Calcium Sensitization Induced by Sodium Fluoride in Permeabilized Rat Mesenteric Arteries

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It was hypothesized that NaF induces calcium sensitization in Ca^{2+}-controlled solution in permeabilized rat mesenteric arteries. Rat mesenteric arteries were permeabilized with β-escin and subjected to tension measurement. NaF potentiated the concentration-response curves to Ca^{2+} (decreased EC_{50} and increased E_{max}). Cumulative addition of NaF (4.0, 8.0 and 16 mM) also increased vascular tension in Ca^{2+}-controlled solution at pCa 7.0 or pCa 6.5, but not at pCa 8.0. NaF-induced vasocontraction and GTPγS-induced vasocontraction were not additive. NaF-induced vasocontraction at pCa 7.0 was inhibited by pretreatment with Rho kinase inhibitors H1152 or Y27632 but not with a MLCK inhibitor ML-7 or a PKC inhibitor Ro31-8220. NaF induces calcium sensitization in a Ca^{2+}-dependent manner in β-escin-permeabilized rat mesenteric arteries. These results suggest that NaF is an activator of the Rho kinase signaling pathway during vascular contraction.

Key Words: Calcium sensitization, Permeabilization, Mesenteric artery, Sodium fluoride, Rho kinase

INTRODUCTION

Smooth muscle contraction is activated by an increase in cytosolic calcium provided with calcium entry through voltage-sensitive calcium channels or with calcium release from the sarcoplasmic reticulum (SR). However, some agonists activating GPCRs can also lead to further contraction at constant level of intracellular calcium, so called calcium sensitization. Accumulating evidence indicates that RhoA/ Rho kinase and protein kinase C (PKC) are the major pathways for calcium sensitization, which involve inhibition of myosin phosphatase activity [1,2]. Several cardiovascular diseases such as coronary vasospasm, cerebral vasospasm, hypertension, atherogenesis and endothelial dysfunction are associated with activation of RhoA-Rho kinase signaling pathway [3].

Sodium fluoride (NaF), an acknowledged Ser/Thr phosphatase inhibitor and G protein activator, has a wide range of effects on cellular metabolism and physiology. Both G protein-dependent and -independent pathways are likely to be involved in the physiological response to fluoride [4]. Fluoride has been reported to activate the stress-response signaling cascade involving MAP kinase [5]. Addition of aluminum fluoride (AlF_4^-) induces calcium sensitization in various smooth muscles such as guinea pig vas deferens and airway smooth muscle [6,7]. However, the mechanism

by which NaF induces calcium sensitization is poorly understood. In a previous study, NaF-induced contraction was inhibited by a Rho kinase inhibitor, but not by inhibitors of MLCK or PKC, suggesting that vascular tension induced by NaF is, at least in part, mediated by Rho kinase [8,9].

β-Escin is reported to permeabilize the plasma membrane of smooth muscle without disrupting receptor function. Smooth muscle preparations treated with β-escin have been shown to respond to excitatory agonists through IP_3-mediated release of Ca^{2+} and to exert calcium sensitization [10].

This study investigated whether NaF induces calcium sensitization in Ca^{2+}-controlled solution by using β-escin-permeabilized rat mesenteric arteries, and which mechanisms are involved in calcium sensitization.

METHODS

Ethical approval

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). Nineteen male Sprague-Dawley rats, weighing 350∼400 g, were used. Animals were anesthetized by sodium pentobarbital (50 mg kg^{-1} i.p.).

ABBREVIATIONS: ANOVA, analysis of variance; CPI17, PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa; MLC20, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase-targeting subunit 1; PDBu, phorbol 12, 13-dibutyrate.

Tissue preparation

Under anesthesia, the mesentery bed was removed and placed in HEPES buffered modified Tyrode's solution composed of (in mM) NaCl, 134; KCl, 5.6; $CaCl_2$, 2.5; $MgCl_2$, 1.0; HEPES, 10 and dextrose, 10. The second or third branches of mesenteric arteries were cleaned of fat and connective tissues, and dissected into small pieces ($200 \sim 300$ μm in diameter, $4 \sim 5$ mm in length) under a microscope. Small ring segments were mounted vertically between two thin wires in organ baths (3.0 ml) filled with HEPES solution, gassed with 100% O_2 and maintained at 37°C. Muscle strips were stretched passively by imposing the optimal resting tension (which was evaluated as maximum tension developed by 70 mM K^+ solution), 0.5 g, which was maintained throughout the experiment as previously described [11]. Each preparation was equilibrated for more than 90 min before permeabilization.

