British Journal of Pharmacology (1999) 126, 1567–1574 © 1999 Stockton Press All rights reserved 0007–1188/99 $12.00
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Relationship between NaF- and thapsigargin-induced endothelium-dependent hyperpolarization in rat mesenteric artery

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1 In isolated rat mesenteric artery with endothelium, NaF caused slowly developing hyperpolarization. The hyperpolarizing effect was unchanged in the presence of N^G-nitro-L-arginine (L-NOARG) and indomethacin, but was markedly reduced by high K^+. In Ca^{2+}-free medium or in the presence of Ni^{2+}, NaF failed to produce hyperpolarization.

2 NaF-induced hyperpolarization was substantially unaffected by deferoxamine, an Al^{3+} chelator, okadaic acid and calyculin A, phosphatase inhibitors, and preincubation with pertussis toxin, suggesting that neither the action of fluoroaluminates as a G protein activator nor inhibition of phosphatase activity contributes to the hyperpolarizing effect.

3 The selective inhibitors of the Ca^{2+}-pump ATPase of endoplasmic reticulum, thapsigargin and cyclopiazonic acid, elicited hyperpolarization, whose properties were very similar to those of NaF. When intracellular Ca^{2+} stores had been depleted with these inhibitors, NaF no longer generated hyperpolarization.

4 In Ca^{2+}-free medium, NaF (or thapsigargin) caused a transient increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured porcine aortic endothelial cells, and subsequent application of thapsigargin (or NaF) failed to increase $[Ca^{2+}]_i$.

5 In arterial rings precontracted with phenylephrine, NaF produced endothelium-dependent relaxation followed by sustained contraction even in the presence of L-NOARG and indomethacin. The relaxant response was abolished by high K^+ or cyclopiazonic acid.

6 These results indicate that NaF causes endothelium-dependent hyperpolarization, thereby leading to smooth muscle relaxation of rat mesenteric artery. This action appears to be mediated by the promotion of Ca^{2+} influx into endothelial cells that can be triggered by the emptying of intracellular Ca^{2+} stores, as proposed for those of thapsigargin and cyclopiazonic acid.

Keywords: NaF; endothelium; hyperpolarization; endothelium-derived relaxing factor; vasorelaxation; thapsigargin; cyclopiazonic acid; vascular smooth muscle

Abbreviations: ACh, acetylcholine; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; EDHF, endothelium-derived hyperpolarizing factor; L-NOARG, N^G-nitro-L-arginine; NO, nitric oxide; PSS, physiological salt solution; PTX, pertussis toxin

Introduction

Many vasodilating substances cause relaxations of smooth muscle cells through the release of non-prostanoid relaxing factors from the endothelium in a variety of blood vessels. The endothelium-dependent relaxations are mediated by nitric oxide (NO), which induces relaxation through increasing vascular cyclic GMP levels (Palmer *et al*., 1987; Ignarro *et al*., 1987), and also by endothelium-derived hyperpolarizing factor (EDHF), which relaxes vascular smooth muscle cells through hyperpolarization as a result of opening of K^+ channels (Chen & Suzuki, 1989; Vanhoutte, 1989).

It has been known for some time that NaF produces strong contractions of smooth muscle in blood vessel preparations (Casteels *et al*., 1981; Nguyen-Duong, 1985; Zeng *et al*., 1989; Adeagbo & Triggle, 1991). NaF has also been reported to induce endothelium-dependent relaxations in canine and porcine coronary arteries (Flavahan & Vanhoutte, 1990; Cushing *et al*., 1990; Shibano *et al*., 1992). The endothelium-dependent relaxant effect of NaF has been considered to be due to interaction with endothelial G proteins, because NaF, in the presence of Al^{3+} (which can come from contamination of glassware), forms a fluoroaluminate complex, which has a similar structure to PO_4^{3-} and is able to interact with the guanosine 5′-diphosphate situated on the α-subunit of G proteins, resulting in activation by mimicking guanosine triphosphate at its binding site (Bigay *et al*., 1985). Indeed NaF is widely accepted to be a useful pharmacological probe for investigating G protein-mediated mechanisms independently of receptor activation. However, NaF has several other cellular effects such as inhibition of protein phosphatase activity (Shenolikar & Nairn, 1991) and inhibition of endoplasmic reticulum Ca^{2+}-pump ATPase (Narayanan *et al*., 1991). Therefore, it has to be considered that interpretation of the results obtained with NaF may be complicated by its multiple actions. In this study, we found that NaF elicited endothelium-dependent hyperpolarization in isolated rat mesenteric artery. We explored the cellular basis for the NaF-induced EDHF responses in order to further understand the mechanism of action of NaF in vascular endothelial cells.

