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CAFFEINE INTERACTION WITH THE Ca-RELEASE CHANNELS OF HEAVY SARCOPLASMIC RETICULUM. EVIDENCE THAT 170 kD Ca-BINDING PROTEIN IS A CAFFEINE RECEPTOR OF THE Ca-CHANNELS

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SUMMARY: The study of Ca^{2+}- and caffeine-induced Ca^{2+} release from heavy sarcoplasmic reticulum vesicles under the different conditions suggests that Ca^{2+} and caffeine can interact with the common receptor of the Ca-release channels. The reticulum membranes were solubilized using nonionic detergent polyoxyethylene 9-lauryl ether, and affinity chromatography on reactive red 120-agarose was carried out. The 170 kD Ca-binding protein which is eluted by caffeine is the most probable candidate for the caffeine receptor of the Ca-channels.

It is well-known that the SR plays a key role in excitation-contraction coupling in skeletal muscle (1,2). The Ca-ATPase pump protein which provides accumulation of calcium into the SR lumen is well-characterized (3,4), whereas the molecular organization and properties of the Ca-channels providing fast Ca^{2+} release from SR are considerably less understood (5,6). According to current concepts, Ca-channels of HSR consist of at least two different components, namely the Ca-channel itself which forms the Ca-conducting pore and the receptors which provide regulation of these channels under the influence of different triggering signals (7). Recent results show that the ryanodine-binding protein with a molecular weight of 450 kD can form a Ca-conducting channel in artificial lipid membranes (8). There are also many indications that the operation and/or regulation of Ca-channels involve some of the other HSR proteins, such as the

ABBREVIATIONS: SR, sarcoplasmic reticulum; HSR, heavy SR; $C_{12}E_9$, polyoxyethylene 9-lauryl ether; RR, ruthenium red; MOPS, 3-(N-morpholino) propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; m.w., molecular weight; $K_{0.5}$, concentration producing 50% effect.

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Ca-ATPase (9,10), calsequestrin (11) and a few other proteins with molecular weights from 30 to 350 kD (12-14).

In the present work we show that Ca^{2+} and caffeine seem to interact with the same Ca-channel receptor, and that the 170 kD Ca-binding protein is the most likely candidate for this receptor.

MATERIALS AND METHODS: **HSR preparation** - A heavy SR fraction from rabbit skeletal muscles was prepared by differential centrifugation (15).

Ca^{2+} accumulation and release - The study of Ca^{2+} uptake and release was carried out in a medium containing 100 mM KCl, 0.5 mM $MgCl_2$, 50 mM PIPES-Tris, pH 7.0, 50 uM antipyrylazo III, 60 uM free Ca^{2+}, 5 mM creatine phosphate, 10 units/ml creatine kinase and 1 mg/ml HSR vesicles. The reaction was started by the addition of 0.5 mM ATP. Extravesicular Ca^{2+} concentration was monitored by the difference in absorbance of antipyrylazo III at 720 and 790 nm (15) with the use of a Hitachi 557 dual wavelength spectrophotometer. Calibration was carried out under the same conditions before addition and after depletion of ATP.

HSR solubilization and reconstitution - HSR vesicles were solubilized at a final concentration of 2 mg/ml in a solution containing final concentrations of 20 mg/ml $C_{12}E_9$, 20% sucrose (w/v), 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM MOPS, pH 7.0 (For details of the original method, see Ref. 16). The solubilized vesicles were then centrifuged at 27,000 x g for 30 min at 4°C. For reconstitution, decanted supernatant was sonicated for 10 min in a bath-type sonicator and then stirred with Bio Beads SM-2 (25 mg of dry resin/mg of protein) for 2 hours at room temperature to remove the detergent (13). The same treatment was repeated two more times. After removal of Bio Beads SM-2 by filtration, the liposome suspension was sonicated for 2 min in a bath-type sonicator and centrifuged at 10,000 x g for 15 min to remove large size particles. The supernatant was dialyzed overnight at 4°C against 500 volumes of buffer (100 mM KCl and 20 mM MOPS, pH 7.0) and was concentrated using Centricon 10 microconcentrators (Amicon).

Isotope flux measurement - In order to measure $^{45}Ca^{2+}$ release, 20 ul of the passively loaded vesicles incubated overnight in a solution containing 100 mM KCl, 20 mM MOPS, pH 7.0 and 5 mM $CaCl_2$ containing $^{45}CaCl_2$ (~10,000 cpm/nmol) at 0°C (7) were diluted 50-fold into a solution containing 100 mM KCl, 20 mM MOPS, pH 7.0, 1 mM EGTA and, where indicated, 5 mM caffeine or 10 uM RR. At various times after dilution, the reaction was quenched by the addition of an equal volume of a solution containing 20 mM $LaCl_3$ and 40 mM $MgCl_2$ (7). The quenched solution was immediately filtered through a 0.45 um HA Millipore filter which was washed twice with 1 ml of solution containing 10 mM $LaCl_3$ and 20 mM $MgCl_2$, and its radioactivity was measured by liquid scintillation counting.

