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The steady-state force–Ca^{2+} relationship in intact lobster (*Homarus americanus*) cardiac muscle

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Abstract The heart of the decapod crustacean is activated by regular impulse bursts from the cardiac ganglion. The cardiac pump function depends on ganglionic burst frequency, burst duration, and burst impulse frequency. Here, we activated isolated lobster cardiac ostial muscle (*Orbicularis ostii* muscle, OOM) by stimulus trains in vitro in order to characterize the response of the contractile apparatus to $[Ca^{2+}]_i$. We employed stimulus trains that generate a steady state between the $[Ca^{2+}]_i$ and force in order to estimate the Ca^{2+} sensitivity of myofilaments. Force and $[Ca^{2+}]_i$ transients were simultaneously recorded using a silicon strain gauge and the fluorescence of iontophoretically microinjected fura-2 salt. We examined the effects of tetanus duration (TD), the interval between trains, and 6 μM cyclopiazonic acid, an inhibitor of the SR Ca^{2+} pump, on the steady-state force–$[Ca^{2+}]_i$ relationship. The instantaneous force–$[Ca^{2+}]_i$ relationships appeared sigmoidal (EC_{50} and Hill coefficient, 98.8 ± 32.7 nM and 2.47 ± 0.20, mean ± SD, respectively), as did the curves superimposed after 500 ms following the start of stimulation, indicating that the force–$[Ca^{2+}]_i$ relationship had reached a steady state at that time. Also, the maximum activated force (F_{max}) was estimated using the steady-state force–$[Ca^{2+}]_i$ relationship. Prolonged stimulus trains, decreasing the interval between recurrent trains from 5 to 2.5 s, and cyclopiazonic acid each increased the measured EC_{50} without changing F_{max}. The EC_{50} correlated strongly with averaged $[Ca^{2+}]_i$ over time. We conclude that the steady-state force–$[Ca^{2+}]_i$ relationships in the OOM indicate cooperation between force generation and Ca^{2+} binding by the myofilaments. Our data also suggest the existence of a novel Ca^{2+}-dependent mechanism which reduces Ca^{2+} sensitivity and accelerates relaxation of lobster cardiac muscle myofilaments.

Keywords *Homerus americanus* · *Orbicularis ostii* muscle · Force–$[Ca^{2+}]_i$ relationship · Train interval · Tetanus duration

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Introduction

The force–$[Ca^{2+}]_i$ relationship in lobster heart is unknown. Cardiac muscle of lobster (*Homarus americanus*) contracts following a burst of action potentials from the cardiac ganglion in vivo (Cooke 2002), and can be tetanized in vitro by a train of stimuli (Yazawa et al. 1999). Cardiac cells respond to the depolarization by a Ca^{2+} influx through the cell membrane and Ca^{2+} release by the sarcoplasmic reticulum, followed by Ca^{2+} reuptake by the sarcoplasmic reticulum and extrusion through the cell membrane (Shinozaki et al. 2002). Contraction and relaxation of these cells are, in turn, determined by the time course and magnitude of the resulting intracellular Ca^{2+} transient and by the response of the contractile apparatus to $[Ca^{2+}]_i$. Here, we employed stimulus trains that generate a steady state between $[Ca^{2+}]_i$ and force in order to estimate the Ca^{2+} sensitivity of myofilaments.

Interaction between Ca^{2+} and troponin-C (Tn-C) triggers a cross-bridge attachment that is responsible for force generation. Activation of striated muscle is modulated by the Ca^{2+} sensitivity of Tn-C. Estimation of myofilament Ca^{2+} sensitivity using skinned fibers requires a steady state of force, muscle length, and Ca^{2+} (Kentish et al. 1986). The slower kinetics of the cross-bridge cycling than those of $[Ca^{2+}]_i$ cycling during the cardiac twitch prevent a steady state of force and $[Ca^{2+}]_i$. Hence, slowing $[Ca^{2+}]_i$ cycling during force generation or during relaxation would allow the establishment of a steady state between force and $[Ca^{2+}]_i$ and permit a measurement of myofilament Ca^{2+} sensitivity in intact cardiac muscle. This was previously achieved by slowing relaxation using ryanodine (Gao et al. 1994) and cyclopiazonic acid (CPA; Dobrunz et al. 1995). These authors reported a close similarity between the steady-state force–$[Ca^{2+}]_i$ relationships and the instantaneous force–$[Ca^{2+}]_i$ relationship during the relaxation phase. Furthermore, it has been shown that isolated mouse trabeculae exhibit a leftward shift of the force–$[Ca^{2+}]_i$ relationship in response to increased stimulus rate (Gao et al. 1998). The purpose of this study was to compare the force–$[Ca^{2+}]_i$ relationship in lobster heart muscle with that of mammalian heart muscle (ter Keurs et al. 2000) and to explore factors that may affect the sensitivity of the filaments to Ca^{2+}.

