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Biochimica et Biophysica Acta 1232 (1995) 217–224

BBA Biochimica et Biophysica Acta

# Modulation of the $Ca^{2+}$,$Mg^{2+}$-ATPase of sarcoplasmic reticulum by the hypothalamic hypophyseal inhibitory factor

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Received 12 April 1995; revised 7 August 1995; accepted 25 August 1995

**Abstract**

We have studied the effect of the endogenous inhibitor of the $Na^+$ and $Ca^{2+}$ pumps, HHIF, on sarcoplasmic reticulum (SR) vesicles. The effect of HHIF on the SR $Ca^{2+}$,$Mg^{2+}$-ATPase activity shows a biphasic pattern. Low HHIF concentrations activate the $Ca^{2+}$,$Mg^{2+}$-ATPase by dissipation of $Ca^{2+}$ gradient across the SR membrane. Higher concentrations irreversibly inhibit this activity following a slow kinetic process both in intact SR membranes and in purified $Ca^{2+}$,$Mg^{2+}$-ATPase. Differential scanning calorimetry shows that the $Ca^{2+}$,$Mg^{2+}$-ATPase is denatured after incubation with HHIF concentrations which produced full inhibition of its activity. Micromolar $Ca^{2+}$ and millimolar $Mg^{2+}$ADP protect against the irreversible inhibition of the $Ca^{2+}$,$Mg^{2+}$-ATPase by HHIF. The concentration of HHIF which produces 50% inhibition depends upon SR membrane concentration and upon the lipid:protein ratio in purified $Ca^{2+}$,$Mg^{2+}$-ATPase. From this we have obtained a partition coefficient for binding of HHIF to SR membranes of 0.6 ($\mu$g SR protein/ml)$^{-1}$.

*Keywords:* Calcium ion; ATPase, $Mg^{2+}$-; Sodium ion; ATPase, $K^+$-, inhibitor; Calcium ion pump; Sarcoplasmic reticulum

## 1. Introduction

In essential hypertension, functional defects in $Ca^{2+}$ handling by vascular smooth muscle cells have been proposed to be an etiologically important factor and may represent a major manifestation of a more generalized membrane defect [1,2]. Actually, there is growing evidence that the alteration of cell $Ca^{2+}$ metabolism seen in hypertension is due to a humoral defect [3,4]. An endogenous inhibitor of both $Na^+$ and $Ca^{2+}$ pumps would thus play an important role in the homeostasis of intracellular $Ca^{2+}$. The existence of an endogenous substance that inhibits the $Na^+$ pump and increases $Ca^{2+}$ intracellular levels has been proposed [5–7]. In addition, alterations in the activity of $Ca^{2+}$ channels and in $Ca^{2+}$ uptake by plasma membrane and sarcoplasmic reticulum (SR) of vascular smooth muscle, leading to increased cytosolic free $Ca^{2+}$ concentration, have been noted [2,8,9]. A dysfunctioning of the $Ca^{2+}$ pump ATPase in the sarcoplasmic reticulum in vascular smooth muscle has been proposed as a contributing factor for the development of genetic hypertension [9].

We have purified to homogeneity from bovine hypothalamus and hypophysis a low-molecular-weight, nonpeptidic, non-lipidic $Na^+$,$K^+$-ATPase inhibitor factor that is different from any other known $Na^+$ pump inhibitor. This factor has been called hypothalamic hypophyseal inhibitory factor (HHIF) [10,11]. We have reported that HHIF also inhibits $Ca^{2+}$,$Mg^{2+}$-ATPase activity and $Ca^{2+}$ uptake of the plasma membrane of synaptosomes [12] and raises the intrasynaptosomal concentration of $Ca^{2+}$ (unpublished results), and increases cell proliferation of rat mesangial cells in culture [13]. Recently, Rodriguez-Barbero et al. [14] have shown that HHIF -nduced prolifer-

Abbreviations: $Ca^{2+}$-ATPase, $Ca^{2+}$,$Mg^{2+}$-ATPase; DPH, 1,6-diphenyl-1,3,5-hexatriene; EGTA, ethylene glycol bis(ß-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HHIF, hypothalamic hypophyseal inhibitory factor; IU, $\mu$mol product per min per mg protein; SR, sarcoplasmic reticulum; Tes, 2((2-hydroxy-1,1-bis(hydroxymethyl) ethyl)amino)ethanesulfonic acid.

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*SSDI* 0005-2728(95)00121-2

ation and contraction of primary cultures of rat mesangial cells is inhibited by endoplasmic reticulum $Ca^{2+}$ release blocker TMB-8. This suggests that HHIF might also act as a modulator of $Ca^{2+}$-transport systems of intracellular stores, such as endoplasmic and sarcoplasmic reticulum, since it is a highly hydrophobic compound [10–12] and also because the increase of cytosolic $Ca^{2+}$ induced by HHIF in rat mesangial cells in culture shows a lag-phase of several minutes, particularly at low HHIF concentrations [14]. In particular, inhibition by HHIF of the endoplasmic reticulum $Ca^{2+}$-pump could account for the observed increase of contraction of rat mesangial cells. The $Ca^{2+}$-pump of endoplasmic reticulum is a P-ATPase closely related to the $Ca^{2+}$-pump of the skeletal muscle SR [15]. Because the SR $Ca^{2+}$ pump from skeletal muscle represents about 80–90% of the total protein of the SR membrane [16], we have studied the effect of HHIF on the SR $Ca^{2+}$-ATPase as a model system, aiming to obtain direct experimental evidences for the proposed modulation of $Ca^{2+}$-pumps of intracellular stores by HHIF.

