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Comparison of the effects of fluoride on the calcium pumps of cardiac and fast skeletal muscle sarcoplasmic reticulum: evidence for tissue-specific qualitative difference in calcium-induced pump conformation

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Abstract

Comparison of the effects of fluoride (NaF, 1–10 mM) on the catalytic and ion transport functions of the Ca^{2+}-ATPase in sarcoplasmic reticulum (SR) vesicles isolated from rabbit cardiac and fast-twitch skeletal muscles revealed similarities as well as striking tissue-specific differences depending on the experimental conditions employed. Short preincubation (3 min at 37°C) of cardiac or fast muscle SR with fluoride in the absence of Ca^{2+} and ATP prior to initiating enzyme turnover by simultaneous addition of Ca^{2+} and ATP to the assay medium resulted in a strong inhibitory effect of fluoride on ATP-energized (oxalate-facilitated) Ca^{2+} uptake and Ca^{2+}-ATPase activity. On the other hand, when turnover was initiated by the addition of ATP to SR preincubated with fluoride in the presence of Ca^{2+} but in the absence of ATP, fluoride caused concentration-dependent stimulation of active Ca^{2+} uptake by fast muscle SR with no appreciable change in Ca^{2+}-dependent phosphoenzyme (EP) formation (from ATP) or Ca^{2+}-ATPase activity but inhibition of active Ca^{2+} uptake by cardiac SR with concomitant inhibition of EP formation and Ca^{2+}-ATPase activity. Exposure of cardiac or fast muscle SR to fluoride in the presence of both Ca^{2+} and ATP resulted in concentration-dependent stimulatory effect of fluoride on Ca^{2+} uptake with no change in EP formation or Ca^{2+}-ATPase activity; this effect diminished substantially at saturating oxalate concentration in the assay. Assessment of the effects of deferoxamine (1 mM) and exogenous aluminum (10 μM) did not indicate a requirement for aluminum in the inhibitory or stimulatory effect of fluoride. These results suggest that (a) the Ca^{2+} and ATP-deprived (E_1/E_2) but not the Ca^{2+} *plus* ATP-liganded (CaE_1ATP) conformation of the SR Ca^{2+}-ATPase is susceptible to inhibition by fluoride in both cardiac and fast muscle; (b) the Ca^{2+}-bound conformation (CaE_1) of the SR Ca^{2+}-ATPase is susceptible to inhibition in cardiac muscle but is refractory to fluoride in fast muscle; and (c) the stimulatory effect of fluoride is largely secondary to its ability to mimic the action of oxalate in intravesicular Ca^{2+} trapping when the fluoride-resistant enzyme is turning over normally. Fluoride inhibited phosphorylation of the Ca^{2+}-free enzyme by P_i in cardiac and fast muscle SR indicating that fluoride sensitivity of the phosphorylation site of the SR Ca^{2+}-ATPase is similar in cardiac and fast muscle. In cardiac SR, disruption of the functional interaction between Ca^{2+}-ATPase and its regulatory protein phospholamban, through phosphorylation of the latter (by cAMP kinase) did not alter the fluoride sensitivity of the Ca^{2+}-bound enzyme (CaE_1). These results, coupled with the refractoriness of CaE_1ATP to fluoride in cardiac and fast muscle SR, suggest that a tissue-specific difference in the accessibility (reactivity) of the nucleotide binding site to fluoride upon Ca^{2+} binding to the enzyme may account for the observed difference in fluoride sensitivity of the cardiac versus fast muscle enzyme – i.e., when the ATPase is in CaE_1 conformation, its ATP binding site is 'fluoride-reactive' in the cardiac enzyme but is 'fluoride-resistant' in the fast muscle enzyme.

Key words: Sarcoplasmic reticulum; Calcium pump; Cardiac muscle; Fast skeletal muscle; Fluoride

1. Introduction

In skeletal and cardiac muscle, the Ca^{2+} pump (Ca^{2+}-ATPase) of SR serves the critical function of

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Abbreviations: SR, sarcoplasmic reticulum; cAMP kinase, cyclic AMP-dependent protein kinase; Gpp(NH)p, guanyl-5'-yl-imidodiphosphate.

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promoting muscle relaxation by sequestering Ca^{2+} from the myoplasm 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 conformational states, E_1 and E_2 (for reviews see [1–3]). These two states differ in that the affinity for Ca^{2+} is high in the E_1 conformation and low in the E_2 conformation, and in that the Ca^{2+} binding sites are exposed to the cytoplasmic side of SR in E_1 but to the luminal side of SR in E_2. The catalytic and ion transport cycle begins with the binding of two Ca^{2+} ions followed by ATP to the E_1 form of the enzyme. An aspartic acid residue in the active site is then phosphorylated resulting in a conformational change of the E_1PCa^{2+} phosphoenzyme intermediate to the E_2PCa^{2+} form which has decreased Ca^{2+} affinity. The Ca^{2+} binding sites are now everted so that they face the SR lumen to which Ca^{2+} is subsequently released. The Mg^{2+}-catalyzed hydrolysis of E_2P devoid of bound Ca^{2+} ions results in the release of P_i into the cytoplasm leaving E_2 which isomerizes to E_1 to complete the cycle.

Although the SR Ca^{2+} pumps of cardiac muscle and fast skeletal muscle are products of separate genes (SERCA2 and SERCA1, respectively, for cardiac and fast muscle), current evidence from structure-function relationships and enzymatic properties supports the same Ca^{2+}-ATPase reaction mechanism and function for cardiac and fast muscle SR. Thus, the cardiac and fast muscle SR Ca^{2+}-ATPase isoforms show striking homology (about 84%) in primary structure [4,5] and consequently, they are predicted to have essentially identical transmembrane topologies and tertiary structures [1–3,6,7]. Extensive site-directed mutagenesis studies of the SERCA1 isoform have defined many amino acid residues which are critical for enzymatic and ion transport functions; these are all conserved in different SERCA isoforms including SERCA2 [3,8]. Also, when the cardiac and fast muscle isoforms were expressed in COS cells they were found to have similar enzymatic properties [9]. Despite these indications of similarity, some degree of intrinsic functional differences among the Ca^{2+}-ATPase isoforms would seem likely in view of their tissue-specific expression as well as differences in the mechanisms known to underlie their physiological regulation. For example, in cardiac SR, interaction of the Ca^{2+}-ATPase with another intrinsic membrane protein, phospholamban, markedly influences enzymatic and ion transport functions; such a mechanism is not operational in fast muscle SR [10–15]. Recently, we have found that the cardiac [16], but not the fast muscle [17], SR Ca^{2+}-ATPase can be phosphorylated and activated by endogenous (membrane-associated) or exogenous Ca^{2+}/calmodulin-dependent protein kinase. Moreover, potential differences in the catalytic cycle of the Ca^{2+}-ATPase in cardiac versus fast muscle SR have been suggested by certain earlier observations. These include slower rate of phosphoenzyme decomposition for cardiac compared with fast muscle enzyme [18,19] and inability of the cardiac (as opposed to fast muscle) enzyme to utilize GTP as substrate for Ca^{2+}-dependent phosphoenzyme formation and Ca^{2+} transport [20].

