

Full Paper

## Inhibition of High $K^+$ -Induced Contraction by the ROCKs Inhibitor Y-27632 in Vascular Smooth Muscle: Possible Involvement of ROCKs in a Signal Transduction Pathway

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**Abstract.** In the isolated rat aorta, a ROCKs (rhoA-dependent coiled coil serine/threonine kinases) inhibitor, Y-27632, inhibited the contractions induced not only by receptor agonists but also by high  $K^+$  with the similar  $IC_{50}$  values (0.8–4.9  $\mu M$ ). However, Y-27632 did not inhibit the increment of cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) due to these stimulants. The Y-27632-induced inhibition of contraction was accompanied by an inhibition of myosin light chain (MLC) phosphorylation, although inhibition of contraction was stronger than that of MLC phosphorylation during the initial phase of contraction. Y-27632 had no effect on the myosin light chain kinase (MLCK) activity. This inhibitor also did not directly change the phosphatase activity. These results suggest that Y-27632 is a selective inhibitor of ROCKs with no direct inhibitory effect on  $[Ca^{2+}]_i$ , calmodulin, MLCK, or phosphatase. Y-27632 disrupted the actin filament network and decreased the filamentous actin, implying that the stronger inhibition by Y-27632 on early phase of contraction than MLC phosphorylation may be explained by this effect. These results suggest that the high  $K^+$ -induced MLC phosphorylation and contraction are mediated not only by the classical  $Ca^{2+}$ /calmodulin-dependent MLCK system but also by a novel MLC phosphorylation pathway involving ROCKs. One of the possibilities is that high  $K^+$  activates ROCKs to inhibit myosin phosphatase resulting in an augmentation of MLC phosphorylation and contraction.

**Keywords:** rho-kinase, Y-27632, rat aorta, A7r5, myosin light chain kinase

### Introduction

Smooth muscle contraction is regulated by  $[Ca^{2+}]_i$  and  $Ca^{2+}$  sensitivity of contractile elements. In the former mechanism,  $Ca^{2+}$  binds to calmodulin and acti-

vates MLCK to phosphorylate MLC, which interacts with actin filaments to induce contraction. The contraction induced by high  $K^+$ , which changes  $K^+$  equilibrium potential to activate the L-type  $Ca^{2+}$  channels, is believed to be regulated solely by  $[Ca^{2+}]_i$  (1). During a receptor agonist-induced contraction, on the other hand, the second mechanism is also activated; i.e., contractile elements are sensitized to  $Ca^{2+}$  to induce a greater

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Abbreviations used are (in alphabetical order):  $[Ca^{2+}]_i$ , cytosolic  $Ca^{2+}$  concentration; DFP, diisopropylfluorophosphate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, *O,O'*-bis (2-amynomethyl) ethylenglychol-*N,N,N',N'*-tetraacetic acid; F-actin, filamentous actin; FITC, fluorescein isothiocyanate; fura-2/AM, acetoxymethyl ester of fura-2; fura-PE3/AM, acetoxymethyl ester of fura-PE3; HEPES, 2-[4-(2-hydroxymethyl) 1-piperazinyl]ethanesulfonic acid; MBS, myosin-binding aubunit; MLC, myosin light chain; MLCK, myosin light chain kinase; PIPES, piperazine-1,4-bis (2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; PSS, physiological salt solution; PVDF, polyvinylidene difluoride; ROCK, rhoA-dependent coiled coil serine/threonine kinase; TCA, trichloroacetic acid; Y-27632, (+)-(R)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridil) cyclohexanecarboxamide-monohydrate.

MLC phosphorylation and greater force at a given  $[Ca^{2+}]_i$  (for review see refs. 2 and 3).

Recent studies have shown that a small GTP-binding protein, rhoA, and ROCKs play a major role in  $Ca^{2+}$  sensitization. The rhoA activated by receptor stimulation (rhoA-GTP) can in turn activate ROCKs. ROCKs phosphorylate MBS of MLC phosphatase and inactivate its activity. As a result, MLCK-induced MLC phosphorylation is augmented to induce a greater contraction at a given  $[Ca^{2+}]_i$  (4–6) (for review see refs. 3 and 7). In addition, it has been reported that ROCKs directly phosphorylate MLC at Ser<sup>19</sup> in vitro (8, 9) and in vivo (10).

Y-27632 has been shown to inhibit a ROCK isoform, p160ROCK (11). An isoquinoline derivative, HA-1077, has also been reported to be a ROCK inhibitor (12). In rat aorta, however, HA-1077 inhibits the increase in  $[Ca^{2+}]_i$  due to high  $K^+$  or norepinephrine, suggesting that HA-1077 has multiple sites of action to relax rat aorta (13). In addition, the  $K_i$  values for Y-27632 and HA-1077 for various serine-threonine kinases demonstrate that Y-27632 is a more selective ROCKs inhibitor than HA-1077 (11). A recent study has revealed that the inhibitory effect of Y-27632 on ROCKs is reversed by ATP in a competitive manner, indicating that Y-27632 inhibits kinases by binding to the catalytic site (14).

Y-27632 has been reported to inhibit various biological functions of smooth muscles, including actin formation,  $Ca^{2+}$  sensitization of contractile elements (11, 15), DNA synthesis, cell migration (16), coronary arterial spasm (17), neointimal formation of balloon-injured arteries (18), and hypertension (11). In addition, Y-27632 inhibits the transcellular invasion of tumor cells (19). Y-27632 is thus likely to be important not only as a tool for studying cell functions, but also for therapeutic applications. However, the pharmacological action of Y-27632 on smooth muscle contractility has not yet been fully understood. The aim of the present study is to investigate the inhibitory mechanisms of Y-27632 on vascular smooth muscle contractions induced by high  $K^+$  depolarization and receptor stimulations.

## Materials and Methods

### *Smooth muscle tissue preparation and measurement of isometric force*

Male Wistar rats (250–300 g) and male white rabbits (2 kg) were killed by a sharp blow to the neck and exsanguination. The thoracic aorta and mesenteric artery were isolated and cut into rings 2–3 mm and 1–2 mm in width, respectively. Isolated muscle strips were placed in a PSS containing 136.9 mM NaCl, 5.4 mM

KCl, 1.5 mM  $CaCl_2$ , 1.0 mM  $MgCl_2$ , 23.8 mM  $NaHCO_3$ , and 5.5 mM glucose. EDTA (0.01 mM) was also added to chelate the contaminated heavy metal ions. The endothelium was removed by gently rubbing the intimal surface with a glass rod moistened with PSS. High  $K^+$  (72.7 mM) solution was made by replacing NaCl with equimolar KCl.  $Ca^{2+}$ -free solution was made by removing  $CaCl_2$  and adding 0.5 mM EGTA. These solutions were saturated with a 95%  $O_2$  and 5%  $CO_2$  mixture at 37°C to maintain the pH at 7.4. Muscle preparations were attached to a holder under a resting tension of 10 mN in rat and rabbit aorta or 5 mN in rat mesenteric artery, and they were equilibrated for 60–90 min in PSS. The muscle force was recorded isometrically. During the recording period, high  $K^+$  was repeatedly applied until the peak force became reproducible.

### *Cell culture*

A7r5 cells (passages 10–25) were purchased from the American Type Culture Collection (Rockville, MD, USA). Rat primary cultured arterial cells were isolated by the explant method. Briefly, the thoracic aorta was isolated from male Wistar rats (200–250 g), and the endothelium was removed aseptically. Small arterial strips were mounted in a dish, and sterile stainless steel mesh was put on top of the strips. Smooth muscle cells migrated onto the dish from the arterial strips after 4–5 days of culture. The cells were grown in DMEM under 95%  $O_2$  and 5%  $CO_2$  in the presence of 10% fetal calf serum, 1.7 mM L-glutamine, streptomycin (30  $\mu$ g  $ml^{-1}$ ), and penicillin (30 U  $ml^{-1}$ ). Sub-confluent cells were used in each experiment. Before starting the experiments, fetal calf serum was removed from the medium for 12–18 h.

