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Rapid Letter

Free Radical-Dependent Ca^{2+} Signaling: Role of Ca^{2+}-Induced Ca^{2+} Release

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ABSTRACT

Previously we have shown that Fe^{3+}/ascorbate-induced Ca^{2+} release from scallop sarcoplasmic reticulum (SR) is due to Ca^{2+}-channel gating by free radicals. This study is aimed at demonstrating that Ca^{2+}-induced Ca^{2+} release (CICR) plays a role in this kind of Ca^{2+} release. Scallop SR vesicles were incubated with fluo-3 and exposed to Fe^{3+}/ascorbate. Fluorimetric recordings showed massive Ca^{2+} release, with maximum rate and 50% release occurring at 30 min after exposure. Conversely, the use of the probe for reactive oxygen species dihydrorhodamine or the assay of malondialdehyde allowed oxyradical production to be traced for ~5 min only. Hence, although Ca^{2+} release started just after exposure to Fe^{3+}/ascorbate, most release occurred after free radical exhaustion. Ruthenium red addition after Fe^{3+}/ascorbate slowed down the Ca^{2+} release, whereas cyclic adenosine 5′-diphosphoribose addition accelerated it, indicating that the free radical-induced Ca^{2+} release from SR vesicles triggers a mechanism of CICR that dramatically increases the initial effect. Antioxid. Redox Signal. 3, 525–530.

INTRODUCTION

It has been shown in various studies that reactive oxygen species can impair intracellular Ca^{2+} homeostasis, but also induce Ca^{2+} signaling when occurring at subtoxic levels (21, 22). In this context, an intensively studied system has been the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle, where oxyradicals, as well as other kinds of free radicals, promote the gating of ryanodine-sensitive SR Ca^{2+} release channels (8, 20). Such a redox sensitivity is due to the presence on SR Ca^{2+} channels of a number of functional sulfhydryls that regulate their closed or open state (23). It has been shown that sulfhydryl oxidation activates the Ca^{2+} release mechanism, whereas reducing agents close down the Ca^{2+} channels (2, 15, 19).

Another important feature in the functioning of the SR Ca^{2+} channel is its sensitivity to Ca^{2+}, which is linked to the well known process of Ca^{2+}-induced Ca^{2+} release (CICR) (9, 12, 17). Such a mechanism is generally known to amplify Ca^{2+} signaling, but it could also enhance the effects of other agents promoting Ca^{2+} release.

In a previous study, we reported that hydroxyl radicals generated by iron/ascorbate

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produced massive Ca^{2+} release from scallop SR vesicles (*Pecten jacobaeus*) (4). Such an effect was significantly decreased by oxyradical scavengers, such as catalase, reduced glutathione, and the specific hydroxyl radical scavenger α-keto-γ-methylbutyric acid, and by the Ca^{2+}-channel blocker ruthenium red (RR), showing that iron/ascorbate caused a free radical-dependent opening of SR Ca^{2+} channels (4). In these experiments, the Ca^{2+} release rate showed a sigmoid trend, suggesting the occurrence of a self-sustained process triggered by free radical production. Moreover, in other previous studies we have shown typical ryanodine receptor (RyR) responses in scallop SR vesicles, also suggesting the occurrence of CICR activity (3, 18). Therefore, we have assumed here that CICR could act as a sustaining process for the Ca^{2+} release induced by free radicals after exposure to iron/ascorbate. To confirm this hypothesis, we have compared the timing of free radical production to that of Ca^{2+} release in the presence of Fe^{3+}/ascorbate. In addition, by adding RR or cyclic adenosine 5′-diphosphoribose (cADPR) after Fe^{3+}/ascorbate, we have also tried to point out Ca^{2+}-channel opening well beyond the end of free radical production.

MATERIALS AND METHODS

Chemicals

ATP, RR, and cADPR, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The fluorescent probes fluo-3 and dihydrorhodamine 123 (DHR) were from Molecular Probes Inc. (Eugene, OR, U.S.A.). All other reagents were of analytical grade. Fe^{3+} was used as ferric chloride.

SR isolation and fluorimetric measurements of Ca^{2+} and oxyradicals

SR-enriched 20,000 *g* fractions were prepared from homogenates of scallop (*Pecten jacobaeus*) adductor muscle and incubated at room temperature in the presence of 4 m*M* ATP and 2.5 μM fluo-3, according to Burlando *et al*. (4). Free Ca^{2+} variations in the incubation medium were recorded with a Perkin Elmer LS 50B spectrofluorimeter (excitation at 510 nm, emission at 530 nm). Ca^{2+} probe calibration was performed according to Minta *et al.* (16) at the end of each experiment. Assessment of oxyradical production was achieved by incubating SR vesicles with 3.2 μM DHR, according to Abele *et al.* (1) (excitation at 505 nm, emission at 534 nm).

