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A role for Rho kinase in vascular contraction evoked by sodium fluoride

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Abstract

Agonist and depolarization-induced vascular smooth muscle contractions involve the activation of Rho-kinase pathway. However, there are no reports addressing the question whether this pathway is involved in NaF-induced vascular contractions. We hypothesized that Rho-kinase plays a role in vascular contraction evoked by sodium fluoride in rat aortae. In both physiological salt solution and calcium-free solution with 2 mM EGTA, cumulative addition of NaF increased vascular tension in concentration-dependent manners. Effects of Rho-kinase inhibitor (Y27632) on phosphorylation of myosin light chain (MLC_{20}) and myosin targeting subunit ($MYPT1_{Thr696}$) of myosin light chain phosphatase as well as NaF-induced contractions were determined using isolated tissue and the Western blot experiments. Y27632 inhibited NaF-induced contractions in a concentration-dependent manner. NaF increased phosphorylation of MLC_{20} and $MYPT1_{Thr696}$, which were also inhibited by Y27632. However, MLCK inhibitor (ML-7) or PKC inhibitor (Ro31-8220) did not inhibit the NaF-induced contraction. These results indicate that activation of Rho-kinase and the subsequent phosphorylation of $MYPT1_{Thr696}$ play important roles in NaF-induced contraction of rat aortae.

Keywords: Fluoride; Contraction; Rho kinase; Y27632

It is generally accepted that the initiation of smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in MLC_{20} phosphorylation [1]. However, other pathways have now been described that may regulate the contractility of smooth muscle by regulating the phosphorylation of MLC_{20} independently of a rise in intracellular Ca^{2+} [2–4].

The phosphorylation of MLC_{20} promotes the interaction of actin and myosin II and the contraction of smooth muscle. The degree of MLC_{20} phosphorylation or contraction does not always parallel the Ca^{2+} concentration. The extent of MLC_{20} phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the Ca^{2+} concentration, a finding explained by so-called Ca^{2+} sensitization [1]. Thus, an additional mechanism of regulation that modulates the levels of MLC_{20} phosphorylation and degree of contraction has been proposed. Subsequent studies have revealed that inhibition of MLC phosphatase is a major pathway in Ca^{2+} sensitization [5].

These pathways are generally stimulated by contractile agonists that activate heterotrimeric G protein-coupled receptors, probably via $G_{12/13}$ stimulation of Rho GEFs [6–8]. Activation of Rho A leads to subsequent activation of a recently isolated downstream target of Rho, a p160 Rho-associated coiled-coil-containing protein kinase (Rho-kinase) [9,10].

Pharmacological manipulation of Rho/Rho-kinase pathway in isolated tissues can be achieved by using agents such as Clostridium botulinum toxin C3, which inhibits Rho, and fasudil or Y27632 that inhibits its effector Rho-kinase [11]. It has been demonstrated that selective Rho-kinase inhibitors fasudil and Y27632 relax pre-contracted smooth muscles and inhibit agonist-induced contractions [12,13].

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In various smooth muscles, F^- has been shown to induce contraction, which may be due to mobilization of cellular Ca^{2+} and/or enhancement of Ca^{2+} sensitivity. Fluoride has been known to induce contraction in blood vessel preparations and is a potent stimulator of G_s, G_i, G_q, and transducin [14–17].

Because of its multiple effects on intracellular signaling, we used NaF as a valuable tool for exploring signaling pathways involved in smooth muscle contraction. It is possible that NaF-induced contractions involve the participation of the RhoA/Rho-kinase pathway. However, there has been no report in the literature as to whether this pathway is activated in NaF-induced vascular smooth muscle contraction. Thus, the aim of the present study was to elucidate the possible role of Rho-kinase in NaF-induced contractions of isolated rat aortae.

