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Mechanisms responsible for the in vitro relaxation of a novel dibenzothiepine derivative (NSU-242) on tracheal and vascular smooth muscles

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Abstract

In our previous general screening experiments, we found that NSU-242, a dibenzothiepine derivative (1–10 mg/kg), inhibited antigen-induced immediate asthmatic response in actively sensitized guinea pigs in a dose-dependent manner. The purpose of the present study was to assess the mechanism of the relaxing effect of NSU-242 on smooth muscle contractions in isolated smooth muscle tissues of the porcine trachea and rat aorta. NSU-242 administration resulted in a concentration-dependent inhibition of the tracheal-tissue contractions induced by carbachol and high K^+ and the aortic-tissue contractions induced by norepinephrine and high K^+. The IC_{50} values of these inhibitions were 1–10 μM, and there was no selectivity for the type of stimulation. In tracheal tissue, the relaxations were accompanied by neither changes in cAMP nor changes in cGMP. Carbachol (1 μM) and high K^+ (59.2 mM) increased myosin light chain (MLC) phosphorylation in the trachea, and NSU-242 (3–30 μM) had no effect on the level of MLC phosphorylation. Furthermore, NSU-242 (300 μM) had no effect on contractions in membrane-permeabilized tracheal tissue. FITC–phalloidin staining of the actin fiber in cultured vascular smooth muscle cells (A7r5) indicated that NSU-242 (10–100 μM) altered the configuration of actin stress fiber in the cytosol. However, unlike cytochalasin D, NSU-242 did not inhibit actin polymerization as assessed by in vitro assay. These results suggest that NSU-242 inhibits smooth muscle contractions without any effect on the Ca^{2+}-dependent MLC phosphorylation. NSU-242 may uncouple the force generated by the activated actomyosin interaction, possibly by modifying the actin assembly in smooth muscle cells without a direct effect on actin molecules.

Keywords: NSU-242; Tracheal smooth muscle; Relaxation; Myosin phosphorylation; Actin assembly

1. Introduction

Compounds with the pharmacological profile of a smooth muscle relaxant have potential value for the treatment of diseases in which smooth muscle becomes hyperreactive to agonists. During the course of our general screening experiments, we found that NSU-242, a novel dibenzothiepine derivative (Fig. 1A), had an inhibitory effect on bronchoconstriction induced by antigen challenge in guinea pigs actively sensitized by ovalbumin and Bordetella pertussis vaccine. We therefore speculated that this compound has the ability to inhibit smooth muscle contraction in vitro, and confirmed this activity in our experiments. In the present study, we attempted to elucidate the mechanisms underlying the relaxing action of this novel smooth muscle relaxant, NSU-242, using tracheal and vascular smooth muscle tissues. Our results showed that NSU-242 is a unique smooth muscle relaxant that inhibits smooth muscle contractions without any effect on the MLC phosphorylation level, and that it may uncouple the force generation from the activated actomyosin interaction, possibly by modifying actin assembly in the cells.

2. Materials and methods

2.1. In vivo study

Hartley guinea pigs (Japan SLC, Sizuoka, Japan; weight range, 280–330 g; age, 5 weeks) were obtained and were

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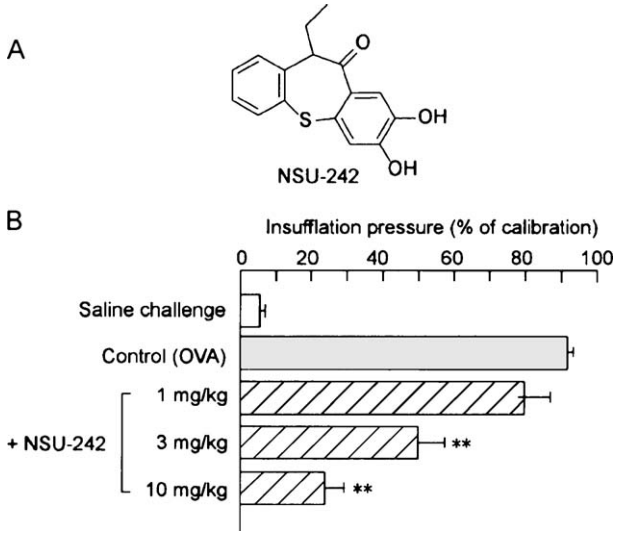

Fig. 1. Chemical structure of NSU-242 (A) and its effect on bronchoconstriction induced by antigen challenge in actively sensitized guinea pigs (B). NSU-242 (1, 3 and 10 mg/kg) was administered (p.o.) 1 h before antigen challenge. Values are expressed as the mean ± S.E.M. ($n = 8$ each). ** Significantly different from the controls (without NSU-242) ($P < 0.01$).

housed at 24 ± 2 °C under 55 ± 10% humidity and a 12-h light/12-h dark cycle. Guinea pigs were actively sensitized with i.m. injection of ovalbumin (1 mg/body) and i.p. administration of Bordetella pertussis vaccine (1.5×10^{10} cell/ml/animal) on two occasions, separated by 7 days. The animals were then challenged by 300 μg/kg of ovalbumin (i.v.) between days 19 and 23 to get a bronchopulmonary constriction. NSU-242 was orally administered via a feeding tube at a dose of 1, 3, or 10 mg/kg at 1 h before the antigen challenge.

