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High-affinity Ca^{2+}-binding site inhibiting Ca^{2+} release from sarcoplasmic reticulum

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Ca^{2+} release from sarcoplasmic reticulum membranes, activated by alkaline pH occurs only when EGTA is present in the release medium. Addition of very low concentrations of Ca^{2+} to the medium inhibits Ca^{2+} release. The concentration of free Ca^{2+} required for 50% inhibition ranges from between 5 and 20 nM in different experiments and/or membrane preparations, irrespective of whether the free Ca^{2+} concentration is controlled by EGTA or CDTA. Other divalent cations such as Mn^{2+}, Ba^{2+}, Cu^{2+}, Cd^{2+} and Mg^{2+} also exert an inhibitory effect on Ca^{2+} release, with higher or lower potency than that of Ca^{2+}. The inactivation of Ca^{2+} release by Ca^{2+} is reversible. We suggest the involvement of high-affinity Ca^{2+}-binding sites in the control of Ca^{2+} release.

Sarcoplasmic reticulum; Ca^{2+} release

1. INTRODUCTION

Ca^{2+} release from the sarcoplasmic reticulum (SR) plays an important role in excitation-contraction coupling [1]. Using skinned muscle fibers and isolated SR membranes, various treatments and stimuli, such as addition of Ca^{2+} [2], depolarization of the membrane [3], alteration in pH [4] or surface charge [5], chemical modifications of the membranes [6,7], or the presence of inositol trisphosphate [8], have been shown to induce Ca^{2+} release.

Recent studies indicate that there is more than one Ca^{2+}-release system in the sarcoplasmic reticulum, involving two or more types of divalent cation channels [6,7,9–16]. These channels differ in: their divalent cation conductivity and specificity, their distribution in the different structural regions of the SR membrane, and also in their activation mechanism. One of these channels located in the SR-T-system junction, is the 'ligand-gated' channel which recently has been identified and purified [11,17,18]. This channel has a high conductance, and is activated by adenine nucleotides, ryanodine and Ca^{2+} and inhibited by Mg^{2+} and ruthenium red [11,14,15]. The presence of a second channel with a low conductance and in all SR structural regions has been demonstrated recently [15,16]. However, the control mechanism of this channel is not yet known.

We have studied Ca^{2+} release in unfractionated native SR membranes and have developed several different conditions for activation of Ca^{2+} release [4–7,19]. This Ca^{2+}-release system is present in all structural regions of the SR membranes, and apparently operates via a channel different from the junctional Ca^{2+}-release channel. Our results suggest that the opening of this non-junctional channel is controlled by a positively charged, internally located, amino group(s).

It is not clear, however, from the different mechanisms proposed for the physiological release

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Abbreviations: DCCD, *N,N'*-dicyclohexyl carbodiimide; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxy-methyl)ethyl]glycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; CDTA, *trans*-1,2-diamino-cyclohexane-*N,N,N',N'*-tetraacetic acid; RR, ruthenium red; TPB^-, tetraphenylboron; SR, sarcoplasmic reticulum

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of Ca^{2+} what is the control mechanism for channel closing. The inactivation of the channel should be different from the usual voltage- and time-dependent inactivation of excitable membranes.

In a previous study [4] we suggested that Ca^{2+} itself controls the closing of Ca^{2+}-release channel(s) in skeletal SR membranes. The control by Ca^{2+} of activation and inactivation of cardiac SR Ca^{2+}-release channel has been suggested by Fabiato [20]. In this communication we report on the involvement of high-affinity Ca^{2+}-binding sites in the inactivation of Ca^{2+} release from SR vesicles.

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, EGTA, Tris, Mops, DCCD and CDTA were obtained from Sigma. [^{45}Ca]$CaCl_2$ was from Radiochemical Center, Amersham, and ruthenium red (97% pure) was from Fluka.

