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ML-9, a myosin light chain kinase inhibitor, reduces intracellular Ca^{2+} concentration in guinea pig trachealis

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Abstract

We investigated the effects of ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine], a myosin light chain kinase (MLCK) inhibitor, on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), contraction induced by high K^+ and an agonist, and capacitative Ca^{2+} entry in fura-2-loaded guinea pig tracheal smooth muscle. ML-9 inhibited both the increase in $[Ca^{2+}]_i$ and the contraction induced by 60 mM K^+, 1 μM methacholine or 1 μM thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase. However, another MLCK inhibitor, wortmannin (3 μM), inhibited the contraction elicited by these stimuli without affecting $[Ca^{2+}]_i$. Under the condition that the thapsigargin-induced contraction was fully suppressed by 3 μM wortmannin, 30 μM ML-9 caused a further decrease in $[Ca^{2+}]_i$. The inhibitory effects of ML-9 on $[Ca^{2+}]_i$ and the contraction elicited by methacholine were similar to those of SKF-96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride), a Ca^{2+} channel blocker. These results indicate that ML-9 acts as a potent inhibitor of Ca^{2+}-permeable channels independently of MLCK inhibition in tracheal smooth muscle.
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Keywords: Ca^{2+} channel; Ca^{2+} entry, capacitative; Fura-2; Myosin light chain kinase; Smooth muscle, airway

1. Introduction

Contraction of airway smooth muscle plays a pivotal role in airway narrowing in bronchial asthma and is regulated by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the sensitivity to Ca^{2+} of the contractile elements (Gerthoffer, 1991). Phosphorylation of 20-kDa myosin light chain (MLC), which is regulated by the Ca^{2+}/calmodulin-dependent MLC kinase (MLCK) pathway and the RhoA/Rho-kinase pathway, is generally considered to be the primary regulatory process for contraction of airway smooth muscle (Somlyo and Somlyo, 2000; Fernandes et al., 2003). MLCK phosphorylates MLC directly in response to an increase in $[Ca^{2+}]_i$ (Gallagher et al., 1997; Kamm and Stull, 2001), whereas Rho-kinase increases MLC phosphorylation levels by suppressing MLC phosphatase (Kimura et al., 1996). Since recent works have demonstrated that MLCK levels and MLCK mRNA of airway smooth muscle cells are higher in asthmatic subjects than in normal subjects, increased MLCK activity may be involved in the bronchoconstriction in asthma attacks (Ammit et al., 2000; Ma et al., 2002).

ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine] (Saitoh et al., 1987) and wortmannin (Nakanishi et al., 1992) are commonly used to block MLCK activity, but are chemically unrelated. Previous reports have shown the different effects of these two inhibitors on $[Ca^{2+}]_i$ in smooth muscle. ML-9 is much more potent in reducing $[Ca^{2+}]_i$ elicited by high K^+-induced contraction than wortmannin at the same contraction levels in aequorin-loaded arterial smooth muscle (Wingard and Murphy, 1999). Moreover, using fura-2, a fluorescent Ca^{2+} indicator, wortmannin abolished both high K^+- and agonist-induced contraction

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without altering $[Ca^{2+}]_i$ in various types of smooth muscle (Asano et al., 1995; Takayama et al., 1996; Burdyga and Wray, 1998; Longbottom et al., 2000). Since Itoh et al. (1989) demonstrated that injection of a constitutively active proteolysed form of MLCK caused contraction without affecting $[Ca^{2+}]_i$ in single stomach smooth muscle cells, MLCK is unlikely to affect the regulation of $[Ca^{2+}]_i$ in smooth muscle. As described above, ML-9 may act as not only an MLCK inhibitor but also a Ca^{2+} channel blocker. However, little is currently known about the relationship between MLCK activity and Ca^{2+} mobilization by this agent.

To determine whether the mechanisms underlying a reduction in $[Ca^{2+}]_i$ by ML-9 are independent of MLCK inhibition, we examined the effects of ML-9 and wortmannin on $[Ca^{2+}]_i$ and the contraction induced by various stimuli, such as high K^+, methacholine, a muscarinic receptor agonist, and thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990), in epithelium-denuded, guinea pig tracheal smooth muscle loaded with fura-2. To evaluate the correlation between $[Ca^{2+}]_i$ and tension in detail, $[Ca^{2+}]_i$ and isometric tension were measured simultaneously.

