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Inhibitory and stimulatory effects of fluoride on the calcium pump of cardiac sarcoplasmic reticulum

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While studying the effects of membrane phosphorylation on active Ca^{2+} transport in cardiac sarcoplasmic reticulum (SR) we used NaF (a conventional phosphatase inhibitor) in the Ca^{2+} transport assay medium to suppress protein dephosphorylation by endogenous phosphatases. Unexpectedly, depending on the experimental conditions employed, NaF was found to cause a strong inhibitory or stimulatory effect on ATP-dependent, oxalate-facilitated Ca^{2+} uptake (Ca^{2+} pump) activity of SR. Investigation of this phenomenon using canine cardiac SR revealed the following. Exposure of SR to NaF in the absence of Ca^{2+} or ATP in the Ca^{2+} transport assay medium (prior to initiating Ca^{2+} transport by the addition of Ca^{2+} or ATP) promoted a striking concentration-dependent inhibitory effect of NaF (50% and 90% inhibition with approx. 4 and 10 mM NaF, respectively) on Ca^{2+} uptake by SR; the magnitude of inhibition did not differ appreciably with varying oxalate concentrations. In contrast, exposure of SR to NaF in the presence of both Ca^{2+} and ATP resulted in a concentration-dependent stimulatory effect of NaF (half-maximal stimulation at approx. 2.5 mM NaF with 2.5 mM oxalate in assay) on Ca^{2+} uptake; the magnitude of stimulation decreased with increasing oxalate concentration (> 2-fold at 1 mM oxalate, 10% at 5 mM oxalate). The inhibitory effect prevailed when SR was exposed to NaF in the presence of Ca^{2+} alone (without ATP) or ATP alone (without Ca^{2+}). Both the inhibitory and stimulatory effects of NaF were specific to fluoride ion, as NaCl (1–10 mM) showed no effect on Ca^{2+} uptake by SR under identical assay conditions. A persistently less active state of the Ca^{2+} pump (evidenced by decreased Ca^{2+} transport rates) resulted upon pretreatment of SR with NaF in the absence of Ca^{2+} or ATP; presence of Ca^{2+} and ATP during pretreatment prevented this transition. The inhibitory action of NaF on the Ca^{2+} pump was accompanied by a two-fold increase in $K_{0.5}$ for Ca^{2+} and decrements in Hill coefficient (n_H) and Ca^{2+}-stimulated ATP hydrolysis, as well as steady-state level of Ca^{2+}-induced phosphoenzyme. The stimulatory effect of NaF, on the other hand, was associated with an increase in the ratio of Ca^{2+} transported/ATP hydrolysed with only minor changes, if any, in the above parameters. These findings imply that the divergent effects of fluoride are dependent on specific conformational states of the Ca^{2+}-ATPase which evolve during the catalytic and ion transport cycle. It is suggested that the inhibitory effect ensures encounter of fluoride with the ground state of the enzyme (E conformation) and its Ca^{2+}-induced E_1 conformation, whereas enzyme conformations which evolve sequentially upon ATP binding to the E_1 conformation are resistant to inhibiton by fluoride. The stimulatory effect is then accounted for by the ability of fluoride to facilitate Ca^{2+} precipitation within the SR lumen, provided the Ca^{2+} pump is functioning normally.

Introduction

In skeletal and cardiac muscle, the Ca^{2+} pump of sarcoplasmic reticulum (SR) plays a central role in promoting muscle relaxation by actively sequestering

Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Ca^{2+} from the myoplasm. The Ca^{2+} pump activity of SR resides in the membrane-bound Ca^{2+}-ATPase which transports Ca^{2+} into the SR lumen at the expense of ATP hydrolysis. The mechanism of active Ca^{2+} transport by the SR Ca^{2+}-ATPase is recognized to involve cyclic transitions between two major conformations (for reviews see Refs. 1–4). In the initial phase of the catalytic cycle, the enzyme interacts with Ca^{2+} and ATP on the cytoplasmic side of the membrane in the E_1 conformation. This is followed by the phosphorylation of an aspartic acid residue in the active site and the occlusion of Ca^{2+} by the ADP-sensitive phosphorylated enzyme intermediate, E_1.P.Ca, into an ADP-insensitive form, E_2.P.Ca, with a decrease in its Ca^{2+} affinity and the subsequent release of Ca^{2+} into the SR lumen. The Mg^{2+}-catalysed cleavage of the acyl-phosphate bond leads to the release of P_i, followed by the isomerization of the enzyme from the E_2 into the E_1 form.

Fluoride is a well known activator of adenylate cyclase [5–7] and a potent inhibitor of protein phosphatase [8,9]. By virtue of its latter property, fluoride is commonly used to sustain the phosphorylated state of proteins in studies examining the role of protein phosphorylation in the regulation of cellular functions. In cardiac muscle, a widely recognized physiological mechanism for the regulation of SR Ca^{2+} pump involves cyclic AMP-mediated phosphorylation of the intrinsic SR protein phospholamban [10–12]. Phosphorylation of phospholamban results in stimulation of active Ca^{2+} uptake by SR, owing to acceleration of the intermediate steps in the catalytic cycle of the Ca^{2+}-ATPase [13,14]. While studying the effects of aging on phospholamban phosphorylation and Ca^{2+} transport in cardiac SR, we used fluoride (NaF), the conventional phosphatase inhibitor, to suppress protein dephosphorylation. Unexpectedly, depending on the experimental conditions employed, fluoride was found to cause marked inhibitory, as well as stimulatory, effects on the ATP-energized Ca^{2+} uptake (Ca^{2+} pump) activity of SR. In general, exposure of SR to fluoride in the absence of Ca^{2+} or ATP caused inhibition in the rates of ATP-dependent, oxalate-facilitated Ca^{2+} up take by the membrane vesicles. In contrast, exposure of SR to fluoride in the presence of Ca^{2+} and ATP resulted in stimulation of Ca^{2+} uptake. The stimulatory but not the inhibitory effect of fluoride was markedly influenced by the concentration of oxalate in the Ca^{2+} transport assay medium. In this report, we describe results of experiments demonstrating these divergent effects of fluoride on the SR Ca^{2+} pump. Based on the observations made it is suggested that the inhibitory and stimulatory effects of fluoride are dependent on specific conformational states of the Ca^{2+}-ATPase which evolve during the catalytic and ion transport cycle.