2.2. *Methods*

2.2.1. Membrane preparations

Sarcoplasmic reticulum vesicles were prepared by the procedure of MacLennan [21], suspended to a final concentration of 20 mg protein/ml in a buffer containing 0.2 M sucrose, 10 mM Tricine, pH 8.0, and 1 mM histidine and stored at −70°C. The protein concentration was determined by the Lowry et al. method [22].

2.2.2. Ca^{2+} efflux

Ca^{2+} efflux from actively loaded SR vesicles was carried out as described in fig.1. The vesicles were loaded with Ca^{2+} for 15 min and then collected by centrifugation at 100000 × *g* for 20 min, and resuspended in 20 mM Mops, pH 6.8, and 100 mM KCl at a protein concentration of about 3 mg/ml. Since TPB^- precipitates K^+, all chemicals in the experiments with TPB^- were used as Na^- salts.

2.2.3. Calculations of free concentrations of cations

Free concentrations were calculated with a computer program using the following apparent binding constants: at pH 6.8, Ca-EGTA 1.94×10^6; Ca-CDTA, 3.26×10^7; Mg-EGTA, 23.8; and at pH 8.0, Ca-EGTA, 4.28×10^8; Ca-CDTA, 6.24×10^8; Mg-EGTA, 8.8×10^2; Mn-EGTA, 5.4×10^9; Cd-EGTA, 7.48×10^{15}; Cu-EGTA, 2.1×10^{15} [23].

3. RESULTS

Dilution of Ca^{2+}-loaded SR vesicles into a basic medium containing EGTA, activates Ca^{2+} release [4]. The amount of Ca^{2+} released is a function of the EGTA concentration in the release medium (fig.1). Under the conditions used in this experiment (pH 8.0 and 0.1 mg SR/ml), maximal Ca^{2+}

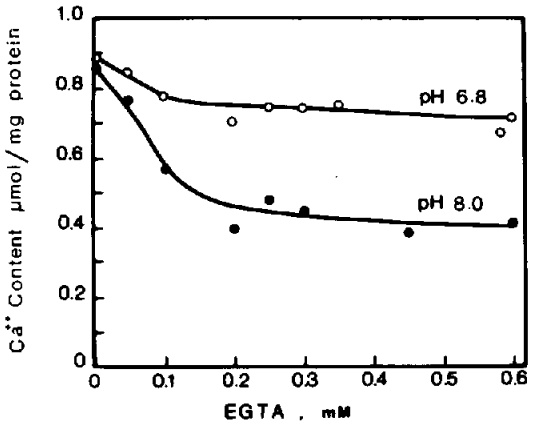


Fig.1. pH-induced Ca^{2+} efflux as a function of EGTA concentration in the release medium. SR vesicles were actively loaded with $^{45}CaCl_2$ as in [24] and collected by centrifugation and resuspended as described in section 2. Ca^{2+} efflux was assayed by 50-fold dilution of the membranes into 100 mM NaCl and 20 mM Mops, pH 6.8 (○) or 20 mM Tris, pH 8.0 (●), in the absence or presence of the indicated concentration of EGTA. Ca^{2+} content was determined 30 s after dilution, by filtering 0.18 ml samples through 0.3 µm nitrocellulose filters and washing with 5 ml of 0.15 M of KCl. Radioactivity on the filters was measured in a liquid scintillation counter.

release was obtained at 0.15 mM EGTA. In the absence of EGTA the amount of Ca^{2+} released at pH 8.0 was similar to that released in control vesicles incubated at pH 6.8.

The Ca^{2+} release, stimulated by alkaline pH, is also a function of the SR concentration in the release medium. About 85% and 7% of trapped Ca^{2+} was released upon 200- and 25-fold dilution, respectively (not shown). These results suggest that external Ca^{2+} is involved in the inhibition of Ca^{2+} release. Fig.2 shows that addition of Ca^{2+} at very low concentrations, to the release medium inhibits Ca^{2+} release. 50% inhibition was obtained at less than 10 nM in some membrane preparations and about 20 nM in others. Assuming that the total EGTA concentration is higher or has about 5% uncertainty, the concentration of free Ca^{2+} required for 50% inhibition ranges between 5 and 15 nM. Similar inhibition was obtained whether the free Ca^{2+} concentration was controlled by the chelator EGTA or CDTA, indicating that it was Ca^{2+} and not the Ca^{2+}-chelator complex that was responsible for the inhibition of Ca^{2+} release. The fact that Ca^{2+} release went from uninhibited to almost completely inhibited upon doubling the

