Cumulative Depletions of Extracellular Calcium in Rabbit Ventricular Muscle Monitored with Calcium-Selective Microelectrodes

Donald M. Bers and Kenneth T. MacLeod

From the Division of Biomedical Sciences, University of California, Riverside, California

SUMMARY. Transient changes of extracellular free calcium in rabbit ventricular muscle under nonsteady state conditions were measured with double-barreled calcium microelectrodes. Resumption of stimulation after a rest interval produces a cumulative decrease of extracellular free calcium often by more than 10% (with bulk extracellular free calcium = 0.2 mM). The extracellular free calcium returns to the bulk value as a new steady state is achieved. The changes of extracellular free calcium recorded presumably represent net calcium uptake and loss by cardiac muscle cells. These cumulative extracellular free calcium depletions are blocked by 0.5 mM cobalt and 1 μM nifedipine and are increased to 167 ± 11% of control by the calcium agonist Bay k 8644 (1 μM) and to 620 ± 150% of control by increasing stimulus frequency from 0.2-2 Hz. Caffeine (10 μM) inhibits the cumulative extracellular free calcium depletions, probably by rendering the sarcoplasmic reticulum unable to accumulate calcium. It is proposed that the extracellular free calcium depletions recorded represent, in large part, calcium which has entered the cells and has been taken up by the sarcoplasmic reticulum (which had become depleted of calcium during the rest interval). Nifedipine and cobalt inhibit these cumulative depletions presumably by preventing the calcium entry which could subsequently be accumulated by the sarcoplasmic reticulum. The net cellular calcium uptake produced by such a post-rest stimulation protocol can also be inhibited by 1-3 μM acetylstrophanthidin and reduction of extracellular sodium to 70 mM. Acetylstrophanthidin and low extracellular sodium would be expected to shift the sodium-calcium exchange in favor of increased calcium uptake, which may, in turn, prevent the loss of sarcoplasmic reticulum calcium during the rest interval. This would limit the amount of calcium which the sarcoplasmic reticulum could take up with subsequent activation. In contrast to the results with caffeine, ryanodine (1 μM) increases the magnitude and rate of calcium uptake after a rest interval, indicative of a fundamental difference in the actions of caffeine and ryanodine. When stimulation is stopped in the presence of ryanodine, extracellular free calcium increases much faster than in control. This suggests that ryanodine may enhance calcium uptake by the sarcoplasmic reticulum during repetitive stimulation and may enhance calcium efflux from the sarcoplasmic reticulum during quiescence. These experiments provide insight into transsarcolemmal calcium movements and certain aspects of cellular calcium regulation.

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all have very different limitations. However, by combining information obtained from all these approaches, a more comprehensive understanding of cardiac cellular calcium movements can be achieved.

Phasic depletions of Ca_{o}^{++} have provided valuable information about calcium influx during individual contractions (Bers, 1983, 1985; Cleemann et al., 1984; Bers and Merrill, 1985; Hilgemann, 1985). Measurement of changes in [Ca_{o}^{++}] over a number of beats (i.e., cumulative changes) can provide information about net cellular uptake and loss of calcium. Knowledge of the net calcium uptake and loss which occur under nonsteady state conditions is crucial in the development of a more comprehensive understanding of cellular calcium fluxes involved in cardiac excitation-contraction coupling, and this is the general focus of the present study.

Activation of cardiac muscle is accompanied by calcium influx and calcium release from the SR. During relaxation, calcium is either taken up by the SR or extruded from the cell. The final fate, in either case, of the calcium which entered the cell is extrusion (if the cell is in a steady state). That is, even the amount of calcium influx which was taken up by the SR must be removed from the cell prior to the next cycle. The two main mechanisms of calcium efflux are probably sarcolemmal sodium-calcium exchange and calcium-ATPase pump (Chapman, 1983; Fabiato and Baumgarten, 1984; Sutko et al., 1986). Under nonsteady state conditions (e.g., changes in stimulation pattern), calcium influx and efflux may not be equal and thus net cellular calcium uptake or loss may occur until a new steady state is established.

The extracellular [Ca_{o}^{++}] is determined by transmembrane calcium fluxes, replenishment from the bulk solution and binding to extracellular sites. When calcium influx exceeds calcium efflux over a period of time, net cellular calcium uptake occurs. Measurement of Ca_{o}^{++} depletions due to cellular calcium uptake requires that these changes of [Ca_{o}^{++}] are faster than can be replenished by diffusion and larger than can be buffered by local calcium-binding sites. Thus, cumulative Ca_{o}^{++} depletions as measured here would tend to underestimate the calcium uptake in the region of the microelectrode tip (due to local calcium buffering and replenishment from the bulk solution).

The two main goals of the present study were to (1) characterize cumulative depletions of Ca_{o}^{++} which probably represent cellular calcium uptake, and (2) evaluate the probable cellular locus of calcium which is taken up by the cells. In order to achieve these goals, we have studied extracellular calcium depletions during post-rest stimulation utilizing interventions which are known to alter cardiac contractility by different primary mechanisms: (1) changes in calcium influx or efflux (i.e., exposure to cobalt, nifedipine, Bay k 8644, and changes in frequency), (2) changes in transsarcolemmal sodium gradient (i.e., reduction of [Na^{+}]), and exposure to acetylstrophanthidin, (3) changes in SR function (i.e., exposure to caffeine and ryanodine).

Methods

General

Papillary muscles or ventricular trabeculae (0.1–0.6 mm in diameter) were obtained from the hearts of adult New Zealand white rabbits after intravenous administration of an overdose of pentobarbital sodium. Loops of fine suture were tied around each end of the muscles, which were then mounted in a superfusion chamber (0.15 ml in volume) over platinum support and stimulating wires. One end was fixed, and the other was attached to a force transducer constructed from a piezo-resistive element (AE 801, Aksjeselskapet Micro-Elektronikk). All experiments were performed at 29°C or 30°C. Temperature variation during any experiment was never more than 0.4°C. Muscles were equilibrated for at least 45 minutes after dissection in normal Tyrode's solution containing 2 mM calcium (see below) while being stimulated at 0.5 Hz before the experiments were started. The flow rate in the chamber was 2 ml/min.

