DIMINISHED INOTROPIC RESPONSE TO AMRINONE IN VENTRICULAR MYOCYTES FROM MYOPATHIC HAMSTERS IS LINKED TO DEPRESSION OF HIGH GAIN Ca^{2+}-INDUCED Ca^{2+} RELEASE

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Running head: Amrinone in cardiomyopathic hearts

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Number of text pages: 34

Number of tables: 0

Number of figures: 11

Number of references: 34

Number of words in abstract: 250

Number of words in introduction: 595

Number of words in discussion: 1615

Non-standard abbreviations: guanylyl 3'5'-cyclic monophosphate (cGMP); Ca^{2+}-induced Ca^{2+} release (CICR); cardiomyopathic (CM); excitation-contraction coupling (EC-coupling); L-type Ca^{2+} current (I_{Ca-L}); phosphodiesterase 3 (PDE-III); cAMP-dependent protein kinase (PKA); sarcoplasmic reticulum (SR).

Recommended section assignment: Cardiovascular

ABSTRACT

This study investigates whether amrinone (100-1000 µM), a phosphodiesterase-III inhibitor, can alleviate depression of contractions in ventricular myocytes from pre-failure cardiomyopathic (CM) hamsters (80-100 days). Cell shortening and ion currents were measured in voltage clamped cells at 37°C. Normal myocytes exhibited low gain Ca^{2+}-induced Ca^{2+} release (CICR) initiated by test steps from -40 mV and high gain CICR initiated from more negative potentials. In normal myocytes, amrinone selectively increased contractions initiated by high gain CICR (fractional shortening increased from 3.6±0.5 to 5.3±0.6%, 300 µM amrinone), but had no effect on low gain CICR. Amrinone decreased L-type Ca^{2+} current (I_{Ca-L}; -5.5±0.8 to -3.7±0.5 pA/pF, 300 µM amrinone). In contrast, in CM myocytes high gain CICR was virtually absent and amrinone had no inotropic effect. Amrinone inhibited I_{Ca-L} less in CM than normal myocytes. Sarcoplasmic reticulum (SR) Ca^{2+} stores, assessed by caffeine, were significantly increased by amrinone in normal but not CM myocytes. Thus, the positive inotropic effect of amrinone in normal hamster myocytes is mediated by selective enhancement of high gain CICR. This effect is not mediated by stimulation of I_{Ca-L}, as I_{Ca-L} is inhibited by this drug in hamster. High gain CICR, which is depressed in CM myocytes, cannot be restored by amrinone. However, minimal stimulation of adenylyl cyclase with forskolin restored the positive inotropic effect of amrinone in CM cells. This positive inotropic effect of amrinone may reflect increased SR Ca^{2+} stores, as increased stores accompanied the positive inotropic effect in normal myocytes but were absent in CM myocytes.

The cardiomyopathic (CM) golden Syrian hamster develops an inherited cardiomyopathy triggered by a large genomic deletion in the delta-sarcoglycan gene (Sakamoto et al, 1997). This gene mutation leads to a deficiency in the dystrophin-associated glycoprotein in the sarcolemma of cardiac and skeletal muscles (Roberds et al., 1993), and leads to deleterious changes in contractile function of these muscles (reviewed in Howlett et al, 1999). In CM hamster heart, focal necrosis is observed at 40-50 days of age and reaches a peak around 90 days of age (Jasmin and Proschek, 1982; Hunter et al., 1984). Necrotic changes are followed by progressive ventricular hypertrophy beginning at about 120 days of age, and heart failure develops between 200-300 days of age (Jasmin and Proschek, 1982; Hunter et al., 1984). Thus, the CM hamster exhibits predictable and progressive cardiomyopathy with hypertrophy and terminal heart failure.

Contractile defects can be observed in cardiac tissues from CM hamsters before development of hypertrophy and heart failure (Bobet et al., 1991; Howlett et al., 1991). This suggests that depression of contraction is an early event which may contribute to the development of heart failure. This defect can be observed in isolated single ventricular myocytes, which suggests that decreased contractile strength represents a defect in excitation-contraction (EC)-coupling (Howlett et al., 1999). In a previous study we showed that the defect in contraction could be observed in voltage-clamped myocytes and therefore could not be attributed to changes in membrane or action potentials (Howlett et al., 1999). Furthermore, our studies showed that a defect in contraction could only be observed when contractions were initiated by voltage steps from negative membrane potentials approaching the resting potential, but not when cells were activated by steps from -40 mV. Contractions activated by steps from -40 mV are believed to be initiated by Ca^{2+}-induced Ca^{2+} release (CICR) coupled to influx of Ca^{2+} by way of L-type Ca^{2+} current (I_{Ca-L}) (Bers, 2001). CICR initiated from depolarized

potentials typically exhibits low gain (Ca^{2+} release/Ca^{2+} current).   In contrast, we have presented evidence that contractions activated by steps from membrane potentials near the resting potential involve a high gain mode of CICR in which very little current is required to initiate sarcoplasmic reticulum (SR) release of Ca^{2+} (Ferrier et al., 2004). Thus, the defect in contraction observed in myocytes from CM hamsters was caused by selective depression of high gain CICR (Howlett et al., 1999).

The contribution of high gain CICR to myocyte contraction is modulated by phosphorylation through the cAMP-dependent protein kinase A (PKA) and Ca^{2+} calmodulin kinase pathways (Hobai et al., 1997; Ferrier et al., 1998; Zhu and Ferrier, 2000; Ferrier and Howlett, 2003). Modulation of high gain CICR by PKA appears to be highly compartmentalized. This was shown in experiments on guinea pig ventricular myocytes where selective phosphodiesterase III (PDE-III) inhibition by amrinone augmented high gain CICR with virtually no effect on low gain CICR, and little effect on I_{Ca-L} (Xiong et al, 2001). As the defect in contraction in CM myocytes is primarily linked to depression of high gain CICR, it is possible that stimulation of high gain CICR with amrinone might restore strength of contraction in myocytes from these animals.

The objectives of this study were: 1) to determine whether amrinone selectively increases high gain CICR in hamster myocytes; 2) to determine whether amrinone can reverse depression of contractile function that occurs in CM hamster myocytes prior to the onset of heart failure; 3) to determine whether the inotropic effects of amrinone in myocytes from normal and/or CM hamsters are mediated by actions on I_{Ca-L} or SR Ca^{2+}.

METHODS

All experiments were performed on isolated ventricular myocytes from 80-100 day old male CM hamsters (CHF 146) and age-matched male normal hamsters (CHF 148), purchased from Canadian Hybrid Farms (Halls Harbour, Nova Scotia, Canada). Experiments were conducted in accordance with the guidelines published by the Canadian Council on Animal Care and this investigation was approved by the Dalhousie University Committee on Animal Care. Hamsters were injected intraperitoneally with heparin (3.3 IU/g) and sodium pentobarbital (80 mg/kg) to induce anesthesia. Following thoracotomy, the ascending aorta was cannulated *in situ* and perfused retrogradely (10 ml/min) with solution gassed with 100% O_2. The solution had the following composition (mM): 120 NaCl, 4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.05 $CaCl_2$, 10 HEPES, 12 glucose (37 ºC, pH 7.4 with NaOH). The heart was then removed from the chest. Following 6-minutes of perfusion with this solution, the heart was perfused for an additional 6 minutes with the same solution but with Ca^{2+} omitted. Hearts were then perfused for 20 minutes with 50 ml of this 0 mM Ca^{2+} solution to which was added: 20 mg collagenase A (Worthington), 15 mg protease (Boehringer Mannheim Corporation), 1.0 mg trypsin (Sigma) and 50 µM $CaCl_2$. After enzymatic dissociation, the ventricles were minced and washed in a substrate-enriched buffer of the following composition (mM): 80 KOH, 50 glutamic acid, 30 KCl, 30 KH_2PO_4, 20 taurine, 10 HEPES, 10 glucose, 3 $MgSO_4$, 0.5 EGTA (pH 7.4 with KOH). This isolation procedure yielded about 60-70% Ca^{2+}-tolerant rod-shaped, striated ventricular cells.

Cells were placed in a chamber mounted on the stage of inverted microscope, and were allowed to adhere to the bottom of the chamber for 15 min. Myocytes were then superfused at 37ºC with oxygenated HEPES-buffered solution of the following composition (mM): 145 NaCl, 4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4 with NaOH). Na^+ current and

transient outward current were inhibited with 200 µM lidocaine and 2 mM 4-aminopyridine, respectively. Most solutions were delivered from a reservoir at 3 ml/min and were changed by switching the inlet to the pump between different solutions. The solution exchange time was about 2 min, as determined by the response to changing extracellular K^+. To assess SR Ca^{2+} stores, caffeine (10 mM) was applied with a heated (37^oC) rapid solution switcher for 4 s. This device allowed exchange of the extracellular solution surrounding the myocyte under study within approximately 300 ms. The rapid solution switcher was controlled by pCLAMP software (version 6.0.3, Axon Instruments, Inc., Foster City, CA, USA) to ensure precise timing.

