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# The effect of pH on the $Ca^{2+}$ affinity of the $Ca^{2+}$ regulatory sites of skeletal and cardiac troponin C in skinned muscle fibres

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**Summary**

It is known that intracellular pH drops rapidly after the onset of ischemia in cardiac muscle and may play some role in the rapid drop in force that ensues. It is also known that $\alpha_1$-adrenoceptor agonists alkalinize intracellular pH by stimulating $Na^+/H^+$ exchange and may represent a mechanism which facilitates recovery of intracellular pH from acidosis. Lowering or raising pH shifts the $Ca^{2+}$ dependence of force development in muscle fibres to higher or lower free $Ca^{2+}$ concentrations, respectively, yet the precise mechanism is unknown. To investigate this phenomenon we have used skinned skeletal or cardiac muscle fibres whose endogenous troponin C (TnC) has been replaced with chicken skeletal TnC labelled with DANZ ($STnC_{DANZ}$) or recombinant cardiac TnC labelled with IAANS ($CTnC3(C84)_{IAANS}$), respectively. The fluorescence of the $STnC_{DANZ}$ or $CTnC3(C84)_{IAANS}$ was enhanced by $Ca^{2+}$ binding to the $Ca^{2+}$-specific (regulatory) site(s) of STnC or CTnC when incorporated into skinned fibres, and was measured simultaneously with force. When the pH was changed from 7.0 to 6.5 or 7.5 the shift in the $Ca^{2+}$ dependence of force paralleled the shift in fluorescence. Since the force and fluorescence shift in parallel as the pH is lowered or raised, it can be concluded that these changes in $Ca^{2+}$ sensitivity are caused by a decrease or increase, respectively, in the $Ca^{2+}$ affinity of the $Ca^{2+}$-specific site(s) of TnC. Since lowering or raising the pH also resulted in lower or higher, respectively, maximal $Ca^{2+}$ activated force while maximal fluorescence remained unchanged, it is possible that $H^+$ may act indirectly, as well, by reducing or increasing, respectively, the number or type of crossbridges attached to actin and thereby alter the crossbridge induced depression or elevation, respectively of the observed TnC $Ca^{2+}$ affinity. Experiments with 2,3-butanedione monoxime, however, where force-generating crossbridges were greatly reduced, indicated that the pH effect may be primarily related to a direct change in the $Ca^{2+}$ affinity to the regulatory sites of TnC.

## Introduction

It is known that intracellular pH drops after the onset of ischemia in cardiac muscle and may play some role in the rapid drop in force that ensues (Lorkovic, 1966; Pannier & Leusen, 1968; Poole-Wilson & Langer, 1975; Williamson *et al.*, 1976). On the other hand $\alpha_1$-adrenoceptor agonists could increase intracellular pH by stimulating the exchange of $H^+$ for $Na^+$ in single cardiac cells, and therefore may represent a mechanism which facilitates recovery of intracellular pH from acidosis (Puceat *et al.*, 1992, 1993; Terzic *et al.*, 1993). It was also shown that decreasing pH results in the rightward shift of the $Ca^{2+}$ dependence of force development towards higher $Ca^{2+}$ concentration both in cardiac myofilaments and skeletal myofibrils (Fabiato & Fabiato, 1978; Ball *et al.*, 1994; Ding *et al.*, 1995). Fabiato and Fabiato (1978), Terzic and colleagues (1992) and Terzic and Vogel (1991) also showed that an increase in pH results in an increased cardiac myofilament sensitivity to calcium.

However, the mechanism of the pH dependent changes in the contractility of cardiac and skeletal muscles remains unknown. Since contraction of cardiac and skeletal muscle is triggered by the binding of $Ca^{2+}$ to the $Ca^{2+}$ specific sites in TnC, it

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is possible that changes in the intracellular $H^+$ concentration may affect the direct binding of $Ca^{2+}$ to the regulatory sites of TnC. Previous studies on isolated cardiac and skeletal TnC (Robertson & Kerrick, 1979; El Saleh & Solaro, 1988; Ogawa 1985; Iida 1988) have demonstrated an effect of pH on the $Ca^{2+}$ affinity of TnC in fibres with the change in the $Ca^{2+}$-sensitivity of force development.

To study this phenomenon we incorporated fluorescently labelled TnC into TnC depleted skinned fibres so that the binding of $Ca^{2+}$ to the $Ca^{2+}$-specific sites in TnC, along with the steady state isometric force development, could be simultaneously measured. Skeletal or cardiac TnCs, specifically labelled with fluorescent probes, underwent significant fluorescence changes upon the binding of $Ca^{2+}$ to the $Ca^{2+}$ specific regulatory sites (I and II) in skeletal TnC or site II in cardiac TnC. The structure of skeletal or cardiac muscle fibre preparations is well maintained and they closely mimic '*in vivo* conditions', and steady state force development can be directly determined. This approach was unique since it allowed us to study the effect of the changing pH on $Ca^{2+}$-activation of muscle contraction while simultaneously estimating $Ca^{2+}$ binding to the regulatory sites of TnC.

Our data clearly show that acidosis in the cardiac and skeletal fibres results in a decreased $Ca^{2+}$ sensitivity of force–pCa dependence, which is paralleled by the same shift in the fluorescence–pCa relationship. Lowering or raising pH also resulted in lower or higher, respectively, maximal $Ca^{2+}$ activated force while maximal fluorescence remained unchanged. To determine the contribution of cycling crossbridges to the pH dependent change in $Ca^{2+}$ affinity of the regulatory site(s) of TnC, simultaneous force and fluorescence measurements were performed in the presence of 2,3-butanedione monoxime (BDM). It has been shown previously that BDM stabilizes the weakly attached state of working crossbridges and significantly inhibits steady state force development (Higuchi & Takemori, 1988; Bagni *et al.*, 1992; Backx *et al.*, 1994; Ikenouchi *et al.*, 1994; Vahl *et al.*, 1994; Zhao & Kawai, 1994). Our data revealed that even though the BDM-treated fibres demonstrated greatly reduced tension development, there was still a profound effect of pH on the fluorescence–pCa relationship. Decreasing pH caused the rightward shift in this dependence towards higher $Ca^{2+}$ concentrations. These results suggest that although changes in the number of attached crossbridges may contribute indirectly to the pH induced shift in the $Ca^{2+}$ dependence of force, the primary effect probably comes from a direct effect of pH on the $Ca^{2+}$ affinity of TnC.

