Selective Abolition of Sarcoplasmic Reticulum Vesicles' Calcium Releasing Mechanisms

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The ability of calcium loaded heavy sarcoplasmic reticulum vesicles to specifically respond to the addition of various agents such as caffeine, calcium ions and calmodulin antagonists to rapidly released calcium can largely be diminished by passing the vesicular suspension in 0.3 M sucrose, 0.6 M KCl, 4 mM $CaCl_2$, pH 7.0 through a Sepharose 6B column or by centrifuging it through a sucrose gradient prepared with the same salt medium. Inactivation of calcium release does neither interfere with calcium uptake nor with the unspecific releasing effect caused by the application of high concentrations of calmodulin antagonists.

Introduction

For muscle activation, calcium ions are released from their intracellular stores in the sarcoplasmic reticulum [1, 2]. Calcium release appears to be the final event in a multi-step reaction sequence. In the living muscle the reaction chain starts with membrane depolarization induced movement of electrical charges in the plasma membranes and their invaginations [3]. The mechanism by which this potential change generates calcium release is unknown. The process can be manipulated by quite a number of pharmacological agents such as caffeine [4, 5]. The action of agents that can modify excitation-contraction coupling, has extensively been studied in skinned and chemically permeabilized muscle fibres [6–9]. The application to these preparations of caffeine [7, 9] and a number of other substances like quercetin which cannot permeate the plasma membrane of living fibres [6, 8], induce calcium release and contraction. The agents prove to also affect calcium release from isolated sarcoplasmic reticulum vesicles [9–16]. These membrane preparations as calcium-storing and releasing structures allow to extensively manipulate the experimental conditions for establishing conditions for calcium release, on the one hand, and the sites of drug action, on the other. In this paper, we report a procedure for preparing an membrane fraction showing a different response to the effect of various reagents as compared to the native membrane. It is shown that the manipulation of the preparation by gradient centrifugation or more reproduceably by gel filtration results in the loss of its caffeine sensitivity and greatly diminishes its calcium sensitivity.

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Materials and Methods

Heavy sarcoplasmic reticulum vesicles were prepared in the presence of protease inhibitors (phenylmethylsulfonylfluoride, benzamidine HCl and benzethonium chloride 0.1 mM each) and actively loaded with $^{45}Ca^{2+}$ as described by Su and Hasselbach [10]. The heavy vesicular fraction was routinely extracted with 0.6 M KCl, 0.3 M sucrose and 3 mM MgATP to remove residual contractile proteins. Desensitization of the preparation was mostly achieved by passing 10 ml of the vesicular suspension (20 mg/ml) in 0.6 M KCl, 0.3 M sucrose, 4 mM $CaCl_2$, 25 mM imidazole, pH 7 through a Sepharose 6B column 5×60 cm. The vesicles eluting with the void volume were concentrated by centrifugation. Initially, desensitization was performed by sedimenting the vesicles through sucrose gradients 0.5–1.5 M containing 0.6 M KCl and 2 mM $CaCl_2$, 20 mM histidine, pH 7.0.

Calcium release was initiated after loading the preparation for 10 min at room temperature by addition of the respective substances, causing a 10% dilution. Calcium release was terminated by filtration through 0.45 μm Sartorius filters. The radioactivity in the filtrate was measured by liquid scintillation counting.

The membrane proteins were separated by gel electrophoresis with a 5–15% gradient of polyacrylamid fixed with sulphosalicilic acid and stained with Coomassie blue. The protease inhibitors and com-

pound 48/80 were purchased from Sigma Chemie GmbH, Deisenhofen, FRG. Calmidazolium was obtained from Janssen Pharmaceutica, Beersen, Belgium. Trifluoperazine was a gift from Röhm and Haas, Darmstadt, FRG.

