Ryanodine as a Tool to Determine the Contributions of Calcium Entry and Calcium Release to the Calcium Transient and Contraction of Cardiac Purkinje Fibers

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SUMMARY. Our object was to assess the relative roles of transsarcolemmal calcium entry and intracellular calcium release in the contraction of cardiac Purkinje fibers. We observed intracellular calcium transients, membrane potential, and contraction in aequorin-injected canine cardiac Purkinje fibers exposed to highly selective pharmacological modifiers of excitation-contraction coupling. To influence selectively the release of calcium from the sarcoplasmic reticulum, we used the plant alkaloid, ryanodine. To influence calcium entry, selectively, we used either the calcium channel antagonist, nitrendipine, or the calcium channel agonist, Bay k 8644. Ryanodine alone (1*10^-6M) reduced both components of the intracellular aequorin luminescence signal (L1 and L2). In three muscles, the luminescence signals were 3% of control in amplitude (standard error of the mean, 2%) without two distinct components and the twitch tension was 2% of control (standard error of the mean, 3%), whereas the action potential was prolonged. The aequorin signal and twitch remaining in ryanodine were abolished by the calcium antagonist nitrendipine (10^-5M), which also lowered the action potential plateau, consistent with the block of functional calcium channels. In two experiments, the calcium-channel agonist, Bay k 8644, in the presence of ryanodine, increased the aequorin luminescence and the contraction, but only to a very small fraction of their control values. Sodium withdrawal in potassium-free, ryanodine-containing solution produced large slow increases in calcium and tension, showing that tension could still be produced, that aequorin remained functional, and that sodium/calcium exchange was not inhibited by ryanodine. Caffeine increased intracellular calcium, showing that calcium stores were not depleted. In summary, the negative inotropic effect of ryanodine is due to a decrease in the calcium released from internal stores, and calcium entry normally contributes only a very small fraction of the total activator calcium. (Circ Res 56:133-138, 1985)

ALTHOUGH a rise in intracellular calcium (Ca++) is known to initiate contraction in cardiac muscle (Allen and Blinks, 1978), the sources of that Ca++, particularly in mammalian tissues, remain unclear. Two concepts are widely held. The first is that insufficient Ca++ enters the cell to account completely for the activation of contraction; estimates of the contribution of entering Ca++ to the activation of contraction vary from none (Fabiato, 1983) to 30% (Morad and Goldman, 1973). The second concept is that the small amount of Ca++ that enters, triggers a much larger release of Ca++ from the sarcoplasmic reticulum (SR) (see Fabiato, 1983).

Support for the idea that entering Ca++ contributes measurably to contractile activation came from voltage-clamp studies in Purkinje fibers (Gibbons and Fozzard, 1975) and ventricular tissue (New and Trautwein, 1972). The first studies on intracellular Ca++ transients in Purkinje fibers (Wier, 1980; Wier and Isenberg, 1982) also suggested that Ca++ entering the cell via surface membrane Ca++ channels accounted for a small component of contraction and an early, rapid rise in aequorin luminescence (component L1 of the aequorin signal). Ca++ currents are now known to be larger than previously thought [in multicellular preparations (Marban, 1981), and in single cells (Isenberg and Klockner, 1982)], further supporting the idea that entering Ca++ may have a significant role in activating contraction.

Nevertheless, recent data (Hess and Wier, 1984) suggest that the L1 component of the aequorin signal may not directly reflect Ca++ entry, and thus the contribution of entering Ca++ remains uncertain. Because caffeine strongly reduces L1 and not slow inward current, Hess and Wier suggested that L1 might arise from Ca++ released from SR that makes junctional contact with surface membrane. (L2 would arise from Ca++ released, via Ca++-induced Ca++ release, from SR deep in the muscle.) The Ca++ that enters would have to be bound rapidly or be taken up by the SR, as suggested by Isenberg (1982) and Fabiato (1983).
To investigate this question further, we attempted to block selectively the release of Ca++ from the SR and to observe the remaining intracellular Ca++ transients. We used the putative blocker of SR calcium release, ryanodine (Jenden and Fairhurst, 1969; Sutko and Kenyon, 1983), on the assumption that this would minimize the contribution of released Ca++ to the Ca++ transient. Ryanodine appears well-suited as a tool for this dissection of the Ca++ transient; whereas it blocks Ca++ release from the SR, entry pathways such as Ca++ channels and Na-Ca exchange are not inhibited (Sutko and Kenyon, 1983; Mitchell et al., 1984). We used Ca++ channel antagonists in the presence of ryanodine to confirm that Ca++ channels remained functional. We used Ca++ channel agonists, also in the presence of ryanodine, to increase Ca++ current in order to observe more clearly any signal directly related to Ca++ entry.

