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Effect of Vanadate on Force and Myosin Light Chain Phosphorylation in Skinned Aortic Smooth Muscle[1]

Sachio Morimoto,[2,3] Osamu Sato,[2] and Yasuo Ogawa

Department of Pharmacology, Juntendo University School of Medicine, Hongo, Bunkyo-ku, Tokyo 113-8421

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The effects of vanadate were examined on Ca^{2+}-activated force and phosphorylation of 20-kDa myosin light chain in membrane-permeabilized rabbit aortic smooth muscle strips. Addition of vanadate during maximum contraction reduced the force in a dose-dependent manner, and inhibited it almost completely at 1 mM. Two-dimensional polyacrylamide gel electrophoretic analyses revealed that vanadate also reduced the phosphorylation of 20-kDa myosin light chain in a dose-dependent manner from ~50% in the absence of vanadate to ~20% in the presence of 1 mM vanadate. The effects of 1 mM vanadate on purified myosin light chain kinase and phosphatase were then examined using purified myosin as substrate, and it was found that vanadate neither inhibited myosin light chain kinase nor activated myosin light chain phosphatase. These results indicate that the reduction in the 20-kDa myosin light chain phosphorylation level by vanadate may be effected through its inhibition of the force generation in skinned smooth muscle strip, as evidenced by the finding that vanadate eliminated the enhancement of myosin light chain kinase activity brought about by the interaction between purified myosin and actin.

Key words: actin-myosin interaction, contraction, myosin light chain kinase, myosin light chain phosphatase, vascular smooth muscle.

Vanadate (Vi) is an inhibitor of myosin ATPase that blocks the force-generating interaction of cross-bridges with actin by forming a stable cross-bridge intermediate, myosin-ADP-Vi (*1, 2*). It inhibits the Ca^{2+}-activated force generation in membrane-permeabilized (skinned) smooth muscle (*3, 4*) as well as in skinned striated muscle (*5-7*), suggesting that a common molecular mechanism underlies the force generation in these two types of muscles with quite different regulatory mechanisms of contraction.

While the contraction of striated muscle is regulated by a specific regulatory protein complex, troponin, located in the thin filaments, the contraction of smooth muscle is generally thought to be activated by phosphorylation of 20-kDa myosin light chain (MLC_{20}), the level of which is regulated by the activity balance between Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (*8*). Although Vi has been shown to inhibit the force-generating interaction of rapidly cycling phosphorylated and slowly cycling unphosphorylated myosin cross-bridges with actin in skinned smooth muscle (*3, 4*), it remains to be determined whether or not Vi has any effect on the regulatory system in smooth muscle. Thus, in the present study, the effects of Vi on the phosphorylation of MLC_{20} and the tension development were examined simultaneously in skinned smooth muscle strips. It was found that Vi inhibited the phosphorylation of MLC_{20} as well as the force generation in these strips. Unexpectedly, however, Vi had no inhibitory effect on purified MLCK and no activating effect on purified MLCP. Using actin and myosin purified from smooth muscles, we further showed that the coexistence of these proteins stimulated the phosphorylating activity of the purified MLCK, and that this stimulation of MLCK activity was suppressed by Vi. These results strongly suggest that the phosphorylation level of MLC_{20} in smooth muscle is increased by the force-generating interaction between myosin and actin so that Vi reduces the phosphorylation level of MLC_{20} by inhibiting the actin-myosin interaction. Part of this work has been reported previously in abstract form (*9, 10*).

MATERIALS AND METHODS

Stock Solutions of Vi—Stock solutions of Vi were prepared from sodium orthovanadate (Na_3VO_4) purchased from Sigma, and the Vi concentration was determined as described by Goodno (*1*).

Preparation of Skinned Strips and Tension Measure-

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[2] The first two authors contributed equally to this work.

[3] To whom correspondence should be addressed to the present address: Department of Clinical Pharmacology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582. Tel: +81-92-642-6081, Fax: +81-92-642-6084, E-mail: morimoto@clipharm.med.kyushu-u.ac.jp

Abbreviations: CaM, calmodulin; DTT, dithiothreitol; IS, ionic strength; MLC_{20}, 20-kDa myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MOPSO, 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid; pCa, $-\log [Ca^{2+}]$; TCA, trichloroacetic acid; Vi, vanadate.

