

RESEARCH ARTICLE

The effects of pH and P_i on tension and Ca^{2+} sensitivity of ventricular myofilaments from the anoxia-tolerant painted turtle

Cornelia E. Fanter¹, Kenneth S. Campbell² and Daniel E. Warren^{1,*}**ABSTRACT**

We aimed to determine how increases in intracellular H^+ and inorganic phosphate (P_i) to levels observed during anoxic submergence affect contractility in ventricular muscle of the anoxia-tolerant Western painted turtle, *Chrysemys picta bellii*. Skinned multicellular preparations were exposed to six treatments with physiologically relevant levels of pH (7.4, 7.0, 6.6) and P_i (3 and 8 $mmol\ l^{-1}$). Each preparation was tested in a range of calcium concentrations (pCa 9.0–4.5) to determine the pCa–tension relationship for each treatment. Acidosis significantly decreased contractility by decreasing Ca^{2+} sensitivity (pCa₅₀) and tension development ($P < 0.001$). Increasing [P_i] also decreased contractility by decreasing tension development at every pH level ($P < 0.001$) but, alone, did not affect Ca^{2+} sensitivity ($P = 0.689$). Simultaneous increases in [H^+] and [P_i] interacted to attenuate the decreased tension development and Ca^{2+} sensitivity ($P < 0.001$), possibly reflecting a decreased sensitivity to P_i when it is present as the dihydrogen phosphate form, which increases as pH decreases. Compared with that of mammals, the ventricle of turtles exhibits higher Ca^{2+} sensitivity, which is consistent with previous studies of ectothermic vertebrates.

KEY WORDS: Acidosis, Calcium, Contractility, Force development, Inorganic phosphate, Reptile

INTRODUCTION

The western painted turtle, *Chrysemys picta bellii*, is the most anoxia-tolerant tetrapod known, having been shown to survive submergence in anoxic water for more than 170 days at 3°C (Ultsch and Jackson, 1982). During anoxia, the turtle heart maintains a reduced level of cardiac function (Farrell et al., 1994; Herbert and Jackson 1985; Hicks and Farrell, 2000; Hicks and Wang, 1998; Jackson, 1987), in contrast to the mammalian heart, which shows rapid and complete cardiac failure (Boutilier, 2001; Gesser and Jorgensen, 1982; Orchard and Kentish, 1990). Functional suppression in turtles is achieved by minimizing heart rate and contractility, allowing for continued function under conditions unfavorable for high rates of ATP synthesis (Shi et al., 1999; Stecyk et al., 2004; Wasser et al., 1990a,b). Avoiding complete circulatory arrest, however, is necessary to ensure adequate convective transport of glucose from the liver to the periphery and transport of lactic acid to the shell for buffering (Jackson, 1997; Jackson and Heisler, 1982; Jackson et al., 1996; Keiver and Hochachka, 1991; Keiver et al., 1992).

The mechanism of anoxia-induced contractile suppression in turtles is thought to be primarily intrinsic, especially at cooler temperatures, and to involve changes in the sarcoplasm that directly affect myofilament function (Jackson et al., 1995; Stecyk et al., 2009). For example, during anoxia, intracellular lactic acidosis results in a large decrease in intracellular pH (pH_i) of the turtle heart (Jackson, 2002; Jackson and Heisler, 1983; Warren and Jackson, 2007). Although the effects of acidosis on ventricular function have been characterized at both the organ and tissue level in painted turtles (Jackson et al., 1995; Shi and Jackson, 1997; Wasser et al., 1990b), the direct effects of pH_i on myofilament function have yet to be characterized.

Even less well understood is how myofilament function is affected by intracellular inorganic phosphate (P_i), which increases during anoxia (Stecyk et al., 2009; Wasser et al., 1990b). In painted turtle hearts, intracellular [P_i] increases by 65% during 4 h of anoxia, suggesting rapid [P_i] increases may also have a role in immediately decreasing contractility (Wasser et al., 1990b). Atrial myofilaments from the relatively anoxia-tolerant slider turtle, *Trachemys scripta*, showed a 47% decrease in maximal force development as well as reduced Ca^{2+} sensitivity with a 6 $mmol\ l^{-1}$ increase in P_i , indicating that [P_i] may play a role in suppressing ventricular function during anoxia in turtles (Jensen and Gesser, 1999). Furthermore, in mammals, skinned ventricular myocytes showed decreased contractile force and calcium sensitivity when exposed to increasing [P_i] (Kentish, 1986). In the heart of slider turtles, intracellular [P_i] increases during anoxia from 3.2 to 7.7 $\mu mol\ g^{-1}$ at 21°C and from 4.1 to 8.8 $\mu mol\ g^{-1}$ at 5°C, suggesting that elevated P_i may account for some of the functional suppression observed during anoxia (Stecyk et al., 2009). The direct effect that this physiologically relevant increase in [P_i] might have on myofilament function in turtle ventricle has yet to be determined.

In the present study, we investigated how physiologically relevant changes in pH and P_i affect intracellular cardiac mechanics in the ventricle of the anoxia-tolerant painted turtle. By skinning multicellular preparations, we controlled intracellular conditions, allowing for isolated measurements of myofilament tension development. We simultaneously varied pH (6.6, 7.0 and 7.4) and P_i concentrations (3 and 8 $mmol\ l^{-1}$) across levels known to exist in the hearts of anoxic turtles while quantifying their effects on myofilament calcium sensitivity (pCa₅₀) and force production. We also compared our results with similar studies in other vertebrates to determine how these properties vary across different taxa.

MATERIALS AND METHODS**Animals**

Western painted turtles, *Chrysemys picta bellii* (Gray 1831) (both sexes, 174–211 g, purchased from Niles Biological, Sacramento, CA, USA) were housed in fiberglass aquaria filled with dechlorinated, St Louis (MO, USA) municipal water (18–22°C) with access to basking platforms bathed with 10 W UV and 90 W incandescent heating lights that followed a weekly updated, natural,

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List of abbreviations

$[Ca^{2+}_{50}]$	free Ca^{2+} concentration required to produce 50% of maximum Ca^{2+} activated tension
E–C coupling	excitation–contraction coupling
l_0	isometric length
n	Hill constant
P_{act}	Ca^{2+} activated tension
P_i	intracellular inorganic phosphate
P_{pas}	passive tension
P_{total}	steady-state isometric tension
pCa ₅₀	calcium sensitivity ($-\log_{10}[Ca^{2+}_{50}]$)
pH _i	intracellular pH
SR	sarcoplasmic reticulum

St Louis photoperiod. They were fed ReptoMin[®] three times a week *ad libitum*, and liver once per week. All housing and animal procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee protocol 2198.

