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THE EFFECTS OF DRUGS ON CALCIUM UPTAKE AND CALCIUM RELEASE BY MITOCHONDRIA AND SARCOPLASMIC RETICULUM OF FROG SKELETAL MUSCLE

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Abstract—The effects of quinidine, chlorpromazine and caffeine on Ca uptake and Ca release by mitochondria and fragmented sarcoplasmic reticulum (FSR) of frog muscle were studied. Quinidine (1–2 mM) released considerable Ca from preloaded mitochondria but had little effect on preloaded FSR. The uptake of Ca both by mitochondria and FSR was inhibited by higher concentrations (2 or 1 mM) of quinidine but the inhibition of mitochondrial Ca uptake was much greater. With lower concentration (0·4 mM), there was no significant effect on Ca uptake by FSR, but a 48 per cent inhibition of mitochondrial Ca uptake was observed.

Chlorpromazine (0·01–0·1 mM) inhibited Ca uptake by both mitochondria and FSR, but the inhibition in the case of FSR was weaker than mitochondria. Only the highest concentration (0·1 mM) of chlorpromazine caused a release of Ca from mitochondria or FSR. Caffeine (2–10 mM) inhibited Ca uptake both by mitochondria and FSR and again the inhibition of Ca uptake by mitochondria was greater than of FSR. Caffeine (10 mM) in contrast to quinidine released Ca from FSR and not from mitochondria. Ca releasing effect of both caffeine and quinidine increased when the ratio of the drug and FSR protein increased.

The Ca releasing concentrations of these drugs were comparable to those reported to elicit contractures of living muscle. The lower concentrations which inhibited Ca uptake were comparable to those which potentiate twitch.

THE ALKALOIDS quinidine and caffeine have rather similar effects on tension development by skeletal muscle. In particular, low concentrations of these drugs cause a potentiation of twitch tension whereas higher concentrations cause contractures.[1–4] Recently chlorpromazine was shown to exert similar effects on the contractile responses of skeletal muscle.[5,6]

It is generally accepted that in skeletal muscle contraction is coupled to excitation through the release of Ca from the sarcoplasmic reticulum.[7–10] There is a growing body of evidence suggesting that agents that modify the contractile states of muscle and have no relevant effects on cell membrane do so through their ability to alter the Ca binding properties of the intracellular stores of the muscles, in particular those of sarcoplasmic reticulum.[9–14]

Both quinidine and caffeine increase the fluxes of ^{45}Ca in living frog muscles in association with their effects on contraction.[9,12,14] Both these drugs as well as chlorpromazine can induce contracture of frog skeletal muscle in the absence of extracellular Ca.[6,15] It has been suggested that these drugs which potentiate twitch and also induce contracture act directly on sarcoplasmic reticulum to cause a release of

Ca or an inhibition of the uptake of Ca, thereby increasing the free myoplasmic Ca concentration. In support of this hypothesis, reports showing that caffeine,[13,16] quinidine[17-19] and chlorpromazine[20] can inhibit the binding of Ca by the isolated sarcoplasmic reticulum have recently appeared.

It should, however, be pointed out that all previous studies on the effects of these drugs with the exception of caffeine which was tested on frog sarcoplasmic reticulum [13,16] were performed on the reticulum isolated from the rabbit skeletal muscle,[17-21] whereas studies dealing with the effects of these drugs on electromechanical properties of muscle were carried out on frog skeletal muscle.[1-4,6,12,14,22,23]

In order to correlate the observed effects of these drugs on the tension development by the living muscle to Ca binding by the isolated subcellular fractions, it must be shown that these drugs have a dual effect on the isolated systems as they do on living muscle, i.e. in low concentrations the drugs can inhibit the Ca transport system resulting in twitch potentiation while in high concentrations only, the drugs are able to release bound Ca, resulting in contractures. With this aim in mind, the present study was performed on mitochondria and fragmented sarcoplasmic reticulum (FSR) isolated from frog skeletal muscle. Mitochondria were studied because in a previous study they were shown to possess as large a capacity for Ca uptake as FSR.[24,25] In addition, the above mentioned drugs have been shown to have profound effects on Ca binding and other metabolic activities of cardiac, liver and brain mitochondria.[26-28]

