

# Ryanodine Modification of Cardiac Muscle Responses to Potassium-free Solutions

## *Evidence for Inhibition of Sarcoplasmic Reticulum Calcium Release*

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**ABSTRACT** To test whether ryanodine blocks the release of calcium from the sarcoplasmic reticulum in cardiac muscle, we examined its effects on the aftercontractions and transient depolarizations or transient inward currents developed by guinea pig papillary muscles and voltage-clamped calf cardiac Purkinje fibers in potassium-free solutions. Ryanodine (0.1–1.0  $\mu\text{M}$ ) abolished or prevented aftercontractions and transient depolarizations by the papillary muscles without affecting any of the other sequelae of potassium removal. In the presence of 4.7 mM potassium and at a stimulation rate of 1 Hz, ryanodine had only a small variable effect on papillary muscle force development and action potential characteristics. In calf Purkinje fibers, ryanodine (1 nM–1  $\mu\text{M}$ ) completely blocked the aftercontractions and transient inward currents without altering the steady state current-voltage relationship. Ryanodine also abolished the twitch in potassium-free solutions, but it enhanced the tonic force during depolarizing voltage-clamp steps. This latter effect was dependent on the combination of ryanodine and potassium-free solutions. The slow inward current was not blocked by 1  $\mu\text{M}$  ryanodine, but ryanodine did appear to abolish an outward current that remained in the presence of 0.5 mM 4-aminopyridine. Our observations are consistent with the hypothesis that ryanodine, by inhibiting the release of calcium from the sarcoplasmic reticulum, prevents the oscillations in intracellular calcium that activate the transient inward currents and aftercontractions associated with calcium overload states.

### INTRODUCTION

Ryanodine is a naturally occurring plant alkaloid that has striking effects on excitation-contraction coupling in skeletal and cardiac muscle (Jenden and Fairhurst, 1969). Its effects are not easily summarized, as they depend on the muscle type, the calcium activity, and the pattern of stimulation (Katz et

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al., 1970; Bianchi, 1977; Hajdu, 1969; Sutko et al., 1979; Sutko and Willerson, 1980). Ryanodine is intriguing because nanomolar concentrations of this compound can dramatically affect the function of intact cardiac cells, which suggests that it may have a specific and functionally important site of action. To date, ryanodine has been demonstrated to affect directly only one process in cardiac muscle: calcium metabolism by the sarcoplasmic reticulum. We have found that ryanodine stimulates calcium accumulation by isolated cardiac sarcoplasmic reticular vesicles, without altering the rate of ATP hydrolysis. This implies that it either increases the efficiency of coupling between the translocation of calcium and the hydrolysis of ATP or that it decreases the efflux of calcium from the vesicle (Jones et al., 1979). Our heuristic hypothesis concerning ryanodine is based on several lines of evidence and, stated simply, is that ryanodine acts on cardiac muscle through a single mechanism, the inhibition of the release of calcium from the sarcoplasmic reticulum (Sutko et al., 1979; Sutko and Willerson, 1980).

In the present report we have tested this hypothesis by investigating the ability of ryanodine to antagonize aftercontractions and transient depolarizations in isolated guinea pig papillary muscles and aftercontractions and transient inward currents in voltage-clamped calf Purkinje fibers exposed to potassium-free solutions (cf. Eisner and Lederer, 1979). Kass et al. (1978*a*, *b*) and Kass and Tsien (1982) have suggested that the aftercontractions and transient inward currents associated with the calcium overload state produced by sodium pump inhibition result from oscillations in cytoplasmic calcium. The sarcoplasmic reticulum has become generally accepted as the source for this oscillatory increase in calcium, although this has yet to be demonstrated directly. If this is the case and if ryanodine blocks the release of calcium from the sarcoplasmic reticulum, then it should selectively inhibit both the aftercontraction and transient depolarization or transient inward current without affecting the development of the calcium overload state. This approach was suggested by the observation that ryanodine abolished aftercontractions in cat papillary muscles that were depolarized in 22 mM potassium and restored to excitability with isoproterenol (Sutko et al., 1979). Additionally, Hajdu and Leonard (1961) and Kahn et al. (1964) found that ryanodine prevented or reversed digitalis-induced arrhythmias in anesthetized dogs. These data coupled with the model of Kass et al. (1978*a*) are consistent with the mechanism of action we have proposed for this agent.

We report here evidence that ryanodine abolishes the oscillatory aftercontractions and transient depolarizations or transient inward currents, while not preventing the calcium overload state caused by potassium removal. Although these findings do not prove our hypothesis concerning the action of this compound, they are clearly consistent with it. A preliminary report of this work has appeared (Kenyon and Sutko, 1983).

#### METHODS

##### *Ventricular Muscle Experiments*

Papillary muscles were excised from the right ventricles of hearts from male Hartley guinea pigs (400–600 g) and were mounted in a tissue bath having a volume of 7 ml.

The perfusate in the bath was circulated internally by a stream of gas bubbles (cf. Blinks, 1965). Tension was recorded using a Statham (Oxnard, CA) UC2 strain gauge and a MECA (Indianapolis, IN) tension-processing amplifier. Action potentials were recorded with microelectrodes filled with 3 M KCl (8–20 M $\Omega$  resistances) suspended from a fine, coiled, chlorided silver wire (cf. Woodbury and Brady, 1956). Membrane potential measurements were made using a commercial electrometer (W-P Instruments, Inc., New Haven, CT) and were displayed on a storage oscilloscope and on a direct writing recorder. The composition of the control perfusate is listed in Table I (solution 1). The potassium-free perfusate was made by omitting the KCl from this solution. The muscles were stimulated through a fine, punctate silver wire cathode at a rate of 1 Hz and equilibrated for 1–2 h before the start of the experiment. During this time they were stretched to  $L_{max}$ ; then multiple microelectrode impalements were made and action potentials were recorded to demonstrate the uniformity of the responses by the preparation. After this, a single impalement was maintained during the change to the test solution. In the test solutions, multiple impalements were again made and action potentials were recorded.

