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Low concentrations of sodium fluoride inhibit Ca^{2+} influx induced by receptor-mediated platelet activation

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Sodium fluoride (NaF) alone below the concentration of 10 mM had no effect on platelet intracellular Ca^{2+} ($[Ca^{2+}]_i$). When platelets were incubated with low concentrations of NaF (< 10 mM) prior to thrombin stimulation, the second phase of $[Ca^{2+}]_i$ elevation which is attributable to Ca^{2+} influx was suppressed, while the initial rapid peak of $[Ca^{2+}]_i$ which is attributable to internal Ca^{2+} release was unaffected. Ca^{2+} influx assessed by the addition of extracellular Ca^{2+} to cells preactivated by thrombin in the absence of extracellular Ca^{2+} was also inhibited by NaF in a dose-dependent manner. NaF was also effective in inhibiting thrombin- or U-46619-induced Mn^{2+} entry. This inhibitory effect of NaF on Ca^{2+} influx occurred after a lag of at least 30 s. However, Ca^{2+} influx induced by ionomycin-induced Ca^{2+} depletion or by thapsigargin, a Ca^{2+}-ATPase inhibitor, was only partially suppressed by NaF treatment. It is suggested that Ca^{2+} entry induced by receptor-mediated activation is NaF-senstive and that the depletion of Ca^{2+} storage sites by artificial procedures facilitates the opening of Ca^{2+} channels via NaF-insensitive pathways.

Introduction

Sodium fluoride (NaF) is a well-known inhibitor of serine/threonine phosphatase, and has been widely used to evaluate the role of phosphatases for various cellular processes [1]. Subsequently, NaF was found to induce cellular activation similar to physiological agonists in a wide variety of cell types. Recent findings that NaF in the form of fluoroaluminate substitutes for the γ-phosphate group of the GTP molecule have stimulated the use of NaF in a number of studies [2], as the important role of GTP-binding proteins being established for signal transduction systems. NaF can interact with inhibitory GTP-binding proteins (Gi) or stimulatory GTP-binding proteins (Gs), depending upon its concentrations and cell types [3]. Moreover, it may interact with the putative Gp which links phospholipase C activation with receptor-ligand interactions [3,4].

With regard to platelets, NaF induces a wide variety of responses, including aggregation, secretion, arachidonic acid metabolism, and elevation of intracellular Ca^{2+} [5–8]. These responses induced by relatively high concentrations of NaF (> 20 mM) with or without the addition of aluminate have been interpreted as representing the activation of GTP-binding proteins. However, a number of conflicting data have rendered the notion controversial that the effects of NaF are solely attributable to GTP-binding proteins.

Recently, low concentrations of NaF that were unable to induce any measurable platelet responses by themselves have been found to inhibit aggregation, Ca^{2+} mobilization, activation of the Na^+/H^+ exchanger induced by other agonists [9,10]. In the present study, we provide evidence for the potent inhibitory effect of NaF on receptor-mediated Ca^{2+} influx but not so much on Ca^{2+} influx facilitated by artificially-induced Ca^{2+} depletion.

Materials and Methods

Materials

Fura-2-acetoxymethyl ester (Fura-2-AM) and ionomycin were obtained from Dojin Laboratories (Kumamoto, Japan) and Calbiochem (CA, USA), respectively. NaF, hirudin, manganese chloride ($MnCl_2$) were purchased from Sigma (MO, USA). Thapsigargin, U-46619 and okadaic acid were from Funakoshi Co. (Tokyo, Japan). A fluorescent membrane potential probe 3,3'-dipropyl-2,3'-thiadicarbocyanine iodide (DiS) was purchased from Molecular Probe (Junction City, OR).

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Unless otherwise stated, platelets were suspended in a modified Hepes-Tyrode's buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 10 mM Hepes (pH 7.2) and 5.5 mM glucose).

Preparation of fura-2-loaded cells

Citrate anti-coagulated blood was obtained by venepuncture from healthy human donors who had not been on any medication for 2 weeks preceding the experiments. The blood was centrifuged at $60 \times g$ for 15 min and the supernatant was used as platelet-rich plasma. Fura-2-AM, at a final concentration of 3 μM, was added to the platelet-rich plasma, and the mixture was incubated at 37°C for 30 min. After incubation, platelets were washed twice with a modified Hepes-Tyrode's buffer and resuspended in the same buffer at a concentration of $1 \cdot 10^5$ cells/μl.

