99. **Conditions Required for Calcium-Induced Release of Calcium from the Sarcoplasmic Reticulum**

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Calcium ion itself has been shown\(^1\)-\(^6\) to be capable of inducing a release of calcium from the sarcoplasmic reticulum (SR) in skinned skeletal muscle fibres.\(^7\) This gives a basis for a certain form of propagation of contraction observed in skinned fibres,\(^8\) and also for more-or-less synchronized repetitive spontaneous release of calcium from the SR in skinned fibres.\(^5\),\(^6\) However, physiological significance of the calcium-induced calcium release mechanism is not yet clear, primarily due to a lack of quantitative information about the effect of calcium, although possible physiological roles of calcium ion in this context in excitation-contraction coupling of cardiac muscle\(^3\) or of skeletal muscle\(^6\) have been pointed out. It seemed, therefore, very important to further investigate the process of stimulation of the SR by calcium ion itself to obtain more quantitative information.

**Methods.** Single fibres were obtained from iliofibularis muscles of African clawed toads, *Xenopus laevis* and skinned in a relaxing solution. Experimental conditions and procedures were almost the same as previously described.\(^5\),\(^9\) Briefly, the amount of calcium in the SR of a skinned fibre at anytime was estimated by releasing it almost completely by means of a high concentration of caffeine, usually 25 mM, and measuring the magnitude of resulting isometric contraction of the fibre. Radioisotope experiments verified that most of calcium in the SR was actually released in this way. To avoid a long-lasting contracture that may damage the fibre seriously, and to prevent the released calcium ions from going back to the SR, caffeine treatment was made in the presence of an appropriate concentration of EGTA (ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid, GEDTA), usually 2 mM.

When the effect of a concentration of free calcium ion was to be observed, a high concentration of calcium buffer with total 10 mM EGTA was used. Otherwise, the free calcium ion concentration in...
the immediate vicinity of the outer surface of the SR membrane might have been appreciably different from that in the bulk of solution, especially when a continuous leak of calcium out of the lumen of the SR was occurring because of, for example, heavy loading.

Results. Raising the free calcium level in the sarcoplasm is expected to have two effects. The SR takes up calcium at a faster rate under a higher level of free calcium, but if the level is also high enough to stimulate the calcium-induced release of calcium, release process must be taking place at the same time. What will be the net result at each fixed level of free calcium?

Figure 1 shows typical time course of net calcium uptake under various free calcium ion concentrations by the SR in a skinned fibre from which calcium was previously depleted. As is seen in the figure, the rate of uptake was increased with the increase in the level of free calcium in the incubating medium up to $3 \times 10^{-5}$ M in this fibre. The steady level of amount of uptake under each concentration of
free calcium seemed to be not markedly different between $10^{-6}$ M and $3 \times 10^{-5}$ M (not shown in Fig. 1). However, when higher concentrations of free calcium were used, while initial rate of uptake was not much different from that of $3 \times 10^{-5}$ M free calcium, the amount of calcium taken up rapidly reached a level over which no further net accumulation occurred.

The above result suggested that with a concentration of free calcium higher than $10^{-4}$ M, calcium-induced calcium release mechanism also operated and as a result the steady level of net uptake of calcium became much lower than that with a lower concentration of free calcium. If this is so, one would expect that after the SR was loaded to a level nearly maximum of its capacity by incubating it with a low concentration of free calcium, a short exposure to a medium containing a concentration of free calcium higher than $10^{-4}$ M should induce a release of calcium, but no release should be expected when exposure was made to a concentration of calcium lower than $3 \times 10^{-5}$ M. This was exactly the case, as shown in Fig. 2. After the SR of a skinned fibre was heavily loaded in $10^{-6}$ M free calcium for 10 minutes, the fibre was treated with various concentrations of free calcium for
15 sec. As is seen, if the treatment was made with free calcium of $3 \times 10^{-5}$ M or lower, the range of concentrations that showed no inhibition of apparent uptake, the magnitude of caffeine response after the brief second calcium treatment remained practically unchanged. However, if the treatment was made with concentrations higher than $10^{-4}$ M (the case of $3 \times 10^{-4}$ M is shown in Fig. 2), which showed inhibition of steady level of uptake (Fig. 1), the magnitude of caffeine response after the calcium treatment was much smaller than in the case of the treatment with a lower free calcium. Since the sensitivity of the contractile system of the fibre to calcium was not altered after the treatments with high concentrations of calcium, the experiments shown in Fig. 2 indicate that the amount of calcium remaining in the SR after a brief treatment with very high free calcium was smaller, and, therefore, calcium must have been released during the very high free calcium treatments.

