and a baseline immediately before cell shortening. The velocity of shortening was determined by dividing the amplitude of contraction by the time to peak contraction and is expressed in micrometers per second. The velocity of relengthening was determined by dividing one-half the amplitude of contraction by the time-to-half relaxation and also is expressed in micrometers per second. ICa-L amplitude was measured as the difference between the peak inward current and a reference point at the end of the voltage step. Time constants for inactivation of ICa-L were measured with Clampfit 8.1 (Axon Instruments). The fast (τf) and slow (τs) time constants of inactivation for ICa-L were determined by fitting the inactivation phase of ICa-L with an exponential function with two exponential components. Cell membrane area was determined by integrating the capacitive transient with pCLAMP software. ICa-L was normalized by cell capacitance and is expressed as current density. Cell length was used to normalize the contraction data.

Data are presented as means ± S.E.M. When two groups were compared, differences between means were assessed by an unpaired Student’s t test. Differences between mean contraction-voltage and current-voltage relationships for βARK-1 and wild-type mice were analyzed by two-way repeated measures analysis of variance. SigmaStat software, version 2.0 (Systat Software, Inc., San Jose, CA) was used for all statistical analyses. No more than two myocytes from the same heart were included in any one data set.

Chemicals. Pentobarbital sodium, trypsin, lidocaine, HEPES, EGTA, MgCl2, 4-aminopyridine, HS9, 8-bromo-cAMP, sodium-cAMP, EDTA, sodium-pyruvate, lactic acid, L-glutamic acid, taurine, and Mg-ATP were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). DMSO was purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada). The dispase II was purchased from Roche Diagnostics (Laval, QC, Canada). Heparin was purchased from Organon (Toronto, ON, Canada). Type I collagenase was purchased from Worthington Biochemicals. All other chemicals were purchased from BDH (Toronto, ON, Canada). HS9 was dissolved in DMSO; the concentration of DMSO in the extracellular buffer was 0.025%. All other chemicals were dissolved in deionized water.

Results

Ventricular cell size and/or membrane area is altered in myocytes from certain strains of transgenic mice (Yatani et al., 1999; Knollmann et al., 2000; Olsson et al., 2004). Therefore, we measured cell length and cell capacitance to determine whether there were differences in these parameters between βARK-1 and wild-type mice. There was no significant difference in mean cell length between myocytes from wild-type and βARK-1 mice (Table 1). However, cell capacitance was significantly greater in myocytes from βARK-1 mice than in myocytes from wild-type mice (Table 1). Because cell capacitance is proportional to cell membrane area, these results suggest that sarcoplasmic area is increased in βARK-1 myocytes. Therefore, in all subsequent experiments, measurements of ICa-L were normalized to cell capacitance to account for differences in cell surface area. We also normalized all contraction data to cell length.

In the first series of experiments, we compared the extent of cell shortening and ICa-L in wild-type and βARK-1 cells impaired with high-resistance microelectrodes. High-resistance microelectrodes were used in these studies to minimize cell dialysis and distortion of differences in cytosolic composition between wild-type and βARK-1 myocytes. In these experiments, cells were voltage-clamped with 250-ms test steps from −40 mV to potentials between −40 and +60 mV, delivered in increments of 10 mV, to elicit contractions and ICa-L (Fig. 1, top). The test step was preceded by five 200-ms conditioning pulses from −80 to 0 mV to provide comparable activation histories in both wild-type and βARK-1 cells. Figure 1, A and B, shows representative contractions and ICa-L elicited by a test step from −40 to −10 mV recorded from wild-type and βARK-1 myocytes, respectively. The extent of cell shortening was larger in the βARK-1 myocyte than the wild-type cell, although amplitudes of ICa-L were similar in the two cells. The extent of cell shortening and ICa-L were plotted as a function of voltage to determine contraction-voltage and current-voltage relations, respectively. The mean contraction-voltage curves were bell-shaped in both wild-type and βARK-1 myocytes (Fig. 1C). However, contractions at the peak of the curve were significantly larger in βARK-1 myocytes than in wild-type cells (Fig. 1C). Figure 1D compares mean current-voltage curves in wild-type and βARK-1 myocytes. Mean amplitudes of ICa-L did not differ between wild-type and βARK-1 myocytes at any membrane potential examined (Fig. 1D). We also compared the inactivation rates for ICa-L in wild-type and βARK-1 myocytes (Table 2). Results showed that τf and τs time constants of inactivation for ICa-L were similar in wild-type and βARK-1 cells (Table 2). We also determined and compared the velocity of shortening and the velocity of relengthening of contractions in wild-type and βARK-1 myocytes. Results showed that there were no significant differences in the rates of contraction and relaxation between wild-type and βARK-1 myocytes (Fig. 1, E and F). Thus, contraction is enhanced in βARK-1 myocytes compared with wild-type controls in the absence of exogenous agonist.