Measurement of fura-2-detected $[Ca^{2+}]_i$ changes

Fura-2 fluorescence was measured with a Hitachi F-2000 fluorescence spectrophotometer with the excitation wavelength being changed alternately every 0.5 s from 340 nm to 380 nm and the emission wavelength set at 510 nm. The platelet suspension (10^5 cells/μl) was kept at 37°C with constant stirring throughout measurement. A computer (NEC 9801 EX2; NEC, Tokyo, Japan) was connected with the spectrophotometer, and fura-2 fluorescence was processed by a computer program developed by Dr. H. Koshi (Hitachi Corporation, Tokyo, Japan). $[Ca^{2+}]_i$ values were determined from the ratio of fura-2 fluorescence intensities at 340 nm excitation and 380 nm excitation, as described by Grynkiewicz et al. [11].

DiS fluorescence studies

DiS fluorescence was measured according to the method described by Friedhoff and Sonenberg [12]. Platelets separated from platelet-rich plasma by centrifugation were suspended in a modified Hepes-Tyrode's buffer at a concentration of $5 \cdot 10^4$ cells/μl. DiS at the final concentration of 2 μM was added to a platelet suspension constantly stirred and maintained at 37°C by a heating device. The fluorescence intensity of the platelet suspension was measured with an excitation wavelength of 620 nm and an emission wavelength of 670 nm. The DiS fluorescence decreased to a constant level within 4 min of addition of dye to the platelet suspension, at which time addition of thrombin or other agents was performed.

Results

Effects of NaF on resting $[Ca^{2+}]_i$

NaF at concentrations higher than 20 mM induced a gradual increase in $[Ca^{2+}]_i$ of platelets, which was more remarkable in the presence of extracellular Ca^{2+}. Below the concentrations of 10 mM, NaF generally had no effect on $[Ca^{2+}]_i$ of unstimulated platelets. The addition of 10 μM $AlCl_3$ alone, or in combination with NaF which is known to form fluoroaluminate, induced no significant changes in $[Ca^{2+}]_i$, when NaF was used at concentrations below 10 mM (data not shown).

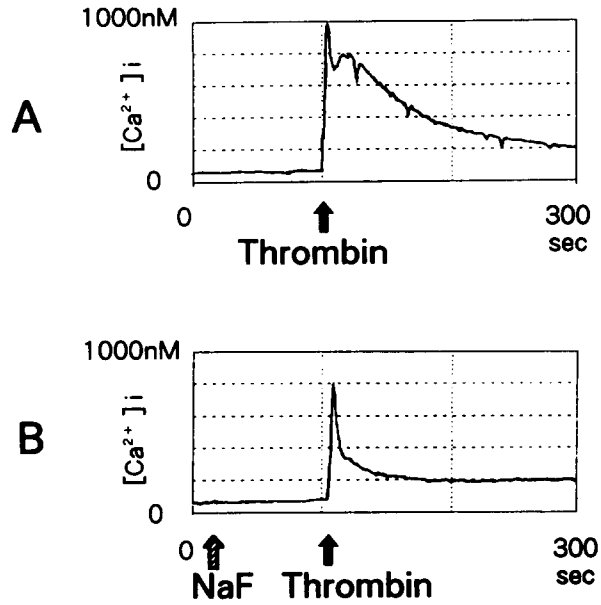

Fig. 1. Effect of NaF on thrombin-induced $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Fura-2-loaded platelets were suspended in a buffer containing 1 mM Ca^{2+}, and the change in $[Ca^{2+}]_i$ was continously measured. Platelets were activated by 0.4 U/ml thrombin at the time indicated by an arrow (A). Platelets were preincubated with 5 mM NaF for 100 s, and 0.4 U/ml thrombin was added (B). The traces are representative of ten experiments.

Effects of NaF pretreatment on $[Ca^{2+}]_i$ elevation induced by thrombin

In the presence of 1 mM extracellular Ca^{2+}, activation of platelets by 0.4 U/ml thrombin induced a rapid rise in $[Ca^{2+}]_i$ which was followed by a second, slow rise in $[Ca^{2+}]_i$. The first rapid peak of $[Ca^{2+}]_i$ elevation is mostly due to Ca^{2+} release from intracellular Ca^{2+} storage sites and the second slower $[Ca^{2+}]_i$ elevation is attributable to Ca^{2+} influx from the extracellular Ca^{2+} pool [13,14]. Pretreatment of platelets with 5 mM NaF for 100 s prior to thrombin stimulation abolished the second rise in $[Ca^{2+}]_i$ while the first rapid phase of $[Ca^{2+}]_i$ elevation was only minimally affected (Fig. 1). NaF at concentrations as low as 2 mM effectively lowered the second phase of $[Ca^{2+}]_i$ elevation attributable to Ca^{2+} influx. Incubation with NaF longer than 30 s was required to fully suppress the second phase of $[Ca^{2+}]_i$ elevation. The addition of 10 μM aluminate did not potentiate the effect of NaF.

