

Intracellular Ca Release in Skinned Smooth Muscle

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ABSTRACT The release of internal Ca from saponin-treated skinned smooth muscle of guinea pig taenia caecum was studied. The amount of Ca released was estimated by the area under the contraction curve during treatment with 25 mM caffeine in the presence of 0.1 mM EGTA. The magnitude of the caffeine response in skinned muscle, after loading with 10^{-6} M Ca for 3 min, was similar to that in the depolarized muscle in the presence of EGTA before treatment with saponin. This suggests that Ca in the skinned muscle was in a physiological range after loading. The release of Ca from the storage site could be facilitated by Ca itself when the skinned muscle was exposed to Ca above 3×10^{-6} M. An increase in environmental Mg concentration suppressed the Ca-induced Ca release mechanism. Sudden replacement of propionate with Cl in the bathing solution made it possible to release Ca from the storage site. This "depolarization"-induced Ca release occurred only immediately after the application of Cl; thereafter, the Ca release mechanism seemed to be inactivated by the prolonged presence of Cl. These results suggest that two mechanisms of Ca release operate in smooth muscle: (a) release induced by Ca itself, and (b) release by "depolarization."

INTRODUCTION

The release of free calcium ions (Ca) from the sarcoplasmic reticulum (SR) is undoubtedly an essential process in excitation-contraction coupling in skeletal muscle (Ebashi and Endo, 1968). The introduction of the skinned fiber preparation, originally devised by Natori (1954), into this field has contributed a great deal to our understanding of the Ca release mechanism. In smooth muscle, the source of Ca serving as a link between excitation and contraction has been postulated to be derived from both extracellular and intracellular sites, but the specific role of an intracellular Ca store in excitation-contraction coupling remains obscure. In particular, no clear evidence for the release of stored Ca from a specific cellular site has been obtained in smooth muscle. This may be due to difficulty in obtaining a suitable preparation, because the sarcotubular system is very poorly developed in smooth muscle as compared with the elaborately organized structure found in skeletal muscle (Porter and Franzini-Armstrong, 1965; Somlyo et al., 1971).

In the present study, a suitable preparation for the study of an intracellular

Ca store could be obtained by skinning smooth muscle with lower concentrations of saponin than that used in our previous study (Saida and Nonomura, 1978). The properties of Ca release were examined using this preparation with the hope of elucidating the role of intracellularly stored Ca in the excitation-contraction coupling process in smooth muscle.

METHODS

Muscle Preparation and Bathing Solution

Skinned smooth muscle was prepared and studied by the methods described in our previous paper (Saida and Nonomura, 1978). The following modifications were made. A thin bundle of muscle (50 μm in width and 1 mm in length) was prepared from taenia caecum of guinea pig in Locke's solution and mounted in an apparatus designed for measurement of isometric tension. Chemically skinned smooth muscle was prepared by saponin treatment ($\sim 40\text{--}60 \mu\text{g/ml}$, for $\sim 20\text{--}30$ min) in a standard relaxing solution containing 130 mM K propionate, 20 mM Tris-maleate, 4 mM MgCl_2 , 4 mM ATP, and 2 mM EGTA, pH 6.8 at 20°C . In each preparation the concentration of saponin was adjusted to ensure that reproducible contractile activity could be generated during the duration of the experiment. Preparations showing deterioration were discarded.

In buffered solutions, Ca concentrations were calculated by assuming an apparent binding constant of the Ca-EGTA complex to be 10^6 M^{-1} at pH 6.8, 20°C (Saida and Nonomura, 1978).