Muscle preparation

All muscle preparations were made following previously published protocols (Campbell et al., 2003; Fitzsimons et al., 2001) from halved ventricles previously flash frozen between liquid nitrogen-cooled tongs and stored at -80°C . Each frozen tissue was mechanically disrupted using a Polytron blender in 10 ml of ice-cooled digestion solution that contained the following (in mmol l^{-1}): 100 KCl, 20 imidazole, 2 EGTA, 4 ATP, 7 MgCl_2 , 0.04 leupeptin and 0.5 phenylmethanesulfonyl fluoride (PMSF). The resulting homogenate was centrifuged at 650–1360 g for 1 min to form a pellet and the supernatant discarded. The pellet was washed by suspension in 10 ml of additional digestion solution, followed by additional centrifugation at 650–1360 g for 1 min until pellet formation. After the supernatant was discarded, the pellet was resuspended in digestion solution containing 1% Triton X-100 and placed on a shaker for 20 min at 8°C . If mechanical disruption yielded muscle fragments larger than approximately 1 mm in length, incubation in Triton X-100 was continued for an additional 10 min to ensure adequate membrane permeabilization. Following incubation, the muscle preparations were centrifuged at 650–1360 g for 1 min to form a pellet, the supernatant discarded, and the pellet resuspended in 10 ml relax solution with a composition identical to that of the digestion solution except it did not contain leupeptin or PMSF. The preparation was washed at least 2–4 additional times, depending on the presence of visible detergent bubbles, and then stored on ice in 10 ml of relax solution until experimentation.

Experimental solutions

The compositions of the pCa solutions used in this study were similar to those from previous studies and are given in Tables S1 and S2 (Campbell and Holbrook, 2007; Campbell and Moss, 2000, 2002; Campbell et al., 2003; Fitzsimons et al., 2001). Six treatments reflected biologically relevant pH levels (7.4, 7.0, 6.6) and P_i levels (3 and 8 mmol l^{-1} added P_i). Maxchelator 2.50 software was used to determine pCa ($-\log_{10}[Ca^{2+}]$) solution compositions as previously described (Campbell and Holbrook, 2007; Campbell et al., 2003), while the following were held constant (in mmol l^{-1}): 20 imidazole, 14.5 creatine phosphate, 7 EGTA, 1 Mg^{2+} free and 4 MgATP bound. KCl was used to maintain 180 mmol l^{-1} ionic strength across batches. The added P_i levels (3 or 8 mmol l^{-1}) augmented the basal contaminant P_i which has been estimated at $\sim 0.7 \text{ mmol l}^{-1}$ (Campbell and Holbrook, 2007).

Solutions were prepared at 22°C , and the appropriate pH was reached using 4 mol l^{-1} KOH. For each treatment, 13 different solutions were made to cover a range in Ca^{2+} ($1\text{--}32,000 \text{ nmol l}^{-1}$). To obtain intermediate calcium concentrations, solutions with pCa 4.5 and 9.0 were mixed according to proportions determined using Maxchelator 2.50 software. Creatine phosphate was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), ATP was obtained from Roche Life Science (Indianapolis, IN, USA) and CaCl_2 was obtained from Thermo-Fisher Scientific (Waltham, MA, USA). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Experiments

Skinned multicellular preparations were held between a force transducer (resonant frequency, 600 Hz; model 403, Aurora Scientific, Aurora, ON, Canada) and a motor (Series 602X Servo System, Cambridge Technology Inc., Lexington, MA, USA). Muscle preparations that were approximately 200 μm in length and 10–40 μm in width were chosen for experimentation. Ideally, unicellular preparations would be used to isolate force production specific to intracellular changes; however, force production could not be isolated from experimental noise in these smaller preparations. Pins attached to the force transducer and the motor were coated with 100% clear silicone sealant and subsequently positioned over and pressed into the muscle preparation for 10–15 min, allowing the sealant to set. Once the preparation was attached, the pins were raised, suspending the preparation in relax solution between the force transducer and motor (Fig. 1). The preparation was moved into storing solution (pCa 9.0, pH 7.0, P_i 3 mmol l^{-1}), where it was stretched to a sarcomere length of $2.2 \pm 0.0034 \mu\text{m}$ (mean \pm s.e.m., $N=24$) and held isometrically (isometric length l_0 , $146 \pm 7.28 \mu\text{m}$, $N=4$) at 22°C . Cross-sectional area (CSA) was estimated ($2.39 \pm 0.246 \text{ nm}^2$), assuming preparations were cylindrically shaped and preparation width was an accurate representation of diameter.

SLControl 4.5.2 software (www.uky.edu/~kscamp3/SLControl/) was used to perform all experimental stretches and shortenings of the muscle preparations (Campbell and Moss, 2003). Prior to activation, passive tension development ($1.92 \pm 0.20 \text{ kN m}^{-2}$, $N=12$), was measured in relax solution (pCa 9.0, pH 7.0, P_i 3 mmol l^{-1}). Preparations underwent two triangular, 0.02 l_0 stretches from l_0

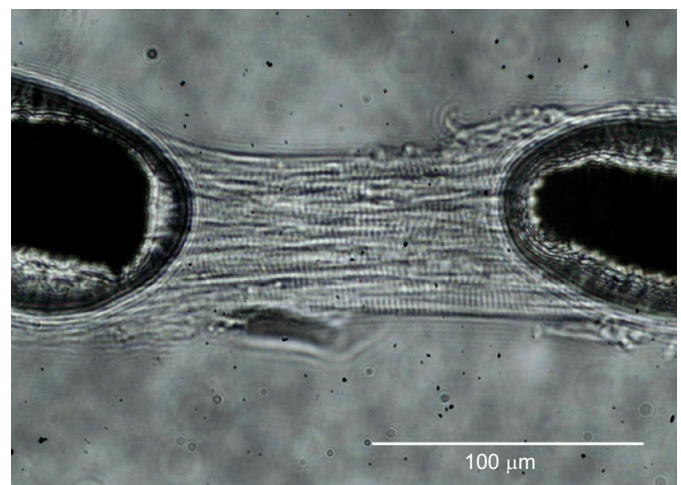


Fig. 1. Loaded multicellular preparations from turtle ventricle. A multicellular preparation attached between a motor and a force transducer and stored in pCa 9.0, pH 7.0, 3 mmol l^{-1} P_i . The preparation has been stretched to a sarcomere length of 2.2 μm .

