Modulation by Monovalent Anions of Calcium and Caffeine Induced Calcium Release from Heavy Sarcoplasmic Reticulum Vesicles

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Sarcoplasmatic Reticulum, Calcium Release, Chloride, Nitrate, Ryanodine

Both calcium and caffeine induced calcium release from actively loaded heavy sarcoplasmic reticulum vesicles were studied to analyze the dependence of both activities on the composition of the release medium with respect to monovalent anions. Calcium is unable to induce net calcium release while caffeine remains effective as releasing agent when the experimental media contain neither chloride nor nitrate ions. Caffeine induced calcium release is not suppressed by chelating residual medium calcium (approximately 0.5–1 μM) with 2 mM EGTA added 15 s prior to 10 μM caffeine. Calcium release from vesicles loaded in media containing 0.2 M gluconate as monovalent anion is induced when the medium is supplemented with chloride or nitrate. The release amplitude increases linearly when K-gluconate is replaced by KCl. At constant ionic strength the release amplitude becomes maximal at a chloride concentration of 0.2 M. The chloride effect completely disappears when 2 mM EGTA are added simultaneously. When chloride is replaced by nitrate, as releasing agent, maximal release is achieved already by addition of 0.1 M K-nitrate. The releasing effect of nitrate can only partially be suppressed by EGTA. The different effectiveness of gluconate, chloride and nitrate as calcium release supporting ions corresponds to their activating effect on the binding of ryanodine to the calcium release channel in the vesicular membranes.

Introduction

The contractile system of muscle is activated by the release of calcium from intracellular stores which are elements of its sarcoplasmic reticulum [1–4]. Various activities of other cells and tissues are controlled, likewise, by the release of calcium, whereby, corresponding intracellular membrane systems function as calcium stores [5]. In contrast to the occurrence of these well established events, the mechanisms by which they are initiated remain to be explained. The close and rapid coupling between the movement of membrane bound charges accompanying membrane potential changes and calcium release in skeletal muscle led to the concept of a direct molecular link between the plasma membrane or its tubular invaginations and the adjacent membranes of the reticular calcium stores [6–8]. The independence of skeletal muscle activity on extracellular calcium is consistent with this concept [9]. In contrast, in other muscles, especially in cardiac muscle, the entry of calcium ions from the extracellular fluid during membrane depolarization seems to be linked rather directly to muscle activation. Since the entering quantities of calcium are not sufficient to saturate the calcium binding sites of the regulatory proteins, it is assumed that the entering calcium ions induce the liberation of larger amounts of calcium from its intracellular stores [10–12]. This mode of activation has been denoted as calcium induced calcium release [13]. Main support for calcium induced calcium release stems from experiments with small fragments of permeabilized cardiac muscle fibers which allow rapid exchange of the calcium concentration in the bath medium [11]. Yet, under in vitro conditions, calcium induced calcium release is not confined to cardiac muscle. It could also be demonstrated in permeabilized skeletal muscle preparations and in experiments performed with isolated membrane fragments of its reticulum [14–16]. Calcium release was monitored as calcium activated tension development of the contractile protein, as liberation of radioactive calcium from preloaded sarcoplasmic reticulum and as changes in absorbance or fluorescence of calcium indicators applied to isolated fibers or to the release medium [17]. Another mode for the activation of the con-
tractile system, which has been discussed more recently, the functioning of IP₃ as calcium releasing agent liberated during activation [18], will not be discussed in this context, because this concept gained only little support in experiments with skeletal muscle preparations [cf. 3]. In this report we focus on the modulation of calcium release from actively loaded heavy sarcoplasmic vesicles isolated from skeletal muscle by the anion composition of the medium. This analysis was prompted when we recognized that nearly all in vitro experiments dealing with calcium release were performed under ionic conditions which are quite different from those prevailing in the myoplasm in the living muscle. The most essential deviation concerns the concentration of the chloride ion, being the main anion in the experimental media, while it is only a minor constituent in most muscle cells. The possible role that anions might play in the mechanism of calcium release had been disregarded in the past although anion specific effects on muscle activation had been reported repeatedly [19–20].