Permeabilization and tension recording

The relaxing solution containing (in mM) potassium methanesulfonate, 74.1; MgATP, 4.5; EGTA, 1.0; PIPES, 30, and creatine phosphate, 10 were neutralized to pH 7.4 with KOH at 25°C as previously described [12]. The free Ca^{2+} concentration was calculated using a computer program and expressed as the negative logarithm (pCa). In the pCa solution, 10 mM EGTA was used to buffer the desired concentration of free Ca^{2+}. Ionic strength was constant at 200 mM by adjusting the concentration of potassium methanesulfonate. Tissues were permeabilized by incubating with 30 μM β-escin in the relaxing solution for 50 min at 27°C. Isometric contractions were recorded using a com-

puterized data acquisition system (PowerLab8/30, AD Instruments, Castle Hill, NSW, Australia).

Drugs

The drugs and chemicals were obtained from the following sources: NaF, H1152, β-escin, ML-7 and Ro31-8220 from Sigma Chemicals (St. Louis, MO, USA); guanosine-5'-O-(3-triphosphate) (GTPγS) from Roche (Indianapolis, IN, USA); Y27632 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); guanosine-5'-O-(2-thiodiphosphate); (GDPβS) from Enzo Life Sciences (Plymouth Meeting, PA, USA). Stock solutions of NaF, H1152, Y27632, GTPγS, GDPβS and Ro31-8220 were prepared in double distilled water; ML-7 was prepared in ethanol:water (1 : 1) mixture. All other reagents were analytical grade.

Statistical analysis

Data are expressed as mean±SEM and were analyzed by repeated measures ANOVA followed by post-hoc Dunnett test or one-way ANOVA, followed by post-hoc Dunnett test. p values of less than 0.05 were regarded as significant.

RESULTS

NaF induces calcium sensitization in permeabilized mesenteric arteries

Concentration-response curves were obtained by changing Ca^{2+}-controlled solution with free calcium of pCa $9.0 \sim$ 5.5 in the absence or presence of NaF (4.0, 8.0 or 12 mM). As shown in Fig. 1, NaF significantly increased the concen-
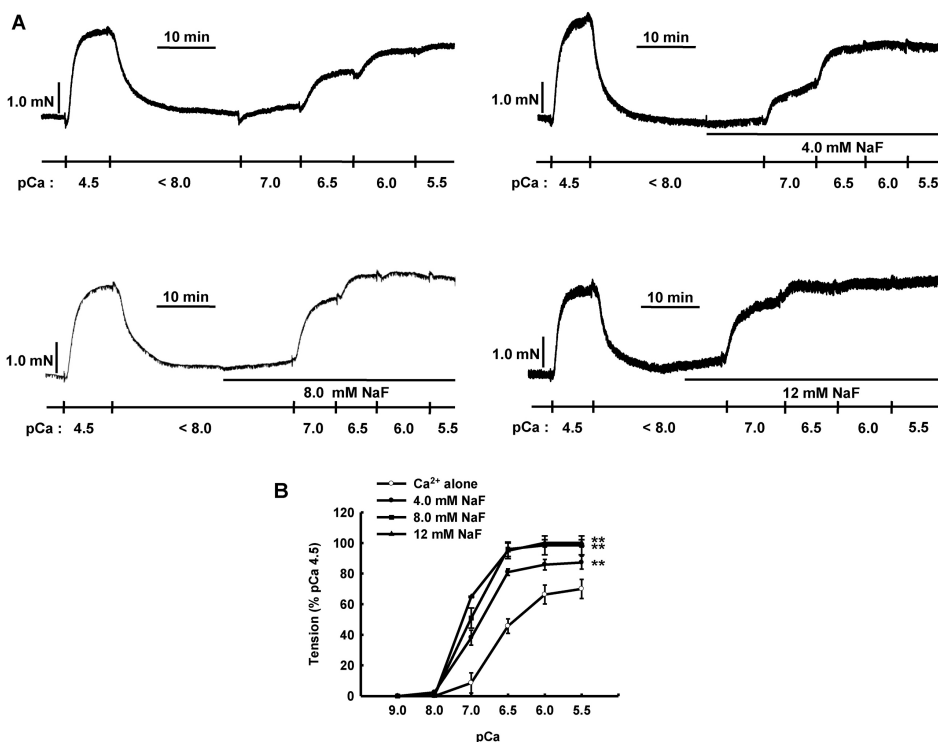