## 2. Materials and methods

SR membranes were purified from rabbit (New Zealand White) hind leg muscle as indicated elsewhere [17]. $Ca^{2+}$-ATPase was purified from SR membranes following the MacLennan procedure [18]. Protein concentration was measured according to Lowry et al. [19], using bovine serum albumin as standard. The phospholipid content of purified ATPase was measured by phosphorus analysis as inorganic phosphate [20], and was found to range between 35 and 45 mol phospholipid per mol of ATPase, in good agreement with earlier reports [18,21].

Lipid reconstitutions were carried out as described by East and Lee [22]. Phosphatidylcholine was dried onto the sides of glass tubes, and then dispersed into 0.5 mg deoxycholate per mg phospholipid and purified ATPase. The mixture was gently vortexed for 30 min and buffer was added (50 mM Tes (pH 7.4), 0.5 M sucrose and 0.1 M KCl) to give a molar ratio of phospholipid:ATPase of 300:1.

HHIF was purified from bovine hypothalamus and hypophysis as previously indicated [10–12]. The inhibition by HHIF of the $Na^+,K^+$-ATPase activity from porcine kidney outer medulla was measured using a coupled assay and one unit (U) was defined as the amount of HHIF required to inhibit by 50% the activity of 8 $\mu$g of purified $Na^+,K^+$-ATPase.

The $Ca^{2+}$-ATPase activity was measured by using the coupled enzyme system [23] with the following assay mixture: 0.1 M Tes (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM ATP, 0.25 mM NADH, 0.42 mM phospho*enol*pyruvate, 50 $\mu$g of pyruvate kinase, 50 $\mu$g of lactate dehydrogenase. The $Ca^{2+}$-independent ATPase activity was measured in the presence of 3.4 mM EGTA, and only SR membrane preparations showing a value of this activity lower than 10% of total uncoupled $Ca^{2+}$-ATPase activity were used in this study. Leakiness of SR vesicles was assessed by measuring the $Ca^{2+}$-ATPase activity in the absence and in the presence of calcimycin (4% w/w). Only those preparations showing a 3–4-fold stimulation of the $Ca^{2+}$-ATPase activity at 20–22°C on the addition of calcimycin have been used in this study. The specific activity of purified $Ca^{2+}$-ATPase ranged typically between 5 and 7 IU at 25°C. Each data point in the figures is the mean activity of at least duplicate determinations from three different experiments ($n \geq 6$), $\pm$S.D.

Calcium uptake was measured using arsenazo III [24,25] from the difference in absorbance between 650 and 700 nm. The conversion of absorbance change into $Ca^{2+}$ concentration change was carried out upon calibration with $Ca^{2+}$-EGTA solutions, taking a dissociation constant of the $Ca^{2+}$-EGTA complex of $10^{-7.2}$ at pH 7.4 [26]. The reaction medium contained 0.1 M Tes (pH 7.4), 0.1 M KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM ATP and 0.2 mg of SR protein/ml. Calcium release has been measured as indicated in the legend of Fig. 8, using $^{45}Ca^{2+}$ (40 000–50 000 dpm/nmol) and Millipore filtration through HAWP-025000 filters, which were counted as indicated in [12]. Aliquots of 50–100 $\mu$g of SR protein were taken for filtration in each data point, which was done by triplicate.

Fluorescence polarization measurements were performed at 25°C with a Perkin-Elmer mod. 650-40 spectrofluorimeter operated in ratio mode and under magnetic stirring. Diphenylhexatriene (DPH) was added to SR membranes at a DPH/lipid molar ratio lower than 1/500. DPH fluorescence measurements were done with excitation and emission wavelengths of 360 and 440 nm, respectively, and after 90 min incubation in darkness to ensure a complete incorporation of the probe in the membrane as previously described [23]. The steady-state anisotropy of the fluorescence of DPH was calculated from polarization measurements as indicated in [23,27].

Scanning calorimetry measurements were carried out with a differential scanning calorimeter MicroCal MC-2, operated at a scanning rate of 60 C°/h and under a nitrogen pressure of 1.5–2 kg/cm$^2$ during the scan. The samples were degassed before loading the calorimeter and a time of 30 min was set for equilibration before starting the scan. The analysis of the calorimetric data was carried out with the Origin™ software developed by MicroCal for the MC-2 differential scanning calorimeter.

### 2.1. Chemicals

ADP, ATP, calcimycin, PK (200 IU), LDH (550 IU), NADH and phospho*enol*pyruvate were obtained from Boehringer Mannheim. $^{45}Ca^{2+}$ was purchased from Du Pont NEN Research Products. All the other chemicals used in this study were obtained from Sigma.

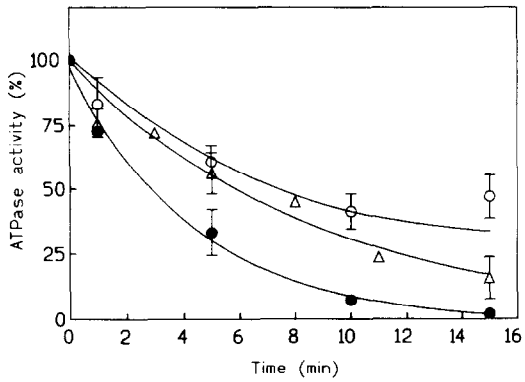


Fig. 1. SR $Ca^{2+}$-ATPase activity in the presence of HHIF as a function of the incubation time. SR vesicles made leaky to $Ca^{2+}$ by addition of 0.04 mg calcimycin/mg protein (●), purified $Ca^{2+}$-ATPase (○) and reconstituted $Ca^{2+}$-ATPase (△). After incubation with HHIF in 0.1 M Tes/Tris buffer (pH 7.4) during the times indicated in the abscissae, the $Ca^{2+}$-ATPase activity was measured in the assay medium indicated in Materials and methods. Leaky SR vesicles, purified $Ca^{2+}$-ATPase and reconstituted $Ca^{2+}$-ATPase were incubated with HHIF at the following HHIF/protein ratios (U of HHIF/$\mu$g of protein) 0.31, 0.375 and 1.38, respectively. The protein and HHIF concentrations in the assay medium were as follows: 4 $\mu$g protein per ml (leaky SR vesicles) and 2 $\mu$g protein per ml (for the case of purified and reconstituted $Ca^{2+}$-ATPase), and the HHIF concentrations were 1.25 U/ml for leaky SR vesicles, 0.75 U/ml for purified $Ca^{2+}$-ATPase and 2.75 U/ml for reconstituted $Ca^{2+}$-ATPase. The reference (100%) is the ATPase activity measured in the absence of HHIF for every experimental condition, $3.0 \pm 0.5$ IU for leaky SR vesicles and $6 \pm 1$ IU for purified and reconstituted $Ca^{2+}$-ATPase.

