*Biochimica et Biophysica Acta*, 735 (1983) 53–66
Elsevier



BBA 71860

# INHIBITION OF SARCOPLASMIC RETICULUM CALCIUM PUMP BY CYTOSOLIC PROTEIN(S) ENDOGENOUS TO HEART AND SLOW SKELETAL MUSCLE BUT NOT FAST SKELETAL MUSCLE

NJANOOR NARAYANAN, MARGERY NEWLAND and DONNA NEUDORF

*Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, S7N 0W0 (Canada)*

(Received January 28th, 1983)
(Revised manuscript received July 15th, 1983)

*Key words: Sarcoplasmic reticulum; $Ca^{2+}$ pump inhibitor; Heart muscle; Slow muscle; Fast muscle; (Rabbit)*

**Cytosol from rabbit heart and slow and fast skeletal muscles was fractionated using $(NH_4)_2SO_4$ to yield three cytosolic protein fractions, viz., CPF-I (protein precipitated at 30% saturation), CPF-II (protein precipitated between 30 and 60% saturation), and cytosol supernatant (protein soluble at 60% saturation). The protein fractions were dialysed and tested for their effects on ATP-dependent, oxalate-supported $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart and slow and fast skeletal muscles. CPF-I from heart and slow muscle, but not from fast muscle, caused marked inhibition (up to 95%) of $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart and from slow and fast muscles. Neither unfractionated cytosol nor CPF-II or cytosol supernatant from any of the muscles altered the $Ca^{2+}$ uptake activity of sarcoplasmic reticulum. Studies on the characteristics of inhibition of sarcoplasmic reticulum $Ca^{2+}$ uptake by CPF-I (from heart and slow muscle) revealed the following: (a) Inhibition was concentration- and temperature-dependent (50% inhibition with approx. 80 to 100 μg CPF-I; seen only at temperatures above 20°C). (b) The inhibitor reduced the velocity of $Ca^{2+}$ uptake without appreciably influencing the apparent affinity of the transport system for $Ca^{2+}$. (c) Inhibition was uncompetitive with respect to ATP. (d) Sarcoplasmic reticulum washed following exposure to CPF-I showed reduced rates of $Ca^{2+}$ uptake, indicating that inhibition results from an interaction of the inhibitor with the sarcoplasmic reticulum membrane. (e) Concomitant with the inhibition of $Ca^{2+}$ uptake, CPF-I also inhibited the $Ca^{2+}$-ATPase activity of sarcoplasmic reticulum. (f) Heat-treatment of CPF-I led to loss of inhibitor activity, whereas exposure to trypsin appeared to enhance its inhibitory effect. (g) Addition of CPF-I to $Ca^{2+}$-preloaded sarcoplasmic reticulum vesicles did not promote $Ca^{2+}$ release from the vesicles. These results demonstrate the presence of a soluble protein inhibitor of sarcoplasmic reticulum $Ca^{2+}$ pump in heart and slow skeletal muscle but not in fast skeletal muscle. The characteristics of the inhibitor and its apparently selective distribution suggest a potentially important role for it in the in vivo regulation of sarcoplasmic reticulum $Ca^{2+}$ pump, and therefore in determining the duration of $Ca^{2+}$ signal in slow-contracting muscle fibers.**

## Introduction

In heart and skeletal muscle, sarcoplasmic reticulum plays a central role in excitation-contraction coupling by releasing $Ca^{2+}$ for the activation of contraction and by sequestering $Ca^{2+}$ from the sarcoplasm to promote muscle relaxation. The $Ca^{2+}$ sequestration function is served by a membrane-associated $Ca^{2+}$ pump represented by the enzyme $Mg^{2+}$-dependent $Ca^{2+}$-ATPase, which transports $Ca^{2+}$ into the sarcoplasmic reticulum lumen at the

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

0005-2736/83/$03.00 © 1983 Elsevier Science Publishers B.V.

expense of ATP hydrolysis (see Refs. 1–6 for recent reviews). While the mechanism of active $Ca^{2+}$ transport across the sarcoplasmic reticulum membrane has been extensively studied and documented (Refs. 1–6 and references therein), relatively less is known about the physiological mechanisms of regulation of sarcoplasmic reticulum $Ca^{2+}$ pump, in addition to the obvious regulation induced by the occupation of $Ca^{2+}$-, ATP- and $Mg^{2+}$-binding sites. The potentially important physiological regulators of sarcoplasmic reticulum $Ca^{2+}$ pump recognized thus far include cyclic AMP and calmodulin, both of which stimulate the $Ca^{2+}$ pump activity of cardiac sarcoplasmic reticulum, apparently through the phosphorylation of a minor hydrophobic membrane protein, phospholamban, catalyzed by specific protein kinases [7–13]. Cyclic AMP-promoted phosphorylation of a phospholamban-like protein and consequent stimulation of $Ca^{2+}$ transport has also been reported in the case of slow skeletal muscle sarcoplasmic reticulum [14]; such a regulatory mechanism involving phospholamban phosphorylation does not appear to be present in fast muscle sarcoplasmic reticulum [14], but cyclic AMP-dependent phosphorylation of membrane proteins other than phospholamban may stimulate $Ca^{2+}$ transport in fast muscle sarcoplasmic reticulum as well [15]. Also, calmodulin does not seem to influence the $Ca^{2+}$ transport activity of fast muscle sarcoplasmic reticulum, although substrates for calmodulin-dependent phosphorylation are present in this membrane [16]. The stimulatory effects of cyclic AMP and calmodulin on the $Ca^{2+}$ transport activity of sarcoplasmic reticulum (at least in the case of heart and slow skeletal muscle) place them as positive modulators of the sarcoplasmic reticulum $Ca^{2+}$ pump. This is also true of a 53-kDa glycoprotein-associated fast muscle sarcoplasmic reticulum [17] which has been recognized to exert a stimulatory influence on the $Ca^{2+}$ pump activity of this membrane by as yet unidentified mechanisms [16]. On the other hand, a variety of exogenous substances such as anesthetics [18–20], photooxidizing agents [21], mercurials [22,23] and the flavanoid quercetin [24] are known to inhibit $Ca^{2+}$ transport activity of sarcoplasmic reticulum vesicles isolated from heart or skeletal muscle. In a recent preliminary report, we showed that a cytosolic protein fraction derived from rabbit heart caused marked inhibition of ATP-dependent $Ca^{2+}$ uptake by rabbit cardiac sarcoplasmic reticulum and suggested the probable involvement of endogenous regulators in the negative modulation of sarcoplasmic reticulum $Ca^{2+}$ pump in heart muscle [25]. In this report we describe the results of detailed studies demonstrating the selective distribution of cytosolic protein inhibitor(s) of sarcoplasmic reticulum $Ca^{2+}$ pump in heart and slow skeletal muscle but not in fast skeletal muscle. Further, the characteristics of inhibition of $Ca^{2+}$ transport as well as certain other properties of the inhibitor are presented. It is suggested that transient inhibition of sarcoplasmic reticulum $Ca^{2+}$ pump by the inhibitor might provide a 'turn off' mechanism for the $Ca^{2+}$ pump in vivo, thus augmenting the duration of $Ca^{2+}$ signal in sarcoplasm during the relatively prolonged contraction phase (compared to fast skeletal muscle) of heart and slow skeletal muscles. Some of these results have appeared in abstract form [26].

## Methods

### *Chemicals*

$^{45}CaCl_2$ (15.94 mCi/mg) and [$\gamma$-$^{32}$P]ATP (36 Ci/mmol) were purchased from New England Nuclear, Montreal, Canada. Ionophore A23187 was from Eli Lilly Co., Indianapolis, IN, U.S.A. [$^{14}$C]Hemoglobin, prepared according to the procedure of Roth and Losty [27], was kindly supplied by Dr. R.L. Khandelwal, Department of Biochemistry, University of Saskatchewan. Reagents for electrophoresis were obtained from Bio-Rad Laboratories, Mississauga, Ontario, Canada. All other chemicals were of highest purity available from Sigma Chemical Co., St. Louis, MO, or Fisher Scientific Co., NJ, U.S.A.

