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Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. I. Ca^{2+} channel blockers

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The effects of Ruthenium red and tetracaine, which inhibit Ca^{2+}-induced Ca^{2+} release from the isolated sarcoplasmic reticulum (e.g., Ohnishi, S.T. (1979) J. Biochem. (Tokyo) 86, 1147–1150), on several types of Ca^{2+} release in vitro were investigated. Ca^{2+} release was triggered by several methods: (1) addition of quercetin or caffeine, (2) Ca^{2+} jump, and (3) replacement of potassium gluconate with choline chloride to produce membrane depolarization. The time-course of Ca^{2+} release was monitored using stopped-flow spectrophotometry and arsenazo III as a Ca^{2+} indicator. Ruthenium red inhibited all of these types of Ca^{2+} release with the same concentration for half-inhibition $C_{1/2}$ = 0.08–0.10 μM. Similarly, tetracaine inhibited these types of Ca^{2+} release with $C_{1/2}$ = 0.07–0.11 mM. Procaine also inhibits both types of Ca^{2+} release induced by method 2 and 3 with $C_{1/2}$ = 0.67–1.00 mM. These results suggest that Ruthenium red, tetracaine and procaine interfere with a common mechanism of the different types of Ca^{2+} release. On the basis of several pieces of evidence we propose that Ruthenium red and tetracaine block the Ca^{2+} channel of sarcoplasmic reticulum.

Introduction

A variety of methods that induce Ca^{2+} release from isolated sarcoplasmic reticulum have been reported in the literature [1–5]. Our recent findings [6,7] as well as others [3] have indicated that there are at least two classes of Ca^{2+} release which are clearly distinguishable in terms of the triggering mechanism. As demonstrated in our recent paper [7], dissociation of the transverse (T)-tubule-sarcoplasmic reticulum complex [8] inhibits rapid Ca^{2+} release ($t_{1/2}$ = 10 ms) induced by ionic replacement ('depolarization'-induced Ca^{2+} release), whereas reformation of the functional complex leads to restoration of depolarization-induced rapid Ca^{2+} release. Furthermore, ionic replacement produces abrupt changes of the T-tubule membrane potential or surface charge which precede the rapid Ca^{2+} release from sarcoplasmic reticulum. Thus, it appears that the rapid Ca^{2+} release induced by ionic replacement involves depolarization of T-tubules followed by signal transmission across the T-tubule/sarcoplasmic reticulum linkage. On the other hand, dissociation of the T-tubules affects neither Ca^{2+}- nor drug-induced Ca^{2+} release, suggesting that these types of Ca^{2+} release are produced by a direct stimulation of the sarcoplasmic reticulum membrane [7]. The rate constants of Ca^{2+} release induced by T-tubule depolarization (k = 70–150 s^{-1}, Ref. 7) are much higher than those of (Ca^{2+} + drug)-induced Ca^{2+}

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Abbreviation: Mes, 4-morpholineethanesulfonic acid.

release ($k = 0.5–3.0\ s^{-1}$, Ref. 6). Thus, these types of Ca^{2+} release are also distinguishable in terms of their rates differing by about two orders of magnitude.

Different types of Ca^{2+} release share some common properties, as described previously [6]. For instance, (a) all types of Ca^{2+} release are followed by Ca^{2+} reuptake, suggesting that the putative Ca^{2+} channels close spontaneously regardless of the method of triggering [6]. (b) High extravesicular [Ca^{2+}] (e.g., 10 μM) inhibits all types of Ca^{2+} release [6]. (c) Almost all types of Ca^{2+} release functions are enriched in heavy sarcoplasmic reticulum fractions [5–7,9–16], which are presumably derived from the terminal cisternae of sarcoplasmic reticulum [17]. These facts suggest that different types of Ca^{2+} release have some steps in common.

In order to account for the dissimilarities as well as similarities of the different types of Ca^{2+} release described above, we have proposed the hypothesis that there is a 'common' Ca^{2+} channel involved in the Ca^{2+} release induced by different mechanisms [5–7,11,18]. A direct way to investigate this hypothesis is to find appropriate blockers of the Ca^{2+} release channel, which according to this hypothesis would inhibit the different types of Ca^{2+} release in the same manner. The finding of such channel blockers would also be useful for identification and characterization of the channel proteins. This study was initiated to search for such Ca^{2+} channel blockers. As described here, it has been found that Ruthenium red [6,12,15,16, 18–21], tetracaine [22–28], and procaine [29–31], which are known inhibitors of Ca^{2+}- and drug-induced Ca^{2+} release, also inhibit the T-tubule-mediated rapid Ca^{2+} release. The inhibition of these three types of Ca^{2+} release occurs in parallel in a characteristic concentration range for each of Ruthenium red and tetracaine. Thus, it appears that these reagents act on the common portion of the different types of Ca^{2+} release mechanisms, viz., the Ca^{2+} channels.

