Extracellular Calcium Transients and Action Potential Configuration Changes Related to Post-Stimulatory Potentiation in Rabbit Atrium

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ABSTRACT Extracellular calcium transients were monitored with 2 mM tetramethylmurexide at low calcium (250 μM total, 130 μM free), and action potentials were monitored together with developed tension at normal calcium (1.3 mM) during the production and decay of post-stimulatory potentiation in rabbit left atrial strips. At normal calcium, the contractile potentiation produced by a brief burst of 4 Hz stimulation is lost in three to five post-stimulatory excitations, which correlate with a negative staircase of the late action potential. At low calcium, stimulation at 4 Hz for 3–8 s results in a net extracellular calcium depletion of 5–15 μM. At the subsequent potentiated contraction (1–45 s rest), total extracellular calcium increases by 4–8 μM. The contractile response at a second excitation is greatly suppressed and results in little or no further calcium shift; the sequence can be repeated immediately thereafter. Reducing external sodium to 60 mM (sucrose replacement) enhances post-rest contractions, supresses the late action potential, nearly eliminates loss of contractility and net calcium efflux at post-rest excitations, and markedly reduces extracellular calcium depletion during rapid stimulation. 4-Aminopyridine (1 mM) markedly suppresses the rapid early repolarization of this preparation at post-rest excitations and the loss of contractility at post-rest stimulation from the rested state; during a post-stimulatory potentiation sequence at low calcium, replenishment of extracellular calcium takes several post-stimulatory excitations. Ryanodine (10 nM to 5 μM) abolishes the post-stimulatory contraction at rest periods of >5 s. If the initial repolarization is rapid, ryanodine suppresses the late action potential, calcium efflux during quiescence is greatly accelerated, and subsequent excitations do not result in an accumulation of extracellular calcium. A positive staircase of the early action potential correlates with the magnitude of net extracellular calcium depletion. These findings demonstrate that negative contractile staircases at post-rest stimulation correspond closely to an accumulation of extracellular calcium at activation and a negative staircase of the late action potential; the correlation of these three events suggests that electrogenic sodium-calcium exchange is the common underlying mechanism.

INTRODUCTION

The experimental definition of cardiac excitation-contraction coupling obviously depends on the means available to characterize trans-sarcolemmal calcium movements in relation to electromechanical events. A potentially powerful new approach is the attempt to monitor extracellular calcium concentration changes, which are associated with activation or inactivation sequences, using extracellularly applied calcium-sensitive dyes (Hilgemann et al., 1983; Hilgemann and Langer, 1984; Cleeman et al., 1984). This spectrophotometric method is attractive because it is noninvasive, it can be applied to intact preparations not suitable for other means of investigation, and it offers potentially both high kinetic and quantitative resolution. The method is limited primarily by the ability to differentiate with confidence light changes caused by muscle movement from light changes caused by calcium-dye interactions. This article describes in rabbit atrium net extracellular calcium shifts associated with the positive inotropic effect produced by a few rapid stimulations (post-stimulatory potentiation) and loss of the enhanced contractility at subsequent excitations.

Non–steady state stimulation of cardiac muscle reveals intrinsic inotropic mechanisms, which have long been considered a "handle" on basic processes involved in excitation-contraction coupling (e.g., Koch-Weser and Blinks, 1963). The analytical description of post-stimulatory potentiation in mammalian cardiac muscle dates to the turn of the century (Woodworth, 1902). Briefly, in mammalian cardiac muscle, "premature" or early, rapid excitations at intervals of ~150–500 ms result in relatively small contractions. A subsequent contraction is enhanced or "potentiated." This type of contractile potentiation is lost with a strong dependence on the number of post-stimulatory excitations, and in rabbit myocardium the potentiation can be lost for the most part at a single excitation (Wohlfart, 1979). The extensive literature pertinent to this contractile pattern cannot be reviewed here, except to point out wide agreement that the contractility changes reflect the function of an internal calcium store and the beat-to-beat exchange of calcium to such a store. Regarding the loss of the potentiation, Rosin and Farah (1955) concluded that in rabbit atrium the contraction "consumes" the contractile potentiation. Wood et al. (1969) suggested from similar observations that a large portion of the calcium activating contraction does not return to release sites at relaxation, and often the loss of potentiated contractility has been interpreted as a loss of activating calcium by sarcolemmal extrusion in competition for re-uptake by the sarcoplasmic reticulum ("recirculation hypothesis"; Morad and Goldman, 1973). The apparent activation dependence of calcium efflux, suggested by these early considerations, has been a latent problem in the study of cardiac excitation-contraction coupling and electrophysiology. Direct evidence for this assumption has recently been provided with extracellular calcium transients measured in rabbit ventricle by the dye method (Hilgemann and Langer, 1984). The loss of post-stimulatory potentiation at individual excitations was associated with a net increment of extracellular calcium several times greater than the net influx found during individual rapid stimulations.

For many years, sodium-calcium exchange has been the primary candidate for calcium extrusion in cardiac muscle (Reuter and Seitz, 1968), although some

authors (e.g., Langer, 1982) stress its possible role in calcium influx. At present there is wide, but not unequivocal (Eisner and Lederer, 1985), agreement that the exchanger is electrogenic, with a 3:1 stoichiometry (Reeves and Hale, 1984). In this context, the activation dependence of calcium efflux would be consistent with the reinterpretation of "slow inward current" in cardiac muscle, as a mixed current with at least a significant component related to calcium extrusion by electrogenic sodium-calcium exchange (Mullins, 1979, 1981; Fischmeister and Vassort, 1981; see Noble, 1984, for a recent review). With regard to the present study, it would be postulated that at potentiated contractions intracellular free calcium rapidly reaches concentrations high enough to favor extrusion, that the exchanger is physiologically fast enough to compete heavily with the sarcoplasmic reticulum for calcium released, and that this mechanism is the primary route of calcium efflux. Calcium extrusion would account for the loss of potentiated contractility by reducing the amount of calcium available for release at a subsequent excitation.

The results of two recent electrophysiological studies in multicellular preparations are indeed consistent with this interpretation. Simurda et al. (1981) described the activation of an inward current at unusually negative potentials during post-stimulatory potentiation sequences in mammalian ventricular muscle. The current was largest at strongly potentiated contractions initiated by small depolarizations. Both the contractile potentiation and inward current decayed within one or two contraction cycles at low-level depolarizations. Brown et al. (1984) interpreted both positive and negative staircases of the slow inward current transient in rabbit sinus node as possible fluctuations of a sodium-calcium exchange current, which would depend on internal release of calcium; this interpretation was based largely on a modeling of the currents. Some of the complexities of these experiments point out the value of an alternative method to approach the problem. The currents reflecting calcium influx and efflux would be of the same sign, would overlap, and would be interdependent (e.g., calcium efflux would depend on previous calcium influx), and multiple ionic interpretations are possible.