More recently, it has been shown by Delay et al. [21] that anions can affect calcium release in the living fibres by modulating calcium currents and charge movement in the plasma membrane. We applied caffeine as calcium releasing agent in our study because of its well established releasing action and its postulated dependence on the calcium content of the medium and its ionic strength [13, 22]. It is shown here that the removal of chloride ions from the medium results in the nearly complete inactivation of calcium induced calcium release and that, in contrast, caffeine induced calcium release was found to remain fully preserved. This observation indicates that calcium induced calcium release cannot be operative at the low levels of chloride as present in most muscle cells and that caffeine induced calcium release is not directly dependent on the functioning of the calcium induced calcium release mechanism. The latter conclusion could be substantiated by inducing calcium release with caffeine after reducing the residual calcium level in the release medium by previously adding EGTA. The apparent discrepancy between these observations and the findings of Rousseau et al. [23], showing that the isolated calcium channel after being incorporated into black lipid membranes is activated by ionized calcium in the absence of chloride, will be dealt with in the Discussion.

**Materials and Methods**

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hind leg muscles as described by Hasselbach and Migala [24]. Calcium uptake and release experiments were performed as described by Su and Hasselbach [22]. The standard medium contained 0.1 mM K-glucanate, 0.1 mM sucrose, 20 mM K-Mops, pH 7.0, 5 mM K- Na-ATP, 3 mM Mg-gluconate, 20 μM CaCl₂, 8 mM phosphoenolpyruvate and 0.04 mg·ml⁻¹ phosphoenolpyruvate kinase and 0.2–0.4 mg·ml⁻¹ vesicular protein. Changes in medium composition are indicated in the legends or in the text. Calcium uptake was started by adding the vesicular protein to the assay. When acetylphosphate instead of ATP was used as energy donor for calcium loading, the medium was supplemented with 5 mM AcP and 3 mM Mg-gluconate. Loading was performed at 30 °C for 10 min followed by cooling to 20 °C. Subsequently, additions were made as described in the legends. For following uptake and release of radioactively labelled calcium, the reaction was interrupted at different times by filtration, using Schleicher and Schuell BA filters 0.45 μm. ⁴⁵Ca was measured by liquid scintillation counting of the filtrate. An ion sensitive electrode from Orion was used to register net calcium movement during uptake and release. Electrodes were used which responded with a potential change of 20–28 mV per concentration decade.

Ryanodine binding to the vesicles was performed at room temperature in solutions containing 0.3 mM sucrose, 0.1 mMimidazole sulfate, 0.1 mM calcium, 1 mg/ml vesicular protein [24]. A salt content of 0.6 M was adjusted by mixing either KCl or KNO₃ with K-glucanate in different ratios as given in the figures. The binding reaction was conducted at a [³H]ryanodine concentration of 50 nM. Unspecific binding was determined in the presence of 2 mM EGTA and subtracted from the binding value obtained in the presence of calcium. The ryanodine reacted vesicles were separated by filtration from the medium and subsequently rinsed with cold medium [24, 25]. 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.). 45CaCl2 and [3H]ryanodine were supplied by Buchler & Co, Frankfurt/Main (F.R.G.).

