Myocardial Force Generation and Anoxia Tolerance in the Common Cockle, *Cerastoderma edule*

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Short running head: ANOXIA TOLERANCE OF THE COCKLE HEART

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ABSTRACT

The myocardium of molluscs exhibits profound anoxia tolerance, however the cellular mechanisms underlying heart performance during normoxia and anoxia are not well understood. In the present study we investigated the role of the sarcoplasmic reticulum (SR) during normoxia and chemical anoxia (2 mM sodium cyanide) in electrically paced ventricle preparations from the common cockle (*Cerastoderma edule*) at ~19°C. Acute anoxia caused a substantial increase in resting tension but did not significantly affect the force of contraction, rate of contraction or rate of relaxation in myocardial preparations. SR inhibition (ryanodine, 10 μM; thapsigargin, 2 μM) attenuated the increase in resting tension, and also caused a significant decrease in the force of contraction during anoxia. During normoxia, SR inhibition reduced the force and rate of contraction by 20-30 % at contraction frequencies of 0.2 Hz and 0.5 Hz. SR inhibition also elicited an increase in resting tension at 0.5 Hz. Our results suggest that the SR plays a role in maintaining cardiac performance during anoxia in cockle myocardium. Furthermore, the SR is operative during normoxia and is relatively more important in the cockle heart than in many ectothermic vertebrates. As efforts to understand the evolution of the SR are advanced, anoxia tolerant invertebrates may serve as valuable model organisms.

INTRODUCTION

Intertidal molluscs experience regular fluctuations in salinity, temperature and oxygen availability and thus serve as potential models for environmental stress tolerance. Upon exposure to air, bivalve molluscs cease ventilation and incur a progressive oxygen debt (Van Dam, 1935), which is met with a slowing of the heart (Helm and Trueman, 1967; Trueman, 1967). Accordingly, profound myocardial anoxia tolerance has been described in several species of molluscs, including whelks (*Busycon contrarium*) (Ellington, 1981) and Tapestry cockles (*Tapes watlingi*) (Jamieson and de Rome, 1979). Despite documented anoxia tolerance, the mechanisms underlying molluscan heart performance during both anoxia and normoxia are not well understood.

The sarcoplasmic reticulum (SR) is an intracellular calcium reservoir with a well-established role in excitation-contraction (E-C) coupling in mammals, birds and some high performance ectotherms (Bers, 2002; Shiels and Galli, 2014). During contraction, Ca^{2+} is released from the SR though ryanodine receptors (RyR) after being induced by Ca^{2+} entering the cell through L-type Ca^{2+} channels (Ca^{2+}-induced Ca^{2+} release (CICR)) (Fabiato, 1983; Fabiato and Fabiato, 1977). This cytosolic Ca^{2+} is pumped out of the cell by the sodium-calcium exchanger or taken up in the SR by the SR Ca^{2+} ATPase (SERCA) during relaxation (Bers, 2002). The role of the SR in the hearts of invertebrates is less well understood, however a combination of ultrastructural (Dykens and Mangum, 1979; Jensen and Tjonneland, 1977) and functional studies (Altimiras, Hove-Madsen and Gesser, 1999; Gesser, *et al*., 1997) have established a prominent role for the SR in cephalopods. Most notably, Gesser and colleagues (1997) demonstrated that ryanodine, which blocks SR calcium release, markedly reduces twitch force (>75%) in octopus (*Octopus vulgaris*) myocardial preparations. This inhibition is of a similar magnitude to that observed in rat ventricles (Bers, 1985), however the hearts of most teleost fish are relatively insensitive to ryanodine (Driedzic and Gesser, 1988; Møller-Nielsen and Gesser, 1992; Tibbits, Hove-Madsen and Bers, 1991). Morphological studies have identified the SR in the hearts of other molluscs (Sanger, 1979; Watts *et al*., 1981) and ryanodine decreases the rate of relaxation in the Atlantic surf clam (*Spisula solidissima*) ventricle (Collis, Sun and Hill, 2006). However, the general importance of the SR in myocardial calcium handling in molluscs remains unclear.