One of the reasons for the choice of rabbit atrium was the description of marked action potential changes (Tanaka et al., 1967; Saito, 1971), which might be related to the mechanisms of interest. Briefly, the action potential plateau is remarkably suppressed after periods of quiescence. This is reflected in a very rapid repolarization, which can be followed by a late, low-level depolarizing phase or "hump" with slow terminal repolarization. During repetitive excitations, the late, low-level phase is suppressed, early repolarization slows, and an action potential plateau develops over 5–10 excitations. A second reason for this choice was that larger net extracellular calcium accumulations could be obtained at single excitations in rabbit atrium than in any other preparation. Three interventions at different levels of excitation-contraction coupling will be described on the post-stimulatory potentiation sequence: (*a*) treatment with ryanodine, which abolishes post-rest contractility in mammalian myocardium (Furchgott, 1964; Hajdu, 1969), presumably by an action on the sarcoplasmic reticulum (Sutko and Kenyon, 1983; Hunter et al., 1983; Hilgemann et al., 1983; Fabiato,

1985); (b) addition of 4-aminopyridine (4-AP), which markedly slows early repolarization at a post-rest excitation in this preparation; (c) a reduction of external sodium by iso-osmotic sucrose replacement, which equilibrates to a modest reduction of the steady state sodium gradient (Sheu and Fozzard, 1982). The effect of β-adrenergic stimulation is described as a control intervention, which increases calcium influx during the production of post-stimulatory potentiation. Many of the extracellular calcium transients described here have been resolved at high temporal resolution (Hilgemann, 1986).

METHODS

Male New Zealand rabbits (1–2.5 kg) were killed for various experimental purposes by intravenous injection of 50 mg/kg pentobarbital with 200 U/kg heparin. The heart was rapidly excised, the left atrium was cut away, and the atrial roof was inspected in an oxygenated dissection chamber for a suitable preparation. If a portion of the atrial roof was nontrabecular, homogeneous, and 0.4–0.7 mm in thickness, atrial strips ~5 mm^2 were prepared. Suitable preparations, found in ~30% of the atria inspected, were mounted horizontally, endocardial side up, in a recirculating, gas lift superfusion chamber similar to that described previously (Hilgemann et al., 1983). The flow speed directed toward the muscle was at least 3 cm/s. The temperature was maintained at 29–31°C. After isolation, atria were equilibrated for 30 min in a 2 mM calcium solution at 0.5 Hz stimulation. Stimulation pulses (1-ms square pulses via a punctate platinum electrode) were set at just above threshold and could be increased by a factor of 3 without inotropic effect.

Action potentials were measured with flexibly mounted glass microelectrodes (tip resistances, 10–30 MΩ) filled with 3 M KCl (Woodbury and Brady, 1956), and were either photographed from a storage oscilloscope record or acquired digitally for later display. Developed tension was monitored with a capacitance force-transducer. In experiments where extracellular calcium transients are described, only light signals are given, for the sake of simplicity. Over the course of three years' experience with this technique, I have found the motion artifact in light signals to be a surprisingly reliable monitor of relative developed tension (see Hilgemann et al., 1983, and Hilgemann and Langer, 1984, for parallel measurements; in the following article, precise temporal relationships between the two signals are given; see also Kass, 1981). In principle, the motion artifact is the more sensitive measure of contractile activity, particularly at the low levels of contractility of this study, since viscous-elastic elements of the muscle and mechanical drag in the experimental apparatus would damp the measurement of small contractile events via developed force at the ends of the muscle.

To avoid calcium buffering by the bath solution, a bicarbonate-free, phosphate-free solution was used. In spite of these omissions, muscle function was essentially stable for several hours. The composition of the solution was (mM): 145 Na$^+$, 138 Cl$^-$, 2.5 K$^+$, 0.5 Mg^{++}, Ca^{++} as given in text and figures, 15 dextrose, 20 HEPES, pH 7.3. The solution was bubbled to saturation with 100% O$_2$. Calcium contamination from the water source and salts was estimated at 25 μmol/liter in the final solutions (calcium electrode measurements), and the contamination was compensated for in the final preparation of solutions.

Photometric measurements were made essentially as described previously (Hilgemann et al., 1983). Briefly, ultraviolet-infrared–blocked light from a highly regulated, 100-W, quartz-halogen source was brought to the underside of the muscle with a fiber optic probe 1.5 mm in diameter. A fiber optic probe 5 mm in diameter, which trifurcated randomly, was used to collect transmitted light by immersing it in the bath to a distance of ~0.3 mm

from the upper muscle surface. The light from the three fiber optic outputs was collimated for interference filtering (20-nm bandpass) and detection with photomultipliers (470-nm signal and, in most cases, the 580-nm signal) or photodiodes (680-nm signal). The individual signals were offset close to electrical ground, amplified, and calibrated via 0.1-A neutral density filtering at the light source. Signals virtually identical to those described here have been obtained using monochromatic illumination and wide-angle photomultiplier detection.

Tetramethylmurexide was used as the calcium-sensitive dye at a concentration of 2 mM, the highest possible concentration that did not result in precipitation over 2–3 h. Fig. 1 shows absorbance spectra of 2 mM tetramethylmurexide with the given total calcium concentrations. In the lower panel, calibration curves are given for the wavelengths routinely monitored in experiments. The differences between the calibrations made in a commercial spectrophotometer (DU-8, Beckman Instruments, Inc., Palo Alto, CA) or made in the experimental set up (1-mm light path) were <5%. An extracellular calcium change measured in experiments would ideally generate absorbance shifts at the calcium-dependent wavelengths, in opposite directions, in an equivalent ratio to these calibrations. In the large majority of experiments, discrepancies were <15% and maximal discrepancies were 25% from the expected ratio. Such discrepancies can be produced predictably by artificially creating an inhomogeneity of the muscle preparation with respect to the light path (the extreme case would be light passing around the edge of the muscle). Inhomogeneity of the illuminated portion of the muscle would also lead to an underestimation of trans-sarcolemmal calcium movements.

Complete calibrations of dye absorbance shifts with various calcium concentrations were performed at five dye concentrations from 3 μM to 2 mM in the muscle bath solution. The results could be very closely fit, assuming a single dye-calcium complex with a K_m of 3.2 mM. This value reflects a somewhat lower affinity than that given by Ohnishi (1978), and the difference is attributable to the sodium concentration of the muscle bath. The extracellular space of tetramethylmurexide was determined by washout of the dye from preparations as described previously. The value obtained was 37 ± 2 ml/100 g wet weight (SE; n = 6). Extracellular calcium transients were also calibrated as described previously (Hilgemann et al., 1983) from the extracellular space of the dye, muscle thickness, and dye calibration curve. Exchange of extracellular calcium with the bathing medium was found to take place with a time constant of 4–6 min (for brevity, no records are presented here), which is still slower than determinations in guinea pig atrium. Muscle thickness was determined both upon isolation of the muscles and at the end of experiments; this measurement is probably the major factor in determining the accuracy of calibrations.