We developed a new preparation of isolated lobster cardiac muscle (the *Orbicularis ostii* muscle, OOM) to measure $[Ca^{2+}]_i$ and force simultaneously (Shinozaki et al. 2002). We have shown that both $[Ca^{2+}]_i$ and force of tetanic contraction in OOM reached steady levels at stimulus trains of tetanus duration (TD) ≥500 ms. Therefore, stimulation of the intact OOM with stimulus trains (TD≥500 ms) should allow measurement of its steady-state force–$[Ca^{2+}]_i$ relationship. Here, we have analyzed the instantaneous force–$[Ca^{2+}]_i$ relationship during both steady force and relaxation. We demonstrate the effect of $[Ca^{2+}]_i$ itself on Ca^{2+} sensitivity of myofilaments.

Materials and methods

Preparation

Cardiac muscles of ostial valves (OOM) were isolated as previously reported (Shinozaki et al. 2002) and kept in standard lobster saline (in mM: 479.6 NaCl, 15.7 KCl, 25.9 $Ca^{2+}Cl_2$, 9.3 $MgSO_4$, 5 Tris, adjusted to a pH of 7.3 with HCl and aerated). After overnight storage at 4 °C, the ends of OOM were attached to a force transducer and a micromanipulator and mounted in a perfusion bath on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan). Platinum electrodes mounted in the sides of the bath were used for electrical stimulation. Stimuli consisted of 500 ms TD of 0.2-ms pulses delivered at 50 Hz, 50% above threshold, at intervals of 5 s in standard lobster saline at 14 °C.

Force and length measurements

The force was measured using a silicon strain gauge (AE801, AME, Sensonor, Horten, Norway). The muscles were allowed to stabilize during stimulation (D4030 stimulator, Digitimer, Elwyn Garden City, UK) for 1 h at 120% of the slack length (1.68 ± 0.34 mm, mean ± SD, $n = 9$). Sarcomere length and thick and thin filament lengths were measured from electron micrographs using the methods that have been described previously (Yazawa et al. 1999). In brief, to prepare tissues for transmission electron microscopy, ostial muscles were fixed in primary fixative for 60 min at room temperature at unloaded length, at a length where the valve had been stretched (by ~40%) to eliminate waviness of the edges, and further stretched by 20% to the length at which the mechanical experiments were performed. The ostial muscles were then immersed in secondary fixative for 60 min at 0 °C, dehydrated, and embedded in Ladd LX-112 epoxy resin. Ultrathin sections (80 nm) were cut parallel to the long axis of the fibers with a diamond knife on a Sorvall MT-6000 ultramicrotome and stained with uranyl acetate and lead citrate. The sections were viewed and photographed with a Hitachi H-7000 transmission electron microscope operated at 75 kV, and calibrated with a carbon replica of a diffraction grating (2,158 lines mm^{-1}).

Electron micrographs (31,050×) of a typical OOM fixed at slack length showed a sarcomere length of 1.63 ± 0.04 and 2.72 ± 0.06 μm ($n = 25$) at the stretched length. Thick filament length was 2.30 ± 0.08 μm ($n = 75$) at all sarcomere lengths and thin filament was 1.49 ± 0.14 μm ($n = 10$). The Z-band in the OOM was 0.14 ± 0.001 μm and the bare zone was 0.39 ± 0.03 μm. Both thick and thin filaments in OOM were substantially longer than in lobster myocardium (thick filament 1.31 ± 0.03 μm, thin filament 0.77 ± 0.3 μm; $n = 10$). These filament lengths predict a plateau of the force–sarcomere length relationship from 3.12–3.51 μm for the OOM (ter Keurs et al. 2000). Our previous work (Yazawa et al. 1999) has indeed shown that the OOM shows an ascending limb of the force–length relationship at extensions up to 20% of length at which the muscle was just taut. Hence, the experiments in this study were performed near optimal sarcomere length.

Fluorescence measurements

Fluorescence measurements employed were similar to those described by Shinozaki et al. (2002). Briefly, the OOM was illuminated with UV light from a 50-W xenon arc lamp (model 6255, Oriel, Stanford, CT, USA) using band-pass filters (Melles Griot, Irvine, CA, USA) at 340

and 380 nm via a 10× UV-Fluor objective lens (Nikon, Tokyo, Japan) using a dichroic mirror (400 DPLC, Omega Opticals, Brattleboro, VT, USA). The fluorescence from the muscle was band-pass filtered (Melles Griot, Irvine, CA, USA) at 510 nm and collected by a photomultiplier tube (PMT; Hamamatsu, Japan). The PMT output (V/lm fluorescence) and force signals (V/N; calibrated using standard weights) were stored in a personal computer (Gateway 2000, North Sioux City, SD, USA) using an AD converter (2801A, Data-Translation, Marlborough, MA, USA).

The free salt form of fura-2 was microinjected electrophoretically into the OOM as reported (Shinozaki et al. 2002). After subtraction of autofluorescence of the OOM measured before fura-2 loading, $[Ca^{2+}]_i$ was calculated according to the following equation (Grynkiewicz et al. 1985):

$$\left[Ca^{2+}\right]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R) \quad (1)$$

where K_d is the effective dissociation constant, R is the ratio of fluorescence at 340-nm excitation over fluorescence at 380-nm excitation, R_{min} is R at zero $[Ca^{2+}]$, and R_{max} is R at saturating $[Ca^{2+}]$. β is the ratio of the fluorescence for Ca^{2+}-free dye to fluorescence with Ca^{2+} bound to the dye at 380 nm. Values for K_d, R_{min}, R_{max}, and β were determined by in vitro calibration; they were 492, 3.45, 0.14, and 8.18 nM, respectively (Shinozaki et al 2002).