Results

In order to register quantitatively calcium induced calcium release in ATP containing media it is necessary to measure net calcium release either by photometric or potentiometric methods. By measuring net increases of the concentrations of calcium in the medium, one avoids that an elevated turnover of calcium in ATP containing media, initiated by calcium addition is taken for a release of calcium when calcium movements are followed with radioactively labeled calcium. This advantage, however, limits the possibility to vary the ionic conditions in the release medium. In this study we, at first, measured calcium release with a calcium selective electrode which allows to follow slow calcium movements as they occur under the prevailing conditions. Fig. 1A demonstrates the time course of calcium uptake and release in an experimental medium containing KCl (0.1 M) as the major salt and K-Pipes (20 mM) as buffer, supplemented with 8 mM phosphoenolpyruvate and pyruvatekinase (0.04 mg·ml⁻¹) for ATP regeneration. Uptake of 20–40 nmol calcium·ml⁻¹ by 0.4 mg vesicular protein·ml⁻¹ is complete in about 2–3 min as it was observed under very similar conditions when the removal of 45Ca from the medium was followed with the filtration method. As reported previously, the addition of 1–2 nmol·ml⁻¹ calcium induces a release of 5–10 nmol·ml⁻¹ calcium which are quite rapidly reaccumulated [25]. Subsequent addition of caffeine (10 mM) releases a similar quantity of calcium which is reaccumulated only slowly and sometimes incompletely. Amount and time course of caffeine induced calcium release are in agreement with the result of corresponding experiments performed with 45Ca. When chloride as major anion is replaced by gluconate, calcium becomes ineffective as releasing agent while caffeine remains fully effective (Fig. 1B). The same results were obtained when K-succinate was used instead of K-gluconate (not shown). The simultaneous exchange of Pipes by Mops is of no importance for the abolition of calcium induced calcium release (Fig. 1C). The finding that caffeine induced calcium release still functions when calcium induced calcium release is abolished indicates that the presence of calcium ions in the medium are not necessarily required for caffeine induced calcium release. This conclusion could in fact be substantiated by experiments in which we monitored caffeine induced calcium release from vesicles actively loaded with 100 nmol·mg⁻¹ radioactive calcium. AcP was used as energy yielding substrate in order to achieve a constant loading of 95 nmol·mg⁻¹ and to be able to manipulate freely the composition of the media. In order to induce calcium release, the medium has to be supplemented by ATP. Calcium removal from the medium was achieved by addition of EGTA. As shown in Fig. 2A a slow calcium efflux is induced when 2 mM EGTA was added together with 4 mM ATP resulting in a free ATP concentration of 1.2 mM. When 15 sec after the induction of this efflux caffeine (10 mM) was added a fast calcium release is induced amounting to 50% of the initial load. It can certainly be assumed that the interval of 15 sec between the addition of EGTA and caffeine, suffices to completely chelate the residual calcium. The release amplitude is reduced when less ATP is added leaving an excess of 1 mM magnesium (Fig. 2B).

The direct involvement of chloride ions in calcium induced calcium release can be recognized most easily when after calcium loading in media of low salt content, the salt concentration is suddenly raised. Fig. 3A demonstrates that the addition of 0.2 M K-gluconate induces only a small release of calcium, which could further be diminished when 2 mM EGTA was added simultaneously. 0.2 M K-Hepes is, likewise, ineffective in supporting calcium release. In contrast, the release becomes large, 40–50 nmol/mg, when the concentration of KCl is raised to 0.2 M KCl. EGTA also reduces this release to about 5–10 nmol·mg⁻¹. The release amplitude increases steadily when ion exchange is performed at a constant ionic strength of 0.2. Osmotic effects that might possibly be involved in this release are considered in the Discussion. The notion that we are dealing with a specific anion effect is supported by the finding shown in Fig. 3B that the replacement of gluconate by nitrate already augments calcium release at a lower degree.
of ion substitution. The release amplitude reaches its maximum at a nitrate concentration of 0.1 M. It is interesting that the releasing effect of nitrate is reduced only little by calcium removal, indicating that its effect, in contrast to that of chloride does not require the presence of calcium ions. These findings suggest that the monovalent anions interfere with the reaction sequence leading to calcium release from the terminal cysternae (compare Discussion). An involvement of the calcium channel protein itself is indicated by the observation showing that the interaction of the calcium release channel protein with its most specific modifier, ryanodine, is activated by the two ions in parallel.
Fig. 2. Caffeine induced calcium release from heavy sarcoplasmic reticulum vesicles in the absence of free calcium. Calcium loading was performed in media containing 3 mM ACP, 3 mM Mg-gluconate, 0.1 mM K-gluconate, 20 mM K-Mops, pH 7.0, 0.1 mM sucrose at 30 °C for 10 min. Subsequently the temperature was lowered to 20 °C. 2 mM EGTA was added to remove medium calcium simultaneously with 4 mM (Fig. 2A) or 2 mM ATP (Fig. 2B). 15 s later 10 mM caffeine were added (●). Calcium release was interrupted by filtration at times given on the abscissa. No caffeine was added to the controls (▲).