The validity of signals, interpreted here as extracellular calcium concentration shifts, is supported by the following arguments. (*a*) The absorbance shifts at the three wavelengths routinely monitored have the expected wavelength dependence. (*b*) Virtually identical signals have been obtained with a second calcium-sensitive dye (antipyrylazo III) with a very different action spectrum. (*c*) The absorbance shifts interpreted as calcium concentration changes can be essentially abolished by the addition of a competing calcium buffer at a high concentration (an example is given in the Results). (*d*) Signals can be greatly decreased by increasing extracellular calcium toward saturating concentrations for the dye, whereas the magnitude of the wavelength-independent motion artifacts is greatly increased (an example is given in the Results). (*e*) The direction of light changes caused by muscle contraction can be inverted in different experiments, whereas calcium-dependent signals are the same.

In this article, the transmitted light intensity signals, calibrated in terms of absorbance, are presented without subtraction of the muscle movement component. Baseline absorb-

ance shifts caused by contraction are either small or negligible in the present work, so that diastolic absorbance shifts at the calcium-dependent wavelengths are closely related to calcium concentration changes. The presentation chosen, while perhaps visually complex, provides the reader with the critical controls of wavelength dependence for each result interpreted as a net extracellular calcium change. Also, it allows the reader to assess fully the possible effects of motion in the signals presented, and thereby eliminates many reservations that could justifiably be raised if only a movement-subtracted extracellular calcium transient were presented.
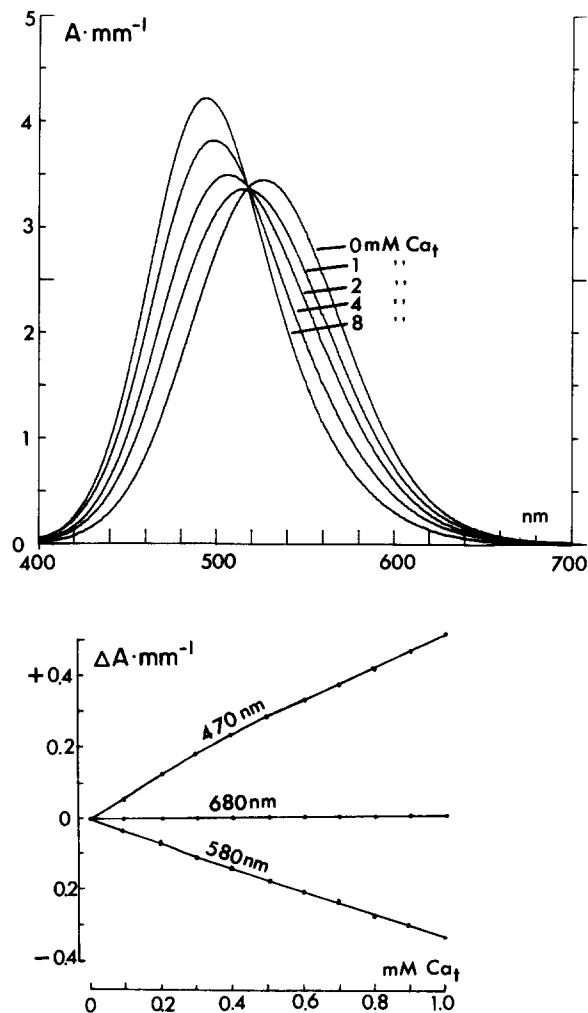

FIGURE 1.   The upper panel shows absorbance spectra of 2 mM tetramethylmurexide in the bath solution used in experiments at the given total calcium concentrations (DU-8 Beckman spectrophotometer; 200-μm cuvette). The lower panel shows differential absorbance shifts at the wavelengths routinely monitored in experiments over the range of 0–1 mM total calcium (1-mm cuvette).

A note on ryanodine pretreatments: Over the course of several years' experience with ryanodine in many different heart muscle preparations, I have found that the onset of action depends strongly upon stimulation of the muscle. Muscles can be pretreated during quiescence for prolonged periods of time (>20 min) with little or no effect on the first several contractions. During stimulation, the typical "ryanodine state" then develops irreversibly, and the degree of the effect achieved seems to depend on the development of a series of strong contractions rather than on excitation per se. Mitchell et al. (1984a) have described similar observations in isolated myocytes. It is apparent that ryanodine pretreatments cannot be standardized on the basis of concentration and time alone. These observations could be relevant to the recent finding that ryanodine binding to muscle triad preparations is strongly dependent on the presence of calcium (Pessah et al., 1985).

4-AP was obtained from Sigma Chemical Co., St. Louis, MO. Other chemical sources were as given previously (Hilgemann et al., 1983).

RESULTS

Action Potential and Contractile Staircases at Normal Calcium

Figs. 2–4 summarize typical action potential configuration changes and contractile responses of rabbit left atria to non–steady state stimulation at normal calcium, and describe the perturbations relevant to the loss of post-stimulatory potentiation mentioned in the Introduction. The calcium concentration (1.3 mM) was chosen so that a post-stimulatory beat after 4 Hz stimulation was still in the intermediate range of contractility for the preparations.

Fig. 2 describes action potential and contractile changes found when a few excitations during a rapid regular train of stimulation were omitted and stimulation at the regular rate was resumed. The muscle was stimulated regularly at 2.5 Hz and a pause of 4 s was interjected. The figure shows the first five contractions and action potentials after renewed stimulation. The first contraction is many times larger than the contractions at the regular, rapid stimulation rate. If the stimulation pause is kept relatively short (4 s here), the previous contractile state is achieved in just one or two excitations. The action potential accompanying the large contraction shows a very rapid repolarization, back to about −40 mV within 15 ms, and a delayed terminal repolarization in comparison with the steady state action potential. The action potential form returns to a steady state similar to the contractile response in just one or two excitations. While nearly all mammalian heart muscle preparations display early action potential shortening and late "trailing off" at potentiated contractions to some extent (see Schouten et al., 1985, for a recent study in rat heart), the magnitudes of both the action potential and contractile changes found in rabbit atrium are unusually large.

The following two figures describe possibilities for "isolating" positive and negative action potential staircases in this preparation. In Fig. 3A, stimulation at 1 Hz was begun after a 5-min rest period. The contractile response, typical for mammalian atrial muscle, is a relatively large rested-state contraction with a loss of contractility for the most part within two beats to a low level (a). The rested-state action potential (A, 1) shows a very rapid repolarization with 8 ms, followed by a small depolarizing phase over ~100 ms, and a final repolarization over 200

ms. During the next three action potentials, the rapid repolarization phase begins to slow, while the late, low-level depolarization is reduced and undergoes little further change. In part B, the stimulation frequency was increased from a steady frequency of 0.2 to 2 Hz. Contractility increased from a very low level to a moderately high level over the course of several excitations (b); a slower tension staircase that took several minutes to equilibrate is not shown here. At 0.2 Hz, the action potential is quite short (action potential [B, 1]; note expanded time scale); at 2 Hz, the plateau increased and repolarization slowed over the course of eight excitations to the typical steady state action potential.