Experimental protocol

Muscles were driven with electrical stimulus trains at TD of 500 ms unless indicated otherwise. To investigate the force–$[Ca^{2+}]_i$ relationship, force and $[Ca^{2+}]_i$ were simultaneously recorded in the following protocol: to test the effect of TD, TD was changed from 100 ms to 300, 500, and 1,000 ms in a stepwise manner ($n = 4$). In another five muscles, TD was fixed at 500 ms to test the effect of train interval, which was changed from 5 to 2.5 s ($n = 1$). Data were recorded 2 min after TD or train intervals had been changed, when force had reached a new steady state. To study the effect of elimination of Ca^{2+} by the sarcoplasmic reticulum (SR), a specific inhibitor of the SR Ca^{2+} pump, 60 μM cyclopiazonic acid (CPA; Sigma Chemical, St Louis, MO, USA) was applied to lobster saline ($n = 1$). Application of CPA required 30 min for a new steady state to become established. A stock solution of CPA (330 mM in DMSO) was used to prepare lobster saline containing 60 μM; the final concentration of DMSO in the saline was 0.01%. This DMSO concentration had no effect on force development (data not shown).

Data analysis and statistics

All recordings were sampled at 500 Hz and stored in a computer. The stored force data were filtered by 100 Hz low pass procedure using MATLAB software (Math Works, Natick, MA, USA). Digitized Ca^{2+} data were averaged to eliminate high-frequency noise using an 11-point running-average procedure in the following analysis. Force was plotted against corresponding $[Ca^{2+}]_i$ (phase–plot analysis). To examine force–$[Ca^{2+}]_i$ relationships in the phase–plot analysis, data were fitted using SigmaPlot (Jandel Scientific, CA, USA) with the following Hill equation:

$$\text{Force} = F_{max} \times \left(\left(\left[Ca^{2+}\right]_i \right)^n / \left[\left(\left[Ca^{2+}\right]_i \right)^n + EC_{50}^n \right] \right) \quad (2)$$

where EC_{50} is the Ca^{2+} concentration giving half of the maximum force activation, n is a Hill coefficient and F_{max} is the maximum activated force. Both EC_{50} and n as well as F_{max} were obtained from the fitted curves. Results are expressed as mean ± SD. Linear regression was used to analyze the relation between EC_{50} and averaged $[Ca^{2+}]_i$. A paired t-test was used to compare mean values. Differences with $p < 0.05$ were considered significant.

Results

Peak $[Ca^{2+}]_i$ and force of tetanic contraction increased with TD, reaching the maximum at 500 ms of TD (Fig. 1A, B). Force plotted against instantaneous $[Ca^{2+}]_i$ at varied TD (100–500 ms) demonstrated superimposable sigmoid curves during relaxation (Fig. 1C). This observation, together with the observation that the force–$[Ca^{2+}]_i$ data of plateau force during tetani at TD≥500 ms lie on the same curve, suggest that the force–$[Ca^{2+}]_i$ relationship reflects a steady state. The steady-state force–$[Ca^{2+}]_i$ relationship shifted slightly rightward at TD of 1,000 ms (Fig. 1C). However, calculated F_{max} at 500 ms of TD was not different from that at 1,000 ms of TD (1.42 ± 0.38 and 1.45 ± 0.35 mM, respectively, $n = 4$). Parameters of the force–$[Ca^{2+}]_i$ relationships at TD of 500 ms are shown in Table 1 ($n = 9$), where calculated F_{max} exceeded measured peak force by 7.2%.

Shortening the train interval from 5 to 2.5 s changed neither resting $[Ca^{2+}]_i$ nor resting force and caused a small decrease in peak $[Ca^{2+}]_i$ and active force (Fig. 2A, B), but accelerated the rate of force decline from 143 to 126 ms without a change in the rate of $[Ca^{2+}]_i$ decline (136 to 139 ms, respectively). Shortening the train interval further from 2.5 to 1 s increased resting $[Ca^{2+}]_i$ due to incomplete decline of $[Ca^{2+}]_i$ with a small decrease in peak $[Ca^{2+}]_i$ (Fig. 2A). In contrast, peak force decreased to 73% at 1-s train intervals with a small increase in resting force. Shortening of train intervals shifted the steady-state force–$[Ca^{2+}]_i$ relationship rightward (EC_{50}: 75, 85, and 130 nM in 5-, 2.5-, and 1-s train intervals, Fig. 2C).