Materials and methods

Chemicals

$^{45}CaCl_2$ and [γ-^{32}P]ATP were purchased from New England Nuclear, Montreal, Canada. All other chemicals were of highest purity available from Sigma, St. Louis, MO, U.S.A. or Fisher Scientific, NJ, U.S.A.

Isolation of membranes

SR-enriched membrane vesicles were isolated from canine hearts according to the procedure of Harigaya and Schwartz [15] with minor modifications as described previously [16]. Following isolation, the membranes were suspended in 10 mM Tris maleate (pH 6.8) containing 100 mM KCl. Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as standard. The experiments described here utilized SR isolated from fresh cardiac muscle as well as cardiac muscle stored frozen at −80°C for up to 2 weeks. The Ca^{2+} transport velocities of SR isolated from fresh muscle were invariably higher compared to SR derived from frozen muscle. However, the effects of fluoride on Ca^{2+} transport were found to be similar with SR preparations from fresh and frozen cardiac muscle (see Results).

Ca^{2+} transport and Ca^{2+}-ATPase assays

ATP-dependent, oxalate-facilitated Ca^{2+} uptake by SR was determined using the Millipore filtration technique as detailed elsewhere [18]. The standard incubation medium (total volume 1 ml) contained 50 mM Tris maleate (pH 6.8), 5 mM $MgCl_2$, 2.5 mM ATP, 120 mM KCl, 2.5 mM potassium oxalate, 5 mM NaN_3, 0.1 mM EGTA, SR (20–30 μg protein) and varying concentrations of $^{45}CaCl_2$ (~8000 cpm/nmol). The free Ca^{2+} concentrations in the assay medium were determined as described previously [18]. Modifications to the standard incubation medium are specificed in the Figure legends and the table. All assays were performed at 37°C; the Ca^{2+} uptake reaction was initiated by the addition of ATP, Ca^{2+}, Mg^{2+} or SR (as specified in the figure legends and the table) following preincubation of the rest of the assay components for 3 min. Ca^{2+}-ATPase activity was determined as described previously [19]. The incubation medium for the assay of Ca^{2+}-ATPase was identical to that used for the Ca^{2+} transport assay except that [γ-^{32}P]ATP was used instead of non-radioactive ATP and non-radioactive $CaCl_2$ was used instead of $^{45}CaCl_2$.

Phosphoenzyme formation

Steady-state levels of Ca^{2+}-induced phosphoenzyme were measured at 23°C using [γ-^{32}P]ATP as described by Hidalgo et al. [20]. The composition of the reaction mixture (total volume 1 ml) was identical to that described for the Ca^{2+} transport assay except that [γ-

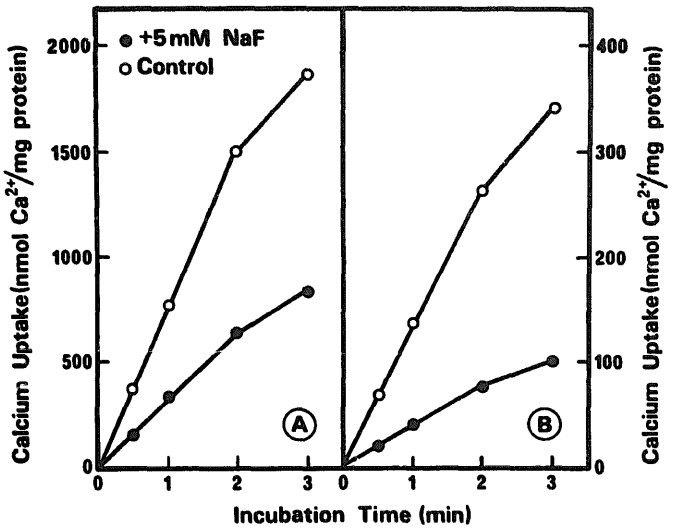

Fig. 1. Time course of ATP-dependent Ca^{2+} uptake by cardiac SR in the absence and presence of NaF. Ca^{2+} uptake was determined using the standard incubation medium (see Materials and Methods) containing 11.9 μM free Ca^{2+}. Ca^{2+} uptake was initiated by the addition of ATP to the incubation medium following preincubation of the rest of the assay components for 3 min at 37°C. Results obtained using SR prepared from fresh (panel A) and frozen (panel B) cardiac muscle are shown.

^{32}P]ATP (25 μM) was used instead of non-radioactive ATP and non-radioactive $CaCl_2$ (free Ca^{2+}, 11.9 μM) was used instead of $^{45}CaCl_2$. The reaction was initiated by the addition of ATP or SR following preincubation of the rest of the assay mixture for 3 min at 23°C. The reaction was allowed to proceed for 15 s and was stopped by adding 0.5 ml of 20% trichloracetic acid. The acid-denatured protein was filtered through HA Millipore filters, previously washed with 1 ml of 0.2 mM ATP. The filters were subsequently washed with 15 ml (5 ml × 3) of 5% trichloroacetic acid containing 0.2 mM KH_2PO_4, dried, and counted in a liquid scintillation counter.

Data presentation

Each experiment described here was repeated at least three times using separate SR preparations, and the results obtained were similar. Data from representative experiments are shown.

Results

Effects of NaF on the time course of Ca^{2+} uptake by SR

Fig. 1 shows the time course of ATP-dependent Ca^{2+} uptake by SR in the absence of NaF and in the presence of 5 mM NaF in the assay medium. In this experiment, the Ca^{2+} uptake was initiated by the addition of ATP to SR preincubated for 3 min with the rest of the assay components. Under these conditions, NaF caused marked decreases (55–70%) in the rates of Ca^{2+} uptake by SR preparations from fresh (panel A) and frozen (panel B) cardiac muscle. The SR preparation from fresh heart tissue exhibited nearly 5-fold higher rates of Ca^{2+} uptake than the SR preparation from frozen tissue. Nevertheless, the effects of NaF were similar with either SR preparation. Subsequent studies utilized membranes derived from fresh as well as frozen heart muscle.