(2000 ms duration each), followed by a $0.1 l_0$ shortening/re-stretch step for 20 ms (Fig. 2). Each preparation was then immersed in a control activation solution of pCa 4.5, pH 7.0, P_i 3 mmol l^{-1} , after which tension development was allowed to stabilize to determine control, steady-state isometric tension ($41.3 \pm 3.8 \text{ kN m}^{-2}$, $N=12$). Each preparation was pseudo-randomly transferred between various pCa solutions (pCa 9.0–4.5) to obtain a Ca^{2+} activation curve. To prevent run-down of the muscle, preparations were not introduced to all pCa solutions, and activation was halted after the Ca^{2+} activation curve was characterized. A pCa–isometric tension relationship was determined for each treatment (pH 7.4, 7.0 or 6.6 combined with an added P_i of either 3 or 8 mmol l^{-1}). To monitor run-down, preparations were transferred to the control activation solution between every two or three experimental activations. When a preparation began to exhibit run-down, reflected by a decrease in control, steady-state isometric force by greater than 15%, a new preparation from the same ventricular tissue was used to obtain pCa–isometric tension relationships for any remaining treatments.

Statistical analysis

Tension development curves using the Hill equation were determined to characterize calcium binding affinity to the contractile apparatus:

$$P_{\text{total}} = \frac{P_{\text{pas}} + P_{\text{act}} [\text{Ca}^{2+}]^n}{[\text{Ca}_{50}^{2+}]^n + [\text{Ca}^{2+}]^n}, \quad (1)$$

where P_{total} is the steady-state isometric tension, P_{pas} is passive tension, P_{act} is Ca^{2+} -activated tension, n is the Hill constant and $[\text{Ca}_{50}^{2+}]$ is the free Ca^{2+} concentration needed to produce 50% of maximum Ca^{2+} activated tension. $[\text{Ca}_{50}^{2+}]$ values are reported in this work as pCa_{50} values where $\text{pCa}_{50} = -\log_{10}[\text{Ca}_{50}^{2+}]$. Relative tension values were calculated by normalizing to the maximum tension measured for the preparation at pH 7.0 and low P_i (3 mmol l^{-1}) in order to remove the influence of differences in absolute force between multicellular preparations.

Model I, two-way ANOVAs were used to determine the effect of pH and P_i on the curve parameters pCa_{50} , n , P_{pas} and P_{act} . When ANOVA revealed a significant treatment effect, *post hoc* analysis was performed using Holm-corrected pairwise *t*-tests to determine differences between treatments. All statistical computations were completed using R package version 3.2.4.

RESULTS

Effects of acidosis and P_i on pCa–tension relationships

P_i - and H^+ -induced changes in the pCa–tension relationship were determined by analyzing the changes in pCa–tension curve

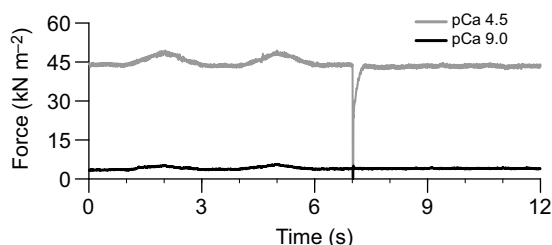


Fig. 2. Mechanical response to experimental stretching and shortening during activation and inactivation. At pH 7.0, 3 mmol l^{-1} P_i , a multicellular preparation underwent two $0.02 l_0$ isometric length (l_0) stretches (2000 ms) followed by a $0.1 l_0$ shortening/re-stretching step (20 ms) at pCa 4.5 (activation) and pCa 9.0 (inactivation). Length changes were separated by 1 s.

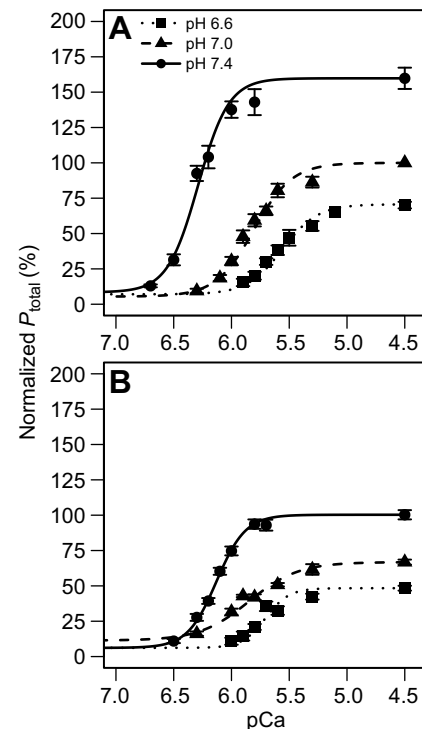


Fig. 3. pCa–tension curves from turtle ventricular preparations. Averaged pCa-normalized tension curves at 3 mmol l^{-1} P_i (A) and 8 mmol l^{-1} P_i (B). Steady-state isometric tension (P_{total}) values are expressed as a percentage of tension developed by the same muscle preparation when exposed to pH 7, 3 mmol l^{-1} P_i , at pCa 4.5. Each curve represents the mean of the 12 curves generated from each preparation. The individual data points on the graphs represent means \pm s.e.m. values where 4–12 measurements were obtained at the same pCa for each of the 12 preparations. Even though 12 curves were constructed for each treatment, measurements were not obtained from every preparation at all of the pCa levels.

parameters (Fig. 3, Table 1). For both P_i levels, as pH decreased, the pCa–tension curve shifted to the right and plateaued at lower tension values. Higher $[P_i]$ (Fig. 3B) resulted in curves plateauing at lower tension values than those at lower $[P_i]$ (Fig. 3A). These changes resulted from significant differences in pCa_{50} and in P_{act} while acidosis and P_i had no effect on the Hill constant (n) or on passive tension (P_{pas}). The effects of pH and P_i accumulation on P_{act} and pCa_{50} were further investigated (see below).

