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Caffeine Indirectly Activates Ca^{2+}-ATPases in the Vesicles of Cardiac Junctional Sarcoplasmic Reticulum

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Abstract: Agents that activate or inhibit the Ca^{2+} release channel in cardiac sarcoplasmic reticulum (SR) were tested for their abilities to affect the activity of the SR Ca^{2+}-ATPase. Vesicles of junctional SR (heavy SR, HSR) from terminal cisternae were prepared from porcine cardiac muscle by density gradient centrifugation. The steady-state activity of Ca^{2+}-ATPases in intact HSR vesicles was 347± 5 nmol/min·mg protein (± SD). When the HSR vesicles were made leaky, the activity was increased to 415± 5 nmol/min·mg protein. This increase is probably due to the uncoupling of HSR vesicles. Caffeine (10 mM), an agonist of the SR Ca^{2+} release channel, increased Ca^{2+}-ATPase activity in the intact HSR vesicle preparation to 394± 30 nmol/min·mg protein. However, caffeine had no significant effect in the leaky vesicle preparation and in the purified Ca^{2+}-ATPase preparation. The effect of caffeine on SR Ca^{2+}-ATPase was investigated at various concentrations of Ca^{2+}. Caffeine increased the pump activity over the whole range of Ca^{2+} concentrations, from 1 μM to 250 μM, in the intact HSR vesicles. When the SR Ca^{2+}-ATPase was inhibited by thapsigargin, no caffeine effect was observed. These results imply that the caffeine effect requires the intact vesicles and that the increase in Ca^{2+}-ATPase activity is not due to a direct interaction of caffeine with the enzyme. We propose that the activity of SR Ca^{2+}-ATPase is linked indirectly to the activity of the Ca^{2+} release channel (ryanodine receptor) and may depend upon the amount of Ca^{2+} released by the channels.
Key words: Ca^{2+}-ATPase, Ca^{2+} release channel, caffeine, sarcoplasmic reticulum, uncoupling.

Sarcoplasmic reticulum (SR) plays a major role in controlling cytoplasmic Ca^{2+} concentration in cardiac myocytes. Cytoplasmic Ca^{2+} is translocated to the lumen of the SR by SR Ca^{2+}-ATPase and stored Ca^{2+} is released, during the myocardial contraction, through the activation of the SR Ca^{2+} release channel (ryanodine receptor). When the release channels are activated, cytoplasmic Ca^{2+} concentration can increase from ~100 nM to ~1 μM (Hasselbach and Oetliker, 1983; Bassani and Bers, 1995). The characteristics of the Ca^{2+}-ATPase and the Ca^{2+} release channel of the SR membrane have been intensively studied. Factors that modulate the activity of Ca^{2+}-ATPase have been identified. For instance, changes in pH and Mg^{2+} concentration modify the apparent affinity and cooperativity of Ca^{2+} binding sites on the enzyme (Forge *et al.*, 1993). Maximal activity of SR Ca^{2+}-ATPase has been obtained at 1~2 mM Mg^{2+} (Hughes *et al.*, 1994) and inhibition of the enzyme has occurred at high concentrations of Mg^{2+}, probably binding to the Ca^{2+} binding sites of the enzyme (Bishop and Al-Shawi, 1988).

The activity of the SR Ca^{2+} release channel has also been investigated using modulators. Caffeine and ATP increase the probability of the channel being open and a high concentration of Mg^{2+} decreases the channel activity (Meissner and Henderson, 1987). Ca^{2+} on the cytoplasmic side of the protein has a biphasic modulatory effect on the channel. Ca^{2+} in the micromolar range activates the channel, mediating Ca^{2+}-induced Ca^{2+} release (CICR), and inhibits the channel activity at higher concentrations, mediating a negative feedback mechanism (Imagawa *et al.*, 1987).

