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Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. II. The effects of dantrolene on Ca^{2+} release induced by caffeine, Ca^{2+} and depolarization

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The effects of dantrolene, which is a known muscle relaxant, on Ca^{2+} release from the isolated sarcoplasmic reticulum induced by several different methods ((1) addition of caffeine, (2) Ca^{2+} jump, and (3) membrane-depolarization produced by choline chloride replacement of potassium gluconate) were investigated. Dantrolene inhibited caffeine-induced Ca^{2+} release with $C_{1/2} = 2.5$ μM, whereas there was no effect on Ca^{2+} release induced by a Ca^{2+} jump. The amount of Ca^{2+} released by depolarization was reduced if Ca^{2+} release was triggered in an earlier phase of the steady state of Ca^{2+} uptake (time elapsed between the addition of ATP and the triggering of Ca^{2+} release, $t_{ATP} < 4$ min); while, if triggered in a latter phase ($t_{ATP} > 4$ min) dantrolene enhanced depolarization-induced Ca^{2+} release. $C_{1/2}$ for the inhibition and that for enhancement of depolarization-induced Ca^{2+} release were 1.0 and 0.3 μM, respectively. These results suggest that dantrolene affects several different steps of the mechanism by which Ca^{2+} release is triggered. The sarcoplasmic reticulum and T-tubule membrane fractions had 7.9 nmol dantrolene-binding sites/mg ($K_{assoc} = 1.0 \cdot 10^5$ M^{-1}) and 21.0 nmol/mg ($K_{assoc} = 1.1 \cdot 10^5$ M^{-1}), respectively. The time-course of dantrolene binding to sarcoplasmic reticulum was monophasic, while that to T-tubules was biphasic.

Introduction

Extensive studies have been carried out on the effects of dantrolene [1], a skeletal muscle relaxant [2], on various functions of the muscle fiber. Dantrolene has little or no effect on the electric properties of the surface membrane (e.g., capacitance [3], resistance [3], resting potential [4–6] and action potential [4]). However, the tension development of the muscle fiber induced by a direct stimulation of the plasma membrane is inhibited by dantrolene [3–10]. Therefore, it has been assumed that the drug acts on some steps of the transverse (T)-tubule/sarcoplasmic reticulum coupling that occur after the T-tubule depolarization [1,9]. Putney and Bianchi [11] have found that Ca^{2+} influx across the T-tubule membrane induced by K^+-depolarization is inhibited by about 20 μM dantrolene, suggesting that the inhibition of influx of 'triggering Ca^{2+}' [12] is one of the major functions of dantrolene (cf. Refs.5, 6, 9, 11, 13, 14). Several functions that may be related to the T-tubule/sarcoplasmic reticulum coupling are

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Abbreviation: Mes 4-morpholineethanesulfonic acid.

also inhibited. For instance, nonlinear charge movement [15] is inhibited by dantrolene [16]. The coupling efficiency between the T-tubule depolarization and tension development is also reduced by dantrolene [1].

On the other hand, caffeine- or halothane-induced contracture, which in the light of our recent knowledge (Refs. 17–19) is produced by a direct stimulation of sarcoplasmic reticulum, is also inhibited by dantrolene [20,21]. Halothane-induced Ca^{2+} release is inhibited by dantrolene [22]. Spontaneous Ca^{2+} release [23] is also inhibited by dantrolene; whereas the effects of dantrolene on the Ca^{2+}-induced Ca^{2+} release [2,7,24–26] and on the passive Ca^{2+} efflux [13,27,28] are contradictory. One of the important observations with the isolated sarcoplasmic reticulum preparations is that dantrolene has little effect on the Ca^{2+} uptake and ATPase activities [22,28]. This would indicate that action of dantrolene as a muscle relaxant is due to inhibition of Ca^{2+} release from sarcoplasmic reticulum rather than activation of Ca^{2+} transport processes.

Recent methodological developments have permitted the resolution of two distinguishable routes for the induction of Ca^{2+} release from the isolated sarcoplasmic reticulum [19,29–31].