In a previous study, we have described both inhibitory and stimulatory effects of fluoride on active Ca^{2+} transport by canine cardiac SR [21]. These divergent effects of fluoride were dependent on the experimental conditions employed and could be attributed to differential sensitivity of the various transitional states of the Ca^{2+}-ATPase to fluoride. In general, the findings suggested that the Ca^{2+}-free ground state of the ATPase (E_1) and the conformation resulting from Ca^{2+} binding (E_1Ca^{2+}) are highly susceptible to the inhibitory action of fluoride. On the other hand, the conformational states evolving sequentially upon ATP binding to the Ca^{2+}-bound enzyme were found to be resistant to inhibition by fluoride. Recently, other investigators have reported inhibitory effect of fluoride on Ca^{2+}-ATPase activity in rabbit fast skeletal muscle SR [22–24] and it was suggested that fluoride stabilizes the Ca^{2+}-free conformation of the Ca^{2+}-ATPase by mimicking P_i binding to the phosphorylation site [22,24], and in addition, acts as an analogue of γ-phosphate of ATP to stabilize the Ca^{2+}-bound form of the enzyme [24]. Although a protective effect of Ca^{2+} and ATP has been noted [22], these studies did not address the potential influence of pre-existing enzyme conformation on the effect of fluoride. Also, the relationship between the effect of fluoride on the enzyme's catalytic versus ion transport function was not examined. In this report, we present the results of studies comparing the effects of fluoride on the catalytic activity as well as Ca^{2+} transport function of Ca^{2+}-ATPase in rabbit cardiac and fast skeletal muscle SR. To our surprise, we have found striking tissue-specific differences in the effects of fluoride on cardiac and fast muscle SR Ca^{2+} pumps. For example, under identical assay conditions, fluoride produced inhibition of Ca^{2+} transport in cardiac SR and stimulation of Ca^{2+} transport in fast muscle SR. Furthermore, under these conditions, fluoride inhibited Ca^{2+}-dependent active site phosphorylation of the cardiac SR Ca^{2+}-ATPase but not the fast muscle SR Ca^{2+}-ATPase. These and other observations reported here suggest a significant qualitative difference between the Ca^{2+}-induced conformational state of cardiac and fast muscle SR Ca^{2+}-ATPase isoforms.

2. Materials and methods

Chemicals. $^{45}CaCl_2$ and [^{32}P]Na_2PO_4 were purchased from New England Nuclear, Montreal. [γ-

^{32}P]ATP was from Amersham Canada, Oakville. All other chemicals were of highest purity available from Sigma, St. Louis, MO, or BDH Chemicals, Toronto.

Preparation of SR vesicles. SR-enriched membrane vesicles were prepared from rabbit heart ventricles and fast (adductor magnus) skeletal muscle as described previously [25]. Following isolation, the SR vesicles were suspended in 10 mM Tris-maleate (pH 6.8) containing 100 mM KCl and stored at −80°C after quick-freezing with liquid N_2. Protein concentration was determined by the method of Lowry et al. [26] using bovine serum albumin as standard.

Ca^{2+} transport and Ca^{2+}-ATPase assays. ATP-dependent, oxalate-facilitated Ca^{2+} uptake was determined using the Millipore filtration technique as detailed elsewhere [27]. The standard incubation medium (total volume 1 ml) contained 50 mM Tris-maleate (pH 6.8), 5 mM $MgCl_2$, 5 mM NaN_3, 2.5 mM ATP, 120 mM KCl, 2.5 mM potassium oxalate, 0.1 mM EGTA, SR (7.5 and 30 μg protein, respectively, for fast muscle and cardiac SR) and 0.1 mM $^{45}CaCl_2$ (~ 12000 cpm/nmol). The initial free Ca^{2+} concentration in the assay medium, determined using the computer program of Fabiato [28], was 8.2 μM. Modifications to the standard incubation medium are specified in the figure legends. Unless indicated otherwise, all assays were performed at 37°C. The Ca^{2+} uptake reaction was initiated by the addition of SR or substrate (ATP/Ca^{2+}) as specified in the figure legends following preincubation of the rest of the assay components for 3 min. Ca^{2+}-ATPase activity was determined as described previously [29]. The incubation medium for the Ca^{2+}-ATPase assay 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 $^{45}CaCl_2$.

Phosphorylation by ATP. Steady-state levels of Ca^{2+}-induced phosphoenzyme were measured using [γ-^{32}P]ATP as described previously [21]. The reaction mixture (total volume 200 μl) contained 50 mM Tris-maleate (pH 6.8), 120 mM KCl, 1 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 0.1 mM EGTA, 0.1 mM $CaCl_2$ and SR (50 μg protein). The Ca^{2+}-dependence of phosphoenzyme formation was monitored in parallel assays in the absence of Ca^{2+} and in the presence of 1 mM EGTA. The reaction, initiated by the addition of ATP or SR following preincubation of the rest of the assay components for 3 min at 37°C, was allowed to proceed for 15s and was stopped by adding 1 ml of ice-cold 10% trichloroacetic acid containing 1 mM ATP and 2 mM KH_2PO_4. The phosphoenzyme was quantified by Millipore filtration and liquid scintillation counting [21]. In some experiments, the acid-denatured protein was recovered by centrifugation, and subjected to lithium dodecyl sulphate-polyacrylamide gel electrophoresis (on slab gels) at 4°C as described by Lichtner and Wolf [30]. Following fixation, staining and destaining as described by Fairbanks et al. [31], the gels were dried and autoradiographed. The phosphoenzyme bands visualized on the autoradiograms were quantified by laser scanning densitometry.

Phosphorylation by P_i. Phosphorylation of SR with P_i was performed in a medium (total volume 200 μl) containing 50 mM Tris-maleate (pH 6.8), 1 mM EGTA, 10% dimethyl sulfoxide, 4 mM [^{32}P]Na_2PO_4 and SR (250 μg). The reaction, initiated by the addition of [^{32}P]phosphate following preincubation of the rest of the assay mixture at 24°C for 3 min, was allowed to proceed for 30 s and was quenched with 200 μl of 10% trichloracetic acid containing 4 mM Na_2HPO_4. The acid-denatured protein was recovered by centrifugation, the pellets were washed with water, digested in 500 μl of 50 mM Tris-HCl (pH 7.5) containing 2% sodium dodecyl sulphate, and the ^{32}P radioactivity was determined by liquid scintillation counting.

Phosphorylation by cAMP-dependent protein kinase. In some experiments the effect of fluoride on Ca^{2+} transport was examined using cardiac SR vesicles subjected to phosphorylation by cAMP-dependent protein kinase. For this, cardiac SR (0.75 mg/ml) was incubated for 3 min at 37°C in a reaction medium containing 50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.8 mM ATP and the catalytic subunit of cAMP-dependent protein kinase (70 μg/ml). Subsequently, aliquots of the phosphorylated membranes were transferred to the Ca^{2+} uptake assay medium (see above) to determine Ca^{2+} transport activity. Membrane vesicles subjected to the same incubation protocol in the absence of protein kinase in the phosphorylation assay medium served as control (unphosphorylated SR) for this experiment. To monitor the SR protein phosphorylation profile, parallel experiments were performed in which [γ-^{32}P]ATP was used in place of nonradioactive ATP in the phosphorylation assay medium and the phosphorylated membranes were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and autoradiography as described previously [32].

Ca^{2+} release assay. $^{45}Ca^{2+}$ release rates from SR vesicles passively loaded with $^{45}CaCl_2$ were determined by Millipore filtration [33]. Passive $^{45}Ca^{2+}$ loading was performed by incubating SR vesicles (1 mg protein/ml) at 24°C for 40 min in a medium containing 50 mM Tris-maleate (pH 6.8), 120 mM KCl, 1 mM $^{45}CaCl_2$ and 1 mM potassium oxalate. To initiate Ca^{2+} release, aliquots of the $^{45}Ca^{2+}$-loaded vesicles were diluted 80-fold into a Ca^{2+} release medium (50 mM Tris-maleate, pH 6.8, containing 2 mM $MgCl_2$ and 1 mM EGTA) that was preincubated for 5 min at 37°C. Subsequently, aliquots of the incubation mixture were filtered through Millipore filters at 30 s intervals for a period of 2 min. The filters were washed with 3 ml of ice-cold 10 mM Tris-maleate (pH 6.8) containing 120