Chromatography - After solubilization of HSR vesicles with $C_{12}E_9$ (see above), the decanted supernatant was applied to a reactive red 120-agarose (Sigma, type 3000CL) column (10 mg of protein/ml of support) which had been prewashed with several column volumes of the solubilization buffer. Elution was done with the same buffer containing 5-20 mM of caffeine.

Miscellaneous - Protein concentration was measured according to Lowry et al. (17) except in the presence of $C_{12}E_9$, where the biuret method (18) with the modifications described elsewhere (16) was used. Lipid concentration was determined by phospholipid phosphorous according to Bartlett (19). SDS-slab gel electrohoresis was performed according to the procedure of Laemmli (20). Gels were stained with Coomassie Blue R-250, with Stains-all (21) or with Bio-Rad Silver Stain (22) and scanned using Helena Laboratories Quick Scan R & D densitometer. All chemicals used were reagent grade or better.

RESULTS AND DISCUSSION: As shown in Fig. 1, addition of ATP to the medium containing all components necessary for the Ca-ATPase operation produces a very

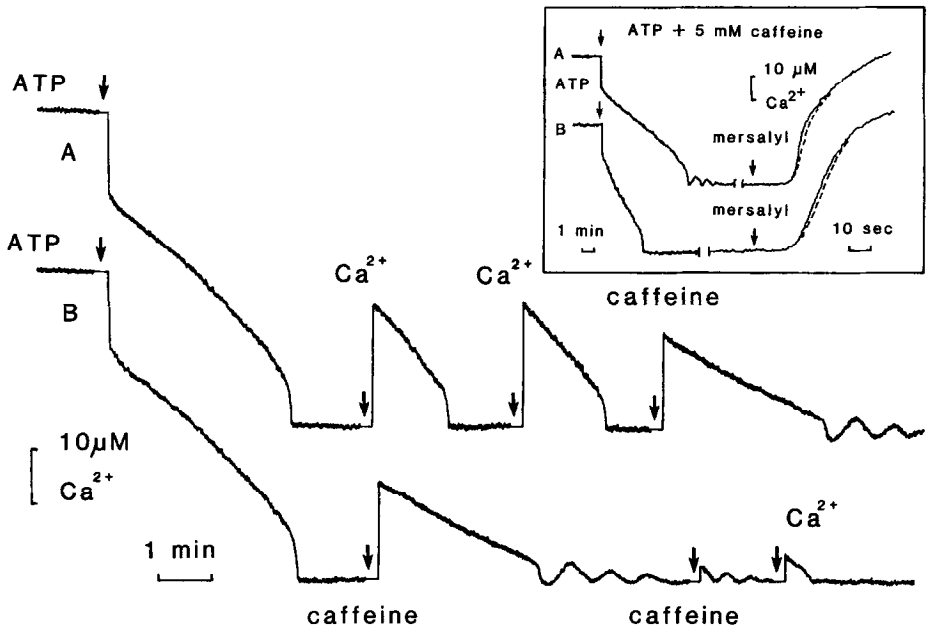

Fig. 1. The time-course of ATP-supported Ca^{2+} uptake and Ca^{2+}- and caffeine-induced Ca^{2+} release from HSR vesicles. Where indicated, ATP at the final concentration of 0.5 mM was added to initiate Ca^{2+} uptake. In the steady state, Ca^{2+} release was triggered by addition of 5 um Ca^{2+} or of 5 mM caffeine. Inset: The time-courses of Ca^{2+} uptake and mersalyl-induced Ca^{2+} release from HSR vesicles in the presence (A) or absence (B) of 5 mM caffeine. Where indicated, ATP (0.5 mM) and mersalyl (0.1 mM) were added. Dotted lines show the mersalyl-induced Ca^{2+} release in the presence of 1 uM RR.

fast decrease of the free Ca^{2+} concentration which reflects mainly Ca^{2+} binding to the added ATP (15). After that, a relatively slow phase of Ca^{2+} accumulation takes place followed by the fast phase of Ca^{2+} uptake. As presently understood, Ca-release channels are open during the slow phase of Ca^{2+} accumulation whereas the fast phase of Ca^{2+} uptake reflects the process of spontaneous closing of Ca-channels (15). The fast Ca^{2+} uptake reaches a steady state when the free Ca^{2+} concentration in the medium becomes nearly zero ($\sim$0.1 uM). In the steady state, the addition of 5 uM Ca^{2+} or 5 mM caffeine to the reaction medium induces rapid release of about 22 nmol Ca^{2+}/mg of protein from HSR vesicles; this is followed by complete reaccumulation of the released Ca^{2+}, again in two phases (Fig. 1). The amount of Ca^{2+} released depends hyperbolically on the caffeine concentration between 0.1 and 5 mM, with $K_{0.5}$ = 0.3 mM (data not shown). This suggests that the caffeine-binding receptor of Ca-channels possesses the properties of saturable binding site.

