Quinine and Caffeine Effects on ^{45}Ca Movements in Frog Sartorius Muscle

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ABSTRACT 1 mM caffeine, which produces only twitch potentiation and not contracture in frog sartorius muscle, increases both the uptake and release of ^{45}Ca in this muscle by about 50%, thus acting like higher, contracture-producing concentrations but less intensely. Quinine increases the rate of release of ^{45}Ca from frog sartorius but not from the Achilles tendon. The thresholds for the quinine effect on ^{45}Ca release and contracture tension are about 0.1 and 0.5 mM, respectively, at pH 7.1. Quinine (2 mM) also doubles the uptake of ^{45}Ca by normally polarized muscle. However, there are variable effects of quinine upon ^{45}Ca uptake in potassium-depolarized muscle. Quinine (2 mM), increases the Ca, Na, and water content of muscle while decreasing the K content. Both caffeine (1 mM) and quinine (2 mM) act to release ^{45}Ca from muscles that have been washed in Ringer's solution from which Ca was omitted and to which EDTA (5 mM) was added. These results, correlated with those of others, indicate that a basic effect of caffeine and quinine on muscle is to directly release activator Ca^{2+} from the sarcoplasmic reticulum in proportion to the drug concentration. The drugs may also enhance the depolarization-induced Ca release caused by extra K^+ or an action potential. In respect to the myoplasmic Ca^{2+} released by direct action of the drugs, a relatively high concentration is required to activate even only threshold contracture, but a much lower concentration, added to that released during excitation-contraction coupling, is associated with the condition causing considerable twitch potentiation.

Caffeine and quinine affect frog skeletal muscle similarly in some respects. In low concentrations (caffeine, 1 mM; quinine, 0.1 mM) they increase the tension output of the twitch; i.e., they are twitch potentiators (for review, see Sandow, 1964, 1965), whereas in somewhat higher concentrations they not only potentiate the twitch but also engender contracture, though with considerable variability (caffeine: Axelsson and Thesleff, 1958; Bianchi, 1961; Frank, 1960, 1962; Feinstein, 1963; Sandow and Brust, 1966; quinine: Krayer and George, 1951; Benoit et al., 1964).

Bianchi (1961) found that movements of ^{45}Ca in frog sartorius muscles were increased by caffeine at relatively high concentrations (2.5 and 5.0 mM). He

therefore suggested that such movements reflected a release into the myoplasm of Ca^{2+} which had been bound at either some superficial membrane or the sarcoplasmic reticulum of the fiber and that this Ca^{2+} acted on the myofibrils to produce contracture effects. Mechanical effects, appeared consistently in Bianchi's tests only when 5 mM caffeine, acting conjointly with 80 mM KCl, strengthened and prolonged the K depolarization contracture. Feinstein (1963) confirmed the effect of caffeine on ^{45}Ca movements. He also found, as had Axelsson and Thesleff (1958) and Frank (1960), that caffeine produced definite contracture when acting alone in concentrations as low as 2.5 mM; and these results indicated clearly, as could only be inferred from Bianchi's work, that the evident ability of caffeine to release Ca^{2+} into the myoplasm could act in the absence of a K contracture to cause an independent contracture of its own. Feinstein showed, furthermore, that certain local anesthetics which suppressed caffeine contracture also inhibited the caffeine-induced ^{45}Ca movements in muscle, in harmony with the suggested mechanism of the contracture.

That the release of free Ca^{2+} into the myoplasm could cause caffeine contracture is strongly indicated by the many studies showing, in general, that Ca^{2+} activates contraction in various myofibrillar systems (Heilbrunn and Wiercinski, 1947; Weber et al., 1963; Portzehl, Caldwell, and Ruegg, 1964; Podolsky and Costantin, 1964). Furthermore, there is considerable evidence that in the intact muscle activator Ca^{2+} originates from a store of bound Ca within the sarcoplasmic reticulum (for review, see Sandow, 1965) and such a source of Ca^{2+} for caffeine contracture, in particular, is indicated by the finding of Herz and Weber (1965) that caffeine causes a release of at least a part (about 30%) of the total Ca sequestered in the vesicles of isolated sarcoplasmic reticulum.

Although the cited studies on Ca movements explicitly attempted to explain the mechanism by which caffeine causes contracture, they omitted consideration of its action as a twitch potentiator. Moreover, they lacked any study of Ca flux when the alkaloid acted at such low concentration as to cause potentiation but not contracture, thus leaving open the questions whether there was a threshold concentration for caffeine to cause increase in Ca movement, and whether the potentiation produced in company with contracture by the higher caffeine concentrations was an effect of the associated increase in Ca movement or of some other action of caffeine. To define a concentration of caffeine, however, that always produces potentiation but never contracture, is rather difficult, because, as already indicated, the sensitivity of muscle in producing caffeine contracture is variable. Thus, Bianchi (1961) reported that 5 mM caffeine, acting alone, caused either no contracture at all (although different but comparable muscles showed large increases in Ca movement), or a slight contracture (in a separate group of muscles tested only for con-

tracture), and this difference evidently depended on a seasonal variation of his frogs. Axelsson and Thesleff (1958) obtained contractures regularly in the relatively low concentration of "0.50 $\times$ 10^{-3} w/v," i.e. essentially 2.6 mM; but they used sartorii from *Rana esculenta* while Bianchi tested *Rana pipiens* sartorii, and thus there may be a species variation factor among amphibia (as there certainly is between amphibia and mammals (Gutmann and Sandow, 1965; Isaacson and Sandow, 1967)). Yet Caputo (1966) has reported that the drug causes threshold contracture in about 2.5 mM concentration in *R. pipiens* semitendinosus muscle, while Frank (1962), using this concentration of the drug, obtained strong contractures in the *R. pipiens* toe muscle, suggesting that there may be variability in respect to different skeletal muscles of the same species. Furthermore, a muscle exposed at room temperature to the ordinarily subcontracture concentration of 1.5 mM caffeine will promptly produce a very strong contracture when its temperature is suddenly lowered to a value below 13°C (Sakai, 1965). In any case, many experiments in our own laboratory show that the threshold concentration necessary for caffeine to cause contracture of *Rana pipiens* sartorii at room temperature is generally about 2.0–3.0 mM (Feinstein, 1963; Sandow and Brust, 1966). Hence, to determine whether or not an increase in Ca flux plays some role in caffeine twitch potentiation, we have worked with the drug in a concentration of 1 mM which always causes potentiation but never produces contracture (see also Brust, 1965, footnote 2).