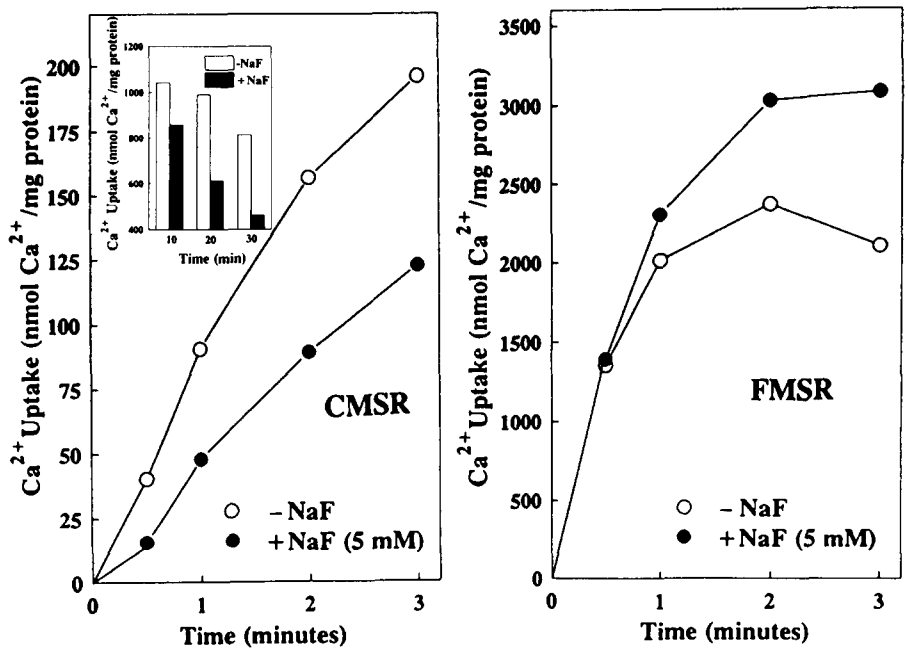


Fig. 1. Time-course of ATP-dependent Ca^{2+} uptake by cardiac muscle SR (CMSR) and fast skeletal muscle SR (FMSR) in the absence and presence of fluoride. Ca^{2+} uptake was determined using the standard incubation medium as described under Materials and methods. Ca^{2+} uptake was initiated by the addition of ATP to the assay medium following preincubation of the rest of the assay components for 3 min at 37°C. The effect of fluoride on maximal levels of Ca^{2+} uptake by CMSR is shown in the inset (left panel).

mM KCl, 10 mM $MgCl_2$ and 10 μM ruthenium red, dried at 60°C and ^{45}Ca radioactivity was determined by liquid scintillation counting.

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.

3. Results

Contrasting effects of fluoride on the Ca^{2+} uptake activity of cardiac and fast muscle SR

Fig. 1 shows the time course of ATP-dependent Ca^{2+} uptake by rabbit cardiac SR and fast skeletal muscle SR in the absence of NaF and in the presence of 5 mM NaF in the assay medium. In this experiment, 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 striking inhibition (38–60%) in the rates of Ca^{2+} uptake by cardiac SR and stimulation (15–46%) in the rates of Ca^{2+} uptake by fast muscle SR. With cardiac SR, the inhibitory effect of NaF could be observed when initial rates or maximal levels of Ca^{2+} uptake were measured. The stimulatory effect of NaF on fast muscle SR appeared to evolve slowly but was clearly evident within 1 min. Thus, the stimulatory effect of NaF on fast muscle SR became more pronounced at maximal levels of Ca^{2+} uptake.

Data on the concentration-dependence of the effects of NaF on ATP-dependent Ca^{2+} uptake by rabbit cardiac and fast muscle SR are summarized in Fig. 2. In these experiments, Ca^{2+} uptake was initiated by the addition of ATP or SR to the preincubated (3 min at

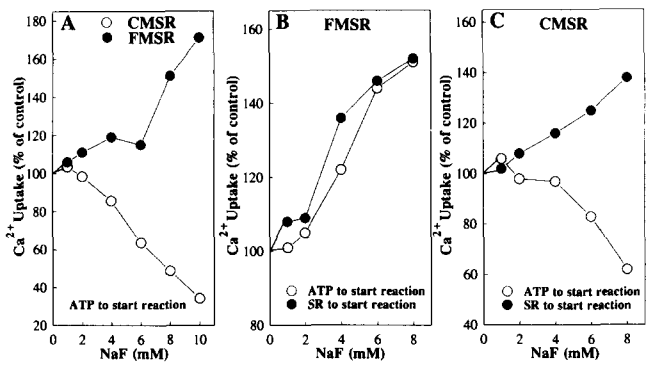


Fig. 2. Concentration-dependence of the divergent effects of NaF on ATP-dependent Ca^{2+} uptake by cardiac muscle SR (CMSR) and fast skeletal muscle SR (FMSR) under different experimental conditions. Ca^{2+} uptake was determined using the standard incubation medium (see Materials and methods) in the absence and presence of varying concentrations of NaF as indicated. Ca^{2+} uptake was initiated by the addition of ATP or SR (as specified inside figure) 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 (activity measured in the absence of NaF, defined as 100%) amounted to: panel A, CMSR 278, FMSR 2810; panel B; ATP to start reaction 5436, SR to start reaction 5602; panel C, ATP to start reaction 426, SR to start reaction 599.

37°C) standard assay medium and Ca^{2+} uptake was allowed to proceed for 3 min. Fig. 2A shows the differential effects of NaF on Ca^{2+} uptake by cardiac and fast muscle SR when the reaction was initiated by the addition of ATP. It is seen that NaF causes concentration-dependent inhibition of Ca^{2+} uptake by cardiac SR (50% inhibition at ~ 8 mM NaF) and stimulation of Ca^{2+} uptake by fast muscle SR (50% stimulation at ~ 8 mM NaF).

In a previous study, we have observed divergent effects of NaF on ATP-dependent Ca^{2+} uptake by canine cardiac SR depending on the reaction conditions employed [21]. An inhibitory effect was observed when SR was initially exposed to NaF in the absence of ATP or Ca^{2+}, and a stimulatory effect was observed when SR was exposed to NaF in the presence of both ATP and Ca^{2+}. The likely explanation for this phenomenon is that the Ca^{2+}-and/or ATP-deprived conformations of the SR Ca^{2+}-ATPase and the enzyme conformations which evolve upon binding of these ligands to the enzyme have differential susceptibility to the effects of NaF [21]. Therefore, the possibility that the effect of NaF on fast muscle SR is also influenced by the conformational state of the Ca^{2+}-ATPase was investigated in experiments where the Ca^{2+} uptake reaction was initiated with either ATP or SR. It was found that the stimulatory effect of NaF on fast muscle SR prevailed regardless of whether Ca^{2+} uptake was initiated by the addition of ATP or SR (Fig. 2, panels A, B). On the other hand, in cardiac SR, NaF produced inhibitory effect when Ca^{2+} uptake was initiated by the addition of ATP and stimulatory effect when Ca^{2+} uptake was initiated by the addition of SR (Fig. 2, panels A, C); these observations with rabbit cardiac SR conform to our previous findings with canine cardiac SR [21]. The stimulatory effect of NaF on Ca^{2+} uptake by fast muscle SR could also be observed following pretreatment of the SR with EGTA (1 mM) and EDTA (1 mM) to remove tightly bound Ca^{2+} and Mg^{2+} (data not shown). In both cardiac and fast muscle SR, the effects of NaF observed under the various assay conditions, were associated with the fluoride anion as similar concentrations of NaCl (1–10 mM) did not influence the Ca^{2+} uptake activity (data not shown). The results described above demonstrate a striking dissimilar effect of fluoride on the Ca^{2+} pump activity of cardiac and fast muscle SR; this tissue-specific difference in the effect of fluoride becomes discernible when SR is initially exposed to fluoride in the absence of ATP (but in the presence of Ca^{2+} and Mg^{2+}).