Methods

Electrophysiological experiments

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Male Wistar rats weighing 250–350 g were anaesthetized with diethyl ether

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and killed by exsanguination. The main branch of the superior mesenteric arteries was carefully excised and placed on a plate containing oxygenated physiological salt solution (PSS) at room temperature. The arteries were dissected free from surrounding connective tissues, cut into rings 3 mm in length, and opened longitudinally. Care was taken to ensure that the endothelial layer was not damaged during the processing of the tissue preparation. Where indicated, the endothelial cells were removed by gently rubbing the intimal surface of the vessel with a moistened cotton ball. The lack of effect of acetylcholine (ACh) was taken as evidence for the complete removal of the endothelium. Some of the preparations were incubated for 120 min in oxygenated PSS containing 400 ng ml^{-1} pertussis toxin (PTX) at 37°C according to the methods by White & Hiley (1997). The tissue was pinned down, intimal side upward, on the bottom of an organ chamber (capacity 3 ml), and superfused with warmed (37°C) PSS aerated with 95% O_2 and 5% CO_2 at a constant flow rate of 7 ml min^{-1} and allowed to equilibrate for at least 60 min before the start of recordings. Glass capillary microelectrodes filled with 3 M KCl (tip resistance 40–80 MΩ) were impaled into the smooth muscle cells from the intimal side. The microelectrode was coupled by an Ag/AgCl junction to a high impedance capacitance neutralizing amplifier (Nihon Kohden, MEZ-8201, Tokyo, Japan). An agar bridge containing 3 M KCl was used as a reference electrode. Electrical signals were continuously monitored on an oscilloscope (Nihon Kohden, VC-10) and recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan). Following stable membrane potentials for at least 2 min, application of NaF or various interventions was commenced. For data analysis we used only the experiments in which a single impalement was maintained.

The composition of PSS was as follows (in mM): NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. For Ca^{2+}-free PSS, Ca^{2+} was omitted and EGTA (0.2 mM) was added. When $NiCl_2$ was used, HCO_3^- was substituted by HEPES. The HEPES solution was gassed with 100% O_2 and the pH of the solution was adjusted to 7.4 with NaOH. The composition of the HEPES solution was as follows (in mM): NaCl 143.0, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.5.

Mechanical experiments

Rat mesenteric arterial rings were prepared as described above. Each ring was suspended by a pair of stainless steel pins in a water-jacketed bath filled with 6 ml of normal PSS. The solution in the bath was gassed with 95% O_2 and 5% CO_2 (pH 7.4) and its temperature was maintained at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by an isometric transducer (Unique Medical, UMTB-1, Tokyo, Japan) and a carrier amplifier (Nihon Kohden, AP-621G). The output of the force transducer was registered on a pen recorder (Rikadenki, R-64, Tokyo, Japan). After the equilibration period, the rings were exposed several times to high K^+ PSS (80 mM $[K^+]_0$) until reproducible contractile responses were obtained. High K^+ PSS was made by substituting NaCl with equimolar KCl. The vessels were precontracted with 1 μM phenylephrine. When N^G-nitro-L-arginine (L-NOARG) (100 μM) and indomethacin (10 μM) were given, the tissue was precontracted with phenylephrine (0.1 μM) in order to match the contraction induced by phenylephrine in control and L-NOARG-pretreated rings (Fukao et al., 1995). After the contraction had reached a plateau level, NaF (10 mM) was applied. Relaxations were expressed as a percentage of the height of the contraction induced by phenylephrine.

Measurement of cytosolic Ca^{2+} concentration in endothelial cells

Porcine aortic endothelial cells were isolated as previously described (Watanabe et al., 1991), by gently scraping the intima of the descending part of porcine aortas. After centrifugation at $250 \times g$ for 10 min in M199 solution (Boehringer, Mannheim, Germany), the pellet of endothelial cells was purified from this suspension, resuspended in M199 solution with Earle's salts, supplemented with 100 IU ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 20% newborn calf serum (GIBCO, New York, NY, U.S.A.), then aliquoted into polybiphenyl dishes fixed on 10 × 10-mm glass cover slips, and incubated at 37°C in 5% CO_2 for 2 days. The medium was renewed every day.