## 3. Results

### 3.1. Inhibition of the SR $Ca^{2+}$-ATPase by HHIF

HHIF inhibits the $Ca^{2+}$-ATPase activity, the extent of inhibition being dependent on the time of incubation with HHIF in 0.1 M Tes/Tris buffer at pH 7.4 (Fig. 1). The inhibition by HHIF was halted at the times indicated in Fig. 1 by dilution of aliquots taken from the incubation medium into the assay medium, owing to the protection against HHIF-induced inactivation provided by 0.1 mM $Ca^{2+}$ and 2.5 mM $Mg^{2+}$-ATP (see below). In the assay medium used for $Ca^{2+}$-ATPase activity measurements, the half-time of the HHIF-induced inactivation of leaky SR vesicles and of purified and reconstituted $Ca^{2+}$-ATPase determined from the slow decay of the ATPase activity with 2 $\mu$g protein/ml and 1.5–2.5 U/ml of HHIF was found to be higher than 30 min. In all cases, control experiments carried out with leaky SR vesicles and with purified and reconstituted $Ca^{2+}$-ATPase in 0.1 M Tes/Tris (pH 7.4) at 25°C at a concentration of 2 $\mu$g protein/ml in the absence of HHIF showed a negligible ($< 10\%$) loss of activity after 20 min incubation.

Fig. 2 shows that at a fixed time of incubation of 5 min with HHIF in 0.1 M Tes/Tris buffer (pH 7.4) at 25°C the extent of inhibition of reconstituted $Ca^{2+}$-ATPase is markedly lower than that of purified $Ca^{2+}$-ATPase. The values of the apparent $K_{0.5}$ with the same protein concentration in the assay medium (2 $\mu$g/ml) are 1 and 2.75 U/ml for purified and reconstituted $Ca^{2+}$-ATPase, respectively. In the presence of HHIF concentrations close to the $K_{0.5}$ values obtained from Fig. 2, the half-time of the HHIF-induced inactivation upon incubation in 0.1 M Tes/Tris (pH 7.4) are 2.5 and 5.5 min for SR membranes and reconstituted $Ca^{2+}$-ATPase, respectively (Fig. 1). Since HHIF is a lipophilic compound [10–12] and the lipid/protein molar ratio of reconstituted $Ca^{2+}$-ATPase (300:1) is higher than in purified $Ca^{2+}$-ATPase ($\approx 45/1$), we considered the simple hypothesis that its relative efficiency as inhibitor of the $Ca^{2+}$-ATPase could be modulated by the lipid bilayer through partition of HHIF in the membrane phase. Thus, we studied whether the inhibition produced by HHIF was also dependent on the SR membrane or $Ca^{2+}$-ATPase concentrations in the assay medium.

As depicted in Fig. 3A, at a fixed time of incubation the concentration of HHIF that produced 50% inhibition of the $Ca^{2+}$-ATPase of leaky SR vesicles induced by HHIF was shifted to higher values when the membrane concentration rose. The titrations of the $Ca^{2+}$-ATPase activity of SR membranes with HHIF were carried out in the presence of the $Ca^{2+}$-ionophore calcimycin, to avoid the large inhibition produced by luminal $Ca^{2+}$ [28] which could mask the effect of HHIF. The inhibition of purified $Ca^{2+}$-ATPase is also dependent on protein concentration (Fig. 3B). From the dependence of $K_{0.5}$ versus membrane concentration, the apparent partition coefficient ($K_p$) for binding of this factor to SR membranes can be calculated according to the method of De Foresta et al. [29]. We obtain a $K_p$ value of 0.6 ($\mu$g SR protein/ml)$^{-1}$ or $(8-10) \cdot 10^5$ (mol lipid/l)$^{-1}$, considering that the $Ca^{2+}$-ATPase amounts to approximately 80% of SR membrane protein and that there is

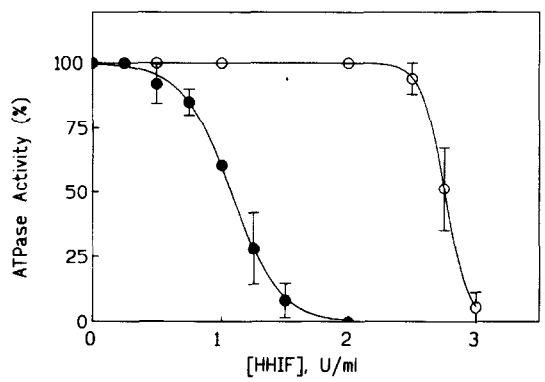


Fig. 2. Dependence of the $Ca^{2+}$-ATPase activity upon the concentration of HHIF. Purified $Ca^{2+}$-ATPase (●) and $Ca^{2+}$-ATPase reconstituted with egg phosphatidylcholine at a molar ratio of phospholipid:ATPase of 300:1 (○). Purified and reconstituted ATPase were incubated in 0.1 M Tes/Tris (pH 7.4) in the absence and in the presence of HHIF for 5 min at 25°C before measuring the $Ca^{2+}$-ATPase activity. The protein concentration in the assay medium was 2 $\mu$g/ml and HHIF concentrations are given in the abscissae. The reference (100%) is the ATPase activity measured in the absence of HHIF ($6 \pm 1$ IU).

