Ryanodine Alteration of the Contractile State of Rat Ventricular Myocardium

Comparison with Dog, Cat, and Rabbit Ventricular Tissues

JOHN L. SUTKOF AND JAMES T. WILLERSON

With the technical assistance of Bonny St. Clair and Starr Wheelan

SUMMARY To test the hypothesis that ryanodine inhibits the release of contractile Ca\(^{2+}\) from intracellular stores, we compared the contractile responses by rabbit, cat, dog, and rat papillary muscles to ryanodine. Results of cumulative ryanodine concentration (10\(^{-8}\) to 10\(^{-4}\) M) response studies indicate the following order of sensitivity to ryanodine: rat > dog = cat > rabbit which mimics the relative dependence of these species on intracellular Ca\(^{2+}\) for force development. In the presence of 2.5 mM [Ca\(^{2+}\)], cumulative additions of ryanodine or a single exposure to 10\(^{-4}\) M concentration produced biphasic contractile responses in rabbit, cat, and dog, but not rat ventricular muscle. The elevation of [Ca\(^{2+}\)]\(_{\text{e}}\) to 5 mM either antagonized the expression of ryanodine’s negative inotropic effect or promoted the positive effect of this agent in all species tested. Ryanodine did produce a biphasic change in contractility in the presence of 2.5 mM [Ca\(^{2+}\)], in K\(^{-}\)-depolarized, isoproterenol-restored rat papillary muscles. In addition, prior exposure of rat myocardium to ryanodine (2.5 mM [Ca\(^{2+}\)]\(_{\text{e}}\)) was similar to a decreased [Ca\(^{2+}\)]\(_{\text{e}}\) in that it permitted inotropic agents, such as increased stimulation rates, hyperosmolality, and ouabain to produce positive contractile responses from this tissue. In contrast, the positive response by rat cardiac muscle to paired electrical stimulation is prevented by ryanodine. Ryanodine also accelerated the rest-decay of force development in rat myocardium, suggesting that it increased the rate of loss of calcium from contractile-dependent Ca\(^{2+}\) stores. The results are consistent with ryanodine effecting a decreased availability of intracellular contractile Ca\(^{2+}\), perhaps through a diminishment of its release.


RYANODINE alters the mechanical performance of mammalian cardiac muscle when present in nanomolar to micromolar concentrations and hence may have a specific cellular site of action (Jenden and Fairhurst, 1969). Recent investigations of the effects of ryanodine on force development by potassium-depolarized, isoproterenol-restored guinea pig ventricles, canine ventricular muscle, and both normal and depolarized cat papillary muscles have revealed that, in addition to its well-documented negative inotropic effects, this drug can also produce positive changes (Frank et al., 1976; Nayler, 1973; Sutko et al., 1979). The expression of these inotropic changes by cat muscles was found to depend on the concentrations of both ryanodine and extracellular Ca\(^{2+}\). Ryanodine also diminished the ability of this preparation to respond to other inotropic interventions such as an increased stimulation frequency, paired electrical stimulation, and hyperosmolality. Perhaps most striking of these was the complete obviation by this drug of the inotropic response to paired pacing (Sutko et al., 1979).

We have also found that ryanodine markedly increased the ability of isolated cardiac sarcoplasmic reticular (SR) membrane vesicles to accumulate Ca\(^{2+}\). This effect which depends, at least partially, on the presence of K\(^{-}\) appears to be due to a blockade by ryanodine of the efflux of Ca\(^{2+}\) from the SR vesicles occurring through specific, K\(^{-}\)-dependent channels (Jones et al., 1979).

Based on the preceding mechanical and biochemical data, we have proposed as a heuristic hypothesis that ryanodine does have a specific effect on cardiac muscle, which results in the reduction of SR Ca\(^{2+}\) release (Sutko et al., 1979). Isolated ventricular cardiac muscle from different species has been reported to vary in the degree to which it depends on contractile Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores to support force development. Rat ventricular muscle appears to depend to a large extent on intracellular sources of this ion. Dog and cat ventricular myocardium seems also to depend on intracellular contractile Ca\(^{2+}\), but is more sensitive to...
contributions from extracellular sources, whereas rabbit cardiac muscle has been suggested to rely more heavily on superficially located Ca\(^{2+}\) stores (Hajdu, 1969; Shine et al., 1971; Henderson et al., 1974; Forester and Mainwood, 1974a; Bodem and Sonnenblick, 1975; Fabiato and Fabiato, 1978). This species order is paralleled by that established by Fabiato and Fabiato (1978) for the threshold Ca\(^{2+}\) concentration needed to demonstrate Ca\(^{2+}\)-induced Ca\(^{2+}\) release in mechanically skinned cells. Consequently, we have investigated the ability of ryanodine to alter the mechanical performance of ventricular tissue from these different species. If this drug does antagonize the release of Ca\(^{2+}\) from intracellular stores then, for the reasons outlined above, it should be most depressant to rat cardiac muscle.