2. Material and methods

2.1. Tissue preparation and solution

The methods were essentially similar to those described previously (Kume et al., 1995). The tracheas were excised from male Hartley guinea pigs (250–350 g) after intraperitoneal injection of pentobarbital (70 mg/kg). The tracheal rings were opened by cutting them longitudinally in the cartilaginous region, and the epithelium was dissected out. The normal bathing solution was composed of (in mM): NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2 and glucose 11.8, bubbled with a mixture of 99% O_2 and 1% CO_2 (pH 7.4). Ca^{2+}-free solution was prepared by replacing 2.4 mM $CaCl_2$ in the normal solution with 2.2 mM NaCl and 0.2 mM EGTA. The bathing solution was perfused in the organ bath at a constant flow of 3 ml/min. The temperature of the organ bath was maintained at 37 °C. All animal procedures were approved by the Animal Care and Use Committee, Research Institute of Environmental Medicine, Nagoya University.

2.2. Isometric tension recording and measurement of fura-2 fluorescence

The methods are similar to those described previously (Ito et al., 2001; Kume et al., 2001). The method for simultaneous recording of $[Ca^{2+}]_i$ and contraction of smooth muscle was as reported by Ozaki et al. (1987). A preparation was placed horizontally in an organ bath (0.6 ml volume). Muscle strips containing three cartilaginous rings, one for isometric tension recording and two for $[Ca^{2+}]_i$ measurements, were prepared. One end of a cartilaginous ring was fixed to the chamber, and the other end was connected to a force-displacement transducer to monitor isometric tension. Both ends of two cartilaginous rings were fixed to the chamber for $[Ca^{2+}]_i$ measurements. Spontaneous tone was abolished by the addition of 2 μM indomethacin, which was present throughout the experiments.

Muscle strips were treated with 10 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 4 h at room temperature (22–24 °C). The non-cytotoxic detergent, pluronic F-127 (0.01% wt/v), was added to increase the solubility of fura-2/AM. After loading, the chamber was perfused with the normal solution at 37 °C for 50 min to wash out extracellular fura-2/AM before the measurements. Isometric tension and the fura-2 fluorescence of muscle strips were measured simultaneously, using a displacement transducer and a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo). The mucosal side of the muscle strips was exposed to the excitation light, and the light emitted from the strip was collected in a photomultiplier through a 500-nm filter. The intensity of fluorescences due to excitation at 340 nm (F340) and that at 380 nm (F380) were measured after background subtraction. The absolute amount of $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} in smooth muscle cytoplasm may be different from that obtained in vitro (Konishi et al., 1988). Therefore, the ratio of F340 to F380 (F340/F380) was used as a relative indicator of $[Ca^{2+}]_i$. Measurements of tension and the F340/F380 ratio were performed just before the solution was changed.

2.3. Experimental protocols

To assess the effects of ML-9 and wortmannin on $[Ca^{2+}]_i$ and the contraction elicited by high K^+ or by an agonist, each agent was applied for 10–15 min to the strips stimulated by 60 mM K^+ or by 1 μM methacholine for 10 min. In each experimental condition, the F340/F380 ratio and tension in response to 1 μM methacholine and 60 mM K^+ were taken as 100% (control). To assess the effects of ML-9 and wortmannin on $[Ca^{2+}]_i$ and the contraction caused by 'capacitative Ca^{2+} entry' (Putney, 1990), 1 μM thapsigargin was applied under the condition that the extracellular medium was nominally Ca^{2+} free (0 mM) to inhibit Ca^{2+} uptake into the sarcoplasmic reticulum, as described previously (Ito et al., 2002). After the depletion of the sarcoplasmic reticulum Ca^{2+} stores, each agent was applied for 10–15 min in the presence of 1 μM thapsigargin at an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) of 2.4 mM. The F340/F380 ratio and tension elicited by 1 μM thapsigargin in response to 2.4 mM $[Ca^{2+}]_o$ were taken as 100% (control). At the end of each experiment, $[Ca^{2+}]_o$ was decreased to 0 mM and the F340/F380 ratio and tension in response to 0 mM $[Ca^{2+}]_o$ were taken as 0%. A previous report showed that both agonist-

related and unrelated contractions are correlated with MLC phosphorylation levels in tracheal smooth muscle (Kai et al., 2000). According to this study, we measured the contraction of tracheal smooth muscle as an index of the levels of MLC phosphorylation.

2.4. Drugs

Indomethacin, methacholine and pluronic F-127 were obtained from Sigma (St. Louis, MO, USA); SKF-96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride) and wortmannin were from Calbiochem (La Jolla, CA, USA); ML-9 was from Seikagaku (Tokyo, Japan); thapsigargin was from Wako (Osaka, Japan); and fura-2/AM was from Dojin Laboratories (Kumamoto, Japan). Fura-2/AM was dissolved in dimethylsulfoxide (DMSO), and the final DMSO concentration did not exceed 0.5%. Neither drug affected the fura-2 fluorescence at the concentrations used.

2.5. Statistical analysis

All data are expressed as means ± S.D.; n is the number of preparations used. Student's t-test or analysis of variance was used to evaluate the statistical significance of differences between means with $P<0.05$ as level of significance.

