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Sodium Fluoride Stimulates Exocytosis at a Late Site of Calcium Interaction in Stimulus-Secretion Coupling: Studies with the RINm5F β Cell Line

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SUMMARY

In the insulin-secreting β cell line RINm5F, sodium fluoride stimulated exocytosis in a concentration (5–15 mM)- and temperature-dependent manner. Depletion of aluminum with the chelator deferoxamine or addition of aluminum to the buffer failed to affect the NaF-stimulated insulin release. This suggests that stimulation of heterotrimeric G proteins or inhibition of phosphatases or other enzymes by fluoroaluminate, an analog of the phosphate moiety, is not involved in the insulinotropic action of NaF. Removal of extracellular Ca^{2+} suppressed the NaF-stimulated insulin release. However, nitrendipine, a blocker of L-type voltage-dependent Ca^{2+} channels, did not inhibit the NaF-stimulated insulin release and NaF did not cause any changes in the cytosolic free calcium concentration ($[Ca^{2+}]_i$). Decreasing $[Ca^{2+}]_i$ with thapsigargin or increasing $[Ca^{2+}]_i$ with ionomycin or a depolarizing concentration of KCl resulted in suppression or enhancement of NaF-stimulated insulin release, respectively. Furthermore, NaF enhanced Ca^{2+}-induced insulin release in electrically permeabilized RINm5F cells. These findings indicate that the effect of NaF on exocytosis is dependent on $[Ca^{2+}]_i$, although NaF itself does not change $[Ca^{2+}]_i$. Inhibitors of protein kinase C, such as staurosporine and bisindolylmaleimide, in concentrations sufficient to block the effects of phorbol esters, did not attenuate the NaF-stimulated insulin release. Neither cellular cAMP content nor [^{3}H]arachidonic acid release was increased by NaF. NaF-stimulated insulin release was synergistically enhanced by the activation of protein kinases A and C. Finally, trifluoperazine, an inhibitor of calmodulin and other Ca^{2+}-binding proteins, inhibited the insulinotropic action of NaF in a concentration-dependent manner. Trifluoperazine (50 μM) and W-7 (100 μM) nullified the 10 mM NaF-stimulated insulin release. It is concluded that NaF evokes exocytosis by a novel mechanism of sensitization to Ca^{2+}, possibly on a Ca^{2+}-responsive protein that is sensitive to trifluoperazine and W-7, leading to exocytosis. Protein kinases A and C also act at this site or at a more distal point.

Much information has been accumulated about initial and early events in stimulus-secretion coupling. In pancreatic β cells, it is thought that increased cellular ATP levels or the ATP/ADP ratio produced by metabolism of glucose and other nutrients has an important role in physiological insulin secretion. The increase in the ATP/ADP ratio contributes to the inhibition of ATP-sensitive K^+ channels and depolarization of the β cell membrane (1). Subsequently, Ca^{2+} influx through voltage-dependent Ca^{2+} channels, which have a pivotal role in nutrient-induced insulin secretion (2), increases the $[Ca^{2+}]_i$. This elevation of $[Ca^{2+}]_i$ triggers the stimulation of insulin release. In addition, other cellular signal transduction systems, some known, including phospholipase C and adenylyl cyclase, and some unknown, have important modulatory enhancing effects on insulin secretion (3–6).

In sharp contrast to these findings on the initial or early events in stimulus-secretion coupling, the mechanisms involved after the elevation of $[Ca^{2+}]_i$, i.e., the late steps of stimulus-secretion coupling, remain largely unknown. Ca^{2+}-binding proteins including annexins (7), calcyclin (8), and other cytosolic protein factors, such as Exo 1 or 2 (9), have been thought to be involved in the exocytotic events after elevation of $[Ca^{2+}]_i$. G proteins are likely to have important roles in the late stages leading to exocytosis because, in permeabilized cells, GTPγS, a nonhydrolyzable analog of GTP, stimulates exocytosis at a distal site beyond the elevation of $[Ca^{2+}]_i$ (10, 11). Additionally, there is evidence that Rab 3a and Rab 3b, low molecular weight G proteins, are

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ABBREVIATIONS: $[Ca^{2+}]_i$, cytosolic free calcium concentration; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; W-7, *N*-(6-aminohexyl)-5-chlorol-1-naphthalenesulfonamide hydrochloride; KRB, Krebs-Ringer bicarbonate; AM, acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IP_3, inositol trisphosphate; GTPγS, guanosine-5'-*O*-(3-thio)triphosphate.

involved in the events leading to exocytosis (12–14). Recently we reported that, in the insulin-secreting β cell line RINm5F, mastoparan, a tetradecapeptide purified from wasp venom, stimulated exocytosis in a Ca^{2+}-independent manner at a late step in stimulus-secretion coupling, possibly by activation of novel G proteins (15).

In an attempt to provide additional information about these late steps in stimulus-secretion coupling, we have studied the effects of sodium fluoride on insulin secretion, again using the RINm5F cell line. Fluoride, by forming a complex with aluminum, mimics the terminal γ-phosphate of GTP and by this means stimulates heterotrimeric G proteins (16, 17). NaF has been used as a general stimulator of heterotrimeric G proteins not only in cell-free systems but also in intact cells (18–23). In some endocrine cells, NaF stimulates exocytosis (24–26). However, it is well known that NaF also acts as a metabolic inhibitor and as a phosphatase or other enzyme inhibitor, so that its actions on cellular functions are probably complex. All of these known effects of NaF require trace amounts of aluminum, as is the case for the stimulation of G proteins, because AlF_4^- ion acts as a high affinity analog of phosphate (17, 27, 28). In this study, we demonstrate that NaF has a unique stimulatory effect on insulin secretion, possibly by sensitizing a Ca^{2+}-binding protein that leads to exocytosis.

Experimental Procedures

Materials. NaF, deferoxamine mesylate, aluminum chloride, sodium orthovanadate, nitrendipine, TPA, staurosporine, carbachol, ionomycin, melittin, trifluoperazine, W-7, sulfinpyrazone, fura-2, and fura-2/AM were from Sigma. Forskolin and bisindolylmaleimide were from Calbiochem. Thapsigargin was from Research Biochemicals (Natick, MA). ^{125}I-Insulin (80–120 μCi/μg) and cAMP radioimmunoassay kits were from DuPont-New England Nuclear. [^{3}H]Arachidonic acid (150–230 Ci/mmol) was purchased from Amersham.

RINm5F cell culture. RINm5F cells were maintained in monolayer culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin, at 37° in a 95% air/5% CO_2 atmosphere. Cells at passages 57–65 were used.

Insulin release. In static incubation experiments, the cells were grown in 16-mm-diameter wells for 3–5 days, and the medium was

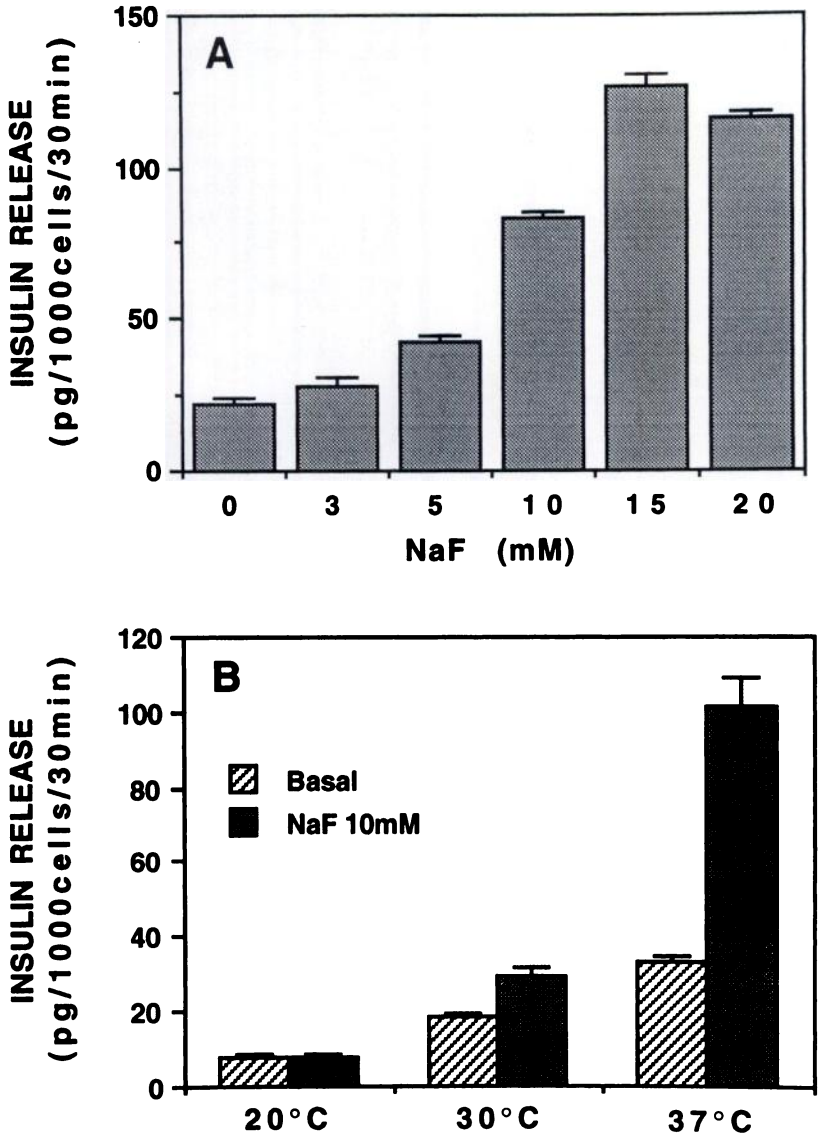

Fig. 1. NaF-stimulated insulin release from RINm5F cells as a function of concentration (A) and temperature (B). Static incubations were performed for 30 min, as described in Experimental Procedures. Values represent the means ± standard errors of four (A) or six (B) determinations.