0.6–0.5 g lipid/g protein in SR membranes [16], with an average molecular weight for lipids of 800. Apparent partition coefficients, $K_p$, in the range of $10^5$ (expressed as (mol lipid/l)$^{-1}$) have been reported for different highly hydrophobic molecules which inhibit the $Ca^{2+}$-ATPase activity [29]. From an operational point of view these results recommended the control of the HHIF/membrane concentration ratio in subsequent experimental handlings.

In spite of the lower lipid/protein ratio of purified $Ca^{2+}$-ATPase with respect to SR membranes, both Figs. 1 and 3 seem to suggest that purified $Ca^{2+}$-ATPase is apparently slightly less sensitive to inhibition by HHIF than SR membranes. However, this needs to be taken cautiously, since HHIF is likely to partition as well in the micelles of the detergent deoxycholate which is added with purified $Ca^{2+}$-ATPase, therefore reducing the HHIF concentration effective for $Ca^{2+}$-ATPase inhibition.

The $Ca^{2+}$-ATPase activity of 80 $\mu$g of SR protein/ml incubated with 25 U of HHIF/ml (approx. 0.31 U of HHIF/$\mu$g of SR protein) for 15 min does not recover with respect to the control value upon removal of HHIF by dialysis for 17 h at 4°C, a treatment that only produces a slight (< 10%) loss of activity of SR membranes in the absence of HHIF. Under these experimental conditions about 80% of the $Ca^{2+}$-ATPase activity was irreversibly lost. This result shows that the inhibition was irreversible and suggested that might be due to protein denaturation. This point was confirmed by differential scanning calorimetry. In native SR membranes the thermal unfolding of the $Ca^{2+}$-ATPase is irreversible and takes place between 47 and 57°C, producing a major endothermic peak centered between 52.5 and 54.5°C depending upon the experimental conditions ([23,30]; and also the control scan, trace a, of Fig. 4), because this protein accounts for approximately 80% of SR membrane protein [16]. SR membranes preincubated with HHIF until the $Ca^{2+}$-ATPase activity was more than 90% lost yielded thermograms lacking the endothermic peak characteristic of the thermal unfolding of the $Ca^{2+}$-ATPase (Fig. 4). Therefore, HHIF produced denaturation of the $Ca^{2+}$-ATPase under the conditions which irreversibly inhibit the ATPase activity.

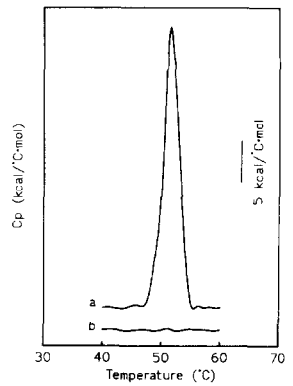


Fig. 4. Differential scanning calorimetry thermograms of SR membranes (1 mg protein/ml). Control of SR membranes in buffer (trace a) and SR membranes incubated for 60 min with 0.15 U of HHIF per $\mu$g of SR protein before loading the calorimeter (trace b). Buffer: 50 mM Tes and 0.1 M KCl (pH 7.4). Scanning rate 60°C/h. The $Ca^{2+}$-ATPase activity of the samples of SR membranes after the incubation with HHIF was lower than 10% of the activity of control samples (not incubated with HHIF). The thermograms shown are representative of the results obtained in triplicate experiments.

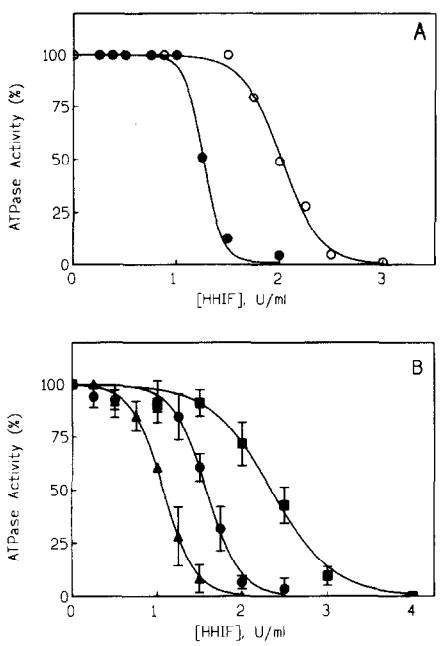


Fig. 3. Dependence of the effect of HHIF on the $Ca^{2+}$-ATPase activity upon the concentration of leaky SR membranes and of purified $Ca^{2+}$-ATPase. SR membranes made leaky by addition of calcimycin (0.04 mg/mg SR protein) and purified $Ca^{2+}$-ATPase were incubated in 0.1 M Tes/Tris (pH 7.4) in the absence and in the presence of HHIF for 5 min at 25°C before measuring the $Ca^{2+}$-ATPase activity. (A) Leaky SR membranes. The reference (100%) is the ATPase activity measured in the absence of HHIF and in the presence of calcimycin (3.0±0.5 IU). The protein concentrations in the assay medium were 4 $\mu$g/ml (●) and 8 $\mu$g/ml (○) and HHIF concentrations are given in the abscissae. The standard deviation of the data is < 10%. (B) Purified $Ca^{2+}$-ATPase. The reference (100%) is the ATPase activity measured in the absence of HHIF (6±1 IU). The protein concentrations in the assay medium were 2 (▲), 4 (●) or 8 (■) $\mu$g/ml and HHIF concentrations are given in the abscissae.