In Fig. 3 C, post-stimulatory potentiation was produced by stimulation at 4 Hz for 6 s; after a 30-s rest period, long enough to attain maximally fast repolarization, the muscle was stimulated at 0.4 Hz and the decay of contractile potentiation
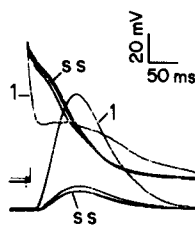

FIGURE 2.   Marked action potential and contraction changes in rabbit atrium during non–steady state stimulation. The muscle was stimulated regularly at 2.5 Hz, then left quiescent for 4 s, and stimulation at 2.5 Hz was resumed. The figure shows the first five excitations. Note the rapid repolarization at the post-rest excitation with delayed terminal repolarization in comparison with subsequent action potentials. Note the large magnitude of the post-rest contraction in relation to subsequent contractions.

was followed (see record c). The action potentials correspond to the first potentiated beat and the following three excitations. The accompanying configuration changes are similar to but more pronounced than those in part A. It should be noted that the late action potential and contractility decayed over several excitations in this case.

The effect of ryanodine on this sequence was studied as a possibly specific means to eliminate the influence of calcium store release at the post-stimulatory excitations. Between recordings C and D, the atrium was incubated for 15 min with 2×10^{-7} M ryanodine at 1 Hz stimulation, and the sequence was repeated. The first contraction after rest was completely abolished, which is typical for this level of ryanodine treatment; the positive contraction staircase at 4 Hz was changed only slightly (a small increase of rest tension during stimulation is typical), and rest periods of 10 s were long enough for contractility to decay back to the negligible rested-state level (record d). Upon stimulation at 0.4 Hz as in part C, the first action potential retained the rapid repolarization, but the late action potential "hump" was abolished (D, 1). During the subsequent four excitations, the slowing of repolarization and the progressive development of a plateau were similar to the control response. With longer rest periods, the first action potential did not change detectably.

Fig. 4 shows the action potentials and contractions of two other atria during post-rest stimulation to illustrate the observations considered most important. Part *A* illustrates a possibility to isolate the late negative action potential staircase, namely by beginning stimulation at 0.2 Hz rather than 1 Hz as described in Fig. 3*A*. Since the initial rate of repolarization approaches a maximum after 5–10 s of quiescence, there is little or no change of the early action potential at 0.2 Hz
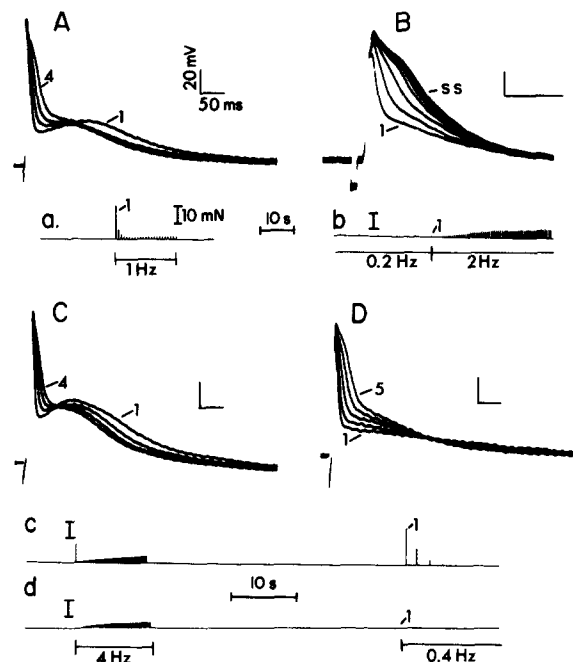

FIGURE 3. Typical action potential and contractile staircases of rabbit left atrium during non–steady state stimulation. (*A*) 1 Hz stimulation after a 5-min rest period. Note the large rested-state contraction and decay of contraction strength in the next two excitations (*a*). The first action potential (1) shows very fast repolarization and a late, low-level depolarization phase; at the next three excitations, the late phase is suppressed and repolarization slows. (*B*) Frequency increment from 0.2 to 2 Hz. Action potential 1 is the last excitation at 0.2 Hz (faster time scale than in *A*) and the next 10 action potentials at 2 Hz (ss = steady state). (*C*) Post-stimulatory potentiation produced by a burst of 4 Hz stimulations (see force record *c*). The figure shows the first four action potentials at 0.4 Hz after a 30-s rest period; contraction strength and the late action potential decay in three excitations. (*D*) Same stimulation pattern as in *C* after 15 min of ryanodine treatment (10^{-7} M). See text for further details.

stimulation; however, 80% recovery of the late action potential phase takes ≥1 min under these conditions (see also Tanaka et al., 1967). The recovery of the rested-state contraction after a single post-rest excitation (decay of the "negative inotropic effect of activation"; Blinks and Koch-Weser, 1961) takes place with a time course almost identical to that of the late action potential in this preparation (no results are presented for brevity). Note that the negative contraction staircase

and the loss of the late action potential phase are both complete in three excitations.

Although it is clearly possible to separate staircases of the early and late action potential, it was striking in this work that a rapid repolarization seemed to be a prerequisite for a well-developed late action potential staircase. This possibility could be verified, since 4-AP (0.3–2 mM) was found to greatly slow the initial
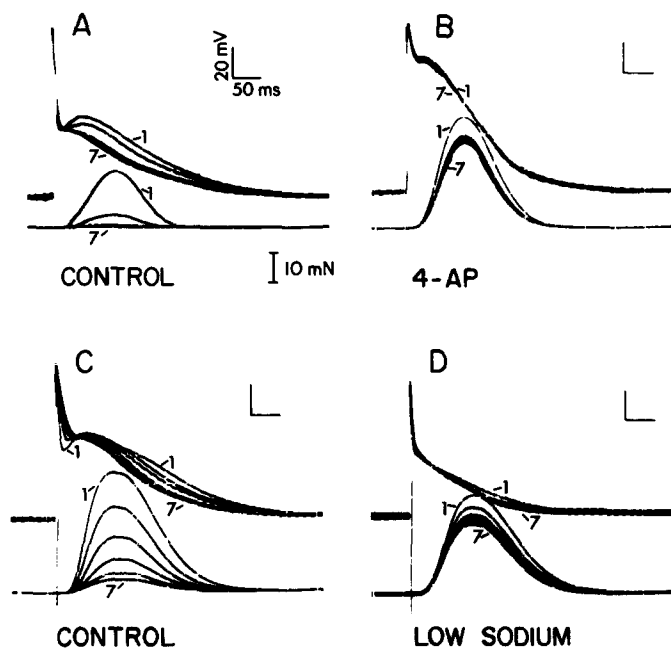

