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Regulation of Ca^{2+}-independent smooth muscle contraction by alternative staurosporine-sensitive kinase

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Abstract

It is well known that inhibition of myosin phosphatase induces smooth muscle contraction in the absence of Ca^{2+}. We characterized the kinase(s) which plays a role in Ca^{2+}-independent, microcystin-LR-induced contraction in permeabilized smooth muscle of the rabbit portal vein. Assessments of various protein kinase inhibitors revealed this kinase(s) (1) was sensitive to staurosporine (1 μM), but resistant to other agents including wortmannin (10 μM), Y-27632 ((*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 100 μM), HA1077 (1-(5-isoquinolinylsulfonyl)-homopiperazine, 100 μM), H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, 100 μM), and calphostin C (100 μM), and (2) induced phosphorylation of 20 kDa myosin light chain at serine-19. We concluded that other kinases exist which phosphorylate myosin light chain at serine-19 and induce Ca^{2+}-independent smooth muscle contraction, distinct from Rho-associated kinase, myosin light chain kinase, and protein kinase C.

Keywords: Smooth muscle, vascular; Contraction, Ca^{2+}-independent; Rho-associated kinase; Microcystin-LR; Myosin light chain phosphorylation

1. Introduction

Myosin phosphatase inhibitors such as okadaic acid, calyculin A, tautomycin, and microcystin containing leucine (L) and arginine (R) residues (microcystin-LR) induce smooth muscle contraction independently from cytosolic calcium ($[Ca^{2+}]_i$), but dependently with respect to an increase in 20 kDa myosin light chain phosphorylation (see review by Karaki et al., 1997). However, increased 20 kDa myosin light chain phosphorylation correlated with myosin phosphatase inhibition could be interpreted as unmasking of some kinase(s) which directly phosphorylates 20 kDa myosin light chain.

Possible candidates for unmasked kinase(s) include the following: Ca^{2+}–calmodulin-independent form of myosin light chain kinase (Suzuki and Itoh, 1993; Walker et al., 1998), Rho-associated kinase (Rho-kinase) (Amano et al., 1996; Kureishi et al., 1997), and p21-associated kinase (van Eyk et al., 1998). In addition, activation of protein kinase C was revealed to sensitize smooth muscle contraction to $[Ca^{2+}]_i$, probably through the inhibition of myosin phosphatase (Masuo et al., 1994). These Ca^{2+}-independent signals may be involved in Ca^{2+}-sensitization of smooth muscle contraction (Somlyo and Somlyo, 1994).

However, the responsible kinase has yet to be identified and the mechanism of Ca^{2+}-independent contraction remains controversial. Here we focused on the effects of various protein kinase inhibitors, including a novel and specific Rho-kinase inhibitor Y-27632 (Uehata et al., 1997), and showed that Ca^{2+}-independent contraction by microcystin-LR is induced by a staurosporine-sensitive kinase(s) distinct from Rho-kinase, myosin light chain kinase, and protein kinase C.

2. Methods

2.1. Force measurement of intact and skinned fibers

Thin strips of male rabbit (body weight 2–3 kg) portal veins (50–100 μm wide and 0.5–1 mm long) were dissected and mounted onto an isometric transducer (UL-2GR,

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Minebea, Japan) in a well (100 μl) on a bubble plate (Kitazawa et al., 1989). The mounted fiber was stretched to 1.2–1.5 times of rest length, incubated for a couple of minutes in normal physiological salt solution, and contracted by the addition of high KCl (118 mM KCl) physiological salt solution or an agonist (0.1 μM endothelin-1). After a normal response was confirmed, the following treatment of the fiber with a selected kinase inhibitor or membrane-permeabilizing agent (α-toxin or Triton X-100) was performed according to each experimental protocol.

Chemical skinned fibers were described previously (Kitazawa et al., 1989). Briefly, each fiber was incubated with 5000 IU/ml α-toxin for 60–75 min at 25°C or 0.5% Triton X-100 for 20 min at 25°C in a well (100 μl) on a bubble-like plate. $[Ca^{2+}]_i$-free conditions were maintained in resting buffer containing 10 mM EGTA ($pCa > 8.0$). The developed force was expressed as a percentage, assuming the values in relaxing solution and pCa 4.5 to be 0 and 100%, respectively.