changed on the day before experiments. Cells were incubated at 37° for 30 min in KRB buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.8 mM glucose, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.4). In some experiments inhibitory agents, such as staurosporine, bisindolylmaleimide, nitrendipine, trifluoperazine, or W-7, were present throughout the experiments. The preincubation solutions were replaced with KRB buffer containing test agents, and cells were incubated at 37°. In temperature dependence experiments, static incubations were also performed at 20° or 30°. The incubation media were sampled 30 min after incubation and centrifuged to remove possible contamination by detached cells, and the supernatants were used for radioimmunoassay of insulin. Cells were counted after incubation, and the rate of insulin release was expressed as picograms/10^3 cells/30 min. In some experiments, cell viability after incubation was evaluated by trypan blue staining. For perifusion, cells were grown to approximately the same density as that used for the static incubations. Cells dispersed in a Ca^{2+}-free solution with EDTA plus trypsin were loaded in each perifusion chamber (10^6 cells in 0.7 ml/chamber) and perifused with KRB buffer at 37°, at a flow rate of 1 ml/min. For Ca^{2+}-free conditions, KRB buffer devoid of Ca^{2+}, with 0.2 mM EGTA, was used. The experiments were started after a 60-min perifusion equilibration period. Samples were collected every 2 min and insulin in the perifusate was measured by radioimmunoassay. The rate of insulin release was expressed as picograms/10^3 cells/minute.

Permeabilization of RINm5F cells. RINm5F cells were electrically permeabilized as described by Vallar *et al.* (11), with minor modifications. In brief, the cell suspension in culture medium was washed twice in Ca^{2+}-free KRB buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM glucose, 0.1% bovine serum albumin, 25 mM HEPES, pH 7.4) and washed once in cold mannitol buffer (270 mM mannitol, 10 mM potassium glutamate, 5 mM Mg-ATP, 0.4 mM EGTA, 20 mM HEPES, pH 6.6). The cells were resuspended in cold potassium glutamate buffer (140 mM potassium glutamate, 5 mM NaCl, 2 mM $MgSO_4$, 5 mM Mg-ATP, 0.4 mM EGTA, 20 mM HEPES, pH 6.6) at a cell density of about 6×10^7 cells/ml. The cells were permeabilized by 12 exposures to an electric field of 3 kV/cm, at 3 μF. After permeabilization, the cells were immediately diluted and washed once in cold potassium glutamate buffer, centrifuged for 4 min at $500 \times g$, resuspended in the same buffer, and immediately distributed to the reaction tubes. Cell permeabilization and subsequent steps were carried out at 4°. After high-voltage discharge 98–99% of the cells were permeable to trypan blue, and at the end of the experiments >90% of the cells remained permeable to trypan blue. Insulin release in the permeabilized cells was measured over a 5-min period.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured fluorimetrically, using the Ca^{2+}-sensitive fluorescent dye fura-2. Cells grown to the same density as those used for the insulin release studies were examined. The cells were dispersed into single cells in a Ca^{2+}-free solution with EDTA plus trypsin. Cells were suspended at 1.5×10^6 cells/ml in RPMI 1640 medium with 5% fetal bovine serum, pH 7.4, and were maintained at room temperature before loading. For fura-2 loading, the cell suspension was centrifuged, resuspended at 4×10^6 cells/ml in KRB buffer containing 0.25 mM sulfinpyrazone and 1 μM fura-2/AM, and incubated at 37° for 30 min, with continuous shaking. The fura-2-loaded cells thus obtained were washed and resuspended at 1.5×10^6 cells/ml in KRB buffer containing 0.25 mM sulfinpyrazone, and 3 ml of suspension were placed in each quartz cuvette. During experiments, and while in the spectrofluorometer (LS-5; Perkin-Elmer-Cetus Instruments), the cell suspensions were continuously stirred with small magnetic bars within the cuvettes. The temperature of the cell suspension was maintained at 36–37° by circulation of warm water through the cuvette holder. An excitation wavelength of 340 nm and an emission wavelength of 510 nm were used to calculate the $[Ca^{2+}]_i$. In many experiments, excitation wavelengths of 340 and 380 nm were used to monitor changes of both Ca^{2+}-sensitive and Ca^{2+}-insensitive fluorescence. The $[Ca^{2+}]_i$ was calculated as we have done previously (15, 29, 30), using the equation

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

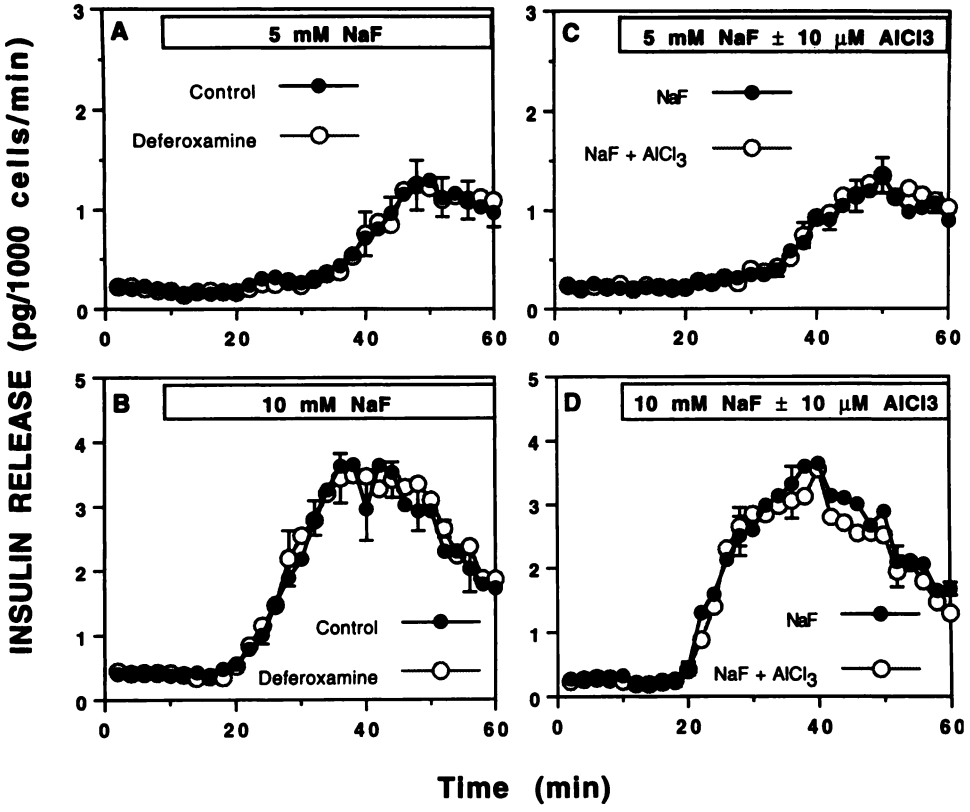

Fig. 2. Effects of deferoxamine (A and B) and $AlCl_3$ (C and D) on 5 mM (A and C) and 10 mM (B and D) NaF-stimulated insulin release. Perifusion experiments were performed as described in Experimental Procedures. A and B, Deferoxamine (200 μM) was present throughout the experiments and during a 60-min equilibration period before the start of the test period. Control experiments were performed in the absence of deferoxamine. C and D, $AlCl_3$ (10 μM) was present in the NaF solutions. Control cells were exposed to normal NaF-containing KRB buffer. Values represent the means ± standard errors of four determinations from two separate paired experiments.