Fig. 1. NaF induces Ca^{2+} sensitization in β-escin-permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to increasing concentration of free calcium after initial high concentration of calcium (pCa 4.5) in the absence or presence of NaF (4.0, 8.0 or 12 mM). Line graphs (B) show the concentration-response curve to increasing concentration of free calcium (pCa $9.0 \sim$ 5.5) in the absence or presence of NaF (4.0, 8.0 or 12 mM) in β-escin-permeabilized mesenteric arteries. Developed tension is expressed as a percentage of the maximum tension induced by pCa 4.5. Data are expressed as means of 5 experiments with vertical bars showing SEM. **p<0.01 vs. Ca^{2+} alone (Repeated measures ANOVA followed by post-hoc Dunnett test).

tration-response curves compared to calcium-alone curve, decreasing the $[Ca^{2+}]$ required to produce half-maximal force (EC_{50}) and increasing the maximum force developed in response to pCa 5.5 (Fig. 1 and Table 1).

Tensions were measured by cumulative addition of NaF (4.0, 8.0 and 16 mM) with calcium clamped at pCa 8.0, pCa 7.0 or pCa 6.5. As show in Fig. 2, cumulative addition of NaF increased vascular tension in Ca^{2+}-controlled solution at pCa 7.0 or pCa 6.5, but not at pCa 8.0. NaF-induced contraction was reversible by washing with pCa 7.0 solutions (data not shown).

Effect of GTPγS on NaF-induced contraction in permeabilized mesenteric arteries

To examine whether GTPγS has an additive effect on NaF-induced vasocontraction, the tension elicited by 100 μM

Table 1. EC_{50} and maximal effect (E_{max}) of the concentration-response curves to Ca^{2+} in the absence or presence of NaF in β-escin-permeabilized rat mesenteric arteries

	EC_{50} (pCa)	E_{max} (% of pCa 4.5)
Ca^{2+} alone	6.6±0.03	69±4.5
4.0 mM NaF	7.0±0.01*	87±4.0*
8.0 mM NaF	7.0±0.01*	98±6.2*
12 mM NaF	7.1±0.06*	100±2.6*

E_{max} is expressed as a percentage of the maximum tension to pCa 4.5. Values are means±SEM of 5 experiments for EC_{50} and E_{max}. *$p < 0.01$ vs. Ca^{2+} alone (One-way ANOVA followed by post-hoc Dunnett test).

GTPγS in the absence or presence of NaF (4.0 or 8.0 mM) was recoded. As shown in Fig. 3, the tension induced by 4.0 or 8.0 mM NaF were not additive to those by 100 μM GTPγS alone ($p > 0.05$).

Effect of GDPβS on NaF-induced contraction in permeabilized mesenteric arteries

To examine whether GDPβS has an inhibitory effect on NaF-induced vasocontraction, we recoded the tension elicited by 8.0 mM NaF before or after treatment with 300 μM GDPβS. Treatment with GDPβS, even at a high concentration (1,000 μM) (data not shown), did not inhibit NaF- or GTPγS-induced vasocontractions (Fig. 4).

Effect of Rho kinases inhibitors, Y27632 or H1152, on NaF-induced vasocontraction in permeabilized mesenteric arteries