Recordings were made from quiescent, rod-shaped ventricular myocytes with clear striations and resting membrane potentials more negative than -85 mV. Discontinuous single-electrode voltage clamp recordings (7-10 kHz) were made with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) and high-resistance microelectrodes (18-25 MΩ, filled with 2.7 M KCl) to minimize cell dialysis. Switching frequency was adjusted to insure adequate settling time for accurate voltage control. A 2.7 M KCl-agar bridge was used as a bath ground to reduce liquid junction potential changes. Voltage clamp protocols were generated, and currents and membrane potentials were recorded with pCLAMP 6.0 software (Axon Instruments, Inc.). Myocyte images were monitored with a closed circuit television camera (Model 1-GP-CD 60, Panasonic) and were displayed on a video monitor. Unloaded cell shortening was sampled at 120 Hz with a video edge detector (Model VED 103, Crescent Electronics, Sandy, UT) coupled to the television camera. Cell length was measured by tracking both ends of each cell with the video edge detector. Contractions, current and voltage were digitized with a Labmaster A/D interface (TL1-125, Axon Instruments) and stored on a computer.

In all experiments, voltage clamp test steps were preceded by trains of 200 ms conditioning pulses from a holding potential of -80 mV to 0 mV delivered at approximately 3 Hz, to maintain consistent activation of the myocyte. Conditioning pulses were followed by a 500 ms long post-conditioning potential of -40 or -60 mV from which the test steps were made. Current, voltage and contractions were measured with pClamp analysis software. Inward I_{Ca-L} was measured as the difference between the peak inward current and a reference point near the end of the voltage step (Li et al., 1995). Peak amplitudes of inward currents were normalized to cell capacitance (membrane area). Cell capacitance was estimated with pClamp software by integrating capacitive transients elicited by test steps from -60 to -50 mV, where little inward current is activated. Specific details of particular voltage clamp protocols are provided in the appropriate results sections. Amplitudes of contraction were measured as the difference between a point immediately before onset of cell shortening and peak cell shortening. Fractional shortening was determined by normalizing contraction amplitudes to diastolic cell length.

The statistical significance of differences between means was tested with a Student's t-test or an analysis of variance. Current-voltage relationships and contraction-voltage relationships were analyzed with a two-way repeated measures analysis of variance. Post-*hoc* comparisons were made with a Student-Newman-Keuls test. All statistical analyses were performed with SigmaStat (Jandel, Version 2.03). Data are presented as means ± SE. The value of "n" represents the number of cells sampled. No more than two cells from one heart were included in each data set.

Amrinone, lidocaine, caffeine, forskolin, 4-aminopyridine and HEPES were purchased from the Sigma-Aldrich Canada Ltd. (Oakville, Ontario). All other chemicals were purchased

from BDH Inc. (Toronto, Ontario). Amrinone was dissolved in HCl in stock solution and the pH

of the final solution of amrinone was adjusted to 7.4 with NaOH.

RESULTS

In a previous study we demonstrated that contractions initiated from negative membrane potentials were selectively depressed in myocytes from CM hamsters (Howlett et al., 1999). Figure 1 compares contractions elicited in voltage clamped myocytes from normal and CM hamsters. The voltage clamp protocol is shown in figure 1A. Following the train of conditioning pulses, the cells were clamped to a post-conditioning potential of -60 mV, followed by sequential steps to -40 and 0 mV. Figure 1B shows records of contractions and currents recorded from a myocyte from a normal heart. The test step to -40 mV initiated a phasic contraction with little accompanying inward current. The subsequent step to 0 mV elicited a second phasic contraction and activated I_{Ca-L}. In contrast, in the CM myocyte the voltage step to -40 mV failed to elicit a contraction (Figure 1C). However, the voltage clamp step to 0 mV initiated a phasic contraction and I_{Ca-L}. Figure 1D shows mean amplitudes of contractions (fractional shortening) and current densities recorded from normal and CM myocytes. The amplitudes of contractions elicited by steps to -40 mV were significantly smaller in CM compared to normal myocytes. There was no difference in the amplitudes of the accompanying small inward currents. However, the step to 0 mV elicited contractions and I_{Ca-L} of comparable magnitudes in myocytes from normal and CM hearts.   Thus, myocytes from CM hearts exhibited marked depression of contractions initiated by steps from -60 to -40 mV, with no difference in contractions initiated by steps from -40 to 0 mV.

The next series of experiments used the same voltage clamp protocol as in figure 1 to investigate the effects of amrinone on contractions and currents in myocytes from normal hamsters. Figure 2A and B, respectively, show records of contractions and currents made before and after superfusion of a normal myocyte with 300 μM amrinone. In this example, amrinone

increased the amplitude of the contraction initiated by the step from -60 to -40 mV, but had little

if any effect on the contraction elicited by the step from -40 to 0 mV. Interestingly, amrinone

decreased the amplitude of I_{Ca-L} activated by the step to 0 mV.   Figure 2C shows mean

amplitudes of contractions and current densities in normal cells exposed to 0, 300, and 1000 μM

amrinone. Amrinone caused a concentration dependent increase in the amplitudes of

contractions initiated by steps from -60 to -40 mV, but did not alter the amplitudes of

contractions initiated by steps from -40 to 0 mV. Mean inward current density initiated by the

step to -40 mV was slightly but not significantly reduced in the presence of amrinone. However,

amrinone caused large and significant decreases in I_{Ca-L} initiated by the step to 0 mV.

We next determined whether comparable effects could be demonstrated in CM myocytes

superfused with amrinone. Figures 3A and B compare recordings of contractions and currents

made from a CM myocyte in the absence and presence of 300 μM amrinone. Little or no

contraction was initiated by the step from -60 to -40 mV in the absence of amrinone, and

superfusion with amrinone had no observable effect on responses to either voltage clamp step,

except for a slight decrease in the amplitude of I_{Ca-L}. Interestingly, contractions initiated by the

step from -60 to -40 mV in CM myocytes were unaffected by amrinone, and remained small in

control and treated myocytes (Figure 3C). Thus, amrinone did not restore contractions initiated

by steps from negative potentials in CM myocytes. Amrinone also had no significant effect on

contractions initiated by the step from -40 to 0 mV in CM myocytes. However, amrinone did

significantly reduce the amplitudes of I_{Ca-L} in CM myocytes, although this effect appeared to be

slightly smaller than in normal myocytes.

It is possible that the lack of inotropic effect of amrinone on CICR initiated by steps from

-40 to 0 mV in both normal and CM myocytes is related to the use of sequential activation steps.

This might occur if the first step from -60 to -40 mV partially depletes SR stores. Therefore, we examined effects of amrinone on contractions and currents initiated by steps -40 to 0 mV with a protocol which omitted the first step (Figure 4, top). In this protocol, the last conditioning pulse was followed by return to a post-conditioning potential of -40 mV. Figure 4A compares contractions and currents recorded from a normal myocyte in the absence and presence of 300 μM amrinone. Amrinone had no effect on the magnitude of contraction although it decreased inward current. Figure 4B presents mean data which show that amrinone had no inotropic effect in normal myocytes activated with this protocol. However, amrinone continued to exert an inhibitory effect on I_{Ca-L} (Figure 4C). Figure 4D shows that amrinone had no effect on contraction in a CM myocyte, but decreased I_{Ca-L}. Mean data demonstrate that amrinone had no significant effect on contraction in CM myocytes, but significantly decreased I_{Ca-L} (Figures 4E and F). Thus, the lack of inotropic effect of amrinone on low gain CICR was not related to the order of activation of contractions. In the next set of experiments we examined the effects of amrinone when steps to 0 mV were made from a post-conditioning potential to -60 mV (Figure 5, top). Figure 5A shows recordings of contractions and currents in a normal myocyte in the absence and presence of 300 μM amrinone. Amrinone increased the amplitude of contraction initiated by a step from -60 to 0 mV and decreased inward current (Figure 5A). Figure 5B and C shows mean data for amplitudes of contraction and current densities recorded in normal myocytes. With this protocol, amrinone caused a significant increase in contraction in normal myocytes, despite continued inhibition of I_{Ca-L} (Figure 5B and C). However, with the same voltage clamp protocol, amrinone still did not exert a positive inotropic effect in CM myocytes, although inhibition of I_{Ca-L} persisted (Figure 5D, E and F). These data suggest that the inotropic

effect of amrinone on normal cells is only available when test steps originate from more negative

potentials. Further, this inotropic effect is not available in myocytes from CM hearts.

It is possible that the presence or absence of effects of amrinone on contractions and

currents could be related to shifts in the voltage-dependence of these responses, rather than

absolute changes in maximum amplitudes. To investigate this we determined contraction-

voltage and current-voltage relations over a wide range of test potentials. Also, we compared

contraction-voltage and current-voltage relations initiated from post-conditioning potentials of

-40 and -60 mV. Mean relations determined in normal cells are shown in Figure 6, and the

voltage clamp protocol is shown at the top. Figure 6A shows that amrinone had no inotropic

effect across the entire activation voltage range tested when the post-conditioning potential was

-40 mV, and that there was no obvious shift in the voltage-dependence of contraction. In

contrast, the current-voltage relations demonstrated a prominent concentration dependent

inhibition of I_{Ca-L} (Figure 6B). Inhibition of I_{Ca-L} occurred without a shift in voltage-dependence.