## Materials and methods

### *$STnC_{DANZ}$ and $CTnC3(C84)_{IAANS}$ preparation*

Skeletal TnC was purified according to Potter (1982) and was labelled with dansylaziridine (Molecular Probes) according to Johnson and colleagues (1978). Chicken CTnC3(C84) and mouse CTnC3(C84) were prepared according to the method of Putkey and colleagues (1989). CTnC3(C84) is a recombinant DNA derived monocysteine mutant of cardiac TnC (CTnC) where the cysteine at position 35 has been changed to serine. Both CTnC3(C84)s were labelled with IAANS at Cys 84 according to Putkey and colleagues (1989).

### *Solutions*

The various pCa solutions were prepared using the room temperature binding constants of Fabiato and Fabiato (1978) and contained: either 1 or 5 mM free $Mg^{2+}$, 7 mM EGTA, 20 mM imidazole, 2 mM MgATP, 15 mM creatine phosphate, and 20 units $ml^{-1}$ phosphocreatine kinase. Ionic strength was then adjusted with potassium propionate (KPr) to 150 mM in all solutions. Three sets of different pCa solutions (from pCa 8 to pCa 4) were prepared depending on the pH of the experiments: (1) pH 7.0; (2) pH 6.5; (3) pH 7.5. The contribution of all ions in these solutions was calculated according to Fabiato and Fabiato (1978). The concentration of all metals was determined by atomic absorption spectroscopy using a Perkin-Elmer model 3030 atomic absorption spectrophotometer. The concentration of stock ATP was determined by the absorbance at 259 nm using a Beckman DU-7 spectrophotometer. EGTA concentration was determined by calcium titration using a calcium electrode. 2,3-butanedione monoxime was purchased from Sigma Chemical Company and used at a concentration of 30 mM in all pCa solutions.

### *Fibre preparation*

All experiments were performed at room temperature. STnC experiments were performed with glycerinated rabbit psoas muscle fibres. Strips of fibres, a few millimeters in diameter and ~5 cm in length were dissected from the rabbit and chemically skinned as previously detailed (Kerrick & Krasner, 1975). After skinning the fibres were tied to sticks, and stored at −20° C for at least overnight but not for more than 3 weeks in 50% relaxing solution (pCa 8.0) and 50% glycerol. Single fibres ~3–4 mm long were dissected from the muscle strips and mounted directly to the force transducer clips.

The cardiac troponin-C (CTnC) experiments were performed with glycerinated porcine ventricular muscle strips. Strips a few millimeters in diameter and ~5 mm in length were dissected from the trabeculae cordis of the left ventricle and extracted for 24 h in 1% Triton X-100 containing (by volume) 49.5% pCa 8.0 relaxing solution and 49.5% glycerol and thereafter transferred to a similar replacement solution without Triton X-100. The strips were stored at −20° C for no longer than 4 weeks.

### *Incorporation of $STnC_{DANZ}$ or $CTnC3(C84)_{IAANS}$ into fibres*

Strips ~2 mm long, and 0.5 mm maximum diameter were

dissected from the strips of muscle immediately before each experiment and mounted to the force transducer clips. The initial steady state force was tested in the pCa 4 buffer followed by the relaxation of the fibres in pCa 8 solution. This was done in order to ensure that the fibres were securely attached to the clips and not damaged in the dissection procedure. Skeletal fibres' endogenous TnC was extracted by 20 min incubation with 5 mM EDTA and 20 mM Tris–HCl buffer (pH 7.8) whereas cardiac endogenous TnC was extracted by incubating with 2 mM CDTA and 10 mM Tris–HCl buffer (pH 8.4) for 30 min to 2 h. Both solutions were renewed automatically every 10 s in a flow-through system (Guth & Potter, 1987). Extracted fibres were then tested for residual force in the contraction, pCa 4 solution. The average residual force was ~30% for skeletal fibres and ~25% for cardiac preparations as compared to force developed by unextracted fibres.

The fibres were then incubated with 1.1 μM $STnC_{DANZ}$ or $CTnC3(C84)_{IAANS}$ for 45 min with the fresh protein solutions renewed every 10 s. $STnC_{DANZ}$ and $CTnC3(C84)_{IAANS}$ reconstituted fibres were subsequently checked for force development in the pCa 4 solution and relaxed in the pCa 8 solution. More than 90% of the original force was recovered after incorporation of fluorescently labelled TnCs into the fibres. Evidence has been provided to show that this procedure results in a direct substitution of the fibre's endogenous TnC with that of the fluorescently labelled TnC (Zot *et al.*, 1986; Guth & Potter, 1987; Putkey *et al.*, 1996).

For $CTnC3(C84)_{IAANS}$ incorporation into fibres employed in the pH 7.0/pH 7.5 experiments, TnC-depleted cardiac preparations were reconstituted with $CTnC3(C84)_{IAANS}$ and stored from 24 h up to 1 month in 50% glycerol, 50% of the pCa 8 buffer (pH 7.0) containing 0.5 μM $CTnC3(C84)_{IAANS}$. After mounting, the cardiac fibres were washed up to 3 h in the flow-through cuvette using relaxing, pCa 8 solution to remove excess $CTnC3(C84)_{IAANS}$. While monitoring force and fluorescence a maximal test contraction was initiated by changing from a relaxing to a contracting solution (pCa 4.0) and then back to a relaxing solution (pCa 8.0) after maximal force was obtained (in about 10 s). This was done in order to ensure that the fibre was securely attached to the clips and that the fibre was not damaged in the dissection procedure, and also to monitor if the fluorescence signal had reached a non-decaying stable value.

### *Mechanical and optical set-up*

The bundle of 2–4 fibres was attached by tweezer clips to a force transducer. A square cuvette, with an inner diameter of 1 mm, was then positioned around the bundle of fibres. The cuvette allowed the fibres to be bathed and solutions changed without any movement of the fibres. The cuvette was positioned under a fluorescence microscope, such that fluorescence and force could be measured simultaneously. The fluorescence microscope and force transducer set-up have been previously described (Guth & Wojciechowski, 1986). The light from a 100 watt HBO super pressure mercury lamp (Osram), filtered with a 340 nm band pass interference filter (Schott) for $STnC_{DANZ}$ or with a 320 nm band pass interference filter for $CTnC3(C84)_{IAANS}$, was directed onto the $STnC_{DANZ}$ or $CTnC3(C84)_{IAANS}$-reconstituted fibres, respectively. The emitted light was collected through the microscope objective and filtered by an OG-515 cut off filter (Schott) for $STnC_{DANZ}$-reconstituted fibres or an SPK-7 450 nm band pass filter for $CTnC3(C84)_{IAANS}$-reconstituted fibres before being converted to a voltage signal by the photomultiplier. A reference signal was obtained from a photodiode mounted in front of the excitation lamp such that it recorded the excitation light after passing through the fibre. This signal was necessary to eliminate noise caused by fluctuations in the excitation light intensity and was used for ratio calculations of the fluorescence signal vs reference signal.