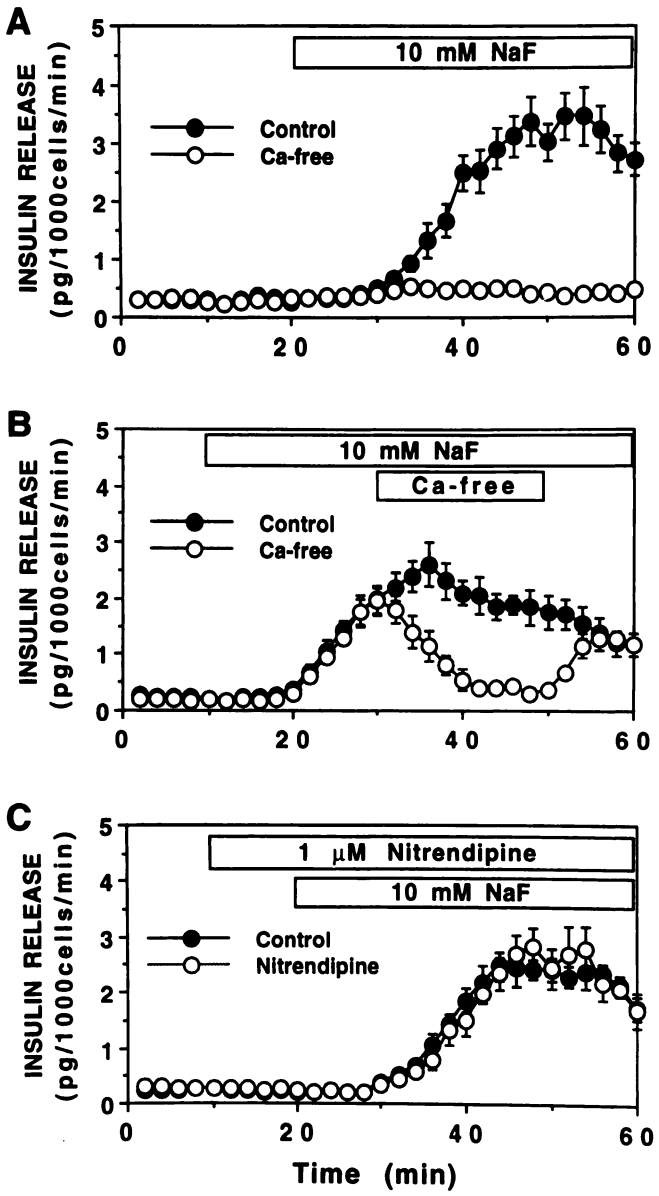

Fig. 3. Time course of 10 mM NaF-stimulated insulin release from RINm5F cells under various conditions. Perifusion experiments were performed as described in Experimental Procedures. NaF (10 mM) was introduced as shown (*horizontal bars*). A, The perifusate in one set of chambers (○) was changed to Ca^{2+}-free KRB buffer with 0.2 mM EGTA at 10 min. B, Perifusion with KRB buffer containing Ca^{2+} in one set of chambers (○) was replaced by perifusion with Ca^{2+}-free KRB buffer with 0.2 mM EGTA between 30 and 50 min, as indicated (*horizontal bar*). C, Nitrendipine at 1 μM was introduced into the perifusate of one set of chambers (○) as shown (*horizontal bar*). Values represent the means ± standard errors of four determinations from two separate paired experiments.

K_d is the effective dissociation constant for Ca^{2+} binding to fura-2 (224 nM), and F, F_{min}, and F_{max} are the fluorescence values at a time of interest, at $[Ca^{2+}]_i = 0$, and at saturated $[Ca^{2+}]_i$, respectively, after correction for extracellular fura-2 and autofluorescence of the cells. Fluorescence due to extracellular fura-2 was determined by adding manganese to the cells at the beginning (in paired cuvettes) and end of each experiment. Because the rate of fura-2 leakage from the cells was constant throughout the experiments, the extracellular fura-2 level at any time in an experiment was obtained by extrapolation. Autofluorescence of the cells was obtained by the addition of manganese after Triton X-100. The cells were lysed with Triton X-100, followed by the addition of EGTA plus Tris·HCl to obtain F_{max} and F_{min}, respectively. For the measurement of changes in the fura-2 fluorescence signal produced by NaF in a cell-free system, KRB buffer containing 0.1 μM fura-2 was used. Excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were used. The fura-2 fluorescence in the presence of 10 mM NaF, under cell-free conditions, was monitored over a 30-min period.

Measurement of cellular cAMP levels. RINm5F cells were grown in 16-mm-diameter wells for 4–5 days. Cells were preincubated in KRB buffer at 37° for 15 min, with gentle shaking. The solution was replaced with KRB buffer containing NaF (10 mM) or forskolin (1 μM), and cells were incubated at 37° for 10 min, 20 min, or 30 min. At the end of the incubation, the assay buffer was removed and kept at −20° until radioimmunoassay for insulin. Ice-cold 6% trichloroacetic acid (0.25 ml) was added to each well to stop the reaction, and cAMP levels were then quantitated by radioimmunoassay according to the manufacturer's instructions.

Arachidonic acid release. RINm5F cells were cultured for 20 hr in medium containing 0.25 μCi/ml [^{3}H]arachidonic acid. Incorporation of isotope into the cells was between 40 and 75% in different batches. Cells were washed three times and incubated for 30 min with 1 ml of KRB buffer containing various concentrations of NaF or melittin, which increases the susceptibility of phospholipids to metabolism by phospholipase A_2 (31). After incubation, the medium was aspirated and immediately placed on ice in Eppendorf tubes. The medium was then centrifuged at 3000 rpm for 2 min at 4° to remove detached cells, and the top 200 μl were aspirated for scintillation counting. Another 100 μl of the medium were kept at −20° until radioimmunoassay for insulin.

Statistical analysis. Results are presented as means ± standard errors. Statistical analysis was performed by one-way analysis of variance, with pairwise comparison by the Bonferroni method; $p < 0.05$ was considered statistically significant.

Results

Concentration and temperature dependence of NaF stimulation of insulin release. NaF stimulated insulin release from RINm5F cells, during 30-min static incubations at 37°, in a concentration-dependent manner (Fig. 1A). The minimum effective concentration of NaF was 5 mM, although 3 mM NaF caused a small, and statistically insignificant, increase in insulin release. The maximum response was observed at 15 mM. Concentrations higher than 20 mM were not tested. The temperature dependence of 10 mM NaF-stimulated insulin release is shown in Fig. 1B. Lowering the ambient temperature dramatically reduced both the basal secretion rates and the insulin release response to NaF. At 37°, 10 mM NaF caused a 2.2-fold increase in insulin release, compared with basal release; however, at 30°, the response was reduced to a 0.6-fold increase. The insulinotropic action of NaF was completely abolished by lowering the ambient temperature to 20°. Incubation with 10 mM NaF for 30 min did not affect the cell viability, as judged by exclusion of trypan blue. Additionally, after the NaF was washed out, the RINm5F cells responded normally to glyceraldehyde (data not shown).

Role of trace amounts of aluminum in the NaF-stimulated insulin release. The role of aluminum ions in NaF-stimulated insulin release was examined using the aluminum chelator deferoxamine. This was done because only small amounts of aluminum are required to stimulate heterotrimeric G proteins or to inhibit enzymes such as phosphatases with fluoride. In an attempt to achieve aluminum-free conditions, we used 200 μM deferoxamine in the KRB

