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PROTEIN KINASE A-MEDIATED PHOSPHORYLATION CONTRIBUTES TO ENHANCED CONTRACTION OBSERVED IN MICE THAT OVEREXPRESS β-ADRENERGIC RECEPTOR KINASE-1

Erin E. Mueller, Scott A. Grandy and Susan E. Howlett

Department of Pharmacology (EEM, SAG, SEH)

5850 College Street

Dalhousie University

Halifax, Nova Scotia

Canada, B3H 1X5

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Correspondence, proofs, and reprints requests should be mailed to:

Susan Ellen Howlett, Ph.D.

Department of Pharmacology

Sir Charles Tupper Medical Building

5850 College Street

Halifax, Nova Scotia, Canada, B3H 1X5

Phone: (902) 494-3552 Fax: (902) 494-1318

E-mail: susan.howlett@dal.ca

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ABSTRACT

Transgenic mice with cardiac specific over-expression 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 over-expression on contraction were examined in mouse ventricular myocytes, studied at 37^{o}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 wild-type myocytes. In contrast, when cells were dialyzed with patch pipette solution (1-3 MΩ; 0 mM NaCl, 70 KCl, 70 K-aspartate, 4 MgATP, 1 $MgCl_2$, 2.5 KH_2PO_4, 0.12 $CaCl_2$, 0.5 EGTA, 10 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, H-89. Interestingly, Ca^{2+} 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 upregulation of βARK-1 on the cAMP-protein kinase-A pathway.

INTRODUCTION

Cardiac contraction is initiated by a rise in intracellular free Ca^{2+}, derived primarily from internal stores in the sarcoplasmic reticulum (SR; Bers, 2001). Ca^{2+} 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, Ca^{2+} cycling between intra- and extracellular compartments is tightly controlled. However, disruptions in Ca^{2+} 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 (Post et al., 1999; Hausdorff et al., 1990).

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 non-myocyte cardiac cells

(Penela et al., 2006). Interestingly, βARK-1 expression and activity are increased in the failing heart (Ungerer et al., 1993). This upregulation of βARK-1 increases phosphorylation of activated βARs, which leads to desensitization, and downregulation of the receptors (Post et al., 1999). This dampens the βAR signalling cascade, which in turn alters Ca^{2+} 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 alpha myosin heavy chain promoter (Koch et al., 1995). This leads to a three-to-five fold over-expression 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 to wild-type controls (Koch et al., 1995; Rockman et al., 1996; Chen et al., 1998). In addition, isoproterenol-stimulated adenylyl cyclase activity is reduced in sarcolemmal membranes from βARK-1 mice compared to wild-type animals (Koch et al., 1995). Interestingly, adenylyl cyclase activity also is reduced in sarcolemmal membranes from βARK-1 mice in the absence of isoproterenol (Koch et al., 1995). This suggests that chronic over-expression of βARK-1 may alter the adenylyl 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 over-expression 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: 1) to compare the extent of cell shortening and magnitude of L-type Ca^{2+} current ($I_{Ca\text{-}L}$) 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) to determine whether the extent of cell shortening and magnitude of $I_{Ca\text{-}L}$ differed between βARK-1 and wild-type myocytes when cells were dialyzed with patch pipette solutions in the absence and presence of various analogues of cAMP; and 3) to 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^{o}C), in the absence of exogenous βAR stimulation.

METHODS

Experimental animals. Experiments were performed on cardiac ventricular myocytes isolated from approximately six-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 pairs were made up of female wild-type (B6SJLF1/J) and male βARK-1 (B6SJL-TgN(BARK12)12Wjk) mice obtained from Jackson Laboratories (Bar Harbour, MA). 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 Jackson Laboratories (www.jax.org/jaxmice/micetech). All experiments respected the guidelines stated in the Canadian Council on Animal Care (CCAC, Ottawa, Ontario; vol. 1, 1980; vol. 2, 1984) and were approved by the Dalhousie University Committee on Animal Care.

Cell isolation. Mice were anaesthetized with an intraperitoneal injection of pentobarbital sodium (200-300 mg/kg), co-injected with heparin (100 units) 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 minutes with oxygenated, Ca^{2+}-free solution of the following composition (mM): 130 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 with NaOH). The solution was maintained at 37^{o}C with a heated circulating water bath. Then, the hearts were perfused with the Ca^{2+} free solution supplemented with 50 μM Ca^{2+}, collagenase (24 mg/30 ml, Worthington Type I), neutral protease dispase II (10 mg/30 ml) and trypsin (1 mg/ml). After approximately 10 mins, the ventricles were excised, minced, and stored at room temperature until use in a high K^+ solution