### *Sarcomere length determination*

Sarcomere length of skeletal fibres was measured for each skeletal fibre immersed in the pCa 8 solution immediately after mounting the fibre to the transducer. The length of the sarcomere was calculated from the diffraction pattern of the illuminated fibre (by a Spectra Physics 155 Helium–Neon laser) according to the following Equation:

$$\text{Sarcomere Length} = \lambda \cdot l/x$$

where $l$ = fibre to observation screen distance, $\lambda$ = laser wavelength (632.8 nm), and $x$ = 0–1st order spacing distance. The average sarcomere length of skeletal muscle fibres was 2.4 ± 0.1 μm. Due to the non-uniform arrangement of cardiac fibres it was not possible to accurately measure the sarcomere length before each experiment. In order to adjust each cardiac muscle preparation to a consistent starting point, we adjusted the resting preload tension to a minimum (zero tension) and then increased the measured length of each preparation by 20%. In skeletal muscle, this change in length resulted in a sarcomere length of ~2.4 μm.

### *Simultaneous fluorescence and force measurements*

Once the endogenous TnC was replaced by either $STnC_{DANZ}$ or $CTnC3(C84)_{IAANS}$ simultaneous fluorescence and force measurements were performed. When both the fluorescence and force reached a stable level for each pCa condition, the solution was changed by flow through the cuvette and the next pCa values of force and fluorescence were determined. Data were normalized to the maximal values of either force or fluorescence and fitted to the Hill equation.

### *Data analysis*

Force and fluorescence curves were fitted to the Hill equation (Hill, 1910):

$$Y_H = 100 \times [Ca^{2+}]^n / ([Ca^{2+}]^n + [Ca^{2+}]_{50}^n)$$

where $Y_H$ is the relative force or fluorescence change expressed as a percentage of the maximal change, $[Ca^{2+}]$ is the free $Ca^{2+}$ concentration, $[Ca^{2+}]_{50}$ is the $Ca^{2+}$ concentration which produces a 50% change in force or fluorescence, and n is the Hill coefficient. The fluorescence signal was collected for each 'pCa' solution and averaged to reduce the noise.

## Results

*Fluorescently labelled skeletal TnC ($STnC_{DANZ}$) and cardiac TnC ($CTnC(C84)_{IAANS}$)*

As was shown by Johnson and colleagues (1978), labelling of skeletal TnC (STnC) with dansylaziridine results in specific labelling of Met-25 located in the $NH_2$-terminus of STnC. Therefore, the binding of $Ca^{2+}$ to the regulatory sites (I and II) of STnC can be monitored by the change (increase) of fluorescence originating from $STnC_{DANZ}$. The binding of $Ca^{2+}$ to sites I and II of $STnC_{DANZ}$ was determined in the reconstituted skinned skeletal muscle fibres, and these measurements were simultaneously collected with the measurements of steady state isometric force.

In order to monitor binding of $Ca^{2+}$ to cardiac TnC (CTnC), a monocysteine CTnC mutant was created (Putkey *et al.*, 1989) in which cysteine 35 was changed to serine. CTnC3(C84) was specifically labelled with IAANS at Cys 84. $CTnC(C84)_{IAANS}$ was shown to undergo a fluorescence increase upon binding of $Ca^{2+}$ to the $Ca^{2+}$-specific site II in CTnC (Putkey *et al.*, 1989). As in the skeletal fibres, the change in fluorescence of $CTnC3(C84)_{IAANS}$-reconstituted cardiac preparation reflected binding of $Ca^{2+}$ to site II of CTnC3- $(C84)_{IAANS}$.

*Simultaneous fluorescence and force measurements*

In Figure 1 we show the protocol for the simultaneous measurements of force and fluorescence from TnC-depleted skinned skeletal or cardiac fibres reconstituted with fluorescently labelled skeletal or cardiac TnCs. Extraction of endogenous TnC from the fibres was completed within 30 min (Fig. 1A) followed by the decrease of isometric force to about 20% of the initial level of unextracted fibres (residual force). Reconstitution of the TnC-depleted fibres with fluorescently labelled TnCs was monitored by the subsequent increase of isometric force to $85 \pm 5\%$ of that of the control fibres (Fig. 1A). As we show in Figure 1B and C, the increase of steady state isometric force was paralleled by the increase of fluorescence intensity originating from $STnC_{DANZ}$ (Fig. 1B) or $CTnC3(C84)_{IAANS}$ (Fig. 1C). As the concentration of free $Ca^{2+}$ increased (from pCa 8 to pCa 4) the force development paralleled the increase of fluorescence intensity, achieving a maximum at pCa 5.2 for skeletal fibres (Fig. 1B) and pCa 4 for the cardiac preparation (Fig. 1C). However, the fluorescence–pCa dependence of the skeletal and cardiac fibres plateaued at pCa 5. Transferring of the reconstituted fibres to the low $[Ca^{2+}]$ solution ($10^{-8}$ M) resulted in the drop of either force or fluorescence for both types of muscle.

*Effect of acidosis on the $Ca^{2+}$ dependence of force and fluorescence*

The effect of the decrease of pH from 7.0 to 6.5 on the force/fluorescence–pCa relationships are shown in Figures 2 and 3. Figure 2 represents data obtained for skeletal fibre experiments where $STnC_{DANZ}$ was used for the fibre reconstitution, whereas Figure 3 shows the effect of the change in pH on the cardiac muscle preparation reconstituted with CTnC3-$(C84)_{IAANS}$. Both types of muscle demonstrate the same tendency of the pH decrease-induced rightward shift in the force/fluorescence relationships to higher $[Ca^{2+}]$. Skeletal muscle fibres bathed in the solution containing 1 mM $MgCl_2$ demonstrated the rightward shift of the force–pCa dependence (by $pCa_{50} = -0.33 \pm 0.07$) which was paralleled by the rightward shift of the fluorescence–pCa relationship by $pCa_{50} = -0.38 \pm 0.1$ (Fig. 2 and Table 1). Cardiac preparations also showed a parallel rightward shift in the force–pCa and fluorescence–pCa relationships by $pCa_{50} = -0.45 \pm 0.13$ and $-0.61 \pm 0.15$, respectively (Fig. 3 and Table 1). The effect of acidosis on the change in both force and fluorescence expressed in $pCa_{50}$ units was respectively 36% and 61% higher for cardiac muscle fibres as compared to the skeletal fibres.

The character of the force/fluorescence–pCa curves was also different for both types of muscles. The cooperativity of the force/fluorescence–pCa dependence of the cardiac preparation was much lower than that of the skeletal fibres (Table 2). The respective Hill coefficients for the fitted curves of the force–pCa relationships at pH 7.0 and 6.5 were 1.2 and 1.1 for cardiac preparation and 2.8 and 2.6 for skeletal muscle fibres. The same was true for the fluorescence–pCa dependence (Table 2).