TABLE I  
Solutions

| Solution | NaCl | KCl | MgCl <sub>2</sub> | CaCl <sub>2</sub> | H <sub>2</sub> PO <sub>4</sub> | HCO <sub>3</sub> | MOPS* | Glucose |
|----------|------|-----|-------------------|-------------------|--------------------------------|------------------|-------|---------|
|          |      |     |                   |                   | <i>mM</i>                      |                  |       |         |
| 1        | 140  | 4.7 | 1.00              | 2.5               | 1.00                           | 28               | —     | 10.0    |
| 2        | 137  | 5.4 | 1.05              | 2.7               | 0.33                           | 15               | —     | 11.1    |
| 3        | 157  | 5.4 | 1.05              | 2.7               | —                              | —                | 10    | 11.1    |

Solutions 1 and 2 were bubbled with a mixture of 95% oxygen plus 5% CO<sub>2</sub>, solution 3 was bubbled with air. The potassium-free solutions were made by omitting the KCl from solutions 1 and 2. All solutions had a pH between 7.3 and 7.4. Ryanodine (Penick Corp., Lyndhurst, NJ) was added from a 0.01-M stock solution. 4-Aminopyridine was weighed out and added to the saline without osmotic correction.

\* MOPS is 3-[*N*-morpholino]propanesulfonic acid.

#### *Calf Purkinje Fiber Experiments*

Calf hearts were obtained at a local slaughterhouse. The hearts were rinsed in cold saline (solution 3 in Table I), and Purkinje fibers were removed. The fibers were stored until use in the normal Tyrode's solution (2 in Table I) at room temperature. Fibers (<500  $\mu$ m total OD) were examined under a dissecting microscope and those that appeared to have only a single column of cells were selected for the voltage-clamp experiments. The fibers were shortened to a length of 1.3–1.5 mm by tying with silk thread. For tension measurements, the fibers were stretched to  $\sim$ 1.4 times this slack length.

The chamber and force transducer were similar to those described by Eisner and Lederer (1979). A slow drift in the output of our force transducer prevented us from reliably recording long-term changes in resting tension. This did not interfere with force measurements made during 250-ms or 4-s voltage-clamp steps and in our figures we have arbitrarily positioned the force records.

Purkinje fibers were voltage-clamped using the two-microelectrode method described by Deck et al. (1964). The voltage-clamp system was a modification of the systems used by Gibbons and Fozzard (1975) and by Kenyon and Gibbons (1979a). Membrane potential was measured as the voltage difference between an intracellular microelectrode and an agar-KCl reference electrode using a commercial electrometer

system (W-P Instruments, Inc.) and this voltage was fed to the summing point of the voltage-clamp amplifier along with the desired command voltage levels. The voltage microelectrodes were filled with 3 M KCl and typically had resistances of 10–20 M $\Omega$ ; the current-passing microelectrodes were filled with 2 M potassium citrate that had been neutralized to pH 7 with HCl and typically had resistances of 30–50 M $\Omega$ . Membrane current was measured using a virtual ground system.

An LSI-11V03 (Digital Equipment Corp., Maynard, MA) computer generated the command pulse sequences and stored the data. The system that interfaced the computer to the experiment was similar to that described by Keynes and Kimura (1976). The tension signal was filtered using an eight-pole Butterworth-type filter (Frequency Devices, Haverhill, MA) with the cutoff frequency ( $f_c$ ) set at 90 Hz. The current signal was filtered using an eight-pole Bessel-type filter (Frequency Devices). The cutoff frequency was chosen according to the experiment: 100–500 Hz for experiments designed to study the slow current changes such as the transient inward current, 1,000 Hz for the experiments designed to study the slow inward current. The filter introduced a delay of  $0.506/f_c$  s (i.e., from 5 to 0.5 ms) over this range.

In the voltage-clamp experiments, the holding voltage was set between –55 and –60 mV to inactivate the sodium current (Colatsky, 1980). More positive holding potentials were not used so as to avoid, as much as possible, inactivating the other membrane current systems. Hyperpolarizing or depolarizing pulses were given at a rate of 2/min. This rate was chosen to allow recovery of membrane currents and tension between pulses.

## RESULTS

### *Papillary Muscle Results*

**ALTERATIONS OF PAPILLARY MUSCLE RESPONSES TO POTASSIUM-FREE SOLUTIONS BY RYANODINE** Guinea pig papillary muscles respond to the removal of extracellular potassium with the following changes (Reiter et al., 1971; and Fig. 1): (a) a pronounced hyperpolarization of the resting membrane potential; (b) a decrease in the overshoot and rate of rise of the action potential; (c) a decreased action potential duration; (d) a transient increase followed by a decrease in developed force; and (e) a gradual and sustained increase in resting force. These changes can be attributed to changes in the trans-sarcolemmal distribution of sodium and potassium ions caused by an inhibition of the sodium pump (Eisner and Lederer, 1979) and a subsequent increase of the intracellular calcium ion activity caused by the activity of the sodium-calcium exchange system. As the muscle becomes calcium overloaded, two calcium-activated events, aftercontractions and transient depolarizations, can be seen (Fig. 1B; and Eisner and Lederer, 1979). These responses are thought to result from an oscillatory release of calcium from the sarcoplasmic reticulum. If ryanodine blocks the release of calcium from this organelle, one would expect it to selectively antagonize those events dependent on this process, such as the aftercontraction and transient depolarization, but not to affect the other changes that result from trans-sarcolemmal ionic alterations.

As illustrated in Fig. 1C–E, 1  $\mu$ M ryanodine selectively abolished aftercontractions and transient depolarizations, while not influencing the other responses to zero potassium. The effects by ryanodine were evident by 1 min