### 3.2. *Protection by $Mg^{2+}$-ADP and $Ca^{2+}$ against inhibition of the $Ca^{2+}$-ATPase by HHIF*

Millimolar concentrations of $Mg^{2+}$-ATP or $Mg^{2+}$-ADP and submillimolar concentrations of $Ca^{2+}$ have been shown to efficiently protect purified delipidated and solubilized monomeric $Ca^{2+}$-ATPase against irreversible loss of activity [31]. Fig. 5A shows that millimolar concentrations of $Mg^{2+}$-ADP in the preincubation medium partially protect the $Ca^{2+}$-ATPase activity of leaky SR membranes against the HHIF inhibition in function of time, in the absence or presence of 1 mM EGTA. Similar results have been obtained using purified and reconstituted $Ca^{2+}$-ATPase, and

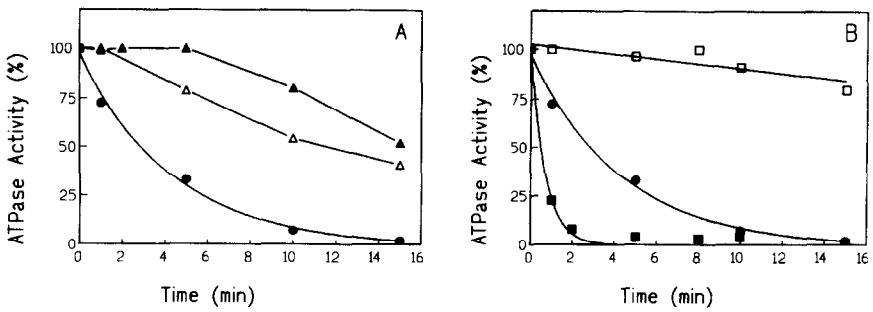


Fig. 5. Protection by $Mg^{2+}$-ADP and $Ca^{2+}$ against inactivation by HHIF of the $Ca^{2+}$-ATPase activity of leaky SR membranes. (A) Effect of $Mg^{2+}$-ADP on the inhibition of the $Ca^{2+}$-ATPase activity by HHIF. Leaky SR vesicles were incubated with HHIF (0.31 U of HHIF/$\mu$g of SR protein) in 0.1 M Tes/Tris (pH 7.4) (●), 2.5 mM $Mg^{2+}$-ADP (▲) or 2.5 mM $Mg^{2+}$-ADP/1 mM EGTA (△). (B) Effect of $Ca^{2+}$ on the inhibition of the $Ca^{2+}$-ATPase activity by HHIF. Leaky SR vesicles were incubated with HHIF (0.31 U of HHIF/$\mu$g of SR protein) in 0.1 M Tes/Tris (pH 7.4) plus 10 $\mu$M $Ca^{2+}$ (●), 0.2 mM $CaCl_2$ (□) or 1 mM EGTA (■). After the times indicated on the abscissae of (A) and (B), the $Ca^{2+}$-ATPase activity was measured in the assay medium given in Materials and methods. The concentrations of SR and HHIF in the assay medium were 4 $\mu$g/ml and 1.25 U/ml, respectively.

with $Mg^{2+}$-ATP instead of $Mg^{2+}$-ADP (data not shown). Furthermore, we have studied the effect of $Ca^{2+}$ in the preincubation medium on the inactivation of the $Ca^{2+}$-ATPase of SR vesicles by HHIF. Free $Ca^{2+}$ concentrations were adjusted using $Ca^{2+}$-EGTA buffers, with an apparent $K_d$ for the $Ca^{2+}$-EGTA complex of $10^{-7.2}$ M at pH 7.4 [26]. Fig. 5B shows that 0.2 mM $Ca^{2+}$ largely protects against the inhibition by HHIF, as it increases more than 60-fold the half-time of inactivation observed in the presence of EGTA. However, it should be noted that 10 $\mu$M $Ca^{2+}$, which saturates the $Ca^{2+}$ transport sites of the $Ca^{2+}$-ATPase at this pH in the absence of HHIF [32], affords only a partial protection against inactivation by HHIF, e.g., it increases approx. 6-fold the half-time of inactivation. Similar results have been obtained using purified $Ca^{2+}$-ATPase (Fig. 6a). The $Ca^{2+}$ concentration dependence of the protection by $Ca^{2+}$ against HHIF inhibition of the activity of purified $Ca^{2+}$-ATPase is shown in the Fig. 6B. 5 min incubation of purified $Ca^{2+}$-ATPase at 25°C in 0.1 M Tes/Tris (pH 7.4) were taken because in the absence of HHIF (control) there is no appreciable loss of activity. From Fig. 6b we obtain an apparent $K_{0.5}$ of protection by $Ca^{2+}$ of $5 \pm 1$ $\mu$M (Fig. 6b), which is only 3- to 4-fold higher than the $K_{0.5}$ of activation of the $Ca^{2+}$-ATPase by $Ca^{2+}$ obtained under these experimental conditions in the absence of HHIF (Fig. 6b; see also [28]). These results indicate that the protection afforded by $Ca^{2+}$ against inactivation by HHIF is probably due to $Ca^{2+}$ binding to the high-affinity $Ca^{2+}$ sites of the $Ca^{2+}$-ATPase, as has been shown to be the case for the protection afforded by $Ca^{2+}$ against denaturation of delipidated and solubilized $Ca^{2+}$-ATPase [31].

