Ryanodine Does Not Affect Calcium Current in Guinea Pig Ventricular Myocytes in Which Ca\(^{2+}\) Is Buffered

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Calcium current in mammalian ventricular muscle is altered in the presence of ryanodine. Previous studies performed on rat ventricular cells have shown a slowing of Ca\(^{2+}\) current inactivation and suggest the hypothesis that ryanodine, by reducing the release of Ca\(^{2+}\) from the sarcoplasmic reticulum, reduces the availability of Ca\(^{2+}\) for inactivation of Ca\(^{2+}\) current (Ca\(^{2+}\)-dependent inactivation). Another hypothesis is that the effects of ryanodine on Ca\(^{2+}\) current are due to a mechanical connection of the ryanodine receptor with the L-type Ca\(^{2+}\) channel. To further test these hypotheses we examined the effect of ryanodine on Ca\(^{2+}\) current in single voltage-clamped guinea pig ventricular myocytes that contained Ca\(^{2+}\) indicator and Ca\(^{2+}\) buffer. We used fura 2 (pentapotassium salt) to confirm that the ryanodine we used was capable of abolishing Ca\(^{2+}\) release from the sarcoplasmic reticulum during the period in which it was present. We perfused the cells with 10 mM EGTA to block changes in intracellular Ca\(^{2+}\) concentration. In the absence of internal EGTA, Ca\(^{2+}\) currents displayed biexponential inactivation and Ca\(^{2+}\)-dependent inactivation (steady-state inactivation curves turned up at positive potentials). Inactivation was slowed by ryanodine at 10 \(\mu\)M. In cells perfused internally with EGTA, however, ryanodine had no effects, and steady-state inactivation curves were not shifted to the right. We conclude that, in guinea pig ventricular myocytes, the effects of ryanodine on Ca\(^{2+}\) current are mediated by Ca\(^{2+}\) and thus the effects of ryanodine do not provide a basis on which to postulate a physical connection between the L-type Ca\(^{2+}\) channel and the ryanodine receptor (sarcoplasmic reticulum Ca\(^{2+}\) release channel). (Circulation Research 1991;68:897–902)

Ryanodine is an important substance for the study of excitation–contraction coupling (E–C coupling) in muscle because it binds with high specificity and affinity to the Ca\(^{2+}\) channel of the sarcoplasmic reticulum (SR). In mammalian cardiac muscle, this interferes with the normal release of Ca\(^{2+}\) from the SR during E–C coupling. L-type Ca\(^{2+}\) current is also altered in cardiac muscle exposed to ryanodine. The question with which we are concerned in the present work is whether or not such changes are the result of a physical interaction or connection between the SR Ca\(^{2+}\) channel and the L-type Ca\(^{2+}\) channel of the sarcolemma, as proposed by Cohen and Lederer. The existence of such a connection would have important implications for the mechanisms of E–C coupling. For example, Cannell et al. have proposed that membrane voltage could modify directly, by mechanical allosteric modification, the release of Ca\(^{2+}\) from cardiac SR during E–C coupling and that a physical connection would be required for such an effect.

Cohen and Lederer reported that in adult rat myocytes ryanodine shifts the relation between membrane voltage and steady-state inactivation of L-type calcium current by 13 mV to more positive potentials. A classical Hodgkin-Huxley type analysis of Ca\(^{2+}\) current was possible in their experiments because the inactivation of Ca\(^{2+}\) current was (reportedly) fit adequately by a single exponential function and because steady-state inactivation curves did not show evidence of Ca\(^{2+}\)-dependent inactivation (no upturn at positive potentials). The time constant of inactivation of Ca\(^{2+}\) current was reported to be decreased at negative potentials and increased at positive potentials by ryanodine. The effect of ryanodine on the steady-state inactivation curve seemed to be indepen-

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dent of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) that is, not blocked by intracellular EGTA or 1,2-bis (2-aminophenoxo)ethane-N,N,N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid, and was absent in myocytes of neonatal rats, in which SR is relatively undeveloped. They suggested that ryanodine did not act directly on the Ca\(^{2+}\) channel and that the effects of ryanodine on Ca\(^{2+}\) current of adult rat myocytes are mediated by a physical connection between the ryanodine receptor and the L-type Ca\(^{2+}\) channel of the surface membrane.