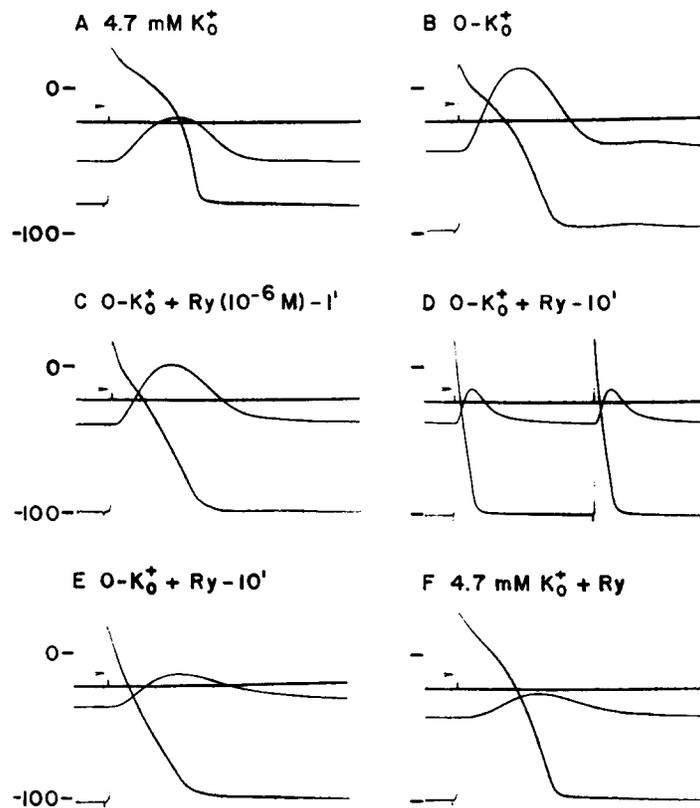


FIGURE 1. Abolition by ryanodine of the aftercontractions and transient depolarizations developed by a guinea pig papillary muscle in potassium-free solution. In each panel, the action potential, the first derivative of the membrane potential, and developed force are displayed. The maximum excursion of the derivative signal, which did not reproduce from the original photographs, is marked by a horizontal arrowhead. In each record, 0 and -100 mV are denoted by horizontal marks. (A) Control recordings in normal potassium solution. (B) Responses obtained 5 min after switching to potassium-free solution. (C-E) Responses observed 1 and 10 min after the addition of 1  $\mu$ M ryanodine to the potassium-free solution. (F) 15 min after the return to normal potassium levels in the continued presence of ryanodine. Time calibration: 200 ms in D, 50 ms in other panels. The vertical calibration bar represents 20 mV, 400 V/s, and 0.2 g for the measurements of membrane potential, the first derivative of membrane potential, and force, respectively. Preparation GPVM 901.

of drug exposure, and aftercontractions and transient depolarizations were markedly decreased by a 0.1- $\mu$ M concentration; 1.0  $\mu$ M ryanodine was routinely used to abolish these events. As previously noted by Reiter et al. (1971) and Eisner and Lederer (1979), the effects of just potassium removal

on the resting and action potentials were partially reversible, while the mechanical depression did not reverse during reperfusion with potassium-containing solution (Fig. 1*F*). The effects exerted by ryanodine were not reversible, whether or not potassium was present.

Because of the rapid onset and largely irreversible nature of the toxic effects produced by exposure of papillary muscles to potassium-free solutions, ryanodine was added as soon as possible after aftercontractions and transient depolarizations were observed. This procedure accounts for the relatively small amplitude of these events (Fig. 1*B*). In experiments where ryanodine was not added, both responses rapidly increased in magnitude and spontaneous activity was often observed (see Fig. 5).

When muscles were exposed to ryanodine before potassium removal, they responded to potassium-free solutions with all of the changes just described for this ionic alteration, with the exception of aftercontractions and transient depolarizations (Fig. 2*C-E*). The selective nature of ryanodine's effects is important because in principle, aftercontractions and transient depolarizations could be abolished independent of an effect on the release of calcium from the sarcoplasmic reticulum, if either the initial sodium loading or the subsequent calcium loading of the cell were prevented. Ryanodine does not appear to produce its effects through either of these alternative mechanisms, since, as illustrated in Fig. 3, exposure to potassium-free solutions resulted in comparable developed and resting force changes in separate control and ryanodine-treated preparations. The ability of ryanodine to prevent or abolish aftercontractions and transient depolarizations was a consistent observation in seven different experiments.

In the presence of 4.7 mM extracellular potassium and stimulation rates of 1 Hz, ryanodine did not consistently alter either action potential characteristics or depress force development (Fig. 4; and see Rumberger, 1976). Ryanodine did alter the time course of the contraction in several of these preparations by decreasing the rates of force development and relaxation and increasing the time-to-peak force interval. These changes are similar to those that have been observed with ryanodine-treated cat papillary muscles. As discussed previously, they are consistent with the proposed alteration of the release of calcium from the sarcoplasmic reticulum by this compound (Sutko et al., 1979). In some preparations, ryanodine did cause a modest increase in action potential duration and a negative inotropic effect (Fig. 2*B*). The variable effect by ryanodine on this tissue may reflect differences between preparations in intracellular calcium loading and the relative contributions made by sarcoplasmic reticular calcium release to contractile activation.

The addition of ryanodine to muscles exposed to zero potassium (but not those in 4.7 mM potassium) often resulted in an additional increase in resting force (Fig. 5). This effect could be secondary to an inhibition of calcium release by ryanodine if it resulted in an accumulation of calcium within the sarcoplasmic reticulum, which would feed back to inhibit further calcium uptake (cf. Hasselbach, 1974). Consequently, an increased fraction of calcium

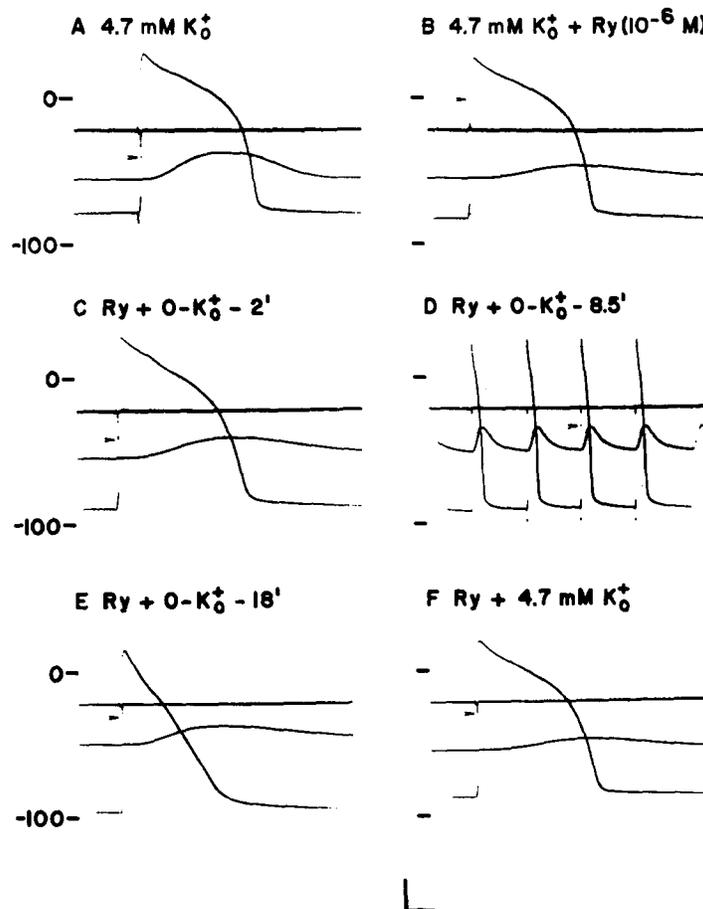


FIGURE 2. Prevention by ryanodine of the development of aftercontractions and transient depolarizations by a guinea pig papillary muscle in potassium-free solution. The records shown are presented in the same format as described for Fig. 1, except that in all panels except *B*, the derivative signal was recorded with the opposite polarity, because of the quality of the storage of the oscilloscope used in these studies. (*A*) Control responses in normal potassium solution. (*B*) Responses obtained 50 min after the addition of 1  $\mu$ M ryanodine. (*C-E*) Records obtained 2, 8.5, and 18 min after the removal of potassium. (*F*) 15 min after the return to normal potassium levels in the continued presence of ryanodine. Time calibration: 500 ms in *D*, 50 ms in other panels. The vertical calibration bar represents 20 mV, 200 V/s, and 0.4 g for the measurements of membrane potential, the first derivative of membrane potential, and force, respectively. Preparation GPVM 904.