CPA slowed the rate of decline of $[Ca^{2+}]_i$ and force (Shinozaki et al. 2002), and shifted the steady-state force–$[Ca^{2+}]_i$ relationship rightward (EC_{50}: from 99 to 250 nM) and increased the steepness of the relationship

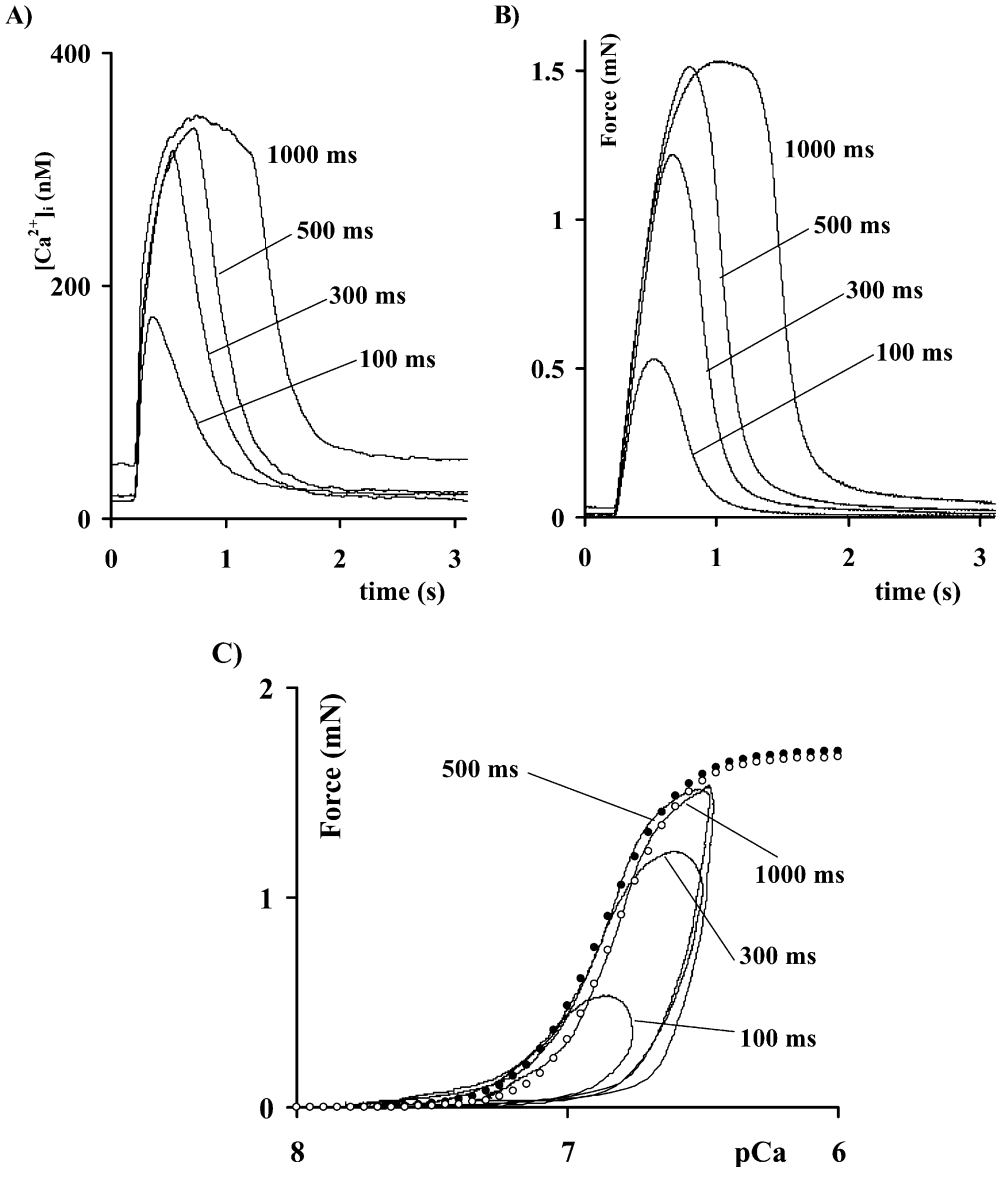

Fig. 1 The effects of varied train duration (TD) on **A** $[Ca^{2+}]_i$ transients and **B** force of tetanic contraction. **C** Force plotted against corresponding $[Ca^{2+}]_i$ expressed as pCa at varied TD. The *superimposed sigmoidal curves* (*closed circles* and *open circles* at 500 and 1,000 ms TD, respectively) are the best fit of a Hill equation, where F_{max}, EC_{50}, and a Hill coefficient are 1.70 and 1.67 mN, 135 and 150 nM, and 3.1 and 3.5, respectively

markedly (Hill coefficient: from 2.1 to 4.0) without change in F_{max} (Fig. 3). CPA increased resting $[Ca^{2+}]_i$ and resting force with stable recordings for more than 1 h. A similar rightward shift was found when we analyzed the data of force development and $[Ca^{2+}]_i$ in the OOM after exposure to ryanodine and caffeine (Shinozaki et al. 2002). The latter interventions led to an increase in resting $[Ca^{2+}]_i$ (from 20 to 40 and 50 nM, respectively) and a rightward shift of the force–$[Ca^{2+}]_i$ relationship (0.36 and 0.57 pCa units respectively; curves not shown).