Ca Loading Procedures and Estimation of Ca Released from Storage Site

It is generally accepted that high concentrations of caffeine release most of the Ca stored in muscle (Endo, 1977). Thus, it was assumed that the magnitude of the contractile response to high caffeine reflects the amount of Ca in the storage site. The following procedures were used for Ca loading and estimation of the amount of Ca released from the storage site: (a) the Ca in the storage site was depleted by 25 mM caffeine in the presence of 2 mM EGTA; (b) the Ca store was loaded with 10^{-6} M Ca by bathing the skinned muscle in a Ca solution weakly buffered with 0.1 mM EGTA for 3 min; (c) the skinned muscle was washed with relaxing solution containing 0.1 mM EGTA; (d) the skinned muscle was exposed to the test stimulus; and (e) 25 mM caffeine was applied to the muscle in the presence of 0.1 mM EGTA. The amount of Ca released was estimated by comparing the area under the caffeine-induced contraction curve with that of the control experiment in which the same procedures were followed except for the application of the Ca-releasing stimulus. Accordingly, an estimate of the amount of Ca released is given by the difference between the area of the caffeine contraction in the control experiment and the area of the caffeine contraction after the application of the test stimulus. The control experiments were carried out before and after the test experiment to examine whether the skinned preparation showed deterioration of the contraction. The response obtained in step d was not used for the estimation of Ca release because the test stimulus did not always act exclusively on the Ca store.

RESULTS

Response of Intact and Skinned Muscles to Caffeine

Caffeine has a strong Ca-releasing action on the SR of striated muscles, whereas it shows complicated effects on smooth muscle, e.g., caffeine can both

stimulate and relax various smooth muscles or even the same smooth muscle under different conditions. I have found a condition in which caffeine will only induce a contraction in smooth muscle. Therefore, the present experiments were carried out in order to compare the caffeine responses on intact and skinned muscles in the same preparation. After the K-induced contracture of the intact muscle was recorded (Fig. 1*a*), the bathing solution was changed to the relaxing solution containing 130 mM K and 2 mM EGTA to avoid possible changes in the membrane potential caused by caffeine and possible participation of external Ca. When 25 mM caffeine was applied to the muscle in the relaxing solution, a transient contraction was induced (Fig. 1*b*). Reapplication of caffeine did not induce a second contraction even after the muscle was bathed in solution containing 130 mM K and 10^{-5} M Ca (Fig. 1*c*).

When the muscle was treated with saponin to obtain a skinned preparation, application of 10^{-5} M Ca, as shown in Fig. 1*c*, caused an appreciable contraction (Fig. 1*d*). 25 mM caffeine did not induce a contraction of the skinned muscle immediately after washing with the relaxing solution (Fig. 1*e*); however, after Ca loading (10^{-6} M Ca), caffeine induced a response in the presence of 0.1 mM EGTA (Fig. 1*f*). The magnitude of the caffeine response of the skinned muscle after loading with 10^{-6} M Ca for 3 min was almost the same as that of the intact muscle (Figs. 1*b* and *f*). Therefore, the skinned muscle was routinely loaded with 10^{-6} M Ca for 3 min.

After a single application of 25 mM caffeine to the skinned muscle, Ca was apparently depleted from the storage site and no response could be obtained by further application. However, when the skinned muscle was reloaded with Ca, the storage site once again accumulated Ca and its ability to respond to caffeine gradually returned. The time course of Ca accumulation by the storage site is shown in Fig. 2.

Ca-induced Ca release

When the skinned smooth muscle was loaded with 10^{-5} M Ca, the amount of Ca in the storage site was smaller than with 10^{-6} M Ca, although the Ca-induced contraction was larger during the Ca loading (Fig. 3*A*). This suggests that Ca-induced Ca release is present. In Fig. 3*B*, the Ca-induced Ca release was more directly examined by the following procedure (Fig. 3*B*, top). The skinned muscle was loaded with a definite concentration of Ca, and then exposed to various concentrations of Ca for a short period of time. The amount of Ca remaining in the storage site after these treatments was estimated by the application of caffeine. As shown in Fig. 3*B* (open circles), the amount of Ca remaining in the storage site decreased after treatment with $\text{Ca} > 3 \times 10^{-6}$ M. This indicates that the Ca-induced Ca release mechanism has been at least partially activated.