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells adhering the glass cover slips was measured as previously described (Watanabe et al., 1998). The cells were incubated for 45 min in a modified Tyrode's solution (composition in mM: NaCl 150.0, KCl 2.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.0 and HEPES 10.0, with pH 7.4 at 25°C) containing 10% newborn calf serum and fura-2/AM (2 μM) (Dojindo, Kumamoto, Japan), a fluorescent Ca^{2+} indicator. The cells were subsequently washed three times with the modified Tyrode's solution without the serum and indicator in order to remove them from the extracellular fluid, and then left unincubated for 20 min before measurements were started. All experiments were performed at 25°C. The absorption shift of fura-2 that occurred upon binding was determined by scanning the excitation spectrum between 340 and 380 nm while monitoring the emission at 510 nm. The resultant fluorescent image was analysed every 30 s from the individual cells with an $[Ca^{2+}]_i$ analyser (Hamamatsu Photonics, Argus 50, Hamamatsu, Japan) using an ultra-high sensitivity television camera (CCD). The fluorescence ratio (F340/F380) was obtained by dividing, after background subtraction, the 340-nm by the 380-nm images on a pixel-by-pixel basis. Intracellular calibration was performed according to the method of Li et al. (1987). In order to obtain the maximum R_{max} or minimum R_{min} value of the fluorescence ratio, after fura-2 loading, the cells were exposed to the modified Tyrode's solution containing ionomycin (10 μM) and Ca^{2+} (3 mM) or EGTA (5 mM), respectively. $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz et al. (1985). None of the NaF, thapsigargin, cyclopiazonic acid and deferoxamine had any effect on fura-2 fluorescence itself and on autofluorescence of unloaded cells when examined at concentrations employed in this study.

Drugs

The compounds used were as follows: NaF (Nakalai, Kyoto, Japan); ACh chloride (Wako, Osaka, Japan); indomethacin, L-NOARG, glibenclamide, deferoxamine mesylate, cyclopiazonic acid and (−)-phenylephrine hydrochloride (Sigma, St. Louis, MO, U.S.A); okadaic acid and thapsigargin (Calbiochem-Novabiochem, San Diego, CA, U.S.A.); calyculin A (Research Biochemicals International, Natick, MA, U.S.A.); and PTX (List Biological Laboratories, Campbell, CA, U.S.A.). PTX was dissolved in 0.9% NaCl. Indomethacin was prepared in Tris (50 mM), L-NOARG in 0.2 N HCl, and glibenclamide in 0.05 N NaOH. Cyclopiazonic acid was dissolved in dimethyl sulphoxide. Thapsigargin, okadaic acid and calyculin A were prepared in dimethyl sulphoxide and

diluted in ethanol. Other drugs were dissolved in distilled water. PSS was used for further dilution to the proper concentrations. Solvents used to dissolve drugs did not, themselves, affect electrical and mechanical responses at their final bath concentrations.

Statistical analysis

All values are presented in terms of the means ± s.e.mean. The data obtained in cultured endothelial cells are expressed as the means ± s.d. Statistical assessment of the data was made by Student's paired and unpaired *t*-test or two-way analysis of variance (ANOVA) with repeated measurements. *P* values less than 0.05 were considered significant.

Results

Endothelium-dependent hyperpolarization by NaF

The resting membrane potentials of vascular smooth muscle cells in rat mesenteric artery were −52.1 ± 0.3 mV ($n = 84$). In tissues with endothelium, NaF (10 mM) hyperpolarized the membrane potential by −16.0 ± 0.6 mV ($n = 6$) (Figure 1a). In a concentration range of 1–20 mM, NaF produced a concentration-dependent hyperpolarizing effect. The maximum hyperpolarization was attained at ⩾10 mM, and the calculated EC_{50} value for NaF was 5.1 mM (data not shown). The hyperpolarizing effect of NaF was not observed in tissues without endothelium (Figure 1b). However, treatment with indomethacin and L-NOARG did not affect the hyperpolarizing response to NaF (Figure 1c). Thus, NaF (10 mM) hyperpolarized the membrane potential by −15.0 ± 0.6 mV ($n = 3$) in the presence of 10 μM indomethacin and 100 μM L-NOARG. When the external K^+ concentration was increased to 30 mM, the membrane potentials were depolarized by 17.3 ± 1.5 mV ($n = 3$) and NaF-induced hyperpolarization was markedly reduced to −1.3 ± 0.2 mV ($n = 3$) (Figure 1d). The ATP-sensitive K^+ channel blocker glibenclamide was without effect on membrane hyperpolarization by NaF (Figure 1e); NaF (10 mM) hyperpolarized by −15.0 ± 0.9 mV ($n = 4$) in the presence of 10 μM glibenclamide.

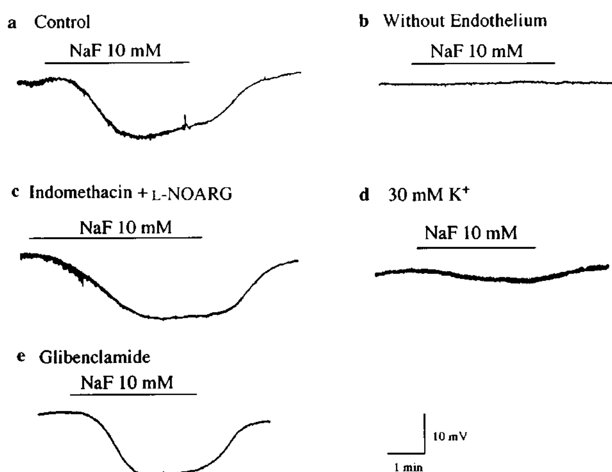

Figure 1 Effect of NaF (10 mM) on cell membrane potential in rat mesenteric artery with intact endothelium in normal PSS (a), and influences of endothelium removal (b), indomethacin (10 μM) plus L-NOARG (100 μM) (c), K^+ PSS (30 mM) and glibenclamide (10 μM) (e) on 10 mM NaF-induced hyperpolarization. Indomethacin, L-NOARG, high K^+ and glibenclamide were added to the bath 15–25 min before NaF.