In this study we have used caffeine to investigate a possible functional relation between the SR Ca^{2+} release channel and the SR Ca^{2+}-ATPase using the vesicles of junctional SR (HSR). The activity of SR Ca^{2+}-ATPase was monitored under three different physical arrangements, intact HSR vesicles, leaky HSR vesicles, and purified enzyme, in order to define the effects of caffeine on the Ca^{2+}-ATPase activity. We found a correlation of the activity of Ca^{2+}-ATPase to the activity

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of the Ca^{2+} release channel, which demonstrates the existence of coupling between Ca^{2+} uptake and Ca^{2+} release in the HSR vesicles.

Materials and Methods

Materials

Sucrose was purchased from Fluka BioChemical Co. (Buchs, Switzerland). Triton X-100 and $MgCl_2$ were supplied by Wako Pure Chemical LTD. (Osaka, Japan). $^{45}CaCl$ was from DuPont-NEN Research Products (Boston, USA). All other chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, USA).

Preparation of cardiac HSR vesicles

HSR vesicles were prepared from the ventricular muscle of pig heart as described for rabbit muscle (Valdivia *et al.*, 1992). Briefly, pig heart (300 g) was cut into small pieces and homogenized. HSR vesicles were precipitated by centrifugation at 10,000×*g*. The pellet was resuspended and incubated in a high-K buffer containing 0.6 M KCl, 5 mM Tris-Mes, pH 6.8. The suspension was centrifuged for 60 min in a type TFT70.38 rotor (Kontorn Instruments, Milan, Italy) at 27,000 rpm. The pellet containing vesicles was resuspended and subjected to a discontinuous sucrose gradient centrifugation in a Kontron TST28.38 rotor for 5 h at 25,000 rpm. Microsomal fractions were recovered from the interfaces of 10, 20, 30, 35, 40% of sucrose layers and HSR vesicles were obtained from the interface between 30% and 35% sucrose layers. Each fraction was centrifuged and resuspended in a solution containing 0.3 M sucrose, 0.1 M KCl, 5 mM Na-Pipes, pH 6.8, frozen in liquid nitrogen, and stored at −80℃ before use. Leaky HSR vesicles were prepared by the addition of 0.001% Triton X-100. The leakage of leaky HSR vesicles was measured by $^{45}Ca^{2+}$ release. The concentration of protein was determined by the Lowry method (Lowry *et al.*, 1951).

Purification of SR Ca^{2+}-ATPase

Ca^{2+}-ATPases from cardiac SR were purified from porcine cardiac muscle by the method of Nakamura *et al.* (1983) as described recently (Kim *et al.*, 1990). Briefly, the SR was solubilized with deoxycholate (0.5 mg/mg·protein) and was centrifuged at 180,000×*g* for 30 min. The supernatant was diluted 8-fold with a solution containing 0.1 M KCl, 10% glycerol, 20 mM Tris-HCl, pH 8.0, and recentrifuged at 156,000×*g* for 40 min. The pellet was suspended in 200 ml of 0.1 M KCl, 0.3 M sucrose, 10 mM histidine/HCl buffer (pH 7.0) and recentrifuged at 70,000×*g* for 25 min. The pellet was washed twice with the sucrose solution and resuspended in the same solution.

Ca^{2+}-ATPase activity

The activity of Ca^{2+}-ATPases of the HSR vesicles or of the purified preparation was measured by the method of Niggli *et al.* (1979). Briefly, the activity was monitored in a physiological solution (control) containing 120 mM KCl, 30 mM Hepes, pH 7.4, 0.5 mM $MgCl_2$, 0.5 mM Na-ATP, 0.4 mM NADH, 50 μM $CaCl_2$, 2 mM phosphoenolpyruvate, 1 IU/ml of pyruvate kinase, and 1 IU/ml of lactate dehydrogenase. The concentration of free Ca^{2+} in the solution was adjusted by using Ca-EGTA buffers calculated by a computer program (Fabiato *et al.*, 1988). The formation of ADP by the activity of Ca^{2+}-ATPase is quantitatively coupled to the oxidation of NADH in the reaction solution resulting in an absorbance decrease at 340 nm. Decrease in absorbance was continuously monitored and the activity was calculated from the slope of the decrease in absorbance.