2.2. Measurement of Rho-kinase activity in vitro

Smooth muscle Rho-kinase was purified from a chicken gizzard and its kinase activity was determined by a phosphorylation assay using 20 kDa myosin light chain as a substrate (Feng et al., 1999).

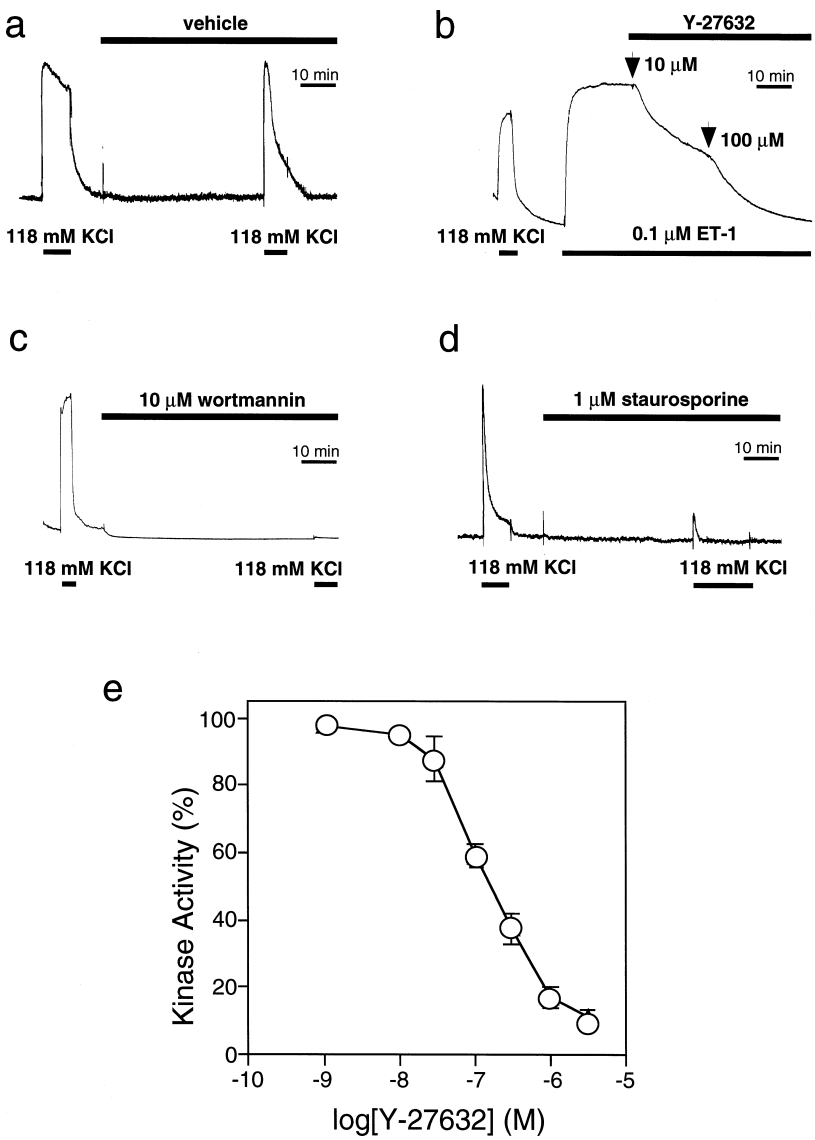

Fig. 1. Effects of protein kinase inhibitors on intact fibers (panels a–d) and effect of Y-27632 on the activity of smooth muscle Rho-kinase (panel e). Each fiber was contracted by 118 mM KCl-induced depolarization (panel a, c and d) and by 0.1 μM endothelin-1 (panel b). The effect of the agent was confirmed by 30-min pretreatment (a, 1% dimethylsulfoxycide as a vehicle; c, 10 μM wortmannin; d, 1 μM staurosporine) or application at a steady state of agonist-induced contraction (b, 100 μM Y-27632). These results are representative of three to five independent experiments. e, Rho-kinase was assayed using 20 kDa myosin light chain as a substrate under the conditions as described under Section 2. The kinase activity in the absence of Y-27632 was expressed as 100%. Error bars indicate means ± S.E. ($n = 3$).