Since NaF is known to be a phosphatase blocker, the effect of NaF was compared with that of okadaic acid, a potent inhibitor of protein phosphatases type 1 and 2A [15]. At the concentrations that significantly inhibited thrombin-induced Ca^{2+} influx ($\geq$ 300 nM), okadaic acid also attenuated the initial phase of $[Ca^{2+}]_i$

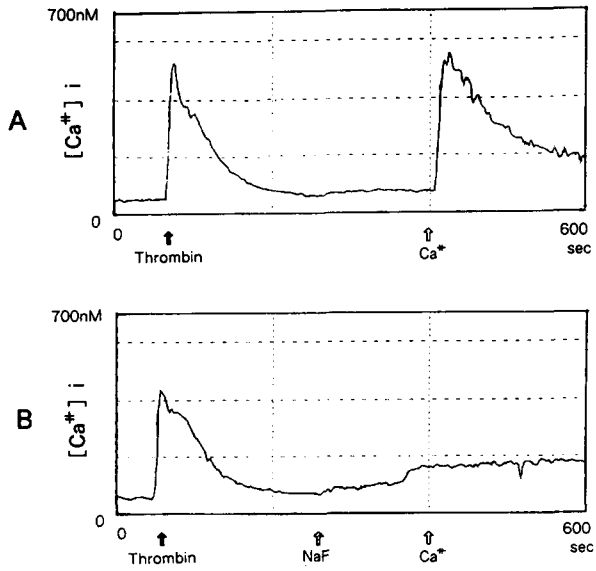

Fig. 2. Effect of NaF on Ca^{2+} influx in thrombin-activated platelets. Platelets were suspended in a buffer containing nominally free Ca^{2+}. Thrombin (0.4 U/ml) was added to the platelet suspension at the time indicated by an closed arrow, and 350 s after thrombin activation, 2 mM Ca^{2+} (an open arrow) was added to the platelet suspension to assess Ca^{2+} influx induced by the Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites (A). NaF at a concentration of 5 mM (a hatched arrow) was added 100 s after thrombin stimulation, and subsequently 2 mM Ca^{2+} (an open arrow) was added to assess Ca^{2+} influx (B). The traces are representative of six experiments.

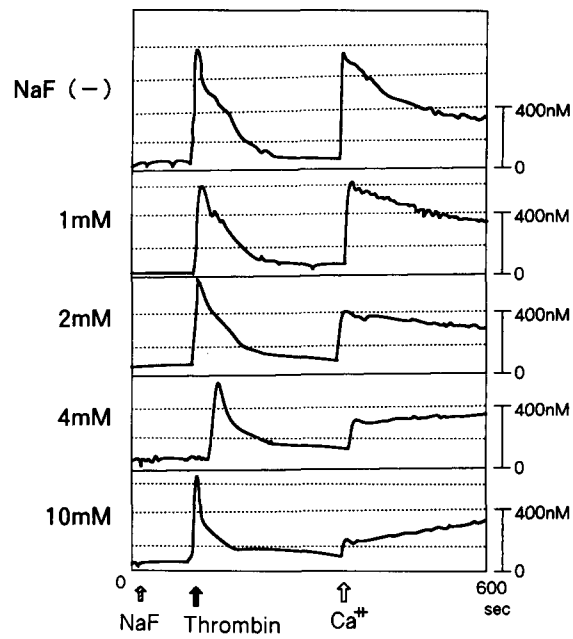

Fig. 3. Dose dependency of inhibitory effects of NaF on Ca^{2+} influx. NaF at various concentrations (a hatched arrow) was added to a platelet suspension containing nominally free Ca^{2+}. 100 s after NaF, thrombin (0.4 U/ml) was added to the platelet suspension (a closed arrow). Ca^{2+} at a concentration of 2 mM (an open arrow) was added 250 s after thrombin stimulation to assess Ca^{2+} influx. The traces are representative of three experiments.

elevation attributable to Ca^{2+} release from intracellular Ca^{2+} storage sites (data not shown). Our findings are in accord with that of Higashihara et al. that okadaic acid inhibits the overall picture of thrombin-induced Ca^{2+} mobilization [16].