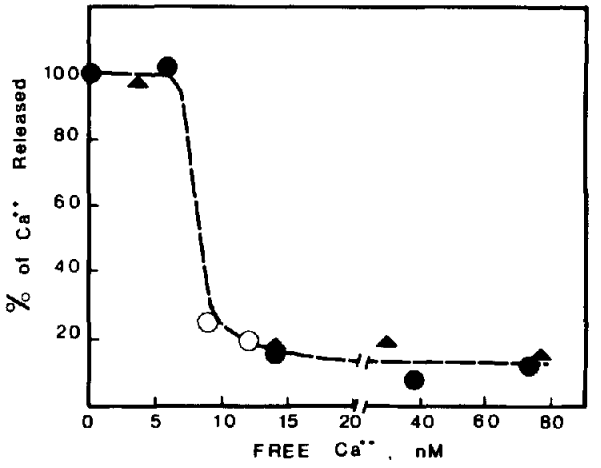


Fig.2. Ca^{2+} inhibition of Ca^{2+} release stimulated by elevated pH. SR vesicles were actively loaded with $^{45}CaCl_2$ and assayed for Ca^{2+} efflux as in table 1 except that free Ca^{2+} at the indicated concentration was present in the release medium and the pH in each case was adjusted to 6.8 or 8.0. Ca^{2+} content was determined 2 min after dilution. Control activity (100%) was 1.4 μmol Ca^{2+}/mg protein. Free Ca^{2+} concentrations were calculated as described in section 2. (○) and (●) indicate two different experiments in which free Ca^{2+} concentration was controlled by EGTA, while in (▲) by CDTA.

Ca^{2+} concentration in the release medium, suggests positive cooperativity in the Ca^{2+} effect and, therefore, on the involvement of two or more Ca^{2+}-binding sites. The results suggest the involvement of high-affinity Ca^{2+}-binding sites in the control of Ca^{2+} release.

Ca^{2+} also inhibits Ca^{2+} release induced by other means such as by TPB^- [19] or by chemical modification of SR vesicles with acetic anhydride [6]. High Ca^{2+} concentrations (mM) were required for the inhibition of TPB^--induced Ca^{2+} release (not shown). This may be due to the TPB^- negative charge incorporated into the membranes [5]. Inhibition of Ca^{2+} released induced by acetic anhydride modification was obtained with micromolar concentrations of Ca^{2+} (not shown).

Table 1 shows that other cations besides Ca^{2+} exert an inhibitory effect on Ca^{2+} release. Mn^{2+} and Ba^{2+} were inhibitory with similar potency to Ca^{2+}. On the other hand, Cu^{2+} and Cd^{2+} were 4–5 orders of magnitude more potent and Mg^{2+} was with about 4 orders of magnitude less effective. However, it is possible that the effect of Cu^{2+} and Cd^{2+} is due to the release of complexed endogenous Ca^{2+} from EGTA.

Table 1

Effect of different divalent cations on Ca^{2+} release

Additions to Ca^{2+} release medium	Free cation concentration, M	Ca^{2+} released % of control
None	–	51
Ca^{2+}, 0.95 mM	4.4×10^{-8}	10
Mg^{2+}, 5 mM	1.4×10^{-3}	10
Ba^{2+}, 0.95 mM	–	39
Ba^{2+}, 1.2 mM	–	7
Mn^{2+}, 0.95 mM	3.4×10^{-9}	10
Mn^{2+}, 1.2 mM	2.0×10^{-4}	3
Cd^{2+}, 0.95 mM	7.5×10^{-15}	2
Cu^{2+}, 0.8 mM	1.9×10^{-15}	36
Cu^{2+}, 0.95 mM	8.8×10^{-15}	10

