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Effect of a cardiotonic agent, MCI-154, on the contractile properties of skinned skeletal muscle fibers

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Abstract

We have studied the effect of a cardiotonic agent, MCI-154 (6-[4-(4-pyridylamino)phenyl]-4,5-dihydro-3(2H)-pyridazinone hydrochloride trihydrate), on the contractile properties and adenosine triphosphatase (ATPase) activity of chemically skinned rabbit skeletal muscle fibers. As in cardiac muscle, MCI-154 potentiated isometric tension and improved isometric tension cost at full Ca^{2+} activation. It showed little Ca^{2+}-sensitizing effect. In contrast to its effect on cardiac muscle, however, MCI-154 decreased all the kinetic parameters tested (shortening velocity, the rate of rise of tension, and actomyosin ATPase activity). All the results are explainable if MCI-154 acts directly on skeletal actomyosin and inhibits a reaction step(s) of the ATPase cycle later than the force-generating event. The qualitative difference between cardiac and skeletal muscles in the responsiveness to this class of cardiotonic agents (MCI-154 and EMD 53998, a thiadiazinone derivative) is most readily understood if the agents have two independent actions, one on troponin and the other on actomyosin itself, the latter being dominant in skeletal muscle. © 1998 Published by Elsevier Science B.V.

Keywords: MCI-154; Skeletal muscle; Isometric tension; Shortening velocity; Ca^{2+} sensitivity; ATPase activity

1. Introduction

Recently a new class of cardiotonic agents, called Ca^{2+} sensitizers, has been developed. Although diverse in structure, these agents share an inhibitory effect on the activity of cardiac type III phosphodiesterase besides increasing Ca^{2+} sensitivity of the contractile machinery. For the treatment of heart failure, these Ca^{2+} sensitizers have clinical advantages over conventional cardiotonic agents, such as Ca^{2+} channel agonists, since they are capable of improving contractile performance without inducing cellular Ca^{2+} overloading, which could result in arrhythmias and myocardiac injury (Abe et al., 1996).

The Ca^{2+} sensitizers include MCI-154 (6-[4-(4-pyridylamino)phenyl]-4,5-dihydro-3(2H)-pyridazinone hydrochloride trihydrate), thiadiazinone derivatives (such as EMD53998), pimobendan, etc. Some of these Ca^{2+} sensitizers increase active tension only at submaximal Ca^{2+} levels, but others are also known to increase active tension at a saturating level of Ca^{2+}, suggesting that the latter have a direct action on actomyosin. These properties suggest potential applications of these Ca^{2+} sensitizers in basic research for elucidation of the mechanism of actomyosin interaction and its regulation by Ca^{2+}, both in cardiac and in skeletal muscle preparations.

Currently we are in search of chemicals which preferentially increase the population of one of the states which constitutes the normal cycle of the ATPase reaction. Study of the properties of the actomyosin complex in each state is essential for understanding the molecular mechanism of contraction, and this kind of chemicals would offer a powerful tool for research. Several chemicals, such as inorganic phosphate (P_i, e.g., Brozovich et al., 1988; Iwamoto, 1995a) and butanedione monoxime (Higuchi and Takemori, 1989; Herrmann et al., 1992; Regnier et al., 1995), are known to reduce the number of force-producing actomyosin complexes by increasing the low force complex. On the other hand, few chemicals are known to *increase* the number of force-producing complexes, information about which is most needed. Interventions such as increasing ADP or decreasing ATP levels may serve the purpose to some extent. These interventions have the shortcoming that they alter the free energy change of the

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whole ATPase reaction. Moreover, especially in the case of ADP, very careful buffering is needed to keep its concentration constant, both spatially and temporally (see Cooke and Pate, 1985; Chase and Kushmerick, 1995). If the cardiotonic agents can increase the number of force-producing complexes, they would be free of such shortcomings and therefore could serve as alternatives superior to those interventions.

MCI-154 may be one of the Ca^{2+} sensitizers most suitable for these purposes, since, at a concentration as low as 100 μM, it is known to increase the tension of skinned cardiac preparations by about 20% at a saturating Ca^{2+} level (Kitada et al., 1989a). MCI-154 is effective in the in vitro motility assay system which consists of cardiac actin, myosin, troponin and tropomyosin (Sata et al., 1995), indicating that its primary target is one (or a few) of the contractile proteins. It has also been reported that MCI-154 enhances Ca^{2+} binding to troponin C complexed with troponin I and troponin T (Kitada et al., 1989b; Liao and Gwathmey, 1994). However, this effect alone would not explain the potentiating effect at saturating Ca^{2+} levels. It is also unclear whether MCI-154 exerts its potentiating effect only on cardiac muscles, or also on other types of muscles.

Here we report that MCI-154 is also effective in skeletal muscle fibers. MCI-154 increased the isometric tension of skinned muscle fibers from rabbit psoas in a dose-dependent manner. Contrary to its effect on cardiac muscles, MCI-154 reduced the ATPase rate during isometric contraction at a saturating Ca^{2+} level. It is concluded that, in skeletal muscle, MCI-154 increases the number of force-producing actomyosin complexes by inhibiting a reaction step which follows the force-generation event.