3. Results

3.1. Effects of MLCK inhibitors on high K^+ depolarization

ML-9 (30 μM) inhibited 60 mM K^+-induced contraction with a significant reduction in the F340/F380 ratio (Fig. 1A). When ML-9 (10–100 μM) was applied, ML-9 caused a concentration-dependent inhibition of tension and $[Ca^{2+}]_i$ induced by 60 mM K^+ (Fig. 1B). Wortmannin (3 μM) inhibited the 60 mM K^+-induced contraction with no

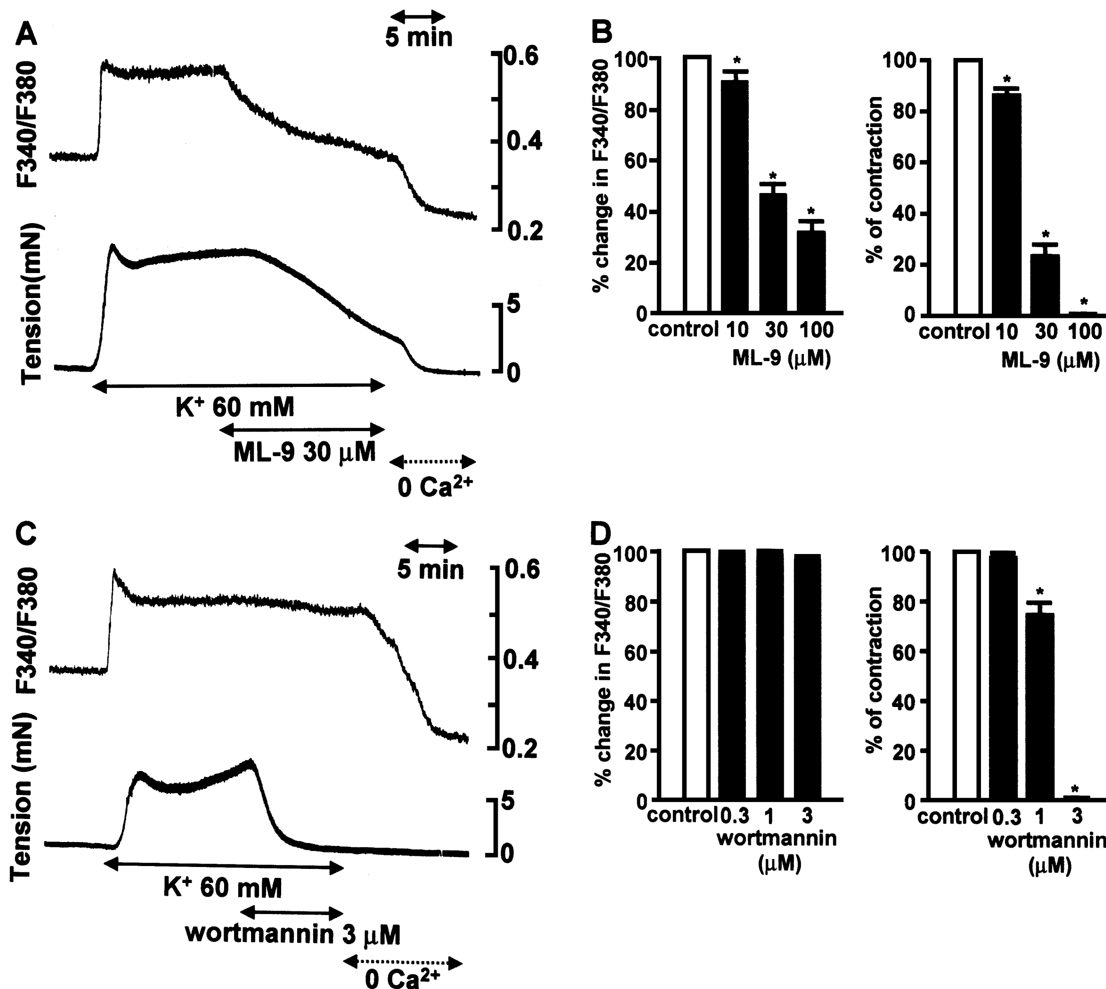

Fig. 1. Representative traces showing the effects of 30 μM ML-9 (A) and 3 μM wortmannin (C) on the F340/F380 ratio and tension induced by 60 mM K^+. Summarized data of the effects of ML-9 (10-100 μM; B) and wortmannin (0.3-3 μM; D) on the F340/F380 ratio and the contraction induced by 60 mM K^+. The F340/F380 ratio and tension in Ca^{2+}-free solution were taken as 0%, and those in response to 60 mM K^+ were taken as 100% (control). Columns represent the means ± S.D. (n = 4). *: Significantly different from the control values ($P<0.05$).

