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Specific Inhibition of Cardiac and Skeletal Muscle Sarcoplasmic Reticulum Ca^{2+} Pumps by H-89

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ABSTRACT. The isoquinolinesulfonamide H-89, an inhibitor of cyclic AMP-dependent protein kinases (EC 2.7.1.37, cAPrK), inhibited the Ca^{2+}-ATPase activity of cardiac and skeletal muscle sarcoplasmic reticulum (SR) with concentrations giving half-maximal inhibition of 8.1 ± 1.3 and 7.2 ± 0.9 μmol/L, respectively. The effect of H-89 on cardiac SR Ca^{2+}-ATPase (EC 3.6.1.38) was the same irrespective of the presence or absence of inhibitors of cAPrK and furthermore, was not affected by a neutralising monoclonal antibody raised against phospholamban. Thus, the action of H-89 in inhibiting SR Ca^{2+}-ATPase would not appear to be mediated by inhibition of cAPrK to reduce the phosphorylation state of phospholamban. In both cardiac and skeletal muscle SR, the inhibition by H-89 was noncompetitive with respect to ATP at a low concentration of ATP (<1 mmol/L) and of a mixed pattern at high concentrations of ATP. H-89 produced a decrease in affinity of the SR Ca^{2+} pump to Ca^{2+} with an increase in the K_m for Ca from 0.52 ± 0.01 to 0.94 ± 0.03 μmol/L ($P < 0.05$) in cardiac SR and from 0.39 ± 0.01 to 0.79 ± 0.02 μmol/L ($P < 0.05$) in skeletal muscle SR. These results suggest that H-89 inhibits SR Ca^{2+}-ATPase by a direct action on the SR Ca^{2+} pump to decrease its affinity to Ca^{2+}. Such an action may contribute to the pharmacological effect of H-89. BIOCHEM PHARMACOL **54**;9: 991–998, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. H-89; Ca^{2+}-ATPase inhibitor; sarcoplasmic reticulum; Ca^{2+}-ATPase; phospholamban; cAMP-dependent protein kinase

In muscle cells, the Ca^{2+}-ATPase (EC 3.6.1.38)‡ of the sarcoplasmic reticulum (SR) is essential to ensure Ca^{2+} pumping by the SR, thereby allowing muscle relaxation. Five distinct isoforms of the Ca^{2+}-ATPase have been identified. The major forms expressed in skeletal muscle and in the heart are SERCA1 (Sarco(endo)plasmic reticulum Ca^{2+}-ATPase) and SERCA2a, respectively [1]. The cardiac SR Ca^{2+} pump is under the control of another cardiac SR protein, phospholamban (PLB), which inhibits the pump while dephosphorylated [2]. The phosphoprotein, PLB, is expressed in cardiac but not in fast skeletal muscle [3]. The major effect of dephosphorylated PLB is to suppress cardiac SR Ca^{2+}-ATPase activity by decreasing its affinity for Ca^{2+} [4, 5, 6]. Although a lowering of the V_{max} of the Ca^{2+}-ATPase by PLB has also been reported [4, 6, 7], this is still controversial [8, 9]. Phosphorylation of PLB by cAMP-dependent protein kinase (EC.2.7.1.37, cAPrK) or by Ca^{2+} calmodulin-dependent protein kinase results in stimulation of Ca^{2+} uptake by the cardiac SR. The relative role of the two kinases is still not clearly understood. However, in permeabilized cardiac myocytes, it has been suggested that cAPrK-dependent phosphorylation of PLB results in an increase in the Ca^{2+} affinity in the SR Ca^{2+} pump, while phosphorylation of PLB by Ca^{2+} calmodulin-dependent protein kinase leads to an increase in the maximum uptake rate [10].

The isoquinolinesulfonamide H-89 is a potent and selective inhibitor of cAPrK, with this being mediated by competition with ATP on the catalytic site of the enzyme [11, 12]. Therefore, it can be hypothesized that the selective inhibition of cAPrK by H-89 should result in an inhibition of Ca^{2+}-ATPase activity in cardiac SR as a consequence of a reduced phosphorylation of PLB, but not in fast skeletal muscle SR. However, this assumes that H-89 has no direct effect on the Ca^{2+}-ATPase. The inhibitory effect of H-89 is thought to be selective for cAPrK, since when tested against other ATP-utilizing enzymes, such as actomyosin ATPase or erythrocyte Ca^{2+}-ATPase, H-89 and related isoquinolinesulfonamides generally showed little or no inhibition [13, 14]. However, since the effect of H-89 on the SR Ca^{2+}-ATPase itself has not been reported yet, we

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‡ *Abbreviations:* cAPrK, cAMP-dependent protein kinase (EC 2.7.1.37); $[Ca^{2+}]_{free}$, Ca^{2+} concentration; Rp-cAMP[S], (*R*)-(*p*)-adenosine 3′,5′-(cyclic)-monophosphorothioate; Pi, inorganic phosphate; PKI, protein kinase inhibitor; PLB, phospholamban; SERCA, Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (EC 3.6.1.38); SR, sarcoplasmic reticulum

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investigated the effect of the compound on the Ca^{2+}-ATPase activities of both cardiac and fast skeletal muscle SR.