Fig. 3. Anion induced calcium release from heavy sarcoplasmic reticulum vesicles. 0.2 ml·ml⁻¹ heavy sarcoplasmic reticulum vesicles were loaded for 10 min in media containing 5 mM ATP, 3 mM Mg-gluconate, 0.1 mM sucrose, 20 mM K-Mops, pH 7.0, 8 mM phosphoenolpyruvate, 0.04 mg·ml⁻¹ pyruvate kinase. Calcium release was induced by stepwise exchanging 0.2 mM K-gluconate by KCl or KNO₃ (●). The sucrose concentration was kept constant. (A) 0.2 mM K-gluconate was exchanged for KCl the concentration of which is given on the abscissa. (B) K-gluconate was exchanged for KNO₃ (●). EGTA was added together with KCl or KNO₃ at a final concentration of 2 mM (▲).
KCL, KN03 (M)

Fig. 4. Anion specific activation of ryanodine binding to heavy sarcoplasmic reticulum vesicles. Heavy sarcoplasmic reticulum vesicles were labelled with 50 nM $[^3]$H]ryanodine for 12 h in media containing 0.6 M salt, 0.1 mM CaCl$_2$, 50 mM imidazole sulfate, pH 7.0, 1 mg·ml$^{-1}$ protein. K-gluconate was stepwise exchanged for KCl (□) or KN03 (○). Note, that ryanodine binding needs higher concentrations of chloride and nitrate for saturation than calcium release.

to their effect on calcium release. The results collected in Fig. 4 demonstrate that at a constant ionic strength of 0.6, ryanodine binding is activated by both ions, yet, considerably more effectively by nitrate than by chloride. Ion effectiveness thus increases in the order gluconate, chloride, nitrate which corresponds to the lyotropic ion series.

Discussion

The results furnished by this study demonstrate a close relationship between calcium induced calcium release from actively loaded heavy sarcoplasmic reticulum vesicles and the composition of the release medium with respect to its ionic components, especially, its monovalent anions. By comparing the effect which different monovalent anions exerts on calcium induced calcium release, a specific activating effect of chloride and its congeners could be ascertained. Our conclusion that we are dealing with a specific interaction between the respective monovalent anions and the calcium release channel gained further support by our finding that the interaction of the channel protein with ryanodine exhibits the same anion specificity. It has been demonstrated previously that considerable quantities of calcium can quite rapidly be released from passively, as well as, actively loaded sarcoplasmic reticulum preparations under quite a variety of conditions by elevating slightly the calcium level in the medium [26–31]. Yet, the phenomenon has been discussed rather controversially concerning its physiological significance. Fabia-to [11] gathered considerable experimental support for his view that calcium release from the sarcoplasmic reticulum induced by calcium influx from the extracellular fluid during membrane depolarization activates the contractile system in the living cardiac muscle. This activation modus corroborates with the well known fact that cardiac activity depends strictly on the presence of calcium ions in the extracellular fluid. Calcium induced calcium release has been proposed also to be involved in skeletal muscle activation since it could be demonstrated to function in in vitro preparations as isolated sarcoplasmic vesicles or skinned fibers [27–29]. However, the ionic conditions as they prevail in the living skeletal muscle seem to impede calcium induced calcium release. The relatively high intracellular magnesium concentration as well as the apparent high calcium threshold of the release mechanism were considered as the main obstacle, further nurished by the fact that skeletal muscle activity only little depends on medium calcium [27]. The main reason for the rather confusing situation must be sought in the fact that calcium induced calcium release can be activated or inhibited under a great variety of conditions which often appear difficult to reconcile with any concept. For instance, calcium induced calcium release from passively loaded vesicles is completely suppressed by the presence of 0.1 mM free magnesium in the absence, as well as, in the presence of ATP [30]. In contrast calcium release from vesicles loaded and suspended in ATP-containing media can be induced by calcium even if the free magnesium concentration exceeds 0.1 mM [27, 28]. Furthermore, caffeine claimed to modulate calcium induced calcium release which has been reported to be effective only in the presence of ATP after active loading [22], can evidently activate the calcium channel in black lipid membranes by increasing its open time probability even in the absence of ATP [31, 32]. These quite different but rather specific effects

