

## Cross-bridge interaction kinetics in rat myocardium are accelerated by strong binding of myosin to the thin filament

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1. To determine the ability of strong-binding myosin cross-bridges to activate the myocardial thin filament, we examined the  $\text{Ca}^{2+}$  dependence of force and cross-bridge interaction kinetics at  $15^\circ\text{C}$  in the absence and presence of a strong-binding, non-force-generating derivative of myosin subfragment-1 (NEM-S1) in chemically skinned myocardium from adult rats.
2. Relative to control conditions, application of  $6\ \mu\text{M}$  NEM-S1 significantly increased  $\text{Ca}^{2+}$ -independent tension, measured at pCa 9.0, from  $0.8 \pm 0.3$  to  $3.7 \pm 0.8\ \text{mN mm}^{-2}$ . Furthermore, NEM-S1 potentiated submaximal  $\text{Ca}^{2+}$ -activated forces and thereby increased the  $\text{Ca}^{2+}$  sensitivity of force, i.e. the  $[\text{Ca}^{2+}]$  required for half-maximal activation (pCa<sub>50</sub>) increased from pCa  $5.85 \pm 0.05$  to  $5.95 \pm 0.04$  (change in pCa<sub>50</sub> ( $\Delta\text{pCa}_{50}$ ) =  $0.11 \pm 0.02$ ). The augmentation of submaximal force by NEM-S1 was accompanied by a marked reduction in the steepness of the force–pCa relationship for forces less than  $0.50 P_0$  (maximum  $\text{Ca}^{2+}$ -activated force), i.e. the Hill coefficient ( $n_2$ ) decreased from  $4.72 \pm 0.38$  to  $1.54 \pm 0.07$ .
3. In the absence of NEM-S1, the rate of force redevelopment ( $k_{tr}$ ) was found to increase from  $1.11 \pm 0.21\ \text{s}^{-1}$  at submaximal  $[\text{Ca}^{2+}]$  (pCa 6.0) to  $9.28 \pm 0.41\ \text{s}^{-1}$  during maximal  $\text{Ca}^{2+}$  activation (pCa 4.5). Addition of NEM-S1 reduced the  $\text{Ca}^{2+}$  dependence of  $k_{tr}$  by eliciting maximal values at low levels of  $\text{Ca}^{2+}$ , i.e.  $k_{tr}$  was  $9.38 \pm 0.30\ \text{s}^{-1}$  at pCa 6.6 compared to  $9.23 \pm 0.27\ \text{s}^{-1}$  at pCa 4.5. At intermediate levels of  $\text{Ca}^{2+}$ ,  $k_{tr}$  was less than maximal but was still greater than values obtained at the same pCa in the absence of NEM-S1.
4. NEM-S1 dramatically reduced both the extent and rate of relaxation from steady-state submaximal force following flash photolysis of the caged  $\text{Ca}^{2+}$  chelator diazo-2.
5. These data demonstrate that strongly bound myosin cross-bridges increase the level of thin filament activation in myocardium, which is manifested by an increase in the rate of cross-bridge attachment, potentiation of force at low levels of free  $\text{Ca}^{2+}$ , and slowed rates of relaxation.

The regulation of myocardial contraction is mediated by the binding of  $\text{Ca}^{2+}$  to a low affinity  $\text{Ca}^{2+}$ -specific binding site on troponin C (TnC) (Johnson *et al.* 1980), which alters the interactions of thin filament proteins and allows myosin to bind to actin (Solaro & Rarick, 1998). In skeletal muscle, several studies (reviewed by Lehrer, 1994) have shown that  $\text{Ca}^{2+}$  alone is unable to fully activate the thin filament, i.e. maximal activation of steady force and the rate of force development results from synergistic actions of  $\text{Ca}^{2+}$  and strong-binding myosin cross-bridges. While similar mechanisms are thought to contribute to the activation of heart muscle (Tobacman, 1996), the roles played by strong-binding cross-bridges in the regulation of myocardial contraction are not known.

McKillop & Geeves (1993) have proposed a generally applicable model for the regulation of muscle contraction

in which the thin filament exists in three distinct states: a blocked state that cannot easily bind myosin cross-bridges, a closed state that weakly binds cross-bridges and an open state in which cross-bridges bind strongly and generate force. In this model,  $\text{Ca}^{2+}$  shifts the thin filament from the blocked to the closed state, whereas strong binding of myosin cross-bridges shifts the thin filament from the blocked state through the closed state to the open state. Therefore, complete activation of the thin filament involves several factors including cooperative binding of myosin cross-bridges to actin and cooperative binding of  $\text{Ca}^{2+}$  to the thin filament regulatory protein troponin (reviewed by Moss, 1992). A model proposed by Campbell (1997) suggests that positive cooperativity is a dynamic component of force generation, i.e. at low  $\text{Ca}^{2+}$  levels the rate of activation is slowed

(compared to the rate at high  $\text{Ca}^{2+}$  levels) by progressive co-operative recruitment of myosin cross-bridges into force-generating states. The effects of co-operative cross-bridge binding on the rate of force development would be minimized at high levels of  $\text{Ca}^{2+}$  since  $\text{Ca}^{2+}$  binding would initially recruit most of the available myosin cross-bridges so that few remained for co-operative binding. The  $\text{Ca}^{2+}$  dependence of the rate of force redevelopment ( $k_{tr}$ ) has been reported in skinned skeletal muscle fibres (Brenner & Eisenberg, 1986; Metzger *et al.* 1989) and both skinned (Wolff *et al.* 1995; Palmer & Kentish, 1998) and intact (Baker *et al.* 1998) myocardial preparations. However, the role of co-operative cross-bridge binding in the activation of myocardial force is not yet known.

In the present study, *N*-ethylmaleimide-modified myosin subfragment-1 (NEM-S1), a strong-binding, non-tension-generating derivative of myosin subfragment-1 (Swartz & Moss, 1992), was applied to chemically skinned myocardium to test the idea that the strong binding of myosin cross-bridges co-operatively activates force and the kinetics of force development. If co-operativity in cross-bridge binding to actin contributes to the rate of activation, saturating the co-operative mechanism with NEM-S1 should speed the rate of force redevelopment and reduce or eliminate its dependence on force and  $\text{Ca}^{2+}$  concentration.

## METHODS

### Experimental animals

Female Sprague-Dawley rats (~3 months of age, weighing 200–224 g) were purchased from Harlan Sprague-Dawley (Madison, WI, USA). Rats were housed in groups of three per cage in temperature- and light-controlled quarters and were provided with food and water *ad libitum*. All rats were inspected daily for signs of distress or discomfort throughout the course of the project. Rats were anaesthetized by placing them in a glass bell jar containing room air and 3–4% methoxyflurane and were subsequently killed by a pneumothorax following the establishment of deep anaesthesia (confirmed by the loss of the pedal reflex and muscular tension of the limbs). Animal use was approved by the University of Wisconsin Animal Care Committee.