Effects of fluoride on Ca^{2+}-induced phosphoenzyme formation and Ca^{2+}-stimulated ATP hydrolysis

The transport of Ca^{2+} by the SR Ca^{2+}-ATPase occurs as part of the reaction cycle of ATP hydrolysis. The mechanism of coupling ATP hydrolysis and Ca^{2+} transport involves sequential transitions in enzyme conformation, with resultant changes in specificity for catalysis, along the reaction pathway [1–3,34]. One of the well characterized intermediate steps in the reaction pathway is the formation of a phosphoenzyme intermediate upon the sequential binding of Ca^{2+} followed by ATP to the ATPase on the cytoplasmic side of SR [1,35]. In examining the mechanism of fluoride action, we determined the effects of fluoride on Ca^{2+}-induced phosphoenzyme formation from ATP under two different experimental conditions. When the reaction was initiated by the addition of SR to the preincubated assay medium (so that fluoride encountered the ATPase in the presence of both Ca^{2+} and ATP), fluoride had no effect on the steady-state levels of phosphoenzyme formed in either cardiac or fast muscle

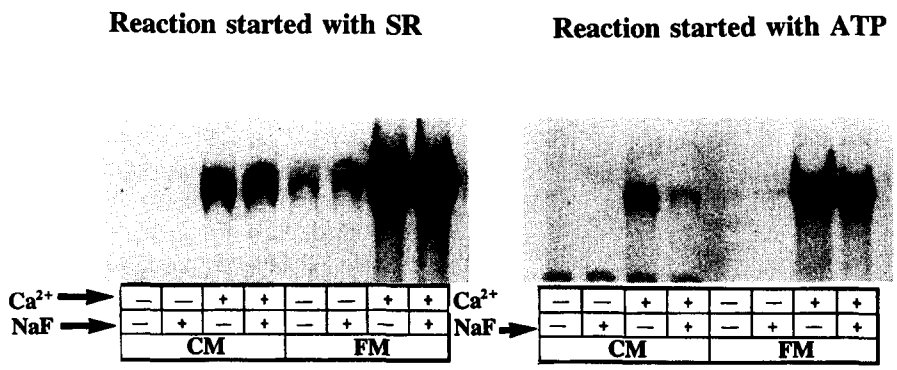


Fig. 3. Autoradiograms depicting the effects of NaF on Ca^{2+}-dependent phosphoenzyme formation from ATP in cardiac muscle (CM) SR and fast skeletal muscle (FM) SR under different experimental conditions. Steady-state levels of phosphoenzyme formed were monitored as described under Materials and methods. The phosphorylation reaction was initiated by the addition of SR (left panel) or ATP (right panel) to the incubation medium following preincubation of the rest of the assay components for 3 min at 37°C. The reaction was quenched 15 s after the addition of SR or ATP. The samples were subjected to electrophoresis and autoradiography. The resulting autoradiograms and laser densitometer scans of the corresponding radioactive bands are shown. Quantitation of phosphoenzyme by Millipore filtration technique [21] yielded similar results on the effects of NaF (data not shown).

SR (Fig. 3, left panel). On the other hand, when the reaction was initiated by the addition of ATP to the preincubated assay medium (under this condition encounter of fluoride with the ATPase occurred in the absence of ATP but in the presence of Ca^{2+}, i.e., with the CaE_1 form), fluoride caused marked decrease in the steady state levels of phosphoenzyme formed in cardiac SR but not fast muscle SR (Fig. 3, right panel). The effect of fluoride on the overall enzymatic cycle (viz Ca^{2+}-stimulated ATP hydrolysis) under the two different experimental conditions noted above are shown in Fig. 4. It can be seen that when the reaction is initiated by the addition of SR to the preincubated assay medium, fluoride has no appreciable effect on Ca^{2+}-stimulated ATP hydrolysis in cardiac or fast muscle SR. However, when the reaction is initiated by the addition of ATP to the preincubated assay medium, Ca^{2+}-stimulated ATP hydrolysis is strongly inhibited by fluoride in cardiac SR but not in fast muscle SR. These tissue-specific differences in the effects of fluoride on Ca^{2+}-induced phosphoenzyme levels and Ca^{2+}-stimulated ATP hydrolysis correspond to the analogous tissue-specific differences in the effects of fluoride on Ca^{2+} transport. It should be noted, however, that the magnitude of tissue-specific difference in the measured Ca^{2+} uptake activities of cardiac and fast muscle SR (~ 10–25-fold higher in fast muscle SR depending on assay conditions, c.f. Fig. 1 and Fig. 2) was much greater when compared with the measured Ca^{2+}-ATPase activities (~ 4-fold higher in fast muscle SR, c.f. Fig. 4). This disparity could arise from the presence of ATP hydrolyzing activity unrelated to the Ca^{2+}-pumping ATPase in cardiac SR and/or due to partial uncoupling of ATP hydrolysis and Ca^{2+} transport. In

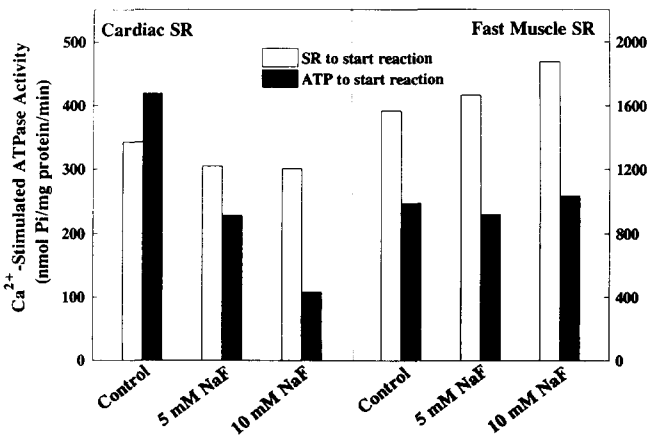


Fig. 4. Divergent effects of NaF on Ca^{2+}-stimulated ATPase activity in cardiac SR and fast skeletal muscle SR under different experimental conditions. Ca^{2+}-stimulated ATPase activity was determined as described under Materials and methods. The ATPase reaction was initiated by the addition of SR or ATP to the assay medium following preincubation of the rest of the assay components for 3 min at 37°C.

any case, such factors cannot account for the qualitatively different effects of fluoride on Ca^{2+} uptake and Ca^{2+}-ATPase activities of cardiac and fast muscle SR.

Influence of preincubation time on the effects of fluoride

It has been reported recently that fluoride inhibits Ca^{2+}-ATPase in rabbit skeletal muscle (presumably fast muscle) SR; the onset of this inhibition is slow and Ca^{2+} and ATP exert protective effects [22–24]. Since no inhibitory effect of fluoride on Ca^{2+} uptake or Ca^{2+}-ATPase activity of fast muscle SR was observed under the experimental conditions described above, further experiments were performed to assess the influence of varying preincubation time as well as the presence or absence of Ca^{2+} and ATP during preincubation with fluoride on Ca^{2+} uptake and Ca^{2+}-ATPase activities of SR. The results are shown in Fig. 5. Preincubation of fast muscle SR with fluoride in the presence of Ca^{2+} prior to initiating Ca^{2+} uptake by the addition of ATP to the assay medium, resulted in preincubation time-dependent stimulatory or inhibitory effects of fluoride. Thus, stimulation of Ca^{2+} uptake was observed when the duration of preincubation with fluoride was less than 10 min (which is in conformity with the data presented earlier, c.f. Fig. 1, right panel; Fig. 2, panel B) and inhibition of Ca^{2+} uptake was observed when the duration of preincubation exceeded 10 min; the extent of inhibition was ~ 40% following 30 min preincubation with fluoride (Fig. 5, panel B). The inhibition of Ca^{2+} uptake was accompanied by inhibition of Ca^{2+}-ATPase activity (Fig. 5, panel B inset). On the other hand, in similar experiments where cardiac SR was preincubated with fluoride in the presence of Ca^{2+}, only the inhibitory effect of fluoride was observed irrespective of the duration of preincubation (Fig. 5, panel A). About 80% inhibition of Ca^{2+} uptake activity occurred within 10 min of preincubation with fluoride and virtually complete inhibition of both Ca^{2+} uptake and Ca^{2+}-ATPase activities resulted within 20 min of exposure to fluoride. In experiments where preincubation with fluoride was carried out in the absence of Ca^{2+} and ATP (prior to initiating Ca^{2+} uptake/Ca^{2+}-ATPase reaction by the addition of Ca^{2+} *plus* ATP) complete inhibition of Ca^{2+} uptake and ATPase activities occurred within 3–5 min of exposure to fluoride in both cardiac SR (Fig. 5, panel C) and fast muscle SR (Fig. 5, panel D). Thus, fluoride has similar inhibitory effect on the Ca^{2+}- and ATP-deprived state (E_1/E_2) of the SR Ca^{2+}-ATPase in cardiac and fast muscle whereas the Ca^{2+}-bound state of the enzyme (CaE_1) shows striking tissue-specific difference in its susceptibility to inhibition by fluoride.