ments—The procedures for measurement of the Ca^{2+}-activated tension development in skinned smooth muscle strips have been described in detail previously (*11*). Briefly, transverse intimal-medial strips were isolated from rabbit thoracic aortas according to the method of Wolinsky and Daly (*12*) and chemically skinned with 1% Triton X-100 and 50% glycerol. A small transverse strip (~1 mm in width and ~4 mm in length) dissected from a stock skinned preparation was then mounted in a thermostatically controlled chamber with a capacity of ~0.9 ml, and isometric tension was measured at 25°C with a semiconductor strain gauge (AE801; Aksjeselskapet Micro-Elektronikk, Hoten, Norway). Bathing solutions contained 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.5 mM dithiothreitol (DTT), 50 mM 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid (MOPSO)/KOH (pH 7.0), 4 mM EGTA, and an appropriate amount of $CaCl_2$ to give a specified free Ca^{2+} concentration.

Protein Preparations—MLCK was purified from chicken gizzard or bovine stomach by the method of Walsh *et al.* (*13, 14*). MLCP (heterotrimeric smooth muscle myosin phosphatase) was purified from chicken gizzard by the method of Alessi *et al.* (*15*). Protein concentrations of MLCK and MLCP were determined according to Kaplan and Pedersen (*16*). Myosin from chicken gizzard was purified by the method of Ebashi (*17*). Myosin similarly prepared from bovine stomach was further purified by a hydroxyapatite column according to Katoh and Morita (*18*). Protein concentration of myosin was determined photometrically using the absorption coefficient $E^{0\,1\%}$ of 0.52 at 280 nm (*19*). CaM from bovine brain was prepared according to Yazawa *et al.* (*20*), and its protein concentration was determined photometrically using the absorption coefficient $E^{0\,1\%}$ of 0.20 at 277 nm. Actin was purified from chicken gizzard by the method of Ebashi (*21*), and desensitized myosin B was prepared from chicken gizzard by the method of Ebashi and Koga (*22*). Protein concentrations of actin and desensitized myosin B were determined by the biuret method using bovine serum albumin as a standard.

Measurements of MLC_{20} Phosphorylation—Two-dimensional isoelectric/SDS-PAGE was carried out according to the method of O'Farrell (*23*), using 2.8% Pharmalyte (pH 4.5-5.4), 5 mM NaOH cathode-electrode solution, and 10 mM glutamate anode-electrode solution on the first dimension, and the phosphorylation levels of smooth muscle MLC_{20} in skinned strips, desensitized myosin B, and isolated myosin were determined as described previously (*11*) using an optical densitometer (MasterScan interpretive densitometer; Scanalytics, Billercia, MA).

Phosphorylation Assay for Purified MLCK—The time course of phosphorylation of MLC_{20} in purified myosin by purified MLCK was determined in the presence or absence of Vi. The assay was carried out at 25°C in 9 ml of a reaction mixture containing 50 mM MOPSO/KOH (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 4 mM EGTA/$CaCl_2$ (pCa 4.6), 0.5 mM DTT, 65 μg/ml of chicken gizzard myosin, 1 μM CaM, 1 μg/ml of chicken gizzard MLCK, and 1 mM or no Vi. After 5 min of preincubation with or without Vi at 25°C, the reaction was started by the addition of ATP, allowed to proceed under gentle stirring for various periods of time, and stopped by pipetting 1-ml aliquots of the reaction mixtures into 0.3 ml of ice-cold 60% (w/v) trichloroacetic acid (TCA). The mixtures were kept on ice for at least 10 min, then centrifuged at 15,000 rpm for 15 min at 4°C (Hitachi Himic CF15D). The resulting precipitates were washed twice to remove TCA by suspending and sedimenting them in 1 ml of 100% acetone. The precipitates were dissolved in 50 μl of lysis buffers [9.5 M urea, 2% (w/v) Nonidet P-40, 2.8% Pharmalyte (pH 4.5-5.4), and 5% 2-mercaptoethanol] for 1 h at room temperature and subjected to the two-dimensional gel electrophoresis.