### Experimental solutions

The compositions of relaxing solution used for the preparation of skinned myocardium and activating solutions used in mechanical measurements were as follows. Relaxing solution contained (mM): KCl, 100; imidazole, 20; MgATP, 4; EGTA, 2; and free  $\text{Mg}^{2+}$ , 1; pH 7.0 at 22°C. Pre-activating solution contained (mM): KCl, 91.9; imidazole, 20; creatine phosphate, 14.5; MgATP, 4; free  $\text{Mg}^{2+}$ , 1; and EGTA, 0.07; pH 7.0 at 15°C and an ionic strength of 180 mM. Activating solution contained (mM): KCl, 79.2; imidazole, 20; creatine phosphate, 14.5; EGTA, 7; MgATP, 4; and free  $\text{Mg}^{2+}$ , 1; with free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{free}$ ) ranging from 1 nM (i.e. pCa 9.0) to 32  $\mu\text{M}$  (i.e. pCa 4.5); pH 7.0 at 15°C and an ionic strength of 180 mM. The compositions of solutions used in the flash photolysis experiments are listed in the legend of Fig. 6. A computer program (Fabiato, 1988) was used to calculate the final concentrations of each metal, ligand and metal–ligand complex using stability constants listed by Godt & Lindley (1982).

### Myocardial preparation

After the rats had been killed, the heart was rapidly excised, placed in a beaker of ice-cold relaxing solution and trimmed free of atria and great vessels. The left ventricle was cut into small pieces and homogenized in ice-cold relaxing solution for 4 s using a Polytron homogenizer to yield multicellular bundles with dimensions of 600–900  $\mu\text{m}$   $\times$  100–180  $\mu\text{m}$ . The homogenate was centrifuged at 120 *g* for 1 min and resuspended in fresh relaxing solution. After the second spin, the pelleted myocardial preparations were chemically skinned by resuspending them for 30 min in fresh relaxing solution containing 1% Triton X-100. The preparations were sedimented by gravity, washed twice with fresh relaxing solution and stored on ice until use.

### Preparation of NEM-S1

Myosin subfragment-1 (S1) was purified from rabbit fast-twitch skeletal muscle and modified with *N*-ethylmaleimide (NEM), as described by Swartz & Moss (1992). While NEM-S1 significantly increases actomyosin ATPase activity in the presence of  $\text{Ca}^{2+}$  (Williams *et al.* 1984; Greene *et al.* 1987), it exhibits little apparent intrinsic ATPase activity (Williams *et al.* 1984). NEM-S1 forms long-lasting complexes with actin in the presence of ATP and in the presence or absence of  $\text{Ca}^{2+}$  (Swartz & Moss, 1992). In this study, the concentration of NEM-S1 was estimated by light absorption at 280 nm (with light-scattering correction performed at 320 nm) using a mass absorptivity value of 0.75 and a molecular weight of 118 000 for S1. Before use, a stock solution of NEM-S1 was dialysed overnight against 20 mM imidazole and 1 mM DTT (pH 7.0). A working solution of NEM-S1 was prepared immediately before use by mixing equal volumes of NEM-S1 stock and a 2 $\times$  stock of pCa 9.0 solution. The NEM-S1 concentration was adjusted to 6  $\mu\text{M}$  by adding the appropriate amount of 1 $\times$  pCa 9.0 solution. Prior to mechanical measurements, skinned myocardial preparations were incubated for 15 min at 15°C in pCa 9.0 solution containing 6  $\mu\text{M}$  NEM-S1. Following 15 min of NEM-S1 treatment, skinned myocardium generated a significant amount of  $\text{Ca}^{2+}$ -independent tension with the rate of force redevelopment ( $k_{tr}$ ) at pCa 9.0 similar to that measured during maximal activation in a solution of pCa 4.5. We observed no further increase in these values when skinned myocardium was treated with NEM-S1 for longer periods (i.e. > 15 min). Although NEM-S1 has little catalytic activity, it still generates phosphate at an appreciable rate (Swartz & Moss, 1992), which would influence both steady-state force and cross-bridge kinetics of skinned myocardium. Therefore, skinned preparations were initially incubated with NEM-S1 in a solution of pCa 9.0 to allow NEM-S1 binding to actin and were subsequently transferred to pre-activating solution for 1 min and then into activating solutions of varying pCa (i.e. pCa 6.6–4.5) without NEM-S1 for 15–45 s to measure steady-state force and the kinetics of cross-bridge interaction. After each mechanical measurement, the preparation was returned to the pCa 9.0 solution containing NEM-S1. Thus, the skinned myocardium was incubated in NEM-S1-free activating solutions for no more than 2 min, during which time negligible amounts of NEM-S1 would be lost since an extensive time (i.e. ~1–2 h) is required to wash out NEM-S1 from the myocardial preparations (data not shown).

### Experimental apparatus

For mechanical measurements, skinned myocardial preparations (dimensions, 600–800  $\mu\text{m}$   $\times$  100–180  $\mu\text{m}$ ) were mounted between a force transducer (model 400A; Aurora Scientific) and a DC torque motor (model 308B; Aurora Scientific) by placing each end (~100  $\mu\text{m}$ ) into stainless steel 25 gauge troughs in an experimental apparatus (Moss, 1979) set on the stage of an inverted microscope (Zeiss). Each myocardial preparation was secured in place by first overlaying each end with a small segment (~1 mm) of 3-0 monofilament suture, which in turn was subsequently tied in place by two loops of 10-0

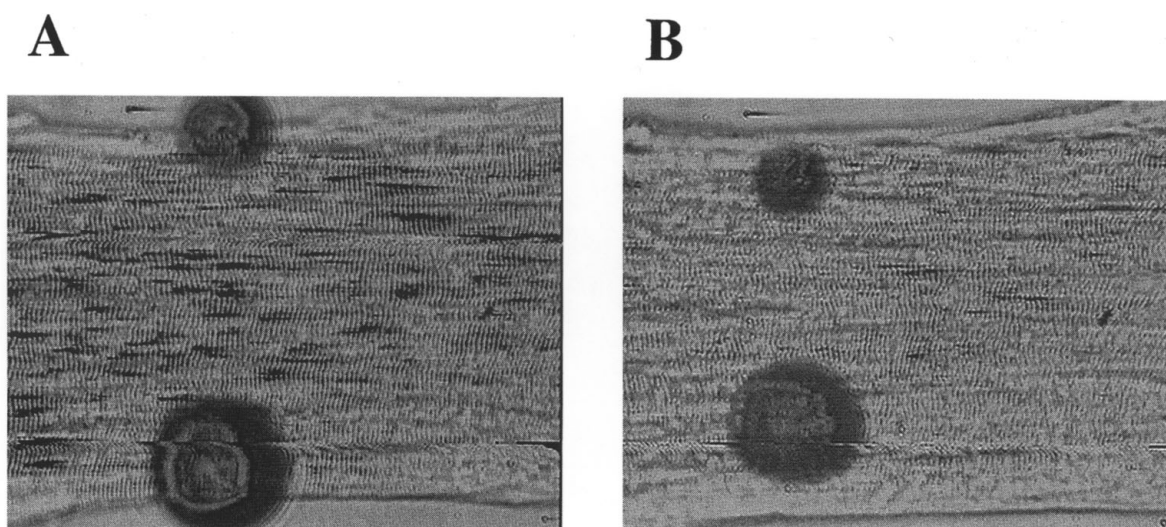
monofilament. Light photomicrographs of a skinned myocardial preparation used in this study are shown in Fig. 1. Length and force signals were digitized at 1 kHz, using a 12-bit A/D converter (model AT-MIO-16F-5; National Instruments Corp.), and displayed and stored on a personal computer using customized software based on LabView Full Development System for Windows (version 5.01; National Instruments Corp.). Length changes were driven by computer-generated voltage commands delivered to the torque motor via a 12-bit D/A converter. All experiments were performed at 15°C with sarcomere length set at  $\sim 2.30 \mu\text{m}$  in relaxing solution. During activation and relaxation, sarcomere length and cell dimensions were recorded on videotape using a video camera (model WV-BL730; Panasonic) and VHS recorder (model SVO-1420; Sony).