Effect of fluoride on phosphorylation by P_i

A characteristic functional difference between the Ca^{2+}-bound and Ca^{2+}-free conformations of the SR

Ca^{2+}-ATPase is that the former undergoes phosphorylation by ATP but not P_i whereas the latter undergoes phosphorylation by P_i but not ATP [1,35]. Since we observed tissue-specific differences in the effects of fluoride on Ca^{2+}-induced phosphoenzyme formation (Fig. 3) and Ca^{2+}-stimulated ATP hydrolysis (Fig. 4) in cardiac versus fast muscle SR, the effect of fluoride on phosphorylation of the Ca^{2+}-free ATPase by P_i was also investigated. It was found that fluoride exerted similar inhibitory effect on enzyme phosphorylation by P_i in both cardiac and fast muscle SR (Fig. 6).

Influence of aluminum and Gpp(NH)p on the effects of fluoride

Several studies have implicated the involvement of aluminum, as fluroaluminate complexes, in the diverse effects of fluoride on various biological systems including adenylate cyclase [36], actin [37], transducin [38] and 'P'-type cation transport ATPases [39–41]. A recent study described inhibitory effect of fluroaluminate complexes on skeletal muscle SR Ca^{2+}-ATPase activity [24] but an absolute requirement for aluminum could not be observed in other studies [22,23]. We investi-

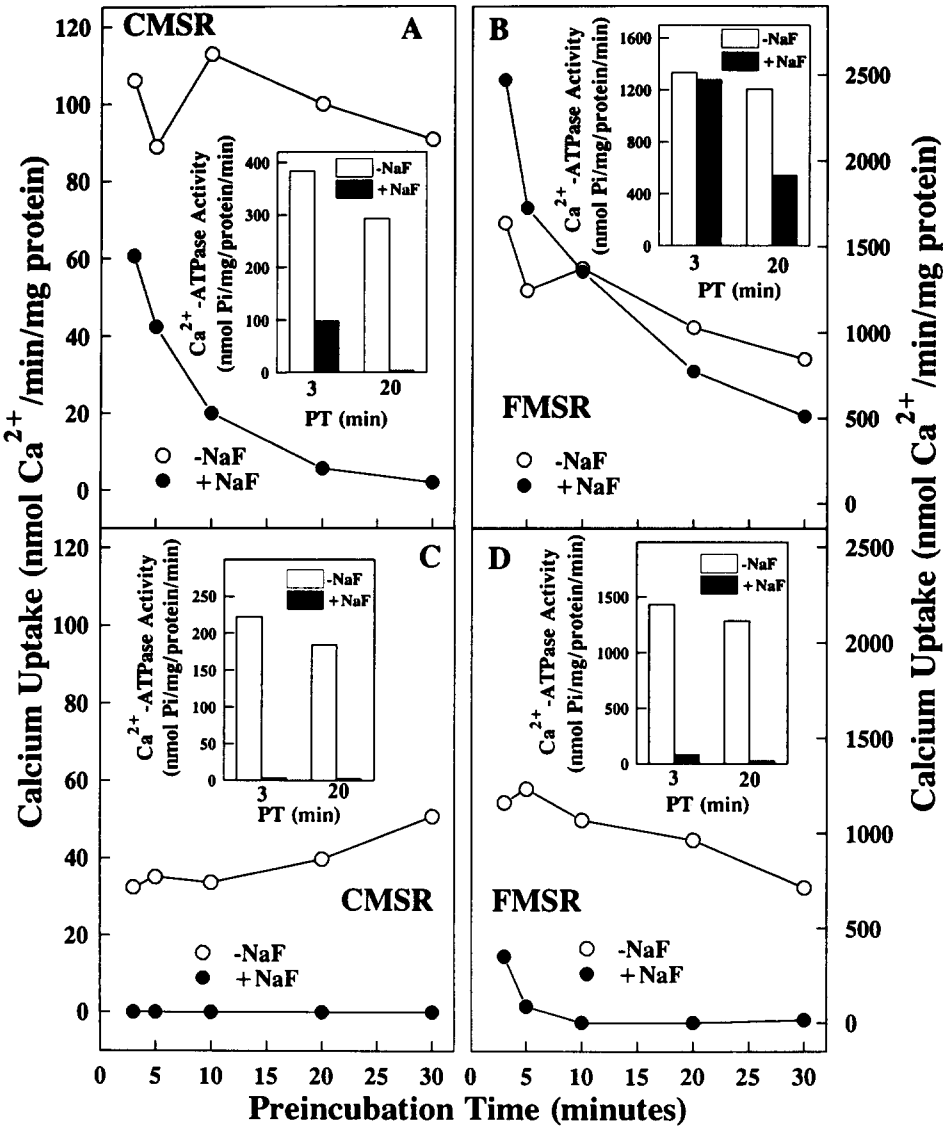


Fig. 5. Effects of varying preincubation time (PT) on ATP-dependent Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activities of cardiac muscle SR (CMSR) and fast skeletal muscle SR (FMSR) under different experimental conditions. Ca^{2+} uptake and Ca^{2+}-ATPase activities were determined using the standard incubation medium as described under Materials and methods. The Ca^{2+} uptake/ATPase reaction was initiated by the addition of ATP (panels A and B) or Ca^{2+} *plus* ATP (panels C and D) to the assay medium following preincubation of the rest of the assay components at 37°C for varying time intervals (3–30 min) as indicated. The reaction was terminated 1 min after addition of ATP or ATP *plus* Ca^{2+}. When present, the concentration of NaF in the assay medium was 10 mM.

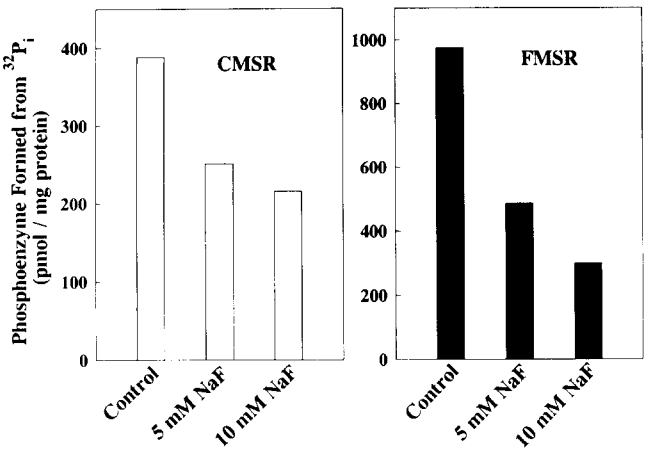


Fig. 6. Effect of NaF on the phosphorylation of the Ca^{2+}-deprived Ca^{2+}-ATPase of cardiac SR (CMSR) and fast skeletal muscle SR (FMSR) by P_i. Steady-state levels of phosphoenzyme formed from [^{32}P]phosphate were determined as described under Materials and methods. The phosphorylation reaction was initiated by the addition of [^{32}P]phosphate following preincubation of the rest of the assay mixture at 24°C for 3 min. The reaction was terminated 30 s after addition of [^{32}P]phosphate.

gated the potential involvement of aluminum in the effects of fluoride on the Ca^{2+} pump activity of cardiac and fast muscle SR by supplementing the Ca^{2+} transport assay medium with exogenous aluminum (10 μM), or deferoxamine (1 mM) to chelate endogenous aluminum. In these experiments, Ca^{2+} uptake was initiated by the addition of ATP to SR preincubated with fluoride (for 3 min), i.e., under conditions where fluoride produced inhibition of Ca^{2+} uptake in cardiac SR and stimulation of Ca^{2+} uptake in fast muscle SR. The presence of aluminum or deferoxamine did not alter appreciably the concentration-dependent stimulatory effect of fluoride on Ca^{2+} uptake by fast muscle SR (Fig. 7, right panel). The inhibitory effect of fluoride on Ca^{2+} uptake by cardiac SR was slightly potentiated by aluminum (50% inhibition with ~ 6 and 4 mM NaF, respectively, in the absence and presence of aluminum), and the effect of deferoxamine was negligible (Fig. 7, left panel). Thus, the divergent effects of fluoride on cardiac and fast muscle SR Ca^{2+} pumps do not seem to have an absolute requirement for aluminum.