2.3. Measurement of the level of 20 kDa myosin light chain phosphorylation

After termination of the contractile response by the addition of frozen-slurry of acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol, the fringe-like strips of the rabbit portal veins permeabilized by Triton X-100 were subjected to glycerol–urea gel electrophoresis with minor modification (Persechini et al., 1986; Kureishi et al., 1997). They were homogenized in urea sample buffer containing 8 M urea. Each aliquot was subjected to glycerol–urea polyacrylamide electrophoresis following an immunoblot using anti 20 kDa myosin light chain antibody or a phosphorylation site-specific antibody for monophosphorylated 20 kDa myosin light chain at serine-19 (pLC1; Sakurada et al., 1998).

2.4. Data analysis

Values were presented as means ± S.E. Data were compared by the Student's *t*-test. $P < 0.05$ was considered to be significant.

2.5. Materials

Y-27632, (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, was kindly provided by Yoshitomi Pharmaceutucal Ind., Osaka, Japan. Other agents were commercially purchased: microcystin-LR, staurosporine, and calphostin C (Wako, Osaka, Japan), HA1077 (1-(5-isoquinolinylsulfonyl)-homopiperazine) (Calbiochem, San Diego, USA), H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) (Research Biochemicals International, Natick, USA). Except for microcystin-LR and H-7, all agents were dissolved with dimethylsulfoxycide. Microcystin-LR was dissolved with ethanol, and H-7 with distilled water. The final concentration of vehicle was kept under 1%.

3. Results

Before each experiment using skinned fibers, we checked whether each inhibitor was active, based on an experiment evaluating the inhibitory effect on the high-K^+-depolarization and/or agonist-induced contraction using intact fibers. As shown in Fig. 1, although the vehicle (1% dimethylsulfoxycide) has no effect on force development of intact fibers (panel a), Y-27632, a Rho-kinase inhibitor, inhibited the endothelin-1 (0.1 μM)-induced contraction in a dose-dependent manner with the concentration producing 50% inhibition (IC_{50}) of 9.1 ± 0.46 μM ($n = 3$). We confirmed that 100 μM Y-27632, at the concentration previously described by Uehata et al., completely inhibited the contraction (panel b). In addition, the inhibitory effect of Y-27632 on the activity of Rho-kinase was confirmed by an in vitro study. As shown in panel e, Y-27632 inhibited the activity of Rho-kinase purified from chicken gizzard in a dose-dependent manner with an IC_{50} value of 0.17 ± 0.01 μM ($n = 3$). Wortmannin (10 μM, panel c), a myosin light chain kinase inhibitor, and staurosporine (1 μM, panel d), a nonspecific protein kinase inhibitor, completely inhibited force development by high-K^+-depolarization to −3.4 ± 1.8 and 1.2 ± 1.0% ($n = 4$), respectively. HA1077 (100 μM) and H-7 (100 μM) also inhibited the

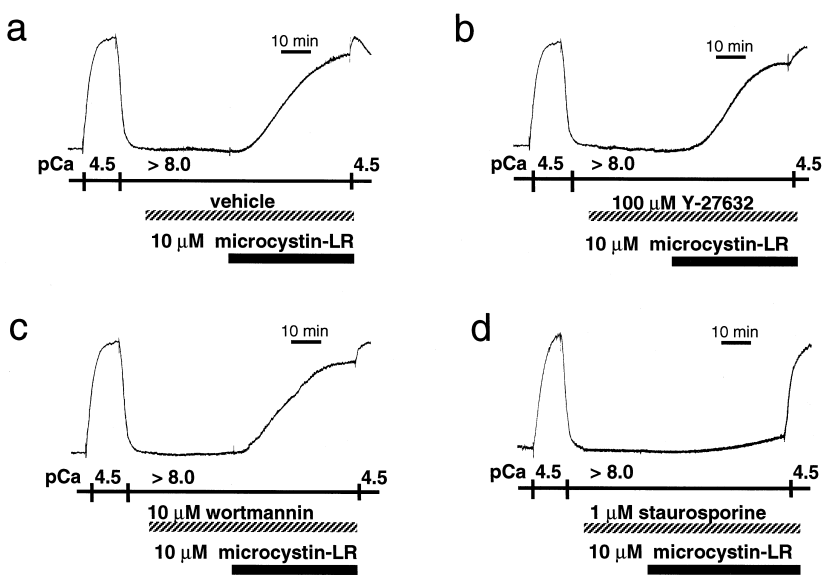

Fig. 2. Representative recording of the effects of protein kinase inhibitors on the 10 μM microcystin-LR-induced contraction in the absence of $[Ca^{2+}]_i$ with 30-min pretreatment of 1% dimethylsulfoxycide as a vehicle (panel a), 100 μM Y-27632 (panel b), 10 μM wortmannin (panel c), and 1 μM staurosporine (panel d). These results are representative of three to five independent experiments.