Bronchoconstriction was recorded as an increase in intratracheal pressure. The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a small cannula was inserted into the trachea. The tracheal cannula was connected to a constant volume respirator, and the animals were ventilated artificially at a volume of 10 ml/kg air at a frequency of 50 strokes/min. Spontaneous breathing was abolished with gallamine (10 mg/kg, i.v.). Changes in insufflation pressure at a constant airflow were measured with a pressure transducer (Validyne, Gould, St. Louis, MO, USA) connected to the side-arm of the tracheal cannula. The increase in insufflation pressure was given as a percentage of the maximal insufflation pressure obtained by clamping off the trachea.

2.2. Muscle tension in vitro

Freshly excised porcine tracheae were obtained from a local abattoir. The smooth muscle was excised from cartilage, and the epithelium and connective tissues were removed. The smooth muscle was then cut into small strips (approximately 1 mm wide and 5 mm long) and placed in a normal physiological salt solution (PSS) that contained (in mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; $NaHCO_3$, 23.8; glucose, 5.5 and EDTA, 0.01. Male Wistar rats (250–300 g) were stunned and bled, and the thoracic aorta was dissected. After removing fat and connective tissues, the aorta was cut into helical strips approximately 2 mm in width and 8 mm in length. The endothelium was removed by gently rubbing the intimal surface with a finger moistened with PSS. A high K^+ solution was established by substituting equimolar KCl for the NaCl. These solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37 °C and pH 7.4. Muscle contraction was recorded isometrically with a force displacement transducer. Each muscle was attached to a holder under a resting force of 10 mN and equilibrated for 60–90 min in a 10-ml tissue bath.

2.3. cAMP and cGMP contents

cAMP and cGMP contents were measured as described by Kwon et al. (2000). Subsequent to incubation with or without the test agent, the tracheal tissues were immediately frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid (TCA) solution. After centrifugation at 2000 × *g* for 15 min at 4 °C, the supernatants were subjected to a cAMP and a cGMP enzyme immunoassay (Amersham, Buckinghamshire, UK), whereas pellets were used to determine protein content. cAMP and cGMP contents are expressed in pmol/mg protein content.

2.4. MLC phosphorylation

The extent of MLC phosphorylation was measured according to Word et al. (1991). Strips of tracheal tissue were quickly frozen in liquid nitrogen and then homogenized with 10% TCA and 10 mM dithiothreithol (DTT). The homogenate was centrifuged at 10,000 × g for 1 min and the pellet was washed with diethyl ether and then suspended in urea–glycerol buffer. Glycerol–poly acrylamide gel electrophoresis (PAGE) was performed for separating phosphorylated MLC, and the electrophoresed proteins were blotted onto poly vinylidene di-fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) to perform Western blotting using a polyclonal antibody raised against bovine tracheal MLC (kindly donated by Dr. J.T. Stull, University of Texas, SWMC, USA). The relative amount of phosphorylated MLC to total MLC was quantified by NIH image software (version 1.61).

2.5. Permeabilized smooth muscle

Permeabilized muscle preparations were obtained according to the method described by Sparrow et al. (1981). A thin bundle (0.3 mm in width and 3 mm in length) of tissue strips from the swine trachea was prepared in the PSS and permeabilized with Triton X-100 (1%, v/v). The relaxing solution contained 50 mM KCl, 4 mM $MgCl_2$,

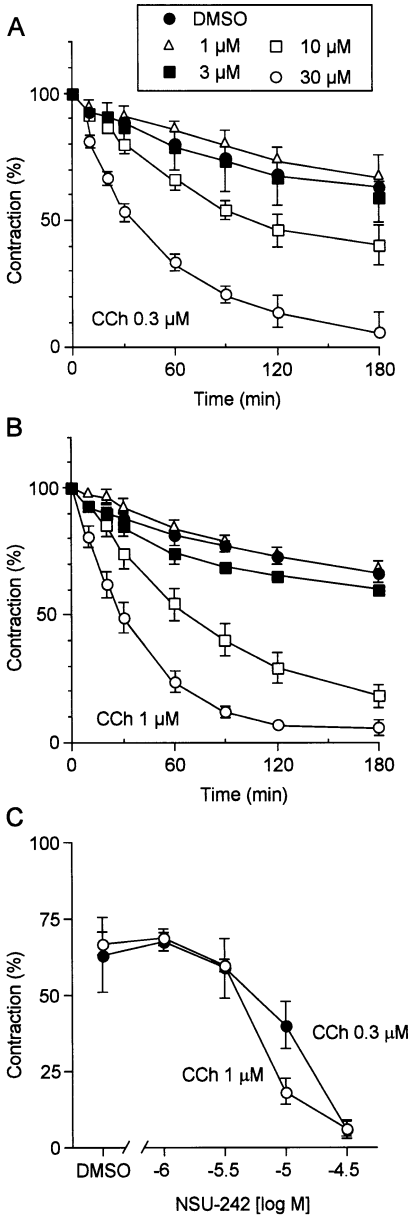

Fig. 2. Effects of NSU-242 on carbachol-induced contraction of porcine tracheal smooth muscle. Panel A (0.3 μM) and panel B (1 μM) show the time course of the relaxation of carbachol-induced contraction after treatment with NSU-242 (1–30 μM). NSU-242 was added after the carbachol-induced contraction had reached a steady state level (after 20 min). Panel C shows the concentration–response relationship for the effect of NSU-242 on contractions induced by carbachol (closed circle 0.3 μM; open circle 1 μM). Points were obtained 180 min after the addition of each concentration of NSU-242. Values are expressed as the mean ± S.E.M. ($n = 3–4$).

