Calcium- and Voltage-activated Plateau Currents of Cardiac Purkinje Fibers

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ABSTRACT We have used the two-microelectrode voltage-clamp technique to investigate the components of membrane current that contribute to the formation of the early part of the plateau phase of the action potential of calf cardiac Purkinje fibers. 3,4-Diaminopyridine (50 μM) reduced the net transient outward current elicited by depolarizations to potentials positive to −30 mV but had no consistent effect on contraction. We attribute this effect to the blockade of a voltage-activated transient potassium current component. Ryanodine (1 μM), an inhibitor of sarcoplasmic reticulum calcium release and intracellular calcium oscillations in Purkinje fibers (Sutko, J. L., and J. L. Kenyon. 1983. *Journal of General Physiology.* 82:385-404), had complex effects on membrane currents as it abolished phasic contractions. At early times during a depolarization (5–30 ms), ryanodine reduced the net outward current. We attribute this effect to the loss of a component of calcium-activated potassium current caused by the inhibition of sarcoplasmic reticulum calcium release and the intracellular calcium transient. At later times during a depolarization (50–200 ms), ryanodine increased the net outward current. This effect was not seen in low-sodium solutions and we could not observe a reversal potential over a voltage range of −100 to +75 mV. These data suggest that the effect of ryanodine on the late membrane current is attributable to the loss of sodium-calcium exchange current caused by the inhibition of sarcoplasmic reticulum calcium release and the intracellular calcium transient. Neither effect of ryanodine was dependent on chloride ions, which suggests that chloride ions do not carry the ryanodine-sensitive current components. Strontium (2.7 mM replacing calcium) and caffeine (10 mM), two other treatments that interfere with sarcoplasmic reticulum function, had effects in common with ryanodine. This supports the hypothesis that the effects of ryanodine may be attributed to the inhibition of sarcoplasmic reticulum calcium release.

INTRODUCTION

The membrane currents that underlie the plateau phase of the action potential of cardiac Purkinje fibers are of interest because they are related to excitation-

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contraction coupling and the genesis of certain cardiac arrhythmias. Work using the two-microelectrode voltage-clamp method has identified several current components important in this regard and given some insight into their gating and regulation. In particular, voltage-clamp depolarizations of Purkinje fibers to potentials positive to −30 mV elicit net outward currents that peak in ~10 ms and then decline to a steady outward current (cf. Deck et al., 1964; Fozzard and Hiraoka, 1973; Kenyon and Gibbons, 1979*a*; Boyett, 1981*b*; Lipsius and Gibbons, 1982; Coraboeuf and Carmeliet, 1982). This net outward current is responsible for the rapid phase 1 of repolarization that characterizes the action potential of this tissue, and, by affecting membrane potential, it will influence calcium entry into the Purkinje fiber cells via the calcium current and sodium-calcium exchange. Consequently, clarification of the nature and regulation of the current components that underlie this behavior is important to the understanding of several key aspects of cardiac function.

Dudel et al. (1967), Fozzard and Hiraoka (1973), and Hiraoka and Hiraoka (1975) reported that exposure to chloride-free solutions reduced the outward current in sheep cardiac Purkinje fibers and concluded that a large phasic chloride current was activated and then inactivated during a depolarization. However, Kenyon and Gibbons (1977) tested the effects on the action potential configuration of a number of large (presumably impermeant) chloride substitutes and found that substitutes that were derived from strong acids and did not lower the calcium activity of the solutions had little effect on phase 1 repolarization. Subsequent voltage-clamp experiments (Kenyon and Gibbons, 1979*a*) confirmed that chloride ions did not carry a major portion of the early outward current. Further experiments showed that 0.5 mM 4-aminopyridine (4-AP), a potassium channel blocker in nerve and skeletal muscle, did reduce the early outward current. It was concluded that the net phasic outward current was composed of at least two current components: one that was sensitive to 4-AP and another that was insensitive to 4-AP but was reduced by chloride replacement (Kenyon and Gibbons, 1979*b*). While 4-AP inhibited only a portion of the net transient outward current, a more complete inhibition of outward currents was reported in experiments using other potassium channel blockers. Intracellular injection of tetrabutylammonium ions (Kass et al., 1982) or loading of Purkinje fibers with cesium (Marban and Tsien, 1980) abolished all time-dependent outward currents, leaving a net membrane current dominated by the calcium current. Thus, these results suggest that there are two transient potassium current components with differing sensitivities to 4-AP. However, repeated observations of the effects of chloride replacement on membrane currents imply that a component of the transient chloride current may also exist (see also Goto and Colatsky, 1982; Colatsky and Goto, 1984).

Work done by Siegelbaum and Tsien (1980) using calf Purkinje fibers provided additional insight into the nature of the transient outward currents. While their experiments did not detect a component of current with a sensitivity to 4-AP similar to that found in sheep Purkinje fibers, they did find a component of phasic outward current activated by intracellular calcium. Siegelbaum and Tsien (1980) and others (cf. Lipsius and Gibbons, 1982) noted that this calcium-

activated current component resembled the 4-AP–resistant current component of sheep Purkinje fibers in its insensitivity to 4-AP, its kinetics, and its sensitivity to chloride replacement. Thus, it seems reasonable to suggest that the calcium-activated current component identified by Siegelbaum and Tsien might be the 4-AP–insensitive component identified by Kenyon and Gibbons (cf. Boyett, 1981*a*; Coraboeuf and Carmeliet, 1982; Lipsius and Gibbons, 1982). However, because Siegelbaum and Tsien did not observe a current component with a sensitivity to 4-AP similar to that observed in experiments done using sheep Purkinje fibers, there has been some uncertainty concerning the extent and significance of species differences. In summary, past work establishes the existence of two components of phasic outward current in sheep Purkinje fibers. In addition, while there has been some question about the existence of an aminopyridine-sensitive current component in calf Purkinje fibers, it seems likely that sheep and calf Purkinje fibers share a component of calcium-activated current.

Recent experiments with ryanodine indicate that this compound is a potent inhibitor of calcium release from cardiac sarcoplasmic reticulum (cf. Sutko et al., 1985), and we have presented evidence that it effectively abolishes intracellular calcium oscillations, as indicated by the abolition of force and calcium-activated current oscillations (Kenyon and Sutko, 1983; Sutko and Kenyon, 1983; Ito et al., 1984; Kenyon et al., 1985). These data and their interpretation have been confirmed by direct observation of the effects of ryanodine on intracellular calcium measured with aequorin (for example, Allen et al., 1984; Marban and Wier, 1985). An important finding that comes from this work is that the sarcoplasmic reticulum is the major supplier of calcium to calcium-activated currents, contraction, and aequorin luminescence in cardiac Purkinje fibers. This is in contrast to the calcium current, which is apparently ineffective in supplying calcium to activate these phenomena (Kenyon and Sutko, 1983).

The experiments described in this article continue the pharmacological dissection and identification of the major currents of the cardiac Purkinje fiber plateau. In particular, we have tried to identify those current components that are dependent on intracellular calcium and establish the relationship between these currents and the release of calcium from the sarcoplasmic reticulum. Such a relationship is of particular interest because it will form a feedback loop linking membrane currents and excitation with calcium release and contraction. Portions of this work have appeared in abstract form (Kenyon and Sutko, 1985, 1986).

MATERIALS AND METHODS

Our implementation of the two-microelectrode method for voltage-clamping cardiac Purkinje fibers (Deck et al., 1964) has been described in detail elsewhere (Kenyon and Gibbons, 1979*a*; Sutko and Kenyon, 1983). In most experiments, the voltage-recording microelectrodes were filled with 3 M potassium chloride and the current-passing microelectrodes were filled with 1.5 M potassium citrate plus 0.66 M potassium chloride (pH adjusted to 7). The ability of individual current electrodes to pass hundreds of nanoamperes of current was routinely tested before impalement and microelectrodes that initially had resistances of 20–35 MΩ usually blocked and failed to pass current. These electrodes were then "beveled" by gently brushing their tips with a finger, reducing their resistances to 10–20 MΩ. In experiments where cesium chloride was used in the superfusate, both

intracellular microelectrodes were filled with a mixture of 2.5 M cesium acetate plus 2 M cesium chloride. When chloride was not changed, the reference electrode was a broken-tipped pipette filled with agar/potassium chloride. In experiments where chloride was replaced, we used a flowing potassium chloride/calomel reference electrode (model 13-639-56, Fisher Scientific Co., Pittsburgh, PA) (cf. Kenyon and Gibbons, 1977). The standard superfusate contained (in mM): 137 NaCl, 5.4 KCl, 1.05 $MgCl_2$, 2.7 $CaCl_2$, 15 $NaHCO_3$, 0.33 NaH_2PO_4, 11.1 glucose. Reagent-grade chemicals (J. T. Baker, Phillipsburg, PA) and glass-redistilled water were used. Modifications to this solution were made by replacing various salts. Sodium chloride substitutes were choline chloride (Mallinckrodt, Paris, KY), lithium chloride (Mallinckrodt), cesium chloride (Sigma Chemical Co., St. Louis, MO), or sodium isethionate (Sigma Chemical Co.); potassium chloride substitutes were cesium chloride, or potassium methanesulfonate made by neutralizing methanesulfonic acid (Aldrich Chemical Co., Milwaukee, WI) with potassium hydroxide. Calcium chloride was substituted with strontium chloride. 4-AP (Sigma Chemical Co.) or 3,4-diaminopyridine (3,4-DAP) (Aldrich Chemical Co.) was dissolved into the superfusate on the day of the experiment. Ryanodine (lot 704-RWP-2, Penick Corp., Lyndhurst, NJ) was added from a 0.01 M aqueous stock solution to the superfusate. The solutions were saturated with a mixture of 95% oxygen plus 5% carbon dioxide, warmed to between 36.5 and 37°C, and had a pH between 7.25 and 7.3.

We tested the effects of isethionate and methanesulfonate on calcium activity using ion-sensitive electrodes (90-02, Orion Research, Inc., Cambridge, MA) (Kenyon and Gibbons, 1977) and murexide absorbance measurements (Dani et al., 1983) in solutions containing various concentrations of calcium chloride, 10 mM HEPES buffer (pH 7.4), and 150 mM of either sodium chloride, sodium isethionate, or sodium methanesulfonate. According to the electrode measurements, both chloride substitutes apparently reduced the calcium activity by ~10%, in agreement with previously reported results (Kenyon and Gibbons, 1977; Colatsky and Goto, 1984; Dani et al., 1983). Most of the measurements made using murexide showed no significant effect of chloride replacement on calcium activity, in agreement with the data of Dani et al. (1983). However, one series of measurements (lot 101F-0601 sodium isethionate) gave anomalous results. In measurements made over a period of 0.5–4 h, the apparent calcium activity fell while the isosbestic point of the dye shifted to longer wavelengths. When stored overnight, these murexide-isethionate solutions lost their color. These effects appear to be due to a reaction between a contaminant in this particular lot of sodium isethionate, since samples from two other lots did not show this effect. Thus, we conclude that neither isethionate nor methanesulfonate lowers calcium activity appreciably.

The voltage-clamp protocols were generated and the data were collected using an LSI-11V03 computer system (Digital Equipment Corp., Maynard, MA) and a direct memory access interface system (Concept 2000, Burlington, VT). The tension signal was filtered using an eight-pole Butterworth filter (Frequency Devices, Haverhill, MA) with the cutoff frequency set at 80 or 90 Hz. The current signal was filtered using an eight-pole Bessel filter with the cutoff frequency set at 1,000 Hz. The figures were prepared by plotting the digitized data with a digital plotter (7470, Hewlett-Packard Co., Palo Alto, CA). The algorithm used to generate the plots connected the data points with straight lines unless a given data point differed by >5% from the preceding point, in which case the point was plotted as a single dot. This method was chosen to de-emphasize the very large and rapid (<5 ms) capacity transients. Essentially all of the data points other than the capacity spikes are plotted as continuous lines. In some cases, the current records have been digitally filtered using a least-squares filter (Hamming, 1983).