We also tested the effect of acidosis (pH 7.0 to pH 6.5) on the $Ca^{2+}$ dependence of force and fluorescence in the skeletal muscle fibres exposed to solutions containing higher $Mg^{2+}$ concentrations (5 mM). As shown in Table 1 and Figure 4, a decrease in pH resulted in the parallel rightward shift of force–pCa and fluorescence–pCa dependence, however the change was lower at 5 mM $Mg^{2+}$ as compared to the skeletal fibres at 1 mM $Mg^{2+}$. The shift of the force–pCa relationship expressed in $pCa_{50}$ was $-0.27 \pm 0.04$ for 5 mM $Mg^{2+}$ while for 1 mM $Mg^{2+}$ it was $pCa_{50} = -0.33 \pm 0.07$ and the fluorescence–pCa dependence was shifted by $-0.22 \pm 0.12$ at 5 mM $Mg^{2+}$ and $-0.38 \pm 0.1$ in the solution of 1 mM $Mg^{2+}$ (Table 1).

*Effect of alkalinization on force–pCa and fluorescence–pCa*

The effect of alkalinization was studied in the cardiac muscle preparation reconstituted with

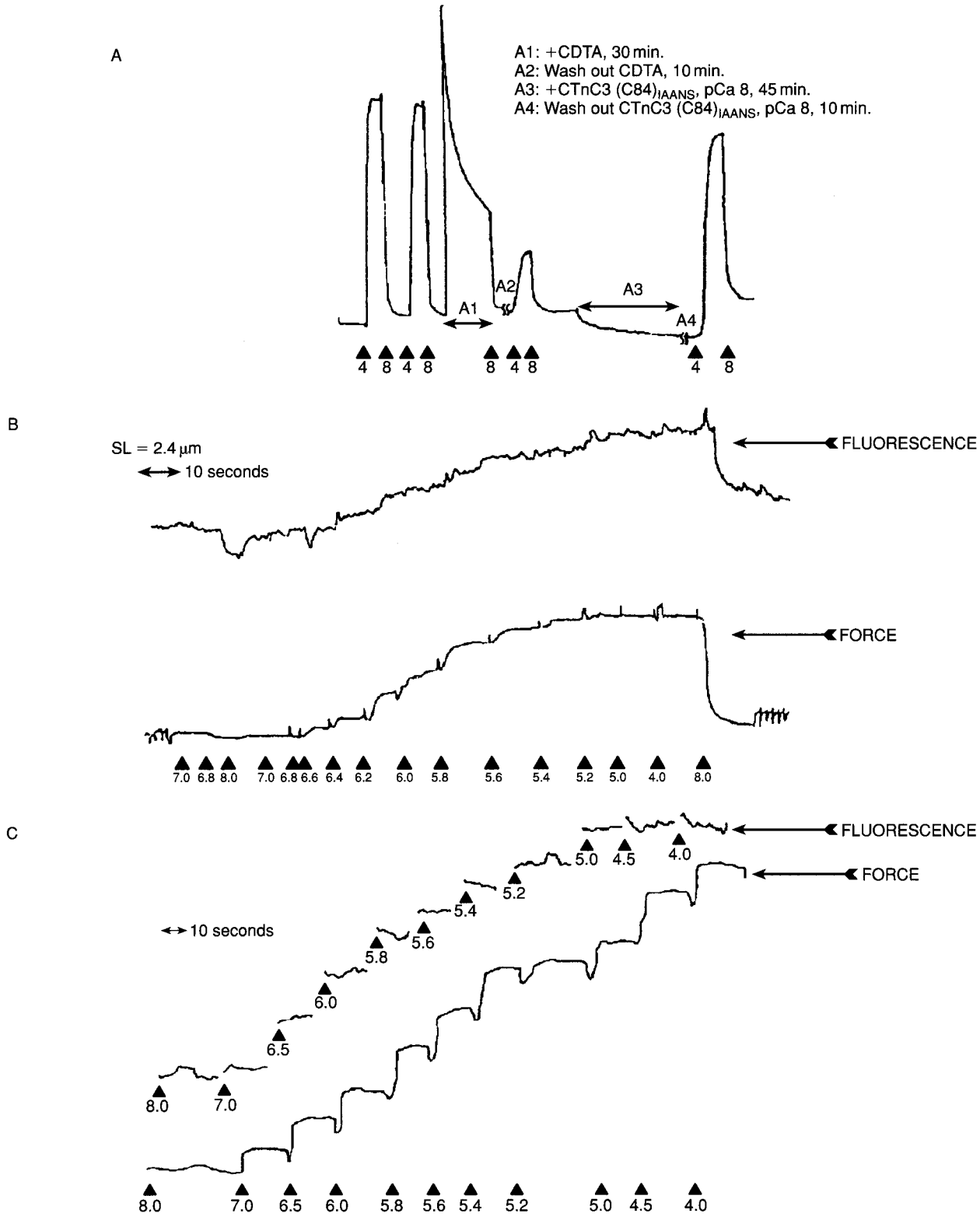


**Fig. 1.** Measurements of steady-state force and fluorescence for skeletal or cardiac muscle fibres reconstituted with $STnC_{DANZ}$ and $CTnC3(C84)_{IAANS}$, respectively. (A) The protocol for $Ca^{2+}$ dependent force development in the cardiac muscle preparation as described under Materials and methods. Briefly, Triton X-100 extracted porcine cardiac ventricular fibres were mounted on force transducer clips and tested for maximal force at pCa 4 (left side of A). CTnC was extracted with CDTA (A1), and after washing with pCa 8 solution (A2), residual force was determined. CTnC-depleted fibres were incubated with $CTnC3(C84)_{IAANS}$ (A3), and finally tested for $CTnC3(C84)_{IAANS}$ reincorporation (right side of A). (B), (C) Parallel fluorescence and force measurements of skeletal and cardiac preparations, respectively. The pCa values are indicated below the graphs by black triangles.