Solutions

The normal Tyrode's solution contained (mm), NaCl, 140; KCl, 6, MgCl2, 1; glucose, 10; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 (pH 7.40). During dissection and equilibration, the normal Tyrode's solution contained 2.0 mM CaCl2. During the experiments, the normal Tyrode's solution contained 0.2 mM CaCl2. All solutions were equilibrated with 100% O2.

When the [Na^{+}] was reduced, it was replaced with equivalent quantities of tris(hydroxymethyl)aminomethane (Tris). Caffeine (Sigma) was added as a solid to the Tyrode's solution. Ryanodine (Pennick Corp.) was dissolved in distilled H2O to produce a stock solution Acetylstrophanthidin (Sigma), nifedipine (Sigma), and Bay k 8644 (a generous gift from Dr. A. Scriabine, Miles Laboratories) were dissolved in ethanol to produce stock solutions. The latter two solutions were kept in dark conditions until immediately before use. After addition of the compounds dissolved in ethanol, the concentrations of ethanol in the final solution did not exceed 0.1%.

Microelectrodes

Double-barreled micropipettes were pulled from 2.0 mm in diameter theta glass, and their tips were broken under microscopic observation to between 4 and 14 μm in diameter. One barrel was silanized by injection of N,N-dimethyl-trimethylsilyllamine (TMSDMA) vapor into one barrel while blowing N2 gas through the other. Both barrels were then backfilled; the silanized barrel with a solution containing 10 mM CaCl2 and 100 mM KCl and the unsilanized barrel with a solution of 140 mM NaCl. Neutral ligand calcium sensor (Oehme et al., 1976) was then sucked into the silanized barrel. The calcium-sensitive barrels of these finished electrodes had resistances of ~1-5 Ω, whereas the reference barrels had resistances of ~500 Ω. Filtration of the reference barrel was sometimes carried out to match impedances of the two barrels (time constants ~2–10 msec). This was done as described by Bers (1983). Electrical and chemical time constants of the electrodes were similar to those given by Bers (1983). The calcium electrodes produced responses of 28–30 mV to a 10-fold change in [Ca_{o}^{++}].
tip diameters exhibit Nernstian behavior over the range 10 µm to 10 mm calcium (Bers and Ellis, 1982). This is adequate for the present purpose. Nifedipine, cobalt, Bay k 8644, acetylstrophanthidin, ryanodine, and caffeine caused only small offsets on the calcium electrodes (<0.4 mV) at the concentrations used. These could be accounted for, and do not affect the interpretations of the results.

Signal Recording and Initial Protocol

The reference barrel potential was measured with respect to a grounded Ag/AgCl wire. The calcium barrel potential was measured either with a varactor bridge operational amplifier (Analog Devices 311 J) wired for unity gain or with a high-impedance electrometer (WPI model FD223). By recording the difference signal between the reference barrel and the calcium-sensitive barrel, we obtained measurements of the calcium activity. The difference signal, reference signal, and force produced by the preparation were recorded on a strip chart recorder, oscillographic recorder, and oscilloscope. The drift on the difference signal was about 0.2 mV/hr. No filtering of the calcium signal was carried out.

After the muscle had stabilized in 2 mM calcium Tyrode's solution, the [Ca++] was decreased to 0.2 mM. Once stability was achieved in 0.2 min calcium Tyrode's solution, the double-barreled electrode was advanced to touch the surface of the muscle and was further advanced so that extracellular action potentials were recorded. The magnitude of the potential changes associated with each action potential decline over 5-20 minutes to become a consistent size (0.4-2.0 mV). Over this stabilization period, the difference signal showed a small transient decrease in the apparent calcium activity. When the electrodes had stabilized, the calcium activity measured was the same as in the superfusing Tyrode's solution. At this point, the experiments were started. The usual protocol was to stop normal stimulation (at 0.5 Hz) and rest the muscle for 30 seconds or 1 minute, then start stimulation (0.2-2 Hz) and study the accompanying decrease in calcium activity. The muscle was then returned to normal 0.5-Hz stimulation. This protocol was carried out before and after experimental interventions.

Results

Figure 1 illustrates the Ca_o++ depletion phenomenon as sensed by the double-barreled calcium microelectrode. The top two traces are the signals recorded from the reference barrel (E_R) and the calcium barrel (E_Ca) of the double-barreled microelectrode. The third trace is the difference signal (E_Ca - E_R) recorded at a higher gain and is a measurement of the [Ca++]o. The bottom trace shows tension development. The spikes on the difference or calcium signal are due to imperfect subtraction of the stimulus artifact and, sometimes, the extracellular action potential upstroke. The spikes are often apparent on the E_Ca and E_R traces and are greatly reduced by subtraction of the signals. In subsequent figures, only the difference signal (calcium signal) will be shown.

At the beginning of Figure 1, stimulation at 0.5 Hz is terminated, and there is a small increase of [Ca++]o during the rest interval. The muscle is then stimulated at 2 Hz for 15 seconds, during which time, peak twitch tension and resting tension increase and then decline. During this period of 2-Hz stimulation, a cumulative depletion of Ca_o++ develops, which reduces [Ca++]o by 11 µM, from the ambient 200 µM [Ca++]o. When stimulation is terminated, the [Ca++]o returns slowly toward the bath level (half time ~10 seconds). If stimulation is continued, [Ca++]o still returns to the bath level, as it.