METHODS

Preparation of subcellular particles. Approximately 5 g of muscle from summer frogs (*Rana temporaria*) was homogenized in ice-cold KCl (150 mM) and imidazole (5 mM) solution (pH 6·8) with an Ultraturrax (Janke & Kunkel) at 20,000 rev/min for 5 × 3 sec periods with intermittent pauses of 30 sec. The homogenate was centrifuged at 900 *g* for 10 min and the pellet discarded. The supernatant was filtered through two layers of gauze and centrifuged at 4000 *g* for 15 min to remove mitochondria. The supernatant was then centrifuged at 8500 *g* for 15 min and the pellet discarded since an electron microscopic examination revealed a marked cross-contamination in this material. The remaining supernatant was spun at 34,000 *g* for 45 min to obtain FSR. The pellets of mitochondria and FSR thus obtained were suspended in 0·6 M KCl and respun. The sediment from each was then washed with the same medium as used for homogenization and suspended in it to a protein concentration of 1–1·5 mg/ml. These fractions, as judged by electron microscopic examination, succinic dehydrogenase assay and inhibition of Ca uptake by sodium azide and dinitrophenol, were relatively free from cross contamination.[25] Approximately 1 mg of FSR or mitochondrial protein per gram muscle was obtained. The above procedure was carried out at 2–4°.

Ca uptake measurements. The assay medium for Ca uptake experiments consisted of 150 mM KCl, 20 mM imidazole buffer (pH 7·0), 5 mM $MgCl_2$, 1 mM ATP and 0·1 mM Ca with 2·5 $\mu Ci^{45}CaCl_2$. The reaction was started by the addition of 0·3–0·4 mg of mitochondrial or FSR protein in a total volume of 1 ml. The reaction was carried out at 20° and was stopped by removal of particles with Millipore filters (0·45 μm dia.) as described previously.[29] Blanks which lacked only the particulate material were filtered simultaneously. Ca uptake was determined by subtracting the

amount of radioactivity in the filtrate of the sample from that of the blank. Radioactivity was determined by a Packard Tri-Carb liquid scintillation counter.

Ca release measurements. The fractions were allowed to take up Ca for 5 min in a medium described under Ca uptake experiments, and 0·1 ml of the appropriate concentration of the drug to be tested was added. Aliquots of the reaction mixture were filtered through the Millipore filters after 2 and 5 min. Ca release was calculated from the increment in the radioactivity of the filtrate after the addition of the drug. Appropriate controls without the drug were run simultaneously. Protein concentration was determined by the method of Lowry *et al.*[30]

Chemicals. Quinidine sulphate and caffeine (anhydrous) were purchased from Sigma Chemical Co. Chlorpromazine chloride was given by Leo, Helsingborg, Sweden. Stock solutions of the drugs and subsequent dilutions thereof were made in 5 mM imidazole (pH 7).

RESULTS

Ca uptake

Quinidine. The effect of various concentrations of quinidine on the rate and capacity of Ca uptake by mitochondria and FSR is shown in Figs. 1 and 2. Ca uptake by mitochondria at the end of 10 min was inhibited by 92 and 23 per cent by 2 and 0·2 mM quinidine respectively (Fig. 1).

In agreement with the previous results[25] Ca uptake by FSR was considerably faster than by mitochondria and was virtually complete after 2 min (Fig. 2). Quinidine inhibition of Ca uptake by FSR was considerably smaller than that of mitochondrial Ca uptake. The highest concentration of quinidine (2 mM) inhibited Ca uptake by

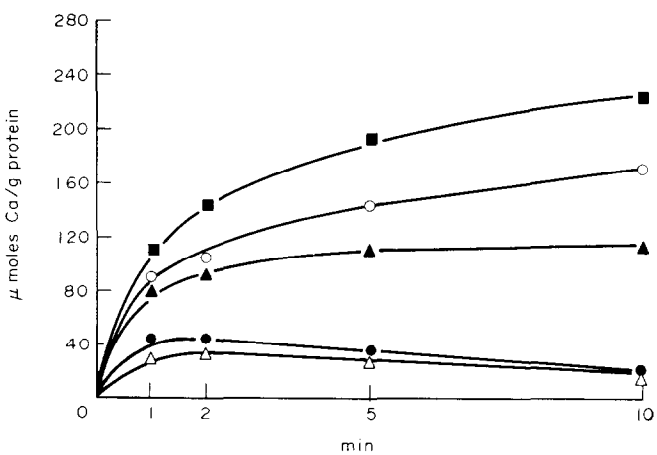

FIG. 1. The effect of quinidine on the kinetics of Ca uptake by frog skeletal muscle mitochondria. Control (■), Quinidine, 0·2 mM (○) 0·4 mM (▲), 1 mM (●), 2 mM (△). Mitochondrial protein concentration in the reaction mixture was 0·42 mg/ml. Other details are given under methods. Each point is the mean of four to seven experiments.