Results

A typical calcium release transient of a native heavy sarcoplasmic reticulum fraction is shown in Fig. 1. On addition of 10 mM caffeine approximately 30% of the calcium load (90 nmol/mg) are released followed by a slow calcium re-uptake. In contrast, gel-filtrated vesicles of the same fraction are nearly completely caffeine insensitive. Even when the caffeine concentration is raised from 10 to 30 mM calcium release is not significantly enhanced. In contrast to the releasing mechanism calcium uptake is relatively little affected by the procedure. The rate of calcium uptake regularly becomes somewhat slower as compared to normal preparations. But the capacity of the preparation for calcium is not significantly reduced. On addition of 0.2 mM calcium/0.3 mM EGTA, corresponding to the adjustment of a free calcium concentration of 0.6 μM, 60% of the stored radioactivity are rapidly released during the first 15 s (Fig. 2). Since the activity of the calcium transport system is largely suppressed by the high internal calcium concentration, the initial rapid calcium release is only little contaminated by the exchange of radioactive against added inactive calcium. The subsequent calcium movement is difficult to monitor due to the changing specific activity of medium calcium. As one can expect from the low rate of net calcium uptake under the prevailing conditions and the low specific activity of medium calcium, no change in the calcium content in the solution can be detected. The calcium-induced like the caffeine-induced calcium release is strongly reduced after gel filtration at all calcium concentrations as shown in Fig. 3.

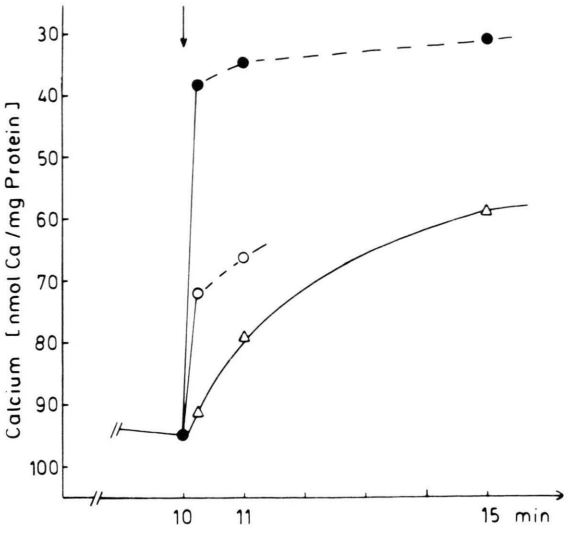

Fig. 2. Calcium-induced calcium release from native and desensitized heavy sarcoplasmic reticulum vesicles. The vesicles were actively loaded as described in Fig. 1. The release was induced by the addition of 0.2 mM calcium/0.3 mM EGTA final concentration. (●) control preparation; (○) desensitized preparation. The slow calcium efflux caused by 0.3 mM EGTA is indicated by (△).

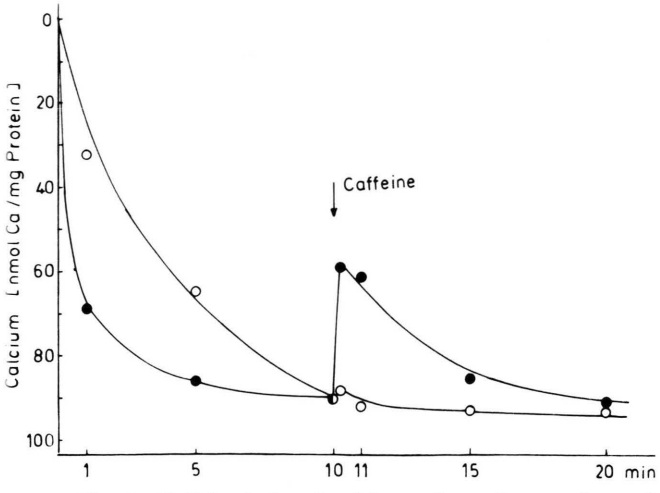

Fig. 1. Caffeine-induced calcium release from native and desensitized heavy sarcoplasmic reticulum vesicles. 0.2 mg vesicular protein/ml were loaded with calcium at 20 °C in media containing calcium, $CaCl_2$ 0.02 mM, ATP 2 mM, phosphoenolpyruvate 4 mM, pyruvate kinase 0.04 mg/ml, KCl 100 ml, sucrose 100 mM, $MgCl_2$ 3 mM and histidine 50 mM, pH 7; for 10 min. Calcium release was induced by the addition of caffeine, 10 mM final concentration. The time course of calcium release was monitored by passing aliquots of the vesicular suspension through Sartorius filters 0.45 μm at given times. (●) control preparation, (○) desensitized preparation.