### Experimental measurements

**Rate of tension redevelopment.** The rate constant of force redevelopment ( $k_{tr}$ ) in skinned myocardium was assessed using a modification of the protocol described previously by Brenner & Eisenberg (1986). Measurement of  $k_{tr}$  involves a mechanical slack–restretch manoeuvre to dissociate bound cross-bridges from actin in a steadily  $\text{Ca}^{2+}$ -activated preparation. The rate of force redevelopment following the slack–restretch manoeuvre reflects the sum of the rate-limiting steps in the transition of myosin cross-bridges to and from (i.e.  $f_{app}$  and  $g_{app}$ , respectively) the strongly bound, force-generating state in steadily activated preparations (Brenner & Eisenberg, 1986). The myocardial preparation was transferred from relaxing solution to activating solutions of varying pCa (i.e. pCa 6.6–4.5) and allowed to develop steady force. The preparation was then rapidly ( $< 2 \text{ ms}$ ) slackened by 20% of its original length, resulting in an abrupt reduction in force to near zero and a brief period of unloaded shortening ( $\sim 20 \text{ ms}$ ). To further disrupt cross-bridges, the preparation was rapidly ( $< 2 \text{ ms}$ ) restretched to its initial length. A  $k_{tr}$ –pCa relationship was obtained by first measuring  $k_{tr}$  in a solution of pCa 4.5 and subsequently in a series of submaximal pCa solutions between pCa 6.6 and 5.6. To assess any decline in maximal  $k_{tr}$ , the preparation was activated in a

solution of pCa 4.5 at the end of each protocol. The reference maximal  $k_{tr}$  value for successive submaximal activations was interpolated between the initial and final measurements of maximal  $k_{tr}$ . Force redevelopment traces were fitted by a single exponential equation,  $F_t = F_o(1 - e^{-k t})$ , where  $F_t$  is force at any time  $t$  after restretch,  $F_o$  is maximum force, and  $k$  is  $k_{tr}$ . The  $k_{tr}$  values reported here were determined without sarcomere length control and may therefore underestimate the true rate constant of tension redevelopment during maximal activation by 15–20% (Chase *et al.* 1994).

**Force–pCa relationship.** During measurements of  $k_{tr}$ , the skinned myocardial preparation was exposed to solutions of varying pCa (pCa 6.6–4.5) and allowed to develop steady-state force. As a consequence of the slack–restretch manoeuvre, a force baseline was obtained. To obtain  $\text{Ca}^{2+}$ -activated force for a given pCa,  $\text{Ca}^{2+}$ -independent force measured in a solution of pCa 9.0 was subtracted from total force (i.e.  $\text{Ca}^{2+}$ -activated force plus  $\text{Ca}^{2+}$ -independent force). Force–pCa relationships were determined by (1) expressing submaximal  $\text{Ca}^{2+}$ -activated force ( $P$ ) at each pCa as a fraction of maximal  $\text{Ca}^{2+}$ -activated force ( $P_o$ ) determined at pCa 4.5, i.e.  $P/P_o$ ; and (2) expressing submaximal total force ( $P^*$ ) at each pCa as a fraction of maximal total force ( $P_{Tot}^*$ ) determined at pCa 4.5, i.e.  $P^*/P_{Tot}^*$ . The apparent co-operativity of force development was inferred from the steepness of the force–pCa relationship at forces less than half-maximal, which was quantified using a Hill plot transformation of the force–pCa data (Strang *et al.* 1994). We focused on this region of the curve because the force–pCa relationship is biphasic, i.e. the curve is steeper at high pCa, indicating that most of the co-operative activation of the thin filament occurs at forces less than  $0.50 P_o$  (Moss, 1992). Force–pCa data were analysed by least-squares regression using the Hill equation:  $\log(P_{rel}/(1 - P_{rel})) = n_2(\log[\text{Ca}^{2+}] + k)$ , where  $P_{rel}$  is force as a fraction of either  $P_o$  or  $P_{Tot}^*$ ,  $n_2$  is the Hill coefficient for forces below half-maximal, and  $k$  is the  $[\text{Ca}^{2+}]$  required for half-maximal activation ( $\text{pCa}_{50}$ ). Using  $\text{pCa}_{50}$  values derived from the Hill equation, force–pCa relationships (Fig. 3A and B) were fitted using the equation  $P_{rel} = [\text{Ca}^{2+}]^n / (k^n + [\text{Ca}^{2+}]^n)$ .



**Figure 1.** Photomicrographs of rat skinned myocardium

Representative skinned myocardial preparation (dimensions:  $430 \mu\text{m} \times 174 \mu\text{m}$ ) while relaxed (A, pCa 9.0) and during maximal activation (B, pCa 4.5). Sarcomere length in pCa 9.0 and 4.5 was 2.35 and  $2.27 \mu\text{m}$ , respectively. Scale bar represents  $50 \mu\text{m}$ .

**Rate of force relaxation.** The rate constant of force relaxation ( $k_{rel}$ ) in skinned myocardium was determined following flash photolysis of diazo-2 (Molecular Probes) as described previously (Patel *et al.* 1996). When exposed to a flash of UV light diazo-2 rapidly ( $> 3000 \text{ s}^{-1}$ ) chelates  $\text{Ca}^{2+}$  due to an increase in the  $\text{Ca}^{2+}$ -binding affinity from  $2.2 \mu\text{M}$  to  $73 \text{ nM}$  (Adams *et al.* 1989). Diazo-2 has been used previously to examine the rate of force relaxation of skinned myocardium (Palmer & Kentish, 1998; Fitzsimons *et al.* 1998). The rate of force relaxation ( $k_{rel}$ ) is a complex process governed by several factors, including the rate of cross-bridge detachment and of  $\text{Ca}^{2+}$  dissociation from troponin C (TnC). Of these, the intrinsic rate of cross-bridge detachment most probably dominates the rate of force relaxation, since the off-rate ( $k_{off}$ ) of  $\text{Ca}^{2+}$  from TnC is approximately  $350 \text{ s}^{-1}$  (Dong *et al.* 1996), which is significantly faster than the rates of relaxation measured in the current study. All photolysis experiments were performed using the solutions listed in the legend of Fig. 6. The amount of force generated prior to flash photolysis of diazo-2 ( $P$ ) was expressed as a fraction of maximal force ( $P_o$ ) determined at pCa 4.5, i.e.  $P/P_o$ . The rate of force relaxation was determined using the following equation:  $k_{rel} = \ln 2/t_{1/2}$ , where  $t_{1/2}$  is the time taken for force to decrease by 50% of the initial value.

