Effects of Rapid Application of Caffeine on Intracellular Calcium Concentration in Ferret Papillary Muscles

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ABSTRACT In this paper we investigate the effects of caffeine (5–20 mM) on ferret papillary muscle. The intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was measured from the light emitted by the photoprotein aequorin, which had previously been microinjected into superficial cells. Isometric tension was measured simultaneously. The rapid application of caffeine produced a transient increase of [Ca\(^{2+}\)]\(_i\), which decayed spontaneously within 2–3 s and was accompanied by a transient contracture. The removal of extracellular Na\(^+\) or an increase in the concentration of intracellular Na\(^+\) (produced by strophanthidin) increased the magnitude of the caffeine response. Cessation of stimulation for several minutes or stimulation at low rates decreased the magnitude of the stimulated twitch and Ca\(^{2+}\) transient. These maneuvers also decreased the size of the caffeine response. These results are consistent with the hypothesis that the caffeine-releasable pool of Ca\(^{2+}\) (sarcoplasmic reticulum) is modulated by maneuvers that affect contraction. Ryanodine (10 \(\mu\)M) decreased the magnitude of the caffeine response as well as that of the stimulated twitch. In contrast, the rapid removal of external Ca\(^{2+}\) abolished the systolic Ca\(^{2+}\) transient within 5 s, but had no effect on the caffeine response. From this we conclude that the abolition of twitch by Ca\(^{2+}\)-free solutions is not due to depletion of the sarcoplasmic reticulum of Ca\(^{2+}\), but may be due to a requirement of Ca\(^{2+}\) entry into the cell to trigger Ca\(^{2+}\) release from the sarcoplasmic reticulum.

INTRODUCTION

It is generally accepted that in mammalian cardiac muscle much of the Ca\(^{2+}\) responsible for contraction comes from the sarcoplasmic reticulum (SR) (Reuter, 1974). Many maneuvers that affect force, such as changing stimulus rate, the application of catecholamines, or changing external Ca\(^{2+}\) concentration, have been suggested to act by changing the Ca\(^{2+}\) content of the SR and thence the amount of Ca\(^{2+}\) released by a subsequent action potential (Beeler and Reuter, 1970; Allen et al., 1976). However, recent work has questioned this conclusion (Isenberg, 1982; Kitazawa, 1984). One problem with investigating the importance of changes of SR Ca\(^{2+}\) content is

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that there is no simple, direct way of measuring the Ca\(^{2+}\) content of the SR in an intact muscle. One way to estimate the SR Ca\(^{2+}\) content would be to try to release all the Ca\(^{2+}\) from the SR and then to measure the resulting increase of tension. This has been done recently by cooling the muscle rapidly (Kurihara and Sakai, 1985; Bridge, 1986). An alternative method is to use caffeine, which has been shown to release Ca\(^{2+}\) from isolated SR (Weber and Herz, 1968). In skeletal muscle caffeine produces both an increase of [Ca\(^{2+}\)], and a contracture (Konishi and Kurihara, 1987). However, in mammalian cardiac muscle most studies find that caffeine does not produce a contracture (e.g., Blinks et al., 1972). Only when [Ca\(^{2+}\)], is artificially elevated does caffeine produce a contracture (Jundt et al., 1975) and, under these conditions much of the contracture may be due to an increase of Ca\(^{2+}\) sensitivity of the contractile apparatus (Wendt and Stephenson, 1983; Eisner and Valdeolmillos, 1985) rather than to an increase of [Ca\(^{2+}\)], itself. Indeed, a recent review (Clusin, 1985) has questioned whether caffeine does produce a rise of [Ca\(^{2+}\)],. Chapman and Leoty (1976) have, however, successfully recorded a caffeine contracture in normal solutions. They found that this contracture was unaffected by changes of [Ca\(^{2+}\)],. Since the twitch is sensitive to [Ca\(^{2+}\)],, this result is hard to reconcile with the SR having a major role in the initiation of contraction.

