Modulation by Ryanodine of Active Calcium Loading and Caffeine Induced Calcium Release of Heavy Sarcoplasmic Reticulum Vesicles

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Sarcoplasmic Reticulum, Caffeine Induced Calcium Release, Ryanodine

The effect of ATP on the calcium release channel in heavy sarcoplasmic reticulum vesicles modulated by ryanodine has been analyzed by monitoring active calcium uptake and caffeine induced calcium release under near physiological conditions. Native as well as ryanodine reacted vesicles display a complex time course of calcium uptake resulting in nearly complete exhaustion of medium calcium when ATP in combination with an ATP-regenerating system, in contrast to ATP alone, or dinitrophenyl phosphate, were used to support calcium uptake. Applying of dinitrophenyl phosphate as energy yielding substrate, not affecting channel activity, allowed to estimate the fraction of light vesicles devoided of calcium channels contaminating the heavy preparation as the fraction that stores calcium after the preparation has been treated with channel opening, low concentrations of ryanodine (1 μM). Calcium uptake by contaminant light vesicles (25%) cannot account for calcium storage, as well as, abolition of caffeine induced calcium release of ryanodine treated heavy vesicles. Calcium uptake of native and ryanodine treated vesicles is accompanied by the uptake of equivalent amounts of inorganic phosphate arising from ATP hydrolysis indicating that calcium is mainly stored as calcium phosphate. The momentary capability of the preparation to accumulate calcium was measured by activating calcium uptake during the calcium storage period with 0.2 mM $^{45}CaCl_2$ and 4 mM phosphate at short time intervals. A significant increase of the momentary uptake activity with time was observed being more pronounced for ryanodine treated than for native vesicles indicating that under regenerating conditions, ATP can induce closing of the native and even more effectively of the ryanodine modified calcium release channels.

Introduction

In recent studies we have demonstrated that caffeine induced calcium release from actively loaded heavy sarcoplasmic reticulum vesicles is blocked when a small number of their ryanodine binding sites is occupied [1, 2]. This result has been explained as to be caused by closing or inactivating the vesicles caffeine sensitive calcium release channels effected by ryanodine binding. Supporting arguments for this interpretation were derived from the accompanying improvement of the ability of the ryanodine treated preparations to concentrate calcium [1]. This explanation is in conflict with the well established finding that low ryanodine concentrations (<10 μM) arrest the calcium release channels of passively loaded vesicles in an open state configuration [3]. The controversy can possibly be resolved if we assume that the vesicular fractions having reacted with ryanodine were unable to store calcium and thus cannot display caffeine sensitivity. This explanation, however, makes it necessary to assume that our heavy vesicular fraction is contaminated with a considerable quantity of vesicles being ryanodine and caffeine insensitive in order to account for the observed active calcium loading. Furthermore, since ATP has been shown to effectively induce calcium release from passively loaded heavy vesicles [4], one has to assume that in ATP-containing media only those vesicles devoided of calcium channels can participate in calcium storage. Therefore, caffeine should be unable to induce calcium release from vesicles loaded in ATP-containing media. This, however, is in conflict with numerous observations [5–7]. The present study was conducted to reevaluate the interrelationship between calcium uptake, caffeine induced calcium release and their modification by ryanodine in the presence of ATP. The effect of inorganic phosphate as a potent physiological calcium precipitating agent, arising during active loading, was included because of its marked effect on calcium accumulation and the resulting interference with

Abbreviations: DnpP, dinitrophenyl phosphate.

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the state of intravesicular calcium [8–11]. DnpP was used as calcium uptake supporting agent to reveal effects of ATP other than energy provision [12]. Calcium release was induced by caffeine since its releasing effectiveness under physiological conditions *i.e.* in media containing calcium, magnesium, phosphate and ATP, has previously well been established [1, 5, 7]. It is shown here that the time course and the extent of calcium storage by heavy sarcoplasmic reticulum vesicles very much depend on the loading conditions with respect to the applied energy yielding substrates ATP *versus* DnpP. Calcium uptake driven by DnpP results in the filling of a calcium release competent vesicular fraction which is in contrast to calcium uptake driven by ATP alone. However when uptake is supported by ATP in combination with an ATP-regenerating system calcium storage leads to the filling of caffeine sensitive vesicular components. This fraction is exempted from calcium loading supported by DnpP after being reacted with low concentration of ryanodine. The momentary rates of calcium uptake measured during ATP-driven loading in media supplemented with an ATP-regenerating system increase with time indicating that under these conditions low concentration of ryanodine can promote channel closing.

Materials and Methods

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hind leg muscles as described by Hasselbach and Migala [13] and modified according to Meissner [4]. 100 g muscle were minced for 4 × 30 s in 400 ml extraction fluid containing 0.1 M KCl, 5 mM potassium phosphate (pH 7.0), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM sodium azide in a mixer at 4 °C. Myofibriles were removed from the suspension by centrifugation in a Serval GS 3 rotor at 6000 rpm for 30 min. The heavy vesicles were separated from the supernatant by subsequent centrifugation in a Spinco type 19 rotor at 10000 rpm for 30 min.