As for the mechanism by which quinine causes its mechanical effects, this has hardly been studied, and, in particular, it is not known whether it produces any changes in Ca movement like those produced by caffeine.

In view of the above, we have studied the indicated questions and have found that caffeine in the subcontracture concentration of 1 mM, and quinine in general, produce movements of Ca qualitatively like those produced by the previously studied higher concentrations of caffeine. However, by correlating our results for caffeine with those of others, and from our own results with quinine, we find certain quantitative differences resulting from action of the drugs at different concentrations, and these are shown to be significant in explaining the role of Ca^{2+} in the mechanisms by which the two alkaloids cause contracture and potentiate the twitch. A preliminary report of some of this work has already appeared (Isaacson, 1966).

METHODS

Paired sartorius muscles were dissected from medium sized frogs, *Rana pipiens*. For isotope experiments, the muscles, at about 110% of rest length in the animal, were tied with 000 nylon monofilament thread to fine glass tubes. In some experiments, frog Achilles tendons were similarly mounted and used as controls to study possible effects of quinine on connective tissue Ca within the muscle (Bianchi, 1961). After

dissection, the muscles were equilibrated for at least 30 min (generally 1 hr) in Ringer's solution made from deionized water, containing 108.4 mM NaCl, 1.6 mM KCl, 1.0 mM $CaCl_2$, and generally 2 mM Tris(hydroxymethyl)aminomethane buffered to pH 7.3. Oxygen was bubbled through the glass tubes during the entire experiment especially to promote mixing of Ca in process of flux and to satisfy respiratory needs. In some experiments, zero Ca Ringer's solution was used, which was the same as the Ringer solution above, except for the omission of $CaCl_2$. When this Ringer's solution containing EDTA (ethylenediaminetetraaceticacid) was needed, the disodium EDTA salt was dissolved in it and neutralized with concentrated NaOH.

We employed the isotope procedures described by Bianchi (1961) and further discussed by us (Isaacson and Sandow, 1967). For muscle uptake of ^{45}Ca, 10 min exposures to the isotope were used, followed by blotting (on Whatman No. 42 filter paper), and rinsing three times in 10 ml of Ringer's (to remove solution adhering to the muscle and rod). Then after a final gentle blotting, the muscles were transferred to seven consecutive test tubes (each containing 5 ml of Ringer's solution). The collection times for the consecutive washouts were 2, 6, 14, 30, 60, 90, and 120 min. For the ^{45}Ca uptake experiments with caffeine (1 mM) we corrected (Bianchi, 1961) both control and experimental muscle ash counts obtained at the end of the 120 min washout, for loss of ^{45}Ca from the slow component during the 120 min washout (correction factor is 1.32 corresponding to the average time constant of 438 min we found on similar muscles during 4 hr washouts). However, it should be noted that this correction procedure does not affect the magnitude of the caffeine effect relative to the controls. Indeed, our later data on quinine (2 mM) effects on ^{45}Ca uptake are presented in terms of the counts in the muscle ash and are uncorrected for the loss of ^{45}Ca during the 120 min washout.

In the efflux experiments, muscles were loaded with ^{45}Ca for 3 hr and then handled as mentioned before for uptake, except that a 4 hr washout was used. For washout, a series of 25 test tubes, each containing 2 ml of Ringer's solution, was used. Muscles were transferred twice at intervals of 5 min and thereafter at intervals of 10 min. At completion of the washout, muscles were lightly blotted and their wet weights were measured on a torsion balance. Then they were placed into tared silica crucibles and dried for 16 hr at 90°C, after which dry weights were obtained. Finally, they were ashed for 16 hr at 500°C in a muffle furnace. To the ash, 5 ml of 0.1 N HCl were added, and the crucibles then were shaken for 3 hr. This procedure following washout also applies to the uptake experiments, in which, however, we used only a 120 min washout.

We detected radioactivity with a Tracerlab windowless gas flow proportional counter. Planchets containing either 2 ml of the washout Ringer or 2 ml of the acidified ash solution were prepared. The muscle ash samples were added to planchets containing 2 ml dried Ringer's solution, so that no correction was needed for self-absorption. Similar procedures were used to count samples of the ^{45}Ca solution. All planchets contained a disc of lens tissue paper and 2 drops of concentrated Zephiran chloride (Winthrop Laboratories, New York); this promoted uniform spreading and sticking of the ^{45}Ca when the planchets were dried before counting.

Washout and rate coefficient curves were prepared (Bianchi, 1961) to describe the

decline in tissue radioactivity. The rate coefficient, in units of per cent per minute, is defined as $100\% \times \frac{\Delta C}{C_m \cdot \Delta t}$ where ΔC is the radioactivity lost from the tissue during the time interval Δt, and C_m is the mean radioactivity in the tissue between the time t and $t + \Delta t$. In some graphs, scatter was reduced by calculating a relative rate coefficient, wherein the relatively constant, slow average release found during the 3rd hour of desaturation was generally taken as 100% and the later rate coefficients were calculated as a percentage of the 3rd hr value.

In some experiments, records of mechanical responses were obtained from muscles dissected with the pelvic bone attached and mounted in a Lucite chamber containing 50 ml of Ringer's solution. Conventional massive stimulation (Sandow and Isaacson, 1966) and tension-recording procedures were used.