Effect of NaF on Ca^{2+} influx induced by thrombin

It is widely accepted that the state of Ca^{2+} depletion of intracellular Ca^{2+} stores facilitates Ca^{2+} channel opening [17]. Platelet activation by 0.4 U/ml thrombin in the absence of extracellular Ca^{2+} should result in Ca^{2+} depletion of their intracellular Ca^{2+} storage sites sensitive to thrombin activation. In accord with this notion, the addition of 2 mM Ca^{2+} to platelets thus treated induced a large increase in $[Ca^{2+}]_i$, which represents Ca^{2+} influx (Fig. 2A). Ca^{2+} influx thus measured may consist of two compartments, one directly coupled with thrombin-receptor interactions and

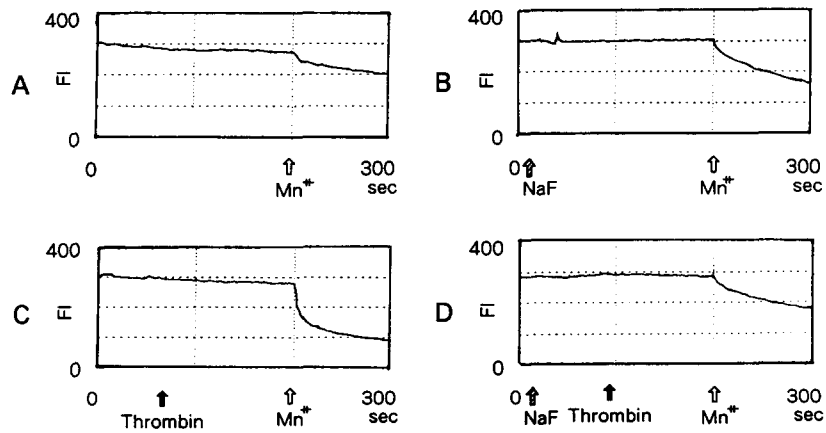

Fig. 4. Effect of NaF on thrombin-induced Mn^{2+} influx. Fura-2-loaded platelets were suspended in a buffer containing nominally free Ca^{2+}, and fura-2 fluorescence was measured at the isosbestic point of fura-2. NaF (5 mM) or saline was added at the time indicated by a hatched arrow. Thrombin (0.4 U/ml) was added to the platelet suspension approximately 100 s after NaF to activate platelets. Subsequently, Mn^{2+} (200 μM) was added to the platelet suspension at the time indicated by an open arrow to assess Mn^{2+} influx. The ordinate represents the fura-2 fluorescence in arbitrary units. The traces are representative of three experiments.

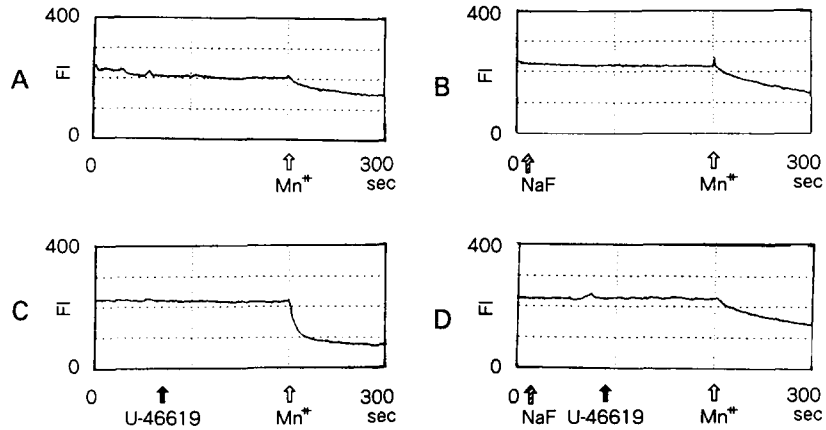

Fig. 5. Effect of NaF on U-46619-induced Mn^{2+} influx. Fura-2-loaded platelets were suspended in a buffer containing nominally free Ca^{2+}, and fura-2 fluorescence was measured at the isosbestic point of fura-2. NaF (5 mM) or saline was added at the time indicated by a hatched arrow. U-46619 (1 μM) was added to the platelet suspension approx. 100 s after NaF to activate platelets. Subsequently, Mn^{2+} (200 μM) was added to the platelet suspension at the indicated by an open arrow to assess Mn^{2+} influx. The ordinate represents the fura-2 fluorescence in arbitrary units. The traces are representative of three experiments.

and the other induced by the Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites. Thus, we employed hirudin, a competitive blocker of thrombin-receptor interactions [18], to exclude the involvement of receptor-linked Ca^{2+} influx. In the same system as described above, the addition of 4 U/ml hirudin, which could completely abolish thrombin-induced platelet activation, 220 s after thrombin activation had only minimal inhibitory effects on $[Ca^{2+}]_i$ elevation induced by the addition of extracellular Ca^{2+} (data not shown). These findings confirm that Ca^{2+} influx assessed by this method correctly represents Ca^{2+} influx induced by the Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites. Thrombin (0.4 U/ml) activation of platelets pretreated with 5 mM NaF in the absence of extracellular Ca^{2+} induced $[Ca^{2+}]_i$ elevation which was only slightly lower than that without NaF pretreatment. However, Ca^{2+} influx induced by the addition of extracellular Ca^{2+} was severely suppressed by NaF pretreatment. NaF was also effective in attenuating Ca^{2+} influx when added after thrombin stimulation (Fig. 2B). For this inhibitory effect of NaF on Ca^{2+} influx to appear, NaF should be given 30–60 s prior to the addition of extracellular Ca^{2+}. Fig. 3 demonstrates dose-dependency of the inhibitory effect of NaF on Ca^{2+} influx when NaF was given prior to thrombin stimulation.