In addition, isolated rat ventricular muscle, in the presence of 2.5 mM extracellular Ca\(^{2+}\), appears to be in a functional state of near Ca\(^{2+}\) overload. Inotropic agents, such as an increased rate of stimulation and hyperosmolality which normally produce positive contractile responses in tissues from other species, result only in negative effects in rat ventricular myocardium. The ability to respond to these inotropic interventions can be induced in rat muscles by reducing the extracellular concentration of Ca\(^{2+}\), which should lead to decreased availability of intracellular Ca\(^{2+}\) (Forester and Mainwood, 1974a; Willerson et al., 1978). If ryanodine acts by antagonizing the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, then it too should modify the behavior attributed to functional Ca\(^{2+}\) overload and also permit the expression of positive responses to these inotropic agents. Therefore, we have also tested the ability of rat papillary muscles to respond to several different inotropic interventions both in the presence and absence of ryanodine.

Methods

Papillary muscles were excised from either the right ventricles of hearts removed from adult cats and dogs of random sex and weight and male, New Zealand rabbits (1.5–2.5 kg) or from the left ventricles of Sprague-Dawley rats, while the animals were anesthetized with sodium pentobarbital (30 mg/kg). Unless otherwise indicated, the rats used in this study were 2–3 months of age. The methods of preparation and the instrumentation used in these studies have been described previously (Willerson et al., 1974; Sutko et al., 1979). The excised muscles were bathed in a Krebs-Henseleit buffer which was maintained at 30°C and which, unless otherwise stated, contained 2.5 mM Ca\(^{2+}\). The rat, cat, and dog preparations were paced electrically at a basal rate of 0.2 Hz and the rabbit preparations at 0.5 Hz, with stimuli of intensities 20% above threshold, applied through platinum electrodes placed parallel to the muscle length. An initial, 2-hour equilibration period was employed routinely, during the first hour of which the muscles were subjected to repeated stretches until the length (L\(_{\text{max}}\)) yielding maximal force development was attained.

The following parameters were used to assess muscle performance: developed force, DF; the maximal rate of force development, dF/dt; the maximal rate of relaxation, —dF/dt; resting force, RF; time-to-peak developed force, tPF, measured from the start of the twitch to the peak DF; and relaxation times, RT\(_{50}\) and RT\(_{90}\), measured at levels 50% and 90% below peak DF, respectively. The time intervals were obtained from responses recorded on a polygraph at a chart speed of 100 mm/sec. Data for DF, ± dF/dt, and RF have been normalized with respect to muscle cross-sectional area, as calculated from muscle length at L\(_{\text{max}}\), and wet weight. Under control conditions, rat, cat, dog, and rabbit muscles developed 6.1 ± 0.3 (n = 85), 6.7 ± 0.4 (n = 44), 5.4 ± 0.8 (n = 7), and 4.8 ± 0.3 (n = 34) g/mm\(^2\) (mean ± SE), respectively. These preparations had mean cross-sectional areas (mm\(^2\)) of 0.72 ± 0.01 (rat), 0.69 ± 0.43 (cat), 0.84 ± 0.14 (dog), and 0.42 ± 0.03 (rabbit).

At the end of the equilibration period, when a stable level of muscle activity had been attained, the experimental period was initiated. The cumulative ryanodine concentration-response studies, the dual drug experiments, and the paired electrical stimulation and K\(^{+}\)-depolarization studies were accomplished as previously described (Sutko et al., 1979). Briefly, the cumulative concentration-response relationships were evaluated by consecutive additions of ryanodine to the tissue bath. A 5-minute period during which the measured parameters did not exhibit a further significant change relative to that observed earlier during the exposure period was required before the drug concentration was increased. When the responses to two drugs were studied, one drug was added and a stable response obtained prior to administration of the second.

The K\(^{+}\)-depolarization studies involved exposing the muscles to a modified bicarbonate buffer solution containing 22 mM KCl with an equivalent reduction in NaCl to maintain a constant osmolality. Contractile activity ceased within 30–60 seconds after the introduction of this solution to the tissue bath. After 5 minutes of inactivity, the stimulation voltage was tripled and an additional 5-minute period of inactivity permitted to ensure that the muscle was completely depolarized. Subsequently, 10 mM isoproterenol was added to the bath, and mechanical activity usually became evident within 60 seconds. Periodically, during the experiment, 2 mM quantities of isoproterenol were added to the bath to ensure that any decrease in contractile activity was not due to an oxidative loss of isoproterenol and a partial return by the tissue to inactivity.

Commercial ryanodine (lot no. 704 RWP-1) used in this study was purchased from S.B. Penick & Co., mannitol from Merck, Sharp & Dohme Research Lab, and l-isoproterenol from Sigma Chem-
ical Co. In our previous studies, we used ryanodine generously supplied by Dr. E.F. Rogers of the Merck Sharp & Dohme Research Lab. The ryanodine obtained from both sources produced quantitatively and qualitatively similar responses from both rat and cat papillary muscles. In addition, ryanodine from these two sources has been found to be equieffective on isolated cardiac muscle SR (Jones et al., 1979). Drugs were solubilized in either distilled water, when additions to the tissue bath were less than 1% of the total bath volume, or in buffer when larger addition volumes were necessary. Isoproterenol solutions also contained 0.1% ascorbic acid to retard the oxidation of this drug.

Statistical analyses of data were done using the t-test for paired observations (Zar, 1974). Results were considered to differ significantly when a P value less than or equal to 0.05 was obtained.