To prevent erratic outflux curves caused by spontaneous twitching (Bianchi, 1961), procaine in amounts of 2 mg/100 ml (0.07 mM) was added to all Ringer's solutions. This procedure is analogous to Bianchi's general use of cocaine to stabilize the muscle preparations and uses the same concentration of procaine (2 mg/100 ml) that Feinstein (1963) likewise found suitable for ^{45}Ca studies on the effects of higher concentrations of caffeine and local anesthetics (including procaine) on frog muscle. We obtained $^{45}Ca\ Cl_2$ in HCl solution from Oak Ridge National Laboratory and neutralized it with NaOH before use. We added ^{45}Ca to give about 1 μc/ml activity in the labeled Ringer solution.

In these experiments we used the following organic chemical forms: caffeine, as the free base (Eastman Organic Chemicals); procaine hydrochloride (Merck, USP, lot number 60191); quinine hydrochloride (S. B. Penick & Co., New York), N. F., lot number LSX-444 and also from Merck, N. F. X1, lot number 63145; and in some related experiments quinidine sulfate (S. B. Penick), USP, lot number LUX 591. In general, we measured the pH of our Ringer's solution prior to and after the addition of these compounds. In each case, although the buffering capacity of the Tris (HCl) buffer at pH 7.2 was limited (as shown for example that when 5 mM Na_2EDTA was added we had to add NaOH to maintain pH 7.2 in the Ringer solution), the organic compounds at the concentrations we used had in general little effect on the pH; e.g., there was only about a 0.1 pH decrease when 2 mM quinine hydrochloride was added to our normal Ringer's solution. Hence, for the compounds tested, the buffer used was adequate to avoid significant changes in pH during an experiment. Also, little change in ^{45}Ca outflow was seen during the exposure of muscles to pure Ringer's solution with pH values $\pm$ 1 pH unit from pH 7.2. However, if quinine effects were tested at the indicated pH values, we noted considerable differences in the magnitude of quinine action at the different pH values.

All experiments were done at room temperature of about 23°C.

RESULTS

I. *Effect of 1 mM Caffeine on ^{45}Ca Influx*

Fig. 1 shows that 1 mM caffeine, which produces twitch potentiation but not contracture, increases the 10 min ^{45}Ca influx in frog sartorius muscles by 51

± 11% (n = 8). (Dispersions are given by standard error.) The absolute values for the Ca uptake by normal and caffeine-treated muscles are given in Table I. Also included in Fig. 1 are data calculated from Bianchi (1961) and Feinstein (1963) showing the relative effect of 2.5 and 5 mM caffeine on ^{45}Ca influx. It is apparent from this figure that the caffeine-mediated increment in ^{45}Ca influx increases with the caffeine concentration over the range covered and that evidently the threshold for this effect on Ca influx is below the 1 mM concentration of our tests.

TABLE I

10 MIN ^{45}Ca UPTAKE* AND INFLUX‡ IN FROG SARTORIUS MUSCLE (n = 8), ±SE

	(C) Ringer's	(E) 1 mM caffeine	(E − C)
1	0.00802 ±0.00108 $\left(\frac{\mu\text{mole Ca}}{\text{g}}\right)$	0.01211 ±0.00112 $\left(\frac{\mu\text{mole Ca}}{\text{g}}\right)$	0.00410 ±0.00089 $\left(\frac{\mu\text{mole Ca}}{\text{g}}\right)$
2	0.0446 ±0.0060 $\left(\frac{\mu\mu\text{mole Ca}}{\text{cm}^2\text{-sec}}\right)$	0.0673 ±0.0052 $\left(\frac{\mu\mu\text{mole Ca}}{\text{cm}^2\text{-sec}}\right)$	0.0228 ±0.0049 $\left(\frac{\mu\mu\text{mole Ca}}{\text{cm}^2\text{-sec}}\right)$ $P<0.01$§
3	100%	151%	51±11%

* Uptake is corrected for loss of ^{45}Ca from slow component during 120 min washout (correction factor is 1.32 corresponding to an average time constant of 438 min).

‡ Influx calculated from uptake using 300 cm²/g wet weight (Bianchi and Shanes, 1959) as the value of surface membrane per unit weight of muscle.

§ P is calculated from t test for paired variates.

II. *Effect of 1 mM Caffeine on ^{45}Ca Outflow*

Fig. 2 shows the average effect on ^{45}Ca outflow of 1 mM caffeine when added after 180 min washout of initial radioactivity in the muscle in normal Ringer's solution. Caffeine (1 mM) thus increases ^{45}Ca outflow (plotted as a rate coefficient) by 40 ± 10%, or by about the same increment as is produced on influx.

We calculated an average normal Ca efflux for the muscles in Fig. 2 (including data from an additional pair of muscles) from the product of the average rate coefficients (expressed as a fraction) during 130–180 min of washout and the total estimated exchangeable Ca in the slow components determined from the outflow curves for these muscles (Bianchi, 1961); this product was divided by 300 cm²/g (Bianchi and Shanes, 1959) and by 60 sec/min to give an efflux. This normal efflux equalled (n = 6) 0.0536 ± 0.0131 $\mu\mu$mole Ca/cm²-sec, and was not significantly different from the influx shown in Table I.

III. *Effect of 1 mM Caffeine on ^{45}Ca Release after Muscles Have Been Exposed to 5 mM EDTA*

Fig. 2 shows a release of ^{45}Ca from muscles treated with 1 mM caffeine. This result does not indicate the source of this ^{45}Ca, however, for either a superficial