20 mM imidazole, 2 mM ATP, 1 mM NaN_3, 0.2 μM calmodulin, 3 mM KH_2PO_4, 3 mM K_2HPO_4 and 2 mM EGTA (pH 6.8 at 25 °C). The free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$ to EGTA (Ca^{2+}–EGTA buffer) considering the apparent binding constant of EGTA for Ca^{2+} as 1 μM at pH 6.8. Muscle tension was measured isometrically under a resting tension of approximately 5 mN.

2.6. Cell culture

A7r5 cells (passaged 10–25 times) were purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum, 1.7 mM L-glutamine, streptomycin (30 μg

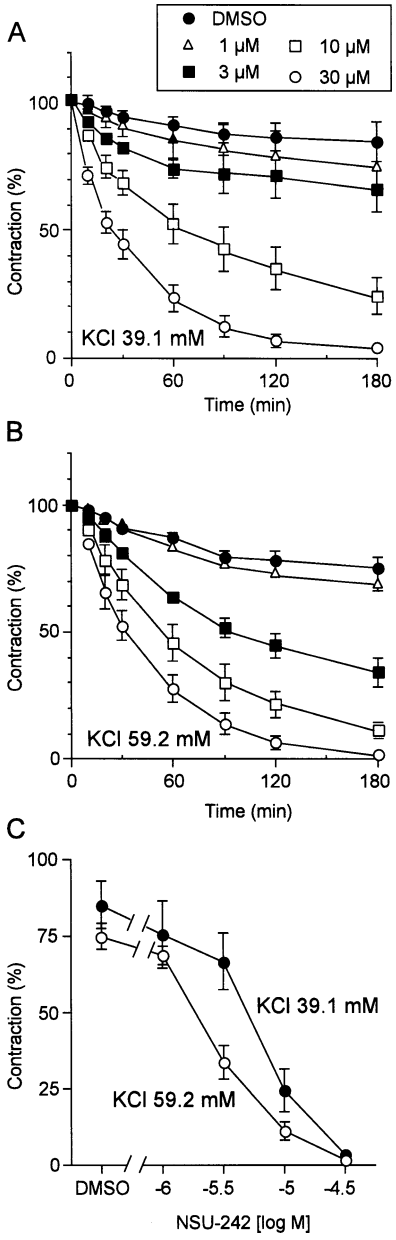

Fig. 3. Effects of NSU-242 on high K^+-induced contraction of porcine tracheal smooth muscle. Panels A (39.1 mM) and B (59.2 mM) show the time course of the relaxation of carbachol-induced contraction after treatment with NSU-242 (1–30 μM). NSU-242 was added after the high K^+-induced contraction had reached a steady state level (after 20 min). Panel C show the concentration–response relationship for the effect of NSU-242 on contractions induced by high K^+ (closed circle 39.1 mM; open circle 59.2 mM). Points were obtained 180 min after the addition of each concentration of NSU-242. Values are expressed as the mean ± S.E.M. ($n = 3–4$).

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.

2.7. Filamentous actin staining

The filamentous actin (F-actin) in A7r5 cells was stained using fluorescein isothiocyanate (FITC)–phalloidin. Briefly, cells were rinsed with PHEM buffer containing (in mM) PIPES 60, HEPES 25, EGTA 10, and $MgCl_2$ 2 at pH 7.3 and 37 °C, 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. The cell membrane was permeabilized by treating the cells with 0.2% Triton X-100 in PHEM buffer for 90 s. FITC–phalloidin (4 U/ml) was added to PHEM buffer for 1 h. The stained F-actin was detected using a confocal microscope (LSM510; Carl Zeiss, Jena, Germany).

2.8. Measurements of polymerization of fluorescent pyrenyl-actin

Skeletal actin was extracted from acetone powder of rabbit fast skeletal muscle using buffer G containing 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and Tris–HCl (pH 8.0) (Spudich and Watt, 1971). Crude G-actin was further purified by Sephadex G-200 gel filtration. Pyrene labeling was performed by a method described previously (Wendel and Dancker, 1986). Actin polymerization was started by the addition of 50 mM KCl and 1 mM $MgCl_2$ to buffer G. The time course of polymerization (or depolymerization) was continuously monitored by measuring the fluorescence of pyrenyl-actin (2.5% of total G-actin) with a fluorometer (FP-2060;