### *Preparations of cytosolic protein fractions*

Male rabbits (2.5–3 kg body weight) were killed by a sharp blow to the base of the skull, and heart (ventricles) and slow (soleus) and fast (adductor magnus) skeletal muscles were quickly excised and transferred to ice-cold homogenizing buffer (10 mM Tris/0.25 M sucrose/0.2 mM dithioerythreitol, pH 7). The tissues (free of connective tissue, fat and blood) were homogenized in 10 vol. (w/v)

of buffer for 50 s using a Polytron PT-20 homogenizer (15, 25 and 10 s bursts, rheostat setting 5.5). The homogenates were centrifuged at 10000 $\times g$ for 20 min and the pellets discarded. The supernatant from this step was centrifuged at 105000 $\times g$ for 2 h to obtain the cytosol fraction. The cytosol was then fractionated using $(NH_4)_2SO_4$ to obtain three cytosolic protein fractions as described below. Solid $(NH_4)_2SO_4$ was added to the cytosol to give 30% saturation and the protein precipitated after 25 min of gentle stirring was sedimented by centrifugation at 10000 $\times g$ for 15 min. This protein fraction is referred to as CPF-I. The concentration of $(NH_4)_2SO_4$ in the supernatant was then raised to 60% and after 25 min, the protein precipitated was sedimented by centrifugation as described above. This protein fraction is referred to as CPF-II. The supernatant from the above step (fraction soluble at 60% $(NH_4)_2SO_4$ saturation) was also collected and is referred to as 'cytosol supernatant'. CPF-I and CPF-II were dissolved in homogenizing buffer to give a protein concentration of 4–5 mg/ml. All three cytosolic protein fractions were dialysed against the homogenizing buffer (for 20 h) and used for experiments. All fractionation procedures were carried out at 4°C.

Heat-treated CPF-I used in some experiments was prepared as follows: CPF-I was first exposed to 75°C in a water bath for 2 min, following which the tube was chilled on ice and the denatured protein was removed by centrifugation; the clear supernatant served as the heat-treated CPF-I.

*Isolation of sarcoplasmic reticulum vesicles and determination of $Ca^{2+}$ transport and ATPase activities*

Sarcoplasmic reticulum-enriched membrane vesicles from rabbit heart (ventricles) and slow (soleus) and fast (adductor magnus) skeletal muscles were prepared according to the procedure of Harigaya and Schwartz [28] with minor modifications as described previously [29]. ATP-dependent oxalate-facilitated $Ca^{2+}$ uptake by sarcoplasmic reticulum was determined using a Millipore filtration technique as detailed elsewhere [30]. 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, sarcoplasmic reticulum vesicles (20–30 $\mu$g protein) and varying concentrations of $^{45}CaCl_2$ (6000–8000 cpm/nmol). Unless indicated otherwise, the assays were performed at 37°C; the $Ca^{2+}$ uptake reaction was initiated by the addition of ATP following preincubation of the rest of the assay components for 3 min. The free $Ca^{2+}$ ion concentrations in the assay medium were determined as described previously [30]. The incubation medium used for the assay of $Mg^{2+}$-dependent $Ca^{2+}$-ATPase was identical to that described for $Ca^{2+}$ uptake, except that [$\gamma$-$^{32}$P]ATP was used instead of non-radioactive ATP and non-radioactive $CaCl_2$ was used instead of $^{45}CaCl_2$. To determine the 'basal' ATPase ($Mg^{2+}$-ATPase) activity, assays were also carried out in the absence of $Ca^{2+}$ and in the presence of 0.2 mM EGTA. The incubations were carried out at 37°C for 3 min and the reaction was stopped by the addition of 1 ml 12% trichloroacetic acid/2 mM $KH_2PO_4$. Following this, 0.1 ml each of 25 mM ATP and 0.1% bovine serum albumin were added to the tubes. The tubes were centrifuged (3000 rpm, 10 min) and the $^{32}P$ released from [$\gamma$-$^{32}$P]ATP was extracted and quantitated as described by Sulakhe and Drummond [31]. The basal ATPase activity was subtracted from the enzyme activity measured in the presence of $Ca^{2+}$ to obtain the $Ca^{2+}$-ATPase activity.

Unless indicated otherwise, the results shown are representative of at least three separate experiments.

*SDS-polyacrylamide gel electrophoresis*

Sarcoplasmic reticulum proteins were fractionated by SDS-polyacrylamide slab-gel electrophoresis as described previously [30].

*Determination of protein*

Protein was determined by the method of Lowry et al. [32] using defatted bovine serum albumin as standard.

## Results and Discussion

*Effects of varying concentrations of cytosolic protein fractions on $Ca^{2+}$ uptake by sarcoplasmic reticulum*

Fig. 1 shows the effects of varying concentrations of different cytosolic protein fractions from

heart and slow and fast skeletal muscles on ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum at a saturating (see below) concentration (11.9 $\mu$M) of $Ca^{2+}$. The cytosolic protein fraction termed CPF-I derived from heart inhibited $Ca^{2+}$ uptake by cardiac sarcoplasmic reticulum in a concentration-dependent manner; unfractionated cytosol and CPF-II from heart showed no appreciable effect on $Ca^{2+}$ uptake (panel A). The effects of cytosolic fractions from slow skeletal muscle on $Ca^{2+}$ uptake by slow muscle sarcoplasmic reticulum were essentially similar to those seen in the case of cardiac muscle, i.e. CPF-I, but not CPF-II and unfractionated cytosol, caused inhibition of $Ca^{2+}$ uptake by sarcoplasmic reticulum (panel B). Interestingly, however, CPF-I (and unfractionated cytosol) derived from fast muscle was found to have no inhibitory effect on $Ca^{2+}$ uptake by fast muscle sarcoplasmic reticulum, whereas CPF-I derived from heart or slow muscle caused inhibition of $Ca^{2+}$ uptake by fast muscle sarcoplasmic reticulum (panel C). In these experiments, where identical amounts of sarcoplasmic reticulum (30 $\mu$g protein) were used in the assay, the concentration of CPF-I required to cause 50% inhibition of $Ca^{2+}$ uptake was similar (75–90 $\mu$g) in the case of heart and slow muscle sarcoplasmic reticulum but relatively higher (130–150 $\mu$g) in the case of fast muscle sarcoplasmic reticulum. In other experiments, the cytosol fraction free of both CPF-I and CPF-II (cytosol supernatant, see Methods) derived from heart and slow and fast muscles was found not to influence the $Ca^{2+}$ uptake activity of sarcoplasmic reticulum from any of these muscles (results not shown). The above findings demonstrate that a protein inhibitor (CPF-I) of active transport of $Ca^{2+}$ across the sarcoplasmic reticulum membrane is present in heart and slow muscle cytosol. While such an inhibitor is not readily detectable in fast muscle cytosol, the inhibitor derived from heart and slow muscle is capable of inhibiting the $Ca^{2+}$ transport activity of fast muscle sarcoplasmic reticulum.

In subsequent studies, we examined the characteristics of CPF-I-mediated inhibition of $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart and