SR vesicles were actively loaded with Ca^{2+} and assayed for Ca^{2+} efflux as in fig.1, except that EGTA concentration was 1 mM and the indicated divalent cations were present in the release medium. Free cation concentrations were calculated as described in section 2. Ca^{2+} content was determined 2 min after dilution. Ca^{2+} content (100%) was between 3.2 and 3.5 μmol/mg protein for all samples except for the Mn^{2+} containing samples which was 3.7 and 4.2 μmol/mg protein for free concentrations of 3.4 nM and 200 μM, respectively

Table 2 shows that the polycationic dye, ruthenium red, is also an inhibitor of Ca^{2+} release through the non-junctional channel activated by alkaline pH. Ruthenium red has been shown to inhibit the Ca^{2+}-induced Ca^{2+} release that operates through the ligand gated-junctional Ca^{2+}-release channel [11].

The inactivation of Ca^{2+} release by Ca^{2+} is reversible. Fig.3 shows the time course of reactivation of Ca^{2+}-inhibited Ca^{2+} release, produced by the addition of EGTA to a Ca^{2+}-containing

Table 2

Ruthenium red inhibition of pH-induced Ca^{2+} release

Ruthenium red (μM)	Ca^{2+} released/min % of Ca^{2+} control
0	46
2.5	35
5	16
15	8

SR vesicles were loaded with Ca^{2+} and assayed for Ca^{2+} efflux as in table 1 except that ruthenium red at the indicated concentration was present in the SR dilution medium. Ca^{2+} content was determined 1 min after dilution. Control activity (100%) was 1.3 μmol Ca^{2+}/mg protein

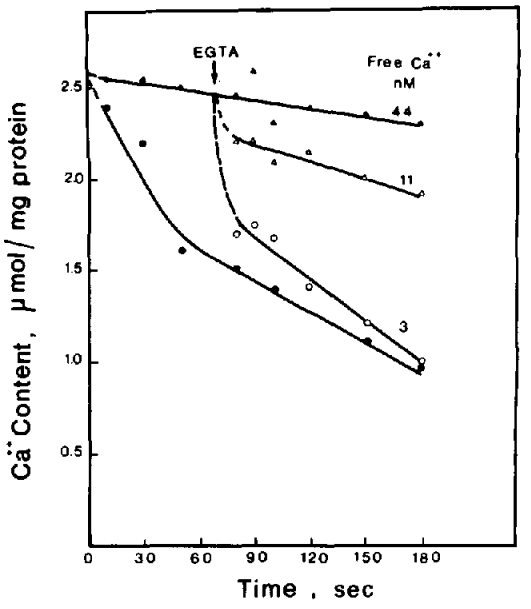


Fig.3. Ca^{2+} inhibition of pH-induced Ca^{2+} release can be reversed by EGTA. SR vesicles were actively loaded with $^{45}CaCl_2$ and assayed for Ca^{2+} efflux as in table 1 except that the loaded vesicles were diluted into release medium containing 1 mM EGTA (●) or 1 mM EGTA and 0.95 mM $CaCl_2$ (▲) (free $[Ca^{2+}]$ is 44 nM). After 70 s EGTA (0.148 or 0.714 mM) was added to the Ca^{2+}-containing sample to lower the free Ca^{2+} concentration to 11 nM (△) or 3 nM (○), respectively.

medium. Ca^{2+}-loaded vesicles, diluted into a medium containing 44 nM free Ca^{2+}, retained most of their internal Ca^{2+}. However, when the free $[Ca^{2+}]$ was decreased to 11 nM or 3 nM, Ca^{2+} is rapidly released. Thus, the results indicate that the transition from an inactive to an active Ca^{2+}-release system is very rapid.