Our results show that at micromolar concentration, H-89 is an inhibitor of both SERCA1 and SERCA2a, and that in cardiac and skeletal muscle SR vesicles, H-89 decreases the apparent affinity of the SR Ca^{2+} pump for Ca^{2+} in both the presence and absence of PLB. A preliminary account of this work has been presented in abstract form [15].

MATERIALS AND METHODS

Animals

All animals used in this study were purchased from an approved vendor, housed, handled, and fed according to the European Community Guidelines (Journal Officiel des Communautés Européennes L. 358 Directive du 24 Nov. 1986). Adult New Zealand White rabbits (3–3.5 kg) were anesthetized by intravenous injection of pentobarbital (40 mg/kg) in the presence of heparin (250 UI/kg). Adult mongrel dogs (20–35 kg) were anesthetized with an injection of pentobarbital (60 mg/kg).

Membrane Preparation

Canine cardiac SR was isolated from left ventricle according to Jones et al. [16]. The membrane vesicles enriched in SR were suspended (5 mg/mL) in 0.6 mol/L KCl and 30 mmol/L histidine, pH 7.0, frozen in liquid nitrogen, and stored at −75° until used. Skeletal muscle SR vesicles were prepared from fast skeletal muscle removed from the back of rabbits. We used the method of Mitchell et al. [17] in which the combination step and continuous gradient were replaced by a sucrose step gradient (sucrose concentrations of 14, 25, 28, 35, 45, 50% (w/v)). The vesicles obtained from the 45/50% interface were homogenized at a concentration of 0.75 mg/mL in 5 mmol/L HEPES (pH 7.1), 0.3 mol/L sucrose, and 0.15 mol/L KCl, frozen in liquid nitrogen, and stored at −75°C until used.

Ca^{2+}-ATPase Activity

The rate of ATP hydrolysis was determined at 30°C by measuring colorimetrically [18] the amount of inorganic phosphate liberated during a 15-min incubation of the SR vesicles (40 μg/mL and 6 μg/mL for cardiac and skeletal muscle, respectively) in a medium containing (in mmol/L) 100 KCl, 2 $MgCl_2$, 50 3-(*N*-morpholino)propanesulfonic acid-KOH (pH 6.8), 2 Na_2ATP, 1 EGTA and different $CaCl_2$ concentrations to yield the desired free Ca^{2+} concentration ($[Ca^{2+}]_{free}$). The calcium ionophore A-23187 (2 μmol/L) was included in the assay system so that Ca^{2+} ions accumulated by SR vesicles during the assay would not inhibit the Ca^{2+} pumps from inner surfaces of the vesicles [19]. The Ca^{2+}-ATPase activity was taken as the difference between the quantity of P_i liberated in the presence and the absence of added $CaCl_2$. The assay was run using a robotic sample processor (RSP 5052, Tecan) which distributed the buffers with or without $CaCl_2$; SR vesicles were then preincubated for 20 min in the assay medium containing H-89 (or DMSO 1% as solvent) before the ATPase reaction was initiated by the addition of Na_2ATP (total volume of the assay = 1 mL). After exactly 15 min, the ATPase reaction was stopped by the addition of the colorimetric reagent before the absorbance in each test tube was read using a spectrophotometer (DU-62, Beckman) connected to a sample processor (Gilson, Nantes, France, model 222). $[Ca^{2+}]_{free}$ was calculated using Fabiato's computer program [20]. Protein content was determined according to Bradford [21] using the Coomassie protein assay kit from BioRad with bovine serum albumin as standard.

Chemicals

H-89 was purchased from Biomol, (*R*)-(*p*)-adenosine 3',5'-cyclic monophosphorothioate sodium salt (Rp-cAMP[S]) from Boehringer Mannheim, and Na_2ATP and bovine heart protein kinase inhibitor (PKI) from Sigma. All other chemicals were of analytical grade, and ultrapure water prepared with Milli-Q and Milli RO-5 equipment (Millipore) was used to prepare all buffers. H-89 was added from stock solution with DMSO and the DMSO concentration never exceeded 1% in the assay media. Monoclonal antibody raised against PLB was obtained from PhosphoProtein Research. All other reagents were of analytical grade.

Data Analysis

The kinetic parameters (K_m and V_{max}) were determined by drawing double reciprocal plots of the data obtained in experiments performed in the presence of varied concentrations of ATP and of H-89. The parameters corresponding to high-affinity and low-affinity sites for ATP were determined from linear regression of the mean data corresponding to 0.2–0.6 mmol/L ATP and 1.2–4.0 mmol/L ATP, respectively. In Ca^{2+} dependency studies to determine the $[Ca^{2+}]_{free}$ required for half-maximal activation (K_m for Ca), the individual experimentally measured values for Ca^{2+}-ATPase at different $[Ca^{2+}]_{free}$ in three independent experiments were fitted to the equation $V = V_{max}/[1 + (K_m/[Ca^{2+}]_{free})^N]$, with N = Hill coefficient [22], by a nonlinear regression analysis using Origin 3.5 software Microcal. Given the bell-shaped dependence of Ca^{2+}-ATPase activity on $[Ca^{2+}]_{free}$, only the points corresponding to the ascending part of each curve were used for the fit. The statistical significance of differences observed between H-89 and DMSO treatment or PKI and buffer pretreatment was tested using Student's *t*-test for paired results. A value of $P \leq 0.05$ was considered significant.