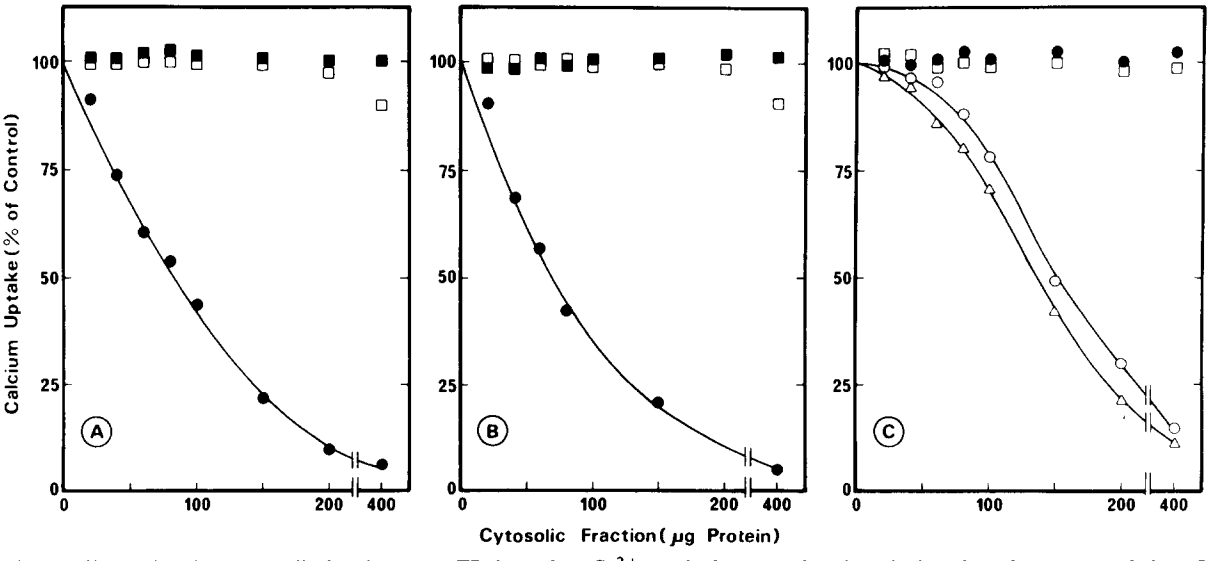


Fig. 1. Effects of various cytosolic fractions on ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart (A) and slow (B) and fast (C) skeletal muscles. The $Ca^{2+}$ uptake reaction was carried out under standard assay conditions (see Methods) in the absence (control) and in the presence of varying amounts of different cytosolic fractions as indicated. The concentration of $Ca^{2+}$ in the assay was 11.9 $\mu$M and that of sarcoplasmic reticulum protein 30 $\mu$g. The results are expressed as percent of control. The $Ca^{2+}$ uptake activity (nmol $Ca^{2+}$/mg protein per min, mean of duplicate determinations) of control (100%) amounted to 54, 712 and 2651 for sarcoplasmic reticulum from heart, slow muscle and fast muscle, respectively. □, unfractionated cytosol. In A and B: ●, CPF-I; ■, CPF-II. In C, ●, fast muscle CPF-I; △, slow muscle CPF-I; ○, heart CPF-I.

slow and fast muscles. In all these studies described below, CPF-I from heart was used to inhibit $Ca^{2+}$ uptake by cardiac and fast muscle sarcoplasmic reticulum, and CPF-I from slow muscle was used to inhibit $Ca^{2+}$ uptake by slow muscle sarcoplasmic reticulum.

*Comparison of the inhibitory effect of CPF-I at varying sarcoplasmic reticulum concentrations*

The concentration-dependence of the inhibitory effect of CPF-I on $Ca^{2+}$ uptake was also examined using varying amounts of sarcoplasmic reticulum in the assay. The results of these experiments indicated that with increasing concentration of sarcoplasmic reticulum in the assay medium, relatively greater concentration of CPF-I was required to cause 50% inhibition of $Ca^{2+}$ uptake. For example, with 10, 30 or 50 $\mu$g heart sarcoplasmic reticulum in the assay 50% inhibition of $Ca^{2+}$ uptake was observed with 29, 82 and 125 $\mu$g CPF-I, respectively; similar results were also obtained in experiments using slow muscle sarcoplasmic reticulum. However, the ratio of 'CPF-I concentration giving 50% inhibition/the amount of sarcoplasmic reticulum in the assay' was found to be almost similar (2.3–2.9) at varying sarcoplasmic reticulum concentrations, suggesting an apparently stoichiometric interaction of the inhibitor with the sarcoplasmic reticulum membrane. The estimated recoveries of CPF-I ($8.3 \pm 0.5$ and $8.7 \pm 0.6$ mg protein/g tissue respectively from heart and slow muscle) and sarcoplasmic reticulum ($3.5 \pm 0.3$ and $4.1 \pm 0.4$ mg protein/g tissue, respectively, from heart and slow muscle) from heart and slow muscle indicated the CPF-I/sarcoplasmic reticulum ratio in these muscles to be about 2.1 to 2.4. Thus, it appears that the concentration of the inhibitor present in the cytosol of heart and slow muscle is sufficient to cause considerable inhibition of the $Ca^{2+}$ transport activity of sarcoplasmic reticulum in vivo. It is worth noting that CPF-I comprised about 22–24% of the total cytosol protein (total cytosol protein amounted to $38 \pm 3$ and $36 \pm 2$ mg/g tissue, respectively, from heart and slow muscle) and therefore, the lack of inhibitory effect of unfractionated cytosol on sarcoplasmic reticulum $Ca^{2+}$ uptake, even at high concentrations (see Fig. 1), seems not to be related to inadequate concentration of the inhibitor in this fraction. On the other hand, it seems likely that factor(s) capable of antagonizing the inhibitory effect of CPF-I might also be present in the cytosol.

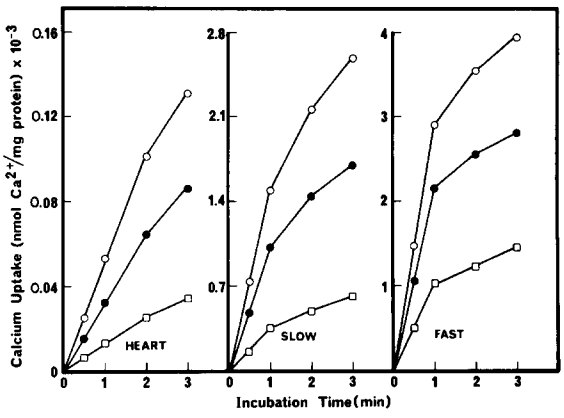


Fig. 2. Time-course of ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart and slow and fast muscles in the absence and presence of CPF-I. $Ca^{2+}$ uptake was determined as described under Methods using 11.9 $\mu$M $Ca^{2+}$ and 20 $\mu$g (fast muscle) or 30 $\mu$g (heart and slow muscle) sarcoplasmic reticulum protein without (○) and with 60 $\mu$g (●) and 120 $\mu$g (□) CPF-I.

*Time-course of $Ca^{2+}$ uptake by sarcoplasmic reticulum in the absence and presence of CPF-I*

Fig. 2 shows the time-course of $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart and slow and fast muscles in the absence and presence of CPF-I. The rates of $Ca^{2+}$ uptake by sarcoplasmic reticulum from all muscles were decreased markedly in the presence of CPF-I (30–35% inhibition with 60 $\mu$g CPF-I, 70–80% inhibition with 120 $\mu$g CPF-I). Under the assay conditions employed, the rates of $Ca^{2+}$ uptake (in the absence and presence of CPF-I) were linear up to 2 min in the case of heart sarcoplasmic reticulum and up to 1 min in the case of slow and fast muscle sarcoplasmic reticulum. The results presented in Fig. 2 were obtained using 11.9 $\mu$M $Ca^{2+}$ in the assay medium; qualitatively similar results could also be obtained using low (0.9 $\mu$M) $Ca^{2+}$ in the assay (not shown). For all the kinetic studies reported here, $Ca^{2+}$ uptake was determined during the initial 30 s (slow and fast muscle sarcoplasmic reticulum) or 1 min (heart sarcoplasmic reticulum) of incubation.

*Effects f CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum at varying concentrations of $Ca^{2+}$*

The results presented in Fig. 3 show the effects of two selected concentrations of CPF-I (50 and 100 μg) on $Ca^{2+}$ uptake by heart sarcoplasmic reticulum at varying concentrations of $Ca^{2+}$. CPF-I inhibited $Ca^{2+}$ uptake by sarcoplasmic reticulum at all $Ca^{2+}$ concentrations (0.5–11.9 μM) tested (Fig. 3, panel A). The degree of inhibition was dependent on the concentration of the inhibitor (approx. 30–40% inhibition with 50 μg CPF-I and 60–65% inhibition with 100 μg CPF-I) and was independent of $Ca^{2+}$ concentration. $Ca^{2+}$ uptake was saturable in the absence and presence of the inhibitor. Double-reciprocal transformation of the data resulted in curved plots concave upward (Fig. 3, panel B), a kinetic feature indicative of cooperative interaction between $Ca^{2+}$ and the transport system [30,33]; CPF-I did not alter this kinetic profile. The effects of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum from slow and fast skeletal muscles at varying $Ca^{2+}$ concentrations were similar to those seen with cardiac sarcoplasmic reticulum (results not shown); the double-reciprocal plots of the data (not shown), however, were linear in the case of skeletal muscle sarcoplasmic reticulum. The kinetic parameters of $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart (derived using the procedure described in Ref. 30) and slow and fast muscles (derived from double-reciprocal plots) in the absence and presence of CPF-I are summarized in Table I. It can be seen that CPF-I reduced the $V_{max}$ of $Ca^{2+}$ uptake without altering appreciably the apparent affinity of the transport system for $Ca^{2+}$. Thus, the inhibition appears to be non-competitive with respect to $Ca^{2+}$.