entering the cell would be available for interaction with the contractile proteins. That this may be the case is suggested by the temporal sequence of these events. As is evident in Fig. 5, ryanodine abolished the aftercontraction before an increase in resting force became evident. This figure also illustrates

## A Control

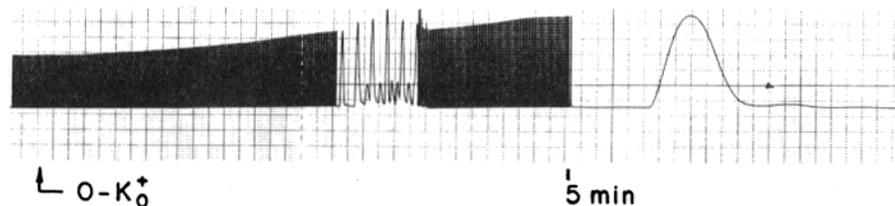
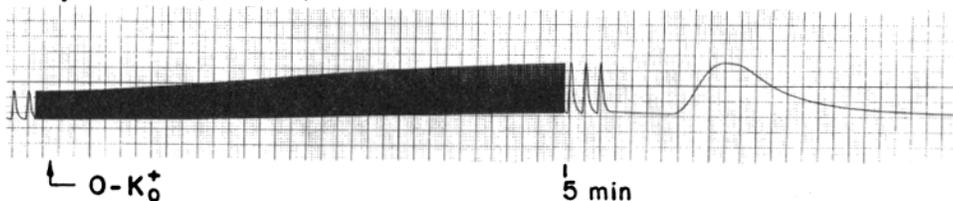
B Ryanodine ( $10^{-6}$  M)

FIGURE 3. The results of two different experiments showing the mechanical responses by guinea pig papillary muscles to potassium-free solution with (B) and without (A) prior exposure to  $1 \mu\text{M}$  ryanodine. Preparation GPVM 906.

the ability of ryanodine to rapidly inhibit the spontaneous activity associated with calcium overload states.

In conclusion, in ventricular myocardium, ryanodine selectively prevents or reverses the aftercontractions and transient depolarizations that are thought to result from a release of calcium from the sarcoplasmic reticulum, while not altering those responses arising directly from changes in the transsarcolemmal distribution of ions.

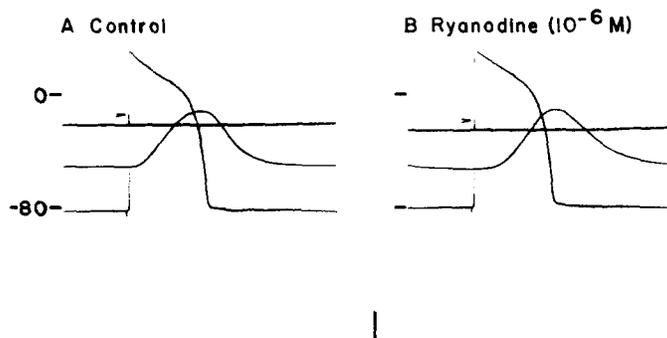
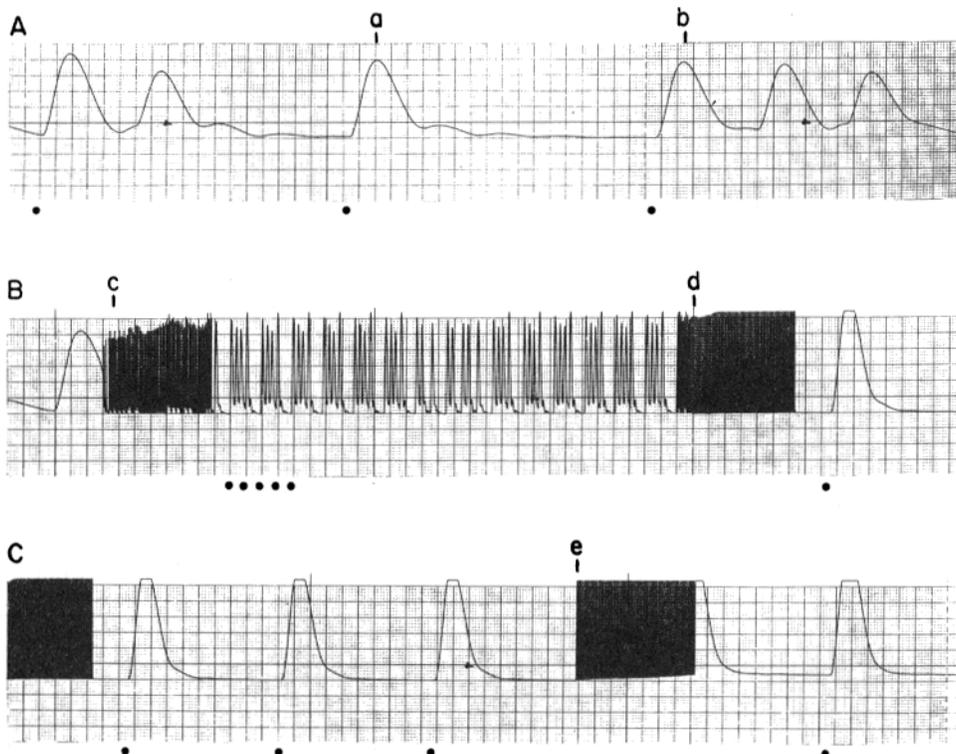


FIGURE 4. Action potentials and force development by a guinea pig papillary muscle in normal potassium-containing solution in the absence (A) and presence (B) of  $1 \mu\text{M}$  ryanodine. The horizontal calibration bar represents 50 ms; the vertical bar denotes 20 mV, 400 V/s, and 0.2 g for the measurements of membrane potential, the first derivative of membrane potential, and force, respectively. Preparation GPVM 909.