In order to address whether NaF-induced contraction involves RhoA/Rho kinase signaling pathway in permeabilized mesenteric arteries, the tension elicited by 8.0 mM NaF 30 min after pretreatment with Rho kinase inhibitors H1152 or Y27632 was recoded. NaF (8.0 mM) induced a sustained contraction, which reached plateaus within 20 min and maintained for at least 20 min. In the present study the same results were obtained as previous studies using intact vascular tissues [8,9] that both H1152 and Y27632 dose-dependently decreased vascular contraction in permeabilized mesenteric arteries (Fig. 5). At a high concentration of H1152 orY27632, NaF-induced contractions were almost completely inhibited.
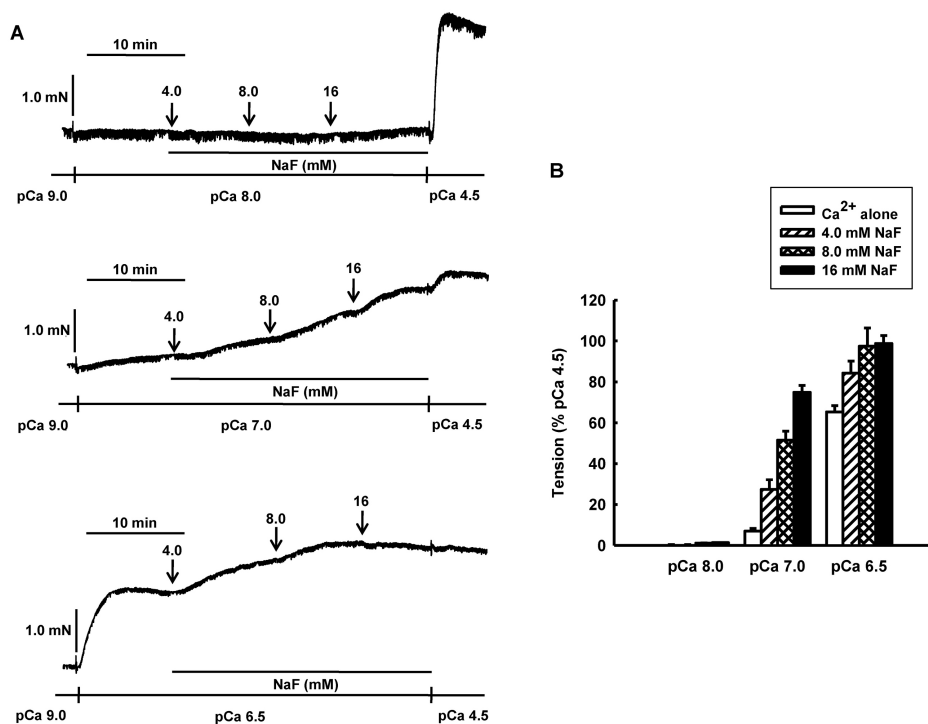