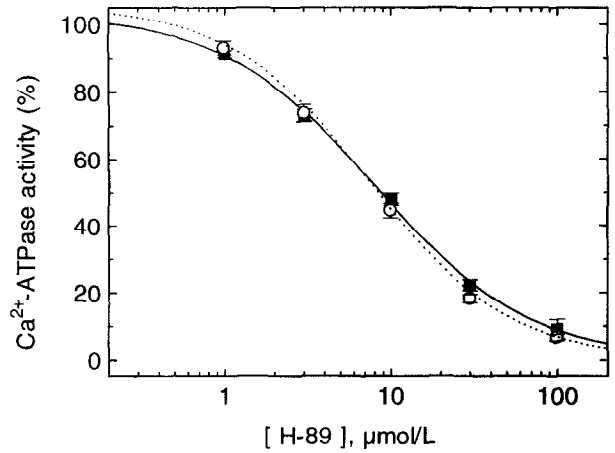

FIG. 1. Inhibition by H-89 of Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activities of canine cardiac (■, solid line) and rabbit skeletal muscle (○, dotted line) SR vesicles were determined at pCa 6.20 in the presence of 2 mmol/L ATP and of various concentrations of H-89. The Ca^{2+}-ATPase activities determined in the absence of H-89 were taken as 100%, and were 0.299 ± 0.032 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ and 3.29 ± 0.27 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ for canine cardiac and for rabbit skeletal muscle microsomes, respectively. Each point represents the mean ± SEM of four determinations.

RESULTS

Concentration-Dependent Inhibition of SR-Ca^{2+}-ATPase Activity by H-89

The effect of H-89 on Ca^{2+}-ATPase activity was investigated in cardiac and skeletal muscle membrane fractions enriched in SR. Figure 1 shows that H-89 inhibited Ca^{2+}-ATPase activity in both preparations to a similar extent. IC_{50} values were almost the same in cardiac and skeletal muscle, 8.1 ± 1.3 and 7.6 ± 1.0 μmol/L, respectively. Furthermore, 100 μmol/L H-89 was able to produce essentially complete inhibition of the ATPase activity in cardiac and skeletal muscle SR. The inhibition was obviously not species-dependent, since the cardiac SR microsomes were prepared from dog and those from fast skeletal muscle were isolated from rabbit.

Effect of H-89 on Ca^{2+}-ATPase Activity of Cardiac SR in the Presence of cAMP-Dependent Protein Kinase Inhibitors

In order to distinguish between a direct inhibition of Ca^{2+}-ATPase and the possible involvement of cAPrK inhibition, the effect of H-89 was investigated in the presence of two inhibitors of cAPrK, Rp-cAMP[S] and PKI. Since the inhibition of cardiac SR-Ca^{2+}-ATPase by phospholamban is dependent on $[Ca^{2+}]_{free}$ [5, 6], the effects of the cAPrK activators were investigated at two different $[Ca^{2+}]_{free}$.

At a $[Ca^{2+}]_{free}$ which produces half-maximal activation of cardiac SR Ca^{2+}-ATPase (pCa 6.20), Rp-cAMP[S] was without any effect whereas PKI produced a significant inhibition (23.4%, $P = 0.035$, df = 6) of the ATPase.