An increase in free magnesium ion (Mg) concentration in the bathing solution suppressed Ca-induced Ca release from the SR in skinned striated muscle (Ford and Podolsky, 1970; Endo, 1975; Fabiato and Fabiato, 1975). In the present study using skinned smooth muscle, the concentration of Ca that elicited Ca-induced Ca-release shifted to a higher Ca level by ~ 0.5 pCa units in the presence of 10 mM Mg. Elevation of Mg could not significantly increase the amount of Ca in the storage site.

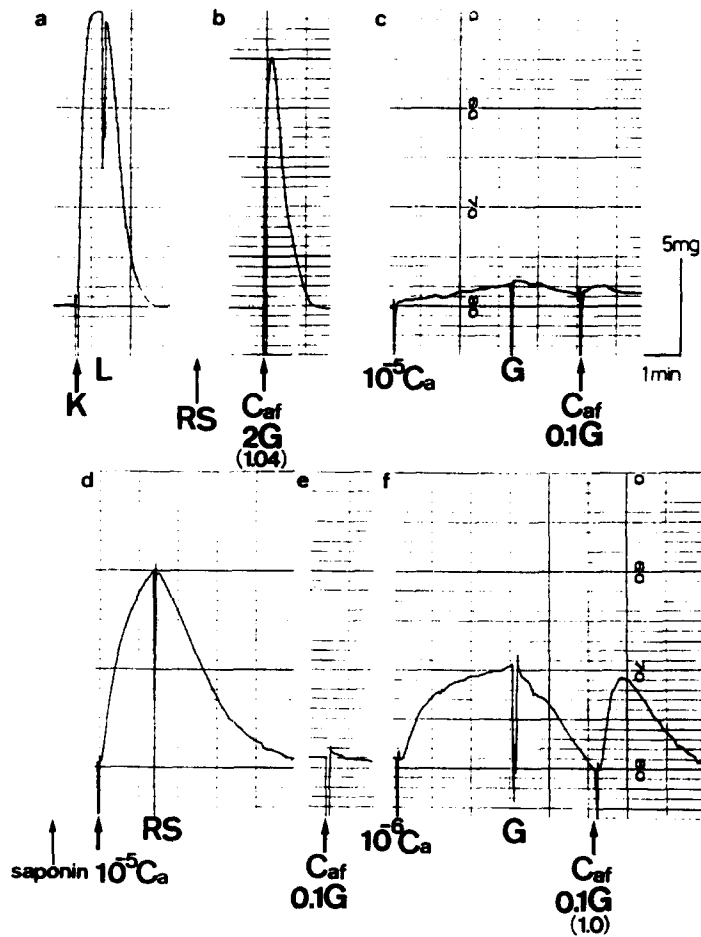


FIGURE 1. The response of caffeine in intact and skinned smooth muscles. Both experiments are from the same preparation. (a) K contracture of the intact muscle induced with K-Locke's solution (K). (b) Caffeine contraction of intact fibers in the presence of 130 mM K and 2 mM EGTA (2G) after bathing in the relaxing solution (RS) for 20 min. (c) The response of intact fibers to caffeine after loading with 10^{-5} M Ca (10^{-5} Ca) and washing with relaxing solution containing 0.1 mM EGTA (0.1G). (d) The contraction of skinned muscle induced with 10^{-5} M Ca (10^{-5} Ca) after treatment with saponin. (e) The response of skinned muscle to caffeine in the presence of 0.1 mM EGTA (0.1G) immediately after washing with the relaxing solution containing 2 mM EGTA. (f) Caffeine contraction in skinned muscle in the presence of 0.1 mM EGTA (0.1G) after loading with 10^{-6} M Ca (10^{-6} Ca) and washing with relaxing solution containing 0.1 mM EGTA (0.1G). The experimental solution was applied as indicated at each arrow. Caf represents the application of 25 mM caffeine. The value of Ca remaining in the store (shown in parentheses) is expressed relative to the control experiment.