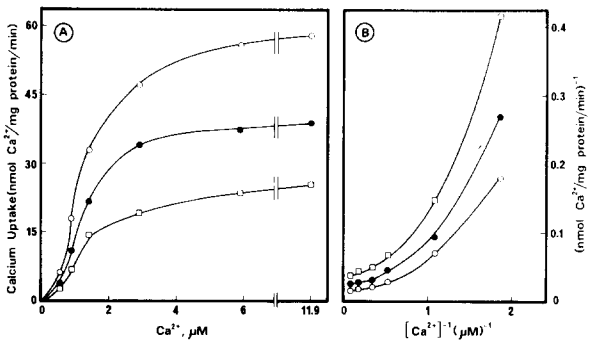


Fig. 3. Effects of CPF-I on ATP-dependent $Ca^{2+}$ uptake by heart sarcoplasmic reticulum at varying concentrations of $Ca^{2+}$. The $Ca^{2+}$ uptake reaction was carried out for 1 min under standard assay conditions (see Methods) using 30 μg sarcoplasmic reticulum protein and varying concentrations of $Ca^{2+}$ in the absence (control; ○) and in the presence of 50 μg (●) and 100 μg (□) CPF-I. In panel A, the $Ca^{2+}$ uptake velocities are plotted against the concentration of $Ca^{2+}$ in the assay. Panel B shows a double-reciprocal plot of the data.

*Effects of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum at varying concentrations of ATP*

The effects of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum was also studied using varying concentrations of ATP in the assay medium. The results obtained using heart sarcoplasmic reticulum are shown in Fig. 4. CPF-I (50 or 100 μg) caused inhibition of $Ca^{2+}$ uptake over a

TABLE I

COMPARISON OF THE APPARENT AFFINITIES OF SARCOPLASMIC RETICULUM CALCIUM PUMP FOR CALCIUM AND MAXIMAL VELOCITIES OF CALCIUM TRANSPORT IN THE PRESENCE AND ABSENCE OF CPF-I

For skeletal muscle sarcoplasmic reticulum, kinetic parameters were derived from double-reciprocal plots (data not shown); for heart sarcoplasmic reticulum, kinetic parameters were derived from the data shown in Fig. 3 using the procedure described in Ref. 30.

| CPF-I (μg) | $K_{0.5}$ app. (μM) | | | $V_{max}$ app. (nmol $Ca^{2+}$/mg per min) | | |
|---|---|---|---|---|---|---|
| | Heart | Slow muscle | Fast muscle | Heart | Slow muscle | Fast muscle |
| 0 | 1.8 | 2.7 | 2.5 | 63 | 2070 | 3478 |
| 50 | 1.5 | 2.8 | 2.2 | 42 | 1515 | 2631 |
| 100 | 1.6 | 3.3 | 2.1 | 27 | 800 | 1290 |

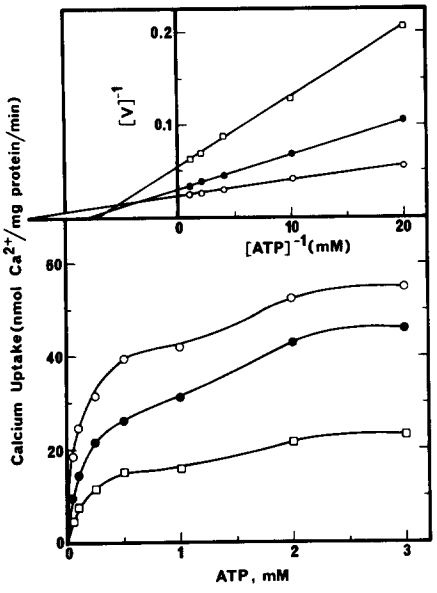


Fig. 4. Effects of CPF-I on ATP-dependent $Ca^{2+}$ uptake by heart sarcoplasmic reticulum at varying concentrations of ATP. The $Ca^{2+}$ uptake reaction was carried out for 1 min under standard assay conditions (see Methods) except that the ATP concentration was varied and assays were performed in the absence (control; ○) and in the presence of 50 μg (●) and 100 μg (□) CPF-I. The concentration of $Ca^{2+}$ in the assay was 11.9 μM and that of sarcoplasmic reticulum protein 30 μg. The bottom panel shows $Ca^{2+}$ uptake velocity as a function of ATP concentration and the top panel a double-reciprocal plot of the data for ATP concentrations between 0.05 and 1 mM.

wide range of ATP concentrations (0.05 to 3 mM) tested; the extent of inhibition varied between 20 and 35% with 50 μg CPF-I, and 55 and 70% with 100 μg CPF-I. In the absence as well as in the presence of the inhibitor, the ATP-dependence curves were biphasic. While an apparent saturation was seen between 0.05 and 1 mM ATP, further increase in $Ca^{2+}$ uptake was evident at higher ATP concentrations. Such complex ATP-dependence of sarcoplasmic reticulum has been observed previously by others [34–37] and is thought to be indicative of separate high-affinity (catalytic) and low-affinity (regulatory) ATP binding sites on the transport system (for a discussion see Refs. 3 and 37). The inhibitory effects of CPF-I (50 or 100 μg) on $Ca^{2+}$ uptake by sarcoplasmic reticulum from slow and fast skeletal muscles at varying ATP concentrations (0.05 to 3 mM) were essentially similar to those described for heart sarcoplasmic reticulum (results not shown). The kinetic constants derived from double-reciprocal plots of the data (from experiments using heart as well as skeletal muscle sarcoplasmic reticulum) covering the ATP concentration range of 0.05 to 1 mM are presented in Table II. These results show that CPF-I causes a large reduction in $V_{max}$ and a modest increase in the $K_m$ for ATP (especially in the case of heart sarcoplasmic reticulum). Thus, inhibition of $Ca^{2+}$ uptake by CPF-I appears to be uncompetitive mixed type with respect to ATP.

*Effect of pretreatment of sarcoplasmic reticulum with CPF-I on $Ca^{2+}$ uptake*

Since the inhibitory effect of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum did not appear to involve competitive inhibition of the binding of $Ca^{2+}$ or ATP to the transport system, in subsequent experiments we examined whether or not pretreatment of sarcoplasmic reticulum with CPF-I

TABLE II

COMPARISON OF THE APPARENT AFFINITIES OF SARCOPLASMIC RETICULUM CALCIUM PUMP FOR ATP AND MAXIMAL VELOCITIES OF CALCIUM TRANSPORT IN THE PRESENCE AND ABSENCE OF CPF-I

The kinetic constants were derived for the ATP concentration range 0.05–1 mM from double-reciprocal plots of the data.

| CPF-I (μg) | $K_m$ app. (μM) | | | $V_{max}$ app. (nmol $Ca^{2+}$/mg per min) | | |
|---|---|---|---|---|---|---|
| | Heart | Slow muscle | Fast muscle | Heart | Slow muscle | Fast muscle |
| 0 | 75 | 72 | 85 | 45 | 952 | 2222 |
| 50 | 125 | 83 | 98 | 33 | 667 | 1667 |
| 100 | 139 | 88 | 115 | 18 | 313 | 1025 |

resulted in persistent inhibition of $Ca^{2+}$ uptake. For this, sarcoplasmic reticulum vesicles were first exposed to CPF-I (sarcoplasmic reticulum: CPF-I ratio 1:3) at 37°C for 3 min. The vesicles were then sedimented by centrifugation and were washed to remove the free inhibitor. Sarcoplasmic reticulum vesicles subjected to the same procedure, but in the absence of the inhibitor, were used as control for these experiments. The $Ca^{2+}$ uptake activities of CPF-I-pretreated and control sarcoplasmic reticulum were determined and compared. The results of such an experiment using sarcoplasmic reticulum from heart and slow muscle are presented in Fig. 5. It can be seen that the rates of $Ca^{2+}$ uptake by CPF-I-pretreated sarcoplasmic reticulum were markedly lower than those of untreated control sarcoplasmic reticulum. Similar findings were obtained in experiments using fast muscle sarcoplasmic reticulum (not shown). These results indicate an apparently 'irreversible' inhibi-