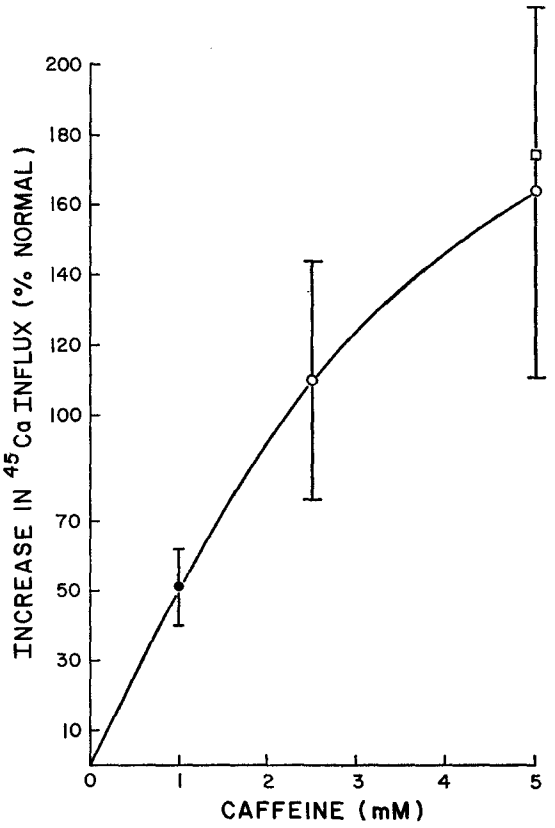

FIGURE 1. The average per cent increase, above controls, in 10 min ^{45}Ca influx rate in frog sartorius muscles as a function of the caffeine concentration in the Ringer solution. The points plotted for 2.5 and 5.0 mM caffeine were calculated from the papers of Bianchi (1961) and Feinstein (1963).

or intracellular store of Ca could conceivably be acted upon by 1 mM caffeine. This is especially difficult to resolve in view of the ready penetrability of this muscle by caffeine, the time constant for release being about 6 min (Bianchi, 1962). To determine the origin of the ^{45}Ca released by caffeine, muscles were washed in zero Ca Ringer's solution for 100 min, then placed in zero Ca Ringer's solution containing 5 mM EDTA (ethylenediaminetetraacetic acid) for the next 80 min, and then placed into zero Ca solution containing 1 mM

caffeine and 5 mM EDTA. Since EDTA is a powerful chelator of Ca and does not penetrate into the intracellular space of the fibers (Bianchi, 1965) during the time they are exposed to it (for longer times, see Feinstein, 1966), it acts to completely remove superficially located Ca and thus prepares the muscle to test selectively for possible action of an agent on intracellular Ca. Fig. 3 shows the results of such an experiment when the average rate coefficient of six pairs of muscles is plotted, the control muscles having been desaturated for 180 min in normal Ringer's solution containing 1 mM Ca^{2+} and then exposed to 1 mM caffeine. These procedures are similar to those used by

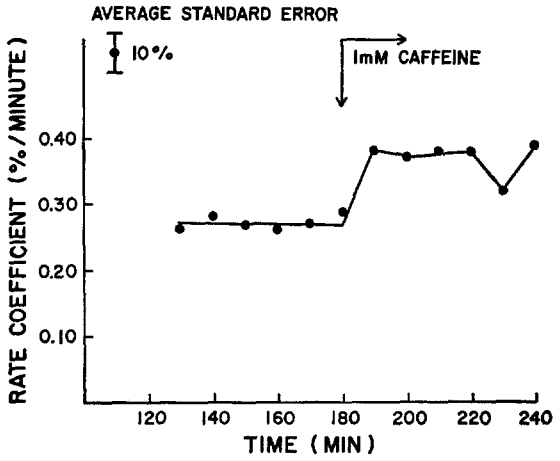

FIGURE 2. Average effect on the ^{45}Ca release of frog sartorii (plotted as a rate coefficient) of 1 mM caffeine added at 180 min to normal Ringer's solution.

Bianchi (1961). However, while he used caffeine in 5 mM concentration, we used it at 1 mM, and we made a direct comparison between the effect of caffeine on ^{45}Ca release from paired muscles pretreated with EDTA and their control muscles in normal Ringer's solution; whereas he compared EDTA control with caffeine + EDTA-treated muscles.

Fig. 3 shows the effect of EDTA on the release of ^{45}Ca, and the rapidity and transitory nature of this effect suggest that EDTA has only removed a superficial fraction of the muscle's Ca, consistent with its lack of penetration into the intracellular space of muscle (Bianchi, 1965). But, Fig. 3 shows that the addition of 1 mM caffeine releases a fraction of the Ca even after EDTA treatment. Moreover, this effect of caffeine is somewhat greater after treatment with EDTA than it is in normal Ringer's solution. Thus, like Bianchi in evaluating his results (1961) relating to the effect of higher concentrations of caffeine, we conclude that 1 mM caffeine acts to release Ca from an intracellular store inaccessible to EDTA and therefore unaffected by it.

IV. *Effect of Quinine on Mechanical Responses*

In a separate series of experiments, the effect of quinine on isometric twitch and tetanus responses of frog sartorius and toe muscles was tested. With toe muscles, twitch potentiation was obtained in concentrations from 0.01 to 0.1 mM quinine. At 0.1 mM, fused 0.4 sec tetani elicited by stimuli at 120 shocks/sec and obtained at about 10 min intervals were not maintained, evidently because of the large increase in the refractory period (Harvey, 1939) caused by quinine.

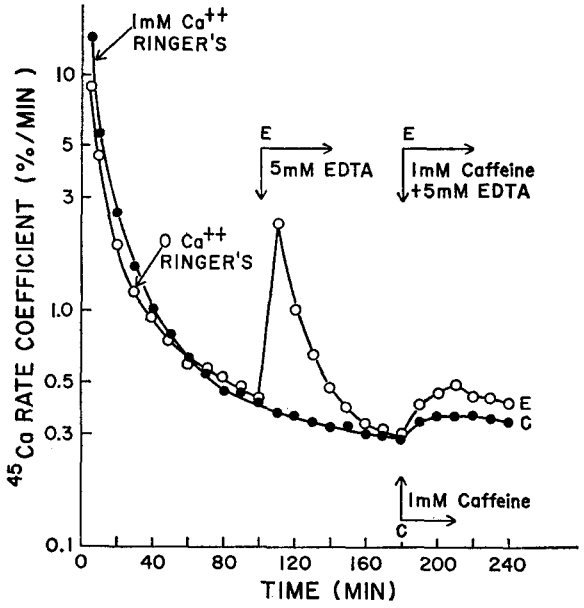

FIGURE 3. Average effect of 1 mM caffeine applied at 180 min on ^{45}Ca efflux (rate coefficient) after sartorius muscles (*E*) have been exposed to zero Ca Ringer's solution plus 5 mM EDTA starting at 100 min. Control muscles (*C*) were desaturated in normal, 1 mM Ca Ringer's solution with only the addition of 1 mM caffeine at 180 min ($n = 6$).