Extracellular Ca^{2+} dependency of NaF-induced hyperpolarization

NaF elicited slowly developing hyperpolarization without apparently producing initial, transient hyperpolarization (Figure 2a). The time required to reach a peak effect after the addition of NaF was 2.7 ± 0.6 min ($n = 6$).

When the tissue was exposed to Ca^{2+}-free PSS, the resting membrane potential was gradually depolarized by 16.4 ± 2.2 mV ($n = 8$) at 10 min after exposure. In Ca^{2+}-free PSS, the hyperpolarizing effect of NaF (10 mM) was not observed in Ca^{2+}-free PSS (Figure 2b). Application of Ni^{2+} (5 mM) caused modest depolarization of the membrane (4.6 ± 1.2 mV, $n = 5$). Treatment with Ni^{2+} completely abolished the hyperpolarizing response to NaF (10 mM) (Figure 2c).

Is NaF-induced hyperpolarization due to G protein activation?

In order to assess the possibility that NaF acts as a fluoroaluminate complex to activate G proteins, we examined whether deferoxamine, an Al^{3+} chelator, affects NaF-induced hyperpolarization. Deferoxamine (100 μM) did not attenuate the peak amplitude (−15.1 ± 0.7 mV, $n = 6$) nor shortened the time to peak (2.5 ± 0.3 min, $n = 6$) of hyperpolarization evoked by NaF (10 mM). In addition, even if 10 μM $AlCl_3$ was given to the bath, the hyperpolarizing effect of NaF (10 mM) remained unchanged (−15.1 ± 1.0 mV, $n = 6$).

Pretreatment of arteries with PTX (400 ng ml^{-1}) did not affect the hyperpolarizing effect of NaF. Thus, NaF (10 mM) caused hyperpolarization by −13.3 ± 0.7 mV ($n = 3$) in arteries incubated for 120 min with PSS in the presence of PTX, a value which was not significantly different from that in arteries incubated in parallel without the toxin (−13.0 ± 1.0 mV, $n = 3$).

Is NaF-induced hyperpolarization due to phosphatase inhibition?

In order to test the possibility that the action of NaF results from inhibition of protein phosphatases, we examined whether okadaic acid and calyculin A, phosphatase inhibitors, can mimic the hyperpolarizing effect of NaF. Neither okadaic acid (50 nM) nor calyculin A (25 nM) altered the membrane potential. In the presence of okadaic acid or calyculin A, NaF (10 mM) produced similar degrees of hyperpolarization to the response in their absence. Thus, the peak amplitudes of hyperpolarization were −16.0 ± 0.4 mV ($n = 3$) and −17.0 ± 0.4 mV

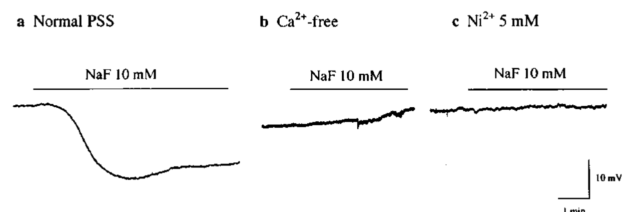

Figure 2 Effect of NaF (10 mM) on cell membrane potential in rat mesenteric artery with intact endothelium in normal PSS (a), in Ca^{2+}-free PSS (b) and in the presence of Ni^{2+} (5 mM). NaF was applied 5 min after exposure to Ca^{2+}-free PSS or Ni^{2+}. The experiments with Ni^{2+} were performed in HEPES solution.

($n=3$) in the presence of okadaic acid and calyculin A, respectively.

Effects of Ca^{2+}-ATPase inhibitors on membrane potential and NaF-induced hyperpolarization

As we have previously demonstrated (Fukao *et al.*, 1995; 1997a), thapsigargin and cyclopiazonic acid, endoplasmic reticulum Ca^{2+}-pump ATPase inhibitors, elicited endothelium-dependent hyperpolarization. The hyperpolarizing responses to these agents were very similar to that to NaF in the time course (Figure 3). Thus, the effects of thapsigargin and cyclopiazonic acid on the membrane potential slowly developed in a monophasic manner, in contrast to ACh whose response appeared immediately after the addition. When thapsigargin and cyclopiazonic acid were applied at relatively lower concentrations (100 nM and 3 μM), the membrane potential returned to the resting level with a very slow time course despite the continuous presence of the agents. After membrane potential had nearly reversed to the resting level (usually 20 min), the addition of NaF (10 mM) had no significant effect on the membrane potential (Figure 3). Thus, treatment with thapsigargin and cyclopiazonic acid caused a complete block of NaF-induced hyperpolarization.