To investigate the possibility that the decreased rates of Ca^{2+} uptake observed in the presence of NaF resulted from NaF-promoted Ca^{2+} efflux from SR vesicles rather than inhibition of active Ca^{2+} transport, the following experiment was performed. After preincubation of SR in the standard Ca^{2+} transport assay medium (free Ca^{2+}, 11.9 μM) in the absence of NaF, Ca^{2+} uptake was initiated by the addition of ATP. When Ca^{2+} uptake approached a steady state, NaF

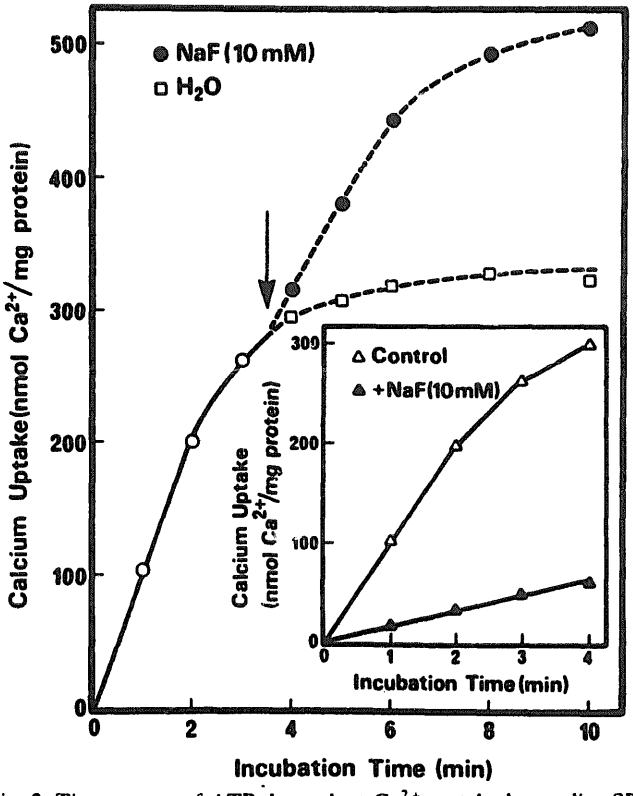

Fig. 2. Time course of ATP-dependent Ca^{2+} uptake by cardiac SR: effect of addition of NaF during active Ca^{2+} sequestration. The results shown in the main figure were obtained as follows. SR was preincubated for 3 min at 37°C in the standard incubation medium (see Materials and Methods) containing 11.9 μM free Ca^{2+} in the absence of NaF. Ca^{2+} uptake was initiated by the addition of ATP (at zero time in figure) and aliquots of the assay medium were removed at selected time intervals to quantitate SR-associated Ca^{2+}. At the time indicated by the arrow (i.e. 3.5 min after the addition of ATP), NaF (0.02 ml) was added to the incubation medium to give a final concentration of 10 mM; control tubes received an identical volume of vehicle solution (0.02 ml water). Subsequently, aliquots of the incubation medium were removed at various time intervals for a period of up to 10 min and the amount of Ca^{2+} remaining in SR was determined. The inset shows the time course of Ca^{2+} uptake by the same SR preparation in the absence of NaF (control) and in the presence of 10 mM NaF (free Ca^{2+}, 11.9 μM) under conditions identical to those described for Fig. 1. SR derived from frozen cardiac muscle was used for these experiments.

was added to the assay medium (to yield final concentration of 10 mM); the control tubes received an equivalent volume of vehicle solution (0.02 ml H_2O). Fig. 2 shows the time course of Ca^{2+} uptake observed under these conditions. Addition of NaF into the assay medium when Ca^{2+} uptake was in progress resulted in enhanced Ca^{2+} uptake; under the influence of NaF, the steady state levels of Ca^{2+} in SR vesicles increased by about 50%. In parallel experiments using the same SR preparation, inclusion of NaF in the assay medium during preincubation prior to the addition of ATP resulted in strong inhibition of the rates of Ca^{2+} uptake (Fig. 2 inset) which is in confirmity with the results shown in Fig. 1.

From the above results it did not seem likely that NaF-induced increase in Ca^{2+} efflux is involved in the inhibitory effect of NaF on Ca^{2+} uptake by SR. On the other hand, the results suggested that NaF can exert either an inhibitory or stimulatory effect on the SR Ca^{2+} pump depending on the experimental conditions employed. Initial exposure of SR to NaF in the absence of ATP seemed to facilitate the inhibitory effect of NaF. In contrast, addition of NaF to SR when the membrane Ca^{2+} pump had already been primed with Ca^{2+} and ATP (and was engaged in active Ca^{2+} transport) appeared to promote the stimulatory effect of NaF. To further investigate these possibilities, in additional experiments, the Ca^{2+} uptake reaction was initiated by the addition of SR or one of the critical ligands of the Ca^{2+} pump (viz., ATP, Ca^{2+} or Mg^{2+}) to the remainder of the transport assay medium preincubated with or without NaF. The results (Fig. 3) showed clearly that the inhibitory effect of NaF ensues when the Ca^{2+} uptake is initiated by the addition of ATP (panel A) or Ca^{2+} (panel D) to rest of the preincubated assay components; the stimulatory effect of NaF is observed when the Ca^{2+} uptake reaction is initiated by the addition of SR (panel B) or Mg^{2+} (panel C) to the rest of the preincubated assay components. These findings demonstrate that (1) exposure of SR to NaF in the absence of ATP or Ca^{2+} promotes the inhibitory effect of NaF on the Ca^{2+} pump, (2) exposure of SR to NaF in the presence of both ATP and Ca^{2+} not only prevents the inhibitory effect of NaF on the Ca^{2+} pump but in fact promotes the stimulatory effect of NaF, and (3) the inhibitory effect of NaF prevails when SR is exposed to NaF in the presence of Ca^{2+} alone (without ATP) or ATP alone (without Ca^{2+}).