Dephosphorylation Assay for Purified MLCP—Bovine stomach myosin (1 mg/ml) was phosphorylated by incubation with bovine stomach MLCK (20 nM) for 10 min at 25°C in a solution containing 20 mM MOPSO/KOH (pH 6.8), 5.5 mM $MgCl_2$, 0.5 mM ATP-Tris, 0.1 mM $CaCl_2$, 0.3 μM CaM, and an appropriate amount of KCl to adjust ionic strength to 0.07 M, then purified by a hydroxyapatite column according to Katoh and Morita (*18*). The extent of phosphorylation was determined to be 93.6 ± 0.4% (mean ± SE, $n = 5$) of total smooth muscle MLC_{20}. The effect of Vi on the dephosphorylating activity of purified MLCP was then determined using the phosphorylated myosin as substrate. The assay was carried out at 25°C in 200 μl of a reaction mixture containing 50 mM MOPSO/KOH (pH 7.0), 117.5 mM KCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mg/ml of phosphorylated myosin, and 55 ng/ml of MLCP with or without 1 mM Vi. The phosphorylated myosin was preincubated in the reaction mixture for 1 min at 25°C. After a further 1 min of incubation with or without Vi, the reaction was started by the addition of MLCP and stopped after 30 s by the addition of 200 μl of 10% (w/v) TCA. The resulting mixture was centrifuged at 15,000 rpm for 5 min at 4°C (Hitachi Himic CF15D) and the precipitate was washed twice with 100% acetone. The washed precipitate was dissolved in 12.5 μl of the lysis buffer and centrifuged at 15,000 rpm for 2 min at room temperature (Eppendorf centrifuge 3200) to remove insoluble materials, and the supernatant was subjected to two-dimensional gel electrophoresis.

Superprecipitation—Superprecipitation of desensitized myosin B was monitored at 25°C by measuring the change in absorbance at 660 nm using a SHIMADZU UV-2100PC spectrophotometer according to the method described by Ebashi (*24*). Reaction mixture consisted of 20 mM MOPSO/KOH (pH 6.8), 5.3 mM $MgCl_2$, 0.3 mM ATP-Tris, 1 mM EGTA or 0.1 mM $CaCl_2$, 1 μM CaM, 0.5 μg/ml of chicken gizzard MLCK, and 60 μg/ml of chicken gizzard desensitized myosin B. Ionic strength was adjusted with KCl.

RESULTS

Figure 1 shows the effect of Vi on the Ca^{2+}-activated tension development of rabbit skinned aortic smooth muscle strip. Cumulative addition of Vi from 0.1 to 1.0 mM suppressed the contraction elicited by maximal Ca^{2+} in a dose-dependent manner, and with subsequent washout of Vi the tension slowly recovered to the original level. The IC_{50} for Vi was about 150 μM (Fig. 2), which is in good agreement with the value reported for porcine skinned carotid artery (~250 μM) (*4*), but is much higher than that for guinea pig taenia coli (23 μM) or trachea (42 μM) (*3*). Vi was also found to inhibit the resting tension in the absence of Ca^{2+} (Fig. 2).

The phosphorylation level of MLC_{20} in the skinned

smooth muscle strips was then checked by two-dimensional isoelectric/SDS-PAGE (Fig. 3). While about 50% of MLC_{20} was phosphorylated in the maximally contracted strip (control), subsequent addition of 1 mM Vi greatly diminished the phosphorylation level to about 20%. This effect of Vi on the MLC_{20} phosphorylation was dose-dependent, and a significant inhibition was also observed at a lower concentration, 0.1 mM Vi (Table I). Vi also appeared to inhibit the MLC_{20} phosphorylation in the relaxed skinned strips, although this was not statistically significant (Table I). These results indicate that Vi inhibits the phosphorylation of MLC_{20} as well as the tension development in skinned aortic smooth muscle.

To see whether the decrease in the phosphorylation level of MLC_{20} is due to inhibition of MLCK and/or activation of MLCP in the skinned aortic smooth muscle, the effects of Vi on the activities of isolated MLCK and MLCP were examined. Figure 4 shows the time courses of MLC_{20} phosphorylation of purified myosin by MLCK in the presence and absence of 1 mM Vi. The phosphorylating reactions were found to proceed in two exponential time courses as reported previously (*25-27*). Sellers *et al.* (*27*) have proposed that this biphasic phosphorylation could be explained by either a random phosphorylation of the two myosin heads with unequal rates because of a pre-existing asymmetry between them, or a negatively cooperative phosphorylation of the two heads that might come from their nonequivalence in the filamentous structure of myosin. Our data were found to be well explained by the negatively cooperative phosphorylation of the two myosin heads described by the equation, $P_t = 100 + (50/(k_1 - k_2))$

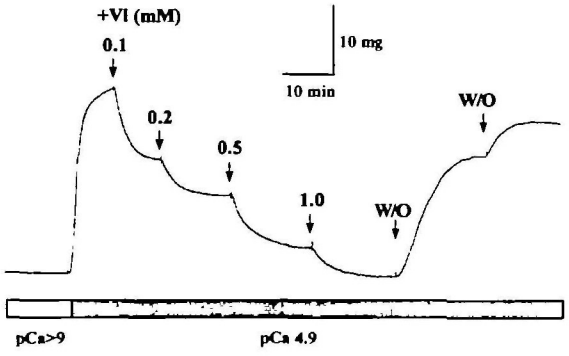

Fig. 1. **Effect of Vi on Ca^{2+}-activated tension development of a skinned aortic smooth muscle strip.** Vi was cumulatively added to a skinned strip maximally contracted in the activating solution of pCa 4.9. Tension was decreased by Vi and reached the steady-state within 10 min. Washout (W/O) of Vi restored tension slowly to the original level.