Effect of NaF on Mn^{2+} influx

Mn^{2+} which quenches fura-2 fluorescence has been widely used to assess the opening state of Ca^{2+} chan-

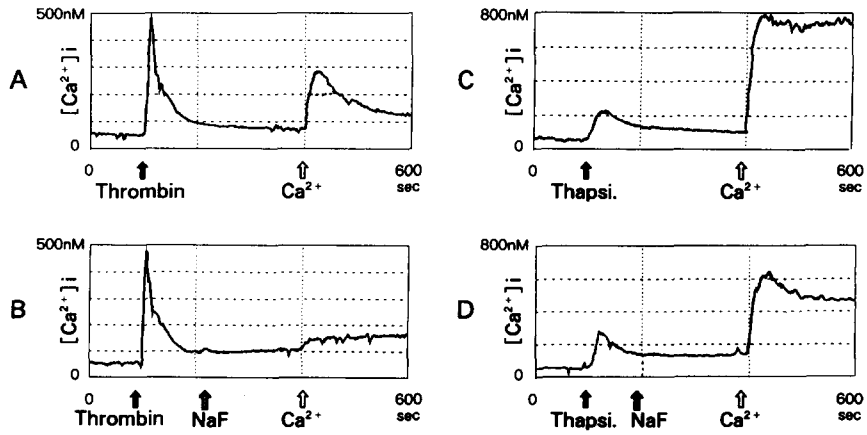

Fig. 6. Differential effects of NaF on thrombin- or thapsigargin-induced Ca^{2+} influx. Platelets were suspended in a buffer containing nominally free Ca^{2+}. Thapsigargin (200 nM) was added to the platelet suspension at the time indicated by an closed arrow, and 300 s after thrombin activation, 2 mM Ca^{2+} (an open arrow) was added to the platelet suspension to assess Ca^{2+} influx induced by the Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites (C). NaF at a concentration of 5 mM (a hatched arrow) was added 100 s after thapsigargin stimulation, and subsequently 2 mM Ca^{2+} (an open arrow) was added to assess Ca^{2+} influx (D). A and B represent the counterparts of C and D, respectively, when thrombin (0.4 U/ml) was used for activating the same platelet preparation. The traces are representative of three experiments.

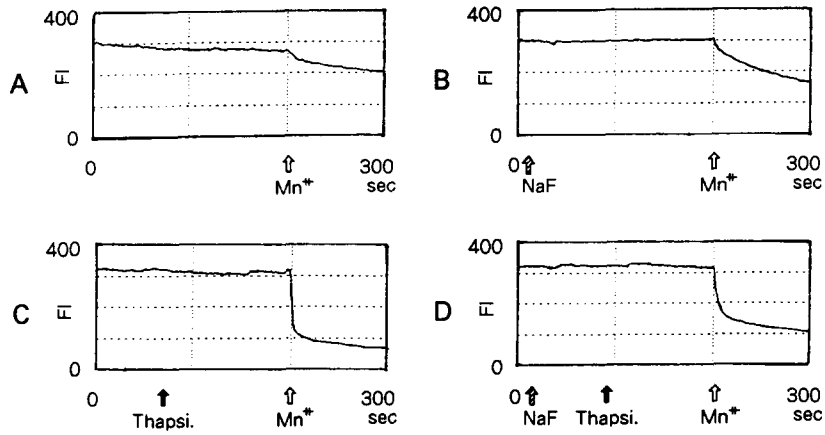

Fig. 7. Effect of NaF on thapsigargin-induced Mn^{2+} influx. Fura-2-loaded platelets were suspended in a buffer containing nominally free Ca^{2+}, and fura-2 fluorescence was measured at the isosbestic point of fura-2. NaF (5 mM) was added at the time indicated by a hatched arrow. Thapsigargin (200 nM) was added to the platelet suspension approx. 100 s after NaF to deplete Ca^{2+} stores. Subsequently, Mn^{2+} (200 μM) was added to the platelet suspension at the time indicated by an open arrow to assess Mn^{2+} influx. The ordinate represents the fura-2 fluorescence in arbitrary units. The traces are representative of three experiments.