Table 1 Steady-state force–$[Ca^{2+}]_i$ relationship[a] at constant tetanus duration of 500 ms

	Peak force (mN)	F_{max} (mN)	EC_{50} (nM)	n
Mean	1.27	1.37	98.8	2.47
SD	0.32	0.38	32.7	0.20

[a] F_{max} Maximum F calculated using the Hill equation, EC_{50} calcium concentration required for half-maximum force, n Hill coefficient, *SD* standard deviation. Number of muscles = 9

The rightward shift of the steady-state force–$[Ca^{2+}]_i$ relationship by these interventions appeared to relate to an increase in a time-averaged $[Ca^{2+}]_i$. To test this hypothesis, EC_{50} was plotted against averaged $[Ca^{2+}]_i$ over time in the muscles tetanized at both 500 and 1,000 ms of TD with 5-s train interval and at TD of 500 ms with 2.5- and 1-s train intervals and in the muscle treated with CPA. EC_{50} of myofilaments was positively correlated to averaged $[Ca^{2+}]_i$ over time ($r = 0.74$, $p < 0.005$, Fig. 4), suggesting an influence of $[Ca^{2+}]_i$ itself on EC_{50}.

Discussion

The present study shows for the first time the instantaneous force–$[Ca^{2+}]_i$ relationship in lobster cardiac

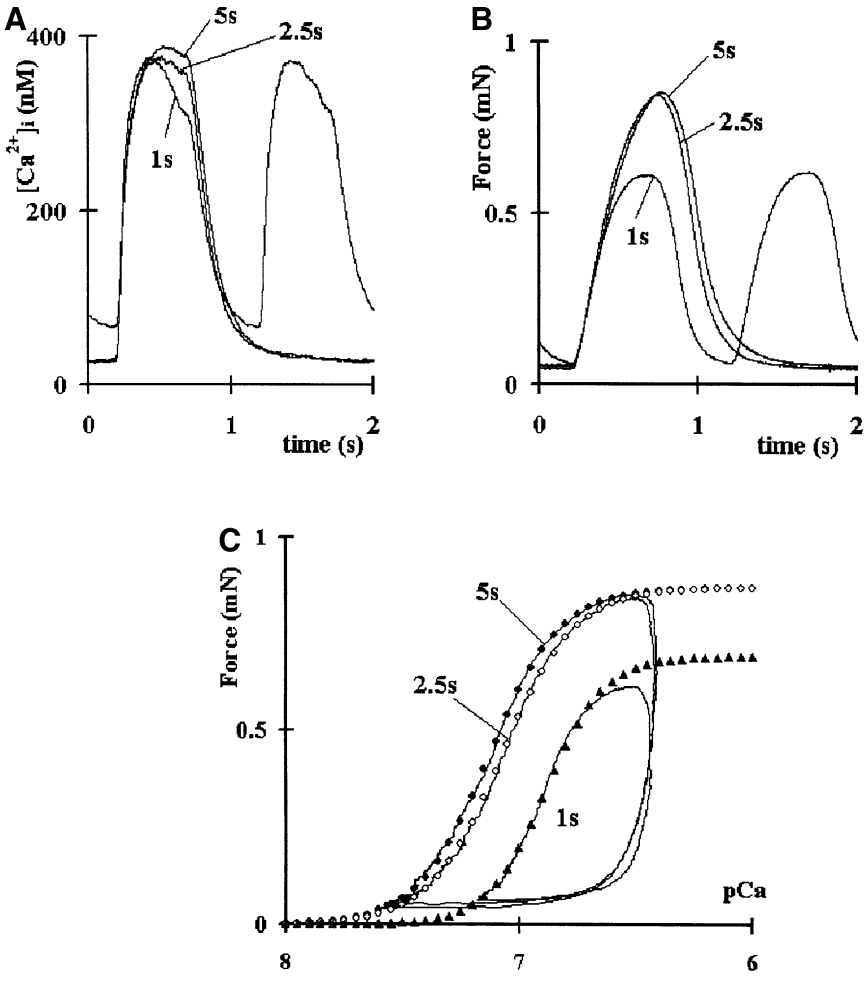

Fig. 2 The effects of varied stimulus train intervals on **A** $[Ca^{2+}]_i$ transients and **B** force of tetanic contraction (TD = 500 ms; 5-, 2.5-, and 1-s intervals). **C** Force plotted against corresponding $[Ca^{2+}]_i$ expressed as pCa in 5-, 2.5-, and 1-s intervals. The *superimposed sigmoidal curves* (*closed circles* for 5-s intervals, *open circles* for 2.5-s intervals and *closed triangles* for 1-s intervals) are the best fits of a Hill equation, where F_{max} is 0.87, 0.87, and 0.69 mN; EC_{50} is 75, 85, and 130 nM; and the Hill coefficient is 2.85, 2.80, and 3.5, respectively

muscle. The instantaneous force–$[Ca^{2+}]_i$ relationship during tetanic contraction at varied TD (100–500 ms) showed that the steady-state data could be superimposed on the data obtained during relaxation. This indicates that 500 ms after the start of the contraction, a steady state of the force–$[Ca^{2+}]_i$ relationship had been reached, and this persisted during subsequent relaxation. Characterization of the steady-state force–$[Ca^{2+}]_i$ relationships showed a Hill coefficient between 2 and 4 and a large variation of the observed EC_{50} for Ca^{2+}. Furthermore, an increase in time-averaged $[Ca^{2+}]_i$ appeared to be responsible for a rightward shift of the steady-state force–$[Ca^{2+}]_i$ relationship without a change in F_{max}, suggesting the existence of a Ca^{2+}-dependent mechanism, which itself regulates the Ca^{2+} sensitivity of myofilaments.