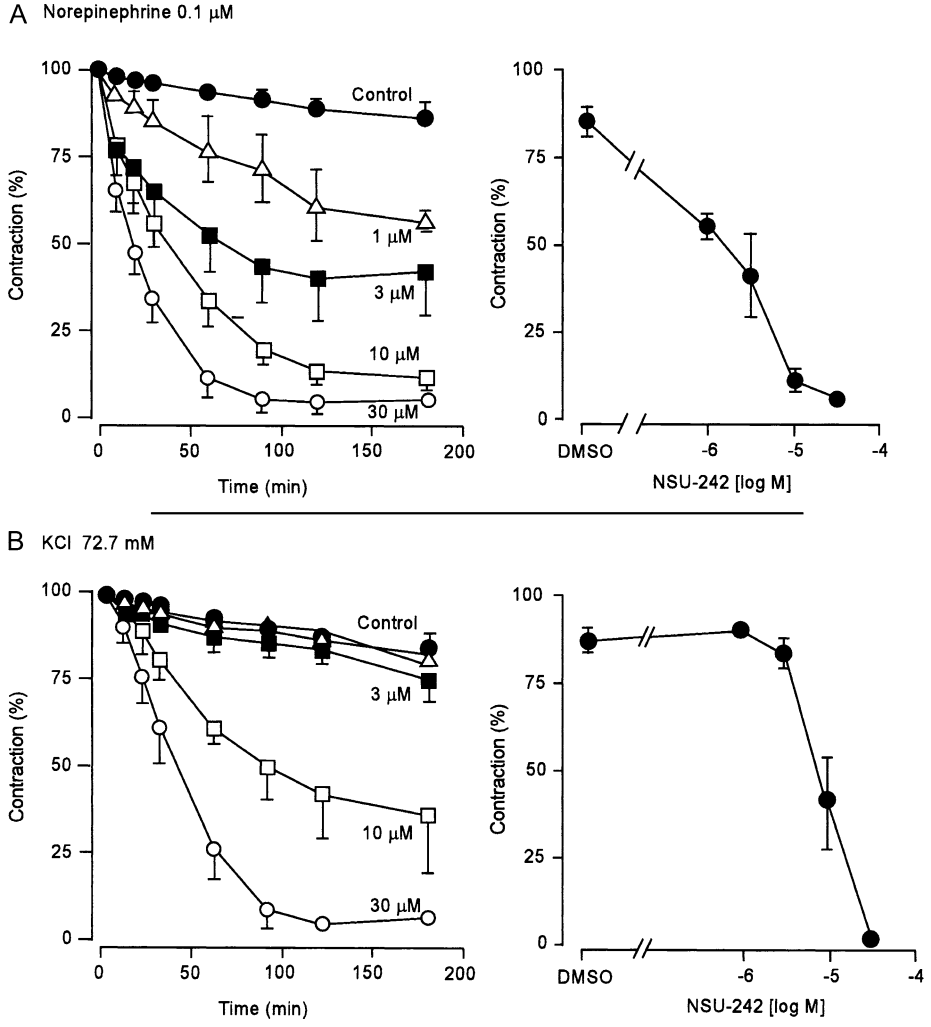

Fig. 4. Effects of NSU-242 on norepinephrine (0.1 μM)-induced contraction (A) and high K^+ (72.7 mM)-induced contraction (B) of rat aorta. The left panel shows the time course of the relaxation of the contractions after treatment with NSU-242 (1–30 μM). NSU-242 was added after the contractions had reached a steady state level (after 180 min). The right panel shows the concentration–response relationship for the effect of NSU-242 on the contractions. Points were obtained 180 min after the addition of each concentration of NSU-242 (0.1–30 μM). Values are expressed as the mean ± S.E.M. ($n=6$).

JASCO, Tokyo, Japan) at 25 °C at 365 nm excitation and 407-nm emission wavelengths.

2.9. Chemicals

NSU-242 was synthesized at Nippon Suisan Kaisha (Tokyo, Japan) and dissolved in DMSO. Other chemicals used were carbachol, forskolin (FK), gallamine, sodium nitroprusside, norepinephrine (Sigma, Tokyo, Japan) and cytochalasin D (Wako, Osaka, Japan).

2.10. Statistics

Student's *t*-test was used to test the significance of results, with values of $P < 0.05$ considered to indicate statistical significance. In some experiments, the results were analyzed by Dunnett's multiple test. Results are presented as the means ± S.E.M.

3. Results

3.1. Bronchial constriction in vivo

We first studied the effect of NSU-242 on immediate asthmatic responses in sensitized guinea pigs (Fig. 1B). In guinea pigs actively sensitized by ovalbumin and Bordetella pertussis vaccine, i.v. administration of ovalbumin produced a remarkable increase in the intratracheal pressure (control group), with a peak response occurring at 3 min. Thereafter, intratracheal pressure gradually decreased. The maximum of intratracheal pressure in the control group was 91.1% on average, and was significantly different from the average pressure of 6.9% in the saline-challenged group. NSU-242 at a dose of 1, 3, or 10 mg/kg, when administered orally 1 h before the antigen challenge, significantly inhibited the increase in the intratracheal pressure by 18%, 50%, and 79%, respectively, as compared with the control group.

3.2. Muscle tension in vitro

In isolated muscle strips from the porcine trachea, carbachol (0.3 and 1 μM) induced contractions which gradually and steadily decreased for over 180 min. NSU-242 (1–30 μM), added after the carbachol-induced contractions had reached steady state, inhibited these responses (Fig. 2). The rate and magnitude of the inhibitory effect of NSU-242 (1–30 μM) was concentration-dependent. Maximum inhibition occurred at a concentration of 30 μM after approximately 180 min incubation. The IC_{50} values of NSU-242 for the contractions induced by 0.3 and 1 μM carbachol were 31.6 ± 0.05 and 9.3 ± 0.38 μM, respectively.

High K^+ solution (39.1 and 59.1 mM) also induced contractions which gradually and steadily decreased for over 180 min. NSU-242 (1–30 μM), added after the high K^+-induced contractions had reached a steady state, inhibited these responses (Fig. 3). The rate and magnitude of the inhibitory effect of NSU-242 (1–30 μM) were concentration-dependent. Maximum inhibition occurred at a concentration of 30 μM after approximately 180 min incubation. The IC_{50} values of NSU-242 for the contractions induced by 39.1 and 59.1 mM K^+ were 8.9 ± 0.21 and 2.8 ± 0.18 μM, respectively.

