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Sulfhydryl oxidation induces calcium release from fragmented sarcoplasmic reticulum even in the presence of glutathione

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Abstract. Alcian blue and plumbagin induced transient Ca^{2+} release from fragmented sarcoplasmic reticulum. Dithiothreitol (DTT) and glutathione (GSH) partially blocked Ca^{2+} release induced by these oxidizing compounds. Pretreatment of alcian blue and plumbagin with DTT or GSH for more than 1 min was required to abolish the ability of the oxidizing compounds to release Ca^{2+}. Mg^{2+} and ruthenium red completely blocked alcian blue- and plumbagin-induced Ca^{2+} release. These results suggest that oxidation of sulfhydryls on Ca^{2+} release channels induces Ca^{2+} release even in the presence of GSH in situ.
Key words. Sarcoplasmic reticulum; Ca^{2+} release; sulfhydryl oxidation; alcian blue; plumbagin.

In skeletal muscle, depolarization of transverse tubular membrane causes Ca^{2+} release from sarcoplasmic reticulum (SR). The mechanism responsible for triggering Ca^{2+} release from the SR remains unknown[1-3]. Redox reactions are involved in many biological systems, and oxidation compounds such as phthalocyanine dye (alcian blue) and naphtholquinone (plumbagin) induce Ca^{2+} release from the SR[4,5]. Salama's group has purified sulfhydryl(SH)-gated Ca^{2+} release channels with similar characteristics to ryanodine binding protein[6,7]. Since the first report by Abramson et al.[8], many studies have shown that heavy metals (Hg^{2+}, Ag^{+}, and Cu^{2+}) at μM concentrations induce rapid Ca^{2+} release from the SR by binding to or by oxidizing SHs. Therefore, the oxidation-reduction response of SHs in Ca^{2+} release channel protein may play a physiological role in triggering Ca^{2+} release from the SR[9]. However, muscle fibres contain enough endogenous GSH to protect SHs from oxidation[10,11]. Brunder et al.[12] reported that treatment of voltage-clamped cut fibres with 10 mM GSH or DTT did not affect the calcium transient obtained by a brief depolarization, while treatment of SR vesicles with such reducing agents inhibited heavy metal-induced Ca^{2+} release. They refuted the hypothesis that SH oxidation plays a crucial role in excitation-contraction (E-C) coupling in situ. However, SH oxidation might be involved in physiological Ca^{2+} release, if the interaction of oxidants with SHs in the Ca^{2+} release channel was much faster than that of oxidants with GSH or DTT. This issue is explored here to evaluate whether SH oxidation is involved physiologically in Ca^{2+} release from the skeletal muscle SR.

Materials and methods

A heavy fraction of SR enriched in terminal cisternae was isolated from leg muscle of the bullfrog (*Rana catesbeiana*) as described previously[13]. Protein concentration was determined by the Biuret method using bovine serum albumin as the standard.

SR (0.2 mg protein/ml) was loaded with 0.05 mM $CaCl_2$ in a solution containing 100 mM KCl, 20 mM Tris-maleate (pH 6.8), 0.5 mM $MgCl_2$, 0.5 mM ATP, 3 mM phosphoenolpyruvate (PEP), and 10 units of pyruvate kinase (PK)/ml at 25 °C, unless otherwise indicated. The SR, which was contaminated with about 50 nmol Ca^{2+}/mg protein, and $CaCl_2$ were added in KCl buffer solution. The mixture of $MgCl_2$, ATP, PEP, and PK was then added to initiate Ca^{2+} uptake. Ca^{2+} release was triggered by adding concentrated drug solutions after about 95% of the Ca^{2+} in the reaction solution was taken up by the SR. The reaction solution (500 μl) was continuously stirred in a chamber with a magnetic stirrer. Extravesicular calcium concentration was measured using a calcium-selective electrode as described[14], except that 20 mM Tris-maleate (pH 6.8) was used instead of 20 mM MOPS (pH 7). The calcium electrode gave a slope of 28.17 ± 0.28 mV (n = 25) per pCa unit between pCa 3 and 7 at 25 °C in calibration solutions containing 100 mM KCl, 20 mM Tris-maleate (pH 6.8), 1 mM $CaCl_2$, and various concentrations of EGTA. In the presence of 10 mM $MgCl_2$, the slope was 25.89 ± 0.35 mV (n = 9) per pCa unit.