However, when the post-conditioning potential was -60 mV (Figure 6C), a marked positive

inotropic effect of amrinone appeared over a wide range of test potentials. This effect was

accompanied by inhibition of I_{Ca-L}, again with no apparent shift in voltage-dependence (Figure

6D). These data show that amrinone can exert a prominent positive inotropic effect over a wide

range of test potentials in normal myocytes, but only when steps are made from negative

membrane potentials. Furthermore, the effects of amrinone cannot be attributed to shifts in

voltage-dependence of either contraction or current.

Figure 7 shows mean contraction-voltage and current-voltage relations determined in CM

myocytes. The data demonstrate that, regardless of post-conditioning potential, amrinone did not

alter either the configuration or amplitudes of the contraction-voltage relations (Figure 7 A and

C). However, amrinone did cause a significant inhibition of I_{Ca-L} initiated by steps from either

-40 or -60 mV. Inhibition of I_{Ca-L} was not accompanied by a shift in voltage-dependence.

Although inhibition of I_{Ca-L} was significant, the effect appeared smaller in CM compared to

normal myocytes. To determine whether inhibition of I_{Ca-L} was greater in normal than in CM

myocytes we compared current-voltage relations from normal and CM myocytes, in the absence

and presence of the highest concentration of amrinone (Figure 8). Figures 8A and B show mean

current-voltage relations elicited by test steps from -40 and -60 mV for normal and CM

myocytes in the absence of amrinone. Control current-voltage relations were not significantly

different between normal and CM myocytes with either post-conditioning potential. Figures 8C

and D compare current-voltage relations in the presence of 1000 μM amrinone. With either post-

conditioning potential, I_{Ca-L} was significantly smaller in normal than CM myocytes.

To determine whether changes in SR Ca^{2+} stores contribute to the inotropic effects of

amrinone, we estimated SR stores by rapid application of 10 mM caffeine. The protocol is

shown at the top of Figure 9. Caffeine application was substituted for the test step normally

following the train of conditioning pulses. Caffeine induces release of SR Ca^{2+} which initiates

an inward Na^+-Ca^{2+} exchange current. The integral of this current provides a measure of Ca^{2+}

released from SR stores (Varro et al., 1993). Figures 9A and B respectively show representative

recordings of caffeine-induced contractures (top) and accompanying Na^+-Ca^{2+} exchange current

(bottom) in a normal myocyte before and after exposure to amrinone. Mean amplitudes of

contractures and currents are shown in Figure 9C. The amplitudes of contractures were not

different in the absence and presence of amrinone. However, amrinone significantly increased

the integral of the Na^+-Ca^{2+} exchange current. This suggests that amrinone increases SR Ca^{2+}

stores in normal hamster myocytes. Figures 10A and B show caffeine-induced contractures and

currents recorded in a CM myocyte in the absence and presence of amrinone. Figure 10C shows

that the mean amplitudes of contractures and integrals of Na^+-Ca^{2+} exchange currents were not

significantly different in myocytes from CM hearts before and after exposure to amrinone. Thus,

amrinone increased SR Ca^{2+} stores in normal but not CM myocytes.

Inhibition of PDE by amrinone would be expected to promote phosphorylation of protein

targets only if there is adequate production of cAMP in the cell. Thus the absence of a positive

inotropic effect of amrinone may indicate that cAMP production is inadequate in CM myocytes.

To investigate this, we used forskolin to stimulate adenylyl cyclase and thus stimulate cAMP

production in myocytes from CM heart. We selected a low concentration forskolin (0.3 µM)

which does not, by itself, cause a significant increase in contraction (Feldman et al, 1987). Then,

we determined whether amrinone (300 µM) could increase contraction in CM myocytes in the

presence of forskolin. The protocol is shown at the top of Figure 11. Figure 11A shows

representative traces of contraction (top) and current (bottom) recorded from a CM myocyte in

response to a test step from -60 to 0 mV. Figure 11B demonstrates that 0.3 µM forskolin had

little effect on magnitudes of either inward current or contraction activated by voltage steps from

-60 mV. However, when 0.3 µM forskolin was added in combination with amrinone,

contraction increased markedly in the CM myocyte (Figure 11C). Mean contraction-voltage and

current-voltage curves are shown in figures 11D and E. These data show that a low

concentration of forskolin had no significant effect on either contraction or current at any voltage

examined. However, in the presence of forskolin, amrinone significantly increased the

amplitudes of contractions (Figure 11D). Interestingly, amrinone plus forskolin had no

significant effect on the magnitude of inward current (Figure 11E). Thus, minimal stimulation of

adenylyl cyclase with forskolin restored the positive inotropic effect of amrinone in myocytes

from CM heart.

DISCUSSION

The objectives of this study were: 1) to determine whether amrinone selectively increases high gain CICR in hamster myocytes; 2) to determine whether amrinone can reverse depression of contractile function that occurs in myocytes from CM hamsters prior to the onset of heart failure; and 3) to determine whether the inotropic effects of amrinone in myocytes from normal and/or CM hamsters are mediated by actions on I_{Ca-L} or SR Ca^{2+}. Our results demonstrated that amrinone caused a concentration-dependent increase in contractions initiated from negative membrane potentials in myocytes from normal hearts. This effect was selective for high gain CICR, as low gain CICR initiated by steps from -40 mV was not affected by amrinone. Surprisingly, we found that amrinone did not exert a positive inotropic effect on either low or high gain CICR in myocytes from CM hearts, except in the presence of forskolin which directly stimulates adenylyl cyclase. Furthermore, we found that amrinone caused a significant inhibition of I_{Ca-L} in both normal and CM myocytes. Interestingly, amrinone caused a significant increase in caffeine-releasable SR Ca^{2+} in normal myocytes. Thus, the positive inotropic effect observed in normal myocytes cannot be attributed to stimulation of I_{Ca-L}, but may involve an increase in SR Ca^{2+} stores.

In the present study we found that amrinone selectively increased contractions initiated by voltage steps from negative membrane potentials in normal hamster myocytes. We previously reported a similar selectivity for contractions initiated from membrane potentials near the resting potential in guinea pig ventricular myocytes (Xiong et al., 2001). Interestingly, earlier studies demonstrating the positive inotropic effects of amrinone in *in vitro* cardiac muscle were conducted in multicellular preparations, where contractions were activated by action potentials triggered from the resting potential (Rosenthal and Ferrier, 1982; Kondo et al., 1983;

Malecot et al., 1985; Morner and Wolfart, 1990). In contrast, our present study and our earlier

study (Xiong et al., 2001) demonstrate that amrinone has virtually no effect on low gain CICR

initiated from more depolarized membrane potentials. Since we have shown that responses

initiated from more negative potentials are initiated by high gain CICR (Richard et al., 2004), our

results indicate that amrinone selectively enhances high gain CICR. Because the main defect in

EC-coupling in myocytes from CM hamsters can be attributed to a defect in high gain CICR

(Howlett et al., 1999), we hypothesized that amrinone would improve function in CM myocytes.

Surprisingly we found that amrinone was unable to restore high gain CICR in CM cells. Thus,

our results suggest that the lack of inotropic effect of amrinone in CM myocytes occurs because

of a defect in CM cells that prevents activation of high gain CICR.

In myocytes from guinea pig (Xiong et al, 2001) and normal hamster hearts, amrinone

caused significant increases in caffeine-releasable SR Ca^{2+} stores. Increased SR Ca^{2+} stores

could result from enhancement of SR Ca^{2+} uptake by amrinone. This is likely, as a closely

related PDE-III inhibitor, milrinone, has been shown to increase SR Ca^{2+} ATPase activity and SR

Ca^{2+} uptake in homogenates and SR vesicles from canine ventricular muscle (Yano et al., 2000).

Thus, it is possible that the key action for the positive inotropic effect of amrinone in normal

myocytes is stimulation of SR Ca^{2+} uptake. However, in myocytes from CM hearts SR Ca^{2+}

stores were not increased by amrinone, which suggests that amrinone does not stimulate SR Ca^{2+}

uptake in CM myocytes. The observation that both the inotropic effect and increase in SR Ca^{2+}

stores were absent in CM myocytes further suggests that these two events are causally linked.

If amrinone produces its positive inotropic effect in normal myocytes by increasing SR

Ca^{2+} stores, it is important to ask why amrinone did not increase CICR initiated by steps from

-40 mV. One possibility is that the positive inotropic effect of increased SR stores is

counteracted by the significant decrease in the amplitude of I_{Ca-L} observed in the present study.

A decrease in I_{Ca-L} would be expected to cause a negative inotropic effect, as SR Ca^{2+} release is

proportional to the magnitude of I_{Ca-L} in conventional CICR (Bers, 2001). Thus, the combined

effects of increased SR Ca^{2+} stores and decreased trigger for release may cancel one another and

result in no net inotropic effect on contractions initiated by conventional CICR. On the other

hand, high gain CICR shows a marked positive inotropic effect. This inotropic effect may

persist because SR Ca^{2+} release triggered by high gain CICR is not proportional to the amplitude

of I_{Ca-L} (Ferrier and Howlett, 2003; Richard et al., 2004). Therefore the positive inotropic effect

of increased SR Ca^{2+} is not countered by the decrease in the magnitude of I_{Ca-L}.

 The mechanism by which amrinone inhibits I_{Ca-L} in hamster myocytes has not been

established. It is possible that the effects of amrinone on I_{Ca-L} are mediated by inhibition of

PDE-III. Inhibition of PDE-III increases both cAMP and cGMP (Movsesian, 2002). If elevation

of cAMP predominates, an increase in I_{Ca-L} would be predicted (Mubagwa et al., 1993).