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of the channels ligands are superimposed by less specific effects exerted by the media's ion constituents. In our context, we have not to consider calcium release as it is induced when less permeant ions are exchanged for more permeant ones, assumed to cause depolarization of the T-tubular membranes [33]. Our preparations have been washed extensively with 0.6 mM KCl in order to separate the small T-tubular vesicles from the large vesicles derived from the terminal cisternae [34]. The preparation thus consists mainly of terminal cisternae membranes into which the calcium release channels are embedded. The absence of T-tubular membranes is in agreement with the observation that the calcium dependent release observed with this preparation is not affected by 0.1 mM ouabain added to block the Na-K-pump in the T-tubular membranes, the activity of which has been suggested to create membrane polarization and via Na-Ca exchange, calcium accumulation inside the T-tubular vesicles (not shown). Our initial observation that calcium induces calcium release in chloride containing media but not in media supplemented with gluconate as major anion, cannot be explained by membrane depolarization. A potential change cannot arise by the addition of 2–4 μM calcium to the ATP-containing medium, in neither the presence of 0.1 mM KCl or 0.1 mM K-gluconate. Even if there should exist a membrane potential in the presence of the less permeant gluconate ions (cf. Hasselbach and Oetliker [35]), it should be changed neither by the addition of calcium nor of caffeine.