**Figure 1.** The effect of stimulation at 2 Hz on the [Ca++]o sensed by the double-barreled microelectrode placed in the extracellular space, as described in Methods. The top trace (E_R) is the potential sensed by the reference barrel, and the second top trace (E_Ca) is the potential sensed by the calcium barrel. The third trace is the difference signal (E_Ca - E_R) recorded at higher gain. A calibration bar showing the respective free [Ca++] is given. The bottom trace shows the changes in force produced by the preparation. See text for experimental protocol. The break in the traces represents a 6-minute period of quiescence.
must, but takes a longer time (~2–3 minutes) to do so.

It should be emphasized that these cumulative changes of [Ca++]o occur only during nonsteady state conditions. The [Ca++]o must return to the bath level in any steady state condition. This is clear when one considers a steady state condition to be one in which calcium influx and efflux are the same so that there is no net movement of calcium. Thus, at steady state, extracellular [Ca++] deficits will have been replenished by superfusate calcium, and Ca++ accumulations will have been washed out to superfusate levels.

When a single stimulation is interposed during the time that [Ca++]o is increasing after the 2-Hz trains in Figure 1, the rate of rise of [Ca++]o is enhanced and the accompanying beat is potentiated. This effect wanes as the time between the 2-Hz train, and the interposed beat (e.g. another 2-Hz train) [Ca++] decreases again.

Another characteristic illustrated in Figure 1 is that when the duration of the rest interval is increased from 30 seconds to 7 minutes, the magnitude of the depletion increases (in this case from 11 to 18 μM). In Figure 1, the conditions prior to the two rest intervals are different, but the result is qualitatively consistent with other calcium depletion experiments in which rest intervals from 10 seconds to 5 minutes were imposed after steady state 0.5-Hz stimulation (Bers and MacLeod, 1985). The last portion of Figure 1 illustrates that when stimulation at 0.5 Hz is resumed after [Ca++]o has returned nearly to the bath level, a smaller and slower cumulative depletion occurs.

It may be noted that, in several of the records presented, when muscles were stimulated at 1–2 Hz after a rest interval, there was inadequate time for complete relaxation to occur between stimuli. The same calcium depletion effects were obtained when there was complete relaxation. The critical point is that, when stimulation rate is decreased or stopped, there is still substantial Ca depletion after tension has returned to the baseline. Thus, there is still a net gain of cellular calcium when there is no evidence of elevated cytoplasmic free [Ca++].

Both low temperature and low [Ca++]o prolong action potentials and contractions. A combination of these effects is responsible for the relatively long contractions observed. In several experiments, cumulative Ca++ depletions were monitored in 2 mM Ca++ medium. These cumulative depletions were qualitatively similar to those obtained at 0.2 mM calcium. The amplitude of the depletions in 2 mM Ca++ was smaller in millivolts, but larger in terms of actual change in [Ca++]o (~80 μM). The stronger contractions at 2 mM Ca++ also made it more difficult to maintain long stable electrode positions. If the depletions represent calcium uptake by the cells, then interventions known to increase or decrease calcium influx (or efflux) would be expected to alter the magnitude or rate of Ca++ depletion.

Protocols with Primary Effects on Calcium Influx or Efflux

Figure 2 illustrates the effect of cobalt on [Ca++]o. Before exposure to 0.5 mM CoCl2, the stimulation frequency was increased for about 10 seconds from 0.5 Hz to 2 Hz. This demonstrates that Ca++ depletions can be produced simply by changing frequency without a rest interval. When cobalt is added, there is a large transient increase in [Ca++]o which peaks at 275 mM and slowly returns to control levels. This effect is not due to cobalt interference with the calcium electrode. This same solution change with the same calcium electrode in the chamber, but not pushed into the muscle produced only a small (<0.2 mV) steady offset in the positive direction. This

**FIGURE 2.** The effect of 0.5 mM CoCl2 on the [Ca++]o, the depletion phenomenon, and on tension. The top trace shows the calcium signal (E_Ca - E_k) and is indicative of free [Ca++]o. The bottom trace shows tension. The break in the traces was for a period of 19 minutes. The basic stimulus frequency was 0.5 Hz, except where shown. When CoCl2 was added, the recorder speed was decreased as shown. The diameter of the preparation was 0.4 mm. Note that the recorder speed was decreased for 3 minutes during cobalt addition, and the gain on the tension record is decreased after removal of cobalt. Similar results were recorded in four other preparations.
increase in [Ca$$^{++}$$], could be due to displacement of calcium bound at extracellular sites or calcium loss from the cell. The former possibility seems more likely to be responsible for most of this rise in [Ca$$^{++}$$], since cobalt is known to compete with calcium at numerous extracellular sites (e.g., Bers and Langer, 1979). The central 2-Hz train in Figure 2 demonstrates the block due to cobalt. The third 2-Hz train shows that 19 minutes after removal of CoCl$_2$, the magnitude of the Ca$_{o}$$^{++}$ depletion is nearly the same as that of the control.

Figure 3 demonstrates the effect of 1 $\mu$M nifedipine, an inhibitor of inward calcium channels, on the Ca$_{o}$$^{++}$ depletion induced by stimulation at 2 Hz following a 1-minute rest interval. A 2.5-minute exposure to 1 $\mu$M nifedipine abolishes the cumulative depletion of Ca$_{o}$$^{++}$, and this effect of nifedipine is almost completely reversible after 23 minutes of control superfusion. That the depletion signal can be eliminated by cobalt and nifedipine, while action potentials continue, is evidence that electrical artifacts are not responsible for the calcium-depletion signals.