In the typical voltage-clamp protocol, the holding potential was set to between −55 and −60 mV and depolarizing steps were delivered at a rate of 2/min. This protocol was

chosen to reduce the sodium current without inactivating the contraction or other currents of interest and to permit their recovery between pulses.

The interpretation of voltage-clamp data obtained from multicellular preparations is limited by possible electrical and chemical nonuniformities caused by the use of a point source of current injection and the presence of intercellular clefts (Johnson and Lieberman, 1971; Kass et al., 1979; Levis et al., 1983). The most recent theoretical and experimental analyses of these problems make it clear that the techniques used in our experiments, including the avoidance of large sodium currents, should result in accurate and rapid (i.e., within 5–8 ms) control of membrane potential and faithful recording of membrane currents and developed force. It is also clear that the accumulation of ions in and the depletion of ions from the intercellular clefts may compromise both the interpretation of current measurements in terms of membrane conductances and the determination of reversal potentials (cf. Levis et al., 1983), although Kass and Sanguinetti (1984) and Bennett et al. (1985, 1986) have reported that under some circumstances these effects are minimal. In interpreting our data, we assume that we had control of membrane potential and have recorded accurate representations of the membrane current and developed force responses to our command steps. We have limited our interpretations to qualitative assessments of what kinds of currents are seen in our records and what factors appear to regulate them.

RESULTS

Effects of Aminopyridines on Membrane Currents and Contractions

Because Siegelbaum and Tsien (1980) reported that low concentrations of 4-AP did not reduce the net outward current in calf Purkinje fibers, while marked effects have been reported in other cardiac preparations, we re-examined the effects of low concentrations of 4-AP and 3,4-DAP on membrane currents and contraction in calf Purkinje fibers. In preliminary experiments, 0.5 mM 4-AP consistently reduced the early outward current in calf Purkinje fibers in a manner similar to that reported for sheep Purkinje fibers (Kenyon and Gibbons, 1979*b*; Boyett, 1981*b*; Coraboeuf and Carmeliet, 1982; Lipsius and Gibbons, 1982). 3,4-DAP, a closely related compound and a more potent blocker of potassium currents in the squid giant axon than 4-AP (Kirsch and Narahashi, 1978), had effects on Purkinje fiber currents that were similar to those of 4-AP (see below) and was significantly more potent. While we have not done an extensive dose-response study using this compound, it has marked effects at concentrations as low as 10 μM and, in one experiment, increasing the concentration from 50 to 100 μM did not further reduce outward currents. These data suggest that 3,4-DAP is at least 10 times more potent than 4-AP. In the experiments described in this article, we have used 50 μM 3,4-DAP as our standard dose and we refer to the affected current component as the aminopyridine-sensitive current to denote its sensitivity to both 4-AP and 3,4-DAP.

The effects of 50 μM 3,4-DAP on net membrane currents and contraction of a calf cardiac Purkinje fiber are shown in Fig. 1. The responses to increasing depolarizations are illustrated in panels *A–D* with control currents marked by arrows. In *A* and *B*, the control current responses immediately after the settling of the step depolarization are net inward currents indicated by brief downward deflections of the current traces. In *C* and *D*, the control currents are net outward (upward deflections) for the entire depolarization. At all potentials shown, the

control currents rise to a peak of outward current between 10 and 20 ms and then decline, remaining net outward, over the course of the 250-ms depolarization. Exposure to 3,4-DAP made the net current less outward (or more inward) at all potentials and over the duration of the voltage-clamp steps shown. Similar results were obtained in every Purkinje fiber tested in normal superfusate (n = 26) as well as in fibers exposed to 3,4-DAP in the course of other experimental protocols. These results clearly demonstrate a significant aminopyridine-sensitive transient outward current component in calf Purkinje fibers.

The aminopyridine-sensitive current component was analyzed in more detail by subtracting the currents remaining in 3,4-DAP from those recorded before exposure to the compound for identical depolarizations. Such difference currents

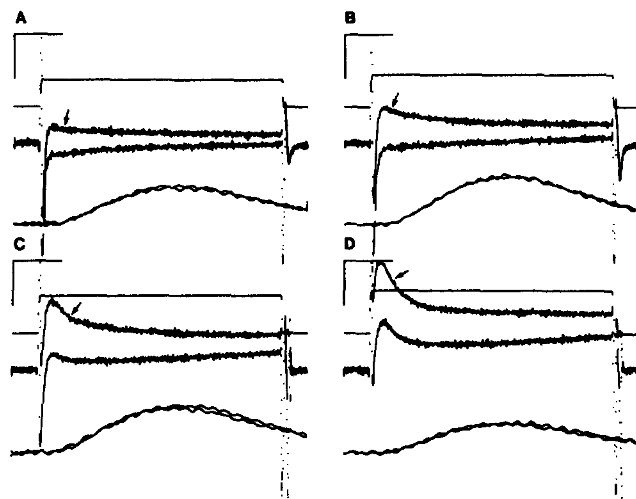

FIGURE 1. Effect of 50 μM 3,4-DAP on membrane currents and contraction. In each panel, the upper trace is the voltage step recorded in the control solution, while the middle traces are the membrane currents before (arrows) and during exposure to 50 μM 3,4-DAP, and the lower traces are developed force before and during exposure to 3,4-DAP. The holding potential was −56 mV. The test potentials were −5 (*A*), +5 (*B*), +15 (*C*), and +25 (*D*) mV. The calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 13885.

for the records shown in Fig. 1 are plotted in Fig. 2. 3,4-DAP, like 4-AP (Kenyon and Gibbons, 1979*b*), had no effect on either the holding current in these experiments or on the resting potentials of fibers studied under current-clamp conditions (data not shown). The aminopyridine-sensitive component was activated by depolarizations to potentials positive to about −30 mV, peaked within 5 ms (the limit of the resolution of the voltage-clamp technique), and then declined over the remainder of the voltage step, with a portion of the sensitive current remaining at the end of 250-ms depolarizations. The decline of the current followed a complex time course that varied from fiber to fiber; this decline was analyzed in seven experiments (including that shown in Figs. 1 and 2) using a least-squares analysis routine to fit the data with one or two exponentials (see Table I). When a single time constant was used, all of the data were well

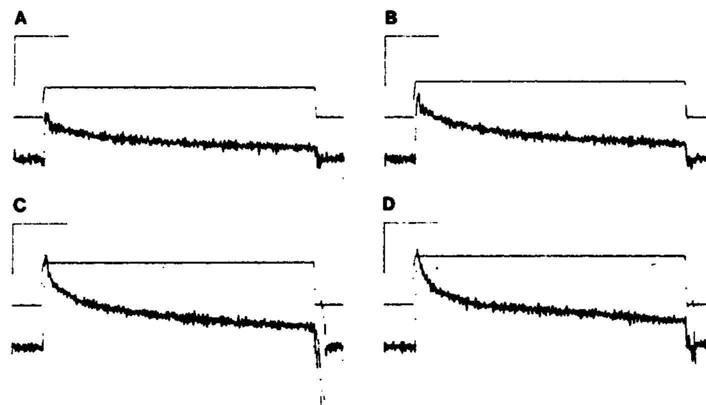

FIGURE 2. The 3,4-DAP–sensitive current components obtained by subtracting the currents recorded in the presence of 50 μM 3,4-DAP from those recorded before exposure to this agent. In each panel, the upper traces are the voltage steps and the lower traces are the difference currents. These data are from the experiment shown in Fig. 1 and the panels in this figure correspond to those in that figure.

described by time constants between 15 and 100 ms, with no consistent dependence on pulse potential. When two time constants were used, the fits were slightly better and the data were described by two time constants, with one in the 15–100-ms range and the other being either much faster (<10 ms) or much slower (>100 ms). Neither of these latter processes could be resolved with confidence by the protocol that we used, and their significance is unclear.

3,4-DAP had small variable effects on the force of contraction. Of the 26 fibers tested, 24 had contractions strong enough to be measured; contraction was unchanged in 8 experiments (e.g., Fig. 1), slightly increased in 2, and decreased in 14. To some extent, the decline in force development seen in these experiments can be attributed to the deterioration of the fibers. This possibility is supported by the number of experiments that showed increases or no change in force development. In any case, while we cannot rule out effects of 3,4-DAP on intracellular calcium metabolism or excitation-contraction coupling, it is clear that the reduction of outward current by 3,4-DAP is not consistently correlated with an inhibition of contraction. This implies that 3,4-DAP does not abolish a

TABLE I

Time Constants for the Decline of the Aminopyridine-sensitive Current from the Experiment Shown in Figs. 1 and 2

Pulse	τ	τ_1	τ_2
mV	*ms*	*ms*	*ms*
−26	48	current too small	
−15	39	3.1	62
−5	61	1.1	74
+5	60	8.9	92
+15	62	6.3	129
+25	44	7.5	132

See text for details of the calculations.

calcium-activated current component by removing the activator calcium, and it further implies that the effect on the net current cannot be attributed to an increased inward calcium current, which would be expected to have a positive inotropic effect. Thus, the effects of 3,4-DAP on the net current can be attributed to the specific reduction of a component of current that is outward at potentials positive to −30 mV and is probably a potassium current activated by depolarization and not by intracellular calcium (see Discussion).

Effects of Chloride Reduction on Membrane Currents and Contraction

In experiments using sheep Purkinje fibers, Kenyon and Gibbons (1979*b*) found that the net transient outward current that remained in the presence of 4-AP was reduced when the chloride in the bathing solution was lowered. This chloride sensitivity was similar to that of the calcium-activated transient outward current described by Siegelbaum and Tsien (1980), which raised the possibility that Purkinje fibers might have a calcium-activated chloride current similar to those described in oocytes (Miledi, 1982; Barish, 1983) and cultured neurons (Owen et al., 1984; Mayer, 1985). However, this interpretation is clouded by the observation by Siegelbaum and Tsien that chloride reduction also inhibited contraction, which suggests that the loss of current might be caused by the loss of activator calcium rather than by the loss of current-carrying ions. We have re-examined this question using isethionate and methanesulfonate as chloride substitutes. We chose sodium isethionate because it is readily available in high purity, it is derived from a strong acid, it does not lower calcium activity, and it has been used to demonstrate chloride-dependent currents in Purkinje fibers (Siegelbaum and Tsien, 1980; Goto and Colatsky, 1982; Colatsky and Goto, 1984). Specifically, we examined the effects of replacing sodium chloride and potassium chloride with sodium isethionate and potassium methanesulfonate on membrane currents and contraction in voltage-clamped cardiac Purkinje fibers. This protocol reduced the chloride concentration of the superfusate by 95%, from 149.9 to 7.5 mM. Fig. 3 shows typical results obtained from fibers tested in the absence (two fibers) or presence (eight fibers) of 50 μM 3,4-DAP in control (arrows) and low-chloride solutions. The data in panels *A* and *B* were obtained in the absence and presence of 3,4-DAP, respectively.

Independently of the presence of 3,4-DAP, the reduction of chloride caused a decrease in the net outward current during depolarizations to potentials positive to −30 mV in 9 of the 10 fibers tested (including both of the fibers tested in the absence of 3,4-DAP). The magnitude of this chloride-sensitive current increased with stronger depolarizations, and in eight fibers it was larger during the earlier part of the voltage steps and then declined, such that at the end of the 250-ms steps, chloride replacement had no effect on membrane current (Fig. 3). In two of the sensitive fibers, the chloride-sensitive current showed very little time dependence; i.e., the current was less outward by a relatively constant amount for the duration of the step. These effects of chloride reduction are similar to those reported by Kenyon and Gibbons (1979*a*) for sheep fibers and by Siegelbaum and Tsien (1980) for calf Purkinje fibers.

Chloride reduction consistently increased the force of contraction in those experiments where it reduced the outward current (i.e., 9 of 10 fibers tested).

In all but one of these fibers, the effect was quite large and ranged from nearly a twofold to a greater-than-threefold increase in the amplitude of the contraction. This positive inotropic effect was voltage dependent; it was small or nonexistent at potentials near −30 or −20 mV (i.e., near the threshold for detectable contractile activation) and greatest for the strongest depolarizations. These observations are dramatically different from those of Siegelbaum and Tsien (1980), who reported reductions of the contraction during low-chloride superfusion.