Since fluoride is known to activate G-proteins [36,42,43], the possibility that the observed effects of fluoride on the SR Ca^{2+} pumps may occur via G-protein activation was examined. For this, the Ca^{2+} uptake assays were performed in the absence and presence of Gpp(NH)p (10 μM), a non-hydrolysable analogue of GTP, which is known to activate G-proteins irreversibly. The results (not shown) demonstrated that Gpp(NH)p does not mimic the effects of fluoride on Ca^{2+} uptake by cardiac or fast muscle SR. Thus it is unlikely that a G-protein is involved in the actions of fluoride on the SR Ca^{2+} pumps.

Influence of oxalate concentration and assay temperature on the stimulatory effect of fluoride

We have noted previously that the stimulatory effect of fluoride on Ca^{2+} uptake by cardiac SR (seen only when SR is exposed to fluoride in the presence of both Ca^{2+} and ATP, c.f. Fig. 2, panel C) is abolished at a saturating concentration of oxalate in the assay medium, and hence, the ability of fluoride to mimic the action of oxalate in precipitating Ca^{2+} inside the SR vesicles may account for its stimulatory effect [21]. To examine if this was also the mechanism for the stimulatory effect of fluoride on Ca^{2+} uptake by fast muscle SR (seen when SR is exposed to fluoride in the presence of Ca^{2+} alone or in the presence of both Ca^{2+} and ATP, c.f. Fig. 2, panel B), the effect of varying oxalate concentration on the stimulatory effect of fluoride on Ca^{2+} uptake by fast muscle SR was determined. As shown in Fig. 8, the stimulatory effect of fluoride declined with increasing oxalate concentration. However, unlike in the case of cardiac SR [21], a small fraction (~ 20%) of the stimulatory effect persisted at saturating oxalate concentration.

The transition from stimulatory to inhibitory effect of fluoride on Ca^{2+} uptake by fast muscle SR required long preincubation (> 10 min) with fluoride when exposure to fluoride was carried out in the presence of Ca^{2+}, and the inhibitory effect was partial (~ 40%) even after 30 min of exposure to fluoride (c.f. Fig. 5,

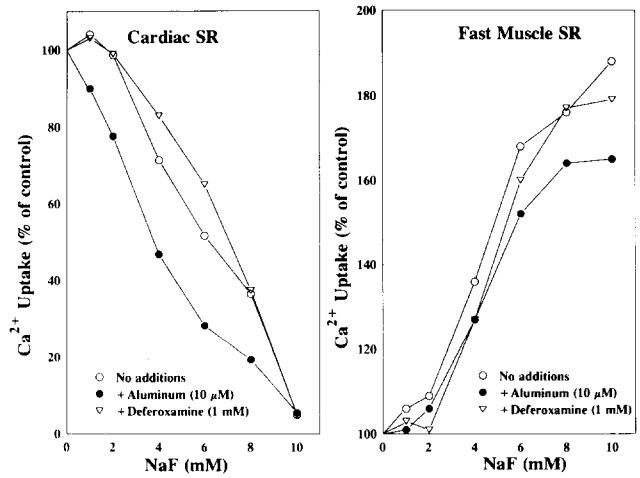


Fig. 7. Influence of aluminum and deferoxamine on the divergent effects of NaF on ATP-dependent Ca^{2+} uptake by cardiac muscle SR and fast skeletal muscle SR. Ca^{2+} uptake was determined using the standard incubation medium (see Materials and methods) in the absence and presence of varying concentrations of NaF with and without aluminum or deferoxamine as indicated. 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. The results are presented as percent of control. Values for Ca^{2+} uptake (nmol Ca^{2+}/mg protein per 3 min) measured in the absence of NaF (control, 100%) were as follows. Cardiac SR: No additions, 642; +aluminum, 732; +deferoxamine, 657. Fast muscle SR: No additions, 3946; +aluminum, 4217; +deferoxamine, 3877.

panel B). In recent reports describing the inhibitory action of fluoride on fast muscle SR Ca^{2+}-ATPase [22,23], the 'fluoride-inhibited ATPase' was obtained following prolonged incubation (60 min at 39°C) with fluoride. We investigated the possibility that slowing the Ca^{2+} pump turnover rate may facilitate fluoride interaction with the ATPase and consequently, expression of the inhibitory effect of fluoride on fast muscle SR Ca^{2+} pump. However, in experiments where the turnover rate of the Ca^{2+} pump was decreased 9-fold by lowering the assay temperature, the stimulatory effect of fluoride on Ca^{2+} uptake by fast muscle SR persisted, and the magnitude of stimulation was undiminished (results not shown).

Effect of fluoride on Ca^{2+} release

The possibility that fluoride may also influence Ca^{2+} release from SR was investigated by determining the effect of fluoride on unidirectional Ca^{2+} release from passively Ca^{2+}-loaded SR vesicles. The results showed a modest potentiation of the rate of Ca^{2+} release from fast muscle SR (depletion of 50% of the Ca^{2+}-load in 85 and 60 s, respectively, in absence of fluoride and in the presence of 10 mM fluoride); such an effect, although evident, was even less marked in cardiac SR (Fig. 9). Obviously, the stimulatory effect of fluoride on Ca^{2+} uptake by fast muscle SR observed in this study cannot be attributed to inhibition of Ca^{2+} release by fluoride. On the other hand, the inhibitory effect of fluoride on Ca^{2+} uptake by cardiac or fast muscle SR observed under different experimental conditions was invariably accompanied by inhibition of Ca^{2+}-ATPase activity. Therefore, it is unlikely that the minor potentiating effect of fluoride on Ca^{2+} release contributes appreciably to the observed inhibitory effect of fluoride on Ca^{2+} uptake by SR.

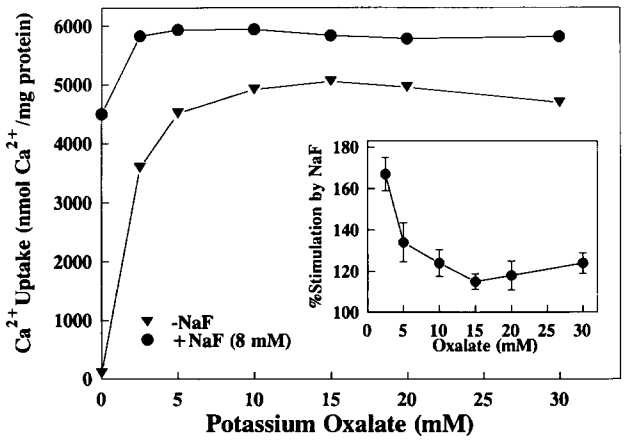


Fig. 8. Effect of varying oxalate concentration on the stimulatory effect of NaF on ATP-dependent Ca^{2+} uptake by fast skeletal muscle SR. The Ca^{2+} uptake assays were performed using the standard incubation medium (see Materials and methods) containing varying concentrations of potassium oxalate in the absence and 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.