Methods

Biological Preparation

Adult mongrel dogs of either sex were anesthetized with T-61 Euthanasia Solution (0.5 ml/kg, iv bolus; American Hoechst Corp.). Each milliliter of this solution contains: 200 mg of N-[2-(m-methoxy-phenyl)-2-ethyl-butyl-(1)γ-hydroxybutyramide; 50 mg of 4,4'-methylenebis(cyclohexyl-trimethyl-ammonium iodide); 5 mg of tetracaine hydrochloride; 0.6 ml of dimethylformamide in distilled water. The hearts were quickly removed through a left lateral thoracotomy and immediately rinsed with tricaine hydrochloride; 0.6 ml of dimethylformamide in 3M KCl-filled microelectrode then was used to measure membrane potential while luminescence was conducted, via a Lucite light guide, onto a photomultiplier tube (EMI 9893) positioned beneath the preparation. Luminescence was collected by photon-counting techniques, and signals were averaged when necessary to improve signal-to-noise ratio, as described by Wier et al. (1983). Maximal luminescence (Lmax) was determined from Triton X-100 lysis at the end of the experiment, and this value was used to calculate fractional luminescence (L/Lmax). Membrane potential and tension were recorded continuously on magnetic tape (Hewlett-Packard 3964A recorder). A sufficient number of aequorin signals was averaged to obtain an acceptable signal-to-noise ratio.

Materials

Ryanodine, a plant-derived alkaloid of molecular weight 495 daltons, was obtained from Penick Corp., and stored at 10°C as 1 mM aqueous stock solution. Nitrendipine and Bay k 8644, supplied by Miles Pharmaceuticals, were kept as 1 mM stock solutions in polyethylene glycol in opaque containers. Drug-containing experimental solutions were prepared immediately before use by appropriate dilution of the stock solutions, or, in the case of caffeine, by addition of drug in powder form and solubilization in the experimental solution.

Results

Effects of Ryanodine on Membrane Potential, Aequorin Luminescence, and Tension

Figure 1A shows an example of the simultaneously recorded action potential, aequorin luminescence, and twitch tension before the addition of drug. Ryanodine (10 nm) decreased twitch tension and aequorin luminescence (Fig. 1B). The aequorin luminescence signal not only decreased in amplitude, but also changed in shape. The two components, L1 and L2, are readily apparent in the presence of ryanodine (Fig. 1B). A selective inhibition of

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\begin{align*}
\text{A} & \quad \text{B} & \quad \text{C} \\
\text{FIGURE 1. Membrane potential, fractional aequorin luminescence and tension in response to ryanodine. Panel A: no drug. L/L_{max} signals are the average of 57 sweeps. Panel B: 30 minutes after washing on 10 nM ryanodine. 57 sweeps averaged. The notch in the aequorin signal separates components L1 and L2. Panel C: Steady state effect (20 min) of 1 µM ryanodine. 528 sweeps averaged. Preparation 1–5.}
\end{align*}
\]
release of Ca**+ from the SR can explain the progression from Figure 1A to 1B: the underlying L1 was "unmasked" by a selective decrease in L2. On this basis, further increase in ryanodine concentration should selectively abolish L2, but not L1. When the ryanodine concentration was increased to 1 μM (Fig. 1C), the aequorin luminescence transient and twitch tension were markedly reduced. Thus, the idea that L1 reflects Ca**+ entry is not supported by these data. Similar effects of ryanodine on twitches and aequorin signals were observed in all other (nine) experiments.

There were three changes in the action potential: (1) a slight increase in the maximum diastolic potential, due at least in part to improved membrane potential recording stability as tension declined; (2) a genuine decrease in the rate of phase 1 repolarization and in the associated "notch" (Kenyon and Gibbons, 1979); and (3) a lengthening of plateau depolarization. The changes in rapid repolarization and in the plateau are consistent with known effects of ryanodine to decrease transient outward current (Sutko and Kenyon, 1983) and to slow the inactivation of Ca**+ current (Mitchell et al., 1984), both of which are attributable (Siegelbaum and Tsien, 1980; Tsien and Marban, 1982) to the observed decrease in Ca**+.

Although the Ca**+ transient and the twitch appear to be abolished by ryanodine, closer inspection of averaged signals reveals tiny Ca**+ transients and twitches, as shown in Figures 2A and 3A. In three muscles, the mean maximal amplitude of the aequorin signals was 3% ± 2% (SEM) of control, whereas the mean peak contraction tension was 2% ± 3% of control (note the change in gain of the calibration bars), making them much smaller than the previously identified L1 or L2. The kinetics of these signals also distinguish them from either L1 or L2: luminescence rises rapidly, but then declines slowly (most evident in Fig. 2A). Correspondingly, twitch relaxation is prolonged.