### *$[Ca^{2+}]_i$ measurement of rat aorta and A7r5 cells*

$[Ca^{2+}]_i$  was measured according to the method described by Sato et al. (20) using the fluorescent  $Ca^{2+}$  indicator fura-PE3. The helical strips isolated from rat aorta were exposed to 5  $\mu$ M fura-PE3/AM in the presence of 0.02% cremophor EL for 5–6 h at room temperature. The muscle strips were then transferred to a muscle bath integrated in the fluorometer (CAF-110; Jasco, Tokyo). The muscle strips were illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm), and the amount of 500-nm fluorescence at each wavelength (F340 or F380, respectively) was measured fluorimetrically. The absolute  $Ca^{2+}$  concentrations in smooth muscle tissue were not calculated because, in a previous study, the dissociation constant of fura-2 for  $Ca^{2+}$  was found to be different from that obtained in vitro (21). Instead, we estimated  $[Ca^{2+}]_i$  according to the F340/F380 ratio, which was set at 0%

in resting muscle and at 100% in  $K^+$ -stimulated muscle.

Sub-confluent A7r5 cells cultured on a round cover glass were treated with 5  $\mu$ M fura-2/AM in the presence of 0.02% cremophor EL for 30 min at 37°C. The cells were then placed in a bath with PSS.  $[Ca^{2+}]_i$  was monitored using the fluorometer (PTI5200; Photon Technology International, Lawrenceville, NJ, USA). At the end of the experiments, ionomycin (10  $\mu$ M), which induced a maximum increase in  $[Ca^{2+}]_i$ , was added in the presence of 4 mM extracellular  $Ca^{2+}$  to obtain the maximum  $[Ca^{2+}]_i$  levels ( $R_{max}$ ). The external  $Ca^{2+}$  was then removed (with 4 mM EGTA) to obtain a minimum  $[Ca^{2+}]_i$  level ( $R_{min}$ ).  $Mn^{2+}$  (5 mM) was then added to quench fura-2 fluorescence and to determine the background fluorescence of the cells. F340 and F380 were calculated after subtracting the background fluorescence. For calculation of the absolute intracellular  $Ca^{2+}$  concentration in A7r5 cells, the dissociation constant of the fura-2- $Ca^{2+}$  complex was assumed to be 224 nM (22).

#### *MLC phosphorylation in intact muscle*

The extent of MLC phosphorylation was measured according to Word et al. (23). Strips of rat aorta were quickly frozen in liquid nitrogen and then homogenized with 10% TCA and 10 mM DTT. The homogenate was centrifuged at  $10,000 \times g$  for 1 min, after which the pellet was washed with diethyl ether and then suspended in a urea-glycerol buffer. Glycerol-PAGE was performed to separate phosphorylated MLC, and electrophoresed proteins were blotted on PVDF membrane (Bio-Rad Lab., Hercules, CA, USA) to perform Western blotting using polyclonal antibody raised against bovine tracheal MLC (kindly donated by Dr. J.T. Stull, University of Texas, Southwestern Medical Center at Dallas, TX, USA). The relative amount of phosphorylated MLC in relation to total MLC was quantified by image-analyzing software (NIH image 1.61).

#### *Reconstituted MLCK activity*

MLCK activity was measured in a contractile protein reconstituted system by estimating  $^{32}P$  incorporation into myosin purified from chicken gizzard (24). MLCK isolated from chicken gizzard or from bovine stomach was used. The reaction buffer (total 50  $\mu$ l) contained myosin (1 mg  $ml^{-1}$ ), isolated MLCK (20  $\mu$ g  $ml^{-1}$ ), 500 nM calmodulin, 30 mM KCl, 10 mM  $MgCl_2$ , 2 mM EGTA or 100  $\mu$ M  $Ca^{2+}$ , and 20 mM Tris-HCl (pH 7.5 at 25°C). The reaction was started by the addition of  $[\gamma\text{-}^{32}P]ATP$  and stopped by 10% TCA and 2%  $NaH_2PO_4$ . The precipitated protein was collected and washed with 10% TCA and 2%  $NaH_2PO_4$  using plastic funnels (Sepacol mini; Seikagaku Kogyo, Tokyo).  $^{32}P$  incorpora-

tion was estimated by Cerencov counting.

#### *Endogenous MLCK or myosin phosphatase activities in intact tissue*

Endogenous MLCK or myosin phosphatase activities were measured as described by Kim et al. (25). Bovine trachea was obtained from a local slaughterhouse. Isolated muscle strips were placed in PSS. After being treated with or without 100  $\mu$ M Y-27632 for 30 min, the muscle strips were frozen by liquid nitrogen and crushed. In a Y-27632 series of experiments, Y-27632 (100  $\mu$ M) was present in all steps of the preparation of endogenous MLCK and myosin phosphatase and also in the reaction medium. The crushed sample was then suspended in Buffer A containing Tris-HCl 20 mM (pH 6.8), 2 mM EGTA, 0.6 M NaCl, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM DFP, 1  $\mu$ g  $ml^{-1}$  leupeptin, and 1 mg  $ml^{-1}$  trypsin inhibitor. The suspended sample was then centrifuged at  $10000 \times g$  for 15 min at 4°C, and the supernatant was collected. To determine the endogenous MLCK activity, calyculin-A (1  $\mu$ M), an inhibitor of type 1 and type 2A phosphatase, was added to the supernatant. To determine the endogenous myosin phosphatase activity, K252a (10  $\mu$ M), a nonselective serine-threonine kinase inhibitor, was added to the supernatant. The reaction medium for measurement of the endogenous MLCK activity contained 30 mM Tris-HCl (pH 7.5), 2 mM  $MgCl_2$ , 60 mM KCl, 0.1 mM  $CaCl_2$ , 1  $\mu$ g  $ml^{-1}$  calmodulin, 0.2 mg  $ml^{-1}$  MLC, and the endogenous MLCK sample (0.8 mg protein  $ml^{-1}$ ). The reaction was started by adding 0.5 mM ATP containing 10  $\mu$ Ci  $[\gamma\text{-}^{32}P]ATP$ . Aliquots of the reaction mixture were picked up, and the reaction was stopped in a mini-column filled with TCA solution (5% TCA and 2%  $NaH_2PO_4$ ). The mini-column was washed four times with TCA solution to remove non-specifically bound ATP, and the radioactivity was counted. For measurement of the endogenous MLC phosphatase activity, MLC was phosphorylated for 6 min in a solution containing 30 mM Tris-HCl (pH 7.5), 2 mM  $MgCl_2$ , 60 mM KCl, 0.5 mM ATP with 10  $\mu$ Ci  $[\gamma\text{-}^{32}P]ATP$ , 100  $\mu$ M  $CaCl_2$ , 0.2 mg  $ml^{-1}$  MLC, 1  $\mu$ g  $ml^{-1}$  calmodulin, and 10  $\mu$ g  $ml^{-1}$  MLCK, and the reaction was stopped by adding 1  $\mu$ M K252a. We confirmed that the MLC phosphorylation levels were not decreased by contaminating myosin phosphatase after stopping the reaction for 30 min. Dephosphorylation of MLC was started by adding the crude myosin phosphatase sample (0.8 mg protein  $ml^{-1}$ ), and the radioactivity of the remaining phosphorylated MLC was counted as described above.

#### *Phosphodiesterase activity*

Phosphodiesterase activity was measured by using

calmodulin-free phosphodiesterase 3':5'-cyclic nucleotide extracted from bovine heart (Sigma Chemical Co., St. Louis, MO, USA). Enzyme activity was measured in 20 mM Tris-HCl (pH 6.8), 10 mM MgCl<sub>2</sub>, 20 mM 0.04 U ml<sup>-1</sup> of phosphodiesterase, 50 nM calmodulin, 0.5 U ml<sup>-1</sup> of 5'-nucleotidase (Sigma), and Ca<sup>2+</sup>-EGTA buffer (2 mM EGTA). The reaction was started by the addition of 2 mM cyclic AMP and terminated by the addition of 10% TCA. The amount of inorganic phosphate liberated during a 30-min incubation at 37°C was measured by an isobutyl alcohol procedure.