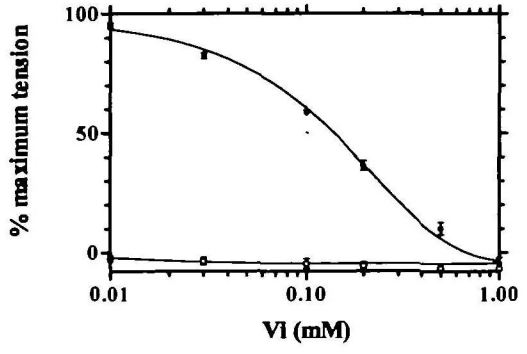

Fig. 2. **Dose-dependent inhibition by Vi of tension development of skinned aortic smooth muscle strips.** Steady-state levels of tension achieved by cumulative addition of Vi to activating solution (pCa 4.9) (●) or to relaxing solution (pCa>9) (○) as shown in Fig. 1 were normalized to the maximal tension produced in the activating solution (pCa 4.9) without Vi and given as means±SE for three determinations.

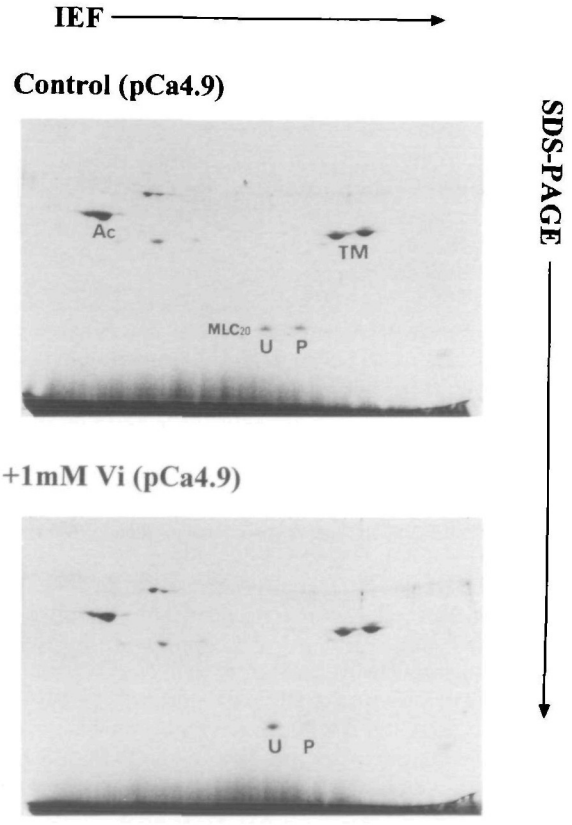

Fig. 3. **Two-dimensional isoelectric/SDS-polyacrylamide gel electrophoresis of extracts from skinned aortic smooth muscle strips.** Strips were first incubated with the relaxing solution (pCa>9) at 25°C for 20 min and then maximally contracted in the activating solution (pCa 4.9) for 10 min. After another 10 min of incubation in the presence (+1 mM Vi) or absence (Control) of Vi, strips were fixed by immersion in ice-cold 15% TCA and subjected to two-dimensional gel electrophoresis. U and P, un- and monophosphorylated smooth muscle MLC_{20}, respectively; Ac, actin; TM, tropomyosin.

TABLE I. **Effect of Vi on phosphorylation level of MLC_{20} in skinned aortic smooth muscle strip.** Strips were first incubated with the relaxing solution at 25°C for 20 min, then maximally contracted in the activating solution (pCa 4.9) or kept in the relaxing solution for a further 10 min. After another 10 min of incubation with or without Vi, strips were fixed by TCA and subjected to two-dimensional gel electrophoresis as shown in Fig. 3. Values are means±SE for three strips.