Experimental procedures

Preparation. A sarcoplasmic reticulum fraction enriched in the T-tubule-sarcoplasmic reticulum complexes was prepared from rabbit leg muscles (fast-twitch white muscle) as described previously [6]. About 150 g muscle were homogenized at a low speed in a Waring blender with 4 vol. of 2.5 mM NaOH solution for 6×20 s at intervals of 3 min. During the homogenization, the pH was adjusted to 6.8 using pH indicator papers. The suspension was centrifuged at $10000 \times g$ for 3 min in a JA-10 rotor (Beckman). The supernatant was filtered through eight layers of cheesecloth and then through Whatman filter papers (No. 4). After readjusting the pH to 6.8, if necessary, the filtrate was centrifuged again at $17000 \times g$ for 30 min in an SS-34 rotor. The pellets were suspended either in a solution containing 0.15 M KCl and 20 mM Mes (pH 6.8) or a solution containing 0.15 M potassium gluconate and 20 mM Mes (pH 6.8), and centrifuged again at $17000 \times g$ for 30 min. The pellets were resuspended in the appropriate solution and the final protein concentration was adjusted to 20–30 $mg \cdot ml^{-1}$. These preparations are designated as KCl vesicles and potassium gluconate vesicles, respectively.

Depolarization-induced Ca^{2+} release. The potassium gluconate vesicles were loaded with Ca^{2+} by ATP-dependent Ca^{2+} accumulation in a reaction mixture containing 1.6 mg protein/ml, 0.15 M potassium gluconate, 200 μM $CaCl_2$, 0.5 mM Mg · ATP, 5.0 mM phospho*enol*pyruvate, 10 units $\cdot ml^{-1}$ pyruvate kinase, 9 μM arsenazo III, and 20 mM Mes (pH 6.8) (solution A). The reaction mixture was then loaded in syringe A of a stopped-flow apparatus (Durrum model D-130). The extravesicular concentration of Ca^{2+} became nearly zero about 2 min after addition of ATP, as determined by separate Ca^{2+} uptake measurements (cf. Ref. 7). The Ca^{2+} taken up was maintained for about 5 min after completion of Ca^{2+} uptake, and then slowly leaked out. At various times, the content of syringe A was mixed with an equal volume of solution B loaded in syringe B to induce Ca^{2+} release. Solution B contained 0.15 M choline chloride, 9 μM arsenazo III and 20 mM Mes (pH 6.8).

Ca^{2+} (or drug)-induced Ca^{2+} release. The KCl vesicles were loaded with Ca^{2+} by ATP-dependent Ca^{2+} accumulation in solution A containing 0.15 M KCl (rather than 0.15 M potassium gluconate), 50 μM $CaCl_2$ and 2.5 mM phospho*enol*pyruvate (otherwise the same as described above). To induce Ca^{2+} release, one part of solution A was

mixed with one part of solution B containing 0.15 M KCl, 100 μM $CaCl_2$, 9 μM arsenazo III, 20 mM Mes (pH 6.8) or 0.15 M KCl, drugs (200 μM quercetin or 4.0 mM caffeine), 9 μM arsenazo III and 20 mM Mes (pH 6.8).

Inhibition studies. In order to investigate the effects of Ruthenium red, tetracaine and procaine, various concentrations of these reagents were included in solution B. Ca^{2+} release was induced by mixing solution A and solution B as described above.

Ca^{2+} release assay. Changes in $\Delta A = A_{650} - A_{680}$ were monitored by a dual-beam system described previously [32], and the data were analyzed by a computer. Calibration of the amount of Ca^{2+} released and measurement and calculation of endogeneous Ca^{2+} concentrations were done as described previously [6]. All Ca^{2+} release measurements were carried out at 27°C. Most experiments described here were carried out on the day after preparation. The kinetic parameters concerning Ca^{2+} release were calculated as described previously [7].

Miscellaneous. Arsenazo III, tetracaine, Ruthenium red and procaine were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade. The protein concentration was determined by the method of Lowry et al. [33]. The concentration of Ruthenium red was determined by the measurements of the absorbance at 533 nm using $\epsilon = 61\,550\ M^{-1} \cdot cm^{-1}$ [34].

Results

We have investigated the effects of increasing concentrations of Ruthenium red (left column of Figs. 1, 2, and 3) and tetracaine (right column) on several different types of Ca^{2+} release: viz., Ca^{2+}-induced Ca^{2+} release (Fig. 1), quercetin-induced Ca^{2+} release (Fig. 2) and depolarization-induced Ca^{2+} release (Fig. 3).