#### *F-actin staining*

The A7r5 cells were treated with *Clostridium botulinum* exoenzyme C3 (10 µg ml<sup>-1</sup>) in the DMEM medium for 72 h. In some experiments, Y-27632 (10 µM) was added to PSS 20 min before staining. The F-actin in A7r5 cells was stained using FITC-phalloidin and that in rat aortic primary cultured cells was stained using rhodamine-phalloidin. Anti-smooth muscle  $\alpha$ -actin antibody was also used to stain F-actin in rat aortic primary cultured cells. Briefly, cells were rinsed with PHEM buffer containing 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl<sub>2</sub> at pH 7.3 and 37°C; and they were then treated with PHEM buffer containing 3.7% formaldehyde for 15 min at 37°C. Formaldehyde was then removed by washing with PHEM buffer at room temperature once every 5 min for 15 min. Cell membrane was permeabilized by treating the cells with 0.2% triton X-100 in PHEM buffer for 90 s. FITC-phalloidin or rhodamine-phalloidin (4 U ml<sup>-1</sup>) was added to PHEM buffer for 1 h. In some experiments, cells were treated with anti-smooth muscle  $\alpha$ -actin antibody (1 100<sup>-1</sup> dilution; Dako, Glostrup, Denmark) in the presence of 5 mg ml<sup>-1</sup> bovine serum albumin in PHEM buffer for 1 h and then treated with FITC-conjugated anti-mouse IgG antibody (1 1000<sup>-1</sup> dilution; Vector, Burlingame, CA, USA). The stained F-actin was detected using a confocal microscope (LSM510; Carl Zeiss, Jena, Germany). In some experiments, the actin filaments were double-stained by anti-smooth muscle  $\alpha$ -actin antibody and rhodamine-phalloidin.

#### *Quantitative analysis of F-actin content*

Quantitative analysis of F-actin was performed according to the method described by Frigeri and Apgar (26) with slight modification. Briefly, sub-confluent A7r5 cells were cultured within 24-well cluster plates at a confluent density. After removing serum for 12 h, the cells were treated with or without 10 or 100 µM Y-27632 for 20 min in PSS. C3 (10 µg ml<sup>-1</sup>) was added to the DMEM medium for 72 h before starting the

quantitation. The cells were immediately fixed with 3.7% formaldehyde in HEPES buffer solution (125.4 mM NaCl, 11.5 mM glucose, 5.9 mM KCl, 1.2 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), and 1.5 mM CaCl<sub>2</sub>) for 15 min at 37°C. The cells were then permeabilized by a treatment with 0.2% Triton X-100 in HEPES buffer solution for 90 s. After washing twice with HEPES buffer solution, cells were stained by FITC-labeled phalloidin (FITC-phalloidin, 6 U ml<sup>-1</sup>) in HEPES buffer solution for 2.5 h in a dark room at room temperature. The cells were then washed three times with HEPES buffer solution, and bound FITC-phalloidin was extracted by incubating the cells with 75% methanol for 2 h in a dark room at 4°C. The relative fluorescence intensity of the extracts in supernatant was measured using a spectrofluorometer (FP-2060; Jasco) with an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The relative F-actin content is shown as a percentage of the F-actin content in the resting state.

#### *Chemicals*

Y-27632 was generously supplied by Mitsubishi Welpharma Corporation (Osaka) and dissolved in water. Calyculin A and mycalolide B were kindly supplied by Dr. Fusetani of the University of Tokyo. The chemicals used were phenylephrine bitartrate, prostaglandin F<sub>2 $\alpha$</sub> , nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, cremophor EL, arginine vasopressin (Sigma); fura-PE3/AM (TEFLABS, Austin, TX, USA); fura-2/AM (Dojindo, Kumamoto), trichloroacetic acid, dithiothreitol (Nacalai Tesque Inc., Kyoto); [ $\gamma$ -<sup>32</sup>P]ATP (Amersham, Arlington Heights, IL, USA); endothelin-1 (Peptide Institute, Osaka); *Clostridium botulinum* exoenzyme C3 (Wako Pure Chemistry, Tokyo); and FITC- or rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA).

#### *Statistics*

Results are expressed as the mean  $\pm$  S.E.M. Comparison between the control and the test group was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. For all evaluations, a *P* value less than 0.05 was considered to be statistically significant.

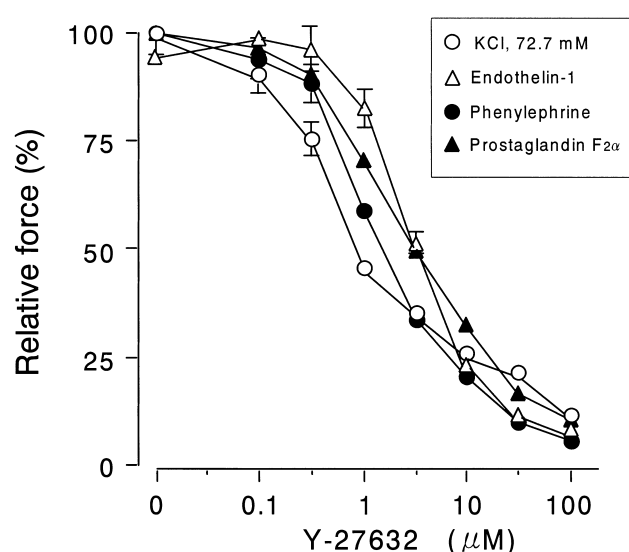
## **Results**

#### *Intact smooth muscle contraction*

In isolated rat aorta, addition of high K<sup>+</sup> (72.7 mM), endothelin-1 (30 nM), prostaglandin F<sub>2 $\alpha$</sub>  (10 µM), or phenylephrine (1 µM) induced sustained contraction. Approximately 20 min after the application of a stimulant, Y-27632 (0.1 – 100 µM) was cumulatively applied.

As shown in Fig. 1, Y-27632 inhibited these contractions in a concentration-dependent manner. The  $IC_{50}$  values and the maximum inhibitions induced by 100  $\mu M$  Y-27632 are shown in Table 1. Y27632 more strongly inhibited the contractions induced by high  $K^+$  ( $IC_{50} = 0.8 \mu M$ ) than those induced by endothelin-1 ( $IC_{50} = 3.8 \mu M$ ) or prostaglandin  $F_{2\alpha}$  ( $IC_{50} = 4.9 \mu M$ ), whereas the effect on phenylephrine ( $IC_{50} = 1.9 \mu M$ ) was intermediate.

In rat mesenteric artery, Y-27632 inhibited contractions induced by high  $K^+$  ( $IC_{50} = 0.3 \mu M$ ), prostaglandin  $F_{2\alpha}$  ( $IC_{50} = 0.4 \mu M$ ), or phenylephrine ( $IC_{50} = 0.5 \mu M$ ) with similar  $IC_{50}$  values and similar maximum inhibition



**Fig. 1.** Effects of Y-27632 on contractions in rat aorta induced by KCl (72.7 mM), endothelin-1 (30 nM), prostaglandin  $F_{2\alpha}$  (10  $\mu M$ ), or phenylephrine (1  $\mu M$ ). Y-27632 was cumulatively added when the contraction reached the steady state. The magnitude of contraction before the addition of Y-27632 was taken as 100%. Each point represents the mean of 4–6 experiments and the S.E.M. is shown by the vertical bar.

levels (Table 1).

In rabbit aorta, in contrast, Y27632 inhibited the contractions induced by phenylephrine ( $IC_{50} = 0.5 \mu M$ ) more strongly than those stimulated by high  $K^+$  ( $IC_{50} = 1.6 \mu M$ ).

#### $[Ca^{2+}]_i$ movement

In the fura-PE3-loaded rat aorta, high  $K^+$  (72.7 mM) induced sustained increases in  $[Ca^{2+}]_i$  and muscle contraction. After the contraction reached a plateau, addition of Y-27632 (10  $\mu M$ ) inhibited muscle contraction to  $14.7 \pm 1.92\%$ , without changing  $[Ca^{2+}]_i$ . Endothelin-1 (30 nM) also induced sustained increases in  $[Ca^{2+}]_i$  and muscle tension. Endothelin-1 was found to induce greater contractions than high  $K^+$  at a given  $[Ca^{2+}]_i$ , indicating that this stimulant increased the  $Ca^{2+}$  sensitivity of contractile elements. Y-27632 (10  $\mu M$ ) inhibited the endothelin-1-induced muscle contraction to  $18.8 \pm 6.1\%$  without changing  $[Ca^{2+}]_i$  ( $n = 4$ ). These results are summarized in Fig. 2.

We further examined the effects of Y-27632 on  $[Ca^{2+}]_i$  in A7r5 cell lines established from rat thoracic aorta (Fig. 3). In the fura-2-loaded A7r5 cells, a transient increase in  $[Ca^{2+}]_i$  followed by a relatively small sustained increase was induced by KCl (72.7 mM), endothelin-1 (30 nM), or arginine vasopressin (1  $\mu M$ ). Pre-incubation of the A7r5 cells with Y-27632 (10  $\mu M$ ) for 15 min had no effects on the  $[Ca^{2+}]_i$  increments due to these stimulants.