FIGURE 4.   Contractions and action potentials during post-rest stimulation. Part A is a control response for part B with 1.5 mM 4-AP. The stimulation protocol was 0.2 Hz stimulation after a 5-min rest period. Part C is a control response for part D with sodium reduction to 60 mM with sucrose replacement. The stimulation protocol was 0.4 Hz stimulation after a 4-Hz stimulation burst as in Fig. 3. Note the negative action potential staircase in A, with virtually no change of the early repolarization phase. Note also that a negative staircase of the late action potential correlates under all conditions with a negative contraction staircase. See text for further details.

repolarization at low frequencies, presumably because of blockade of the early outward current (Kenyon and Gibbons, 1979). Between parts A and B of the figure, 1.5 mM 4-AP was applied and the stimulation pattern was repeated. The first contraction was somewhat enhanced in relation to the pretreatment level, and subsequent contractions decreased very little in magnitude. The corresponding action potentials are almost superimposable. The effect of 4-AP on the first action potential is essentially similar to the action potential changes described after a brief stimulation pause in Fig. 2; when the early repolarization was inhibited, the late action potential was suppressed and the action potential

duration at 80% repolarization was decreased in comparison with part *A*. It should be noted that 4-AP did not suppress the post-stimulatory potentiation produced by brief high-frequency stimulation, but it did increase the number of excitations needed to dissipate the potentiation (described subsequently at low calcium).

As described in Fig. 4*C* (different preparation), loss of potentiation in one to three excitations was not invariable; most notably, prolonged steady stimulation promoted the loss of post-stimulatory potentiation over more excitations. Part *C* shows the first seven contractions and action potentials at 0.4 Hz, after a potentiating train of 4 Hz stimulation as described in Fig. 3. Note that the decay of potentiation took place over about seven excitations, and the late action potential also decayed over the seven post-stimulatory excitations. From 20 preparations studied, a large negative staircase of tension at post-rest stimulation was never found without a negative staircase of the late action potential over a comparable number of excitations.

Between parts *C* and *D* of Fig. 4, the bath sodium was reduced to 60 mM with iso-osmotic sucrose replacement for 15 min, and the post-stimulation potentiation sequence was repeated. The first contraction (1) after 4 Hz stimulation is actually slightly smaller than the potentiated contraction (1) under control conditions. The negative staircase of tension is very small in comparison with part *C*; the steady state contractions at 0.4 Hz (approximately the level of contraction 7) are greatly increased in comparison with the control in part *C*. With long rest periods, the contraction magnitude virtually did not change, and stimulation at 0.1–0.4 Hz from the rested state resulted in contraction changes similar to those shown here. In low sodium, the late action potential was very markedly reduced and underwent little change during the low-frequency post-rest stimulation. It should be mentioned that low external sodium does not suppress the drop in contractility associated with a premature excitation ("restitution" of contractility), as described in Fig. 10.

Extracellular Calcium Transients During Post-Stimulatory Potentiation

Fig. 5 describes the development of extracellular calcium depletion/replenishment signals associated with post-stimulatory potentiation and the typical effect of isoproterenol on the sequence (250 μM total calcium, 130 μM free calcium). In part *A*, signals are presented after a 30-min period of quiescence; the muscle was stimulated for 8 s at 4 Hz, followed by renewed quiescence and the addition of a single excitation during the quiescent period. The virtual absence of muscle movement (see the calcium-independent 680-nm signal) is typical after such a long rest period at this calcium concentration. The 0.0012-*A* increase of absorbance at 580 nm and the 0.0025-*A* decrease of absorbance at 470 nm would correspond to a fall of mean extracellular calcium from 250 to 230 or 225 μM, respectively, based on muscle dimension (0.5 mm maximum thickness), extracellular dye space (35 ml/100 g), and dye calibration curves. During quiescence after the stimulation train, the return of calcium-dependent light signals toward baseline is very slow and still not complete after 2.5 min, as shown here. It is slow enough that diffusion of calcium from the muscle bath into the depleted extracellular space can be expected to contribute significantly to the replenish-

ment of calcium. As shown, the post-stimulatory excitation made after the first stimulus train typically resulted in neither a calcium nor a movement response.

Between parts A and B of the figure, the stimulation sequence (4 Hz for 8 s with 1 min quiescence) was repeated five times, and the signals reflected a cumulative depletion of extracellular calcium at each burst of rapid stimulation. In the course of the repeated stimulation sequence, the pattern of light changes described in Fig. 5B became stable and typically remained stable for several hours. Part B shows the effects of an 8-s rapid stimulation at 4 Hz, followed by
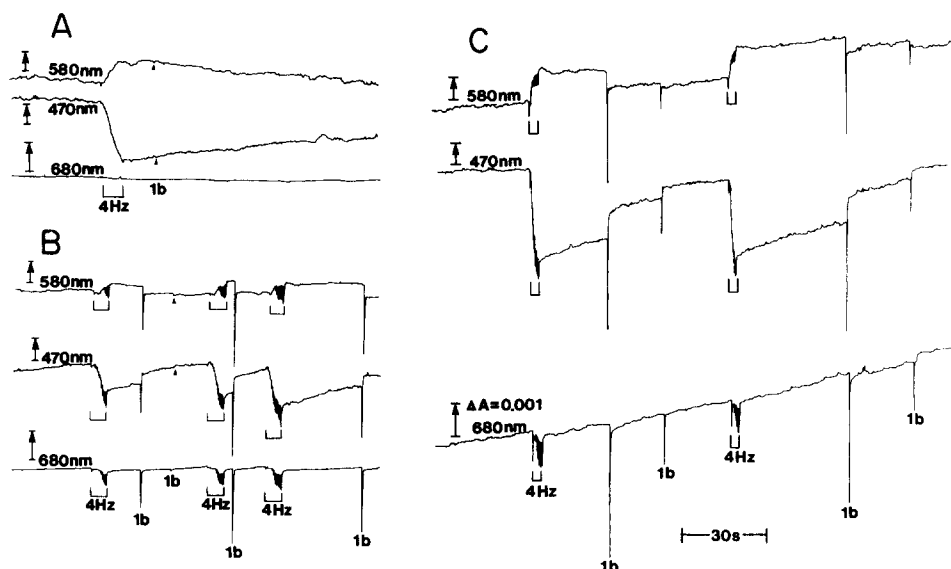

FIGURE 5. (A) Light signals obtained after 30 min quiescence. The muscle was stimulated at 4 Hz for 8 s. Note that a post-stimulatory excitation is without effect (1b = 1 beat). (B) Signals for a repetitive sequence of rapid stimulation and single post-stimulatory excitations. Note that the depletion responses began with a delay of several excitations, that a second post-stimulatory excitation was almost without effect, and that potentiation has decayed very little even after 40 s quiescence (last stimulation). Between parts B and C, 3×10^{-8} M isoproterenol was added. Same stimulation pattern. Note that a second post-stimulatory beat was partially potentiated. See text for further explanations.

a variable rest period and then a post-stimulatory excitation. The rapid stimulation period produced small, increasing motion artifacts at all wavelengths, and the 680-nm baseline shows a small downward shift. The first rest period was 20 s, and the first excitation produced a relatively large contraction artifact (see the 680-nm signal) that was similar in all the signals. Afterward, the opposite baseline absorbance shifts at the calcium-dependent wavelengths were largely reversed. A second excitation made 20 s later resulted in almost no further effect. After the second burst of 4 Hz stimulation, the rest period was 4 s, and after the final 4-Hz run, the rest period was 40 s. The baseline absorbance shifts during rapid stimulation averaged -0.0012 A at 470 nm and 0.0007 A at 580 nm, which

would correspond to an ~ 9-μM depletion of the mean total extracellular calcium concentration. Averaging quantitations of the 470- and 580-nm signals for the replenishment at the first post-stimulatory excitations gives a net calcium increment of 6 μM. It is pointed out that the return shifts of absorbance are not exactly in the ratio expected from the dye calibrations, the 580-nm absorbance decrease being 20% greater than expected. This discrepancy was reversed if the motion artifacts were positive, and it is addressed in Fig. 6.