Evaluation of the steady-state force–$[Ca^{2+}]_i$ relationship in intact mammalian cardiac muscles requires tetanic contractions elicited in the presence of CPA or ryanodine—to eliminate the function of the SR—together with non-physiologically elevated $[Ca^{2+}]_o$ (Gao et al. 1994), which may change the intracellular milieu, e.g. pH (Hoerter et al. 1986). In addition, in mammalian myocytes a Ca^{2+} leak out of the SR induced by ryanodine increases Ca^{2+} uptake by the SR Ca^{2+} pump, leading to excessive ATP consumption and production of inorganic phosphate. The latter may decrease Ca^{2+} sensitivity of the contractile filaments (Kentish 1986). In this study, we made use of the fact that the OOM can be tetanized without any drugs and can generate the F_{max} at physiological $[Ca^{2+}]_o$ (25.9 mM; Shinozaki et al. 2002). Thus, the OOM is a preparation naturally suited to analysis of factors that influence the steady-state force–$[Ca^{2+}]_i$ relationship.

In the present study, the instantaneous force–$[Ca^{2+}]_i$ relationship during relaxation at varied TD (100∼500 ms) showed overlapping sigmoidal relationships ∼500 ms after the onset of force development (Fig. 1C). The force–$[Ca^{2+}]_i$ data points of the steady state during contractions ≥500 ms decreased according to this relationship. These observations suggest that a steady state between force and $[Ca^{2+}]_i$ is achieved in ∼500 ms of tetanic contraction and after that force and $[Ca^{2+}]_i$ decline in keeping with a pseudo-equilibrium. This is consistent with the assumption that the rate of $[Ca^{2+}]_i$ extrusion from cytosol under these conditions is slower than the dissociation rate of Ca^{2+} from Tn-C, similar to intact rat trabeculae treated with CPA (Dobrunz et al. 1994). Our previous work (Shinozaki et al. 2002) suggests that the rate of $[Ca^{2+}]_i$ decline in the