"Depolarization"-induced Ca Release

A sudden change in the ionic composition of the solution bathing skinned skeletal muscle fibers alters the membrane potential of the SR (Costantin and Podolsky, 1967; Ford and Podolsky, 1970; Nakajima and Endo, 1973). If a less permeant anion is quickly replaced by a more permeant anion, the lumen of the SR should become more negative with respect to the outside.

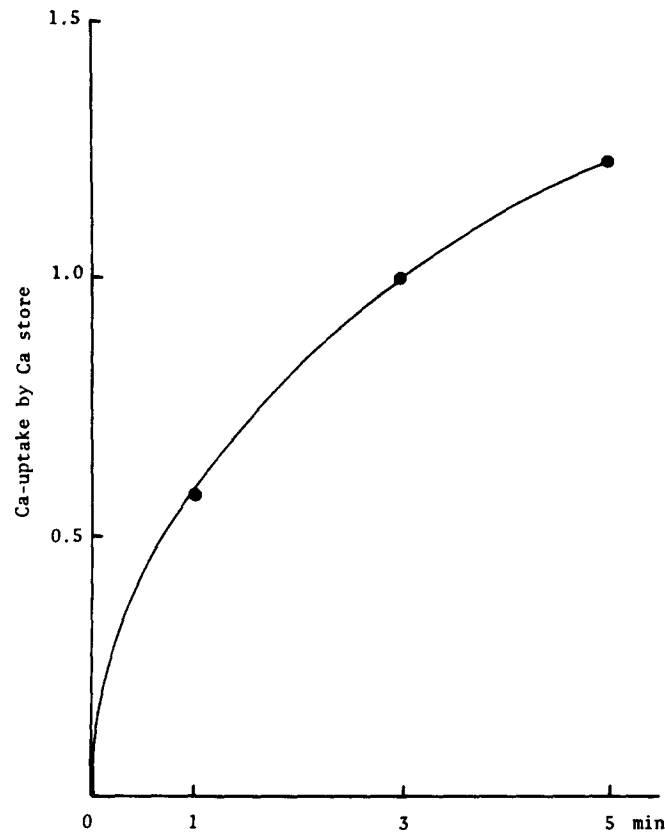


FIGURE 2. Time course of Ca uptake by the Ca store. Areas under the contractile response to 25 mM caffeine were plotted relative to values obtained after loading with 10^{-6} M Ca for 3 min (control). 1.0 (ordinate) corresponds to the area under the caffeine contraction of the control experiment.

The present experiments were carried out to examine whether ionic replacement induces Ca release from the storage site of skinned smooth muscle. When 130 mM propionate in the bathing solution was quickly replaced with chloride (Cl) in the presence of 0.1 mM EGTA, the skinned muscle showed a small transient contraction, as shown in Fig. 4. After the ionic replacement, the amount of Ca remaining in the storage site was $\sim 25\%$ of that before the