The inactivation of the calcium induced calcium release mechanism by the absence of chloride, with or without ionic compensation cannot be referred to the possibility that the calcium pump might remove more rapidly calcium from the medium in the absence of chloride than in its presence. The small differences of calcium uptake activity observed in control experiments, however, cannot account for the nearly complete abolition of the releasing effect of calcium. The inactivation of the calcium induced release mechanism can also not be explained by the observed differences in the residual calcium level in the medium at the end of calcium loading. Usually the residual total calcium concentrations are higher in chloride (1 μM) than in gluconate (0.5 μM) containing medium [cf. 22]. The opposite would be conform with the assumption that high residual calcium might counteract calcium induced calcium release. It can, however, not be excluded that a small release of calcium might persist which cannot be detected. Since an elevation of the calcium concentration in the gluconate uptake medium after cessation of calcium uptake by the addition of 4 nmol·mg⁻¹ is not registered by the electrode, a release of this quantity must be considered as the limit of detection. This is in agreement with observations of Mori et al. [26] who used arsen-azo III to monitor calcium release. The results demonstrating considerable differences in the magnitude of the calcium release amplitude when calcium release was initiated by addition of either chloride or gluconate to an uptake medium of low salt content agree with the assumption of an anion specific activation of the release mechanism. Chloride induced usually a three times greater release than gluconate. The fact that the amplitude of calcium release was reduced to the same low level under both conditions when the salts were added together with 2 mM EGTA indicates the involvement of calcium ions, on the one hand, and on the other hand, excludes that the addition of salts might have caused calcium release by shrinkage or swelling of the preloaded vesicles. If such a mechanism would be effective alone, calcium release should not be reduced by EGTA. A similar reduction of calcium release by the simultaneous addition of salt and EGTA was observed by Stephenson, who studied calcium release from skinned fibers and tried to separate the occurrence of diffusion potentials from calcium specific and osmotic effects, as they might contribute to chloride induced calcium release [33]. In our experiments, osmotic effects might account for the calcium insensitive release of 7 nmol·mg⁻¹ occurring in the absence or presence of chloride. Anion specificity is further stressed by our observation showing that the residual calcium independent release was much greater when nitrate instead of chloride was applied. The fact that the releasing effect of nitrate is much less calcium sensitive than that of chloride goes along with its more powerful releasing action, as indicated by the fact that the release amplitude becomes maximal already at 0.1 mM nitrate as compared to 0.2 mM required for chloride. The order of the effectiveness of the anions parallels the lyotropic series. The idea that we are dealing with an anion specific effect is sup-
ported also by our observation that the known activation of the reaction between the calcium release channel protein in the membrane with its most specific modifier, ryanodine, exhibits the same anion specificity. These findings suggest that the anions directly affect the calcium channel's protein. Yet, this appears to apply only as long as the channel protein is in its natural membrane surrounding. We observed that the anion specific activation of ryanodine binding completely disappears after the channel protein has been isolated (not shown). This loss of anion sensitivity suggests an explanation for the finding of Rousseau et al. [23] showing that the calcium release channel in black lipid membranes can be activated by ionized calcium in the presence of high concentrations of Hepes-buffer alone. However, the anions appear not to interact with the reaction directly coupled to calcium release as it emerges from our finding that the caffeine induced calcium release can take place irrespectively of the anion composition of the medium. As shown by Su and Hasselbach [22] caffeine induced calcium release is augmented when the ionic strength of the medium was raised, but no further anion specific activation of the release has been observed here, where the release was induced by high concentrations of caffeine (10 mM). At low, non saturating concentrations of caffeine, however, a distinct anion specific activation of caffeine induced calcium release has been observed. Nitrate appears to increase significantly the receptors affinity for caffeine. The different action mode of caffeine and anions is stressed also by the finding that caffeine induced calcium release persists after calcium has been removed completely from the medium. Effects of residual calcium were excluded by adding EGTA (2 mM) 15 s prior to caffeine. Even if calcium binding by EGTA should proceed slowly in media of physiological pH, the binding reaction should be completed. This finding agrees with the observations made by Rojas and Jaimovich [36] in control experiments of their studies on the IP$_3$ induced calcium release from frog muscle fibers performed in the presence of glutamate as major anion. These observations are at variance with the notion that caffeine sensitizes the calcium release channel für the releasing effect of calcium or, vice versa, that calcium sensitivity of the calcium channel is increased by caffeine. The concept of calcium or caffeine sensitization was derived from experiments in which calcium release was monitored indirectly by measuring force development or fiber shortening [26, 3]. The possible interrelation between calcium independent caffeine induced activation of the calcium release channel in black lipid membranes observed by Sipesan and Williams [31] and the results of our release experiments made with native vesicles in ATP-containing media needs further evaluation.

The reason for overlooking the specific role of chloride ions for calcium induced calcium release might be seen in the fact that nearly all experiments dealing with calcium induced calcium release were mostly done in media containing quite high concentration of chloride and the effect of the ion specificity was not explored [39]. For instance, in the experiments of Fabiato the ionic strength of the medium imitating the intracellular fluid was adjusted with KCl [11, 12]. In most studies performed with isolated vesicles or the receptor complex, 0.1 mM KCl was used as the main ionic constituent [38]. The results obtained under these conditions are difficult to apply to living muscle fibers or cells. Only in a few muscles is the intracellular chloride concentration high enough to activate calcium induced calcium release [40, 41]. Yet, the role of chloride could be integrated into the concept of calcium induced calcium release by assuming that the calcium release channel in the terminal cysternae might be in contact or get into contact during excitation with extracellular chloride.

Acknowledgements

We are grateful for the support provided by the Max-Planck-Society after the retirement of W.H. We are indebted to Mr. A. Pfandke for his permanent help in preparing the sarcoplasmic reticulum vesicles.