Next, we determined whether EC coupling was altered in ventricular myocytes isolated from βARK-1 mice when cells were dialyzed with a standard potassium-based patch pipette solution (Fig. 2). The experimental protocol is shown at the top of the figure. Figure 2, A and B, shows representative contractions and currents recorded from a wild-type and βARK-1 myocyte, respectively. Contractions and ICa-L were similar in the two cell types. We next determined whether the voltage dependence of contraction and ICa-L differed in wild-type and βARK-1 myocytes when cells were dialyzed with pipettes containing a standard potassium-based solution. Mean amplitudes of cell shortening were similar in wild-type and βARK-1 cells over a wide range of voltages (Fig. 2C). Figure 2D compares mean current-voltage relations in wild-type and βARK-1 myocytes. Mean amplitudes of ICa-L did not differ between wild-type and βARK-1 myocytes until the voltage exceeded +40 mV. At membrane potentials more positive than +40 mV, ICa-L was larger in βARK-1 cells than in wild-type cells (Fig. 2D). However, the τf and τs time constants of inactivation of ICa-L were similar in wild-type and βARK-1 cells dialyzed with standard pipette solution.
Table 2. The velocities of shortening and relengthening also were compared in wild-type and βARK-1 myocytes dialyzed with standard pipette solution. Results showed that the rates of contraction and relaxation were similar in wild-type and βARK-1 myocytes (Fig. 2, E and F). These experiments demonstrate that the increase in the extent of cell shortening in βARK-1 myocytes is abolished when βARK-1 and wild-type cells are dialyzed with the same potassium-based patch pipette solution. These observations suggest that a factor or factors required to increase contraction amplitude in βARK-1 cells may be removed by intracellular dialysis with pipette solutions.

Previous studies have shown that dialysis with pipette solutions can disrupt the adenylyl cyclase-protein kinase A pathway and alter contractile function and that inclusion of cAMP in pipette solutions can restore contractile function in ventricular myocytes (Ferrier et al., 1998; Ferrier and Howlett, 2003). Therefore, we next determined whether inclusion of cAMP in patch pipette solutions would restore the differences in contraction observed in undialyzed cells between wild-type and βARK-1 myocytes. Cells were voltage-clamped with pipettes that contained the phosphodiesterase-resistant analog of cAMP, 8-bromo-cAMP (50 μM; Meyer and Miller, 1974). Figure 3, A and B, shows representative con-

![Undialyzed myocytes](image)
tractions and ICa-L recorded from wild-type and βARK-1 myocytes in the presence of 50 μM 8-bromo-cAMP in the pipette. The contraction was larger in the βARK-1 cell compared with the wild-type myocyte, whereas ICa-L was similar in wild-type and βARK-1 myocytes. Current-voltage relationships showed that the magnitude of ICa-L was similar in wild-type and βARK-1 myocytes, except for a small increase in the current in βARK-1 cells at +50 and +60 mV. E and F, the average velocities of cell shortening and relengthening in response to a voltage step from −40 to 0 mV were similar in wild-type and βARK-1 myocytes dialyzed with standard pipette solution. n = 10 wild-type myocytes and n = 17 βARK-1 myocytes. *, p < 0.05, denotes significantly different from wild type.

These results show that contraction amplitudes were significantly larger in βARK-1 cells compared with wild-type cells when 8-bromo-cAMP was included in the pipette solution.

We also determined whether differences in contraction between βARK-1 and wild-type myocytes were present when cells were dialyzed with the phosphodiesterase-sensitive analog of cAMP, sodium-cAMP (50 μM; Meyer and Miller, 1974). Figure 4, A and B, shows representative contractions and ICa-L recorded from wild-type and βARK-1 myocytes. In these examples, magnitudes of contractions and ICa-L were similar in wild-type and βARK-1 myocytes dialyzed with sodium-cAMP (Fig. 4, A and B). We compared the extent of cell shortening and relengthening of ICa-L between wild-type and βARK-1 myocytes dialyzed with sodium-cAMP over a range of membrane potentials (Fig. 4, C and D). Mean contraction-voltage and current-voltage relations were similar in the two groups (Fig. 4, C and D). Furthermore, the time constants of
inactivation of I_{Ca-L} (Table 2) as well as the rates of contraction and relaxation were similar in wild-type and βARK-1 myocytes (Fig. 4, E and F). Thus, differences in the extent of cell shortening between wild-type and βARK-1 myocytes were observed only when cells were dialyzed with solution that contained a phosphodiesterase-resistant analog of cAMP or when high-resistance electrodes were used to minimize cell dialysis.