Materials and methods

Organ bath study. The investigation is in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH 1996). Male Sprague–Dawley rats, weighing 320–350 g, were used. With animals under anesthesia (sodium pentobarbital 50 mg kg^{-1} i.p.), the thoracic aorta was immediately excised and immersed in an ice-cold, modified Krebs' solution composed of (in mM) NaCl, 115.0; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and dextrose, 10.0. To make a Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mM EGTA was added. The aorta was cleaned of all adherent connective tissue on wet filter paper, soaked in the Krebs'-bicarbonate solution, and cut into four ring segments (4 mm in length) as described by Kim's method [18]. Some of the rings were denuded of endothelium by gently rubbing the internal surface with a forceps edge. Two stainless-steel triangles were inserted through each vessel ring. Care was taken to avoid rubbing the endothelial surface of the vessels that had intact endothelium. Each aortic ring was suspended in a water-jacketed organ bath (20 ml) maintained at 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2. One triangle was anchored to a stationary support, and the other was connected to an isometric force transducer (Grass FT03C, Quincy, MA, USA). The rings were stretched passively by imposing the optimal resting tension, 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 90 min before the experiment involving the contractile response to 50 mM KCl addition. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia). To study the dose-dependent effects and to determine the maximal concentration of NaF, the drug was added cumulatively. A maximal concentration of NaF (8 mM) was selected for further experiments. The time course of responses to NaF (8 mM) was monitored for 1 h.

The effect of Y27632 (10^{-8}–$10^{-5.5}$ M) was determined by cumulative addition of it after NaF (8 mM)-, phenylephrine (1 μM)-, KCl (50 mM)-, or PDBu (1 μM)-induced contractions reached plateaus in normal Krebs' solution or Ca^{2+}-free Krebs' solution. To determine the effect of myosin light chain kinase inhibitor ML-7 on NaF-induced contraction, NaF was added cumulatively to elicit tension in the presence or absence of 10 μM ML-7 for 30 min. To determine the effect of protein kinase C inhibitor Ro31-8220 on NaF-induced contraction, Ro31-8220 was added after NaF (8 mM)- or PDBu (1 μM)-induced contractions reached plateaus.

Western blot. After a functional study, muscle strips were quickly immersed in acetone containing 10 mM dithiothreitol (DTT) pre-cooled to −80 °C, air-dried, and kept at −20 °C. Previously, stored samples were homogenized in a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors leupeptin (10 μg/ml), trypsin inhibitor (10 μg/ml), aprotinin (2 μg/ml), and phenylmethylsulfonyl fluoride (PMSF; 100 μg/ml). Protein-matched samples (Bradford assay) were electrophoresed (SDS–PAGE), transferred to nitrocellulose membranes, and subjected to an immunoblot with an pMYPT1 antibody (1:1000, Upstate Biotechnology, Lake Placid, NY, USA) that detects phosphorylated MYPT1. Anti-rabbit IgG (goat), conjugated with horseradish peroxidase, was used as a secondary antibody (1:2000, Sigma, St. Louis, MO, USA). The bands containing pMYPT1 were detected with enhanced chemiluminescence (ECL) visualized on films. The nitrocellulose membranes were stripped off the pMYPT1 antibody and reblotted with regular MYPT1 antibody (1:1000, BD Biosciences Pharmingen, San Diego, CA, USA).

MLC_{20} phosphorylation. Muscle strips were quick frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) pre-cooled to −80 °C. Muscles were washed four times with acetone containing 5 mM DTT for 15 min each to remove TCA and were soaked for 1 h with frequent vortex in 60 μl of sample buffer containing 20 mM Tris base/23 mM glycine (pH 8.6), 8.0 M urea, 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue. Polyacrylamide gels containing 40% glycerol were pre-electrophoresed for 30 min at 200 V. The running buffer consists of 20 mM Tris base/23 mM glycine (pH 8.6), 2.3 M thioglycolate, and 2.3 mM DTT. The urea-extracted samples (15 μl) were electrophoresed at 300 V for 6 h, transferred to nitrocellulose membranes, and subjected to immunoblotting with a specific myosin light chain antibody (1:2000, Sigma, St. Louis, MO, USA). Anti-mouse IgM (goat), conjugated with horseradish peroxidase, was used as a secondary antibody (1:2000, Stressgen, Victoria, BC Columbia, Canada). The bands containing myosin light chains were visualized with enhanced chemiluminescence (ECL) on films and then analyzed by NIH image as described [18].