Results

Mechanical Responses to Cumulative Additions of Ryanodine

Ventricular muscles obtained from rat, dog, cat, and rabbit were found to differ greatly in the sensitivity of their mechanical activity to the inotropic effects of ryanodine. As illustrated in Figures 1 and 2, exposure to increasing drug concentrations diminished the contractile abilities of rat muscles most dramatically; dog and cat muscles were intermediate in this respect, while the overall performance of rabbit preparations was relatively unaffected by ryanodine. In these studies, the muscles usually were exposed to each ryanodine concentration for approximately 1 hour. Rabbit, dog, and cat preparations exhibited similar threshold sensitivities, with 1-5 nM concentrations of ryanodine producing significant alterations of the measured parameters. In contrast, force development by rat muscles was consistently decreased by as little as 0.1 nM ryanodine (the lowest concentration tested, data not shown), and the rate of force development was reduced to 50% of the pre-drug values by 1-10 nM ryanodine.

It has been reported previously that the depressant effects of ryanodine can, at least partially, be reversed by increases in the frequency of stimulation (Hajdu and Leonard, 1961; Nayler et al., 1970). Consequently, the relative insensitivity of rabbit papillary muscles to ryanodine could, in part, have been due to the higher basal stimulation rate used with these preparations. To investigate this possibility, three muscles were paced at a frequency of 0.2 Hz and exposed to 1 μM ryanodine. This drug level reduced the dF/dt of these muscles to 80.7 ± 5.8% (mean ± se) of pre-ryanodine control levels, which is not different from the responses obtained to this drug concentration from muscles paced at 0.5 Hz. This result is consistent with our previous finding with cat papillary muscles that, although in the presence of 1 μM ryanodine at a doubling of the stimulation rate from 0.2 to 0.4 Hz resulted in a positive inotropic response, essentially the same degree of depression was observed at both rates when compared to pre-drug control responses (Sutko et al., 1979).

Several of the contractile parameters studied were altered in a biphasic manner by increasing concentrations of ryanodine, which involved an initial decrease followed by a rebound or return to-
First, the preparations used in these earlier studies were paced at 60 contractions/min at 37°C, whereas in our studies lower stimulation frequencies and temperatures were employed. Ryanodine has been reported to be less effective in some systems at lower temperatures (Jenden and Fairhurst, 1969). Second, of the 19 rabbit muscles we have tested in these studies, 2 were found to be more sensitive to ryanodine than were the other 17. Similarly, a few of the rat muscles tested were less sensitive than the majority. Consequently, depending on the species population available the evaluation of a relatively small number of preparations could result in a different conclusion concerning the susceptibility of these species to ryanodine. Third, there could be some variability in the "ryanodines" used in the different studies.

Effect of Exposure to A Single High Ryanodine Concentration

In our previous work we found that a single high concentration (100 μM) of ryanodine produced mechanical responses from cat papillary muscles that mimicked both qualitatively and quantitatively those obtained in the cumulative addition experiments. This similarity in response was also observed with rat papillary muscles, although perhaps due to the much shorter exposure time the extent of the depression resulting from the single high concentration was not as severe as that observed with cumulative additions (Figs. 1 and 3). In contrast to the latter experiments, a significant rebound in tPF was observed with a single exposure to 100 μM ryanodine. Rabbit papillary muscles responded somewhat differently to the single high concentration of ryanodine than they did to the cumulative additions, with the majority of the muscles tested not undergoing the initial negative phase but rather only showing a progressive positive response in both DF (7 of 9 muscles) and dF/dt (6 of 9 muscles). Once again tPF underwent only a steady prolongation.

Effects of the Extracellular Calcium Concentration on the Responses to Ryanodine

In our earlier studies we observed that the extent of the negative phase of cat papillary muscle responses to 100 μM ryanodine was sensitive to the extracellular concentration of Ca²⁺ ([Ca²⁺]o) present (Fig. 3). Therefore, we investigated whether the responses to this drug obtained from other species exhibited a similar sensitivity. Rat muscle responses also were altered by the presence of 5 mM [Ca²⁺]o. Although, unlike cat muscle responses, the extent of the early negative DF and dF/dt responses was not changed, the overall response became biphasic with a small but significant rebound occurring in both of these parameters following 60 minutes of drug exposure. In contrast to the results obtained
in the cumulative addition experiments, the time-to-peak force interval underwent a significant bi-phasic change in the presence of 100 μM ryanodine and 2.5 mM [Ca^{2+}]_o. This response was further modified to just a progressive monophasic increase in the presence of 5 mM [Ca^{2+}]_o. When tested in five preparations a further increase in [Ca^{2+}]_o to 7.5 mM produced no additional changes in the responses obtained from rat muscles. In the presence of either 2.5 or 5 mM [Ca^{2+}]_o, ryanodine produced primarily the positive response from rabbit papillary muscle described above, whereas, in the presence of 0.625 mM [Ca^{2+}]_o, a significant initial negative effect in the rate of force development was observed. The apparently greater rebound obtained with 0.625 mM [Ca^{2+}]_o (Fig. 3) is probably the result of the lower initial level of both DF and dF/dt, which were reduced by 55 and 65%, respectively, by the lowering of [Ca^{2+}]_o.