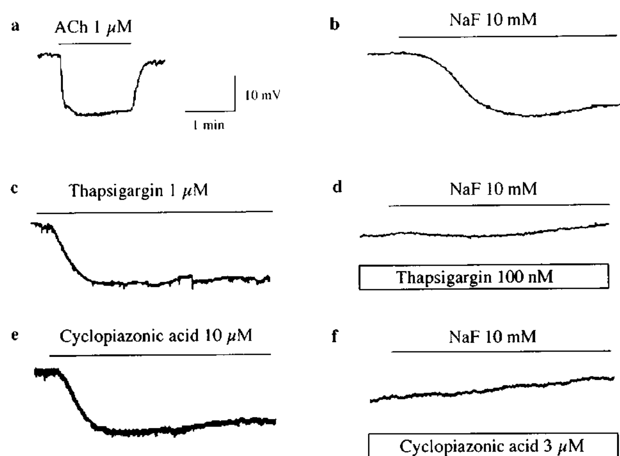

Figure 3 Effects of ACh (1 μM) (a), NaF (10 mM) (b), thapsigargin (1 μM) (c), cyclopiazonic acid (10 μM) (e) on cell membrane potential in rat mesenteric artery with intact endothelium, and influences of thapsigargin (100 nM) (d) and cyclopiazonic acid (3 μM) (f) on 10 mM NaF-induced hyperpolarization. NaF was applied 20 min after the addition of thapsigargin or cyclopiazonic acid.

NaF- and thapsigargin-stimulated $[Ca^{2+}]_i$ responses in cultured endothelial cells

In order to determine whether NaF and thapsigargin produced endothelium-dependent hyperpolarization by acting on the common site, the interacting effects of these agents on $[Ca^{2+}]_i$ were examined in cultured porcine aortic endothelial cells. In Ca^{2+}-free medium, NaF (10 mM) and thapsigargin (1 μM) both caused a transient increase in $[Ca^{2+}]_i$ in endothelial cells (Figure 4). A preceding challenge with NaF completely abolished the $[Ca^{2+}]_i$ response to thapsigargin (Figure 4a). In addition, prior exposure to thapsigargin rendered the cells incapable of responding upon subsequent exposure to NaF (Figure 4b). The NaF-induced increase in $[Ca^{2+}]_i$ was entirely unaffected by treatment with 100 μM deferoxamine ($n=20$ cells from three separate experiments).

NaF-induced relaxation

In the endothelium-intact arterial ring preparations which had been precontracted with phenylephrine, NaF (10 mM) produced marked relaxation with a short delay after the addition which was followed by sustained contraction (Figure 5a). In the presence of indomethacin (10 μM) and L-NOARG, (100 μM), the relaxant response to NaF (10 mM) was reduced from $90\pm5\%$ ($n=5$) to $67\pm6\%$ ($n=4$, $P<0.05$). In addition, treatment with indomethacin and L-NOARG resulted in a more transient relaxation to NaF (Figure 5b). Thus, the relaxant response to NaF in the presence of indomethacin and L-NOARG showed a more rapid return to the precontracted level in comparison with that in their absence; a two way ANOVA test indicated the return curves of the tone in the absence and presence of indomethacin and L-NOARG to be significantly different from each other. Removal of the endothelium virtually abolished the relaxant responses to NaF regardless of whether indomethacin and L-NOARG were present (Figure 5c). The relaxant effect of NaF observed in the presence of indomethacin and L-NOARG was not seen when the external K^+ concentration was increased to 30 mM (Figure 5d). Deferoxamine (100 μM) was without effect on NaF-induced relaxation ($70\pm4\%$, $n=4$) (Figure 5e). On the other hand, NaF no longer caused relaxations in the presence of cyclopiazonic acid (3 μM) (Figure 5f).