Mitchell et al.\(^{10}\) found that the decline of Ca\(^{2+}\) current in rat ventricular myocytes was slowed by ryanodine. In their experiments, as in those of others,\(^{10}\) the decline of Ca\(^{2+}\) current was fit best by the sum of two exponential functions. Ryanodine did not change the time constants but increased the relative amplitude of the initial value of the one with the larger time constant (the slower one). Inactivation curves were turned up at positive potentials, and ryanodine decreased the amount of inactivation present in a test pulse to 0 mV after a short (5-msec) prepulse to potentials ranging from \(-40\) mV to \(+60\) mV. Although the curves thus obtained were not true steady-state inactivation curves, they did show the phenomenon generally believed to be Ca\(^{2+}\)-dependent inactivation.\(^{11-13}\) These authors further noted that the reduction in the rate of inactivation was consistent with the idea that ryanodine reduced the amount of intracellular Ca\(^{2+}\) available for inactivation of Ca\(^{2+}\) current.

In the present experiments, we focused on the issue of a possible [Ca\(^{2+}\)]-independent shift in steady-state inactivation of Ca\(^{2+}\) current, as reported by Cohen and Lederer.\(^7\) We used fura 2 to confirm that ryanodine was acting on the SR. We perfused the cells internally with EGTA at high concentration (10 mM) to eliminate changes in [Ca\(^{2+}\)]. We used sodium-free solutions (inside the pipette and outside the cell) to eliminate interference from Na\(^{+}\) currents and from Na-Ca exchange currents. In guinea pig ventricular cells under these conditions, ryanodine did not have any effect on steady-state inactivation of Ca\(^{2+}\) current. Therefore, our results do not support the suggestion of Cohen and Lederer\(^7\) that the effects of ryanodine on Ca\(^{2+}\) current are mediated by a physical connection between the ryanodine receptor (SR Ca\(^{2+}\) release channel) and the L-type Ca\(^{2+}\) channel. Our results further differ from theirs in that ours have shown that the decline of Ca\(^{2+}\) current was fit best by the sum of two exponential functions and that the steady-state inactivation curves turned up at positive potentials; these findings are consistent with those of others\(^{11-13}\) and are usually interpreted as evidence for Ca\(^{2+}\)-dependent inactivation.\(^6,10\)

**Materials and Methods**

Single guinea pig ventricular cells were obtained by an enzymatic dispersion technique similar to that already described.\(^{14}\) After isolation, the cells were stored in a physiological salt solution consisting of (mM) NaCl 135, dextrose 10, HEPES 10, KCl 4, NaH\(_2\)PO\(_4\) 0.33, MgCl\(_2\) 1.0, and CaCl\(_2\) 1.0. The pH was adjusted with NaOH to 7.30.

External physiological salt solutions contained (mM) tetraethylammonium chloride 140 or NaCl 135, dextrose 10, HEPES 10, CsCl 10, MgCl\(_2\) 1.0, and CaCl\(_2\) 1.0. Sodium-containing versions of this solution also contained NaH\(_2\)PO\(_4\) at 0.33. The pH of these solutions was adjusted with either NaOH or CsOH to 7.30.

Solutions used for filling the micropipette electrodes consisted of (mM) cesium glutamate 130, HEPES 10, NaCl 10, CsCl 10, MgCl\(_2\) 1.0, and Na\(_2\)ATP 3.0. The pH was adjusted to 7.20 with CsOH. This solution also contained, depending on the experiment, EGTA at 10 or 0.070 mM or fura 2 at 0.100 mM. When Na\(^{+}\)-free conditions were used, NaCl was replaced by CsCl, Na\(_2\)ATP was replaced by MgATP, and no other magnesium was added.

Ca\(^{2+}\) currents were recorded using standard whole-cell recording techniques and a single electrode continuous voltage-clamp device (Axopatch 1C, Axon Instruments). Micropipette electrodes had resistances between 0.5 and 2.0 MΩ. Data were sampled at 1 kHz. Typically, 80–90% compensation of series resistance could be achieved.

Under these ionic conditions and with a test pulse amplitude of \(+10\) mV, the estimated contribution of an outward leak current was 0.06 nA. Therefore, the peak L-type Ca\(^{2+}\) current was underestimated by 0.06 nA or 8–9%, which was not significant for subtraction.

Recording of fura 2 fluorescence and calibration of the fura 2 fluorescence were by methods described in detail elsewhere.\(^{15}\)