Drugs. The drugs and chemicals were obtained from the following sources: NaF, KCl, PDBu, phenylephrine, ML-7, and Ro31-8220 from Sigma Chemicals (St. Louis, MO, USA); Y27632 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis. The data were expressed as means ± SE of the mean and were analyzed by Student's *t* test. *P* values < 0.05 were regarded as statistically significant.

Result

NaF-induced contraction

In both normal Krebs' solution and Ca^{2+}-free Krebs' solution, cumulative addition of NaF increased vascular tension in concentration-dependent manners (Fig. 1A). The maximal effects of E_{max} and ED_{50} for NaF were 156.4 ± 8.2% and 4.7 ± 0.1 mM in normal Krebs' solution and 36.0 ± 3.5% and 5.5 ± 0.4 mM in Ca^{2+}-free Krebs' solution. NaF-induced contractions reached plateaus within 20–30 min in normal Krebs' solution but reached plateaus more slowly in Ca^{2+}-free condition.

Effects of Rho-kinase inhibitor Y27632 on NaF-induced contractions

To determine whether Rho-kinase play a role in NaF-induced contractions in rat aorta, the effects of Rho-kinase inhibitor Y27632 were examined. In normal Krebs' solution, Y27632 dose-dependently decreased vascular tension induced by 50 mM KCl, 1.0 μM phenylephrine, or 8 mM NaF, but not by 1.0 μM phorbol dibutyrate (Fig. 2A). Y27632 also decreased vascular tension induced by 8 mM

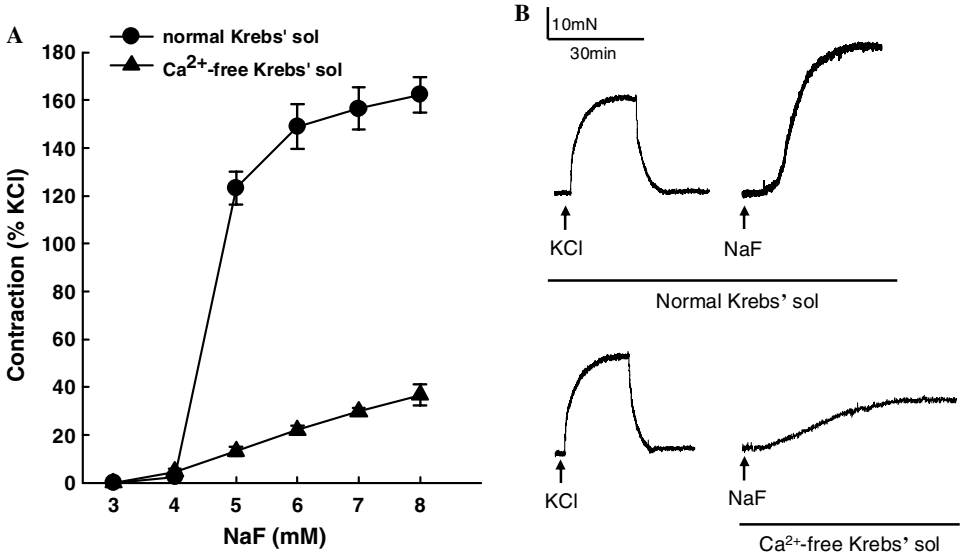

Fig. 1. Vascular smooth muscle contraction by NaF. (A) Cumulative concentration-responses for the contractile response to NaF in the normal Krebs' solution and Ca^{2+}-free Krebs' solution. Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as means of 5 experiments with vertical bars showing SEM. (B) Representative tracings of isometric tension development of NaF (8 mM) in the normal Krebs' solution and Ca^{2+}-free Krebs' solution.