Next, we examined the effect of the pretreatment with NSU-242 on tracheal contraction induced by carbachol. Pretreatment of the tissue with NSU-242 (1 μM) for 180 min, the contraction induced by carbachol (1 μM) was inhibited to 10.7 ± 2.5% ($n = 9$). After the wash out of the tissue with normal PSS for 30 min, the carbachol-induced contraction recovered to 60.4 ± 7.3% of the control response.

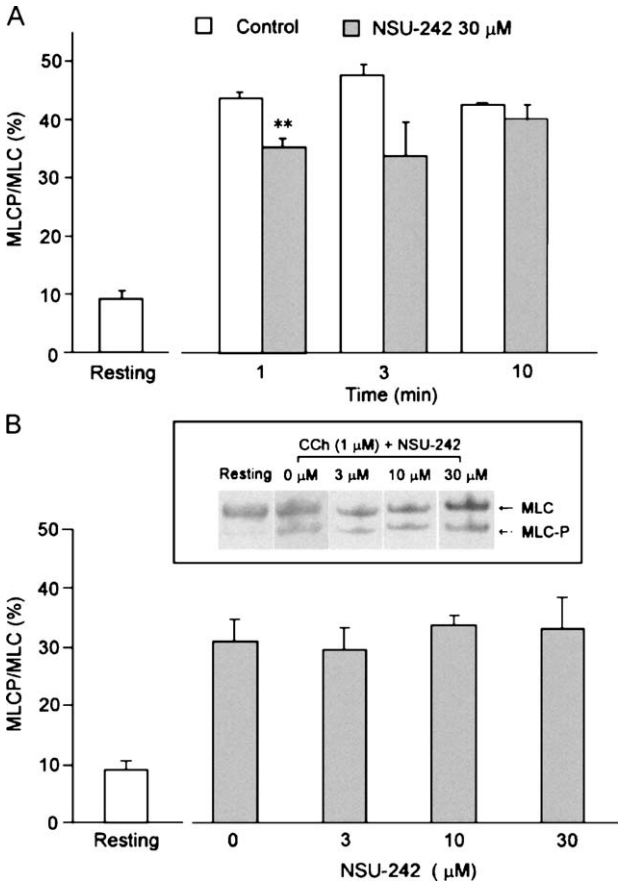

Fig. 5. Effects of NSU-242 on myosin light chain (MLC) phosphorylation in the absence or presence of carbachol. (A) Porcine tracheal smooth muscle strips were treated with 30 μM NSU-242 for 180 min before adding carbachol (1 μM) for 1, 3, or 10 min. (B) Muscle strips were treated with 3–30 μM NSU-242 for 180 min before adding carbachol for 10 min. Each column represents the mean phosphorylated MLC/total MLC ratio ($n = 4$–13). S.E.M. is shown by the vertical bars. ** Significantly different from the controls (without NSU-242) ($P < 0.01$). The inset in panel (B) shows typical patterns of Western blotting for unphosphorylated and phosphorylated MLC.

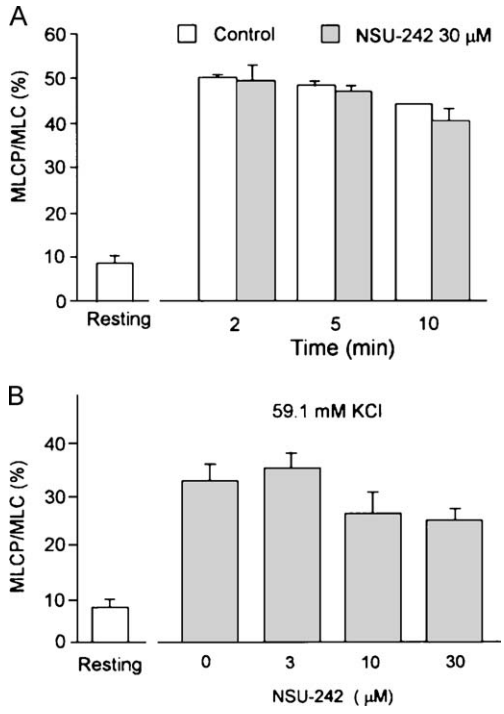

Fig. 6. Effects of NSU-242 on myosin light chain (MLC) phosphorylation in the absence or presence of high K^+. (A) Porcine tracheal smooth muscle strips were treated with 30 μM NSU-242 for 180 min before adding high K^+ (59.1 mM) for 2, 5, or 10 min. (B) Muscle strips were treated with 3–30 μM NSU-242 for 180 min before adding high K^+ for 10 min. Each column represents the mean phosphorylated MLC/total MLC ratio ($n=4$). S.E.M. is shown by the vertical bars.

In isolated aortic strips from rats, norepinephrine (0.1 μM) induced contractions which gradually and steadily decreased for over 180 min. NSU-242 (1–30 μM), added after the carbachol-induced contractions had reached a steady state, inhibited these responses (Fig. 4A). The rate and magnitude of the inhibitory effect of NSU-242 (0.1–30 μM) were concentration-dependent. Maximum inhibition occurred at a concentration of 30 μM after approximately 180 min incubation. The IC_{50} value of NSU-242 was 1.53 ± 0.23 μM. High K^+ (72.7 mM) also induced contractions which gradually and steadily decreased for a minimum of 180 min. NSU-242 (1–30 μM), added after the norepinephrine-induced contractions had reached a steady state, inhibited these responses (Fig. 4B). The rate and magnitude of the inhibitory effect of NSU-242 (1–30 μM) were concentration-dependent. Maximum inhibition occurred at a concentration of 30 μM after approximately 180 min incubation. The IC_{50} value of NSU-242 was 4.8 ± 0.21 μM.