The effects of the calcium channel agonist, Bay k 8644 (1 $\mu$M), on the depletion of Ca$_{o}$$^{++}$ are shown in Figure 4. Addition of Bay k 8644 increased steady state twitch tension (800%), but did not produce any apparent change of [Ca$$^{++}$$], during steady state stimulation. After a 1-minute rest interval, 1-Hz stimulation produced a 20 $\mu$M (or 10%) decrease in [Ca$$^{++}$$], under control conditions. In the presence of Bay k 8644, the magnitude of Ca$_{o}$$^{++}$ depletion with this protocol increased to 27 $\mu$M, and the maximum rate of depletion increased approximately 2.3-fold. Similar results were seen in six experiments with Bay k 8644.

Figure 5 illustrates the effect of stimulation frequency on the cumulative Ca$_{o}$$^{++}$ depletion. In both of the experiments shown, the muscles were stimulated at 0.5 Hz, and then after a 30-second (panel...
**Figure 5.** The effect of stimulation frequency on the cumulative Ca\(^{++}\) depletion. Results from two separate preparations are shown. Muscles were stimulated at 0.5 Hz and, after a rest interval of 30 seconds (panel A) or 1 minute (panel B), stimulation was resumed at the frequencies shown. Panel A top traces: \(E_{Ca}-E_{Na}\), bottom traces: tension. Panel B shows \(E_{Ca}-E_{Na}\).

A) or 1-minute (panel B) rest interval, stimulation was resumed at 0.2, 0.5, 1, or 2 Hz. Figure 5A shows depletions on a faster time scale than in Figure 5B. In the experiment shown in Figure 5B, increasing the post-rest frequency from 0.2 to 2 Hz increased the magnitude of the Ca\(^{++}\) depletion from 0.4 to 1.5 mV, and the maximum rate of depletion increased approximately 22-fold. Figure 6 illustrates the dependence of the magnitude and maximum rate of Ca\(^{++}\) depletion on stimulation frequency. The results have been normalized to the values at 1 Hz for each experiment. Alterations in the transmembrane sodium gradient are known to affect cellular calcium fluxes and would thus be expected to alter Ca\(^{++}\) depletions.

**Protocols with Primary Effects on Transmembrane Sodium Gradient**

When the extracellular [Na\(^+\)] is reduced to 70 mM, there is a transient decrease in [Ca\(^{++}\)]\(_o\) (not shown). This may be due to both increased calcium binding in the extracellular matrix and net calcium uptake by the cells. Figure 7 shows two examples of the effect of [Na\(^+\)]\(_o\) reduction on the cumulative depletion of Ca\(^{++}\). It can be seen that the Ca\(^{++}\) depletion can be inhibited almost completely by this degree of

**Figure 6.** The effect of stimulus frequency on the magnitude (○) and maximum rate (●) of the Ca\(^{++}\) depletions. Results have been calculated with respect to those found at 1 Hz. The points plotted are means ± SEM of between four and nine separate preparations.
reduction in $[\text{Na}^+]_o$. The degree of depression is graded with respect to reduction of $[\text{Na}^+]_o$ and is not always complete at 70 mM sodium. It is usually completely reversible. The results in Figures 1–6 have shown that the magnitude of the depletion signal becomes larger with interventions which increase contractile force. Low $[\text{Na}^+]_o$ and acetylstrophanthidin (below) illustrate that the magnitude of the depletion signal is decreased while contractile force is increased. This dissociation of contractile force and calcium depletion again provides evidence that the $\text{Ca}^{2+}$ depletions recorded are neither due to, nor affected by, mechanical artifacts.

Figure 8 demonstrates the effects of acetylstrophanthidin (ACS) on the cumulative $\text{Ca}^{2+}$ depletion induced by 1-Hz stimulation after 30-second (panel A) and 1-minute (panel B) rest intervals. In the experiment in Figure 8B, 2 nM acetylstrophanthidin reduced the $\text{Ca}^{2+}$ depletion to less than half that seen in control, and there was no significant increase in resting force. The effects of this exposure to acetylstrophanthidin were largely, but not completely, reversible. The experiment in Figure 8A is an example in which 3 nM acetylstrophanthidin eliminated the $\text{Ca}^{2+}$ depletion and produced, if anything, a net loss of calcium from the cells upon stimulation after a 30-second rest interval. This muscle exhibited a significant increase in resting tension and did not recover completely from exposure to acetylstrophanthidin. Addition of acetylstrophanthidin to the superfusate did not produce any significant initial transient change of the $[\text{Ca}^{2+}]_o$ during steady state stimulation (in comparison to those seen with cobalt and low sodium superfusion).

If, indeed, these $\text{Ca}^{2+}$ depletions represent cellular calcium uptake, it is important to evaluate the intracellular sites responsible for calcium uptake under these conditions. The SR is a likely possibility, and, as such, the effects of two agents known to alter SR calcium handling (caffeine and ryanodine) have been examined on $\text{Ca}^{2+}$ depletions.

### Agents with Primary Effects on Sarcoplasmic Reticulum

When 10 mM caffeine is added to the superfusate during steady stimulation at 0.5 Hz, there is a transient increase in twitch tension, and after a brief delay, there is a transient increase in $[\text{Ca}^{2+}]_o$. This is shown in Figure 9. The transient increase in contractile force may be due to the initial enhancement of calcium loss from the SR to the cytoplasm induced by caffeine. The subsequent rise in $[\text{Ca}^{2+}]_o$ may represent the transsarcolemmal loss of this calcium which had been stored in the SR. Equilibration of muscles with 10 mM caffeine inhibits the cumulative $\text{Ca}^{2+}$ depletion normally seen upon post-rest stimulation. Figure 9 shows an experiment in which a 5-minute exposure to 10 mM caffeine reduced the $\text{Ca}^{2+}$ depletion by about 50%. Figure 10A shows an experiment in which a 15-minute exposure to 10 mM caffeine virtually eliminated the $\text{Ca}^{2+}$ depletion induced by 1-Hz stimulation following a 30-second rest interval. The effects of caffeine were slowly but usually completely reversible.