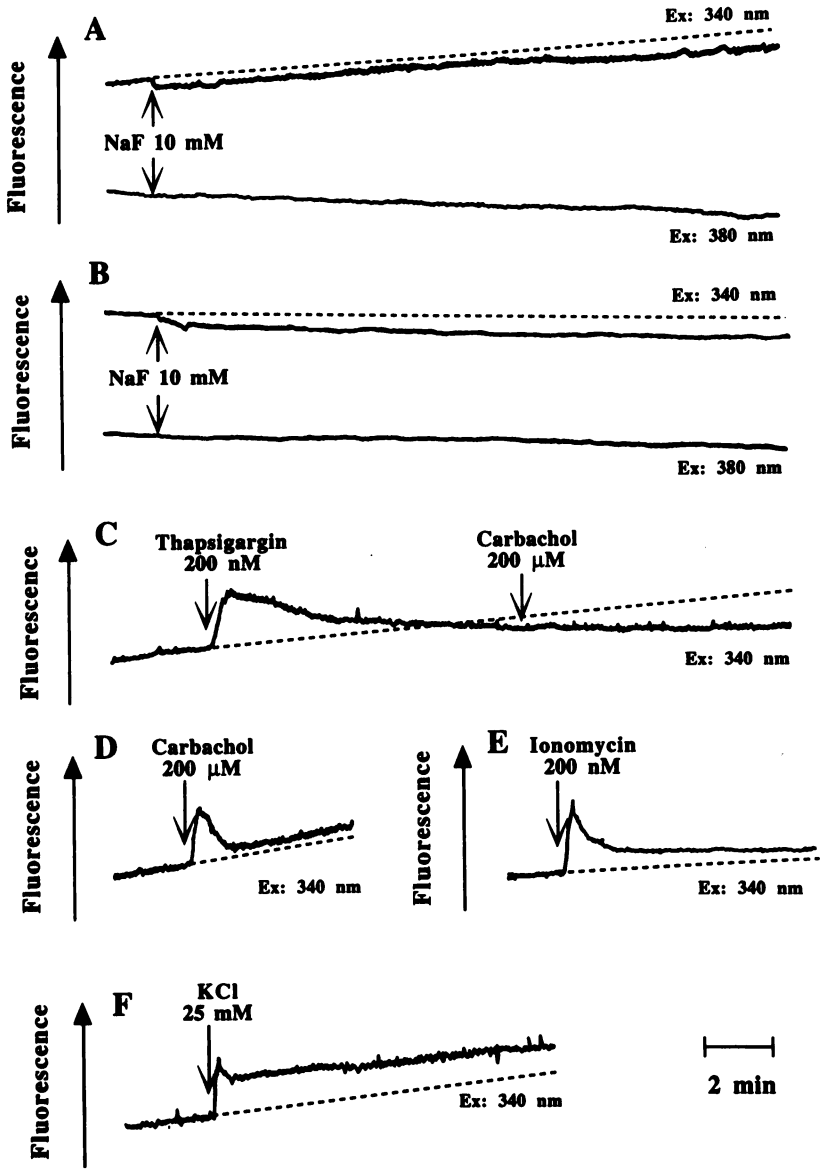

Fig. 4. Fluorescence measurement of $[Ca^{2+}]_i$ and responses to 10 mM NaF, 200 nM thapsigargin, 200 μM carbachol, 200 nM ionomycin, and 25 mM KCl. RINm5F cells were loaded with fura-2/AM as described in Experimental Procedures. A, Fluorescence was monitored in a single cuvette using two excitation wavelengths, 340 nm and 380 nm, and an emission wavelength of 510 nm. NaF (final concentration, 10 mM) was introduced into the cuvette as indicated (*arrows*). B, No cells were present in the cuvette. The fluorescence from KRB buffer containing 0.1 μM fura-2 was monitored using two excitation wavelengths, 340 nm and 380 nm, and an emission wavelength of 510 nm. NaF (final concentration, 10 mM) was introduced into the cuvette as indicated (*arrows*). C, Effects of thapsigargin and subsequent addition of carbachol are shown. D-F, Carbachol (D), ionomycin (E), and KCl (F) effects on $[Ca^{2+}]_i$ were monitored with excitation and emission wavelengths of 340 nm and 510 nm, respectively. *Dashed lines*, extrapolations from basal fluorescence values obtained from paired control cuvettes. All of the experiments shown were repeated at least three times, with similar results.

buffer and perifused the cells for 60 min before studying the effect of NaF on insulin release. These conditions have been used successfully by others to demonstrate the need for aluminum in some fluoride-stimulated intact cells (18, 21, 22). In these experiments, 5 mM NaF was used, in addition to 10 mM, because of the reported hypersensitivity of the 5 mM stimulus to deferoxamine treatment and to aluminum addition. As shown in Fig. 2, A and C, 5 mM NaF caused a monophasic increase in the rate of insulin release. The rates of insulin release began to increase after a lag period of 10 min after the exposure to NaF. Fig. 2, B and D, illustrates the temporal profile of 10 mM NaF-stimulated insulin release. The rate of insulin release also began to increase after a lag period of 10 min after the stimulation, and the peak of insulin release was observed at 30 min after stimulation. Fig. 2, A and B, shows that deferoxamine treatment had no effect on either 5 mM (Fig. 2A) or 10 mM (Fig. 2B) NaF-stimulated insulin release. We then sought other evidence for the involvement of aluminum. It was reported that low concentrations of $AlCl_3$ (in the low micromolar range) potentiated the effects of NaF on several cell systems (18, 20, 22, 32). These stimulatory effects were interpreted as effects of increased amounts of AlF_4^- and increased activation of G proteins. Therefore, we also examined the effects of NaF, with or without 10 μM $AlCl_3$ (as used by others successfully) (18, 20–22), on insulin release. The addition of 10 μM $AlCl_3$ with NaF had no effect on either 5 mM (Fig. 2C) or 10 mM (Fig. 2D) NaF-stimulated insulin release. Thus, we were unable to increase or decrease the response to NaF by increasing or decreasing the availability of aluminum. Additionally, vanadate, another phosphate analog, could not mimic the insulinotropic effect of NaF. In perifusion experiments, 10 mM orthovanadate stimulated negligible amounts of insulin release in RINm5F cells [10 mM NaF, 4.9-fold increase (37 determinations); 10 mM vanadate, 0.3-fold increase (four determinations) in 60-min perifusion periods]. Therefore, these data provide no evidence for activation of G proteins by NaF and no evidence for inhibition of enzymes such as phosphatases or ATPases by NaF in the stimulation of insulin release in RINm5F cells.

Effects of extracellular Ca^{2+} removal and nitrendipine on NaF-stimulated insulin release. To evaluate the

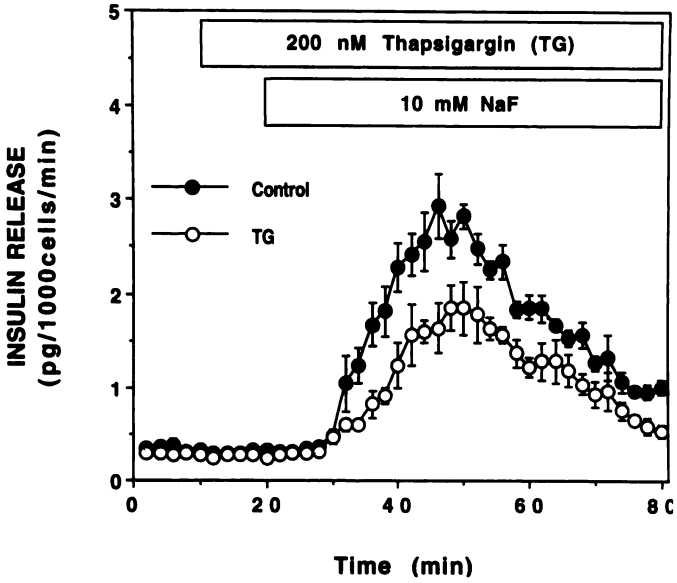

Fig. 5. Effect of pretreatment with 200 nM thapsigargin on the time course of 10 mM NaF-stimulated insulin release from RINm5F cells. Perifusion experiments were performed as described in Experimental Procedures. NaF (10 mM) was introduced as shown (*horizontal bar*). Thapsigargin was present in the perifusion solution of one set of chambers (○) 10 min before and throughout the period of NaF stimulation. Values represent the means ± standard errors of four determinations from two separate paired experiments.

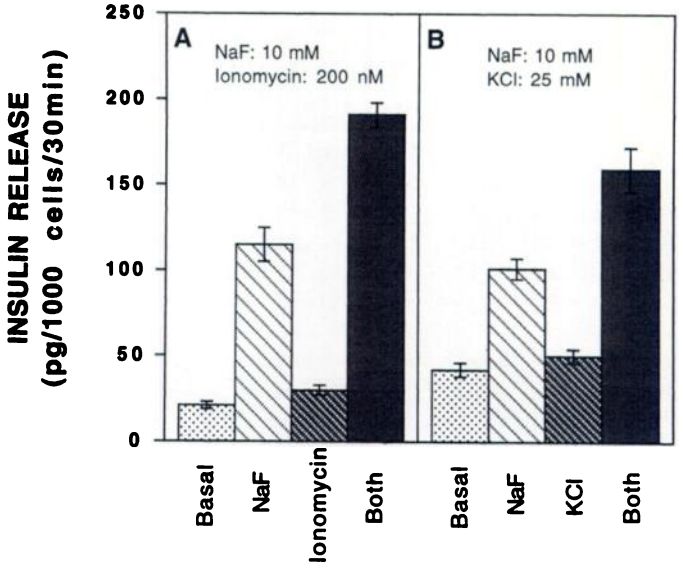

Fig. 6. Effects of ionomycin and a depolarizing concentration of KCl on NaF-stimulated insulin release. Static incubations, as described in Experimental Procedures, were performed. KRB buffer only was used in a 30-min equilibration period before the test and control incubations. Values represent the means ± standard errors of six determinations.

role of extracellular Ca^{2+} in NaF-stimulated insulin release, perifusion experiments were performed with normal Ca^{2+}-containing KRB buffer and with Ca^{2+}-free KRB buffer plus 0.2 mM EGTA. Under the Ca^{2+}-free conditions, in which Ca^{2+} was removed 10 min before the addition of NaF, 10 mM NaF produced only the slightest response (Fig. 3A). When extracellular Ca^{2+} was removed during the stimulation with NaF, as can be seen from the results in Fig. 3B, the rate of insulin release declined to nearly basal values; restoration of the Ca^{2+} in the perifusate allowed the rate of insulin release to recover to the stimulated values seen in control cells within 4 min. To determine whether Ca^{2+} entry via L-type voltage-dependent Ca^{2+} channels was involved in this dependence of NaF-stimulated insulin release on extracellular Ca^{2+}, the effect of nitrendipine, a specific and potent inhibitor of these channels, was examined. As shown in Fig. 3C, 1 μM nitrendipine did not affect NaF-stimulated insulin release.