In the present paper we have estimated the rate of release of Ca\(^{2+}\) ions from the SR produced by caffeine. This was done by measuring the resultant increase of [Ca\(^{2+}\)],, using the photoprotein aequorin, in addition to the tension for the following reasons. (a) The effects of caffeine on tension reflect a complicated balance of changes of both [Ca\(^{2+}\)],, and Ca\(^{2+}\) sensitivity. (b) Aequorin is only injected into cells near the surface of the preparation and therefore [Ca\(^{2+}\)], is only measured from cells that are in rapid diffusional contact with the superfusing solution. The results show that caffeine produces a transient increase of [Ca\(^{2+}\)],, and that the magnitude of this increase is altered by maneuvers that change contraction. This is consistent with the idea that changes in SR Ca\(^{2+}\) content are involved in the regulation of contraction.

Preliminary accounts of some of the present results have appeared in abstract form (Allen et al., 1986; Valdeolmillos et al., 1987).

**METHODS**

The experiments were performed on papillary muscles isolated from the right ventricle of ferrets. The muscles, whose diameters varied from 0.4 to 0.8 mm, were mounted in the experimental chamber and superfused with a modified Tyrodes solution (see below). All experiments were performed at room temperature (20–22°C). This temperature was used because it is much easier to perform very rapid solution changes at room temperature as changes of flow rate do not change the temperature of the experimental bath, and because, as noted previously (Chapman and Leoty, 1976), the caffeine response is much more obvious at these lower temperatures.

The experimental chamber had a volume of 0.25 ml, a transparent base, and was covered with a coverslip. The solution was superfused with a peristaltic pump. To apply caffeine rapidly the inflowing solution flow was stopped. Then 1 ml of a caffeine-containing solution was injected down another inflow line using a syringe. This syringe was driven by air pressure that was regulated by an electrically operated valve. The syringe took 200 ms to complete its travel. Using this apparatus the solution change in the bath took 200 ms to go from 10 to
90% completion. This time was measured by injecting solutions of different optical density into the bath and measuring the transmission of light from a light standard above the bath to a photomultiplier tube (PMT) below (Fig. 1B).

The \([\text{Ca}^2+]\) was measured by recording the light emitted from the photoprotein aequorin. The aequorin had previously been microinjected into 20–30 superficial cells. Care was taken to inject only superficial cells to minimize the diffusion time to the cells during the application of caffeine. This was done by attempting to inject only the first cell encountered by any microelectrode penetration (as judged by the resting potential). The light emitted by aequorin was measured with a PMT. In the earlier experiments the PMT was placed above the experimental chamber and connected to the coverslip with a perspex light guide. A disadvantage of this technique was that any air bubbles that collected above the preparation interfered with light collection. Therefore in later experiments the PMT and light guide were placed below the transparent base of the bath. The PMT output was connected to an amplifier discriminator and photon counter. The counter was used to measure the total number of photons in the caffeine response. Measurement of the total number of photons was a much more reliable method of measuring the response than the amplitude. Nevertheless, typically only \(\sim 100\) photons were recorded despite efforts to inject as much aequorin as possible into the preparation. On average the total amount injected corresponded to \(4 \pm 1\) million counts \((n = 11)\) as measured by the photon counter.

At the end of the experiment the total amount of aequorin remaining in the preparation was measured by adding 1% (vol/vol) Triton X-100 to the superfusing solution. From this the fractional luminescence \(((L/L_{\text{max}}))\) that corresponded to a given light output in counts per second could be estimated as follows:

\[
\frac{L}{L_{\text{max}}} = \frac{\text{counts per second}}{(\tau \times \text{counts on exposure to Triton})} \tag{1}
\]

where \(\tau = 1.0\ \text{s}^{-1}\) is the rate constant of aequorin consumption measured in 150 mM K\(^+\), 10 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\), pH 7.0, at 22°C, using aequorin equilibrated in the same solution but without the Ca\(^{2+}\) (see Moore and Blinks, 1984). Fractional luminescence was then converted to \([\text{Ca}^{2+}]\), using the formula

\[
[\text{Ca}^{2+}] = \frac{(L/L_{\text{max}})^{0.55}(1 + K_{TR}) - 1}{[K_R - K_R(L/L_{\text{max}})^{0.55}] - [K_R(L/L_{\text{max}})^{0.55}]} \tag{2}
\]