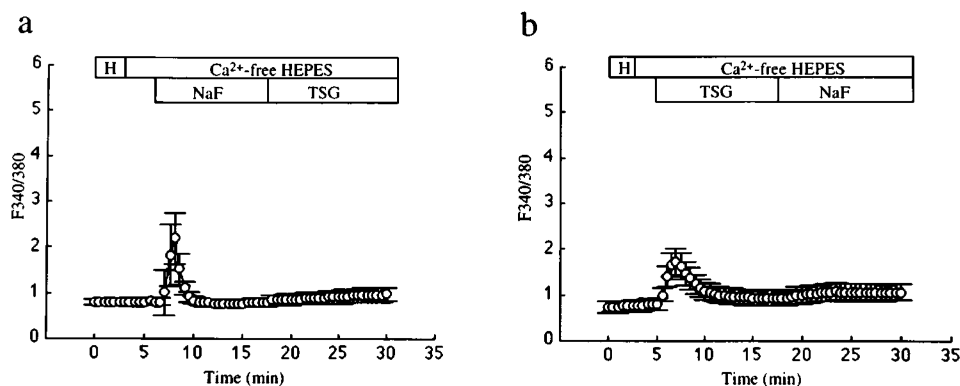

Figure 4 Effects of NaF (10 mM) and thapsigargin (TSG) (1 μM) on $[Ca^{2+}]_i$ in cultured porcine aortic endothelial cells. Endothelial cells loaded with fura-2/AM were exposed to Ca^{2+}-free HEPES-Tyrode's solution after incubation with the solution containing Ca^{2+} (1 mM) (H). Then, cells were challenged with NaF (a) or thapsigargin (b), and subsequently stimulated with thapsigargin (a) or NaF (b). Values are the means$\pm$s.d.; $n=21$ cells from three separate experiments.

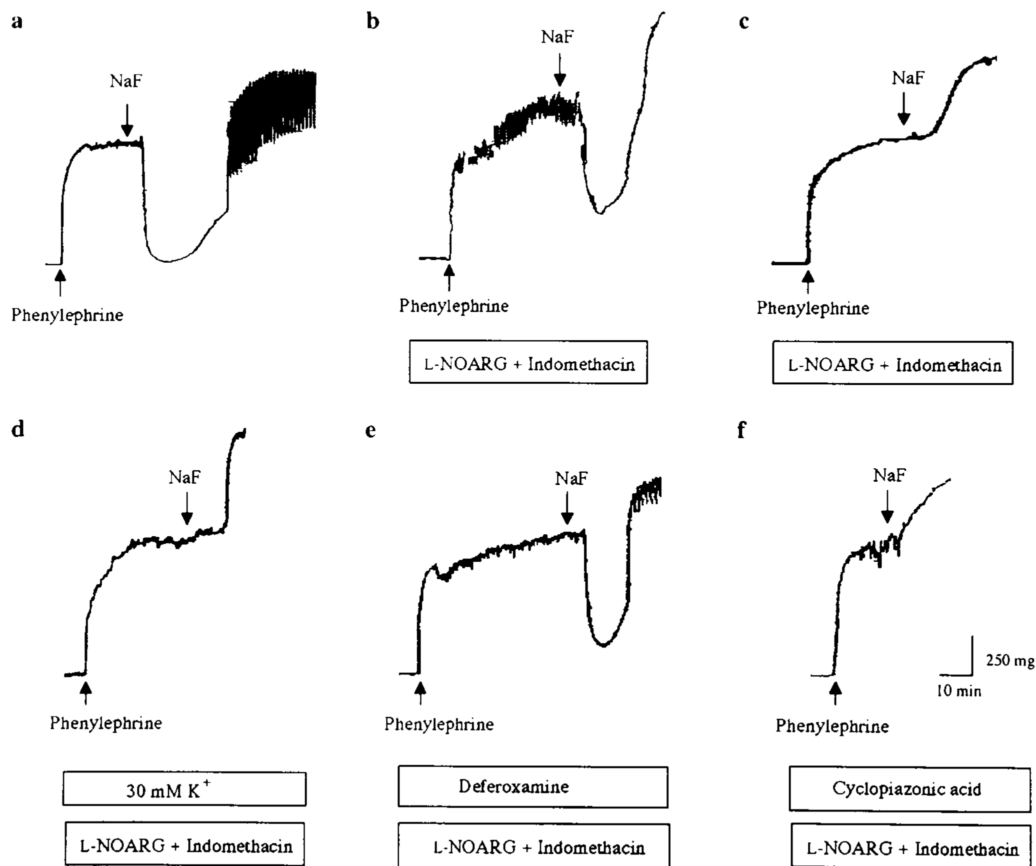

Figure 5 The relaxant effect of NaF (10 mM) in rat mesenteric arterial rings precontracted with phenylephrine. In traces (b) to (f), L-NOARG (100 μM) and indomethacin (10 μM) were added to the bath 15–20 min before application of phenylephrine. In trace (c), the endothelium was mechanically removed. Rings were pretreated with K^+ (30 mM) (d), deferoxamine (100 μM) (e) and cyclopiazonic acid (3 μM) (f) 10 min after the addition of L-NOARG and indomethacin.