Even larger releases of calcium than those produced by calcium itself can be induced by a number of agents known as calmodulin antagonists like calmidazolium, compound 48/80 or trifluoperazine [17] (Fig. 4). In contrast to caffeine, all calmodulin antagonists interfere with the activity of the calcium pump. Yet their releasing action cannot be related to pump inhibition. The effects of the substances differ with respect to maximum calcium release as well as

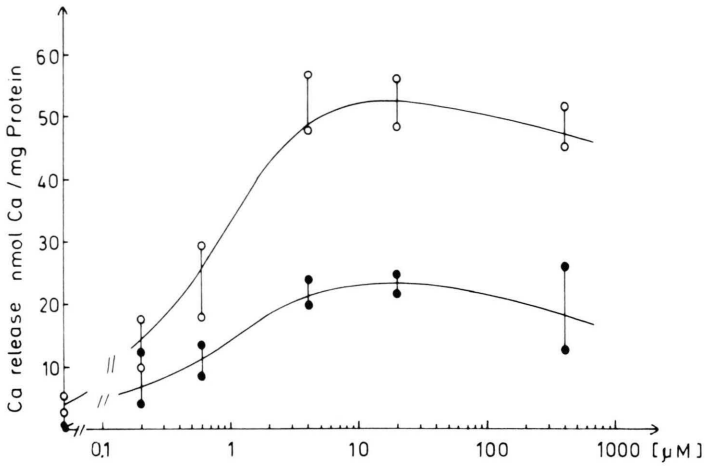

Fig. 3. Dependence on the free calcium concentration of calcium-induced calcium release from native and desensitized heavy sarcoplasmic reticulum vesicles. Active calcium loading of the sarcoplasmic reticulum vesicles was performed as described in Fig. 1. Release was induced by the addition of calcium EGTA solutions giving free calcium concentrations in the medium as indicated on the abscissa. On the ordinate, the quantity of calcium released after 15 s is plotted. The amount of calcium is calculated from the specific activity of the stored calcium. The uncertainty concerning the specific activity used for the calculation resulting from the uptake of cold calcium during the releasing period does not exceed 10%. (○) control preparations; (●) desensitized preparations.

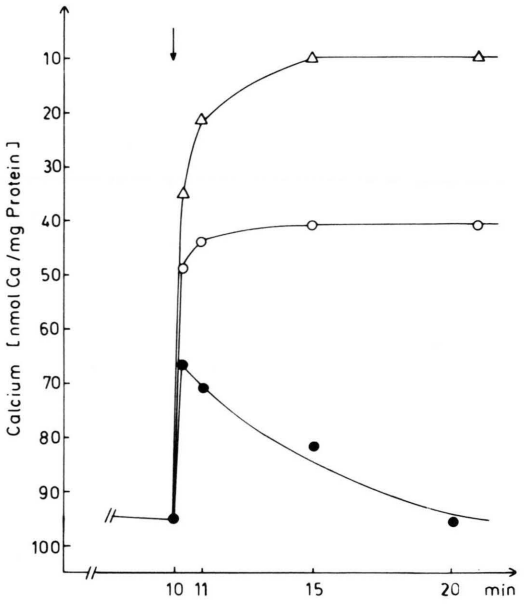

Fig. 4. Calcium release from heavy sarcoplasmic reticulum vesicles induced by various calmodulin antagonists. The vesicles were actively loaded as described in Fig. 1. Release was induced by drug addition – 0.1 mM and terminated by filtration. Calmidazolium (△), trifluoperazine (○), compound 48/80 (●).

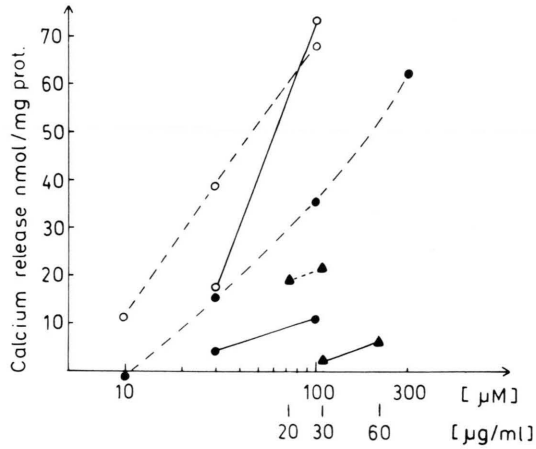

Fig. 5. Dependence on the drug concentration of calcium release from native and desensitized heavy sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were actively loaded with calcium as described in Fig. 1. Calcium release was initiated by drug addition giving the final concentration plotted on the abscissa. The amount of calcium released after 15 s is given on the ordinate. Trifluoperazine (●), calmidazolium (○), compound 48/80, μg/ml (▲); – – – – control preparation, ——— desensitized preparation.