Malondialdehyde (MDA) assay

SR vesicles were incubated with Fe^{3+}/ascorbate for different times. The reaction was stopped with 1 volume of acetonitrile, centrifuged at 6,000 *g* for 20 min at 4°C, and run on a Waters HPLC carbohydrate column. Peak absorbance was monitored at 268 nm by diode array detector (Waters SpA, Milan, Italy). The elution buffer contained 30 m*M* Tris-HCl, pH 7.4/acetonitrile (9:1), and 0.1 m*M* H_2O_2 to avoid interference of contaminating ascorbate peaks.

Protein assay

The protein content of the 20,000 *g* supernatant was assayed by the method of Hartree (10), using bovine serum albumin as standard.

Statistics

Data were analyzed by the Systat 8.0 software (SPSS Inc., Evanston, IL, U.S.A.).

RESULTS AND DISCUSSION

As mentioned above, we previously showed the occurrence of typical RyR Ca^{2+} dynamics in scallop SR (18). In the present study, we first wanted to obtain direct evidence of CICR, before trying to establish a correlation between SR response to Fe^{3+}/ascorbate and CICR. An experiment was therefore accomplished, where fixed aliquots of Ca^{2+} were added to SR vesicles under control conditions, or in the presence of the Ca^{2+}-channel blocker RR (Fig. 1). This also showed Ca^{2+} dynamics in the absence of perturbing factors, such as Fe^{3+}/ascorbate. As already observed using this experimental system (3, 4, 18), Ca^{2+} addition to the incubation medium produced a sudden fluorescence peak that was followed by a decrease down to basal values within a few minutes, due to the

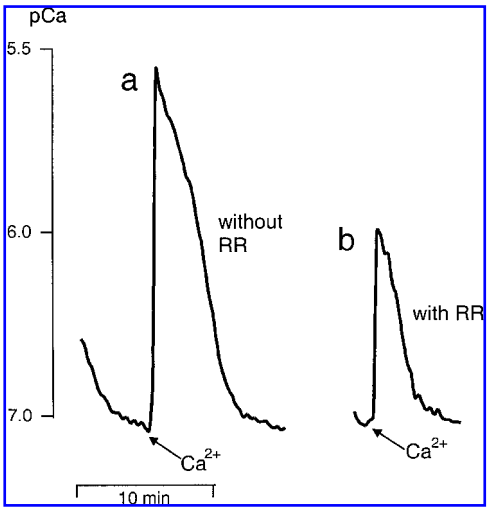

FIG. 1. Fluorimetric recordings showing free Ca^{2+} variations after incubation of SR vesicles from scallop adductor muscle (1 mg of protein) with 4 mM ATP and 2.5 μM fluo-3 (final volume, 2 ml). (**a**) Addition of 100 nmol of Ca^{2+} results in free [Ca^{2+}] rise followed by a decrease due to Ca^{2+} sequestration into SR vesicles. (**b**) In the presence of 5 μM RR, an equivalent Ca^{2+} addition results in a much lower free [Ca^{2+}] rise. In this and the following figures, traces are representative of at least five experiments.

Ca^{2+} sequestering activity of the sarcoplasmic-endoplasmic Ca^{2+}-ATPase pump. However, after calibration of the probe fluorescent signal, the resulting peak of free Ca^{2+} was 3,122 ± 1,022 pmol/ml ($n = 9$) in the control and 984 ± 165 pmol/ml ($n = 12$) in the presence of 5 μM RR ($p < 0.001$). The higher free Ca^{2+} rise observed in controls, compared with RR-exposed vesicles, can be explained as deriving from the sum of external Ca^{2+} addition plus a process of CICR elicited in SR vesicles by added Ca^{2+} itself. By contrast, in the presence of RR the mechanism of CICR does not occur due to the blockage of Ca^{2+} channels.