Fig. 2. NaF induces vasocontraction in Ca^{2+}-controlled solution in β-escin- permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to cumulative addition of NaF (4.0, 8.0 and 16 mM) at constant concentration of calcium of pCa 8.0, 7.0 or 6.5. Bar graphs (B) show the developed tension elicited by cumulative addition of NaF (4.0, 8.0 and 16 mM) at constant concentration of calcium of pCa 8.0, 7.0 or 6.5 in β-escin-permeabilized mesenteric arteries. When NaF (16 mM)-induced contraction reached plateau, the bathing solutions were replaced with pCa 4.5 solution to obtain maximum contraction. Developed tension is expressed as a percentage of the maximum tension to pCa 4.5. Data are expressed as means of 5 experiments with vertical bars showing SEM.
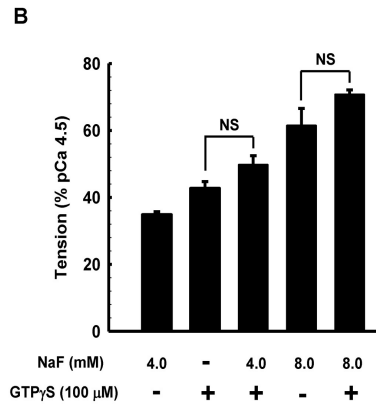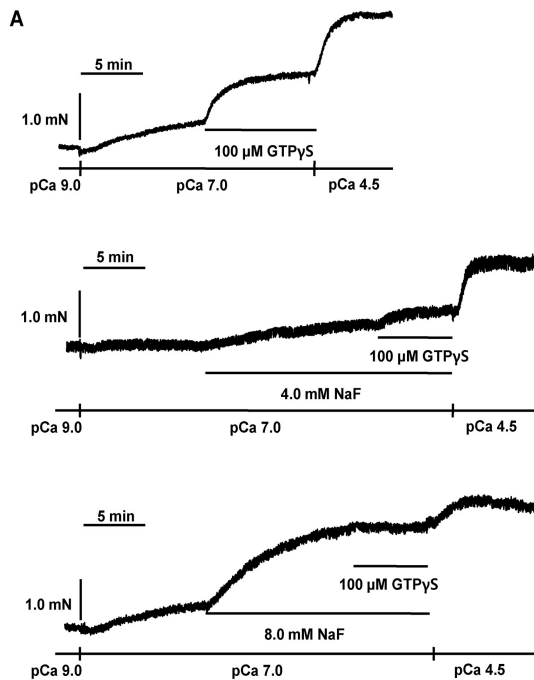
Fig. 3. Effect of GTPγS on NaF-induced contraction in β-escin-permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to addition of 100 μM GTPγS in the absence or presence of NaF (4.0 or 8.0 mM) at constant concentration of calcium of pCa 7.0. Bar graphs (B) show the developed tension elicited by addition of 100 μM GTPγS in the absence or presence of NaF (4.0 or 12 mM) at constant concentration of calcium of pCa 7.0. When GTPγS (100 μM)-induced contraction reached plateau, the bathing solutions were replaced with pCa 4.5 solutions to obtain maximum contraction. Developed tension is expressed as a percentage of the maximum tension to pCa 4.5. Data are expressed as means of 5 experiments with vertical bars showing SEM. NS, not significant (p$>$0.05) (One-way ANOVA followed by post-hoc Dunnett test).
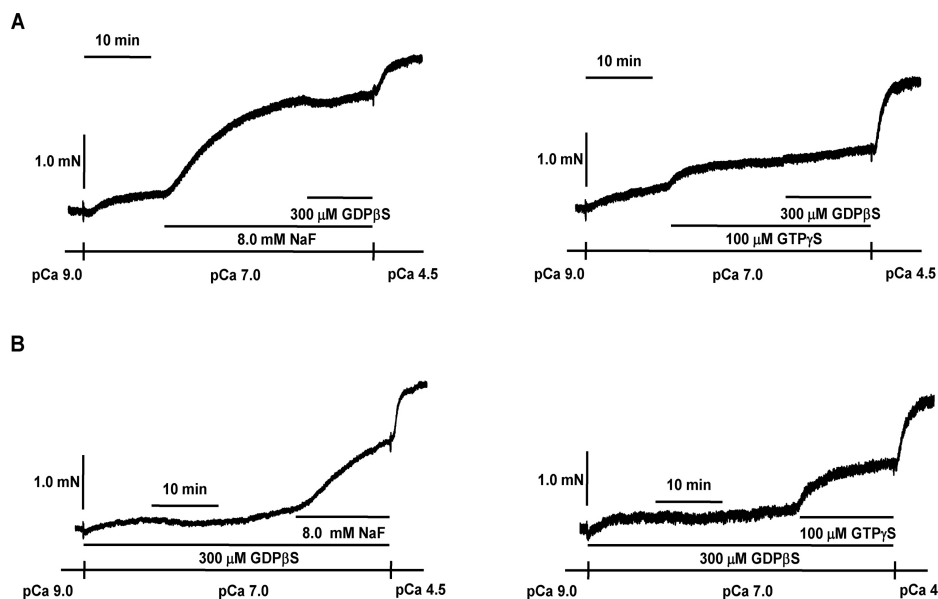

Fig. 4. Effect of GDPβS on NaF-induced contraction in β-escin-permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to addition of 8.0 mM NaF or 100 μM GTPγS following 300 μM GDPβS at constant concentration of calcium of pCa 7.0. Representative traces (B) show tension development when β-escin-permeabilized mesenteric arteries were exposed to addition of 8.0 mM NaF or 100 μM GTPγS in the absence or presence of 300 μM GDPβS at constant concentration of calcium of pCa 7.0. When GTPγS (100 μM)- or NaF (8.0 mM)-induced contraction reached plateau, the bathing solutions were replaced with pCa 4.5 solutions to obtain maximum contraction. The traces are representative of four experiments.

DISCUSSION

Effect of ML-7 or Ro31-8220 on NaF-induced vasocontraction in permeabilized mesenteric arteries

To examine the involvement of MLCK or PKC on NaF-induced vasocontraction in permeabilized mesenteric arteries, the effects of each inhibitor, ML-7 or Ro31-8220, on NaF-induced vasocontraction were compared. As shown in Fig. 6, ML-7 or Ro31-8220 did not significantly inhibit the contraction induced by NaF (p$>$0.05).

This study demonstrates that NaF induces calcium sensitization in a Ca^{2+}-dependent manner in β-escin-permeabilized rat mesenteric arteries. NaF-induced calcium sensitization involves the Rho kinase pathway.