The aim of the present paper is first to investigate the effect of dantrolene on these types of Ca^{2} release, and secondly to investigate the characteristics of dantrolene binding to the isolated membrane fractions in an attempt to localize the site of drug action. As shown here, dantrolene inhibits caffeine-induced Ca^{2+} release, whereas there is no effect on Ca^{2+}-induced Ca^{2+} release. Dantrolene produces dual effects (inhibition and activation) on depolarization-induced Ca^{2+} release. Thus, the drug's effects are different depending upon the triggering method. In this study, we have confirmed previous reports [32,33] that the isolated sarcoplasmic reticulum membrane has a considerable capacity of dantrolene binding. However, high concentrations of caffeine affect only a very small fraction of the total dantrolene-binding sites. Characteristics of dantrolene binding to the isolated T-tubules are clearly distinguishable from those of the binding to sarcoplasmic reticulum in that T-tubulus have (a) larger capacity of binding, (b) higher specific fluorescence intensity of the bound dantrolene than the sarcoplasmic reticulum, and (c) two kinetically distinguishable classes of binding sites while there is only one class in the sarcoplasmic reticulum. The slow-binding class, but not the fast-binding class, appears to be related to the dantrolene effects on depolarization-induced Ca^{2+} release. The present results suggest that dantrolene has differential effects on different types of Ca^{2+} release. Thus, dantrolene belongs to a category of inhibitors that is different from the channel blockers described in the accompanying paper [34].

Experimental procedures

Preparation of sarcoplasmic reticulum. The Ca^{2+}-releasing sarcoplasmic reticulum fraction was prepared as described in the preceding paper [34].

Preparation of T-tubules. About 150 g rabbit leg muscle (fast-twitch white muscle) was homogenized at a low speed in a Waring blender with 4 vol. of 0.15 M KCl and 20 mM Mes (pH 6.8) for two times of 30 s at an interval of 30 s. The homogenate was centrifuged at $10000 \times g$ for 20 min in a JA-10 rotor (Beckman). The supernatant fraction was filtered through eight layers of cheesecloth, and centrifuged at $100000 \times g$ for 60 min in a Ti-45 rotor (Beckman ultracentrifuge). The resulting pellet was suspended in a solution containing 0.6 M KCl and 20 mM Mes (pH 6.8) and centrifuged again at $100000 \times g$ for 60 min. The pellet was suspended in 0.15 M KCl and 20 mM Mes (pH 6.8), and treated twice with a French press (3000 lb/in^2). A discontinuous sucrose gradient was prepared, which consisted of four layers of 3.5 ml sucrose solution in 0.15 M KCl and 20 mM Mes (pH 6.8): 50, 35, 27.5 and 25% sucrose. The French press-treated sample was loaded on it, and centrifuged in an SW-27 rotor overnight. The fractions collected from the top of the 25% sucrose and from the interface between the 25 and 27.5% sucrose layers were combined and sedimented for 60 min at $100000 \times g$. The pellet was homogenized in 0.15 M KCl and 20 mM Mes (pH 6.8). In some cases, further purification was carried out by loading the sample on the top of a second sucrose gradient made of 35, 27.5, 25% sucrose and centrifuged in the SW-40 rotor for 5–6 h. The purity of the resulted T-tubule was

checked by measuring the cholesterol content [35].

Ca^{2+} release assays. Induction and measurements of various types of Ca^{2+} release were done as described in the accompanying paper [34].

Dantrolene-binding assays. Dantrolene-binding studies were done according to the fluorometric method described by Dehpour et al. [33]. To determine the intrinsic fluorescence of the heavy sarcoplasmic reticulum and the purified T-tubule preparation, different amounts of protein were added to a solution containing 5 μM dantrolene, 0.15 M KCl, 20 mM Mes (pH 6.8) (and various concentrations of caffeine if necessary), and the fluorescence intensity was measured at 25°C at excitation wavelength of 400 nm and emission wavelength of 520 nm. For the determination of the maximal binding capacity and binding affinity 0.5 mg/ml protein was used, and the dantrolene concentration was varied in the range of 1–30 μM. In some experiments, different amounts of caffeine were added to the reaction mixture to see the inhibitory effect of caffeine.