Results

The ATPase activities of the microsomal fractions from a discontinuous sucrose gradient are shown in Fig. 1. The activity of ATPase was determined by the time-dependent decrease in absorbance at 340 nm representing steady-state activity. The average activity of each fraction was taken from 3 to 5 experiments. Higher activities were obtained from the fraction F-3 and the fraction F-4 and were 347± 5 nmol/min·mg protein and 395± 52 nmol/min·mg protein, respectively, approximately twice as high as the activities of the lighter microsomal fractions. The activities of fractions F-1 and F-2 were 201± 28 nmol/min·mg protein and 126 ± 13 nmol/min·mg protein, respectively.

In order to investigate the functional relations between the SR Ca^{2+}-ATPase and the Ca^{2+} release channel, we used the fraction F-3, which contains mostly HSR vesicles derived from the terminal cisternae of SR. Activities of non-SR membrane ATPases have been minimized because HSR vesicles were separated from the sarcolemma, which may have sarcolemmal Ca^{2+}-ATPases, and the transverse tubules (T-tubule), which may have Na^+, K^+-ATPases, during vesicle preparation (see Methods). Sarcolemma and T-tubule vesicles were dissociated from HSR vesicles by salt treatment and then separated from the HSR vesicles by density gradient centrifugation. Fraction F-4 was not used in this study because it may still contain vesicles originated from T-tubules, which are undissociated from HSR vesicles by salt treatment. ATPases of contaminated mitochondria were inhibited by cyanide treatment during

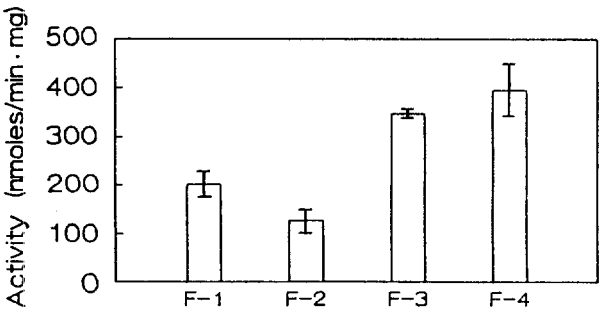

Fig. 1. ATPase activities of porcine cardiac microsomes. Fractions, F-1 to F-4 were obtained from the interfaces of a discontinuous sucrose gradient (see Methods) and numbered from the top interface of the gradient. Samples of 60~160 μg protein were used in each experiment. Activity of each fraction was calculated from the slope of absorbance decrease. Values are means± SD (n=3~5).

the analysis.

The effects of caffeine, an agonist of the SR Ca^{2+} release channel, and high concentration of Mg^{2+}, an antagonist of the SR Ca^{2+} release channel, on the SR Ca^{2+}-ATPase activity are shown in Fig. 2. Caffeine (10 mM) increased the activity approximately 14%. The effects of caffeine appeared slowly, but after about three minutes the ATPase activity was significantly increased (Fig. 2A). The average activity for the control, as mentioned above, was 347± 5 nmol/min·mg protein. With 10 mM caffeine this increased to 394± 30 nmol/min·mg protein (Fig. 2B). Low concentrations of Mg^{2+}, 1~2 mM, were required for SR Ca^{2+}-ATPase activity, but a high concentration (20 mM) was inhibitory as shown in this figure. At 20 mM Mg^{2+} the average activity of the enzyme was reduced to 272± 5 nmol/min·mg protein.