**Cumulative Ryanodine Concentration-Response Relationship in Potassium-Depolarized Isoproterenol-Restored Rat Papillary Muscles**

The appearance of the secondary positive phase or rebound in the responses obtained from rat papillary muscles in the presence of an elevated [Ca^{2+}]_o could be due to an increased secondary inward current (Frank et al., 1976; Sutko et al., 1979). To investigate this possibility further, we determined the effects of ryanodine on rat muscles in which, following depolarization produced by an elevation of extracellular potassium, mechanical and electrical functions were restored by the administration of isoproterenol. Under these experimental conditions, the presence and extent of both mechanical and electrical activities are thought to be closely related to the presence of the secondary inward current (Watanabe and Besch, 1974). The potassium-depolarized muscles used in our studies were paced at 0.1 Hz. Unlike normally polarized and potassium-depolarized cat papillary muscles from which we obtained quantitatively and qualitatively similar responses to ryanodine, potassium-depolarized rat muscles responded differently from the preparations bathed in a normal potassium concentration (Fig. 4). Although the negative phases of the DF and dF/dt responses were similar in both preparations, they differed in that the depolarized muscles responded in a biphasic manner in the presence of 2.5 mM [Ca^{2+}]_o. In addition, a marked increase in the tPF interval now was evi-
SPECIES COMPARISON OF RYANODINE INOTROPIES/ Sutko and Willerson

The contractile responses obtained to cumulative additions of ryanodine from K⁺-depolarized, isoproterenol-restored rat papillary muscles are presented. The developed force (DF), the maximal rate of force development (dF/dt), and the time-to-peak force interval (TPF) of the contractile responses obtained are shown in the upper, center, and lower graphs, respectively. The values obtained before and after K⁺-depolarization, isoproterenol-restoration are indicated on the abscissa by C and H-K, respectively. Following the completion of the cumulative ryanodine additions, the effects of D600 were tested on each preparation. The values shown represent the mean ± SE of 6 experiments. The small circles (°) indicate a significant difference between the indicated response and the pre-ryanodine (H-K) control value. The presence of + denotes a significant difference with the preceding minimal value.

**Figure 4**

The contractile responses obtained to cumulative additions of ryanodine from K⁺-depolarized, isoproterenol-restored rat papillary muscles are presented. The developed force (DF), the maximal rate of force development (dF/dt), and the time-to-peak force interval (TPF) of the contractile responses obtained are shown in the upper, center, and lower graphs, respectively. The values obtained before and after K⁺-depolarization, isoproterenol-restoration are indicated on the abscissa by C and H-K, respectively. Following the completion of the cumulative ryanodine additions, the effects of 1 μM D600 were tested on each preparation. The values shown represent the mean ± SE of 6 experiments. The small circles (°) indicate a significant difference between the indicated response and the pre-ryanodine (H-K) control value. The presence of + denotes a significant difference with the preceding minimal value.

Two of the five muscles studied in this series developed a single, weak, but consistent, aftercontraction following exposure to the elevated potassium concentration. As was found with potassium-depolarized cat papillary muscles (Sutko et al., 1979), in both of the present cases the aftercontraction disappeared after the addition of ryanodine (10 nM) to the tissue bath. Upon completion of the ryanodine concentration response, D600 (1 μM) was added to the bath to verify that the observed mechanical events were dependent on slow channel activity. In contrast to the relative ineffectiveness of this agent on normally polarized adult rat ventricular muscle (Willerson et al., 1978), in every case this concentration of D600 abolished all contractile activity in the potassium-depolarized preparations.

**Alteration by Ryanodine of Other Inotropic Responses of Rat Papillary Muscle**

The results described above suggest that ryanodine can dramatically depress the basal contractile state in rat ventricular muscle. These changes could be the consequence of this drug acting either to reduce the availability of contractile Ca²⁺ or to diminish the sensitivity or responsiveness of Ca²⁺-binding elements participating in the contraction cycle. If the latter were the case, then it would be anticipated that normally negative inotropic interventions would become more negative in the presence of ryanodine. In the presence of 2.5 mM [Ca²⁺], both an increase in the frequency of stimulation and an increase in bathing medium osmolality produce negative inotropic effects on rat ventricular myocardium. The nature of these responses appears to be sensitive to the intracellular Ca²⁺ load since a reduction in [Ca²⁺] results in both interventions producing positive responses (Forester and Mainwood, 1974a, Willerson et al., 1978). If ryanodine acts to reduce the intracellular availability of Ca²⁺, then prior exposure to this drug should also permit the expression of positive responses to increases in stimulation frequency and bathing medium osmolality.

**Treppe**

The normal diminution in rat ventricular muscle dF/dt produced by elevation of the stimulation rate from 0.2 to 0.6 Hz is shown in Figure 5A. Following exposure to 1 nM ryanodine, a transient positive response was obtained to the rate change (Fig. 5B). In addition, in the presence of ryanodine the steady state responses obtained at the two simulation rates were not significantly different, whereas prior to drug exposure the 0.6 Hz steady state dF/dt values were significantly less than those obtained at 0.2 Hz (Fig. 5).