However, if an increase in cGMP predominates in the vicinity of the L-type Ca^{2+} channel in

hamster myocytes, a decrease in I_{Ca-L} may result. Elevation of cGMP can inhibit I_{Ca-L} through

activation of protein kinase G, which can phosphorylate the L-channel at an inhibitory site (Mery

et al., 1991; Sumii and Sperelakis, 1995). Alternatively, protein kinase G may activate protein

phosphatases, which would dephosphorylate the PKA-phosphorylated site on the L-channel

(Shen and Pappano, 2002). Inhibition of I_{Ca-L} was greater in normal myocytes compared to CM

myocytes. The basis for this difference is unknown, but one could speculate that less inhibition

of I_{Ca-L} might occur if the levels of PDE-III are less in CM myocytes compared to normal

hamster myocytes (Yu et al., 1996). Interestingly, previous studies in guinea pig and ferret

ventricular myocytes have shown that amrinone either has no effect on I_{Ca-L} or actually increases

the magnitude of I_{Ca-L} (Xiong et al, 2001; Malecot et al., 1985; Kondo et al., 1983). Thus, effects of amrinone on I_{Ca-L} appear to vary with species and experimental conditions and are not well correlated with inotropic effects.

A central finding in the present study is that amrinone can increase high-gain CICR in normal myocytes, but these effects are absent in CM myocytes where high gain CICR is depressed (Howlett et al., 1999). The mechanisms underlying depression of high gain CICR and the lack of a stimulatory effect of amrinone in myocytes from CM animals are not certain. However, cAMP content is significantly reduced in CM hamster hearts (Wikman-Coffelt et al., 1986). We have shown previously that high-gain CICR is promoted by the cAMP-PKA pathway (Ferrier et al., 1998; Ferrier and Howlett, 2003; Richard et al., 2004). Thus, lower levels of cAMP in CM myocytes might lead to weaker activation of high-gain CICR. A reduction in cAMP levels in CM myocytes might result through reduced synthesis or greater degradation. The lack of effect of amrinone suggests that enhanced degradation is not responsible for depression of high-gain CICR. On the other hand, reduced synthesis of cAMP would explain the lack of effect of amrinone in CM myocytes. Indeed, a low concentration of forskolin, which did not in itself increase contraction, restored the stimulatory effect of amrinone in CM myocytes. Thus, it is likely that the absence of high gain CICR in CM myocytes reflects a reduction in basal cAMP production in CM myocytes. Interestingly, reduced synthesis of cAMP also has been shown to underlie reduced sensitivity and maximum effect of PDE-III inhibitors in trabeculae from hearts of patients in end-stage heart failure (Feldman et al., 1987; Bohm et al., 1988). As CM hamsters eventually develop heart failure (Jasmin and Proschek, 1982; Hunter et al., 1984), our results suggest that a defect in the cAMP-PKA pathway is present before the onset of overt failure, and therefore may contribute to development of failure.

There is evidence that an increase in myofilament Ca^{2+} sensitivity may contribute to the

positive inotropic effects of certain PDE inhibitors (Schmitz et al., 1989), although it is not clear

whether this occurs with amrinone. The related PDE III inhibitor milrinone, either has no effect

or actually decreases sensitivity to Ca^{2+} (Gwathmey and Morgan, 1985; Alousi et al., 1988).

Thus, it is unlikely that sensitization of myofilaments to Ca^{2+} contributes to the positive inotropic

effects of amrinone we observed in normal myocytes. It also is unlikely that changes in

myofilament Ca^{2+} sensitivity are responsible for the lack of effect of amrinone in CM myocytes,

as our results suggest that reduced cAMP production is primarily responsible for the insensitivity

of CM myocytes to PDE inhibitors. However, down-regulation of PDE, which occurs in heart

failure (Smith et al., 1997), also might contribute to the reduced efficacy of PDE inhibitors in

CM myocytes.

In summary, our results demonstrate that amrinone selectively enhances high gain CICR

in normal hamster myocytes, but is not able to restore high gain CICR, which is depressed in

myocytes from CM hamsters, except in the presence of forskolin which stimulates adenylyl

cyclase. Further, the positive inotropic effect of amrinone in normal myocytes cannot be

attributed to effects on I_{Ca-L}, which is inhibited in hamster myocytes by this drug. However, the

positive inotropic effect may reflect increased SR Ca^{2+} stores, as increased SR stores

accompanied the positive inotropic effect in normal myocytes but were absent in CM myocytes.

Interestingly, loss in sensitivity to PDE-III inhibitors has been observed in end-stage heart failure

in humans (Feldman et al., 1987; Bohm et al., 1988). As the loss of sensitivity to PDE-III

inhibitors in CM hamster myocytes occurred prior to the onset of heart failure, our results

suggest that this defect may contribute to the development of heart failure in these animals.

ACKNOWLEDMENTS

We thank Peter Nicholl and Cindy Mapplebeck for providing excellent technical

assistance.

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FOOTNOTES

a) This work was supported in part by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Nova Scotia. Dr. W. Xiong was a Killam Scholar. This work has been presented in part previously in the following abstracts:

Xiong W, Ferrier GR and Howlett SE (1998) Amrinone potentiates contractions initiated by a voltage sensitive mechanism in ventricular myocytes from normal but not cardiomyopathic hamsters. *Biophys J* 74:A54.

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Figure 1. Contractions initiated by test steps from negative potentials are selectively depressed in myocytes from CM hamsters. The voltage clamp protocol is shown in **Panel A**.

Panel B. Traces show representative recordings of contractions (top) and currents (bottom) activated by sequential test steps from -60 to -40 and 0 mV in a normal myocyte. Contractions were initiated by both steps. **Panel C.** Contraction was absent on the step from -60 to -40 mV in a representative recording from a CM myocyte, although contraction still occurred with the step to 0 mV. **Panel D.** Mean data compare amplitudes of contractions initiated by steps to -40 and 0 mV in normal and CM myocytes. Contractions initiated by the step to -40 were significantly decreased in amplitude in CM compared to normal myocytes. Contractions initiated by the step to 0 mV were not significantly different between CM and normal myocytes. Inward currents initiated by both steps were similar in amplitude in the two groups. * Denotes significantly different from normal ($p < 0.05$); n = 5 normal and 14 CM myocytes.

Figure 2. Amrinone selectively enhances high gain CICR initiated by steps from -60 to -40 mV, but inhibits I_{Ca-L} in normal hamster myocytes. The voltage clamp protocol is shown at top. **Panel A.** Representative recordings of contractions and currents from a normal myocyte in the absence of amrinone. **Panel B.** Representative recordings from a normal myocyte exposed to 300 μM amrinone shows that amrinone selectively increased the contraction initiated by the step from -60 to -40 mV. In addition, amrinone inhibited I_{Ca-L} activated by the step to 0 mV. **Panel C.** Mean data show that amrinone exerted a concentration dependent positive inotropic effect on high gain CICR initiated by steps from -60 to -40 mV. Amrinone had no effect on low gain CICR initiated by steps from -40 to 0 mV, but significantly decreased I_{Ca-L} initiated by this

step. * Denotes significantly different from control in the absence of drug (p<0.05); n= 9

myocytes.

Figure 3. Amrinone does not restore contractions initiated by high gain CICR in CM

myocytes. The voltage clamp protocol is shown at the top. **Panels A and B.** Representative

recordings of contractions and currents elicited in a CM myocyte before and after exposure to

300 μM amrinone. Amrinone did not restore contraction for the step from -60 to -40 mV. **Panel**

C. Mean data demonstrate that amrinone did not enhance contractions initiated by high gain

CICR triggered by steps to -40 mV. Amrinone also did not increase contractions initiated by the

step to 0 mV, but did significantly decrease I_{Ca-L}. * Denotes significantly different from control

in the absence of drug (p<0.05); n= 6-10 myocytes.

Figure 4. Amrinone does not increase amplitudes of contractions initiated by low gain

CICR regardless of order of activation. A voltage clamp protocol (top) was used to activate

low gain CICR with a step from -40 to 0 mV, without prior activation of high gain CICR. **Panel**

A. Representative traces illustrate contraction (top) and current (bottom) recorded from a normal

myocyte in the absence and presence of 300 μM amrinone. Amrinone had no effect on the

magnitude of contraction but decreased the amplitude of inward current. **Panel B.** Mean data

for contractions in normal myocytes show that amrinone caused no significant change in

amplitudes of contraction. **Panel C.** Amrinone caused a significant decrease in the peak

amplitudes of I_{Ca-L} in normal myocytes. **Panel D.** Representative traces of contraction and

inward current recorded from a CM myocyte illustrate that amrinone had no effect on the

magnitude of contraction but decreased the amplitude of inward current. **Panels E and F.** In

CM myocytes, amrinone was without effect on mean amplitudes of contraction. However, amrinone caused a significant decrease in amplitudes of I_{Ca-L}. Denotes significantly different from control in the absence of drug (p<0.05); n = 9 - 11 normal, and 4 - 11 CM myocytes.