containing (mM): 80 KOH, 50 glutamic acid, 30 KCl, 30 KH_2PO_4, 20 taurine, 10 HEPES, 10 glucose, 3 $MgSO_4$, and 0.5 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 mins. Then, the cells were superfused at 3 ml/min with a standard buffer solution of the following composition (mM): 145 NaCl; 10 glucose; 10 HEPES; 4 KCl; 1 $CaCl_2$; 1 $MgCl_2$ (pH 7.4 with NaOH). This solution was supplemented with 0.3 mM lidocaine and 4 mM 4-aminopyridine 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 (mM): 0 NaCl, 70 KCl, 70 K-aspartate, 4 MgATP, 1 $MgCl_2$, 2.5 KH_2PO_4, 0.12 $CaCl_2$, 0.5 EGTA, 10 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, Axon Instruments) and an Axoclamp 2B amplifier (Axon Instruments). Unloaded cell shortening was recorded with a video edge detector (Crescent Electronics, Sandy, UT) and video camera (model TM-640, Pulnix America) operating at 120 Hz. In some experiments, the protein kinase inhibitor N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89; 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 H-89 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 mV to 0 mV to provide a consistent history of activation. Cells were then repolarized to -40 mV for 500 msec. 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 utilized to elicit peak inward $I_{Ca\text{-}L}$ and contraction in experiments with H-89. A single test step was used in these experiments because preliminary experiments showed that the concentration of DMSO used to dissolve the H-89 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 prior to cell shortening. The velocity of shortening was determined by dividing the amplitude of contraction by the time to peak contraction and was expressed in μm/sec. The velocity of relengthening was determined by dividing half the amplitude of contraction by the time-to-half relaxation and also was expressed in μm/sec. $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ were measured with Clampfit 8.1 (Axon Instruments, Union City, CA). The fast (τ_f) and slow (τ_s) time constants of inactivation for $I_{Ca\text{-}L}$ were determined by fitting the inactivation phase of $I_{Ca\text{-}L}$ with an exponential function with two exponential components. Cell membrane area was determined by integrating the capacitive transient with pCLAMP software. $I_{Ca\text{-}L}$ was normalized by cell capacitance and expressed as current density. Cell length was used to normalize the contraction data.

Data are presented as means +/- SEM. 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 ANOVA. Sigma Stat software (Jandel version 2.0) 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, $MgCl_2$, 4-aminopyridine, H-89, 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 Molecular Probes (Eugene, OR). The protease II was purchased from Roche Diagnostics (Laval, QC, Canada). Heparin was purchased from Organon (Toronto, ON, Canada). Type I collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals were purchased from BDH (Toronto, ON, Canada). H-89 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). As cell capacitance is proportional to cell membrane area, these results suggest that sarcolemmal area is increased in βARK-1 myocytes. Therefore, in all subsequent experiments, measurements of $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ in wild-type and βARK-1 cells impaled 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 msec test steps from -40 mV to potentials between -40 and +60 mV, delivered in increments of 10 mV, to elicit contractions and $I_{Ca\text{-}L}$ (Figure 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ were similar in the two cells. The extent of cell shortening and $I_{Ca\text{-}L}$ 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 (Figure 1*C*). However, contractions at the peak of the curve were significantly larger in βARK-1 myocytes than in wild-type cells (Figure 1*C*). Figure 1*D* compares mean current-voltage curves in wild-type and βARK-1 myocytes. Mean amplitudes of $I_{Ca\text{-}L}$ did not differ between wild-type and βARK-1 myocytes at any membrane potential examined (Figure 1*D*). We also compared the inactivation rates for $I_{Ca\text{-}L}$ in wild-type and βARK-1 myocytes (Table 2). Results showed that the fast (τ_f) and slow (τ_s) time constants of inactivation for $I_{Ca\text{-}L}$ 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 (Figure 1*E* and *F*). Thus, contraction is enhanced in βARK-1 myocytes when compared to 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 (Figure 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 $I_{Ca\text{-}L}$ were similar in the two cell types. We next determined whether the voltage-dependence of contraction and $I_{Ca\text{-}L}$ 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 (Figure 2*C*). Figure 2*D* compares mean current-voltage relations in wild-type and βARK-1 myocytes. Mean amplitudes of $I_{Ca\text{-}L}$ did not differ between wild-type

and βARK-1 myocytes until the voltage exceeded +40 mV. At membrane potentials more positive than +40 mV, $I_{Ca\text{-}L}$ was larger in βARK-1 cells than in wild-type cells (Figure 2*D*). However, the fast (τ_f) and slow (τ_s) time constants of inactivation of $I_{Ca\text{-}L}$ 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 (Figure 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 undialysed cells between wild-type and βARK-1 myocytes. Cells were voltage-clamped with pipettes that contained the phosphodiesterase-resistant analogue of cAMP, 8-bromo-cAMP (Meyer & Miller, 1974; 50 μM). Figure 3, *A* and *B*, shows representative contractions and $I_{Ca\text{-}L}$ 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 to the wild-type myocyte, while $I_{Ca\text{-}L}$ was similar in the two cells. Contraction-voltage and current-voltage relations in wild-type and βARK-1 cells dialyzed with 8-bromo-cAMP are shown in Figure 3*C* and *D*. The extent of cell