Dantrolene binding kinetics. Sarcoplasmic reticulum or T-tubule protein (0.2–0.5 mg/ml) in a solution containing 0.15 M KCl and 20 mM Mes (pH 6.8) was loaded into the A syringe of a Durrum stopped-flow apparatus and mixed with an equal volume of 10 μM dantrolene solution. The reaction mixture was illuminated through a monochromator at 400 nm, and the emitted light was collected through a filter that cut the light below 500 nm.

Miscellaneous. Dantrolene was a generous gift from Norwich Eaton Pharmaceuticals, Inc.

Results

I. Effects of dantrolene on various types of Ca^{2+} release

Caffeine-induced Ca^{2+} release

In the experiment shown in Fig. 1, various concentrations of dantrolene were added to the sarcoplasmic reticulum after ATP-dependent Ca^{2+} accumulation was completed, and then the Ca^{2+}-loaded sarcoplasmic reticulum was mixed with caffeine in a stopped-flow apparatus to induce Ca^{2+} release. Further improvement of the time resolution of the stopped-flow measurements has permitted us to resolve some new features of the kinetics of Ca^{2+} release. There is an appreciable lag phase before Ca^{2+} release (Fig. 1). If the data points in the lag phase are omitted from fitting, the subsequent Ca^{2+} release can be fitted by single exponential. The kinetic parameters calculated from iterative fitting (size of Ca^{2+} release (A), rate constant (k) and the length of lag period (t_L; for the method of determination, see the legend to Fig. 2)) are plotted as a function of the concentration of dantrolene (Fig. 2). As seen here, increasing concentrations of dantrolene lead to a sharp decrease of k and increase of t_L in the range of 1.0–10.0 μM (half-maximal inhibition ($C_{1/2}$) = 2.5 μM). There is a small but significant decrease in the size of Ca^{2+} release in the same range of dantrolene concentration.

Ca^{2+}-induced Ca^{2+} release

Experiments similar to those of Fig. 1 were carried out to investigate the effects of dantrolene on Ca^{2+}-induced Ca^{2+} release. Dantrolene has no effect on any parameters of Ca^{2+} release induced by a Ca^{2+} jump [29] (not shown). It is interesting that dantrolene does not inhibit caffeine-induced Ca^{2+} release when the extravesicular Ca^{2+} concentration is high due to a slow leakage of the accumulated Ca^{2+}. Similarly, there is no dantrolene inhibition when Ca^{2+} release is induced by the simultaneous addition of caffeine and Ca^{2+}. Thus, it appears that inhibitory effects of dantrolene on caffeine-induced Ca^{2+} release are antagonized by higher concentrations of extravesicular Ca^{2+}.

Depolarization-induced Ca^{2+} release

Dilution of the sarcoplasmic reticulum, incubated previously in Ca^{2+}, ATP and 0.15 M potassium gluconate, into 0.15 M choline chloride, leads to the rapid Ca^{2+} release from sarcoplasmic reticulum as determined by stopped-flow spectrophotometry (Fig. 3, cf. Ref. 19). Dantrolene affects depolarization-induced Ca^{2+} release in a time-dependent fashion. Fig. 3 is a plot of the amounts of Ca^{2+} released in the rapid phase versus the time of triggering after the addition of ATP for Ca^{2+} loading (t_{ATP}). As shown here, 20 μM dantrolene inhibits depolarization-induced Ca^{2+} release when Ca^{2+} release is submaximal ($t_{ATP} < 4$ min), but