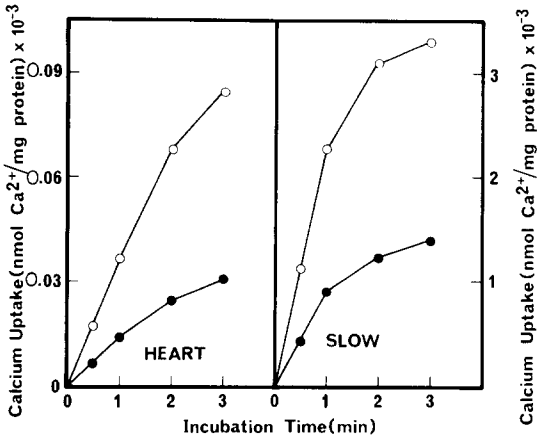


Fig. 5. Comparison of the time-course of ATP-dependent $Ca^{2+}$ uptake by CPF-I-treated (●) and untreated (control; ○) sarcoplasmic reticulum from heart (left panel) and slow muscle (right panel). CPF-I-treated sarcoplasmic reticulum was prepared as follows. Sarcoplasmic reticulum vesicles (1 mg protein) were incubated at 37°C for 3 min in a reaction medium (total volume, 2 ml) containing 10 mM Tris-maleate (pH 6.8), 100 mM KCl and 2.8 mg CPF-I (sarcoplasmic reticulum: CPF-I ratio, 1:2.8). Following incubation, the tubes were chilled on ice and centrifuged at 40000 × *g* for 15 min. The supernatant was discarded and the membrane pellet washed twice by resuspension in buffer (4 ml of 10 mM Tris-maleate/100 mM KCl (pH 6.8)) and subsequent centrifugation as before. Control sarcoplasmic reticulum was prepared by subjecting the membrane vesicles to the same procedure with the exception that CPF-I was omitted from the incubation medium. $Ca^{2+}$ uptake by control and CPF-I-treated sarcoplasmic reticulum was determined under standard assay conditions (see Methods) using 11.9 μM $Ca^{2+}$ and 30 μg membrane protein. The results shown are typical of two separate experiments.

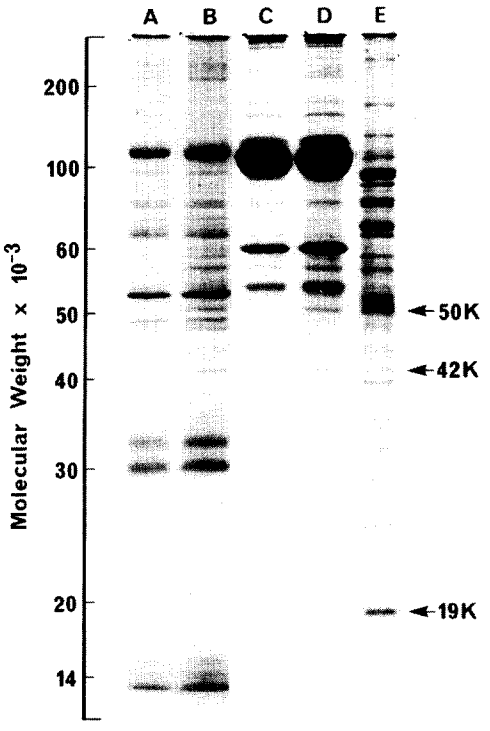


Fig. 6. Comparison of the protein profiles of control sarcoplasmic reticulum, CPF-I-pretreated sarcoplasmic reticulum and CPF-I. (A) Control heart sarcoplasmic reticulum; (B) CPF-I-pre-treated heart sarcoplasmic reticulum; (C) control fast muscle sarcoplasmic reticulum; (D) CPF-I-pretreated fast muscle sarcoplasmic reticulum; (E) cardiac CPF-I. Control and CPF-I-pretreated sarcoplasmic reticula were prepared as described in legend to Fig. 5. The sarcoplasmic reticulum preparations (30 μg heart sarcoplasmic reticulum and 40 μg fast muscle sarcoplasmic reticulum) and cardiac CPF-I (30 μg) were fractionated by SDS-polyacrylamide slab gel (10%) electrophoresis. The gels were stained with 0.025% Coomassie blue and destained in 10% acetic acid. The procedures used for electrophoresis and determination of apparent molecular weights of protein bands were as detailed in Ref. 30. Note that compared to control sarcoplasmic reticulum, CPF-I-pretreated sarcoplasmic reticulum (from heart and fast muscle) contains additional protein bands of apparent molecular weights 50000, 42000 and 19000 (another faintly stained protein band of apparent molecular weight 36000 present in CPF-I-pretreated sarcoplasmic reticulum did not appear in the photograph); protein bands of corresponding molecular weights are present in CPF-I.

tion of the $Ca^{2+}$ transport system upon exposure of sarcoplasmic reticulum to CPF-I. In additional experiments, the protein composition of control and CPF-I-pretreated sarcoplasmic reticulum was examined following SDS-polyacrylamide gel electrophoresis. The results obtained using sarcoplasmic reticulum from heart and fast muscle are shown in Fig. 6. When compared to control sarcoplasmic reticulum, CPF-I-pretreated sarcoplasmic reticulum (from both heart and fast muscle) contained additional protein bands of approx. 50, 42, 36 and 19 kDa (the protein band of 36 kDa was very faintly stained and did not photograph well). Electrophoretic profiles of cardiac CPF-I showed about 35 protein bands ranging from approx. 280 kDa to 12 kDa; peptide bands having apparent molecular weights of 50000, 42000, 36000 and 19000 were also present among these. Thus, it appears that certain proteins of CPF-I associate with sarcoplasmic reticulum upon exposure of the membrane to CPF-I. Whether or not the association of these CPF-I constituents with the sarcoplasmic reticulum is involved in the inhibitory effect of CPF-I on membrane $Ca^{2+}$ transport remains to be determined.

*Influence of other cytosolic fractions on the inhibitory effect of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum*

As noted above, pretreatment of sarcoplasmic reticulum vesicles with CPF-I caused persistent inhibition of $Ca^{2+}$ uptake, suggesting apparently irreversible inhibition of the membrane $Ca^{2+}$ transport system by this cytosolic fraction. If the inhibitory effect of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum observed in vitro is to bear any physiological relevance, such inhibition has to be transient and potentially reversible in vivo. Therefore, we examined whether some component other than the inhibitor present in the cytosol is able to antagonize the inhibitory effect of CPF-I on sarcoplasmic reticulum $Ca^{2+}$ uptake. Such a study seemed particularly worthwhile, since unfractionated cytosol was found not to inhibit $Ca^{2+}$ uptake by sarcoplasmic reticulum (see Fig. 1). Thus, two cytosolic fractions from heart, viz. CPF-II and cytosol supernatant (see Methods), were tested for their effects on $Ca^{2+}$ uptake by cardiac sarcoplasmic reticulum in the absence and presence of CPF-I. Addition of varying amounts (20–200 μg protein) of CPF-II in the assay medium did not alter the inhibition of $Ca^{2+}$ uptake observed with 60 or 180 μg CPF-I (results not shown). Also in agreement with the previous finding (see Fig. 1), $Ca^{2+}$ uptake in the absence of CPF-I was unaffected by CPF-II. However, as shown in Fig. 7, cytosol supernatant substantially (but not completely) antagonized the inhibitory effect of CPF-I on sarcoplasmic reticulum $Ca^{2+}$ uptake in a concentration-dependent manner. Further, in the absence of CPF-I, this cytosolic fraction had no discernible effect on $Ca^{2+}$ uptake by sarcoplasmic reticulum. These results indicate that factor(s) capable of counteracting the inhibitory effect of CPF-I might also be present in the cytosol.

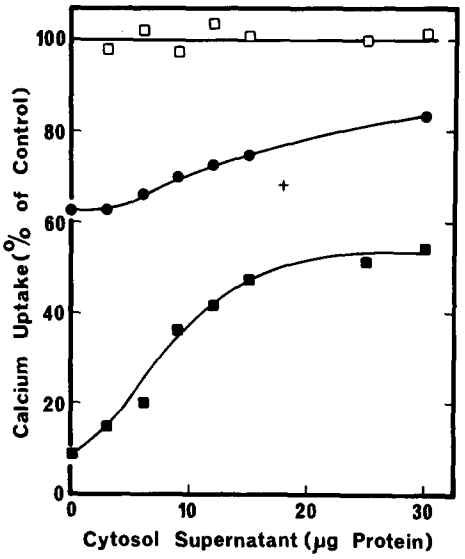


Fig. 7. Effects of cytosol supernatant on $Ca^{2+}$ uptake by heart sarcoplasmic reticulum in the absence and presence of CPF-I. The $Ca^{2+}$ uptake reaction was carried out under standard assay conditions (see methods) in the absence of cytosolic fractions (control) and in the presence of varying amounts of cytosol supernatant, either alone (□) or in combination with selected amounts of CPF-I: 60 μg (●); 180 μg (■). The concentration of $Ca^{2+}$ in the assay was 11.9 μM and that of sarcoplasmic reticulum protein 30 μg. The results are expressed as percent of control. The $Ca^{2+}$ uptake activity of control (100%) amounted to 48 nmol $Ca^{2+}$/mg protein per min).