3.3. MLC phosphorylation

Next, we examined the effect of NSU-242 on MLC phosphorylation induced by carbachol and high K^+. Application of carbachol (1 μM) increased MLC phosphorylation from a resting level of 8.7 ± 3.2% ($n=13$) to 43.3 ± 1.8%, 43.4 ± 2.3%, and 42.5 ± 0.87% ($n=4$) at 1, 3, and 10 min, respectively. NSU-242 (30 μM), added 180 min before stimulation, inhibited the carbachol-induced MLC phosphorylation by only 8% at 1 min, and had no effect on the MLC phosphorylation at 3 and 10 min (Fig. 5A). Fig. 5B shows the effect of various concentrations of NSU-242 on carbachol-induced MLC phosphorylation at 10 min. NSU-242 (3–30 μM) had no effect on these responses.

Application of high K^+ (59.1 mM) also increased MLC phosphorylation from a resting level of 8.7 ± 3.2% ($n=13$) to 49.8 ± 1.1%, 48.1 ± 1.52%, and 44.0 ± 0.33% ($n=4$) at 2, 5, and 10 min, respectively. NSU-242 (30 μM), added 180 min before stimulation, had no effect on the MLC phosphorylation at 2, 5, or 10 min (Fig. 6A). Fig. 6B shows the effects of various concentrations of NSU-242 on high K^+-induced MLP phosphorylation at 10 min. NSU-242 (3–30 μM) again had no effect on these responses.

3.4. cAMP and cGMP contents

We also investigated the effect of NSU-242 on cAMP and cGMP contents. Forskolin (FK, 10 μM) increased the cAMP content by 13.5-fold. On the other hand, addition of 30 μM NSU-242 did not change the resting level of cAMP content (Fig. 7A). Addition of sodium nitroprus-

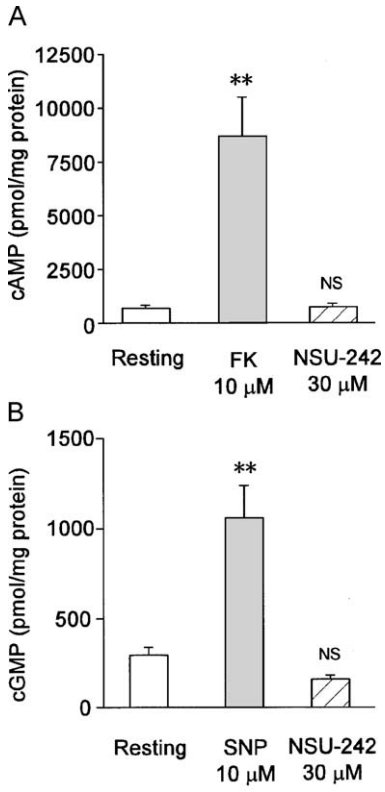

Fig. 7. Effect of NSU-242, forskolin (10 μM, FK), and sodium nitroprusside (10 μM, SNP) on cAMP and cGMP contents of porcine trachea. Each column represents the mean of 4–5 experiments, and S.E.M is shown by vertical bar. ** Significantly different from the controls at $P<0.01$. NS; not significantly different.

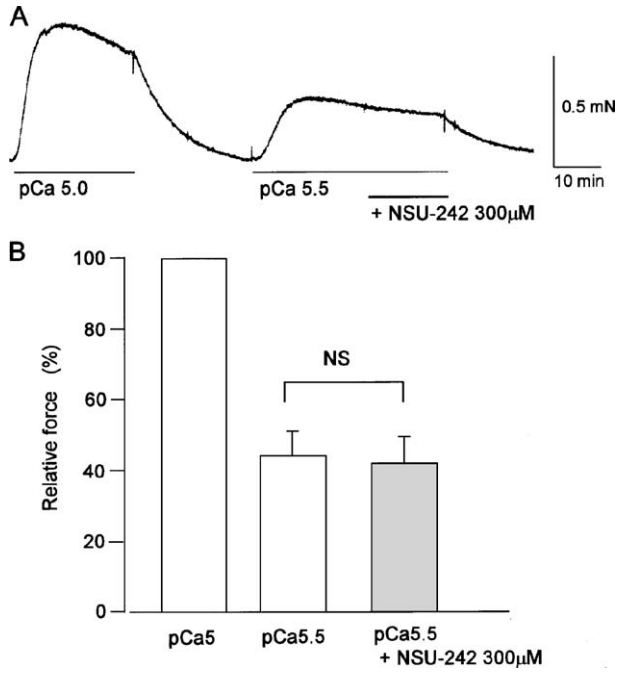

Fig. 8. Effects of NSU-242 on contraction of the permeabilized porcine trachea. After the contraction evoked by 3 μM Ca^{2+}, 300 μM NSU-242 was added (A). Panel (B) summarizes the results ($n=4$ each). NS; not significantly different.

side (SNP, 10 μM) induced an increase in the resting level of cGMP content. On the other hand, addition of NSU-242 (30 μM) did not change the resting level of cGMP (Fig. 7B).