nels irrespective of $[Ca^{2+}]_i$ levels [19,20]. The addition of 200 μM Mn^{2+} to non-activated platelets quenched fura-2 fluorescence to some extent (Fig. 4A). When Mn^{2+} was added after thrombin stimulation, the reduction in fura-2 fluorescence was significantly greater than when Mn^{2+} was added to non-stimulated cells (Fig. 4B). While NaF (5 mM) pretreatment slightly potentiated the quenching effect of Mn^{2+} on non-stimulated platelets (Fig. 4C), NaF pretreatment significantly attenuated fura-2 quenching by Mn^{2+} of thrombin-stimulated cells (Fig. 4D). The effect of NaF was also evaluated on U-46619-, a thromboxane A_2 analogue, induced Mn^{2+} influx. NaF pretreatment completely abolished fura-2 quenching by Mn^{2+} that was attributable to U-46619 stimulation (Fig. 5).

Effects of NaF on Ca^{2+} influx induced by mechanisms other than receptor-mediated platelet activation

Since the foregoing experiments provided evidence for the inhibitory effect of NaF on Ca^{2+} influx induced by thrombin and U-46619 which both activate platelets via receptor-mediated pathways, we sought to evaluate the effect of NaF on Ca^{2+} influx induced by other mechanisms. Thapsigargin is known to inhibit Ca^{2+}-ATPase of intracellular Ca^{2+} storage sites with resultant depletion of Ca^{2+} contents in these storage sites [21,22]. The Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites leads to the opening of Ca^{2+} channels, although the mechanism of this action remains to be elucidated. The addition of 2 mM Ca^{2+} to platelets which had been pretreated with 200 nM thapsigargin in

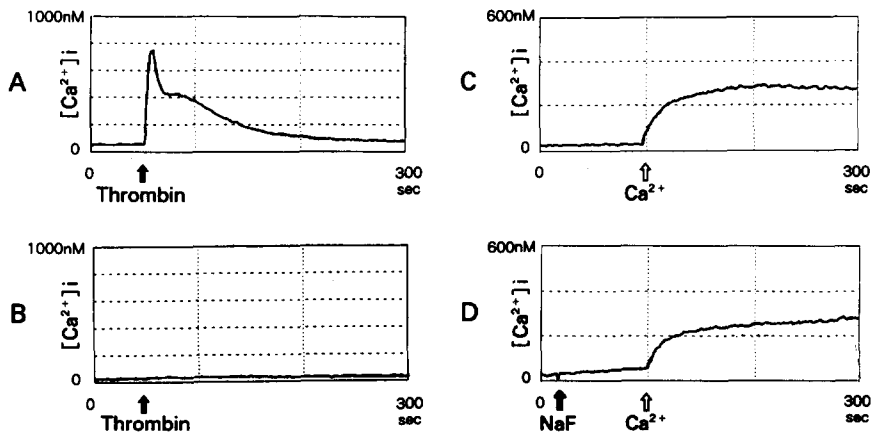

Fig. 8. Effect of NaF on Ca^{2+} influx in ionomycin-treated platelets. Platelets were treated with 0.2 μM ionomycin in the presence of EGTA to deplete intracellular Ca^{2+} storage sites. The cells were washed twice, and resuspended in a buffer containing 100 μM EGTA. The control consisted of the platelets not pretreated with ionomycin but otherwise processed in the same manner. (A) thrombin activation (0.4 U/ml) of the control cells not pretreated with ionomycin. (B) Thrombin activation (0.4 U/ml) of the cells whose intracellular Ca^{2+} storage sites had been depleted of Ca^{2+}. (C) The addition of extracellular Ca^{2+} induced Ca^{2+} influx in ionomycin-pretreated cells. (D) The effect of 5 mM NaF on Ca^{2+} influx in ionomycin-pretreated cells. The traces are representative of three experiments.

the absence of extracellular Ca^{2+} induced a remarkable rise in $[Ca^{2+}]_i$ (Fig. 6C), suggesting that the Ca^{2+}-depleted state of intracellular Ca^{2+} storage sites contributed to the opening of Ca^{2+} channels. When 5 mM NaF was added to the platelets 100 s after the addition of 200 nM thapsigargin, Ca^{2+} influx was only minimally suppressed (Fig. 6D). In contrast, Ca^{2+} influx which occurred after thrombin activation was almost completely inhibited by 5 mM NaF (Fig. 6A and B, platelets from the same donor as Fig. 6C and D). Relatively weak inhibitory effects of NaF on thapsigargin-induced Ca^{2+} influx were also confirmed in the subsequent experiments using Mn^{2+} quenching of fura-2. Mn^{2+} influx, detected by the degree of quenching in fura-2 fluorescence, was remarkably facilitated by 200 nM thapsigargin (Fig. 7A and C). NaF (5 mM) pretreatment only minimally suppressed the increase in Mn^{2+} influx induced by 200 nM thapsigargin treatment.