The difference in the extent of cell shortening between βARK-1 and wild-type cells was observed with the same concentration of 8-bromo-cAMP in the pipette (e.g., 50 μM). Thus, the increase in the extent of cell shortening in βARK-1 cells is not likely to be due to differences in cAMP levels between wild-type and βARK-1 cells. However, it is possible that a component or components of the cAMP/protein kinase A pathway might be sensitized by chronic overexpression of βARK-1. Therefore, we next determined whether enhanced protein kinase A-mediated phosphorylation might contribute to differences between wild-type and βARK-1 myocytes. In these experiments, cells were dialyzed with 8-bromo-cAMP and superfused with buffer in the absence and presence of the protein kinase A inhibitor H89 (Bassani et al., 1995; Ferrier et al., 1998). The experimental protocol involved a test step from −40 to 0 mV delivered after a train of conditioning pulses as shown at the top of Fig. 5. Figure 5A shows representative examples of cell shortening and I_{Ca-L} Recorded from wild-type and βARK-1 myocytes initiated by a voltage step from −40 to 0 mV. Contraction was larger in the βARK-1 cell compared with the wild-type cell. C, the peak of the contraction-voltage relationship was significantly larger in βARK-1 compared with wild-type myocytes. D, current-voltage relationships were similar in wild-type and βARK-1 myocytes. E and F, mean velocities of shortening and velocities of relengthening elicited by a voltage step from −40 to 0 mV did not differ between wild-type and βARK-1 myocytes when cells were dialyzed with 8-bromo-cAMP. n = 8 wild-type and 10 βARK-1 myocytes. *p < 0.05, denotes significantly different from wild type.
compared with wild-type cells, although magnitudes of $I_{\text{Ca,L}}$ were similar in the two groups (Fig. 5B). H89 abolished the increase in contraction amplitude in βARK-1 myocytes, but it had no effect on amplitudes of $I_{\text{Ca,L}}$ in the two groups (Fig. 5C). Furthermore, the time constants of inactivation for $I_{\text{Ca,L}}$ were similar in wild-type and βARK-1 myocytes in the presence and absence of H89 (Table 2). Velocities of cell shortening and relengthening also were compared in wild-type and βARK-1 myocytes in the absence and presence of H89. The rates of contraction and relaxation also were similar in wild-type and βARK-1 myocytes in the absence and presence of H89 (Fig. 5, D and E).