Ryanodine, another agent which interferes with normal SR function, produces results which are opposite to those of caffeine. Figure 10B shows that ryanodine (1 $\mu$M) increases the magnitude of $\text{Ca}^{2+}$ depletion induced by 1-Hz stimulation following a 30-second rest interval. The addition of 1 $\mu$M ryanodine increases the magnitude of the $\text{Ca}^{2+}$ depletion often more than 2-fold (as in this example). The effects of ryanodine are only slightly reversible.

Figure 11 shows an experiment in which the following protocol was performed in control conditions (panel A), in the presence of 1 $\mu$M ryanodine (panel B) and in the presence of 1 $\mu$M ryanodine plus 10 mM caffeine (panel C). The protocol was steady state stimulation at 0.5 Hz, 30-second rest interval, 1-minute stimulation at 1 Hz, 1-minute rest interval, and, finally, 0.5-Hz stimulation. Ryanodine increases the magnitude of the cumulative $\text{Ca}^{2+}$ de-
Discussion

Character of Cumulative Depletions of Ca⁺⁺

The cumulative depletions of Ca⁺⁺ reported in the present study probably represent net cellular uptake (where transiently, calcium influx exceeds calcium efflux) under the nonsteady state stimulation protocols. Similarly, the increases of [Ca⁺⁺]o seen during rest intervals (e.g., Fig. 11B) and when caffeine is added (Fig. 9) probably represent net cellular losses of calcium. These conclusions are complicated, however, since any decrease of [Ca⁺⁺]o will eventually be replenished by diffusion, ultimately, from the superfusate. Thus, the ability to measure Ca⁺⁺ depletions requires that there be some diffusional limitation to calcium replenishment. It is also apparent that any Ca⁺⁺ depletion measured is an underestimate of the depletion which would be measured if there were no replenishment (both at 1 and 0.5 Hz). When caffeine is added, the Ca⁺⁺ depletions are reduced to levels below that seen in the pre-ryanodine control. When stimulation is stopped, [Ca⁺⁺]o, rises back toward the bath [Ca⁺⁺] (Fig. 11A). Ryanodine accelerates this calcium rise (Fig. 11B), and this may represent an increased loss of cellular calcium induced by ryanodine during quiescence. Caffeine (10 mM) exerts a very strong positive inotropic effect when applied in the continued presence of ryanodine (note the change in tension scale between panels B and C in Fig. 11). This is in contrast to the modest positive or negative inotropic effect of caffeine alone (e.g., see Figs. 9 and 10A, and Bers, 1985). The muscle shown in Figure 11 had been previously exposed to 10 mM caffeine (Fig. 9). In this experiment, 10 mM caffeine alone increased contractile force by 80%, but in the presence of ryanodine, 10 mM caffeine increased contractile force by 680%.

FIGURE 9. The effect of 10 mM caffeine on the Ca⁺⁺ and tension Top trace, E₆₃-E₉; bottom trace, tension. The first break in the trace was for a period of about 15 minutes and the second for a period of about 8 minutes. The diameter of the muscle bundle was 0.12 mm.
stration. In the steady state situation, calcium influx and calcium efflux must be the same, so that there is no net change in cellular calcium over one complete cardiac cycle. Thus, the $[\text{Ca}^{++}]_o$, must always return to the bath level as the steady state is approached, and the rate at which it returns will depend upon diffusional limitations. When a cumulative depletion is seen, the minimum $[\text{Ca}^{++}]_o$, observed will depend not only upon the rate of net calcium uptake, but also upon the rate of replenishment. In addition, the extracellular matrix probably buffers $[\text{Ca}^{++}]_o$ changes to some degree. For example, just the outer surface of the plasma membrane can bind more calcium than is required for myofilament activation (Bers, 1983). These same considerations apply to net calcium losses, where any increase of $[\text{Ca}^{++}]_o$, would be washed out by the superfusate calcium. Thus, both diffusion from the bulk solution and buffering by extracellular calcium-binding sites would tend to restore the local $[\text{Ca}^{++}]_o$ toward that in the bulk solution.

Whereas the changes of $[\text{Ca}^{++}]_o$, are usually interpreted to reflect cellular calcium gain or loss, caution must be exercised, as there are other possible explanations for some of the observed $[\text{Ca}^{++}]_o$, changes. For example, there may be changes in the amount of calcium bound to extracellular moieties and the effect of cobalt addition to displace calcium (see Fig. 2) illustrates the possible magnitude of such effects. The changes in extracellular $[\text{Ca}^{++}]_o$ induced by such displacements of extracellular calcium are transient due to replenishment by the bulk solution and would not necessarily be expected to influence the cumulative $\text{Ca}_{o}^{++}$ depletions which are induced by activation during continued exposure to an agent. Furthermore, unless such activation-dependent increases in calcium binding were specifically inhibited by cobalt, nifedipine, caffeine, acetyl strophanthidin, and low $[\text{Na}^+]_o$, or were enhanced by Bay k 8644, increased frequency, and ryanodine, the most probable interpretation of these cumulative $\text{Ca}_{o}^{++}$ depletions is that they represent cellular calcium uptake. These cumulative $\text{Ca}_{o}^{++}$ depletions occur

* In experiments with isolated rabbit ventricular sarcolemmal vesicles, we have found that, at least, sarcolemmal calcium binding is not significantly altered by membrane potential (Mansier and Bers, 1984), acetyl strophanthidin, nifedipine, Bay k 8644, caffeine, or ryanodine (Bers, et al., 1986) Cobalt and changes of $[\text{Na}^+]_o$, did, however, affect calcium binding It may be noted that, whereas steady state exposure to both cobalt and nifedipine block the cumulative $\text{Ca}_{o}^{++}$ depletion, only cobalt appears to displace $\text{Ca}_{o}^{++}$ when it is applied (see Figs 2 and 3).
under conditions where cellular calcium uptake may be expected. They are also inhibited by exposure to agents known to inhibit calcium influx (nifedipine and cobalt) and enhanced by agents which increase calcium influx (Bay k 8644) and calcium uptake (increased frequency). Although the interpretations of the results with caffeine, acetylstrophanthidin, low [Na\(^+\)]\(_o\), and ryanodine (see below) are somewhat more complicated; they are completely consistent with this interpretation and, also, with what is known about how these agents work.