Effects of acidosis and P_i on pCa_{50}

Skinned ventricular myocytes exposed to pH 6.6, 7.0 and 7.4 exhibited pH-induced changes in pCa_{50} ($P < 0.001$). At pH 7.4, pCa_{50} was 6.29 ± 0.03 at low P_i and decreased to 6.13 ± 0.01 at high P_i (Fig. 4A). When pH was decreased from 7.4 to 7.0, pCa_{50} significantly decreased to 5.80 ± 0.03 at low P_i and to 5.85 ± 0.03 at high P_i ($P < 0.001$ at both P_i levels). From pH 7.0 to 6.6, pCa_{50} decreased further to 5.56 ± 0.03 ($P < 0.001$) and to 5.70 ± 0.03 ($P = 0.002$), at low and high P_i , respectively. Taken together, these data show that acidosis decreases pCa_{50} and decreases myofilament Ca^{2+} sensitivity (Fig. 4B,C).

We also detected a significant interaction between P_i and pH on pCa_{50} ($P < 0.001$), which showed variation in the effects of P_i on pCa_{50} across pH (Fig. 4A). At pH 7.4, low P_i resulted in a significantly higher pCa_{50} at high P_i ($P < 0.001$). At pH 7.0, there was no significant difference between the two P_i levels ($P = 0.298$). At pH 6.6, pCa_{50} was again significantly different across P_i levels

Table 1. Force–pCa relationship curve parameters

Treatment	P_{act}	P_{pas}	pCa ₅₀	n
pH 7.4, 3 mmol l ⁻¹ P _i	159.79±7.93	8.42±1.87	6.29±0.03	3.27±0.37
pH 7.0, 3 mmol l ⁻¹ P _i	100.00±0.00	5.42±0.62	5.81±0.03	2.64±0.23
pH 6.6, 3 mmol l ⁻¹ P _i	70.65±2.48	7.05±1.40	5.56±0.04	2.85±0.32
pH 7.4, 8 mmol l ⁻¹ P _i	100.30±3.27	6.15±1.06	6.13±0.01	3.54±0.24
pH 7.0, 8 mmol l ⁻¹ P _i	66.81±1.79	11.37±1.57	5.85±0.03	2.04±0.20
pH 6.6, 8 mmol l ⁻¹ P _i	48.37±1.72	6.17±2.09	5.70±0.03	4.28±0.75

P_i, inorganic phosphate; P_{act} , Ca²⁺ activated tension; P_{pas} , passive tension; pCa₅₀, calcium sensitivity ($-\log_{10}[\text{Ca}^{2+}_{50}]$); n , Hill constant. All values are means±s.e.m. ($n=12$ preparations).

($P=0.002$), but in this case, low P_i resulted in a lower pCa₅₀ at high P_i, the opposite of the observation at pH 7.4.

Effects of acidosis and P_i on P_{act}

Skinned ventricular preparations exposed to pH 6.6, 7.0 and 7.4 showed pH-induced changes in maximum tension development ($P<0.001$). Between pH 7.4 and 7.0, P_{act} showed a 36% and 41% decrease at low and high P_i, respectively. A further decrease down to pH 6.6 caused additional P_{act} decreases of 33% and 24% at low and high P_i, respectively. Overall, the decrease in pH from 7.4 to 6.6 produced a 58% decrease in P_{act} at low P_i; and a 55% decrease at high P_i (Figs. 5 and 6).

Skinned ventricular preparations were also introduced to high or low [P_i] to determine whether maximum tension development was affected. Increasing [P_i] by 5 mmol l⁻¹ significantly decreased P_{act} at all pH levels ($P<0.001$). A higher [P_i] resulted in P_{act} decreases of 38%, 41% and 34% at pH 7.4, 7.0 and 6.6, respectively. Furthermore, we exposed skinned ventricular preparations to [H⁺] and [P_i] increases simultaneously to determine whether H⁺ and P_i interacted to modify P_{act} . The interaction between [H⁺] and [P_i] significantly attenuated further decreases in P_{act} ($P<0.001$).

DISCUSSION

The present study is the first to characterize the contractility of ventricular myofilaments in any reptile, thereby furthering our understanding of how ventricular contractility varies across vertebrate species. We have also elucidated the likely roles of physiologically relevant changes in pH and P_i on the contractile properties of ventricular myofilaments in mediating the intrinsic functional suppression during anoxia in the most anoxia-tolerant tetrapod, the painted turtle. Contractile properties were characterized by comparing pCa–tension relationship differences resulting from changes in tension development (P_{act}) and calcium sensitivity (pCa₅₀). P_{act} decreased in the presence of higher H⁺ or higher P_i. A decrease in pCa₅₀ was only observed in the presence of higher H⁺. The interaction between P_i and pH decreased P_{act} and pCa₅₀ to a lesser degree compared with acidosis alone.

Effect of acidosis and P_i on tension development (P_{act})

Unsurprisingly, acidosis had a significant negative effect on P_{act} , supporting previous suggestions that acidosis is a primary factor mediating functional suppression *in vivo* during anoxia (Shi and Jackson, 1997). The pH effects were quantitatively similar to previous measurements in rats at the same temperature (Fig. 7A). In turtles at both P_i levels, increasing pH from 7.0 to 7.4 increased tension by ~65% while decreasing pH from 7.0 to 6.6 reduced tension by ~28% (Fig. 7A). Rat ventricular myofilaments showed a 60% increase in P_{act} when pH was increased from 7.0 to 7.4–7.5 while a decrease in pH from 7.0 to 6.5–6.6 decreased P_{act} by 25–40% (Fabiato and Fabiato, 1978; Kentish, 1991; Smith and Steele, 1994; Solaro et al., 1988).