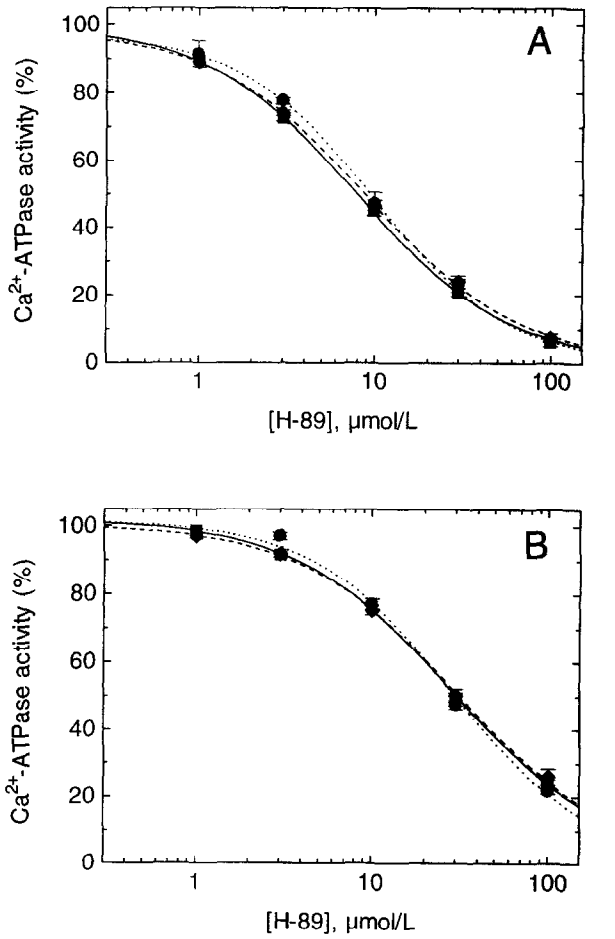

FIG. 2. Effect of inhibitors of cAMP-dependent protein kinase on the inhibition by H-89 of the Ca^{2+}-ATPase activity of canine cardiac SR. The cardiac microsomes were incubated at pCa 6.20 (A) or at pCa 5.66 (B) in the presence of 2 mmol/L ATP and of various concentrations of H-89 and without inhibitor of cAPrK (■, solid line), with 100 μmol/L Rp-cAMP[S] (●, dotted line), and with 200 μg/mL PKI (◆, dashed line). The Ca^{2+}-ATPase activities determined in the absence of H-89 were taken as 100%, and were, at pCa 6.2, 0.301 ± 0.026, 0.291 ± 0.023, and 0.231 ± 0.020 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ without cAPrK inhibitor, with Rp-cAMP[S], and with PKI, respectively. At pCa 5.66, the Ca^{2+}-ATPase activities measured in the absence of H-89 were 0.480 ± 0.032, 0.466 ± 0.032, and 0.403 ± 0.035 μmol $P_i \cdot mg^{-1}$ protein $\cdot$ min^{-1} without cAPrK inhibitor, with Rp-cAMP[S], and with PKI, respectively. The results are expressed as mean ± SEM of two determinations for assays with Rp-cAMP[S] and four determinations for assays with PKI and in the absence of cAPrK inhibitors.

Ca^{2+}-ATPase activities with the solvent, 100 μmol/L Rp-cAMP[S], and 200 μg/mL PKI were 0.301 ± 0.026, 0.291 ± 0.023 and 0.231 ± 0.020 μmol $P_i \cdot mg^{-1}$ protein $\cdot$ min^{-1}, respectively. Figure 2A shows that the extent of the inhibition by H-89 was the same irrespective of the presence of cAPrK inhibitors. The IC_{50} values were 7.93 ± 0.69, 9.91 ± 1.55, and 9.04 ± 0.88 μmol/L with the solvent, Rp-cAMP[S], and PKI, respectively.

In the presence of a $[Ca^{2+}]_{free}$, which maximally activates cardiac SR Ca^{2+}-ATPase (pCa 5.66), both cAPrK

inhibitors were without significant effect on Ca^{2+}-ATPase activity (0.480 ± 0.032, 0.465 ± 0.033, and 0.403 ± 0.035 μmol P_i · mg^{-1} protein · min^{-1}, with the solvent, 100 μmol/L Rp-cAMP[S], and 200 μg/mL PKI, respectively). The results in Fig. 2B show that the potency of H-89 was decreased at this pCa. However, the IC_{50} values were the same regardless of the presence or absence of cAPrK inhibitors (29.2 ± 1.2, 28.9 ± 1.4, and 31.1 ± 1.8 μmol/L with the solvent, Rp-cAMP[S], and PKI, respectively). Thus, both at high and at low $[Ca^{2+}]_{free}$, the inhibition of cardiac Ca^{2+}-ATPase activity by H-89 was not affected by the presence of cAPrK inhibitors.

Effect of H-89 on the Dependence of ATPase Activity on the Concentration of ATP

The dependence of SR ATPase activity on ATP concentration is complex, with a first activation between 1 and 100 μmol/L ATP and a further activating effect at millimolar ATP concentrations. In this study, for both cardiac and skeletal muscle SR ATPase, the double reciprocal plots (Fig. 3) showed a transition near 1 mmol/L ATP, as previously reported [23]. Such a behaviour is assumed to correspond to the interaction of ATP with two sites: a high-affinity (catalytic) site and a low-affinity (regulatory) site [24, 25].

For both cardiac and skeletal muscle SR microsomes, in a concentration range of 0.2–0.6 mM ATP, the slopes and intercepts on the vertical axis were increased by H-89 (10 and 30 μmol/L), and the three lines crossed at a single point on the horizontal axis. With H-89 (30 μmol/L), the V_{max} decreased from 0.161 to 0.049 μmol P_i · mg^{-1} protein · min^{-1} for the cardiac enzyme and from 1.34 to 0.256 μmol P_i · mg^{-1} protein · min^{-1} for the skeletal enzyme, while the K_m for ATP was unchanged (0.167 and 0.182 mmol/L in the cardiac and skeletal muscle SR ATPase, respectively). This indicates that H-89 showed noncompetitive inhibition of the ATPase with respect to the ATP concentration, both in cardiac and skeletal muscle SR microsomes, at concentrations of ATP lower than 1 mmol/L.