In the fibers exposed to low-chloride solutions containing 3,4-DAP, the time course of the net current resembled that shown in Fig. 3*B*. In particular, the net

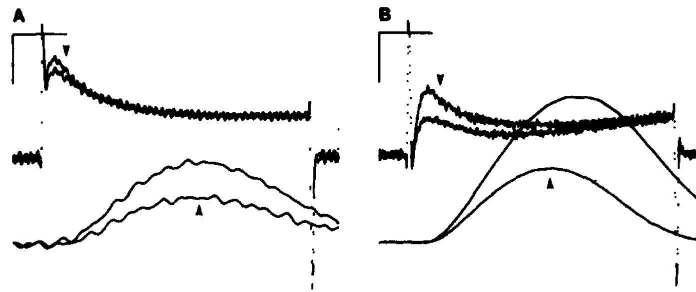

FIGURE 3. The effect of chloride reduction on membrane currents and contraction recorded in the absence (*A*) or presence (*B*) of 50 μM 3,4-DAP. In each panel, the upper traces are the membrane currents in control (arrows) and during exposure to low-chloride solutions, and the lower traces are developed force in control and test solutions. The membrane currents and contractions were recorded first in the control solution and again using identical voltage-clamp depolarizations in the presence of low chloride. In *A*, control solution was the standard superfusate, the holding voltage was −57 mV, the step was to +14 mV, and the calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 30885. In *B*, the control solution was the standard superfusate plus 50 μM 3,4-DAP, the test solution was low-chloride solution plus 50 μM 3,4-DAP, the holding potential was −53 mV, the step was to +17 mV, and the calibration bars are 50 ms, 200 nA, and 60 μN. Experiment 19985.

current traces did not look like those expected if the records were dominated by the calcium current, which suggests that a component of transient outward current remained under this condition. One possibility suggested by these data is that the concentration of 3,4-DAP was not high enough and the chloride replacement not complete enough to fully remove these two components of current. An alternative possibility is that there is at least one component of phasic outward current that is insensitive to both the aminopyridine and chloride reduction. The subsequent results obtained with ryanodine suggest that the latter interpretation is correct (see below).

Effects of Ryanodine on Membrane Currents and Contraction

Ryanodine inhibits the release of calcium from the sarcoplasmic reticulum of cardiac cells (Sutko et al., 1979, 1985), and we have reported preliminary observations of its effects on membrane currents and contractions in calf Purkinje

fibers (Kenyon and Sutko, 1983; Sutko and Kenyon, 1983). Those experiments revealed that ryanodine abolished phasic force development and reduced the early outward current in the presence of 0.5 mM 4-AP, and suggested that ryanodine is useful in the study of the influence of calcium release by the sarcoplasmic reticulum on membrane currents. We extended these observations by examining whether the current components sensitive to ryanodine contributed to the configuration of the action potential as well as the effects of ryanodine on membrane currents over a wide range of potentials in the absence and in the presence of 3,4-DAP.

Representative results of the effects of increasing concentrations of ryanodine on action potentials and contractions in unshortened (>3 mm long) Purkinje fibers are shown in Fig. 4. In four experiments, action potentials were evoked

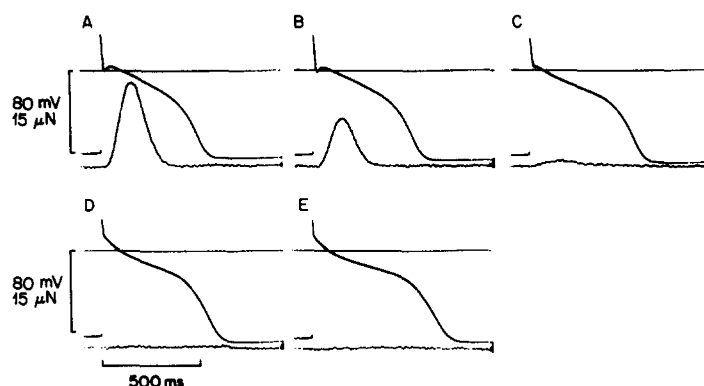

FIGURE 4. The effect of ryanodine on the action potential configuration and contraction. Action potentials were stimulated at 0.5 Hz and typical action potentials and twitches were recorded approximately every 30 min as the ryanodine concentration was increased. In each panel, the action potential is shown as the upper trace, with a horizontal line drawn at 0 mV, and the contraction is shown as the lower trace. See text for further details.

by stimulation through intracellular microelectrodes at a rate of 0.5 Hz and the fibers were exposed to increasing concentrations of ryanodine. While the effects of ryanodine became apparent within minutes of exposure to the compound, long exposure times were used to be sure that the effects at each concentration had fully developed. Panel *A* was recorded in normal superfusate (control), while panels *B*, *C*, and *D* were recorded after 35 min in 1 nM ryanodine, 1 h in 10 mM ryanodine, and 20 min in 1 μM ryanodine, respectively. These concentrations of ryanodine prolonged the time course of repolarization (particularly phase 1) as they abolished contraction. Panel *E* was recorded after a 50-min superfusion with ryanodine-free solution and shows that the effects of ryanodine are poorly reversible in this tissue as in other cardiac preparations (cf. Sutko et al., 1979; Sutko and Kenyon, 1983). Ryanodine had no significant effects on the resting potential or action potential amplitudes. These data indicate that ryanodine is a very potent negative inotropic agent on Purkinje fibers that also modifies

a component or components of membrane current important in the formation of the plateau phase of the action potential. Similar results were obtained by Marban and Wier (1985) using canine cardiac Purkinje fibers.

We have examined the effects of ryanodine in voltage-clamp experiments in the absence (eight fibers) and presence (six fibers) of 3,4-DAP, and in low-chloride solutions containing 3,4-DAP (six fibers). Figs. 5 and 6 illustrate the effects on membrane currents and contraction of 1 μM ryanodine in chloride-containing superfusates in the absence (Fig. 5) or presence (Fig. 6) of 3,4-DAP.

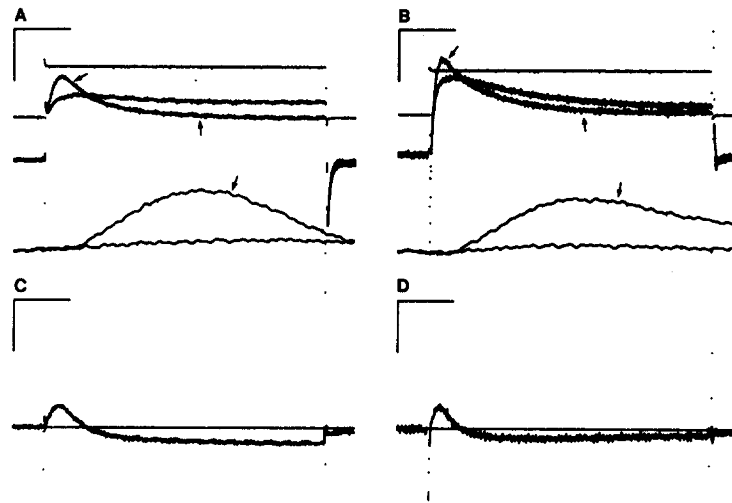

FIGURE 5. The effect of ryanodine on membrane currents and contraction in two experiments (*A* and *B*) and the difference currents obtained by subtracting the currents recorded in the presence of ryanodine from those recorded before exposure to the compound (*C* and *D*). The upper traces in *A* and *B* are the voltage steps recorded in the control solutions, the middle traces are membrane currents in standard superfusate (arrows) and during exposure to 1 μM ryanodine, and the lower traces are developed force in control (arrows) and during exposure to 1 μM ryanodine for identical depolarizations. Panel *C* is the difference current obtained from *A*, and *D* is the difference current obtained from *B*; the continuous line in these panels is zero current. In *A*, the holding potential was −66 mV and the step potential was +13 mV. Experiment 29684. In *B*, the holding potential was −55 mV and the step potential was +13 mV. Experiment 03784. The calibration bars are 50 ms, 200 nA, and 19 μN (*A* and *B* only).

Responses obtained before (arrows) and during exposure to 1 μM ryanodine are shown. At potentials positive to −30 mV, exposure to ryanodine reduced the net outward current at early times (between 5 and 20 ms), while it increased the net outward current at later times (between 20 and 250 ms). Thus, the currents recorded before and during exposure to ryanodine cross over. The difference currents obtained by subtracting the currents recorded in the presence of ryanodine from those recorded before exposure to the drug (for example, Fig. 5, *C* and *D*) showed a characteristic early positive phase followed by a later

negative phase. This behavior was seen in 6 of 8 experiments done in standard superfusate and in all 12 of the other experiments. In the two experiments where the cross-over was not seen, the early net outward current was reduced but the late currents were not affected by ryanodine. Exposure to ryanodine abolished the phasic contraction (twitch) at all potentials in all experiments. However, treatment with ryanodine did not inhibit all force development, as indicated by

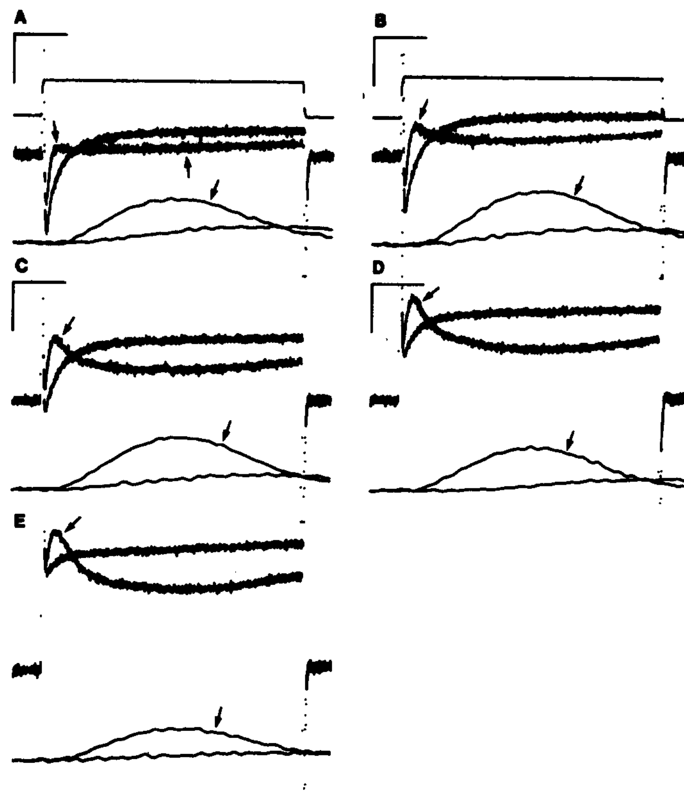

FIGURE 6. The effect of ryanodine on membrane currents and contraction in the presence of 50 μM 3,4-DAP. In *A* and *B*, the upper traces are the voltage steps recorded in the presence of 3,4-DAP. The middle traces in *A* and *B* and the upper traces in *C–E* are membrane currents in 3,4-DAP (arrows) and during exposure to 3,4-DAP plus 1 μM ryanodine, and the lower traces are developed force in 3,4-DAP (arrows) and during exposure to 3,4-DAP plus ryanodine for identical depolarizations. The holding potential was −57 mV and the step potentials were +4 (*A*), +14 (*B*), +24 (*C*), +34 (*D*), and +44 (*E*) mV. The calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 061185.

the small tonic forces elicited by step depolarizations in some experiments (e.g., Fig. 6, *B–D*). This force development was unlike phasic contraction in that it did not relax during the step depolarization.