In the experiments shown in Fig. 1, various concentrations of Ruthenium red (0–4 μM) or tetracaine (0–4 mM) were added to solution B containing 100 μM $CaCl_2$ to trigger Ca^{2+}-induced Ca^{2+} release, and mixed with an equal volume of solution A containing sarcoplasmic reticulum that had been loaded actively with Ca^{2+} (the increase in the extravesicular [Ca^{2+}] was 12.5 μM, cf. Ref. 6). The time-course of Ca^{2+} release was monitored using arsenazo III as a Ca^{2+} indicator in the presence of various concentrations of blockers as indicated (final concentration after mixing). As shown here, an increase of the reagent concentration leads to the progressive reduction of the size of Ca^{2+} release (upward excursion). Further increase of the reagent concentration results in the activation of Ca^{2+} uptake (downward excursion). The time-course of Ca^{2+} uptake in the presence of high concentrations of blocker is about the same as the Ca^{2+} uptake time-course in the absence of blockers (not shown). These results suggest that blockers exert their action in a two-step fashion; blocking of Ca^{2+} release in the first step, and reactivation of the Ca^{2+} pump in the second step. These results suggest the existence of a mechanism by which the Ca^{2+} pump and Ca^{2+} release are controlled in an alternate fashion.

Fig. 2 depicts the time-course of quercetin-induced Ca^{2+} release in the presence of various concentrations of blockers. An increase of the blocker concentration leads to a sharp reduction of the size of Ca^{2+} release. In this case, there is no reactivation of Ca^{2+} uptake owing to the facts that no Ca^{2+} jump was made and that quercetin inhibits Ca^{2+} uptake [35].

The effects of Ruthenium red and tetracaine on the T-tubule-mediated rapid Ca^{2+} release produced by choline chloride replacement of potassium gluconate (Ref. 7) are shown in Fig. 3. Increasing concentrations of Ruthenium red and tetracaine reduce the amounts of Ca^{2+} released in both the rapid and slow phases.

Fig. 4 depicts the plots of the amount of Ca^{2+} released by Ca^{2+} jump and depolarization versus the concentration of blockers. It is important to note that in each case of Ruthenium red and tetracaine both types of Ca^{2+} release described above are inhibited in the same concentration range, though the inhibitory concentration ranges are three orders of magnitude different between Ruthenium red and tetracaine (Table I). The inhibition of quercetin-induced Ca^{2+} release and caffeine-induced Ca^{2+} release occurs also in the same concentration ranges of blockers as does that of Ca^{2+}-induced and depolarization-induced Ca^{2+} release (Table I). These results clearly indicate that