**Hyperosmolality**

As illustrated in Figure 6, the normally negative inotropic effect of hyperosmolality (50 mOsm mannitol) in adult rat ventricular muscles was converted to a positive response in the presence of ryanodine. In contrast to the rat preparations, the normally positive response obtained from dog and cat muscles to this intervention was not evident following exposure of these tissues to 1 μM ryanodine (a concentration which produced a depression in the latter two tissues comparable to that observed with rat muscles and 1–5 nM ryanodine). In addition, elevation of [Ca²⁺], to 5 mM also prevented the positive inotropic response to hyperosmolality by
obtained from muscles from these animals, but did the animal, we decided to test older rats (12 months). In an attempt to obtain more clear-cut differences, the ability of rat ventricular tissue to respond to cardiac glycosides declines with increasing age of the animal. We examined the effects of 100 nM ouabain on muscles obtained from rats approximately 12-14 weeks of age. Prior to ryanodine exposure, this concentration of ouabain failed to produce a significant increase in either DF or dF/dt responses obtained from muscles of these animals, but did produce a small (less than 10%) increase in seven of nine preparations tested. Following exposure to 1 nM ryanodine, ouabain did produce a significant (15-20%) increase in both of these parameters. Since Langer et al. (1975) have demonstrated that the ability of rat ventricular tissue to respond to cardiac glycosides declines with increasing age of the animal, we decided to test older rats (12 months) in an attempt to obtain more clear-cut differences. The results of these studies are presented in Figure 7A. Under control conditions these muscles were insensitive to cumulative additions of ouabain ranging from 10 nM to 1 μM. In the presence of 10 and 100 μM ouabain, two of the six muscles tested developed a slight and reversible (upon washout of the ouabain) increase in resting force, with an associated loss of DF. This decline is at least partially responsible for the small reduction in DF recorded in the presence of these two ouabain concentrations. Since the muscles were exposed to each ouabain concentration for 30-50 minutes, the aging of the muscle in the tissue bath is probably responsible for the slow progressive decline in DF observed with lesser concentrations of ouabain. Following exposure to 10 nM ryanodine, 10 and 100 μM ouabain produced a significant increase in DF which was paralleled by a similar change in dF/dt (data not shown). If our hypothesis that ryanodine's permissive action is due to a decreased intracellular availability of contractile Ca^{2+} is correct, then, as was the case with treppe and responses to hyperosmolality, a lowering of the [Ca^{2+}], also should permit a positive inotropic response to ouabain. As shown in Figure 7B, this was the case. There have been conflicting prior reports concerning the ability of cardiac glycosides to produce a positive inotropic response in ryanodine-treated tissues. Hajdu and Leonard (1961) found no effect of strophanthidin in rat right ventricular slices previously exposed to ryanodine. Similarly, Procita (1958) found ouabain cat papillary muscles. These results suggest that the ability of hyperosmolar solutions to produce a positive inotropy in cardiac muscle may be dependent on the intracellular Ca^{2+} load.

Ouabain

Ouabain is another inotropic agent which produces a positive inotropic response in other mammalian species such as cat and rabbit at concentrations of 0.1-1.0 μM, but is relatively ineffective in rat cardiac muscle. To determine if the intracellular Ca^{2+} load might partially underlie the resistance to ouabain, we tested the ability of this drug to produce a positive response in rat papillary muscles following exposure to ryanodine. In our initial studies we examined the effects of 100 μM ouabain on muscles obtained from rats approximately 12-14 weeks of age. Prior to ryanodine exposure, this concentration of ouabain failed to produce a significant increase in either DF or dF/dt responses obtained from muscles of these animals, but did produce a small (less than 10%) increase in seven of nine preparations tested. Following exposure to 1 nM ryanodine, ouabain did produce a significant (15-20%) increase in both of these parameters. Since Langer et al. (1975) have demonstrated that the ability of rat ventricular tissue to respond to cardiac glycosides declines with increasing age of the animal, we decided to test older rats (12 months) in an attempt to obtain more clear-cut differences. The results of these studies are presented in Figure 7A. Under control conditions these muscles were insensitive to cumulative additions of ouabain ranging from 10 nM to 1 μM. In the presence of 10 and 100 μM ouabain, two of the six muscles tested developed a slight and reversible (upon washout of the ouabain) increase in resting force, with an associated loss of DF. This decline is at least partially responsible for the small reduction in DF recorded in the presence of these two ouabain concentrations. Since the muscles were exposed to each ouabain concentration for 30-50 minutes, the aging of the muscle in the tissue bath is probably responsible for the slow progressive decline in DF observed with lesser concentrations of ouabain. Following exposure to 10 nM ryanodine, 10 and 100 μM ouabain produced a significant increase in DF which was paralleled by a similar change in dF/dt (data not shown). If our hypothesis that ryanodine's permissive action is due to a decreased intracellular availability of contractile Ca^{2+} is correct, then, as was the case with treppe and responses to hyperosmolality, a lowering of the [Ca^{2+}], also should permit a positive inotropic response to ouabain. As shown in Figure 7B, this was the case. There have been conflicting prior reports concerning the ability of cardiac glycosides to produce a positive inotropic response in ryanodine-treated tissues. Hajdu and Leonard (1961) found no effect of strophanthidin in rat right ventricular slices previously exposed to ryanodine. Similarly, Procita (1958) found ouabain...
and digoxin to be ineffective in protecting intact dogs and cats from ryanodine’s cardiovascular effects. In contrast, Hillyard and Procita (1959) found prior administration of ouabain to slow, but not alter, the contractile depression exerted by ryanodine on kitten atria, and Sleator et al. (1964) observed strophanthin-K to partially reverse ryanodine depression of guinea pig atria.