shortening was significantly increased in βARK-1 myocytes compared to wild-type myocytes at voltages between -20 and +20 mV (Figure 3*C*). In contrast, amplitudes of $I_{Ca\text{-}L}$ were similar in wild-type and βARK-1 cells at all membrane potentials examined (Figure 3*D*) and the time constants of inactivation of $I_{Ca\text{-}L}$ were similar in the two groups (Table 2). The velocities of cell shortening and relengthening in wild-type and βARK-1 myocytes also were examined in these experiments. As shown in Figures 3*E* and *F*, the rates of contraction and relaxation were similar in wild-type and βARK-1 myocytes. These results show that contraction amplitudes were significantly larger in βARK-1 cells when compared to 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 dialysed with the phosphodiesterase-sensitive analogue of cAMP, sodium-cAMP (50 μM; Meyer & Miller, 1974). Figure 4, *A* and *B*, shows representative contractions and $I_{Ca\text{-}L}$ recorded from wild-type and βARK-1 myocytes. In these examples, magnitudes of contractions and $I_{Ca\text{-}L}$ were similar in wild-type and βARK-1 myocytes dialyzed with sodium-cAMP (Figure 4*A* and *B*). We compared the extent of cell shortening and magnitude of $I_{Ca\text{-}L}$ between wild-type and βARK-1 myocytes dialyzed with sodium-cAMP over a range of membrane potentials (Figure 4*C* and *D*). Mean contraction-voltage and current-voltage relations were similar in the two groups (Figure 4*C* and *D*). Furthermore, the time constants of inactivation of $I_{Ca\text{-}L}$ (Table 2) as well as the rates of contraction and relaxation were similar in wild-type and βARK-1 myocytes (Figure 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 dialysed with solution that contained a phosphodiesterase-resistant analogue of cAMP or when high-resistance electrodes were used to minimize cells 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, H-89 (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 Figure 5. Figure 5*A* shows representative examples of cell shortening and $I_{Ca\text{-}L}$ recorded from wild-type and βARK-1 myocytes in the absence and presence of 5 μM H-89. The contraction was larger in the βARK-1 cell than in the wild-type cell in the absence of H-89. However, H-89 abolished the difference between the wild-type and βARK-1 myocytes (Figure 5*A*). Mean amplitudes of cell shortening were significantly larger in βARK-1 myocytes when compared to wild-type cells, although magnitudes of $I_{Ca\text{-}L}$ were similar in the two groups (Figure 5*B*). H-89 abolished the increase in contraction amplitude in βARK-1 myocytes, but had no effect on amplitudes of $I_{Ca\text{-}L}$ in the two groups (Figure 5*C*). Furthermore, the time constants of inactivation for $I_{Ca\text{-}L}$ were similar in wild-type and βARK-1 myocytes in the presence and absence of H-89 (Table 2). Velocities of cell shortening and relengthening also were compared in wild-type and βARK-1 myocytes in the absence and presence of H-89. The rates of contraction and relaxation also were similar in wild-type and βARK-1 myocytes in the absence and presence of H-89 (Figure 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 wild-type cells. However, when cells were dialyzed with pipettes filled with standard solution or a phosphodiesterase-sensitive analogue 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 analogue of cAMP, contractions were larger in βARK-1 than wild-type cells. However, the amplitudes and rates of inactivation of $I_{Ca\text{-}L}$ 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, H-89, abolished differences between βARK-1 and wild-type myocytes. These results demonstrate that contractile function is augmented in βARK-1 myocytes when the intracellular milieu is not disrupted by dialysis with pipette solutions, or when phosphorylation pathways are preserved with phosphodiesterase-resistant cAMP. Furthermore, these results suggest that protein kinase-A-mediated phosphorylation contributes to enhanced contractile function in myocytes that chronically overexpress βARK-1.

When intracellular dialysis was minimized with high-resistance microelectrodes, contractions were larger in βARK-1 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. As dialysis with pipette solutions can disrupt the cAMP/protein kinase-A pathway and affect contractile function

in ventricular myocytes (Ferrier et al., 1998; Ferrier & Howlett, 2003), we hypothesized that removal of signalling 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 wild-type cells. As 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. As 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. Additionally, Korzick et al. (1997) conducted their studies at 23^{o}C, whereas our experiments were conducted at 37^{o}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 & 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 when compared to 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 H-89 (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 Ca^{2+} channels, phospholamban,

ryanodine receptors and protein phosphatase-1 are substrates for protein kinase-A-mediated phosphorylation (Kapiloff, 2002; El-Armouche et al., 2003; Yoshida et al., 1992). Protein kinase-A-mediated phosphorylation of L-type Ca^{2+} channels increases channel conductance (Striessnig, 1999). This could increase the size of $I_{Ca\text{-}L}$ that triggers SR Ca^{2+} release (Bers, 2001). However, it is unlikely that changes in Ca^{2+} channel phosphorylation increase the extent of cell shortening in βARK-1 myocytes, as we found $I_{Ca\text{-}L}$ 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 Ca^{2+} ATPase, increase the rate of SR Ca^{2+} uptake and thereby increase SR Ca^{2+} (Frank and Kranias, 2000; Frank et al., 2003). This would increase Ca^{2+} 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 Ca^{2+} uptake accounts for increased contraction in βARK-1 myocytes. Alternatively, phosphorylation of ryanodine receptors increases their open probability and increases SR Ca^{2+} 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 Ca^{2+} 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 $I_{Ca\text{-}L}$. The magnitude of $I_{Ca\text{-}L}$ was increased in βARK-1 compared to 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 $I_{Ca\text{-}L}$ is unlikely to impact upon EC-coupling under physiological conditions. However, in most experiments we found that amplitudes of $I_{Ca\text{-}L}$ were similar in βARK-1 and wild-type cells. This finding was unexpected, as protein kinase-A-mediated phosphorylation of the Ca^{2+} channel increases the magnitude of $I_{Ca\text{-}L}$ (Kapiloff, 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 Ca^{2+} channel also requires a macromolecular complex, which is disrupted in βARK-1 myocytes.

Cell capacitance was increased in βARK-1 myocytes when compared to 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, as 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 upregulation of βARK-1 in failure (Ungerer et al., 1993). Upregulation of βARK-1 plays a key role in phosphorylation and desensitization of βARs, which further impairs contractile function in the failing heart (Post et al., 1999; Hausdorff et al., 1990). Our results show that chronic βARK-1 over-expression 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 upregulation of βARK-1 on components of the cAMP-protein kinase-A pathway.