**Relationship to Ca**+ Entry Via Ca**+ Channels or Na-Ca Exchange**

To investigate the possible relationship of these signals to surface membrane Ca**+ conductance, we exploited the new highly specific dihydropyridine Ca**+ channel agonist, Bay k 8644 (Schramm et al., 1983), and antagonist, nitrendipine (Lee and Tsien, 1983). Figure 2 shows the effects of nitrendipine (10 μM) on membrane potential, aequorin luminescence, and tension during exposure to ryanodine (1 μM). The action potential plateau is markedly depressed by nitrendipine, as expected from the blockade of functional Ca**+ channels. Concomitantly, the luminescence and tension signals become undetectable during exposure to nitrendipine (Fig. 2B). Our conclusion that Ca**+ channels remain functional in ryanodine agrees with the results of voltage clamp experiments in calf Purkinje fibers (Sutko and Kenyon, 1983) and in rat ventricular cells (Mitchell et al., 1984).

In two experiments, the effects of an increase in Ca**+ influx were tested using Bay k 8644, an agonist compound known to increase Ca**+ current in Purkinje fibers (Sanguinetti and Kass, 1984; Cohen and Chung, 1984). In preliminary dose-response experiments, we found that 300 nM Bay k 8644 maximized twitch tension. With ryanodine present, this concentration of Bay k 8644 elevates the action potential plateau and augments the luminescence transient and tension (Fig. 3).

Although Na-Ca exchange is generally believed to extrude Ca**+ from heart cells, under certain conditions the mechanism can reverse and actually cause Ca**+ entry (e.g., Coraboueuf et al., 1981).
Ryanodine does not affect Na-Ca exchange as measured in sarcolemmal membrane vesicles (Sutko and Kenyon, 1983), but we sought independent confirmation that this mechanism remains functional in our preparation. Figure 4 shows aequorin luminescence, derived Ca++, and force during exposure to K+-free solution for 3 minutes just prior to washing in K+-free, low-Na+ solution. After 1 minute, caffeine (10 mM) was added. Luminescence, [Ca++], and tension decreased when the fiber was re-exposed to physiological saline solution.

Another feature of Figure 4 should also be noted. In other systems (e.g., Chapman, 1974; Fabiato and Fabiato, 1975), caffeine has been used as a tool to “dump” Ca++ from the SR to determine its level of Ca++ loading. Using the same strategy, 10 min caffeine was applied to the fiber in Figure 4. Caffeine evoked a large increase in Ca++ and force, above and beyond those induced by Na+ withdrawal alone. Under these conditions, the SR appears to contain a large amount of Ca++ even in the presence of ryanodine, which has been suggested (under different conditions) to decrease the Ca++ loading of the SR (Hilgemann et al., 1983). Similar effects of Na+ withdrawal in the presence of ryanodine were seen in six other preparations; the caffeine effect was confirmed in one other fiber.

Upper-Limit Estimates for Ca++ Transients in Ryanodine

If aequorin is uniformly distributed throughout the cytoplasm, the observed luminescence sets an upper limit (Blinks et al., 1982) on the spatial average [Ca++]. In three experiments, luminescence signals of the type shown in Figure 2A, when referred to a standard calibration curve (Wier and Hess, 1984), corresponded to a mean peak [Ca++] of 109 ± 25 nM (SEM).

Discussion

We find that Ca++ entry results in only a very small rise in Ca++ in the presence of ryanodine. It is not immediately clear that this is true under control conditions, since in the absence of ryanodine (control) the amount of entering Ca++ and/or its intracellular distribution will be somewhat different than in its presence, as discussed below.

At least one factor will tend to increase Ca++ entry in the presence of ryanodine, compared to control. Recent voltage clamp experiments indicate that Ca++ influx is increased by ryanodine (Mitchell et al., 1984), perhaps as a consequence of reduced inactivation of Ca++ current by Ca++. This may explain the increased depletion of extracellular Ca++ caused by ryanodine (Hilgemann et al., 1983).