The SR also has a lesser-appreciated role in maintaining long-term calcium homeostasis

during oxygen deprivation. In a study of isolated rat cardiomyocytes, Allshire *et al*. (1987) showed that intracellular Ca^{2+} concentration increases during anoxia, but this is reversible providing that the concentration does not become too high. However, in the presence of caffeine, which causes Ca^{2+} to leak out of the SR, cardiomyocytes could not recover from the anoxia-induced calcium overload (Allshire, *et al*., 1987). Further, in a study on isometric ventricular tissue preparations from armoured catfish (*Pterygoplichthys pardalis*), MacCormack *et al*. (2003) showed that SR inhibition exacerbated the loss of contractile force during anoxia (MacCormack, *et al*., 2003). The role of the SR during environmental stress clearly warrants further investigation, as it is possible that the SR contributes to anoxia tolerance in the hearts of bivalves and other molluscs.

The aim of the present study was to investigate the effect of chemical anoxia (2 mM sodium cyanide) on myocardial preparations from the common cockle (*Cerastoderma edule*), with particular emphasis on the importance of SR calcium cycling. We further investigated the role of the SR during normoxia at two physiologically relevant pacing frequencies (Trueman, 1967). Compared to cephalopods, the cockle is a relatively sedentary mollusc, thus we hypothesised the SR would be less well developed than in octopuses (Gesser, *et al*., 1997), in accordance with the pattern in ectothermic vertebrates (Galli and Shiels, 2012; Shiels and Galli, 2014).

MATERIALS AND METHODS

Animals

Sixteen common cockles (*Cerastoderma edule*), weighing 21.62 ± 10.09 g (mean ± SD), were collected from Fairhaven, Isle of Cumbrae and maintained at the nearby Millport Marine Station in filtered sea water for up to five days.

Myocardial Preparations

Cockles were prised open and the major ganglia were severed as the heart was rapidly excised. The entire ventricle was dissected and served as a single preparation. Preparations were hung vertically between two clips, the uppermost of which was attached to a force

transducer, whilst the other provided a stable anchor. The force transducers were attached to an amplifier (Transbridge 4M; World Precision Instruments), A/D converter (LT4/16-S; World Precision Instruments) and then attached to a computer running DataTrax acquisition software (World Precision Instruments). The preparations were then lowered into organ baths containing artificial sea water of the following mM concentrations: NaCl, 432; KCl, 9.1; $MgCl_2$, 23.4; $MgSO_4$, 25.6; $CaCl_2$, 9.2; $NaHCO_3$, 2.19; glucose, 5 (following Gesser, *et al.*, 1997). All organ baths were bubbled with compressed air and experiments took place at room temperature (19±1 °C). Preparations were left to stabilize for 20 minutes before stimulation begun. A Grass SD9 electrical stimulator was attached to two electrodes either side of the preparations and provided 10 ms pulses at 90-100 V. Preparations were initially stimulated at 0.2 Hz, which is in accordance with previous studies on mollusc myocardium (Driedzic, 1985; Gesser *et al.*, 1997). After 10 minutes of pacing, myocardial preparations were stretched with a micrometer screw until the maximum force of contraction was attained. After the preparations had stabilized at peak isometric force, the experimental protocol commenced.

Anoxia

5-10 minutes before anoxia, the solution was changed to fresh artificial sea water, and the percentage changes in force of contraction, rate of contraction, rate of 50 % relaxation and resting tension after five minutes were recorded to provide a control condition. Chemical anoxia was then implemented by changing the solution to 2 mM sodium cyanide dissolved in artificial sea water. The change in force of contraction, rate of contraction, rate of 50 % relaxation and resting tension, as a percentage of that immediately before the cyanide solution change, was recorded five minutes after cyanide treatment. The five-minute interval was chosen based on previous studies demonstrating that cyanide rapidly ceases oxidative phosphorylation in the mollusc heart (Jamieson and de Rome, 1979). This protocol was repeated with separate preparations that had undergone 30 minutes of SR inhibition (ryanodine (10 μM) and thapsigargin (2 μM)), in which case the cyanide solution also contained ryanodine and thapsigargin in the same respective concentrations.