Figure 5. The positive inotropic effect of amrinone can be observed with steps to 0 mV when cells are activated by steps from negative potentials in normal but not CM myocytes. A voltage clamp protocol (top) was used to activate high gain CICR with a step from -60 to 0 mV. **Panel A.** Representative traces illustrate contraction (top) and current (bottom) recorded from a normal myocyte in the absence and presence of 300 µM amrinone. Amrinone increased the magnitude of contraction but decreased the amplitude of inward current. **Panel B** shows that amrinone caused a significant increase in the mean amplitudes of contractions initiated by steps from -60 to 0 mV in normal myocytes. **Panel C** shows that amrinone caused a significant inhibition of mean peak I_{Ca-L}. **Panel D.** Representative traces of contraction and inward current recorded from a CM myocyte illustrate that amrinone had no effect on the magnitude of contraction but decreased the amplitude of inward current. **Panels E and F.** In CM myocytes amrinone did not have a significant effect on amplitudes of contraction, but caused a significant decrease in the amplitudes of peak I_{Ca-L}. Denotes significantly different from control in the absence of drug (p<0.05); n = 9 - 11 normal, and 4 - 11 CM myocytes.

Figure 6. Effects of amrinone on contraction-voltage and I-V relations in myocytes from normal hamsters. Contraction-voltage and I-V relations were determined with the voltage clamp protocol shown at top. **Panel A** shows mean contraction-voltage relations for low gain CICR determined with steps from -40 mV. The relation was bell-shaped. Amrinone had no

significant effect on the configuration or amplitude of this relationship. **Panel B.** Mean I-V relations also were bell-shaped. Amrinone caused significant inhibition of mean amplitudes of I_{Ca-L} without altering the voltage dependence. **Panel C** shows mean contraction-voltage relations for contractions initiated from -60 mV. Amrinone significantly increased the mean amplitudes of contraction over a wide range of membrane potentials. **Panel D** shows that amrinone significantly inhibited mean amplitudes of I_{Ca-L} without changing the configuration of the I-V relation. * Denotes 300 µM amrinone significantly different from control in the absence of drug (p<0.05); [†] denotes 1000 µM amrinone significantly different from control in the absence of drug (p<0.05); n = 9 – 11 myocytes.

Figure 7. Effects of amrinone on contraction-voltage relations and I-V relations in myocytes from CM hamsters. Voltage clamp protocol at top. **Panel A** shows contraction-voltage relations for contractions initiated by low gain CICR activated by steps from -40 mV. Amrinone had no significant effect on these contractions in CM myocytes. **Panel B.** Amrinone significantly decreased the mean amplitudes of peak I_{Ca-L} but did not alter the voltage dependence of I_{Ca-L}. **Panel C.** Amrinone also had no effect on contraction-voltage relations initiated by steps from -60 mV in CM myocytes. **Panel D.** Inhibitory effects of amrinone on I_{Ca-L} were similar to those observed with steps from -40 mV. * Denotes 300 µM amrinone significantly different from control in the absence of drug (p<0.05); [†] denotes 1000 µM amrinone significantly different from control in the absence of drug (p<0.05); n = 4 – 11 myocytes.

Figure 8. Inhibitory effects of amrinone on amplitudes of I_{Ca-L} were greater in normal compared to CM myocytes. Data are from experiments illustrated in figures 6 and 7. **Panel A** shows that the amplitudes and configuration of I-V relations determined with steps from -40 mV were similar in normal and CM myocytes in the absence of drug. **Panel B** shows that I-V relations determined from -60 mV in normal and CM myocytes were very similar. **Panel C and D** show mean I-V relations recorded in the presence of 1000 μM amrinone. Amrinone caused significantly greater inhibition of I_{Ca-L} in normal myocytes than in CM myocytes. * Denotes significantly different from normal ($p < 0.05$); n = 4 - 11 CM, and 9 - 11 normal myocytes.

Figure 9. Amrinone increases caffeine-releasable SR Ca^{2+} stores in normal hamster myocytes. Caffeine (10 mM) was applied for 4 seconds with a rapid solution switcher as shown in the protocol (top). **Panel A** shows a caffeine-induced contracture (top) and associated inward current (bottom) recorded in a normal myocyte in the absence of amrinone. **Panel B** shows a representative contracture and current from the same cell in the presence of 300 μM amrinone. **Panel C** shows mean amplitudes of caffeine-induced contractures and integrals of inward Na^+-Ca^{2+} exchange currents in the absence and presence of amrinone. Although amrinone did not affect mean amplitudes of contractures, it caused a significant increase in inward Na^+-Ca^{2+} exchange current. Denotes significantly different from control in the absence of drug ($p < 0.05$); n = 5 myocytes.

Figure 10. Amrinone had no effect on caffeine-releasable SR Ca^{2+} stores in CM hamster myocytes. Caffeine (10 mM) was applied for 4 seconds with a rapid solution switcher as shown in the protocol (top). **Panel A** shows a caffeine-induced contracture (top) and associated inward

current (bottom) recorded in a CM myocyte in the absence of amrinone. **Panel B** shows a

representative contracture and current from the same cell in the presence of 300 μM amrinone.

Panel C shows mean amplitudes of caffeine-induced contractures and integrals of inward Na^+-

Ca^{2+} exchange currents in the absence and presence of amrinone. Amrinone did not affect mean

amplitudes of contractures or the mean integrals of inward Na^+-Ca^{2+} exchange currents. n = 14

myocytes.

Figure 11. Minimal stimulation of adenylyl cyclase with forskolin restores the positive

inotropic effect of amrinone in CM myocytes. The voltage clamp protocol is shown at the top.

Panel A. Representative traces illustrate contraction (top) and current (bottom) recorded from a

CM myocyte in response to a depolarizing test step from -60 to 0 mV. **Panel B.** Contraction

was not affected by superfusion with buffer that contained 0.3 μM forskolin. Although in this

example the current appeared to decrease, the mean data indicate that forskolin did not change

inward current magnitude. **Panel C.** In the presence of 0.3 μM forskolin, amrinone (300 μM)

markedly increased contraction with little effect on inward current. **Panel D.** Mean contraction-

voltage curves demonstrate that amrinone plus forskolin significantly increased contraction in

CM myocytes. **Panel E.** Mean I-V relations demonstrate that neither forskolin, nor forskolin

plus amrinone significantly affected the magnitude of inward current in myocytes from CM