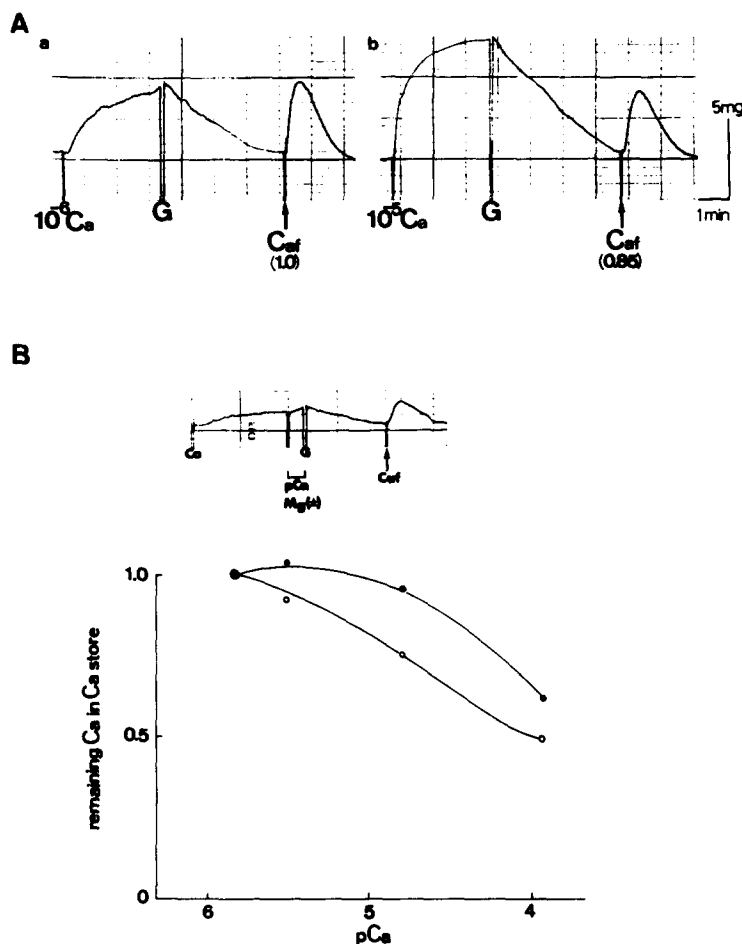


FIGURE 3. Ca-induced Ca release in skinned smooth muscle. A. Caffeine response of skinned fibers after loading with 10^{-6} M Ca (a) and 10^{-5} M Ca (b). 25 mM caffeine (Caf) applied to the muscle in the presence of 0.1 mM EGTA. G represents washing with relaxing solution containing 0.1 mM EGTA. B. Top: Experimental procedure. The skinned muscle was loaded with 10^{-6} M Ca (Ca) for 3 min and then exposed to various concentrations of Ca (pCa) with or without 10 mM Mg (Mg \pm) for 30 s. The amount of Ca remaining in the storage site was estimated by the 25 mM caffeine (Caf) contraction after washing with relaxing solution containing 0.1 mM EGTA (G). Bottom: Ca remaining in the store as a function of pCa. Ca values were plotted relative to that in which the muscle was loaded with 10^{-6} M Ca with (closed circles) or without (open circles) 10 mM Mg.

application of the Ca-releasing stimulus. The effect of Cl on Ca release from the storage site is shown in Fig. 5, which indicates that the amount of released Ca increased with the Cl concentration.

When the Ca store of skinned muscle was treated successively with 65 mM

and then 130 mM Cl, the amount of Ca remaining in the storage site was almost the same as that after single treatment with 65 mM Cl (Figs. 6*b* and *c*), whereas the higher concentration of Cl was effective in releasing Ca when applied without pretreatment with 65 mM Cl.