The rate of Ca^{2+} uptake during the slow phase of accumulation in the presence of caffeine is about one-half that in its absence (Fig. 1A). Since caffeine does not affect the Ca-ATPase activity at the concentrations used (23), this slow rate of Ca^{2+} accumulation seems to connect with the increase of Ca^{2+} efflux from HSR vesicles through the open Ca-channels during the Ca-pump operation. This is in accordance with the fact that caffeine increases the frequency and duration of single channel events in HSR membranes (24). But the biphasic character of the Ca^{2+} accumulation in the presence of caffeine shows that the Ca-channels convert to a closed state even in the presence of high concentrations of this drug (Fig. 1).

The repeated addition of 5 mM caffeine to the reaction medium does not induce Ca^{2+} release (Fig. 1B). This suggests that all caffeine-binding sites are already occupied by caffeine. However, Ca^{2+} addition in the presence of caffeine also does not induce Ca^{2+} release (Fig. 1B). This seems to indicate that the caffeine and Ca^{2+} interact with the same receptor in the Ca-release channels or that the caffeine and Ca^{2+} receptors interact very strongly.

The addition of the thiol blocker mersalyl to the medium during the steady state produces relatively fast release of the accumulated Ca^{2+} from HSR vesicles both in the presence (Fig. 1, inset A) and in the absence of caffeine (Fig. 1, inset B). According to the literature (23), mersalyl does not affect Ca-channels but inhibits the Ca-pump of HSR, so the Ca^{2+} release after mersalyl addition occurs not through the Ca-channels. This is supported by our finding that addition of 1 uM of the Ca-channel blocker, RR, into the medium before or simultaneously with the mersalyl addition practically does not decrease the rate of Ca^{2+} efflux (Fig. 1, inset). This effect of RR suggests that at the steady state Ca-channels are already closed both in the presence and in the absence of caffeine.

The data obtained therefore allow us to conclude that Ca^{2+} cannot induce Ca^{2+} release when the caffeine-binding sites of the Ca-channels are occupied by caffeine (Fig. 1B) , although the Ca-channels are closed during the steady state in the presence of caffeine (Fig. 1,inset A). This possibly indicates that Ca^{2+} and caffeine have a common receptor in the Ca-release channels.

In an attempt to isolate the caffeine receptor of the Ca-channels, we solubilized the HSR membranes with nonionic detergent $C_{12}E_9$ using the approach which was developed earlier for the purification of the Ca-ATPase protein (16). As shown in Fig. 2, the decanted supernatant after solubilization of HSR vesicles contains the majority of proteins (Fig. 2B) which are present in HSR membranes (Fig. 2A), whereas protein with m.w. 200 kD (probably myosin), calsequestrin and some others are pelleted in the presence of 1 mM Ca^{2+} (Fig. 2C). The supernatant usually contained 50-55% of protein and 95-100% of phospholipid of the original HSR membranes under the solubilization conditions used.

The solubilized material was applied to the reactive red 120-agarose column, and then a solution containing caffeine was passed through the column. As shown in Fig. 2D, caffeine elutes a number of proteins. Increase of the caffeine concentration in the eluent (5, 10 and 20 mM) leads to the progressive increase of