The amount of Ca^{2+} in the SR was calculated from the pCa value by using the following binding constants: $2.45 \times 10^4\ M^{-1}$ for Mg-ATP[15]; $8.5 \times 10^3\ M^{-1}$ for Ca-ATP[15]; $3.16 \times 10^6\ M^{-1}$ for H-ATP[16]. The maximum amount of released Ca^{2+} and the maximum rate of Ca^{2+} release were expressed as mean ± SEM (n = number of preparations) in nmol Ca^{2+}/mg SR protein and nmol Ca^{2+}/mg SR protein/s, respectively.

ATPase activity was measured in a solution containing 100 mM KCl, 20 mM Tris-maleate (pH 6.8), 0.5 mM $MgCl_2$, 0.5 mM ATP, 3 mM PEP, 10 units of PK/ml, 0.2 mg SR protein/ml, 2 μM A23187, and 0.05 mM $CaCl_2$ or 1 mM EGTA at 25 °C. The reaction

was initiated by addition of Mg-ATP, PEP, and PK, and terminated by adding ice-cold 5% trichloroacetic acid. Pi liberated was measured by the method of LeBel et al.[17] Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity (EGTA was added) from Ca^{2+}, Mg^{2+}-ATPase activity ($CaCl_2$ was added). Alcian blue, plumbagin, ruthenium red, dithiothreitol, glutathione, ATP, phosphoenolpyruvate, pyruvate kinase, superoxide dismutase, and catalase were obtained from Sigma Chemical Co., St. Louis, MO. A23187 was from Calbiochem-Behring Corp., La Jolla, CA. Other reagents were of analytical grade. Plumbagin (20 mM) and A23187 (2 mM) were dissolved in 99.5% ethanol as stock solutions.

Results and discussion

Addition of ATP to the reaction solution suddenly decreased the Ca^{2+} concentration due to the chelation of Ca^{2+} by ATP. This was followed by a gradual decrease in Ca^{2+} concentration due to Ca^{2+} uptake by the fragmented SR (fig. 1 a). The amount of Ca^{2+} taken up 7 min after addition of ATP was 230.4 ± 3.5 nmol/mg protein ($n = 6$). Addition of 10 μM alcian blue led to a transient increase in extravesicular Ca^{2+} concentration due to Ca^{2+} release from and re-uptake by the SR (fig. 1 a). The maximum amount of released Ca^{2+} and the maximum rate of Ca^{2+} release were 20.3 ± 4.3 nmol/mg and 2.42 ± 0.87 nmol/mg/s ($n = 6$), respectively. The release of Ca^{2+} induced by 10 μM alcian blue was almost completely inhibited by 10 mM $MgCl_2$ or 2 μM ruthenium red, blockers of Ca^{2+}-induced Ca^{2+} release channel (fig. 1 b, c). Interestingly, 5 mM DTT (fig. 1 d) and 5 mM GSH (data not shown) only partially inhibited Ca^{2+} release induced by the oxidizing compound. Plumbagin (100 μM) also induced a transient Ca^{2+} release from the SR, which took up 234.6 ± 3.5 nmol/mg ($n = 6$) 7 min after ATP addition (fig. 2 a). The maximum amount of released Ca^{2+} and the maximum rate of Ca^{2+} release were 20.2 ± 9.5 nmol/mg and 4.75 ± 1.34 nmol/mg/s ($n = 6$), respectively. Ca^{2+} release induced by 100 μM plumbagin (fig. 2 a) was almost completely inhibited by 10 mM $MgCl_2$ or 2 μM ruthenium red (fig. 2 b, c), but only partially by 5 mM GSH (fig. 2 d) or 5 mM DTT (data not shown), similar to Ca^{2+} release induced by alcian blue.

When alcian blue and plumbagin were pretreated with DTT or GSH, these oxidizing compounds lost the ability to release Ca^{2+} in a time-dependent manner. Fig. 3 shows that pretreatment of alcian blue with DTT for 10 s only slightly decreased the amount of Ca^{2+} released by 10 μM alcian blue (22% decrease), but pretreatment for 1 min inhibited Ca^{2+} release almost completely (99.6% decrease). These results indicate that DTT and GSH can reduce alcian blue and plumbagin on a time scale of 1 min, and reduced alcian blue and plumbagin have no ability to release Ca^{2+}. This suggests that interaction of oxidizing compounds with Ca^{2+} release channel protein is faster than that of oxidants with reducing reagents.