2. Materials and methods

2.1. Preparation

Rabbits (body weight, 1.5–2.0 kg) were killed after anesthesia with 4 ml/kg of 25% urethane. Bundles (diameter, $\sim$2 mm) of fibers were excised from the psoas muscle, skinned in relaxing solution containing 0.5% (w/v) Triton X-100, stored in a 50% mixture of glycerol and relaxing solution. Thin fiber bundles containing 2–3 fibers were excised from the thick bundle and mounted in the experimental chamber with aluminum T-clips at both ends, as described previously (Iwamoto, 1995a).

2.2. Solutions

Bathing solutions for mechanical measurements were similar to those described previously (Iwamoto, 1995a,b): the relaxing solution had the following composition in mM: potassium propionate, 80; EGTA, 10; $MgCl_2$, 5; Na_2ATP, 4; phosphocreatine, 20; creatine phosphokinase, 125–300 u/ml; dextran T-500, 4% (w/v); imidazole, 20 (pH = 7.2). Dextran was added to reverse the filament lattice swelling caused by the skinning procedure. When 20 mM inorganic phosphate (P_i) was added, the concentration of potassium propionate was reduced to 40 mM to keep the ionic strength constant. In the preactivating solution, the EGTA concentration was reduced to 0.1 mM. The contracting solution contained up to 10.1 mM $CaCl_2$ in addition to the components in the relaxing solution. The free Ca^{2+} concentration was calculated with an algorithm of Goldstein (1979) with stability constants from Chase and Kushmerick (1988). For ATPase activity measurement, the solutions also contained 3 μM cyclopiazonic acid, 2 mM NaN_3 and 330 μM diadenosine pentaphosphate, to suppress Ca^{2+}-ATPase (Kurebayashi and Ogawa, 1991), mitochondrial ATPase and myokinase, respectively. MCI-154 was added to the relaxing, preactivating and activating solutions to obtain the desired final concentrations (up to 600 μM). Phosphocreatine and creatine phosphokinase were obtained from Sigma.

2.3. Apparatus for mechanical measurements

The length of the specimens was servo-controlled, and the tension was recorded with a semiconductor strain gauge, as described previously (Iwamoto, 1995a).

2.4. Protocol for mechanical measurements

The fibers were activated and relaxed as described (Iwamoto, 1995a). The solution exchange was achieved by exchanging solution chambers made of anodized aluminum blocs. Various concentrations of MCI-154 were applied in an ascending, descending or random order. The effect of MCI-154 was reversible and the results did not seem to be affected by the order of the MCI-154 concentrations.

Fiber stiffness was measured by applying small sinusoidal vibrations (frequency, 500 Hz, peak-to-peak amplitude, 0.1–0.2% of fiber length). The force–velocity relation was obtained by allowing the fibers to shorten under various amounts of load. The steady state shortening velocity (V) was plotted against the load (P) normalized to the level of isometric tension (P_o) and fitted to the hyperbolic function of Hill (1938), i.e.,

$$(P + a)(V + b) = (P_o + a)b,$$

where a and b are constants. The rate of tension recovery after shortening (k_{tr}) was measured according to the method of Brenner (1988): After a period of isotonic shortening under a light load ($< 0.1P_o$), the fibers were stretched back to their original length and held isometric for the rest of the activation period (in some cases the restretch was omitted). The tension approached its pre-release level, and the process of tension recovery was fitted to a single exponential association by nonlinear least-

squares regression (Prism software package, Graphpad). All experiments for mechanical measurements were done at 3–5°C.

2.5. Protocol for ATPase measurements

Ca^{2+}-activated actomyosin ATPase activity of fibers was measured by quantitating the amount of free creatine released during isometric contraction in exchange for ATP regeneration from ADP through the action of creatine phosphokinase. The quantitation utilized the α-naphthol–diacetyl reaction (e.g., Wong, 1971; Chaen and Sugi, 1982). The fiber bundles used in this experiment were thicker than those for mechanical measurements (containing ~ 5 fibers). As soon as the Ca^{2+}-activated isometric tension reached a plateau (15–20 s after the onset of contraction), the fibers were transferred to another chamber containing a fixed amount (135 μl) of activating solution for ATPase measurement. The fibers were made to contract for a predetermined period (0, 30, 60 or 90 s) before they were transferred to the chamber containing relaxing solution. After five contraction–relaxation cycles, the activating solution was collected, and after addition of α-naphthol and diacetyl, the amount of colored complex with a molar extinction coefficient of $\sim 1.3 \times 10^4$ M^{-1} cm^{-1} was determined colorimetrically at 520 nm using a Hitachi U-3210 spectrophotometer. The rate of ATP hydrolysis per myosin head was calculated by assuming that the concentration of myosin head in the fiber was 200 μM (see Yates and Greaser, 1983). The volume of the fiber bundle was determined by multiplying the fiber length by the cross-sectional area obtained by the method of Blinks (1965).

2.6. Drug

MCI-154 (6-[4-(4-pyridylamino)phenyl]-4,5-dihydro-3(2H)-pyridazinone hydrochloride trihydrate) was from Mitusbishi Chemical Corporation. It was stocked in a refrigerator in the form of a 10 mM aqueous solution. Although the effect of MCI-154 seemed to increase monotonically with concentration, the highest concentration was limited to 600 μM because of its limited solubility in the presence of other solutes.