(for derivation see Allen et al., 1977). The value of the two constants, \(K_{TR} = 130\) and \(K_R = 3.5 \times 10^8\), were obtained by fitting the above expression to an in vitro calibration curve. This was determined using the methods described by Allen et al (1977) with the modification of Moore and Blinks (1984) to take into account the necessity to preequilibrate aequorin with Mg\(^{2+}\). The calibrating solution contained 150 mM K\(^+\), 1.0 mM Mg\(^{2+}\), pH 7.0, and was kept at 22°C.

Note that the value of Mg\(^{2+}\) used was 1 mM in contrast to the value of 3 mM used in earlier studies from this laboratory. The value of 1 mM is taken from Blatter and McGuigan, 1986. This has the effect of lowering our estimates of \([\text{Ca}^{2+}]\) by a factor of \(\sim 2\) in comparison with our earlier studies from this laboratory. Because of the uncertainty about the level of [Mg\(^{2+}\)] and other intracellular factors that might affect the sensitivity of aequorin to \([\text{Ca}^{2+}]\), the calibration procedure should be regarded as giving a general indication of the level of \([\text{Ca}^{2+}]\) rather than a precise measure.

Solutions

The experimental solution had the following composition (in millimolars): 135 Na\(^+\), 5 K\(^+\), 1 Mg\(^{2+}\), 2 Ca\(^{2+}\), 100 Cl\(^-\), 20 acetate, 5 Hepes, 10 glucose, and \(4 \times 10^{-5}\) insulin. In low-Ca\(^{2+}\) solutions 20 mM Cl\(^-\) was replaced by 10 mM EGTA. In zero Na\(^+\) solutions, Li\(^+\) was used instead of Na\(^+\).
RESULTS

The experiment of Fig. 1 shows the effects of a sudden application of 20 mM caffeine on a ferret papillary muscle. This produces a transient increase of $[\text{Ca}^{2+}]_i$ that reaches a peak within $\sim 1$ s and then decays away over 2 s. The increase of $[\text{Ca}^{2+}]_i$ is accompanied by a transient contracture. Under the control conditions used here the caffeine contracture relaxes fully.

![Diagram](image)

**Figure 1.** The effects of caffeine on $[\text{Ca}^{2+}]_i$ and the resting force. (A) Specimen effects of caffeine. The top trace shows aequorin light and the bottom one shows tension. Caffeine (20 mM) was applied for the period shown above. Note that the first deflection of the contraction record is an artefact of the rapid solution change. (B) Time course of onset of caffeine response. The top trace shows the period over which the syringe moved to expel fluid. The second trace shows the increase of $[\text{Ca}^{2+}]_i$ produced by caffeine application. The initial spike is an artefact. The bottom trace was obtained subsequently by injecting a dilute solution of a colored compound into a more concentrated one while optical transmission was measured as an index of the extent of the solution change.

The Effects of Maneuvers that Alter $[\text{Ca}^{2+}]_i$

The magnitude of the caffeine response was increased by maneuvers that increased $[\text{Ca}^{2+}]_i$. Fig. 2A shows the results of a control exposure to caffeine. In Fig. 2B, the same preparation had been exposed to a solution in which all the Na$^+$ in the bathing solution has been replaced by Li$^+$. This increased the magnitude of the Ca$^{2+}$ transient produced by stimulation. Correspondingly, the effects of caffeine on both
[Ca\textsuperscript{2+}], and tension were then considerably greater than in the control. In addition to the increase in the magnitude of the caffeine response, there was substantial prolongation of its decay. The effects of Na\textsuperscript{+} removal were reversible (not shown). In agreement with much previous work (Allen and Blinks, 1978; Wier and Hess, 1984), the addition of strophanthidin increased the magnitude of the stimulated Ca\textsuperscript{2+} transient and twitch (Fig. 3). This figure also shows that strophanthidin increases the magnitude of the response to caffeine.