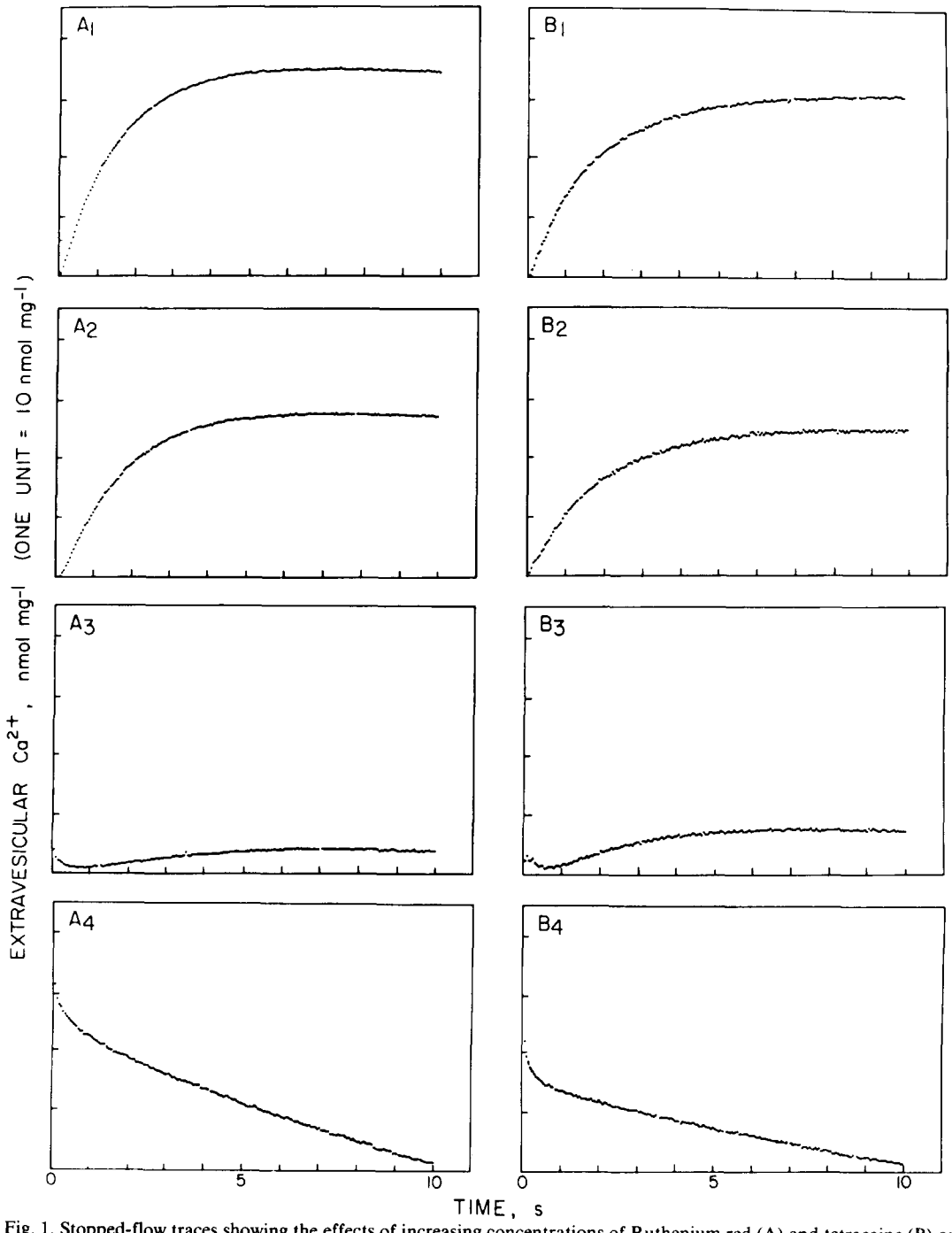

Fig. 1. Stopped-flow traces showing the effects of increasing concentrations of Ruthenium red (A) and tetracaine (B) on Ca^{2+}-induced Ca^{2+} release. For active Ca^{2+} loading, KCl vesicles (1.6 $mg \cdot ml^{-1}$) were incubated in a solution containing 0.15 M KCl, 50 μM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ATP, 2.5 mM phospho*enol*pyruvate, 10 $units \cdot ml^{-1}$ pyruvate kinase, 9 μM arsenazo III, and 20 mM Mes (pH 6.8) (solution A). To induce Ca^{2+} release, one part of solution A was mixed in a stopped-flow apparatus with one part of solution B containing 0.15 M KCl, 100 μM $CaCl_2$, 9 μM arsenazo III, 20 mM Mes (pH 6.8), and various concentrations of inhibitors, whose final concentrations upon mixing are as follows. Ruthenium red, μM: A_1, 0; A_2, 0.05; A_3, 0.10; A_4, 0.3. Tetracaine, mM: B_1, 0; B_2, 0.05; B_3, 0.25; B_4, 0.50. About ten traces, which were collected in the period of 2–7 min after addition of ATP, were signal-averaged for each figure.

these blockers react with a common component regardless of the triggering method.

Earlier experiments with the skinned muscle fiber preparations [3,31] have suggested Ca^{2+}-induced Ca^{2+} release and depolarization (Cl^- replacement of methanesulfonate$^-$)-induced Ca^{2+} release are distinguishable in that procaine inhibits the former but not the latter. We have found that procaine inhibits depolarization-induced Ca^{2+} release as well as Ca^{2+}-induced Ca^{2+} release from the isolated sarcoplasmic reticulum, and that $C_{1/2}$ is about the same for both types of Ca^{2+} release (Table I). Thus, it appears that procaine also blocks a common mechanism for different types of Ca^{2+} release.

Although higher concentrations of blocker produce complete inhibition of Ca^{2+}- and quercetin-induced Ca^{2+} release, a portion of depolarization-induced Ca^{2+} release (e.g., 20–25%) remained uninhibited in many experiments. We have found