Another set of experiments was aimed at investigating the effects of oxyradicals by using Fe^{3+}/ascorbate as a redox cycling model system (4). To point out the timing of free radical production in our experimental system, SR vesicles were incubated with DHR, a fluorescent probe for reactive oxygen species (7, 11), and then exposed to 25 μM Fe^{3+}/100 μM ascorbate. The DHR signal showed initially a slight basal slope, which suddenly increased upon Fe^{3+}/ascorbate addition. Such a fluorescence rise lasted for ~70 s and was eventually followed by a recovery of the basal slope (Fig. 2a). We also checked for lipid peroxidation rates by measuring MDA production at 0, 5, 10, 20, 300, and 3,600 s after incubation of SR vesicles with Fe^{3+}/ascorbate. The regression curve calculated from MDA levels against time showed saturation kinetics, characterized by a t_{50} of 13.3 ± 2.6 s (Fig. 2b) and by a plateau at ~5 min. Moreover, a second addition of Fe^{3+}/ascorbate after 30 min of incubation led to further MDA production (Fig. 2b), indicating that the kinetics of MDA production was not related to a depletion of membrane unsaturated fatty acids, but rather to the timing of free radical

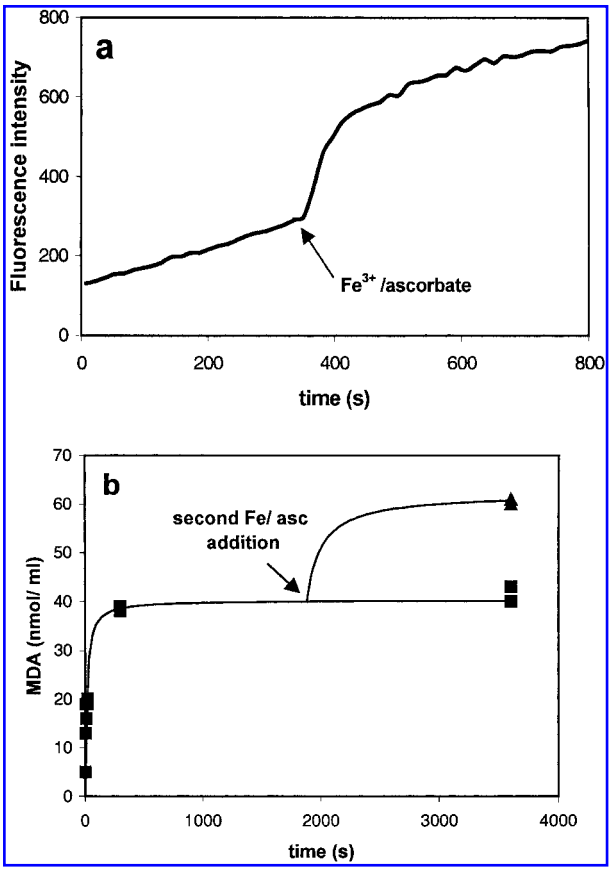

FIG. 2. Detection of free radical production after SR treatment with 25 μM Fe^{3+}/100 μM ascorbate. (**a**) SR incubation with the probe DHR and fluorimetric recording show initial basal fluorescence signal, sudden fluorescence rise upon Fe^{3+}/ascorbate addition, and basal slope recovery after ~70 s. (**b**) MDA evaluation obtained by HPLC assay at different times after SR exposure to Fe^{3+}/ascorbate. The nonlinear regression curve fitting experimental points shows saturation kinetics, reaching a plateau after ~5 min ($R^2 = 0.98$). A second Fe^{3+}/ascorbate addition at 1,800 s results in further MDA production, as detected at 3,600 s. Note that the upper curve in (b) is hypothetical.

TABLE 1. SUMMARY STATISTICS OF SR Ca^{2+} RELEASE RATES INDUCED BY 25 μM Fe^{3+}/100 μM ASCORBATE, FOLLOWED OR NOT BY ADDITION OF 20 μM cADPR

	$t(v_{max})$	t_{50}	v_{max}
No cADPR	1,768 ± 550	1,767 ± 402	16.0 ± 7.7
With cADPR	1,534 ± 533*	1,508 ± 326*	16.5 ± 6.6

$t(v_{max})$, time of maximum Ca^{2+} release rate (s); t_{50}, time of 50% [Ca^{2+}] rise (s); v_{max}, maximum Ca^{2+} release rate (pmol/s/mg of protein).
*$p < 0.01$, paired t test, $n = 5$ parallel runs.

formation. Hence, data from DHR fluorescence and from MDA assay consistently indicate that in our experimental system oxyradical production lasted for no longer than 5 min after treatment with Fe^{3+}/ascorbate.