*Purkinje Fiber Results*

**RYANODINE ABOLISHES TRANSIENT INWARD CURRENT AND AFTERCONTRACTION** In preliminary concentration-response experiments (data not shown), we found that nanomolar concentrations of ryanodine reduced the twitch of cardiac Purkinje fibers. To be sure of a maximal drug effect, we



**FIGURE 5.** The abolition by  $1 \mu\text{M}$  ryanodine of the spontaneous activity observed in a guinea pig papillary muscle exposed to potassium-free solution. (A) Spontaneous contractions can be seen interposed between stimulated contractions. Points *a* and *b* indicate the normal cycle length. (B) The administration of  $1 \mu\text{M}$  ryanodine was begun at point *c*;  $\sim 2$  min later, at point *d*, a normal stimulated rhythm was restored. (C) Exposure to ryanodine has continued for an additional 2 min and at the end of this period the aftercontractions evident in *A* and *B* are absent. Note that an increase in resting force is not observed until point *e* and that the top of the force record is off scale after point *d*. The timing of some of the stimuli is indicated as circles at the bottom of each panel. Preparation GPVM 912.

routinely used a  $1\text{-}\mu\text{M}$  concentration in the Purkinje fiber experiments reported in this paper, a concentration that consistently abolished the twitch in our concentration-response experiments.

Fig. 6 shows records of membrane currents and developed forces during 4-s-long voltage-clamp depolarizations from  $-59$  to  $0$  mV in control, potas-

sium-free, and potassium-free plus ryanodine solutions. In 5.4 mM potassium Tyrode's solution (Fig. 6A), depolarization to 0 mV elicited an early outward current, after which the current declined to a minimum before settling to a steady outward current. Fig. 6A also shows the normal twitch response, which is characteristically followed by little or no maintained tonic tension. Fig. 6B shows the effects of perfusion with a potassium-free Tyrode's solution on the membrane currents and tension. In contrast to the hyperpolarization observed with the papillary muscles, an inward shift in the holding current that corresponds to a depolarization of the unclamped Purkinje fiber membrane was seen. A marked increase in twitch force and the appearance of a

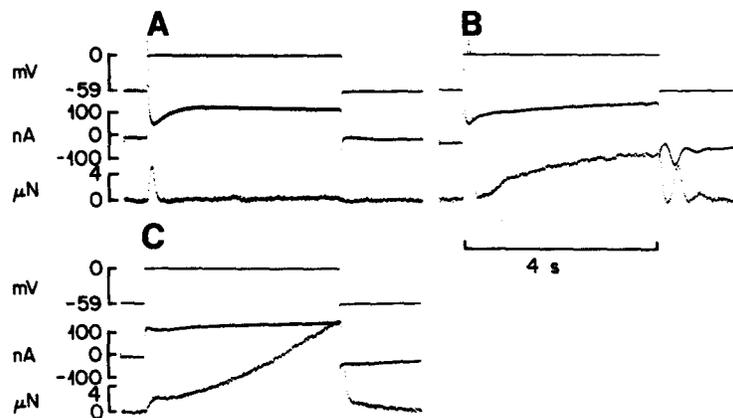


FIGURE 6. Calf Purkinje fiber voltage-clamp records obtained from a fiber bathed in normal (A), potassium-free (B), and potassium-free plus 1  $\mu$ M ryanodine (C) solutions. In each case, the membrane potential was held at  $-59$  mV and then stepped to various levels for 4 s. Only the steps to 0 mV are shown. In each panel, the upper trace is the membrane potential, the middle trace is the current, and the lower trace is developed force. In B, obtained after 7 min in potassium-free solution, the potentiated twitch is nearly off scale, peaking just above the voltage record. The record shown in C was obtained 9 min after the addition of ryanodine. Preparation 29682.

significant tonic tension during the depolarization were also observed. Repolarization elicited an oscillatory transient inward current and aftercontraction. Ryanodine (1  $\mu$ M) added to the potassium-free perfusate abolished the twitch, as well as the aftercontraction and transient inward current, but increased the tonic tension (Fig. 6C; also see Fig. 8 below). Both the "creep current" (Eisner and Lederer, 1979) observed during depolarizations and an inward "tail" current seen upon repolarization remained in the presence of ryanodine. The significance of these observations is considered in the Discussion.

The current-voltage relationship obtained from the experiment shown in Fig. 6 is presented in Fig. 7. Currents were measured at the holding potential and at the end of the 4-s-long voltage-clamp steps. As described by Eisner

and Lederer (1979), potassium removal caused the loss of the normal N shape of the current-voltage relationship. In general, ryanodine had no further influence on this relationship, but we did notice in other experiments that for strong depolarizations the steady state currents in potassium-free solutions with ryanodine were often slightly more outward than those recorded in the absence of the compound. This may have been due to the continued exposure to zero potassium (and elevated intracellular calcium) and not an effect of the ryanodine. Effects of ryanodine on the earlier time-dependent currents are discussed below.

**TONIC TENSION IN POTASSIUM-FREE SOLUTION WITH RYANODINE** As shown in Fig. 6C, the addition of 1  $\mu\text{M}$  ryanodine to potassium-free perfusate

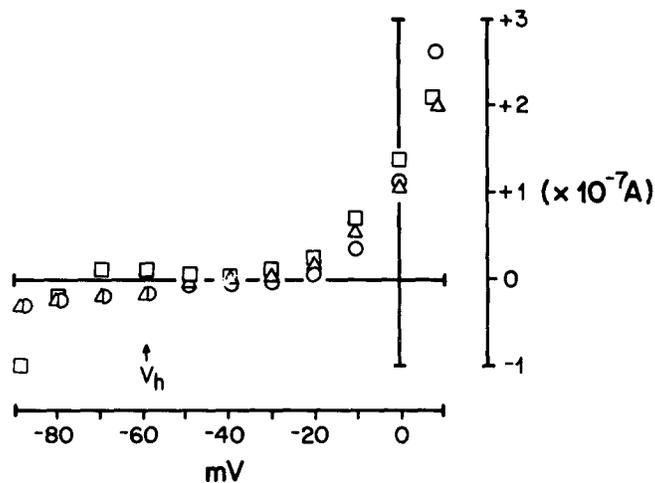


FIGURE 7. Current-voltage relations in normal (squares), potassium-free (circles), and potassium-free plus 1  $\mu\text{M}$  ryanodine (triangles) solutions. Membrane currents were measured at the end of 4-s-long voltage-clamp polarizations from a holding potential of  $-59 \text{ mV}$ . The data are from the same experiment shown in Fig. 6. Preparation 29682.

caused a marked change in the pattern of force development during voltage-clamp polarizations. The potentiated twitch disappeared and the bumpy tonic force became larger and smooth. Fig. 8 shows the voltage dependence of this tonic force during an experiment similar to that shown in Fig. 6. Membrane currents and developed force were recorded during 4-s-long voltage-clamp steps from the holding potential of  $-57 \text{ mV}$ . The data in Fig. 8 were taken after 21 min of perfusion in potassium-free Tyrode's solution with 1  $\mu\text{M}$  ryanodine. Fig. 8A shows that a 30-mV hyperpolarization caused a small but clear relaxation (note the higher tension gain in Fig. 8A). Fig. 8B shows depolarizations from the same voltage-clamp run. Stronger depolarizations caused greater tonic force development. Similar changes in force were observed under voltage clamp by changing the holding potential such that

negative holding potentials caused relaxation, while positive holding potentials caused contraction. Over the duration of the 4-s-long clamp steps used in these experiments the tonic tension was steady and showed no sign of relaxing.