*Effect of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum at varying pH and temperature*

Using sarcoplasmic reticulum from heart and

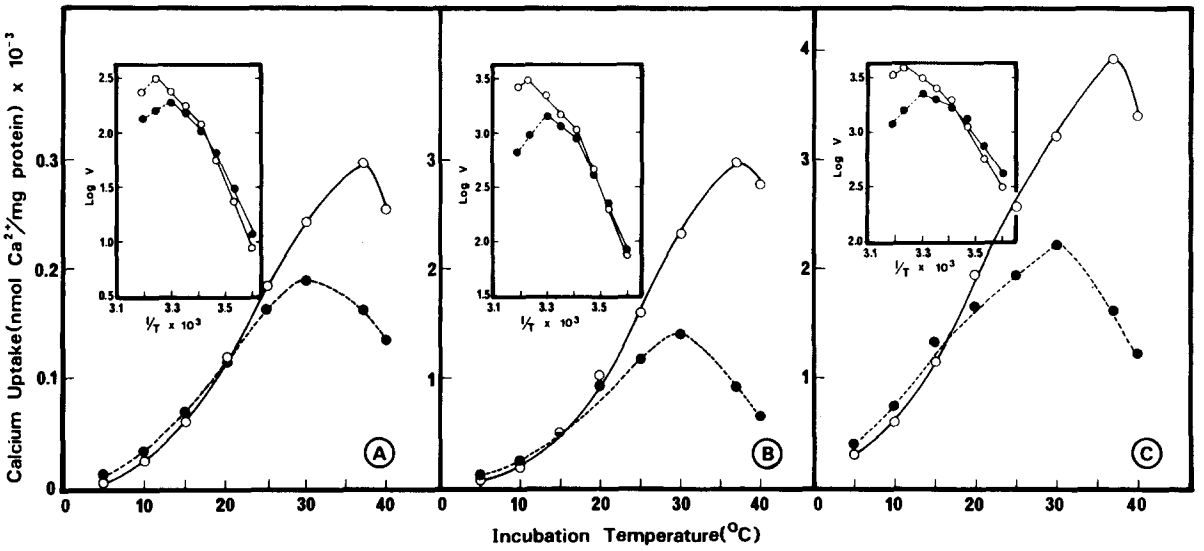


Fig. 8. Effects of CPF-I on ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum from heart (panel A), slow muscle (panel B) and fast muscle (panel C) at varying temperatures. The $Ca^{2+}$ uptake reaction was carried out for 5 min in the absence (control; ○) and in the presence of 100 μg CPF-I (●) under standard assay conditions (see Methods) with the exception that the assay temperature was varied as indicated. The concentration of $Ca^{2+}$ used was 11.9 μM and that of sarcoplasmic reticulum protein 20 μg (fast muscle) or 30 μg (heart and slow muscle). The main figure of each panel shows the $Ca^{2+}$ uptake velocities as a function of assay temperature; the inset of each panel shows Arrhenius plot of the data.

slow muscle, the inhibitory effect of CPF-I on $Ca^{2+}$ uptake could be observed at varying pH (pH 5 to 8); the pH profile was similar in the presence and absence of the inhibitor (results not shown).

The effects of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum was also examined at varying temperatures. In these experiments $Ca^{2+}$ uptake assays were performed at varying temperatures in the absence of CPF-I (control) and in the presence of 100 μg CPF-I. With sarcoplasmic reticulum from heart, and slow and fast skeletal muscles, the inhibitory effect of CPF-I on $Ca^{2+}$ uptake was seen only at assay temperatures above 20°C (Fig. 8). Arrhenius plots of the data (Fig. 8, insets) showed discontinuity at 20°C which is considered to be the transition temperature of the sarcoplasmic reticulum membrane [38]. Thus, the inhibitor appears to be effective only at temperatures above transition. Comparison of the values for energy of activation ($E_a$) indicated that CPF-I caused a decrement in $E_a$ of the transport system, especially at temperatures above transition (estimated $E_a$ values (kcal/mol, above transition) were: CPF-I absent: heart, 11.39; slow muscle, 14.48; fast muscle, 11.39; CPF-I present: heart, 8.67; slow muscle, 8.91; fast muscle, 5.73). This phenomenon of decrement in $E_a$ upon inhibition of sarcoplasmic reticulum $Ca^{2+}$ transport by CPF-I constrasts with the reported incremenet in $E_a$ following cyclic AMP-mediated activation of $Ca^{2+}$ transport in cardiac sarcoplasmic reticulum [39]. It may be noted, however, that a shift in the temperature optimum for $Ca^{2+}$ uptake by sarcoplasmic reticulum was evident in the presence of CPF-I (30°C in the presence of CPF-I and 37°C in its absence) and hence, the inhibitor may render the membrane $Ca^{2+}$ transport system more thermolabile.

*Effects of CPF-I, EGTA and ionophore A23187 on $Ca^{2+}$ release from $Ca^{2+}$-preloaded sarcoplasmic reticulum*

The results described thus far have documented the powerful inhibitory effect of CPF-I on ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum. In other experiments we examined whether this $Ca^{2+}$ transport inhibitor was also capable of releasing $Ca^{2+}$ from $Ca^{2+}$-preloaded sarcoplasmic reticulum vesicles. The results of such an experiment using cardiac sarcoplasmic are summarized

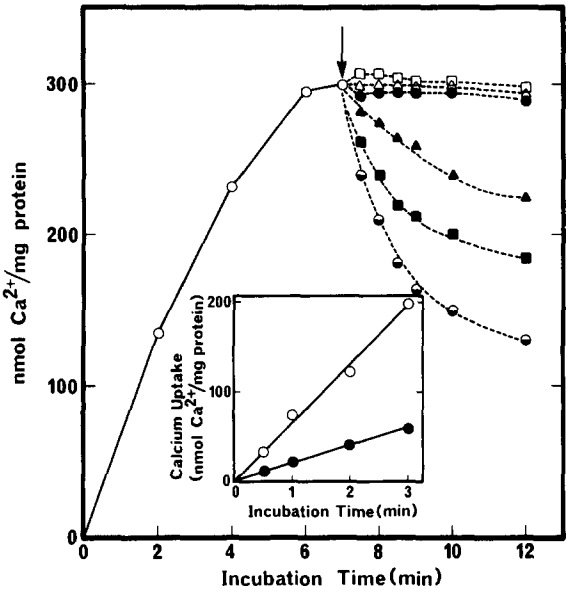


Fig. 9. Comparison of the effects of CPF-I, EGTA and ionophore A23187 on $Ca^{2+}$ release from $Ca^{2+}$-preloaded heart sarcoplasmic reticulum. The results shown in the main figure were obtained as follows. Cardiac sarcoplasmic reticulum was incubated under standard assay conditions (see Methods) with 11.9 $\mu$M $Ca^{2+}$ in the absence of CPF-I. When $Ca^{2+}$ uptake had reached a steady state (at 7 min, as shown by arrow), 120 $\mu$g CPF-I (□), 2.5 mM EGTA (■) or 0.05 ml ionophore A23187 (1 $\mu$M (▲) or 3 $\mu$M (◒) was added as indicated; the control tubes received an equivalent volume of the vehicle solution (buffer (●) or ethanol (△)). Subsequently, aliquots were removed at short time intervals for a period of 5 min and the amount of $Ca^{2+}$ remaining in the sarcoplasmic reticulum vesicles was determined. The results presented in the inset compare the rates of $Ca^{2+}$ uptake by the same cardiac sarcoplasmic reticulum preparation when incubated in the absence (control; ○) and in the presence of 120 $\mu$g CPF-I (●) under standard assay conditions (see Methods) with 11.9 $\mu$M $Ca^{2+}$. The results shown are typical of two separate experiments.

in Fig. 9. It can be seen that addition of CPF-I to sarcoplasmic reticulum, following steady-state $Ca^{2+}$ accumulation, did not result in $Ca^{2+}$ release from the sarcoplasmic reticulum. On the other hand, addition of EGTA or the ionophore A23187 did promote $Ca^{2+}$ release, as expected. In the same sarcoplasmic reticulum preparation, CPF-I strongly inhibited the rates of $Ca^{2+}$ uptake (see Fig. 9, inset). These results show that the observed inhibitory effect of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum cannot be ascribed to disruption of the structural integrity of sarcoplasmic reticulum vesicles or enhanced sarcoplasmic reticulum $Ca^{2+}$ permeability caused by CPF-I. Further, CPF-I was found to have no protease activity when tested with either $^{14}$C-labelled hemoglobin or $^{3}$H-labelled sarcoplasmic reticulum (prepared from rabbit heart 18 h following the administration of a mixture of $^{3}$H-labelled amino acids) as substrate (results not shown).