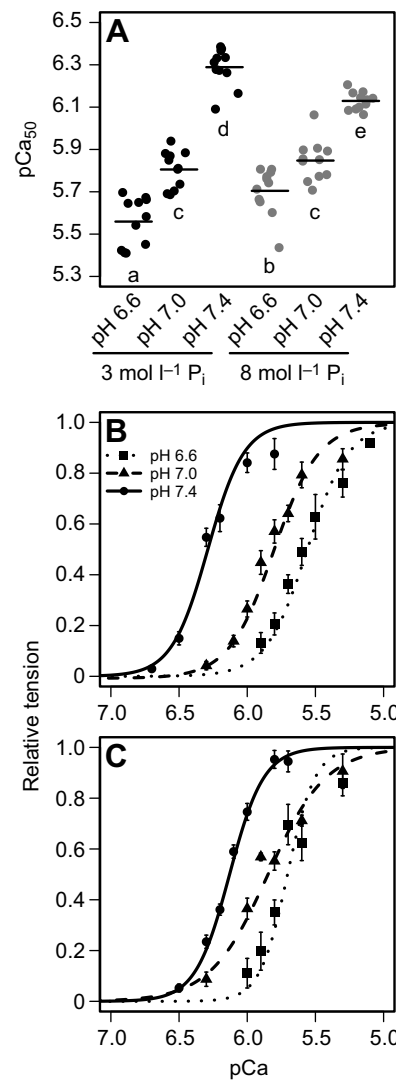


Fig. 4. Effects of increased H⁺ and P_i on pCa₅₀. (A) pCa₅₀ values ($-\log_{10}[\text{Ca}^{2+}_{50}]$, a measure of calcium sensitivity) are depicted for all pH levels at 3 and 8 mmol l⁻¹ P_i, where significant differences are indicated by different letters ($P<0.05$, $n=12$). (B, C) Averaged pCa-normalized tension curves ($N=12$ preparations) for pH 6.6, 7.0 and 7.4 at 3 mmol l⁻¹ P_i (B) and 8 mmol l⁻¹ P_i (C). Each curve on the graphs represents the mean of the 12 curves generated from each of the 12 preparations. The individual data points on the graph represent mean±s.e.m. values where 4–12 measurements were obtained at the same pCa level for each of the 12 preparations. Even though 12 curves were constructed for each treatment, measurements were not obtained from every preparation at all of the pCa levels.

Although turtle and rat exhibit similar responses to pH changes at room temperature, the physiological temperatures at which they experience pH changes are different. Mammalian muscle experiences intracellular acidosis during exercise at 37°C, and studies performed at 30°C indicated that P_{act} is less sensitive to acidosis than at lower temperatures (Knuth et al., 2006; Nelson et al., 2014; Nelson and Fitts, 2014; Orchard et al., 1991; Orchard and Kentish, 1990; Pate et al., 1995). This has led to the general belief that acidosis may only have minor *in vivo* effects on P_{act} in mammalian muscle at the physiologically relevant temperature of 37°C (Debold, 2012). In contrast, turtles experience a broad range of body temperatures, from 3°C while overwintering to more than 30°C in summer. Anoxia-induced intracellular acidosis is most likely to occur during winter, so the more pronounced

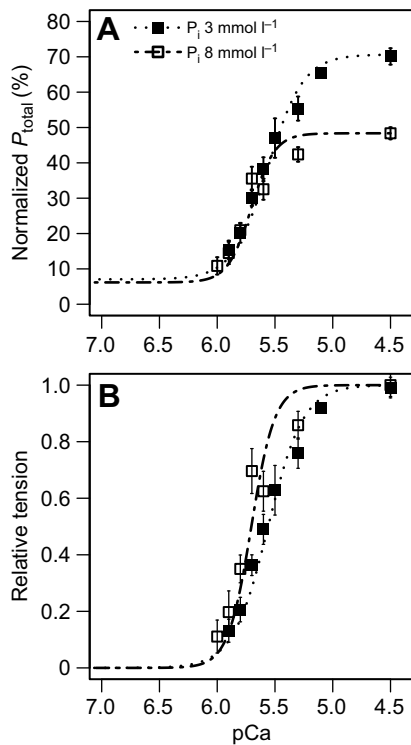


Fig. 5. Effect of P_i at pH 6.6. All values are means \pm s.e.m. from 12 preparations. (A) Averaged pCa-normalized tension curves at 3 and 8 mmol l⁻¹ P_i . P_{total} values are expressed as a percentage of tension developed by the same muscle preparation when exposed to pH 7, 3 mmol l⁻¹ P_i , at pCa 4.5. (B) Relative tension curves for pH 6.6 are depicted at 3 and 8 mmol l⁻¹ P_i .

pH-dependent changes in mammalian P_{act} observed at lower temperatures suggest that acidosis may be especially important in reducing P_{act} and, therefore, suppressing contractility of cardiac myofilaments *in vivo* in the anoxic turtle. Clearly, additional studies at temperatures colder than 20°C are needed to confirm this hypothesis.

Increasing $[P_i]$ also decreased P_{act} in turtle ventricular myofilaments. We observed a ~40% decrease in P_{act} across all pH levels with a 5 mmol l⁻¹ increase in P_i (Fig. 3), a response that is

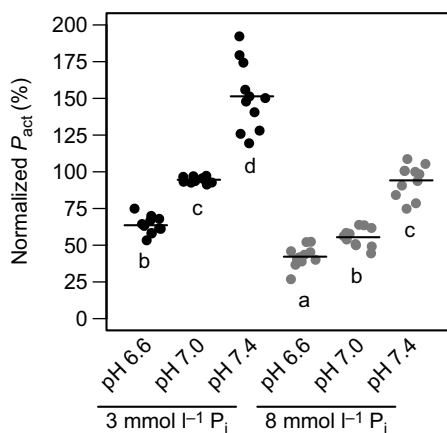


Fig. 6. Effects of H^+ and P_i elevation on active tension at pCa 4.5. Ca^{2+} -activated tension (P_{act}) values are expressed as a percentage of tension developed by the same muscle preparation when exposed to pH 7, 3 mmol l⁻¹ P_i , at pCa 4.5. Significant differences are indicated by different letters ($P < 0.05$, $n = 12$ preparations).