Fig. 5C presents signals obtained after moderately strong β-adrenergic stimulation (3 $\times$ 10^{-8} M isoproterenol). The stimulation pattern is similar to the previous panel and is given twice: 4 Hz for enough excitations to produce a nearly maximal depletion response (estimated at 20 μM or 8% of the total 250 μM extracellular calcium), then a rest for 20 s, one stimulus, a rest for 20 s, one stimulus, a rest for 15 s, one stimulus, and another rest for 15 s. The depletion response was increased two- to threefold in comparison with part B. Note that a second post-stimulatory excitation resulted in some further replenishment, which is consistent with the idea that cAMP-dependent mechanisms would favor calcium uptake by the sarcoplasmic reticulum over sarcolemmal calcium extrusion (e.g., Morad et al., 1978).

Abolition of Signals by Competing Calcium Buffer

Fig. 6 presents a control for this method, which removes the calcium-dependent signal component for the most part, but preserves the baseline absorbance of the dye and muscle contractility. The experimental dye-containing solution was buffered to roughly the same pCa with a competing calcium buffer at a high concentration, in this case 25 mM citrate. This is particularly useful when the calcium-dependent signal components are as small as 10% of the contraction artifact signals, as in this figure. Fig. 6A presents light signals obtained during repeated sequences of 4 Hz stimulation for 5 s, followed by a rest period of a few seconds and a single post-stimulatory excitation (2 mM tetramethylmurexide, 0.25 mM total calcium, 0.13 mM free calcium). The responses were the same as those described in Fig. 5, except that the motion artifact signal component at 4 Hz was somewhat smaller (almost absent), whereas the potentiated motion artifact was relatively large. Calibrations of the depletion/replenishment responses gave an exchange value of 5.5 μM. Fig. 6B presents signals obtained 2 min after switching to a citrate-buffered solution (25 mM citrate, 7 mM total calcium; the dye concentration and pH were maintained, the final sodium concentration was 15 mM higher, and the chloride concentration was 50 mM lower than in the control solution). Free calcium measured with a calcium electrode (93-20-00, Orion Research, Inc., Cambridge, MA) was 0.18 mM, but this value may not be accurate (see Dani et al., 1983; Ginsburg and Rhamimoff, 1984). The buffer capacity of the citrate solution reduced absorbance shifts to small changes of the total calcium by a factor of 7 in comparison with the control solution. Note that the contraction artifacts generated by the stimulation sequence are very similar in magnitude to the control responses, but the diastolic absorbance shifts interpreted as extracellular calcium concentration changes in part A are largely abolished; the baseline absorbance at 580 and 680 nm shifts slightly downward at the potentiated contractions.

Action Potential Sequence at Low Calcium

Fig. 7 shows action potential configuration changes under the conditions of the previous experiments, which demonstrate that the basic features described at high calcium are also present at low calcium. For brevity, only a 4-Hz run is shown after a 10-s pause, without a delay period after the potentiated contraction. The action potential marked "1" corresponds to the first potentiated post-
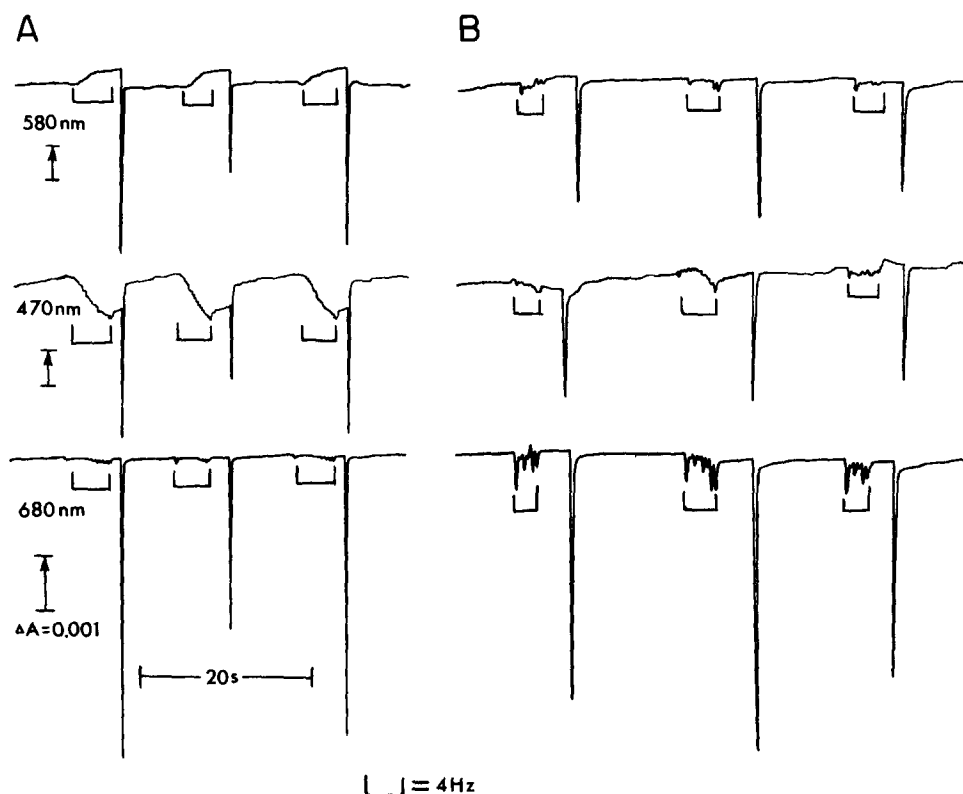

FIGURE 6. (*A*) Typical light signals at 580, 470, and 680 nm for repetitive sequences of 4 Hz stimulation for 4 s, followed by quiescence for 1–3 s and a single post-stimulatory excitation. Between parts *A* and *B*, the solution was exchanged for a calcium-buffered solution with 25 mM citrate. Note the suppression of baseline absorbance shifts at 580 and 470 nm. See text for further details.

stimulatory excitation, and subsequent action potentials up to the steady state ("ss") correspond to 4 Hz stimulation. Note that the first action potential crosses through the subsequent action potentials at a negative potential. From 10 observations, it can be summarized that the positive action potential staircases during post-rest stimulation generally accumulated over more excitations at low calcium; negative staircases of the late action potential were less pronounced than at normal calcium, but could invariably be induced by a prior 4-Hz stimulation.