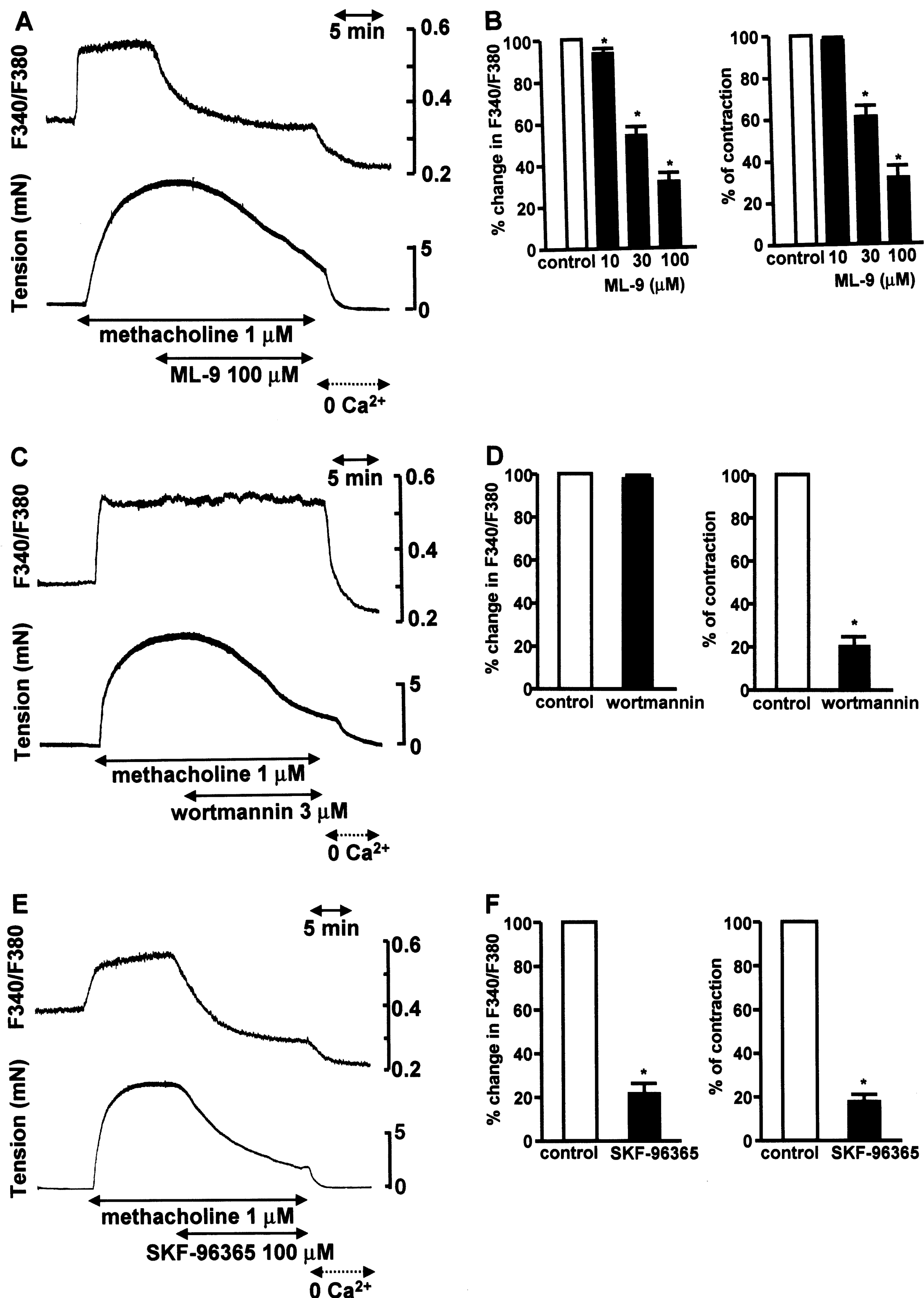

Fig. 2. Representative traces showing the effects of 100 μM ML-9 (A), 3 μM wortmannin (C) and 100 μM SKF-96365 (E) on the F340/F380 ratio and tension induced by 1 μM methacholine. Summarized data of the effects of ML-9 (10-100 μM; B), 3 μM wortmannin (D) and 100 μM SKF-96365 (F) on the F340/F380 ratio and the contraction induced by 1 μM methacholine. The F340/F380 ratio and tension in Ca^{2+}-free solution were taken as 0%, and those in response to 1 μM methacholine were taken as 100% (control). Columns represent the means ± S.D. ($n=4$). *: Significantly different from the control values ($P<0.05$).

reduction in the F340/F380 ratio (Fig. 1C). When wortmannin (0.3–3 μM) was applied to tissues precontracted by 60 mM K^+, wortmannin caused a concentration-dependent inhibition of the 60 mM K^+-induced contraction (Fig. 1D). However, the F340/F380 ratio was not affected by wortmannin (Fig. 1D). After the removal of extracellular Ca^{2+}, the F340/F380 ratio was lower than that under resting conditions (Fig. 1A and C).