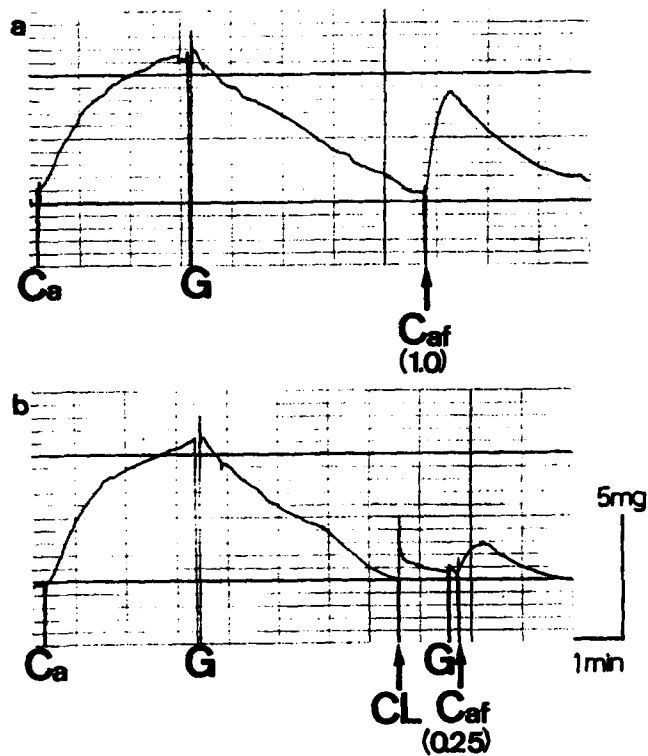


FIGURE 4. "Depolarization"-induced Ca release in skinned smooth muscle. (a) Control. Ca release from the store by caffeine after loading with 10^{-6} M Ca for 3 min in the presence of 0.1 mM EGTA. (b) Ca release from the store induced by "depolarization." The "depolarization" was produced by replacing 130 mM propionate with an equimillimolar concentration of Cl in the relaxing solution containing 0.1 mM EGTA (CL). Ca, G, and Caf represent Ca loading of 10^{-6} M Ca, washing with relaxing solution with 0.1 mM EGTA, and application of 25 mM caffeine, respectively. The value of Ca remaining in the store is shown in parentheses and expressed relative to control.

DISCUSSION

It is well known that caffeine is one of the most useful drugs for revealing a key step in excitation-contraction coupling in striated muscles because of its strong Ca-releasing action on the SR. In the present study using smooth muscle, 25 mM caffeine caused contraction of depolarized intact muscle in the presence of EGTA and contraction of skinned muscle only after loading with Ca. These results indicate that caffeine acts intracellularly in smooth

muscle and that the functionality of the caffeine-sensitive internal Ca store seems to be preserved after skinning the muscle with saponin. These possibilities are also supported by the fact that the caffeine-sensitive Ca store was refilled by Ca in the micromolar range in the skinned muscle, not in the intact muscle. There was a major difference between intact and skinned muscles in the speed of contraction induced by caffeine. This is probably caused by an environmental difference of the Ca store between two muscle preparations, e.g., Ca released by caffeine may partly diffuse out of cells in the case of

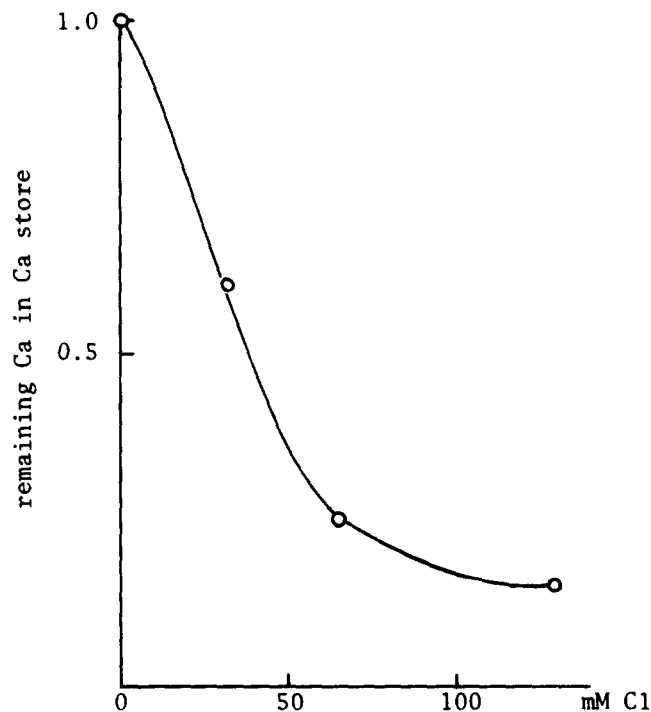


FIGURE 5. Effect of Cl concentration on Ca release from the storage site. The value of Ca remaining in the store (ordinate) was plotted relative to the control experiment in which 25 mM caffeine was applied to the skinned smooth muscle after loading with 10^{-6} M Ca for 3 min without treatment with Cl. 1.0 corresponds to the area under the caffeine contraction of the control experiment. Abscissa: concentration of Cl in the test solution.

skinned muscle. Despite this diffusion, the magnitude of the caffeine response in skinned muscle after loading with 10^{-6} M Ca for 3 min was almost the same as that in the depolarized muscle in the presence of EGTA before treatment with saponin. These results suggest that Ca in the skinned smooth muscle is in the physiological range after loading with 10^{-6} M Ca for 3 min.