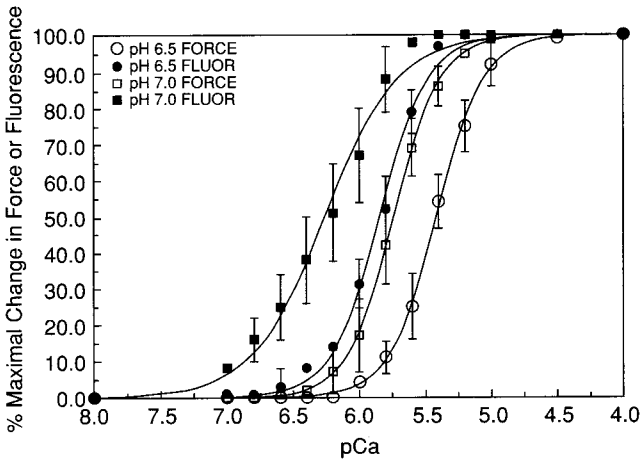


**Fig. 2.** The effect of a decrease in pH from 7.0 to 6.5 on the $Ca^{2+}$ dependence of force and fluorescence for skeletal fibres reconstituted with $STnC_{DANZ}$. Simultaneous force and $STnC_{DANZ}$ fluorescence were measured at 1 mM $Mg^{2+}$ as described under Materials and methods. The data were plotted as the mean of eight experiments ± SE. No error bars are shown when the SE is less than or equal to the size of the symbol. The solid curves represent data fitted to the Hill equation.

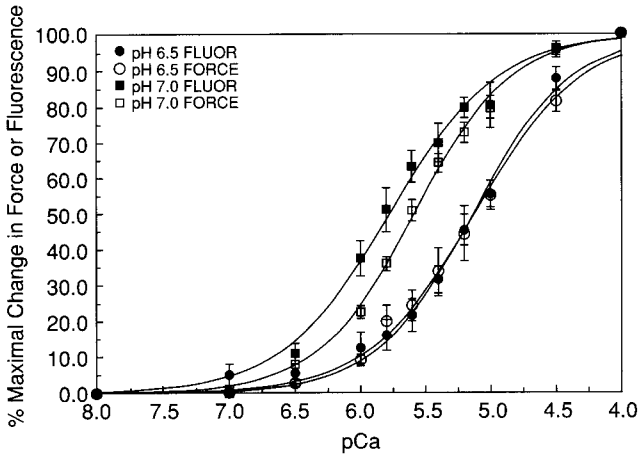


**Fig. 3.** The effect of a decrease in pH on the $Ca^{2+}$ dependence of force and fluorescence for a cardiac muscle preparation reconstituted with $CTnC3(C84)_{IAANS}$ at 1 mM $Mg^{2+}$. Simultaneous force and $CTnC3(C84)_{IAANS}$ fluorescence were measured as described under Materials and methods. The data of eight experiments were analysed and plotted as described in Fig. 2. No error bars were shown when the SE was less than or equal to the size of the symbol.

**Table 1.** Average $pCa_{50}$ values (± SE) for skinned fibre experiments as described under Materials and methods.

| *Tissue* | *pH* | *[$Mg^{2+}$] (mM)* | *Force $pCa_{50}$* | *Fluorescence $pCa_{50}$* | *Fluorescence minus force* |
|---|---|---|---|---|---|
| Skeletal | 7.0 | 1.0 | 5.75 ± 0.04 | 6.23 ± 0.07 | 0.48 ± 0.11 |
| Skeletal | 6.5 | 1.0 | 5.42 ± 0.03 | 5.85 ± 0.03 | 0.43 ± 0.06 |
| Shift of $pCa_{50}$ | – | – | −0.33 ± 0.07* | −0.38 ± 0.10* | – |
| Skeletal | 7.0 | 5.0 | 5.42 ± 0.02 | 5.63 ± 0.06 | 0.21 ± 0.08 |
| Skeletal | 6.5 | 5.0 | 5.15 ± 0.02 | 5.41 ± 0.06 | 0.26 ± 0.08 |
| Shift of $pCa_{50}$ | – | – | −0.27 ± 0.04* | −0.22 ± 0.12* | – |
| $Cardiac_1$ | 7.0 | 1.0 | 5.58 ± 0.04 | 5.76 ± 0.08 | 0.18 ± 0.12 |
| $Cardiac_1$ | 6.5 | 1.0 | 5.13 ± 0.09 | 5.15 ± 0.07 | 0.02 ± 0.16 |
| Shift of $pCa_{50}$ | – | – | −0.45 ± 0.13* | −0.61 ± 0.15* | – |
| $Cardiac_2$ | 7.0 | 1.0 | 5.70 ± 0.03 | 5.80 ± 0.04 | 0.10 ± 0.07 |
| $Cardiac_2$ | 7.5 | 1.0 | 5.98 ± 0.08 | 5.99 ± 0.04 | 0.01 ± 0.12 |
| Shift of $pCa_{50}$ | – | – | 0.29 ± 0.11* | 0.19 ± 0.08* | – |

*Indicates significant ($p < 0.05$, Student's *t*-test) shifts in $pCa_{50}$ (± SE) caused by the changes in pH. $CARDIAC_1$ and $CARDIAC_2$ indicate data obtained for the shift in pH from 7.0 to pH 6.5 and from pH 7.0 to pH 7.5, respectively.

**Table 2.** Average Hill coefficient (*n*) values ± SE for the skinned fibre experiments.

| *Tissue* | *pH* | *[$Mg^{2+}$] (mM)* | *Hill coefficients for force curves* | *Hill coefficients for fluorescence curves* |
|---|---|---|---|---|
| Skeletal | 7.0 | 1.0 | 2.8 ± 0.3 | 2.0 ± 0.2 |
| Skeletal | 6.5 | 1.0 | 2.6 ± 0.1 | 2.5 ± 0.1 |
| Skeletal | 7.0 | 5.0 | 3.1 ± 0.1 | 2.8 ± 0.4 |
| Skeletal | 6.5 | 5.0 | 3.1 ± 0.2 | 2.4 ± 0.1 |
| $Cardiac_1$ | 7.0 | 1.0 | 1.2 ± 0.06 | 1.1 ± 0.05 |
| $Cardiac_1$ | 6.5 | 1.0 | 1.1 ± 0.06 | 1.2 ± 0.09 |
| $Cardiac_2$ | 7.0 | 1.0 | 1.9 ± 0.13 | 1.6 ± 0.19 |
| $Cardiac_2$ | 7.5 | 1.0 | 1.5 ± 0.14 | 1.4 ± 0.06 |

$CARDIAC_1$ and $CARDIAC_2$ indicate paired data of the shift in pH 7.0 to pH 6.5 and pH 7.0 to pH 7.5, respectively.