SR Inhibition

To ascertain the effect of sarcoplasmic reticulum inhibition, myocardial preparations underwent force-frequency trials (an increase in stimulation frequency from 0.2 Hz to 0.5 Hz) before and after incubation for 20 minutes with ryanodine (10 μM) and thapsigargin (2 μM), which are specific blockers of RyR and SERCA, respectively (Rousseau, Smith and Meissner, 1987; Sagara and Inesi, 1991). To account for time-related decrease in contractility over the 25-minute experiment, control preparations (n=4) were run separately, with the same changes in solution and frequency, but no SR inhibition (Shiels and Farrell, 1997). Deterioration in experimental preparations was accounted for by normalizing the average percentage changes in experimental preparations relative to controls at both 0.2 Hz and 0.5 Hz. On average, 'fatigue' caused resting tension to fall to 92.59 (± 7.90) %, force of contraction 90.67 (± 5.48) %, rate of contraction 84.58 (± 11.37) % and rate of relaxation 88.00 (± 8.59) % of initial values during the course of the experiment.

Statistical Analysis

During analysis, resting tension at the start of the experiment was normalized to 10 mN to mitigate for exaggerated relative changes. Paired t-tests were used to investigate percentage changes in force of contraction, rate of contraction, rate of 50 % relaxation and resting tension following control solution changes and cyanide solution changes. Unpaired t-tests were then used to investigate the percentage changes in all four variables elicited by cyanide in the presence or absence of SR inhibition. A regression analysis was performed to investigate the relationship between the change in resting tension and the change in force following cyanide treatment. A repeated measures two-way analysis of variance (ANOVA) was used to investigate the effect of SR inhibition on preparations paced at 0.2 Hz and 0.5 Hz. A significance level of $p<0.05$ was set and all values are presented as mean ± SE.

RESULTS

Anoxia

Original and representative traces showing change in resting and peak tension after cyanide treatment with and without SR inhibition are presented in Figure 1. In control conditions resting tension and force of contraction did not change after solution change. Application of 2 mM sodium cyanide caused mean resting tension to significantly increase (t=4.52,

P=0.0014), although no significant changes were elicited in the force of contraction (t=1.09, P=0.30), rate of contraction (t=0.73, P=0.48) or rate of 50% relaxation (t=1.25, P=0.24) (Fig. 1 and 2). However, in preparations in which the SR was inhibited, both the mean force (t=2.43, P=0.029) and rate of contraction (t=2.21, P=0.045) decreased significantly more than in the non-SR inhibited condition (Fig. 1 and 3). A regression analysis further revealed a significant positive relationship (r^2=0.32; P=0.02) between the relative change in resting tension and the change in force during anoxia when data from SR-inhibited and non-SR inhibited preparations were pooled (Fig. 4).

SR Inhibition

At both 0.2 Hz and 0.5 Hz, SR inhibition significantly attenuated relative twitch force by 20-30% (Fig. 5A). When preparations were paced at 0.2 Hz, there was no statistically significant difference in the baseline resting tension before and after SR inhibition (Fig. 5B). However, when stimulation frequency was increased to 0.5 Hz, SR inhibition led to a relatively larger increase in resting tension (Fig. 5B). SR inhibition significantly depressed the rate of contraction at both 0.2 Hz and 0.5 Hz (Fig. 5C). There were no statistically resolvable differences in the rate of relaxation before and after SR inhibition, although there was a trend towards slower rates of relaxation following SR inhibition, particularly at 0.5 Hz (Fig. 5D).

DISCUSSION

Sarcoplasmic Reticulum

In the cockle ventricle, the SR is necessary both to reduce diastolic calcium levels and to maintain contraction, as evidenced by the increase in resting tension (Fig. 5B) and decrease in force of contraction (Fig. 5A), respectively, following SR inhibition. Although its relative contribution to force production is minor (20-30 %), the SR is clearly operative in the cockle myocardium, thus corroborating early ultrastructural studies on molluscan hearts (Sanger, 1979; Watts, *et al.*, 1981). The SR appears to play a greater role in the cockle heart than in many ectothermic vertebrates, for example reptiles (Galli, *et al.*, 2006) and many teleost fish (Driedzic and Gesser, 1988; Møller-Nielsen and Gesser, 1992; Tibbits, *et al.*, 1991), where