Another procedure to facilitate Ca^{2+} influx by depleting intracellular Ca^{2+} storage sites is the use of low concentrations of ionomycin in the absence of extracellular Ca^{2+} [14]. Platelets were incubated with 0.2 μM ionomycin in the presence of 1 mM EGTA and no extracellular Ca^{2+} for 10 min at 37°C. After incubation, the platelets were washed twice in a buffer containing 200 nM PGI_2 and 0.1% bovine serum albumin. The platelets were then resuspended in a buffer containing 100 μM EGTA. The control consisted of the platelets not pretreated with ionomycin but otherwise treated in the same manner. While the platelets which had not been treated with ionomycin responded to thrombin stimulation with a relatively large $[Ca^{2+}]_i$ elevation (Fig. 8A), the response was almost completely absent with those pretreated with ionomycin (Fig. 8B), confirming that intracellular Ca^{2+} storage sites had been successfully depleted of Ca^{2+} after ionomycin treatment. The addition of 2 mM Ca^{2+} to Ca^{2+}-depleted cells resulted in a large increase in $[Ca^{2+}]_i$ representing Ca^{2+} (Fig. 8C). Pretreatment with 5 mM NaF only partially attenuated Ca^{2+} influx (Fig. 8D). Higher doses of NaF (up to 20 mM) did not potentiate the inhibitory effects of NaF on Ca^{2+} influx facilitated by ionomycin-induced Ca^{2+} depletion.

Effects of NaF on thrombin-induced changes in the membrane potential

We then sought to evaluate whether the inhibitory effects of NaF on thrombin-induced Ca^{2+} influx might be associated with changes in the membrane potential. The membrane potential of quiescent platelets suspended in a Ca^{2+}-free buffer was estimated to be approx. -55 mV by the use of valinomycin and altered extracellular potassium concentrations according to the method described by Friedhoff and Sonenberg [12]. Thrombin stimulation (0.4 U/ml) induced a biphasic change in DiS fluorescence, an initial rapid rise and

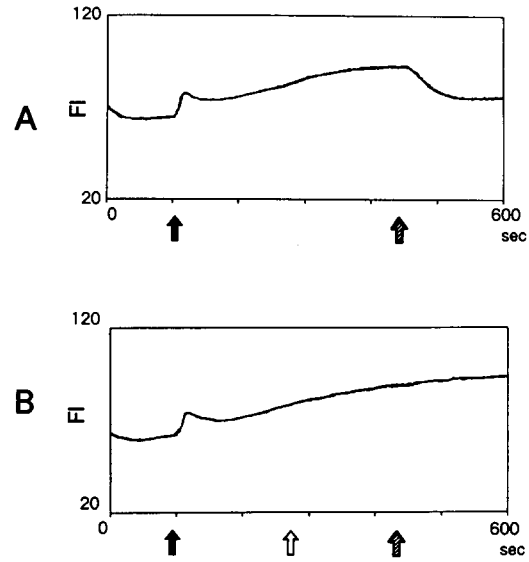

Fig. 9. Effect of NaF on thrombin-induced changes in membrane potential. DiS at the final concentration of 2 μM was added to a platelet suspension, and the DiS fluorescence was measured at an excitation wavelength of 620 nm and an emission wavelength of 670 nm. (A) Thrombin (0.4 U/ml) was added at the time indicated by a closed arrowhead, and 2 mM Ca^{2+} at a hatched arrowhead. (B) 5 mM NaF (indicated by an open arrowhead) was added 100 s after thrombin (0.4 U/ml), and subsequently 2 mM Ca^{2+} was added at the time indicated by a hatched arrowhead. The traces are representative of three experiments.

the second gradual increase that started about 75 s after thrombin stimulation. Six min after thrombin stimulation, the platelet membrane as assessed by DiS fluorescence was depolarized to approx. -20 mV. The addition of 2 mM Ca^{2+} to the platelet suspension preactivated by thrombin reverted the membrane potential to a more hyperpolarized state (Fig. 9A). The addition of 5 mM NaF 100 s after thrombin activation did not alter the membrane potential, but completely inhibited the hyperpolarization caused by the subsequent addition of 2 mM Ca^{2+} (Fig. 9B). Under the same condition using fura-2-loaded cells, 5 mM NaF totally inhibited Ca^{2+} influx of thrombin-activated platelets. These findings suggest that NaF inhibits Ca^{2+} influx independently of the membrane potential.