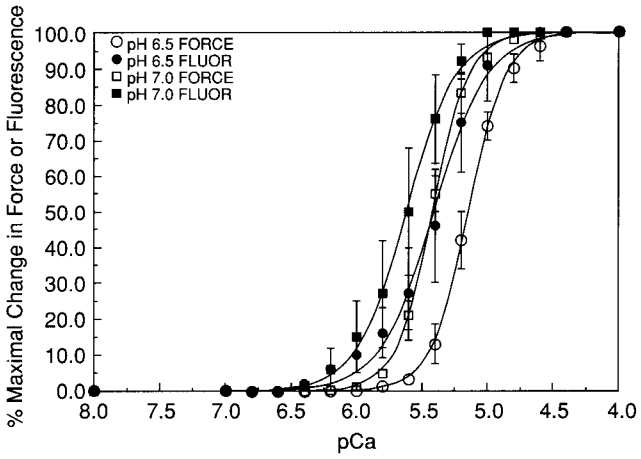


**Fig. 4.** The $Ca^{2+}$ dependence of skeletal fibre force and $STnC_{DANZ}$ fluorescence at pH 7.0 and pH 6.5 at 5 mM $Mg^{2+}$. Simultaneous force and $STnC_{DANZ}$ fluorescence were measured as described under Materials and methods. The data were plotted as the mean of six experiments ± SE. The solid lines represent the best fit to the Hill equation.

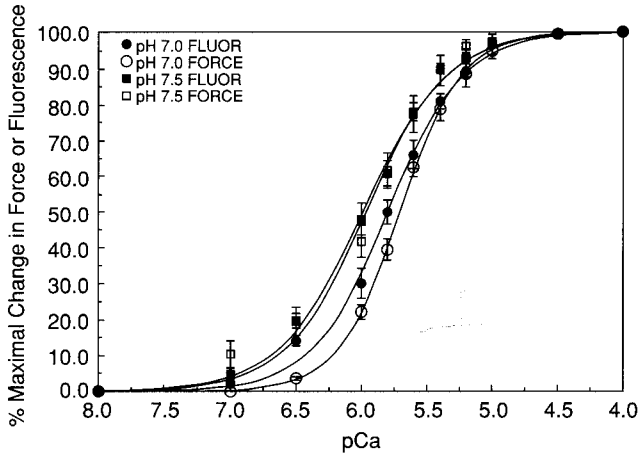


**Fig. 5.** The effect of an increase in pH (from 7.0 to 7.5) on the $Ca^{2+}$ dependence of force and fluorescence in cardiac muscle preparations reconstituted with $CTnC3(C84)_{IAANS}$. Simultaneous force and $CTnC3(C84)_{IAANS}$ fluorescence were measured at pH 7.0 and at pH 7.5 at 1 mM $Mg^{2+}$, as described under Materials and methods. The data were plotted as the mean of five experiments ± SE. Solid curves represent the best fit to the Hill equation.

$CTnC3(C84)_{IAANS}$. Unlike in the case of acidosis, a leftward shift in both force–pCa and fluorescence–pCa dependence was observed upon the change in pH from pH 7.0 to pH 7.5 (Fig. 5). The changes in $pCa_{50}$ were 0.29 ± 0.11 and 0.19 ± 0.08 for the force development and fluorescence curves, respectively (Table 1).

*Effect of the change in pH on maximal force and fluorescence responses in skeletal and cardiac muscle fibres*

Table 3 demonstrates single experiments on the effect of a change in pH (from 7.0 to 6.5 or from 7.0 to 7.5) on the maximal force and fluorescence measured at high $Ca^{2+}$ concentration (pCa 4) in skeletal and cardiac muscle fibres. No statistics were obtained in this comparison performed on the same skeletal or cardiac preparations at different pH and $[Mg^{2+}]$. As pH changed from 7.0 to 6.5, the decrease of maximal force was observed for both skeletal and cardiac fibre preparations exposed to the solution containing 1 mM $Mg^{2+}$. The percent ratio of the maximal force at pH 6.5 to the force at pH 7.0 was 59.8% and 70.0% for skeletal and cardiac fibres, respectively (Table 3), while at the same time, there was no change in the observed fluorescence signal. In addition, no change in fluorescence measurements was seen upon the shift in pH from 7.0 to 7.5, yet there was a 58% increase in the maximal force.

**Table 3.** Ratios (%) of force (pCa 4) and fluorescence of skeletal (at 1 mM and 5 mM $Mg^{2+}$) and cardiac (at 1 mM $Mg^{2+}$) preparations obtained for different pH values.

| *Tissue* | *$[Mg^{2+}]$ (mM)* | *Maximal force ratio (%)* | *Maximal fluorescence ratio (%)* |
|---|---|---|---|
| Skeletal (pH 6.5/pH 7.0) | 1.0 | 59.8 | 100.4 |
| Skeletal (pH 6.5/pH 7.0) | 5.0 | 73.8 | 100.6 |
| $Cardiac_1$ (pH 6.5/pH 7.0) | 1.0 | 70.0 | 100.0 |
| $Cardiac_2$ (pH 7.5/pH 7.0) | 1.0 | 158 | 100.0 |

SKELETAL and $CARDIAC_1$ indicate the percentage ratios at pH 6.5/pH 7.0. $CARDIAC_2$ indicates the same ratios at pH 7.5/pH 7.0. The values represent the data from single paired experiments of fluorescence/force measured at pH 7.0 to pH 6.5 and at pH 7.0 to 7.5.

*Effect of BDM on force and fluorescence measurements*

In order to estimate the contribution of strongly-bound crossbridges to the pH-induced change in the $Ca^{2+}$ affinity of cardiac TnC, simultaneous force and fluorescence originating from the CTnC3(C84)$_{IAANS}$-reconstituted cardiac preparation were measured in the presence or absence of BDM. 2,3-Butanedione monoxime has been shown to decrease the number of the strongly bound crossbridges and to stabilize the weakly attached state (Higuchi & Takemori, 1988; Bagni *et al.*, 1992; Zhao & Kawai, 1994; Kagawa *et al.*, 1995). As shown in Figure 6A, the BDM-treated fibres developed about 20–27% of the maximal (pCa 4)