Contaminating contractile proteins were extracted by treating the pellet with 10 volumes of 0.6 M KCl, 0.3 M sucrose, 3.0 mM ATP, 3.0 mM $MgCl_2$, 0.1 mM benzethonium-Cl and 0.1 mM benzamidine-HCl, 0.1 mM dithiothreitol for 20 min.

Subsequently, the vesicles were sedimented by centrifugation in a Spinco rotor Ti 60 at 55000 rpm for 50 min and resuspended in 0.1 M KCl (30–50 mg/ml).

Light sarcoplasmic reticulum vesicles were isolated from the supernatant of the 10000 rpm × 30 min centrifugation step as described by de Meis and Hasselbach [14].

Calcium uptake and release experiments were performed as described by Su and Hasselbach [7]. The standard media contained 50 mM potassium gluconate, 50 mM KCl, 0.1 M sucrose, 50 mM imidazole-sulfate, pH 7.0. 3 mM magnesium-gluconate, 20 μM $^{45}CaCl_2$, 2 mM ATP or 2 mM DnpP, 8 mM phosphoenolpyruvate and 0.04 mg/ml phosphoenolpyruvate kinase were added when ATP was to be regenerated.

In some experiments 0.1 M K-gluconate was used instead of 50 mM K-gluconate + 50 mM KCl. Changes in medium composition are mentioned in legends or text. Calcium uptake was started by adding 3 mg vesicular protein to 15 ml of the assay solution. Aliquots were taken at appropriate times and filtered through Schleicher & Schuell BA filters 0.45 μm. Caffeine-induced calcium release was initiated by the addition of 10 mM caffeine. Release was interrupted at different times by filtration. ^{45}Calcium was measured by liquid scintillation counting of the filtrate.

Ryanodine binding to the vesicles was performed at room temperature, 20–22 °C in solutions containing 0.6 M KCl, 0.3 M sucrose, and 5–10 mg/ml vesicular protein. Medium pH was adjusted to 7.0 with 0.1 M imidazole sulfate buffer. Medium pCa was measured with a calcium sensitive electrode and adjusted to 4.0. The binding reaction was conducted at ryanodine concentrations as given in the legends.

ATP hydrolysis was determined by measuring phosphate liberation either colorimetrically [15] or by liquid scintillation counting, when ^{32}P-ATP was used as substrate [16].

Ryanodine was purchased from Cal Biochem. Frankfurt (F.R.G.). ATP was obtained from Pharma-Waldhof, Mannheim (F.R.G.).

Phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer, Mannheim (F.R.G.). All other reagents were analytical grade and bought from E. Merck, Darmstadt; Sigma Chemical Company, Deisenhofen (F.R.G.) and Serva,

Heidelberg (F.R.G.). $^{45}CaCl_2$ and ^{32}P ATP were supplied by Buchler & Co., Frankfurt/Main (F.R.G.).

Results and Discussion

Modulation by ryanodine of ATP- and DnpP-supported calcium uptake

Fig. 1 illustrates that ATP-supported calcium uptake by heavy vesicles remains incomplete when performed in media containing 2 mM ATP as energy yielding substrate. Uptake cannot be improved by applying higher concentrations of ATP (not shown). This is different from the uptake pattern of heavy vesicles supported by DnpP or AcP which both effect a steadily proceeding calcium uptake tending to completion Fig. 1 and [7, 17]. These different patterns of calcium uptake displayed by heavy vesicles are in line with the notion that in these preparations the amount of actively accumulated calcium remaining inside the vesicles largely depends on the state of their calcium releasing channels, apart from pump activity. The steadily proceeding quite rapid DnpP-supported uptake indicates that the channels are closed and that there is no change in their state of calcium ion conductance. This assumption agrees with the fact that DnpP-driven uptake is neither enhanced by ruthenium red (5 μM) an agent long known to block calcium release [18] nor by concentrations of ryanodine higher than 10 μM which have been shown by Meissner [3] to effect channel closing in passively loaded heavy vesicles (compare Fig. 1 and Fig. 2). The incomplete calcium uptake of heavy vesicles supported by ATP can be rationalized by assuming that ATP does not only function as energy donor of the calcium pump but that it also modifies channel function as a most effective calcium releasing agent [4, 19, 20]. If this effect of ATP would be preponderant, ATP-driven calcium uptake could only occur in preparations the channels of which had been shut. Thus, in fact, ATP-driven calcium uptake by heavy vesicles could previously be augmented by low concentration of ruthenium red [18] and more recently by high concentrations of ryanodine 10 μM [21].

Channel closing caused by high concentration of ryanodine transiently passes through an open state configuration which is maintained when ryanodine concentrations below 1 μM are applied. The existence of such an open state had first been demonstrated by Meissner [3] who studied calcium efflux from passively loaded vesicles. As shown in Fig. 2 this effect also prevails under conditions of

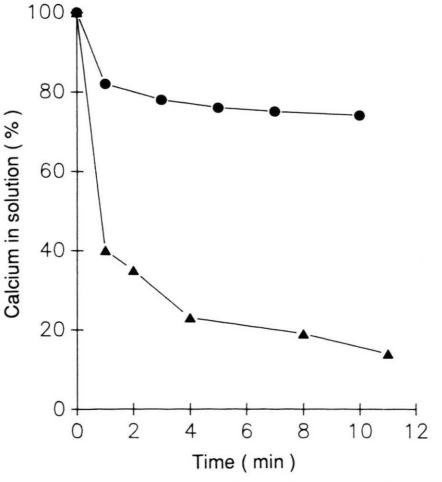


Fig. 1. Time course of calcium uptake of heavy sarcoplasmic reticulum vesicles supported by ATP or DnpP. 2 mM ATP (●) or 2 mM DnpP (▲) were used to drive calcium uptake as described in Materials and Methods. Calcium uptake was terminated by filtration at times given on the abscissa. Ordinate: calcium remaining in the solution related to its protein content. Initial calcium concentration 20 μM, 0.2 mg vesicular protein · ml^{-1}.