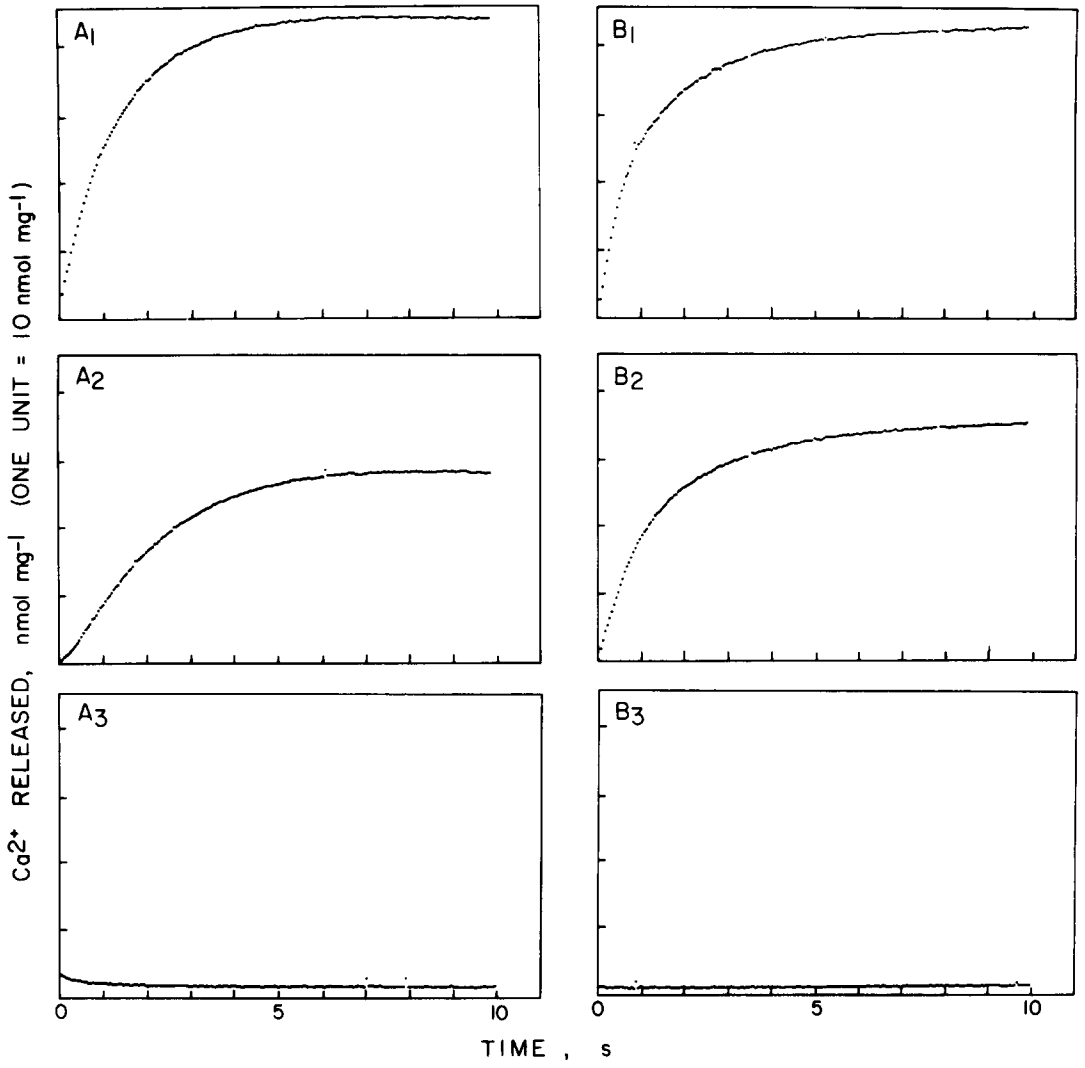

Fig. 2. Stopped-flow traces showing the effects of increasing concentrations of Ruthenium red (A) and tetracaine (B) on quercetin-induced Ca^{2+} release. Active Ca^{2+} loading of KCl vesicles was done as described in the legend to Fig. 1, and Ca^{2+} release was induced by mixing one part of solution A with one part of solution B containing 0.15 M KCl, 200 μM quercetin, 9 μM arsenazo III, 20 mM Mes (pH 6.8), and various concentrations of inhibitors, whose final concentrations upon mixing are as follows. Ruthenium red, μM: A_1, 0; A_2, 0.05; A_3, 0.15. Tetracaine, mM: B_1, 0; B_2, 0.05; B_3, 0.15. About ten traces were signal-averaged.

that the resistant portion varies depending upon the time elapsed between triggering of Ca^{2+} release and the addition of ATP. Tetracaine and Ruthenium red produce almost complete inhibition of depolarization-induced Ca^{2+} release if they are applied in the earlier phase of the steady state of Ca^{2+} uptake (2.0–3.5 min after addition of ATP), whereas the extent of inhibition of depolarization-induced Ca^{2+} release becomes less if applied in the latter phase of the steady state of Ca^{2+} uptake (3.5–7.0 min after addition of ATP). These results suggest that some factors (e.g., phosphorylation of membrane components, and slight changes in the extravesicular Ca^{2+} concentration during the post-steady state of Ca^{2+} uptake may affect the mode of inhibition. In contrast, there is