234 the contribution is negligible. In fact, the role is comparable to that of some endotherms, such
235 as guinea pig ventricle (Agata, Tanaka and Shigenobu, 1994). As hypothesised, the SR plays
236 a less dominant role in cockle myocardium than in the octopus (Gesser, *et al*., 1997), which is
237 likely due to the higher aerobic demands, and therefore cardiovascular capacity, of the
238 cephalopod. As evolutionary explanations for the function of the SR are advanced (Shiels and
239 Galli, 2014) it is clearly important to consider invertebrates, which have received
240 comparatively little attention.
241
242 *Anoxia*
243
244 Cyanide treatment caused a large increase in resting tension (Fig. 2A) but did not affect the
245 force of contraction, rate of contraction or rate of relaxation (Fig. 2B-D) in cockle ventricular
246 myocardium. In similar heart preparations from another bivalve, *Tapes watlingi*, the force of
247 contraction fell by up to 60 %, with the maximum decrease occurring seven minutes after
248 cyanide treatment (Jamieson and de Rome, 1979). Cyanide also depressed contraction in
249 cephalopod systemic ventricles after five minutes, although the decrease in contractility
250 continued following longer exposure (up to 30 minutes) (Driedzic, 1985). In perfused whelk
251 hearts, severe hypoxia had minimal effects on contraction amplitude for up to two hours
252 (Ellington, 1981). Although we only sought to investigate the effect of acute anoxia (five
253 minutes), it appears that the common cockle is as tolerant, if not more tolerant to oxygen
254 deprivation than other molluscs.
255
256 The greatest manifestation of anoxia was the rise in resting tension (Fig. 1 and 2B). This
257 implies that exudation of calcium during diastole may be compromised when oxygen is
258 limited. In the working heart, increased resting calcium levels may result in a decreased end-
259 diastolic volume, thereby reducing stroke volume during hypoxia (Gesser and Overgaard,
260 2009). Thus, an increase in resting tension is often regarded as a negative outcome that is
261 characteristic of anoxia-sensitive species (e.g. Bailey, *et al*., 1999). However, it is also
262 possible that the increase in resting levels of calcium represents a beneficial response to
263 anoxia, at least in the cockle. During oxygen deprivation, a build-up of inorganic phosphates
264 renders myofilaments less sensitive to calcium (Driedzic and Gesser, 1994; Jensen and
265 Gesser, 1999; Kentish, 1986). Further, increasing extracellular calcium concentration
266 ameliorates the negative inotropic effect of anoxia in the myocardium of ectothermic

vertebrates (Nielsen and Gesser, 1983; Nielsen and Gesser, 1984; Overgaard, Gesser and Wang, 2007; Overgaard, *et al*., 2005). Cyanide caused no apparent increase in resting tension in *Tapes watlingi*, whilst the force of contraction clearly subsided (Jamieson and de Rome, 1979). This suggests that the greater contractile performance during anoxia in cockles may go hand in hand with the increased diastolic calcium, which is strongly supported by the positive relationship observed between the change in resting tension and the change in the force of contraction following cyanide treatment (Fig. 4). The generality of this notion, however, requires further investigation. For example, the relationship between tension and calcium concentration deserves special consideration. The binding of calcium to troponin is co-operative, resulting in a sigmoidal force-calcium relationship (Sun and Irving, 2010; Sun, Lou and Irving, 2009). This means that a small change in calcium concentration may elicit small or large changes in force, depending on the initial calcium concentration. Further, the force-calcium relationship varies between species and is highly temperature sensitive (Harrison and Bers, 1990). As a result, the consequence of an increased intracellular calcium concentration during anoxia may vary greatly between species in different environmental conditions.

In SR-inhibited preparations, resting tension tended to increase less and the force of contraction fell significantly more than in uninhibited myocardium (Fig. 3). This suggests that the SR may be responsible for the increased diastolic calcium levels and remarkable contractile performance during anoxia. Indeed, in mammalian preparations it has previously been shown that cyanide increases calcium release from the SR and/or mitochondria (Jundt, *et al*., 1975), and further, calcium release from the SR is essential for the recovery of force production during acidosis (Orchard, 1987). This supports our hypothesis that the SR is vital during anoxia in the cockle, and also lends support to the developing view that the SR is a key regulator of calcium homeostasis during environmental stress (Galli and Shiels, 2012). Thus it appears that the SR may be more dynamic and plastic than has hitherto been appreciated.