The Effects of Altering the Pattern of Stimulation

In the experiment shown in Fig. 4, the frequency of stimulation was altered before adding caffeine. The muscle was stimulated at the desired rate and, after a 10-s rest,

![Graph showing the effects of altering the pattern of stimulation](image)

FIGURE 2. The effects of removal of external Na\textsuperscript{+} on the stimulated and caffeine responses. (A) Control. The top two traces show aequorin light. The top light record was recorded at a higher gain than the lower one. The bottom trace is tension. The preparation was initially stimulated at 1 Hz but then stimulation stopped while caffeine (90 mM) was added. (B) The response was obtained after removing external Na\textsuperscript{+} (replaced by Li\textsuperscript{+}). The high-gain light record is not shown here.

caffeine was applied. This protocol was then repeated at a different rate of stimulation. Fig. 4 B shows the steady-state twitch and aequorin transient at various frequencies of stimulation. Increasing the stimulus frequency increases the magnitude of both the transient and twitch. Furthermore, the twitch is markedly prolonged at the slower rate (cf., Allen et al., 1976). Fig. 4 A shows that increasing the rate of stimulation increases the magnitude of the caffeine response.

Previous work has shown that in many cardiac preparations, if regular stimulation is interrupted with a delay of several minutes, the next stimulated twitch is much smaller than those before the rest. This behavior has been attributed to a decrease of the Ca\textsuperscript{2+} content and thence of the Ca\textsuperscript{2+} released from the SR (Allen et al., 1976). An example of this phenomenon is shown in Fig. 5 B, which shows that, although rests of up to 1 min increase both the Ca\textsuperscript{2+} transient and the twitch, longer
duration rests decrease the transient and the twitch. In the same experiment we investigated the effects of applying caffeine after a delay long enough to decrease the twitch to low levels. Fig. 5 A shows that the caffeine response after a 5-min rest was much smaller than that after a 10-s rest, thus supporting the hypothesis that the SR Ca$^{2+}$ content is decreased. Although not shown, the response to caffeine after a 10-s rest was slightly greater than that with no rest (see Fig. 6).

Fig. 5 B also shows that although both the twitch and Ca$^{2+}$ transient after a long rest are smaller than those at a regular stimulation rate, a shorter rest increases the size of the twitch and Ca$^{2+}$ transient. The same phenomenon is illustrated in more detail in Fig. 6. The data in Fig. 6 A show the effects on both the stimulated twitch and the caffeine response to varying the rest duration. Increasing the rest from 1 to 10 s produces a small increase of both the twitch and caffeine response. However, further increase of rest duration decreases both. The addition of strophanthidin increased both the twitch and caffeine response at all rest durations. However, the effects of rest duration on both twitch and caffeine response are now greatly atten-

![Figure 3](image-url)
uated. This point is emphasized by Fig. 6 B, which shows the same data now normalized to the values after a 10-s rest.

In the experiment illustrated in Fig. 7, the response to a 10-s rest was atypical. Fig. 7 A shows that, after such a rest, the twitch is considerably smaller than that during steady-state 1-Hz stimulation. The effects of caffeine were also tested after either a 1- or 10-s rest (Fig. 7 B). The caffeine response is barely detectable after a 10-s rest but is obvious after 1 s. Thus, in this experiment also, the behavior of the caffeine response parallels that of the twitch.