Effect of 4-AP on Depletion/Replenishment Responses

Fig. 8 shows the effect of 1.5 mM 4-AP on depletion/replenishment signals during post-stimulatory potentiation. Part *A* is the control response; part *B* is with 4-AP. The arrowheads correspond to individual excitations made at ~0.3 Hz. The control response shows the usual depletion produced by 4 Hz stimulation and replenishment at the first post-stimulatory excitation, with little further effect at subsequent post-stimulatory excitations. After addition of 4-AP (part *B*), motion artifacts at the low-frequency excitations were markedly increased. The depletion produced by 4 Hz stimulation was of roughly the same magnitude as before, but it accumulated in fewer excitations. The first potentiated contraction artifact was of roughly the same magnitude as before; however, the return baseline absorbance shifts accompanying the first potentiated contraction were
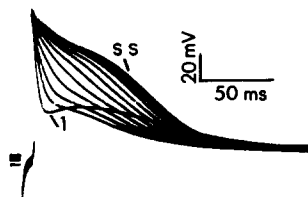

FIGURE 7.   Action potential configuration changes at low calcium (150 μM) during 4 Hz stimulation after a 10-s rest period. The first action potential corresponds to a potentiated contraction as in previous figures; note that the action potential crosses through the subsequent action potential at a low potential. This pattern is similar to but less pronounced than changes found at normal calcium (Fig. 3 C).

~30% smaller than before, and the subsequent excitation generated a relatively potentiated contraction with a further shift of the baseline absorbances back toward control. The motion artifacts decreased markedly in magnitude between the second and third excitations.

Extracellular Sodium Reduction and Depletion/Replenishment Responses

Figs. 9 and 10 present results obtained with replacement of extracellular sodium by iso-osmotic sucrose concentrations. Similar results have been obtained with other interventions thought to act on the sarcolemmal sodium gradient (reduction of external sodium, reduction of external potassium, and application of heart glycosides; see Koch-Weser and Blinks, 1962, and Toda, 1969, for descriptions of the inotropic patterns in atrial muscle). With moderate sodium reduction by 55 mM (90 mM final concentration), a contractile pattern develops at low calcium (0.35 mM total calcium, 0.2 mM free calcium) that is similar to the pattern described in Fig. 3 at normal calcium. The rested-state contraction artifact is relatively large, and a subsequent contraction is greatly suppressed. A single stimulus was applied at each arrowhead in Fig. 9. The 580-nm signal in this record has a slow, continuous downward drift (note the position of the first and last motion artifact tips).

The first stimulation corresponds to a 15-min rest period, and this contraction resulted in absorbance shifts at 580 and 470 nm of −0.0006 and +0.0008 *A*,

respectively. There was a smaller downward shift of the baseline absorbance at 680 nm that did not reverse. After 4 s, a single excitation resulted in a greatly reduced motion artifact and little further shift of baseline absorbances. Over the course of ~30 s, signals returned to nearly the prestimulatory level, which would correspond to a decreasing extracellular calcium concentration and presumably a net uptake of extracellular calcium. An excitation made at that point generated
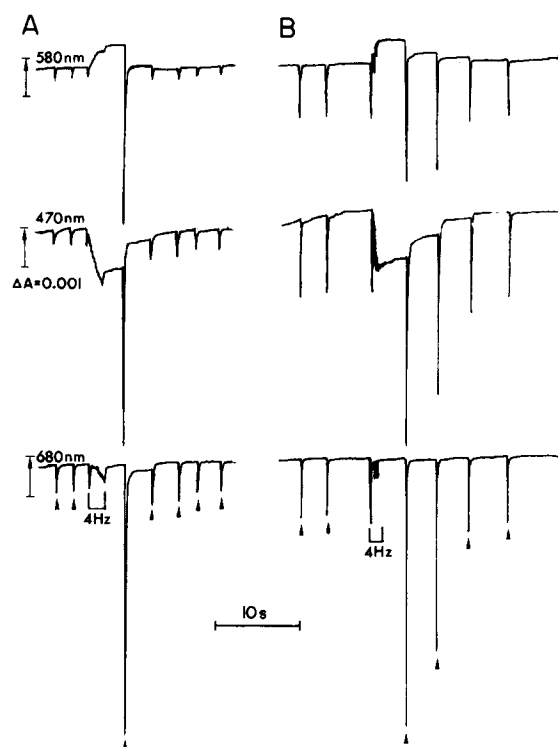

FIGURE 8. (A) Control post-stimulatory potentiation sequence as described previously (0.25 mM total calcium), but started from low-frequency stimulation. Single excitations are indicated with arrowheads below the 680-nm signal. (B) After 1.5 mM 4-AP. Note that the magnitude of depletion during 4 Hz stimulation is similar in A and B, but it develops more rapidly in B. The return shifts of absorbance at post-stimulatory excitations are almost complete in one excitation in A, but take three excitations in B.

a contraction artifact of nearly the rested-state magnitude. In the late portion of the records, excitations were made after an intermediate time period during re-equilibration of extracellular calcium; note that motion artifacts were also of an intermediate magnitude. The net calcium increments at the recovered contractions in this figure correspond to a 5-μm increase by the 580-nm signal, and to a 4-μM increase by the 470-nm signal, based on calibrations of the sucrose-containing solution, muscle thickness, and extracellular space of the dye. This result demonstrates that a net accumulation of extracellular calcium at activation does not depend on prior depletion of calcium in the extracellular space, and

suggests a relationship between net uptake of calcium and recovery of contractility in this sequence. In 6 of 14 preparations studied, the negative staircase of motion artifacts took three to four excitations, accompanied by smaller increments of extracellular calcium; in 3 of the 14 preparations, the rested-state contraction artifacts did not decrease during low-frequency stimulation after rest, and net increments of extracellular calcium were not found. With greater reductions of external sodium, as described below, this was invariably the result.