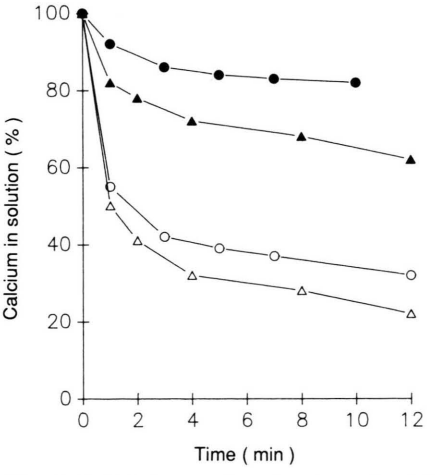


Fig. 2. Inhibition and activation of calcium uptake of heavy sarcoplasmic reticulum vesicles by ryanodine. Heavy vesicles were reacted with ryanodine in 0.6 M KCl as described in Materials and Methods (●, ▲) 1 μM ryanodine; (○, △) 0.2 mM ryanodine. Calcium uptake was started by adding 0.2 mg vesicular protein · ml^{-1} to standard uptake medium containing 20 μM calcium. Uptake was supported by 2 mM ATP (●, ○) or 2 mM) DnpP (▲, △).

active loading when DnpP and ATP were used for energy provision. The large DnpP-supported calcium storage is strongly suppressed by ryanodine while calcium uptake driven by ATP is only little further reduced because the channel opening by ATP can hardly further be augmented by ryanodine.

Modulation by ryanodine of calcium uptake supported by an ATP-regenerating system

A complete different pattern of calcium uptake emerges when it is supported not by ATP alone but by ATP in combination with a regenerating system for energy provision. As shown in Fig. 3 native heavy vesicles as well as vesicles treated with low channel-opening concentrations of ryanodine (1 μM) take up calcium from media containing ATP in combination with a regenerating system. The calcium uptake of the ryanodine-treated preparations appears to be retarded as compared to the controls performed with native vesicles. Yet, after an uptake period of 8–10 min, ryanodine-treated preparations often accomplish lower residual calcium levels than those reached by the controls ([1], see below). The effect of ATP regeneration on calcium uptake is impressively shown when the uptake medium is supplemented with phosphoenolpyruvate after calcium uptake of native, as well as, ryanodine-treated vesicles (1 μM) has come to an halt in the presence of ATP alone (Fig. 4). Calcium uptake is accelerated without delay. Its progress depends on the concentration of ATP and the activity of the regenerating system (not shown). In most previous studies dealing with calcium release from actively loaded vesicles, ATP was used for loading in combination with a regenerating system [5–7, 21]. These conditions have tacitly been adopted due to the encountered weak and irregular uptake activity of heavy preparations in the presence of ATP alone [5–7].

The fact that in spite of the releasing effect of ATP and of the channel-opening effect of low concentration of ryanodine, heavy vesicles take up calcium from media in which ATP is regenerated, might be based on different mechanisms which in the following will be considered. 1) Our preparation of heavy vesicles might highly be contaminated with light vesicles devoid of ATP-sensitive calcium channels. 2) Inorganic phosphate which has

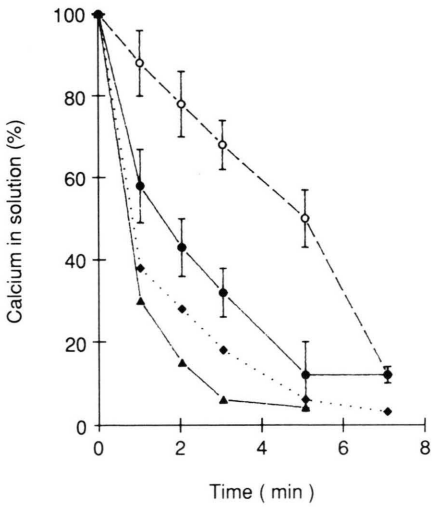


Fig. 3. Calcium uptake of heavy sarcoplasmic reticulum vesicles supported by ATP regenerated from phosphoenolpyruvate and its modification by ryanodine and ruthenium red. Calcium uptake was measured in standard uptake media. Native vesicles (●). Vesicles were reacted with either 1 μM ryanodine (○); or 0.2 mM ryanodine (◆) in 0.6 M KCl for 3 h and subsequently diluted into the uptake media. (▲) 5 μM ruthenium red was added to the uptake medium. Ordinate: calcium remaining in solution.