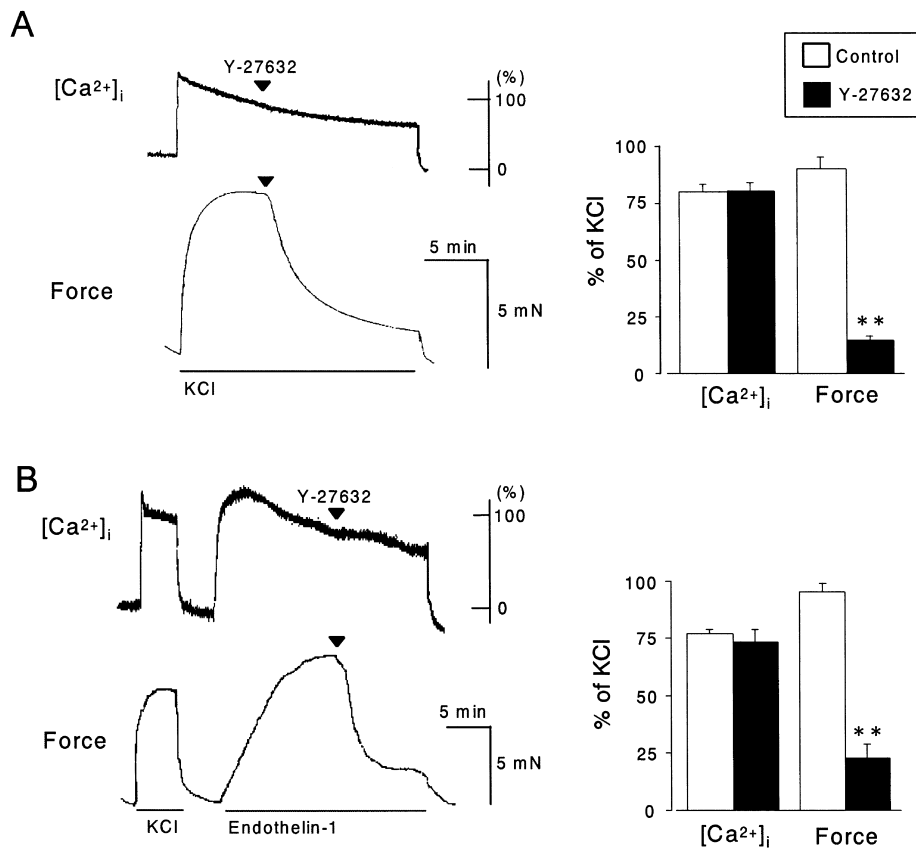
#### MLC phosphorylation in intact muscle

Figure 4 shows the effects of Y-27632 on muscle force and MLC phosphorylation in isolated rat aorta. High  $K^+$  increased muscle force in a time dependent manner (Fig. 4A). Pretreatment of the muscle strips with Y-27632 (1 or 10  $\mu M$ ) for 20 min significantly decreased muscle force stimulated with high  $K^+$  at 1, 3, and 10 min. In the resting condition, 7–12% of total

**Table 1.** Maximum inhibition and  $IC_{50}$  values for Y-27632 on the contractions induced by various stimulants in vascular smooth muscles

	High $K^+$ (72.7 mM)	Phenylephrine (1 $\mu M$ )	Prostaglandin $F_{2\alpha}$ (10 $\mu M$ )	Endothelin-1 (30 nM)
Rat aorta (n = 6)	$0.8 \pm 0.17$ (88.5 $\pm$ 2.36)	$1.9 \pm 0.53$ (94.5 $\pm$ 1.48)	$4.9 \pm 0.80^*$ (89.8 $\pm$ 2.40)	$3.8 \pm 0.72^*$ (91.7 $\pm$ 3.50)
Rat mesenteric artery (n = 4)	$0.3 \pm 0.04$ (86.3 $\pm$ 7.9)	$0.5 \pm 0.05$ (83.4 $\pm$ 8.6)	$0.4 \pm 0.07$ (89.5 $\pm$ 6.1)	Nd
Rabbit aorta (n = 4)	$1.6 \pm 0.43$ (45.0 $\pm$ 5.0)	$0.5 \pm 0.04^*$ (83.2 $\pm$ 3.2)	Nd	Nd

Each value is the mean  $\pm$  S.E.M. from 4–6 experiments. The  $IC_{50}$  value (upper numeral) and maximum inhibition (lower numeral) for 100  $\mu M$  Y-27632 are shown in  $\mu M$  and as a percentage of the contractions induced by each agonist in the absence of Y-27632, respectively. Nd, not determined. \* $P < 0.05$  vs high  $K^+$ .



**Fig. 2.** Effects of 10  $\mu$ M Y-27632 on  $[Ca^{2+}]_i$  transient and muscle force in rat aorta. Rat aorta loaded with fura-PE3 was stimulated with KCl (72.7 mM) (A) or endothelin-1 (30 nM) (B). The left panels show typical traces out of 4–6 experiments. The right column shows the summarized results of  $[Ca^{2+}]_i$  and muscle force 10 min after the addition of Y-27632. \*\* $P < 0.01$  compared with the control. Muscle force and  $[Ca^{2+}]_i$  are shown as a percentage of the KCl-induced increase in muscle force and  $[Ca^{2+}]_i$  in the same preparation.

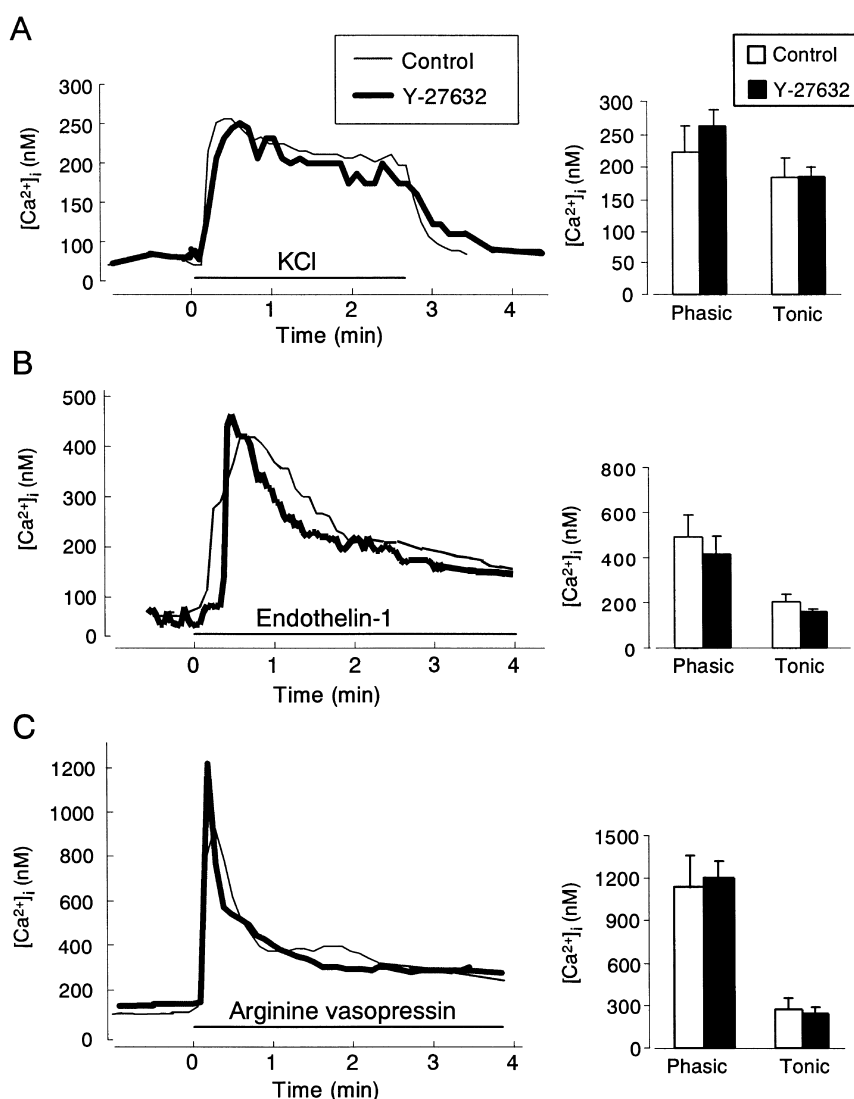
MLC was phosphorylated. Stimulation with high  $K^+$  (72.7 mM) for 1, 3, and 10 min increased MLC phosphorylation to 41.8%, 39.5%, and 37.5%, respectively, as shown in Fig. 4C. Pretreatment of the muscle strips with Y-27632 (1 or 10  $\mu$ M) for 20 min significantly decreased MLC phosphorylation stimulated with high  $K^+$ .