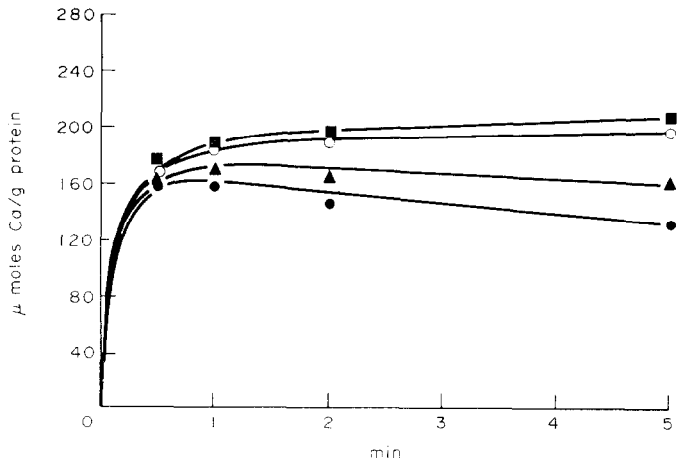

FIG. 2. The effect of quinidine on the kinetics of Ca uptake by FSR (0·41 mg/ml). Control (■), Quinidine, 0·4 mM (○), 1 mM (▲), 2 mM (●).

only 10 and 35 per cent after 30 sec and 5 min respectively. There was no significant inhibition with 0·4 mM quinidine, a concentration which inhibited mitochondrial Ca uptake by 48 per cent. With the highest concentration of quinidine (2 mM) there was an indication of Ca release, both from mitochondria (Fig. 1) and FSR (Fig. 2). Comparative inhibition by quinidine of Ca uptake by mitochondria and FSR is illustrated in Fig. 3.

Chlorpromazine. Chlorpromazine (0·1 mM) also strongly inhibited Ca uptake by mitochondria (Fig. 4). With lower concentrations the inhibition was correspondingly smaller. At 0·01 mM there was a small (about 10%) but statistically significant ($P < 0·05$) inhibition of Ca uptake by mitochondria. Chlorpromazine inhibited Ca uptake by FSR but the inhibition was weaker than that observed with mitochondria

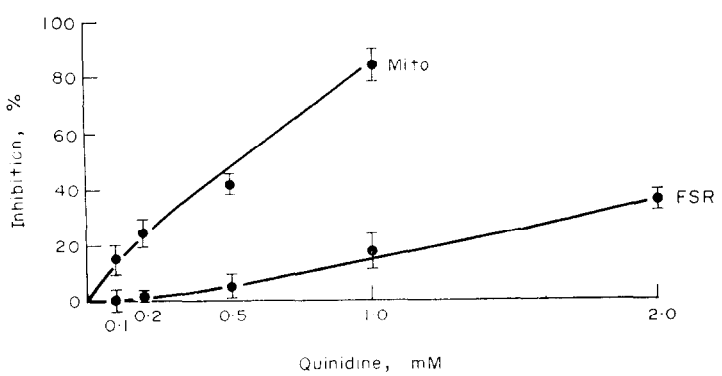

FIG. 3. Comparative inhibition by quinidine of Ca uptake by frog skeletal muscle mitochondria (0·39 mg/ml) and FSR (0·38 mg/ml). Ca uptake was measured after 5 min of incubation. Each point is the mean of four experiments. Vertical bars represent S.E.M.

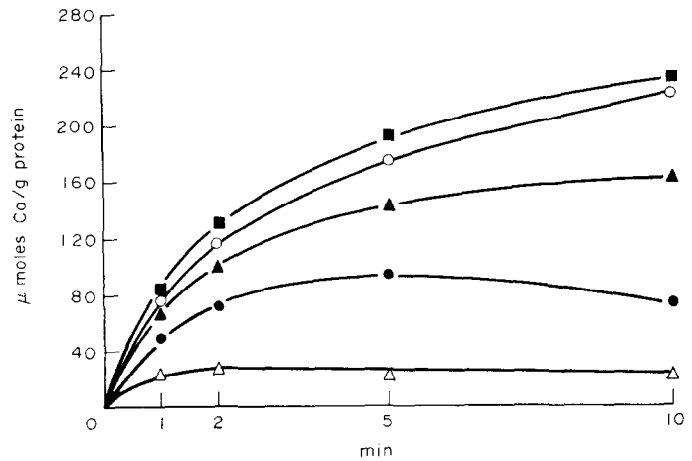

FIG. 4. The effect of chlorpromazine on the kinetics of Ca uptake by frog skeletal muscle mitochondria. Control (■), Chlorpromazine 0·01 mM (○), 0·02 mM (▲), 0·05 mM (●), 0·1 mM (△). Mitochondrial protein concentration in the reaction mixture was 0·41 mg/ml. Each point is the mean of four to six experiments.