2.7. Statistical analysis

Data are expressed as means $\pm$ S.D. All the data subjected to statistical analysis were paired, i.e., in each specimen, measurements were made under different experimental conditions. The significance of the dose–response curves (Fig. 3) and the tension–pCa relation was tested with the repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni's post test, utilizing the Prism software package (Graphpad). Other differences between the data with and without MCI-154 treatment were evaluated with a paired *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of MCI-154 on isometric tension, force–velocity characteristics and the rate of tension redevelopment after shortening

Although MCI-154 effectively potentiates the contractility of skinned cardiac preparations at concentrations ≤ 100 μM, higher concentrations were needed to obtain comparable effects on skeletal muscle fiber preparations. Fig. 1a shows superposed traces of length and tension during a single contraction–relaxation cycle of a skinned fiber bundle in the presence and absence of 600 μM MCI-154. The bundle was fully Ca^{2+}-activated ($pCa \equiv -\log[Ca^{2+}] = 4.49$). Isometric tension was enhanced by an average of 27% in the presence of 600 μM MCI-154. After the tension had reached a plateau, the bundle was allowed to shorten against a light load ($\sim 0.05 \times$ isometric tension level P_o), and then restretched to its original length.

The time course of fiber shortening is shown on an expanded time scale in Fig. 1b. In the presence of 600 μM MCI-154, the shortening velocity was visibly slower (20% reduction on average). The steady state force–velocity relation was obtained from shortening velocities recorded under a variety of loads. The force–velocity curve in the presence of 600 μM MCI-154 is compared with that in its

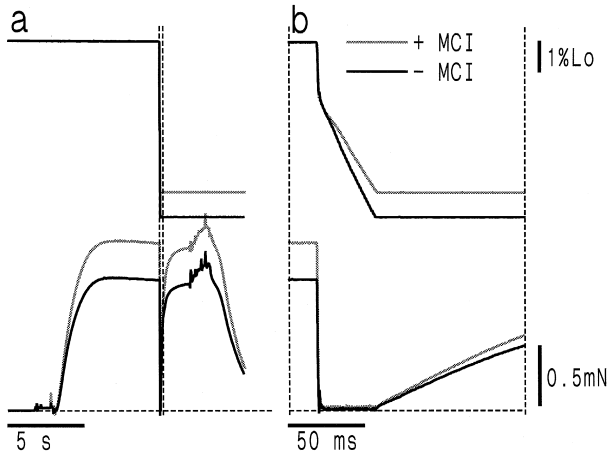

Fig. 1. Superimposed traces for tension and length during isometric contractions of a rabbit skeletal muscle fiber bundle. (a) slower time base; (b) faster time base (the portion of (a) between the two vertical broken lines is expanded). Solid line, in the absence of MCI-154, stippled line, in the presence of 600 μM MCI-154. Upper traces, length (shortening downward); lower traces, tension. Traces with greater tension and slower shortening represent the records in the presence of MCI-154. The noise in the tension records is due to solution exchange. Scale bars: time (left horizontal), 5 s in (a) and 50 ms in (b); length (upper vertical), 1% of fiber length (L_o); tension (lower vertical), 0.5 mN.

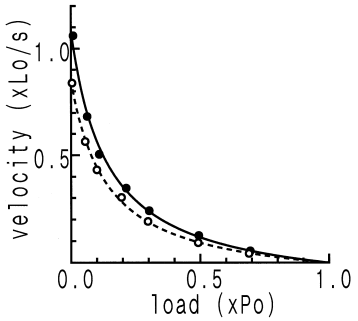

Fig. 2. An example of the force–velocity curves obtained from a rabbit skeletal muscle fiber bundle in the presence and absence of 600 μM MCI-154. Filled circles, control; open circles, in the presence of MCI-154. The load held during shortening is expressed as a fraction of the isometric tension level (P_o), and the steady state shortening velocity is expressed as fiber lengths/s. The curves represent best-fit hyperbolic function of Hill (1938).

absence in Fig. 2. In the presence of MCI-154, the velocities were smaller at all loads.

The rate constant for the recovery of tension following the restretch (k_{tr}) reflects the kinetics of the force generation of cross-bridges and their dissociation from actin (see Brenner, 1988). Specifically, k_{tr} is the sum of the rate constants for these two events. The value of k_{tr} was also reduced in the presence of 600 μM MCI-154 (25% reduction on average). The force–velocity parameter b, the proportionality constant for velocity, also decreased monotonically with increasing concentrations of MCI-154. On the other hand, MCI-154 did not show any clear effect on the parameter a/P_o, which represents the curvature of the force–velocity relation.

The dose dependence of these effects is summarized in Fig. 3. Except for the parameter a/P_o, all the effects increased monotonically with increasing concentrations of MCI-154. Analysis of variance showed that the overall effect was highly significant ($P = 0.0003$ in V_{max} and $P < 0.0001$ in isometric tension, k_{tr} and parameter b). At concentrations below 100 μM, no appreciable effect was observed (data not shown).

The increase in isometric tension and the decreases of k_{tr} and the shortening velocity were also observed at a higher temperature (23°C) on application of MCI-154 (data not shown). Also, MCI-154 had no effect on the resting stiffness of the fiber. Therefore, the observed effects of MCI-154 to reduce the kinetic parameters were not due to the low temperature or the alteration of the passive mechanical properties.