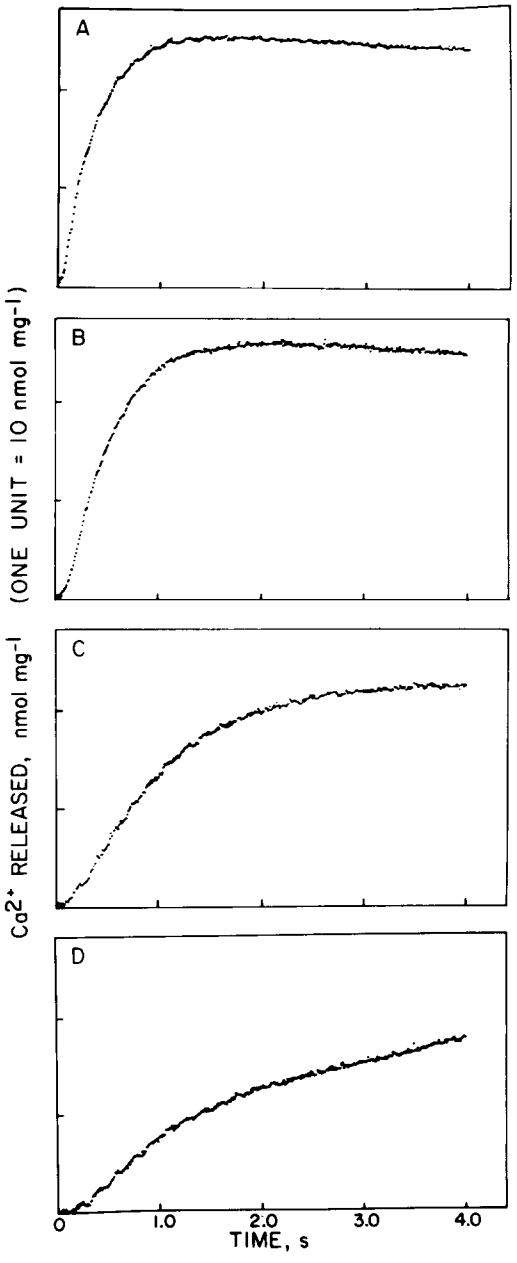

Fig. 1. The effect of dantrolene on caffeine-induced Ca^{2+} release. The heavy sarcoplasmic reticulum vesicles (1.6 mg/ml) were incubated at 27°C for 2.5 min in a solution containing 0.15 M KCl, 20.0 mM Mes, 0.5 mM $MgCl_2$, 9 μM arsenazo III, at pH 6.8. The Ca^{2+} uptake reaction was started by addition of 50 μM $CaCl_2$, 10 units/ml pyruvate kinase, 2.5 mM phospho*enol*pyruvate and 0.5 mM ATP. 4 min after the addition of ATP, 0 μM (A), 2.5 μM (B), 10.0 μM (C), 20.0 μM (D) dantrolene was pipetted into the reaction solution, and the mixture was loaded into the A syringe of a Durrum stopped-flow apparatus. The B syringe contained 0.15 M KCl, 20.0 mM Mes,

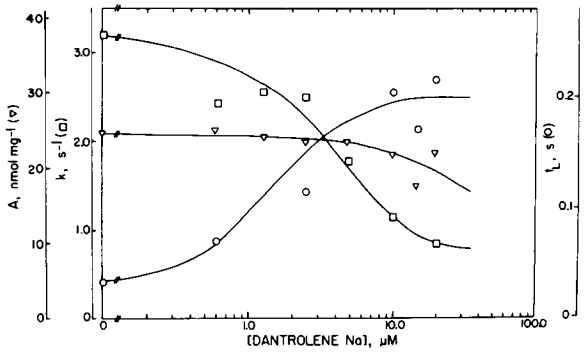

Fig. 2. Inhibition of the caffeine-induced Ca^{2+} release by different concentrations of dantrolene. Experimental conditions were the same as described in the legend of Fig. 1. The amount of Ca^{2+} released (▽), the rate constant of the Ca^{2+} release (□) and the duration of the lag phase (○) are plotted as a function of dantrolene concentration. For iterative computer-fitting, appropriate truncation of the data points in the initial phase of the Ca^{2+} release reaction was made in order to obtain satisfactory fitting by a single exponential model. A and k were calculated from a single exponential, $y = A[1-\exp(-kt)]$, where y is the amount of released Ca^{2+}. t_L was obtained from the intersect of the fitted curve to the abscissa.

enhances release after the amount of Ca^{2+} released has reached the maximal level ($t_{ATP} > 4$ min).

Fig. 4 illustrates the [dantrolene] dependence of inhibition in the earlier phase ($t_{ATP} < 4$ min) and that of activation in the later phase ($t_{ATP} > 4$ min). Inhibition of depolarization-induced Ca^{2+} release takes place in a lower concentration range ($C_{1/2} = 1.0$ μM) than that for the inhibition of caffeine-induced Ca^{2+} release ($C_{1/2} = 2.5$ μM). The activation by dantrolene ($t_{ATP} > 4$ min) takes place in an even lower concentration range ($C_{1/2} = 0.3$ μM).