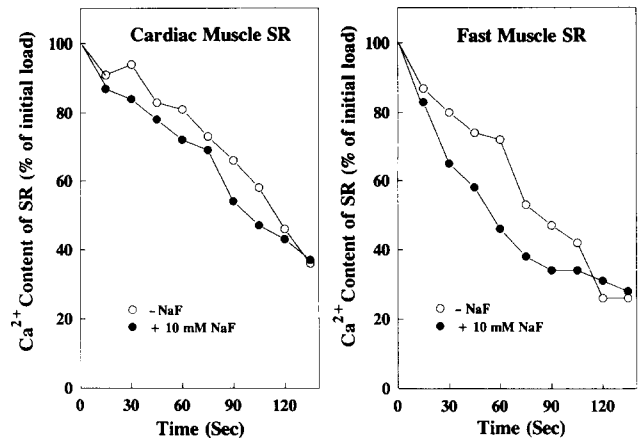


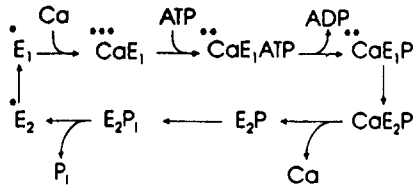
Fig. 9. Effect of NaF on Ca^{2+} release from Ca^{2+}-loaded SR vesicles. SR vesicles from cardiac muscle and fast skeletal muscle were passively loaded with $^{45}CaCl_2$ and the rates of Ca^{2+} release were monitored in the absence and presence of NaF in a Ca^{2+}-release medium as described under Materials and methods. The initial Ca^{2+} load (nmol/mg/protein) of SR vesicles (prior to initiating release) was 253 for cardiac SR and 1485 for fast muscle SR.

Influence of the interaction between Ca^{2+}-ATPase and phospholamban on the effect of fluoride on cardiac SR Ca^{2+} pump

In cardiac SR, but not in fast muscle SR, the Ca^{2+}-ATPase is subject to regulation by the intrinsic membrane protein, phospholamban [10–15]. Current models of Ca^{2+}-ATPase regulation by phospholamban depict dephospho-phospholamban as an inhibitor of the Ca^{2+}-ATPase. Inhibition is exerted by association between the two proteins and is diminished by dissociation of the two proteins when phospholamban is phosphorylated by cAMP kinase or Ca^{2+}/calmodulin-dependent protein kinase [11–15,44]. In order to assess whether interaction between Ca^{2+}-ATPase and phospholamban influenced the action of fluoride on SR Ca^{2+} pump function, we compared the effects of fluoride on Ca^{2+} uptake activities of cardiac SR with phospholamban in its unphosphorylated or phosphorylated state. The sensitivity of Ca^{2+} pump to inhibition by fluoride was found to be essentially similar in cardiac SR containing phospho- or dephospho form of phospholamban (results not shown) suggesting that interaction between Ca^{2+}-ATPase and phospholamban does not influence the effect of fluoride.

4. Discussion

In this study we have compared the effects of fluoride on the enzymatic and ion transport functions of



Scheme 1. The conformational states evolving during the catalytic cycle of the Ca^{2+}-ATPase. * Susceptible to inhibition by fluoride. ** Resistant to inhibition by fluoride. *** Susceptible to inhibition by fluoride in cardiac muscle but not fast muscle.

the Ca^{2+}-ATPase in cardiac and fast skeletal muscle SR. The results obtained demonstrate similarities as well as striking tissue-specific differences in the actions of fluoride on the two Ca^{2+} pumps. These include: (i) a stimulatory effect of fluoride on active Ca^{2+} uptake by cardiac and fast muscle SR under experimental conditions which permitted encounter of fluoride with Ca^{2+}-ATPase in the presence of both Ca^{2+} and ATP (Fig. 2, panels B and C); (ii) an inhibitory effect of fluoride on active Ca^{2+} uptake by cardiac and fast muscle SR under experimental conditions where encounter of fluoride with the Ca^{2+}-ATPase occured in the absence of Ca^{2+} and ATP (Fig. 5, panels C and D); and (iii) striking dissimilar effects of fluoride on active Ca^{2+} uptake by cardiac and fast muscle SR under experimental conditions where fluoride encountered the Ca^{2+}-ATPase in the presence of Ca^{2+} but in the absence of ATP (e.g., Figs. 1, 2, 7). Plausible explanations for these divergent effects of fluoride and the basis of tissue-specific differences in its action can be sought by examining the requirements for inhibition or stimulation in the context of the predominant conformational state of the ATPase present at the time of introduction of fluoride into the reaction medium. The conformational states evolving during the catalytic cycle of the Ca^{2+}-ATPase are shown in Scheme 1. The divergent effects of fluoride seen under diverse experimental conditions likely stem from differential susceptibility of various conformational states of the ATPase to fluoride. Our observation that exposure of cardiac or fast muscle SR to fluoride in the absence of Ca^{2+} and ATP results in strong inhibitory effect of fluoride on both Ca^{2+} uptake and Ca^{2+}-ATPase activity (Fig. 5, panels C, D) suggests that the Ca^{2+}- and ATP-deprived conformations of the ATPase (E_1 and E_2 in Scheme 1) are highly susceptible to the inhibitory action of fluoride. On the other hand, exposure of either cardiac or fast muscle SR to fluoride in the presence of Ca^{2+} and ATP resulted in stimulation of Ca^{2+} uptake (Fig. 2, panels B, C) with no appreciable change in Ca^{2+}-ATPase activity (Fig. 4). These findings imply that in both cardiac and fast muscle SR, the CaE_1ATP conformation of the ATPase is immune to the inhibitory action of fluoride. Consistent with these observations, Murphy and Coll [22] have reported recently that Ca^{2+} and ATP exert protective effect against inhibition of fast muscle SR Ca^{2+}-ATPase activity by fluoride. Despite the above overall similarities in the effects of fluoride on cardiac and fast muscle SR, striking tissue-specific differences emerged under the experimental condition where Ca^{2+} pump turnover was induced by the addition of ATP to SR preincubated with fluoride in the presence of Ca^{2+} but in the absence of ATP. Under this condition, fluoride caused stimulation of Ca^{2+} uptake by fast muscle SR with no appreciable change in Ca^{2+}-stimulated ATP hydrolysis, and inhibition of Ca^{2+} uptake by cardiac SR with concomitant inhibition of Ca^{2+}-stimulated ATP hydrolysis (Figs. 1, 2, 4, 7). These findings imply that at least one steady-state intermediate of the SR Ca^{2+}-ATPase should differ qualitatively in cardiac and fast muscle with respect to its fluoride sensitivity. Evidence from our data, summarised below, points to the Ca^{2+}-liganded E_1 conformation (CaE_1, Scheme 1) of the Ca^{2+}-ATPase as the intermediate depicting such a tissue-specific qualitative difference. (a) Since encounter of fluoride with the ATPase (in cardiac and fast muscle SR) in the presence of both Ca^{2+} and ATP did not result in inhibition of Ca^{2+} uptake (Fig. 2, panels B and C), or ATPase activity (Fig. 4), the conformational states $CaE_1ATP \rightarrow CaE_1P$ are 'fluoride-resistant' in both cardiac and fast muscle. (b) Encounter of fluoride with the ATPase in the presence of Ca^{2+} alone (i.e., ATP absent) resulted in inhibition of Ca^{2+} uptake and ATPase activity in cardiac but not fast muscle SR (e.g., Figs. 1, 2, 4, 7). Since the possible enzyme conformations prevailing in the presence of Ca^{2+} (and in the absence of ATP) are $E_2 \rightarrow E_1 \rightarrow CaE_1$, the difference in fluoride sensitivity between the cardiac and fast muscle SR ATPases should relate to one or more of these enzyme conformations. (c) Encounter of fluoride with the ATPase in the absence of Ca^{2+} and ATP resulted in inhibition of Ca^{2+} uptake and ATPase activity in both cardiac and fast muscle SR (Fig. 5, panels C and D) suggesting that fluoride reactivity of the prevailing enzyme conformations $E_2 \rightarrow E_1$ is similar in both muscle types. This leaves the conformational state CaE_1 as 'fluoride-sensitive' in cardiac and 'fluoride-resistant' in fast muscle. Further evidence in support of this view comes from our observations that (i) in cardiac SR, but not in fast muscle SR, fluoride inhibited phosphoenzyme formation from ATP (Fig. 3) by the Ca^{2+}-liganded Ca^{2+}-ATPase (CaE_1), and (ii) fluoride did not inhibit phosphoenzyme formation from ATP by the Ca^{2+} *plus* ATP-liganded Ca^{2+}-ATPase (CaE_1ATP) in cardiac or fast muscle SR (Fig. 3).