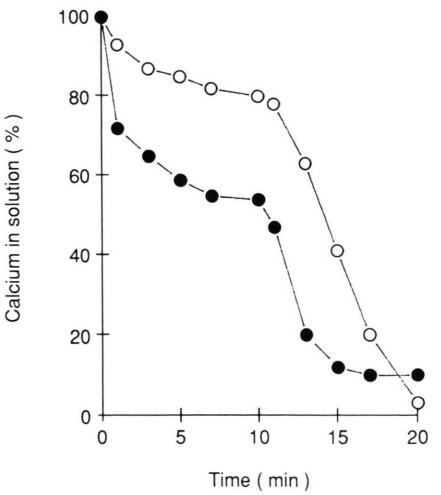


Fig. 4. Activation of calcium uptake of native and ryanodine-reacted heavy vesicles by initiating ATP regeneration. Heavy vesicles were reacted with 1 μM ryanodine in 0.6 M KCl. Calcium loading of native vesicle (●) and ryanodine-reacted vesicles (○) was performed in standard media containing 2 mM ATP without regenerating system, for 10 min. Subsequently the assays were completed by adding 8 mM phosphoenolpyruvate and 0.04 mg · ml^{-1} phosphoenolpyruvate kinase.

previously been shown to support effectively calcium uptake and which is produced by the vesicular ATPases, might overcome the releasing effect of ATP and channel opening by ryanodine [8, 9, 11]. 3) ATP in addition to its action as energy donor and calcium-releasing agent might alter the functional state of the calcium release channel in a time-dependent manner leading to its closure.

Contaminating fraction of light vesicles in the heavy vesicular preparations

As to the first alternative, a reasonable estimate of the size of the light fraction which contaminates our heavy preparation can be inferred from the results illustrating DnpP-supported calcium uptake by a native (Fig. 1) and a ryanodine (1 μM) treated (Fig. 2) heavy vesicular preparation. We can expect that, after the preparation has been treated with 1 μM ryanodine in 0.6 M KCl at pH 7.0 for 3 h, all calcium channels of the preparation have been opened and hence calcium could not be stored. Fig. 1 shows that while DnpP-driven calcium uptake of the native preparation continuously proceeds up to 85 nmol·mg^{-1}, at a possible maximum of 100 nmol·mg^{-1}, calcium storage of the ryanodine-treated preparation levels off after it has reached 20–30% of the maximal load obtainable under the applied conditions (Fig. 2). This demonstrates that 20–30% of our preparation are not affected by ryanodine and hence have to be considered as light vesicles. A somewhat smaller fraction stores calcium when ATP, alone, is applied as energy-yielding substrate (Fig. 2). Calcium uptake driven by both substrates is markedly accelerated when the channels are blocked by treating the preparation with 0.2 mM ryanodine (see below).

Modulation of calcium uptake by phosphate

As to our second option, concerning the role which inorganic phosphate might play as calcium uptake supporting agent, we have monitored the elevation of the phosphate concentration during calcium accumulation and the activation of ATP-supported uptake by added phosphate. Fig. 5 shows that on addition of 20 nmol/ml calcium to the medium to establish standard uptake conditions a transient liberation of phosphate is initiated. The calcium-activated ATPase of native preparations levels off already after 1 min, while the extrasplitting of ryanodine-treated preparations last approximately 4 min. In both cases the activity returns to the basic level observed in the absence of calcium [8]. The phosphate concentrations in the media reach 0.5 mM and 1.5 mM, respectively, after 5 min. With these endogenous phosphate concentrations a threshold is reached above which calcium uptake of native heavy and light and ryanodine-treated heavy vesicles becomes detectably enhanced as illustrated by Fig. 6. The extent of uptake measured after 10 min is more affected than its initial rate. The observed threshold for the enhancement of calcium uptake by phosphate well agrees with the concentration of 1 mM reported by Beil *et al.* [9]. It is considerably lower than those used by Mitchel *et al.* (100 mM) for studying spontaneous calcium release [10].

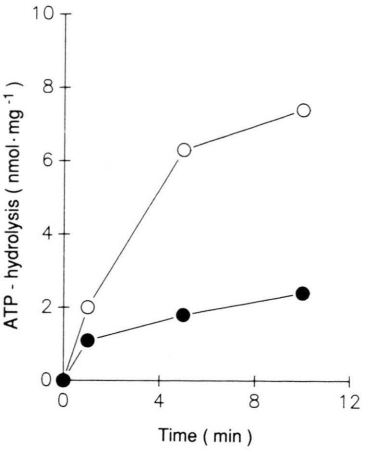


Fig. 5. Transient calcium activation of ATP hydrolysis of native and ryanodine-treated heavy sarcoplasmic reticulum vesicles. ATPase activity was measured in standard media by determining the liberation of inorganic phosphate. ATP hydrolysis was initiated by adding 0.2 mg prot.·ml^{-1} to the assay containing 20 nmol calcium ·ml^{-1}. (●) Native vesicles; (○) vesicles treated with 1 μM ryanodine for 3 h in 0.6 M KCl.

As previously shown the enhancement of calcium uptake following the addition of oxalate or phosphate to the medium is linked to the co-accumulation of calcium and the two anions [8, 9].