With frog sartorius muscles, quinine in somewhat higher concentrations, e.g., 0.5 mM, produced potentiation only transitorily, and then the twitch declined within an hour to a few per cent of its initial value. A weak contracture of about 1 g developed in about half the muscles treated with 0.5 mM quinine. Increasing the concentration of quinine to 1, 2, and 10 mM produced additional contracture tension up to about 20% of the initial tetanus tension. The above results are essentially in agreement with previous observations (Krayer and George, 1951; Benoit et al., 1964).

Quinine, like caffeine (Axelsson and Thesleff, 1958), caused contractures in sartorius muscles depolarized with KCl for 30 min. Thus, muscles that had

been depolarized by 80 mM KCl in Ringer's solution and had produced the associated transient contracture, produced contracture anew on addition of quinine (2 mM) to the medium, which developed slowly during the next 30 min and generally produced, at maximum, a tension of about 40% of that previously produced in the KCl contracture. Similar results have been obtained with muscles depolarized for 30 min by 95 mM K_2SO_4 (buffered to pH 7.2 with 2 mM Tris HCl) and then exposed to the isotonic K_2SO_4 solution containing 1.6 mM quinine. Thus quinine contracture probably is not mediated by way of a surface membrane depolarization. Contradictory results on the effectiveness of quinine in producing contracture after potassium depolarization have been reported by Benoit et al. (1964) in work with frog toe muscle; however, Conway and Sakai (1960) state results in this regard which agree with our observations.

In confirmation of the findings of Benoit et al. (1964), 2 mM quinine has been shown to produce contracture even in zero Ca Ringer's. In this experiment twitch and tetanus responses of a sartorius muscle were abolished after exposure for about 1 hr to zero Ca plus 2 mM EDTA Ringer's solution. At that time 2 mM quinine was added and a generally normal contracture was obtained. Thus, as is the case for caffeine (Axelsson and Thesleff, 1958), external Ca^{2+} is not needed to produce quinine contracture.

V. *Effect of Quinine on Release of ^{45}Ca from Muscle and Tendon*

In view of the previously mentioned similarities between the effects of quinine and caffeine, the following experiments were performed to see whether quinine released Ca from muscle. Fig. 4 shows the average effects of 0.1, 0.5, and 2 mM quinine on ^{45}Ca release from frog sartorius muscles, plotted as a relative rate coefficient. At each of these concentrations, an increase in outflow occurs when the quinine is added after 180 min of desaturation of ^{45}Ca from the muscles. Expressed as a percentage of either a portion (for 0.1 mM), or the entire 3rd hr average rate coefficient, the peak ^{45}Ca release rises to 121, 420, and 610% after 0.1, 0.5, and 2 mM quinine respectively. At all concentrations tested the increase in ^{45}Ca release was sustained with little decrease during the 60 min of exposure to the quinine.

Similar experiments with frog Achilles tendon showed no effect of 0.5 or 2 mM quinine on ^{45}Ca release. Therefore we conclude that in the whole muscle the action of quinine is confined to the muscle fibers. This specificity of quinine for Ca in muscle fibers is like that previously shown for caffeine (Bianchi, 1961).

VI. *Effect of Quinine on Release of ^{45}Ca after Muscles Have Been Exposed to 5 mM EDTA*

The results shown in Fig. 4, like those in Fig. 2 for caffeine, are inconclusive as to the source of the ^{45}Ca emerging from the muscle fibers. To get further

information on this question, sartorius muscles were treated with zero Ca Ringer's solution containing 5 mM EDTA after 100 min of washout in zero Ca Ringer's solution, and then at 180 min quinine hydrochloride (2 mM) was added to the zero calcium Ringer's solution with 5 mM EDTA. Fig. 5 shows the results of such an experiment in which the average rate coefficients of six

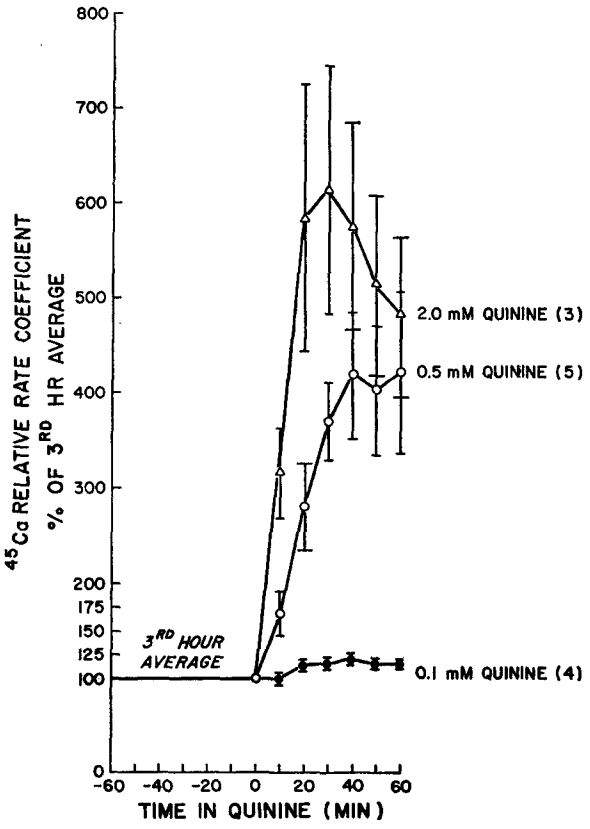

FIGURE 4. The average effects of 0.1, 0.5, and 2 mM quinine on ^{45}Ca release (plotted as a relative rate coefficient) from frog sartorius muscles. The SE for the normalized 100% points, the 3rd hr average, was about 10%. For the muscles in 0.1 mM quinine, the 100% level was taken as the average of the 170 and 180 min collections; i.e., the two collections just preceding the addition of the quinine.

pairs of muscle are plotted; the control muscles were washed in normal Ringer's solution containing 1 mM Ca and finally were exposed to Ringer's solution plus 2 mM quinine at 180 min.