The characteristic tonic forces seen in potassium-free solution with ryanodine could have been caused by ryanodine, by the continued exposure to potassium-free solution, or by the combination of the treatments. The experiment shown in Fig. 9 demonstrates that this phenomenon results from the combination of these treatments. The membrane potential was stepped from the resting potential of  $-55$  mV to various test potentials for 4-s pulse durations. Fig. 9 shows the membrane currents and developed force during

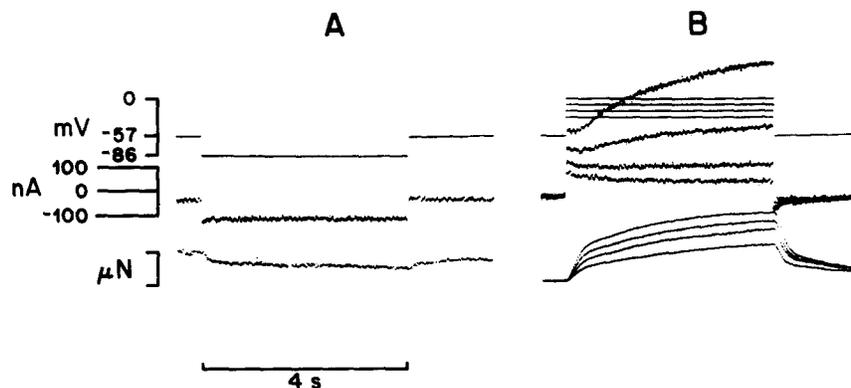


FIGURE 8. Calf Purkinje fibre voltage-clamp records obtained in potassium-free solution containing  $1 \mu$ M ryanodine. (A) A hyperpolarization from the holding potential of  $-57$  to  $-86$  mV. (B) Depolarizations to  $-27$ ,  $-17$ ,  $-8$ , and  $+1$  mV. Each voltage-clamp polarization was 4 s long. In each panel, the upper trace is membrane potential, the middle trace is current, and the lower trace is developed force. The force calibration is  $1.4 \mu$ N in A and  $4.5 \mu$ N in B. Preparation 12582.

voltage-clamp steps to  $+12$  mV. The responses shown in A were obtained 5 min after switching from  $5.4$  mM potassium Tyrode's solution to potassium-free Tyrode's solution. The enhanced twitch, bumpy tonic force, and oscillatory transient inward current and aftercontraction were well developed at that time. The responses shown in B were obtained after 1 h in the potassium-free solution. Except for a slight decline in twitch amplitude and a more pronounced aftercontraction and transient inward current, there was little change after the longer exposure to potassium-free solution. This implies that potassium removal alone cannot cause the strong, smooth tonic force described previously. Fig. 9C was obtained 20 min after the addition of  $1 \mu$ M ryanodine to the potassium-free perfusate. As in previous experiments, ryanodine abolished the twitch, the transient inward current, and aftercontraction, while increasing the tonic tension. Fig. 9D was obtained 15 min

after the return to 5.4 mM potassium Tyrode's solution (without ryanodine). The effects of the zero-potassium perfusion reversed, but those of ryanodine did not and no force was developed at any potential. In other experiments we found that the effects of ryanodine were not reversed after 1 h of washout (see also Sutko et al., 1979). Thus, the characteristic tonic force seen in potassium-free solutions containing ryanodine is a consequence of both treatments. The possible significance of this observation is discussed below.

**RYANODINE DOES NOT BLOCK THE SLOW INWARD CURRENT** A variety of slow channel blockers have been found to reduce the transient depolarizations or transient inward currents and aftercontractions caused by sodium pump inhibition (Ferrier and Moe, 1973; Kass et al., 1978a). Therefore, we ex-

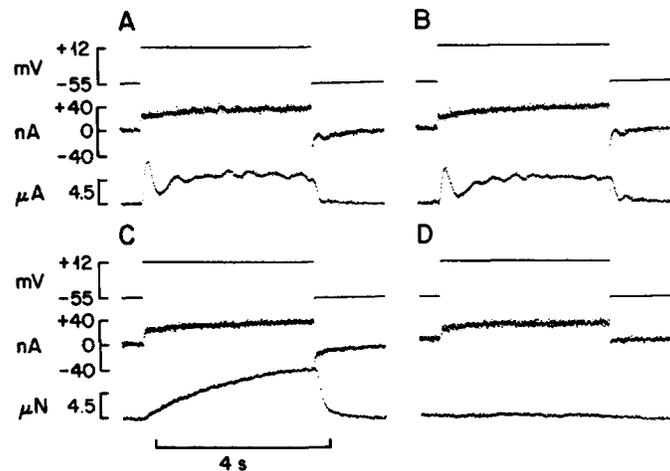


FIGURE 9. Voltage-clamp records obtained after 5 min of exposure of a calf Purkinje fiber to potassium-free solution (A), after 60 min of exposure to potassium-free solution (B), after 20 min of exposure to potassium-free solution containing 1  $\mu$ M ryanodine (C), and after 15 min in normal potassium solution (D). In each case, the membrane potential was held at  $-55$  mV and then stepped to various potentials for 4-s-long pulses. Only the steps to  $+12$  mV are shown. In each panel, the upper trace is membrane potential, the middle trace is current, and the lower trace is developed force. Preparation 20582.

amined the effects of ryanodine on the early plateau currents, including the slow inward current, to see if ryanodine might be acting as a slow channel blocker. These experiments were done in 5.4 mM potassium Tyrode's solution containing 0.5 mM 4-aminopyridine (4-AP) to reduce the outward current transient that overlaps the slow inward current (Kenyon and Gibbons, 1979b). Voltage-clamp depolarizations of 250 ms duration were used to activate membrane currents and twitches. Fig. 10 shows results from two of those experiments. The traces marked with open circles were obtained in 5.4 mM potassium Tyrode's solution, the traces marked with crosses were obtained in the presence of 0.5 mM 4-AP, and the traces marked with boxes were obtained in the presence of 0.5 mM 4-AP plus 1  $\mu$ M ryanodine. Fig.