To summarize, MCI-154 potentiates isometric tension but reduces the kinetic parameters of contractile events in skeletal muscle preparations.

3.2. Effects of inorganic phosphate on the contractile properties in the presence and absence of MCI-154

With both skeletal and cardiac muscles, addition of inorganic phosphate (P_i) to the bathing solution reduces the isometric tension (e.g., Cooke and Pate, 1985; Kentish, 1986; Hoar et al., 1987; Brozovich et al., 1988; Martyn and Gordon, 1992; Iwamoto, 1995a), by reversing the phosphate-release step which is closely associated with force-producing events (Hibberd et al., 1985; Dantzig et al., 1992). It is also known that P_i reduces the sensitivity of the contractile machinery to Ca^{2+} (Hoar et al., 1987; Martyn and Gordon, 1992). Like EMD 53998 (Strauss et al., 1992, 1994), MCI-154 reverses these inhibitory effects

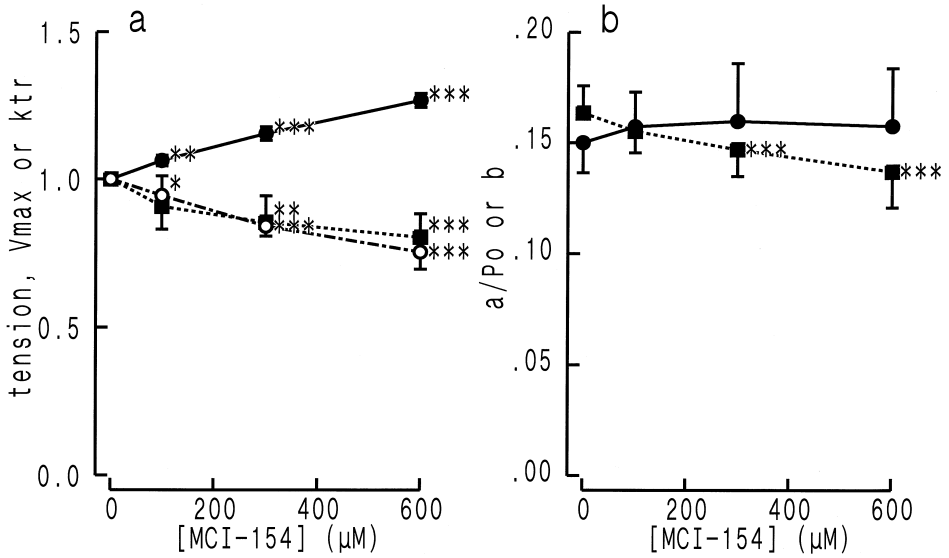

Fig. 3. Dose-dependence of the kinetic parameters of rabbit skeletal muscle fibers. (a) Isometric tension (filled circles), V_{max} (shortening velocity extrapolated to zero load, filled squares) and k_{tr}, the rate constant for the rise of tension after shortening (open circles). Normalized to control values. (b) Parameters describing the force–velocity function of Hill (1938). Filled circles, a/P_o; filled squares, b. Expressed as absolute values. Error bars represent S.D. ($n = 6$). Asterisks represent the degree of statistical significance of the difference between control (without MCI-154) and test values ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Table 1
Effect of 20 mM inorganic phosphate on the contractile properties of skinned skeletal muscle fibers in the presence and absence of 600 μM MCI-154

	0 MCI-154, 0 P_i	600 MCI-154, 0 P_i	0 MCI-154, 20 P_i	600 MCI-154, 20 P_i
Isometric tension ($\times 10^5$ N m^{-2})	1.21 ± 0.17 (1.00 ± 0.00)	1.51 ± 0.22[b] (1.25 ± 0.02)	0.61 ± 0.09 (0.50 ± 0.01)	0.84 ± 0.12[b] (0.69 ± 0.02)
Shortening velocity (L_o s^{-1})	1.00 ± 0.15 (1.00 ± 0.00)	0.78 ± 0.09[b] (0.78 ± 0.04)	1.04 ± 0.13 (1.05 ± 0.07)	0.82 ± 0.07[b] (0.83 ± 0.07)
k_{tr} (s^{-1})	8.26 ± 0.54 (1.00 ± 0.00)	7.13 ± 0.13[a] (0.87 ± 0.08)	9.74 ± 0.35 (1.18 ± 0.07)	8.78 ± 0.36[a] (1.06 ± 0.05)
Stiffness ($\times 10^7$ N m^{-2})	1.73 ± 0.32 (1.00 ± 0.00)	1.84 ± 0.34[a] (1.07 ± 0.03)	1.21 ± 0.23 (0.70 ± 0.03)	1.32 ± 0.26[a] (0.76 ± 0.02)
Phase lead (°)	7.18 ± 1.91 (1.00 ± 0.00)	7.13 ± 1.77[c] (1.03 ± 0.29)	12.67 ± 2.85 (1.91 ± 0.64)	6.64 ± 2.17[a] (0.98 ± 0.38)

Mean ± S.D. ($n = 6$). The values in parentheses are normalized to the control values (0 MCI-154, 0 P_i). Letters represent statistical significance of the differences between the values with and without MCI-154 ([a]$P < 0.01$; [b]$P < 0.001$; [c]not significant).

of P_i (Kitada, 1997) in skinned cardiac muscle preparations. To test whether MCI-154 was also effective to reverse the inhibitory effects of P_i in skeletal muscle fiber preparations, we examined how contractile properties were affected by 600 μM MCI-154 in the presence and absence of 20 mM P_i.