3.5. Permeabilized smooth muscle

To determine whether NSU-242 directly inhibits the contractile elements, we further examined its effect on porcine tracheal smooth muscle permeabilized with Triton X-100. In the permeabilized trachea, Ca^{2+} (10 μM followed by 3 μM) induced sustained contractions. Addition of NSU-242 (300 μM) had no effect on the Ca^{2+}-induced contraction (Fig. 8A and B).

3.6. Actin organization

A7r5 cells have a meshwork of contractile actin–myosin filaments (actin stress fibers), as shown in Fig. 9A. After treating A7r5 cells with NSU-242 (10 μM) for 30 min, the meshwork of actin filaments was decreased and clusters of actin fibers appeared in the smooth muscle myoplasm (Fig. 9B). In the presence of 100 μM NSU-242, actin fiber bundle became thin and the actin components concentrated in the nucleus (Fig. 9C). On the other hand, cytochalasin D (3 μM), a known actin-depolimerizing agent, almost completely disrupted the actin fibers, as shown in Fig. 9D.

3.7. Actin polymerization

We further examined whether NSU-242 directly inhibits the actin polymerization. In the analysis of pyrenyl-actin fluorescence, addition of 1 mM $MgCl_2$ polymerized 5 μM G-actin. When NSU-242 (30 and 100 μM) was added at 30

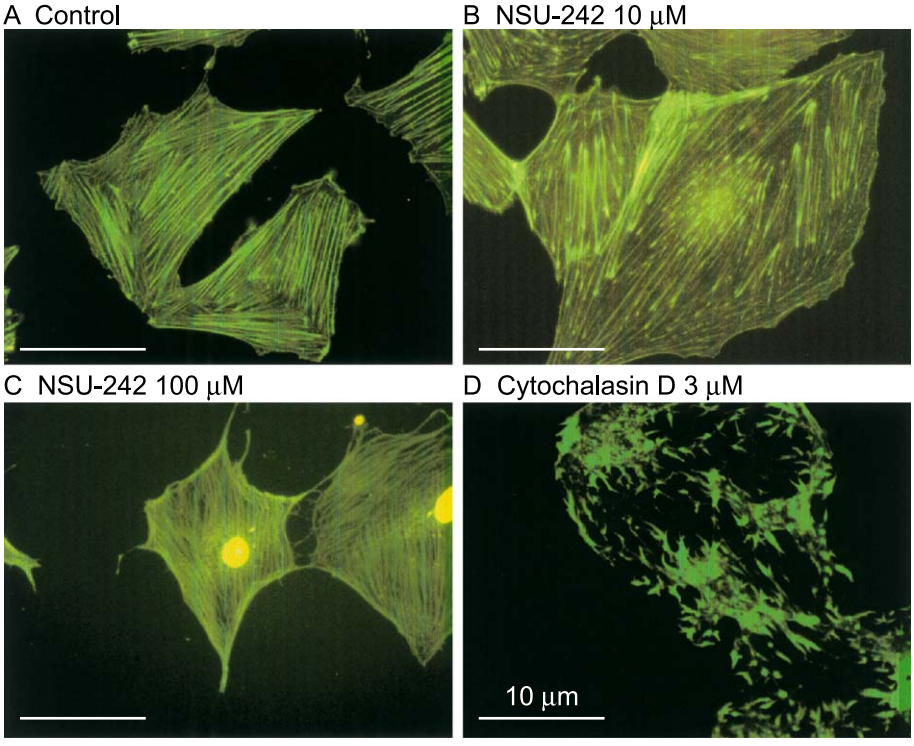

Fig. 9. Effect of NSU-242 and cytochalasin D on actin filaments in A7r5 cells. Cells were treated without (A) or with NSU-242 (10 μM, B; 100 μM C) and cytochalasin D (1 μM) (D) for 30 min. Actin fibers were stained with FITC–phalloidin.

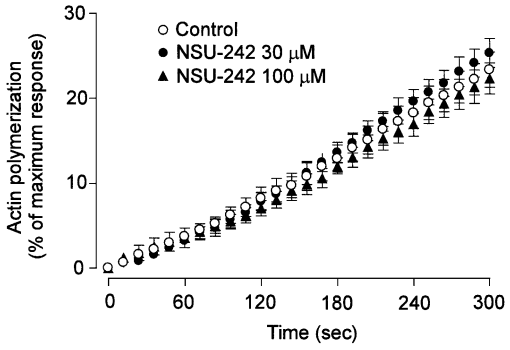

Fig. 10. Effect of NSU-242 on actin polymerization. The degree of actin polymerization was monitored by measuring the fluorescent intensity of pyrenyl-actin. The reaction medium contained 5 μM actin in the buffer with or without NSU-242 (30 and 100 μM). The medium was preincubated for 30 min without $MgCl_2$, and then the reaction was initiated by adding 1 mM $MgCl_2$.

min before the addition of $MgCl_2$, the actin polymerization induced by addition of $MgCl_2$ was not changed (Fig. 10). In a previous study using this assay system, cytochalasin D was shown to inhibit the actin polymerization (Saito et al., 1994).