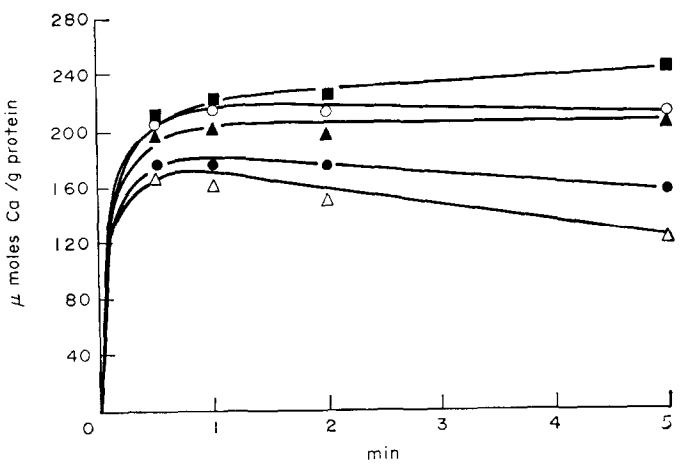

FIG. 5. The effect of chlorpromazine on the kinetics of Ca uptake by FSR (0·30 mg/ml). Symbols and conditions as in Fig. 4.

(Fig. 5); Ca uptake being inhibited by 90 and 48 per cent respectively in mitochondria and FSR with the highest concentration (0·1 mM) of the drug used. The Ca releasing effect of this drug on both mitochondria and FSR is apparent from these results (Figs. 4 and 5). The results clearly indicate that with this drug, as with quinidine, the rate and capacity of Ca uptake were lowered. The inhibition, both in the case of mitochondria and FSR, appears to be a linear function of the concentration of chlorpromazine (Fig. 6).

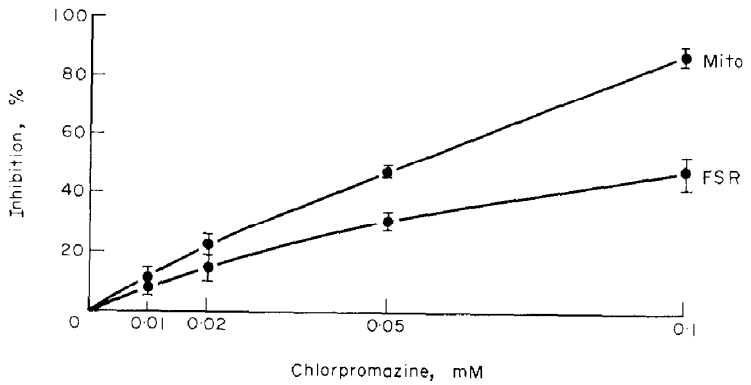

FIG. 6. Comparative inhibition by chlorpromazine of Ca uptake by frog skeletal muscle mitochondria (0·39 mg/ml) and FSR (0·38 mg/ml). Ca uptake was measured after 5 min of incubation. Each point is the mean of four experiments. Vertical bars represent S.E.M.

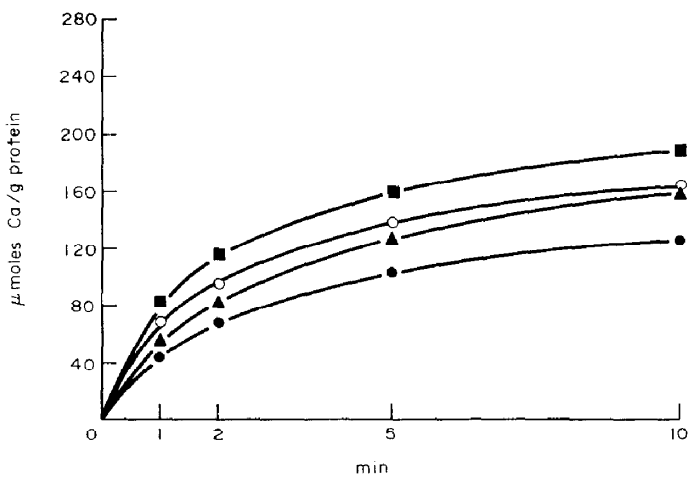

FIG. 7. The effect of caffeine on the kinetics of Ca uptake by frog skeletal muscle mitochondria. Control (■), Caffeine, 2 mM (○), 5 mM (▲), 10 mM (●). Mitochondrial protein in the reaction mixture was 0·35 mg/ml. Each point is the mean of three to five experiments.