Accumulating evidence demonstrates that agonists induce calcium sensitization in permeabilized smooth muscle tissues of different species. Sphingosylphosphorylcholine (SPC), a biologically active sphingomyelin metabolite, aug-
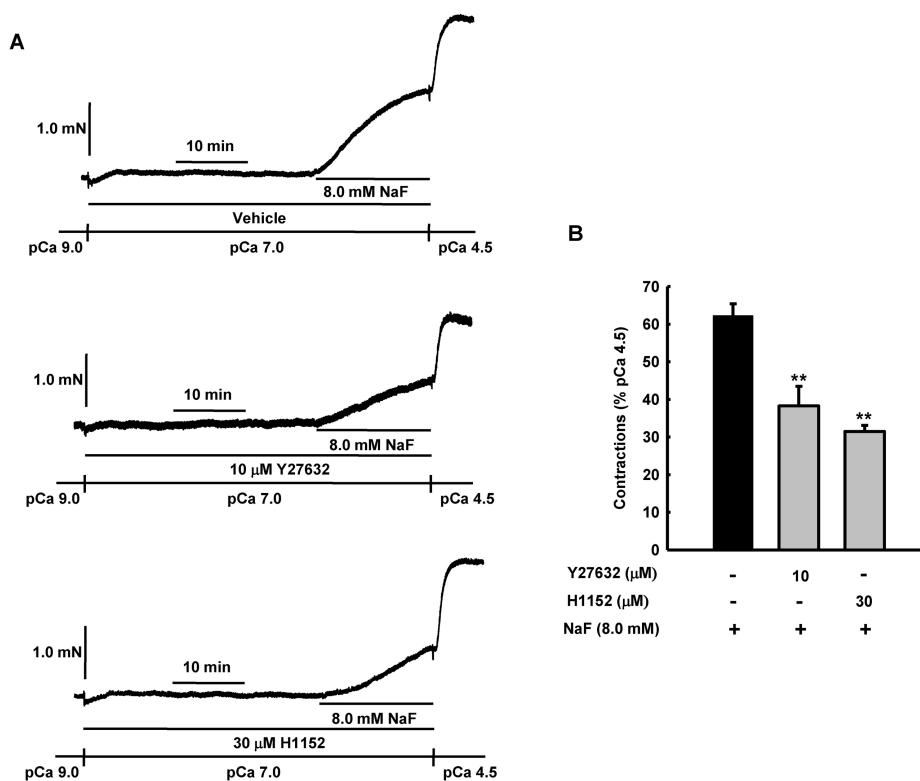
Fig. 5. Effect of Rho kinase inhibitors, Y27632 and H1152 on NaF-induced vasocontraction in β-escin-permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to addition of 8.0 mM NaF in the absence or presence of Rho kinase inhibitors, Y27632 or H1152, at constant concentration of calcium of pCa 7.0. Bar graphs (B) show the developed tension elicited by addition of 8.0 mM NaF in the absence or presence of Rho kinase inhibitors, Y27632 or H1152, at constant concentration of calcium of pCa 7.0 in β-escin-permeabilized mesenteric arteries. When NaF (8.0 mM)-induced contraction reached plateau, the bathing solutions were replaced with pCa 4.5 solution to obtain maximum contraction. Developed tension is expressed as a percentage of the maximum tension to pCa 4.5. Data are expressed as means of 5 experiments with vertical bars showing SEM. **p<0.01 vs. vehicle (One-way ANOVA followed by post-hoc Dunnett test).

mented contractile force at pCa 6.3 in β-escin-permeabilized rabbit coronary arteries [12]; a muscarinic agonist induces calcium sensitization in β-escin-permeabilized rat and guinea-pig detrusor smooth muscles [13]; histamine, ET-1 and PDBu induce calcium sensitization in α-toxin-permeabilized rabbit femoral artery [14]; a thromboxane A$_2$ agonist, U46619-induced contraction involves Ca^{2+} entry and calcium sensitization in rat caudal arterial smooth muscle [15]. However, whether NaF induces calcium sensitization is poorly understood. In a previous study it was reported that NaF enhanced vasocontraction in intact rat aorta in normal Krebs' solution or in EGTA-treated solution. The present study was able to demonstrate that NaF induces calcium sensitization (Fig. 1). Cumulative addition of NaF increased vascular tension in Ca^{2+}-controlled solution at pCa 7.0 or pCa 6.5, but not at pCa 8.0 (Fig. 2), showing that NaF enhances vasocontraction in a Ca^{2+}-dependent manner.