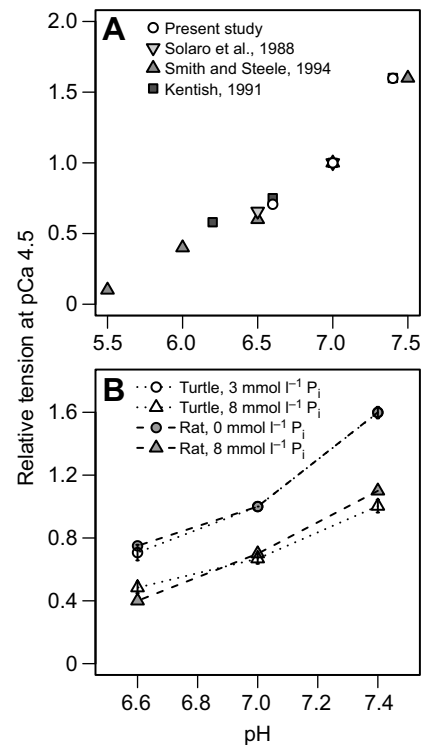


Fig. 7. Effects of H^+ and P_i on maximum tension development in skinned myocardium from mammals and turtle. (A) Comparison of the effect of acidosis on relative tension development between turtle and rat ventricle (Solaro et al., 1988; Smith and Steele, 1994; Kentish, 1991). (B) The effect of acidosis on relative tension at low and high P_i levels (5 mmol l⁻¹ increase) in both rat and turtle ventricle. All values are normalized to pH 7.0, pCa 4.5 at low $[P_i]$.

similar to that observed in mammalian striated muscle (Nelson and Fitts, 2014; Palmer and Kentish, 1994). It is hypothesized that increasing intracellular $[P_i]$ drives a reversal in the cross-bridge cycle, which shifts cross-bridges from a state of high tension development to a state of lower tension development where a greater proportion of myosin heads are bound to P_i (Palmer and Kentish, 1994; Takagi et al., 2004). This is likely the mechanistic cause of the P_i -induced P_{act} decrement observed in this study.

Although increased P_i decreased P_{act} in turtle, the physiologically relevant P_i increase did not decrease P_{act} by the same magnitude as the physiologically relevant decrease in pH, indicating acidosis is probably the more important factor during anoxia. For example, at pH 7.4, increasing P_i by 5 mmol l⁻¹ only decreased tension by 38%, while the physiologically relevant decrease in pH at 3 mmol l⁻¹ P_i decreased it by 58%. In turtles, studies of whole ventricle and ventricular strips have suggested that acidosis contributes to cardiac functional suppression during anoxia (Jackson, 1987; Overgaard et al., 2005; Wasser et al., 1991); however, other work has suggested that the changing levels of energetic components, such as increasing $[P_i]$, may account for the cardiac functional suppression observed during anoxia (Stecyk et al., 2009). This is the first study to compare the effects of intracellular pH and P_i changes on ventricular myofilament function in turtles, and the large decrease in tension development due to acidosis further supports the notion that acidosis plays a more significant role in functional suppression of the turtle heart during anoxia.

The statistical interaction between $[P_i]$ and pH indicates that when both P_i and H^+ are at high concentrations, the decrease in P_{act} may be slightly attenuated in turtles (Fig. 6), which differs from mammalian

striated muscle, where no such attenuation occurs (Eisner et al., 1987; Kentish, 1991; Wilkie, 1986; Wilson et al., 1988). In a previous study of rat ventricle, relative tension was measured at pH 6.6, 7.0 and 7.4 at two levels of P_i differing by 5 mmol l^{-1} (Kentish, 1991), the results of which we have plotted alongside the results from our present study in Fig. 7B. For this comparison, maximum tension values were normalized to pH 7.0 of the lower P_i concentration for the two studies (0 and 3 mmol l^{-1} for rat and turtle, respectively). Although no statistical comparisons can be made, the effect of increased P_i on pH-dependent P_{act} appears strikingly similar between the two species, despite the unique attenuation effect observed in turtle preparations. Further studies at identical $[P_i]$ levels are required to make comparisons across species regarding the P_i -pH interaction effect on P_{act} .

Effect of acidosis and P_i on calcium sensitivity

A second mechanism by which pH and P_i can alter cardiac contractility is by changing the myofilament sensitivity to Ca^{2+} . This can be assessed by measuring the tension–pCa relationship and thus the $p\text{Ca}_{50}$ under different conditions. We found a clear right-shift in the tension–pCa relationship curve (increased $p\text{Ca}_{50}$) as $[\text{H}^+]$ increased (Fig. 4), indicating a decrease in the Ca^{2+} sensitivity of turtle ventricular myofilaments with acidosis, an affect long-known to occur in mammals (Fabiato and Fabiato, 1978; Orchard et al., 1991; Orchard and Kentish, 1990; Solaro et al., 1988). In contrast, increasing P_i from 3 to 8 mmol l^{-1} at pH 7.0 had no statistically significant effect on Ca^{2+} sensitivity in the present study (Fig. 4A). This differs somewhat from rats, which showed significant decreases in Ca^{2+} sensitivity with much larger P_i increases (20 mmol l^{-1}) (Palmer and Kentish, 1994). Turtle atrial trabeculae also exhibited decreased Ca^{2+} sensitivity with increasing P_i concentration (Jensen and Gesser, 1999). Although we cannot rule out the possibility that higher levels of P_i might also have decreased Ca^{2+} sensitivity, we are able to conclude that physiologically relevant increases in P_i are insufficient to cause any change in myofilament Ca^{2+} sensitivity and that *in vivo* contractile suppression in anoxic turtles is at least partly related to decreases in $p\text{Ca}_{50}$ by acidosis.

Just as there were interactive effects of P_i and pH on P_{act} , we also found interactive effects on $p\text{Ca}_{50}$ (Fig. 4A). At pH 7.4, increasing P_i by 5 mmol l^{-1} decreased $p\text{Ca}_{50}$, while at pH 6.6, the 5 mmol l^{-1} P_i increase rescued $p\text{Ca}_{50}$. Although there was no statistical effect of P_i or pH on the Hill constant (n), closer examination of the relative tension curves at pH 6.6 indicates that there may be a difference in n at pH 6.6, which in turn may impact $p\text{Ca}_{50}$ (Fig. 5B). Additionally,

the pH– P_i interaction may impact myofilament cooperativity, which could cause a change in myofilament tension kinetics that determine $p\text{Ca}_{50}$. Regardless, the results suggest that intracellular acidosis is the primary cause of decreased myofilament Ca^{2+} sensitivity in this preparation and probably accounts for contractile suppression, *in vivo*, in the anoxic turtle ventricle.