Effects of NaF, thapsigargin, and ionomycin on changes in $[Ca^{2+}]_i$. As shown in Fig. 4A, the addition of 10 mM NaF to a suspension of fura-2-loaded RINm5F cells caused an abrupt small decrease in the fluorescence signal (using an excitation wavelength of 340 nm); thereafter the signal remained stable for at least 30 min. The fluorescence level gradually increased due to the continuous leakage of fura-2 from the cells. As can be seen in Fig. 4A, a paired control cuvette exhibited a parallel increase in fluorescence due to leakage of the dye. The administration of 10 mM NaF did not affect the fluorescence derived from an excitation wavelength of 380 nm. The fluorescence gradually decreased as a result of the leakage of fura-2. The effects of 10 mM NaF on the fluorescence signal in the absence of RINm5F cells are shown in Fig. 4B. NaF was introduced into the cuvette with regular KRB buffer in the presence of 0.1 μM fura-2. The change in the fluorescence signal in this cell-free system was similar to that seen in Fig. 4A, in the presence of RINm5F cells. This, and the lack of effect of NaF on the signal obtained using 380 nm, indicates that the initial decrease in fluorescence from an excitation wavelength of 340 nm was not due to a NaF-induced change in $[Ca^{2+}]_i$. We conclude at this point that the dependence of the effect of NaF on extracellular Ca^{2+}, without a NaF-induced increase in $[Ca^{2+}]_i$, could involve a dependence upon $[Ca^{2+}]_i$. Therefore, we sought ways of decreasing or increasing $[Ca^{2+}]_i$ as a means of testing whether the NaF responses could be modulated by changes in $[Ca^{2+}]_i$. For this purpose thapsigargin, ionomycin, and KCl were investigated. Thapsigargin, a specific inhibitor of microsomal Ca^{2+}-ATPase (33), at a concentration of 200 nM produced a rapid but transient (6-min) increase in $[Ca^{2+}]_i$, as shown in Fig. 4C. The basal and peak values of $[Ca^{2+}]_i$ were 54 nM and 101 nM, respectively. Ten minutes after the start of exposure to thapsigargin, when the $[Ca^{2+}]_i$ had returned to below basal values (47 nM), 200 μM carbachol was introduced into the cuvette. As expected under these conditions, carbachol failed to increase the $[Ca^{2+}]_i$. In the control cuvette without thapsigargin, carbachol caused its usual transient increase in $[Ca^{2+}]_i$, in this case from 88 nM (basal) to 135 nM (peak) (Fig. 4D). These results indicate that 200 nM thapsigargin caused depletion of the IP_3-sensitive intracellular Ca^{2+} pool in the RINm5F cells. Interestingly, $[Ca^{2+}]_i$ declined further, to well below the basal value, for the remainder of the observation period. As shown in Fig. 4E, 200 nM ionomycin, a Ca^{2+} ionophore, caused a brief peak of $[Ca^{2+}]_i$, which was followed by a continuous but small elevation of $[Ca^{2+}]_i$ above basal values. The basal value in this experiment was 79 nM, the peak value induced by ionomycin was 201 nM, and the sustained elevated $[Ca^{2+}]_i$ level was approximately 90 nM. Finally, a depolarizing concentration of KCl produced a rapid increase of $[Ca^{2+}]_i$, as expected, from the basal value of 59 nM to a peak value of 123 nM (Fig. 4F). An increase in fluorescence induced by 25 mM KCl reached its

peak only 15 sec after the exposure, followed by a gradual decrease of the fluorescence. However, the absolute value of $[Ca^{2+}]_i$ remained well above basal levels over the next 20 min.

Effects of thapsigargin, ionomycin, and a depolarizing concentration of KCl on NaF-stimulated insulin release. To test the possibility that the NaF response is dependent on $[Ca^{2+}]_i$, we modulated $[Ca^{2+}]_i$ down (with thapsigargin) and up (with ionomycin and a depolarizing concentration of KCl) and examined the effects upon NaF-stimulated insulin release. Pretreatment with 200 nM thapsigargin, which depleted the IP_3-sensitive intracellular Ca^{2+}-store and produced a sustained decrease in $[Ca^{2+}]_i$, caused a reduction in NaF-stimulated insulin release (Fig. 5). The elevation of $[Ca^{2+}]_i$ produced by ionomycin (Fig. 6A) and by a depolarizing concentration of KCl (Fig. 6B) clearly enhanced the NaF-stimulated insulin release, despite their having only small effects alone. Thus, although the effect of NaF is exerted at a site distal to the elevation of $[Ca^{2+}]_i$ in stimulus-secretion coupling (because it fails to increase $[Ca^{2+}]_i$), the insulin release response to NaF is modulated by $[Ca^{2+}]_i$.

Effects of NaF on insulin release in permeabilized RINm5F cells. To confirm that the insulinotropic action of NaF is modulated by $[Ca^{2+}]_i$, we examined the effect of NaF on insulin release in electrically permeabilized cells. In permeabilized cells, "$[Ca^{2+}]_i$" is easily manipulated and fixed by the free Ca^{2+} concentration of the medium. In Fig. 7A are shown the rates of insulin release from permeabilized RINm5F cells in the presence of 0.1, 1.0, and 10 μM free Ca^{2+} and the effects of 10 mM NaF on these release rates. Concentration-dependent increases in insulin release were observed with increasing concentrations of Ca^{2+}. NaF at 10 mM had no effect on the release rate in the presence of 0.1 μM Ca^{2+}. However, marked stimulation was seen at 1.0 μM Ca^{2+} and even greater stimulation at 10 μM Ca^{2+}. These data confirm that the effect of NaF is to magnify the effect of Ca^{2+} to stimulate insulin release.

Because of easy access of small molecules to the cell interior, the permeabilized cell preparation offers the opportunity to more rigorously test the potential role of aluminum in the effect of NaF than is possible in intact cell studies. Consequently, we studied the effect of NaF to stimulate insulin

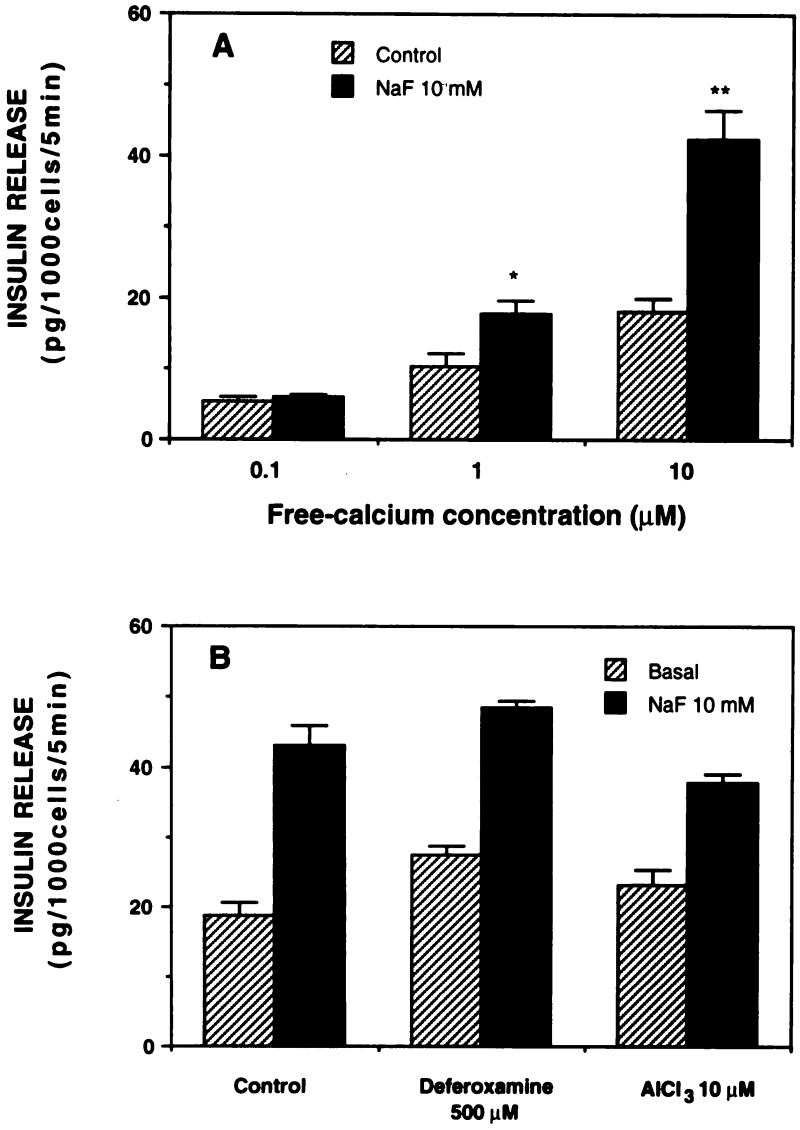

Fig. 7. Effects of NaF on insulin release in electrically permeabilized RINm5F cells. The cells were permeabilized as described in Experimental Procedures. After 15 min of preincubation at 4°, the cells were incubated under various conditions for 5 min at 37°. Supernatants were kept for insulin radioimmunoassay after centrifugation of the tubes. Values represent the means ± standard errors of five determinations. *, $p < 0.005$; **, $p < 0.000$, versus respective controls.

release in permeabilized cells, in the presence or absence of 500 μM deferoxamine or 10 μM $AlCl_3$. These results are shown in Fig. 7B. It can be seen in Fig. 7B, *left*, that NaF enhanced the rate of insulin release due to 10 μM Ca^{2+}. As can be seen from the data in Fig. 7B, *center* and *right*, deferoxamine did not attenuate the effect of NaF, and $AlCl_3$ did not potentiate the effect of NaF. These data provide additional evidence that the effect of NaF to stimulate insulin release is not due to AlF_4^-.