Endothelin-1 (30 nM) also increased muscle force in a time dependent manner as shown in Fig. 4B. Y-27632 (10  $\mu$ M) partially inhibited the endothelin-1-induced contraction. Endothelin-1 (30 nM) also increased MLC phosphorylation in a time-dependent manner as shown in Fig. 4D (19.6%, 40.1% and 46.5% at 1, 3, and 10 min, respectively). Y-27632 (10  $\mu$ M) significantly inhibited the MLC phosphorylation at 3 and 10 min but not at 1 min (25.2%, 21.9%, and 27.9% at 1, 3, and 10 min, respectively).

Stimulation with phenylephrine (1  $\mu$ M) for 5 min increased MLC phosphorylation to  $53.4 \pm 1.2\%$  ( $n = 5$ ). Y-27632 (10  $\mu$ M) decreased the phenylephrine-induced

MLC phosphorylation to  $16.5 \pm 3.4\%$  ( $n = 10$ ).

Figure 5 shows the time-dependent changes in relationship between MLC phosphorylation and muscle force. Each data of Fig. 5, A and B, was plotted from Fig. 4, A and C, or Fig. 4, B and D, respectively. In the absence of Y-27632, high  $K^+$  and endothelin-1 increased force in accordance with increment of MLC phosphorylation (Fig. 5, control). In the presence of 1  $\mu$ M Y-27632, correlation between these two parameters was almost identical to that obtained in the absence of Y-27632, suggesting that Y-27632 inhibited MLC phosphorylation and force in parallel. In the presence of a high concentration (10  $\mu$ M) of Y-27632, however, the early phase of high  $K^+$ -induced contraction (1 min after the addition of high  $K^+$ ) was smaller than that predicted from MLC phosphorylation, suggesting the dissociation of force from MLC phosphorylation (Fig. 5A). Similar dissociation was observed with muscles stimulated with endothelin-1 for 1 min in the presence of 10  $\mu$ M Y-27632 (Fig. 5B).



**Fig. 3.** Effects of Y-27632 (10  $\mu$ M) on  $[Ca^{2+}]_i$  transient in A7r5 cells induced by KCl (72.7 mM) (A), endothelin-1 (30 nM) (B), or vasopressin (1  $\mu$ M) (C). Y-27632 was added 15 min before the addition of each stimulant. The left panel shows typical traces out of 4 experiments. The right column shows the summarized results of peak  $[Ca^{2+}]_i$  (Phasic) and sustained  $[Ca^{2+}]_i$  at 2 min after stimulation (Tonic), respectively. Absolute cytosolic  $Ca^{2+}$  concentration was calculated as described in the Materials and Methods.

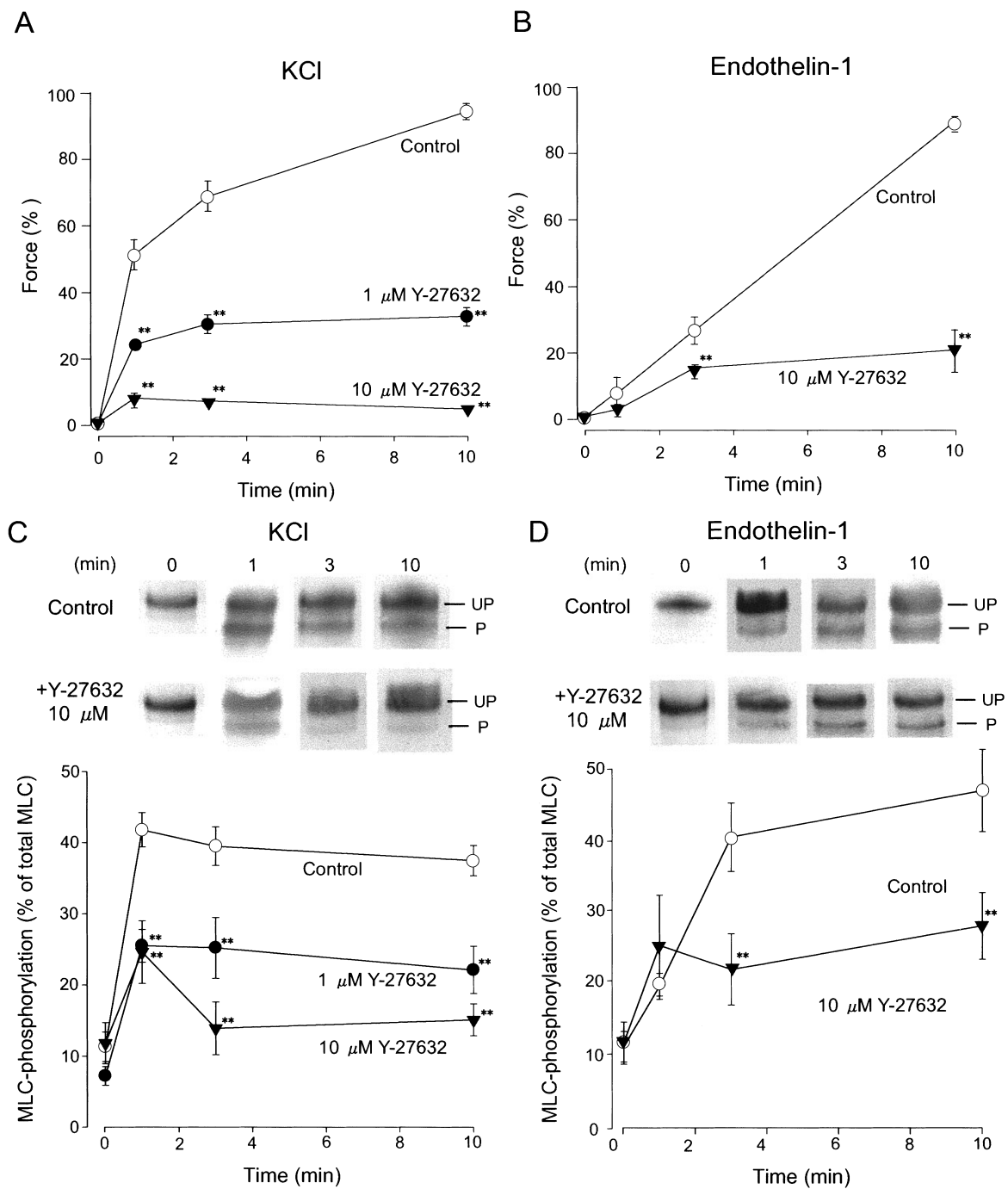
### Enzymatic activities

Avian MLCK has been widely used in the study of smooth muscle biochemistry. Bovine stomach MLCK has also been used because it has a mammalian-specific site in its N-terminus regulatory domain (27). In the present study, we used short-form smooth muscle MLCK isolated from bovine stomach and chicken gizzard to compare the effects of Y-27632 on the respective kinase activities. Table 2 summarizes the effects of Y-27632 on  $V_{max}$  and  $K_m$  for ATP on both kinases. The results indicate that Y-27632 does not affect the activities of isolated avian or mammalian MLCK.

We further examined the effects of Y-27632 on endo-

genous MLCK and myosin phosphatase activity of bovine trachea. We confirmed that Y-27632 inhibited high  $K^+$ -induced contraction in accordance with the decrease in MLC-phosphorylation in bovine trachea ( $n = 4$ , data not shown). As shown in Fig. 6, however, Y-27632 changed neither the endogenous MLCK activity nor the myosin phosphatase activity.