Calcium sensitivity across species

Our study provides further evidence that high calcium sensitivity is a general property of the ectothermic myofilament apparatus in ventricular tissue (Table 2). Comparison of the properties of the myofilaments from turtle ventricle with previously published values for mammalian ventricle reveals that turtles have a higher calcium sensitivity. At pH 7.0, mammalian ventricular $p\text{Ca}_{50}$ ranges from 5.29 to 5.57, while turtle $p\text{Ca}_{50}$ was 5.81 (Bers, 1991; Harrison and Bers, 1989, 1990; Palmer and Kentish, 1994). Only at pH 6.6, 3 mmol l^{-1} P_i , did turtle ventricular myofilaments exhibit a $p\text{Ca}_{50}$ within the range of mammals, where $p\text{Ca}_{50}$ decreased to 5.56. Similar to turtle cardiomyocytes, frog ventricular myocytes exhibited a $p\text{Ca}_{50}$ of 5.87 at pH 7.0, 20°C , indicating a higher Ca^{2+} sensitivity than in mammals (Brandt et al., 1998). Studies of frog and rainbow trout ventricular myofilaments have also shown higher sensitivity to Ca^{2+} than mammalian ventricular myofilaments, regardless of temperature (Churcott et al., 1994; Harrison and Bers, 1990). It is reasonable to suspect that differences in $p\text{Ca}_{50}$ across species are caused by variation in the isoforms of troponin C (TnC) and troponin I (TnI), which accounts for tissue-specific $p\text{Ca}_{50}$ differences in mammals (Debold, 2012).

The higher ventricular myofilament calcium sensitivity of ectotherms makes sense when considering the differing roles of the sarcoplasmic reticulum (SR) in excitation–contraction (E–C) coupling across vertebrates and their relative heart rates. Mammals have a well-developed, highly organized SR that allows for large calcium transients during E–C coupling (Barceñas-Ruiz and Wier, 1987; DuBell and Houser, 1989) and their high heart rates necessitate a lower pCa to insure rapid relaxation and return to diastole. In contrast, turtle ventricular myocardium relies comparatively less on the SR for Ca^{2+} during contraction (Galli et al., 2006a,b), as does that of frogs (Fabiato, 1982; Morad et al., 1981) and fish. (Hove-Madsen et al., 1999; Shiels and Galli, 2014; Tibbits et al., 1991; Vornanen, 1989; Vornanen and Haverinen, 2013). It has recently been proposed that the SR in ectotherms may be more important during times of physiological stress (Cros et al., 2014) or in supporting the contractile requirements of more aerobic

Table 2. Ventricular myofilament Ca^{2+} sensitivity of different species at pH 7.0

Citation	Species	Common name	Temperature ($^\circ\text{C}$)	$p\text{Ca}_{50}$
Churcott et al., 1994	<i>Oncorhynchus mykiss</i>	Rainbow trout	21.0	6.49
Churcott et al., 1994	<i>Rattus norvegicus</i>	Rat	21.0	6.24
Brandt et al., 1998	Frog*	Frog	20.0	5.87
Present study	<i>Chrysemys picta bellii</i>	Western painted turtle	22.0	5.81
Godt et al., 1991	<i>Gallus gallus</i>	Chicken	22.0	5.65
Harrison and Bers, 1990	<i>Rana pipiens</i>	Frog	22.0	5.62
Palmer and Kentish, 1994	<i>Oryctolagus cuniculus</i>	Rabbit	25.0	5.57
Harrison and Bers, 1989	<i>Oryctolagus cuniculus</i>	Rabbit	29.0	5.49
Harrison and Bers, 1990	<i>Rattus norvegicus</i>	Rat	29.0	5.33
Harrison and Bers, 1990	<i>Cavia porcellus</i>	Guinea pig	29.0	5.29
Harrison and Bers, 1990	<i>Rana pipiens</i>	Frog	1.0	5.02
Harrison and Bers, 1990	<i>Rana pipiens</i>	Frog	1.0	5.02
Harrison and Bers, 1990	<i>Cavia porcellus</i>	Guinea pig	1.0	4.74
Harrison and Bers, 1989	<i>Oryctolagus cuniculus</i>	Rabbit	1.0	4.73
Harrison and Bers, 1990	<i>Rattus norvegicus</i>	Rat	1.0	4.68

*Species name was not stated.

ectothermic species, such as varanid lizards (Galli et al., 2009; Warren et al., 2010). Interestingly, the chicken ventricle shows a pCa_{50} of 5.65 at 22°C, which occupies an intermediate calcium sensitivity between mammals and ectothermic vertebrates (Godt et al., 1991), although birds do utilize the SR as a calcium source during contraction (Gwathmey and Morgan, 1991).

Calcium sensitivity within the heart

Our study suggests that cardiac myofilament Ca^{2+} sensitivity varies not only across species but also within the turtle heart. Turtle atrial trabeculae exhibited less sensitivity to calcium with a pCa_{50} of 5.67 (pH 7.35–7.45) (Jensen and Gesser, 1999), compared with that for a pCa_{50} of 6.29 (pH 7.4) observed in this study. Atrial tissue also exhibits a reduced Ca^{2+} sensitivity in both pigs and humans (Morano et al., 1988). This is not true of all vertebrates, however, as frog atrial and ventricular tissues have similar Ca^{2+} sensitivities (Brandt et al., 1998).

Conclusions

We have shown that acidosis has profound negative effects on both tension development and Ca^{2+} sensitivity in skinned ventricular cardiomyocytes from turtles, while increased $[P_i]$ has smaller effects only on tension development, and none on Ca^{2+} sensitivity. By extending these functional data to the tissue and organ level in the anoxic painted turtle, we can conclude that the intrinsic functional suppression in the ventricular myocardium is primarily from intracellular acidosis. This agrees with previous studies at the organ and tissue level that support a larger role for acidosis in functional suppression of cardiac function in anoxic turtles (Jackson, 1987; Overgaard et al., 2005; Wasser et al., 1991). It is noteworthy that during 12 weeks of anoxia, plasma-free Ca^{2+} levels gradually increase to 25 mequiv l^{-1} and total intracellular Ca^{2+} in heart increases from 1.5 to 5.7 mmol kg^{-1} (Jackson and Heisler, 1982, 1983). Additional studies measuring intracellular Ca^{2+} transients during anoxia and acidosis are needed to determine whether these extracellular and intracellular calcium changes are functionally important.