Table 1
Summary of the effects of protein kinase inhibitors on microcystin-LR-induced contractions in α-toxin-permeabilized fibers. In the presence of each reagent, values shown in the table indicate the percentage of the maximal Ca^{2+}-induced contraction in α-toxin skinned fibers

Reagent (concentration)	Microcystin-LR-induced contraction in permeabilized fibers (%)
Vehicle (1%)	88.4 ± 2.3
HA1077 (100 μM)	90.1 ± 2.1
Y-27632 (100 μM)	88.5 ± 2.6
Wortmannin (10 μM)	84.9 ± 4.6
H-7 (100 μM)	85.9 ± 1.6
Calphostin C (100 μM)	83.5 ± 5.9
Staurosporine (1 μM)	7.7 ± 1.8

contraction by high-K^+-depolarization to 14.5 ± 6.8 and 0.7 ± 0.15% ($n = 4$), respectively.

To clarify which kinase is responsible for Ca^{2+}-independent contraction with resultant myosin phosphatase inhibition induced by 10 μM microcystin-LR, we examined the effects of various kinase inhibitors on microcystin-LR-induced contraction in α-toxin-permeabilized rabbit portal vein strips (Fig. 2, Table 1). As shown in Fig. 2, Y-27632 (100 μM) and wortmannin (10 μM) were ineffective, whereas staurosporine (1 μM) inhibited microcystin-LR-induced contraction. One micromolar staurosporine decreased microcystin-induced contraction from 88.4 ± 2.3% ($n = 4$, notice that force normalized to maximal Ca^{2+} (pCa 4.5)-induced contraction) to 7.7 ± 1.8% ($n = 4$, $P < 0.05$), indicating that staurosporine inhibited the microcystin-induced contraction by more than 90% (Table 1). When staurosporine was loaded at the steady state of microcystin-LR-induced contraction, there was no change in the level of force development (data not shown). Staurosporine (1 μM) also completely suppressed the contraction induced by 10 μM okadaic acid, an alternative protein phosphatase inhibitor (data not shown). Another Rho-kinase inhibitor, HA1077 (100 μM), and two kinds of protein kinase C inhibitors, H-7 (100 μM) and calphostin C (100 μM), also had little affect on microcystin-LR-induced contraction (Table 1). All these effects were reproducible not only in Ca^{2+}-independent contraction induced by 10 μM okadaic acid, but also in 0.5% Triton-X-100-permeabilized rabbit portal vein strips (data not shown).

To confirm the correlation between the changes in levels of 20 kDa myosin light chain phosphorylation and contraction, the levels of myosin light chain phosphorylation under each condition were determined. As shown in Fig. 3a, almost 100% of the detectable bands of 20 kDa myosin light chain were monophosphorylated in the presence of 10 μM microcystin-LR, and this monophosphorylation was inhibited only by staurosporine. In the presence of 1 μM staurosporine, only 8.9 ± 2.7% ($n = 5$, $P < 0.05$) of the total amount of 20 kDa myosin light chain was detected as a monophosphorylated form. These results were consistent with those of the effects on the contractile responses. As shown in Fig. 3b, anti-phosphorylated serine-19-specific antibody also reacted to these bands producing the same pattern as in Fig. 3a.

4. Discussion

Here, we showed the existence of a staurosporine-sensitive kinase(s) as a possible regulator of Ca^{2+}-independent smooth muscle contraction induced by a myosin phos-