Conclusion

Intertidal molluscs represent an inexpensive and often accessible model for myocardial anoxia tolerance. Here, we have demonstrated that SR calcium cycling appears fundamental

in maintaining performance during both anoxia and normoxia in the heart of the common cockle. Whilst much of our understanding of myocardial calcium handling is based on studies in vertebrates, invertebrates such as the cockle may offer novel perspectives on the evolution of the heart.

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Figure Legends

Figure 1. Representative traces from separate cockle myocardial preparations demonstrating the change in resting tension and contractile force production over time in three different treatments. Initial increase in force is due to solution change (arrow). Resting tension was normalized at 0 for all traces to facilitate the comparison.

Figure 2. Relative changes in twitch force (A), resting tension (B), rate of contraction (C) and rate of 50% relaxation (D) in cockle myocardial preparations (n=10) five minutes after a control solution change (control; grey bars) and five minutes after the addition of a solution containing 2mM sodium cyanide (cyanide; black bars). Positive values indicate an increase and negative values indicate a reduction in each parameter measured. Asterisks denote significant differences between control and cyanide solution changes as evaluated by paired t-tests. Values are means ± SEM.

Figure 3. Relative changes in twitch force (A), resting tension (B), rate of contraction (C) and rate of 50% relaxation (D) in cockle myocardial preparations in the absence (n=10) or presence of sarcoplasmic reticulum inhibition (n=6) five minutes after the addition of a solution containing 2mM sodium cyanide (cyanide; black bars). Positive values indicate an increase and negative values indicate a reduction in each parameter measured. Asterisks denote significant differences between non-inhibited and SR-inhibited preparations as evaluated by unpaired t-tests. Values are means ± SEM.

Figure 4. The relationship between the relative changes in resting tension and force of contraction in cockle myocardium following sodium cyanide (2mM) treatment. Non-SR inhibited preparations are black dots (n=10), SR inhibited preparations are represented by white dots (n=6). A regression analysis on the pooled data revealed a significant positive relationship between the variables (r^2=0.32; P=0.02).

Figure 5. The effect of SR inhibition (ryanodine, 10 μM; thapsigargin, 2 μM) on resting tension (A), force production (B), rate of contraction (C) and rate of relaxation (D) in cockle heart preparations paced at 0.2 Hz or 0.5 Hz (n=6). All data are represented as relative changes from the initial value at 0.2 Hz normalized to 100 %. Asterisks denote significant

differences between control and SR inhibited preparations ($p<0.05$). Values are means ± SEM.