The effects of caffeine and Mg^{2+} were further analyzed in the leaky vesicle preparation. When the HSR vesicles were made leaky by treating them with Triton X-100 (0.001%), the average activity increased to 415 ± 5 nmol/min·mg protein, ~20% increase (Fig. 2B, Con). This was probably due to the uncoupling of HSR vesicles, since we had observed the formation of a $^{45}Ca^{2+}$ gradient and the release of stored $^{45}Ca^{2+}$ by Triton X-100 treatment (unpublished observations). In order to test this idea, the activity of the SR Ca^{2+} release channel was inhibited by 20 mM Mg^{2+} (Anderson *et al.*, 1989) and Triton X-100 was simultaneously treated. The uncoupling effect was still observed in the solution containing 20 mM Mg^{2+}, although Mg^{2+} partially inhibited the SR Ca^{2+}-ATPase as shown by Hughes *et al.* (1994). The enzyme activity in the leaky vesicle preparation was increased to 298± 3 nmol/min·mg protein a ~10% increase, compared to 272± 5 nmol/min·mg protein, the activity of the intact vesicle preparation (Fig. 2B, Mg, n=10). However, 10 mM caffeine did not

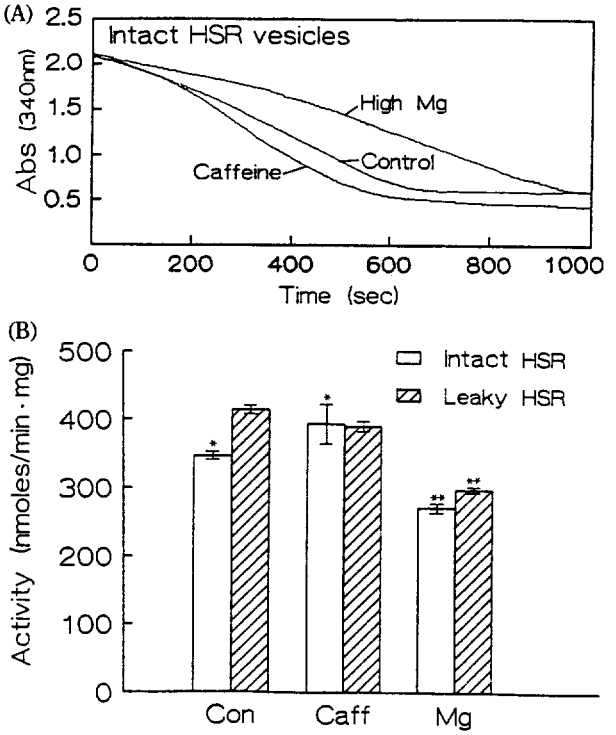

Fig. 2. Effects of modulators on the activity of SR Ca^{2+}-ATPases. (A) Continuous scanning of absorbance at 340 nm. Each experiment was done with 110 μg/ml protein. The effects of 10 mM caffeine (Caff) and 20 mM Mg^{2+} (High Mg or Mg) are shown in the intact HSR vesicle preparation. (B) The effects of modulators on the SR Ca^{2+}-ATPases in above conditions. Effects were measured in the intact HSR vesicles (blank) and in the leaky vesicles (slashed). Leaky vesicles were prepared by the addition of Triton X-100 (0.001%). Values are means± SD (n=3~10). *p<0.001, **p<0.002 by Anova test.

Table 1. Activities of SR Ca^{2+}-ATPases in various conditions

	Control	Thapsigargin[a]	Thapsigargin +Caffeine[b]
Intact HSR vesicles	409± 10[c]	258± 9	227± 8
Leaky HSR vesicles	511± 15	255± 10	229± 5
Purified Ca^{2+}-ATPase	857± 20	0	0

[a]Thapsigargin, 10 μM.
[b]Caffeine, 10 mM.
[c]Unit of activity: nmol/min·mg protein.
Values are means± SD (n=3~5).

increase the enzyme activity in the leaky vesicle preparation (Fig. 2B, Caff, n=7). The caffeine-induced increase in SR Ca^{2+}-ATPase activity requires intact vesicles and this may be due to the caffeine-induced activation of the Ca^{2+} release channels in intact HSR vesicles.