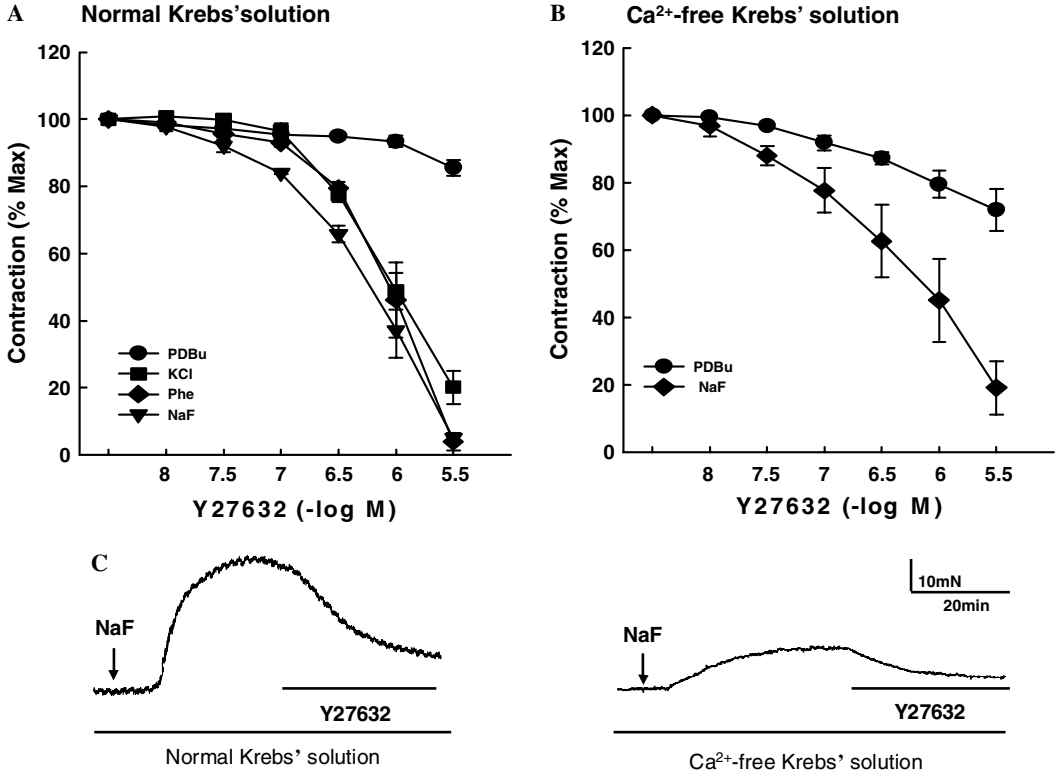

Fig. 2. Effect of Rho-kinase inhibitor Y27632 on various contractions in normal Krebs' solution and Ca^{2+}-free Krebs' solution. (A) Y27632 was added cumulatively when NaF (8 mM)-, phenylephrine (1 μM)-, KCl (50 mM)-, or PDBu (1 μM)-induced contractions reached plateaus in normal Krebs' solution. (B) Y27632 was added cumulatively when NaF (8 mM)- or PDBu (1 μM)-induced contractions reached plateaus in Ca^{2+}-free Krebs' solution. Developed tension is expressed as a percentage of the maximum contraction. Data are expressed as means of five experiments with vertical bars showing SEM. (C) Representative tracings of isometric tension change after challenge with NaF (8 mM) followed by Y27632 (1 μM) in the normal Krebs' solution and Ca^{2+}-free Krebs' solution.

NaF, but not by 1.0 μM phorbol dibutyrate in Ca^{2+}-free Krebs' solution (Fig. 2B).

Effect of NaF on MLC_{20} phosphorylation of rat aortae

Phosphorylation of the regulatory myosin light chains (MLC_{20} phosphorylation) is a key event in the activation of both Ca^{2+}-induced contraction and Ca^{2+} sensitization in smooth muscle [1]. We next examined the effect of NaF (8 mM) on MLC_{20} phosphorylation. NaF (8 mM) caused a time-dependent increase in MLC_{20} phosphorylation, with a maximum of an approximately fivefold increase compared with control values (Fig. 3A). To determine whether Rho-kinase plays a role in NaF-induced MLC_{20} phosphorylation in rat aorta, the effect of Rho-kinase inhibitor Y27632 was examined. Y27632 (1 μM) inhibited NaF-induced MLC_{20} phosphorylation (Fig. 3B).

Phosphorylation of myosin targeting subunit of MLC phosphatase ($MYPT1_{Thr696}$)

In smooth muscle, the GTP-RhoA activates Rho-kinases, which in turn phosphorylate $MYPT1_{Thr696}$ to inactivate MLC phosphatase activity. Therefore, we examined whether the $MYPT1_{Thr696}$ phosphorylation occurred during NaF-induced contraction in rat aortae. Fig. 4A shows phosphorylation of $MYPT1_{Thr696}$ by NaF (8 mM) in time-dependent manner. The phosphorylation of $MYPT1_{Thr696}$ in the presence of NaF was inhibited by Y27632 (Fig. 4B).