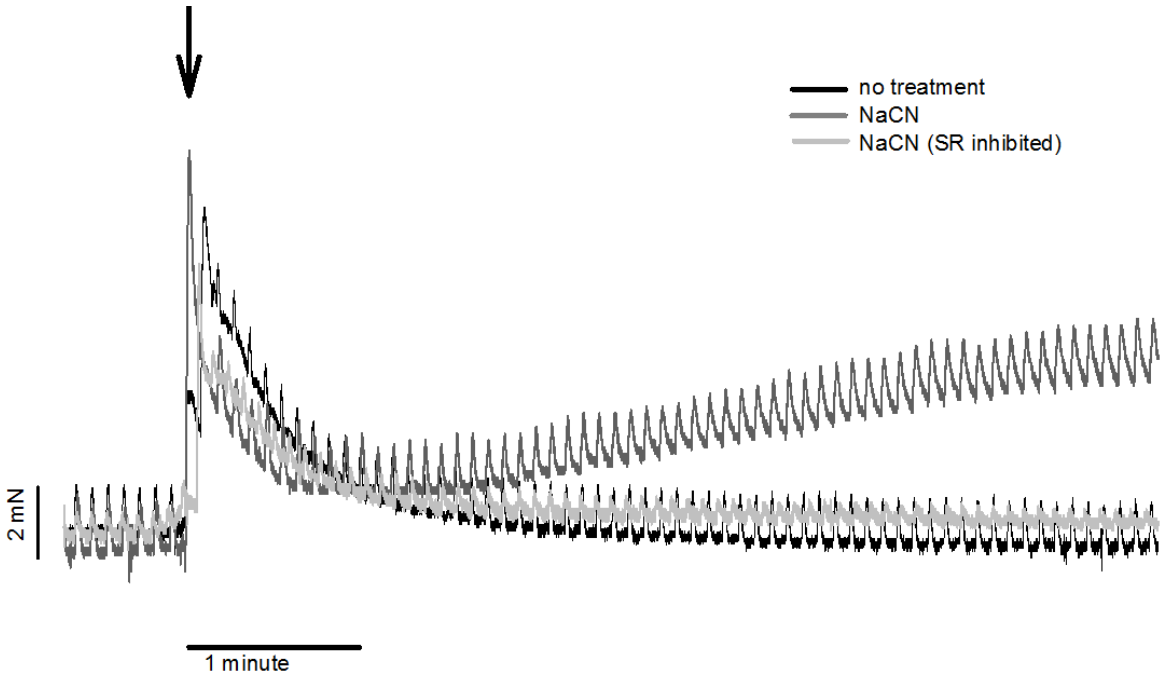
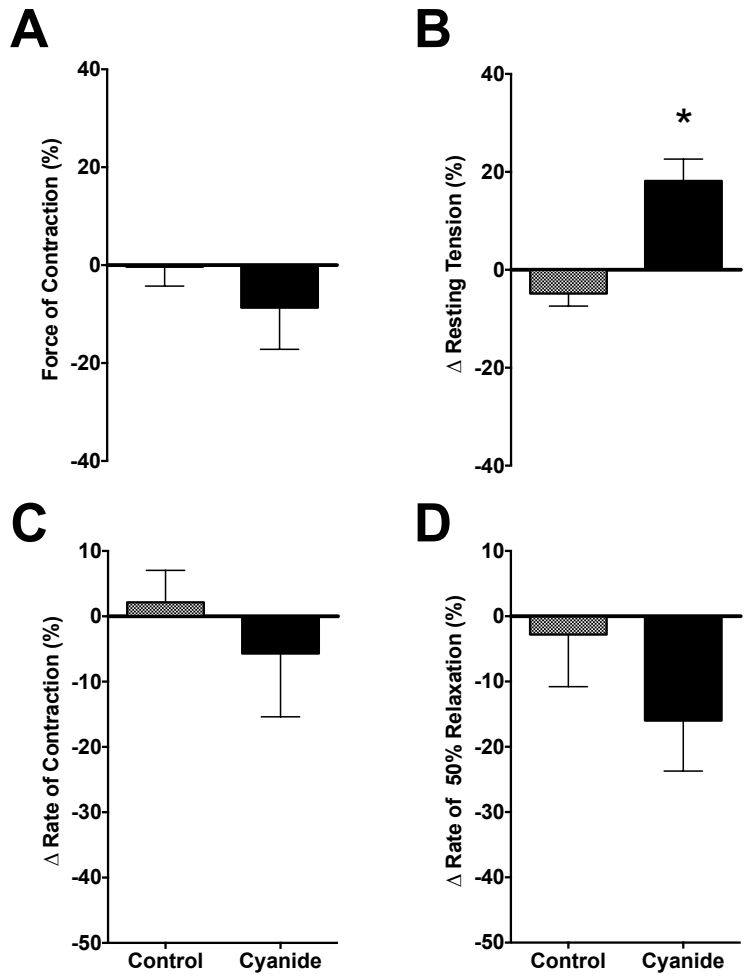


Figure 1.