Fig. 6 shows the effects of ryanodine on membrane currents and contraction during depolarizations to potentials ranging from +4 to +44 mV. The addition of 3,4-DAP to the superfusates reduced the outward currents, thus facilitating

voltage-clamping to very positive potentials. The data obtained in the presence of 3,4-DAP were similar to those obtained in its absence (compare Figs. 5 and 6 and see Fig. 7). In particular, exposure to ryanodine caused the early net current to be less outward, while the late net current was more outward. However, in the presence of 3,4-DAP, the addition of ryanodine removed all obvious components of phasic outward current, and the net currents quantitatively resembled the inward currents described by Marban and Tsien (1982) and Kass and Sanguinetti (1984) and were blocked by manganese and cadmium (data not shown), which suggests that under this condition, time-dependent outward currents are effectively inhibited and the net current is dominated by the calcium current (see Discussion). The aminopyridine-sensitive difference currents obtained in the presence or absence of ryanodine were similar (data not shown). In addition, the ryanodine-sensitive currents obtained in the presence of 3,4-DAP resembled the difference currents shown in Fig. 5 (without 3,4-DAP). These observations suggest that these current components are independent. We also point out that in those cases in which tonic tension could be seen during exposure to ryanodine plus 3,4-DAP, it was correlated with the magnitude of the inward current, i.e., for depolarizing voltage-clamp steps of increasing magnitude, the amplitudes of the inward current and the force development increased (−40 to 0 mV) and then decreased (positive to 0 mV) together (e.g., Fig. 6). This suggests that the calcium current is the major source of activator calcium for this tonic force, although a contribution from the sodium-calcium exchanger is possible. Such a contribution has been implicated in the activation of tonic force under the condition of increased intracellular sodium (Sutko and Kenyon, 1983).

Thus, ryanodine has three distinct effects on calf Purkinje fibers: the abolition of the twitch, the reduction of the early net outward current, and the increase of the late net outward current. To investigate possible relationships between these effects, we have compared their dependences on pulse potential. Qualitatively, at potentials negative to −40 mV (i.e., negative to the threshold for detectable contraction), ryanodine had no clear effect on membrane currents, while at potentials positive to −20 mV (positive to the contraction threshold), ryanodine treatment consistently decreased the net early outward current and increased the net late outward current as it abolished the twitch. Over an intermediate potential range (i.e., −20 to −40 mV, near contraction threshold), there was considerable variability in the effects of ryanodine. In particular, in some experiments, there were effects on currents at potentials just negative to the threshold for detectable contraction, and we also saw reductions of the early net outward current without increases in the net late outward current and vice versa.

Fig. 7 illustrates the voltage dependences of the effects of ryanodine quantitatively by plotting the amplitude of the ryanodine-sensitive contraction, the maximum decrease in early net outward current, and the maximum increase in the net late outward current caused by ryanodine as functions of step potential for two experiments. The data were normalized relative to the maximum values attained during the experiment. Panels A and *B* show data obtained in the absence and presence of 3,4-DAP, respectively, and both plots are typical of the 18 experiments that we analyzed in this manner. All three variables are triggered

by depolarizations to potentials positive to about −40 mV and increase fairly steeply with depolarizations up through 0 mV. In general, the contraction (circles) and the amplitude of the reduction of the early net outward current (squares) increased over a similar voltage range. This behavior is similar to that reported by Siegelbaum and Tsien (1980, their Fig. 7). For depolarizations to potentials positive to about +10 mV, both of these variables decreased. The

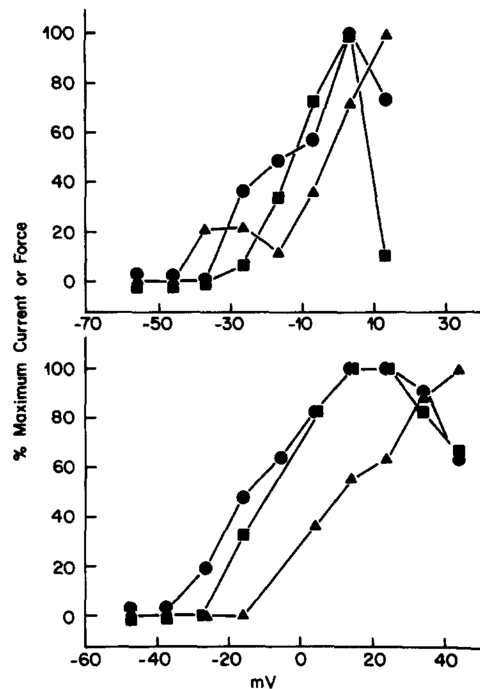

FIGURE 7. Voltage dependence of the early ryanodine-sensitive current, the late ryanodine-sensitive current, and the ryanodine-sensitive contraction. The ryanodine-sensitive currents and contractions were obtained by subtracting records obtained in the presence of 1 μM ryanodine from those recorded before exposure to the compound for matching voltage-clamp depolarizations. The normalized data are plotted vs. step potential. The data in the top panel were obtained in the standard superfusate (holding potential, −66 mV; experiment 29684), and those in the bottom panel were obtained in the presence of 50 μM 3,4-DAP (holding potential, −57 mV; experiment 061185). The squares are the early ryanodine-sensitive current, the triangles are the late ryanodine-sensitive current, and the circles are the ryanodine-sensitive contraction. See text for additional details.

relationship between pulse potential and the increase in the late outward current (triangles) component was shifted to the right of those for contraction and the early current component. In addition, the late current component increased for even the largest depolarizations.

These data show a good correlation between the abolition of the phasic contraction and the reduction of the early net outward current by ryanodine. Siegelbaum and Tsien (1980) also observed correlations between the amplitudes

of the early net outward current and contractions and interpreted them as evidence that the calcium transient that activated the contraction also activated an outward current. We think that the current component responsible for the reduced early outward current in ryanodine is the same current component described by Siegelbaum and Tsien. Obvious possible mechanisms that could underlie such a current include calcium-activated potassium or chloride conductances. In contrast, the correlation between the increase in the net late outward current and contraction is not as good. This observation, and others described below, suggest that the "early" and "late" effects of ryanodine on the net membrane currents represent at least two different ryanodine-sensitive current components. For convenience, we will refer to them as the early and late ryanodine-sensitive current components; however, we point out that we have no detailed information on their kinetics, but infer that one predominates at early times, while the other predominates at later times.

We have investigated the ionic nature of the late ryanodine-sensitive current component by attempting to observe reversal potential behavior. In these studies, we anticipated that, for membrane potentials approaching the reversal potential of the late ryanodine-sensitive current component, the magnitude of the effect of ryanodine on the net membrane current would decrease and change sign as applied potential crossed the reversal potential. We considered that a sodium, potassium, chloride, or a nonselective conductance were the most likely candidates to underlie the late ryanodine-sensitive current component, with +70 mV as the sodium equilibrium potential (Ellis, 1977), −43 mV for the chloride equilibrium potential (Vaughan-Jones, 1979), 0 mV for the reversal potential of a nonselective current (Kass et al., 1978), and −77 mV for the potassium equilibrium potential (Lee and Fozzard, 1975). The calcium equilibrium potential in cardiac muscle is positive to +100 mV, but current flow through calcium channels will show a reversal potential negative to that value by virtue of the permeation of ions other than calcium through the calcium channels. Thus, we take the calcium channel reversal potential of +65 mV reported by Kass and Sanguinetti (1984) as a reasonable estimate for the reversal potential of current flow through a hypothetical ryanodine-sensitive calcium conductance. From these considerations, the possibility that the late effect of ryanodine on the membrane current is due solely to an effect on a nonselective cation conductance is ruled out by the results shown in Fig. 7, where the late ryanodine-sensitive current component increases monotonically over the potential range from −20 to +40 mV.

To examine the later ryanodine-sensitive current component at the extremes of potential required by the sodium, calcium, and potassium reversal potentials, we adopted a two-step protocol in which a 125-ms conditioning pulse to a potential between 0 and +10 mV was used to activate the late ryanodine-sensitive current component before stepping to the desired test potential. This protocol allowed us to examine the behavior of the ryanodine-sensitive current at potentials where it is not activated by a simple step polarization. Fig. 8 shows results typical of those obtained in two experiments in which the late ryanodine-sensitive current component was examined at very positive potentials. Records obtained in the presence of 50 μM 3,4-DAP before (arrows) and during exposure to 1 μM

ryanodine are shown. The records in panel *A* were obtained during a maintained pulse to +9 mV, while those in *B–D* were obtained when the potential was stepped after 125 ms to test potentials of +29, +40, or +75 mV. At each of these test potentials, ryanodine caused an increase in the late net outward current. The observation that ryanodine treatment consistently increased the net outward current at potentials approaching and beyond the sodium equilibrium potential and the reversal potential for a calcium current implies that this change in the current is not due solely to the reduction of either a sodium or calcium current component.

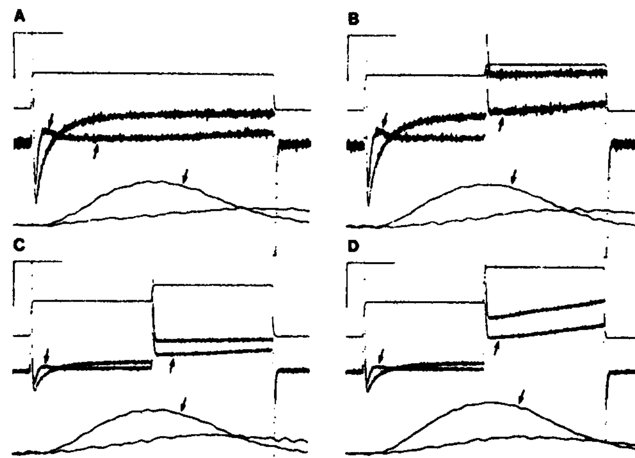

FIGURE 8. The late ryanodine-sensitive current at very positive potentials in the presence of 50 μM 3,4-DAP. In each panel, the upper traces are membrane potential recorded in 3,4-DAP, the middle traces are membrane currents in 3,4-DAP (arrows) and during exposure to 3,4-DAP plus 1 μM ryanodine, and the lower traces are developed force in 3,4-DAP (arrows) and during exposure to 3,4-DAP plus ryanodine for identical depolarizations. The holding potential was −57 mV. In *A*, the potential was stepped to +9 mV for 250 ms. In *B–D*, the potential was stepped to +9 mV for 125 ms and then stepped to +29 (*B*), +40 (*C*), and +75 (*D*) mV. Note the reduction of the current gain in *C* and *D*. The calibration bars are 50 ms, 200 nA (*A* and *B*), 630 nA (*C* and *D*), and 19 μN. Experiment 061185.

Fig. 9 shows the results of an experiment designed to test the possibility that ryanodine treatment increased a potassium or chloride conductance. In this and two other experiments, the late ryanodine-sensitive current component was examined at very negative potentials. Records obtained before (arrows) and during exposure to 1 μM ryanodine are shown. The inset shows the force records obtained before and during exposure to ryanodine and the voltage protocol, which was a 125-ms prestep from the holding potential of −59 to +11 mV, followed by a test step to various potentials, −99 mV in this instance. In the control superfusate, the conditioning step elicited a large outward current that briefly saturated the analog-to-digital converter before declining over the re-

mainder of the step. The hyperpolarization to −99 mV elicited an inward current that declined over 125 ms. In the presence of ryanodine, the current during the conditioning step (i.e., at +11 mV) was less outward at early times but was more outward at the end of that step. At −99 mV, the current in ryanodine was less inward (i.e., more outward, just as it was at +11 mV). Identical results were obtained in all three experiments over a potential range of −50 to −100 mV. These data are similar to those for the inhibition of the inward current tails shown by Kenyon and Sutko (1983). The observation that ryanodine treatment consistently made the net currents less inward at potentials approaching and beyond the reversal potentials of chloride and potassium ions implies that this

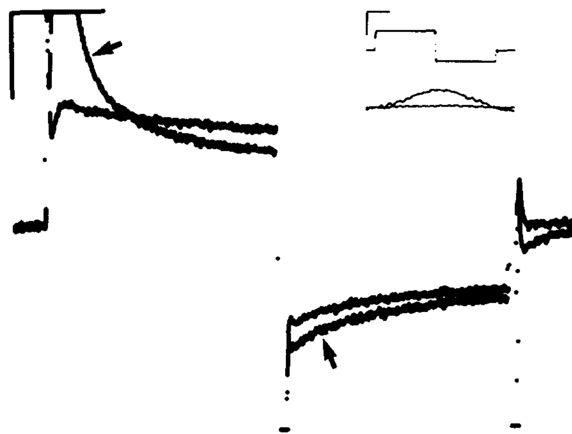

FIGURE 9. The late ryanodine-sensitive current at a very negative potential. The inset shows the voltage-clamp protocol and the developed force before (twitch) and during exposure to 1 μM ryanodine (flat line). The main part of the figure is the current record obtained in standard superfusate (arrows) and during exposure to ryanodine. The peak of the outward current in control is off scale. The holding voltage was −59 mV, the conditioning voltage step was to +11 mV for 125 ms, and the test voltage step was to −99 mV for 125 ms. The calibration bars in the main part of the figure are 50 ms and 200 nA. The calibration bars in the inset are 50 ms and 19 μN. Experiment 18386.

current cannot be attributed solely to an increase in a potassium or chloride current component.