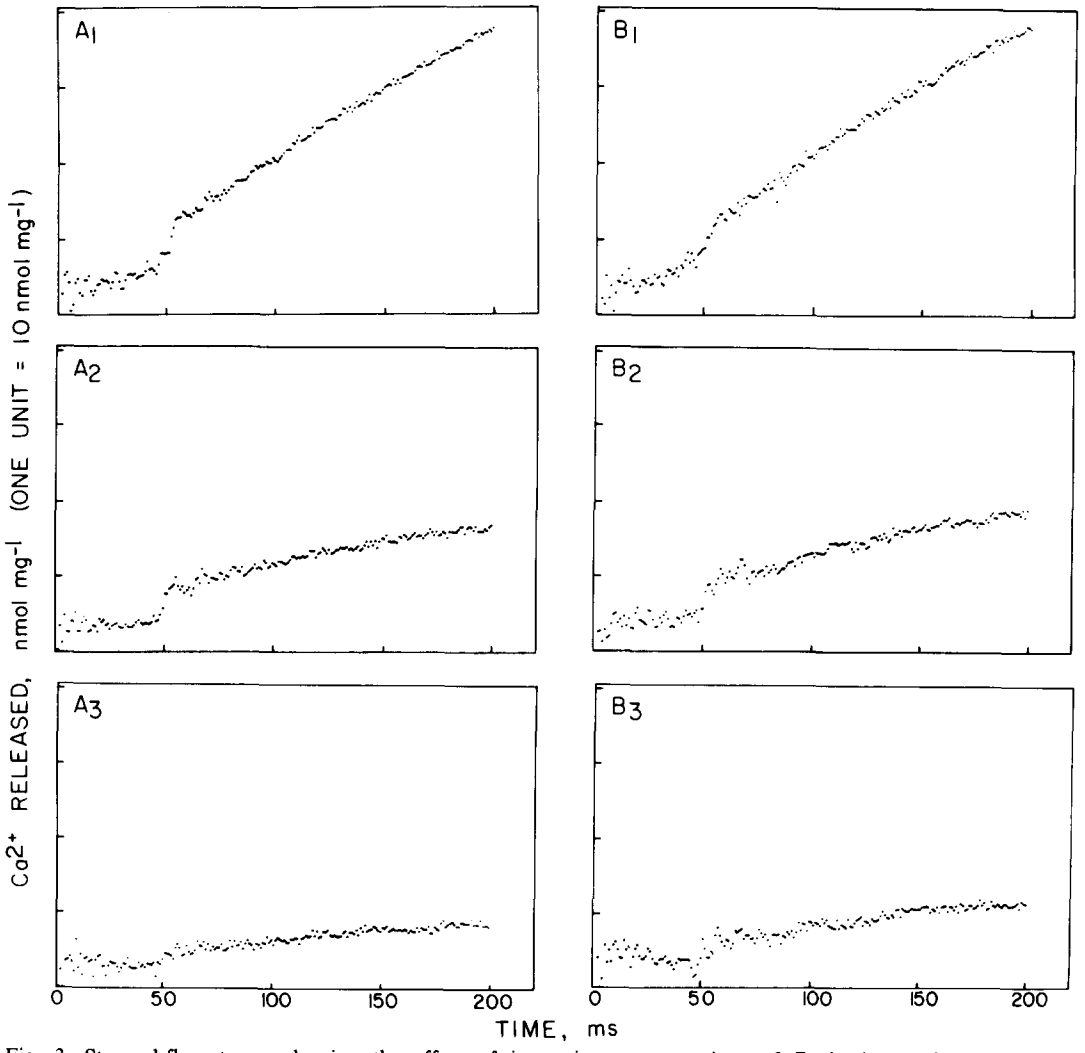

Fig. 3. Stopped-flow traces showing the effect of increasing concentrations of Ruthenium red (A) and tetracaine (B) on depolarization-induced Ca^{2+} release. The potassium gluconate vesicles (1.6 mg·ml^{-1}) were actively loaded with Ca^{2+} in a reaction mixture containing 0.15 M potassium gluconate, 200 μM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ATP, 5.0 mM phospho*enol*pyruvate, 10 units·ml^{-1} pyruvate kinase, 9 μM arsenazo III, and 20 mM Mes (pH 6.8) (solution A). To produce depolarization-induced Ca^{2+} release, one part of solution A was mixed with one part of solution B containing 0.15 M choline chloride, 9 μM arsenazo III, 20 mM Mes (pH 6.8), and various concentrations of inhibitors, whose final concentrations upon mixing are as follows. Ruthenium red, μM: A_1, 0; A_2, 0.05; A_3, 0.5. Tetracaine, mM: B_1, 0; B_2, 0.05; B_3, 1.0. About ten traces collected during the period of 2–7 min after the addition of ATP were signal-averaged.