The above effects (both inhibition and activation) could not be observed when dantrolene was placed in solution B and mixed with sarcoplasmic reticulum concurrently with ionic replacement. This suggests that binding of dantrolene to the inhibitory or activating sites mentioned above requires an incubation.

9 μM arsenazo III and 4 mM caffeine (pH 6.8). The Ca^{2+} release was triggered by mixing the content of the A syringe with the content of the B syringe at the 1:1 volume ratio, and the changes in the transmittance of arsenazo III were recorded at 680 and 650 nm. The Ca^{2+} release data obtained between 5.0 and 6.5 min after the addition of ATP were signal-averaged.

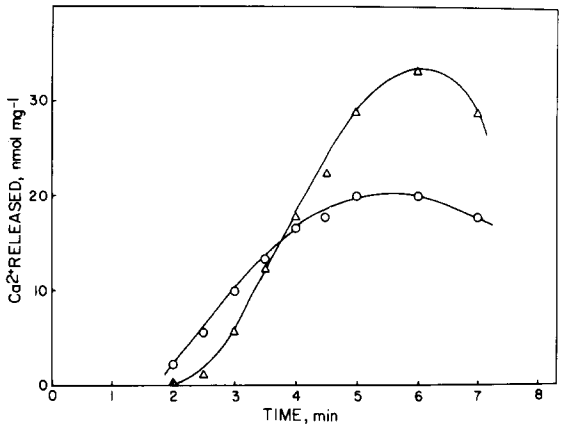

Fig. 3. Effect of dantrolene on depolarization-induced Ca^{2+} release as a function of time elapsed between triggering of Ca^{2+} release and the addition of ATP. For active loading, the sarcoplasmic reticulum vesicles (homogenized in 0.15 M potassium gluconate and 20.0 mM Mes (pH 6.8) were incubated in a reaction solution containing 1.6 mg/ml protein, 0.15 M potassium gluconate, 20.0 mM Mes, 200.0 μM $CaCl_2$, 5.0 mM phospho*enol*pyruvate, 10 units/ml pyruvate kinase, 0.5 mM Mg·ATP and 9 μM arsenazo III. At 1.5 min after the addition of ATP, 20 μM dantrolene was pipetted into the reaction mixture, and the solution was loaded into the A syringe of a Durrum stopped-flow apparatus. Ca^{2+} release was triggered by mixing the content of the A syringe with an equal volume of B solution containing 0.15 M choline chloride, 20.0 mM Mes and 9 μM arsenazo III (pH 6.8), and the changes of the absorbance of arsenazo III were recorded at 680 and 650 nm. As control, no dantrolene was added to the reaction mixture. ○, Control; △, 20 μM dantrolene.