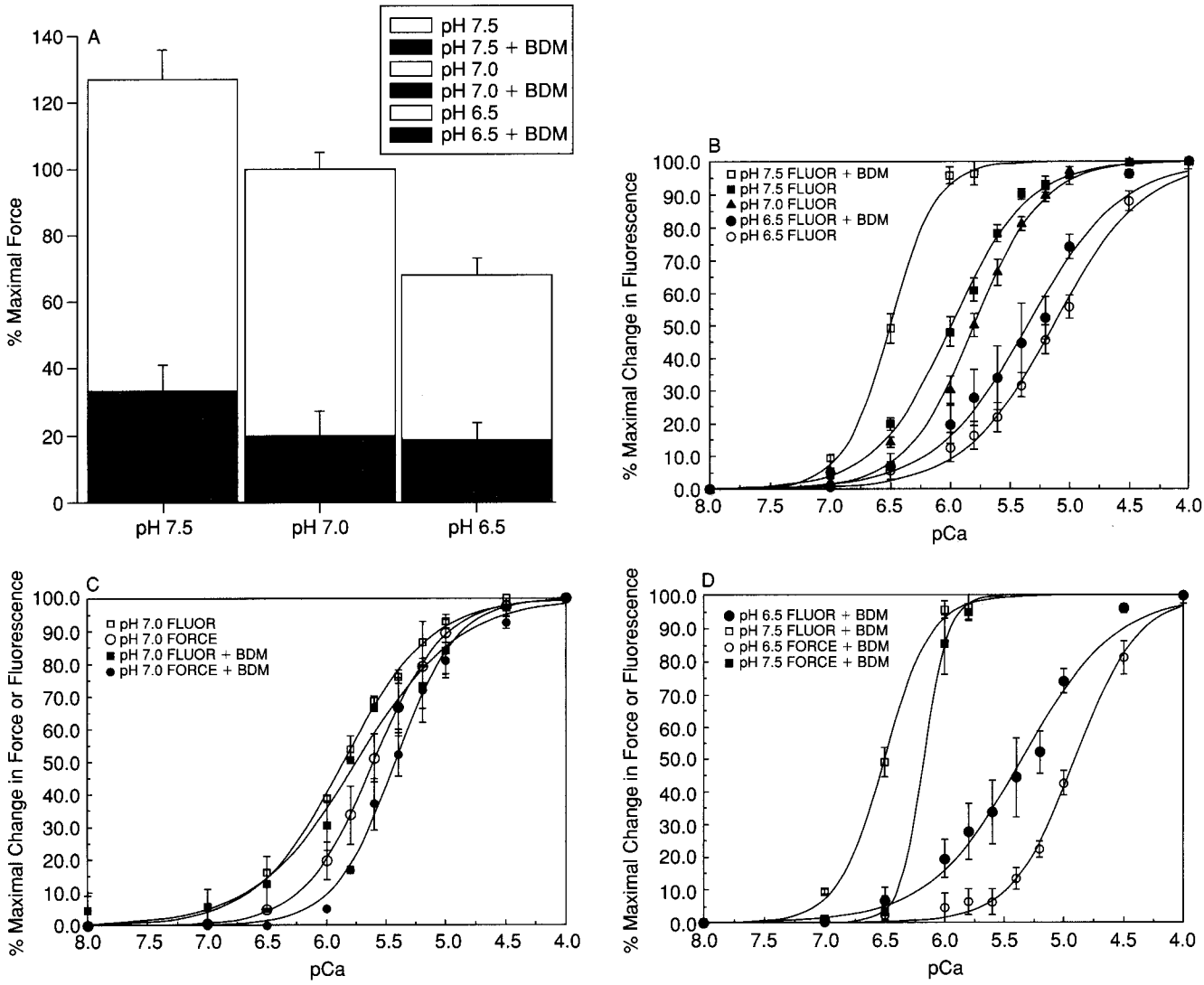


**Fig. 6.** (A) The effect of BDM on the maximal force in cardiac muscle preparation reconstituted with CTnC3(C84)$_{IAANS}$. The experiments were performed at pH 7.5, 7.0 and 6.5. The data were plotted as the mean of three experiments ± SE. (B) The effect of BDM on the $Ca^{2+}$ dependence of fluorescence in cardiac muscle preparations reconstituted with CTnC3(C84)$_{IAANS}$. CTnC3(C84)$_{IAANS}$ fluorescence was measured in the absence and presence of 30 mM BDM at pH 7.5 and pH 6.5 at 1 mM $Mg^{2+}$ (the fluorescence–pCa dependence determined at pH 7.0 is also included as a reference) as described under Materials and methods. The data from three experiments were normalized as a percentage of the maximal response and plotted as a mean ± SE. The solid curves represent the best fit to the Hill equation. (C) The effect of BDM on the $Ca^{2+}$ dependence of force and fluorescence of cardiac muscle preparations reconstituted with CTnC3(C84)$_{IAANS}$. Simultaneous force and CTnC3(C84)$_{IAANS}$ fluorescence were measured in the presence and absence of 30 mM BDM at pH 7.0 and at 1 mM $Mg^{2+}$ as described under Materials and methods and in Fig. 6B. (D) The effect of BDM on the $Ca^{2+}$ dependence of force and fluorescence of cardiac muscle preparations reconstituted with CTnC3(C84)$_{IAANS}$. Simultaneous force and CTnC3(C84)$_{IAANS}$ fluorescence were measured in the presence of 30 mM BDM at pH 7.5 and pH 6.5 at 1 mM $Mg^{2+}$ as described under Materials and methods and in Fig. 6B and C. The data from three experiments were normalized as a percentage of the maximal response and plotted as a mean ± SE. The solid curves represent the best fit to the Hill equation.

steady state force observed for untreated fibres at pH 7.5, pH 7.0, and pH 6.5. Under the same experimental conditions the maximal fluorescence intensity of BDM-treated fibres bathed in the pCa 4 solution did not change as compared to untreated fibres (data not shown). As shown in Figure 6B and C, the effect of BDM on the fluorescence–pCa dependence was not significant at pH 7.0 or 6.5. However, at pH 7.5 the BDM-treated fibres were more sensitive to $Ca^{2+}$ than the untreated fibres, and the fluorescence–pCa relationship was shifted towards lower $Ca^{2+}$ concentrations by $pCa_{50} = 0.5 \pm 0.18$ (Fig. 6B). Interestingly, the net shift in fluorescence or force brought about by the change in pH from 7.5 to 6.5 was larger in the presence (Fig. 6D) than in the absence (Table 1; Figs 3 and 5) of BDM. As shown in Figure 6B and D, the fluorescence–pCa dependence changed by $\sim pCa_{50} = -1.15 \pm 0.17$ for BDM-treated fibres transferred from pH 7.5 to pH 6.5 solution compared to a change in $pCa_{50}$ of approximately $-0.86 \pm 0.08$ for untreated fibres. Similarly, the force-pCa dependence was shifted by $pCa_{50} = -1.27 \pm 0.19$ in the presence of BDM as compared with a change of $pCa_{50} = -0.87 \pm 0.09$ for the untreated cardiac preparation (data are the mean ± SE of three experiments). This large net effect of the BDM treatment was primarily due to the leftward shift in the force/fluorescence–pCa dependence at pH 7.5. For example, the BDM treatment at this pH (7.5) resulted in an increase in the $Ca^{2+}$ sensitivity of fluorescence by as much as $pCa_{50} = 0.5 \pm 0.12$ (Fig. 6B). This suggests that in the absence of strongly bound crossbridges (+BDM) increasing pH resulted in strengthened $Ca^{2+}$ binding, possibly due to ionization of side chains which affects some component(s) in the $Ca^{2+}$ binding process, e.g. thin or thick filament proteins, etc. The presence of strongly bound crossbridges (−BDM) attenuates these effects. In summary, the BDM experiments demonstrate that the pH-induced changes in the fluorescence–pCa relationship are primarily related to direct changes in the $Ca^{2+}$ affinity to the regulatory site of CTnC.