Since in the above experiment only net changes in the amount of calcium in the SR during the second calcium treatment were measured, it cannot be denied that by treatment with lower levels of free calcium, the release of calcium might also have occurred but have been counterbalanced by a higher rate of uptake. While whether the release also occurs at a lower level of free calcium or not cannot be determined until unidirectional fluxes of calcium across the SR membrane are measured, the facts that the initial rate of uptake of calcium was not reduced with concentration of free calcium higher than $10^{-4}$ M (Fig. 1) and that the release experiments shown in Fig. 2 was done on the SR loaded to its nearly maximum capacity seem to suggest that an appreciable release occurs only with concentrations of free calcium higher than $10^{-4}$ M, under the experimental conditions of Fig. 2.

The result of experiments shown in Fig. 1 also suggested another condition required for net release of calcium by calcium: the SR must be preloaded with calcium at least to a level that could be attained by the SR in the steady state under the stimulating concentration of free calcium. This was also shown to be the case. In the experiments shown in Fig. 3, the level of loading of the SR was altered by altering the duration of incubation with the loading medium, and for each loading the effect of exposing the SR to a high enough concentration of free calcium was examined. As is shown in the figure, when the loading of the SR was light (say, less than 2 minutes), a brief treatment with $10^{-4}$ M calcium only resulted in an additional accumulation of calcium, but if the SR was heavily loaded (say, 5–8 minutes), the same concentration of calcium caused a release of calcium, the final level of calcium in the SR becoming definitely lower than in the
case of a lighter loading (3 minutes). Thus, there seems to be a certain level of loading for the net release of calcium to occur, and above the level, the heavier the loading is, the larger fraction seems to be released by the treatment with the same concentration of free calcium.

The level of free magnesium in the medium profoundly affected the calcium-induced calcium release as already noted. On lowering free magnesium from about 0.9 mM in the normal medium used in the experiments described above (4 mM total magnesium, 4 mM total ATP: following affinity constants are used for calculation: Mg ATP $10^4$ M$^{-1}$, CaATP $4 \times 10^5$ M$^{-1}$, KATP 10 M$^{-1}$) to, for example, 0.05 mM (1 mM total magnesium, 4 mM total ATP), initial phase of calcium uptake by the SR under lower concentration range of free calcium ($3 \times 10^{-6}$ M or lower) was not altered, but uptake under the concentration of free calcium higher than $10^{-5}$ M was strongly inhibited. As a result, in the presence of 0.05 mM free magnesium, steady level
of uptake of calcium attained under $10^{-5}$ M or higher free calcium was much lower than that under lower free calcium concentrations. A similar phenomenon was observed only with $10^{-4}$ M or higher free calcium in the presence of 0.9 mM free magnesium (Fig. 1). In agreement with this, calcium-induced release of calcium similar to that shown in Fig. 2 could be evoked with $10^{-5}$ M free calcium, if free magnesium was 0.05 mM (ref. 10).

The effect of the reduction of total magnesium from 4 mM to 1 mM must be due to the reduction in free magnesium, since if free magnesium was kept constant, the changes in MgATP or free ATP concentration did not appreciably alter the effectiveness of calcium to produce a release of calcium.

Raising free magnesium produced an opposite effect. With an increase in the level of free magnesium, the free calcium ion concentration necessary to induce release of calcium increased.

**Discussion.** Two conditions required for calcium-induced calcium release were found: i.e., free calcium ion concentration must be higher than a certain level, and calcium must be preloaded to a certain level, both of which one could naturally expect. These requirements are profoundly affected by the level of free magnesium. The physiological significance of the calcium-induced calcium release mechanism will be discussed in the accompanying papers.10,11)

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