10A shows currents and tensions resulting from voltage-clamp depolarizations from a holding potential of  $-63$  to  $+4$  mV. In the  $5.4$  mM potassium Tyrode's solution, the membrane current (after a brief capacity transient that is not well shown in this figure) was dominated by a transient outward current. 4-AP reduced the outward current and revealed an inward current. The

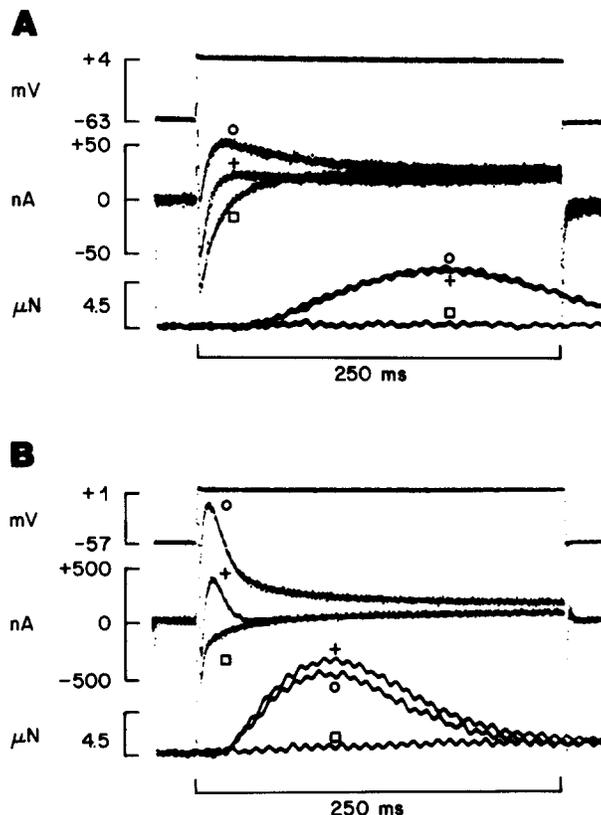


FIGURE 10. Calf Purkinje voltage-clamp records from two different experiments (*A* and *B*). In both panels, the traces marked by the circles were obtained in standard potassium-containing solution; the traces marked by the crosses were obtained after the addition of  $0.5$  mM 4-AP and the traces marked by the squares were obtained after the further addition of  $1$   $\mu$ M ryanodine. See text for full details of the protocol used. In each panel, the upper trace is membrane potential, the middle trace is current, and the lower trace is developed force. *A*: preparation 23682. *B*: preparation 14982.

addition of  $1$   $\mu$ M ryanodine, which completely abolished the twitch, did not block the inward current in these studies or in a preliminary experiment in which the fiber was loaded with cesium via the method of Marban and Tsien (1982) (Fig. 10 and unpublished observations). Further addition of  $2$   $\mu$ M D600 or  $4$  mM manganese blocked this inward current, identifying it as the slow inward calcium current (data not shown). The action of ryanodine was

apparently to increase the slow inward current or, alternatively, to reduce a component of outward current that was not always apparent as net outward current in the presence of 4-AP. The experiment shown in Fig. 10B, done in the presence of 5.4 mM CaCl<sub>2</sub> in all solutions, suggests that ryanodine does in fact reduce an outward current. In this and other experiments done in 2.7 mM CaCl<sub>2</sub>, 0.5 mM 4-AP was clearly only partially effective in reducing the outward current transient (Kenyon and Gibbons, 1979b; Coraboeuf and Carmeliet, 1982), while the addition of ryanodine appeared to complete the blockade of the outward current as it abolished the twitch. This interpretation of the effect of ryanodine is consistent with the suggestion of Siegelbaum and Tsien (1980) that a component of outward current is activated by an increase in intracellular calcium ion activity related to the activation of the twitch. These experiments do not rule out that ryanodine may have subtle effects on the magnitude or kinetics of the slow inward current, a possibility we are currently investigating. In any case, it is clear that the ability of ryanodine to abolish the twitch, aftercontraction, and transient inward current is not due to a blockade of the slow inward current.

#### DISCUSSION

Sodium pump inhibition by either cardiac glycosides or low-potassium solutions has been shown to cause an increase in intracellular sodium ion activity (Ellis, 1977) and an increase in intracellular calcium ion activity (Sheu and Fozzard, 1982). These two events are presumably linked by a membrane sodium-calcium ion-exchange mechanism. A continued sodium pump block causes the development of arrhythmogenic phenomena, the transient inward current, or transient depolarization (also known as the delayed afterdepolarization), which are coupled with aftercontractions. These events were linked to an increase in intracellular calcium activity by Kass et al. (1978a), who proposed that when cardiac cells are overloaded with calcium there are oscillations in intracellular calcium activity and that these oscillations activate both the transient inward current and the aftercontraction. They further suggested that the sarcoplasmic reticulum might be the source of these oscillations (see also Kass and Tsien, 1982), an idea that is consistent with the calcium-induced calcium release phenomena observed by Fabiato and Fabiato (1975). The central role of the sarcoplasmic reticulum in controlling the transient inward current and aftercontraction in Purkinje fibers and ventricular muscle has become widely accepted (see reviews by Ferrier [1977] and by Hoffman and Rosen [1981]), but remains to be experimentally established.

The results presented in this paper show that ryanodine prevents or abolishes the effects of sodium pump blockade that have been attributed to the sarcoplasmic reticulum. In principle, this effect could be achieved by altering any step in the chain of events proposed by Kass et al. (1978a, p. 204): our observations that exposure to potassium-free solutions produces comparable developed and resting force changes in control and ryanodine-treated papillary muscles and that ryanodine increases the tonic tension of Purkinje fibers under these ionic conditions strongly suggest that ryanodine

does not prevent cellular calcium loading and may in fact increase the availability of calcium to the myofibrils under these conditions.

We have also considered the possibility that ryanodine might prevent the cellular responses to calcium released from the sarcoplasmic reticulum, i.e., both the calcium-myofibril interaction and the calcium regulation of membrane conductance. Such a scheme is more complicated, involving multiple sites of action, and we think it is less likely. Moreover, we have shown that ryanodine-depressed muscles respond to a variety of inotropic interventions that increase intracellular calcium (Sutko and Willerson, 1980) and that ryanodine does not affect the calcium-stimulated ATPase activity of isolated rat cardiac myofibrils (Sutko, Markham, and Willerson, unpublished observations). Furthermore, ryanodine does not affect the pCa-tension curve of skinned rat cardiac myocytes (A. Fabiato, personal communication). The possibility that ryanodine might directly block the membrane channels responsible for the transient inward current cannot be eliminated at this time. We are currently examining this possibility using the isolated membrane patch-clamp technique (Hamill et al., 1981).