Discussion

In 1991, Kondo et al. reported that low concentrations of NaF inhibited the second phase of thrombin-induced Ca^{2+} elevation which was attributable to Ca^{2+} influx [9]. We confirmed their findings and have extended investigation to include the inhibitory effect of NaF on Ca^{2+} influx induced by various mechanisms. In the present study, we have found that low concentrations of NaF (< 10 mM) suppress Ca^{2+} influx induced by receptor-mediated platelet activators including thrombin and U-46619, a thromboxane A_2 analogue.

NaF appears to be less effective on Ca^{2+} influx induced by artificial measures including ionomycin and thapsigargin.

The inhibitory effect of NaF on Ca^{2+} influx was observed when administered at least 30 s prior to agonist-induced platelet stimulation in the presence of extracellular Ca^{2+} or prior to the addition of extracellular Ca^{2+} when platelets had been activated in the absence of extracellular Ca^{2+}, suggesting that NaF does not act as a Ca^{2+} channel blocker; Ca^{2+} channel blockers should act almost instantly after being added to platelet suspensions. That the inhibitory effect of NaF on Ca^{2+} influx occurs only after a lag of at least 30 s may represent the time required for NaF to penetrate the cell. This partially agrees with an early report that NaF-induced aggregation and release occurred after a lag of several minutes [23]. The difference in the time required for the various effects of NaF to appear may be due to the different requirement for NaF concentrations within cells to elicit these responses.

Ca^{2+} influx induced by agonist-receptor interactions consists of two components. One is the Ca^{2+} channel opening directly linked with receptor occupancy. The involvement of GTP-binding proteins which link receptors with effectors has been suggested in the regulation of the opening state of Ca^{2+} channels in various types of cells [24]. In platelets, direct coupling of receptor occupancy with Ca^{2+} channel opening has been suggested to occur with ADP stimulation [25]. The other is caused by the Ca^{2+}-depleting state of Ca^{2+} storage sites which occurs after agonist stimulation [17,26,27]. A large body evidence has supported this concept, while the precise mechanism by which the emptied state of Ca^{2+} storage sites results in the opening of Ca^{2+} channels remains largely unknown.

NaF pretreatment completely suppressed the second phase of thrombin-induced $[Ca^{2+}]_i$ elevation, which is attributable to Ca^{2+} influx. This Ca^{2+} influx consists of Ca^{2+} influx directly linked with receptor occupancy and Ca^{2+} influx resulting from the Ca^{2+} depletion of intracellular Ca^{2+} storage sites, as described above. That this phase of Ca^{2+} influx was completely inhibited by NaF pretreatment suggests that NaF affects both routes of Ca^{2+} entry. However, whether NaF truly interferes with the opening of Ca^{2+} channels directly linked with receptor occupancy was not clearly addressed in this study, since we had no accurate method of differentiating this route of Ca^{2+} influx from that induced by Ca^{2+} depletion.

In comparison with the inhibitory effect on receptor-mediated Ca^{2+} influx, NaF was less effective in suppressing Ca^{2+} influx induced by artificially-induced Ca^{2+} depletion of intracellular Ca^{2+} storage sites. Based on the differential effects of NaF on Ca^{2+} influx, we suggest that the Ca^{2+} storage sites that can be emptied by artificial measures send the signal of their Ca^{2+} content depletion to Ca^{2+} channels partly via NaF-insensitive pathways. Whether these findings imply the presence of multiple Ca^{2+} storage sites within platelets as suggested for a variety of cell types [28–30] awaits further evaluation.

The mechanism by which NaF inhibits receptor-mediated Ca^{2+} influx remains largely unknown. While the membrane potential may have a profound effect on Ca^{2+} influx [31], we found that NaF attenuated Ca^{2+} influx without affecting the membrane potential changes induced by thrombin. Okadaic acid, a phosphatase inhibitor, inhibited both Ca^{2+} release from intracellular Ca^{2+} storage sites and Ca^{2+} influx, while NaF selectively affected the latter process. It is interesting to note that Siffert et al. demonstrated with human platelets that low concentrations of NaF inhibited thrombin- or phorbol myristate acetate-induced Na^+/H^+ exchanger activation, while NaF had no effect on acid-induced Na^+/H^+ exchanger activation [10]. This analogy implies that receptor-mediated Na^+/H^+ exchanger activation and Ca^{2+} influx induced by Ca^{2+} depletion of Ca^{2+} storage sites may be governed by the same mechanism. Siffert et al. suggested that a novel GTP-binding protein that is activated by NaF functions as a negative regulator of the Na^+/H^+ exchanger in platelets. Although it is an attractive idea that GTP-binding proteins are involved in regulation of receptor-mediated Ca^{2+} influx, we have no concrete evidence now to attribute the inhibitory effect of NaF on Ca^{2+} influx to activation of GTP-binding proteins.

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