Another aspect which bears on the quantitation of these Ca\(_{0}^{++}\)-depletion signals is that the precise extracellular location of the microelectrode tip is not known. This adds to the difficulty of extrapolating with quantitative precision from the depletions measured to transsarcolemmal fluxes per unit tissue. It is worth noting that the time course and magnitude of the cumulative Ca\(_{0}^{++}\) depletions reported here using calcium microelectrodes are very similar to those reported by Hilgemann and Langer (1984) who used extracellular calcium-sensitive dyes which are likely to be distributed uniformly in the extracellular space. This similarity is remarkable, considering the difference of technique and technical limitations. The disadvantages of the calcium dye technique are that the dyes add substantial calcium buffering to the extracellular space; it is difficult to use other divalent cations such as cobalt, and the optical signals are very sensitive to motion. The biggest disadvantages with the calcium microelectrode are the lack of knowledge of its precise location in the extracellular space and the small signals which require extremely stable double-barreled calcium microelectrodes. Mechanical artifacts are not responsible for the signals recorded, since the signals can be eliminated by acetylstrophanthidin, [Na\(^+\)]\(_o\), reduction, and caffeine when contractile force is increased. Electrical artifacts are also not responsible for the signals recorded, since the depletion signals can be eliminated by cobalt, nifedipine, low [Na\(^+\)]\(_o\), acetylstrophanthidin, and caffeine, while electrical activity and action potentials (sometimes modified) continue. The rapid electrical time constants of the electrodes used in these experiments (e.g., see Bers, 1983) would also make this potential problem unlikely.

The magnitude of the cumulative depletions often amount to 20 \(\mu\)M or 10% of the bath [Ca\(^{++}\)] (this would correspond to a 1.3-mV decrease in the calcium signals in the figures). Using extracellular calcium dyes and similar protocols in rabbit ventricle, Hilgemann and Langer (1984) also reported cumulative Ca\(_{0}^{++}\) depletions of 10% at comparable free [Ca\(^{++}\)]. Based upon this quantitative similarity, it may be reasonable to assume that the Ca\(_{0}^{++}\) depletion measured by the calcium electrode is roughly representative of the mean extracellular space. If it is further assumed that the volume of the extracellular space is 0.33 liter/g wet weight (Lee and Fozzard, 1975), this calcium uptake would be 6.6 \(\mu\)mol/kg wet weight. This amount of calcium uptake is of the same order as that which would be required to produce the small twitches observed in 0.2 mM Ca\(_{0}^{++}\) (Solaro et al., 1974). However, it should be emphasized that the cumulative Ca\(_{0}^{++}\) depletions reported here do not reflect specific aspects of the complex intracellular calcium transients. The cumulative Ca\(_{0}^{++}\) depletions simply represent calcium which has entered the cells and has not come back out at the same beat. Where this calcium might be located within the cell during diastole is considered in the next section.

In addition to these cumulative depletions which represent net calcium uptake, it has been reported previously that phasic calcium depletions represent calcium influx during individual beats (Bers, 1983, 1985). These studies of phasic Ca\(_{0}^{++}\) depletion indicated that the calcium influx associated with each contraction increased monotonically from small values at the first post-rest beat to a steady state value. The steady state value of the phasic Ca\(_{0}^{++}\) depletions under the same experimental conditions used in the present study were 8.5 \(\mu\)M (Bers, 1983). Thus, it would seem that the amount of net calcium uptake is only of the order of 2–3 times the amount of calcium which cycles into and back out of the cell during each beat.

The cumulative Ca\(_{0}^{++}\) depletion is usually small at the first post-rest beat, but the net gain of calcium per beat increases rapidly so that the maximum rate of depletion occurs after just a few beats. It seems probable that the cells retain a larger percentage of the calcium which entered during the first few beats, and that it takes many beats for efflux to match the levels of influx. It is also possible that the slow phase of tension recovery after rest or change of stimulation frequency may be partly due to the slow recovery of [Ca\(^{++}\)]\(_o\) back to the bath level after the initial depletion.

Increasing frequency of post-rest stimulation from 0.2 to 2 Hz increases both the magnitude and maximum rate of cumulative Ca\(_{0}^{++}\) depletions (see Fig. 6). This increase in calcium uptake with increasing frequency could be attributed to either solely increased calcium influx or solely decreased calcium efflux per unit time. It seems more likely that there is some combination of these two effects, since there would be an increased number of beats where depolarization-dependent calcium influx would occur and a decrease in diastolic interval during which at least some part of cellular calcium efflux occurs. In addition, if intracellular sodium activity increases with these frequency increases (Cohen et al., 1982; Ellis, 1985), this would be expected to shift the sodium-calcium exchange system in the direction of increased calcium uptake (which again could be due to some combination of increased calcium influx during systole and decreased calcium efflux during diastole). It seems probable that a decrease of calcium efflux contributes significantly to the frequency dependent enhancement of the Ca\(_{0}^{++}\) depletion for...
the following reason. In six experiments, 1 μM Bay k 8644 increased force to 903 ± 172% (n = 6) of control, presumably due to increased calcium influx [and not inhibition of calcium efflux (Schramm et al., 1983)]. This large increase in force is associated with an increase in \( \text{Ca}^{++} \) depletion to only 167 ± 11% (n = 6) of control. On the other hand, this large inotropy is probably due primarily to increased calcium influx via calcium channels. Efflux may nearly compensate for this enhanced influx, so there is only a modest net gain of calcium by the cell. Increasing frequency of stimulation from 0.2 to 2 Hz after a rest interval increases twitch tension to ≤200% of control, but increases \( \text{Ca}^{++} \) depletion magnitude to 620 ± 150% (n = 4) of the control value. Thus, it seems that, in addition to increased calcium influx, decreased calcium efflux is a major factor in the frequency-dependent increase in \( \text{Ca}^{++} \) depletion magnitude where there is only modest positive inotropy. Restated, a primary increase in calcium influx can produce a dramatic increase in tension, with only a modest increase in calcium content. Shifting the balance of influx and efflux to produce a larger increase in calcium content does not necessarily imply greater inotropy.