heart.   * Denotes significantly different from control in the absence of drug ($p < 0.05$); n = 5
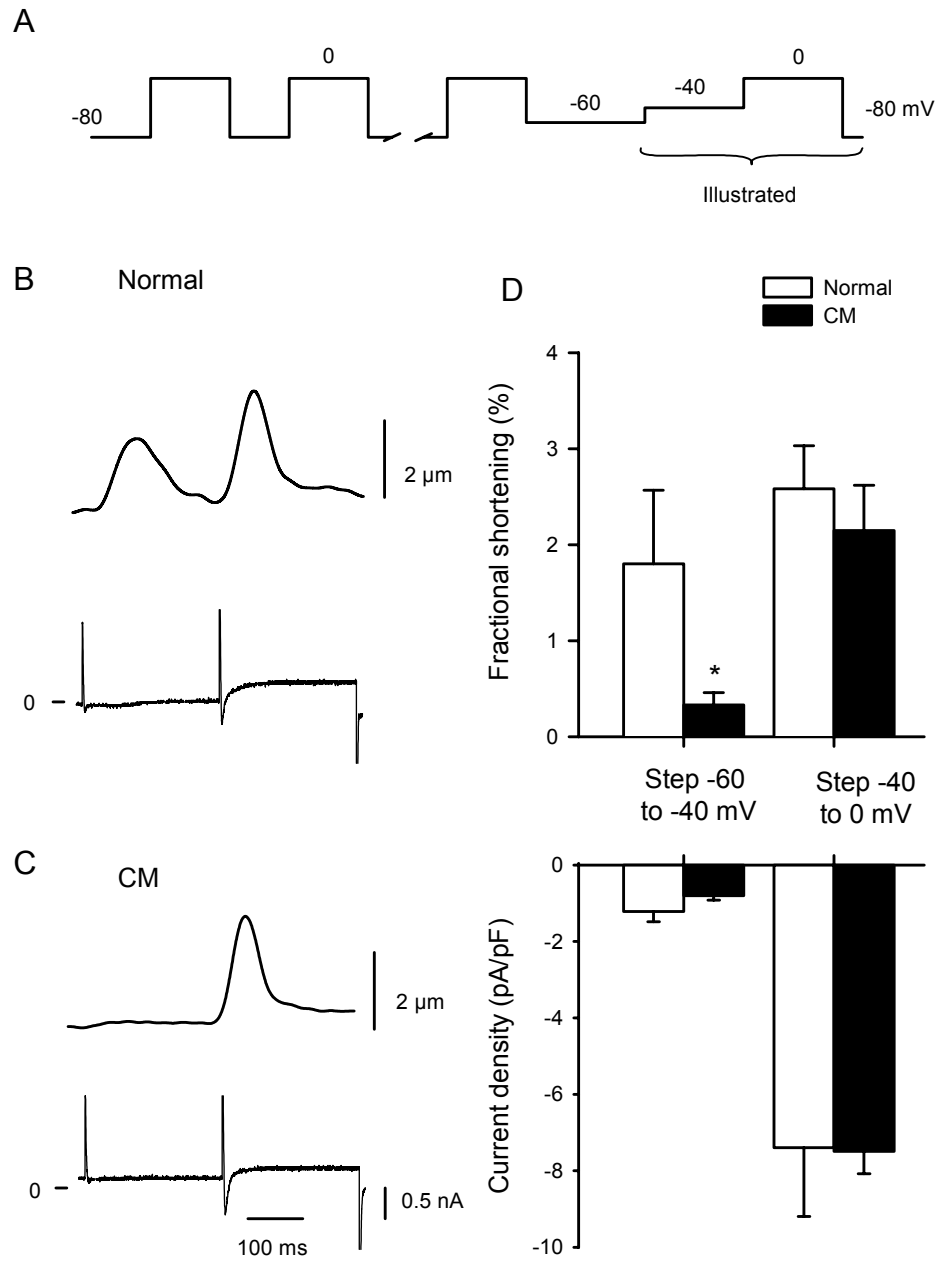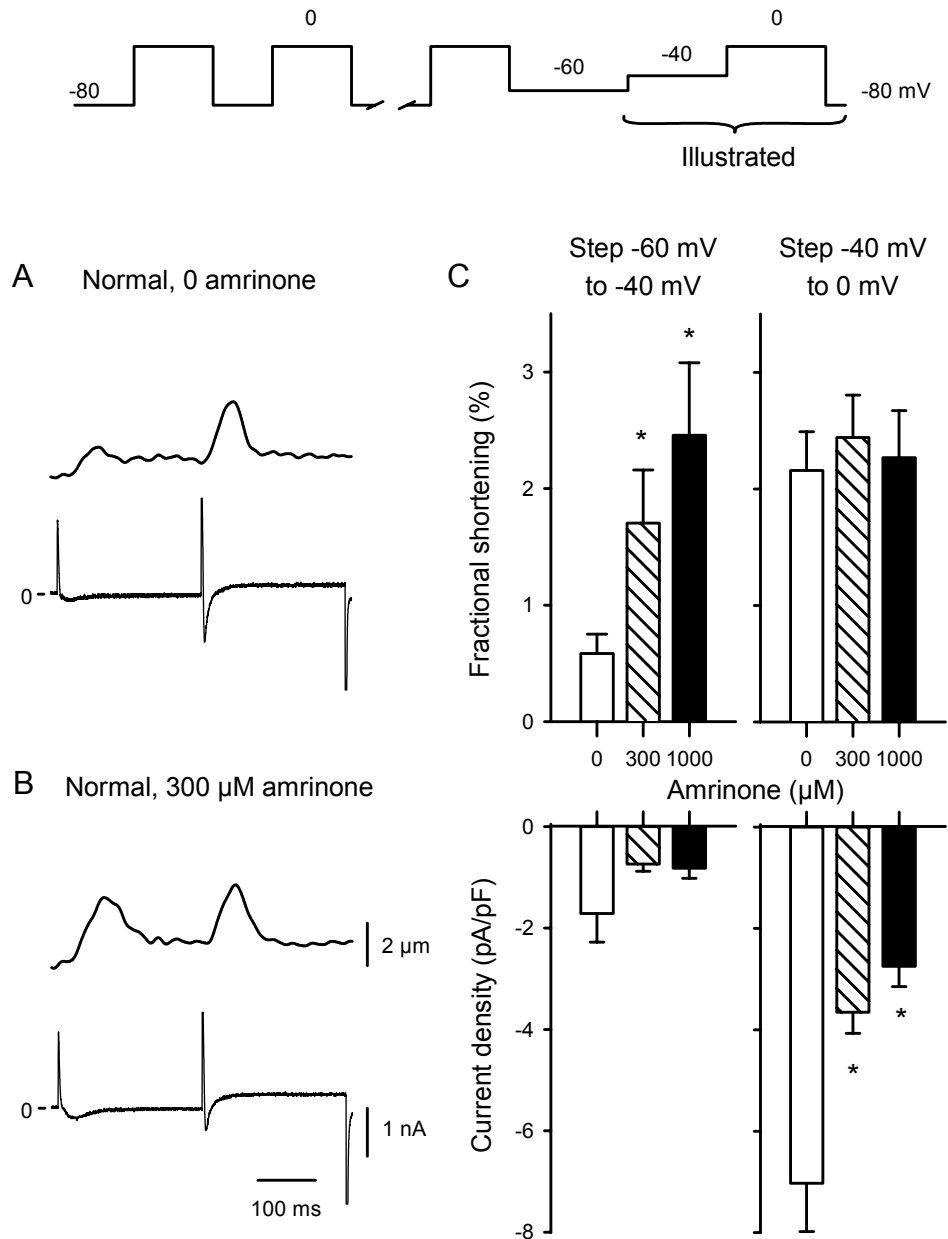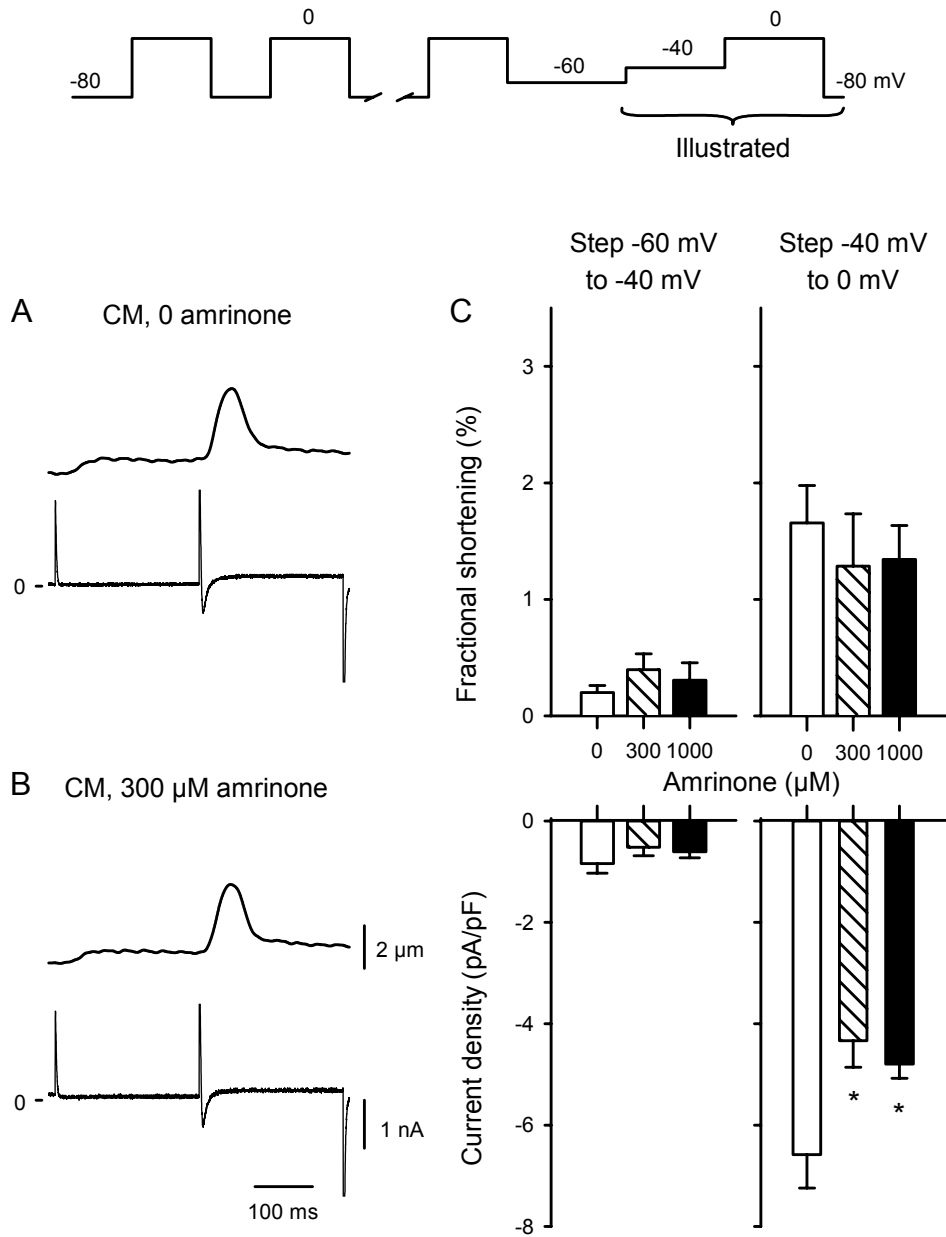
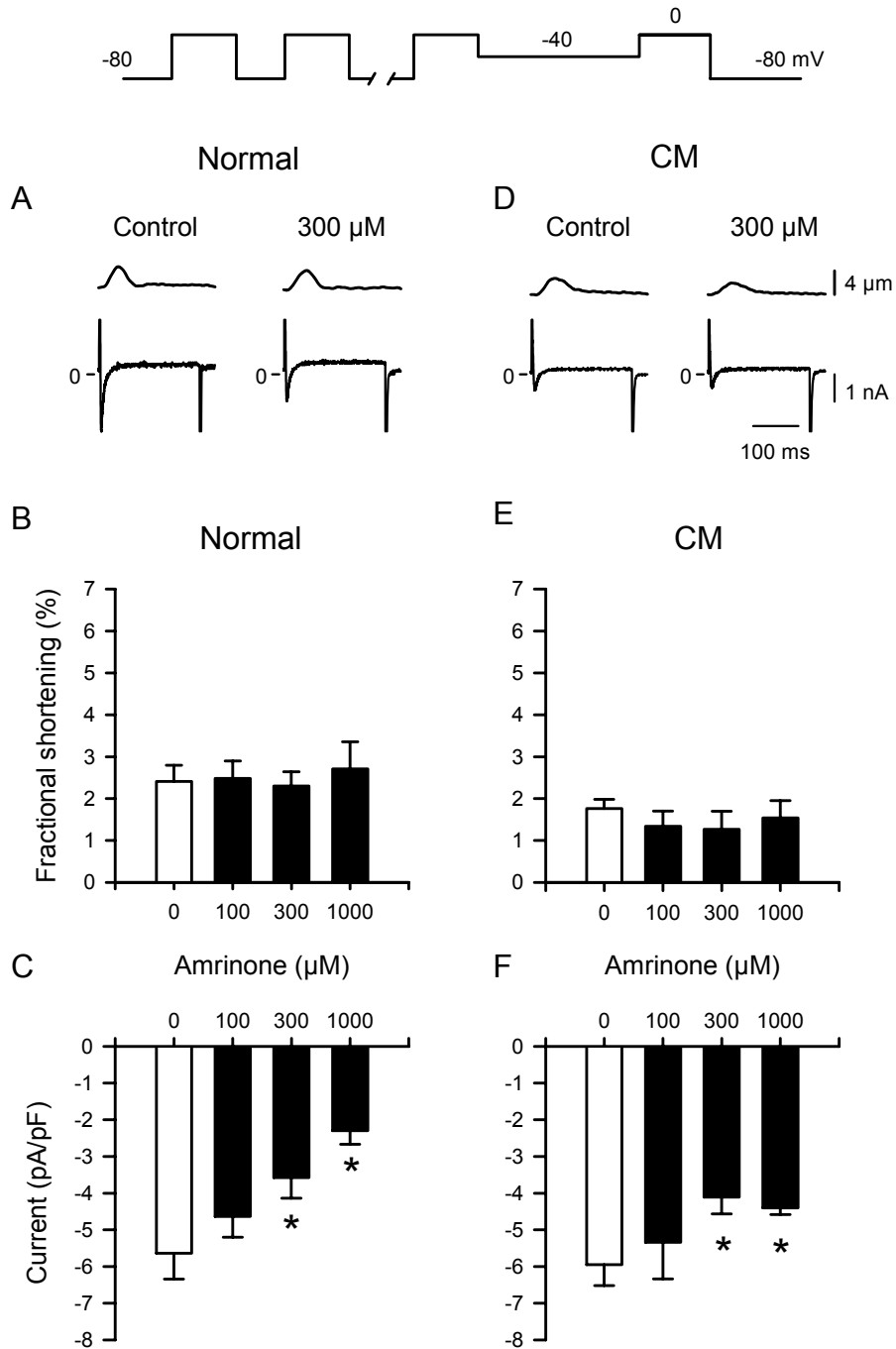myocytes.