**Discussion**

We determined whether alterations in the cAMP-protein kinase A pathway in βARK-1 myocytes might alter contractile function in the absence of exogenous βAR agonist. When high-resistance microelectrodes were used to minimize intracellular dialysis, contractions were larger in βARK-1 than in wild-type cells. However, when cells were dialyzed with pipettes filled with standard solution or a phosphodiesterase-sensitive analog of cAMP, the extent of cell shortening was similar in wild-type and βARK-1 myocytes. In contrast, when cells were dialyzed with solutions that contained a phosphodiesterase-resistant analog of cAMP, contractions were larger in βARK-1 than in wild-type cells. However, the amplitudes and rates of inactivation of $I_{\text{Ca,L}}$ were similar in βARK-1 and wild-type cells in all experiments, and rates of contraction and relaxation did not differ between the two groups. Interestingly, the protein kinase A inhibitor H89 abolished differences between βARK-1 and wild-type myocytes. These results demonstrate that contractile function is augmented in βARK-1 myocytes when the intracellular mi-
The protein kinase A inhibitor H89 abolished the difference in extent of cell shortening between wild-type and βARK-1 cells. Cells were dialyzed with patch pipettes filled with 50 μM 8-bromo-cAMP. The voltage-clamp protocol is shown at the top of the figure. A, representative recordings of contractions and I_{Ca-L} initiated by a test step from −40 to 0 mV. The extent of cell shortening was greater in the βARK-1 cell compared with the wild-type myocyte in the absence of H89. However, this difference was not observed in the presence of H89. B, contractions were significantly increased in βARK-1 cells compared with wild-type cells under control conditions. In contrast, amplitudes of I_{Ca-L} were similar in the two groups. C, when cells were superfused with 5 μM H89, the increase in contraction amplitude in βARK-1 myocytes was abolished. D and E, the mean velocities of shortening and relengthening did not differ between wild-type and βARK-1 myocytes in the absence or presence of H89. n = 3 to 10 myocytes per group. *p < 0.05 denotes significantly different from wild type.
myocytes than wild-type cells. However, when wild-type and βARK-1 cells were dialyzed with identical K+-based intracellular solutions, differences in contractions were abolished. This suggests that a component required to increase the extent of cell shortening in βARK-1 myocytes was removed from the cells by dialysis. Because dialysis with pipette solutions can disrupt the cAMP-protein kinase A pathway and affect contractile function in ventricular myocytes (Ferrier et al., 1998; Ferrier and Howlett, 2000), we hypothesized that removal of signaling components important in this pathway might reduce the extent of cell shortening in βARK-1 cells. Therefore, myocytes were dialyzed with pipette solutions supplemented with phosphodiesterase-resistant 8-bromo-cAMP. Under these conditions, contractions were larger in βARK-1 myocytes than in wild-type cells. Because increased cell shortening was observed in βARK-1 myocytes exposed to the same concentration of cAMP as wild-type cells, it is unlikely that increased intracellular cAMP was responsible for differences in contractions between βARK-1 and wild-type myocytes. However, these findings suggest that the cAMP-protein kinase A pathway is involved in the increase in contraction in βARK-1 cells.

Korzick et al. (1997) previously showed that the extent of cell shortening was similar in field-stimulated βARK-1 and wild-type cells, in the absence of exogenous catecholamines. This contrasts with our results, which showed that the extent of cell shortening was greater in undialyzed βARK-1 myocytes. There are several possible reasons for the differences between these two studies. Korzick et al. (1997) used field-stimulated myocytes where contractions were initiated by action potentials. Because action potential characteristics may differ between βARK-1 and wild-type cells, the stimuli used to initiate contraction may differ between the two cell types (Korzick et al., 1997). In contrast, our study used voltage-clamp steps of fixed duration, so identical stimuli were used to initiate contraction in wild-type and βARK-1 cells. In addition, Korzick et al. (1997) conducted their studies at 23°C, whereas our experiments were conducted at 37°C. It is well established that temperature can dramatically alter cardiac contractile function (Bers, 2001; Ferrier et al., 2003). Therefore, methodological differences may explain differences between our study and previous work (Korzick et al., 1997).

We found no difference in the extent of cell shortening between wild-type and βARK-1 myocytes dialyzed with phosphodiesterase-sensitive sodium-cAMP (Meyer and Miller, 1974). This suggests that a reduction in phosphodiesterase activity does not account for the enhanced contractions we observed in βARK-1 myocytes. If phosphodiesterase activity were reduced in βARK-1 myocytes, this would reduce degradation of sodium-cAMP and thereby promote accumulation of intracellular cAMP (Francis et al., 2001). Thus, we would have expected to see an increase in the extent of cell shortening in βARK-1 cells dialyzed with sodium-cAMP compared with wild-type cells. However, we found no differences in contractions between wild-type and βARK-1 myocytes dialyzed with sodium-cAMP. Consequently, a reduction in phosphodiesterase activity in βARK-1 cells is unlikely to be responsible for increased cell shortening.

One explanation for our results is that downstream components of the cAMP-protein kinase A pathway might become sensitized by a chronic increase in βARK-1 activity. We hypothesized that enhanced protein kinase A-mediated phosphorylation might account for differences in contraction between wild-type and βARK-1 myocytes. Indeed, the selective protein kinase A inhibitor H89 (Bassani et al., 1995; Ferrier et al., 1998) abolished the difference in cell shortening between βARK-1 and wild-type myocytes when cells were dialyzed with 8-bromo-cAMP. These results suggest that enhanced protein kinase A-mediated phosphorylation of components important in cardiac EC-coupling can explain the increase in cell shortening in βARK-1 myocytes dialyzed with 8-bromo-cAMP. This explanation also may account for the increase in contractions in undialyzed βARK-1 cells, although this was not addressed here.