Other factors will tend to reduce the Ca++ transient due to Ca++ entry in ryanodine compared to that in control. For example, an important factor that influences the Ca++ transient, and which may be different in ryanodine compared to control is the Ca++-buffering capacity of the cytoplasm. Ryanodine does not itself a Ca++ buffer, as it does not affect Ca++ activity in solution (Jones et al., 1979), but it may have indirect effects on Ca++ buffering. Ca++ released from the SR under normal conditions will compete with entering Ca++ for buffer sites. In the presence of ryanodine, more of the entering Ca++ will be buffered, because more Ca++-binding sites will be available. However, we calculate that this effect is small: even if sufficient Ca++ is released from the SR to elevate free [Ca++] to 10^-6 M, then the additional [Ca++] increment resulting from entry could be at most twice that in the absence of Ca++ release from the SR (assuming steady state buffering, calculated from data in Table 4 of Fabiato, 1983). A second factor to be considered is that, in the absence of the release process, the SR may have a somewhat increased ability to take up the Ca++ that enters, because it may not be loaded with Ca++, as suggested by Hilgemann et al. (1983). However, this would require a fairly large leak of Ca++ from the SR in the presence of ryanodine, a phenomenon for which we can find no supporting evidence. Furthermore, the experiment in Figure 4 shows that the SR can contain Ca++ when ryanodine is present (although the conditions for Ca++ loading in this experiment are quite different than during twitches). Consistent with our observations, there is no direct effect of ryanodine on Ca++ ATPase activity in SR vesicles (Sutko et al., 1979).

On balance, the data support the conclusion that, under control conditions, only a very small fraction of the Ca++ transient could be due directly to Ca++ entry. Thus, the components of the aequorin signal, L1 and L2, may arise from Ca++ released from intra-
cellular storage sites. This possibility had already been raised, based on the effects of caffeine, another inhibitor of SR function which decreases L₁ and L₂ while not blocking Ca++ channels (Hess and Wier, 1984). However, the effects of caffeine are difficult to interpret, in that it not only affects the SR, but also sensitizes the myofilaments to Ca++ (Wendt and Stephenson, 1984; Hess and Wier, 1984) thereby increasing Ca++ buffering.

The small aequorin signal that remains in the presence of ryanodine could be due to Ca++ entry, or it could be due to an incomplete effect of ryanodine to inhibit Ca++ release from the SR. In either case, the figure of 100 nm (see Results) is an upper-limit on the increase in cytoplasmic [Ca++] which is due to Ca++ entry. This result agrees reasonably well with estimates of Ca++ influx based on contemporary measurements of Ca++ current. Using voltage clamp data from Ca⁺⁺ loaded calf Purkinje fibers (Marban and Tsien, 1982), Fabiato and Baumgarten (1984) have calculated that total Ca++ rises approximately 1 µM during the first 20 msec of maximal Ca++ current. When intracellular Ca++ buffering is considered, the maximum [Ca++] increase in this time interval should be equal to or less than 100 nm.

Implications for Excitation-Contraction Coupling

Would Ca++ transients of the magnitude measured in ryanodine suffice to trigger Ca++-induced Ca++ release under normal conditions? Our upper-limit estimate for spatial average [Ca++] due to Ca++ entry is lower than the minimum concentration (400 nm) required to trigger Ca++-induced Ca++ release in skinned dog Purkinje cells (Fabiato, 1982). A number of factors might explain the apparent discrepancy. The Ca++ transient we detect could arise from a subcellular compartment in which local [Ca++] exceeds spatial average [Ca++]. Some of the entering Ca++ does reach the myofilaments, however, as twitches invariably accompany our luminescence signals. Furthermore, Ca++-induced Ca++ release may propagate along the SR; if so, only a fraction of the SR need be exposed to the “trigger” [Ca++]. Finally, even a small absolute rise in [Ca++] may suffice to trigger release, if the rate of change of [Ca++], Δ[Ca++] /Δt is important, as suggested by Fabiato (1983).

A small increment of 100–200 nm [Ca++] would not produce much force when added on to the low resting [Ca++], but the steepness of the Ca++-tension relation at higher [Ca++] predicts that such an increment would result in more force if superimposed on the [Ca++]* rise associated with release from the SR.

Finally, we noted a tendency for estimated diastolic [Ca++] to be lower in ryanodine than in control. If real, such a decrease in diastolic [Ca++]* could be due to a decrease in the basal Ca++ "leak" from the SR and would agree with the observation that ryanodine decreases Ca++-dependent phosphorylase a activity in cardiac muscle (Jones et al., 1979).

Ryanodine does not affect the amphibian heart-beat (Chapman, 1979), and only partially inhibits the twitch of mammalian ventricular muscle (Sutko et al., 1979). Additional experiments will be required to establish whether the effect of ryanodine on the SR is less complete, or if, as expected (Morad et al., 1983), Ca++ entry is relatively more important in these tissues.

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