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REFERENCES

Bassani RA, Mattiazzi A and Bers DM (1995) CaMKII is responsible for activity-dependent acceleration of relaxation in rat ventricular myocytes. *Am J Physiol* **268**: H703-712.

Bers DM (2001) *Excitation-Contraction Coupling and Cardiac Contractile Force* (2^{nd} ed.) Kluwer Academic Publishers, Boston, MA.

Chen EP, Bittner HB, Akhter SA, Koch WJ and Davis RD (1998) Myocardial recovery after ischemia and reperfusion injury is significantly impaired in hearts with transgenic overexpression of beta-adrenergic receptor kinase. *Circulation* **98**: II249-253.

El-Armouche A, Rau T, Zolk O, Ditz D, Pamminger T, Zimmermann WH, Jackel E, Harding SE, Boknik P, Neumann J and Eschenhagen T (2003) Evidence for protein phosphatase inhibitor-1 playing an amplifier role in beta-adrenergic signaling in cardiac myocytes. *Faseb J* **17**: 437-439.

Ferrier GR and Howlett SE (2003) Differential effects of phosphodiesterase-sensitive and -resistant analogs of cAMP on initiation of contraction in cardiac ventricular myocytes. *J Pharmacol Exp Ther* **306**: 166-178.

Ferrier GR, Smith RH and Howlett SE (2003) Calcium sparks in mouse ventricular myocytes at physiological temperature. *Am J Physiol* **285**: H1495-1505.

Ferrier GR, Zhu J, Redondo IM and Howlett SE (1998) Role of cAMP-dependent protein kinase A in activation of a voltage-sensitive release mechanism for cardiac contraction in guinea-pig myocytes. *J Physiol (Lond)* **513**: 185-201.

Fiset C, Clark RB, Larsen TS and Giles WR (1997) A rapidly activating sustained K^+ current modulates repolarization and excitation-contraction coupling in adult mouse ventricle. *J Physiol (Lond)* **504**: 557-563.

Francis SH, Turko IV and Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* **65**: 1-52.

Frank KF, Bolck B, Erdmann E and Schwinger RH (2003) Sarcoplasmic reticulum Ca^{2+}-ATPase modulates cardiac contraction and relaxation. *Cardiovasc Res* **57**: 20-27.

Frank K and Kranias EG (2000) Phospholamban and cardiac contractility. *Ann Med* **32**: 572-578.

Hausdorff WP, Caron MG and Lefkowitz RJ (1990) Turning off the signal: desensitization of beta-adrenergic receptor function. *Faseb J* **4**: 2881-2889.

Kapiloff MS (2002) Contributions of protein kinase A anchoring proteins to compartmentation of cAMP signaling in the heart. *Mol Pharmacol* **62**: 193-199.

Keys JR and Koch WJ (2004) The adrenergic pathway and heart failure. *Recent Prog Horm Res* **59**: 13-30.

Knollmann BC, Knollmann-Ritschel BE, Weissman NJ, Jones LR and Morad M (2000) Remodelling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. *J Physiol (Lond)* **525**: 483-498.

Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA and Lefkowitz RJ (1995) Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a ßARK inhibitor. *Science* **268**: 1350-1353.

Korzick DH, Xiao RP, Ziman BD, Koch WJ, Lefkowitz RJ and Lakatta EG (1997) Transgenic manipulation of beta-adrenergic receptor kinase modifies cardiac myocyte contraction to norepinephrine. *Am J Physiol* **272**: H590-596.

Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ (1990) Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* **265**:

3202-3211.

Marks AR (2001) Ryanodine receptors/calcium release channels in heart failure and sudden cardiac death. *J Mol Cell Cardiol* **33**: 615-624.

Marks AR, Marx SO and Reiken S (2002) Regulation of ryanodine receptors via macromolecular complexes: a novel role for leucine/isoleucine zippers. *Trends Cardiovasc Med* **12**: 166-170.

Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR and Kass RS (2002) Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science* **295**: 496-499.

Meyer RB Jr and Miller JP (1974) Analogs of cyclic AMP and cyclic GMP: general methods of synthesis and the relationship of structure to enzymic activity. *Life Sci* **14**: 1019-1040.

Olsson MC, Palmer BM, Stauffer BL, Leinwand LA and Moore RL (2004) Morphological and functional alterations in ventricular myocytes from male transgenic mice with hypertrophic cardiomyopathy. *Circ Res* **94**: 201-207.

Penela P, Murga C, Ribas C, Tutor AS, Peregrin S and Mayor F Jr (2006) Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. *Cardiovasc Res* **69**: 46-56.

Pieske B, Maier LS and Schmidt-Schweda S (2002) Sarcoplasmic reticulum Ca^{2+} load in human heart failure. *Basic Res Cardiol* **97**: I63-71.

Post SR, Hammond HK and Insel PA (1999) Beta-adrenergic receptors and receptor signaling in heart failure. *Annu Rev Pharmacol Toxicol* **39**: 343-360.

Rockman HA, Choi DJ, Rahman NU, Akhter SA, Lefkowitz RJ and Koch WJ (1996) Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic

mice. *Proc Natl Acad Sci* **93**: 9954-9959.

Rockman HA, Koch WJ, and Lefkowitz RJ (2002) Seven-transmembrane-spanning receptors and heart function. *Nature* **415**: 206-212.

Striessnig J (1999). Pharmacology, structure and function of cardiac L-type Ca^{2+} channels. *Cell Physiol Biochem* **9**: 242-269.