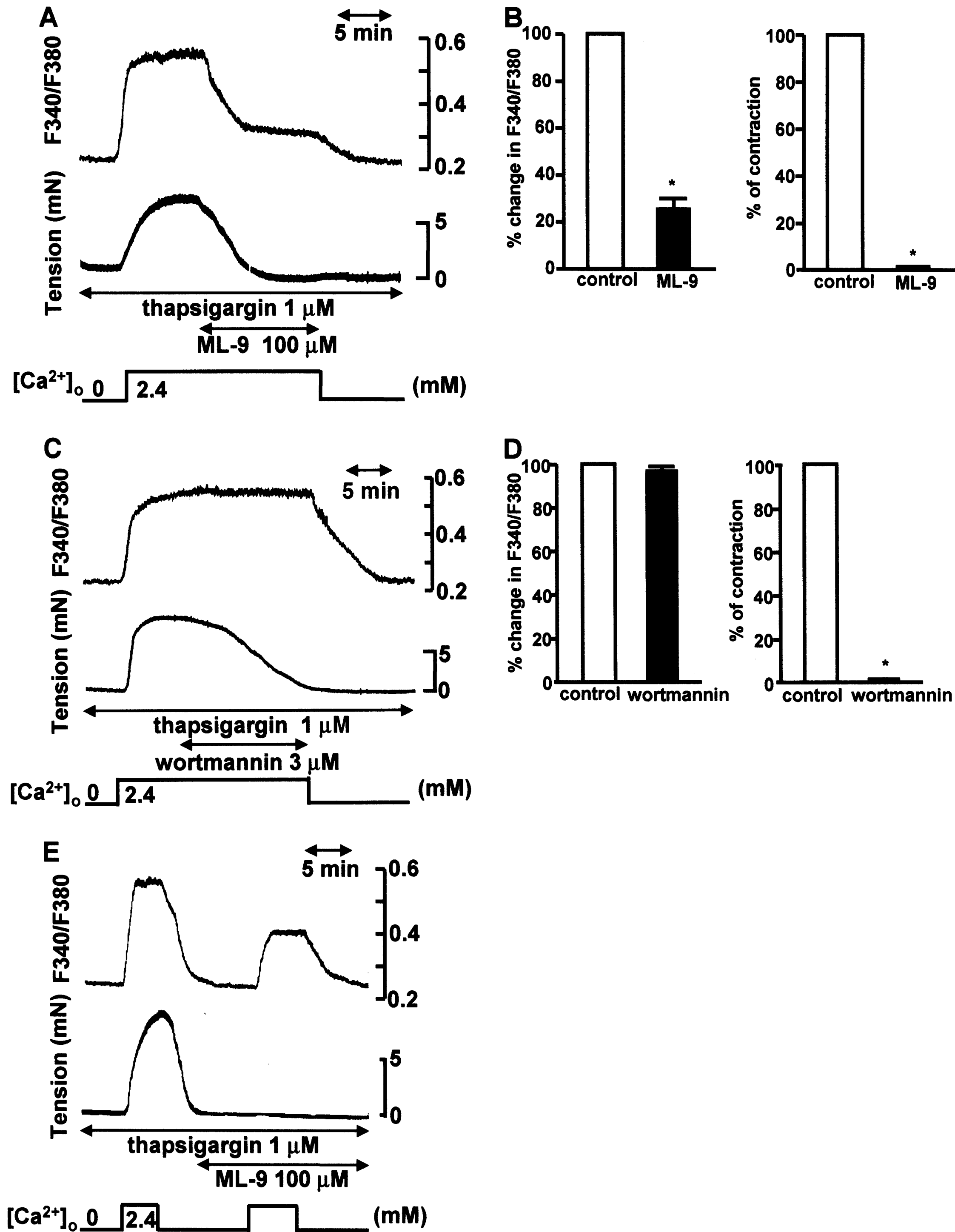

Fig. 3. Representative traces showing the effects of 100 μM ML-9 (A) and 3 μM wortmannin (C) on the F340/F380 ratio and tension induced by 1 μM thapsigargin. Summarized data of the effects of 100 μM ML-9 (B) and 3 μM wortmannin (D) on the F340/F380 ratio and the contraction induced by 1 μM thapsigargin. E: ML-9 (100 μM) was applied in the absence of extracellular Ca^{2+}, and then the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was changed from 0 to 2.4 mM. The F340/F380 ratio and tension in Ca^{2+}-free solution were taken as 0%, and those in response to 2.4 mM $[Ca^{2+}]_o$ were taken as 100% (control). Columns represent the means ± S.D. ($n=4$). *: Significantly different from the control values ($P<0.05$).