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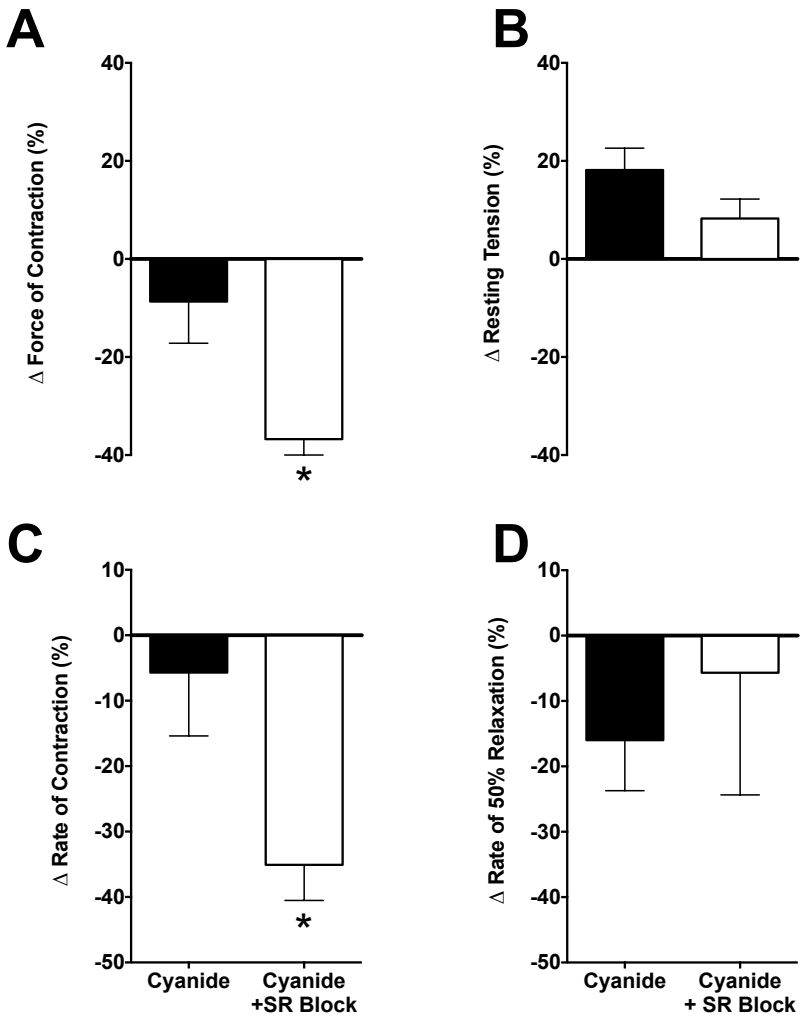
594 **Figure 2.**



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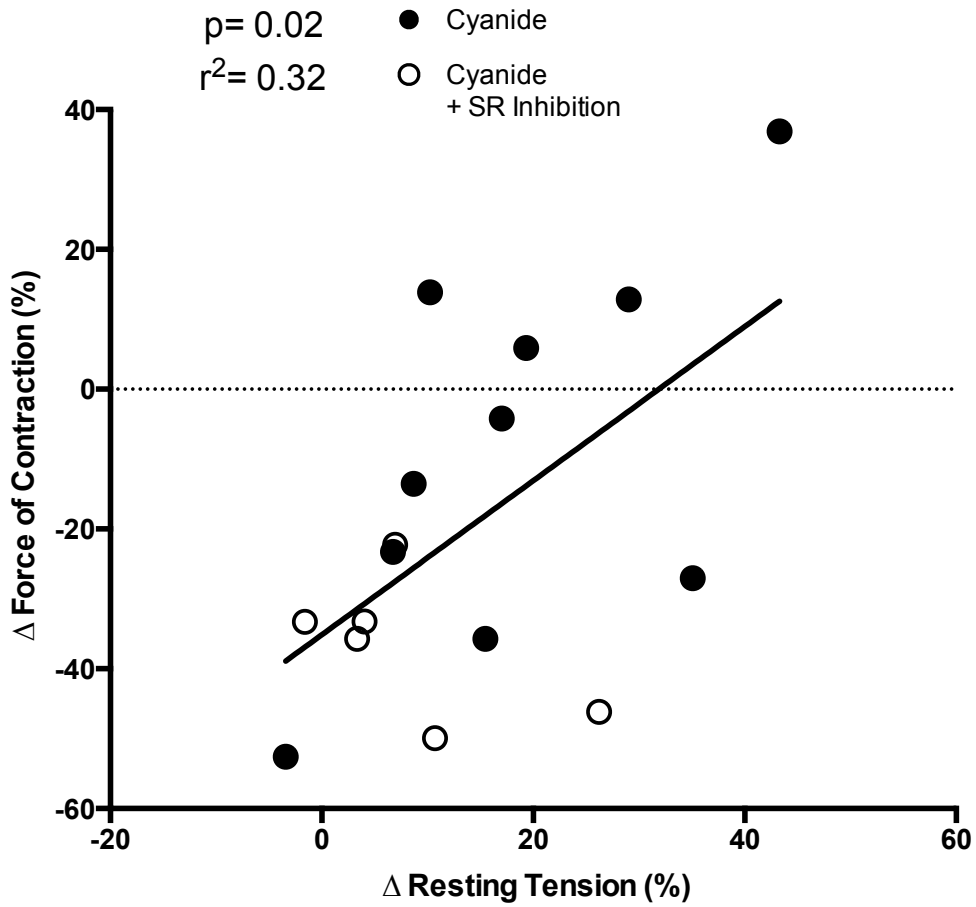

Figure 4.