Several investigators (Ford and Podolsky, 1970; Endo et al., 1970; Endo, 1975) reported that Ca induces a Ca release from the SR of skinned skeletal muscle fibers. Because they used conditions facilitating the Ca-induced Ca

release mechanism (e.g., low concentrations of Mg, addition of caffeine; or high concentrations of Ca), the Ca-releasing effect of Ca itself seems weak under physiological conditions in skeletal muscle. Using skinned cardiac

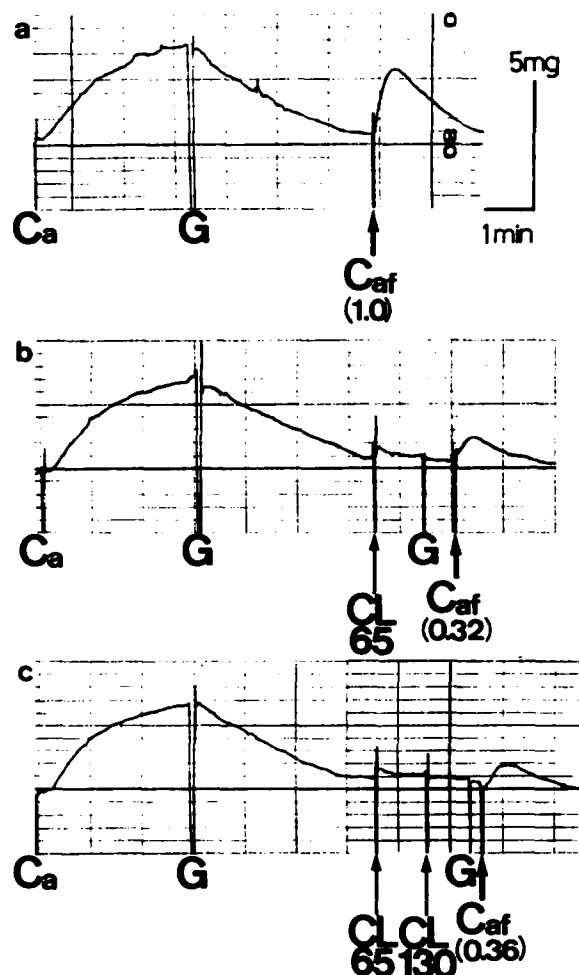


FIGURE 6. The effect of repetitive application of Cl on the Ca store. (a) Control. (b) The release of Ca induced by 65 mM Cl. (c) Effect of 130 mM Cl on the Ca present in the store after the application of 65 mM Cl. Ca, G, and Caf represent Ca loading with 10^{-6} M Ca, washing with relaxing solution with 0.1 mM EGTA, and application of 25 mM caffeine, respectively. The concentration of Cl in bathing solutions is shown in millimoles. The Ca remaining in the store is shown in parentheses relative to control levels.

preparations, Fabiato and Fabiato (1972) showed that Ca-induced Ca release can be more easily evoked with a lower level of Ca than in skeletal muscle. In the present study using skinned smooth muscle, Ca-induced Ca release starts operating at 3×10^{-6} M Ca without additional conditions facilitating

the release mechanism, i.e., Ca release from the storage site can be triggered in skinned smooth muscle by much smaller concentrations of Ca than that required in skinned skeletal muscle fibers. In this respect smooth muscle is similar to cardiac muscle rather than to skeletal muscle. It should be desirable to use a high concentration of Ca buffer to obtain the precise concentration of Ca in the skinned muscle preparation. When a low concentration of Ca buffer was used in the skinned skeletal muscle fibers, the level of Ca in the immediate vicinity of the SR membrane could be altered by the SR activity. In this study, a relatively low concentration of Ca buffer (0.1 mM) was used because the amount of Ca in the storage site of skinned smooth muscle was significantly decreased in the presence of EGTA in the millimolar range, unlike the SR of skinned skeletal muscle fibers. This implies that the Ca store of smooth muscle is more leaky than that of skeletal muscle. In the case of skinned smooth muscle, it is unlikely that the level of Ca in the immediate vicinity of the Ca store could be altered by the Ca store activity.