### *3.3. HHIF produced leakiness to $Ca^{2+}$ of SR vesicles at concentrations lower than those effectively inhibiting the $Ca^{2+}$-ATPase*

As shown in Fig. 7, the dependence of the $Ca^{2+}$-ATPase activity of sealed SR vesicles on the concentration of HHIF is complex, with two distinct phases, showing stimulation of the $Ca^{2+}$-ATPase activity at low HHIF concentrations followed by an abrupt inhibition at higher HHIF concentrations (apparent Hill coefficient $13 \pm 2$). How-

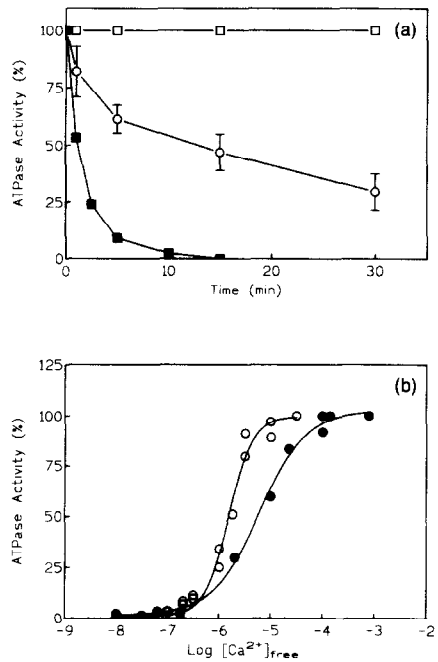


Fig. 6. Protection by $Ca^{2+}$ against inactivation by HHIF of purified $Ca^{2+}$-ATPase. (a) Effect of $Ca^{2+}$ on the kinetics of inhibition of the $Ca^{2+}$-ATPase activity by HHIF. Purified $Ca^{2+}$-ATPase was incubated at 25°C with HHIF (0.38 U of HHIF/$\mu$g of SR protein) in 0.1 M Tes/Tris (pH 7.4) plus 10 $\mu$M $Ca^{2+}$ (○), 0.2 mM $CaCl_2$ (□) or 1 mM EGTA (■). After the times indicated on the abscissae the $Ca^{2+}$-ATPase activity was measured in the assay medium given in Materials and methods. The concentrations of purified $Ca^{2+}$-ATPase and of HHIF in the assay medium were 4 $\mu$g/ml and 1.5 U/ml, respectively. (b) Activity of purified $Ca^{2+}$-ATPase (4 $\mu$g/ml) remaining after 5 min incubation at 25°C with HHIF (0.375 U/$\mu$g protein) as a function of the free $Ca^{2+}$ concentration in the incubation medium (filled circles). The $Ca^{2+}$-ATPase activity was measured in the assay medium given in Materials and methods with a saturating $Ca^{2+}$ concentration (30 $\mu$M). Empty circles, $Ca^{2+}$ dependence of the ATPase activity of the purified $Ca^{2+}$-ATPase without HHIF at pH 7.4 and 25°C. The standard deviation of the data is < 10%.

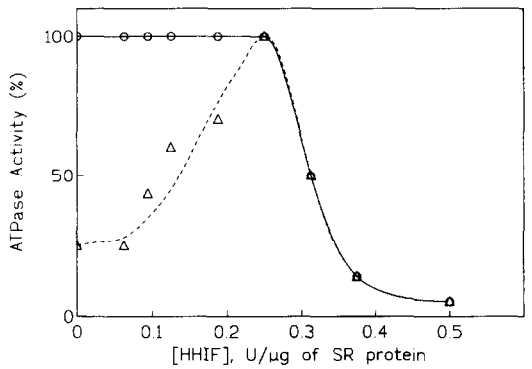


Fig. 7. Dependence of the $Ca^{2+}$-ATPase activity upon the concentration of HHIF. Sealed SR vesicles (△) and SR vesicles made leaky to $Ca^{2+}$ with calcimycin (0.04 mg/mg protein) (○). SR were incubated in 0.1 M Tes/Tris (pH 7.4) in the absence and in the presence of HHIF for 5 min at 25°C before measuring the $Ca^{2+}$-ATPase activity. The concentration of SR protein in the assay mixture was 4 μg/ml and HHIF concentrations are given in the abscissae. The reference (100%) is the ATPase activity measured in the absence of HHIF and in the presence of calcimycin (3.0±0.5 IU). The standard deviation of the data is < 10%.

ever, no stimulation of the $Ca^{2+}$-ATPase activity is observed in the presence of the calcium ionophore calcimycin (4% w/w) and the $Ca^{2+}$-ATPase activity of sealed SR membranes at maximal stimulation by HHIF approaches that of SR membranes in the presence of calcimycin, suggesting that stimulation by HHIF of the ATPase activity of sealed SR membranes is due to dissipation of the $Ca^{2+}$ gradient produced by operation of the $Ca^{2+}$ pump, which largely inhibits the $Ca^{2+}$-ATPase activity [28].

The results presented in Fig. 8A demonstrated that the steady-state level of $Ca^{2+}$ accumulation by SR vesicles decreases as the concentration of HHIF is increased, becoming negligible at the HHIF concentration which produced maximal stimulation of the $Ca^{2+}$-ATPase activity of SR membranes. Because this effect of HHIF could be due to uncoupling of the $Ca^{2+}$ transport from ATP hydrolysis in the $Ca^{2+}$-ATPase or alternatively to an increased permeability to $Ca^{2+}$ of SR membranes in the presence of HHIF, we studied the permeability of SR membranes to $Ca^{2+}$ in the presence of HHIF. HHIF at concentrations which inhibit > 90% of $Ca^{2+}$ uptake by SR vesicles, 0.05 U of HHIF per μg of SR protein, largely increases the permeability to $Ca^{2+}$ of SR membranes (Fig. 8B). This point was assessed using two different experimental protocols, as indicated in detail in the legend of Fig. 8B. In the ATP-loading protocol the SR vesicles were loaded with $Ca^{2+}$ by the activity of the $Ca^{2+}$-pump, under experimental conditions identical to those used in Fig. 8A. In parallel experiments SR vesicles were passively loaded by incubation at pH 7.4 with a buffered solution containing 2 mM $Ca^{2+}$. Both experimental approaches gave the same results within experimental error. In addition, the half-time for the $Ca^{2+}$ release from SR membranes in the absence of HHIF (controls) which we obtained from these results is in good agreement with the values reported by other laboratories [33,34]. The HHIF-induced increase of the permeability to $Ca^{2+}$ of the SR membrane does not appear to require preincubation of SR membranes with HHIF, at difference to the HHIF-induced inhibition of the $Ca^{2+}$-ATPase. Therefore, these results suggest that the HHIF-induced