**Results**

**Effects of Ryanodine on Ca\(^{2+}\) Current and Ca\(^{2+}\) Release in Absence of Internal EGTA**

In external solutions containing Na\(^{+}\) and pipette solutions with no EGTA, ryanodine had several effects on membrane current, as shown in Figure 1: 1) The peak amplitude of the Ca\(^{2+}\) current was either unchanged (as in Figure 1) or slightly increased in individual experiments (average increase of 8%, \(+0.1\) nA). 2) The decline of current during the pulse was slowed, particularly near the time of peak current. 3) The slow inward tail of the current after repolarization was abolished. Similar effects were seen in 10 cells. The relatively small effect on peak amplitude of the current and the slowing of the decay are similar to the effects described by others in rat cells\(^6,7\) but not in guinea pig cells,\(^8\) in which ryanodine reduced the peak Ca\(^{2+}\) current (\(\sim 50\%\)). The large slow tail of inward current after repolarization is thought to be carried largely by the Na-Ca exchanger.\(^15\) In our experiments, this inward current tail was abolished by internal EGTA, by ryanodine, or by use of Na\(^{+}\)-free solutions. The inward tail current was not detectable in the recordings published by Cohen and Lederer.\(^7\) It was large and abolished by ryanodine and internal EGTA in rat ventricular myocytes in the experiments.
of Mitchell et al.\textsuperscript{6} and was large, abolished by EGTA, but unaffected by ryanodine in the guinea pig ventricular myocytes studied by Fedida et al.\textsuperscript{8}

It was important to establish that the ryanodine we used actually had an effect on the SR. Previous work has shown that ryanodine strongly reduces the rapid rise in $[\text{Ca}^{2+}]_i$, that is normally elicited by an action potential\textsuperscript{8} or by voltage-clamp depolarization to $+10$ mV.\textsuperscript{5,14,16} In a recent study\textsuperscript{17} in which the net flux of Ca\textsuperscript{2+} from the SR was calculated, it was shown that this rapid rise in Ca\textsuperscript{2+} on depolarization results mainly from release of Ca\textsuperscript{2+} from the SR. Therefore, we examined the effect of ryanodine on the rapid rise in $[\text{Ca}^{2+}]_i$, elicited by depolarization to $+10$ mV, on the assumption that a reduction indicates a direct, specific effect of ryanodine on the SR. Exposure to ryanodine at a concentration of 10 $\mu$M for 20 minutes abolished the rapid initial rise of the $[\text{Ca}^{2+}]_i$, transient, as illustrated in Figure 1; therefore, we conclude that the ryanodine we used was capable of affecting the SR and that 20 minutes was sufficient for this effect to develop. However, the binding of ryanodine to its receptor requires Ca\textsuperscript{2+}\textsuperscript{3} and thus, this experiment does not exclude the possibility that ryanodine does not act on the SR when EGTA is present inside the cell. We could not examine this possibility, because the inclusion of EGTA in the filling solution abolishes the free $[\text{Ca}^{2+}]_i$, transient, by which the effect of ryanodine is measured.

\textbf{Ca\textsuperscript{2+} Current in Na\textsuperscript{+}-Free Solutions With High Internal EGTA Concentration}

It was necessary to characterize Ca\textsuperscript{2+} currents under the conditions of our experiments, which were somewhat different from those of others. Measurements of the “control” characteristics were made 20 minutes after establishing the whole-cell recording and changing to Na\textsuperscript{+}-free external solution. Figure 2 illustrates a typical Ca\textsuperscript{2+} current (panel A) under these conditions and an analysis of its time course (panel B). Decline of the current during the pulse could be described as the sum of two exponential functions of approximately equal initial magnitude. The time constant of the faster one typically ranged from 10 to 20 msec, and the time constant of the slower one typically ranged from 100 to 200 msec. Panels C and D illustrate the membrane currents and steady-state inactivation curves typically obtained under these conditions. The inactivation curve has an “upturn” at positive membrane potentials, similar to that observed in the experiments of others.\textsuperscript{11-13} These results indicate that a Hodgkin-Huxley-type analysis would be inappropriate, since inactivation of Ca\textsuperscript{2+} current under these conditions cannot be described simply by the kinetic parameter ($i$, as introduced by Beeler and Reuter\textsuperscript{18}).

A difficulty we encountered was that the Ca\textsuperscript{2+} current usually tended to increase with time under the conditions of our experiments. When external Na\textsuperscript{+} was present, Ca\textsuperscript{2+} currents usually decreased slowly (not shown); when external Na\textsuperscript{+} was absent, Ca\textsuperscript{2+} currents changed initially, but at the time of the actual recordings, Ca\textsuperscript{2+} currents were relatively stable or increased slowly. However, even in experiments in which changes in peak Ca\textsuperscript{2+} current occurred with time, these changes were not associated with shifts in the steady-state inactivation curves. We do not know what is responsible for these changes.