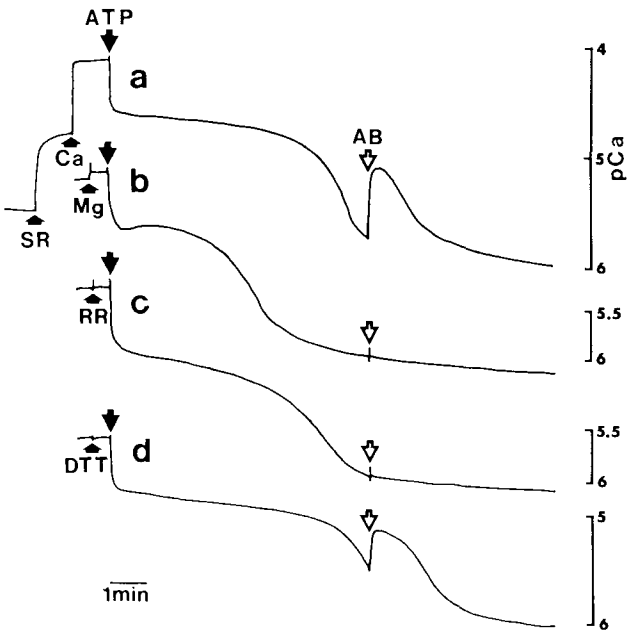

Figure 1. Effects of Mg^{2+}, ruthenium red, and dithiothreitol on alcian blue-induced Ca^{2+} release from actively Ca^{2+}-loaded SR. Ca^{2+} uptake was carried out as described in 'Materials and methods'. Alcian blue (10 μM) was added 7 min after addition of ATP (a). After addition of 10 mM $MgCl_2$ (b), 2 μM ruthenium red (c), or 5 mM dithiothreitol (d), Ca^{2+} uptake was initiated, and then 10 μM alcian blue was added (at open arrows). In b, c, and d, initial parts of pCa changes produced by applications of SR and Ca^{2+} were omitted. Similar results were obtained in 6 experiments.

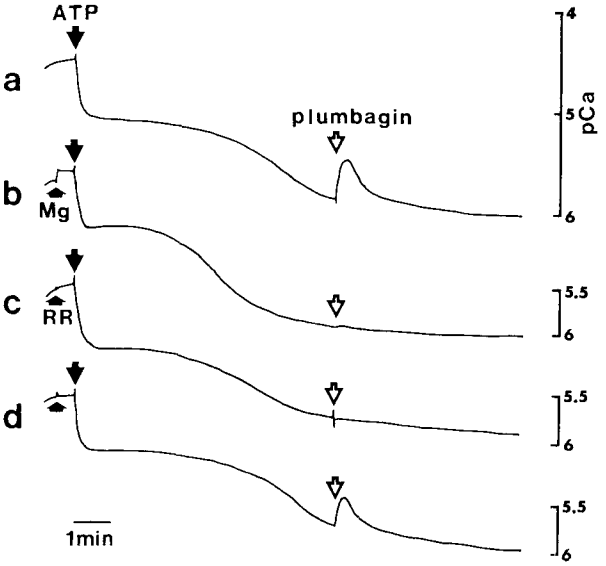

Figure 2. Effects of Mg^{2+}, ruthenium red, and glutathione on plumbagin-induced Ca^{2+} release. Ca^{2+} uptake was carried out as described in 'Materials and methods'. Plumbagin (100 μM) was added 7 min after addition of ATP (a). After addition of 10 mM $MgCl_2$ (b), 2 μM ruthenium red (c), or 5 mM glutathione (d), Ca^{2+} uptake was initiated, and then 100 μM plumbagin was added (at open arrows). Changes in pCa produced by applications of SR and Ca^{2+} were omitted. Similar results were obtained in 6 experiments.