**Paired Electrical Stimulation**

We have previously demonstrated that ryanodine prevents the usually potent positive inotropic effects of paired electrical stimulation (PES) in cat papillary muscles (Sutko et al., 1979). Since PES is thought to involve intracellular stores of Ca\(^{2+}\), these results suggested that ryanodine diminishes the availability of contractile Ca\(^{2+}\) from these stores. If ryanodine is acting on these same stores of Ca\(^{2+}\) in rat cardiac muscles, then it should alter the responses to PES by this tissue in a manner similar to that observed for cat cardiac muscle. Prior to ryanodine exposure, PES produced a small (30%) but significant increase in both DF and dF/dt responses (Fig. 8). As was found in isolated cat cardiac muscle (see Sutko et al., 1979, Fig. 9), in the presence of 1 nM ryanodine the relative potentiation produced by PES was accentuated, whereas, following exposure to 1 or 100 μM drug concentrations, the inotropic effect of PES was prevented. In addition, rat muscles mimicked the cat muscle responses in that in the presence of the higher ryanodine levels (10-100 μM) the second mechanical response, not
the first, was augmented. Similar results also were obtained with dog and rabbit preparations.

**Post-Rest Decay**

A reduction in the intracellular availability of Ca$^{2+}$ could be the consequence of an increased cellular efflux of Ca$^{2+}$. Ryanodine has been reported by others to alter Ca$^{2+}$ efflux from both guinea pig atria and canine papillary muscles (Nayler et al., 1970; Frank and Sleator, 1975). The rest-decay relationship, that is the ability of the muscle to develop force following a pause in rhythmic stimulation, may be a functional reflection of this cellular Ca$^{2+}$ efflux (Allen et al., 1976). As illustrated in Figure 9, exposure to ryanodine greatly accelerated the rest-decay of developed force in rat ventricular myocardium. Since during a rest period the entry of Ca$^{2+}$ related to the action potential is inoperative, the level of Ca$^{2+}$ remaining in the cell to be released becomes primarily a function of the rate at which Ca$^{2+}$ is leaving the cell. If ryanodine did result either directly or indirectly in a stimulation of this Ca$^{2+}$ loss, then results such as those pictured in Figure 9 would be anticipated, since following a period of quiescence less Ca$^{2+}$ would remain in the intracellular stores to be released and consequently less force would be developed by the post-rest contraction. These results are confirmatory to the findings by Hajdu and Leonard (1961) and Hajdu (1969) that ryanodine abolishes the Woodworth staircase phenomenon in rat ventricular muscle.

**Discussion**

The order of sensitivity to ryanodine's depressant effects observed for the species tested in the present study is consistent both with our working hypothesis that ryanodine acts on cardiac muscle to diminish the release of contractile Ca$^{2+}$ from intracellular (primarily sarcoplasmic reticular) stores and with previous suggestions as to the extent to which these species rely on intracellular Ca$^{2+}$ to support force development. As mentioned earlier, the species order we have observed also parallels that found by Fabiato and Fabiato (1978) for the threshold concentration of Ca$^{2+}$ necessary to initiate Ca$^{2+}$-induced Ca$^{2+}$ release. In addition, Fabiato and Fabiato (1978) have been unable to demonstrate the cyclic tension oscillations characteristic of this release in skinned frog ventricular cells, and Ciofalo (1973) has found ryanodine concentrations as high as 1 µM to be ineffective in altering the contractile response of this tissue. Furthermore, from data present in the literature, it appears that the sensitivity of mammalian atrial muscle to ryanodine approximates that of rat ventricular tissue (Hillyard and Procia, 1959; Sleator et al., 1964; Ciofalo, 1973; Frank and Selator, 1975). Fabiato and Fabiato (1978) have reported relative ease in demonstrating Ca$^{2+}$-induced Ca$^{2+}$ release in both of these tissues. Although these parallels are consistent, at present, they offer only circumstantial support for the preceding hypothesis. A more definitive interpretation of these data must await a clearer definition of the cellular basis underlying the interspecies contractile differences.

The presence of a biphasic contractile response and the ability of an elevated extracellular Ca$^{2+}$ to accentuate the positive inotropic effects of ryanodine also exhibits species differences. Two possible secondary cellular changes which could be responsible for this positive inotropic response and which are consistent with an inhibition of SR Ca$^{2+}$ release by ryanodine previously have been discussed (Sutko et al., 1979). First, as originally suggested by Frank et al. (1976), ryanodine's actions may result in an enhanced secondary inward Ca$^{2+}$ current. An augmented electrogenic Ca$^{2+}$ influx would be a logical secondary effect of a decreased intracellular Ca$^{2+}$ availability, if the decreased levels of this ion also occur at the cellular sites at which Ca$^{2+}$ ions...
act to control the K+ conductance responsible for membrane repolarization and consequently action potential duration (Brady, 1964; Bassingthwaighte et al., 1976). Second, an additional, or perhaps alternative, explanation is that a diminished SR Ca2+ release could lead to an accumulation of Ca2+ within the interior of the SR. This increased SR Ca2+ load may then alter the Ca2+ release characteristics of this organelle (Fabiato and Fabiato, 1978) and lead to an increased release, thereby reversing the effects of the initial inhibition by this drug. An increased [Ca2+]o would enhance the development of a positive response due to both of these mechanisms.