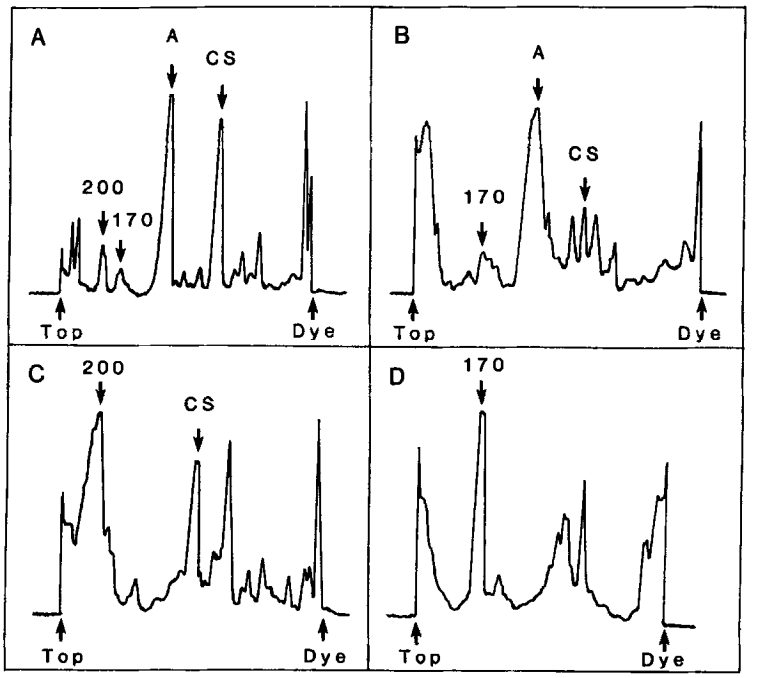

Fig. 2. Densitometric scans after separation by SDS-gel electrophoresis of HSR membranes proteins (A), decanted supernatant (B), Ca-pelleted proteins (C) and caffeine-eluted material (D). The gels were stained with Coomassie Blue (A-C) or with silver stain (D). The gels were loaded with 30 ug (panels A-C) or 2 ug (panel D) of protein. A, Ca-ATPase (m.w. ~100 kD); CS, calsequestrin (m.w. 65 kD); 200, 200 kD protein (probably myosin heavy chain); 170, 170 kD Ca-binding protein.

the 170 kD protein percentage in the eluted material (data not shown). With the use of 20 mM caffeine in the elution buffer, the content of 170 kD protein in the eluted material is about 25 to 30 times more than in the supernatant applied to the column (Fig. 2, B and D). The content of other caffeine-eluted proteins does not vary significantly with the change of the caffeine concentration in the elution buffer (data not shown). This allows us to propose that the 170 kD protein is the caffeine-binding protein and possibly the caffeine receptor of the Ca-release channels.

Comparison of $^{45}Ca^{2+}$ release from the native HSR vesicles (Fig. 3A) with vesicles reconstituted from the supernatant after detergent removal with Bio Beads SM-2 (Fig. 3B) suggests that reconstituted vesicles contain both the Ca-release channel and caffeine receptor. The total capacity of the reconstituted vesicles for Ca^{2+} is only about 10% of that of native HSR vesicles. This possibly reflects the smaller size of the reconstituted vesicles, but the Ca^{2+} release in the reconstituted system is sensitive to RR and to caffeine (Fig. 3B). This allows us to conclude that under the conditions used $C_{12}E_9$ solubilizes both the Ca-release channel and the caffeine receptor associated with this channel.

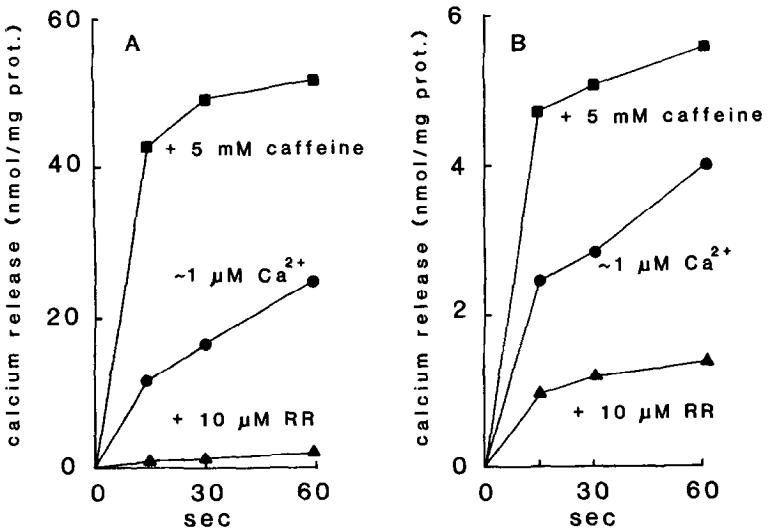

Fig. 3. The time-course of efflux of passively loaded $^{45}Ca^{2+}$ from native HSR vesicles (A) and from reconstituted vesicles (B). Experimental conditions are described in Materials & Methods.

It is interesting to note that after SDS-gel electophoresis of the native HSR membranes and the $C_{12}E_9$-solubilized material the 170 kD protein stains blue with the carbocyanine dye Stains-all (data not shown). This indicates that this protein is probably a Ca-binding protein (21). As was shown earlier (12), the 170 kD protein can bind doxorubicin which increases Ca^{2+} release from HSR. Moreover, doxorubicin binding to the 170 kD protein is inhibited by caffeine and depends on Ca^{2+} concentration (12). This supports our suggestion that the 170 kD Ca-binding protein is a caffeine-binding protein and is the most probable candidate for caffeine receptor of Ca-release channels.

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