In smooth muscles, the major mechanism for calcium sensitization is inactivation of MLCP. There are two main pathways known to control independently the activity of MLCP. One of them is RhoA/ Rho kinase signaling pathway. The involvement of RhoA in agonist-induced calcium sensitization has been reported in many tissues such as vas deferens, cerebral vasculatures, intestine and airways [6,16-18]. RhoA also causes a concentration-dependent calcium sensitization in β-escin-permeabilized smooth muscles [19]. U46619-incuced Ca^{2+} sensitization involves Rho kinase mediated phosphorylation of MYPT1 at Thr855, but not Thr697, in rat caudal arterial smooth muscle [15]. Rho kinase is responsible for the calcium sensitization during arterial myogenic response through phosphorylation of MYPT1$^{\text{thr855}}$

[16]. In a previous study we observed that NaF dose-dependently increased the amount of GTP RhoA and the phosphorylation level of CPI17 and MYPT1 which are downstream effectors of Rho kinase. Furthermore, both H1152 and Y27632 dose-dependently decreased vascular contraction and the phosphorylation level of CPI17 and MYPT1 induced by NaF in intact rat aorta (unpublished data). The present study also obtained the same results that both H1152 and Y27632 decreased vascular contraction in β-escin-permeabilized mesenteric arteries (Fig. 5). These results suggest that NaF is an activator of Rho kinase signaling pathway during vascular contraction in permeabilized mesenteric arteries.

Another signaling pathway for calcium sensitization is the inactivation of MLCP mediated by PKC and CPI17. PDBu-induced calcium sensitization which involves PKC/ CPI17 can be almost completely inhibited by pretreatment with a PKC inhibitor GF109203X but slightly inhibited by a Rho kinase inhibitor Y27632 [14]. NaF-induced vasocontraction was not inhibited by pretreatment with a PKC inhibitor Ro31-8220 (Fig. 6). PKC-induced Ca^{2+} sensitization may be absent in permeabilized arteries where CPI-17 can be washed out [20]. In the experimental conditions in this study, PDBu also did not produce significant tension in the permeabilized mesenteric arteries (data not shown), suggesting that some signaling molecules involved in PKC pathway may be missed in rat mesenteric arteries after permeabilization. PKC, at least in the current experiment, is not a main pathway in NaF-induced calcium sensitization in β-escin-permeabilized mesenteric arteries so that the involvement PKC in NaF-induced vasocontraction can not be ruled out. NaF-induced vasocontraction was not in-
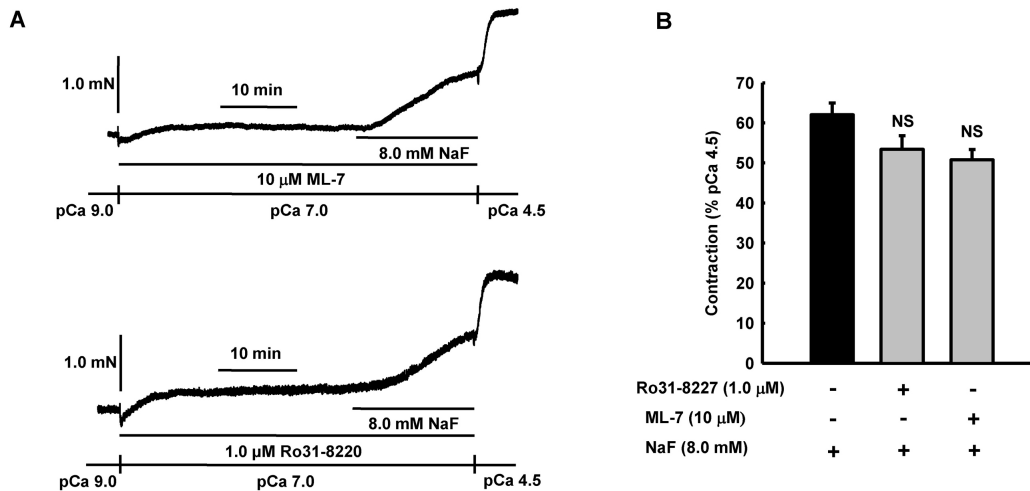
Fig. 6. Effect of ML-7 or Ro31-8220 on NaF-induced vasocontraction in β-escin-permeabilized mesenteric arteries. Representative traces (A) show tension development when β-escin-permeabilized mesenteric arteries were exposed to addition of 8.0 mM NaF in the absence or presence of MLCK inhibitor ML-7 or PKC inhibitor Ro31-8220 at constant concentration of calcium of pCa 7.0. Bar graphs (B) show the developed tension elicited by addition of 8.0 mM NaF in the absence or presence of MLCK inhibitor ML-7 or PKC inhibitor Ro31-8220 at constant concentration of calcium of pCa 7.0 in β-escin-permeabilized mesenteric arteries. When NaF (8.0 mM)-induced contraction reached plateau, the bathing solutions were replaced with pCa 4.5 solution to obtain maximum contraction. Developed tension is expressed as a percentage of the maximum tension to pCa 4.5. Data are expressed as means of 5 experiments with vertical bars showing SEM. NS, not significant (p > 0.05) (One-way ANOVA followed by post-hoc Dunnett test).