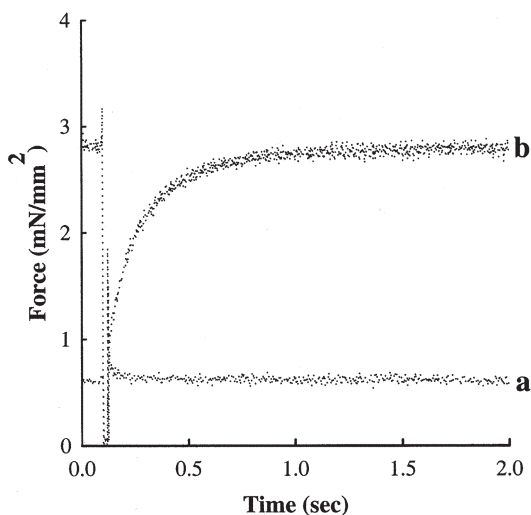
### Statistics

All data are expressed as means  $\pm$  S.E.M. Where appropriate, either Student's two-tailed  $t$  test for independent samples or Student's paired  $t$  test was used as a *post hoc* test of significance, with significance set at  $P < 0.05$ .

## RESULTS

### Influence of NEM-S1 on steady-state force

Previous experiments on skinned skeletal muscle fibres (Swartz & Moss, 1992) and cardiac myocytes (Fitzsimons



**Figure 2. NEM-S1 increases  $\text{Ca}^{2+}$ -independent force**

A mechanical release–restretch manoeuvre was used to measure the rate of force redevelopment in a solution of pCa 9.0 in the absence and presence of  $6 \mu\text{M}$  NEM-S1. This skinned myocardial preparation generated  $\text{Ca}^{2+}$ -independent force of  $0.05 P_o$  in the absence of NEM-S1 in pCa 9.0 (trace a), whereas  $\text{Ca}^{2+}$ -independent force increased to  $0.22 P_o$  following NEM-S1 treatment (trace b).

& Moss, 1998) showed that  $6 \mu\text{M}$  NEM-S1 elicited a steady increase in submaximal  $\text{Ca}^{2+}$ -activated force, without reducing maximum  $\text{Ca}^{2+}$ -activated force ( $P_o$ ). However, higher levels of NEM-S1 caused a progressive reduction in  $P_o$ , most probably due to competitive inhibition of endogenous cross-bridge binding (Swartz & Moss, 1992). Therefore, in the present study of the activating effects of strong-binding myosin cross-bridges in myocardium, skinned preparations were treated with  $6 \mu\text{M}$  NEM-S1. Control experiments showed no effects of unmodified S1 (i.e. no NEM modification) on steady force or cross-bridge kinetics because of the rapid cycling rate of unmodified exogenous S1 (data not shown).

The effects of  $6 \mu\text{M}$  NEM-S1 on the maximum total tension ( $P_{Tot}$ ), maximum  $\text{Ca}^{2+}$ -activated tension ( $P_o$ ),  $\text{Ca}^{2+}$  sensitivity of tension and Hill coefficient ( $n_2$ ) in rat skinned myocardium are summarized in Table 1. Although  $6 \mu\text{M}$  NEM-S1 did not alter total maximum tension,  $\text{Ca}^{2+}$ -independent tension at pCa 9.0 was significantly increased and there was a concomitant reduction in maximal  $\text{Ca}^{2+}$ -activated tension. In the absence of NEM-S1,  $\text{Ca}^{2+}$ -independent tension accounted for  $\sim 4\%$  of total tension ( $4.4 \pm 0.7\%$ ) in maximally activated preparations, which increased nearly 4-fold after NEM-S1 treatment ( $16.5 \pm 1.4\%$ ) (Fig. 2). This large NEM-S1-induced increase in  $\text{Ca}^{2+}$ -independent force ( $3.7 \pm 0.8$  vs.  $0.8 \pm 0.3 \text{ mN mm}^{-2}$ ) is unique to myocardium since skinned skeletal muscle fibres treated with  $6 \mu\text{M}$  or higher concentrations of NEM-S1 exhibited much smaller increases in  $\text{Ca}^{2+}$ -independent tension (Swartz & Moss, 1992). The increase in  $\text{Ca}^{2+}$ -independent force appeared to be the result of increased binding of endogenous cross-bridges due to NEM-S1-induced activation of the thin filament (as discussed below).

NEM-S1 also increased submaximal  $\text{Ca}^{2+}$ -activated force (Fig. 3A). Mean pCa<sub>50</sub> increased significantly after NEM-S1 treatment ( $\Delta\text{pCa}_{50} = 0.11 \pm 0.02$ ,  $P < 0.05$ ), indicating an increase in the  $\text{Ca}^{2+}$  sensitivity of force. These results are consistent with the idea that NEM-S1 promotes the formation of strongly bound force-generating cross-bridges at low levels of  $\text{Ca}^{2+}$ . At progressively higher  $\text{Ca}^{2+}$  concentrations (i.e. pCa  $< 5.8$ ), NEM-S1 had negligible effects on  $\text{Ca}^{2+}$ -activated tension compared to control. Commensurate with the increase in force at low  $[\text{Ca}^{2+}]$ , NEM-S1 significantly reduced the steepness ( $n_2$ ) of the force–pCa relationship, in agreement with results reported previously in skinned ventricular myocytes (Fitzsimons & Moss, 1998) and skeletal muscle fibres (Swartz & Moss, 1992). Similar effects of NEM-S1 were evident in the total force–pCa relationships obtained from these same preparations (Fig. 3B). Prior to NEM-S1 treatment, mean pCa<sub>50</sub> was  $5.86 \pm 0.05$ , which increased to  $6.09 \pm 0.05$  in the presence of NEM-S1 ( $\Delta\text{pCa}_{50} = 0.23 \pm 0.03$ ,  $P < 0.05$ ). The Hill coefficient for the total force–pCa data was markedly reduced in the presence of NEM-S1 ( $n_2$  was  $0.93 \pm 0.22$  vs.  $4.50 \pm 0.69$  in untreated myocardium;  $P < 0.05$ ), which suggests that

**Table 1. Steady-state mechanical measurements in skinned left ventricular myocardium in the absence and presence of 6 μM NEM-S1**