Ungerer M, Bohm M, Elce JS, Erdmann E and Lohse MJ (1993) Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation* **87**: 454-463.

Wallukat G (2002) The beta-adrenergic receptors. *Herz* **27**: 683-690.

Yatani A, Frank K, Sako H, Kranias EG and Dorn GW 2nd (1999) Cardiac-specific overexpression of Gαq alters excitation-contraction coupling in isolated cardiac myocytes. *J Mol Cell Cardiol* **31**:1327-1336.

Yoshida A, Takahashi M, Imagawa T, Shigekawa M, Takisawa H and Nakamura T (1992) Phosphorylation of ryanodine receptors in rat myocytes during beta-adrenergic stimulation. *J Biochem* (*Tokyo)* **111**: 186-190.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. The extent of cell shortening was larger in βARK-1 myocytes than in wild-type cells, when cells were voltage clamped with high-resistance electrodes to minimize intracellular dialysis with pipette solutions. Cells were impaled with high-resistance microelectrodes. The voltage clamp protocol is shown at the top. *A* and *B*: Representative contractions (top) and $I_{Ca\text{-}L}$ (bottom) recorded from wild-type and βARK-1 myocytes in response to a voltage step from -40 to -10 mV. Contraction was larger in the βARK-1 cell than in the wild-type cell. *C*: Contraction amplitudes were significantly larger near the peak of the contraction-voltage curve in βARK-1 myocytes compared to wild-type cells. *D*: Current-voltage relationships were similar in wild-type and βARK-1 myocytes. *E* and *F*: Mean velocities of shortening and relengthening were determined for contractions elicited by a step from -40 to 0 mV. The rates of contraction and relaxation were similar in wild-type and βARK-1 myocytes when intracellular dialysis with pipette solutions was minimal. *N* = 12 wild-type and 7 βARK-1 myocytes; * denotes significantly different from wild-type, $p<0.05$.

Figure 2. The extent of cell shortening and magnitudes of $I_{Ca\text{-}L}$ were similar in βARK-1 and wild-type myocytes when cells were dialyzed with pipettes filled with standard potassium-based solution. The voltage clamp protocol is shown at the top. *A* and *B*: Representative contractions and $I_{Ca\text{-}L}$ recorded from wild-type and βARK-1 cells in response to a voltage step from -40 to 0 mV. *C*: Contraction-voltage relationships were similar in wild-type and βARK-1 myocytes. *D*: Current-voltage relationships showed that the magnitude of $I_{Ca\text{-}L}$ 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 17 βARK-1 myocytes; * denotes significantly different from wild-type, $p<0.05$.

Figure 3. In cells dialyzed with a phosphodiesterase resistant analogue of cAMP, the extent of cell shortening was greater in βARK-1 myocytes than in wild-type myocytes. Cells were dialyzed with 50 μM 8-bromo-cAMP. The voltage clamp protocol is shown at the top. *A* and *B*: Representative recordings of contractions and $I_{Ca\text{-}L}$ 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 to the wild-type cell. *C*: The peak of the contraction-voltage relationship was significantly larger in βARK-1 compared to 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; * denotes significantly different from wild-type, $p<0.05$.

Figure 4. In cells dialyzed with phosphodiesterase-sensitive sodium-cAMP, the extent of cell shortening was similar in wild-type and βARK-1 cells. Cells were dialyzed with 50 μM sodium-cAMP. The voltage clamp protocol is shown at the top. *A* and *B*: Representative recordings of contractions and $I_{Ca\text{-}L}$ initiated by a test step from -40 to 0 mV were similar in wild-type and βARK-1 myocytes. Contraction-voltage (*C*) and current-voltage (*D*) relationships were similar in wild-type and βARK-1 myocytes when cells were dialyzed with sodium-cAMP. *E* and

F: Average velocities of shortening and relengthening initiated by a voltage step from -40 to 0 mV were similar in wild-type and βARK-1 myocytes dialyzed with sodium-cAMP. *N* = 6 wild-type and 4 βARK-1 myocytes.

Figure 5. The protein kinase A inhibitor, H-89, abolished the difference in extent of cell shortening between wild-type and βARK-1 cells. Cells were dialyzed with patch pipettes filled with 8-bromo-cAMP (50 μM). The voltage clamp protocol is shown at the top of the figure. *A*: Representative recordings of contractions and $I_{Ca\text{-}L}$ initiated by a test step from -40 to 0 mV. The extent of cell shortening was greater in the βARK-1 cell compared to the wild-type myocyte in the absence of H-89. However, this difference was not observed in the presence of H-89. *B*: Contractions were significantly increased in βARK-1 cells compared to wild-type cells under control conditions. In contrast, amplitudes of $I_{Ca\text{-}L}$ were similar in the two groups. *C*: When cells were superfused with 5 μM H-89, 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 H-89. *N* = 3-10 myocytes per group; * denotes significantly different from wild-type, $p<0.05$.

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TABLE 1

CHARACTERISTICS OF WILD-TYPE AND βARK-1 MICE

	Wild-type	**βARK-1**
Age (months)	5.60 ± 0.40 (13)	6.47 ± 0.33 (24)
Weight (g)	36.06 ± 1.89 (11)	34.85 ± 1.45 (22)
Cell Length (μm)	137.45 ± 2.18 (99)	135.97 ± 3.26 (47)
Cell Capacitance (pF)	208.27 ± 9.70 (36)	247.51 ± 12.83* (39)

* Denotes $p<0.05$; values represent the mean ± SEM. The number in parentheses represents the number of animals (age, weight) or myocytes (cell length, cell capacitance).