Like caffeine (Fig. 3), quinine releases ^{45}Ca from muscles previously treated with EDTA to remove superficial calcium. Indeed, it produces a somewhat greater relative increase in the rate coefficient of ^{45}Ca from muscles after EDTA treatment than from muscles in normal Ringer's solution. As was con-

cluded for caffeine, these results indicate that 2 mM quinine acts to release Ca from an intracellular store inaccessible to EDTA.

VII. *Effect of Quinine on ^{45}Ca Uptake, Total Ca, Na, K, and Water Content*

In an experiment similar to that used to measure ^{45}Ca uptake in the presence of caffeine, we found that 2 mM quinine increased the 10 min ^{45}Ca influx 111 ± 10% (n = 4) over the normal ^{45}Ca influx. The normal influx in this experiment, uncorrected for loss of ^{45}Ca from the slow component during the 120 min desaturation before analysis for ^{45}Ca in the muscle, was 0.0451 ± 0.0066 $\mu\mu$mole Ca/cm²-sec.

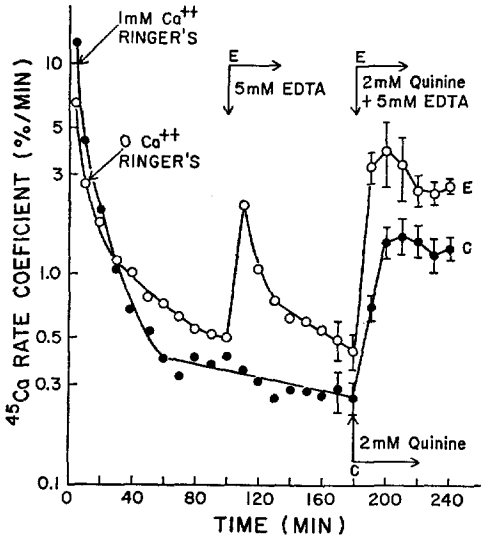

FIGURE 5. Average effect of quinine (2 mM) applied at 180 min on ^{45}Ca efflux (rate coefficient) after frog sartorius muscles (E) have been exposed to EDTA (5mM) in zero Ca Ringer's solution starting at 100 min. Control muscles (C) were desaturated in normal, 1 mM Ca Ringer's solution with only the addition of 2 mM quinine at 180 min (n = 6, SE are shown for later portions of efflux).

Total calcium analyses (Table II) were performed with a Perkin-Elmer absorption spectrophotometer, as described by Carvalho (1966), in a duplicate series of experiments to see whether 2 mM quinine had any effect on total Ca in muscle. In this experiment, five pairs of muscles were equilibrated in normal Ca Ringer's solution and the experimental muscles were then placed in Ringer's solution plus 2 mM quinine for 10 min, after which they were transferred through seven sets of test tubes containing normal Ringer's solution for a total of 120 min. Total Ca analyses gave 2.07 ± 0.006 for normal and 2.44 ± 0.10 μmoles Ca/g (n = 5) for quinine-treated muscles. This increase of 18% is statistically significant (t test for paired variates, $0.01 < P < 0.05$). This increase of 18% in total Ca in quinine-treated muscle is much smaller than the increase of 111% in ^{45}Ca uptake by muscles similarly exposed to quinine for 10 min and then washed out for 2 hr in normal Ringer's solution. Thus, it is likely that the increase in ^{45}Ca content represents mostly an increase in exchange of ^{40}Ca for ^{45}Ca in quinine-treated muscles. However,

the possibility remains, as noted in similar experiments with caffeine (Feinstein, 1963), that a larger increase in total muscle Ca may occur during the 10 min exposure to quinine. The subsequent 2 hr washout in normal Ringer's solution may possibly obscure effects of quinine on total muscle Ca.

Table II also contains data on total Na, K, and water content of the muscles analyzed for total Ca after exposure to quinine for 10 min. Standards were prepared for Na and K containing 1 mM of either Na (in K) or K (in Na) to approximate the interference with absorption spectroscopy caused by having

TABLE II

CATION AND WATER CONTENT OF NORMAL AND QUININE-TREATED FROG SARTORIUS MUSCLES

No. of muscles	Experimental conditions*	Concentration			Water content
		Ca	Na	K	Per cent of total muscle
		μmoles/g wet wt. ± (S E)			
5	Control (normal Ringer's)	2.07 (±0.06)	32.5 (±1.1)	74.8 (±2.1)	79.1 (±0.3)
5	Experimental (10 min in Ringer's + 2 mM quinine)	2.44 (±0.10)	49.1 (±3.2)	54.4 (±3.9)	82.0 (±0.5)
10	(Experimental − control)	+0.37 (±0.12)	+16.6 (±2.7)	−20.4 (±2.7)	+3.0 (±0.5)
		[$P<0.05$]‡	[$P<0.01$]‡	[$P<0.01$]‡	[$P<0.01$]‡

* Control and experimental muscles were equilibrated for about 2 hr in normal Ringer's prior to the 10 min exposure to quinine (experimental) after which time all muscles were washed out in normal Ringer's for an additional 2 hr.

‡ P is based on the SE of differences from paired muscles.

a mixture of Na and K in the samples of muscle ash (Carvalho, 1966). Quinine can be seen to cause a 51% gain in Na (16.6 μmoles/g) and a 27% decrease in K (−20.4 μmoles/g) in the experimental muscles relative to the muscles soaked in normal Ringer's solution. Likewise, the water content is increased by 3.7% in the quinine-treated muscles. All these changes are statistically significant ($P < 0.01$).