Vi (mM)	MLC_{20} phosphorylation level (%)	
	$-Ca^{2+}$(pCa>9)	$+Ca^{2+}$ (pCa 4.9)
0	5.3±1.6	52.5±0.2
0.1		36.0±2.0*
1.0	0.0±1.1	20.0±2.2*

*$p<0.01$ *vs.* 0 mM Vi (control) (Dunnett's multiple-comparison test).

$(\exp(-k_1t)(2k_2-k_1)-k_1\exp(-k_2t))$ (*27*), where P_t is the phosphorylation level (%) at time t, and the rate constants k_1 and k_2 were determined to be 1.41 and 0.23 min^{-1} in the absence of Vi and 1.51 and 0.26 min^{-1} in the presence of 1 mM Vi, respectively (Fig. 4). The F test indicated that this model fitted significantly ($p<0.05$) better than a single exponential model described by the equation, $P_t=100(1-\exp(-kt))$, or the model with pre-existing asymmetry between the two myosin heads described by the equation, $P_t=50(1-\exp(-k_1t))+50(1-\exp(-k_2t))$ (*27*). The rate constants of $k_1=1.41$ and $k_2=0.23$ min^{-1} were in excellent agreement with those reported by Sellers *et al.* (*i.e.*, $k_1=1.4$ min^{-1} and $k_2=0.225$ min^{-1}) (*27*). No effects of Vi were found on these kinetic parameters (1.51 and 0.26 min^{-1}, respectively), indicating that Vi has no direct effects on the phosphorylating activity of MLCK. Vi also had no effect on the dephosphorylating activity of MLCP up to 0.3 mM (data not shown), but was found to have a significant inhibitory effect at 1 mM Vi (Fig. 5). This inhibitory effect of Vi on MLCP was unexpected, because it would result in the increase in the MLC_{20} phosphorylation level in the skinned smooth muscle strips rather than the decrease in its level which has been observed. Thus, the Vi-induced reduction in the phosphorylation level of MLC_{20} cannot be explained by any direct effect of Vi on the phosphorylating or dephosphorylating system in the skinned smooth muscle strips.

We next examined the effect of actin-myosin interaction on the phosphorylation level of MLC_{20} in a more simplified system of desensitized myosin B (an actomyosin preparation lacking MLCK activity) or purified (MLCK- and MLCP-free) actin and myosin (Fig. 6). The desensitized myosin B developed no superprecipitation accompanied with the phosphorylation of MLC_{20} even in the presence of Ca^{2+}, and the addition of purified MLCK and CaM restored a Ca^{2+}-induced superprecipitation and the phosphorylation of MLC_{20}, indicating that MLCK and CaM are lost in this preparation. Furthermore, removal of Ca^{2+} by EGTA after the superprecipitation reached a plateau did not reduce the phosphorylation of MLC_{20} (data not shown), indicating that the desensitized myosin B used in the present study also shows little, if any, MLCP activity. Using the desensitized myosin B supplemented with a given amount of purified MLCK and CaM, we determined simultaneously the phosphorylation level of MLC_{20} and the extent of superprecipitation in a medium with varied ionic strength, because superprecipitation is considered to well reflect the contractile activity *in vitro*, and the ionic strength is known to affect the interaction of myosin and actin (Fig. 7). At an ionic strength (IS) of 0.05 M, the superprecipitation (elevation of the turbidity caused by actin-myosin interaction) of desensitized myosin B occurred in a Ca^{2+}-dependent manner, while the steady-state level of MLC_{20} phosphorylation increased from 6% (1 mM EGTA) to 90% (0.1 mM $CaCl_2$). The Ca^{2+}-dependent superprecipitation was almost completely inhibited by increasing the ionic strength to 0.09 M, indicating that the actin-myosin interaction is

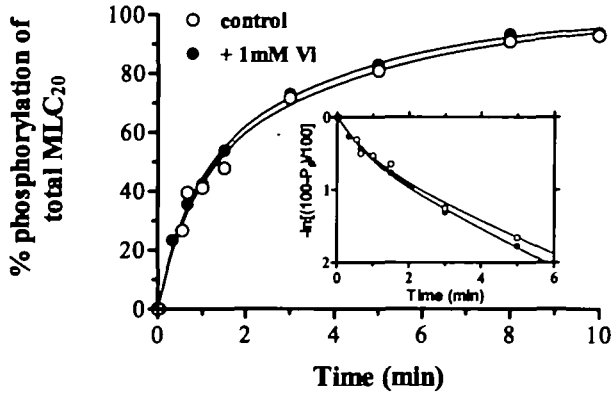

Fig. 4. **Effect of Vi on the time course of MLC_{20} phosphorylation in isolated myosin by purified MLCK.** The lines in the figure represent the best fit to the data using the following equation on the assumption of a negatively cooperative phosphorylation of the two myosin heads by MLCK (see Ref. *27*): $P_t=100+(50/(k_1-k_2))(\exp(-k_1t)(2k_2-k_1)-k_1\exp(-k_2t))$, where P_t is the phosphorylation level (%) at time t and the rate constants k_1 and k_2 were determined to be 1.41 and 0.23 min^{-1} in the absence of Vi (control) and 1.51 and 0.26 min^{-1} in the presence of 1 mM Vi, respectively. Inset shows a semilogarithmic plot of the data.