Effects of NaF at varying concentrations of oxalate

The effects of NaF on Ca^{2+} uptake by SR described above were obtained when the Ca^{2+} uptake assays were performed with a selected concentration of oxalate (2.5 mM) in the assay medium. Like oxalate, fluoride might facilitate precipitation of Ca^{2+} inside the SR vesicles (especially if the oxalate concentration used is submaximal) and this may contribute to the observed stimulatory effect of NaF on Ca^{2+} uptake. To examine this possibility, the effects of NaF on Ca^{2+} uptake by SR were determined at varying concentrations (1–15 mM) of oxalate in the assay medium. In experiments where the Ca^{2+} uptake reaction was initiated by addition of SR to the assay medium, the stimulatory effect of NaF could be observed only at submaximally effective concentrations (< 10 mM) of oxalate (Fig. 4). On the other hand, in experiments where the Ca^{2+} uptake reaction was initiated by the addition of ATP to SR preincubated with the rest of the assay components, the inhibitory effect of NaF prevailed at all oxalate concentrations and the magnitude of inhibition (60–75% with 5 mM NaF) did not differ appreciably at varying (1–15 mM) oxalate concentrations (Fig. 5). Subsequent studies were performed with an intermediate concentration of oxalate

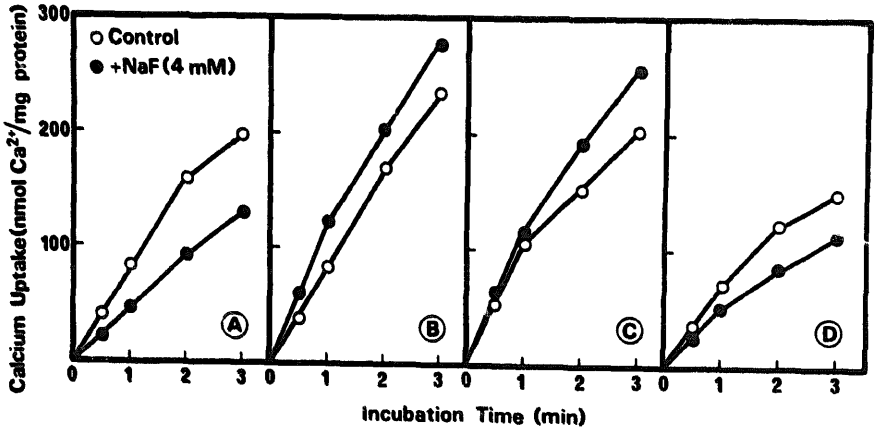

Fig. 3. Time course of ATP-dependent Ca^{2+} uptake by cardiac SR: divergent effects of NaF under different experimental conditions. The Ca^{2+} uptake assays were performed using the standard incubation medium (see Materials and Methods) containing 11.9 μM free Ca^{2+} in the absence (control) and in the presence of NaF. Following preincubation of the assay medium for 3 min at 37°C, Ca^{2+} uptake was initiated (at zero time in figure) by the addition of ATP (panel A) SR (panel B), Mg^{2+} (panel C) or Ca^{2+} (panel D). SR derived from frozen cardiac muscle was used for this experiment.

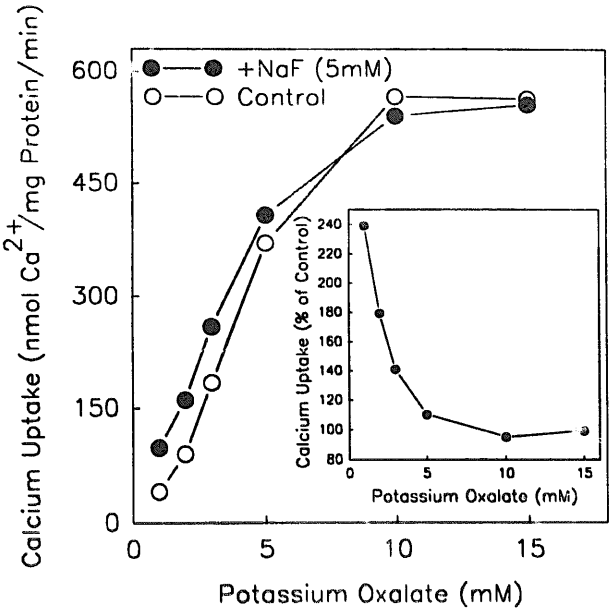

Fig. 4. Effect of varying oxalate concentration on the stimulatory action of NaF on ATP-dependent Ca^{2+} uptake by cardiac SR. The Ca^{2+} uptake assays were performed using the standard incubation medium containing varying concentrations of potassium oxalate in the absence (control) and in the presence of NaF. The concentraton of free Ca^{2+} was 11.9 μM. Ca^{2+} uptake was initiated by the addition of SR following preincubation of the rest of the assay components for 3 min at 37°C. SR derived from frozen cardiac muscle was used for this experiment.

(2.5 mM) in the assay medium which permitted further analysis of both the inhibitory and stimulatory effects of NaF on Ca^{2+} uptake by SR.

Effects of NaF pretreatment

The effects of NaF on Ca^{2+} uptake described above were obtained when NaF was added directly into the Ca^{2+} transport assay medium. To examine whether the effects of NaF were persistent or reversible, experiments were performed where SR was pretreated with NaF in the absence and presence of critical Ca^{2+} pump ligands (Ca^{2+}, Mg^{2+}, ATP), centrifuged and washed to remove free NaF (and other unbound ligands), and then assayed for Ca^{2+} uptake in the standard Ca^{2+} transport assay medium. The results (Fig. 6) showed decreased rates of Ca^{2+} uptake by SR pretreated with NaF in the absence of Ca^{2+} pump ligands (panel A), or in the presence of Mg^{2+} (panel B), or Mg^{2+} and ATP (panel C). In contrast, SR pretreated with NaF in the presence of Ca^{2+}, ATP and Mg^{2+} showed increased rates of Ca^{2+} uptake. These results (which conform to the observations described in Fig. 3) suggest that pretreatment of SR with NaF in the absence (but not in the presence) of Ca^{2+} and ATP resulted in stabilization of a less active conformation (NaF-inhibited state) of the Ca^{2+} pump.