The increase in extent of cell shortening in βARK-1 myocytes might be due to changes in the regulation of proteins involved in EC-coupling. L-type Ca2+ channels, phospholamban, ryanodine receptors, and protein phosphatase-1 are substrates for protein kinase A-mediated phosphorylation (Yoshida et al., 1992; Kapiloff, 2002; El-Armouche et al., 2003). Protein kinase A-mediated phosphorylation of L-type Ca2+ channels increases channel conductance (Striessnig, 1999). This could increase the size of I_{Ca-L} that triggers SR Ca2+ release (Bers, 2001). However, it is unlikely that changes in Ca2+ channel phosphorylation increase the extent of cell shortening in βARK-1 myocytes, because we found I_{Ca-L} amplitudes and inactivation rates were similar in βARK-1 and wild-type myocytes. Nevertheless, protein kinase A-mediated phosphorylation of phospholamban, ryanodine receptors, and/or protein phosphatase-1 might contribute to the increase in contractions in βARK-1 myocytes. Phosphorylation of phospholamban would relieve inhibition of SR Ca2+ ATPase, increase the rate of SR Ca2+ uptake, and thereby increase SR Ca2+ (Frank and Kranias, 2000; Frank et al., 2003). This would increase Ca2+ available for release and thus increase contraction in βARK-1 myocytes. However, we found that the rates of relaxation of contraction were similar in wild-type and βARK-1 myocytes. Thus, it is unlikely that increased SR Ca2+ uptake accounts for increased contraction in βARK-1 myocytes. Alternatively, phosphorylation of ryanodine receptors increases their open probability and increases SR Ca2+ release, which would produce a larger contraction (Marks, 2001; Marks et al., 2002). However, we found that wild-type and βARK-1 myocytes had similar rates of contraction, which makes it unlikely that an increased rate of Sr Ca2+ release enhances contraction in βARK-1 cells. Finally, phosphorylation of protein phosphatase-1 would reduce dephosphorylation of targets such as phospholamban and/or ryanodine receptors (El-Armouche et al., 2003). Decreases in phosphorylation would amplify the cAMP-protein kinase A cascade and increase the extent of cell shortening in βARK-1 myocytes. Further experiments will be required to distinguish between these possibilities.

Chronic βARK-1 overexpression had little effect on the magnitude or inactivation rates of I_{Ca-L}. The magnitude of I_{Ca-L} was increased in βARK-1 compared with wild-type cells.
when cells were dialyzed with standard solutions, but only at very positive potentials. However, these potentials are much more positive than the plateau of the murine action potential (Fiset et al., 1997). Thus, this increase in ICa-L is unlikely to affect upon EC-coupling under physiological conditions. However, in most experiments we found that amplitudes of ICa-L were similar in βARK-1 and wild-type cells. This finding was unexpected, as protein kinase A-mediated phosphorylation of the Ca2+
 channel increases the magnitude of ICa-L (Kapillof, 2002). However, it previously has been shown that protein kinase A regulation of slow outward potassium current requires a macromolecular complex that includes protein kinase A, protein phosphatase-1, and a targeting protein called yotiao (Marx et al., 2002). Therefore, one explanation for our data is that protein kinase A-dependent regulation of the Ca2+
 channel also requires a macromolecular complex, which is disrupted in βARK-1 myocytes.

Cell capacitance was increased in βARK-1 myocytes compared with wild-type cells, which suggests that cell membrane area is increased in βARK-1 cells. This increase in membrane area does not result from an increase in cell length, because cell length was similar in βARK-1 and wild-type myocytes. However, an increase in membrane surface area in βARK-1 cells could arise from an increase in cell width, cell volume, t-tubule area, and/or caveolae area. Whether chronic βARK-1 overexpression alters one or more of these factors to increase membrane area will require further investigation.

The results of the present investigation have important implications for studies of contractile function in myocytes isolated from models of heart failure. The sympathetic nervous system is activated in heart failure to compensate for diminished contractile function through activation of βARs (Keys and Koch, 2004). This chronic adrenergic stimulation results in up-regulation of βARK-1 in failure (Ungerer et al., 1993). Up-regulation of βARK-1 plays a key role in phosphorylation and desensitization of βARs, which further impairs contractile function in the failing heart (Hausdorff et al., 1990; Post et al., 1999). Our results show that chronic βARK-1 overexpression also may sensitize components of the cAMP-protein kinase A pathway. Thus, changes in contractile function observed in failing myocytes may reflect, at least in part, effects of chronic up-regulation of βARK-1 on components of the cAMP-protein kinase A pathway.

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