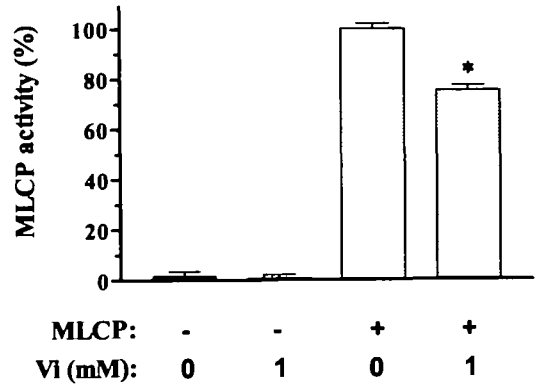

Fig. 5. **Effect of Vi on dephosphorylation of phosphorylated MLC_{20} in isolated myosin by purified MLCP.** The extent of dephosphorylation within the initial 30 s of the reaction was normalized to that in the presence of MLCP without Vi and expressed as relative MLCP activity. Data represent means±SE for three determinations. $^*p<0.001$ *vs.* 0 mM Vi+MLCP (unpaired t-test).

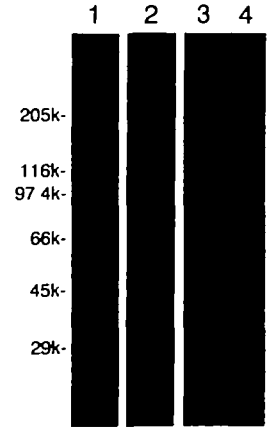

Fig. 6. **SDS-PAGE of myosin B (lane 1), desensitized myosin B (lane 2), purified myosin (lane 3), and purified actin (lane 4) prepared from chicken gizzard.** Electrophoresis was carried out by the method of Laemmli (*45*), and the gel was stained with Coomassie Brilliant Blue R-250. Note that the 130-kDa band corresponding to MLCK was selectively removed in desensitized myosin B.

substantially inhibited at this ionic strength. Under these conditions, steady-state levels of MLC_{20} phosphorylation were about 5% (1 mM EGTA) and about 70% (0.1 mM $CaCl_2$). The extents of superprecipitation and MLC_{20} phosphorylation were intermediate at the ionic strength of 0.07 M. Thus, the phosphorylation level of MLC_{20} of myosin B in the presence of Ca^{2+} decreased by about 20% when ionic strength was increased from 0.05 to 0.09 M. On the other hand, the MLCK activity determined by using purified myosin as substrate was almost unchanged (the phosphorylation level of ~70%) in the ionic strength range of 0.05-0.11 M (Fig. 8). These results suggest that the phosphorylation level of MLC_{20} is enhanced by the actin-myosin interaction and that the inhibition of actin-myosin interaction does reduce the apparent MLCK activity. Furthermore, the extent of the MLC_{20} phosphorylation of purified myosin by MLCK was found to be directly enhanced by the addition of purified actin (Table II); the phosphorylation levels of MLC_{20} of purified myosin in the presence of 0.1 mM Ca^{2+}/CaM and MLCK were dose-dependently elevated by increasing the concentration of actin; tropomyosin had little effect on this enhancement of MLC_{20} phosphorylation of purified myosin by actin (data not shown). While Vi had no effects on the MLC_{20} phosphorylation of purified myosin by MLCK in the absence of actin, it suppressed the extra phosphorylation of MLC_{20} generated in the presence of actin (Fig. 9). These *in vitro* studies demonstrate that the MLCK activity is increased by actin-