Concentration dependence of the effects of NaF

The concentration dependence of the inhibitory effect of NaF was determined in experiments where Ca^{2+} uptake was initiated by adding ATP to SR preincubated with varying concentrations (1–10 mM) of NaF in the standard Ca^{2+} transport assay medium. The concentration dependence of the stimulatory effect of NaF was examined in experiments where Ca^{2+} uptake was initiated by adding SR to Ca^{2+} transport assay medium preincubated with varying concentrations (1–10 mM) of NaF. Both the inhibitory and stimulatory effects of NaF increased with increasing concentrations of NaF (Fig. 7). The concentration of NaF required for 50% inhibition of Ca^{2+} uptake was approx. 4.3 mM (panel A); the stimulatory effect was half-maximal with approx. 2.5 mM NaF (panel B). Under identical assay conditions, NaCl (1–10 mM) had no effect on Ca^{2+} uptake by SR (Fig. 7, panels A and B). Thus, both inhibitory and stimulatory effects of NaF are associated with the fluoride anion.

Effects of NaF at varying Ca^{2+}

The inhibitory effect of NaF at varying Ca^{2+} was evaluated in experiments where Ca^{2+} uptake was initiated by adding ATP to SR preincubated with or without NaF in the standard Ca^{2+} transport assay medium containing varying amounts of free Ca^{2+} (0.5–11.9 μM). At the submaximally effective concentration of NaF used (4 mM, cf. Fig. 7A), inhibition of Ca^{2+}

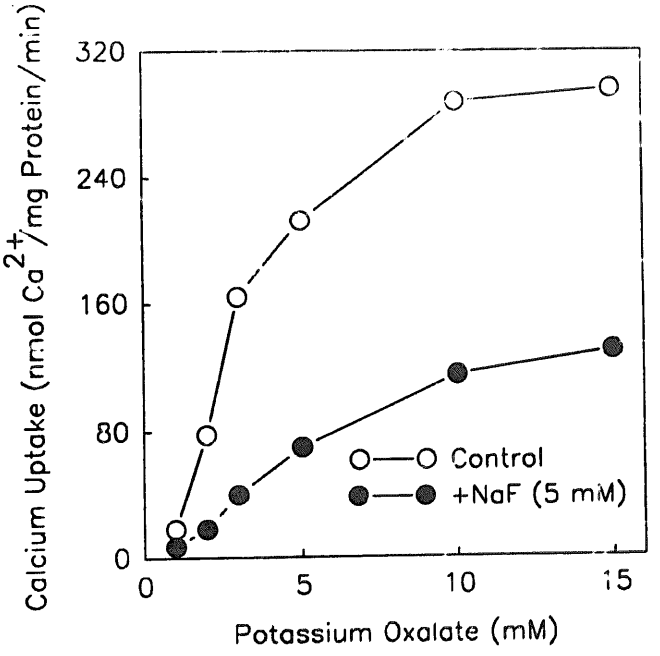

Fig. 5. Effect of varying oxalate concentration on the inhibitory action of NaF on ATP-dependent Ca^{2+} uptake by cardiac SR. The assay conditions were as described for Fig. 4 except that Ca^{2+} uptake was initiated by the addition of ATP following preincubation of the rest of the assay components for 3 min at 37°C. SR derived from frozen cardiac muscle was used for this experiment.