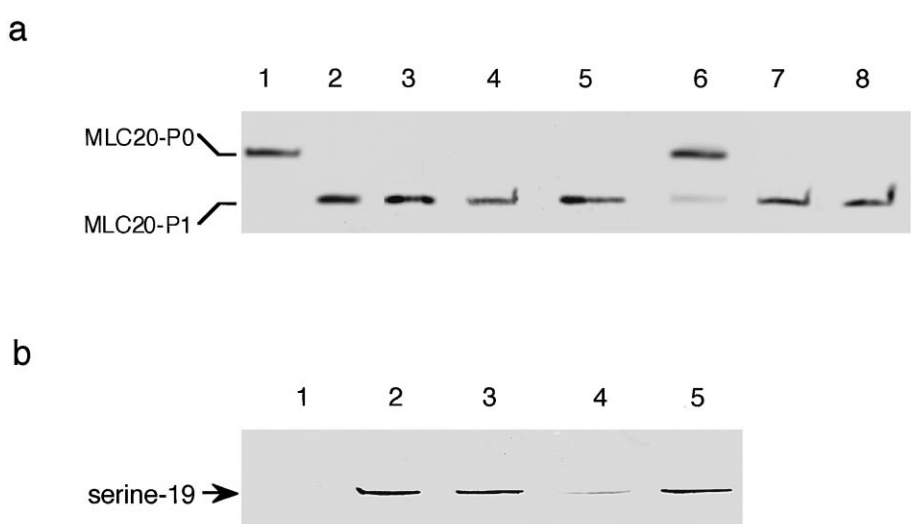

Fig. 3. Effects of protein kinase inhibitors on 10 μM microcystin-LR-induced increases in the levels of monophosphorylation of 20 kDa myosin light chain (MLC20-P1) at pCa > 8.0 (panel a). Nonphosphorylated form of 20 kDa myosin light chain is represented as MLC20-P0. Experimental conditions for each lane are the same as those for the force measurements in Fig. 2. Lane 1, in the absence of microcystin-LR with 1% vehicle; lanes 2–8, in the presence of 10 μM microcystin-LR with 1% vehicle, 100 μM Y-27632, 100 μM HA1077, 10 μM wortmannin, 1 μM staurosporine, 100 μM H-7, and 100 μM calphostin C, respectively. These results are representative of four independent experiments. Identification of phosphorylation sites of 20 kDa myosin light chain using pLC1 antibody (panel b). Lane 1, in the absence of microcystin-LR with 1% vehicle; lanes 2–5, in the presence of 10 μM microcystin-LR with 1% vehicle, 100 μM Y-27632, 1 μM staurosporine, and 10 μM wortmannin, respectively.

phatase inhibitor. This staurosporine-sensitive kinase induced monophosphorylation of 20 kDa myosin light chain at serine-19, which is known to be accompanied by activation of actomyosin ATPase activity, and the inhibitory effects of staurosporine on the force development were correlated with the decrease in the level of 20 kDa myosin light chain monophosphorylation at serine-19. It was reported that almost all phosphatases were inhibited to a submaximal extent in an irreversible manner by microcystin-LR (MacKintosh et al., 1990). The complete inhibition of myosin phosphatase was confirmed by the findings that staurosporine did not relax the permeabilized fiber which had been contracted by microcystin-LR. The effects of staurosporine on microcystin-LR-induced contraction should be pharmacomechanically uncoupled and nonspecific to microcystin-LR, because they were reproducible under the following conditions: (1) contraction induced by microcystin-LR in Triton-X-100-permeabilized rabbit portal vein strips, in which pharmacomechanical coupling of receptor-G-protein signaling was completely abolished (Somlyo and Somlyo, 1994), and (2) contraction induced by okadaic acid, an alternative protein phosphatase inhibitor, in fibers permeabilized by α-toxin and Triton X-100.

Among the inhibitors examined, only pretreatment by staurosporine suppressed the microcystin-LR-induced contraction of the rabbit portal vein. The activities of the inhibitors used in this study were supported by the findings that each inhibitor at the indicated dose showed almost complete inhibition on high-K^+-depolarization or endothelin-1-induced contraction in intact fibers. The concentrations of the inhibitors used in this study, namely 100 μM Y-27632 (Uehata et al., 1997), 100 μM HA1077 (Asano et al., 1989), 10 μM wortmannin (Nakanishi et al., 1992), 100 μM H-7 (Schramm and Grunstein, 1995), 100 μM calphostin C (Jensen, 1996) and 1 μM staurosporine (Asano et al., 1995), were appropriate and sufficient to inhibit each respective kinase.

Rho-kinase is considered to be involved in so-called Ca^{2+}-independent contraction via inhibition of myosin phosphatase and/or direct phosphorylation of 20 kDa myosin light chain (Amano et al., 1996; Kureishi et al., 1997). However, its involvement in microcystin-LR-induced contraction appears unlikely in this case, based on the ineffectiveness of two kinds of specific inhibitors, Y-27632 and HA1077 (Uehata et al., 1997).