Fig. 7 demonstrates that co-accumulation of calcium and phosphate does not require supplementation of the medium with the anion. Calcium uptake by heavy vesicles supported by ATP alone

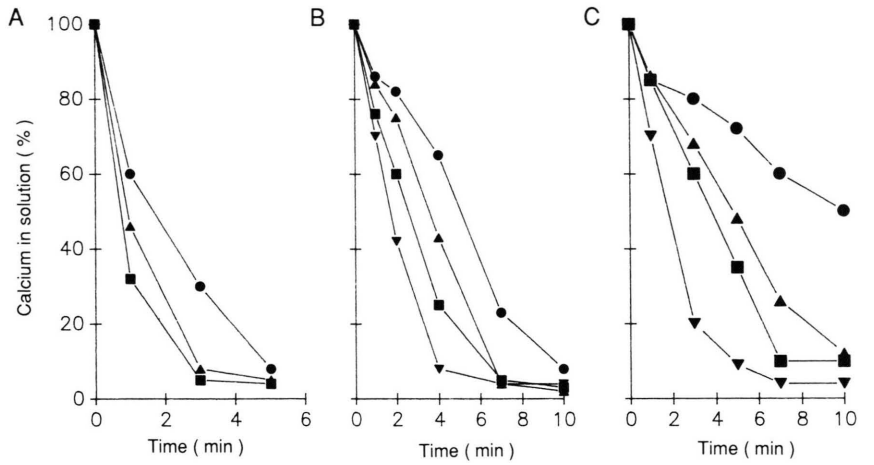
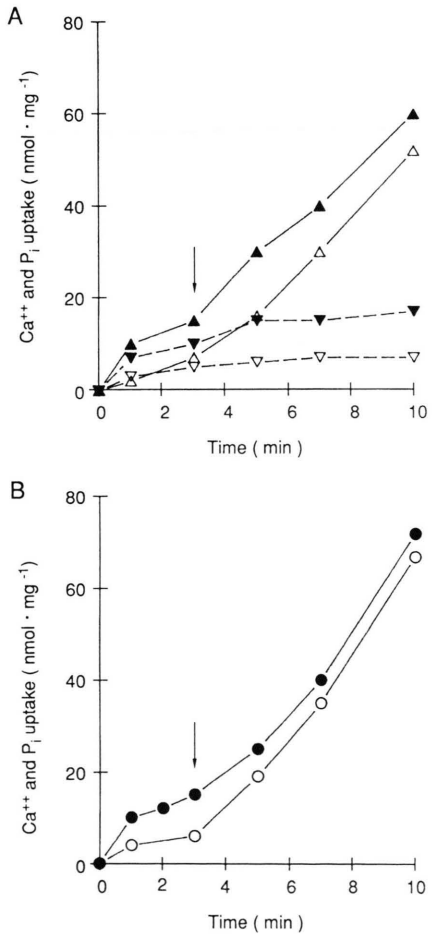


Fig. 6. Acceleration of calcium uptake by inorganic phosphate of native heavy vesicles (A), light vesicles (B) and ryanodine-treated heavy vesicles (C). Calcium uptake was measured under standard conditions at 20 °C in media containing 0.1 M K-gluconate. Protein concentrations: A and C, 0.2 mg·ml^{-1}; B, 0.04 mg·ml^{-1}. Phosphate concentrations: (●) no added phosphate; (▲) 1 mM; (■) 2 mM; (▼) 4 mM. Calcium uptake was terminated by filtration at times given on the abscissae. Ordinates: calcium remaining in solution measured in the filtrate.



or in the presence of a regenerating system proceeds as simultaneous uptake of calcium and phosphate liberated from ^{32}P-ATP. Calcium and phosphate uptake remain marginal when ATP is not regenerated (Fig. 7A) in agreement with the results shown in Fig. 1. When ATP is regenerated calcium uptake starts with a lag period of 3–4 min after which the rate of uptake gets faster and simultaneously the vesicles start to accumulate phosphate in parallel with calcium. Calcium uptake exceeds phosphate uptake by a small and approximately constant increment during this phase. This pattern does not essentially change when the experiments

Fig. 7. Simultaneous uptake of calcium and endogenously produced phosphate by native and ryanodine-reacted heavy vesicles. A. Calcium and phosphate (Pi) uptake by native vesicles was supported by 2 mM ATP alone (▼, ▽) and by 2 mM ATP + 8 mM phosphoenolpyruvate and pyruvate kinase 0.04 mg·ml^{-1} (▲, △). Full symbols: calcium uptake, empty symbols: phosphate uptake. B. Uptake of calcium and phosphate by ryanodine (1 μM) reacted vesicles supported by ATP and phosphoenolpyruvate and phosphoenolpyruvate kinase. For monitoring co-uptake of calcium and phosphate parallel assays were run either supplemented with ^{45}calcium, and cold ATP or with cold calcium and ^{32}P-ATP. The decline of the specific activity of inorganic phosphate liberated from ^{32}P-ATP resulting from the regeneration of ATP by cold phosphoenolpyruvate was not taken into account for calculating phosphate uptake. It is therefore underestimated at later times. Calcium uptake (●); phosphate uptake (○). The arrows in A and B indicate the termination of the lag phases.

are performed with vesicles pretreated with low concentration (1 μM) of ryanodine to effect opening of their calcium channels (Fig. 7B). We can therefore conclude that in the period of accelerated calcium uptake calcium and phosphate are simultaneously taken up by heavy vesicles and that this uptake mode is not detectably affected by ryanodine. Hence, we must suppose that the content of native and ryanodine pretreated preparations should have the same composition when calcium and phosphate uptake cease after 10–15 min.