Figure 1

Figure 2

Figure 3

Figure 4

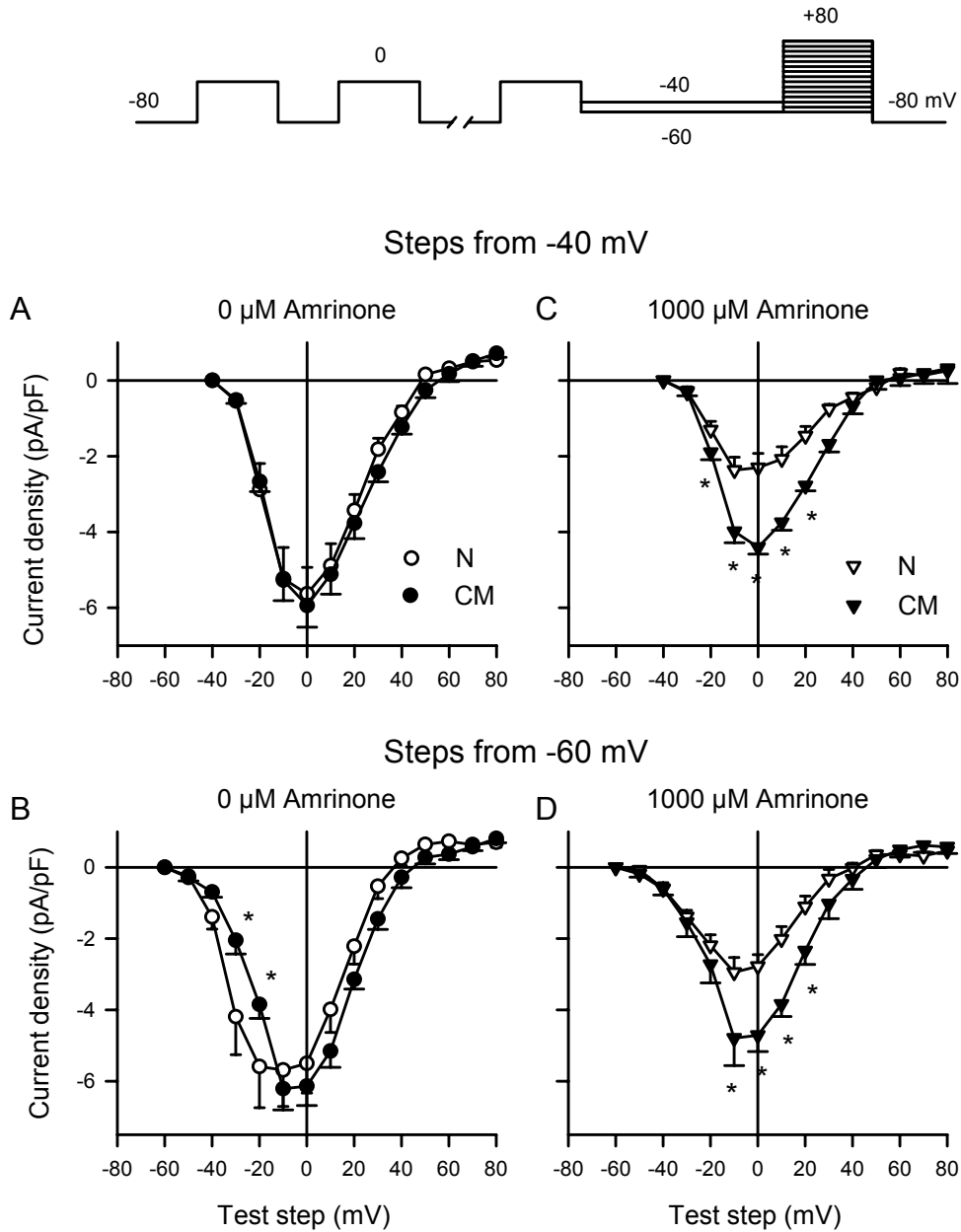# Figure 5
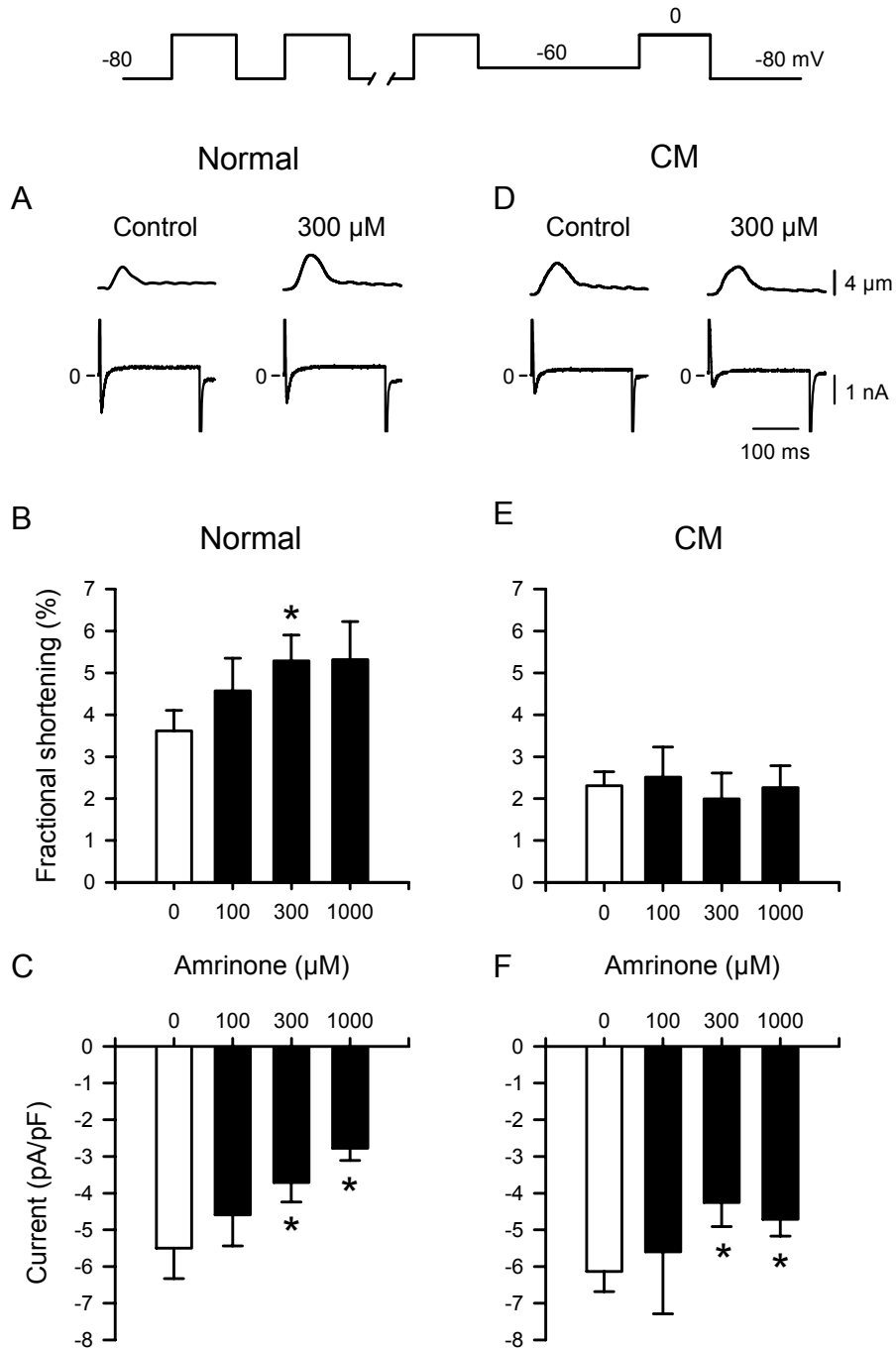
Figure 6