The results are summarized in Table 1. In the absence of MCI-154, 20 mM P_i reduced isometric tension by 50%, reduced fiber stiffness by 30% and increased k_{tr} by 18%, but hardly affected the shortening velocity. In the presence of 20 mM P_i, addition of 600 μM MCI-154 affected the contractile properties in the same manner as in the absence of P_i: It increased isometric tension and stiffness and reduced k_{tr}. Although the effect on isometric tension seemed to be somewhat greater in the presence of 20 mM P_i, other effects of 600 μM MCI-154 were comparable to those in the absence of P_i. This is in marked contrast to the effect in cardiac muscle preparations, in which concentrations greater than 20 mM of P_i are required to obtain its inhibitory effects in the presence of another Ca^{2+} sensitizer, EMD 53998 (Strauss et al., 1992). It is concluded, therefore, that MCI-154 has only modest if any effects to reverse the inhibitory effects of P_i in skeletal muscle preparations.

An interesting finding is that of the effect of MCI-154 on the relative phase of tension with respect to length during 500 Hz sinusoidal oscillation. In the presence of 20 mM P_i, a greater phase lead is observed (Iwamoto, 1995a). This is ascribed to fast configurational fluctuations of low-force, attached myosin cross-bridges (Iwamoto, 1995a). Addition of 600 μM MCI-154 completely abolished this extra phase lead, although the inhibitory effect of P_i on tension still existed. It is possible that MCI-154 also acts on low-force cross-bridges to stabilize their motion.

3.3. Effect of MCI-154 on tension–p_{Ca} characteristics

MCI-154 has a strong Ca^{2+}-sensitizing effect on cardiac muscle preparations. To examine whether MCI-154 also acts as a Ca^{2+} sensitizer on skeletal muscle preparations, isometric tension was measured at various Ca^{2+} concentrations in the presence and absence of 600 μM MCI-154. Fig. 4 compares the tension–pCa curves in the presence and absence of MCI-154 in the range of pCa in which isometric tension is most sensitive to Ca^{2+} levels. MCI-154 slightly shifted the tension–pCa curve to the higher p_{Ca} end (lower Ca^{2+} concentrations). The shift was much smaller than in the case of cardiac preparations, and the effect was seen only at high pCa. It is concluded, therefore, that MCI-154 is a poor Ca^{2+}-sensitizer for skeletal muscle preparations.

3.4. Effect of MCI-154 on the activity of creatine phosphokinase

The MCI-154-induced potentiation of tension and reduction of shortening velocity were similar to those induced by the application of ADP (Cooke and Pate, 1985; Hoar et al., 1987). Although a sufficient amount of creatine phosphokinase had been added to the solutions to keep the ADP level low (see Chase and Kushmerick, 1995), ADP could still accumulate in the interior of the fibers if

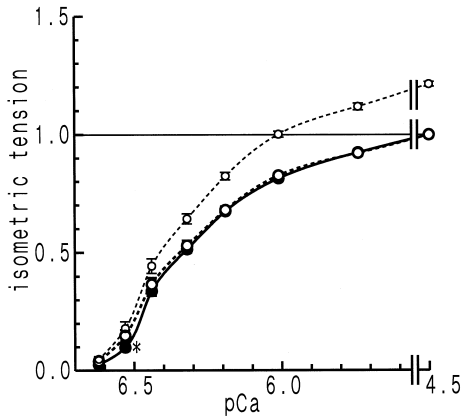

Fig. 4. Tension–pCa curves of rabbit skeletal muscle fibers in the presence and absence of 600 μM MCI-154. The region of the curves is shown in which isometric tension is most sensitive to pCa. Filled circles, control; open circles, in the presence of MCI-154. Greater circles represent data normalized to the fully activated tension (pCa = 4.49) in the absence of MCI-154, and the smaller circles, normalized to the fully activated tension in the presence of MCI-154. The error bars represent S.D. ($n = 6$) At pCa = 6.53 (asterisk), the potentiation induced by MCI-154 was significantly ($P < 0.05$) greater than that at other Ca^{2+} levels.

the activity of creatine phosphokinase was suppressed by MCI-154. To test this possibility, we assayed the activity of creatine phosphokinase (the same lot as used in fiber experiments) in the presence and absence of 600 μM MCI-154, by colorimetrically quantitating the creatine liberated through the reaction. Fig. 5 shows that MCI-154 has little effect on the activity of the creatine phosphokinase. Therefore, it is concluded that the observed effects of MCI-154 on the contractile properties were not caused by the accumulation of ADP within the fibers.