Time courses of ATP-induced channel closing

The documented ATP-supported calcium and phosphate uptake by our heavy vesicular fraction being only relatively little contaminated by light vesicles led us to assume that the calcium release channels cannot have permanently been kept open by ATP but must rather have been closed in a time-dependent reaction. When loading is performed in ATP-containing media supplemented with an ATP-regenerating system the channels should initially be open when the vesicles are added to the medium as it is the case when ATP is not regenerated (see above). The lag phase in the time course of uptake which follows the short initial uptake effected by the small fraction of vesicles having no channels is presumably caused by the channel-opening effect of ATP. The lag phase becomes progressively longer when the concentration of pyruvate kinase is reduced (not shown). Treating the vesicles with 1 μM ryanodine in 0.6 M KCl likewise prolongs the lag phase of calcium and phosphate uptake. Yet, uptake resumes like in native preparations and residual calcium levels are approached lower than those reached by untreated preparations. Evidently, ATP-supported calcium uptake in the presence of ATP-regenerating systems might initiate a reaction that closes the channels of native vesicles. For ryanodine-treated vesicles the situation is more complex and will be dealt with in the following section. Such a channel-closing reaction has previously been proposed by Morii *et al.* [22] to explain the complex time course of calcium uptake by native heavy vesicles and the time-dependent inactivation of calcium-induced calcium release. The time course of the closing reaction can be inferred by comparing the time course of calcium uptake of vesicles the channels of which were blocked by ruthenium red with that of native of ryanodine-treated preparations (Fig. 3). As to the mechanism of time-dependent channel closing occurring in ATP-regenerating media, one might think of a slowly proceeding phosphorylation of the channel protein which should counteract ATP-induced channel opening. Without ATP regeneration, the rising level of ADP must be assumed to counteract phosphorylation. Yet, it is difficult to assure specific phosphorylation of the channel protein or of a protein that modifies channel function. A somewhat similar phosphorylation mechanism involving calmodulin has been discussed by MacLennan *et al.* [23] and by Morii *et al.* [24].

Caffeine-induced calcium release from actively loaded heavy vesicles

The filling of a caffeine-sensitive vesicular fraction is a most crucial point favouring the assumption that calcium is accumulated by vesicles the channels of which have been closed, and not, or not only, by the subfraction of light vesicles under conditions of ATP regeneration [1]. The light vesicular fraction separated from the same preparation displays only a very minute caffeine sensitivity and its pattern of calcium uptake is neither affected by ryanodine nor by ruthenium red (not shown). When calcium uptake of the heavy fraction is driven by DnpP, which does not interfere with channel activity, the same caffeine-sensitive compartment is filled. For the elicitation of calcium release by caffeine, it must be applied together with ATP as shown in Fig. 8. The small effects of ATP and caffeine alone are considerably augmented when they are simultaneously applied. This sensitizing effect of ATP on caffeine action was first observed by Su and Hasselbach [7] when they used acetylphosphate instead of ATP as energy donor for loading. The application of caffeine as calcium-releasing agent allows us to monitor the state of the calcium release channel in the presence of ATP *i.e.* under physiological conditions, after having been reacted with channel opening, low or channel closing, high concentrations of ryanodine. Fig. 9 demonstrates that rising concentrations of ryanodine (10 μM) activate ATP-driven calcium uptake. Since light vesicles are not affected by ryanodine its activating effect on uptake must be

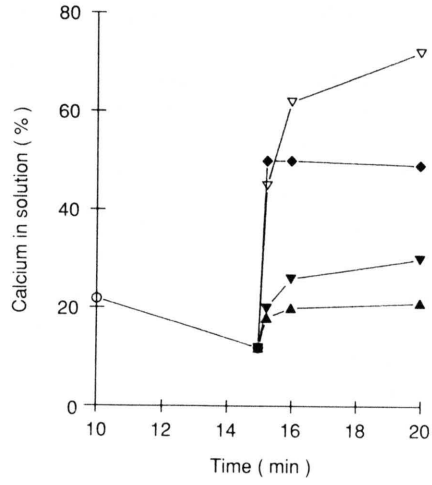


Fig. 8. Calcium release from heavy sarcoplasmic reticulum vesicles actively loaded using DnpP as energy-yielding substrate. Heavy vesicles were loaded in standard uptake medium containing 2 mM DnpP for 10 min. Release was induced by adding 10 mM caffeine (▲), 1 mM ATP (▼), 1 mM ATP + 10 mM caffeine (◆) and 1 mM ATP-γ-S (▽). Release was terminated by filtration. Ordinate: calcium in solution.