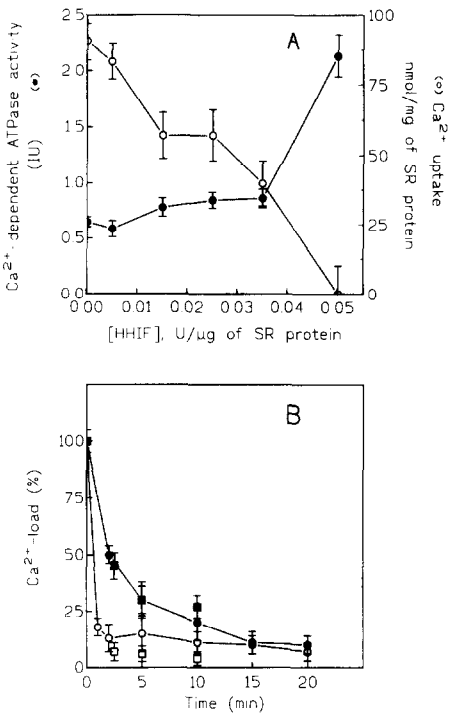


Fig. 8. Effect of HHIF on $Ca^{2+}$ uptake and on $Ca^{2+}$ release. (A) Dependence of $Ca^{2+}$-uptake and of $Ca^{2+}$-ATPase activity upon the concentration of HHIF. SR membranes (2 mg protein/ml) were incubated with the indicated concentrations of HHIF for 15 min at 25°C in 0.1 M Tes/Tris (pH 7.4), and then aliquots were pooled for $Ca^{2+}$-uptake and $Ca^{2+}$-ATPase activity measurements. $Ca^{2+}$-uptake and $Ca^{2+}$-ATPase activity measurements were done at 25°C as indicated in Materials and methods. (B) $Ca^{2+}$ efflux from SR vesicles in the absence (●,■) and in the presence of HHIF (○,□). SR vesicles were loaded with $Ca^{2+}$ in the absence (■,□) or in the presence (●,○) of ATP as described below. *ATP-dependent $Ca^{2+}$ loading protocol:* SR vesicles (2 mg/ml) were loaded with $Ca^{2+}$ by 1 min incubation at 25°C with the ATPase assay mixture indicated in the Materials and methods section. $Ca^{2+}$-uptake was stopped by addition of 10 mM glucose/100 μg of hexokinase and 0.3 mM EGTA. After 30 s, HHIF (100 U/ml) was added and this was taken as the zero time. Aliquots were pooled at the times indicated in the abscissae after the addition of HHIF (○). The results obtained in control experiments carried out in the absence of HHIF are also presented (●). The $Ca^{2+}$-load of SR membranes 30 s after the addition of the $Ca^{2+}$-stopping solution (50–55 nmol $Ca^{2+}$ per mg of SR protein) was taken as the 100% value for these experimental series. *Passive $Ca^{2+}$-loading protocol:* SR vesicles (20 mg/ml) were loaded by 2 h incubation at 25°C with a solution containing 50 mM M Tes/0.1 M KCl/0.25 M sucrose and 2 mM $CaCl_2$ (pH 7.4). At zero time, the vesicles were diluted up to 2 mg/ml in a medium containing 50 mM Tes (pH 7.4)/0.1 M KCl/1 mM EDTA/0.25 M sucrose and 100 U/ml of HHIF (□), and aliquots were pooled at the times indicated in the abscissae. Control experiments (■) were done by dilution of SR vesicles in the same medium minus HHIF. The 100% value for these series were 4–5 nmol $Ca^{2+}$ per mg of SR protein.

increase of the permeability to $Ca^{2+}$ of SR membranes is not due to the interaction of HHIF with the $Ca^{2+}$-ATPase.

Since HHIF behaves as a lipophilic compound [10–12], we considered the possibility that the effects of HHIF on $Ca^{2+}$ permeability of SR membranes and/or on the activity of the SR $Ca^{2+}$-ATPase could be due, at least in part, to large long-range structural perturbations of the lipid bilayer. However, HHIF at up to 0.4 U of HHIF per $\mu$g of SR protein did not solubilize the SR membranes, as indicated by light-scattering measurements at 400 nm (data not shown). The lack of a detergent action of HHIF has been noticed elsewhere with synaptosomal membranes [12]. Owing to the modulation of the SR $Ca^{2+}$-ATPase by the long-range organization and dynamic state of the lipid bilayer [35,36], we have measured the effect of HHIF on the polarization of the fluorescence of the lipid state probe DPH incorporated in SR membranes. We have found that with up to 0.4 U of HHIF per $\mu$g of SR protein (e.g., 10 U/ml of HHIF and 25 $\mu$g/ml of SR protein), the steady-state anisotropy of fluorescence of DPH incorporated in SR membranes ($0.130 \pm 0.004$) is not significantly different from that of control SR membranes ($0.136 \pm 0.004$). These results, therefore, exclude the possibility that the modulation of the SR $Ca^{2+}$-ATPase activity by HHIF arose from a long-range structural perturbation of the lipid bilayer, like an altered lipid microviscosity.

## 4. Discussion

Recent results from this laboratory reported elsewhere [14] suggested a direct action of HHIF on the $Ca^{2+}$ handling by endoplasmic reticulum of rat mesangial cells in culture. In this communication, we have investigated the effect of the endogenous inhibitor of the plasma membrane $Na^+$ and $Ca^{2+}$ pumps, HHIF, on the $Ca^{2+}$-ATPase activity of SR membranes from skeletal muscle. The presence of a phenolic ring in HHIF structure has been recently suggested [11,12], and different phenolic compounds are known to be potent inhibitors of the $Ca^{2+}$-ATPase activity of SR membranes [37,38] and of $Na^+$,$K^+$-ATPase [39]. These compounds have a high tendency to partition into the membrane, giving an effect dependent on membrane concentration.