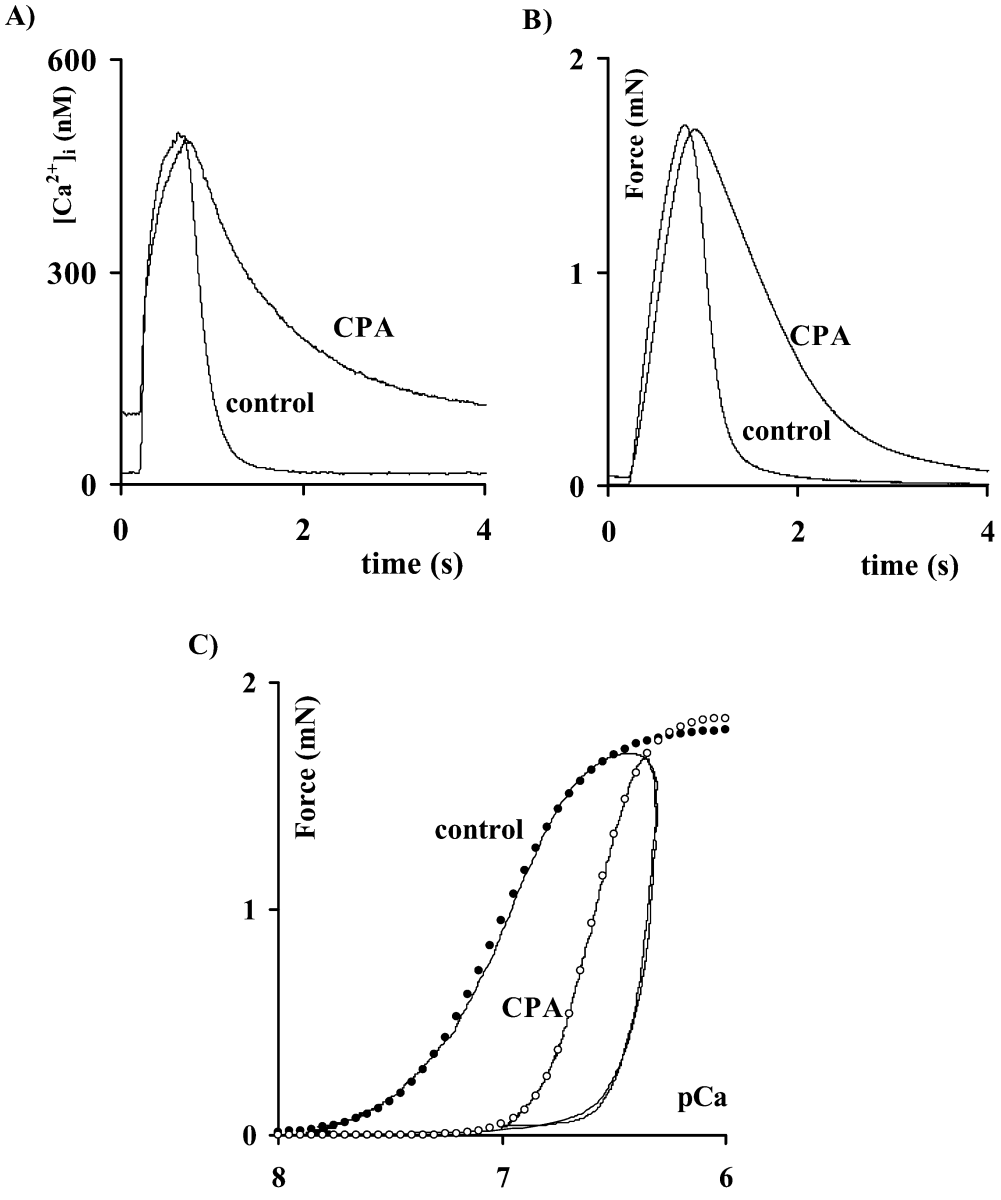

Fig. 3 The effects of cyclopiazonic acid (CPA) on **A** $[Ca^{2+}]_i$ transients and **B** force of tetanic contraction at TD = 500 ms; *control* indicates the response to stimulation in the drug-free state. **C** Force plotted against corresponding $[Ca^{2+}]_i$ expressed as pCa before and after application of CPA. The *superimposed sigmoidal curves* (*closed circles* in control and *open circles* in CPA) are the best fits of a Hill equation, where F_{max} is 1.80 and 1.88 mN, EC_{50} is 43.7 and 114.8 nM, n is 2.31 and 3.99, respectively

OOM is faster than that in mammalian cardiac trabeculae even after correction by the Q_{10} (time constant: 180 ms at 14 °C in the OOM and 300 ms at 26 °C in the rat; Stuyvers et al. 1997). Hence, the above observation, together with a rapid $[Ca^{2+}]_i$ decline in the OOM, suggest that the dissociation of Ca^{2+} from Tn-C in lobster heart is faster than that in mammalian heart.

The contractile filaments in OOM appear to be more sensitive to Ca^{2+} (Table 1; EC_{50} ~0.1 μM) compared to those of intact rat cardiac muscle in our laboratory (EC_{50} 0.5–1 μM; ter Keurs et al. 2000) and in other laboratories (Gao et al. 1994; Backx et al. 1995). This conclusion is consistent with the observation that skinned skeletal muscle of crayfish is more sensitive to Ca^{2+} (EC_{50} 0.6 μM; Stephenson and Williams 1980) than mammalian skeletal muscle (EC_{50} 1–10 μM; Kerrick et al. 1980). These differences in EC_{50} between crustaceans and mammals might be explained by structural differences in Tn-C. Lobster skeletal muscle Tn-C has only 5% amino acid sequence homology with other phyla (Garone et al. 1991). The significance of differences in the composition of the cytosol between the OOM and mammalian cardiac muscle to the observed higher Ca^{2+} sensitivity in this study remains to be investigated.

Tn-C of lobster skeletal muscle has one Ca^{2+}-specific binding site (Nakamura et al. 1994). Hence, the observed large (2–4) Hill coefficient suggests cooperation between Ca^{2+} binding to Tn-C and the steps that lead to force development in OOM. Thus, even though the structure of the Tn-C of crustacean and mammalian heart differs (Garone et al. 1991), a cooperative mechanism between force generation and Ca^{2+} binding to cardiac Tn-C seems to be common among species.

Interventions that increased time-averaged $[Ca^{2+}]_i$, such as an increase in TD, a decrease in train interval, or

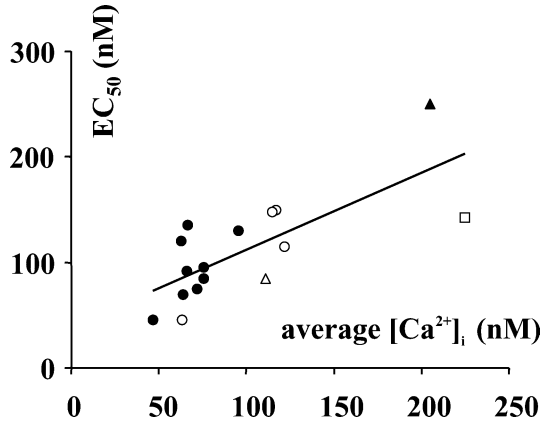

Fig. 4 EC_{50} of the steady-state force–$[Ca^{2+}]_i$ relationship was plotted against averaged $[Ca^{2+}]_i$ over time during the different protocols. *Closed circles* and *open circles* indicate data of train stimulation at 5-s intervals at 500 and 1,000 ms TD, respectively. *Closed triangles* indicate data after application of CPA at 5-s intervals at 500 ms TD. *Open triangles* and *open squares* indicate data of train stimulation at 2.5- and 1-s intervals at 500 ms TD, respectively. *Linear regression line* is superimposed on the data ($r = 0.74$, $p < 0.005$)

application of CPA (as well as ryanodine and caffeine) shifted the steady-state force–$[Ca^{2+}]_i$ relationship rightward without change in F_{max}. CPA itself does not change Ca^{2+} sensitivity in mammalian cardiac muscle (Hollander 1995). The strong correlation between EC_{50} and averaged $[Ca^{2+}]_i$ over time (Fig. 4) suggests that $[Ca^{2+}]_i$ itself may modulate Ca^{2+} sensitivity of myofilaments.