The ability of the slow current antagonist, D600 to completely block the mechanical responses of the depolarized rat muscles at a concentration that is relatively ineffective in normally polarized preparations also supports the notion that this species primarily depends on contractile Ca2+ derived from intracellular sources. Since both the magnitude of the mechanical response and the amplitude of the action potential obtained from K+ depolarized, isoproterenol-restored preparations correlate with the extracellular level of Ca2+ (Watanabe and Beach, 1974), the observation of a significant rebound phase in the presence of 2.5 mM [Ca2+]o, in depolarized, but not in normally polarized rat muscles, is consistent with the involvement of the slow inward Ca2+ current in this response. The depolarized state may also alter the characteristics of SR Ca2+ release, since recent findings suggest that SR membrane potential changes occur at the time of Ca2+-induced release (Fabiato and Fabiato, 1977). This latter possibility precludes a comparison of the increased time-to-peak force interval of the mechanical responses obtained from the K+-depolarized muscles during the rebound phase with a similar increase in this interval observed with rest state contractions (Allen et al., 1976; Beresewicz and Reuter, 1977; Reiter et al., 1978). Beresewicz and Reuter (1977) have suggested that these responses rely primarily on Ca2+ supplied by the slow inward current, although others have disagreed with this interpretation (Allen et al., 1976; Reiter et al., 1978).

The relative functional insensitivity of rabbit ventricular muscle to ryanodine could be due to ryanodine not being effective in this tissue or, alternatively, the cellular events which are affected by ryanodine may not be functionally important to the contractile state of rabbit ventricular myocardium. That the latter may be the case is suggested by the following observations. First, the contractile changes produced in rabbit muscles by ryanodine have a threshold drug concentration similar to that observed for the more sensitive species. Second, it is likely that the species tested in the present study differ in their contractile Ca2+ dependencies by a matter of degree, such that a small percentage of each species will functionally resemble another species more than its own kind. Consistent with this view, we found two of the 19 rabbit muscles tested in the cumulative addition studies to be as sensitive as the dog and cat preparations to the depressant effects of ryanodine. Third, frog ventricular tissue has been found to bind a large amount of 3H-ryanodine, but not to exhibit any alteration of contractile performance (Ciofalo, 1973). This tissue has been demonstrated to depend primarily on transsarcolemmal ion movements for both initiation and termination of contractile activity (Anderson et al., 1977; Vassort, 1978). Toad ventricular tissue, which may under certain conditions rely to a slightly greater extent on contributions of contractile Ca2+ from the SR, has been reported to respond to ryanodine with only a rapidly reversible positive inotropic effect (Ciofalo, 1973).

The similar abilities of both ryanodine and a lowered [Ca2+]o, to permit positive inotropic responses to increases in stimulation rate, hyperosmolality, and ouabain by adult rat heart muscles are likely to share a common basis, namely the diminishment of the intracellular load of contractile Ca2+. The ability of an elevated [Ca2+]o, to block the positive inotropic effects of hypertonic mannitol in cat ventricular muscle is also consistent with this interpretation. The existence of an intracellular near functional Ca2+ overload appears to partially explain the well known insensitivity of adult rat heart muscles to cardiotonic steroids. In this regard, our findings are confirmatory to those reported by Masuoka and Saunders, 1960; Forester and Mainwood, 1974b; and Gerstenblith et al., 1979. Additionally, the abolishment of the potentiating effects of paired electrical stimulation by 1 μM ryanodine in all of the tissues tested further supports an influence of this drug on intracellular Ca2+ stores (see Sutko et al., 1979 for references supporting this view).

As demonstrated by the present data, the mechanical responses obtained under a variety of conditions from cardiac muscles from several different species are consistent with our working hypothesis. As we have noted previously, a major inconsistency with this interpretation of the data is that in intact tissues a threshold ryanodine concentration of 1-5 nM is observed, whereas in isolated SR vesicles 1-10 μM ryanodine concentrations are required to produce an effect (Sutko et al., 1979). Thus, it appears that either the responses obtained from the isolated SR membranes are not representative of those of this organelle in situ or that an additional site(s) of action must exist for this drug.

In regard to the first possibility, the work of others suggests that ryanodine's effects may be both superficial (Frank and Sleator, 1975) and restricted to a specific region of the SR (Fairhurst, 1974). Thus, a specialized part of the SR, such as the peripheral coupling sites, may be more sensitive to this drug. In addition, the in situ correlates of the binding and uptake of Ca2+ measured with different fractions of isolated SR vesicles in vitro need further clarification. Furthermore, current attempts to
study SR Ca\(^{2+}\) release in vitro may not involve the appropriate models for the actual intracellular event (H.R. Besch Jr., personal communication; Fabiato and Fabiato, 1979). The performance of isolated SR membranes in vitro is labile, and thus this organelle may be altered during the isolation procedures. Perhaps a factor or condition responsible for conferring ryanodine sensitivity on this organelle is also modified during the preparative manipulations.

In considering possible additional cellular sites of action for ryanodine, we have initially taken as our starting conditions that ryanodine diminishes the functional intracellular availability of contractile Ca\(^{2+}\). Ryanodine could directly produce this decrease by attenuating the entry of Ca\(^{2+}\) into the cell; by preventing the release of Ca\(^{2+}\) from intracellular stores; by causing a redistribution of intracellular Ca\(^{2+}\) to stores not involved in supplying Ca\(^{2+}\) for force development; by decreasing the capacity of intracellular Ca\(^{2+}\) stores; and/or by increasing the efflux of Ca\(^{2+}\) from these stores.