To make sure that the effect of caffeine is on the SR Ca^{2+}-ATPase in the HSR preparation, we inhibited the SR Ca^{2+}-ATPases by thapsigargin, a specific antagonist, and measured the effect of caffeine. In both intact and leaky vesicle preparations thapsigargin (10

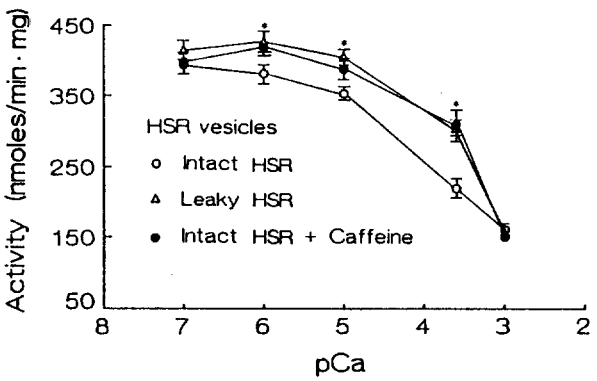

Fig. 3. Effect of caffeine on SR Ca^{2+}-ATPase at various concentrations of Ca^{2+}. The Ca^{2+}-dependence of ATPase activity was measured in the intact HSR vesicles (open circle) and in the leaky vesicles (triangle). Effect of 10 mM caffeine in the intact vesicles is shown (filled circle). Values are means± SD (n=3~5). The enzyme activities obtained in the concentrations of free Ca^{2+} between 1 μM and 250 μM were analyzed by Anova test (*). The p-values were less than 0.002 between the activities of intact HSR vesicles and leaky HSR vesicles or between the activities of intact HSR vesicles and caffeine-treated HSR vesicles.

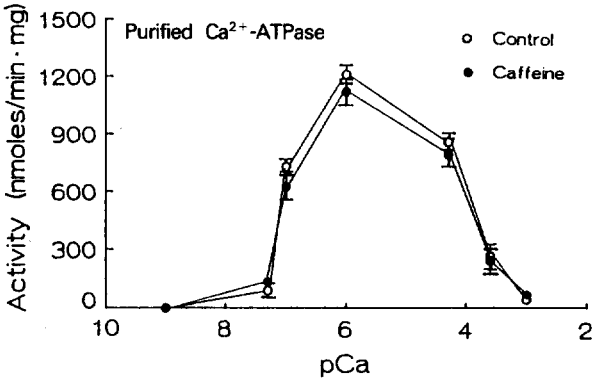

Fig. 4. Effect of caffeine on the purified SR Ca^{2+}-ATPase. The activity of the purified SR Ca^{2+}-ATPase was measured at various concentrations of free Ca^{2+} (open circle). Caffeine (10 mM) was added in each condition (filled circle). Values are means± SD (n=3~7).

μM) decreased the activity 40~50% (Table 1). Further addition of caffeine did not increase the enzyme activity and it even further decreased the activity through an unknown mechanism. These findings indicate that the caffeine-sensitive ATPase in the intact vesicles is inhibited by thapsigargin and suggest that the caffeine-induced increase in the ATPase activity is mediated by the SR Ca^{2+}-ATPase. Thapsigargin completely inhibited the purified SR Ca^{2+}-ATPase in low concentration, ~100 nM, and additional caffeine effect was not measurable (Table 1).