Group	$P_{Tot}$ (mN mm <sup>-2</sup> )	$P_o$ (mN mm <sup>-2</sup> )	$P_{rest}$ (mN mm <sup>-2</sup> )	$n_2$	pCa <sub>50</sub>	ΔpCa <sub>50</sub>
Control (7)	25.8 ± 1.8	24.7 ± 1.7	0.8 ± 0.3	4.72 ± 0.38	5.85 ± 0.05	
6 μM NEM-S1 (7)	23.7 ± 2.1	19.7 ± 1.6*	3.7 ± 0.8*	1.54 ± 0.07*	5.95 ± 0.04*	0.11 ± 0.02

All values are expressed as means ± S.E.M., with the number of ventricular preparations given in parentheses.  $P_{Tot}$ , maximal total tension (maximal Ca<sup>2+</sup>-activated tension plus Ca<sup>2+</sup>-independent tension);  $P_o$ , maximal Ca<sup>2+</sup>-activated tension;  $P_{rest}$ , Ca<sup>2+</sup>-independent tension at pCa 9.0;  $n_2$ , Hill coefficient for Ca<sup>2+</sup>-activated force below 0.50  $P_o$ ; pCa<sub>50</sub>, pCa required for half-maximal activation; ΔpCa<sub>50</sub>, change in pCa<sub>50</sub> following addition of 6 μM NEM-S1. \*Significantly different from control ( $P < 0.05$ ).

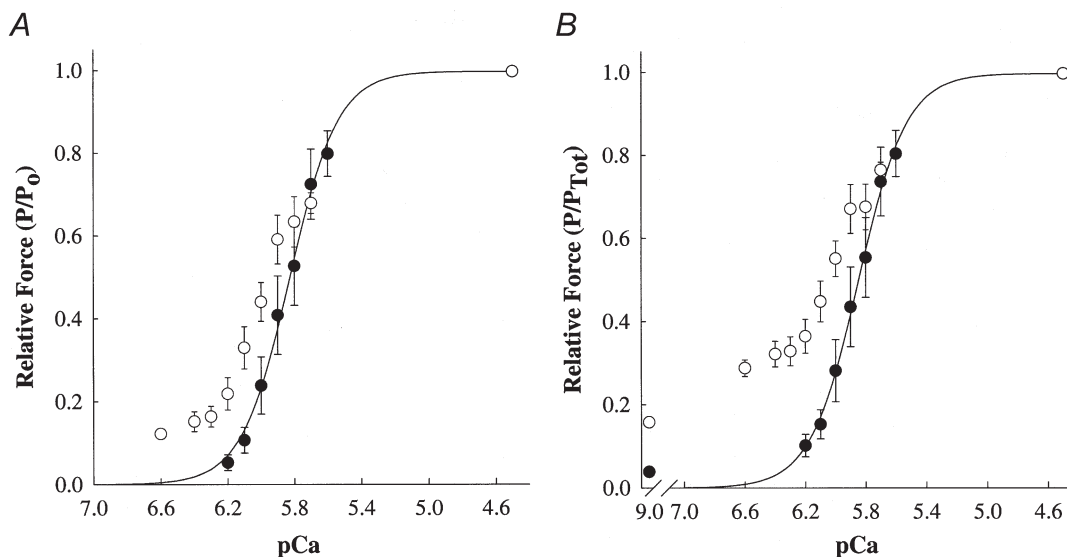
6 μM NEM-S1 saturated the co-operativity evident in the activation of tension in the absence of NEM-S1.

**Acceleration of cross-bridge kinetics by NEM-S1**

**Rate of force redevelopment.** The rate constant of force redevelopment ( $k_{tr}$ ) following rapid release and restretch (Brenner & Eisenberg, 1986) has been used to estimate the rate of weak- to strong-binding cross-bridge transitions in muscle. We recorded force redevelopment in skinned myocardium in the absence and presence of NEM-S1 to determine whether the NEM-S1-induced potentiation of submaximal Ca<sup>2+</sup>-activated force was associated with an acceleration of the transition(s) to strongly bound cross-bridge states. Examples of the effects of NEM-S1 on the time course and rate of force development are shown in Fig. 4. In the absence of NEM-S1, maximum Ca<sup>2+</sup>-activated

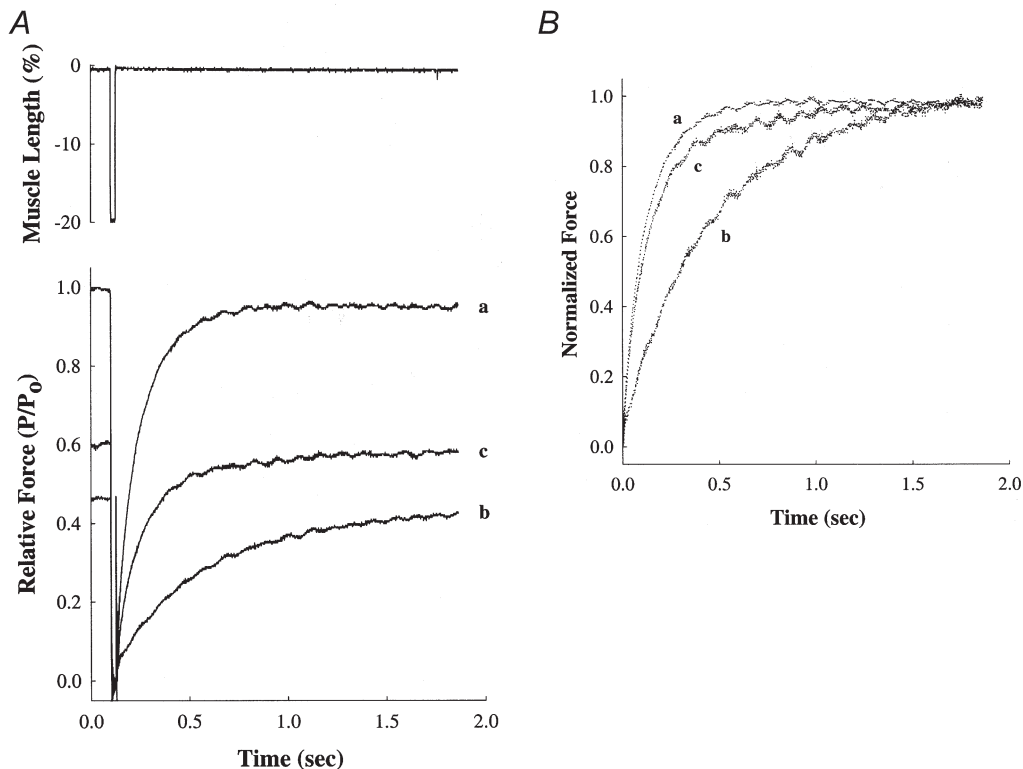
force ( $P_o$ ) developed in a solution of pCa 4.5 with an apparent rate constant ( $k_{tr}$ ) of 8.25 s<sup>-1</sup>, whereas force in a solution of pCa 5.8 developed to a steady level of 0.47  $P_o$  with a  $k_{tr}$  of 2.00 s<sup>-1</sup>. However, addition of NEM-S1 increased the steady-state force at pCa 5.8 to 0.61  $P_o$  and increased  $k_{tr}$  to a near-maximal value of 7.41 s<sup>-1</sup>.