TABLE I

THE CONCENTRATION OF BLOCKERS FOR HALF-INHIBITION ($C_{1/2}$) OF VARIOUS TYPES OF Ca^{2+} RELEASE

Various types of Ca^{2+} release were induced as described in Experimental Procedures and $C_{1/2}$ was determined from the plot of A (size of Ca^{2+} release) versus the concentration of Ruthenium red, tetracaine and procaine.

Method of triggering	Ruthenium red (μM)	Tetracaine (mM)	Procaine (mM)
Ca^{2+}	0.10	0.11	1.00
Quercetin	0.08	0.09	-
Caffeine	0.10	0.10	-
Depolarization	0.08	0.07	0.67

little or no time-dependent change in the extent of inhibition of Ca^{2+}-induced Ca^{2+} release by Ruthenium red or tetracaine.

Discussion

A number of methods have been reported to induce Ca^{2+} release from sarcoplasmic reticulum in vitro: e.g., (a) jump of extravesicular Ca^{2+} concentration [6,11,13,15,16,36,37], (b) the addition of drugs such as caffeine [6,12,15,16,38,39], quercetin [6,37,40], halothane [11,15,16], and enflurane [15], (c) combination of a and b, (d) replacement of impermeable anions with permeable ones or re-

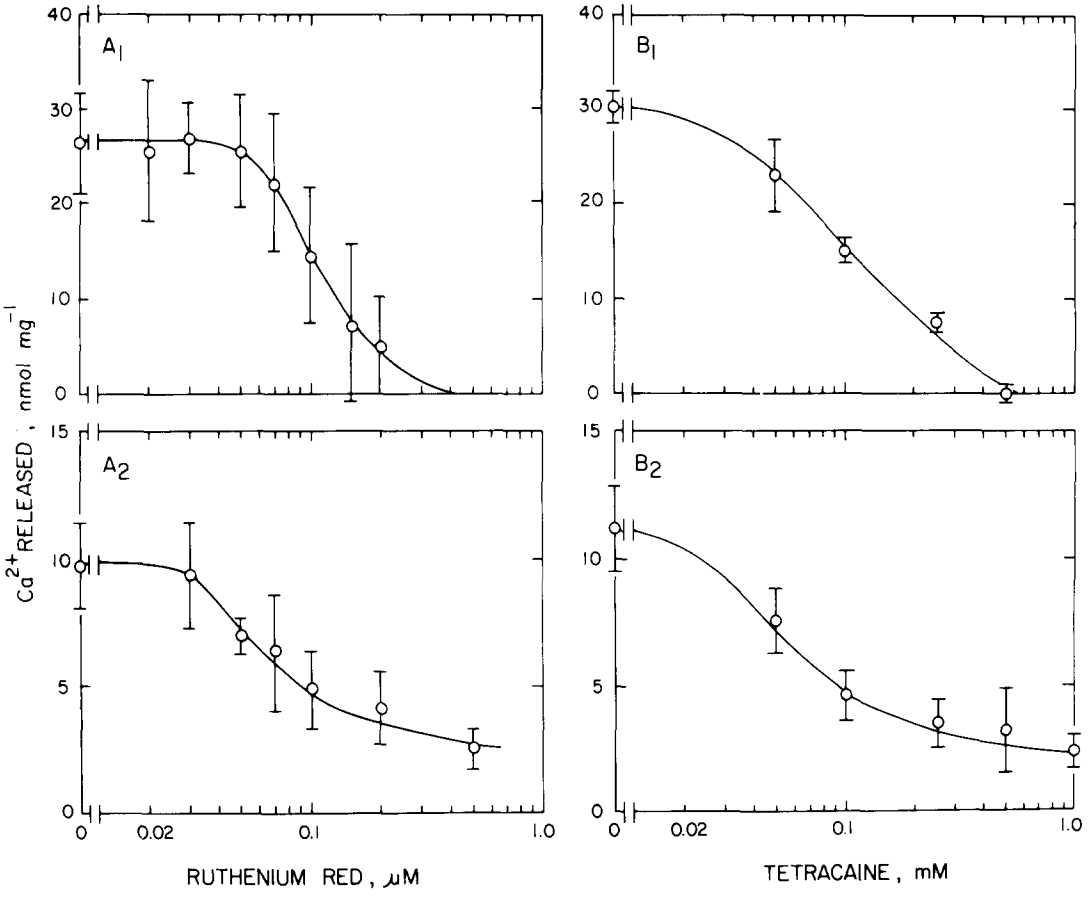

Fig. 4. Plots of the relative activity of Ca^{2+} release versus the concentration of Ruthenium red (A) and tetracaine (B). Two types of Ca^{2+} release – viz., Ca^{2+}-induced Ca^{2+} release (A_1, B_1), and depolarization-induced Ca^{2+} release (A_2, B_2) – were induced as described in the legends to Figs. 1–3, and the amount of Ca^{2+} release was determined as a function of the final concentration of inhibitors. Note that determination of the extent of inhibition of depolarization-induced Ca^{2+} release was made on the rapid phase. The rapid phase represents the T-tubule-mediated Ca^{2+} release which is clearly distinguishable from the second slow phase that is induced by the Ca^{2+} released in the rapid phase (cf. Ref. 7). Therefore, the possibility that inhibition of Ca^{2+}-induced Ca^{2+} release might be involved in the inhibition of depolarization-induced Ca^{2+} release can be excluded. Each data point represents an average of 3–12 experiments $\pm$ S.D.