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TABLE 2

FAST AND SLOW TIME CONSTANTS FOR INACTIVATION OF $I_{Ca\text{-}L}$

	τ_f (msec)		τ_s (msec)	
	Wild-type	**βARK-1**	**Wild-type**	**βARK-1**
Undialyzed cells	8.5 ± 1.0	5.9 ± 1.8	27.1 ± 4.8	19.7 ± 7.4
Standard solution	8.0 ± 0.6	8.0 ± 1.0	31.1 ± 5.0	23.3 ± 5.3
8-bromo-cAMP	7.1 ± 0.9	7.1 ± 0.4	26.8 ± 10.4	18.4 ± 3.6
Sodium-cAMP	5.4 ± 0.6	5.3 ± 0.7	15.5 ± 2.4	14.3 ± 2.2
Absence of H-89	4.7 ± 0.8	5.0 ± 0.8	10.5 ± 0.4	12.8 ± 1.6
Presence of H-89	4.8 ± 0.5	5.1 ± 0.8	14.8 ± 2.1	13.5 ± 0.9

Values represent the mean ± SEM. Values of n are presented in the appropriate figure legends.

There were no significant differences in either τ_f or τ_s between wild-type and βARK-1 myocytes.

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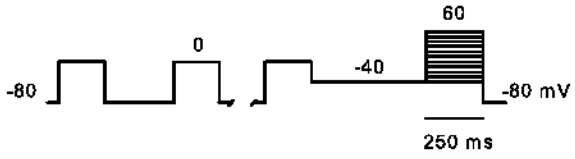
Figure 1