A summary of the  $k_{tr}$  and relative  $k_{tr}$  values for several levels of Ca<sup>2+</sup> activation is presented in Table 2, and cumulative  $k_{tr}$ -pCa relationships obtained in the presence or absence of NEM-S1 are presented in Fig. 5A. In the absence of NEM-S1,  $k_{tr}$  varied with the level of Ca<sup>2+</sup>, increasing progressively from 1.11 ± 0.21 s<sup>-1</sup> at pCa 6.0 to 9.28 ± 0.41 s<sup>-1</sup> at pCa 4.5. The Ca<sup>2+</sup> dependence of  $k_{tr}$  in skinned myocardium observed here is consistent with the results of several earlier studies (Wolff *et al.* 1995; Palmer

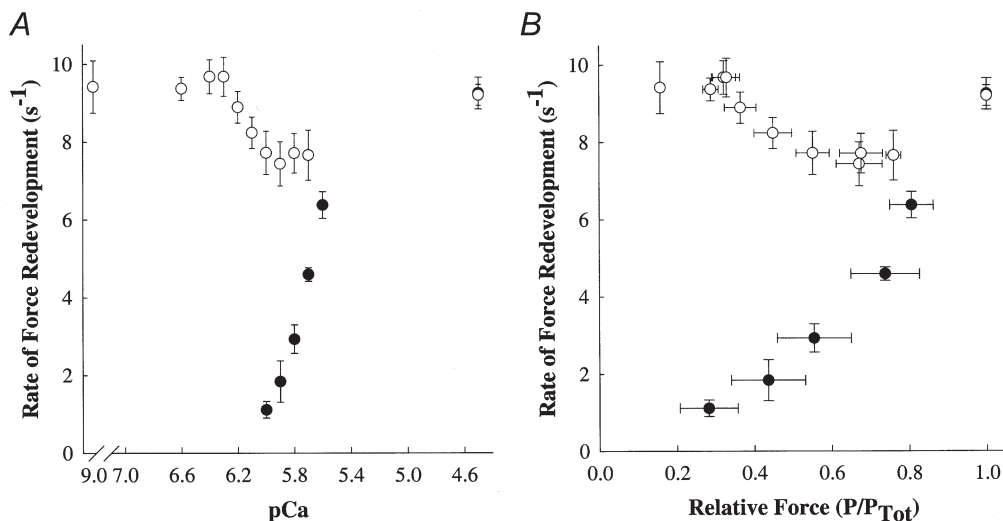


**Figure 3. Effects of 6 μM NEM-S1 on force-pCa relationships in skinned myocardium**

Force-pCa relationships were determined from skinned ventricular myocardium ( $N = 7$ ) in the absence (●) and presence (○) of NEM-S1. Smooth lines were generated by fitting the mean data with the equation:  $P_{rel} = [Ca^{2+}]^n / (k^n + [Ca^{2+}]^n)$ , where  $P_{rel}$  is force as a fraction of maximal Ca<sup>2+</sup>-activated force ( $P_o$ ) or maximal total force ( $P_{Tot}$ ),  $n$  is the Hill coefficient, and  $k$  is the  $[Ca^{2+}]$  required for half-maximal activation (i.e. pCa<sub>50</sub>). Data points are means and the error bars are S.E.M. A, Ca<sup>2+</sup>-activated force-pCa relationship in the absence (pCa<sub>50</sub> = 5.85 ± 0.05,  $n_2$  = 4.72 ± 0.38) and presence of NEM-S1 (pCa<sub>50</sub> = 5.95 ± 0.04,  $n_2$  = 1.54 ± 0.07). B, total force-pCa relationship in the absence (pCa<sub>50</sub> = 5.86 ± 0.05,  $n_2$  = 4.50 ± 0.69) and presence of NEM-S1 (pCa<sub>50</sub> = 6.09 ± 0.05,  $n_2$  = 0.93 ± 0.22). Total force is the sum of Ca<sup>2+</sup>-independent and Ca<sup>2+</sup>-activated forces at each pCa.



**Figure 4. NEM-S1 potentiates submaximal force and the rate of force redevelopment**  
*A*, force records showing redevelopment of submaximal force developed by a skinned ventricular preparation at pCa 5.8 both in the absence (trace b) and in the presence of 6  $\mu\text{M}$  NEM-S1 (trace c). Force in each case is scaled to the maximum force ( $P_0$ ) generated by the same preparation when exposed to a solution of pCa 4.5 (trace a). The upper panel shows the mechanical release–restretch manoeuvre used to measure  $k_{tr}$ . *B*, to illustrate differences in the rate of force redevelopment, the force records in *A* are replotted with the peak force at each pCa scaled to 1.0.



**Figure 5. NEM-S1 nearly eliminates the activation dependence of the rate of force redevelopment**  
 Force redevelopment following rapid release and restretch was measured in rat skinned myocardium ( $N = 7$ ) in the absence ( $\bullet$ ) and presence ( $\circ$ ) of NEM-S1. Data points are means and the error bars are the S.E.M. *A*, the  $\text{Ca}^{2+}$  dependence of the rate constant of force redevelopment,  $k_{tr}$ . *B*, the rate constant of force redevelopment ( $k_{tr}$ ) as a function of force (as a fraction of total force) measured at each pCa. Total force is the sum of  $\text{Ca}^{2+}$ -independent and  $\text{Ca}^{2+}$ -activated forces at each pCa.

**Table 2. Summary of  $k_{tr}$  and  $Ca^{2+}$ -activated force at maximal and submaximal concentrations of  $Ca^{2+}$  before and after treatment with  $6 \mu M$  NEM-S1 in skinned left ventricular myocardium**

Control (7)		pCa 6.0	pCa 5.8	pCa 4.5
$P/P_o$		$0.24 \pm 0.07$	$0.53 \pm 0.09$	$1.00 \pm 0.00$
$k_{tr} (s^{-1})$		$1.11 \pm 0.21$	$2.93 \pm 0.37$	$9.28 \pm 0.41$
Relative $k_{tr}$		$0.10 \pm 0.01$	$0.25 \pm 0.03$	$1.00 \pm 0.00$
6 $\mu M$ NEM-S1 (7)	pCa 6.4	pCa 6.2	pCa 5.9	pCa 4.5
$P/P_o$	$0.15 \pm 0.02$	$0.22 \pm 0.04$	$0.59 \pm 0.06$	$1.00 \pm 0.00$
$k_{tr} (s^{-1})$	$9.69 \pm 0.44$	$8.91 \pm 0.40^*$	$7.45 \pm 0.57^*$	$9.23 \pm 0.27$
Relative $k_{tr}$	$1.02 \pm 0.06$	$0.96 \pm 0.03$	$0.88 \pm 0.02$	$1.00 \pm 0.00$