In view of the evidence that ryanodine does not prevent the steps preceding or following the release of calcium from the sarcoplasmic reticulum, we conclude that ryanodine can effectively block this event. As noted earlier, the source of this calcium release is thought to be the sarcoplasmic reticulum, and we believe that this is the site of ryanodine's action. At present, there is no way to directly demonstrate this point. However, in addition to the results presented in this paper, a wide variety of experiments are consistent with this view: (a) Ryanodine abolishes the normal contractile potentiation caused by paired electrical stimulation, generally accepted to depend on the increased release of calcium from the sarcoplasmic reticulum (Sutko et al., 1979; Sutko and Willerson, 1980). (b) Cardiac tissues that depend more strongly on the release of calcium from the sarcoplasmic reticulum are relatively more sensitive to ryanodine (Sutko and Willerson, 1980). (c) Ryanodine strongly inhibits the first contraction after a rest, but does not prevent the *treppé* response (Frank and Sleator, 1975; Sutko and Willerson, 1980). The magnitude of the post-rest response is thought to depend primarily on the amount of calcium released from the sarcoplasmic reticulum. (d) Ryanodine abolishes variations in the intensity and amplitude of light scattered by cardiac muscle preparations (Sutko et al., 1983). This signal is related to contractile protein interactions that occur in response to a low-level oscillatory release of calcium from the sarcoplasmic reticulum (Lakatta and Lappe, 1981). (e) Ryanodine blocks the aequorin and tension signals caused by calcium-induced calcium release in skinned cardiac myocytes (A. Fabiato, personal communication). Although none of these results prove that ryanodine blocks the release of calcium from the sarcoplasmic reticulum, they do provide a large body of evidence consistent with this view.

Since lidocaine, caffeine, and strontium ions have also been reported to reduce both transient inward currents and aftercontractions, it is pertinent to consider whether they and ryanodine might share a common mechanism

of action. Lidocaine reverses the increase in intracellular sodium as it reduces the transient inward current and aftercontraction and thus may act by indirectly reducing the cellular calcium overload (Sheu et al., 1982). As just discussed, ryanodine does not appear to prevent cellular calcium loading and therefore does not act like lidocaine. Caffeine and ryanodine may reduce transient inward currents and aftercontractions through different mechanisms. We have observed that caffeine increases and ryanodine decreases myoplasmic calcium levels in resting rat ventricular myocardium, as judged from the effects of these agents on two calcium-sensitive processes, phosphorylase *a* activation and resting force (Sutko et al., 1983). These results suggest that caffeine may deplete the sarcoplasmic reticulum of releasable calcium, whereas ryanodine causes this organelle to retain calcium. Strontium ions apparently act as a low-affinity calcium substitute and appear to preclude transient changes in the cytoplasmic availability of activator cation by slowing the kinetics of both release and uptake processes at the sarcoplasmic reticulum and of the activation of the contractile proteins (Eisner and Lederer, 1979; Kass and Tsien, 1982). In addition, strontium ions appear incapable of activating calcium-gated sarcolemmal channels (Siegelbaum and Tsien, 1980) and this may in part explain the absence of the transient inward current. It is unlikely that strontium ions and ryanodine act via the same primary mechanism, but it is possible that both may alter the time course of the release of calcium from the sarcoplasmic reticulum so as to obviate transient or oscillatory changes in this event. We simply do not sufficiently understand either the mechanism of action of ryanodine or that of the release of calcium from the sarcoplasmic reticulum to speculate further at this time.

Although ryanodine abolishes the oscillatory transient inward current and aftercontractions, it does not seem to affect either the monotonic current and tension creeps described by Eisner and Lederer (1979) or the inward tail currents. The nature of these current and tension creeps and of the current tails is not clear. These events could reflect slow changes in intracellular calcium activity caused by either a kinetically altered release of calcium from the sarcoplasmic reticulum or by a system other than that responsible for the oscillations in intracellular calcium that underlie the transient inward current and aftercontraction. For example, both of these events could reflect the activity of the sodium-calcium exchange process (see below). In this view, the monotonic current creep might represent current flow through the conductance mechanism responsible for the transient inward current in a situation where that conductance is responding to a monotonic (as opposed to an oscillatory) change in intracellular calcium activity. Consequently, the insensitivity of these monotonic changes to ryanodine is not inconsistent with the hypothesis that ryanodine abolishes the release of calcium from the sarcoplasmic reticulum.

Two additional observations of interest were made during the course of these studies. First, in the presence of potassium-free solution and ryanodine, calf Purkinje fibers develop large, sustained tonic tensions that are voltage dependent. One possible mechanism for this is the action of the electrogenic

sodium-calcium exchange diffusion system (Reeves and Sutko, 1980). This is consistent with the observation by Eisner et al. (1982) that the development of tonic tension in voltage-clamped sheep Purkinje fibers in potassium-free solutions was correlated with the increase in intracellular sodium activity. The increased tensions seen in the presence of ryanodine may be a secondary consequence of the ryanodine block of calcium release from the sarcoplasmic reticulum. For example, it is possible that in the presence of ryanodine, the sarcoplasmic reticulum loads with calcium, and in the absence of a calcium release, further calcium uptake is inhibited. This mechanism is speculative and further investigation is required.

A second observation is that in 5.4 mM potassium, ryanodine removes a portion of outward current that is insensitive to 0.5 mM 4-AP as it abolishes the twitch. This current may be the calcium-activated outward current described by Siegelbaum and Tsien (1980). The reduction of this current in the presence of an apparently undiminished slow inward current suggests that the calcium that activates the conductance comes from the sarcoplasmic reticulum. Recently, Coraboeuf and Carmeliet (1982) reached a similar conclusion from experiments in which they showed that 10 mM caffeine reduced the outward current remaining in the presence of 1.0 mM 4-AP. As in the case of the transient inward current, isolated membrane patch-clamp experiments will be necessary to test the possibility that ryanodine and caffeine might have direct effects on the membrane conductances in addition to their actions on the sarcoplasmic reticulum. These data do point to the possibility that calcium released from the sarcoplasmic reticulum can influence sarcolemmal conductances (Kenyon and Sutko, 1983) and play a role in excitation-contraction coupling.

Finally, ryanodine's ability to abolish the arrhythmogenic oscillatory changes associated with the calcium overload state make it a valuable probe of this arrhythmogenic mechanism and a prototype for a new class of antiarrhythmic agents.

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