Caffeine. Caffeine inhibited Ca uptake both by mitochondria and FSR and in either case there was a decrease in the rate and the capacity of Ca uptake (Figs. 7 and 8). Again Ca uptake by mitochondria (Fig. 7) was depressed more than Ca uptake by FSR (Fig. 8). With 5 and 10 mM caffeine, the inhibition of mitochondrial Ca uptake was 15 and 33 per cent respectively. The corresponding figures for the inhibition of Ca uptake by FSR were 12 and 20 per cent. With 2 mM caffeine the inhibition of Ca uptake by mitochondria was still about 13 per cent which was statistically significant. The small inhibition (5 per cent) observed in the case of FSR was not significant. As seen in these results (Figs. 6 and 7) there was no indication of a Ca releasing effect of caffeine.

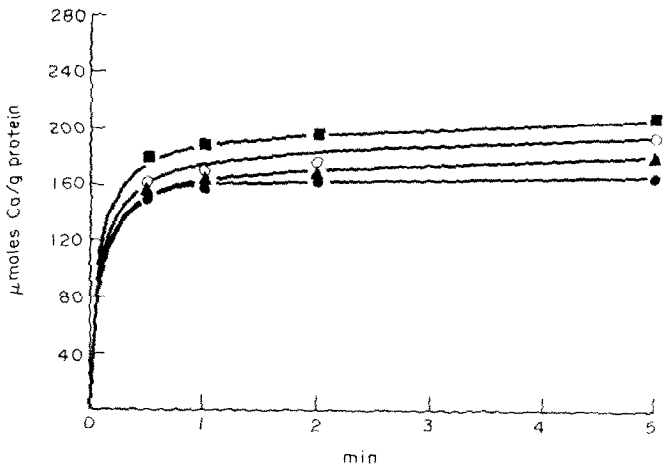

FIG. 8. The effect of caffeine on the kinetics of Ca uptake by FSR (0·36 mg/ml). Symbols and conditions as in Fig. 7.

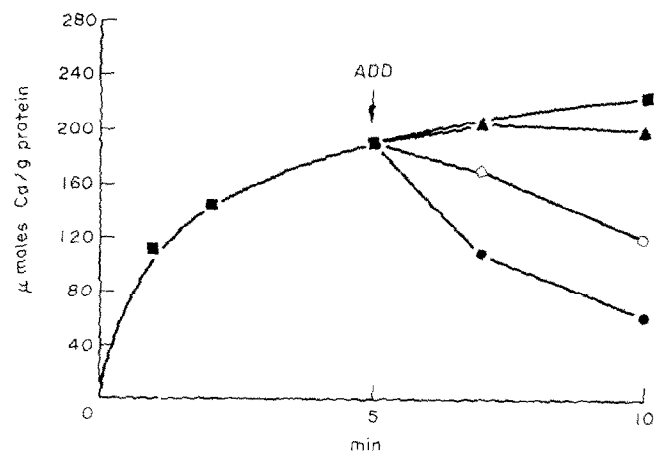

FIG. 9. Ca release by different concentrations of quinidine from loaded mitochondria. Mitochondria (0·39 mg/ml) were allowed to take up Ca for 5 min and the appropriate concentration of the drug added (arrow). Ca release was measured as described under methods. Control (■), Quinidine, 0·4 mM (▲), 1 mM (○), 2 mM (●). Each point is the mean of four to six experiments.

Ca release

Quinidine. The effect of three different concentrations of quinidine added after 5 min of Ca accumulation by mitochondria is shown in Fig. 9. When Ca loaded mitochondria were exposed to 2 or 1 mM quinidine, a considerable amount of Ca was released. At the end of 5 min, mitochondria had lost 67 and 36 per cent of their Ca with 2 and 1 mM quinidine respectively. In this period, the control preparation took up an additional 18%. With 0·4 mM quinidine there was no release of the accumulated Ca but the additional uptake of Ca was completely inhibited.

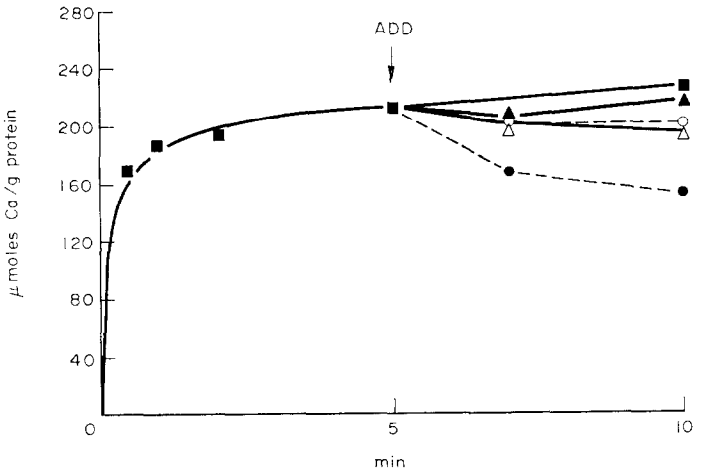

FIG. 10. Ca release by quinidine (solid lines) and chlorpromazine (dotted lines) from loaded FSR (0·40 mg/ml). Control (■), Quinidine, 1 mM (▲), 2 mM (△), Chlorpromazine 0·05 mM (○), 0·1 mM (●). Details as in Fig. 9. Each point is the mean of four to six experiments.