Inactivation of active Ca^{2+} release was also obtained by the addition of Ca^{2+} to an EGTA-containing release medium. Inactivation of Ca^{2+} release could be halted immediately at any time during its time course by the addition of Ca^{2+} (not shown). The rate constants for activation and inactivation of Ca^{2+} release could not be determined due to experimental limitations in resolving the kinetics of these two processes.

4. DISCUSSION

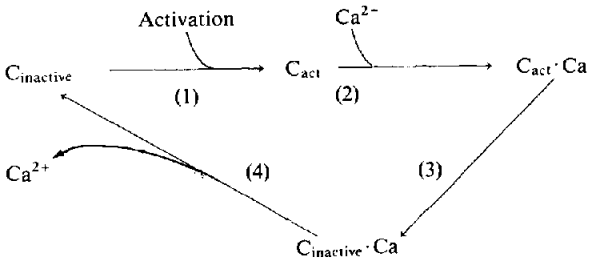
The study presented here suggests the involvement of high-affinity Ca^{2+}-binding sites in the control of Ca^{2+} release. Less than 20 nM of Ca^{2+} are required to inhibit Ca^{2+} release activated by alkaline pH. However, this concentration is dependent on the SR concentration in the release media.

The source of the inactivating Ca^{2+} could be intra- or extravesicular. Even in the presence of low SR concentrations and 50 μM EGTA no Ca^{2+} release was observed (fig.1), suggesting that inhibition can be caused by the small amount of Ca^{2+} released upon activation. On the other hand, addition of Ca^{2+} to the external medium also prevented the release of Ca^{2+} (fig.2). The inhibitory effect of Ca^{2+} is reversible (fig.3).

Our previous studies [5–7,19] suggest that Ca^{2+} release, activated by various treatments, probably operate through one or more types of Ca^{2+}-release channels, present in SR membranes [12–16]. We suggest, therefore, that Ca^{2+} by its binding to specific sites led to inactivation of Ca^{2+}-release channel(s), and that the non-junctional channel is much more sensitive to Ca^{2+}.

The Ca^{2+}-binding sites are probably located within a protein because internal Ca^{2+} did not prevent the release of Ca^{2+} upon activation and this Ca^{2+} release is inhibited by DCCD but not with the water-soluble carboxylic group reagents [24]. We propose, therefore, that a carboxylic group (or groups) exposed on activation of Ca^{2+} release is involved in the inhibition of Ca^{2+} release.

The Ca^{2+}-binding sites, controlling the inactivation of the Ca^{2+} channel(s), have a very high affinity (K_d < 10 nM). However, this high affinity for Ca^{2+} might be expected to lead to channel closing immediately following the first burst of Ca^{2+} released. This, however, would not be the case if there is a rate limiting step such as the slow binding of Ca^{2+} (reaction (2) in the model of scheme 1) or if the complex channel·Ca must undergo a slow conformational change(s) (reaction (3) in the model) in order to reach its closed state. We tried to examine these two possibilities by following: (i) the rate of Ca^{2+} inhibition of preactivated Ca^{2+} release and (ii) the rate of activation of Ca^{2+}-inactivated Ca^{2+} release, produced by the addition of EGTA (fig.3). In both experiments the rate was too fast to follow by the filtration technique. However, this does not rule out the involvement of such rate limiting step(s) in the Ca^{2+}-dependent inactivation.



Scheme 1. for time-and Ca^{2+}-dependent inactivation of Ca^{2+} release. $C_{inactive}$ and C_{act} indicate inactivated and activated channel, respectively.

It should be mentioned that it is possible that the inactivation proceeds through a multi-state reaction. A similar mechanism for time- and Ca^{2+}-dependent inactivation of Ca^{2+}-induced Ca^{2+} release in cardiac SR has been suggested by Fabiato [20]. A Ca^{2+}-dependent inactivation of the voltage-gated Ca^{2+} channel has been demonstrated in a variety of excitable cells [25].

Studies of the Ca^{2+} binding and inactivation of Ca^{2+} release in SR membranes could provide further insight into the control mechanism for the closing of the Ca^{2+} release channel(s).

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