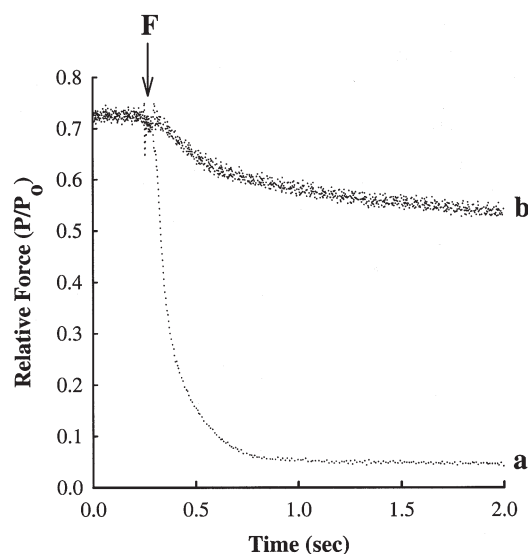
All values are expressed as means  $\pm$  S.E.M., with the number of ventricular preparations given in parentheses.  $P/P_o$ , relative  $Ca^{2+}$ -activated force;  $k_{tr}$ , rate of tension redevelopment; relative  $k_{tr}$ , relative rate of force redevelopment normalized to the maximal rate obtained at pCa 4.5 under the same conditions (i.e. either control or in the presence of NEM-S1). \*Significantly different from control for given level of force ( $P < 0.05$ ).

& Kentish, 1998; Baker *et al.* 1998). However, addition of NEM-S1 significantly reduced the  $Ca^{2+}$  dependence of  $k_{tr}$ ; that is, mean  $k_{tr}$  was  $9.38 \pm 0.30 s^{-1}$  at pCa 6.6 as compared to  $9.23 \pm 0.27 s^{-1}$  at pCa 4.5 (Fig. 5A). Even at very low  $[Ca^{2+}]$ , i.e. pCa 9.0, the induction of  $Ca^{2+}$ -independent force by NEM-S1 was accompanied by an increase in  $k_{tr}$  to  $9.42 \pm 0.67 s^{-1}$ .

The rate constant of force redevelopment was also plotted *vs.* total steady-state isometric force at each pCa, in order to assess the variation of  $k_{tr}$  with a variable that reflects the effects of both  $Ca^{2+}$  and strong-binding cross-bridges on thin filament activation (Fig. 5B). In the absence of NEM-S1,  $k_{tr}$  increased as a function of increasing isometric force, gradually at low forces and more steeply at high forces, an observation that is consistent with previous studies in skeletal (Brenner, 1988; Metzger *et al.* 1989; Swartz & Moss, 1992) and cardiac muscles (Wolff *et al.* 1995; Palmer & Kentish, 1998; Baker *et al.* 1998). However, the relationship between  $k_{tr}$  and steady-state isometric force was substantially altered by NEM-S1. At very low forces and at maximum force,  $k_{tr}$  was similar to values obtained during maximal activation of the same preparation in the absence of NEM-S1. At intermediate forces, NEM-S1 increased  $k_{tr}$  to values greater than control at similar relative forces but still less than the values obtained at both low and maximum forces. These data are consistent with the idea that NEM-S1 cooperatively promotes the binding of endogenous cross-bridges at low and intermediate levels of activation.

**Rate of force relaxation ( $k_{rel}$ ) following flash photolysis of diazo-2.** To investigate the possible involvement of strong-binding cross-bridges in determining the rate of thin filament inactivation, the relaxation of steady-state force was recorded in the presence and absence of NEM-S1 following photolysis of the photolabile  $Ca^{2+}$  chelator diazo-2 (Fig. 6). The conditions used to determine the rate of relaxation were designed so that control preparations would relax by greater than 90% from pre-photolysis steady-state force. When

incubated in loading solution containing 0.12 mM  $CaCl_2$  and 0.25 mM diazo-2, each preparation generated a pre-flash steady force of  $\sim 0.70 P_o$ . Following flash photolysis of diazo-2, there is an almost instantaneous decrease in intracellular  $[Ca^{2+}]$  (Adams *et al.* 1989), such that relaxation, and hence the rate of thin filament

**Figure 6. NEM-S1 markedly slows the rate of force relaxation**

Myocardial preparations were bathed for 15 min in a pCa 9.0 solution in the absence or presence of  $6 \mu M$  NEM-S1 and subsequently incubated for 2 min in loading solution containing (mM): Bes, 100; potassium propionate, 40.9; creatine phosphate, 15; dithiothreitol, 5; MgATP, 4; free  $Mg^{2+}$ , 1;  $CaCl_2$ , 0.12; and diazo-2, 0.25; pH 7.0 at  $15^\circ C$ . Force relaxation following flash photolysis of diazo-2 was recorded either in the absence (trace a) or in the presence of  $6 \mu M$  NEM-S1 (trace b), where F refers to the time point at which the flash lamp was triggered. Each of the myocardial preparations generated  $\sim 0.70 P_o$  in the loading solution prior to photolysis of diazo-2.

inactivation, most probably reflects the rate of cross-bridge detachment (Palmer & Kentish, 1998). Although steady force decreased in each preparation following flash photolysis of diazo-2, the extent and rate of force relaxation were dramatically reduced by NEM-S1. In the absence of NEM-S1, steady-state force declined to near-zero active force with an apparent rate constant ( $k_{\text{rel}}$ ) of  $6.9 \text{ s}^{-1}$ , whereas force decreased by only 20% with a  $k_{\text{rel}}$  of  $\sim 0.9 \text{ s}^{-1}$  in the NEM-S1-treated preparation.

## DISCUSSION

The main objective of the present study was to determine the role of strong-binding myosin cross-bridges in the activation of myocardial force. Our results show that application of NEM-S1 to skinned myocardium co-operatively promotes the formation of endogenous strongly bound force-generating cross-bridges, as manifested by: (1) significant increases in  $\text{Ca}^{2+}$ -independent tension at pCa 9.0, (2) potentiation of submaximal  $\text{Ca}^{2+}$ -activated tension and a corresponding increase in the  $\text{Ca}^{2+}$  sensitivity of force (i.e. a leftward shift in the force–pCa relationship), (3) significant reductions in the  $\text{Ca}^{2+}$  dependence of the rate of force redevelopment ( $k_{\text{tr}}$ ), and (4) marked slowing of the rate of relaxation ( $k_{\text{rel}}$ ).