A more sensitive test of the relationship between the twitch and the caffeine response is shown in Fig. 8. Fig. 8 A shows that, after a 1-min rest, the first stimulated twitch and Ca\(^{2+}\) transient are large, whereas the second stimulated twitches are small. A plausible explanation for the small second twitch is that the SR is depleted of Ca\(^{2+}\) after the first twitch. In Fig. 8 B and C we have investigated the effect of applying caffeine either at a time corresponding to the first stimulus (i.e., after a 1-min rest) or at the time of the second stimulus (i.e., after a 1-min rest and then one stimulus). The results show that the occurrence of the first stimulus decreases

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**Figure 4.** The effects of stimulation rate on the response of \([\text{Ca}^{2+}]_i\) and tension to both stimulation and caffeine. (A) Caffeine response. In both panels traces show aequorin light on the top, and tension on the bottom. Caffeine (20 mM) was applied for the period shown. The muscle has been stimulated at either 1 (a) or 0.1 Hz (b). Then, after a 10-s rest, caffeine was applied. (B) Stimulated response. Averaged (n = 16) records of aequorin light (top) and tension (bottom) are shown at 0.1 and 1 Hz. The stimulus marker is shown below.
the magnitude of the caffeine response 1 s later. Therefore, after a rest, one twitch depresses both the next twitch and a caffeine response.

The Effects of Ryanodine on the Caffeine Response

Ryanodine has been reported to interfere with Ca\(^{2+}\) release from the SR. It is therefore of interest to know whether it blocks the ability of caffeine to release Ca\(^{2+}\).

![Diagram](image)

**FIGURE 5.** The effects of rest duration on the stimulated and caffeine responses. (A) Caffeine response. In both panels the preparation has initially been stimulated at 1 Hz. Stimulation was then discontinued for either 10 s (a) or 5 min (b) before adding caffeine (20 mM). The record shows only the last few seconds of these rests and the caffeine exposure. In both panels traces show aequorin light on the top and tension on the bottom. (B) Response to stimulation. The preparation was stimulated at 1 Hz and rested for the periods shown (from left to right 10 s, 1 min, and 5 min). The upper trace shows the integral of the aequorin response in each stimulated transient. 1 unit corresponds to 10\(^{-4}\) of the total counts in the preparation. This is a less noisy signal than the magnitude of the response. The lower trace shows tension.

Previous work has found that ryanodine sometimes inhibits the effects of caffeine, depending on the experimental conditions (Fabiato, 1985; Marban and Wier, 1985). Fig. 9 A shows the effects of 10 \(\mu\)M ryanodine on the Ca\(^{2+}\) transient and twitch. In this experiment ryanodine decreased twitch tension to 26% and the aequorin light to 33% of control. In agreement with previous work (Marban and Wier, 1985; Wier et al. 1985), ryanodine decreased the aequorin transient to
Figure 6. Interactions between strophanthidin and rest on the stimulated and caffeine responses. (A) The effects of rest duration on the stimulated twitch (circles) and caffeine response (triangles). The preparation was initially stimulated at 1 Hz before being rested for a test period. After this period either caffeine (20 mM) was applied or stimulation was recommenced. The open symbols were obtained under control conditions and the closed symbols after 20 min of exposure to strophanthidin. (B) Normalized data. The data from A are normalized to the values at a 10-s-duration rest.
20 ± 5% (n = 4) and the tension to 28%. In contrast to these partial effects of ryanodine on the stimulated aequorin transient and tension, Fig. 9 B shows that ryanodine abolishes the caffeine response. It has been suggested (Fabiato, 1985) that ryanodine may have less effect on Ca\textsuperscript{2+}-overloaded preparations. However, we found that ryanodine abolished the caffeine response even in a Na\textsuperscript{+}-free solution (not shown).

![Graphs showing aequorin light and tension responses](image)

**FIGURE 7.** Comparison of stimulated and caffeine responses in a preparation in which a 10-s rest decreased twitch tension. (A) Effect of rest on the stimulated response. The preparation had been stimulated at 1 Hz and stimulation was discontinued for the 10-s period shown. Traces show aequorin light on the top and tension on the bottom. (B) Effects of rest on the caffeine response. Caffeine (20 mM) was applied for the period indicated. In both panels the preparation had been stimulated at 1 Hz. Stimulation was stopped and caffeine applied after a delay of either 1 (a) or 10 s (b).