To investigate the timing of the free-radical induced Ca^{2+} release, SR vesicles were incubated with fluo-3 and exposed to 25 μM Fe^{3+}/100 μM ascorbate. Five minutes after treatment, *i.e.*, concomitantly with the observed extinction of free-radical generation, the Ca^{2+} release rate was 0.74 ± 0.47 pmol/s/mg of protein, whereas the percentage of total [Ca^{2+}] rise was only 0.9 ± 0.4% ($n = 7$). Thereafter, the Ca^{2+} release increased exponentially until ~30 min after treatment, when both maximum Ca^{2+} release rate and 50% of total [Ca^{2+}] rise occurred (Table 1). Hence, the maximum effect in terms of Ca^{2+} release was observed several minutes after the exhaustion of free radical production.

We also tried to modulate the Ca^{2+} release by using compounds acting on SR Ca^{2+} channels. Addition of 15 μM RR at ~20 min after Fe^{3+}/ascorbate resulted in a significant slowing down of the Ca^{2+} release rate (Fig. 3), confirming an involvement of Ca^{2+} channels in the exponential Ca^{2+} release occurring beyond the end of free radical production. In addition, the use of 20 μM cADPR after 5 min of the Fe^{3+}/ascorbate treatment significantly reduced the time of maximum Ca^{2+} release rate and the time of 50% [Ca^{2+}] rise, whereas the value of maximum Ca^{2+} release rate was not modified (Table 1). The average time shifts in Ca^{2+} release induced by cADPR were slight, but they nonetheless were highly significant according to paired t test, as data derived from pairs of parallel experiments. Considering that cADPR is a CICR modulator that has been found to act also in the scallop SR (18), this latter result supports the assumption that CICR plays a role in the Ca^{2+} release triggered by free radicals. The unchanging maximum Ca^{2+} release rate in the presence of cADPR is quite in line with this view, as it suggests that cADPR itself did not increase SR permeability to Ca^{2+}, but instead it quickened the Ca^{2+}-channel recruitment typical of CICR.

In summary, our previous and present data indicate that SR exposure to a free radical pulse lasting a few minutes is able to induce Ca^{2+} release through Ca^{2+}-channel oxidation. This produces a limited Ca^{2+} release rate, which is nevertheless able to trigger CICR-dependent Ca^{2+}-channel recruitment, eventually leading to exponentially increasing Ca^{2+} release through a self-sustained process. Hence, although free radical production provides the initial stimulus, the mechanism of CICR seems actually responsible for most of Ca^{2+} loss from SR vesicles.

Ca^{2+}-channel alterations in striated muscle cells have been reported as the source of different diseases (14). For instance, malignant hyperthermia has been related either to free radical-induced Ca^{2+} homeostasis impairment (6) or to RyR alteration causing excessive CICR stimulation (24). Evidence from our data suggests a unifying explanation for the above pathological conditions in terms of CICR am-

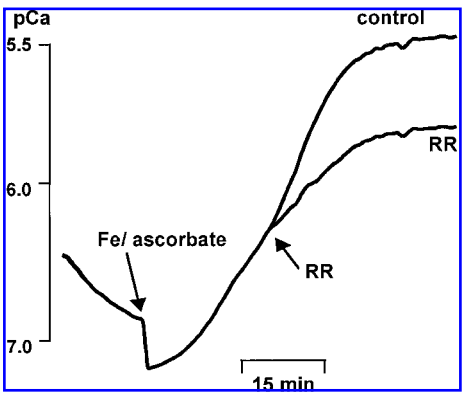

FIG. 3. Modulatory effect of RR on Fe^{3+}/ascorbate-induced Ca^{2+} release. SR exposure to 25 μM Fe^{3+}/100 μM ascorbate elicits a sigmoid Ca^{2+} release (control). Addition of 15 μM RR after Fe^{3+}/ascorbate results in a considerable reduction of Ca^{2+} release compared with control.

plification of oxyradical-dependent Ca^{2+} release. Moreover, oxyradical production (5) and Ca^{2+}-dependent cytotoxicity (13) have been reported as common pathways for the cellular effects of environment contaminants. Therefore, it can also be speculated that, at least in some cases, CICR could mediate the effects of oxidative stress triggered by prooxidant compounds. As a corollary, CICR is generally known as a pathway for Ca^{2+} signaling amplification, yet evidence is accumulating that such a mechanism of intracellular Ca^{2+} release could also increase the sensitivity of specific cell types to oxidative stress.

ACKNOWLEDGMENTS

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ABBREVIATIONS

cADPR, cyclic adenosine 5′-diphosphoribose; CICR, Ca^{2+}-induced Ca^{2+} release; DHR, dihydrorhodamine 123; MDA, malondialdehyde; RR, ruthenium red; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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