In summary, if we consider the results of the experiments using the single-step protocols (e.g., Fig. 5) and those obtained using the two-step protocols (e.g., Figs. 8 and 9), we find that ryanodine treatment caused the net current 125 ms after a depolarization to be more outward (or less inward) over a potential range from −103 to +75 mV. These observations imply that the effect of ryanodine on the late net outward current cannot be attributed to a specific effect on a single sodium, potassium, calcium, or chloride conductance. One possibility is that the late effect of ryanodine represents the sum of the effects on two or more such components (e.g., increased potassium conductance at depolarized potentials plus decreased sodium conductance at negative potentials). A second possibility is

that the late current effect is mediated through an indirect effect of ryanodine on the sodium-calcium exchange current. This possibility is supported by the sodium sensitivity of the effect on the late current component described below (see Discussion).

Effects of Ryanodine on Membrane Currents and Contraction in Low-Chloride Solution

The observation by Siegelbaum and Tsien (1980) that the calcium-activated component of transient outward current was reduced in low-chloride solution

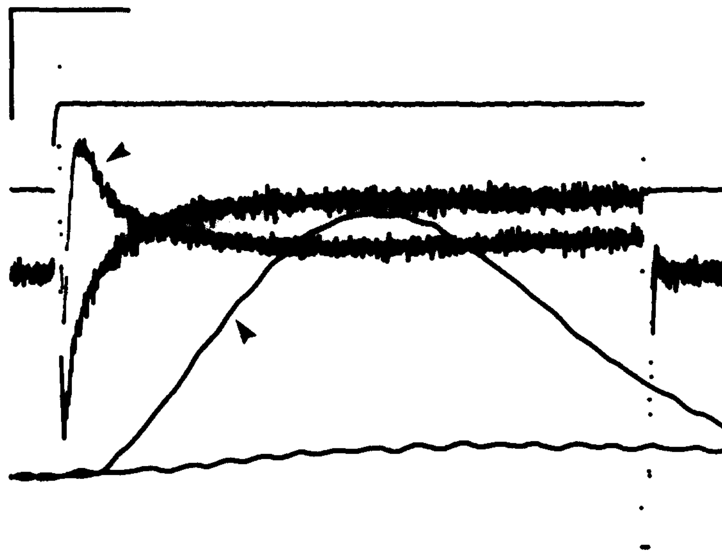

FIGURE 10. The effect of ryanodine on membrane currents and contraction in low-chloride solution containing 50 μM 3,4-DAP. The upper trace is the membrane potential recorded in low-chloride solution with 3,4-DAP, the middle traces are membrane current recorded in low-chloride solution with 3,4-DAP solution (arrows) and during exposure to ryanodine plus 3,4-DAP, and the lower traces are developed force in 3,4-DAP (arrows) and during exposure to ryanodine plus 3,4-DAP for identical depolarizations. The holding potential was −58 mV and the potential was stepped to +8 mV. The calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 19985.

raised the possibility of a calcium-activated chloride current in Purkinje fibers. We have considered the possibility that the early ryanodine-sensitive current might be a chloride current and tested this by observing the effects of ryanodine in low-chloride solutions. Fig. 10 shows membrane currents and contractions recorded in low-chloride solution containing 50 μM 3,4-DAP before (arrows) and during exposure to 1 μM ryanodine. The effects of ryanodine in low-chloride solution were the same as in standard superfusate. In particular, ryanodine abolished the contraction, reduced the net outward current at early times, and increased the net outward current at later times. These results were seen in six Purkinje fibers and demonstrate that the major effects of ryanodine are inde-

pendent of chloride ions. This suggests that the ryanodine-sensitive currents are not likely to be chloride currents.

Effects of Ryanodine on Membrane Currents and Contractions in Low-Sodium Solutions

If ryanodine modifies the net membrane current by affecting the sodium-calcium exchange current, the current modification should be dependent on the superfusate sodium concentration. Therefore, we examined the effects of ryanodine treatment during exposure of Purkinje fibers to low-sodium superfusates in

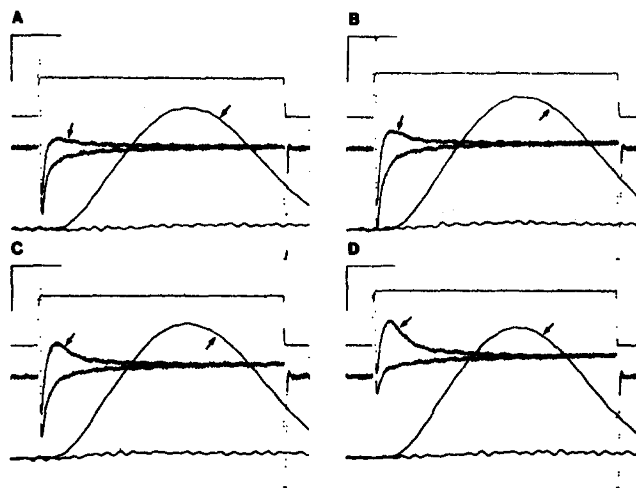

FIGURE 11. The effect of ryanodine in low-sodium (choline) solution on membrane currents and contraction. In each panel, the upper traces are membrane potential recorded in low-sodium solution containing 3,4-DAP, the middle traces are membrane currents in low-sodium solution plus 3,4-DAP (arrows) and during exposure to 1 μM ryanodine in low-sodium solution plus 3,4-DAP, and the lower traces are developed force in low-sodium solution plus 3,4-DAP (arrows) and during exposure to 1 μM ryanodine in low-sodium solution plus 3,4-DAP for identical depolarizations. The holding potential was −76 mV, the steps were to −5 (*A*), +5 (*B*), +15 (*C*), and +26 (*D*) mV. The calibration bars are 50 ms, 200 nA, 19 μN (before ryanodine), and 60 μN (after ryanodine). Experiment 23885.

which the sodium chloride was replaced by either choline, lithium, or cesium. The fibers were superfused with low-sodium solution for at least 15 min; during this time, a contracture developed that relaxed approximately halfway back to the steady state tension observed in sodium solution.

Fig. 11 shows membrane currents and contractions recorded during depolarizations in choline solution containing 50 μM 3,4-DAP before (arrows) and during exposure to 1 μM ryanodine. As in normal solutions, ryanodine caused a decrease in the early outward current as it abolished contraction. However, the current at later times did not become more outward in the presence of ryanodine. Fig. 12 shows similar results in experiments using lithium (*A*) or cesium (*B*) as sodium replacements. In three successful experiments using choline, three with

cesium, and one with lithium (plus another with lithium chloride mistakenly replacing both sodium and potassium chlorides), ryanodine reduced the net outward current at early times and abolished the twitch but never caused an increase in the late outward current. These data imply that the effects of ryanodine on the early current can be dissociated from those on the later currents in low-sodium solutions, that at least two different current components are involved, and that the effect on the later current requires sodium ions and none of the replacements that we tried can replace sodium.

Effects of Strontium and Caffeine on Membrane Currents and Contraction

We have described how ryanodine reduced the early net outward current and increased the late outward current as it abolished the phasic contraction of

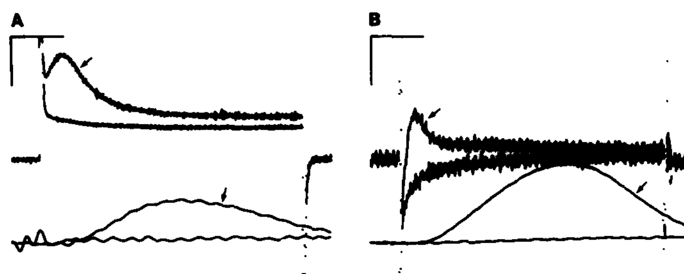

FIGURE 12. The effects of ryanodine in low-sodium (lithium or cesium) solution. In *A*, sodium chloride was replaced with lithium chloride; in *B*, sodium chloride and potassium chloride were replaced with cesium chloride; 50 μM 3,4-DAP was included in all solutions. In each panel, the upper traces are membrane currents recorded before (arrows) and during exposure to 1 μM ryanodine and the lower traces are developed force before (arrows) and during exposure to ryanodine. In *A*, the holding potential was −57 mV and the step was to +34 mV. Experiment 11386. In *B*, the holding potential was −56 and the step was to +35 mV. Experiment 16785. The calibration bars are 50 ms, 200 nA, and 19 μN.

cardiac Purkinje fibers. Our hypothesis is that all the effects of ryanodine on membrane currents can be attributed to an inhibition of calcium release by the sarcoplasmic reticulum and the consequent loss of the intracellular calcium transient that activates calcium-dependent currents (Sutko and Kenyon, 1983; Kenyon and Sutko, 1983; Sutko et al., 1985). As a test of this hypothesis, we assessed the extent to which other interventions that alter the function of the sarcoplasmic reticulum have similar effects on membrane currents. In particular, we investigated two such treatments: the replacement of calcium in the superfusate with strontium (Siegelbaum and Tsien, 1980; Coraboeuf and Carmeliet, 1982) and exposure to 10 mM caffeine (Coraboeuf and Carmeliet, 1982).

Fig. 13 shows membrane currents and contraction recorded in the presence of 50 μM 3,4-DAP before (arrows) and after the replacement of the superfusate calcium with strontium. For depolarizations to −19 (*A*) and +1 mV (*B*), strontium treatment made the net membrane currents more inward, apparently revealing a noninactivating strontium current flowing through the membrane calcium

channels (Siegelbaum and Tsien, 1980; Coraboeuf and Carmeliet, 1982; Thomas et al., 1985). The effect of strontium at more depolarized potentials was different and qualitatively resembled the effects of ryanodine; that is, the net outward current was reduced at early times and increased at later times. These results are similar to those shown by Siegelbaum and Tsien (1980) but are slightly different from those reported by Coraboeuf and Carmeliet (1982), who did not find an increased late outward current. The effects of strontium exposure on contraction were complex. Strontium inhibited force development during depolarizations near the threshold for detectable force development. For stronger depolarizations, phasic twitches were replaced by tonic force development, with

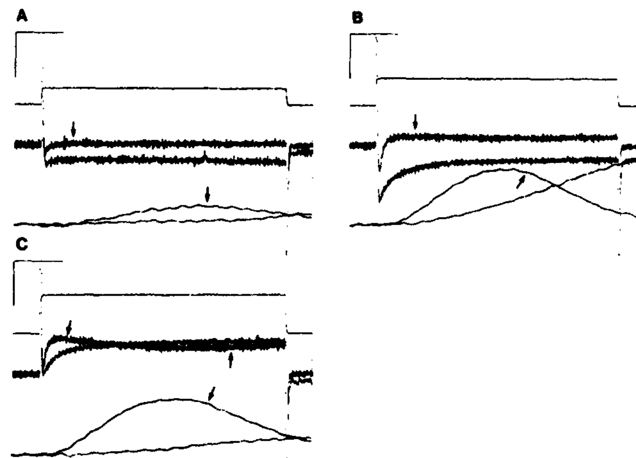

FIGURE 13. The effect of the replacement of superfusate calcium with strontium on membrane currents and contraction in the presence of 50 μM 3,4-DAP. In each panel, the upper traces are the membrane potential recorded in standard superfusate containing 3,4-DAP, the middle traces are membrane currents recorded in 3,4-DAP solution (arrows) and during exposure to strontium plus 3,4-DAP, and the lower traces are developed force in 3,4-DAP solution (arrows) and during exposure to strontium plus 3,4-DAP for identical depolarizations. The holding voltage was −50 mV, and the depolarizations were to −19 (*A*), +1 (*B*), and +21 (*C*) mV. The calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 24985.

the amplitude of this force increasing and then decreasing for very strong depolarizations. Thus, at the extremes of voltage, the effects on force development of exposing Purkinje fibers to strontium were similar to those caused by ryanodine. Similar results were seen in two other experiments in which Purkinje fibers were exposed to strontium in the presence of 3,4-DAP.