Undialyzed myocytes

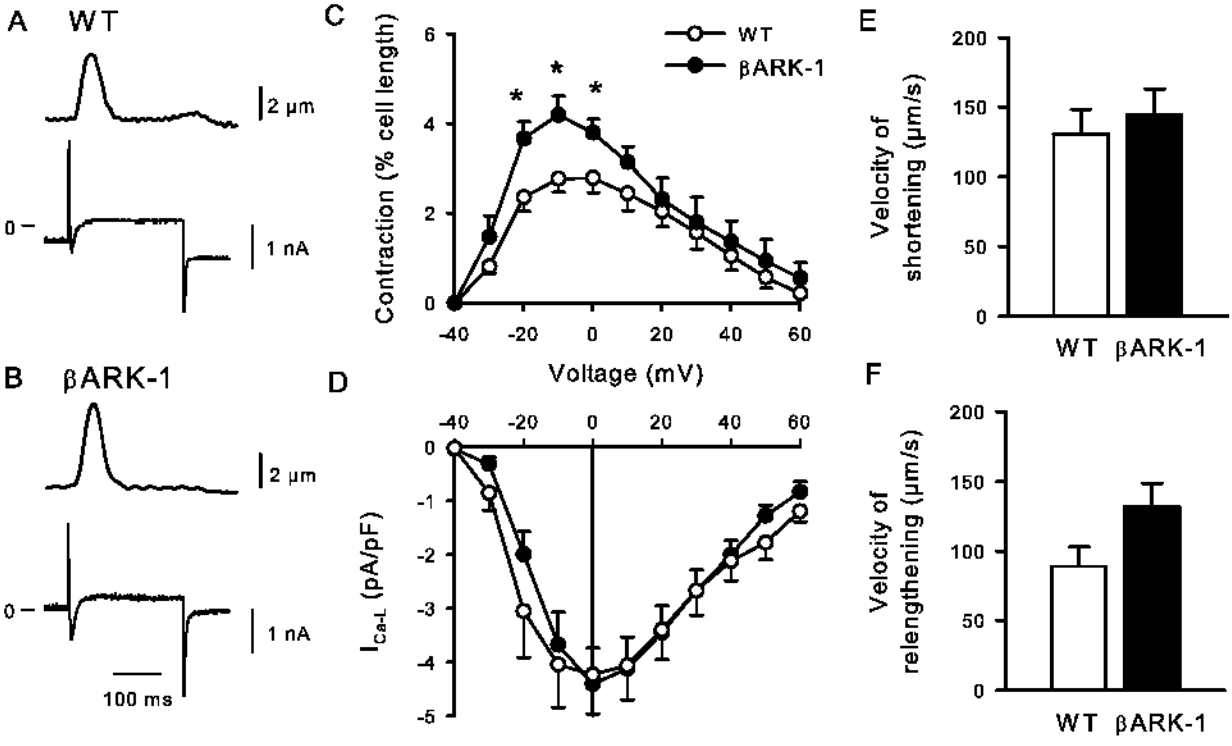


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Figure 2

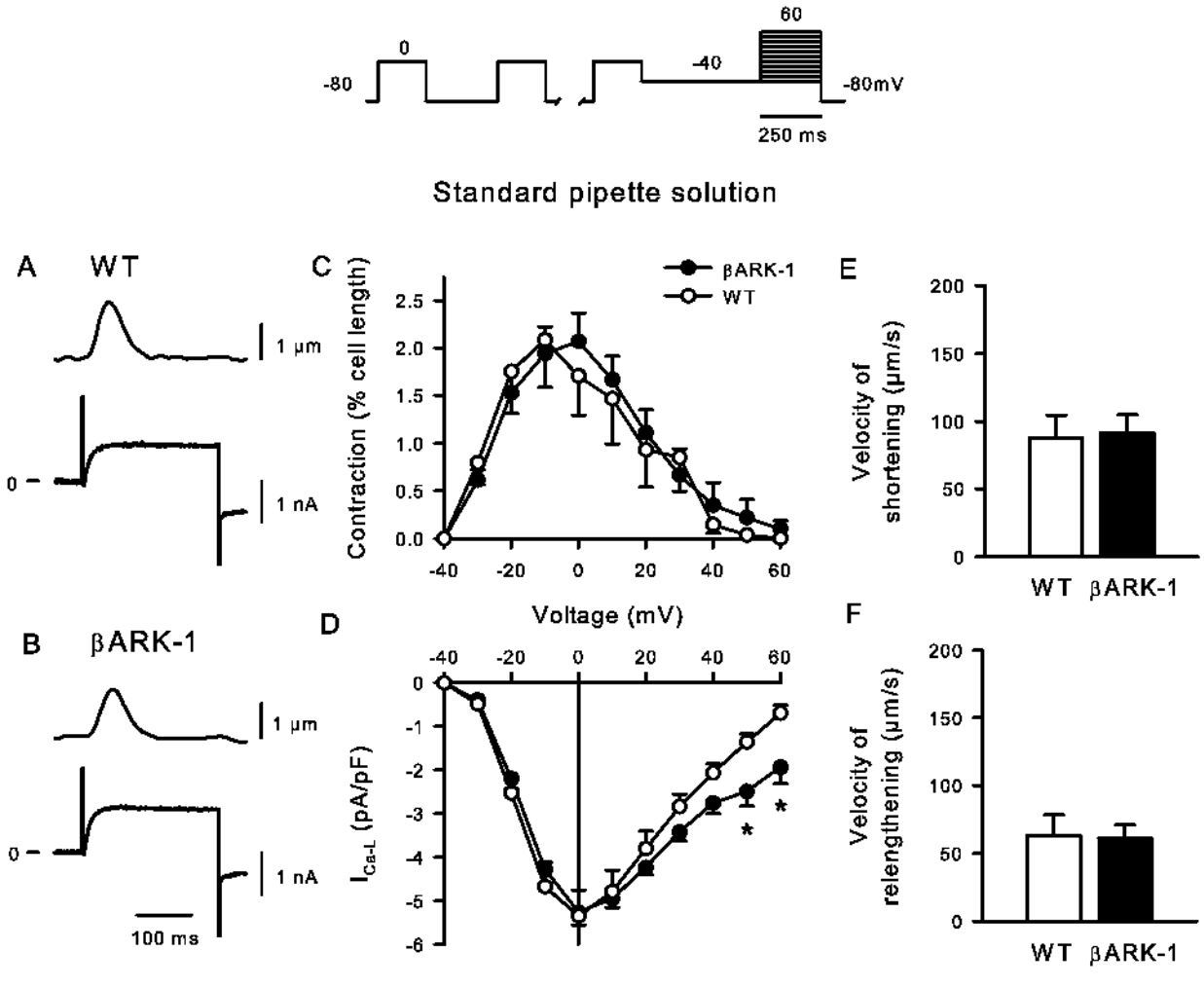

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Figure 3