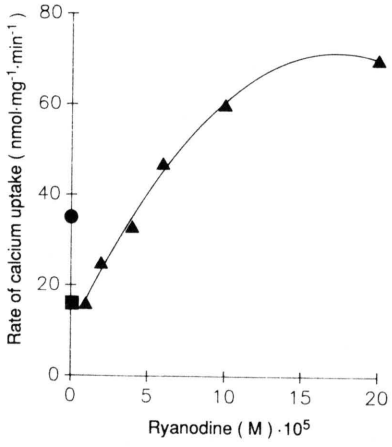


Fig. 9. Activation of calcium uptake by ryanodine. Calcium uptake was measured under standard conditions. Heavy vesicles were reacted with ryanodine in 0.6 M KCl, 0.1 mM $CaCl_2$, pH 7.0 for 1 h. (■) 1 μM ryanodine; (▲) concentrations of ryanodine on the abscissa; (●) control.

assigned to the filling of heavy vesicles the channels of which have been closed permanently by ryanodine. The thus treated preparation is completely caffeine-insensitive indicating that caffeine cannot counteract the channel-closing effect of ryanodine. This effect of high concentrations of ryanodine is most likely related to the occupation of the channel protein's unspecific binding sites of approximately 200 pmol·mg^{-1} which is not calcium-dependent, in contrast to specific binding occurring below 1 μM. The occupation of specific ryanodine-binding sites occurring at concentrations of ryanodine below 10 μM effecting channel opening in the absence of ATP, appears to result only in a transient impediment of calcium uptake under conditions of ATP regeneration (comp. Fig. 3 and [1]). The thus treated preparations have lost completely their caffeine sensitivity when 3 pmol·mg^{-1} are bound to sites having a dissociation constant of 4 nM [1, 25]. Under the applied conditions with respect to the concentration of vesicular protein in the assays (0.2 mg·ml^{-1}) and the limited amount of storable calcium (20 nmol·ml^{-1}), it is difficult to decide which of the vesicular compartments, the preponderant heavy or the contaminating, light vesicles contributes preferentially to calcium storage since the criterion for the filling of the heavy fraction, its caffeine sensitivity, is lost. The problem is complicated, furthermore, by the fact that the uptake activity of the light vesicular fraction in the assays (0.05 mg·ml^{-1}) would be large enough to account for calcium storage alone, especially when uptake is activated by rising phosphate concentrations as they are produced by the more rapidly proceeding hydrolysis of ATP by the heavy vesicles having opened channels. In the following we try to decide which of the two vesicular components is involved in calcium storage after treatment with low concentrations of ryanodine. If the channels in the heavy fraction would be closed during loading the total fraction would participate in calcium uptake. We assumed that the temporal change in the state of the calcium channels could be probed by measuring initial calcium uptake at every moment during ATP-supported calcium storage in absence and presence of ryanodine. This has been done by adding 0.2 mM $^{45}CaCl_2$ and 4 mM phosphate (pH 7.0) at various times after starting calcium loading. The selected concentrations can be expected to optimally activate calcium uptake. As shown in Fig. 10 the momentary rates of calcium uptake significantly increase with incubation time and this increase is considerably augmented when the vesicles were pretreated with ryanodine. (Such changes are only rudimentarily seen when the same experiments were performed with light

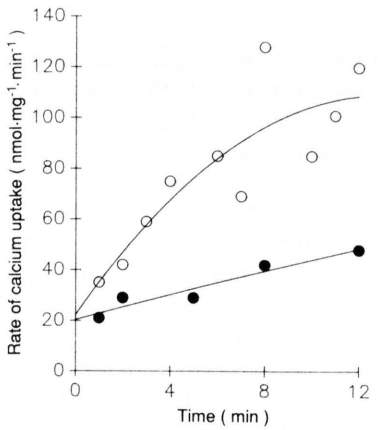


Fig. 10. Increase of momentary calcium uptake activity during calcium loading of native and ryanodine-treated heavy sarcoplasmic reticulum vesicles. Treatment with 1 μM ryanodine was performed in 0.6 M KCl. Standard medium with cold calcium and radioactive calcium, as control, was used for loading. The momentary uptake activity was initiated by adding 0.2 mM $^{45}CaCl_2$ + 4 mM K-phosphate (pH 7.0) at the times given on the abscissa to the uptake medium containing 10 μM cold calcium. The momentary rates were calculated from the uptake of ^{45}calcium determined 20, 40 and 60 s after the start of the reaction and are given on the ordinate. (●) Native vesicles, (○) ryanodine-treated vesicles.

vesicles as controls.) The increase in the momentary rate of calcium uptake with time is difficult to reconcile with a constant fraction of calcium-storing vesicles in the preparation. This fraction should, furthermore, be smaller in ryanodine treated than in native preparations which, however, contradicts the observed augmentation of the momentary uptake rate in the presence of ryanodine. Yet, one might envisage that this behaviour results from a more remote complication, the formation of calcium phosphate nucleii in the lumen of the light vesicular fraction during loading favouring subsequent calcium phosphate precipitation and thus accelerating calcium uptake [13]. The higher momentary rates of calcium uptake of ryanodine-treated preparations would then be related mainly to the fact that the phosphate level of the loading medium is raised more steeply by ryanodine treated than by native preparations. This mechanism would underline the role of small changes in the concentration of phosphate for calcium storage. As illustrated in Fig. 6B. The calcium uptake of ryanodine-treated heavy vesicles as well as of heavy and light vesicles is augmented already at quite low concentrations of phosphate. The effect of phosphate can be accentuated in these experiments by reducing the concentration of ATP for driving calcium loading (not shown). Phosphate similarly activates calcium uptake by light vesicles (Fig. 6C). The contribution to calcium uptake of the light vesicular fraction as present in heavy preparations (25%) can be calculated from the results of Fig. 6B and Fig. 6C. The data compiled in Fig. 11 show that light and heavy vesicles compete for medium calcium. In the initial phase of uptake calcium is stored only by the light component. After about 3 min uptake by the heavy fraction speeds up and after 10 min calcium is equally distributed between the two fractions. Thus even after having taken into account the considerable contribution of the relatively small fraction of the light vesicular component to calcium storage, the resulting large residual uptake is partaken by the heavy fraction indicating that the ryanodine-reacted channels cannot have remained open. This result is further strengthened when calcium uptake of na-