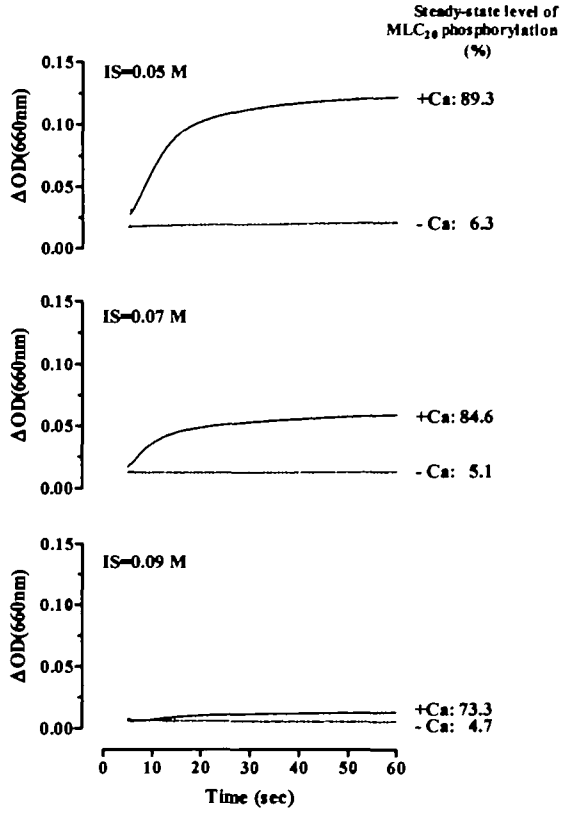

Fig. 7. **Effects of the ionic strength on superprecipitation and MLCK activity of desensitized myosin B.** Superprecipitation of desensitized myosin B in the presence of CaM and MLCK was determined in the presence (solid lines) or absence (dashed lines) of 0.1 mM Ca^{2+} at varied ionic strengths (IS) as described in "MATERIALS AND METHODS." After 5 min of preincubation at 25°C, the reaction was started by the addition of MgATP. After 1 min of the recording of superprecipitation, 5% (w/v) TCA was added to the reaction medium to determine the phosphorylation level of MLC_{20} (see also "MATERIALS AND METHODS").

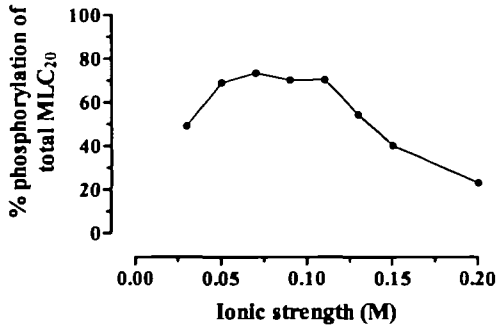

Fig. 8. **Effect of ionic strength on the intrinsic MLCK activity.** Effect of ionic strength on the MLC_{20} phosphorylation by MLCK in the presence of 0.1 mM Ca^{2+}/CaM was examined using purified chicken gizzard myosin as substrate under otherwise the same conditions as those for the MLC_{20} phosphorylation of desensitized myosin B in Fig. 7. The protein concentration of purified myosin was adjusted to 36 μg/ml, which was equivalent to the myosin concentration in the desensitized myosin B as determined by SDS-PAGE analysis.

TABLE II. **Effect of actin on MLC_{20} phosphorylation of purified myosin.** Effect of actin on MLC_{20} phosphorylation of purified myosin by MLCK was examined by increasing the concentration of actin in the reaction mixture under conditions similar to those in Fig. 8. Ionic strength was 0.05 M.

Actin/myosin (molar ratio)	MLC_{20} phosphorylation level (%)	
	$-Ca^{2+}$	$+Ca^{2+}$
0	4.0	63.0
0.33	3.3	67.8
1.0	4.2	72.3
3.3	3.7	80.5
10	3.8	80.4

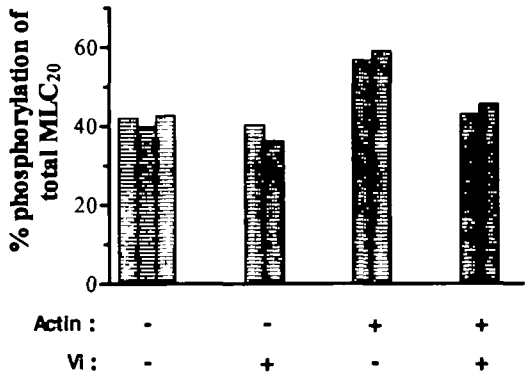

Fig. 9. **Effect of Vi on MLC_{20} phosphorylation of purified myosin in the presence or absence of actin.** Effect of Vi (1 mM) on MLC_{20} phosphorylation of purified myosin by MLCK was examined in the presence of 0.1 mM Ca^{2+} at an actin/myosin molar ratio of 0 (Actin: −) or 20 (Actin: +) under conditions similar to those in Table II except that the protein concentration of MLCK was reduced to half (0.25 μg/ml) and 10 nM microcystin-LR, an MLCP inhibitor, was included. Note that the MLC_{20} phosphorylation levels in the absence of Vi were considerably lower than those in Table II because of the MLCK concentration being reduced to half.

myosin interaction, supporting the idea that the inhibition of MLC_{20} phosphorylation by Vi in skinned aortic smooth muscle is due to the inhibition of the force-generating interaction between myosin and actin.