Fig. 14 shows membrane currents and contractions recorded in the presence of 50 μM 3,4-DAP and strontium before (arrows, same data shown in Fig. 13) and during exposure to 1 μM ryanodine. Ryanodine had only modest effects on membrane currents in this protocol; it made the net current slightly more inward at early times but did little or nothing at later times. In another experiment, ryanodine had no effect on the current recorded in the presence of strontium

plus 3,4-DAP. Ryanodine reduced force development in strontium-containing solutions.

Coraboeuf and Carmeliet (1982) demonstrated that 10 mM caffeine, another agent that disrupts calcium release by the sarcoplasmic reticulum, inhibited a component of outward current in sheep cardiac Purkinje fibers that was insensitive to 1 mM 4-AP. We have repeated those experiments in calf Purkinje fibers using 50 μM 3,4-DAP and extended the observations to include the effects of caffeine and caffeine plus ryanodine on tension. Fig. 15 shows membrane currents and contractions recorded in the presence of 50 μM 3,4-DAP before (arrows) and during exposure to 10 mM caffeine. The records obtained in the

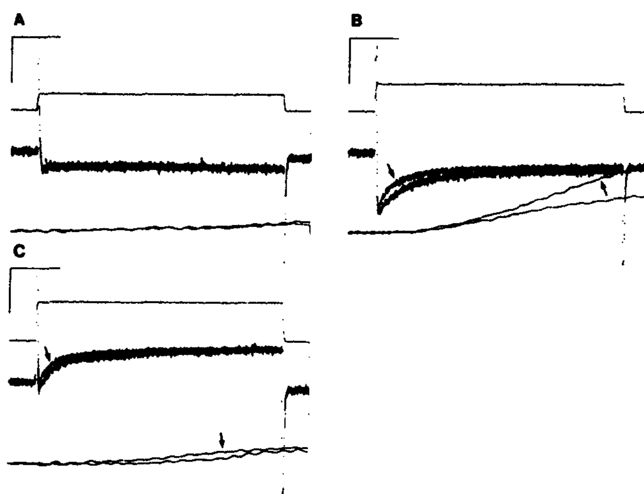

FIGURE 14. The effect of ryanodine in the presence of strontium (replacing calcium) and 50 μM 3,4-DAP on membrane currents and contraction. In each panel, the upper traces are the membrane potential recorded in strontium plus 3,4-DAP solution, the middle traces are membrane currents in strontium plus 3,4-DAP (arrows, same data shown in Fig. 13) and during exposure to 1 μM ryanodine plus strontium plus 3,4-DAP, and the lower traces are developed force in strontium plus 3,4-DAP (arrows, same data shown in Fig. 13) and during exposure to ryanodine plus strontium plus 3,4-DAP. The potentials and calibrations are the same as in Fig. 13. Experiment 24985.

presence of 3,4-DAP are similar to those shown earlier and there is a clear net transient outward current. Treatment with 10 mM caffeine caused the holding current to shift to more negative values (corresponding to a depolarization of an unclamped preparation; cf. Clusin, 1983) and, during depolarizations, made the net early current more inward but did not affect the later currents. Thus, the net current appeared to be dominated by the calcium current, as previously shown by Coraboeuf and Carmeliet (1982). Similar results were obtained in one other experiment using this protocol.

Fig. 15 (panel *A*) also shows that caffeine had marked effects on the amplitude and kinetics of force development. Caffeine increased the amount and rate of force development during depolarizations to potentials just above the threshold

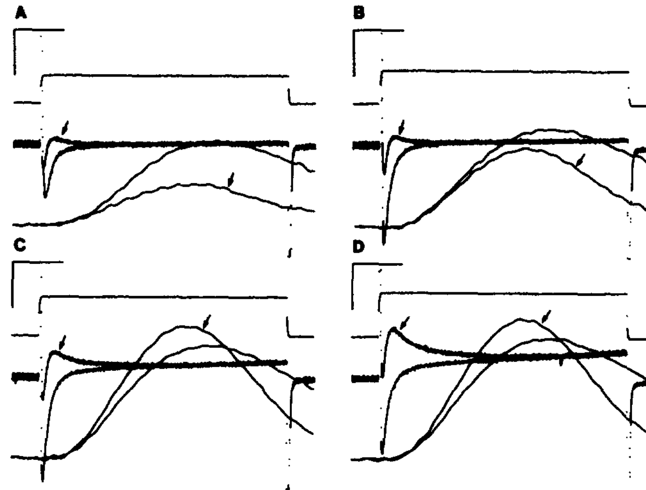

FIGURE 15. The effect of 10 mM caffeine on membrane currents and contraction in the presence of 50 μM 3,4-DAP. In each panel, the upper trace is membrane potential recorded in the standard superfusate plus 3,4-DAP, the middle traces are membrane currents in 3,4-DAP (arrows) and during exposure to caffeine plus 3,4-DAP, and the lower traces are developed force in 3,4-DAP (arrows) and during exposure to caffeine plus 3,4-DAP for identical voltage steps. The holding potential was −63 mV, and the steps were to −13 (*A*), −3 (*B*), +7 (*C*), and +17 (*D*) mV. The calibration bars are 50 ms, 200 nA, and 19 μN. Experiment 011085.

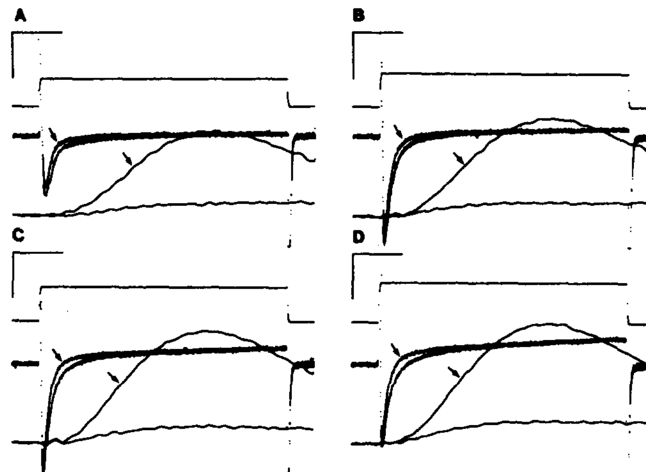

FIGURE 16. The effect of ryanodine in the presence of caffeine plus 3,4-DAP on membrane currents and contraction. In each panel, the upper traces are membrane potential recorded in caffeine plus 3,4-DAP, the middle traces are membrane currents in caffeine plus 3,4-DAP (arrows, same data shown in Fig. 13) and during exposure to 1 μM ryanodine plus caffeine plus 3,4-DAP, and the lower traces are developed force in caffeine plus 3,4-DAP (arrows, same data shown in Fig. 15) and during exposure to 1 μM ryanodine plus caffeine plus 3,4-DAP for identical depolarizations. The potentials and calibrations are the same as in Fig. 15. Experiment 011085.

for detectable force development. During stronger depolarizations, caffeine treatment reduced the peak amplitude, whereas it slowed the rise and fall of the contractions (Fig. 15, *B–D*). Note that while treatment with either ryanodine or strontium caused the normal phasic contraction to be replaced by a tonic force development, treatment with caffeine resulted in a force development that was largely phasic; i.e., relaxation clearly started before the end of the voltage-clamp step.

Fig. 16 shows membrane currents and contractions recorded in the presence of 50 μM 3,4-DAP plus 10 mM caffeine before (arrows, same data as Fig. 15) and during exposure to ryanodine. As seen in the experiments with strontium, the addition of ryanodine had only small effects on membrane current, apparently increasing the net inward current at early times, while having no visible effect on the later currents. These modest effects on the currents were accompanied by the abolition of phasic contraction.

DISCUSSION

Aminopyridine-sensitive Current

Our data indicate the presence of a significant component of aminopyridine-sensitive current in calf Purkinje fibers. The sensitivity to aminopyridines of this current component is similar to that found in a variety of cardiac tissues, including sheep Purkinje fibers (Kenyon and Gibbons, 1979*b*; Boyett, 1981*b*; Lipsius and Gibbons, 1982; Coraboeuf and Carmeliet, 1982), calf Purkinje fibers (Marban and Tsien, 1982), ventricular muscle (Kukushkin et al., 1983; Josephson et al., 1984; Mitchell et al., 1984*a*; Ito et al., 1984), as well as specialized cardiac fibers (Nakayama and Irisawa, 1985; Giles and van Ginneken, 1985). Our observations contrast somewhat with those of Siegelbaum and Tsien (1980), who reported that the outward currents of calf cardiac Purkinje fibers were insensitive to low concentrations (0.5–1 mM) of 4-AP, while higher concentrations (5–10 mM) that reduced the outward current also caused contracture and cellular uncoupling. We do not understand the failure of Siegelbaum and Tsien (1980) in this regard and can only speculate that some aspect of their experimental protocol designed to enhance the observation of the calcium-activated outward current may have impaired the observation of the aminopyridine-sensitive current. We do point out that the current records shown in their article closely resemble the currents that we and others (cf. Lipsius and Gibbons, 1982) have recorded in the presence of aminopyridines, which suggests that the transient outward current that they analyzed corresponds to the aminopyridine-insensitive component of transient outward current.

Many details of the aminopyridine-sensitive current of cardiac Purkinje fibers are unknown. The data we have presented using subtraction analysis (Fig. 2) indicate that the activation of this current component is at least as rapid as the ability of the two-microelectrode voltage-clamp to charge the membrane capacitance (see also Hart et al., 1982; Coraboeuf and Carmeliet, 1982). During a maintained depolarization, the current amplitude declines with a multiexponential time course, with time constants that are not strongly related to membrane

potential. Interpretation of this decline is difficult because it is probably due in part to the accumulation of potassium ions in the intercellular clefts of the Purkinje fibers. Measurements of the current decline in isolated myocyte preparations (Josephson et al., 1984; Giles and van Ginneken, 1985; Nakayama and Irisawa, 1985) show a simpler time course and suggest that a true reduction of the membrane conductance contributes to the decline of the current. Thus, one expects that the decline of the current in Purkinje fibers reflects a reduction in conductance as well as the effects of ion accumulation. We conclude that a portion of the decline of the aminopyridine-sensitive current of Purkinje fibers may be attributed to a process with a time constant in the range of 15–100 ms and that other slower and possibly faster processes are possible. We note that the properties that we have described for the aminopyridine-sensitive current in calf Purkinje fibers, particularly with regard to the time constants of the decline of the current, are similar to those described for the 4-AP–sensitive current of sheep (Kenyon and Gibbons, 1979*b*; Coraboeuf and Carmeliet, 1982). This suggests that differences in the outward currents of calf and sheep Purkinje fibers are quantitative (and probably small) rather than qualitative.

As shown in Fig. 2, there was a significant amount of aminopyridine-sensitive current remaining at the end of the 250-ms pulses that we used to study this current. This observation is similar to results reported by Kenyon and Gibbons (1979*b*) and Coraboeuf and Carmeliet (1982) and is consistent with the single channel observations of Nakayama and Irisawa (1985). These data suggest that inactivation of the aminopyridine-sensitive current is either incomplete or very slow.