TABLE 1. CALCIUM RELEASE BY QUINIDINE (2 mM) AND CAFFEINE (10 mM) WITH DIFFERENT CONCENTRATIONS OF FSR PROTEIN IN THE REACTION MIXTURE

FSR protein (mg/ml)	Ca load (μmoles/g protein)	Per cent change in Ca load		
		Control	Quinidine	Caffeine
0·390	210·6	+10·6	−5·8	−10·6
0·195	201·8	−9·8	−24·7	−15·7
0·097	204·3	+3·2	−44·3	−25·2

Ca uptake (Ca load) was measured after 5 min of incubation at which time the drug was added. The amount of Ca lost or gained after 5 min is shown as per cent change.

Quinidine was unable to cause a significant release of Ca stored by FSR and only slightly depressed the additional uptake of Ca which was about 8 per cent between 5 and 10 min (Fig. 10). There was, however, a greater variation observed in the experiments with quinidine on Ca release from FSR and occasionally a release was observed. It was also noted that in the experiments where quinidine was able to cause a release of Ca, the FSR protein concentration in the uptake medium was relatively low. It was therefore of interest to test the effect of this drug on Ca release by using different concentration of FSR protein in the medium. These results are shown in Table 1. As suspected, quinidine was able to cause a greater release of the stored Ca when the FSR protein concentration was lowered which, in effect, resulted in an increased ratio of the concentration of the drug and FSR protein. A similar effect of caffeine on Ca release was observed (Table 1). The total uptake of Ca with various FSR protein concentrations did not change significantly.

Chlorpromazine. Ca was released both from loaded mitochondria (Fig. 11) and FSR (Fig. 10) only by the highest (0·1 mM) concentration of chlorpromazine used. With lower concentrations although there was no release of Ca stored by either FSR or mitochondria, the additional Ca uptake by both mitochondria and FSR was inhibited.

Caffeine. Ten mM caffeine caused about 11 per cent release of Ca taken up by FSR (Fig. 12). However, as observed with quinidine, release of Ca by caffeine was greater when the ratio of the drug and FSR protein was increased (Table 1). At a very low concentration of FSR protein, as much as 25 per cent of the Ca load was released by caffeine (Table 1). These values for Ca release by caffeine are in good agreement

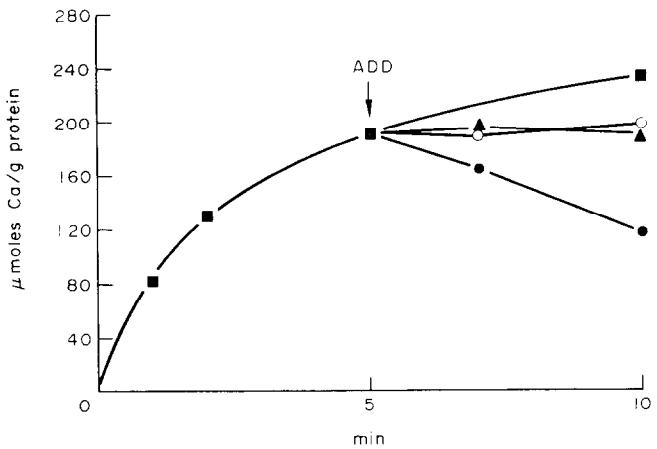

FIG. 11. Ca release by different concentrations of chlorpromazine from loaded mitochondria (0·38 mg/ml). Control (■), Chlorpromazine, 0·01 mM (▲), 0·05 mM (○), 0·1 mM (●). Details as in Fig. 9. Each point is the mean of four to five experiments.