Although under physiological conditions the exact intracellular concentration of Mg in muscle tissues is still unknown, the concentration has been estimated to be ~ 1 mM. In the presence of this concentration of Mg, $>10^{-4}$ M Ca is necessary to induce Ca release from the SR in skinned skeletal muscle fibers (Endo, 1975; Saida and Suzuki, 1981), which is unlikely to be physiologically relevant. However, in the presence of 5 mM Mg, Ca release from the SR can be triggered in skinned cardiac cells by 10^{-7} M Ca, a much smaller concentration than that required in skinned skeletal muscle fibers (Fabiato and Fabiato, 1975). On the other hand, we have previously reported that the physiological intracellular concentration of Mg seems to be in the range of 1–2 mM in smooth muscle (Saida and Nonomura, 1978). In the present study, Ca release from the storage site required $>3 \times 10^{-6}$ M Ca in the skinned smooth muscle with physiological concentrations of Mg. The threshold of Ca to trigger release was increased when the concentration of Mg was raised in the skinned smooth muscle, like the effect seen in the skinned cardiac cells (Fabiato and Fabiato, 1975). According to Fabiato and Fabiato (1975), elevation of Mg increased the capacity and rate of binding for Ca by the SR in the skinned cardiac cells. In skinned smooth muscle, however, elevation of Mg did not significantly increase the amount of Ca in the storage site, which suggests that competition may occur between the two divalent cations.

Because it is impossible to use microelectrodes in the SR due to its small size, the only effective way to alter the membrane potential of the SR in a direct manner is to change quickly the ionic composition of the solution bathing the skinned muscle preparation. In the skinned skeletal muscle fibers, replacement of methanesulfonate or propionate with Cl caused a Ca release from the SR probably caused by “depolarization” of the SR (Costantin and Podolsky, 1967; Ford and Podolsky, 1970; Nakajima and Endo, 1973; Saida and Suzuki, 1981). In skinned cardiac cells, Fabiato and Fabiato (1977) have shown that the effect of Cl replacement was very weak, whereas Endo and Kitazawa (1978) showed that replacement of K with choline caused Ca release from the SR. In the present study using skinned smooth muscle, the skinned

preparation showed only a small transient contraction when propionate was replaced by Cl whereas ~75% of the stored Ca was released from the storage site by the ionic replacement. The unexpectedly small contraction is probably caused by an acceleration of the Ca sequestration by EGTA in the solution containing Cl. The amount of Ca released by the ionic replacement was dependent on the Cl concentration in the solution. The Ca release occurred only immediately after the application of Cl; thereafter, the Ca release mechanism seemed to be inactivated by the prolonged presence of Cl. This inactivation may be difficult to explain as a direct action of Cl but may be reconciled as a "depolarization"-induced Ca release. However, it still remains unresolved whether the release of Ca induced by Cl replacement is caused exclusively by depolarization of the Ca store. Other effects of the ionic replacement cannot be ruled out at present.

Although the increase in intracellular Ca concentration associated with a single action potential was suggested to be 4×10^{-6} M by Goodford (1967), the functional increase in Ca concentration may be lower because not all the Ca binds to the contractile proteins. The activation of the contractile system by 4×10^{-6} M Ca would be no more than 30% of the full activation according to the pCa tension curve described in our previous paper (Saida and Nonomura, 1978). Therefore, it is quite possible that Ca released from the storage site by either regenerative mechanisms or "depolarization," or both, contributes to full activation, but it is still unclear to what extent these mechanisms participate in excitation-contraction coupling in smooth muscle.

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