Several lines of evidence suggest that the aminopyridine-sensitive current component is a potassium current. These include its insensitivity to chloride reduction (see also Kenyon and Gibbons, 1979*a*; Nakayama and Irisawa, 1985; Giles and van Ginneken, 1985), the inhibition of depolarization-induced potassium efflux from Purkinje fibers by 1 mM 4-AP (Vereecke et al., 1980), and the reversal potential observations made by Giles and van Ginneken (1985) on isolated crista terminalis cells. However, Nakayama and Irisawa (1985) concluded that the single channels mediating the transient outward current had a potassium-to-sodium permeability ratio of ~5:1; i.e., sodium ions could carry a significant current through these channels. This conclusion was based in large part on the results of cell-attached patch-clamp experiments, where the replacement of the sodium in the pipette solution caused a reduction in the single channel slope conductance and shifted the extrapolated reversal potential nearer but still positive to the potassium equilibrium potential (Fig. 10 of Nakayama and Irisawa, 1985). We find this result unexpected and are unsure of its proper interpretation. In particular, one expects that the removal of extracellular (i.e., pipette) sodium at a constant intracellular sodium will shift the equilibrium potential for sodium ions to an indeterminate large negative value and cause the reversal potential of the currents to shift to a value negative to the potassium equilibrium potential. Thus, we are unsure of the permeability ratio calculated from these experiments and suggest that Nakayama and Irisawa (1985) may have underestimated the selectivity of these channels for potassium ions.

The observation that the aminopyridine-sensitive current is unaffected by concentrations of ryanodine that abolish contraction and strongly attenuate intracellular calcium transients (Marban and Wier, 1985) is evidence that the activation of this current is not triggered by intracellular calcium but is probably gated by membrane potential. There is a considerable body of data that supports this conclusion. In a variety of cardiac preparations, the transient outward current is not inhibited by treatments that reduce or abolish the calcium current and contraction (Coraboeuf and Carmeliet, 1982; Hart et al., 1982; Josephson et al., 1984; Giles and van Ginneken, 1985; Nakayama and Irisawa, 1985). Furthermore, the kinetics of reactivation of this current in sheep Purkinje fibers are different from those of the twitch (Boyett, 1981*a*; Lipsius and Gibbons, 1982).

A major finding of our experiments is that 3,4-DAP is at least 10 times more potent than 4-AP at inhibiting the aminopyridine-sensitive current component of cardiac Purkinje fibers. Because we have not done rigorous dose-response experiments, the precise potency of this compound is unclear, but we suspect that the dose that we used may have been higher than necessary, and the potency of this compound in cardiac tissues is similar to that described by Kirsch and Narahashi (1978) in squid giant axons.

In summary, there is considerable evidence suggesting that the conductance that underlies the aminopyridine-sensitive current of cardiac cell is a voltage-gated potassium conductance and that the selectivity for potassium may be quite high. The similarity of this current with the transient outward currents observed in a variety of neurons has been pointed out by a number of workers (Kenyon and Gibbons, 1979*b*; Rogawski, 1985).

Effects of Chloride Reduction on Membrane Currents and Contraction

The interpretation of results from chloride-reduction experiments is difficult because chloride reduction has a variety of physiological effects in addition to simple reduction of that ionic species. For example, in Purkinje fibers, chloride replacement reduces membrane potassium permeability by an unknown mechanism (Carmeliet and Verdonck, 1977) and causes an increased intracellular pH through a membrane chloride-bicarbonate exchange mechanism (Vaughan-Jones, 1979). Either of these effects is expected to alter membrane currents other than chloride currents, and to influence intracellular calcium activity, and hence affect calcium-activated currents as well as excitation-contraction coupling. Thus, changes in membrane currents caused by chloride replacement cannot be attributed simply to the loss of a chloride current component. On the other hand, if particular current components, such as the ryanodine-sensitive current components, can be observed in low-chloride solutions, it is reasonable to conclude that they are not chloride currents. In our work, we have limited our experiments and their interpretation with this problem in mind; we have found that chloride replacement does not alter the essential effects of ryanodine on membrane currents and conclude that these currents are not chloride currents. To what extent chloride ions may carry current is not clear; the significant effects of chloride reduction that we and others have seen (Kenyon and Gibbons, 1979*a*, *b*; Siegelbaum and Tsien, 1980; Goto and Colatsky, 1982; Colatsky and

Goto, 1984) suggest that chloride currents may contribute to the net membrane current of cardiac Purkinje fibers.

Initially, we considered it likely that cardiac Purkinje fibers had a calcium-activated chloride current resembling those described for oocytes (Miledi, 1982; Barish, 1983) and cultured neurons (Owen et al., 1984; Mayer, 1985), and that this current would correspond to the early ryanodine-sensitive (transient outward) current component. However, our data clearly showed a substantial net transient outward current in low-chloride solutions plus 3,4-DAP and the addition of ryanodine reduced that outward current, revealing the calcium current. This implies that most, if not all, of the early ryanodine-sensitive current component is not carried by chloride ions and that it is likely to be a calcium-activated potassium current. We repeat that the possibility of a significant chloride current, and particularly a calcium-activated chloride current, cannot be ruled out by these data. It is possible that the positive inotropic effect of low-chloride solutions reflected an increased intracellular calcium transient and that this increased calcium could have compensated for the loss of the current-carrying species.

The effects of chloride replacement on membrane currents that we saw are in qualitative and quantitative agreement with the results of Kenyon and Gibbons (1979*a, b*). They reported that in low-chloride solutions, the peak outward current was reduced to ~20% of control (see also Table I of that article), a value that compares well with the 15% reduction shown in Fig. 3*A* of this report. Our data are in qualitative agreement with those of Siegelbaum and Tsien (1980), although those workers saw rather larger effects of chloride reduction in three of the five experiments they reported.

The effects of chloride replacement on developed force that we saw are very different from those described by Siegelbaum and Tsien (1980). They reported consistent reductions in this parameter, while we saw consistent and large increases. We are unable to explain this difference. It is not due to the use of isethionate as a chloride substitute, because Siegelbaum and Tsien (1980) also used isethionate and found a 30% reduction in twitch force. We also point out that a positive inotropic effect of chloride reduction has been observed by others in mammalian and frog ventricles (Nosek, 1979; Horackova and Vassort, 1982) and in smooth muscle (Kamm and Casteels, 1979). The cellular basis for this effect is not known, but it may include an increased calcium influx (Nosek, 1979; Horackova and Vassort, 1982; Kamm and Casteels, 1979), a shift in the pCa-tension relationship, increased calcium release from the sarcoplasmic reticulum caused by intracellular alkalinization (Fabiato, 1985), or direct effects of chloride reduction on either the myofilaments (Ashley and Moisescu, 1977) or other excitation-contraction processes. In any case, it is clear that many questions remain concerning the effects of chloride reduction on cardiac muscle.

Ryanodine-sensitive Currents

The effects of ryanodine on the early and late currents of cardiac Purkinje fibers can be distinguished in a number of ways. The voltage dependences of the early ryanodine-sensitive current and the twitch are very similar and both phenomena

are seen in low-sodium solutions. In contrast, the voltage dependence of the late ryanodine-sensitive current is not as closely correlated with the twitch and the current is not seen in low-sodium solutions. For these reasons, we have concluded that the early and late effects are mediated by effects on at least two different components of ryanodine-sensitive current.

The early ryanodine-sensitive current. The early ryanodine-sensitive current component resembles the calcium-activated transient outward current proposed by Siegelbaum and Tsien (1980) and the caffeine-sensitive current (i_{bo}) described by Coraboeuf and Carmeliet (1982). The similarities include common sensitivities to caffeine or the replacement of calcium by strontium and correlations with intracellular calcium transients. We agree with these workers in their conclusion that this current is mediated by a calcium-activated conductance that is activated by the same calcium transient that activates contraction. While a direct blockade of the calcium-activated channels by ryanodine cannot be eliminated by our data, the compound does not block the calcium-activated potassium channels located in the transverse tubules of rabbit skeletal muscle (Miller, C., personal communication). Thus, the sensitivity to ryanodine of a component of the transient outward current in Purkinje fibers is most simply explained by the inhibition of the intracellular calcium transient (Sutko and Kenyon, 1983; Marban and Wier, 1985) and the consequent loss of activator calcium. If this hypothesis is correct, it implies that the main source of activator calcium for this current is the sarcoplasmic reticulum because ryanodine does not inhibit the calcium current (see below; Sutko and Kenyon, 1983). On the other hand, to the extent that the calcium current is able to provide activator calcium, one expects to observe calcium-activated potassium currents during treatment with ryanodine. The observation that the time course of the net inward current recorded in the presence of ryanodine and 3,4-DAP is similar to the time course of the inward current recorded in cesium-loaded preparations (Marban and Tsien, 1982) or in preparations injected with tetrabutylammonium (Kass et al., 1982) suggests that calcium-activated outward currents are very small in the absence of calcium release from the sarcoplasmic reticulum and that the calcium current is unable to provide significant activator calcium by itself (see further discussion below).

We are able to shed some light on the ionic nature of the early ryanodine-sensitive current component. If the current is an outward current over the potential ranges we have examined, then the most likely current-carrying species are potassium or chloride ions. Because we can observe a ryanodine-sensitive current in low-chloride solutions, it is probably not a calcium-activated chloride current. In addition, the observation of this current component in low-sodium solutions indicates that it is not mediated by sodium-calcium exchange. We think that this current is probably a calcium-activated potassium current. This is consistent with the ability of intracellular tetrabutylammonium injection (Kass et al., 1982) or cesium ions (Marban and Tsien, 1982) to block both the aminopyridine-sensitive and the ryanodine-sensitive components of the transient outward current. This interpretation is also consistent with the observations by Callewaert et al. (1986) regarding potassium-selective single channels that are transiently activated by depolarizations in the presence of elevated calcium at the intracellular surface.

Net membrane currents recorded in the presence of both aminopyridine and ryanodine resembled those observed in Purkinje fibers loaded with intracellular cesium or injected with tetrabutylammonium (Figs. 6 and 8; see also Kenyon and Sutko, 1983; Sutko and Kenyon, 1983). This similarity is apparent in the voltage dependence of activation and the persistence of net inward currents at potentials as positive as +20 mV. A detailed analysis of the results of two experiments showed that the decline of the net inward current for pulse potentials negative to +25 mV was described by two exponential processes, a fast process with time constants in the range of 6–20 ms and a slower process with time constants in the range of 35–100 ms (data not shown). Thus, the kinetics of the net inward current are similar to those reported for the calcium current in Purkinje fibers injected with tetrabutylammonium (Kass and Sanguinetti, 1984). These data suggest that, under this condition, the net current is dominated by the calcium current and that treatment with aminopyridine plus ryanodine is very effective in inhibiting the time-dependent outward currents that overlap the calcium current. This raises the possibility that this protocol may provide a way to examine the properties of the calcium current in the absence of the normal intracellular calcium transient. In this regard, preliminary experiments re-examining the question of the calcium-dependent inactivation of the calcium current in ryanodine-treated Purkinje fibers using a two-pulse protocol (Kass and Sanguinetti, 1984; Lee et al., 1985) found a clear reduction in the amount of inactivation after very large depolarizing presteps. These data suggest that calcium-dependent inactivation is an important process in Purkinje fibers and that the calcium current itself can supply the necessary calcium.

To the extent that the sarcoplasmic reticulum is able to supply the calcium for calcium-mediated inactivation of calcium currents, one expects that the inhibition of calcium release from the sarcoplasmic reticulum will cause an increased amplitude and slowed inactivation of the calcium current. Thus, treatment with ryanodine might reduce net outward currents indirectly by reducing the inactivation of the calcium current. Such an effect has been observed by Mitchell et al. (1984*b*) in voltage-clamp experiments in isolated rat ventricular myocytes under conditions where contamination of the net currents by outward current components was minimized. They found that as ryanodine inhibited contraction, the kinetics of inactivation of the calcium current were slowed, and they concluded that the loss of the intracellular calcium transient caused by ryanodine resulted in a reduced calcium-mediated inactivation of the calcium current; i.e., the release of calcium by the sarcoplasmic reticulum makes a crucial contribution to the inactivation of the calcium current. The extent to which a similar effect might contribute to the ryanodine-sensitive currents in Purkinje fibers is unclear. It seems unlikely that the entire effect of ryanodine shown in experiments like those shown in Figs. 5 and 6 could be attributed to a modulation of the calcium current of the sort shown by Mitchell et al. (1984*b*). This suggestion is supported by preliminary measurements showing that ryanodine has, at most, only a very small effect on the calcium current recorded in cesium-loaded Purkinje fibers (unpublished data). The execution and interpretation of this sort of experiment are difficult because there is no independent method of assessing the completeness of the block of outward currents by cesium. Thus, any effect of ryanodine

on the net inward current may reflect a true effect of ryanodine on the calcium current or on a residual component of calcium-activated outward current. In any case, it seems that effects of ryanodine on calcium currents are quantitatively too small to contribute significantly to the ryanodine-sensitive currents described in this article.