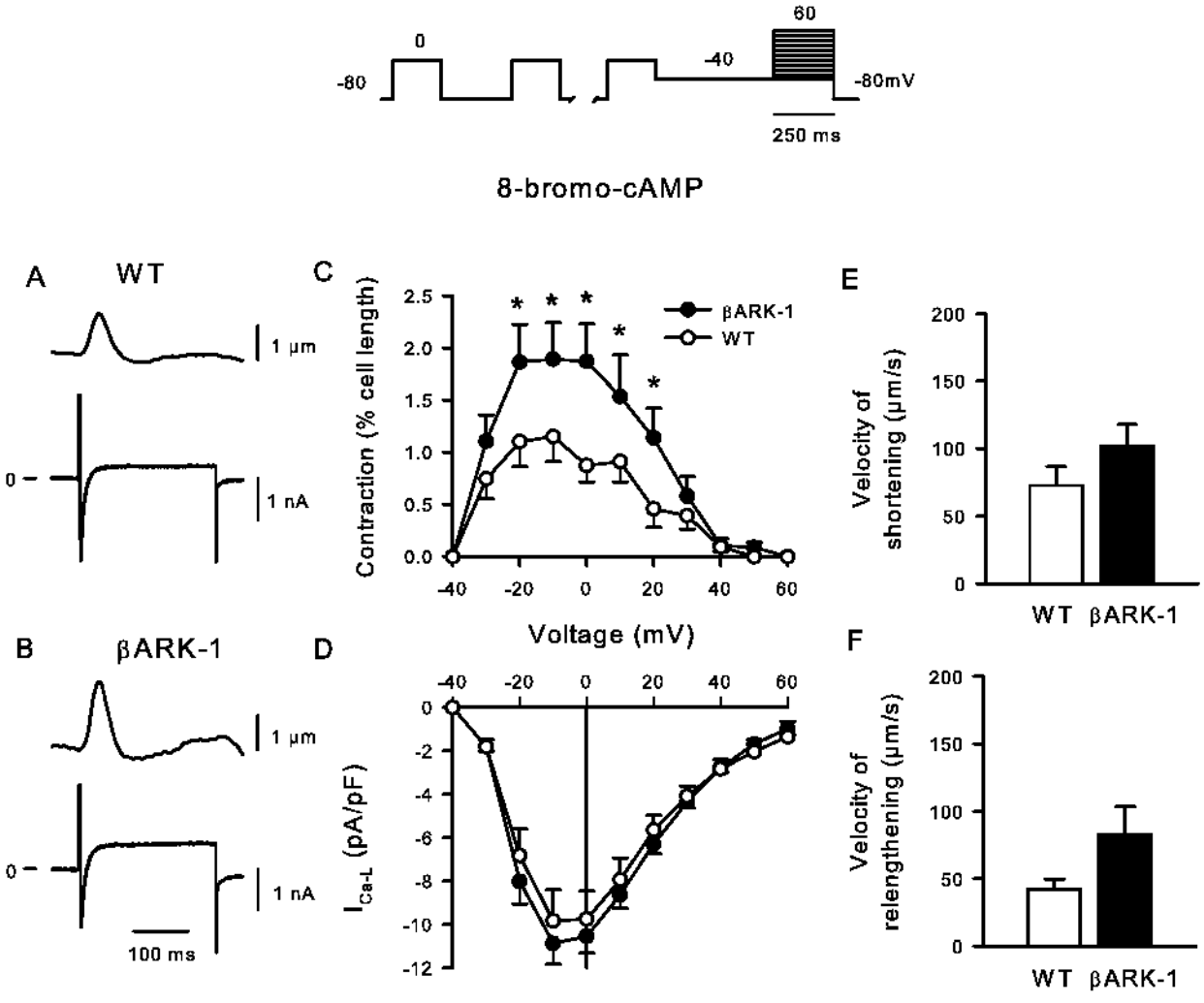

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Figure 4

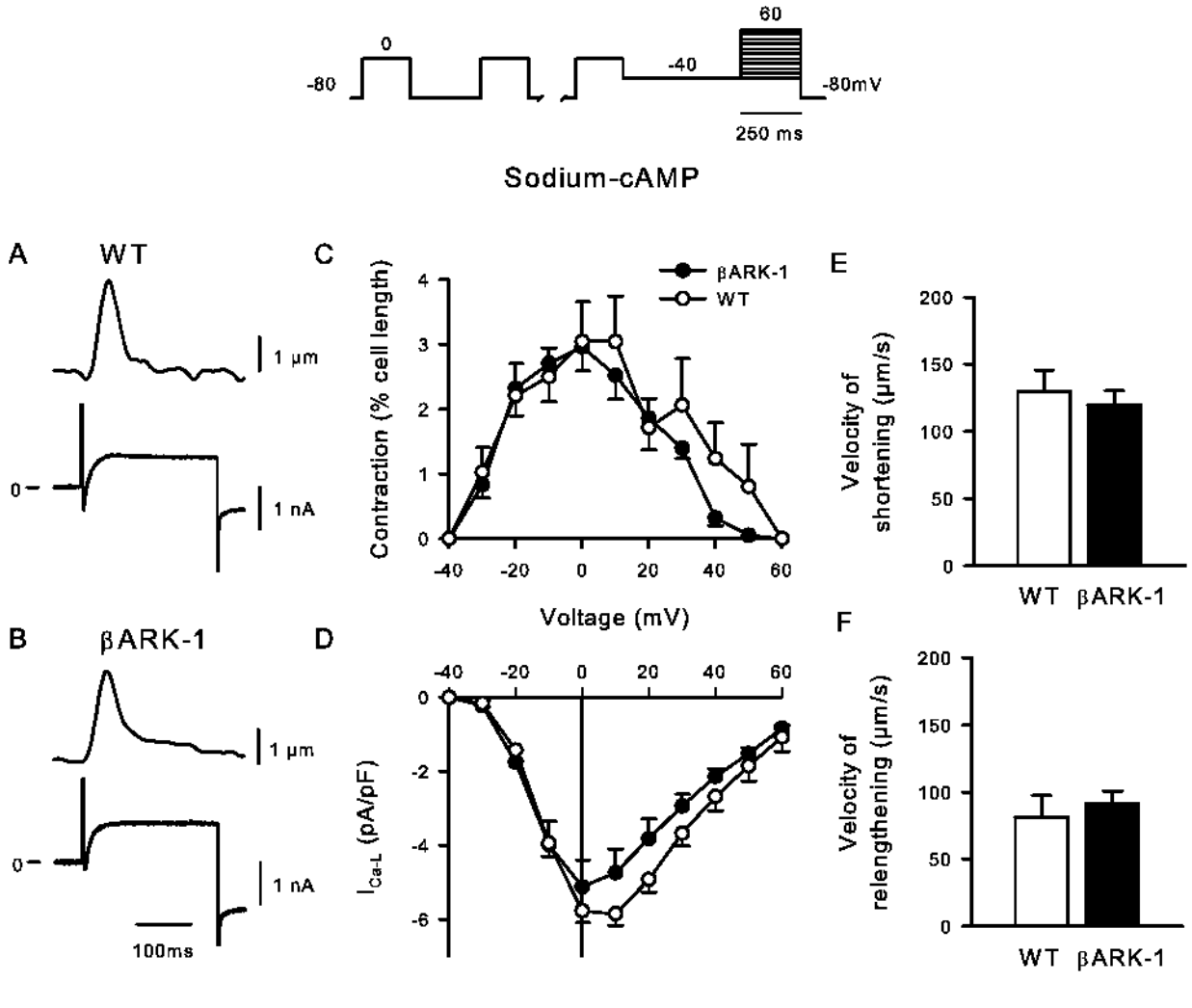

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Figure 5