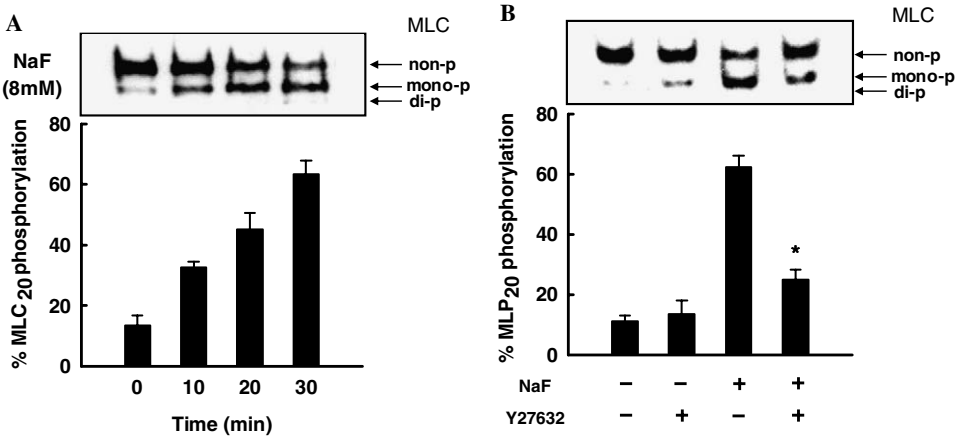

Fig. 3. Effect of NaF on MLC_{20} phosphorylation of rat aorta. (A) MLC_{20} phosphorylation was measured in 0 (control), 10, 20, and 30 min after NaF (8 mM) treatment. (B) Y27632 (1 μM) was applied when NaF (8 mM)-induced contraction had reached plateaus. Graphs show mean of MLC_{20} phosphorylation expressed as a percentage to the total MLC_{20}. Data are expressed as means of five experiments with vertical bars showing SEM. $^*P < 0.01$ as compared to the stimulation with NaF only.

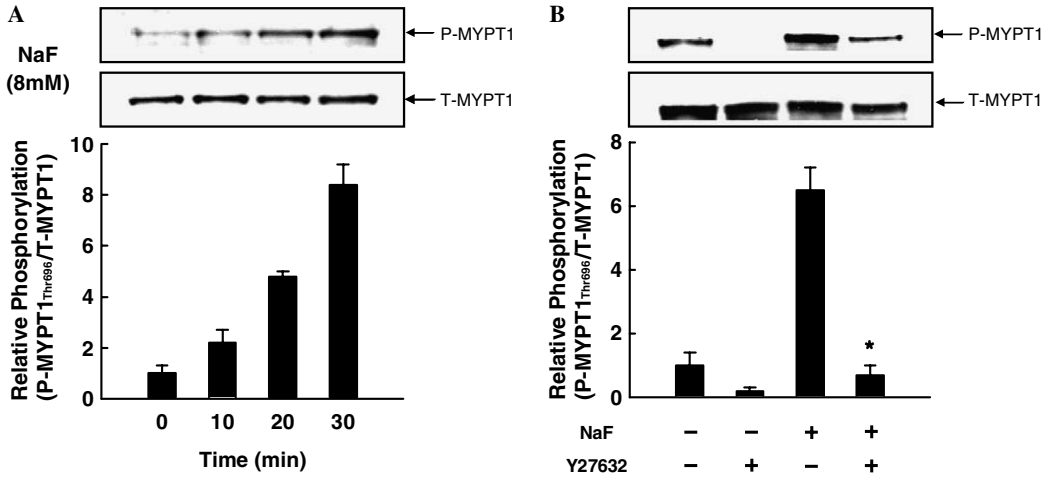

Fig. 4. Effect of NaF on $MYPT1_{Thr696}$ phosphorylation of rat aorta. (A) $MYPT1_{Thr696}$ phosphorylation was measured in 0 (control), 10, 20, and 30 min after NaF (8 mM) treatment. (B) Y27632 (1 μM) was applied when NaF (8 mM)-induced contraction had reached plateau. Top and middle representative Western blots were probed with anti-p$MYPT1_{Thr696}$ and total-MYPT1 antibody, respectively. Bottom, Densitometry shows that mean density of phosphorylated $MYPT1_{Thr696}$ (top) versus the total MYPT1 density (middle) for the control was expressed as 1 arbitrary unit. Data are expressed as means of five experiments with vertical bars showing SEM. $^*P < 0.01$ as compared to the stimulation with NaF only.