We have also provided further evidence that the ventricles from ectothermic vertebrates have a higher myofilament Ca^{2+} sensitivity compared with those of mammals. Although turtle Ca^{2+} sensitivity is intrinsically different from that of mammals, the effect of acidosis on Ca^{2+} sensitivity is not qualitatively different across species. Likewise, the effects of decreased pH and increased $[P_i]$ on decreased tension development in turtles were not different from those measured in mammals. However, a decrease in Ca^{2+} sensitivity due to increased $[P_i]$ previously observed in mammals was not observed in our studies of turtle. This suggests that P_i plays a larger role in mammals in decreasing both tension development and Ca^{2+} sensitivity, while acidosis is the main contributing factor in turtles.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.E.F., D.E.W.; Methodology: C.E.F., K.S.C., D.E.W.; Formal analysis: C.E.F., K.S.C.; Investigation: C.E.F., K.S.C., D.E.W.; Writing - original draft: C.E.F., D.E.W.; Writing - review & editing: C.E.F., K.S.C., D.E.W.; Supervision: D.E.W.; Project administration: D.E.W.; Funding acquisition: D.E.W.

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Supplementary information

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Table S1: *pCa 9.0 Solution Composition*

Treatment	ATP (mM)	CaCl ₂ (mM)	Creatine Phosphate (mM)	EGTA (mM)	Imidazole (mM)	KCl (mM)	KH ₂ PO ₄ (mM)	MgCl ₂ (mM)
pH 7.4, 3 mM Pi	4.61	0.0899	14.5	7.00	20.0	38.0	3.14	6.09
pH 7.0, 3 mM Pi	4.71	0.0156	14.5	7.00	20.0	38.0	3.11	5.63
pH 6.6, 3 mM Pi	4.99	0.00265	14.5	7.00	20.0	38.0	3.07	5.41
pH 7.4, 8 mM Pi	4.63	0.0899	14.5	7.00	20.0	29.0	8.35	6.27
pH 7.0, 8 mM Pi	4.71	0.0156	14.5	7.00	20.0	27.0	8.28	5.80
pH 6.6, 8 mM Pi	5.00	0.0025	14.5	7.00	20.0	29.0	8.18	5.52

Table S2: *pCa 4.5 Solution Composition*

Treatment	ATP (mM)	CaCl ₂ (mM)	Creatine Phosphate (mM)	EGTA (mM)	Imidazole (mM)	KCl (mM)	KH ₂ PO ₄ (mM)	MgCl ₂ (mM)
pH 7.4, 3 mM Pi	4.70	7.08	14.5	7.00	20.0	38.0	3.16	5.35
pH 7.0, 3 mM Pi	4.78	6.99	14.5	7.00	20.0	38.0	3.13	5.35
pH 6.6, 3 mM Pi	5.05	6.52	14.5	7.00	20.0	38.0	3.08	5.35
pH 7.4, 8 mM Pi	4.68	7.08	14.5	7.00	20.0	24.0	8.41	5.53
pH 7.0, 8 mM Pi	4.79	7.00	14.5	7.00	20.0	26.0	8.32	5.48
pH 6.6, 8 mM Pi	5.06	6.53	14.5	7.00	20.0	29.0	8.20	5.43

Fig. S1

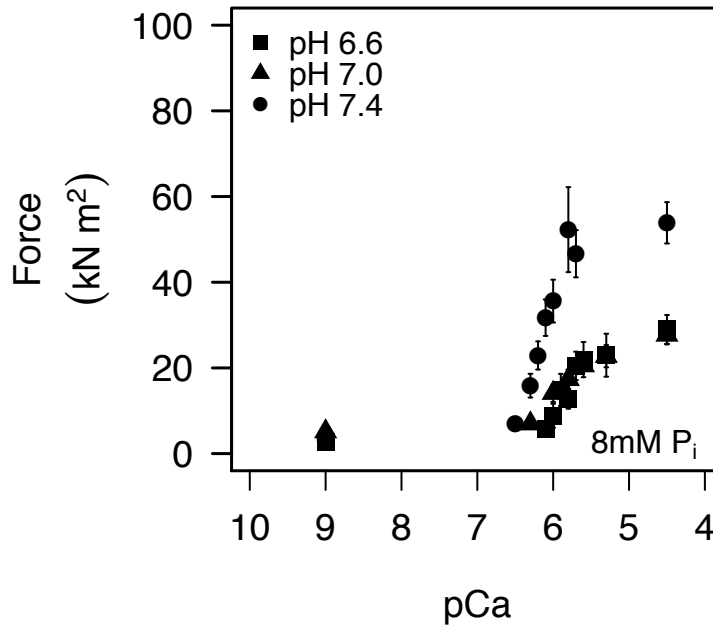
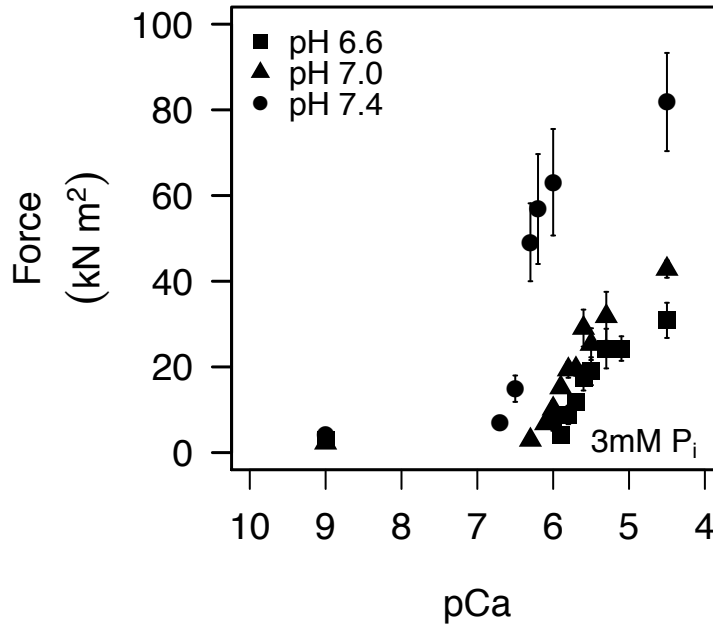


Fig. S1. The pCa-Tension Relationship from Turtle Ventricular Preparations. Averaged tension development at 3 mM Pi (A), and 8 mM Pi, (B). Data points represent means \pm SEM values where 4-12 measurements were obtained at the same pCa for each treatment. Measurements were not obtained from every preparation at all of the pCa levels.