### Effects of NEM-S1 on steady-state force

Our initial hypothesis was that an increase in the number of strong-binding cross-bridges would increase the co-operative activation of the thin filament, which would be manifested by increases in submaximal  $\text{Ca}^{2+}$ -activated tension and in the  $\text{Ca}^{2+}$  sensitivity of tension. The approach to test this idea was to treat chemically skinned myocardium with NEM-S1, a strong-binding non-tension-generating derivative of myosin sub-fragment-1, as a means to increase the number of strongly bound bridges and thereby presumably promote the formation of endogenous strong-binding cross-bridges (Swartz & Moss, 1992). NEM-S1 elicited a marked increase in  $\text{Ca}^{2+}$ -independent tension (Fig. 2 and Table 1), as measured in the virtual absence of  $\text{Ca}^{2+}$ , i.e. pCa 9.0 ( $[\text{Ca}^{2+}]_{\text{free}} \approx 1 \text{ nM}$ ). The amount of  $\text{Ca}^{2+}$ -independent tension observed in these experiments suggests that the cardiac-specific thin filament proteins confer greater activating effects of strongly bound cross-bridges in myocardium, since a similar concentration of NEM-S1 had little or no effect on  $\text{Ca}^{2+}$ -independent tension in skeletal muscle fibres (Swartz & Moss, 1992). This result would imply that relatively few NEM-S1 heads are required to switch on the cardiac thin filament, in contrast to the case for the skeletal muscle thin filament. In addition, both submaximal tension (Fig. 3) and the  $[\text{Ca}^{2+}]$  required for half-maximal activation (pCa<sub>50</sub>) (Table 1) were significantly increased in the presence of NEM-S1. These NEM-S1-induced increases in  $\text{Ca}^{2+}$ -independent force, submaximal force and  $\text{Ca}^{2+}$  sensitivity of force could be due to NEM-S1-induced effects to either co-operatively promote the formation of endogenous strong-binding cross-bridges or

increase the  $\text{Ca}^{2+}$ -binding affinity of troponin C. However, the latter possibility seems unlikely since recent studies have suggested that force and not the number of cross-bridges *per se* leads to increases  $\text{Ca}^{2+}$  binding to TnC (Wang & Fuchs, 1994; Fuchs & Wang, 1996), and NEM-S1 does not generate force.

### NEM-S1 eliminates the $\text{Ca}^{2+}$ dependence of the rate of force development

In the absence of NEM-S1, the rate of force redevelopment ( $k_{\text{tr}}$ ) was highly variable with the level of activating  $\text{Ca}^{2+}$ , increasing as  $\text{Ca}^{2+}$  was increased from submaximal to maximal concentrations (Fig. 5A). The  $\text{Ca}^{2+}$  dependence of  $k_{\text{tr}}$  observed in the present study is consistent with previous results from both skeletal (Brenner, 1988; Metzger *et al.* 1989) and cardiac muscle preparations (Wolff *et al.* 1995; Palmer & Kentish, 1998; Baker *et al.* 1998). A recent model proposed by Campbell (1997) provides a plausible explanation for such a relationship by predicting that  $k_{\text{tr}}$  should be slow at low levels of activation due to inter-molecular co-operation in the activation process. According to Campbell, cross-bridges exist in non-cycling and cycling pools, and the distribution between these pools is determined by activating effects on the thin filament due to  $\text{Ca}^{2+}$  and strong-binding of cross-bridges. At saturating levels of  $\text{Ca}^{2+}$ , virtually all cross-bridges are part of the cycling pool so that the rate of force development is determined by the sum of the rate constants of cross-bridge attachment ( $f$ ) and detachment ( $g$ ), i.e. there is little effect of cross-bridge binding on thin filament activation. However, at low levels of  $\text{Ca}^{2+}$ , only a small fraction of cross-bridges are initially recruited into the cycling pool by the binding of  $\text{Ca}^{2+}$  to the thin filament, leaving a large fraction in the non-cycling pool. In such a case, the first cross-bridges that bind would co-operatively recruit additional cross-bridges from the non-cycling pool, which would then lead to successive cycles of recruitment. Thus, the rate of force development would be slow at low  $[\text{Ca}^{2+}]$  and would progressively increase as  $[\text{Ca}^{2+}]$  is increased due to a progressive reduction in the size of the non-cycling pool.

In the context of Campbell's model (Campbell, 1997), application of NEM-S1 should increase the number of strongly bound cross-bridges and thereby accelerate  $k_{\text{tr}}$  at low levels of  $\text{Ca}^{2+}$ , which is the result we observed. The rates of force redevelopment increased to maximal or near-maximal levels in the presence of NEM-S1 at all levels of  $\text{Ca}^{2+}$  activation, significantly reducing the  $\text{Ca}^{2+}$  dependence of  $k_{\text{tr}}$  (Fig. 5A). Even at very low  $[\text{Ca}^{2+}]_{\text{free}}$  (i.e. pCa 9.0), NEM-S1-treated myocardium exhibited maximal rates of force redevelopment (Fig. 2), similar to those observed during maximal  $\text{Ca}^{2+}$  activation. Furthermore, the relationship between  $k_{\text{tr}}$  and steady-state isometric force prior to the release–restretch protocol, which is an index of the total activation of thin filaments by  $\text{Ca}^{2+}$  and strongly bound cross-bridges, was



dramatically altered by NEM-S1 (Fig. 5B). In the presence of NEM-S1,  $k_{tr}$  values at very low forces and at maximal force were similar to values obtained during maximal activation in the absence of NEM-S1. At intermediate levels of force, NEM-S1 increased  $k_{tr}$  to values greater than control but not as great as those observed at both low and high relative forces.

The fact that NEM-S1 did not completely eliminate the activation dependence of  $k_{tr}$  could have several explanations, which we have addressed in another study done on skinned skeletal muscle fibres (D. P. Fitzsimons, J. R. Patel, K. S. Campbell & R. L. Moss, manuscript in preparation). One possibility is that the amount of NEM-S1 we applied was insufficient to bind to all of the functional groups (1 troponin, 1 tropomyosin and associated actins) within the thin filament. While we cannot completely rule out this possibility, we still observed a minimum in  $k_{tr}$  at intermediate levels of activation when NEM-S1 concentration was increased to 10  $\mu\text{M}$  (data not shown). It is also possible that other mechanisms contribute to  $k_{tr}$ , which seems likely in view of results on skeletal muscle fibres showing that the residual depression of  $k_{tr}$  at intermediate levels of activation is eliminated by partial extraction of TnC from the thin filament. Such an intervention is thought to disrupt co-operation between adjacent functional groups within the thin filament (Moss *et al.* 1985), suggesting that NEM-S1 does not significantly facilitate such co-operation. We have discussed these mechanisms in detail in the above study (D. P. Fitzsimons, J. R. Patel, K. S. Campbell & R. L. Moss, manuscript in preparation), but whatever the cause of the lesser effects of NEM-S1 on  $k_{tr}$  at intermediate levels of activation, the present results show clearly that strong-binding cross-bridges accelerate the kinetics of cross-bridge binding in myocardium.

Additional results from the present study further support the idea that activation of the myocardial thin filament is modulated by strong binding of myosin cross-bridges. Application of NEM-S1 to skinned myocardium reduced both the extent and rate of relaxation from a steady force of 0.70  $P_0$  (Fig. 6) and 0.4  $P_0$  (data not shown) following photolysis of diazo-2. By forming long-lasting complexes with actin, NEM-S1 appears to promote the attachment of endogenous cross-bridges to actin, thereby slowing the rate of relaxation. A co-operative mechanism similar to this has recently been proposed as an explanation for the slower rates of relaxation observed in mouse hearts expressing significant amounts of  $\alpha$ -tropomyosin on a normal  $\beta$ -tropomyosin background (Wolska *et al.* 1999; Moss, 1999).

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