placement of permeable cations with impermeable ones, or both [5–7,9–11,14,42–44], (e) the addition of proton ionophores [45] and organic anions [46,47], (f) the addition of noncovalently reacting or covalently reacting -SH reagents [48], and (g) prolonged incubation of sarcoplasmic reticulum in Ca^{2+}, ATP and inorganic phosphate (spontaneous Ca^{2+} release, Ref. 28). The most important task of the studies of isolated sarcoplasmic reticulum is to elucidate how these in vitro types of Ca^{2+} release are related to the mechanism operating in situ.

Endo [3] has suggested that the two major classes of Ca^{2+} release (viz., (a) Ca^{2+}-induced Ca^{2+} release including drug-induced Ca^{2+} release and (b) depolarization-induced Ca^{2+} release) are governed by different mechanisms, as evidenced from the fact that they differ in several criteria, such as Ca^{2+}-loading requirement and sensitivities to Mg^{2+}, ATP, sugars and procaine. It has been reported that several mM procaine inhibits Ca^{2+}-induced Ca^{2+} release, but has no effect on depolarization-induced Ca^{2+} release [31]. In contrast to this observation, we have found that procaine inhibits both Ca^{2+}-induced and depolarization-induced Ca^{2+} release, suggesting that Cl^--induced Ca^{2+} release observed in the skinned muscle fibers and depolarization-induced Ca^{2+} release described in this paper (also see Ref. 7) are governed by different mechanisms. It is also possible that the apparent discrepancy is due to the differences in the experimental conditions between the in situ (e.g., the use of EGTA) and in vitro studies.

The present results suggest that the two classes of Ca^{2+} release (a and b, see above) share some common properties. The most important point in this paper is the finding that both classes of Ca^{2+} releases are inhibited by either Ruthenium red or tetracaine in the same concentration range for each drug. It is unlikely that different components involved in different triggering mechanisms would have the same binding affinity to two different reagents. Therefore, it appears that these blockers react with a common component involved in both Ca^{2+} (or drug)-induced Ca^{2+} release and depolarization-induced Ca^{2+} release. In view of our hypothesis that an identical set of proteins serves as the Ca^{2+} channel for different types of Ca^{2+} release (see Introduction), the sites with which Ruthenium red, tetracaine and procaine react are presumably located within the putative Ca^{2+} channel.

The addition of blockers concurrently with the triggering of Ca^{2+} release produces inhibition as effectively as the addition of blocker before triggering. This would indicate that the blockers enter the Ca^{2+} channel as soon as it opens, leading to an instantaneous blocking of Ca^{2+} flow through the channel. It is interesting in this context that procaine inhibits caffeine contracture in an intact single fiber [31,49], while it has no effect or even slight potentiation effect on the initial rate of potassium contracture [31,49]. However, procaine produces considerable shortening of the duration of potassium contracture [49,50] probably by acceleration of the inactivation process of Ca^{2+} release [49]. Thus, the reduction of the amount of Ca^{2+} release by procaine described in the present study may in fact be due to an accelerated closing of the opened channel rather than blocking of the Ca^{2+} flow through the opened channels.

Although it appears that Ruthenium red, tetracaine and procaine react with the Ca^{2+} channel as discussed above, these reagents also likely react with the other components. In fact, Ruthenium red was reported to inhibit functions of many different systems: e.g., the spontaneous miniature endplate potential at synaptic junctions ($(2.5–10.0)\cdot 10^{-6}$ M, Ref. 51), mitochondrial Ca^{2+} transport ($C_{1/2} = 3.0\cdot 10^{-8}$ M, Ref. 52), and sarcoplasmic reticulum Ca^{2+} binding ($(1.0–2.5)\cdot 10^{-5}$ M, Ref. 53). Tetracaine inhibits a charge movement induced by the T-tubule depolarization [54], which probably represents a mechanism other than the function of the Ca^{2+} channel per se. Procaine is also known to affect the potassium current [3]. Therefore, one cannot a priori expect that these reagents serve as specific markers of the Ca^{2+} channel, though they are useful tools for the studies of behavior of the Ca^{2+} channel.

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