A Ca^{2+}–calmodulin-independent form of myosin light chain kinase has been proposed to regulate Ca^{2+}-independent contraction induced by myosin phosphatase inhibitor, based on the effects of ML-9 (1-(5-chloronaphthalenesulfonyl)-1*H*-hexahydro-1,4-diazepine) and/or a inhibitory peptide for myosin light chain kinase, SM-1, which corresponds to residues 783 to 804 of chicken gizzard myosin light chain kinase (Gong et al., 1992; Suzuki and Itoh, 1993; Uehata et al., 1997). However, wortmannin had no effect on Ca^{2+}-independent smooth muscle contraction induced by microcystin-LR. On the other hand, it completely inhibited high-K^+-depolarization-induced contraction in intact fibers. Wortmannin is a more specific inhibitor for myosin light chain kinase than ML-9, and it inhibits not only Ca^{2+}–calmodulin-dependent activity of myosin light chain kinase but also Ca^{2+}-independent activity induced by its proteolytic degradation (Nakanishi et al., 1992). In addition, wortmannin inhibited autophosphorylation of myosin light chain kinase in vitro (data not shown), which induced slight Ca^{2+}-independent activation (Tokui et al., 1995) and might be a Ca^{2+}-independent form of myosin light chain kinase in vivo (Walker et al., 1998). Our preliminary experiments showed that ML-9 (300 μM) inhibited the Ca^{2+}-independent contraction induced by microcystin-LR as previously reported (Gong et al., 1992; Suzuki and Itoh, 1993; Uehata et al., 1997). However, ML-9 also inhibited microcystin-LR-induced contraction even at the steady level of force already induced by microcystin-LR. Since phosphatase may not dephosphorylate myosin light chain in the microcystin-LR-treated permeabilized fibers, these effects of ML-9 might be unrelated to the levels of 20 kDa myosin light chain phosphorylation. This speculation was supported by our preliminary data that ML-9 (300 μM) completely inhibited microcystin-LR-induced contraction in the presence of ATPγS instead of ATP. In permeabilized smooth muscle, ATPγS treatment results in thiophosphorylation of myosin light chain, which is then a very poor substrate for phosphatase activity (Hoar et al., 1979). All these results suggest that the inhibitory effect of ML-9 (300 μM) is due not only to the inhibition of myosin light chain kinase and the resultant inhibition of 20 kDa myosin light chain phosphorylation but also to some unknown effects which induce smooth muscle contraction regardless of the level of myosin light chain phosphorylation. Although we have not obtained conclusive data to distinguish the effects of wortmannin from those of other myosin light chain inhibitors, the inconsistency of the effects between wortmannin and ML-9 on the microcystin-LR-induced fibers suggests that a staurosporine-sensitive kinase is distinct from myosin light chain kinase.

Protein kinase C has also been reported to mediate smooth muscle contraction (see review by Somlyo and Somlyo, 1994; Walsh et al., 1996; Karaki et al., 1997). Phorbol ester (PDBu), an activator of protein kinase C, has also been found to induce Ca^{2+}-independent contraction via myosin phosphatase inhibition (Masuo et al., 1994). Our findings that the protein kinase C inhibitors, namely H-7 and calphostin C, were insensitive to microcystin-LR-induced contraction were reasonable because myosin phosphatase should be completely and irreversibly inhibited in the microcystin-LR-treated fiber and additional inhibition of myosin phosphatase by protein kinase C-catalyzed pathway will not enhance the contraction. Therefore, the results suggested that protein kinase C is not responsible for microcystin-LR-induced contraction.

Among the kinases involved in smooth muscle contraction, p21-activated protein kinase was recently found to induce Ca^{2+}-independent contraction (van Eyk et al., 1998). However, no pharmacological inhibitor of p21-activated protein kinase has been reported. Considering that p21-activated protein kinase induced Ca^{2+}-independent contraction without an increase in 20 kDa myosin light chain phosphorylation (van Eyk et al., 1998), p21-activated protein kinase does not appear involved in microcystin-LR-induced contraction.

In conclusion, we suggest that the kinase which induces Ca^{2+}-independent contraction is distinct from Rho-kinase, myosin light chain kinase, and protein kinase C.

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