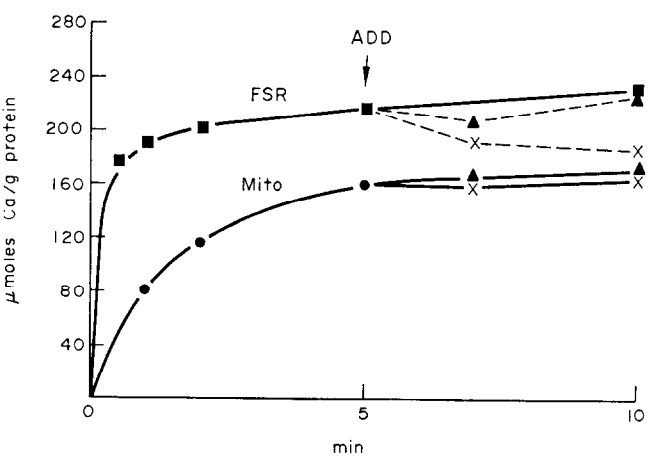

FIG. 12. Ca release by caffeine, 5 mM (▲) and 10 mM (×), from preloaded mitochondria (0·39 mg/ml) and FSR (0·40 mg/ml). Details as in Fig. 9. Each point is the mean of four to five experiments.

with those recorded by others.[13,16] Caffeine failed to cause a release of Ca from mitochondria but somewhat depressed the additional Ca uptake (Fig. 12).

DISCUSSION

The chemical agents which modify the mechanical responses of skeletal muscle may do so by their action on some part of the excitation–contraction coupling or the mechanism underlying relaxation. For example, heavy metal potentiators of twitch increase the duration of the active state by prolonging the action potential,[9,31,32] whereas certain anions, caffeine and quinidine lower the mechanical threshold of the muscle fiber.[3,6,33] In addition all of these agents are believed to modify to some extent the Ca release and uptake by the sarcoplasmic reticulum.[9,33]

Both caffeine and quinidine in low concentrations are potentiators of twitch tension while in high concentration they cause contractures.[1–4] Both drugs can readily pass through cell membranes, including those of muscle,[34,35] and have also been shown to cause contractures in depolarized muscles.[1,4,6] To explain these observations on the intact muscle Sandow[9] and Isaacsson and Sandow[4] hypothesized that alkaloids such as quinidine and caffeine cause twitch potentiation and contracture of skeletal muscle by virtue of their ability to release Ca from sarcoplasmic reticulum into the myoplasm. They further suggested that low concentrations of quinidine caused the release of an amount of Ca which by itself was subthreshold for the activation of contraction but which, when added to Ca, normally released by an action potential, caused a prolongation of the active state and thereby increased the twitch tension. With higher concentrations of these drugs, the amount of Ca released was greater and was sufficient to activate the contractile mechanism and thus produce contractures. However, a prolongation of the active state could also result from an inhibition of the Ca pump leading to an elevated Ca level in the myoplasm for a longer time. On the other hand, to explain contractures which are caused by high concentration of these drugs, one is almost compelled to postulate a direct effect of these drugs on the intracellular Ca stores, resulting in a release of Ca in amounts sufficient to activate the contractile mechanism. In this respect, the results of the present investigation showing that the drugs caused an inhibition of Ca uptake by mitochondria and FSR when employed in low concentrations, while in higher concentrations caused a release of Ca from mitochondria or FSR, are significant.

The finding that these drugs, particularly quinidine, had a very pronounced effect on mitochondrial Ca uptake and Ca release and a relatively small effect on FSR, is provocative inasmuch as, in the past, little importance has been attached to the role of mitochondrial Ca uptake in skeletal muscle contraction. One of the major reasons for this negative view of mitochondrial Ca uptake in muscle contraction is that Ca accumulation by mitochondria of skeletal muscle is slower and its affinity lower than that of sarcoplasmic reticulum.[8] This was also shown in a recent comparative study from this laboratory on Ca uptake by mitochondria and FSR isolated from frog skeletal muscle.[25] This observation, however, only suggests that sarcoplasmic reticulum, in contrast to mitochondria, by virtue of its high affinity for Ca binding and speed of accumulation, would fulfil the requirement for a relaxing system in muscle.[25] Since muscle mitochondria have a large capacity to store Ca,[24,25] there is no reason to suppose that any agent which has been implied in the release of Ca from an intra-

cellular site would do so only from Ca stores in sarcoplasmic reticulum and not from the stores in mitochondria. Evidence for the participation of mitochondria in controlling myoplasmic Ca of skeletal,[36,37] smooth[38,39] and cardiac[40,41] muscles has been reported. The effects of the individual drug are discussed below.