Figure 7

Figure 8

Steps from -40 mV

Steps from -60 mV
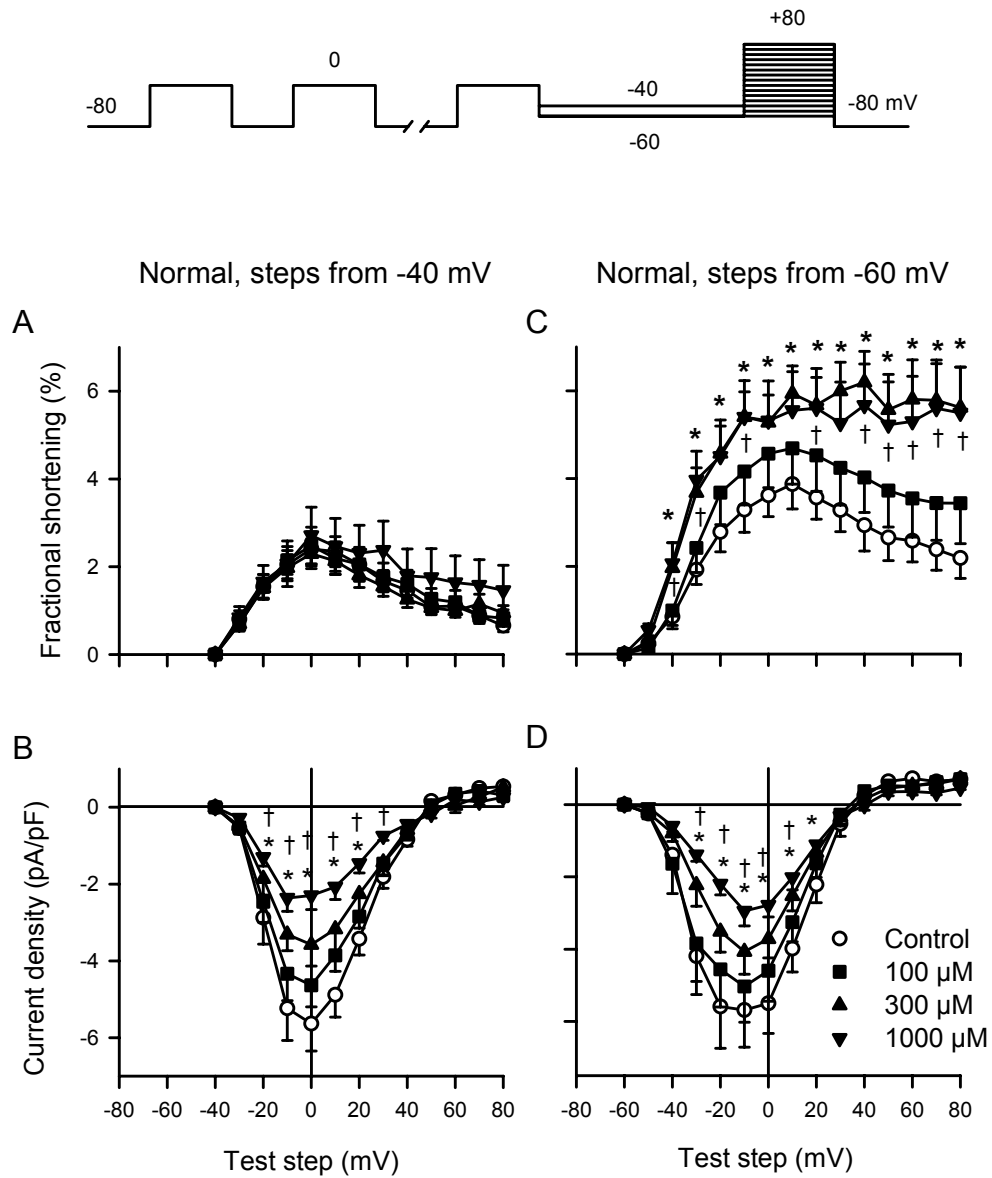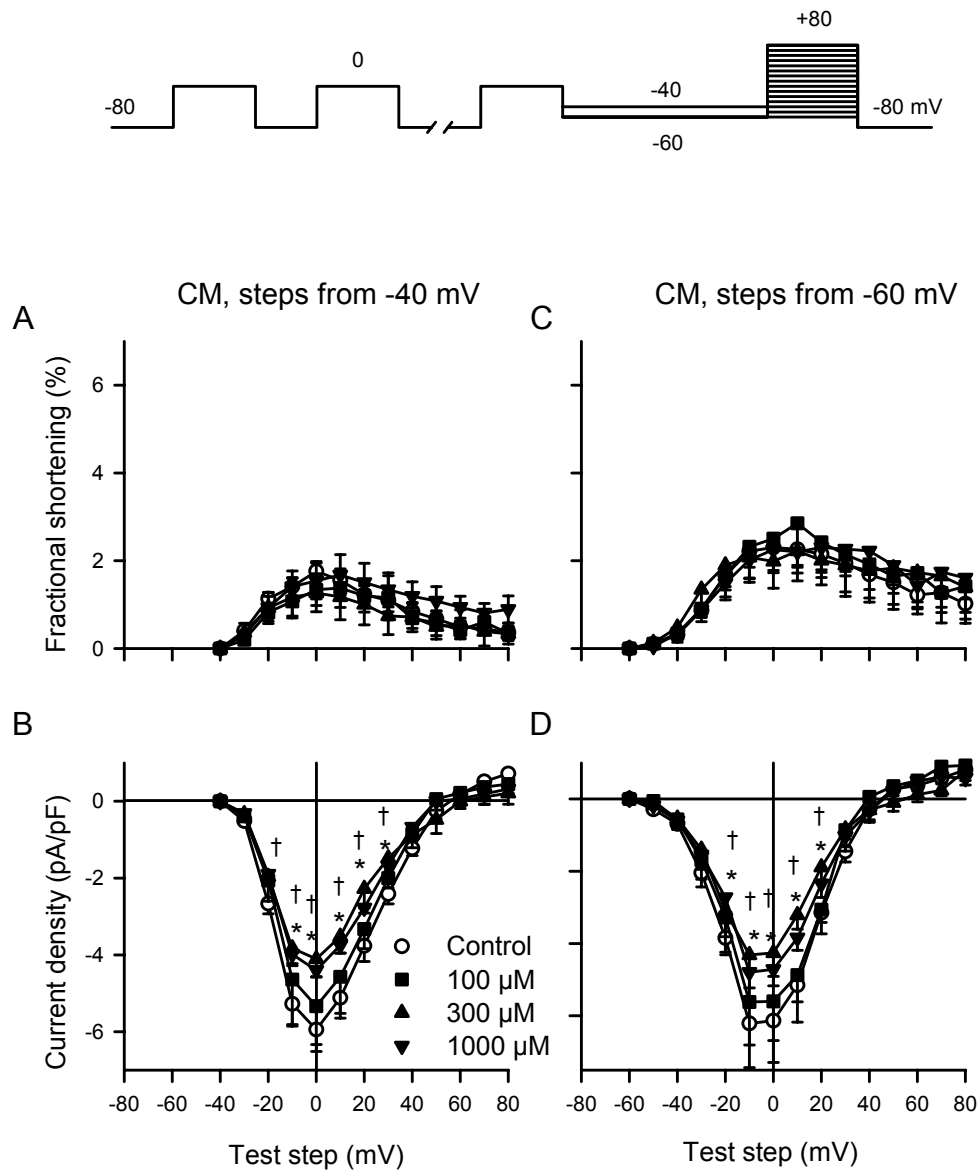
Figure 9

Figure 10

Figure 11