Effects of NaF and forskolin on cellular cAMP contents in RINm5F cells. In an examination of the involvement of cAMP as a potential second messenger in NaF-stimulated insulin release, cellular cAMP contents were quantified under static incubation conditions for 10, 20, and 30 min. As shown in Fig. 8A, 10 mM NaF did not increase, and in fact slightly decreased, cellular cAMP contents at all time points studied. The fact that the cells could respond to an adequate stimulus was shown by the increase of cellular cAMP levels induced by 1 μM forskolin. Simultaneous measurements of insulin release by these cells confirmed that 10 mM NaF stimulated insulin release in a time-dependent manner (Fig. 8B). Forskolin, as expected under these basal conditions, had only slight effects on insulin release.

Effects of inhibitors of protein kinase C on NaF-stimulated insulin release. The effect of staurosporine, a potent inhibitor of protein kinase C (34), on 10 mM NaF-stimulated insulin release was tested in 30-min static incubations. As can be seen in Table 1, staurosporine did not suppress NaF-stimulated insulin release at any concentration tested. Staurosporine (1 μM), as expected, greatly inhibited 100 nM TPA-stimulated insulin release from RINm5F cells (basal, 34 ± 3 pg of insulin/10^3 cells/30 min; TPA, 129 ± 6 pg of insulin/10^3 cells/30 min; basal plus staurosporine, 25 ± 0 pg of insulin/10^3 cells/30 min; TPA plus staurosporine, 42 ± 2 pg of insulin/10^3 cells/30 min; six determinations). The effects of another protein kinase C inhibitor, bisindolylmaleimide (35), were also examined. This inhibitor (at concentrations up to 1 μM), like staurosporine, did not attenuate 10 mM NaF-stimulated insulin release (data not shown), whereas this inhibitor effectively suppressed TPA-induced insulin release in RINm5F cells, as we reported previously (15).

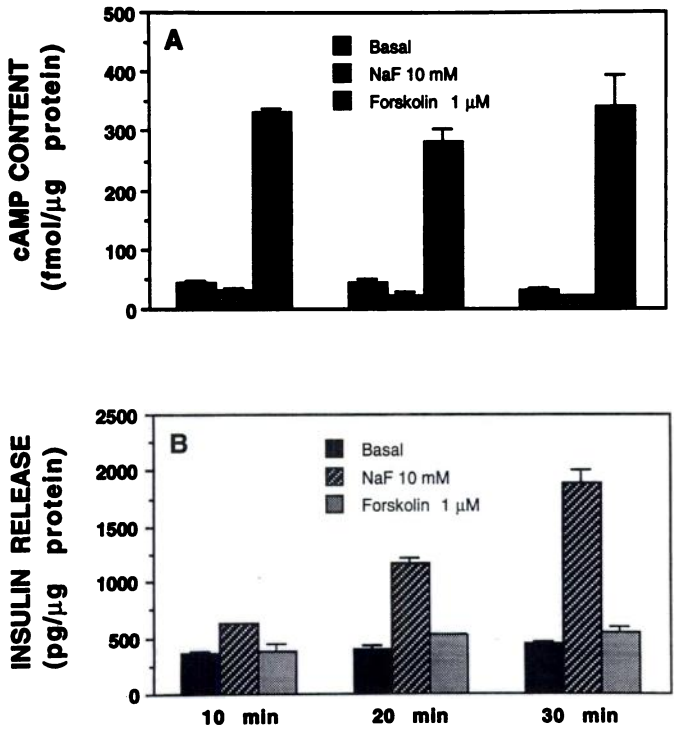

Fig. 8. Effects of NaF and forskolin on cellular cAMP content and insulin release. After 15 min of preincubation in KRB buffer, the cells were incubated with 10 mM NaF or 1 μM forskolin at 37° for 10, 20, or 30 min, with gentle shaking. Cellular cAMP and released insulin were determined using radioimmunoassays, as described in Experimental Procedures. Values are the means ± standard errors from a single experiment performed in triplicate.

TABLE 1

Lack of effect of staurosporine on 10 mM NaF-stimulated insulin release

Static incubations, as described in Experimental Procedures, were performed. Staurosporine was present throughout the preincubation and incubation periods. Values represent the means ± standard errors of four determinations.

	Insulin release			
	0 μM[a]	0.01 μM	0.1 μM	1 μM
	pg/10^3 cells/30 min			
Basal	23 ± 1	ND[b]	ND	18 ± 1
NaF (10 mM)	51 ± 8	48 ± 10	54 ± 9	43 ± 3

[a] Staurosporine concentration.
[b] ND, not determined.

Effects of NaF and melittin on arachidonic acid release from RINm5F cells. Because arachidonic acid stimulates Ca^{2+}-independent insulin release (36) and NaF is known to stimulate arachidonic acid release from human platelets (37), [^{3}H]arachidonic acid release from [^{3}H]arachidonic acid-preloaded RINm5F cells was measured. Melittin, which increases the phospholipid susceptibility to phospholipase A_2 (31), was used to show that [^{3}H]arachidonic acid-preloaded cells could release ^{3}H by the activation of phospholipase A_2. The results are shown in Table 2. Although 5 and 10 mM NaF stimulated insulin release in a concentration-dependent manner, both concentrations of NaF failed to increase arachidonic acid release. In sharp contrast to these findings, melittin strongly stimulated arachidonic acid release concomitant with increased insulin release.

TABLE 2

Effects of NaF and melittin on ^{3}H and insulin release by RINm5F cells preloaded with [^{3}H]arachidonic acid

RINm5F cells were loaded with 0.25 μCi/ml of [^{3}H]arachidonic acid in culture medium for 20 hr. Cells were washed three times with KRB buffer. Then, 30-min incubations with various concentrations of NaF or melittin were carried out. Released radioactivity was determined by liquid scintillation counting. Insulin release was determined by radioimmunoassay. Both ^{3}H release and insulin release were expressed as percentages of basal response which were designated 100%. The absolute value of basal ^{3}H release was 199 ± 14 cpm. The absolute value of basal insulin release was 53 ± 3 pg/10^3 cells/30 min. Values indicate the means ± standard errors of eight (in the case of NaF) or four (in the case of melittin) determinations.

	Release			
	NaF		Melittin	
	5 mM	10mM	0.5 μg/ml	2 μg/ml
	% of basal			
Arachidonic acid release	106 ± 25	107 ± 23	707 ± 80[a]	1435 ± 110[a]
Insulin release	201 ± 37[b]	368 ± 45[a]	241 ± 9[a]	495 ± 36[a]

[a] $p < 0.01$ versus basal values.
[b] $p < 0.05$.

Effects of forskolin and TPA on NaF-stimulated insulin release. Fig. 9 shows the effects of forskolin and TPA on NaF-stimulated insulin release measured in 30-min static incubations. As shown in Fig. 9A, 1 μM forskolin (a stimulator of adenylyl cyclase), as expected with the cells under basal conditions, did not stimulate insulin release. However, it clearly enhanced NaF-stimulated insulin release. TPA (30 nM), an activator of protein kinase C, and NaF (10 mM) caused 0.6-fold and 1.5-fold increases in insulin release, respectively. Simultaneous stimulation with TPA and NaF produced a synergistic 5.9-fold increase in insulin release (Fig. 9B). In RINm5F cells, TPA causes membrane depolarization and activation of L-type voltage-dependent Ca^{2+} channels, leading to an elevation of $[Ca^{2+}]_i$ (29, 38). To exclude this effect and to detect the effect of TPA-induced protein kinase C activation that is not caused by elevation of $[Ca^{2+}]_i$, we examined the effect of TPA in the presence of a high concentration (5 μM) of the calcium channel antagonist nitrendipine. As shown in Fig. 9C, TPA and NaF, even in the presence of this maximally effective concentration of nitrendipine, still showed a synergistic effect on the stimulation of insulin release. It is concluded that both protein kinase A and protein kinase C are able to enhance the effect of NaF to stimulate insulin release.

Inhibitory effects of Trifluoperazine and W-7 on NaF-stimulated insulin release. Although NaF itself did not change the $[Ca^{2+}]_i$, its insulinotropic effect was synergistically increased by an elevation of $[Ca^{2+}]_i$ and was decreased by a reduction of $[Ca^{2+}]_i$. Therefore, it is possible that NaF acts at the same site as $[Ca^{2+}]_i$ in stimulus-secretion coupling or at a related site where $[Ca^{2+}]_i$ exerts its effect on (presumably) a Ca^{2+}-binding protein. Under normal circumstances an elevation of $[Ca^{2+}]_i$ is required for the stimulation of release. The action of NaF would be to sensitize the Ca^{2+}-binding protein such that basal $[Ca^{2+}]_i$ is stimulatory. To test this possibility, we examined the effect of trifluoperazine, a calmodulin antagonist, on NaF-stimulated insulin release. Fig. 10 shows the effect of 30 μM (Fig. 10A) and 50 μM (Fig. 10B) trifluoperazine on the rates of insulin release induced by stimulation with 10 mM NaF. Basal insulin release was not affected by trifluoperazine, whereas NaF-stimulated insulin release was greatly suppressed. Fig. 11A shows the inhibitory effects of various concentrations of trifluoperazine on NaF-stimulated insulin release under static incubation conditions. Trifluoperazine inhibited the NaF-stimulated insulin release in a concentration-dependent manner, and at a concentration of 50 μM it completely inhibited the insulinotropic action. We examined also the effects of another calmodulin antagonist, W-7, on the NaF-stimulated insulin release. As shown in Fig. 11B, W-7, at concentrations up to 30 μM, had no effect on NaF-stimulated insulin release. W-7 at 50 μM inhibited release by 27% and at 100 μM by 90%.