VIII. *Effect of 2 mM Quinine on ^{45}Ca Uptake or Release in Muscles Depolarized with Potassium*

In view of the effectiveness of quinine in producing contracture in muscles depolarized for 30 min by KCl (80 mM added to Ringer's solution), similar experiments were performed in which ^{45}Ca uptake was measured. Paired muscles were first exposed for 10–30 min in Ringer's solution plus 80 mM KCl; then the experimental muscles were placed for 10 min in ^{45}Ca-labeled Ringer's

solution plus 80 mM KCl containing 2 mM quinine. The control muscles were exposed to the same labeled high potassium Ringer's solution, but without quinine. After the soak in ^{45}Ca the muscles were washed in normal Ringer's for 120 min and the radioactivity remaining in the muscles was determined as described earlier. There was no significant difference between the average ^{45}Ca content of five pairs of experimental and control muscles after a 10 min presoak in high potassium Ringer's solution. Similar results were obtained in an experiment with a 30 min presoak in high potassium Ringer's; however, there was a tendency for the quinine-treated muscles to take up less ^{45}Ca than their controls (experimental − control = -0.006 ± 0.004 μmole Ca/g [$n = 5$, $P = 0.2$]).

Corresponding experiments on release are of interest and preliminary results indicate that in muscles labeled with ^{45}Ca for 3 hr, quinine (1 mM) consistently caused the usual sustained two- to threefold increase in the rate coefficient of ^{45}Ca release, regardless of whether the muscles had been previously depolarized with K_2SO_4 (95 mM).

DISCUSSION

A major presumption underlying the interpretation of the present results in general, and earlier ones especially regarding caffeine (Bianchi, 1961; Feinstein, 1963; Isaacson and Sandow, 1967), is that the increased movement of Ca^{2+} caused by the alkaloids reflects the presence of a proportionately increased level of myoplasmic Ca^{2+} which has been internally released and which is responsible for the ability of the drugs to produce contracture and other mechanical effects. The drugs might also increase the permeability of the membrane to Ca^{2+}. This is unlikely for caffeine since Feinstein (1963) observed no change in Ca content of frog sartorii subjected to 5 mM caffeine for 10 min and then washed for 2 hr; but it might be true for quinine since our comparable experiments indicated that this alkaloid did increase the muscles' Ca content. However, such permeability changes, in general, should play no role in producing at least the contractures of interest, for our results and those of others (Frank, 1960, 1962; Bianchi, 1961; Feinstein, 1963; Benoit et al., 1964; Caputo, 1966) showing that both drugs cause contracture and increased ^{45}Ca movement in muscles exposed to zero calcium, even after EDTA treatment, strongly indicate that contracture production does not depend on the entrance of Ca^{2+} from the external medium at all, but on release of the ion internally. Furthermore, the findings that caffeine (Herz and Weber, 1965) and quinine also (for details, see later) both release a portion of the Ca sequestered within extracted vesicles of the sarcoplasmic reticulum (SR), suggest a definite mechanism for the supposed internal action of the drugs.

We have found that 1 mM caffeine, which does not produce contracture, does cause increases in both ^{45}Ca inflow and outflow, and in outflow even

after treatment with EDTA, which are all qualitatively like those produced by caffeine at higher concentrations. Thus we infer that 1 mM caffeine causes an internal release of Ca^{2+} qualitatively like that it produces at higher concentrations. This indicates that the inability of caffeine at this concentration to produce contracture is not because it was acting below some absolute threshold connected with its action, as such, to cause release of Ca^{2+}. But the correlation of our results on influx with those obtained by others at higher concentrations, as shown by Fig. 1, indicates that the level of free Ca^{2+} produced in the myoplasm is lower, the smaller the concentration of the caffeine. A similar result appears in respect to release of ^{45}Ca, for calculation shows that in Bianchi's (1961) work, 5 mM caffeine caused the rate coefficient of release to increase over the normal by 3.2 times, but the corresponding increase in our work with 1 mM caffeine was only 1.4 times. Thus we infer that the reason contracture is always absent in muscles subjected to 1 mM caffeine is that the level of myoplasmic Ca^{2+} it produces is below the threshold of about 10^{-7} M generally needed to activate contraction, as demonstrated in various other contractile systems (for review see Sandow, 1965; Weber, 1966). Our analysis suggests, furthermore, that the levels of this Ca^{2+} obtained at 2.5 and 5.0 mM and still higher caffeine concentrations are generally sufficiently great to either attain or exceed this threshold. Our results, however, do not explain the great variability in the sensitivity of muscles to produce caffeine contracture.

As for causing twitch potentiation—and, incidentally, also production of the special increased speed of contraction obtained during the earliest part of both isotonic (Sandow and Seaman, 1964) and isometric (Sandow and Preiser, 1964) twitches (see also Sandow, Taylor, and Preiser, 1965; Sandow, 1965; Sandow and Brust, 1966)—1 mM caffeine might act so that, during excitation-contraction (E-C) coupling, there would be increased permeability or alteration of some other function of the transverse tubules involving Ca^{2+}, or increased release of Ca^{2+} from the sarcoplasmic reticulum (SR), thus supernormally raising the level of activator Ca^{2+} which would prolong the active state and augment the twitch. But available evidence is inadequate to decide whether such processes occur. However, the amount of Ca^{2+} we infer is already released in the resting muscle by 1 mM caffeine, even though subthreshold for contracture, would be available to add on to that normally released by E-C coupling during a twitch and thus provide the supernormal amount of free Ca^{2+} needed to produce the various features of a potentiated response. It is possible that this mechanism may also account for the positive inotropic effect of caffeine on cardiac muscle (Nayler, 1963; de Gubareff and Sleator, 1965), especially since Nayler (1963) found that caffeine increased both inflow and outflow of ^{45}Ca in hearts. At any rate, according to this mechanism for potentiating the twitch, there would be more extra Ca^{2+} and therefore greater potentiation, the greater the caffeine concentration; and