The late ryanodine-sensitive current. Our initial hypothesis concerning the late ryanodine-sensitive current component was that the increased outward current was caused by the loss of a calcium-activated inward current component mediated by the calcium-activated conductance responsible for the transient inward current (Kass et al., 1978). While this notion was supported by the observed sodium sensitivity of the current, the failure to observe a reversal potential at even very positive potentials implied that such a simple hypothesis was incorrect. In addition, the failure to observe a reversal potential at very negative potentials raises the complex possibility that the late effect is caused by multiple effects of ryanodine on both inward and outward current components.

We think that a more attractive possibility is that the late ryanodine-sensitive current results from a change in the electrogenic sodium-calcium exchange current caused by the loss of the intracellular calcium transient. This effect can be illustrated by a simple calculation based on an exchange ratio of three sodium ions for each calcium ion (Reeves and Hale, 1984) and the intracellular sodium and calcium ion activities at rest and during contraction. We consider that intracellular sodium activity is ~7 mM, extracellular sodium activity is ~105 mM, and hence the sodium equilibrium potential (E_{Na}) is +70 mV (Ellis, 1977). Intracellular calcium in a resting Purkinje fiber is ~0.2 μM and it rises to ~1.7 μM during contraction (Hess and Wier, 1984). Thus, the calcium equilibrium potential (E_{Ca}) is about +127 mV at rest and falls to +99 mV during the twitch. To predict the effect of ryanodine on the sodium-calcium exchange current, one calculates the equilibrium potential of a 3:1 exchange system (E_c) using the equation, $E_c = 3E_{Na} - 2E_{Ca}$ (Reeves, 1985). For a control depolarization (with a normal twitch), a value of +12 mV is calculated for E_c. During exposure to ryanodine, we expect little or no increase in intracellular calcium upon depolarization (this work; see also Marban and Wier, 1985), and a value of −44 mV is calculated for E_c using +127 mV as the calcium equilibrium potential. Thus, in control, a pulse to 0 mV will be negative to the equilibrium potential of the exchanger and the exchange current will be inward, while during exposure to ryanodine, the same pulse will be positive to the equilibrium potential and the exchange current will be outward. A complete consideration of the effect of changing the equilibrium potential for sodium-calcium exchange on the magnitude of the exchange current depends on the details of the exchange mechanism. Eisner and Lederer (1985) discuss several quantitative models for this process. Their "simple" model (their Fig. 2) predicts effects on the exchange current recorded under voltage-clamp in response to a change in intracellular calcium that are identical to those reported here. That is, a reduction in intracellular calcium causes an increased outward (or decreased inward) current at all potentials. While more complicated models (e.g., Fig. 3 of Eisner and Lederer) can provide for a reversal of the current change, it is clear that simple models of

sodium-calcium exchange can account for the failure to observe a reversal potential and the sodium sensitivity of the effect. We also point out that such a scheme is supported by the observation that lithium ions, which can substitute for sodium ions in most channels (including calcium-activated nonselective channels; Yellin, 1982) but not in sodium-calcium exchange (Reeves and Sutko, 1979), do not substitute for sodium in the late ryanodine-sensitive current (see also Schouten and ter Keurs, 1985). Moreover, this scheme is appealing because it does not require an action of ryanodine beyond the inhibition of the intracellular calcium transient (see below).

The role of the sodium-calcium exchange current in cardiac excitation has been considered on theoretical grounds (Mullins, 1979; Fischmeister and Vassort, 1981; DiFrancesco and Noble, 1985; Eisner and Lederer, 1985), with various conclusions about how such a current might contribute to the action potential and the net currents of a voltage-clamp experiment, particularly the transient inward current and the calcium-activated component of the transient outward current. Recently, Hume and Uehara (1986*a*, *b*), Kimura et al. (1986), and Mechmann and Pott (1986) have measured membrane currents showing characteristics expected of sodium-calcium exchange current in sodium-loaded, isolated ventricular and atrial myocytes. Of particular interest are the data of Mechmann and Pott (1986), which show time-dependent oscillations in the exchange current, apparently driven by fluctuations in intracellular calcium caused by the activity of the sarcoplasmic reticulum. If our interpretation of the late ryanodine-sensitive current component as a sodium-calcium exchange current is correct, then it is of some interest that our data, obtained at normal intracellular sodium activity, are quite compatible with the results obtained from sodium-loaded, perfused myocytes. In particular, both our results and those of Mechmann and Pott (1986) suggest that the sodium-calcium exchange current is physiologically important and that the activity of the sarcoplasmic reticulum can regulate this current.

In summary, we think that ryanodine abolishes the intracellular calcium transient that is normally triggered by depolarization. As a consequence, the calcium-activated potassium current and inward sodium-calcium exchange current are not activated, which causes a reduced early net outward current and an increased late net outward current. This scheme can be compared with the model of DiFrancesco and Noble (1985), which includes a calcium-activated potassium conductance, a sodium-calcium exchange current, and a modeled intracellular calcium transient, but does not include an aminopyridine-sensitive current component. Accordingly, Fig. 5 of their article, which models the effect on the net membrane current of removing the calcium-activated currents, can be compared with Fig. 6 of this article. The results of the model and the experiment are quite similar. In particular, the model predicts the increased outward current we described as the late ryanodine-sensitive current, and supports our interpretation of the effects of ryanodine. This interpretation is further strengthened by the experiments using strontium and caffeine.

In this regard, the model of DiFrancesco and Noble (1985) also predicts that at early times during depolarization, the sodium-calcium exchange current is

outward and that it may contribute to the aminopyridine-insensitive component of the transient outward current. Our data do not rule out such a contribution; indeed, the sensitivity of the aminopyridine-insensitive transient outward current to ryanodine, caffeine, strontium, and EGTA is consistent with this hypothesis. On the other hand, Figs. 11 and 12 show large, apparently normal, aminopyridine-insensitive transient outward currents in low-sodium superfusates. This implies that a mechanism other than sodium-calcium exchange mediates at least a portion of this current and supports the notion of a calcium-activated potassium current discussed earlier.

Role of the Ryanodine-sensitive Currents in the Purkinje Fiber Action Potential

Fig. 4 shows that exposure to 1 μM ryanodine causes a slowing of the rate of phase 1 repolarization, an abolition of the notch separating phase 1 from the plateau, little change in the amplitude of the plateau, and a prolongation of the action potential duration. Figs. 5–7 show that during 250-ms step depolarizations to potentials positive to −40 mV, exposure to ryanodine causes a reduction of outward current at early times and an increased outward current during the middle and latter portion of the depolarizations. The reduction of the early outward current caused by ryanodine is easily reconciled with the slowing of the rate of phase 1 and the loss of the notch; these are straightforward consequences of the changes in membrane currents (cf. McAllister et al., 1975; DiFrancesco and Noble, 1985). On the other hand, the action of ryanodine to increase the net outward current does not have the expected effect of shortening the action potential duration. Several factors may contribute to this. First, during an action potential, the time course of the voltage is not a square step, and the prolonged depolarization (i.e., slowed repolarization) caused by the effect of ryanodine on the early current may affect the activation of other currents in such a way that they compensate for the increased outward current caused by ryanodine. In addition, the late ryanodine-sensitive current is relatively small at potentials negative to −10 mV (i.e., potentials near the plateau), and thus only small compensations are required to obscure the effects of such currents. Finally, it should be noted that we have not examined the effects of ryanodine on membrane currents at times longer than 250 ms, i.e., durations rather shorter than the typical action potential duration. It seems likely that if our suggestion that the late ryanodine-sensitive current is mediated by sodium-calcium exchange, such a current will show time dependence and will decrease or even change sign after the intracellular calcium transient has declined. In some of our records, such a decrease is suggested at 250 ms (e.g., Figs. 5*B* and 6, and data not shown). We admit that such arguments are invoked in order to explain the failure of an expected effect and note that, in general, predictions of the effects of alterations in plateau currents on action potential configuration are difficult (cf. Kass and Tsien, 1976).

Our results can be compared with the effects of ryanodine on the action potential configuration in ventricular preparations. We have previously reported that 1 μM ryanodine had little or no effect on the action potential or contraction of guinea pig ventricular muscle, which suggests that ryanodine-sensitive pro-

cesses play little role in these events, at least under the conditions of our measurements (Sutko and Kenyon, 1983). In ventricular myocytes isolated from rat (Mitchell et al., 1984*a*, *b*; Ito, K., J. L. Kenyon, and J. L. Sutko, manuscript in preparation), cat, or rabbit (Ito et al., manuscript in preparation) hearts, ryanodine typically slows early repolarization and speeds later repolarizations. These observations are consistent with the effects on membrane currents we observed and raise the possibility that ryanodine-sensitive current components similar to those described here may be found in many tissues. Further experiments are necessary to examine this possibility.

Comparison of the Effects of Ryanodine with Those of Strontium and Caffeine

For strong depolarizations, the replacement of the superfusate calcium by strontium ions has qualitatively similar effects on both contraction and membrane current as exposure to ryanodine. In particular, the rate of force development in strontium was dramatically slowed, such that force was essentially abolished for most of a 250-ms depolarization and the early net outward current was reduced, while the late net outward current was increased. These effects are like those reported by Siegelbaum and Tsien (1980) for both strontium and intracellular injection of EGTA. Furthermore, the relative lack of effect of ryanodine when added in the presence of strontium is additional evidence that the two treatments affect the same cellular processes. These data suggest that the effects of ryanodine can be attributed to the abolition of the normal intracellular calcium transient and that it is not necessary to invoke other mechanisms.

Caffeine, on the other hand, reduced the early net outward current without changing the late currents (this article; see also Coraboeuf and Carmeliet, 1982) and had complex voltage-dependent effects on the twitch. Because caffeine increases cytoplasmic cyclic AMP concentrations and the calcium sensitivity of the myofilaments to calcium, these data are difficult to interpret in terms of primary effects on the intracellular calcium transient. These uncertainties notwithstanding, our results show that caffeine neither abolished force development in the later part of a 250-ms depolarization nor caused an increase in the late outward current. This is in contrast to the effects of ryanodine, strontium, and EGTA injection, which attenuate contraction and increase the late outward current, and supports the hypothesis of a cause-and-effect relationship between the reduction of the intracellular calcium transient and the increased outward current. This is consistent with our suggestion that the late current effect might be mediated by the sodium-calcium exchange current. However, a prediction of this thought is that the abolition of phasic contraction by the addition of ryanodine in the presence of caffeine should cause an increase in outward current. Our failure to see such an increase in the late outward current may mean that ryanodine and caffeine interact on the mechanism that mediates this effect by ryanodine.

To the extent that they are comparable, our results concerning the effects of caffeine on membrane currents and contraction agree with those of Coraboeuf and Carmeliet (1982) and the more detailed study of Hess and Wier (1984). Clarification of the effects of caffeine will require more detailed measurements

of intracellular calcium activity and consideration of direct effects of caffeine on membrane currents (including channels and exchangers) as well as indirect effects, including those mediated by elevated intracellular cyclic AMP levels.

Summary

Our data indicate the existence of at least four different current components that contribute to the formation of the plateau phase of the Purkinje fiber action potential. These include the calcium current; the aminopyridine-sensitive component of transient outward current, which is probably a voltage-gated potassium current; the aminopyridine-insensitive component of transient outward current, which is sensitive to ryanodine and other treatments that reduce the intracellular calcium transient and is probably a calcium-activated potassium current; and the late ryanodine-sensitive current component, which may reflect a contribution of sodium-calcium exchange current to the normal action potential. The sensitivity of the latter two components to ryanodine is interpreted as evidence that current flow is dependent upon an elevation of intracellular calcium and that the major portion of the activating calcium is supplied by the sarcoplasmic reticulum. Other components of membrane current, including chloride currents, a delayed rectifier current, and a current mediated by a nonselective cation conductance, are not excluded by our data.

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