Quinidine. Quinidine in concentration of 1–2 mM which has been shown to cause contracture in the living muscle[4,6] released a considerable amount of Ca from mitochondria but had little effect on Ca release from FSR. Carvalho[17] reported a release of Ca from rabbit FSR by quinidine, however, higher concentrations (2–5 mM) than used in the present experiments were needed to cause a significant release of Ca. Balzer[19] observed a considerable release of Ca by 1 mM quinidine from preloaded FSR of rabbit skeletal muscle and Fuchs *et al.*[18] reported a greater inhibition of Ca uptake than observed in the present experiments. However, in both these studies since oxalate was included in the uptake medium, very low concentrations of FSR protein were used. As shown in present results, when the concentration of the drug with respect to the FSR protein is increased, the amount of Ca released by the drugs is correspondingly increased. It is therefore important to consider this factor, which is often overlooked, when comparing data on Ca uptake from experiments with and without oxalate in the medium. Since in the present results, using similar protein concentrations of mitochondria and FSR in the Ca uptake experiments, a much greater effect of quinidine was observed on both mitochondrial Ca release and uptake than that on FSR, it is inferred that the preferred site of the action of this drug to release Ca in the myoplasm would be the mitochondria.

Although quinidine failed to cause release of Ca from preloaded FSR, it appeared to release Ca when present from the start in the incubation medium as shown in the Ca uptake experiments. This observation may be related to that reported recently by Balzer[19] who found that the amount of Ca released by quinidine decreased as the uptake incubation was prolonged. His data showed that quinidine released 85 per cent of the Ca-load of vesicles when washed with 1 mM quinidine solution after 1 min of uptake, but released only 27% Ca when washed after 8 min. Balzer explained these results by proposing that oxalate entered the vesicles at later moments and reduced the concentration of free Ca inside the vesicles and therefore its release by quinidine. Since in the present study, the incubation medium did not contain oxalate, the above explanation would not be applicable. The present results indicate that somehow quinidine action to release Ca is facilitated when present during the active Ca transport in FSR. Any speculation as to the mechanism of the action at this stage is unwarranted[19].

Chlorpromazine. The observation that this drug was able to inhibit Ca uptake by FSR is in agreement with the results of Balzer *et al.*[20] However, in the present results both the rate and capacity of Ca uptake by FSR were inhibited by chlorpromazine which is at variance with the results of Balzer *et al.*[20] who showed that only the rate of Ca uptake by (0·1 mM) chlorpromazine was lowered. Their data indicated that after 8 min of incubation, the amount of Ca taken up in the presence of the drug was virtually the same as in its absence. In the present results the amount of Ca taken up in the presence of the drug was in fact decreased as the incubations were prolonged since the drug released a portion of Ca which was taken up in the initial phase (1 min) at a very rapid rate. The contradiction between the present results and those of Balzer *et al.*[20] may be explained as being due to a difference in the media used for Ca

uptake in the two studies. While oxalate was present in the medium used by Balzer *et al.*[20] it was absent from the medium used in the present experiments (also see ref. 42). Oxalate, by precipitating Ca, would lower the concentration of Ca inside the vesicles and thus prevent its efflux.

In the present results it was further shown that high concentrations of chlorpromazine released Ca from both FSR and mitochondria. To my knowledge, there are no other reports showing the effect of this drug on skeletal muscle mitochondria, although reports on its effects both on Ca binding and respiration by brain,[28] liver[26,27] and cardiac[26] mitochondria have recently appeared. In these studies chlorpromazine in concentrations similar to those used in the present investigation inhibited Ca uptake by mitochondria.

Chlorpromazine also had a greater effect on Ca uptake and release by mitochondria than by FSR. However, the effect of this drug on FSR was considerable. From the results of the present *in vitro* study one can only say that high concentration of chlorpromazine reaching the myoplasm would release Ca both from mitochondria and sarcoplasmic reticulum, whereas lower concentrations would only inhibit reaccumulation of Ca. The most significant finding with this drug as with quinidine, in the present study, is the duality of action of these drugs. In addition, the concentrations of these drugs which released Ca either from mitochondria or FSR or both, in the present experiments, are strikingly close to those reported to induce contractures in the intact muscle.

Caffeine. Although caffeine caused a greater inhibition of Ca uptake by mitochondria than by FSR it was unable to release Ca from mitochondria. This suggests that the contracture producing effect of this drug is probably due to a release of Ca from the sarcoplasmic reticulum, which is in agreement with the suggestions made in the literature.[9,13,16] Thus in contrast to quinidine which predominantly released Ca from mitochondria, caffeine caused no release of Ca from mitochondria but from FSR. Several authors have reported differences in the contractures produced by quinidine and caffeine. Andersson[6] and Huddart[43] observed differences in the time course of contractures by quinidine and caffeine. Benoit *et al.*[44] found that, at a time when caffeine contracture could no longer be obtained, quinidine contracture could still be induced. Procaine was able to block caffeine contracture but not the quinidine contracture.[14] Huddart[43,45] who recently studied in detail the effects of caffeine and quinidine on crab skeletal muscle, concluded that the two drugs induce Ca release from reticulum in different ways. The present results indicate that the two drugs in fact act on different intracellular Ca stores.

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