to its reversal. Reversal is observed only at low concentrations of trifluoperazine and compound 48/80. The different calmodulin antagonists affect native and desensitized preparations differently (Fig. 5). While the action of trifluoperazine is strongly diminished, the effect of calmidazolium is significantly reduced at low concentrations only. Thus, desensitization of the vesicles appears to make the differences in release response of the agents more distinct.

Centrifugation of the heavy vesicular fraction through a KCl sucrose gradient or the passage through a Sepharose column most obviously removes some membrane constituents. Since pump activity is not affected by the two procedures, neither the pump protein nor essential lipid constituents should be affected. In various reports high molecular weight constituents were claimed to be characteristic for the membrane fraction enriched in triads. We, too, found these proteins, but could not ascertain significant and reproduceable change of these components by the modification procedure. The only polypeptides which regularly got lost during gel filtration migrated in polyacryl gel electrophoresis with an apparent molecular weight of 42 KDa, actin, and 94 KDa, phosphorylase.

Discussion

In this study we have demonstrated that the calcium releasing structures present in a heavy sarcoplasmic reticulum membrane fraction can be inactivated or removed by a simple procedure without affecting the membrane's calcium transport system. The described sucrose gradient centrifugation or more reliably gel filtration make the membranes completely irresponsive to caffeine or related substances (quercetin) and largely diminish the releasing effect of calcium, ATP analogues and calmodulin antagonists. The finding that calcium release can be abolished without affecting pump activity strongly supports the view that the calcium pump is not involved in calcium release. This view is in line with the observation that calcium is released by caffeine only from a subpopulation of the vesicles and with the finding that the releasing mechanism remains intact when the pump is irreversibly blocked [18]. A very low caffeine sensitivity has been reported by Meissner [12] for a heavy membrane fraction which however, is not identical with the fraction considered in this report or that deduced by Su and Hasselbach. The reason for the ineffectiveness of caffeine under the conditions applied by Meissner is the absence of ATP – which is an essential cofactor for the releasing effect of caffeine [11] – in the releasing medium. The calcium releasing channels in our releasing membrane fraction are probably not contained or confined to structures in the T-tubules or in structures connecting T-tubules and sarcoplasmic reticulum vesicles. The releasing fraction has been extracted with 0.6 M KCl and 5 mM Mg-ATP to remove contractile proteins present as contaminants. This treatment results in dissociation of sarcoplasmic reticulum vesicles and T-tubules and removes the structures connecting sarcoplasmic reticulum and T-tubules [19]. The electron microscopic inspection did not reveal any significant difference between releasing and non-releasing membranes. The releasing structures are therefore most likely attached or constituents of the sarcoplasmic reticulum membranes proper. In our attempt to identify the constituents of the releasing structure by comparing the peptide pattern of releasing and non-releasing membranes, we could find no changes among the protein species which recently were connected with calcium release. The great number of agents the application of which induces calcium release indicates a relatively low specificity of the releasing structures. Since desensitization concerns the effect of all agents, we must assume that they interact with the same target structure. Although desensitization affects the action of all agents, there are some significant quantitative differences with respect to the extent of desensitization. While caffeine becomes completely ineffective at all applicable concentrations, the calcium-induced response is only reduced by maximally 60%. Also the releasing effect of the calmodulin antagonists is not equally affected by desensitization. This might be related to the fact that all calmodulin antagonists exert, when applied at higher concentrations, unspecific effects due to their hydrophobic interaction with various membrane constituents. Thus, all calmodulin antagonists quite effectively inhibit calcium transport ATPase and calcium transport. Hence, part of the residual calcium release of the desensitized preparations may be due to unspecific effects exerted by the drugs on the sarcoplasmic reticulum calcium pump (*cf.* [20]).

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