At ATP concentrations above 1 mmol/L, the slopes were enhanced by H-89 (10 and 30 μmol/L), and the three lines crossed at a point to the left of the vertical axis and above the horizontal axis in both cardiac and skeletal muscle SR. A 30 μmol/L H-89 moderately increased the K_m for ATP for the cardiac SR ATPase from 1.35 to 1.92 mmol/L, whereas the K_m for ATP was strongly increased, from 1.49 to 5.88 mmol/L, for the skeletal muscle SR ATPase. Simultaneously, the V_{max} was decreased from 0.413 to 0.147 μmol P_i · mg^{-1} protein · min^{-1} for the cardiac enzyme and from 3.51 to 1.75 μmol P_i · mg^{-1} protein · min^{-1} for the skeletal muscle enzyme. Thus, H-89 showed a mixed pattern of inhibition of both cardiac and skeletal muscle SR ATPases with respect to ATP in concentrations above 1 mmol/L.

For both high and low concentrations of ATP, a 10-fold smaller V_{max} was observed for cardiac ATPase compared to skeletal muscle ATPase. This can presumably be attributed to the lower content of Ca^{2+}-ATPase per unit weight of total protein in cardiac SR vesicles compared to skeletal muscle SR vesicles [26].

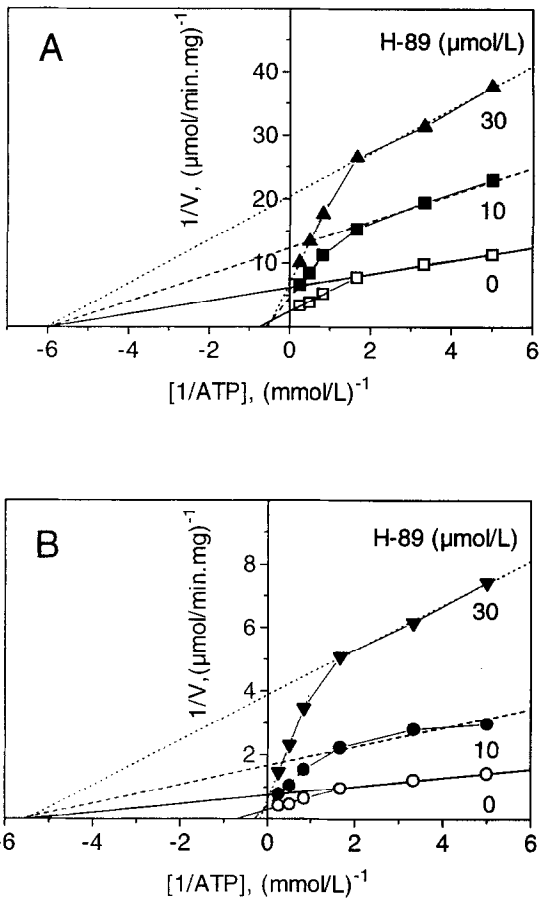

FIG. 3. Double reciprocal plots showing the effect of H-89 on the ATP concentration-dependence of SR Ca^{2+}-ATPase. Canine cardiac (A) and rabbit fast skeletal muscle (B) SR vesicles were incubated in an assay medium whose ATP concentration varied between 0.2 and 4 mmol/L. The $CaCl_2$ concentration was maintained at 0.4 mmol/L, which resulted in pCa 6.20 ± 0.01. Each data point is the mean of three experiments with different microsome preparations. The Dixon plot (1/V vs. [H-89]) of the data shown in (B) and corresponding to high concentrations of ATP (1.2–4.0 mmol/L) gave a K_i value of 3 μmol/L for H-89 on skeletal muscle ATPase.

Effect of H-89 on Ca^{2+} Dependence of SR-Ca^{2+}-ATPase

Since the major effect of H-89 in cardiac myocytes was to decrease the affinity for Ca^{2+} of SR Ca^{2+} pump [10], the effect of H-89 on the Ca^{2+}-dependency of SR-ATPase was examined. Figure 4A shows the typical bell-shaped dependence of cardiac ATPase activity on $[Ca^{2+reset}]_{free}$ and how it was affected by H-89. In the absence of H-89, maximum ATPase activity was observed near 2.2 μmol/L $[Ca^{2+}]_{free}$. This value was slightly increased to 3.7 μmol/L in the presence of 10 μmol/L H-89, and maximum ATPase