*Effect of CPF-I on sarcoplasmic reticulum $Ca^{2+}$-ATPase*

Since CPF-I inhibited ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum, in subsequent experiments the effect of CPF-I on $Mg^{2+}$-dependent, $Ca^{2+}$-stimulated ATPase ($Ca^{2+}$-ATPase) activity of sarcoplasmic reticulum was investigated. In these experiments, the same sarcoplasmic reticulum preparations from heart and slow and fast muscles were used to determine $Ca^{2+}$-ATPase as well as $Ca^{2+}$ uptake activities under identical assay conditions in the absence and presence of CPF-I.

TABLE III

COMPARISON OF THE EFFECTS OF CPF-I ON CALCIUM-STIMULATED ATP HYDROLYSIS AND ATP-DEPENDENT CALCIUM UPTAKE BY SARCOPLASMIC RETICULUM

$Ca^{2+}$-stimulated ATP hydrolysis and ATP-dependent $Ca^{2+}$ uptake were measured under identical assay conditions (see Methods) using the same sarcoplasmic reticulum preparation (30 $\mu$g protein in the case of heart and slow muscle; 20 $\mu$g protein in the case of fast muscle) in the absence of CPF-I and in the presence of 100 $\mu$g CPF-I. The concentration of $Ca^{2+}$ used was 11.9 $\mu$M. Data from three separate experiments are presented as mean ± S.E. The numbers in parentheses denote percentage inhibition caused by CPF-I.

| Sarcoplasmic reticulum from | $Ca^{2+}$-stimulated ATP hydrolysis (nmol $P_i$/mg protein per min) | | ATP-dependent $Ca^{2+}$ uptake (nmol $Ca^{2+}$/mg protein per min) | |
|---|---|---|---|---|
| | CPF-I absent | CPF-I present | CPF-I absent | CPF-I present |
| Heart | 188 ± 9 | 79 ± 8 (58) | 51 ± 4 | 24 ± 3 (53) |
| Slow muscle | 721 ± 53 | 371 ± 32 (49) | 1151 ± 92 | 512 ± 53 (55) |
| Fast muscle | 1649 ± 146 | 857 ± 80 (48) | 2884 ± 149 | 1549 ± 127 (46) |

The results obtained showed that the $Ca^{2+}$-ATPase activity of sarcoplasmic reticulum was also inhibited by CPF-I; the degree of this inhibition was more-or-less similar to that seen on $Ca^{2+}$ uptake (Table III). These findings indicate that CPF-I interferes with the enzymatic as well as $Ca^{2+}$ ion translocation functions of the sarcoplasmic reticulum $Ca^{2+}$ pump.

*Effects of CPF-I on $Ca^{2+}$ uptake by sarcoplasmic reticulum in the presence of calmodulin*

The effect of CPF-I on $Ca^{2+}$ uptake by cardiac sarcoplasmic reticulum was also examined in the presence of varying concentrations of calmodulin, which is reported to stimulate $Ca^{2+}$ uptake by dog heart sarcoplasmic reticulum [11–13]. The results of these experiments are presented in Table IV. The inhibitory effect of CPF-I (even at the submaximally effective concentration used) on $Ca^{2+}$ uptake was not reversed by calmodulin. Also, no appreciable stimulatory effect of calmodulin (with or without CPF-I) on $Ca^{2+}$ uptake by rabbit heart sarcoplasmic reticulum could be observed under the assay conditions employed. These results make it very unlikely that the inhibitory effect of CPF-I on sarcoplasmic reticulum $Ca^{2+}$ uptake described here is mediated by a calmodulin-binding protein, which is reported to antagonize the stimulatory effects of calmodulin on $Ca^{2+}$ uptake and ATPase activities of erythrocyte membranes [40].

TABLE IV

COMPARISON OF THE EFFECTS OF CPF-I ON ATP-DEPENDENT CALCIUM UPTAKE BY HEART SARCOPLASMIC RETICULUM IN THE PRESENCE AND ABSENCE OF CALMODULIN

$Ca^{2+}$ uptake reaction was carried out for 5 min under standard assay conditions (see Methods) using 30 μg sarcoplasmic reticulum and 11.9 μM $Ca^{2+}$. When present, the concentration of CPF-I was 60 μg. The values shown are means of duplicate determinations (duplicates agreed within 10%) from a single experiment. Qualitatively similar results were obtained in an additional experiment using a lower concentration of $Ca^{2+}$ (2.9 μM) in the assay.

| Concentration of calmodulin in the assay (μM) | Calcium uptake (nmol $Ca^{2+}$/mg protein per 5 min) | |
|---|---|---|
| | CPF-I absent | CPF-I present |
| 0 | 431 | 258 |
| 0.56 | 448 | 224 |
| 1.12 | 467 | 253 |
| 5.6 | 438 | 278 |
| 11.2 | 493 | 251 |

*Effects of heat and trypsin treatment on the inhibitor activity of CPF-I*

CPF-I prepared from cardiac muscle was used to study the effects of heat and trypsin treatment on the inhibitor activity. The results presented in Fig. 10 compare the rates of $Ca^{2+}$ uptake by cardiac sarcoplasmic reticulum in the absence of CPF-I and in the presence of (60 and 120 μg) heat-treated as well as original CPF-I. Original CPF-I, but not heat-treated CPF-I, caused inhibition of $Ca^{2+}$ uptake by sarcoplasmic reticulum, indicating that the inhibitor activity of CPF-I is heat-labile. When stored frozen at −20°C, the inhibitor activity of CPF-I was, however, stable (at least up to 2 months). Interestingly, mild treatment of CPF-I with trypsin (trypsin: CPF-I ratio,

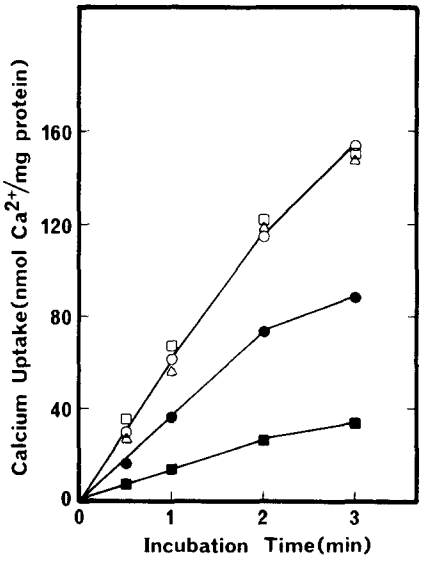


Fig. 10. Effect of heat treatment of CPF-I on its ability to inhibit $Ca^{2+}$ uptake by heart sarcoplasmic reticulum. ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum was determined under standard assay conditions (see Methods) in the absence of CPF-I (control; ○) and in the presence of 'original' (●, 60 μg; ■ 120 μg) as well as 'heat-treated' (□, 60 μg; △, 120 μg) CPF-I. The concentration of $Ca^{2+}$ in the assay was 11.9 μM and that of sarcoplasmic reticulum protein 30 μg. The results shown are typical of two separate experiments.

1 : 10) resulted in a moderate increase (approx. 50% as judged from the concentration required to cause 50% inhibition of $Ca^{2+}$ uptake by sarcoplasmic reticulum) in its inhibitor activity (results not shown). Further studies using purified inhibitor protein are required to understand the significance of this observation.