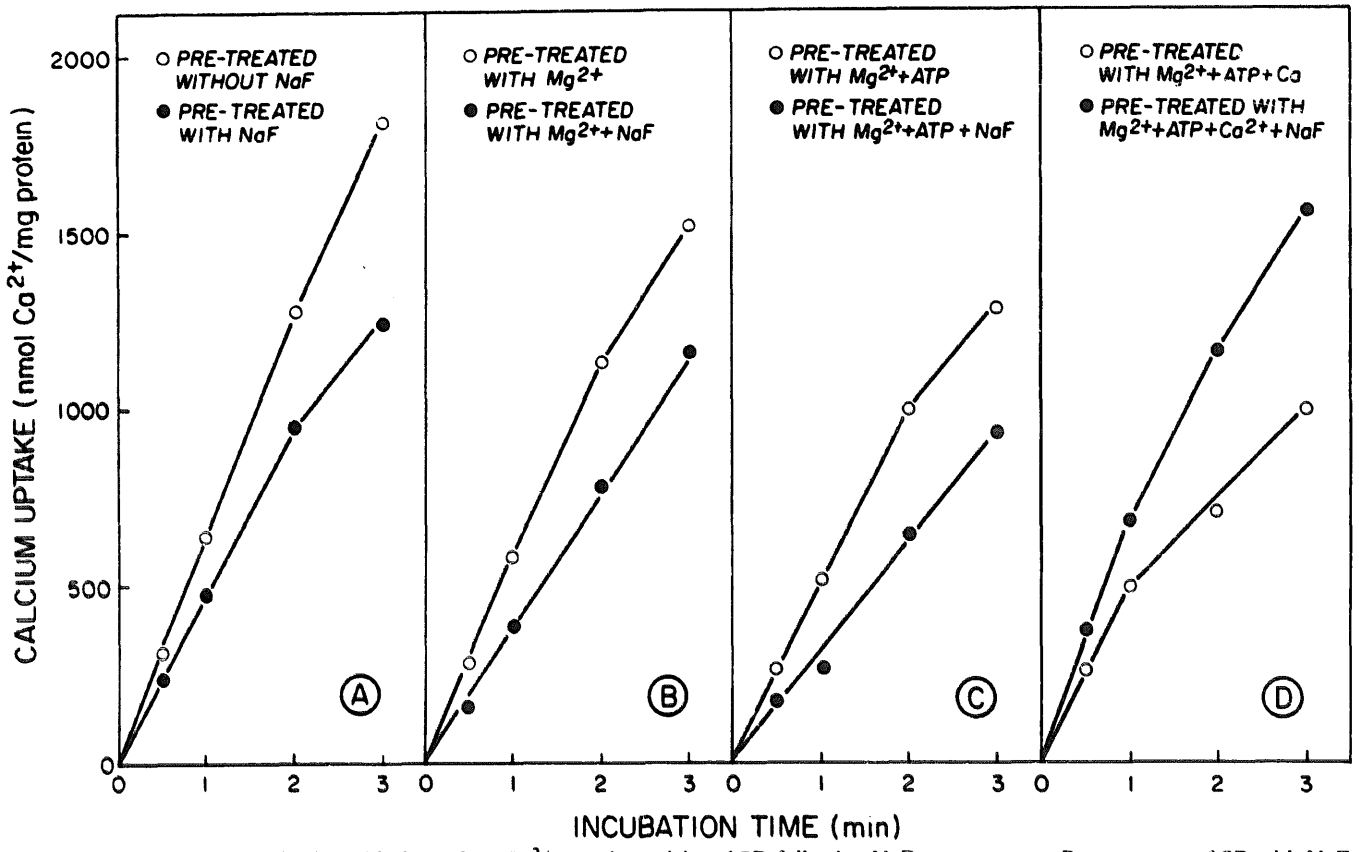

Fig. 6. Persistent alterations in the ATP-dependent Ca^{2+} uptake activity of SR following NaF-pretreatment. Pretreatment of SR with NaF was performed by incubating the membranes (0.8 mg protein) at 37°C for 3 min in a medium (total volume 2 ml) containing 10 mM Tris-maleate (pH 6.8) 100 mM KCl and 5 mM NaF in the absence or in the presence of Mg^{2+} (5 mM), ATP (2.5 mM) and Ca^{2+} (10 μM) as indicated. The tubes were subsequently chilled on ice (for 10 min), the membranes were sedimented by centrifugation (40000× g for 15 min), washed once with 4 ml buffer (10 mM Tris-maleate containing 100 mM KCl, pH 6.8) and used for Ca^{2+} uptake assays in the standard assay medium (free Ca^{2+}, 11.9 μM) as described in Fig. 1. SR subjected to the same protocol but without NaF in the pretreatment medium served as control. SR derived from fresh cardiac muscle was used for this experiment.

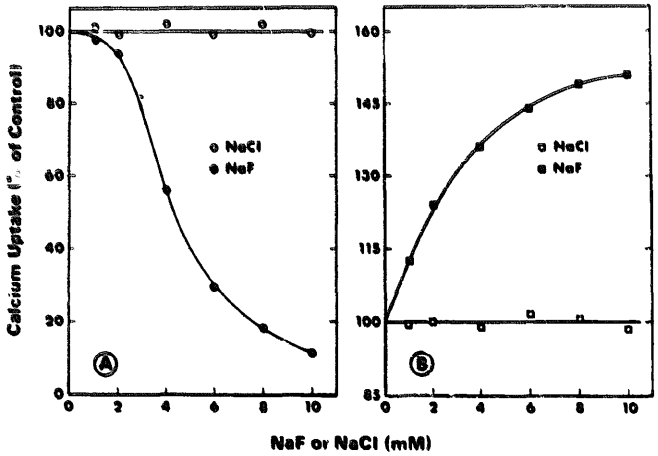

Fig. 7. Concentration dependence of the divergent effects of NaF on ATP-dependent Ca^{2+} uptake by cardiac SR. Ca^{2+} uptake was determined using the standard incubation medium (see Materials and Methods) in the absence and presence of varying concentrations of NaF or NaCl as indicated. The concentration of free Ca^{2+} was 11.9 μM. Ca^{2+} uptake was initiated by the addition of ATP (panel A) or SR (panel B) to the incubation medium following preincubation of the rest of the assay components for 3 min at 37°C. The results are presented as percent of control. The Ca^{2+} uptake activity (nmol Ca^{2+}/mg protein per 3 min) of control amounted to 282 for panel A and 329 for panel B. SR derived from frozen cardiac muscle was used for these experiments.

uptake was observed at all Ca^{2+} concentrations tested (Fig. 8); the magnitude of inhibition varied between 37 and 62% at varying Ca^{2+} concentrations. Double-reciprocal transformation of the data resulted in curved plots concave upward (not shown), a kinetic feature indicative of cooperative interactions between Ca^{2+} and the transport system. Analysis of the kinetic parameters (using the procedure described in Ref. 18), indicated that the inhibitory effect of NaF on Ca^{2+} uptake was accompanied by decrements in V_{max} (V_{max} (nmol Ca^{2+}/mg protein per min): control, 719; +NaF, 454) and Hill coefficient (n_H) (control, 2; +NaF, 1.48) for Ca^{2+} as well as an increase in $K_{0.5}$ for Ca^{2+} ($K_{0.5}$ (μM): control, 1.78; +NaF, 3.85).

The stimulatory effect of NaF at varying Ca^{2+} concentrations was assessed in experiments where Ca^{2+} uptake was initiated by adding SR to the Ca^{2+} transport assay medium (containing 0.5 to 11.9 μM free Ca^{2+} and 2.5 mM oxalate) preincubated with and without NaF. Under these conditions, NaF (4 mM) stimulated Ca^{2+} uptake (30–33%) at all Ca^{2+} concentrations tested); the stimulatory effect of NaF was