## Discussion

Results presented in this study show that the changes in the $H^+$ concentration within the muscle fibres affect the affinity of the regulatory sites of TnC for $Ca^{2+}$. The drop in pH from 7.0 to 6.5 resulted in a rightward shift towards higher $Ca^{2+}$ concentrations of the $Ca^{2+}$-dependence of force development in skeletal and cardiac muscle preparations. The pH-dependent changes in the contractility of cardiac and skeletal muscle have been observed also by others (Fabiato & Fabiato, 1978; Robertson & Kerrick, 1979; El-Saleh & Solaro, 1988; Solaro *et al.*, 1988; Gulati & Babu, 1989; Hofmann *et al.*, 1993; Kawashima *et al.*, 1995) but the mechanism responsible for these effects has not yet been solved. In this study we tested the hypothesis that the observed decreases in the sensitivity of the force–pCa relationship in muscle fibre preparations can be directly related to the lowered $Ca^{2+}$ affinity of the $Ca^{2+}$ specific regulatory sites (I and II) of TnC. To accomplish this we utilized simultaneous measurements of force development and $Ca^{2+}$ binding. The parallel shift of both the force–pCa dependence and the $Ca^{2+}$ binding to the regulatory sites in TnC indicated that acidic pH affects the binding of $Ca^{2+}$ to the regulatory sites of TnC in muscle. A drop in pH from 7.0 to 6.5 resulted in a significant decrease in the $Ca^{2+}$ sensitivity of force development for skeletal fibres and the cardiac muscle preparation. The pH dependent decrease in $Ca^{2+}$-sensitivity of striated myofilaments was also observed by Ball and colleagues (1994), Wattanapermpool and colleagues (1995), Ding and colleagues (1995) and Kawashima and colleagues (1995) but the molecular mechanism of diminished $Ca^{2+}$ sensitivity of steady-state force in acidic conditions has not been confirmed. In the studies of Kawashima and colleagues (1995) it has been proposed that troponin plays an important role in the pH-induced decrease in the $Ca^{2+}$ sensitivity of the myofibrillar ATPase activity, with slow skeletal myofibrils being more resistant to these effects than fast skeletal muscles. Moreover, consistent with our observations, it has been shown that acidic pH induces a greater reduction of $Ca^{2+}$ sensitivity in cardiac muscle than in skeletal muscle (Palmer & Kentish, 1994; Ding *et al.*, 1995). The authors of the latter paper suggest that the different response of skeletal and cardiac muscle to the change in pH may be linked to the TnC–TnI interaction. They postulate the dominant role of CTnI in the overall acidotic mechanism (Ding *et al.*, 1995). The TnI contribution to pH-sensitivity was first shown by El-Saleh and Salaro (1988) and later confirmed by Wattanapermpool and colleagues (1995). Our results suggest that the mechanism responsible for the myofilament deactivation to $Ca^{2+}$ upon acidosis may be directly related to the altered transmission of the TnC–$Ca^{2+}$ signal, which triggers contraction in striated muscle. Similar effects of the altered ability to bind $Ca^{2+}$ upon decreased pH were also observed in solution studies, where acidic pH decreased the $Ca^{2+}$ binding to both cardiac and skeletal TnC (Robertson & Kerrick, 1979; Ogawa, 1985; El-Saleh & Solaro, 1988; Iida, 1988; Palmer *et al.*, 1994).

Decreasing pH has also been shown to greatly decrease the maximal force in skinned muscle fibres (Fabiato & Fabiato, 1978; Solaro *et al.*, 1988; Gulati & Babu, 1989; Metzger & Moss, 1990). As was shown by Guth and Potter (1987), strongly attached cross-bridges increase the affinity of the $Ca^{2+}$-specific sites

of TnC by ~tenfold. Thus, the pH-dependent drop in force, which results from a decrease in the number of strongly attached cycling crossbridges, would also be expected to affect the affinity of TnC for $Ca^{2+}$. The BDM experiments were designed to address this question directly. By decreasing the number of strongly attached crossbridges (+BDM) we were able to observe the direct effect of the change in pH on the $Ca^{2+}$ affinity to the regulatory sites of TnC. The finding that BDM had little effect on the pH-induced shift in either the $Ca^{2+}$ dependence of force or fluorescence between pH 7.0 and 6.5 suggests that the primary effect of pH is on the affinity of the $Ca^{2+}$ specific sites for $Ca^{2+}$. Interestingly, at pH 7.5, the BDM treatment resulted in a profound leftward shift towards lower $[Ca^{2+}]$ in the fluorescence–pCa dependence compared with the untreated cardiac muscle preparation. This is the opposite of what one would expect and suggests an independent mechanism that appears to involve an ionization of some side chains, only at alkaline pH. It is not clear where these side chains are located, e.g. thin or thick filaments, but suggests a new line of inquiry.

Interestingly, increasing $[Mg^{2+}]$ concentration from 1 to 5 mM attenuated the pH-dependent shift in force–pCa relationship and also fluorescence–pCa dependence. This attenuation by $Mg^{2+}$ was also observed by others (Donaldson *et al.*, 1978) in skinned skeletal muscle fibres.

In summary, we have shown that the $Ca^{2+}$ affinity of the regulatory sites (I and II) of TnC strongly depends on pH. The pH dependence could arise from the change of the total charge of the protein which varies with pH due to the presence of titratable side chains (Linse & Forsen, 1995). Increased pH (from 7.0 to 7.5) could result in strengthened $Ca^{2+}$ binding due to higher negative charge and a decrease in pH (from 7.0 to 6.5) could lead to lower $Ca^{2+}$ binding due to the decrease in the negative charge of the $Ca^{2+}$ loop. The higher $H^+$ concentration (lower pH) has been suggested to decrease the reactivity of all $Ca^{2+}$ coordinating oxygens within the regulatory $Ca^{2+}$ specific sites of TnC (Linse & Forsen, 1995). It is also possible that higher $H^+$ concentrations decrease the interaction of TnC with the target proteins (TnI, TnT) and therefore decrease the affinity of $Ca^{2+}$ binding to TnC. The free energy coupling of $Ca^{2+}$ binding is higher when TnC is complexed with other proteins (Potter & Gergely, 1975; Linse & Forsen, 1995). Reduced $Ca^{2+}$ affinity may thus result from the higher exposure of hydrophobic protein surfaces to the solvent.

In conclusion, we have demonstrated that the pH effect is primarily related to the direct change in the $Ca^{2+}$ affinity to the $Ca^{2+}$-specific site(s) of CTnC and STnC in both cardiac and skeletal muscle fibres.

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