3.2. Effects of MLCK inhibitors on muscarinic stimulation

ML-9 (100 μM) inhibited both the increase in the F340/F380 ratio and the contraction elicited by methacholine (Fig. 2A). Application of ML-9 (10–100 μM) reduced the F340/F380 ratio and tension induced by 1 μM methacholine in a concentration-dependent manner (Fig. 2B). Wortmannin (3 μM) markedly inhibited the 1 μM methacholine-induced contraction with no change in the F340/F380 ratio (Fig. 2C and D). SKF-96365 (100 μM) inhibited the contraction induced by 1 μM methacholine with a marked reduction in the F340/F380 ratio (Fig. 2E and F). SKF-96365 decreased the F340/F380 ratio below the resting value at this concentration.

3.3. Effects of MLCK inhibitors on thapsigargin-induced capacitative Ca^{2+} entry

As shown in Fig. 3A, when $[Ca^{2+}]_o$ was changed from 0 to 2.4 mM following the depletion of the sarcoplasmic reticulum Ca^{2+} stores by 1 μM thapsigargin, an increase in the F340/F380 ratio and in force generation was observed. Subsequent application of ML-9 (100 μM) caused a significant reduction in the F340/F380 ratio with a complete inhibition of contraction (Fig. 3A and B). Wortmannin (3 μM) completely inhibited the thapsigargin-induced contraction with a modest reduction in the F340/F380 ratio (Fig. 3C and D). Next, ML-9 (100 μM) was applied in the absence of the extracellular Ca^{2+} and the basal F340/F380 signal was not affected by ML-9 (Fig. 3E). ML-9 significantly inhibited both the increase in the F340/F380 ratio and the contraction induced by Ca^{2+} re-application to the extracellular side (Fig. 3E).

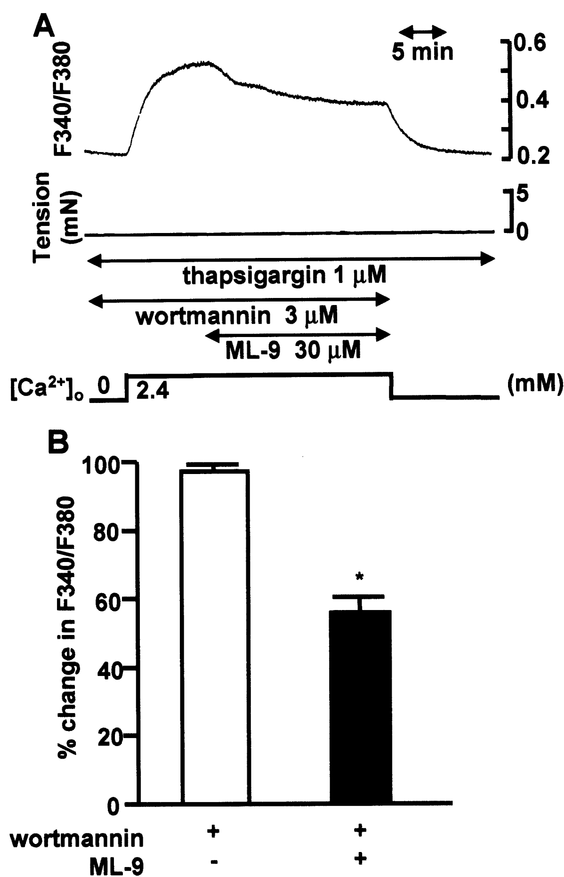

Fig. 4. A representative trace (A) and summarized data (B) of effects of 30 μM ML-9 on the F340/F380 ratio and tension induced by 1 μM thapsigargin in the presence of 3 μM wortmannin. $[Ca^{2+}]_o$ were changed from 0 to 2.4 mM. Columns represent the means ± S.D. ($n = 4$). *: Significantly different between two groups ($P < 0.05$).

3.4. Effects of ML-9 on capacitative Ca^{2+} entry in the presence of wortmannin

To investigate whether ML-9 inhibits the increase in $[Ca^{2+}]_i$ independently of MLCK inhibition, 30 μM ML-9 was applied to strips treated with 1 μM thapsigargin under the condition that the contraction had been completely suppressed by 3 μM wortmannin. After incubation with 3 μM wortmannin for 15 min in Ca^{2+}-free solution, application of 2.4 mM Ca^{2+} elevated the F340/F380 ratio without causing contraction in the presence of 1 μM thapsigargin (Fig. 4A). In this condition, 30 μM ML-9 caused a reduction in the F340/F380 ratio with no change in the contraction.