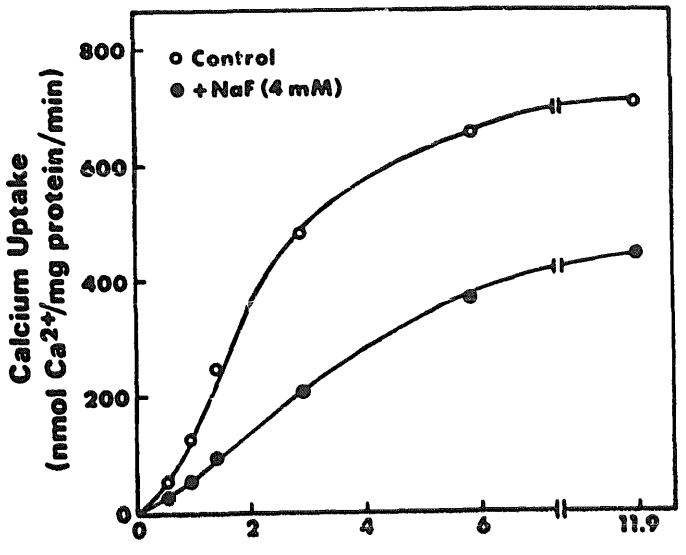

Fig. 8. Inhibitory effect of NaF on ATP-dependent Ca^{2+} uptake by cardiac SR at varying Ca^{2+} concentrations. The Ca^{2+} uptake assays were performed using the standard incubation medium (see Materials and Methods) containing varying concentrations of Ca^{2+} in the absence (control) and in the presence of NaF. Ca^{2+} uptake was initiated by the addition of ATP following preincubation of the rest of the assay components for 3 min at 37°C. SR derived from fresh cardiac muscle was used for this experiment.

accompanied by an increase in V_{max} without any appreciable change in n_H or $K_{0.5}$ for Ca^{2+} (data not shown).

Effects of NaF on Ca^{2+}-stimulated ATPase activity

The effect of NaF on Ca^{2+}-stimulated ATPase activity of SR was determined under assay conditions where NaF caused inhibitory and stimulatory effects on Ca^{2+} uptake. In these experiments Ca^{2+} uptake was also determined concurrently using the same SR preparation. The data from such experiments are summarized in Table I. Under the assay conditions where NaF inhibited Ca^{2+} uptake (Table I, Expt. A), Ca^{2+}-stimulated ATPase activity was also inhibited to a similar degree such that the ratio of Ca^{2+} transported/ATP hydrolysed remained virtually unaltered. Under the assay conditions where NaF caused stimulation of Ca^{2+} uptake (Table I, Expt. B), no stimulatory effect of NaF on Ca^{2+}-ATPase activity was observed, rather a slight (10–15%) decrease in enzyme activity was noted. Thus, the stimulatory action of NaF on Ca^{2+} uptake was accompanied by an apparent improvement in the stoichiometric ratio of Ca^{2+} transported/ATP hydrolysed.

Effects of NaF on phosphoenzyme levels

Under the experimental conditions where NaF caused inhibition of Ca^{2+} uptake (and Ca^{2+}-ATPase activity), the steady state level of Ca^{2+}-dependent phosphoenzyme was reduced substantially (to 30% of control at 10 mM NaF) and in a concentration-dependent manner by NaF (Ca^{2+}-dependent EP (nmol/mg protein per 15 s): control, 0.298; +5 mM NaF, 0.197; +10 mM NaF, 0.092). A small decrease (< 25% with 10 mM NaF) in the steady-state level of Ca^{2+}-dependent phosphoenzyme was noted under the experimental conditions where NaF stimulated Ca^{2+} uptake (Ca^{2+}dependent EP (nmol/mg protein per 15 s): control, 0.310; +5 mM NaF, 0.245; +10 mM NaF 0.230).

Discussion

The results presented here demonstrate both inhibitory and stimulatory effects of NaF on ATP-energized Ca^{2+} pump activity of SR isolated from heart muscle. These divergent effects of NaF depend on the Ca^{2+} transport assay conditions employed. The inhibitory effect is observed when SR is initially exposed to NaF in the absence of ATP or Ca^{2+}; the stimulatory effect is observed when SR is exposed to NaF in the presence of both ATP and Ca^{2+}. Plausible explanations for this phenomenon can be sought by examining the requirements for inhibition or stimulation by NaF in the context of the reaction mechanisms underlying

TABLE I

Comparison of the effects of NaF on calcium-stimulated ATP hydrolysis and ATP-dependent calcium uptake by cardiac sarcoplasmic reticulum

Ca^{2+}-stimulated ATP hydrolysis and ATP dependent Ca^{2+} uptake were measured under identical assay conditions using the same SR preparation in the absence and in the presence of NaF. The concentration of Ca^{2+} used was 11.9 μM. In Expt. A, the reaction was initiated by the addition of ATP to the assay medium: in Expt. B, the reaction was initiated by the addition of SR to the assay medium. The data represent mean ± S.D. of duplicate determinations using two separate SR preparations from fresh heart. The numbers in parentheses denote the ratio of Ca^{2+} uptake/ATP hydrolysis.

Additions to the assay medium	Experiment A		Experiment B	
	Ca^{2+}-stimulated ATP hydrolysis (nmol P_i/mg protein per min)	ATP-dependent Ca^{2+} uptake (nmol Ca^{2+}/mg protein per min)	Ca^{2+}-stimulated ATP hydrolysis (nmol P_i/mg protein per min)	ATP-dependent Ca^{2+} uptake (nmol Ca^{2+}/mg protein per min)
None	634 ± 63	706 ± 51(1.11)	672 ± 67	807 ± 59(1.20)
NaF, 4 mM	372 ± 37	368 ± 27(0.99)	610 ± 61	1040 ± 76(1.70)
NaF, 8 mM	95 ± 10	85 ± 6(0.89)	568 ± 57	1129 ± 82(1.99)

ATP hydrolysis and Ca^{2+} transport by the SR Ca^{2+} ATPase. The ATP-energized Ca^{2+} transport by the SR Ca^{2+}-ATPase is recognized to involve several steps, including the binding of Ca^{2+} and ATP, formation of a covalently linked phosphoenzyme intermediate, Ca^{2+} translocation, and finally, breakdown of the phosphorylated intermediate [1–4]. The intermediate steps in the catalytic cycle can be condensed as follows:

$$*E + Ca_o \rightleftharpoons *ECa_o + ATP \rightleftharpoons **ECa_oATP \rightleftharpoons ADP\text{-}P \sim **ECa_i$$

$$\rightleftharpoons *E + Ca_i + ADP + P$$

where E = ATPase enzyme (Ca^{2+} pump protein); o = outside membrane; i = inside membrane; * susceptible to inhibition by NaF; ** resistant to inhibition by NaF.