4. Discussion

Bronchial asthma is an atopic disease characterized by bronchoconstriction, airway hyperreactivity, and an influx of inflammatory cells into the airways. In this study, we first found that NSU-242 inhibited antigen-induced immediate asthmatic response in actively sensitized guinea pigs (Fig. 1). Thus, we next examined whether this compound would affect the tracheal smooth muscle contraction in vitro. In the isolated porcine trachea, NSU-242 non-selectively inhibited the contractions elicited by carbachol and high K^+, and the inhibitory effect of NSU-242 was not changed by changing the concentration of stimulants (Figs. 2 and 3). In the rat aorta, NSU-242 similarly inhibited the contractions induced by norepinephrine and high K^+ (Fig. 4). In addition, the relaxation of smooth muscle contraction by NSU-242 was associated with neither an increase in cAMP nor an increase in cGMP (Fig. 7). These results suggest that NSU-242 may inhibit smooth muscle contraction via a mechanism that is responsible for both membrane depolarization (high K^+) and stimulation with receptor agonists. However, NSU-242 did not inhibit the Ca^{2+}-induced contraction in permeabilized muscle (Fig. 8). From these results, it is speculated that NSU-242 may inhibit smooth muscle contraction without any direct action on the contractile apparatus.

Smooth muscle contraction is believed to be activated by MLC phosphorylation through the Ca^{2+}/calmodulin/MLC kinase system. Unexpectedly, NSU-242 did not decrease the Ca^{2+}-dependent MLC phosphorylation in the muscles activated by high K^+ and carbachol (Figs. 5 and 6). As for the possible mechanism for the dissociation between MLC phosphorylation and force production, NSU-242 may inhibit the mechanical force by altering the cytoskeletal components of the force transmission system (Saito et al., 1996). As expected, NSU-242 changed the configuration of actin stress fibers in cultured vascular smooth muscle cells (Fig. 9). Distinct from the known actin depolymerizer, cytochalasin D, NSU-242 failed to directly inhibit actin polymerization in vitro (Fig. 10), and its effects on the actin stress fibers were mild. We have recently reported that Y27632, an inhibitor of Rho-dependent coiled coil serine/threonine kinase (ROCK), inhibited smooth muscle contraction by modifying the actin disassembly (Sakamoto et al., 2003). However, as distinct from NSU-242, Y27632 inhibits smooth muscle contraction by decreasing MLC phosphorylation. Because NSU-242 had no effect on contraction in the membrane-denuded skinned smooth muscle fiber, its action may be mediated by interaction with some membrane-associated signal transduction system connected to the actin assembly. Further study is needed to clarify this point. In addition, because actin dynamics play an important role in the immune cells (May and Machesky, 2001; Oka et al., 2002) that might participate in the in vivo responses, the effect of NSU-242 on this pathway should be considered in future studies.

It would also be of interest to measure whether NSU-242 alters the intracellular Ca^{2+} level. However, we were not able to measure the Ca^{2+} signals in smooth muscle cells because NSU-242 interfered with the fluorescence property of fura-2.

In summary, NSU-242 is a unique smooth muscle relaxant that inhibits smooth muscle contractions without any effect on the Ca^{2+}-dependent MLC phosphorylation. Although the precise mechanism is not yet known, NSU-242 may uncouple the force generation from the activated actomyosin interaction, possibly by modifying actin assembly in the cells. Although NSU-242 inhibits antigen-induced immediate asthmatic response in actively sensitized guinea pigs, it does not change systemic blood pressure in our previous bronchoconstriction study (unpublished observation). Thus, this novel compound is expected to be an effective therapeutic agent for clinical treatment of bronchial asthma.

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References

Kwon, S.C., Ozaki, H., Karaki, H., 2000. NO donor sodium nitroprusside inhibits excitation–contraction coupling in guinea pig taenia coli. Am. J. Physiol. 279, G1235–G1241.

May, R.C., Machesky, L.M., 2001. Phagocytosis and the actin cytoskeleton. J. Cell Sci. 114, 1061–1077.

Oka, T., Sato, K., Hori, M., Ozaki, H., Karaki, H., 2002. FcεRI cross-linking-induced actin assembly mediates calcium signaling in RBL-2H3 mast cells. Br. J. Pharmacol. 136, 837–846.

Saito, S., Watabe, S., Ozaki, H., Fusetani, N., Karaki, H., 1994. Mycalolide B, a novel actin depolymerizing agent. J. Biol. Chem. 269, 29710–29714.

Saito, S.Y., Hori, M., Ozaki, H., Karaki, H., 1996. Cytochalasin D inhibits smooth muscle contraction by directly inhibiting contractile apparatus. J. Smooth Muscle Res. 32, 51–60.

Sakamoto, K., Hori, M., Izumi, M., Oka, T., Kohama, K., Ozaki, H., Karaki, H., 2003. Inhibition of high K^+-induced contraction by a ROCKs inhibitor Y-27632 in vascular smooth muscle; possible involvement of ROCKs in a signal transduction pathway. J. Pharmacol. Sci. 92, 56–69.

Sparrow, M.P., Mrwa, U., Hofmann, F., Ruegg, J.C., 1981. Calmodulin is essential for smooth muscle contraction. FEBS Lett. 125, 141–145.

Spudich, J.A., Watt, S., 1971. The regulation of rabbit skeletal muscle contraction: I. Biochemical studies of the interaction of the tropomyosin–troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. 246, 4866–4871.

Wendel, H., Dancker, P., 1986. Kinetics of actin depolymerization: influence of ions, temperature, age of F-actin, cytochalasin B and phalloidin. Biochim. Biophys. Acta 873, 387–396.

Word, R.A., Casey, M.L., Kamm, K.E., Stull, J.T., 1991. Effects of cGMP on $[Ca^{2+}]_i$, myosin light chain phosphorylation, and contraction in human myometrium. Am. J. Physiol. 260, C861–C886.