**Cellular Locus of Calcium Uptake Responsible for \( \text{Ca}^{++} \) Depletions**

Thus far, the intracellular locus for the calcium which is taken up by the cells under conditions where cumulative depletions are seen has not been discussed. We hypothesize that the cumulative calcium depletions represent calcium taken up by the cell and stored primarily by the SR. The increase in calcium uptake after longer rest intervals (e.g., Fig. 1) is consistent with the frequently used explanation for rest decay of force. Many others have attributed the decline in the first post-rest beat with increasing rest duration to the loss of releasable calcium from the SR (e.g., Wood et al., 1969; Allen et al., 1976; Edman and Johannsson, 1976; Beresewicz and Reuter, 1977; Lewartowski et al., 1978; Reiter et al., 1984; Bers, 1985; Sutko et al., 1986). Thus, the longer the rest interval, the more empty the SR, and thus the greater the potential for refilling upon resumption of stimulation. The increases of \( \text{Ca}^{++} \) depletion with increased frequency and Bay k 8644 are consistent with this hypothesis. That is, an increase of calcium influx and/or a decrease of calcium efflux would be expected to increase the level of SR calcium loading. It would also be expected that the SR would be better loaded after a train of 1-Hz or 2-Hz stimulation. Thus, the next contraction after a suitable interval would be enhanced, reflecting greater SR calcium release. This can be seen in Figure 1, 2 (at right), 3 (at left), and 9 (at left). When calcium influx is blocked with cobalt or nifedipine, refilling of the SR may be inhibited and, hence, no \( \text{Ca}^{++} \) depletion is seen.

The post-rest calcium uptake is also inhibited by caffeine. This would be consistent with the SR being the locus of this calcium uptake. Exposure to 10 μM caffeine may inhibit the ability of the SR to accumulate calcium (figures 9 and 10A). It can also be seen in Figure 9 that caffeine inhibits the potentiation of the first contraction after the train of 1-Hz stimulation (where this potentiation was attributed to SR calcium loading above). In addition, it should be noted that caffeine inhibits the cumulative \( \text{Ca}^{++} \) depletions despite the fact that it has been reported to increase calcium influx (Blinks et al., 1972; Yatani et al., 1984; Bers, 1985). This emphasizes the distinction between calcium influx and calcium uptake (as monitored by cumulative \( \text{Ca}^{++} \) depletions). The greater amount of calcium which entered in the presence of caffeine must be extruded before the next beat. This may occur because, in the presence of caffeine, the SR is unable to take up calcium and hence compete with calcium efflux mechanisms. Caffeine also increases the calcium sensitivity of the myofilaments (Fabiano, 1981; Wenda and Stephen- son, 1983). When SR function is already compromised by exposure to ryanodine, caffeine exerts a strong inotropic effect (see discussion of Fig. 11, pp. 775 and 776). This may result from the positive inotropic effects of enhanced calcium influx and myofilament sensitivity acting without the negative inotropic effect of SR depression.

When \([\text{Na}^{+}]_{o}\) is reduced or acetylstrophanthidin is applied, the inwardly directed sodium electrochemical gradient is reduced. This, in turn, shifts the sacrolemmal sodium-calcium exchange system in the direction favoring calcium uptake. Sometimes a transient decrease in the \([\text{Ca}^{++}]_{o}\) can be seen when \([\text{Na}^{+}]_{o}\) is reduced or acetylstrophanthidin is added, and this may reflect a net gain of cell calcium. The shift of the sodium-calcium exchange has been suggested to limit the efflux of cellular calcium during rest intervals and to be responsible for the dramatic slowing of rest decay of tension (Sutko et al., 1986). In other words, when diastolic calcium efflux via the sodium-calcium exchange is inhibited, the SR is not depleted of calcium during rest. Thus, the SR remains relatively loaded with calcium, and the first post-rest contractions are large (see Figs. 7 and 8). Cardiac glycosides and low \([\text{Na}^{+}]_{o}\) have also been shown to increase both steady state cellular calcium content (Langer and Serena, 1970; Wenda and Langer, 1977) and resting intracellular calcium activity (Lee et al., 1980; Marban et al., 1980; Bers and Ellis, 1982). Thus, the SR is likely to be relatively loaded with calcium and will not be depleted greatly during rest. This may explain the finding that low \([\text{Na}^{+}]_{o}\) and acetylstrophanthidin inhibit the depletion of \( \text{Ca}^{++} \) induced by post-rest stimulation (Figs. 7 and 8). The SR may still contain the amount of calcium which would be associated with steady stimulation, and thus no additional calcium is taken up when stimulation is resumed.
With both acetylstrophanthidin and low [Na\(^+\)]\(_{o}\), we occasionally saw a small net loss of calcium during resumption of stimulation (e.g., Fig. 8A). This might simply be a mass action effect, where mean intracellular calcium activity is very high during post-rest stimulation due to calcium loading, and therefore calcium efflux is enhanced. Bay k 8644 may increase calcium influx and availability to the myofilaments, but probably does not shift the sodium-calcium exchange system dramatically and does not limit calcium efflux during rest intervals.