4. Discussion

ML-9 was developed and is widely used as a selective inhibitor of MLCK (Saitoh et al., 1987; Ishikawa et al., 1988). In the present study, we have shown for the first time that ML-9 inhibits Ca^{2+} mobilization induced by a high concentration of K^+, methacholine and thapsigargin in guinea pig tracheal smooth muscle (Figs. 1–4). These stimuli are known to activate different types of Ca^{2+} channels in smooth muscle (Karaki et al., 1997; Barritt, 1999). Ca^{2+} channels are divided into three major groups, i.e. voltage-operated Ca^{2+} channels (VOCs), store-operated Ca^{2+} channels (SOCs) and store-independent receptor-operated Ca^{2+} channels (ROCs) (Barritt, 1999). In airway smooth muscle, L-type Ca^{2+} channels of VOCs are mainly activated via membrane depolarization by higher concentrations of K^+ in the extracellular side. The SOCs, which are the channels for capacitative Ca^{2+} entry, are activated by depletion of the sarcoplasmic reticulum Ca^{2+} stores or by inositol 1,4,5,trisphosphate-mediated Ca^{2+} release from the sarcoplasmic reticulum (Putney, 1990; Barritt, 1999). Thapsigargin is used as a direct pharmacological activator of the SOCs without inositol 1,4,5,trisphosphate production. In the present study, ML-9 significantly inhibited the Ca^{2+} mobilization induced by thapsigargin (Figs. 3 and 4). However, it is possible that Ca^{2+} removal depolarizes the muscle, opening VOCs. A previous report has indicated that nifedipine, a VOC inhibitor, does not inhibit Ca^{2+} entry or contraction induced by Ca^{2+} re-application in guinea pig

tracheal smooth muscle when nifedipine was applied with Ca^{2+} after removal of extracellular Ca^{2+} in the presence of thapsigargin (Ito et al., 2002). Similar results were also reported for pulmonary arterial smooth muscle (Doi et al., 2000). These findings suggest that VOCs are not activated by Ca^{2+} re-addition following Ca^{2+} removal and that the Ca^{2+} influx pathway inhibited by ML-9 is via SOCs under this condition (Fig. 3).

The ROCs are activated by receptor agonists independently of store depletion (Barritt, 1999) and are also involved in agonist-induced Ca^{2+} entry in airway smooth muscle cells (Murray and Kotlikoff, 1991; Ito et al., 2002). The present results indicate that ML-9 inhibits all these Ca^{2+} channels in tracheal smooth muscle (Figs. 1–3). Interestingly, the inhibitory effects of ML-9 on $[Ca^{2+}]_i$ and the contraction elicited by methacholine and thapsigargin were similar to those of SKF-96365 (Fig. 2 in the present study; Ito et al., 2002). SKF-96365 is a nonselective Ca^{2+} channel inhibitor and does not inhibit the release of Ca^{2+} from the sarcoplasmic reticulum (Merritt et al., 1990). This agent was also shown to inhibit high K^+-, methacholine-, and thapsigargin-induced contraction in guinea pig tracheal smooth muscle (Li et al., 1997; Ito et al., 2002). Therefore, the present results indicate that ML-9 inhibits Ca^{2+} mobilization by blocking Ca^{2+} channels nonselectively in tracheal smooth muscle.

Additionally, ML-9 (100 μM) was applied after the sarcoplasmic reticulum Ca^{2+} stores had been depleted by thapsigargin (1 μM) in the absence of extracellular Ca^{2+}. ML-9 significantly inhibited the Ca^{2+} mobilization induced by Ca^{2+} re-application to the extracellular surface (Fig. 3E), indicating that cytosolic Ca^{2+} is mobilized mainly as a result of Ca^{2+} entry through the plasma membrane. These findings indicate that ML-9 blocks Ca^{2+} influx, and that the uptake of Ca^{2+} into the sarcoplasmic reticulum is not involved in the effects of this agent. Ca^{2+} influx may be mediated by Ca^{2+} channels.

As shown in Figs. 1 and 2, it has been reported that $[Ca^{2+}]_i$ of the resting state (about 2 mM $[Ca^{2+}]_o$ concentrations) is higher than that in the absence of extracellular Ca^{2+} in intact tracheal smooth muscle tissue (Ozaki et al., 1990; Ito et al., 2002). The resting $[Ca^{2+}]_i$ was reduced by both ML-9 and SKF-96365. Therefore, the resting $[Ca^{2+}]_i$ is maintained by Ca^{2+} influx passing through Ca^{2+} channels and is reduced by these agents via nonselective inhibition of Ca^{2+} channels.