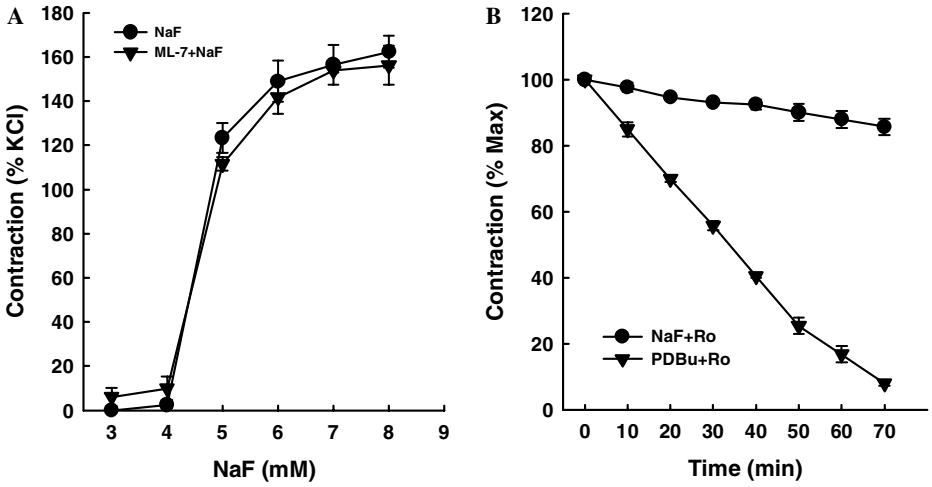

Fig. 5. Effect of myosin light chain kinase inhibitor ML-7 and protein Kinase C inhibitor Ro31-8220 on NaF-induced contraction. (A) NaF was added cumulatively to elicit tension in the presence or absence of 10 μM ML-7 for 30 min. Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. (B) Ro31-8220 was added when NaF (8 mM)- or PDBu (1 μM)-induced contractions reached plateaus. Tension is expressed as a percentage of the maximum contraction to NaF (8 mM) or PDBu (1 μM). Data are expressed as means of five experiments with vertical bars showing SEM.

Sensitivity to a MLCK inhibitor and a PKC inhibitor of the contraction induced by NaF or PDBu

To examine the involvement of MLCK and PKC, we compared the effects of each inhibitor, ML-7 or Ro31-8220, on NaF-induced contraction. After a pre-incubation period of 30 min with ML-7 (10 μM), NaF was applied cumulatively in the presence or absence of ML-7. ML-7 did not affect the NaF-induced contraction (Fig. 5A). During the sustained phase of NaF (8 mM)- or PDBu (1 μM)-induced contractions, addition of Ro31-8220 had little effect on the time course of the change in tension (Fig. 5B).

Discussion

This study demonstrated that NaF increased vascular tension in concentration-dependent manners in both normal Krebs' solution and Ca^{2+}-free Krebs' solution, and that Rho-kinase, but not protein kinase C, plays an important role in NaF-induced vascular contraction.

Two mechanisms are postulated for the development of contraction and MLC_{20} phosphorylation in smooth muscle. The first mechanism is that elevation in $[Ca^{2+}]_i$ activates a Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK), which in turn catalyzes phosphorylation of MLC_{20}, leading to cross bridge cycling and contraction [19]. A decrease in the concentration of intracellular Ca^{2+} inactivates MLCK and allows MLC phosphatase to dephosphorylate MLC_{20}, resulting in muscle relaxation. A second mechanism of VSM contraction involves Rho-kinase [20], whose activity is dependent on activation of the small GTP-binding protein RhoA. The RhoA/Rho-kinase pathway plays a key role in the Ca^{2+} sensitization of smooth muscle contraction, which is mainly induced by MLC phosphatase inhibition [21,22].