We then examined the effects of Y-27632 on calmodulin activity using a  $Ca^{2+}$ /calmodulin-dependent enzyme, phosphodiesterase 3':5'-cyclic nucleotide from bovine heart. Trifluoperazine (100  $\mu$ M), a known calmodulin inhibitor, strongly inhibited this enzyme activity ( $14.4 \pm 6.0\%$  of control,  $n = 4$ ). However, Y-27632 (300  $\mu$ M)



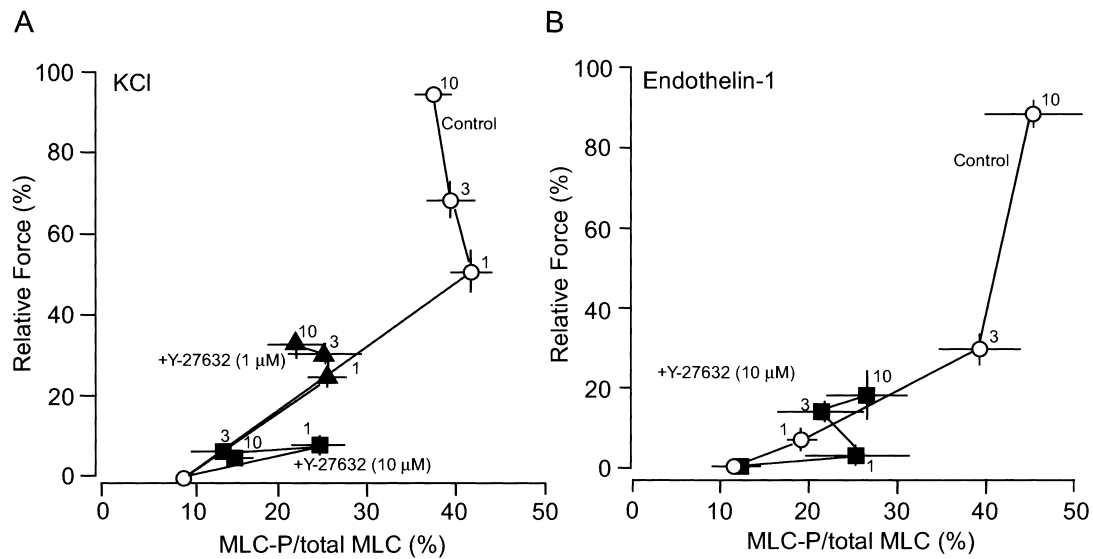
**Fig. 4.** Effects of Y-27632 on muscle force and MLC phosphorylation in rat aorta stimulated with high  $K^+$  (A, C) or endothelin-1 (B, D). Muscles were treated with 1 or 10  $\mu$ M Y-27632 for 20 min before adding KCl (72.7 mM) or endothelin-1 (30 nM). Muscle force and MLC phosphorylation were measured in the resting state and at 1, 3, and 10 min after stimulation with KCl or endothelin-1. Muscle force is shown as a percentage of the KCl-induced increase in muscle force in the same preparation. Each plot in C and D represents mean phosphorylated MLC/total MLC ( $n = 5-9$ ). S.E.M. is shown by the vertical bars. \*\* $P < 0.01$ , compared with the control. UP: unphosphorylated MLC, P: phosphorylated MLC.

did not affect the activity of this enzyme ( $97.5 \pm 1.0\%$  of control,  $n = 4$ ).

#### Actin organization

A7r5 cells have a meshwork of contractile actin-myosin filaments (actin stress fibers) as shown in Fig. 7A. After a 3-day culture of A7r5 cells with





**Fig. 5.** The relationship between muscle force and MLC-phosphorylation in rat aorta stimulated with 72.7 mM KCl (A) or 30 nM endothelin-1 (B) in the presence or absence of Y-27632. The data of MLC-phosphorylation and muscle force were plotted from Fig. 4. Each number shows min after stimulation with high  $K^+$  or endothelin-1. Each plot showed results in the absence of Y-27632 (open circle), or in the presence of 1  $\mu$ M (closed triangle) or 10  $\mu$ M (closed square) of Y-27632.

**Table 2.** Effect of Y-27632 on  $V_{max}$  and  $K_m$  for ATP values for the short smooth muscle MLCK activities isolated from chicken gizzard and bovine stomach

	Chicken gizzard MCLK		Bovine stomach MCLK	
	( $V_{max}$ )	( $K_m$ for ATP)	( $V_{max}$ )	( $K_m$ for ATP)
Control	$8.3 \pm 0.30$	$45.1 \pm 1.60$	$8.2 \pm 0.14$	$36.4 \pm 5.95$
Y-27632	$9.9 \pm 0.38$	$53.8 \pm 2.04$	$8.2 \pm 0.06$	$28.8 \pm 2.95$

$V_{max}$ :  $\mu$ mol myosin  $\text{min}^{-1} \text{mg}^{-1}$  MLCK.  $K_m$  for ATP: 50% of binding constant for ATP ( $\mu$ M). Samples were treated with Y-27632 (100  $\mu$ M) for 20 min before adding ATP.  $V_{max}$  and  $K_m$  for ATP were calculated by the Hanes-Woolf plot method. Each value represents the mean  $\pm$  S.E.M. ( $n = 4$ ).

*Clostridium botulinum* exoenzyme C3 (10  $\mu\text{g ml}^{-1}$ ), the meshwork of actin filaments was decreased and the cells become round (Fig. 7B). Addition of Y-27632 (10  $\mu$ M, 20-min treatment) to A7r5 cells induced morphological changes similar to those by C3 (Fig. 7C).

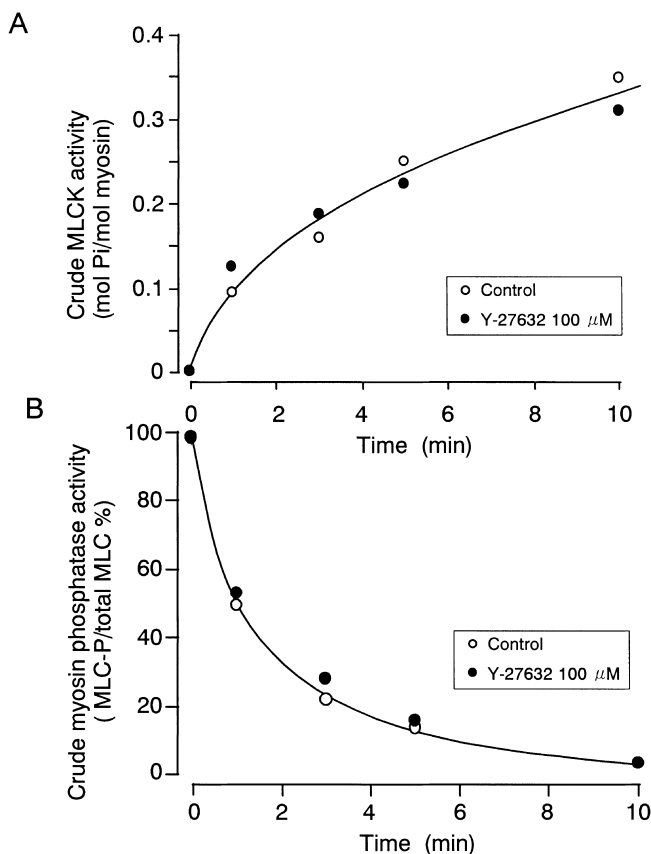
In the rat aortic primary cultured cells, the assembly of actin filaments was stained with either anti- $\alpha$ -actin antibody (Fig. 8A) or rhodamine-phalloidin (Fig. 8B). Similar to the results in A7r5 cells, the meshwork of actin filaments was disrupted after a 20-min treatment with Y-27632 (10  $\mu$ M) (Fig. 8C).

In a quantitative analysis of F-actin content, an actin depolymerizing agent, mycalolide-B (300 nM), decreased the F-actin content to 89.2% ( $P < 0.01$ ). Filamentous actin content was also decreased in the A7r5 cells treated with C3 toxin (10  $\mu\text{g ml}^{-1}$ ) for 72 h. In the presence of 10  $\mu$ M or 100  $\mu$ M Y-27632 for 20 min, F-actin content significantly decreased to 92.6% and

84.8%, respectively). These results are summarized in Fig. 9.

## Discussion

It has been considered that high  $K^+$ -induced contraction is attributable simply to the elevation of  $[\text{Ca}^{2+}]_i$  due to activation of the voltage-dependent L-type  $\text{Ca}^{2+}$  channels, but not to the changes in  $\text{Ca}^{2+}$  sensitivity of smooth muscle contractile elements (2, 3). Receptor agonists, on the other hand, activate the rhoA/ROCKs pathway to inhibit myosin phosphatase and to increase the  $\text{Ca}^{2+}$  sensitivity of contractile elements and thus induce a greater contraction at a given  $[\text{Ca}^{2+}]_i$  than that induced by high  $K^+$  (see Introduction). If Y-27632 is a selective inhibitor of ROCKs, it is expected to selectively inhibit the receptor agonists-induced contractions without inhibiting the high  $K^+$ -induced contraction.