\textbf{Effects of Ryanodine on Ca\textsuperscript{2+} Current in Na\textsuperscript{+}-Free Solutions With High Internal EGTA Concentration}

Figure 3A illustrates the peak amplitude of the Ca\textsuperscript{2+} current throughout a typical experiment. Voltage-clamp pulses to $+10$ mV were given at 10-second intervals except during the brief periods required to obtain steady-state inactivation curves (evidenced by gaps in the recording). During the 12 minutes after whole-cell recording had been first established and preceding the first actual recordings, the Ca\textsuperscript{2+} current changed (not shown) as EGTA diffused into the cell (evidenced by the rapid abolition of contraction) and as the Na\textsuperscript{+}-free external solution was introduced. Ryanodine (10 $\mu$M) was introduced after 20 minutes in Na\textsuperscript{+}-free external solutions, and recordings were made throughout the next 60 minutes. Original recordings of membrane current immediately before and after 20 minutes of exposure to ryanodine are shown (Figure 3B), as are the steady-state inactivation...
curves (Figure 3C) derived from them. In this experiment, Ca\textsuperscript{2+} current was increased slightly after 20 minutes of exposure to ryanodine, and the steady-state inactivation curve was shifted slightly to the left. The inactivation curves were not characterized by a Boltzmann distribution function because of the upturn at positive potentials. The curves were characterized by the voltage (V\textsubscript{1/2}) at which steady-state inactivation was 0.5, when the points were simply connected by lines. In nine experiments, V\textsubscript{1/2} was -24.7\textpm{}8.3 mV (mean\textpm{}SD) in control conditions and -28.2\textpm{}8.9 mV after 20 minutes of exposure to 10 \textmu{}M ryanodine. Statistical analysis with a paired-sample t test showed that the mean shift to the left of 3.5 mV was significant (p<0.001). However, this leftward shift is not attributable to ryanodine. A leftward shift of identical magnitude (3.4 mV) occurred over the same period of time in four control experiments that were performed exactly the same way except that ryanodine was not used. In these four control experiments, V\textsubscript{1/2} was -24.2\textpm{}5.8 mV in control conditions and -27.6\textpm{}5.8 mV after an additional 20 minutes in the absence of ryanodine. Thus, there is no possibility that the leftward shift that occurred with time masked a rightward shift induced by ryanodine. These small shifts may be due to a change in the tip potential of the electrode. These changes were larger when using micropipette electrodes containing Cl\textsuperscript{-} (not shown), in which such shifts would be expected to be larger. To minimize this problem, cesium glutamate filling solutions were used in all experiments cited above.\textsuperscript{19}

**Discussion**

Our results can be summarized as follows: ryanodine did interfere with SR function, but in cells in which changes in [Ca\textsuperscript{2+}]\textsubscript{i} were abolished by high internal EGTA concentration, there was no effect of ryanodine on the Ca\textsuperscript{2+} current. In particular, in cells containing high internal EGTA concentration, there was no rightward shift of the steady-state inactivation of L-type Ca\textsuperscript{2+} current, as reported by Cohen and Lederer.\textsuperscript{7}

A possible explanation for the differences between our experiments and those of Cohen and Lederer\textsuperscript{7} is a species difference in the mechanism of E-C cou-
plunging between the rat and the guinea pig. It is known that there are differences in the Ca²⁺ currents recorded from the rat compared with the guinea pig. The principal difference between the calcium currents measured in adult guinea pig ventricular cells and adult rat ventricular cells is that rat ventricular cells showed a more rapid and complete inactivation of the L-type Ca²⁺ current. In contrast to these differences, there are numerous similarities between the Ca²⁺ currents measured in both adult rat and guinea pig ventricular cells: 1) similar peak amplitudes of L-type Ca²⁺ currents, 2) similar description of L-type Ca²⁺ current inactivation by the sum of two exponential functions, and 3) similar evidence of Ca²⁺-dependent inactivation using a double-pulse protocol (upturn at positive potentials). However, in their adult rat ventricular cells, Cohen and Lederer did not find evidence for either the two time constants of inactivation or Ca²⁺-dependent inactivation. Although we cannot entirely rule out a species difference in the mechanisms of E-C coupling between the rat and the guinea pig, a more likely explanation for the differences between our experiments and theirs is that their adult rat ventricular cells are different in some important way from the rat cells used by others and from the guinea pig cells used by us.

Thus, in guinea pig ventricular cardiac cells, our results do not provide a basis on which to postulate a direct connection between the SR and the sarclemma that can be affected by ryanodine. This conclusion requires the assumption that ryanodine acts on the SR in the presence of high internal EGTA concentration, just as it does in the absence of internal EGTA (Figure 1). This assumption may be questioned on the basis that the binding of ryanodine to its receptor is Ca²⁺ dependent. This assumption is impossible to verify with our experimental means, since our assay for an effect of ryanodine is the abolition of SR Ca²⁺ release, but the presence of high EGTA concentration precludes measuring this. However, this limitation of the experimental conditions used by us also applies to the experimental conditions used by Cohen and Lederer and therefore, it cannot account for the differences between their experimental results and ours.

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