The effect of caffeine on the SR Ca^{2+}-ATPase was investigated at various concentrations of Ca^{2+} in the HSR vesicle preparation. The Ca^{2+}-dependent profiles of the SR Ca^{2+}-ATPase activity are shown in Fig. 3. As reported previously (Sasaki *et al.*, 1992; Hughes *et al.*, 1994), maximal activities were observed at micromolar Ca^{2+} (~pCa=6). At higher concentrations of Ca^{2+} the enzyme activity gradually decreased. The uncoupling effect was observed over the whole range of Ca^{2+} from pCa=7 to pCa=3 since the activities in the leaky vesicle preparation were higher than those in the intact vesicle preparation. The increase in enzyme activity with a range of 5~30% was dependent on the concentration of Ca^{2+}. When 10 mM caffeine was added to the intact HSR vesicle preparation, the enzyme activity increased up to the level of activity of the leaky vesicle preparation. The activities were analyzed by an Anova test and no significant differences were obtained in the enzyme activities between caffeine-treated vesicles and leaky vesicles. However, these activities were significantly higher than those of the intact HSR vesicles ($p<0.002$ by Anova test). The effect of caffeine on the purified SR Ca^{2+}-ATPase was also investigated at various concentrations of Ca^{2+}. As shown in Fig. 4, maximal activity was observed at 1 μM free Ca^{2+} and no activities were observed at the concentrations of free Ca^{2+} below 10 nM or above 1 mM. Caffeine had no significant effect on the purified enzyme. In the near-physiological range of Ca^{2+}, 100 nM~50 μM, caffeine rather slightly decreased the enzyme activity.

Discussion

This study demonstrates that the efflux of luminal Ca^{2+} from the SR activates the SR Ca^{2+}-ATPases probably by dissipating the Ca^{2+} gradient across the SR membrane. Since the SR contains Ca^{2+}-sequestering proteins which are known as calsequestrin (Slupsky *et al.*, 1987), these proteins may influence the activity of SR Ca^{2+}-ATPases. However, the buffering effect of calsequestrin in our results may not be significant, because we are actually measuring the steady-state activity of SR Ca^{2+}-ATPases in the intact or leaky HSR vesicles. We are currently investigating the Ca^{2+}-buffering capacity of HSR vesicles and evaluating the contribution of luminal calsequestrin to the kinetics of Ca^{2+} release and uptake using a radioactive isotope, $^{45}Ca^{2+}$.

Caffeine (10 mM) increased the SR Ca^{2+}-ATPase activity in the intact HSR vesicles. Since there is no evidence that caffeine binds to the SR Ca^{2+}-ATPase, it may have increased Ca^{2+}-ATPase activity by activating the Ca^{2+} release channel (ryanodine receptor), a well established effect. Activation of the release channels would increase the Ca^{2+} efflux through the channels and could help the dissipation of the Ca^{2+} gradient established by the Ca^{2+} pumping activity of the SR

Ca^{2+}-ATPase. Gradient dissipation (uncoupling), if it occurs, would increase the pump activity and this is the simplest model. The caffeine-induced Ca^{2+} gradient dissipation model is consistent with the results obtained with leaky vesicles and purified Ca^{2+}-ATPase. In the leaky vesicles a Ca^{2+} gradient would be difficult to maintain and in the purified SR Ca^{2+}-ATPase no gradient could exist. In both leaky vesicles and purified enzyme preparations caffeine had no effect on the enzyme activity (Fig. 2~4). The absence of the caffeine effects in these experiments suggests that caffeine does not influence SR Ca^{2+}-ATPase activity directly.

It has been reported that cardiac myocytes contain $InsP_3$-sensitive Ca^{2+} pools (Gorza *et al.*, 1993; Kijima *et al.*, 1993). Our cardiac HSR vesicle preparation may contain the vesicles originated from the $InsP_3$-sensitive Ca^{2+} stores, although the major part of these vesicles were removed by densitiy gradient centrifugation. Thapsigargin can also block Ca^{2+} influx into $InsP_3$-sensitive Ca^{2+} stores but caffeine can only dissipate the luminal Ca^{2+} of the caffeine-sensitive Ca^{2+} pools. Therefore, the effect of caffeine is limited to the HSR vesicles containing the caffeine-sensitive Ca^{2+} release channels. The activity of Ca^{2+}-ATPases in the leaky preparation may represent the total enzyme activity of these two types of vesicles. However, the contribution of $InsP_3$-sensitive pools may not be significant in our preparation because the increased enzyme activity in the caffeine-treated intact HSR vesicles was close to the enzyme activity measured in the leaky vesicles (Fig. 4). These results indicate that 10 mM caffeine is enough to empty the caffeine-sensitive stores, as reported by Bassani and Bers (1995).

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