*Physiological significance of sarcoplasmic reticulum $Ca^{2+}$ pump inhibitor*

The studies reported here show that a cytosolic protein fraction (termed CPF-I) derived from heart and slow skeletal muscle strongly inhibits ATP-dependent $Ca^{2+}$ uptake and $Ca^{2+}$-sensitive ATP-hydrolysis by sarcoplasmic reticulum from heart and slow and fast skeletal muscles. The ATP-supported $Ca^{2+}$ uptake activity of isolated sarcoplasmic reticulum is widely accepted as the in vitro manifestation of the $Ca^{2+}$ pump function of this membrane in vivo. Hence, the inhibition of sarcoplasmic reticulum $Ca^{2+}$ transport by CPF-I raises the possibility of the participation of this inhibitor in the regulation of sarcoplasmic reticulum $Ca^{2+}$ pump in vivo. Conceptually, an inhibitor of sarcoplasmic reticulum $Ca^{2+}$ pump might provide a 'turn off' mechanism for the $Ca^{2+}$ pump, thereby determining the duration of $Ca^{2+}$ signal in the cell and at the same time preventing unwanted hydrolysis of ATP. In order to be physiologically relevant, such inhibition has to be transient and readily reversible. The persistent inhibition of the $Ca^{2+}$ uptake activity of CPF-I-pretreated sarcoplasmic reticulum observed here, however, suggested apparently irreversible inhibition of the membrane $Ca^{2+}$ transport system by this cytosolic fraction. This observation notwithstanding, the potential reversibility of $Ca^{2+}$ pump inhibition in vivo cannot be discounted. In this regard, it is of considerable interest that unfractionated cytosol failed to inhibit $Ca^{2+}$ uptake by sarcoplasmic reticulum. Further, one of the cytosolic fractions (cytosol supernatant) was found to antagonize the inhibitory effect of CPF-I on sarcoplasmic reticulum $Ca^{2+}$ uptake. Thus, it appears that factor(s) capable of counteracting the inhibitory effect of CPF-I might also be present in the cytosol. A sequential interaction of the inhibitor and its antagonist with sarcoplasmic reticulum $Ca^{2+}$ pump, conceivably, could provide a physiologically important regulatory mechanism. For example, soon after the release of $Ca^{2+}$ from the sarcoplasmic reticulum, a transient blockade of the $Ca^{2+}$ pump by the inhibitor would prevent re-uptake of $Ca^{2+}$ during the period of active tension development in muscle, thus effectively increasing the duration of $Ca^{2+}$ signal. After the tension development has peaked, an inhibitor antagonist would lift the pump blockade to initiate re-uptake of $Ca^{2+}$ so as to promote muscle relaxation. Future studies using purified $Ca^{2+}$ pump inhibitor protein and its putative antagonist are, undoubtedly, necessary to establish the validity of a regulatory mechanism such as that proposed here. Investigations in this regard are currently in progress in our laboratory. Preliminary findings have shown that the $Ca^{2+}$ pump inhibitor (from heart muscle) can be adsorbed to an ion-exchange (DEAE-cellulose) column and can be eluted with a linear salt gradient [25].

It is intriguing that although CPF-I from heart and slow muscle caused inhibition of $Ca^{2+}$ uptake by fast muscle sarcoplasmic reticulum, such a $Ca^{2+}$ pump inhibitor was not readily detectable in fast muscle cytosol. This apparently selective distribution of the inhibitor in heart and slow muscle but not in fast muscle may be related to the intrinsic differences in contractile characteristics of these muscle types. The contraction phase (period of tension development) is much greater in heart and slow muscle compared to fast muscle, and it would be functionally advantageous to have the $Ca^{2+}$ pump turned off, especially during the relatively long contraction phase. Thus, if the sarcoplasmic reticulum $Ca^{2+}$ pump inhibitor is conceived to function during the period of tension development in muscle, clearly, the requirement for such an inhibitor might be much more stringent physiologically in heart and slow muscle than in fast muscle. Finally, in view of the presence of a $Ca^{2+}$ pump inhibitor in heart and slow muscle cytosol, it seems possible that sarcoplasmic reticulum isolated from these muscles may have the inhibitor associated with the membrane; this may explain why sarcoplasmic reticulum preparations from heart and slow muscle always exhibit lower $Ca^{2+}$ transport activities than sarcoplasmic reticulum preparations from fast muscle.

## Acknowledgements

We thank Dr. P.V. Sulakhe and Dr. R.L. Khandelwal for helpful discussions. This work was supported by a grant from the Saskatchewan Heart Foundation. Dr. N. Narayanan is a Research Scholar of the Canadian Heart Foundation.

## References

1 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–79
2 DeMeis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292
3 Hasselbach, W. (1981) in Membrane Transport (Bonting, S.L. and Dupont, Y., eds.), pp. 183–208, Elsevier Biomedical Press, Amsterdam
4 Inesi, G. (1981) in Cell and Muscle Motility, Vol. 1 (Dowben, R.M. and Shay, J.W., eds.), pp. 63–97, Plenum Press, New York
5 Ikemoto, N. (1972) Annu. Rev. Physiol. 44, 297–317
6 Berman, M.C. (1982) Biochim. Biophys. Acta 694, 95–121
7 Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) J. Biol. Chem. 249, 6166–6173
8 Tada, M., Yamada, M., Ohmori, F., Kuzuya, T., Inui, M. and Abe, H. (1980) J. Biol. Chem. 244, 4406–4412
9 Kranias, E.G., Mandel, F., Wang, T. and Schwartz, A. (1980) Biochemistry 19, 5434–5439
10 Tada, M. and Katz, A.M. (1982) Annu. Rev. Physiol. 44, 401–423
11 Lopaschuk, G., Richter, B. and Katz, S. (1980) Biochemistry 19, 5603–5607
12 Lepeuch, C.J., Haiech, J. and Damaille, J.G. (1979) Biochemistry 18, 5150–5157
13 Kirchberger, M.A. and Antonetz, T. (1982) J. Biol. Chem. 257, 5685–5691
14 Kirchberger, M.A. and Tada, M. (1976) J. Biol. Chem. 251, 725–729
15 Galani-Kranias, E., Bick, R. and Schwartz, A. (1980) Biochim. Biophys. Acta 628, 438–450
16 Chiesi, M. and Carafoli, E. (1982) J. Biol. Chem. 257, 984–991
17 Michalak, M., Campbell, K.P. and MacLennan, D.H. (1980) J. Biol. Chem. 255, 1317–1326
18 Pang, D.C. and Briggs, F.N. (1973) Biochemistry 12, 4905–4911
19 Noack, E., Kurzmack, M., Verjovski-Almeida, S. and Inesi, G. (1978) J. Pharmacol. Exp. Ther. 206, 281–288
20 Messineo, F.C. and Katz, A.M. (1979) J. Cardiovasc. Pharmacol. 1, 449–459
21 Yu, B.P., Mosoro, E.J. and Bertrand, H.A. (1974) Biochemistry 13, 5083–5087
22 Hasselbach, W. and Sheradarian, K. (1966) Biochem. Z. 345, 159–172
23 Murphy, A. (1976) Biochemistry 15, 4492–4496
24 Shoshan, V. and MacLennan, D.H. (1981) J. Biol. Chem. 256, 887–892
25 Narayanan, N., Lee, P., Newland, M. and Khandelwal, R.L. (1982) Biochem. Biophys. Res. Commun. 108, 1158–1164
26 Narayanan, N. (1982) J. Mol. Cell. Cardiol. (Suppl. 1) 14, 52 (abstract)
27 Roth, J.S. and Losty, T. (1971) Anal. Biochem. 42, 214–221
28 Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 781–794
29 Sulakhe, P.V. and Narayanan, N. (1978) Biochem. J. 175, 171–180
30 Narayanan, N. (1981) Biochim. Biophys. Acta 678, 442–459
31 Sulakhe, P.V. and Drummond, G.I. (1974) Arch. Biochem. Biophys. 161, 448–455
32 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
33 Froehlich, J.P., Lakatta, E.G., Beard, E., Spurgeon, H.A., Weisfeldt, M.L. and Gerstenblith, G. (1978) J. Mol. Cell. Cardiol. 10, 427–438
34 Yamamoto, T. and Tonomura, Y. (1967) J. Biochem. (Tokyo) 62, 558–575
35 DeMeis, L. and DeMello, M.C.F. (1973) J. Biol. Chem. 248, 8222–8226
36 Noack, E., Kurzmack, M., Verjovski-Almeida, S. and Inesi, G. (1978) J. Pharmacol. Exp. Ther. 206, 281–288
37 Verjovski-Almeida, S. and Inesi, G. (1979) J. Biol. Chem. 254, 18–21
38 Inesi, G., Millan, M. and Elter, S. (1973) J. Mol. Biol. 81, 483–504
39 Chiesi, M. (1979) J. Mol. Cell. Cardiol. 11, 245–259
40 Larsen, F.L., Raess, B.V., Hinds, T.R. and Vincenzi, F.F. (1978) J. Supramol. Struct. 9, 269–274