hibited by pretreatment with ML-7, either (Fig. 6), indicating that MLCK is not a main pathway in NaF-induced calcium sensitization in β-escin-permeabilized mesenteric arteries. In the present study we examined the involvement of Ca^{2+} on NaF-induced contraction in permeabilized rat mesenteric arteries. NaF-induced vasocontraction was not inhibited by pretreatment with a sarcoplasmic reticular Ca^{2+}-ATPase inhibitor cyclopiazonic acid (data not shown), suggesting that NaF induces vasocontraction regardless of Ca^{2+} release from sarcoplasmic reticulum.

It is known that GTPγS activates both the membrane-associated heterotrimeric G protein and the small G protein, resulting in Ca^{2+}-independent contraction of smooth muscle. Many agonists such as histamine and norepinephrine (NE) induce calcium sensitization in the presence of GTP [6,10], whereas SPC does in the absence of GTP [10]. The facts that NaF induced vasocontraction in the absence of GTPγS and that GTPγS did not enhance the contraction induced by NaF suggest that GTPγS has no additive effect on NaF-induced contraction (Fig. 3). GDPβS reversed agonist- or GTP-induced contraction in many permeabilized tissues such as rabbit femoral artery and guinea pig vas deferens [6,14]. As shown in Fig. 4, GDPβS did not inhibit NaF- or GTPγS-induced vasocontraction. It may be due to a high concentration of GTPγS in this condition because GDPβS competes with GTPγS for its binding [21]. Fluoride has been known to be a potent stimulator of G proteins including Gs, Gi and transducin [22-26]. The structural similarity of AlF$_4^-$ to phosphate allows it to bind next to the β-phosphate of GDP and to mimic the terminal γ-phosphate of GTP. This eliminates the normal requirement for GDP-GTP exchange to cause conformational change and consequent activation of G proteins. The bound AlF$_4^-$ cannot be hydrolyzed so that the G protein complex is maintained in its active, dissociated state, thus interrupting its

normal cycling between GDP- and GTP-bound states [27]. Together, it seems likely that NaF and GTP activate signaling pathway in the similar way at a targeting site on G protein. However, the mechanism by which NaF activates G protein remains to be elucidated.

Rho-kinase was first reported to be involved in development of hypertension in 1997. Y27632 decreased the blood pressure of all three hypertensive animal models including spontaneously hypertensive rat (SHR), renovascular hypertensive rats and deoxycorticosterone acetate (DOCA) salt-induced hypertensive rats, while it did a little in normotensive WKY rats [28]. Furthermore, conditional deletion of G12-13 did not alter normal blood pressure but blocked the development of salt-induced hypertension [29]. Rho kinase has been identified as an important target for treatment of several cardiovascular diseases [3,30]. Since NaF is an activator of Rho kinase signaling pathway during vascular contraction, it may be an important chemical tool for development of therapeutic drugs.

In conclusion, NaF induces calcium sensitization in a Ca^{2+}-dependent manner in β-escin-permeabilized rat mesenteric arteries. Rho kinase is the major signaling pathway in NaF induced calcium sensitization.

ACKNOWLEDGEMENTS

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