3.5. Effect of MCI-154 on ATPase activity during isometric contraction

Since MCI-154 is known to increase the ATPase activity of cardiac muscle preparation at a saturating Ca^{2+} level, it was of interest to test whether MCI-154 also increases the ATPase activity of skeletal muscle preparations. The previous experiment had demonstrated that the creatine quantitation is a valid method for ATPase activity measurement in the presence of MCI-154. Fig. 6 compares the time courses of creatine liberation during isomeric contraction in the presence and absence of 600 μM MCI-154. Both in the presence and absence of MCI-154, the amount of creatine liberated increased linearly with time. The ATPase activity per myosin head was 1.13 ± 0.07 s^{-1} ($n = 6$) in the absence of MCI-154, whereas in the presence of MCI-154, it was $\sim$20% lower (0.92 ± 0.06 s^{-1}). The difference was statistically significant ($P < 0.005$). This inhibitory effect of MCI-154 on ATPase activity is contrary to reports on cardiac muscle preparations (Kitada et al., 1989b; Liao and Gwathmey, 1993), but not unexpected since MCI-154 has been shown to reduce the kinetic parameters of contraction (Fig. 3).

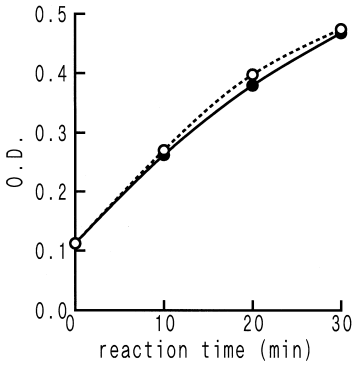

Fig. 5. Time course of creatine liberation from phosphocreatine by an ATP-regenerating reaction catalyzed by creatine phosphokinase in the presence and absence of 600 μM MCI-154. Filled circles, control; open circles, in the presence of MCI-154. The solution contained 5 mM creatine phosphate, 2.83 mM ADP and 0.6 U/ml creatine phosphokinase. The reaction was carried out at 37°C and stopped by adding *p*-hydroxymercuribenzoic acid to the solution. For the method of creatine quantitation see the section of ATPase measurements in Section 2.

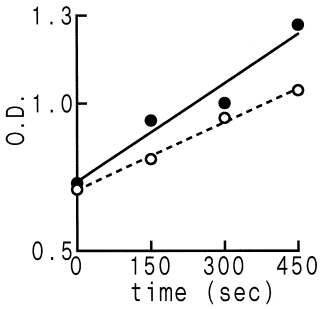

Fig. 6. An example of the measurement of ATPase activity during isometric contraction in the presence and absence of 600 μM MCI-154. Filled circles, control; open circles, in the presence of MCI-154. The lines were drawn by least squares regression. The high background level of absorbance may be due to contaminating ADP and/or spontaneous hydrolysis of ATP.

4. Discussion

4.1. Mechanism of action of MCI-154 on skeletal muscle

The present study showed that, at concentrations higher than 100 μM, MCI-154 increases the isometric tension of skinned skeletal muscle fibers at a saturating Ca^{2+} level (pCa = 4.49). On the other hand, only at pCa 6.3 did MCI-154, 600 μM, enhance the Ca^{2+} sensitivity of the skeletal muscle fibers to a far lesser extent than that for skinned cardiac preparations. The striking feature of the effect of MCI-154 on skeletal muscle fibers is that the tension enhancement at a saturating Ca^{2+} level is accompanied by an overall decrease of kinetic parameters, i.e., decreased velocity, decreased rate of rise of tension after shortening (k_{tr}) and decreased actomyosin MgATPase activity. This inhibitory effect of MCI-154, along with its very small Ca^{2+}-sensitizing effect, is not to be expected if MCI-154 increases the affinity of skeletal troponin-C for Ca^{2+}. Instead, all these effects are explainable if MCI-154 has a direct action on actomyosin. In particular, the concomitant increase of isometric tension and decrease of ATPase rate are readily explained if MCI-154 decreases the rate constant for a reaction step which limits the rate of dissociation of force-producing myosin heads from actin. At saturating ATP concentrations (millimolar range), this step would be the ADP release from myosin or an isomerization preceding it (cf., Dantzig et al., 1991; Fig. 7).

The effect of decreasing the rate constant for ADP release is clear if one considers a two-state model, such as the one by Huxley (1957), in which the rate constants for the association of myosin heads with actin and their dissociation are represented as f and g, respectively. In this model (in which association means force generation), the fraction of associated myosin heads (hence tension and

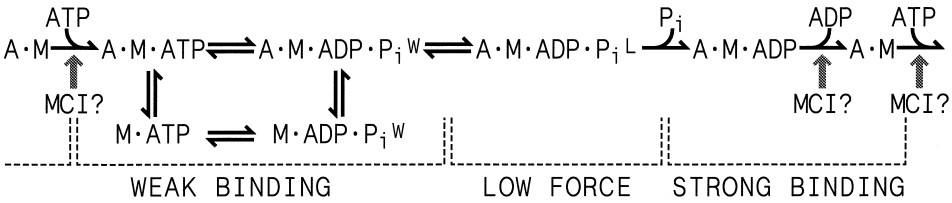

Fig. 7. Scheme for actomyosin ATPase cycle in skeletal muscle. Modified from Iwamoto, 1995b. A, actin; M, myosin. Possible steps to be inhibited by MCI-154 are indicated by thick stippled arrows.