The mechanism by which the Rho-kinase inhibits MLC phosphatase is via phosphorylation of the MYPT1 subunit at Thr696 [23]. Rho-kinase phosphorylates the regulatory subunit of the smooth muscle myosin light chain phosphatase (MLCP) [23] either directly or via an additional myosin phosphatase-associated kinase [24]. MLCP is a trimeric protein consisting of a catalytic subunit (protein phosphatase type 1, PP1c), a myosin-binding subunit (MBS, also known as MLCP targeting subunit, MYPT), and a 20 kDa subunit of unknown function [25]. Recently, several isoforms of MYPT have been described: MYPT1, MYPT2, MBS85, MYPT3, and TIMAP [26]. Activation of G-proteins has been shown to result in MYPT1 phosphorylation, and Rho-kinase has been demonstrated to phosphorylate MYPT1 at Thr695 in the avian [27], or Thr697 in the mammalian sequence [28], which results in an inhibition of MLC phosphatase activity and a Ca^{2+}-independent increase in MLC_{20} phosphorylation and force [29].

Several compounds, such as Y27632 and fasudil (HA-1077), have been developed and reported to inhibit the Rho-kinase specifically and competitively with ATP. Y27632 decreased vascular tension induced by NaF as well as KCl or phenylephrine, both of which are known to activate Rho-kinase [30], whereas Y27632 hardly decreased vascular tension induced by phorbol dibutyrate, which is known to activate protein kinase C, but not Rho-kinase (Fig. 2). These results suggest that vascular tension induced by NaF is, at least in part, mediated by Rho-kinase. It took about 20–30 min for vascular tension induced by NaF to reach a plateau. The phosphorylation of MLC_{20} and $MYPT1_{Thr696}$ also slowly increased for 30 min, which were inhibited by Y27632 (Figs. 3 and 4). These results indicate that $MYPT1_{Thr696}$ is phosphorylated through activation of Rho-kinase during NaF-induced contractions in rat aortae.

Pre-treatment of MLCK inhibitor ML-7 did not affect NaF-induced contractions (Fig. 5A) while the same concentration of ML-7 decreased KCl-induced contractions (data not shown). Therefore, it seems that MLCK does not play a major role in NaF-induced contractions as it does in KCl-induced contractions. The PKC also participates in the inhibition of MLCP through mechanisms involving CPI-17 [31]. To test this possibility, the PKC inhibitor Ro31-8220 was applied on NaF- or PDBu-induced contractions. Ro31-8220 decreased the tension induced by PDBu, but not NaF (Fig. 5B). If PKC had played a significant role in the NaF-induced contraction, the contraction should have been sensitive to the PKC inhibitor Ro31-8220 in rat aortae. In the present study, Ro31-8220 hardly affected the NaF-induced contraction (Fig. 5B). These data suggest that PKC is not likely to be responsible for the NaF-induced contraction.

The mechanism by which F^- activates G-proteins has been clearly established [14–17]. It has been reported that the effect of NaF on heterotrimeric G protein is the result of formation of AlF_4^- from fluoride and trace amounts of aluminum, which can come from contamination of glassware [32,33]. The cellular mechanism by which fluoroaluminates activate G proteins is based on the structural similarity of AlF_4^- to PO_4^{3-}, enabling the former to interact with GDP situated on the α-subunit of the G proteins where it can mimic GTP [34]. NaF is also a classical Ser/Thr phosphatase inhibitor [35] and is routinely included in extraction buffers to prevent dephosphorylation of proteins on Ser and Thr residues by endogenous phosphatases. Since it has been known that Rho-kinase plays a role in hypertension, coronary artery spasm, and basilar artery vasospasm [3,36,37], NaF is a useful chemical to activate Rho-kinase pathway for research.

In conclusion, NaF increased vascular tension in concentration-dependent manners in both normal Krebs' solution and Ca^{2+}-free Krebs' solution, and that Rho-kinase, but not protein kinase C, plays an important role in NaF-induced vascular contraction. Activation of Rho-kinase and the subsequent phosphorylation of $MYPT1_{Thr696}$ play important roles in NaF-induced contraction.

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