It is unlikely that the use of fura-2 caused a confounding effect in these observations. Fura-2 may bind to cytosolic proteins, slowing the binding kinetics of fura-2 to free Ca^{2+} ion (Baylor and Hollingworth 1988). However, this laboratory has shown that the K_d of fura-2 for Ca^{2+} in rat cardiac muscle in vitro and in vivo are similar (Backx and ter Keurs 1993). In addition, an increase in averaged $[Ca^{2+}]_i$ over time increased both the rate of $[Ca^{2+}]_i$ decline and the rate of force decline in the OOM (Shinozaki et al. 2002), indicating that the change in fura-2 signal reflected a true change in $[Ca^{2+}]_i$.

The EC_{50} estimated for the OOM in these experiments may deviate slightly from the true EC_{50} if sarcomere shortening influences Ca^{2+} sensitivity of lobster cardiac muscle as has been shown for mammals. Sarcomere shortening during contractions at constant OOM length is expected to be modulated by parallel and series elastic structures in the preparation. We could not measure sarcomere length because of light scatter by the perimysium and cannot, therefore, correct for length dependence of Ca^{2+} sensitivity (Kentish et al. 1986). In our preparations, the connective tissue that attached the muscle cells to the force transducer appeared to be stretched by ~5% of the resting muscle length during contraction. Sarcomere length at the plateau of the force–sarcomere length relationship in lobster OOM ranged from 2.34–2.60 μm, which is slightly longer than in mammalian cardiac muscle (Kentish et al. 1986; ter Keurs et al. 2000); hence, 5% shortening would correspond to ~0.13 μm shortening in sarcomere length, which is expected to increase EC_{50} by 0.12 pCa units in intact rat cardiac muscle (Kentish et al. 1986; ter Keurs et al. 2000). There are no data on length-dependent Ca^{2+} sensitivity in lobster cardiac muscle. However, it is unlikely that a similar, small length-dependent change of the EC_{50} can explain the observed variation of the EC_{50} in response to time-averaged changes of $[Ca^{2+}]_i$ in these experiments.

An increase in $[Ca^{2+}]_i$ has been shown to reduce pH_i and to increase P_i (Hoerter et al. 1986), both of which decrease Ca^{2+} sensitivity and F_{max} (Kentish 1986; Solaro et al. 1988; Orchard and Kentish 1990). Increased $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin-dependent (CAM) protein kinase II, leading to intracellular acidosis through activation of Na/H exchanger (Fliegel et al. 1992). These processes, however, probably did not occur in the present experiments because F_{max} did not change. An increase in $[Ca^{2+}]_i$ may activate phosphorylation of myosin light chain kinase by CAM protein kinase II (Morano et al. 1985), leading to an increase in Ca^{2+} sensitivity. Hence, this mechanism cannot explain our results. CAM kinase II could induce cAMP-dependent Tn-I phosphorylation, and thereby decrease Ca^{2+} sensitivity. The iso-enzymes required for this second messenger pathway (Iyengar 1993), however, have not been demonstrated in lobster cardiac tissue.

PKC phosphorylates Tn-I and Tn-T in vitro and reduces Ca^{2+} sensitivity of the myofilaments (Noland et al. 1996). Conversely, inhibition of PKC by chelerythrine increases Ca^{2+} sensitivity of tetanic force markedly in the rabbit heart (Stamm et al. 2001). PKC has been identified in lobster skeletal muscle (Kuo et al. 1996) and the Ca^{2+}-activated form translocates from cytosol to cell membrane, allowing interaction with phosholipid and DAG. If PKC is fully integrated at the cell membrane, it can be activated by an increase in $[Ca^{2+}]_i$ without interaction with DAG (Shabb and Corbin 1992). Thus, activation of PKC by $[Ca^{2+}]_i$ may play a role in the modulation of myofilament Ca^{2+} sensitivity by Ca^{2+} itself.

The rightward shift of the force–$[Ca^{2+}]_i$ relationship may serve a functional role in control of cardiac output by lobster heart in vivo. At rest, the lobster heart is activated ~60 times min^{-1} by bursts of impulses from the cardiac ganglion lasting 200–300 ms. During physical activity of the animal, both the rate of firing of impulse bursts by the cardiac ganglion and the duration of the bursts increase (Cooke 2002). This increased firing rate may lead to an increase in average $[Ca^{2+}]_i$ in cardiac cells and a decrease in myofilament sensitivity to activating Ca^{2+} ions. On the one hand, this effect could mitigate the increase in cardiac force brought about by the increased firing rate and longer impulse bursts generated by the ganglion; on the other hand, this effect could accelerate relaxation (see Fig. 1). Accelerated relaxation could prolong the filling time of the heart and increase cardiac output at the shortened diastolic inter-

val in the active lobster, similar to the effects of adrenergic stimuli in mammalian heart (Bers 2001).

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