**The Effects of Ca\textsuperscript{2+}-free Solutions**

It has been known for many years that the removal of Ca\textsuperscript{2+} from the external solution abolishes the force of contraction of cardiac muscle (Ringer, 1883). There are two possible explanations for this result: (a) removal of extracellular Ca\textsuperscript{2+} will indirectly deplete the SR of Ca\textsuperscript{2+} or (b) removal of external Ca\textsuperscript{2+} will abolish the inward Ca\textsuperscript{2+} current, which will, either directly or via the elimination of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, abolish contraction. One way to distinguish these explanations is to remove Ca\textsuperscript{2+} rapidly. On hypothesis (a) contraction should disappear slowly as the SR Ca\textsuperscript{2+}
is depleted, whereas on (b) contraction should be eliminated instantaneously. One problem with performing this experiment in multicellular preparations is the time taken to wash out the extracellular space. This problem is minimized in the present experiments where \([\text{Ca}^{2+}]_{i}\) is only measured from superficial cells.

In the present series of experiments we decreased \([\text{Ca}^{2+}]_{o}\) rapidly by changing to a \([\text{Ca}^{2+}]_{o}\)-free solution containing 10 mM EGTA. Fig. 10 A shows that \([\text{Ca}^{2+}]_{o}\) removal abolishes the \([\text{Ca}^{2+}]_{o}\) transient within seconds. Contraction, however, decreases more slowly presumably because it takes time for \([\text{Ca}^{2+}]_{o}\) to diffuse out of the bulk of the preparation. Other experiments (not shown) showed that the action potential was not affected over these short periods of \([\text{Ca}^{2+}]_{o}\) removal. The averaged data of Fig. 10 B show that it takes ~5 s for the \([\text{Ca}^{2+}]_{o}\) transient to disappear. In terms of the above hypotheses this rapid abolition of the \([\text{Ca}^{2+}]_{o}\) transient is only consistent with hypothesis (a) if the SR is depleted over a similar rapid time course. We have

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**Figure 8.** The effects of a single twitch on the caffeine response. In all three panels the upper trace shows aequorin light and the lower tension. The stimulus marker is indicated below. The preparation had been stimulated at 1 Hz before the records began. In each panel stimulation was stopped for 1 min. At the end of this period the following happened: (A) stimulation was recommenced, (B) caffeine (20 mM) was applied, and (C) one stimulus was applied and then caffeine was added.
attempted to assess the degree of loading of the SR by the rapid application of caffeine. A typical result is shown in Fig. 11. Fig. 11 A shows a control exposure to caffeine. In Fig. 11 B EGTA was added 10 s before caffeine. This removal of \([Ca^{2+}]_o\) abolished the Ca\(^{2+}\) transient. However, the increase of \([Ca^{2+}]_i\) produced by caffeine is similar to that in the control. In six experiments the increase of \([Ca^{2+}]_i\) produced by caffeine in the presence of EGTA was 80% of that in control solution. Similar experiments were performed using Ni\(^{2+}\) to block Ca\(^{2+}\) entry into the cell instead of using EGTA to lower external Ca\(^{2+}\). The application of 10 mM Ni\(^{2+}\) blocked the Ca\(^{2+}\) transient and the twitch with a time course similar to that of EGTA. Similarly, the caffeine response was only slightly attenuated after 10 s in Ni\(^{2+}\).