Considerable evidence indicates that distinct sequential transitions in the conformational state of the ATPase occur coupled with the formation of the reaction intermediates shown above (cf. Refs. 1–4). It is likely that different conformational states of the ATPase have differential susceptibility to the effects of NaF. The enzyme conformation which prevails in the absence of Ca^{2+} is regarded as the ground state or E conformation, and is characterized by the inability to undergo phosphorylation with ATP (cf. Refs. 1–4). Transition of the ATPase from E to E_1 conformation ensues upon Ca^{2+} binding to the enzyme and the E_1 conformation (ECa_o) is characterized by the ability to form the phosphorylated intermediate, EP, from metal-ATP (generally Mg-ATP) but not P_i (cf. Refs. 1–4). Our observation that exposure of SR to NaF in the absence of Ca^{2+} promotes the strong inhibitory effect of NaF suggests that the Ca^{2+}-deprived, ground state of the enzyme (E) is highly susceptible to inhibition by NaF. Also, the inhibitory effect is manifested when SR is exposed to NaF in the presence of Ca^{2+} alone (i.e. in the absence of ATP) implying that the Ca^{2+}-induced conformational state E_1 (ECa_o) is equally susceptible to inhibition by NaF. On the other hand, exposure of SR to NaF in the presence of both Ca^{2+} and ATP resulted in stimulation of Ca^{2+} uptake indicating that the Ca^{2+} + ATP-induced conformational state (ECa_oATP) and the succeeding phosphorylated state ($ADP\text{-}P \sim ECa_i$) are resistant to the inhibitory effect of NaF.

The data from concurrent analyses of the effects of NaF on the enzymatic and ion transport functions of the Ca^{2+}-ATPase also support the view that the contrasting effects of NaF are dependent on the enzyme conformation. Thus, under the experimental conditions which promoted formation of E (i.e. when SR was exposed to NaF in the absence of Ca^{2+}) or E_1 (i.e. when SR was exposed to NaF in the presence of Ca^{2+} but in the absence of ATP) conformation of the ATPase, the inhibitory effect of NaF on Ca^{2+} transport was accompanied by concomitant inhibition of Ca^{2+}-stimulated ATP hydrolysis as well as a parallel decrease in the Ca^{2+}-induced steady-state level of the phosphoenzyme intermediate. It is reasonable, therefore, to conclude that the encounter of NaF with the E or E_1 state of ATPase results in a blockade of the sequential conformational transitions which normally ensure binding of Ca^{2+} to the E state and ATP (as Mg-ATP) to the E_1 state, thus paralysing both the enzymatic and ion transport functions of the Ca^{2+} pump. Under the experimental conditions which favored transition of the ATPase to its phosphoenzyme conformation (i.e. when SR was exposed to NaF in the presence of Ca^{2+}, ATP and Mg^{2+}), NaF caused stimulation of Ca^{2+} transport without influencing substantially Ca^{2+}-stimulated ATP hydrolysis or the steady-state level of Ca^{2+}-induced phosphoenzyme. This stimulatory effect of NaF on Ca^{2+} transport is likely due to the ability of fluoride to mimick the action of oxalate in precipitating Ca^{2+} within the SR lumen. Such a conclusion is supported by the observation that the stimulatory effect of NaF is observed only at subsaturating concentrations of oxalate in the assay.

Both the inhibitory and stimulatory effects of NaF are associated with the fluoride anion as NaCl (under identical assay conditions and similar concentration range) did not influence Ca^{2+} uptake by SR. In analogy with the mechanisms postulated to underlie the actions of fluoride on other enzyme systems, it can be suggested that fluoride interaction at the nucleotide (ATP) binding site (i.e. phosphorylation site) of the Ca^{2+}-ATPase may be involved in its inhibitory action. For example, the effects of fluoride on hormone-sensitive adenylate cyclase systems are thought to occur as a result of the interaction of fluoride with the nucleotide (GTP)-binding site of the α-subunit of G proteins [5–7,21]. In this case, fluoride complexed with aluminum (AlF_4^-) is considered to be the effective species [6], and apparently, AlF_4^- mimicks the γ-phosphate of GTP when bound to the nucleotide binding site in conjunction with GDP [22]. Such mimicry, it has been suggested, could account for the effects of fluoride on other enzyme systems involving phosphate groups, such as phosphatases [22]. Fluoride has been shown to irreversibly inhibit ($Na^+ + K^+$) ATPase [23,24]; this inhibition is potentiated by aluminum and appears to proceed via interaction of AlF_4^- at the phosphorylation site of the enzyme [24]. While the involvement of aluminum in the effects of fluoride has not been specifically addressed in the present report, we have observed a marked potentiating effect of aluminum on the inhibitory effect of NaF on Ca^{2+} uptake by SR (in the presence of 30 μM $AlCl_3$, the concentration of NaF required for 50% inhibition of Ca^{2+} uptake is decreased 5-fold; N. Narayanan, unpublished results) suggesting potential similarity in mechanism of action.

Also, it is noteworthy that certain characteristics of the inhibitory action of fluoride on the SR Ca^{2+} pump resemble those described for vanadate which inhibits $(Na^+ + K^+)$-ATPase [25–27] and Ca^{2+}-ATPase [28–32] by interacting at the phosphorylation site of these enzymes. For example, like vanadate [30–32], fluoride readily inhibits the Ca^{2+}-free conformation of the Ca^{2+}-ATPase and produces decrements in the enzyme's Ca^{2+}-binding affinity and ability to form phosphoenzyme from ATP. In view of the above observations and the potential of fluoride to interact with the phosphate sites of proteins, it seems reasonable to propose that the phosphorylation site of the Ca^{2+}-ATPase is a putative site for the inhibitory action of fluoride on the SR Ca^{2+} pump.

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