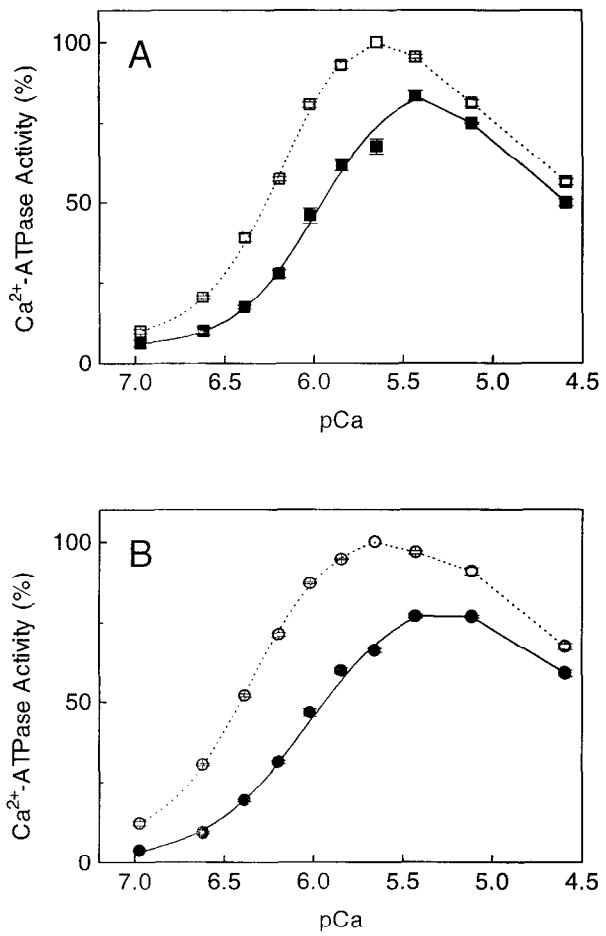

FIG. 4. Effect of H-89 on cardiac SR Ca^{2+}-ATPase activity as a function of the concentration of $[Ca^{2+}]_{free}$. Cardiac (A) or skeletal muscle (B) SR microsomes were incubated in the presence of 2 mmol/L ATP and with 10 μmol/L H-89 (●) or (■), or with the solvent alone (○) or (□). The results are expressed as % of the maximum ATPase activity reached in each experiment in the absence of H-89. Maximum ATPase activities in the absence of H-89 were 0.822 ± 0.024 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ and 7.01 ± 0.10 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ for canine cardiac and rabbit skeletal muscle microsomes, respectively. Each point represents the mean ± SEM of three experiments with different microsome preparations.

activity was reduced by 16.7 ± 1.6% (0.822 ± 0.024 and 0.683 ± 0.012 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ without and with 10 μmol/L H-89, respectively). In addition, H-89 clearly increased the K_m for Ca, from 0.52 ± 0.01 to 0.94 ± 0.03 μmol/L ($P = 0.003$, df = 4), which indicates that the K_m value for the high-affinity (activation) Ca^{2+} sites was nearly doubled. Simultaneously, the Hill coefficient was slightly decreased, with values of 1.91 ± 0.04 and 1.66 ± 0.06 ($P = 0.002$, df = 4) in the absence and the presence of H-89, respectively.

Figure 4B shows the effect of 10 μmol/L H-89 on the Ca^{2+}-dependency of fast skeletal muscle SR ATPase. In this case also, a typical bell-shaped curve is observed with a K_m for Ca of *ca.* 0.39 ± 0.01 μmol/L in the absence of H-89. This higher affinity for Ca^{2+} of the skeletal muscle relative to the cardiac SR Ca^{2+} pump can be explained by the absence of PLB in that tissue [3]. In skeletal muscle SR vesicles, maximum ATPase activity was decreased by 23.0 ± 0.6% by H-89 (7.01 ± 0.10 and 5.40 ± 0.11 μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$ without and with 10 μmol/L H-89, respectively). Maximum ATPase activity occurred at *ca.* 3.7 μmol/L $[Ca^{2+}]_{free}$ compared with 2.2 μmol/L $[Ca^{2+}]_{free}$ in the presence of the solvent alone. As previously observed in the cardiac preparation, the major effect of H-89 was an approximate doubling of the K_m for Ca, which reached 0.79 ± 0.02 μmol/L ($P = 0.001$, df = 4) in the presence of 10 μmol/L H-89. However, in this case, the increase in the apparent K_m value for the high-affinity Ca^{2+} sites was not associated with any clear effect on the Hill coefficient (1.77 ± 0.02 and 1.74 ± 0.04 without and with 10 μmol/L H-89, respectively).

These results demonstrate that in both cardiac and skeletal muscle SR vesicles, H-89 affected the Ca^{2+}-dependency of SR ATPase predominantly by a decrease in the apparent affinity for Ca^{2+}.

Effect of H-89 on ATPase Activity of Cardiac SR in the Presence of Monoclonal Antibodies Raised against Phospholamban

The possible involvement of PLB in the effect of H-89 on cardiac SR Ca^{2+}-ATPase was investigated further by comparing the effect of H-89 in the presence and the absence of a monoclonal antibody raised against PLB (mAbA1) [27]. In the presence of this neutralising antibody, the Ca^{2+}-ATPase activity of cardiac microsomes was increased by 29% (Table 1). This resulted from the removal of the inhibitory effect of PLB on cardiac SR Ca^{2+}-ATPase.

TABLE 1. Effect of phospholamban antibodies on the inhibition of cardiac SR Ca^{2+}-ATPase by H-89

Condition	Ca^{2+}-ATPase activity (μmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$)	Inhibition by H-89 (%)
DMSO	0.710 ± 0.036	
H-89	0.158 ± 0.028	77.9 ± 2.8
mAb A1 + DMSO	0.918 ± 0.043	
mAb A1 + H-89	0.228 ± 0.014	75.0 ± 2.6

Cardiac SR microsomes (20 μg/mL) were preincubated with 2.0 mg mAb A1 per mg microsomal protein or with the corresponding solvent. Ca^{2+}-ATPase activity of the microsomes was determined after incubation at pCa 6.20 in the presence of H-89 (30 μmol/L) or of DMSO (1%). Values are mean ± SEM of two different experimentations.