Discussion

The present study demonstrated that NaF caused endothelium-dependent hyperpolarization and relaxation in rat mesenteric arteries. The hyperpolarizing effect of NaF was unaffected by pretreatment with L-NOARG and indomethacin, indicating that the production of NO and prostanoids is not involved in this hyperpolarizing effect. On the other hand, in the presence of L-NOARG and indomethacin, the relaxant effect of NaF was significantly reduced in the magnitude and time course. However, the large part of the relaxation was resistant to L-NOARG and indomethacin. NaF-induced hyperpolarization was markedly reduced in high K^+ (30 mM) solution and the relaxant response to NaF observed in the presence of L-NOARG and indomethacin was also abolished. These properties are in good agreement with those of EDHF (Chen & Suzuki 1990; Nagao & Vanhoutte, 1992; Waldron & Garland, 1994). Therefore, it seems most likely that NaF releases EDHF together with NO from endothelial cells and results in endothelium-dependent hyperpolarization, thereby leading to smooth muscle relaxation of rat mesenteric artery.

The lack of NaF-induced hyperpolarization in high K^+ solution suggests that the hyperpolarizing effect of NaF is caused by opening of K^+ channels on smooth muscle cells. Several types of K^+ channels have been shown to mediate EDHF responses in different arteries from different species (Garland *et al.*, 1995). In the present study, the EDHF responses of rat mesenteric artery induced by NaF were insensitive to glibenclamide, a compound considered to be a specific blocker of ATP-sensitive K^+ channels (Nelson *et al.*, 1990), indicating no involvement of ATP-sensitive K^+ channels. Our recent observations have also shown that glibenclamide is without effect on endothelium-dependent hyperpolarization induced by ACh (Fukao *et al.*, 1997b). Thus, K^+ channels other than ATP-sensitive ones may be responsible for endothelium-dependent hyperpolarization in rat mesenteric artery.

Based on the information that fluoroaluminates are an activator of G proteins (Bigay *et al.*, 1985), NaF with added or contaminating aluminum, forming aluminum fluorides, has been shown to interact with G proteins to stimulate a variety of cellular effector systems (Sternweis & Gilman, 1982; Blackmore *et al.*, 1985; Hall *et al.*, 1990; Hattori *et al.*, 1995). Thus, it has been believed that the mechanism by which NaF causes endothelium-dependent relaxation may involve interaction with G proteins (Flavahan & Vanhoutte, 1990; Cushing *et al.*, 1990; Shibano *et al.*, 1992). In this study, NaF-induced hyperpolrization was observed regardless of whether the significant amount of $AlCl_3$ was added. More importantly, neither the hyperpolarizing response nor the L-NOARG- and indomethacin-resistant relaxant response to NaF was blocked by deferoxamine, a chelator of Al^{3+} (Ackrill & Day, 1984). At the concentration employed in this study deferoxamine can completely eliminate the positive inotropic effect of NaF in rabbit left atrial muscles (Hattori *et al.*, 1995). These findings imply that the EDHF responses induced by NaF are not the result of the fluoroaluminate complex activating G proteins. In contrast, recent work has demonstrated that NaF produces a sustained production of inositol 1,4,5-trisphosphate in cultured porcine coronary artery endothelial cells probably

due to direct activation of G proteins (Graier *et al*., 1998), although the present study showed that deferoxamine had no effect on the increase in $[Ca^{2+}]_i$ evoked by NaF in cultured porcine aortic endothelial cells and that NaF did not produce an initial transient hyperpolarization related to a mobilization of Ca^{2+} from endothelial intracellular stores by inositol 1,4,5-trisphosphate as seen with ACh in rat mesenteric artery. The reason for this discrepancy is not clear, but may be related to the different experimental models used. Flavahan & Vanhoutte (1990) have reported that the endothelium-dependent relaxations evoked by NaF could be attenuated by PTX, which is capable of blocking the activity of several G proteins by ADP-ribosylation of the proteins (Dolphin, 1987) in canine coronary artery. However, we found that pretreatment of rat mesenteric artery with PTX caused no significant change in NaF-induced hyperpolarization. It is therefore concluded that NaF releases EDHF from endothelial cells of rat mesenteric artery in a manner independent of G protein activation.

NaF is one of the classical phosphatase inhibitors (Shenolikar & Narin, 1991). It is possible that endothelium-dependent hyperpolarization may result from indirect actions on the EDHF generating and releasing processes in endothelial cells *via* phosphatase inhibition. However, okadaic acid and calyculin A, both of which are potent and highly selective inhibitors of protein phosphatases (Takai *et al*., 1987; Ishihara *et al*., 1989), failed to elicit hyperpolarization in rat mesenteric artery. Furthermore, even in the presence of these phosphatase inhibitors, any alteration in NaF-induced hyperpolarization was not seen. These inhibitors at the concentrations used in this study could significantly increase phosphorylation of the myosin light chain in cultured porcine aortic endothelial cells (H. Watanabe, unpublished observations), indicating that the concentrations we used inhibit effectively protein phosphatases in endothelial cells. We thus suggest that inhibition of phosphatase activity could not contribute to the EDHF effects of NaF in rat mesenteric artery.