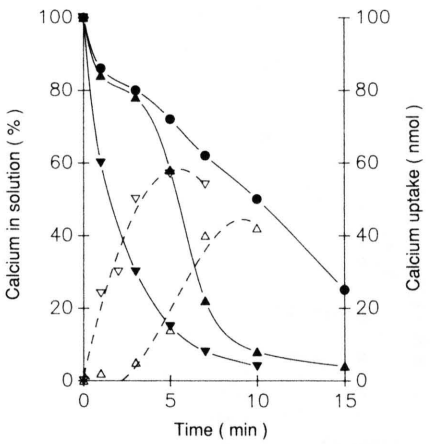


Fig. 11. Contributions to total calcium uptake of light and heavy vesicular fractions contained in the preparation. Calcium uptake was measured under standard conditions as calcium removal from the solution. (▼) Native heavy vesicles, 0.2 mg·ml^{-1}; (▲) heavy vesicles treated with 1 μM ryanodine in 0.6 M KCl, 0.2 mg·ml^{-1}; (●) native light vesicles, 0.05 mg·ml^{-1}. The contribution to calcium uptake of the channel bearing fraction in the heavy vesicular preparation was calculated under the assumption that the preparation was contaminated by 25% of light vesicles and that the concentration of calcium in the media did not affect uptake activity. (▽) Calcium uptake by the native heavy fraction; (△) calcium uptake by the heavy fraction after treatment with 1 μM ryanodine.

tive and ryanodine-treated vesicles are compared at low protein concentrations (0.02 mg·ml^{-1}). Under these conditions the phosphate concentration in the assay remains nearly all the time (10 min) under the limit where uptake by the light fraction can become significant (0.4 mM). In these experiments calcium uptake of native and ryanodine-treated vesicles proceed with similar rates for quite a long time because of the large calcium supply (Fig. 12). This finding, like the results described above, is not conceivable with the presence of a large fraction of ryanodine-opened vesicles. Calcium uptake impaired by the channel-opening effect of low concentrations of ryanodine can not be compensated by the uptake activity of the small light fraction at the prevailing low phosphate level. Hence we must conclude that caffeine-induced calcium release of ryanodine-treated vesicles is impeded by the combined effect of ryanodine and ATP on the calcium channel bearing heavy vesicles and not by the preferential filling of the light vesicular fraction without channels.

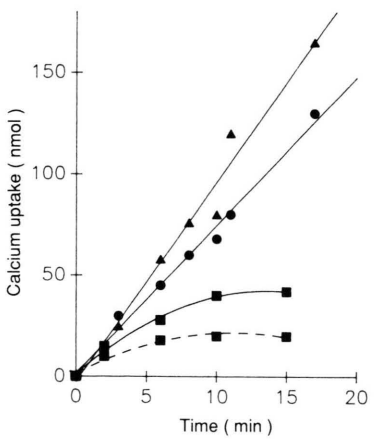


Fig. 12. Calcium uptake of native and ryanodine-treated vesicles at low protein 0.02 mg·ml^{-1} (▲, ●) or light vesicles, 0.005 mg·ml^{-1} (■). Heavy vesicles reacted with 1 μM ryanodine in 0.6 M KCl (▲); native heavy vesicles (●); light vesicles without added phosphate (■– –■); 0.4 mM phosphate added (■—■).

Conclusion

We have shown that our heavy vesicular sarcoplasmic reticulum membrane fraction is contaminated by 20–30% of light vesicles having no ryanodine or ATP-sensitive calcium release channels as the fraction that stores calcium after the preparation had been treated with low concentrations of ryanodine which were known to arrest the calcium release channel in an open state configuration [3, 26]. This fraction of light vesicles only partially accounts for the active uptake of calcium by our heavy vesicular preparation treated with ryanodine when energy is provided by an ATP-regenerating system. Under these conditions calcium uptake always occurs in conjunction with the uptake of comparable amounts of phosphate, whereby the phosphate level attained by ATP hydrolysis suffice to support uptake. The reported close coupling between calcium and phosphate uptake suggests that it might be of great importance for calcium storage *in vivo* by increasing the limited storing capacity of the vesicular calcium-binding proteins [2, 27]. The finding that native preparations actively loaded with calcium under conditions of ATP regeneration are caffeine-sensitive indicates that channel activation by ATP is offset during loading. No direct evidence could be provided for an ATP-induced protein phosphorylation discussed as a possible mechanism for channel closing of native heavy vesicles [23, 24]. The light as well as the heavy fraction of our vesicular preparation treated with low concentrations of ryanodine contribute about equally to calcium uptake supported by an ATP-regenerating system. This finding is considered to result from a slowly proceeding ATP-dependent closing reaction of the ryanodine-opened calcium release channels in the heavy vesicular fraction.

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