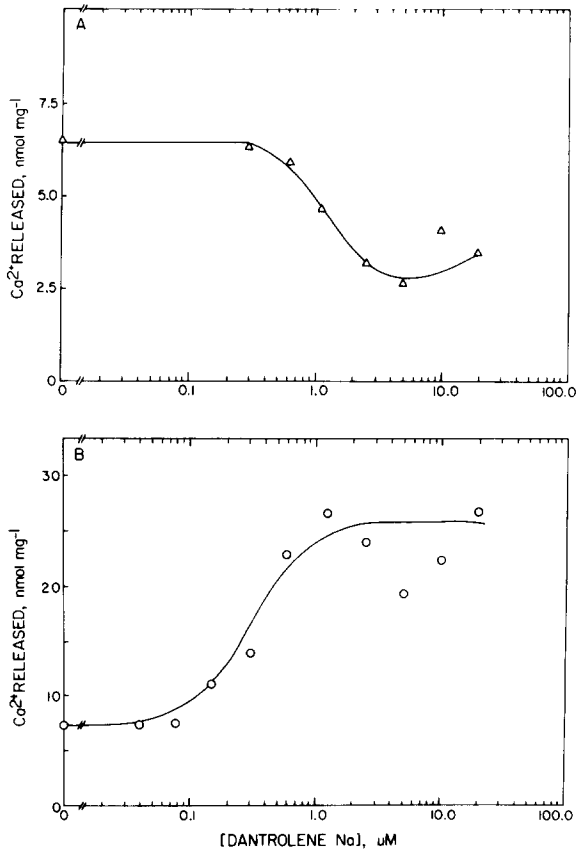

Fig. 4. Inhibition of depolarization-induced Ca^{2+} release by different concentrations of dantrolene. Experimental conditions were same as described in the legend to Fig. 3. The stopped-flow traces of Ca^{2+} release triggered between 2–4 min after addition of ATP were signal-averaged. (B) Acceleration of the depolarization-induced Ca^{2+} release by different concentrations of dantrolene. Experimental conditions were same as described in the legend to Fig. 3. Ca^{2+} release traces between 4–5 min after addition of ATP were signal-averaged.

II. Characteristics of dantrolene binding

We have carried out studies of dantrolene binding to the two types of membrane preparations (the sarcoplasmic reticulum preparation used for the Ca^{2+} release studies described above, and purified T-tubules) using the fluorometric method [33]. The fluorescence intensity of added dantrolene (5 μM) increases upon the addition of increasing concentrations of protein. The $1/F$ (F= fluorescence intensity of dantrolene) versus 1/[protein] plot is linear in both preparations. It is interesting that the fluorescence intensity per unit amount of membrane-bound dantrolene (the specific fluorescence intensity), as determined by extrapolation of the fitted line to the ordinate, is about 2-times higher in the T-tubule membrane than in sarcoplasmic reticulum. Scatchard plots of the binding dats showed that the total binding capacity is also significantly larger in T-tubules (21.0 nmol/mg) than in sarcoplasmic reticulum (7.9 nmol/mg); whereas the affinity of binding (T-tubule, $1.1 \cdot 10^5$ M^{-1}; sarcoplasmic reticulum, $1.0 \cdot 10^5$ M^{-1}) is about the same for both membrane preparations. Addition of increasing concentrations of caffeine (0–3 mM) to the sarcoplasmic reticulum preparation produced a small but significant decrease of the affinity of binding (slope). Although caffeine decreased the total binding capacity in many experiments, the extent of

the change was within the experimental error. Further increases of the caffeine concentration produced no further change in the Scatchard plot. The results suggest that the number of dantrolene-binding sites to which caffeine competes represents only a very small portion of the total dantrolene-binding sites. It is interesting in this context that considerable amounts of dantrolene are bound to bovine serum albumin (6.0 nmol · mg^{-1}) and soybean phospholipid azolectin (9.0 nmol · mg^{-1}), K_{assoc} being about $1.0 \cdot 10^5$ M^{-1} in both cases. Therefore, it appears that a large portion of the dantrolene binding represents nonspecific sites which are not related to the observed effects on Ca^{2+} release.

Fig. 5 depicts the time-course of dantrolene binding to sarcoplasmic reticulum preparation (A) and that to the isolated T-tubules (B), as determined by stopped-flow fluorometry. Binding to sarcoplasmic reticulum is rapid and monophasic, while binding to the isolated T-tubule occurs in two clearly distinguishable phases.

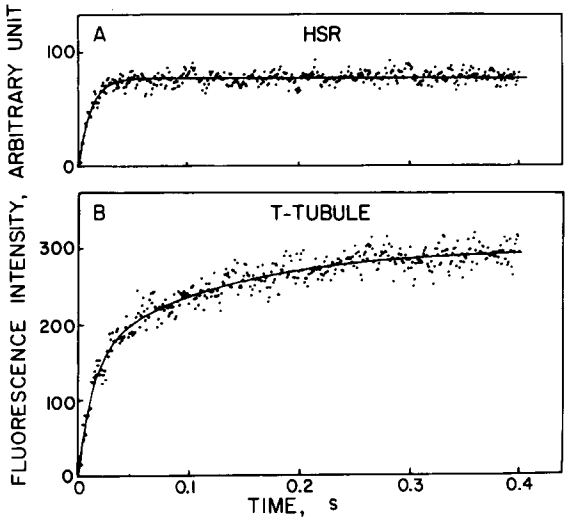

Fig. 5. Stopped-flow fluorometric trace of dantrolene binding to sarcoplasmic reticulum (A) and to purified T-tubules (B). Solution A containing 0.5 mg/ml protein, 0.15 M KCl and 20.0 mM Mes (pH 6.8) was mixed with an equal volume of solution B containing 0.5 M KCl, 20.0 mM Mes (pH 6.8) and 10 μM dantrolene in a Durrum stopped-flow apparatus at 25°C. The reaction mixture was illuminated through a monochromator at excitation wavelength of 400 nm, and the emitted light was collected through a filter that cut the light below 500 nm.