There were striking similarities between the actions of NaF and those of thapsigargin and cyclopiazonic acid. First, NaF, thapsigargin and cyclopiazonic acid all produced slowly developing endothelium-dependent hyperpolarization in rat mesenteric artery, in contrast to ACh which generated hyperpolarization composed on an initial transient and following sustained phase (Fukao *et al*., 1995; 1997a). Second, the hyperpolarizing effects of the three agents were not observed in Ca^{2+}-free PSS even if the endothelium was intact (the present study; Fukao *et al*., 1995). Finally, NaF as well as thapsigargin and cyclopiazonic acid can inhibit the Ca^{2+}-pump ATPase of endoplasmic reticulum (Narayanan *et al*., 1991). Indeed, in Ca^{2+}-free medium, the transient increase in $[Ca^{2+}]_i$ elicited by thapsigargin was completely eliminated by a preceding challenge with NaF in cultured porcine aortic endothelial cells. This transient increase in $[Ca^{2+}]_i$ in response to thapsigargin appears to be due to acceleration of Ca^{2+} leak from endothelial intracellular stores (Baró & Eisner, 1992). Prior exposure to thapsigargin also resulted in complete abolishment of NaF-induced increase in $[Ca^{2+}]_i$. This suggests that NaF and thapsigargin act on the intracellular Ca^{2+} stores *via* a common mechanism. Thapsigargin and cyclopiazonic acid deplete the rapidly exchanging intracellular Ca^{2+} stores by blocking the refilling of Ca^{2+} stores, possibly due to inhibition of activity of the Ca^{2+}-pump ATPase located on the endoplasmic reticulum (Georger *et al*., 1988; Seidler *et al*., 1989; Thastrup *et al*., 1990). Based on the hypothesis referred to as the capactitative model (Putney, 1990), depletion of intracellular Ca^{2+} stores is thought to trigger Ca^{2+} influx through some unknown mechanism (Jakob, 1990; Byron *et al*., 1992; Hoth & Penner, 1992). Thus, we have proposed that both thapsigargin and cyclopiazonic acid deplete intracellular Ca^{2+} stores in endothelial cells and the empty of the Ca^{2+} stores generates an intracellular signal to trigger Ca^{2+} influx from the extracellular medium, thereby leading to the production and release of EDHF (Fukao *et al*., 1995). In view of the notion that the actions of NaF are very similar to those of thapsigargin and cyclopiazonic acid, the same mechanism may operate for endothelium-dependent hyperpolarization and relaxation induced by NaF.

When intracellular Ca^{2+} stores had been depleted with thapsigargin or cyclopiazonic acid, NaF-induced hyperpolarization did no longer occur. In addition, after treatment with cyclopiazonic acid, NaF failed to produce L-NOARG- and indomethacin-resistant relaxation. These findings suggest that the release of EDHF caused by NaF is dependent on the extent of filling of intracellular Ca^{2+} stores in endothelial cells. Nevertheless, the hyperpolarizing response to NaF was not observed in Ca^{2+}-free medium, in which ACh generated a transient hyperpolarization associated with Ca^{2+} release from intracellular stores (Fukao *et al*., 1995; 1997a). The possible pathway for Ca^{2+} entry into endothelial cells is thought to be nonspecific cation channels (Nilius, 1990). It has been shown that depletion of intracellular Ca^{2+} stores with thapsigargin or cyclopiazonic acid activates nonspecific cation channels in human umbilical vein endothelial cells (Gericke *et al*., 1993; Zhang *et al*., 1994). In the presence of Ni^{2+}, NaF failed to generate hyperpolarization and ACh produced only a transient hyperpolarization, findings which were the same as those obtained in Ca^{2+}-free medium. This could be explained by assuming that Ni^{2+} may block the Ca^{2+} influx pathway through nonspecific cation channels. Therefore, we propose that NaF depletes intracellular Ca^{2+} stores in endothelial cells possibly due to inhibition of endoplasmic reticulum Ca^{2+}-pump ATPase activity and the emptying of the Ca^{2+} stores triggers the promotion of Ca^{2+} influx through nonspecific cation channels. Opening of the channels could supply sufficient Ca^{2+} into the endothelial cells to initiate the production and release of EDHF.

In conclusion, this study showed that NaF elicited endothelium-dependent hyperpolarization and relaxation in rat mesenteric artery. The hyperpolarization and the large part of the relaxation were due to release of EDHF. A striking similarity with the effects of thapsigargin and cyclopiazonic acid suggests that the mechanism may be related to increased Ca^{2+} influx into endothelial cells triggered by depletion of intracellular Ca^{2+} stores due to inhibition of endoplasmic reticulum Ca^{2+}-pump ATPase activity.

The authors wish to thank Miss Megumi Nakadate for her technical assistance. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science, Sports and Culture of Japan.

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(Received October 14, 1998
Revised December 14, 1998
Accepted January 13, 1999)