Discussion

In this study we demonstrate that NaF stimulates insulin secretion in a unique way. Increasing $[Ca^{2+}]_i$ with ionomycin or with a depolarizing concentration of KCl or decreasing $[Ca^{2+}]_i$ with thapsigargin enhanced or diminished the response, respectively. Lowering the $[Ca^{2+}]_i$ by removal of extracellular Ca^{2+} markedly inhibited the response. Furthermore, in electrically permeabilized RINm5F cells, NaF enhanced Ca^{2+}-induced insulin release. Therefore, NaF, which does not change $[Ca^{2+}]_i$ itself, is influenced by $[Ca^{2+}]_i$. These results indicate that the site of action of Ca^{2+} in stimulus-secretion coupling may be the site of action of NaF. One obvious possibility is that, whether this is direct or indirect, the action of NaF at this site increases the sensitivity of the site to Ca^{2+}. This notion is supported by the fact

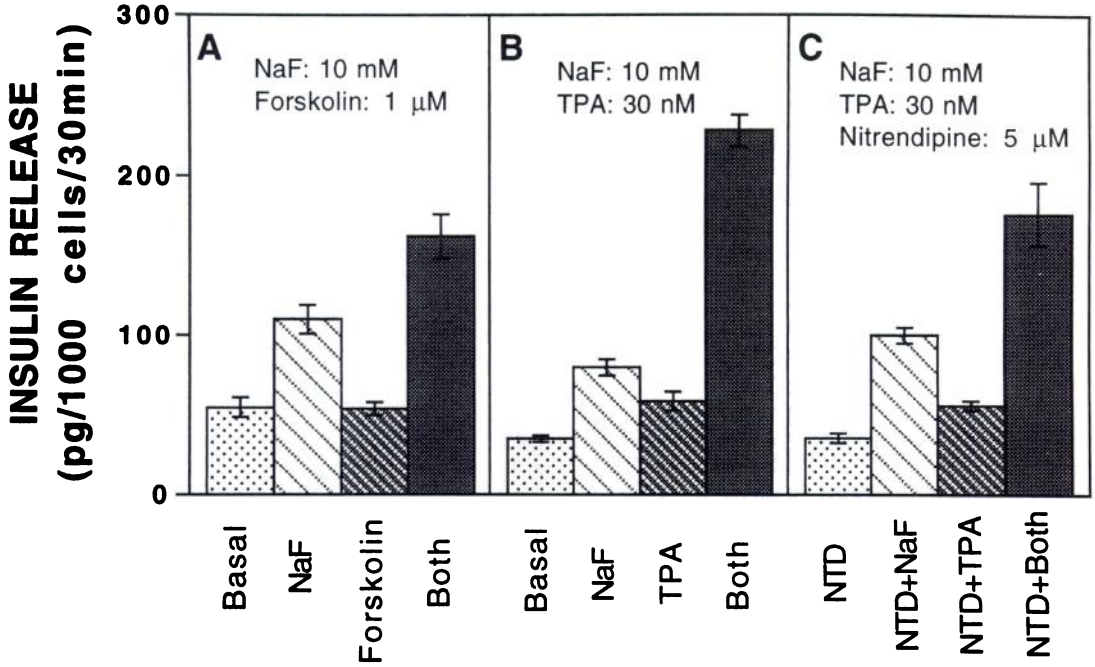

Fig. 9. Effects of forskolin and TPA on NaF-stimulated insulin release. Static incubations, as described in Experimental Procedures, were performed. KRB buffer only was used in a 30-min equilibration period before the test and control incubations, except in C, where nitrendipine (*NTD*) was present through the equilibration and incubation periods. Values represent the means ± standard errors of six determinations.

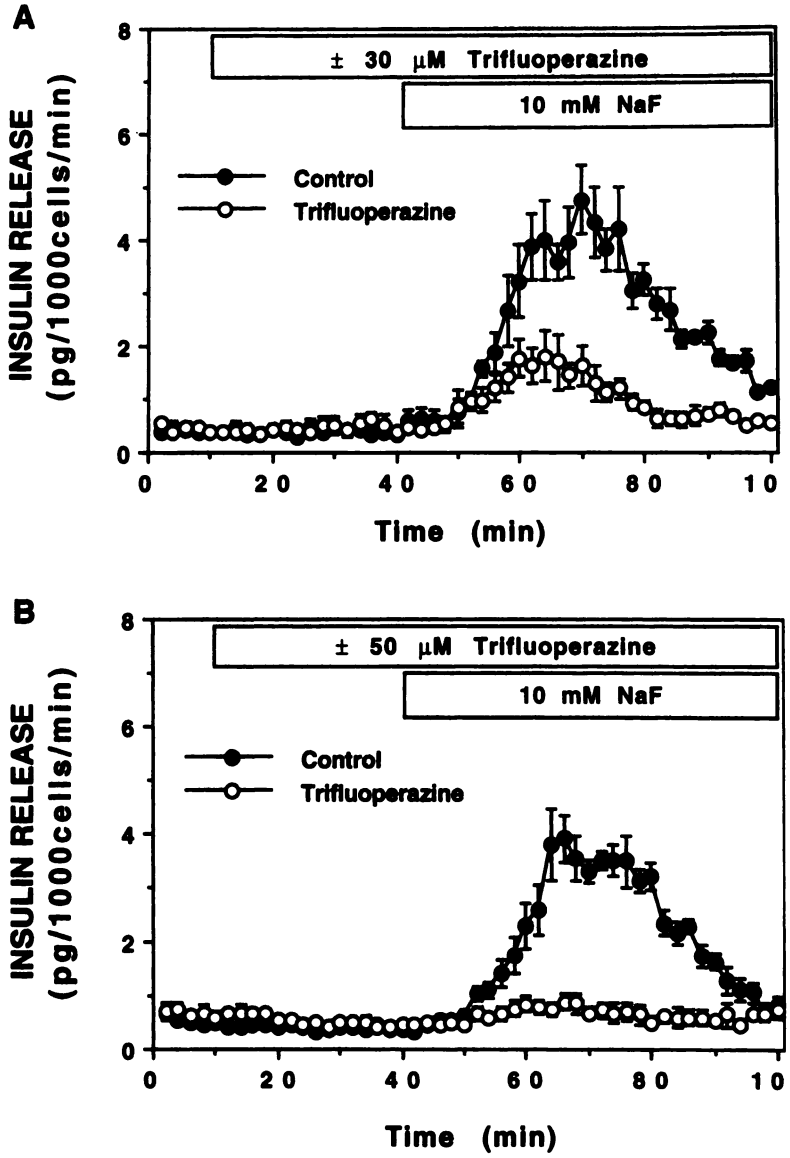

Fig. 10. Inhibitory effects of trifluoperazine on 10 mM NaF-stimulated insulin release. Perifusion experiments were performed as described in Experimental Procedures. Trifluoperazine (30 μM in A and 50 μM in B) was introduced into the chambers (○) as shown (*horizontal bars*). At 40 min, 10 mM NaF was introduced into all chambers. Values represent the means ± standard errors of four determination from two separate paired experiments.

that trifluoperazine and W-7 inhibited the NaF-stimulated insulin release.

The stimulation of insulin release appears to be "physiological," in the sense that it is temperature sensitive, enhanced by protein kinases A and C, and reversible (by washing away the NaF). The viability of the cells was unchanged after treatment with NaF, and the cells responded to glyceraldehyde similarly to control cells that had not been exposed to NaF. Finally, the stimulation of release by NaF was potentiated by activators of protein kinase A and C, a finding that leads to another important conclusion. The site of action at which the two kinases potentiate the rate of insulin release is at or beyond the site of action of Ca^{2+} (and NaF) in stimulus-secretion coupling. This conclusion is compatible with previous reports that activation of protein kinase A (39, 40) and C (29, 39) could directly stimulate exocytosis beyond the site of Ca^{2+}.