However, the effect of H-89 on cardiac SR Ca^{2+}-ATPase was the same in the presence or absence of mAbA1 (77.9 ± 2.8 and 75.0 ± 2.6% inhibition without and with mAbA1, respectively. This result suggests that the inhibition of cardiac SR Ca^{2+}-ATPase by H-89 does not involve PLB.

DISCUSSION

H-89 was found to produce a clear inhibition of both cardiac and skeletal muscle cardiac SR Ca^{2+}-ATPase activities (Fig. 1). It has previously been reported [10, 28] that H-89 reduces SR Ca^{2+} transport in cardiac myocytes by decreasing cAPrK-dependent PLB phosphorylation. The same action could explain the effect of H-89 that we observed on cardiac SR vesicles. However, this action cannot account for the inhibitory effect produced by H-89 in skeletal muscle SR microsomes. Indeed, PLB is not expressed in fast skeletal muscle SR [3, 29], which was confirmed in our study, using a rabbit preparation, by electrophoresis followed by immunodetection (data not shown).

To determine whether the inhibition of cardiac SR Ca^{2+}-ATPase produced by H-89 may be related to inhibition of cAPrK, the effect of two cAPrK inhibitors was investigated. Rp-cAMP[S], which selectively inhibits cAPrK by competing with cAMP on its regulatory subunit [30], was without any effect on cardiac SR Ca^{2+}-ATPase activity. This can be explained by the absence of cAMP in the assay used to assess Ca^{2+}-ATPase activity. In contrast, PKI, which inhibits cAPrK by binding to its catalytic subunit [31], significantly decreased cardiac SR Ca^{2+}-ATPase activity at pCa 6.20, though it was inactive at pCa 5.66. Such an effect is in agreement with a decrease in PLB phosphorylation which predominantly affects the Ca^{2+} affinity of the SR Ca^{2+} pump, leading to a more pronounced inhibition at lower $[Ca^{2+}]_{free}$ [5, 6]. However, neither at high nor low $[Ca^{2+}]_{free}$ did the two cAPrK inhibitors used alter the effect of H-89 on cardiac SR Ca^{2+}-ATPase (Fig. 2). Therefore, it appears unlikely that the inhibition of SR Ca^{2+}-ATPase activity produced by H-89 on cardiac microsomes could result from a reduced phosphorylation of PLB as a consequence of cAPrK inhibition. The lower potency of H-89 at pCa 5.66 than at pCa 6.20 (Fig. 2) can be related to the pattern of inhibition shown by this compound, which indicates a predominant decrease in Ca^{2+} affinity of the SR Ca^{2+} pump (Fig. 4).

Since H-89 and related isoquinolinesulfonamides inhibit cAPrK by competing with ATP on the catalytic subunit of the enzyme [11], a possible explanation for the inhibitory effect of H-89 on the SR Ca^{2+} pump could be a competition with ATP. Although the pattern of inhibition by H-89 on the cardiac and skeletal enzymes was similar (Fig. 4), at ATP concentrations above 1 mmol/L the effect of the compound on the K_m for ATP was clearly more pronounced on skeletal than on cardiac muscle SR ATPase. This different behaviour could result from the presence of PLB in the cardiac SR. Indeed, it has been proposed that in the cardiac SR, PLB interferes with the acceleratory effect of ATP at concentrations above those necessary for binding to the high-affinity catalytic site [32]. However, the pattern of inhibition of Ca^{2+}-ATPase by H-89 in both cardiac and skeletal muscle SR is clearly different from that observed for the inhibition of the catalytic subunit of cAPrK, suggesting different mechanisms for the two inhibition processes.

The major effect of H-89 on the Ca^{2+}-dependent activity of the cardiac SR was an apparent decrease in the affinity of the pump for Ca^{2+}, associated with a small decrease in cooperativity between the two Ca^{2+} binding sites of the enzyme (Fig. 4A). There was only a moderate alteration of the maximum ATPase activity. Such an effect of H-89 on the Ca^{2+}-dependency of cardiac SR ATPase can be explained by a decrease in cAPrK-dependent PLB phosphorylation as a result of the inhibition of SR-bound cAPrK by H-89, as previously suggested [10, 28]. Indeed, a type II cAPrK has been identified in canine cardiac SR [33], and PLB is a substrate for this kinase [34]. Furthermore, several studies have shown that the regulation of the SR Ca^{2+} pump by PLB involves an inhibition of pump activity when PLB is in the unphosphorylated state, with a relief of this inhibition upon phosphorylation. The inhibition is associated with a decrease in the apparent K_m of the pump for Ca^{2+} [4, 5, 6]. A few studies have also reported increases in the V_{max} when PLB is phosphorylated by cAPrK [4, 6], although this effect is still a matter of debate [8, 9]. Thus, the effects we have observed with H-89 on Ca^{2+}-ATPase activity in cardiac SR microsomes are consistent with a possible decrease in the level of PLB phosphorylation. However, the inability of cAPrK inhibition to alter the effect of H-89 on SR Ca^{2+}-ATPase suggests a direct effect of H-89 on the SR-Ca^{2+} pump which would not involve PLB.