**DISCUSSION**

*The Response to Caffeine*

The results of the present paper show that the application of caffeine produces an increase of both \([Ca^{2+}]_i\) and tension that lasts for \(~2\) s. The brevity of the caffeine response is in agreement with tension measurements (Chapman and Leoty, 1976). Under control conditions the mean \([Ca^{2+}]_i\) at the peak of the caffeine response was
FIGURE 10. The effects of Ca\textsuperscript{2+} removal on the stimulated response. (A) Original record. The top two traces represent aequorin light and tension. External Ca\textsuperscript{2+} was removed and 10 mM EGTA was added for the period indicated above. The bottom trace shows the aequorin light records on an expanded time scale. The stimulus marker is shown below this record and the solution change indicator at the bottom. (B) Averaged data. This shows the time course of decay of the twitch magnitude as a function of time after Ca\textsuperscript{2+} removal. Error bars show SEM (n = 6).
considerably less than that at the peak of the stimulated Ca\textsuperscript{2+} transient. If caffeine releases Ca\textsuperscript{2+} from the SR by the same mechanism as does the action potential, one might expect the peak Ca\textsuperscript{2+} value to be the same in both cases. The smaller caffeine response may, however, be explained by the slower time course of the caffeine response. It is possible that if the caffeine could be applied instantaneously to the SR, that the response would be considerably larger.

Rapid cooling of cardiac muscle produces an increase of resting force (Kurihara and Sakai, 1985; Bridge, 1986), which has been attributed to release of Ca\textsuperscript{2+} from the SR. Like the caffeine contracture presented in this paper, these cooling contractions were affected in a similar way to the twitch by changing the frequency of stimulation.

![Graph](image)

**Figure 11.** The effects of Ca\textsuperscript{2+} removal on the caffeine response. In both panels the top trace is aequorin light and the bottom is tension. (A) Control caffeine response. The preparation was stimulated at 1 Hz and stimulation was then discontinued for 10 s before caffeine (20 mM) was added. The bottom panel shows an expanded version of the effects of caffeine on aequorin light. (B) Ca\textsuperscript{2+}-free. Ca\textsuperscript{2+} was removed (and 10 mM EGTA added) for the period shown. Stimulation was stopped 1 Hz later as indicated by the termination of contraction. Finally, caffeine (20 mM) was added.

The observation that the caffeine contracture is large and slower in Na\textsuperscript{+}-free than in Na\textsuperscript{+}-containing solutions has been demonstrated to be because of the Ca\textsuperscript{2+} released from the SR being removed from the cell by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in the presence of Na\textsuperscript{+} (Jundt et al., 1975). Our observation that the decay of the caffeine response is greatly slowed in a Na\textsuperscript{+}-free solution is also consistent with this hypothesis. This suggests that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange can decrease [Ca\textsuperscript{2+}], within seconds. This is much faster than the rate constant of radioactive Ca\textsuperscript{2+} efflux from the cell, suggesting that the rate of radioactive Ca\textsuperscript{2+} efflux may actually be limited by efflux from intracellular compartments. This effect of [Na\textsuperscript{2+}]\textsubscript{o} on the rate of fall of [Ca\textsuperscript{2+}], is, however, reminiscent of the effects of extracellular Na\textsuperscript{+} removal. When Na\textsuperscript{+} is
removed there is an increase in $[\text{Ca}^{2+}]_i$, which then spontaneously decreases. This spontaneous decrease of $[\text{Ca}^{2+}]_i$ is, however, much slower than that seen when $\text{Na}^+$ is returned to the solution (Allen et al., 1983).