Discussion

A considerable body of accumulated evidence as summarized in the Introduction suggests that dantrolene serves as a muscle relaxant by acting on several different steps of the T-tubule/sarcoplasmic reticulum coupling leading to the blocking of Ca^{2+} release from sarcoplasmic reticulum. In an attempt to identify and characterize such sites, several workers have carried out studies of dantrolene binding to the isolated sarcoplasmic reticulum membrane [32,33], and found that the sarcoplasmic reticulum membrane has in fact an appreciable capacity of dantrolene binding within the concentration range in which the drug produces definitive effects on the physiological functions. However, little information is available about the characteristics of dantrolene binding to the T-tubule membrane. On the other hand, reports concerning the effects of dantrolene on the Ca^{2-} release functions in the isolated sarcoplasmic reticulum are contradictory. The purpose of this study is to gain further insights into (a) the modes of dantrolene action on various types of Ca^{2+} release in vitro using the recently developed methods that permit induction and monitoring of rapid Ca^{2+} release, and (b) characteristics of dantrolene binding to both the sarcoplasmic reticulum and T-tubule membranes.

As described here, dantrolene has no effect on Ca^{2+}-induced Ca^{2+} release, but has a strong inhibitory effect on caffeine-induced Ca^{2+} release and dual effects (inhibition and activation) on the depolarization-induced Ca^{2+} release. Thus, dantrolene produces differential effects on Ca^{2+} release, depending upon the methods of induction of Ca^{2+} release.

In view of the close similarities between Ca^{2+}-induced Ca^{2+} release and caffeine-induced Ca^{2+} release [17,29], it has been assumed that these types of Ca^{2+} release are governed by the same mechanism. One of the new aspects emerging from the present study is that Ca^{2+}-induced Ca^{2+} release and caffeine-induced Ca^{2+} release are clearly distinguishable in terms of their sensitivity to dantrolene. In the light of the previously described receptor-channel concept [19,24,29–31], these data suggest that receptors for caffeine and Ca^{2+} are different, though there may be strong interactions between these two kinds of receptors.

Since depolarization-induced Ca^{2+} release is

triggered via T-tubules [19], the dantrolene-binding sites that are responsible for the dual effects on depolarization-induced Ca^{2+} release are probably located in the T-tubule membrane. On the other hand, the dantrolene-binding sites responsible for the inhibition of caffeine-induced Ca^{2+} release are presumably located in the sarcoplasmic reticulum, since caffeine-induced Ca^{2+} release is produced by a direct stimulation of sarcoplasmic reticulum. Some binding data described in this paper can be interpreted in the light of the above views. The effects of dantrolene on depolarization-induced Ca^{2+} release could be observed only when the membrane was incubated with the drug before triggering of Ca^{2+} release. Thus, it appears that the dantrolene-binding sites that represent the slow phase of binding are involved in the drug's effects. However, an almost negligible portion of dantrolene-binding sites is affected by caffeine. Thus, it is likely that that dantrolene interferes with caffeine-induced Ca^{2+} release without affecting caffeine binding.

As shown here, dantrolene inhibits depolarization-induced Ca^{2+} release if it is applied to the sarcoplasmic reticulum in the earlier phase of the steady state of Ca^{2+} uptake, whereas activation occurs if applied in the later phase. Furthermore, $C_{1/2}$ for the activation is much lower than that for the inhibition. These data suggest that at least two different dantrolene-binding components are involved in the T-tubule/sarcoplasmic reticulum coupling. Characterization of these components is one of the most important topics to be investigated in order to gain further insight into the key steps of the T-tubule/sarcoplasmic reticulum coupling.

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