stiffness) is expressed as $f/(f+g)$, ATPase rate $fg(f+g)$, and k_{tr} $f+g$. Decreasing g results in an increase of tension and a decrease of the ATPase rate and k_{tr}. The increase in stiffness upon addition of 600 μM MCI-154 was smaller than that of isometric tension, but the difference between them was not as great as reported for EMD 57033 (Kraft and Brenner, 1997). During shortening, a decreased rate of dissociation implies that a myosin head remains attached to actin for a while after completion of the power stroke, providing resistance to shortening. Force-producing (strong binding) myosin heads are also known to activate the thin filament in a cooperative manner (e.g., Lehrer, 1994). Thus, increased population of the force-producing myosin heads in the presence of MCI-154 is expected to shift the force–pCa curve leftward (towards the high pCa end) even if MCI-154 does not interact with troponin directly.

It is believed that the step of force generation is associated with the release of P_i from myosin (Hibberd et al., 1985; Fig. 7). The decreased isometric tension in the presence of P_i is therefore ascribed to the effect of P_i to shift the equilibrium of this step toward the form of myosin with bound P_i ($A \cdot M \cdot ADP \cdot P_i$). It has been suggested that the reversal of this step by P_i is due to an increase of the reverse rate constant for P_i release, and the increase occurs in a concentration-dependent manner (Dantzig et al., 1992). The value of k_{tr} is expected to increase, because in the aforementioned two-state model, k_{tr} is now expressed as $f_+ + f_- + g$, where f_+ and f_- are the forward and reverse rate constants for the force-generation step, respectively. The $A \cdot M \cdot ADP \cdot P_i$ was previously thought to represent a weak binding, rapidly equilibrating actomyosin complex. Later studies have shown that the $A \cdot M \cdot ADP \cdot P_i$ complex (more accurately, actomyosin complex in one of the $A \cdot M \cdot ADP \cdot P_i$ states) has slow dissociation kinetics, thus represents another class of intermediate (Iwamoto, 1995a,b). The $A \cdot M \cdot ADP \cdot P_i$ complex seems to repeat rapid configurational fluctuations, and this is reflected in the greater lead of tension over length during 500 Hz oscillation (Iwamoto, 1995a). In the presence of 20 mM P_i, parameters of the contractile performance of the skeletal muscle fibers were affected by MCI-154 to an extent comparable to that in the absence of MCI-154 (Table 1). This is consistent with the idea that the influence of MCI-154 is mainly on the dissociation of the force-producing myosin heads of skeletal muscle. This is in marked contrast to cardiac muscle preparations, in which MCI-154 and EMD 53998 are known to reverse the inhibitory effect of P_i (in other words, their potentiating effect is much greater in the presence of P_i than in its absence: Strauss et al., 1992, 1994; Kitada, 1997).

These effects of MCI-154 on skeletal muscle fibers are reminiscent of those of ADP. ADP is believed to compete with ATP for the nucleotide binding site on myosin (e.g., Lu et al., 1993). Since myosin heads with bound ADP have high affinity for actin, addition of ADP is expected to, and actually does, decrease the rate of dissociation of myosin heads from actin (Dantzig et al., 1991; Simnett et al., 1993). When applied to contracting skinned muscle fibers, ADP is known to increase isometric tension, decrease shortening velocity (Cooke and Pate, 1985) and shift the tension–pCa curve leftward (Hoar et al., 1987). The present results ruled out the possibility that MCI-154 raises the ADP concentration inside the fiber by inhibiting creatine phosphokinase activity. Therefore, one can say that MCI-154 acts as a non-metabolite inhibitor of actomyosin ATPase with effects equivalent to those of ADP. At present, however, the possibility that MCI-154 binds to nucleotide binding sites in a competitive manner cannot be excluded.

The effect of MCI-154 on skeletal muscle is inhibition in terms of enzymology, but another expression would be that it can improve the tension cost, i.e., the energy consumed to support a given amount of tension. At concentrations effective for cardiac muscles ($\sim$100 μM), MCI-154 has little effect on skeletal muscle fibers. If an appropriate method is found to load high concentrations of MCI-154 into skeletal muscle fibers, it may prove beneficial for improving the energetic performance of posture muscles.

4.2. Difference in the responsiveness of cardiac and skeletal muscle preparations to Ca^{2+} sensitizers

The most intensively studied Ca^{2+} sensitizer of this class is EMD 53998 (or its positive enantiomer, EMD 57033), a thiadiazinone derivative (referred to as EMD). The effects of MCI-154 on skinned cardiac and skeletal muscle preparations are compared with those of EMD in Table 2.

The effects of EMD on cardiac muscle preparations are similar to those of MCI-154. Both drugs have potentiating

Table 2
Current status of MCI-154 and EMD effects on the contractile properties of skinned cardiac and skeletal muscle preparations

	MCI-154		EMD	
	cardiac	skeletal	cardiac	skeletal
Isometric tension	↑↑[a]	↑↑[b]	↑↑[c]	↑↑[d]
Shortening velocity	→[e]	↓↓[b]	↑↑[f]	→[g]
Rate of rise of tension	?	↓↓[b]	↑[h]	→[g]
Rate of relaxation	?	?	↑[i]	→[i]
ATPase activity	↑[j]	↓↓[b]	↑↑[k]	→[g]
Reversal of P_i effect	↑[l]	→[b]	↑↑[m]	?
Calcium sensitization	↑↑[n]	↑[b]	↑↑[o]	?