this, at least up to 3 mM caffeine, is indeed the case (Sandow and Brust, 1966). We attempted to obtain further information regarding this point by using procaine, in experiments like those of Feinstein (1963), to inhibit the increased movement of ^{45}Ca caused by 1 mM caffeine. The procaine was in 0.7 mM concentration (thus, it was in essentially the same ratio to the 1 mM concentration of our caffeine as Feinstein had employed (1 mg/ml) in relation to his 5 mM caffeine), and we found that it considerably delayed and reduced the magnitude of the increase in ^{45}Ca efflux produced by the 1 mM caffeine. However, the effects of the procaine on the influx of Ca caused by 1 mM caffeine were erratic. Futhermore, in attempting to relate the clear effect of procaine on the caffeine-induced Ca efflux to its effect on caffeine twitch potentiation, it has been found in various experiments in this laboratory (unpublished results) that procaine itself, depending on its concentration and time of action, either depresses or potentiates the twitch. Thus, although it is of interest that procaine inhibits the Ca efflux produced by 1 mM caffeine, more work is needed to permit clear evaluation of our results with procaine in regard to caffeine potentiation of the twitch. In any case, the general features of potentiation by caffeine have been attributed to its capacity to lower the mechanical threshold (Etzensperger and Gasciolli, 1963; Sandow, Taylor, Isaacson, and Sequin, 1964; Sandow, Taylor, and Preiser, 1965). In view of the preceding discussion, this effect could be due to the presence of the free myoplasmic Ca^{2+} produced by caffeine in the resting muscle, or to some effect on the SR or the T tubule that causes a given depolarization to release a supernormal amount of Ca^{2+} from the SR into the myoplasm.

In considering our results involving quinine we must note that it potentiates the twitch by a dual action on electromechanical coupling: it not only lowers the mechanical threshold, like caffeine, but it also considerably prolongs the action potential, this being in contrast to the slight and mechanically insignificant ability of caffeine, especially at only 1 mM concentration, to prolong the action potential (Sandow, Taylor, Isaacson, and Sequin, 1964; Isaacson, Taylor, and Sandow, unpublished results). Our present results show, however, that quinine, though acting at much smaller concentrations, produces effects which, in general, are like those produced by caffeine; i.e., it causes contracture (not only in normal but also in Ca-deprived and depolarized muscles), increases Ca flux of various types (including the increase occurring in presence of EDTA), and produces certain concentration changes of muscle Na and K (and, anomalously, of Ca also: compare Feinstein's (1963) results with caffeine). Thus, we conclude that these effects reflect the influence on electromechanical coupling that quinine has in common with caffeine; i.e., the reduction in the mechanical threshold. Furthermore, we infer that the mechanism by which quinine reduces the mechanical threshold is essentially similar to that by which caffeine reduces the threshold; i.e., as

indicated above, either by increasing the effectiveness of a depolarization in releasing myoplasmic Ca^{2+}, or by a release of Ca^{2+} from the SR in the unstimulated muscle.

In the case of quinine acting directly on the SR, it would be necessary that it be able to penetrate into the muscle like caffeine. But, this should be true, for quinine is a weak base with $pK_a = 8.4$ (Schanker et al., 1957), and when dissolved in Ringer's solution at about pH 7.2 it should have about 10% of its molecules uncharged and therefore penetrable. Concordant with our views as to the mechanisms by which quinine causes contracture are the results of Ashley (1965) and Ashley et al. (1965) on crab fibers, which show that injections of the Ca chelator EGTA (ethylene glycol bis(β-amino-ethyl-ether)-*N*,*N*'-tetraacetic acid) delay and markedly reduce contractures produced by external application of quinine (and of caffeine, as well). Benoit (personal communication, 1966) has suggested that the site where quinine releases intracellular Ca may be different from that where caffeine acts. This is based on the observations in Benoit et al. (1964) that quinine contractures in zero Ca Ringer's solution can still be obtained after caffeine contractures have been abolished by repeated application of the caffeine. In respect to this question, quinine evidently releases activator Ca from reticular vesicles, as does caffeine, since it causes contraction (as does caffeine, also) of extracted models of muscle fibers, provided they contain reticular material (Hasselbach and Weber, 1955). Bondani and Karler (1966) showed that quinidine (2 mM) causes release of Ca bound (evidently passively; i.e., independently of adenosinetriphosphate [ATP]) by microsomes of skeletal and cardiac muscle. Furthermore, according to Carvalho[1] 1 mM quinine or quinidine at pH 7.0 can release about 33% of the Ca bound by SR in the presence of ATP. However, these alkaloids appear to exert an action more general than that of caffeine, for they also reduce the binding by the SR of Mg and K. Moreover, caffeine has no effect on at least the ATP-dependent fraction of the SR's bound Ca, although other types of experiments (Weber, 1966) have demonstrated that caffeine affects a relatively small labile fraction of the total Ca that can be maximally loaded into SR preparations. Thus, though both caffeine and quinine (or quinidine) may act on Ca bound to SR, they probably affect differently characterized fractions of the SR's Ca-binding sites, and this may account for the quantitative differences between the actions of the two drugs on muscle.

General consideration of our results with both quinine and caffeine indicates that our conclusions on the Ca-dependence of both contracture and twitch potentiation of the drug-treated muscles are in general harmony with those derived from experiments involving artificial addition of Ca^{2+} to activate

[1] Carvalho, A. 1967. Unpublished results.

various contractile systems, either extracted (Weber et al., 1964) or intact (Caldwell and Walster, 1963; Portzehl, Caldwell, and Ruegg, 1964; Hellam and Podolsky, 1966). This suggests that essentially the same mechanisms of Ca dependence which determine activation by artificially added Ca^{2+} occur in intact fibers when, under the action of the drugs, the Ca^{2+} needed to activate their contraction is obtained by release from the normal store of Ca sequestered within the sarcoplasmic reticulum.

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