It must be noted that prolonged preincubation (> 10 min) of fast muscle SR with fluoride in the presence of Ca^{2+} led to the onset of partial inhibition of Ca^{2+} uptake and Ca^{2+}-stimulated ATP hydrolysis (Fig. 5,

panel B). This slowly evolving partial inibition is likely due to the interaction of fluoride with 'Ca^{2+}-free' rather than 'Ca^{2+}-bound' enzyme for the following reasons. (i) Prolonged preincubation leads to appreciable decrease in extravesicular free Ca^{2+} due to passive Ca^{2+} loading of SR (under the nonturnover conditions of preincubation). Since the free Ca^{2+} concentration at the start of preincubation is in the low micromolar range (initial free $Ca^{2+} = 8.2$ μM), decrease in extravesicular Ca^{2+} with increasing preincubation time would increase the probability of fluoride encountering the Ca^{2+}-free enzyme, thus causing inhibition. Accordingly, we have observed that the onset of inhibition could be prevented by adding a small amount of Ca^{2+} periodically (100 nmol Ca^{2+} at 5 min intervals during preincubation) to compensate for the fall in extravesicular free Ca^{2+} (results not shown). (ii) Recent kinetic data on the effect of fluoride on fast muscle SR Ca^{2+}-ATPase activity suggest that the Ca^{2+}-bound enzyme is not fluoride-reactive in fast muscle SR [22].

As pointed out in the 'Results' section, for the most part, the stimulatory effect of fluoride on Ca^{2+} uptake by SR observed under specific experimental conditons described in this study is secondary to the ability of fluoride to mimic the action of oxalate in trapping Ca^{2+} inside the SR vesicles when the fluoride-resistant enzyme (Ca^{2+}- and ATP-bound conformation) turns over normally. The inhibitory effect of fluoride, on the other hand, must involve interaction of some form of fluoride with the Ca^{2+}-ATPase. Combination of fluoride with aluminum has been implicated in the effects of fluoride on a number of proteins [36–38]. The effects of fluoroaluminate complexes on Na^+/K^+-ATPase [39] and SR Ca^{2+}-ATPase [24,40,41] have also been reported and it has been suggested that aluminum fluoride (AlF_4^-) is the effective species. However, recent kinetic studies on the effects of fluoride on fast muscle SR Ca^{2+}-ATPase [22] and on kidney Na^+/K^+-ATPase [45] showed that fluoride inhibits these 'P-type' transport ATPases independent of aluminum and it was suggested that inhibition results from interaction of free fluoride with the Mg^{2+}-bound enzyme. Our data showing negligible effect of deferoxamine (aluminum chelator), also suggest no essential requirement for aluminum in the action of fluoride on the SR Ca^{2+} pumps although a potentiating effect of aluminum would seem likely. Regardless of the form of fluoride involved, it has been suggested that fluoride inhibits the SR Ca^{2+}-ATPase by stabilizing the Ca^{2+}-free form of the enzyme through occupation of the phosphorylation site [22–24], and in addition, acts as an analogue of γ-phosphate of ATP (when bound to the nucleotide binding site in conjunction with ADP) to stabilize the Ca^{2+}-bound from of the enzyme [24]. Our finding that fluoride inhibits phosphorylation of the Ca^{2+}-deprived enzyme from P_i in both cardiac and fast muscle SR (Fig. 6) suggests that fluoride-sensitivity of the phosphorylation site of SR Ca^{2+}-ATPase is similar in cardiac and fast muscle. On the other hand, we have found that phosphorylation of the Ca^{2+}-bound enzyme from ATP is inhibited by fluoride in cardiac but not fast muscle SR (Fig. 3). In view of the above observations and the finding that Ca^{2+} *plus* ATP-liganded enzyme (CaE_1ATP) is immune to inhibition by fluoride in both cardiac and fast muscle SR, it can be postulated that a tissue-specific difference in the accessibility (reactivity) of the nucleotide binding site to fluoride upon Ca^{2+} binding to the enzyme may account for the observed difference in fluoride-sensitivity of the cardiac versus fast muscle enzyme – i.e., when the ATPase is in CaE_1 conformation, the ATP binding site is accessible (reactive) to fluoride in the cardiac enzyme but not in the fast muscle enzyme. According to current models describing the structure-function relationship of SR Ca^{2+}-ATPase, the nucleotide binding domain and the phosphorylation domain containing the aspartyl residue (Asp 351) phosphorylated from ATP are located at the opposite sides of the Ca^{2+} binding cavity, and the transfer of γ-phosphate of ATP to the protein is brought about in connection with a conformational change (initiated by Ca^{2+} binding) that closes the Ca^{2+} cavity thereby occluding the bound Ca^{2+} [2,3,6,7,45,47]. Interestingly, despite the high degree of homology in primary structure [4,5,48] and predicted similarities in transmembrane topologies and tertiary structures [2,3,6,7] between the cardiac and fast muscle SR Ca^{2+}-ATPase, limited yet apparently unique, differences are seen in amino acid residues comprising some regions (including predicted alpha helix and beta strand regions) of the nucleotide binding domain of these two ATPases (for sequence comparison, see Refs. 4–6). On the other hand, the amino acid sequence within the phosphorylation domain is virtually identical in the cardiac and fast muscle SR Ca^{2+}-ATPase [4–6]. Thus, there exists a structural basis probably sufficient to impart subtle differences in functional properties of the nucleotide binding site associated with a Ca^{2+}-induced change in enzyme conformation affecting the nucleotide binding domain. Such structure-related, conformation-dependent, differences in the functional properties of the nucleotide binding domain in cardiac versus fast muscle SR Ca^{2+}-ATPases may also explain the previously reported differences in catalytic properties and substrate (nucleotide triphosphate) utilization by these two Ca^{2+} pumps [18–20,49]. Muscle-specific difference in the structure/function property of the nucleotide binding domain may be important physiologically as this domain has been implicated in the interaction between cardiac SR Ca^{2+}-ATPase and its physiological regulator, phospholamban [50]; the fast muscle SR Ca^{2+}-ATPase is not subject to regulation by phospho-

lamban. In the present study, we, however, did not observe alteration in the fluoride-sensitivity of the cardiac SR Ca^{2+}-ATPase upon perturbation of the functional interaction between the ATPase and phospholamban through phosphorylation of the latter.

Finally, in agreement with our finding, in recent kinetic studies, Murphy and Coll [22] have found that the Ca^{2+}-bound conformation of the fast muscle SR Ca^{2+}-ATPase does not react with fluoride. In contrast, in another study on the effects of fluroaluminate complexes on fast muscle SR Ca^{2+}-ATPase, Troullier et al. [24] reported that fluoride inhibits the Ca^{2+}-bound enzyme in the presence of aluminum and ADP. We did not observe inhibition of the Ca^{2+}-bound fast muscle SR Ca^{2+}-ATPase by fluoride even in the presence of aluminum (Fig. 7, right panel). This apparent discrepancy may be explained by the requirement for ADP for inhibition of the Ca^{2+}-bound enzyme by fluoride [24]. In our experiments, no ADP was present during preincubation of SR with Ca^{2+} and fluoride prior to initiating enzyme turnover with ATP. Since ATP protects against fluoride inhibition of the enzyme (present study and Ref. 22), ADP formed during turnover would be ineffective in promoting inhibition by fluoride. Our observations underscore the critical influence of the pre-existing Ca^{2+} pump conformation encountered by fluoride in determining its effects on Ca^{2+} pump function.

Acknowledgements

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