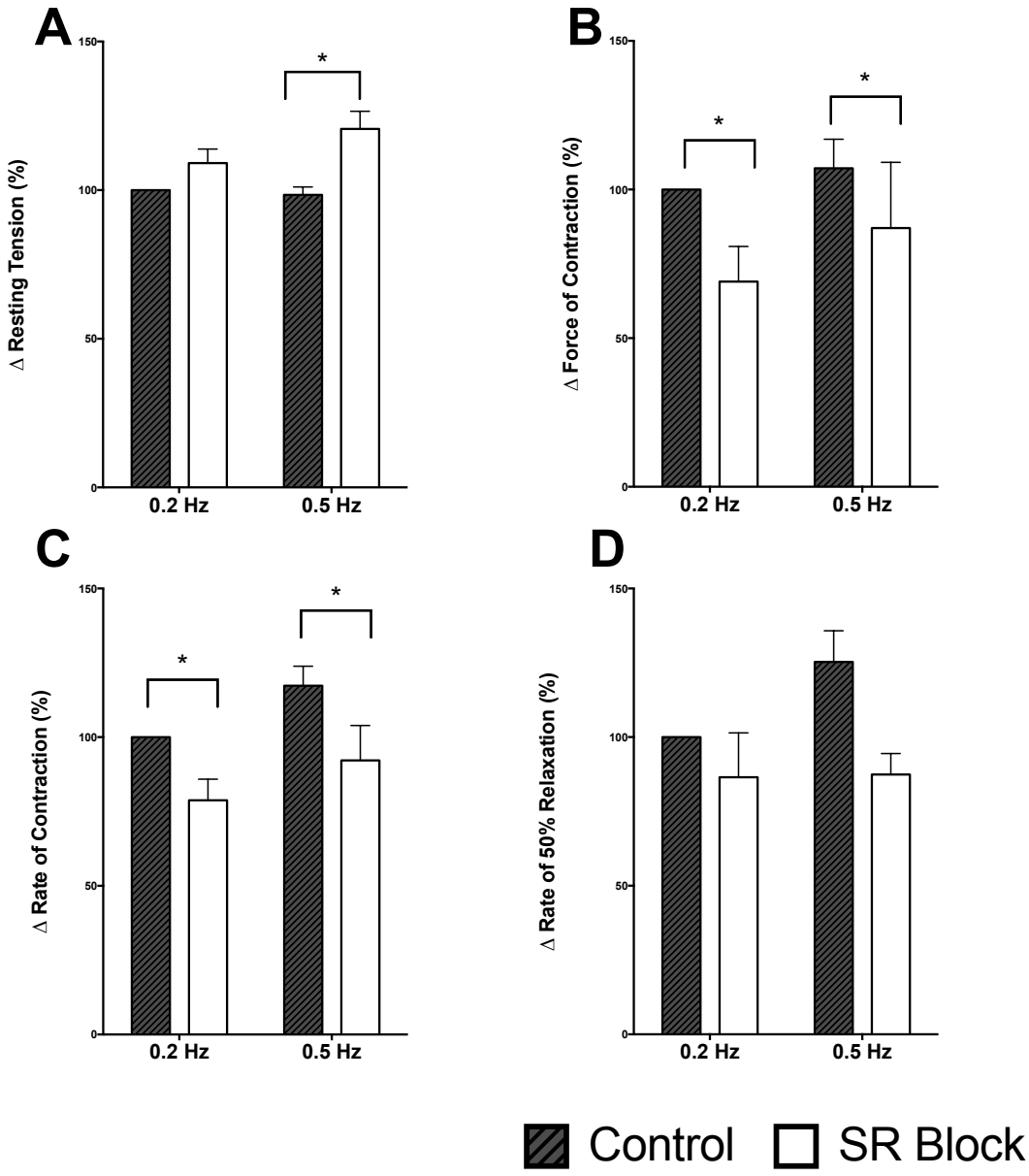

Figure 5.