In regard to the first possibility, we have previously argued against an inhibitory effect by ryanodine on the electronegenic entry of Ca\(^{2+}\) (Sutko et al., 1979), a position further supported by the relative species sensitivities observed in the present work. Possible effects by ryanodine on the electroneutral entry of Ca\(^{2+}\) are not supported by preliminary tests on Na\(^+-\)Ca\(^{2+}\) exchange diffusion measured in isolated canine sarcolemmal vesicles using recently reported methods (Reeves and Sutko, 1979; Reeves and Sutko, unpublished observations). The second possibility that ryanodine reduces the release of Ca\(^{2+}\) from intracellular stores has already been considered, as our current working hypothesis. In regard to the third possibility, mitochondria represent the most likely intracellular site capable of binding sufficient Ca\(^{2+}\) to significantly alter the intracellular distribution of the ion. Ryanodine has been found to be ineffective in altering Ca\(^{2+}\) uptake by this organelle (Jenden and Fairhurst, 1969; Amal Mukherjee, personal communication). The fourth possibility, the presence of a diminished intracellular Ca\(^{2+}\)-binding capacity, is not supported by our previous findings of increased Ca\(^{2+}\) accumulated by isolated cardiac muscle SR vesicles in the presence of ryanodine (Jones et al., 1979), although a complete evaluation of this possibility must await an extension of these studies to both membranes isolated from specific SR regions and to sarcolemmal membranes.

The last and perhaps most likely possibility, that ryanodine may alter the cellular efflux of contractile Ca\(^{2+}\) has been suggested by the previous findings of Nayler et al., 1970, and Frank and Sleator, 1975. To test this possibility further, we investigated the effects exerted by this drug on the rest-decay responses obtained from rat papillary muscles. Our findings that ryanodine markedly accelerates the rest-decay in this tissue, a response which should functionally reflect the rate of contractile-Ca\(^{2+}\) removal from the cell, are consistent with such an effect. These results are also in agreement with the abolition by ryanodine of both the post-rest (10-second pause) contraction in guinea pig atria (Frank and Sleator, 1975) and the Woodworth staircase (Hajdu, 1969). As discussed above, preliminary findings do not support an effect of this drug on Ca\(^{2+}\) efflux produced by Na-Ca exchange diffusion. Whether ryanodine alters the Ca\(^{2+}\) efflux occurring via the activity of a sarcolemmal Ca\(^{2+}\) ATPase remains to be established. The observed apparent increase in Ca\(^{2+}\) efflux also could be a secondary consequence of a diminished release of Ca\(^{2+}\) from intracellular stores, if the uptake of Ca\(^{2+}\) into this store is an obligatory step in both the pathway through which Ca\(^{2+}\) is removed from the cell as well as in that through which contractile Ca\(^{2+}\) is returned to the primary release sites (Bassingthwaighte and Reuter, 1972). Under these conditions, the processes involved in repriming the release sites and those responsible for Ca\(^{2+}\) removal would compete for Ca\(^{2+}\). A blockade of Ca\(^{2+}\) release would result in an accumulation of Ca\(^{2+}\) and in an increased amount of this ion being made available to efflux mechanisms.

Ryanodine may also act to indirectly alter the availability of contractile Ca\(^{2+}\) by altering the binding of this ion by elements involved in contractile events. Although, as discussed in the “Results” section, the inotropic responses obtained from adult rat cardiac muscle in the presence of ryanodine argue against such indirect effects, more direct experimental evidence is needed to further evaluate this possibility.

An additional potential complication is presented by the fact that the commercial ryanodine preparation presently available consists of two closely related compounds (Dr. R. Harmetz, personal communication). If these two compounds differ in their influence on myocardial contractility, then complex responses could result. To test this possibility, we are currently involved in the separation and characterization of these two components.

Acknowledgments

We wish to thank Drs. John P. Reeves and H.R. Besch, Jr., for helpful discussions and Dr. Gordon Templeton for the generous loan of both equipment and space. The expert secretarial assistance of Belinda Lambert and Laurie Grey is gratefully acknowledged.

References

SPECIES COMPARISON OF RYANODINE INOTROPIES/Suto and Willerson

Bassingthwaighte JB, Fry CH, McGuian JAS (1976) Relationship between internal calcium and outward current in mammalian ventricular muscle; a mechanism for the control of the action potential duration. J Physiol (Lond) 262: 15-57

Beresnewicz A, Reuter H (1977) The effects of adrenaline and theophylline on action potential and contraction of mammalian ventricular muscle and "rested-state" and "steady-state" stimulation. Naunyn Schmiedebergs Arch Pharmacol 301: 99-107


Fabiato A, Fabiato F (1977) Variations of the membrane potential of the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle detected with a potential-sensitive dye (abstr). J Gen Physiol 70: 6a

Fabiato A, Fabiato F (1978) Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rat and frog hearts and from fetal and newborn rat ventricles. Ann NY Acad Sci 307: 492-522


Naylor WG (1973) Effect of inotropic agents on canine trabecular muscle rendered highly permeable to calcium. Am J Physiol 225: 918-924


Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat, and rabbit ventricular tissues.
J L Sutko and J T Willerson

Circ Res. 1980;46:332-343
doi: 10.1161/01.RES.46.3.332

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/46/3/332.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/