It is conceivable that mitochondria are in part responsible for the calcium uptake seen during post-rest stimulation. However, this seems unlikely, since low [Na\(^+\)]\(_{o}\), and acetylstrophanthidin might be expected to have rather different effects due to the Na\(^+\) dependence of calcium efflux from mitochondria (Crompton et al., 1978). Both interventions increase cellular calcium loading, but acetylstrophanthidin increases intracellular sodium activity, while [Na\(^+\)]\(_{o}\) reduction decreases intracellular sodium activity. Thus, acetylstrophanthidin might be expected to deplete mitochondrial calcium, while [Na\(^+\)]\(_{o}\) reduction might be expected to enhance mitochondrial calcium, but both interventions produced essentially the same result. The Ca\(^{2+}\) depletions induced by post-rest stimulation, even under control conditions, are only a small fraction of the calcium uptake induced by acetylstrophanthidin (Langer and Serena, 1970) or reduction of [Na\(^+\)]\(_{o}\) (Wendt and Langer, 1977) (these studies were carried out on rabbit ventricular muscle using radioactive calcium and 5-10 times higher [Ca\(^{2+}\)]\(_{o}\)). It is possible that most of the net calcium uptake during steady state exposure to acetylstrophanthidin or low [Na\(^+\)]\(_{o}\) is accumulated in the mitochondria, and this would agree with quantitative estimates of SR and mitochondrial calcium capacities discussed by Chapman (1983). These mitochondrial calcium changes may not be as directly activation dependent, and may be too slow to be detected by the extracellular calcium electrode (due to relatively rapid diffusional replenishment). It is certainly still possible that slower changes in mitochondrial calcium loading are occurring, but it is not apparent in our records.

When a single stimulus is given shortly after a train of stimuli at 2 Hz (as in Fig. 1), the accompanying contraction is very large, and the loss of calcium from the cell is accelerated transiently. This large single beat probably is caused by a very large SR calcium release (since the SR was loaded by the 2-Hz train), although only a relatively small influx appears to occur with this first beat after a short rest interval (Bers, 1983; 1985). Calcium efflux would thus be enhanced due to the elevated intracellular calcium activity. Using calcium-sensitive dyes and this protocol, Hilgemann and Langer (1984) showed that the single beat after a 2-Hz train produced an instantaneous and complete replenishment of Ca\(^{2+}\)\(_{o}\). We have never seen a larger replenishing effect with this protocol than that illustrated in Figure 1. It is possible that this difference is a reflection of physiologically relevant differences in the techniques employed. For example, the calcium dye is presumably in transverse tubules. If the calcium efflux seen by Hilgemann and Langer (1984) occurred primarily into transverse tubules, the calcium microelectrode (which cannot be in a transverse tubule) would sense a smaller or slower efflux.

Ryanodine, in contrast to caffeine, enhanced the magnitude of the Ca\(^{2+}\)\(_{o}\) depletions induced by post-rest stimulation. These effects of caffeine and ryanodine on post-rest Ca\(^{2+}\)\(_{o}\) depletions have also been reported by Hilgemann et al. (1983) in guinea pig atrium using extracellular calcium dyes. These results demonstrate that these two agents which inhibit SR function do so by different mechanisms. Caffeine probably makes the SR unable to accumulate calcium, and thus is unable to release calcium (Weber and Herz, 1968). Ryanodine, on the other hand, appears to inhibit SR calcium release (Fabiato, 1985; Sutko et al., 1985; Sutko and Kenyon, 1983), but has been shown to increase SR calcium uptake (Jones et al., 1979). This mechanism of action is consistent with the result that ryanodine increased the cellular calcium uptake during post-rest stimulation. Ryanodine also appears to enhance calcium loss when stimulation is stopped. Figure 11B shows that, in the presence of ryanodine, there is a large loss of cell calcium which starts within the first 3 seconds after stimulation is stopped and [Ca\(^{2+}\)]\(_{o}\) transiently exceeds the bath level. It seems that, during continuous stimulation, ryanodine enhances the uptake of calcium by the SR and, thereby, increases the magnitude of the cumulative Ca\(^{2+}\)\(_{o}\) depletion. Only after stimulation is terminated does ryanodine appear to accelerate calcium loss from the cell. It is tempting to speculate that this represents the enhancement of calcium efflux from the SR directly into the extracellular space, without passing through the cytoplasm. It seems unlikely that this calcium has leaked from the SR into the cytoplasm, and has then been pumped out of the cell, since there is no transient rise in tension (in fact, resting tension is lower than in control). Sutko et al. (1983) have also demonstrated that, under resting conditions where the SR is heavily calcium loaded (zero Na\(^+\)), the application of ryanodine produces relaxation, whereas caffeine produces large contractures. Ryanodine may enhance Ca\(^{2+}\)\(_{o}\) depletions by: (1) increasing the amount of calcium accumulated by the SR during stimulation (which could, via mass action, increase the amount of calcium lost via the normal pathways responsible for depleting the SR during rest), (2) enhancing loss of SR calcium from the cell during rest (such that the SR can accumulate more calcium during stimulation when this calcium efflux pathway does not appear to function), (3)
some combination of these two possible primary effects. The present results do not distinguish between these possibilities.

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Address for reprints: Dr. Donald M. Bers, Division of Biomedical Sciences, University of California, Riverside, California 92521-0212.

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INDEX TERMS. Excitation • Contraction coupling • Cobalt • Nifedipine • Bay k 8644 • Caffeine • Ryanodine • Frequency
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D M Bers and K T MacLeod

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