DISCUSSION

It was shown that pretreatment with Vi produced a Ca^{2+}-independent force without MLC_{20} phosphorylation in Triton X-100-permeabilized taenea coli fibers, probably due to the oxidation of regulatory or contractile proteins (*28*). This phenomenon occurred at relatively high concentrations of Vi (threshold at 1-2 mM) in the absence of reducing agent such as DTT and was reversed by DTT. In the present study, the effects of Vi on the force and MLC_{20} phosphorylation in permeabilized aorta fibers were examined in the presence of 0.5 mM DTT, and no Ca^{2+}-independent force was elicited after Vi treatment under these conditions (data not shown). Thus, the effects of Vi observed in the present study should be independent of the Vi oxidation.

Several layers of regulation of activities of MLCK and MLCP involving cAMP-dependent protein kinase, Ca^{2+}/CaM-dependent protein kinase II, small GTP-binding protein, and protein kinase C have been suggested to exist in smooth muscle cells (*29-31*). However, it is unlikely that Vi changes the phosphorylation level of MLC_{20} in the Triton-X100-demembranated smooth muscle preparations used in the present study by affecting one or more of these regulatory mechanisms, because these mechanisms may operate only in smooth muscle preparations that were permeabilized more mildly by the use of α-toxin or β-escin.

In striated muscle, it has been demonstrated that the force-generating cross-bridge interaction with actin enhances the Ca^{2+}-binding affinity of troponin C through the activation of the thin filaments (*32-37*), and that Vi inhibition of the force generation reduces the Ca^{2+}-binding affinity of troponin C (*7*). The present study provides the first evidence that the force-generating cross-bridge interaction with actin in smooth muscle increases the phosphorylating activity of MLCK so that the Vi inhibition of the force generation reduces the enhancement of MLCK activity. Since MLCK is thought to be predominantly associated with the thin filaments (*19, 38, 39*), the force-generating cross-bridge interaction with actin might increase the Ca^{2+}-binding affinity of CaM which is associated with MLCK, just as in the case of the Ca^{2+}-binding affinity of troponin C in striated muscle. However, this could not explain the inhibitory effects of Vi or ionic strength on the MLC_{20} phosphorylation in the skinned smooth muscle strips, myosin B, and purified actin-myosin system, because these effects have been observed in the presence of a saturating concentration of Ca^{2+}. An X-ray diffraction study has shown that most myosin-ADP-Vi complexes are detached from actin in skinned skeletal muscle fiber (*40*). Thus, the force-generating cross-bridge interaction with actin may increase the apparent MLCK activity by maintaining the close proximity of MLC_{20} on myosin cross-bridge and MLCK on the thin filaments, *i.e.*, by increasing the effective concentrations of MLC_{20} and/or MLCK. This idea is supported by the finding that the level of MLC_{20} phosphorylation of purified myosin by MLCK is elevated by increasing the concentration of actin, and Vi suppresses this elevated phosphorylation. Although there is some uncertainty about the true form of MLCP responsible for the dephosphorylation of MLC_{20} *in vivo* as well as its localization (*15, 41, 42*), the heterotrimeric smooth muscle myosin phosphatase, a strong candidate for MLCP in smooth muscle, contains subunits that bind myosin regardless of the presence or absence of MLC_{20} phosphorylation (*43*). This suggests that MLCP may be localized in the thick filaments. Thus, the localization of MLCK and MLCP in the thin and thick filaments, respectively, may provide a basis for the dynamic change in the phosphorylation level of MLC_{20} in smooth muscle during the transition from relaxed to force-generating state in response to Ca^{2+}.

Finally, it is noteworthy that stretching the depolarized or agonist-stimulated intact smooth muscle preparations has been reported to increase the MLC_{20} phosphorylation in the ascending limb of the force-length relation (*44*). The findings of the present study suggest that an increase in the apparent MLCK activity by cross-bridge interaction with actin may be involved in this length-dependent change in the phosphorylation level of MLC_{20}.

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