Comparison of the Caffeine and Stimulated Responses

The experiments have shown that maneuvers, such as the application of strophanthidin or the removal of external $\text{Na}^+$, that increase the stimulated $\text{Ca}^{2+}$ transient and twitch also increase the response to caffeine. This is consistent with the hypothesis that these inotropic maneuvers operate by increasing the $\text{Ca}^{2+}$ content of the SR. This conclusion is strengthened by the results of experiments in which the pattern of stimulation was varied. First, increasing the rate of stimulation over a range that increased the stimulated $\text{Ca}^{2+}$ transients also increased the caffeine response. Secondly, when regular stimulation was interrupted by a rest, then the magnitude of the next stimulated twitch depended on the duration of the rest. A similar dependence of the rest duration was also found for the caffeine response. In all muscles a sufficiently long rest decreased both the twitch and the caffeine response. In many muscles a short (10 s) rest increased both. The correlation is further emphasized by Fig. 7, which shows that in one muscle a 10-s rest decreased both the twitch and the caffeine response. Perhaps the most graphic demonstration of the correlation between the caffeine response and the twitch was given by the effects of applying caffeine either after a 1-min rest or after the first twitch after a 1-min rest. The latter caffeine response was considerably smaller than the former. A stimulus applied after the first twitch (i.e., at the same time as the latter would have occurred) produced a twitch that was smaller than control.

The results in the present paper are somewhat at variance with those of Chapman and Leoty (1976). Those authors found that even a 30-min exposure to $\text{Ca}^{2+}$-free solutions (with 1 mM EGTA) did not decrease the size of the caffeine contracture significantly below control levels. In our experiments, resting the muscle for as short a time as 5 min could abolish the caffeine response (even in the presence of external $\text{Ca}^{2+}$). We have no explanation for this discrepancy since the experiments were performed on the same preparation and temperature as Chapman and Leoty used.

The Effects of Caffeine in the Presence of Ryanodine

It is generally accepted that, in the presence of ryanodine, the normal systolic release of $\text{Ca}^{2+}$ ions from the SR is abolished. It is, however, still somewhat controversial as to whether this results directly from ryanodine inhibiting SR $\text{Ca}^{2+}$ release or, alternatively, ryanodine actually making the SR leaky to $\text{Ca}^{2+}$ ions (see Bers and MacLeod, 1986; Hilgemann, 1986a, b; and Hansford and Lakatta, 1987 for recent discussion). We found that, in the presence of ryanodine, caffeine did not increase either tension or $[\text{Ca}^{2+}]_i$. These inhibitory effects of ryanodine on the caffeine response are in agreement with the results of Fabiato (1985) on skinned cardiac muscle but differ from those of Marban and Wier (1985), who found that caffeine increased $[\text{Ca}^{2+}]_i$, even in the presence of ryanodine. It should be noted that the work of Marban and Wier was performed in a Na$^+$-free solution in which the preparation would have been overloaded with $\text{Ca}^{2+}$. Under such conditions, ryanodine has less effect (Fabiato, 1985). However, in the present experiments ryanodine inhibited the caffeine effect even in a Na$^+$-free solution.
**The Effects of Ca\textsuperscript{2+}-free Solutions**

We have seen that Ca\textsuperscript{2+} removal abolishes the Ca\textsuperscript{2+} transient within seconds. In contrast, the caffeine response was not greatly affected by Ca\textsuperscript{2+} removal. Similar results on tension have been reported by Chapman and Leoty (1976). This dissociation between the stimulated Ca\textsuperscript{2+} transient and the caffeine response contrasts with the parallel behavior of the two parameters upon changing the pattern of stimulation. If the decrease of the Ca\textsuperscript{2+} transient were to be accounted for by depletion of the SR, then one would expect the caffeine response to be reduced in parallel with the stimulated Ca\textsuperscript{2+} transient. The observation that the Ca\textsuperscript{2+} transient is abolished while there is still Ca\textsuperscript{2+} in the SR (as judged by the caffeine response) suggests that the decrease of the Ca\textsuperscript{2+} transient is due to a failure of Ca\textsuperscript{2+} release from the SR. This is consistent with the hypothesis that the normal mechanism of Ca\textsuperscript{2+} release from the SR involves Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release triggered possibly by the Ca\textsuperscript{2+} current (Fabiato, 1983; London and Krueger, 1986; Barcenas-Ruiz and Wier, 1987). It is, however, less easily reconciled with other hypotheses of Ca\textsuperscript{2+} release such as depolarization-induced Ca\textsuperscript{2+} release (Cannell et al., 1987).

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