Effects of superoxide dismutase and catalase on oxidizing compound-induced Ca^{2+} release

Oxidizing compound	Control	+SOD	+SOD + catalase
Alcian blue (25 μM)	R[a] 1.05 ± 0.19	1.37 ± 0.31	1.31 ± 0.22
	A[b] 10.6 ± 2.1	13.1 ± 3.9	12.4 ± 2.9
Plumbagin (200 μM)	R 4.20 ± 0.45	3.43 ± 0.55	3.36 ± 0.99
	A 27.6 ± 1.6	24.2 ± 2.9	25.3 ± 4.8

Active Ca^{2+} uptake was performed as described in 'Materials and methods', except 0.5 mg SR protein/ml was used. The amount of Ca^{2+} taken up was 95.0 ± 0.72 nmol/mg (n = 4). Ca^{2+} release was induced by the addition of alcian blue or plumbagin. Superoxide dismutase (SOD) and catalase were added 60 s and 30 s, respectively, before addition of oxidizing compounds. Values given represent the mean ± SEM of four preparations.
[a]Rate of Ca^{2+} release (nmol/mg/s); [b]amount of Ca^{2+} release (nmol/mg).

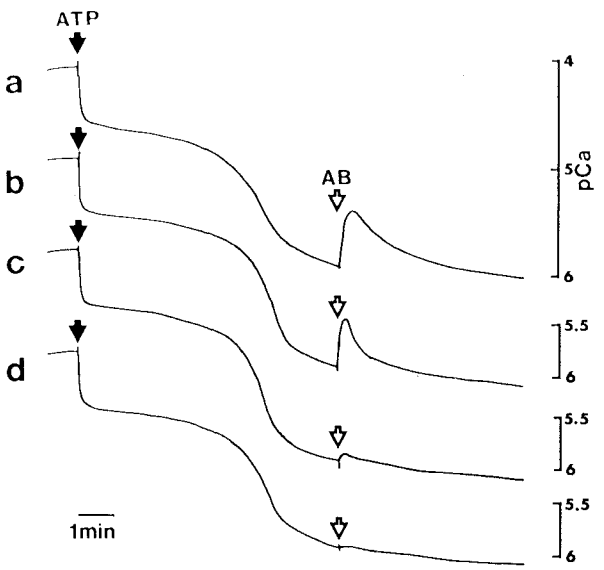

Figure 3. Effect of pretreatment of alcian blue with dithiothreitol on Ca^{2+} release induced by alcian blue. Ca^{2+} uptake was carried out as described in 'Materials and methods'. Alcian blue (10 μM) was added 7 min after addition of ATP (a). Alcian blue (2 mM) was mixed with an equal volume of dithiothreitol (1 M) for 10 s (b), 30 s (c), or 1 min (d), and 5 μl of the mixture was added to 500 μl of the reaction solution. Changes in pCa produced by applications of SR and Ca^{2+} were omitted. Similar results were obtained in 3 experiments.

Alcian blue and plumbagin can produce superoxide and hydroxy free radicals, which may interact in the SR membrane with SH groups and unsaturated fatty acids, respectively, to increase ion permeability. However, these free radicals played no critical role in alcian blue- and plumbagin-induced Ca^{2+} release, since the Ca^{2+} release was hardly affected by 100 units/ml superoxide dismutase which reduces superoxide radicals, or 100 units/ml catalase, which prevents the production of hydroxy radicals (table).

Neither 10 μM alcian blue nor 100 μM plumbagin inhibited SR Ca^{2+}-ATPase (data not shown), indicating that Ca^{2+} efflux through Ca^{2+} release channels, but not Ca^{2+} leakage caused by Ca^{2+} pump inhibition, is involved in the Ca^{2+} release induced by alcian blue or plumbagin.

Abramson and Salama[9] have proposed an involvement of SH oxidation in E-C coupling from evidence that various SH reagents induce Ca^{2+} release from the terminal cisternae of the SR. However, the finding that sufficient endogenous GSH was present in the muscle fibre and that intracellularly applied GSH did not inhibit E-C coupling led to disagreement[12]. In the present study, we assert that alcian blue and plumbagin induce Ca^{2+} release from the SR through oxidation of SH groups in Ca^{2+}-induced Ca^{2+} release channels, even in the presence of 5 mM DTT or 5 mM GSH (figs 1 and 2). The amounts of SH reducing reagent used (5 μmol/0.2 mg SR protein = 250 μmol/g muscle) were much larger than the amount of GSH present in skeletal muscle in situ, 0.5–1.1 μmol/g wet weight[10,11]. Thus, our findings provide evidence that highly reactive SH groups are probably located in the Ca^{2+} release channel, and those groups can be oxidized even in the presence of endogenous GSH. Therefore, SH oxidation may be involved in the physiological E-C coupling in skeletal muscle.

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