In an attempt to definitively exclude a lower PLB phosphorylation resulting from cAPrK inhibition as the mechanism by which H-89 inhibits cardiac SR Ca^{2+}-ATPase, the effect of a monoclonal antibody raised against PLB [27] was investigated. As expected, the neutralising antibody increased SR Ca^{2+}-ATPase activity, by relieving the inhibitory effect of PLB. The antibody did not affect the inhibition of cardiac SR Ca^{2+}-ATPase by H-89 (Table 1), providing clear evidence for the action of H-89 being independent of PLB. Even more evidence for this conclusion is provided by the inhibition by H-89 of the SR Ca^{2+}-ATPase in fast skeletal muscle which lacks PLB [3, 29]. Thus, irrespective of the presence or absence of PLB and the SR Ca^{2+}-ATPase isoform (SERCA1 or SERCA2a), H-89 clearly decreases the apparent Ca^{2+} affinity of the SR Ca^{2+} pump and, though to a lower extent, decreases maximum enzyme activity. It can be concluded that this is the result of a direct interaction of H-89 with the Ca^{2+}-ATPase in both cardiac and fast skeletal muscles.

One may wonder about the relevance of the direct inhibition of SR Ca^{2+}-ATPase by much higher concentrations of H-89 (K_i of 3 μmol/L) than those required to

inhibit isolated cAPrK (K_i of 0.048 μmol/L [12]). In some studies, concentrations of H-89 up to 10–30 μmol/L were used to inhibit cAPrK-dependent processes in isolated cells [12]. The discrepancy regarding the effective dose of H-89 between *in vitro* and *in vivo* systems was proposed to result from the poor permeability of H-89 in cell membranes and from the high concentration of ATP in these experiments [12]. Likely for similar reasons, concentrations of H-89 as high as 40–65 μmol/L were used to determine the effect of cAPrK on SR Ca^{2+} uptake in permeabilized myocytes [10]. However, it cannot be excluded that the lower Ca affinity of the SR Ca^{2+} uptake process produced by H-89 in this study results partly at least from a direct inhibition of SR Ca^{2+}-ATPase. In a recent study [35], 0.1 μmol/L H-89 maximally inhibited cAPrK-mediated effects in artery rings. Therefore, the need to use high concentrations of H-89 to pharmacologically manipulate cellular cAPrK-mediated processes should be examined cautiously. Otherwise, it would be difficult to discriminate between the cellular effects resulting form cAPrK inhibition and those resulting from nonspecific effects such as Ca^{2+}-ATPase inhibition.

It is noteworthy that the inhibitory profile displayed by H-89 on the SR Ca^{2+} pumps is different from that reported for the most commonly studied inhibitors, namely, thapsigargin and cyclopiazonic acid. In contrast to H-89, these two compounds are reported to exhibit little or even no effect on the K_m for Ca, whereas they significantly reduce the V_{max} of the enzyme [36, 37, 38]. Moreover, the inhibition by H-89 differs from that of thapsigargin and cyclopiazonic acid with respect to the ATP concentrations. Cyclopiazonic acid has been shown to inhibit the ATPase in a competitive manner [39, 40, 37], while thapsigargin did not significantly affect the values of K_m for ATP (for both the catalytic and regulatory sites), but strongly decreased the V_{max} of the enzyme [36, 40, 38].

Concerning other compounds, H-89, 3′,3″,5′,5″-tetraiodophenolsulfonephtalein, like H-89, showed a noncompetitive inhibition pattern with respect to ATP at the concentration range of 0.25–1 mmol/L, but differed in showing an uncompetitive inhibition pattern with respect to $[Ca^{2+}]_{free}$ [23]. Bis-phenol and nonylphenol, which potently inhibit the SR Ca^{2+} pump, also show a different pattern of inhibition from H-89 in that neither of these compounds affects the K_m for Ca of the enzyme [41, 42, 43].

Thus, it can be considered that H-89 belongs to a novel class of inhibitors of the SR Ca^{2+} pumps (SERCA1 and SERCA2) characterised by an ability to decrease the apparent affinity for Ca^{2+} of the catalytic site. Such an action suggests that H-89 may interact with the hinge region of the ATPase, which is essential for determining Ca^{2+} affinity [44]. However, further studies are required to define the molecular mechanism of action of H-89 more precisely.

In conclusion, our results clearly demonstrate that H-89 is able to directly inhibit the Ca^{2+}-ATPase activity of the SERCA1 and SERCA2a isoforms of the SR Ca^{2+} pumps. Although the potency of H-89 is lower than that of the well-known SR Ca^{2+}-ATPase inhibitors cyclopiazonic acid or thapsigargin, the inhibition of the Ca^{2+}-ATPase by H-89 occurs at concentrations in the same range as those usually used to inhibit cAPrK activity in several isolated cell systems [12, 10]. Thus, in cell systems needing high concentrations of H-89 to pharmacologically modulate cAPrK-mediated effects, the possible involvement of SR-Ca^{2+}-ATPase inhibition in cellular effects should be considered. Finally, owing to the novel profile of inhibition displayed by H-89, this compound constitutes an interesting tool to investigate the catalytic and transport mechanisms of Ca^{2+} by the purified SERCA pumps.

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