**Fig. 6.** Effects of Y-27632 (100  $\mu$ M) on MLCK (A) and myosin phosphatase activities (B) in the crude tissue homogenate. MLCK activity is shown by phosphorylated myosin (mol Pi per mol myosin) and myosin phosphatase activity as a percentage of phosphorylated MLC taking the total MLC as 100%, respectively. Typical results out of 3 experiments are shown.

In the present experiments, we found that Y-27632 inhibited the contractions induced by receptor-agonists (Fig. 1) with a decrease in MLC phosphorylation (Fig. 4), supporting the suggestion that ROCKs are involved in signal transduction activated by receptor agonists and that Y-27632 is an inhibitor of ROCKs. To our surprise, however, Y-27632 also inhibited high  $K^+$ -induced contraction. Mita et al. (28) also indicate the similar results in rat caudal artery. Furthermore, the inhibitory effect on high  $K^+$ -induced contraction, in the present study, was stronger than that on the contractions induced by endothelin-1 and prostaglandin  $F_{2\alpha}$  (Fig. 1 and Table 1). The inhibitory effect on high  $K^+$ -induced contraction was accompanied by an inhibition of MLC phosphorylation.

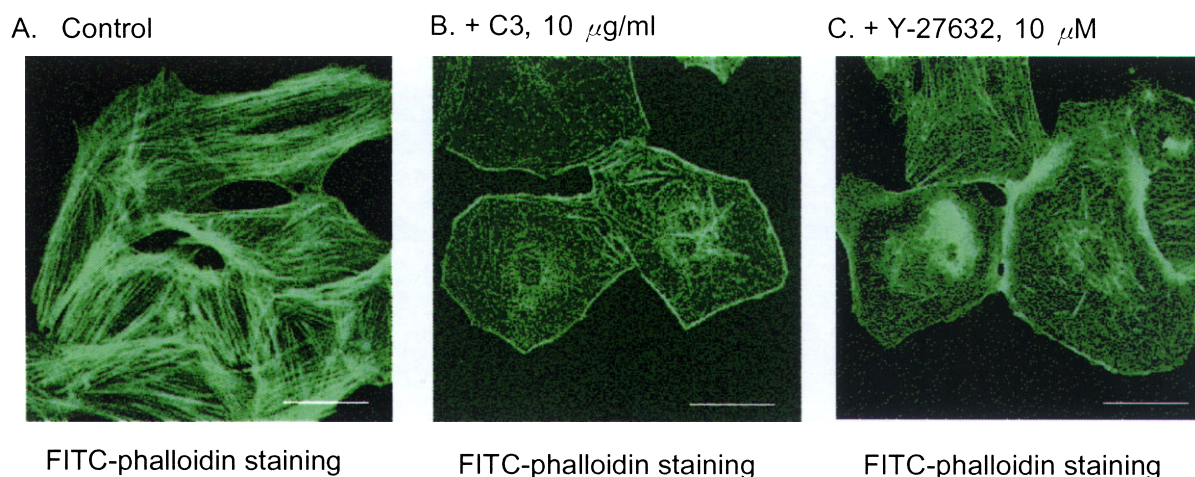
To confirm the results on rat aorta, we examined the effect of Y-27632 on other types of vascular smooth muscle. It was found that Y-27632 inhibited the contractions induced by high  $K^+$ , phenylephrine, or

prostaglandin  $F_{2\alpha}$  with similar  $IC_{50}$  values in the rat mesenteric artery (Table 1). In rabbit aorta, it has been reported that Y-27632 inhibited the phenylephrine-induced contraction more strongly than the high  $K^+$ -induced contraction (11). We confirmed their results, although the difference in  $IC_{50}$  values was only three-fold (Table 1). In one of the phasic type of smooth muscles, rat caudal artery, Y-27632 selectively inhibited the tonic component of high  $K^+$ -induced contraction without affecting the phasic component (28). In chicken gizzard, a typical phasic type of smooth muscle, in contrast, Y-27632 showed only a slight inhibitory action on high  $K^+$ - and carbachol-induced contractions even at a high concentration of 100  $\mu$ M (29). These results suggest that although the sensitivity of smooth muscle to Y-27632 is variable among the animal species, tissues, and stimulants, Y-27632 inhibits not only the contraction induced by receptor agonists but also the contraction induced by high  $K^+$  in vascular smooth muscle. These variable effects of Y-27632 on muscle contractions may be related to kinase-phosphatase balance in phasic and tonic types of smooth muscles (30).

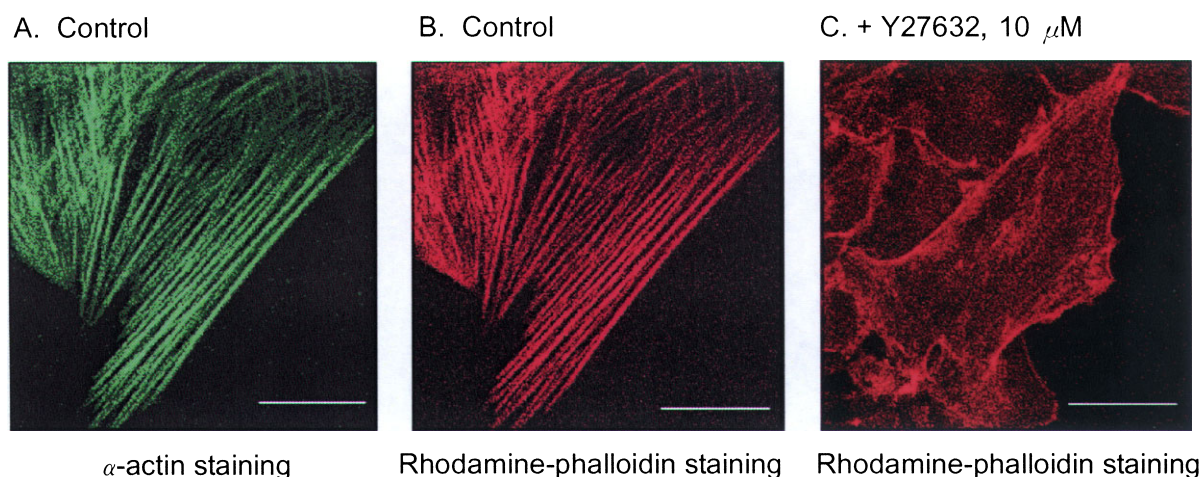
We then closely examined the effects of Y-27632 on the  $[Ca^{2+}]_i$ /calmodulin/MLCK/phosphatase system in rat aorta. It was found that Y-27632 (10  $\mu$ M) did not change the increase in  $[Ca^{2+}]_i$  stimulated by high  $K^+$  or endothelin-1 (Fig. 2). In A7r5 cells, Y-27632 also showed no effect on the increase in  $[Ca^{2+}]_i$  stimulated by high  $K^+$ , endothelin-1, or arginine vasopressin (Fig. 3). These results suggest that the site of action of Y-27632 is not the  $Ca^{2+}$  metabolism but the MLCK/myosin phosphatase system.

To evaluate this possibility, we measured the smooth muscle MLCK activity in a re-constituted system using MLCK purified from chicken gizzard and bovine stomach. The molecular masses of gizzard MLCK and bovine stomach MLCK are slightly different (27). Bovine stomach MLCK has a mammalian MLCK-specific amino acid region in the regulatory domain of the N-terminal end, although bovine MLCK is homologous to gizzard MLCK (31). Our results indicated that Y-27632 (100  $\mu$ M) changed neither  $V_{max}$  nor  $K_m$  for ATP values for MLCK preparations (Table 2). Similar results have been reported in chicken gizzard MLCK (11).

We further examined the effects of Y-27632 on crude MLCK and MLC phosphatase activities in smooth muscle tissue homogenate. Results indicated that Y-27632 did not change the time course of MLC phosphorylation or MLC-dephosphorylation (Fig. 6), suggesting that Y-27632 does not have direct effects on MLCK or myosin phosphatase. We also confirmed that Y-27632 does not inhibit the  $Ca^{2+}$ -dependent phosphodiesterase activity, an indicator of calmodulin acti-



**Fig. 7.** Effects of C3 and Y-27632 on actin filaments in A7r5 cells stained by FITC-phalloidin. A7r5 cells were treated with *Clostridium botulinum* exoenzyme C3 ( $10 \mu\text{g ml}^{-1}$ ) in DMEM for 72 h (B) or with Y-27632 ( $10 \mu\text{M}$ ) for 20 min before staining (C). Typical results out of 3 experiments are shown. Scale bar represents  $15 \mu\text{m}$ .