The addition of HHIF produced a biphasic effect on the $Ca^{2+}$-ATPase activity of SR membrane preparations. The first phase, observed at low HHIF concentrations, is correlated with the loss of calcium accumulation by SR vesicles, which releases the inhibition by high free $Ca^{2+}$ concentrations in the luminal space achieved during active transport [28]. Low concentrations of HHIF produced an enhanced $Ca^{2+}$ permeability of SR membranes, which accounts for the inhibition of $Ca^{2+}$ uptake by SR vesicles. At higher concentrations, HHIF inhibits the $Ca^{2+}$-ATPase activity of SR membranes. $Ca^{2+}$ and $Mg^{2+}$-ADP, which shift the ATPase to the E1 state, protect against inhibition by HHIF.

The concentration dependence of HHIF inhibition and activation varies with the concentration of SR membranes in the reaction mixture and also with the lipid to protein ratio in purified $Ca^{2+}$-ATPase. This indicated a large partitioning of HHIF in the SR membrane. The quenching of the intrinsic fluorescence by HHIF (not shown) supports this hypothesis, because 11 out of the 13 Trp of the ATPase are located in the intramembranous hydrophobic portion of the protein [40]. The partition coefficient of HHIF in SR membranes calculated in this paper, 0.6 ($\mu$g protein/ml)$^{-1}$, agrees well with the one measured as indicated elsewhere for synaptosomal membranes [12]. Because HHIF also has a high tendency for partition on liposomes made from lipids extracted from SR membranes, we concluded that it incorporates into the lipid bilayer. However, the inhibition of the $Ca^{2+}$-ATPase is not correlated with a significant alteration of the lipid microviscosity of the SR membrane nor with solubilization of the membranes and, thus, the possibility that it could be due to a long-range mediated lipid bilayer perturbation can be excluded.

A region of the $Ca^{2+}$-ATPase where lipophilic molecules bind is at the lipid/protein interface [25,37,38, 41], and it is well established that disruption of the $Ca^{2+}$-ATPase lipid annulus, e.g., delipidation, largely enhances the rate of the $Ca^{2+}$-ATPase irreversible inactivation [42,43]. In addition, it has been shown that the lipophilic local anaesthetics increase the instability of the $Ca^{2+}$-ATPase in SR membranes by disruption of the lipid annulus of this membrane protein [23,44]. The very abrupt dependence of the $Ca^{2+}$-ATPase inhibition on HHIF concentration (showing apparent Hill coefficients of $13 \pm 2$) supports the hypothesis that the inhibition of the $Ca^{2+}$-ATPase activity by HHIF is related to the disruption of the lipid annulus, because it has been shown that the activity of intrinsic membrane proteins shows allosteric behavior with respect to the saturation of their annular binding sites by lipids [45]. This hypothesis can account for the fact that the sensitivity of purified $Ca^{2+}$-ATPase to inhibition by HHIF is clearly higher than that of reconstituted $Ca^{2+}$-ATPase with larger lipid to protein ratio, and also that the inhibition of the $Ca^{2+}$-ATPase by HHIF is a slow, irreversible process leading to protein unfolding or denaturation, as shown by the differential scanning calorimetry data. In addition, $Ca^{2+}$ and $Mg^{2+}$-ADP, which protect against thermal denaturation of purified $Ca^{2+}$-ATPase [30,46], are also effective antagonists of the irreversible inactivation by HHIF of the $Ca^{2+}$-ATPase. In the case of $Ca^{2+}$, the $K_{0.5}$ of protection against inactivation by HHIF is only 3- to 4-fold higher than the $K_{0.5}$ of $Ca^{2+}$-ATPase activation by $Ca^{2+}$ in the absence of HHIF, which is due to saturation of the high-affinity $Ca^{2+}$ sites of the $Ca^{2+}$-ATPase [16,28,31]. A decreased affinity for $Ca^{2+}$ binding to the high-affinity sites of the $Ca^{2+}$-ATPase has been reported in the presence of other lipophilic compounds, such as pentobarbital [25], procaine [47], thapsigargin and

2,5-di(*tert*-butyl)-1,4-benzohydroquinone [48] or detergents like Triton X-100 below its critical micelle concentration [49]. Therefore, these results suggest that the protection afforded by $Ca^{2+}$ against the irreversible inactivation produced by HHIF can be accounted for binding of $Ca^{2+}$ to the high-affinity sites of the $Ca^{2+}$-ATPase, as has been suggested previously for $Ca^{2+}$ protection against the thermal denaturation of solubilized and delipidated $Ca^{2+}$-ATPase [46] and of the $Ca^{2+}$-pump of SR membranes [50].

In conclusion, at low concentrations HHIF stimulates $Ca^{2+}$ release from SR vesicles and at higher concentrations produces irreversible inactivation by denaturation of the $Ca^{2+}$-ATPase of SR membranes. Therefore, interaction of HHIF with SR membranes largely decreases the ability of these membranes to store and sequester $Ca^{2+}$, and could account for the increased cytosolic $Ca^{2+}$ and contractility reported for muscle cells in culture upon incubation with HHIF [14].

## Acknowledgements

We are indebted to J. Colilla for excellent technical assistance and to Dr. Jaime M. Merino for his help with differential scanning calorimetry measurements. This work has been supported, in part, by the Spanish D.G.I.C.Y.T. (Grant No. PB91-0311), by the Spanish F.I.S., and by Science Project No. SC1*-CT92-0783 of the EC. M.R. has been the recipient of fellowships from the Spanish F.I.S. and the Rich Foundation.

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