The lack of effect of deferoxamine treatment and addition of $AlCl_3$ to the KRB buffer to modulate the availability of AlF_4^- was surprising. Although we cannot be sure that treatment with 200 μM deferoxamine was sufficient to completely chelate the extracellular and intracellular Al^{3+}, in other studies similar treatment with deferoxamine successfully suppressed the effects of NaF (18, 21, 22). Furthermore, we performed experiments using higher concentrations of deferoxamine (up to 500 μM) without seeing any effect on the NaF-stimulated insulin release. Similarly, there are several reports that addition of $AlCl_3$ enhances the effects of NaF (18, 20, 22, 32). In addition to the data in Fig. 2, we examined the effects of concentrations of $AlCl_3$ up to 100 μM, and none of these additions potentiated the insulinotropic action of NaF. Perhaps more rigorously, in the permeabilized cells neither 500 μM deferoxamine treatment nor the addition of 10 μM $AlCl_3$ affected the insulinotropic action of NaF. Considering these data, we conclude that the insulinotropic action of NaF in RINm5F cells is unlikely to require Al^{3+} ions. This conclusion suggests a novel mechanism of action of NaF. Among the known effects of NaF are inhibition of enzymes such as phosphatases or ATPases, inhibition of metabolism, and stimulation of heterotrimeric G proteins. However, all of these effects of NaF required trace amounts of Al^{3+}, because the structure of AlF_4^- is very similar to that of a phosphate

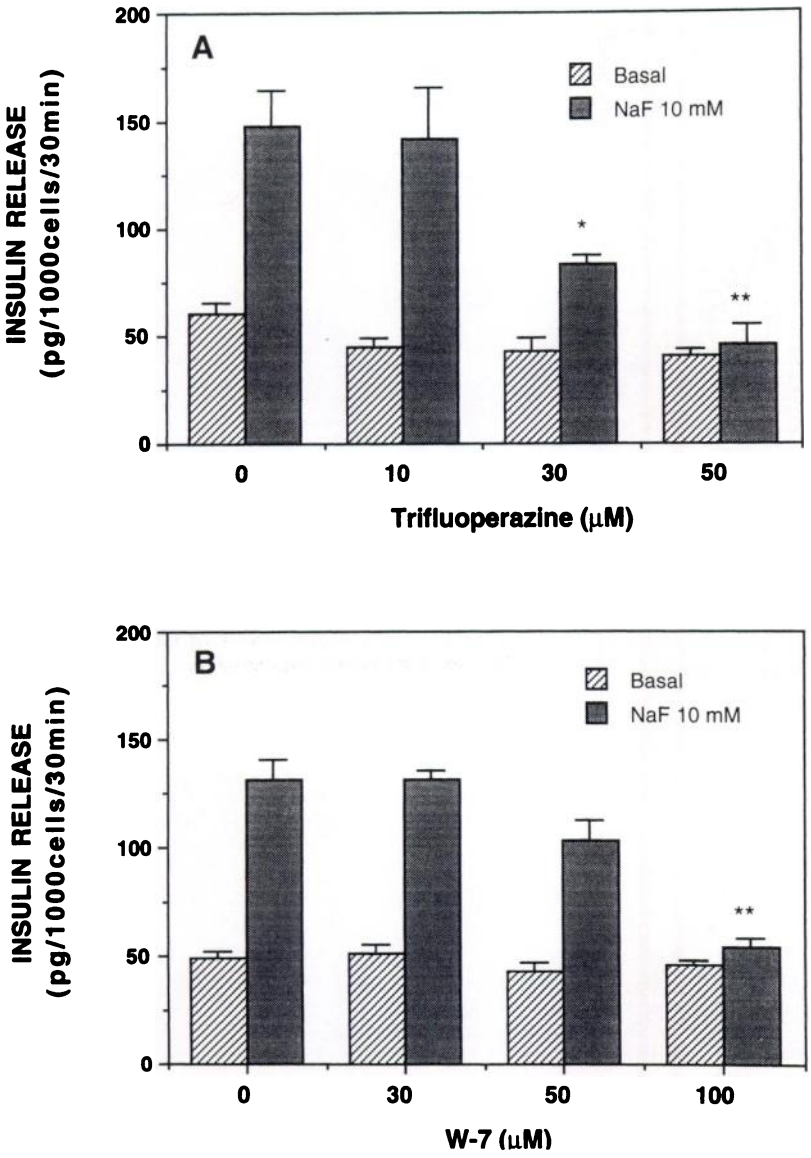

Fig. 11. Effects of various concentrations of trifluoperazine (A) and W-7 (B) on 10 mM NaF-stimulated insulin release. Static incubation experiments were performed as described in Experimental Procedures. Values represent the means ± standard errors of six determinations. *, $p < 0.05$; **, $p < 0.01$, versus 10 mM NaF alone.

group and AlF_4^- acts as a high affinity analog of phosphate (17, 27, 28). Supportive evidence that the insulinotropic action of NaF is not due to its structural analogy to a phosphate group comes from the fact that vanadate could not mimic the effect of NaF in RINm5F cells, because, in some situations, vanadate acts as a phosphate analog and exhibits effects similar to those of AlF_4^- (17, 28).

Previous studies have shown stimulation of exocytosis by NaF in different cell types (24–26) and have suggested a possible mechanism of action. All suggest G protein involvement, because NaF is a well known G protein activator. In one study using the RINr cell line (25), NaF stimulated both insulin release and phosphoinositide hydrolysis. This led to the conclusion that activation of G proteins, with subsequent activation of phospholipase C and a resulting increase in $[Ca^{2+}]_i$ and protein kinase C activity, was a sufficient explanation for the stimulation of secretion. This mechanism is not involved in the stimulation of insulin release demonstrated in our studies, because NaF did not elevate $[Ca^{2+}]_i$, nor was its effect eliminated by depletion of the IP_3-sensitive intracellular Ca^{2+} store with thapsigargin. Furthermore, inhibitors of protein kinase C at concentrations shown to inhibit the effects of TPA had no effect on the response to NaF. NaF (as AlF_4^-) is well known to stimulate adenylyl cyclase in membrane preparations (21); however, in intact RINm5F cells cellular cAMP content was not increased by NaF. In permeabilized platelets, NaF (as AlF_4^-) was reported to stimulate arachidonic acid release (37); however, NaF did not increase arachidonic acid release from RINm5F cells. Therefore, we exclude the possible involvement of these second messengers in the mechanism of NaF-stimulated insulin release.

In view of the results of this study, what are the possible mechanisms by which NaF could stimulate exocytosis in RINm5F cells? The insulinotropic action of NaF was enhanced by increases in $[Ca^{2+}]_i$ and decreased by reductions in $[Ca^{2+}]_i$. In the permeabilized RINm5F cells, NaF also enhanced (sensitized) Ca^{2+}-induced insulin release. These findings suggest the involvement of Ca^{2+}-binding proteins in the mechanism of NaF-stimulated insulin release. Indeed, trifluoperazine inhibited NaF-stimulated insulin release in a concentration-dependent manner, and 50 μM trifluoperazine

completely abolished the insulinotropic action of NaF. In general, it is rather difficult to interpret the inhibitory effects of trifluoperazine on exocytosis, because this compound also inhibits Ca^{2+} influx over a similar concentration range. However, NaF did not cause Ca^{2+} influx and a Ca^{2+} channel blocker did not affect the NaF-stimulated insulin release in RINm5F cells. Inhibition of Ca^{2+} influx by trifluoperazine, therefore, is not the cause of the inhibition of NaF-stimulated insulin release. In view of the inhibitory effect of trifluoperazine, calmodulin is one of a number of possible candidates for the Ca^{2+}-binding protein responsible for the action of NaF. However, the inhibitory effect of W-7 occurred at higher concentrations than would be expected for an action on calmodulin, as was also the case for trifluoperazine. It is, therefore, likely that a Ca^{2+}-binding protein that is sensitive to trifluoperazine but is less sensitive to W-7 is a target of NaF in RINm5F cells.

Summarizing these data, NaF stimulates insulin release in a concentration-dependent, temperature-dependent, and reversible manner. The stimulated release is dependent upon extracellular Ca^{2+} but does not influence $[Ca^{2+}]_i$. Rather, it appears to modulate the effect of $[Ca^{2+}]_i$ on the rate of insulin secretion. Thus, increasing $[Ca^{2+}]_i$ enhances the effect of NaF and decreasing $[Ca^{2+}]_i$ diminishes the effect of NaF. The best explanation for these data appears to be that NaF acts on the same site in stimulus-secretion coupling as does Ca^{2+} and that it sensitizes the site to the action of Ca^{2+}. This explanation is supported by the fact that both trifluoperazine and W-7 effectively inhibited the insulinotropic action of NaF. In this way, the basal $[Ca^{2+}]_i$ becomes a stimulatory concentration in the presence of NaF. In studying the mechanism of action of NaF, it appears unlikely that activation of a heterotrimeric G protein or inhibition of enzymes such as phosphatases are involved. Also, no evidence for an action through phospholipase A_2 or C or protein kinase A or C activation was detected. In fact, both protein kinase A and protein kinase C were shown to enhance the action of NaF. These findings lead us to believe that the effects of Ca^{2+} and NaF are exerted at the same site or related sites in stimulus-secretion coupling and that kinases A and C act either at these sites or at a more distal site. The actions of NaF will be important for understanding the late steps, after the elevation of $[Ca^{2+}]_i$, that lead to stimulated exocytosis. Although the current studies do not explain the mechanism of action of fluoride to stimulate insulin release, the characterization provided and the unusual nature of the dependence on $[Ca^{2+}]_i$ provide an important starting point for studies on the mechanisms involved.

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