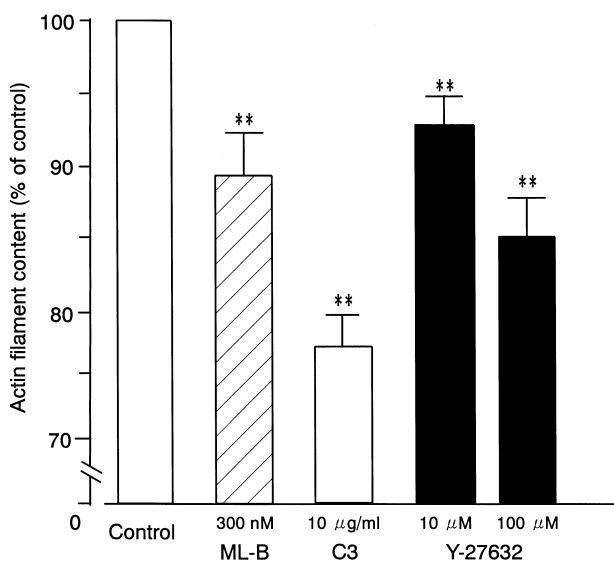


**Fig. 8.** Effects of Y-27632 ( $10 \mu\text{M}$ ) on actin filaments in rat aortic primary cultured cells. In (A) and (B), actin filaments were simultaneously stained with anti- $\alpha$  actin antibody (A) and rhodamine-phalloidin (B). Cultured cells were treated with Y-27632 ( $10 \mu\text{M}$ ) for 20 min before staining with rhodamine-phalloidin (C). Typical results out of 3 experiments are shown. Scale bar represents  $20 \mu\text{m}$ .

vity.

All of these results suggest that Y-27632 is a selective inhibitor of ROCKs with no direct effect on  $[\text{Ca}^{2+}]_i$ , calmodulin, MLCK, and myosin phosphatase. If Y-27632 is a selective inhibitor of ROCKs as shown in the present results and also in previous reports (11, 15, 32), and if high  $\text{K}^+$ -induced contraction is due solely to the increase in  $[\text{Ca}^{2+}]_i$  with no effect on  $\text{Ca}^{2+}$  sensitivity, it is quite difficult to explain the mechanism of the inhibitory effect of Y-27632 on high  $\text{K}^+$ -induced contraction. One of the possibilities to solve this problem is to presume that high- $\text{K}^+$  activates not only the L-type  $\text{Ca}^{2+}$  channels but also  $\text{Ca}^{2+}$  sensitivity of contractile element. Support-

ing this possibility, we have observed that increase in  $[\text{Ca}^{2+}]_i$  by a  $\text{Ca}^{2+}$  ionophore, ionomycin, induced much smaller contraction than high  $\text{K}^+$  at a given  $[\text{Ca}^{2+}]_i$  in rat aorta (100 nM ionomycin:  $83.4 \pm 6.48\%$  and  $31.5 \pm 1.43\%$  of KCl-induced  $[\text{Ca}^{2+}]_i$  increase and contraction;  $1 \mu\text{M}$  ionomycin:  $186.7 \pm 21.9\%$  and  $102.9 \pm 24.3\%$  of KCl-induced  $[\text{Ca}^{2+}]_i$  increase and contraction, respectively,  $n = 6$  each). From these results, we suggest that high  $\text{K}^+$ -depolarization activates not only L-type  $\text{Ca}^{2+}$  channels but also a  $\text{Ca}^{2+}$  sensitizing mechanism involving ROCKs in vascular smooth muscle (5, 6). The original idea is advocated by Mita et al. (28). They suggested, in rat caudal artery, that both activation of MLCK and



**Fig. 9.** Quantitative analysis of actin filaments content in A7r5 cells treated with mycalolide-B, C3 or Y-27632. Mycalolide-B (ML-B), a potent actin depolymerizing agent, (300 nM) or Y-27632 (10 or 100  $\mu$ M) were treated for 20 min at 37°C. Cells were also treated with *Clostridium botulinum* exoenzyme C3 (10  $\mu$ g ml<sup>-1</sup>) in the DMEM medium for 72 h before staining. \*\* $P$ <0.01, compared with the control. Each column shows the mean  $\pm$  S.E.M. (n = 6 each).

ROCKs-induced inhibition of myosin phosphatase are necessary to maintain the high K<sup>+</sup>-induced contraction. Although this novel suggestion was based on the assumption that Y-27632 selectively inhibits ROCKs without changing MLCK activity, the authors did not examine the action of Y-27632 on MLCK activity in intact vascular tissues. In the present study, we for the first time established that Y-27632 does not directly inhibit MLCK activity not only in purified MLCK but also in the extract from tissue homogenate, which supported their suggestion. As shown in Fig. 6, Y-27632 did not inhibit the Ca<sup>2+</sup>/calmodulin-activated MLC phosphorylation in the presence of crude kinases extracted from bovine trachea, which is expected to contain not only MLCK but also ROCKs. This result indicates that ROCKs may not directly phosphorylate MLC. However, we can not rule out the possibility that ROCKs directly phosphorylate MLC at Ser<sup>19</sup> (8). Further study is necessary to evaluate this possibility.

There are evidences supporting the possibility that the rhoA-mediated cell signaling pathway is involved in high K<sup>+</sup>-induced contraction. In the guinea pig ileum, for example, translocation of rhoA is induced by high-K<sup>+</sup> stimulation (33). Furthermore, membrane permeable inhibitors of rhoA, *Clostridium difficile* toxin B (34, 35), and chimeric toxin (DC3B) consisting of C3 and the noncatalytic B fragment of diphtheria toxin (36), have been shown to partially inhibit the high K<sup>+</sup>-induced

contractions. These results indicate the possibility that high K<sup>+</sup>-induced contraction is, at least in part, regulated by a rhoA-mediated cell signaling pathway.

In phasic type smooth muscle, it has been reported that the ROCKs mediate Ca<sup>2+</sup> sensitization mainly during the sustained phase rather than the phasic phase of contraction induced by receptor agonists (28, 34, 35, 37). In rat aorta, in contrast, we found that 10  $\mu$ M Y-27632 inhibited the early phase of contraction more strongly than MLC-phosphorylation or delayed the rate of force production compared to the rate of MLC phosphorylation (Fig. 5). This result is quite interesting because it suggests the possibility that a high concentration (10  $\mu$ M) of Y-27632 has an inhibitory effect other than MLC-phosphorylation to dissociate force from MLC phosphorylation. To address this problem, we paid our attention to actin filaments. As shown in Figs. 7–9, 10  $\mu$ M Y-27632 significantly decreased the actin filament network in the arterial smooth muscle cell line A7r5. Similar results are obtained in rat aortic primary cultured cells. The Y-27632-induced actin disrupting effect was similar to that induced by *Clostridium botulinum* exoenzyme C3. There are several reports showing the effects of contractile stimulants on actin filaments. In primary cultured human tracheal smooth muscle cells maintaining the contractile phenotype, muscarinic stimulation activates actin organization (38) and rhoA regulates sustained contraction through cytoskeletal reorganization of an actin-binding protein HSP27 (39). Organization of the actin network facilitates myosin filament formation that in turn causes cross-linking and bundling of the actin network into actomyosin fibrils (40). The resulting rigidly cross-linked actomyosin network would have an increased capacity to transduce muscle tension (40). These and the present results indicate one possibility that the actin filaments may be important for the rate of force development but not force maintenance. Disassembly of actin filament by Y-27632 may decrease the rate of force development in the early phase of contraction compared to the increase in MLC phosphorylation. Further studies are necessary to clarify the point.

In summary, Y-27632 inhibited contractions elicited not only by receptor agonists but also by high K<sup>+</sup>. Although this effect was accompanied by a decreased MLC phosphorylation, Y-27632 changed neither [Ca<sup>2+</sup>]<sub>i</sub> nor the activities of calmodulin, MLCK, and myosin phosphatase. These results suggest the presence of two different regulatory pathways for vascular smooth muscle contraction: a classical Ca<sup>2+</sup>/calmodulin/MLCK/myosin phosphatase system and a novel myosin phosphorylation pathway including ROCKs. Not only receptor agonists but also high K<sup>+</sup> seems to activate both

of these pathways. In addition, Y-27632 has an effect to decrease filamentous actin. The decrease in actin filaments may induce a stronger inhibition on the rate of force production than the rate of MLC phosphorylation.

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