Wortmannin, another MLCK inhibitor, inhibited the contraction without affecting the increase in $[Ca^{2+}]_i$ elicited by high K^+, methacholine and thapsigargin (Figs. 1–3). Previous reports have also demonstrated that wortmannin inhibits the contraction induced by high K^+ and agonists without changing $[Ca^{2+}]_i$ in various smooth muscles (Asano et al., 1995; Takayama et al., 1996; Burdyga and Wray, 1998; Longbottom et al., 2000). In the present study, the effects of ML-9 on Ca^{2+} mobilization were not mimicked by wortmannin. These results indicate that MLCK is essential for contraction but is not involved in the regulation of $[Ca^{2+}]_i$ in tracheal smooth muscle, as described in a previous report for stomach smooth muscle cells (Itoh et al., 1989). Even under the condition that the contraction was completely inhibited by 3 μM wortmannin, 30 μM ML-9 reduced thapsigargin-induced Ca^{2+} mobilization (Fig. 4). Under this experimental condition, MLCK activity should be inhibited by 3 μM wortmannin before ML-9 application as described in the next paragraph. These findings further indicate that ML-9 inhibits Ca^{2+} mobilization, independently of MLCK inhibition.

Wortmannin is also known to be a potent inhibitor of phosphatidylinositol 3-kinase (PI-3 kinase) at lower concentrations, and the EC_{50} values for PI-3 kinase and MLCK are 3.0 and 200 nM, respectively, in vitro (Yano et al., 1993). Here, we used a relatively high concentration (3 μM) of wortmannin, enough to inhibit MLCK activity, as shown in previous studies of smooth muscle (Burdyga and Wray, 1998; Longbottom et al., 2000). Since thapsigargin and high K^+ mobilize $[Ca^{2+}]_i$ without causing PI-3 kinase activation, wortmannin acts as a specific inhibitor of MLCK under these experimental conditions. Although it is possible that PI-3 kinase is activated during methacholine-induced contraction, wortmannin did not affect $[Ca^{2+}]_i$ (Fig. 2). Therefore, PI-3 kinase is unlikely to affect Ca^{2+} mobilization induced by methacholine in tracheal smooth muscle. Similarly, Ethier and Madison (2002) reported that wortmannin did not affect $[Ca^{2+}]_i$ in airway smooth muscle. In addition, Bain et al. (2003) and Davis et al. (2000) have shown that wortmannin is a more specific inhibitor of MLCK than ML-9.

Recently, MLCK has been proposed to participate in Ca^{2+} entry pathways in endothelial cells (Watanabe et al., 1998, 2000). Watanabe et al. (1998, 2000) have reported that the inhibition of MLCK activity by ML-9, wortmannin, and MLCK antisense blocks both thapsigargin and agonist-induced Ca^{2+} mobilization in endothelial cells. However, Kuroiwa-Matsumoto et al. (2000) have reported that ML-9 (30–100 μM) inhibits thapsigargin-induced Ca^{2+} entry, but wortmannin (100 μM) does not do so in endothelial cells, similar to results shown in this study. In endothelial cells, the role of MLCK in the regulation of $[Ca^{2+}]_i$ is still controversial. In an electrophysiological study, ML-9 (15 μM) blocked SOCs, inward Ca^{2+} currents induced by thapsigargin, in endothelial cells (Norwood et al., 2000). In aequorin-loaded arterial smooth muscle, ML-9 reduced both high K^+- and histamine-induced Ca^{2+} mobilization (Wingard and Murphy, 1999). Therefore, ML-9 is considered to inhibit Ca^{2+} mobilization by various stimuli, such as high K^+, agonists, and thapsigargin, both in smooth muscle and in endothelial cells. Moreover, another MLCK inhibitor, ML-7 [1-(5-iodonaphthalene-1-sulfonyl)- 1*H*-hexahydro-1,4-diazepine], a compound related to ML-9 (Saitoh et al., 1987), also inhibited increases in $[Ca^{2+}]_i$ induced by 60 mM K^+, methacholine and thapsigargin in guinea pig tracheal smooth muscle (data not shown), suggesting that common structures of ML-9 and ML-7 may affect various Ca^{2+} channels in a nonselective

manner. However, further studies are needed to elucidate these molecular mechanisms, and electrophysiological and radioisotopic techniques may offer more direct information on the Ca^{2+} entry blocking activity of ML-9.

In summary, ML-9 inhibits Ca^{2+} mobilization through VOCs, SOCs, and ROCs independently of MLCK inhibition in guinea pig tracheal smooth muscle. The chemical structure of ML-9 and related compounds may be important for the reduction in $[Ca^{2+}]_i$ induced by these agents. Our results suggest that ML-9 should be used with care when examining the regulation of $[Ca^{2+}]_i$ because this agent acts as a nonselective Ca^{2+} channel blocker.

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