Except for Ca^{2+} sensitization, items refer to parameters at saturating Ca^{2+} levels. ↑↑ denotes a substantial (≥20%) increase; ↑, a modest increase; →, little or no effect; ↓↓, substantial decrease; ?, not in the literature. Rate of rise of tension includes k_{tr} and the rate of rise after flash photolysis of caged Ca^{2+} (a photolabile Ca^{2+} chelator).
[a]Kitada et al., 1989a; [b]present paper; [c]Leijendekker and Herzig (1992), Ventura et al. (1992), Gross et al. (1993), Lues et al. (1993), Simnett et al. (1993); [d]Simnett et al. (1993), Kraft and Brenner (1997); [e]Sata et al. (1995) (in vitro motility assay); [f]Solaro et al. (1993) (in vitro motility assay); [g]Kraft and Brenner (1997); [h]Simnett et al. (1994), Arner et al. (1995); [i]Simnett et al. (1993); [j]Kitada et al. (1989b), Liao and Gwathmey (1993); [k]Ventura et al. (1992), Lues et al. (1993); [l]Kitada (1997), [m]Strauss et al. (1992, 1994); [n]Kitada et al. (1989a,b), Perreault et al. (1989), Sata et al. (1995) (in vitro motility assay); [o]Leijendekker and Herzig (1992), Ventura et al. (1992), Gross et al. (1993), Lues et al. (1993), Solaro et al. (1993), Barth et al. (1995). [n] and [o] papers which show force–pCa curves.

effects on almost all aspects of contraction listed in Table 2. Both can cancel the inhibitory effect of P_i. EMD improves the tension cost under potentiating conditions (Leijendekker and Herzig, 1992; Gross et al., 1993; Strauss et al., 1994). Although EMD is suggested to have direct effects on cardiac actomyosin (Solaro et al., 1993), there is also evidence which strongly suggests direct involvement of the troponin subunits in the effects of EMD (Barth et al., 1995). MCI-154 is also known to increase Ca^{2+} binding to cardiac troponin C (Kitada et al., 1989b).

EMD is known to accelerate the rise of tension in cardiac preparations (Leijendekker and Herzig, 1992; Simnett et al., 1994). This acceleration may be due to either the direct action of EMD on actomyosin kinetics, or the further activation of the thin filament. It is known that an increased level of activation results in an increased rate of rise of tension (e.g., Brenner, 1988), probably because of the increased rate constant for the transition from weak binding, rapidly dissociating actomyosin complex to low force, slowly dissociating complex (Regnier et al., 1995; Iwamoto, 1996).

The effects of EMD and MCI-154 on skinned skeletal muscle fibers are similar, and distinct from their effects on cardiac muscle preparations. Both potentiates isometric tension at saturating Ca^{2+} levels. However, they have only slight (EMD, Kraft and Brenner, 1997) or even negative (MCI-154, this work) effects on kinetic parameters including ATPase activity. As a result, tension cost is improved just as in cardiac muscle preparations. Slight or negative kinetic effects imply that, in skeletal muscle, isometric tension is not potentiated through further activation of the thin filament. Absence of the reversal of the inhibitory effect of P_i (in the case of MCI-154) may also support this assumption, since P_i is known to reduce the sensitivity of the contractile machinery to Ca^{2+}.

To summarize, of the two mechanisms of action of MCI-154 and EMD, the one through troponin seems to be less functional in skeletal muscle preparations. It is likely that, through the direct action of these drugs, the number of force-producing myosin heads (and tension per force-producing myosin) is increased but the kinetic parameters of the actomyosin are not. In general, skeletal muscle seems to be less sensitive to these Ca^{2+} sensitizers, probably because only one of the potentiating mechanisms (direct action on actomyosin) is working. It is likely that this difference between skeletal and cardiac muscle preparations reflects the difference in the properties of troponin isoforms (Barth et al., 1995). In skeletal muscles, the force of a muscle is regulated by the number of excited motor units and all that is needed for each muscle fiber is to contract in an all-or-none fashion. On the other hand, this way of regulation is unachievable for a functionally syncytial tissue of cardiac muscle, yet a wide spectrum of regulation of force is required to meet various physiological demands. In order to ensure this, cardiac muscle may keep its thin filament submaximally activated even at saturating Ca^{2+} levels under basal conditions.

5. Conclusion

We showed that MCI-154 increases isometric tension of skeletal muscle fibers at a saturating Ca^{2+} concentration, and suggested that this is due to its direct action on the kinetics of the actomyosin ATPase reaction. This property emphasizes the usefulness of MCI-154 as a tool for studying the mechanism of actomyosin interaction, since it acts as a non-metabolite inhibitor to increase the proportion of the force-producing actomyosin intermediates. There is a tendency to focus the action of Ca^{2+} sensitizers on the thin filament regulatory system, but the present results make it essential to focus on their direct action on actomyosin as well, to fully account for their potentiating effects. Further studies, including studies using isolated proteins from both skeletal and cardiac muscles, are needed to understand the mechanism of action of these Ca^{2+} sensitizers at a molecular level.

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