Fig. 10 shows the typical effect of sodium reduction by 85 mM (60 mM final concentration) with iso-osmotic sucrose replacement for the usual stimulation sequence. The control signals are given in *A*, and are very similar to those described previously. The first stimulation in part *B* corresponds to a rested-state
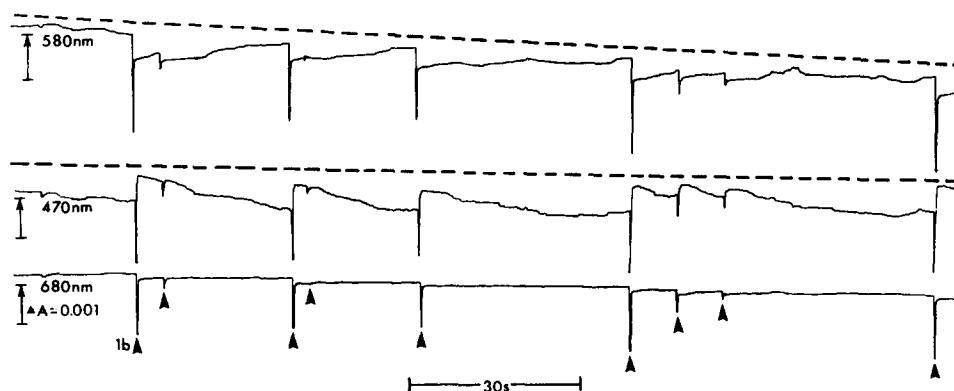

FIGURE 9. Net increment of extracellular calcium at rested-state contractions. Continuous signals are shown with single excitations as indicated by the arrowheads after 55 mM reduction of sodium with iso-osmotic sucrose replacement (0.35 mM total calcium, 0.18 mM free calcium). The first excitation was made after a 3-min rest period. Note that the excitations made at early points in the re-equilibration of the calcium-dependent signals resulted in very small contraction artifacts; excitations made at intermediate points in the re-equilibration period resulted in intermediate contraction artifacts.

contraction (10-min rest period). With further low-frequency stimulation, contraction artifacts remained at roughly the same magnitude, and there was no detectable extracellular calcium shift. The low-frequency contraction artifacts in part *B* are of roughly the same magnitude as the potentiated contraction artifact under control conditions. The burst of 4 Hz stimulation resulted in almost no baseline absorbance shifts, and the subsequent contraction artifacts were not potentiated over the previous level. The contractile response, monitored here by light signals, is virtually identical to that described at normal calcium in Fig. 4, although the level of contractility is only ~15% of that at 1.5 mM calcium.

Extracellular Calcium Transients in Ryanodine-pretreated Atria

In previous work it was found that submicromolar ryanodine pretreatments at normal calcium for 10–20 min substantially increase cumulative depletions of extracellular calcium during post-rest stimulation and greatly accelerate the

replenishment of extracellular calcium during a quiescent period; after prolonged stimulation, cessation of stimulation results in a rapid and substantial increase of extracellular calcium above baseline (Hilgemann et al., 1983). These findings have since been verified in every type of mammalian heart preparation used (arterially perfused right ventricles of rabbit, guinea pig, rat, and cat; superfused atria of guinea pig rat and rabbit). In frog ventricle, no effects were found at any concentration. In rabbit atrium, the pattern described in Fig. 11 was obtained with pretreatment at normal calcium for 1 h using 10^{-8} M ryanodine, and with
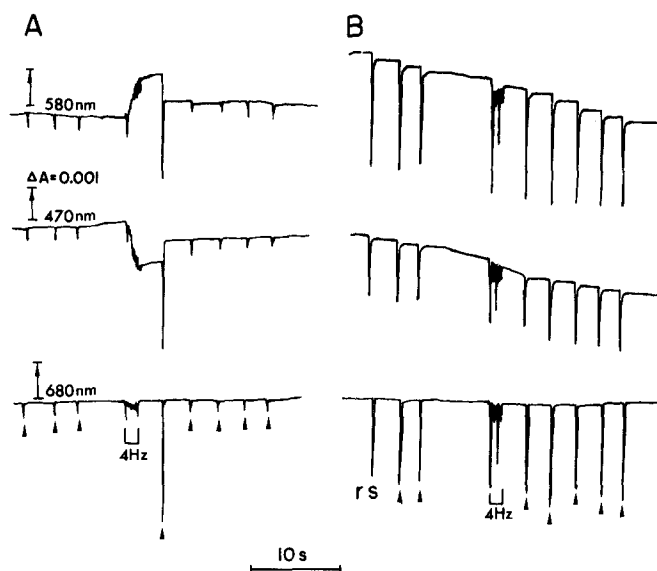

FIGURE 10. (A) Control post-stimulatory potentiation sequence similar to that in Fig. 8 (0.25 mM total calcium). (B) After 85 mM reduction of external sodium to a final concentration of 60 mM. The first contraction artifact in B (rs = rested state) corresponds to a 10-min rest period. Note that the magnitude of subsequent contraction artifacts was not reduced as in Fig. 9, that during 4 Hz stimulation baseline absorbances did not shift, and that subsequent contraction artifacts were not potentiated.

just 10 min pretreatment with 10^{-7} M ryanodine. The result shown is for continuous incubation with 1 μM ryanodine, a very high concentration for intact muscle. The stimulation pattern is the same as described for post-stimulatory potentiation in previous figures: a burst of 4 Hz stimulation to deplete extracellular calcium, followed by a test excitation after a variable rest period. The test period was varied in a random order. In the central portion of the figure and again at the end of the observation period, extracellular calcium was allowed to re-equilibrate; the apparent $t_{1/2}$ of replenishment was ~6 s (compared with replenishments taking several minutes under control conditions; see Fig. 5). Total depletions at the end of the 4-Hz simulation bursts are estimated at 45 μM of the total 250 μM calcium used in the experiment.

At the responses marked 1 and 5, the test intervals were 1.5 and 0.5 s,

respectively. The test excitations resulted in a contractile response (see 680-nm motion artifacts) of essentially the control magnitude obtained in this preparation (omitted for brevity). At longer rest periods, where replenishment of extracellular calcium had progressed, the contractile response was negligible. Response number 2, which corresponds to a test interval of 3 s, showed no contractile response. At an interval of 2 s (responses 6, 8, and 9), the motion artifacts were substantially decreased, whereas calcium efflux was just beginning. Although the rate of calcium replenishment here is 10–20 times faster than under control conditions, it is apparent that the ability to contract, as monitored via the light
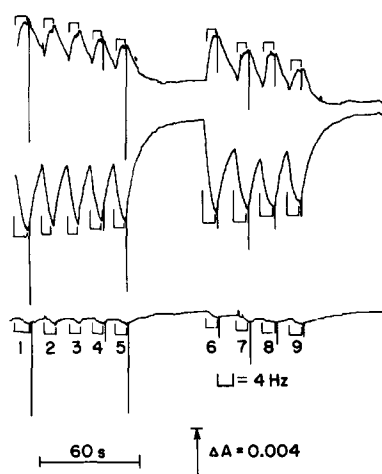

FIGURE 11. Post-stimulatory potentiation in an atrium incubated continuously with 1 μM ryanodine (0.25 mM total calcium). As indicated, bursts of 4 Hz stimulation were applied, and then a test stimulation was given after a variable rest interval. Rest intervals for the numbered responses were (in seconds): (1) 1.5, (2) 3, (3) 5, (4) 2, (5) 0.5, (6) 2, (7) 1.5, (8) 2, (9) 2. Between the fifth and sixth responses and after the last response, extracellular calcium was allowed to equilibrate. Note the speed of replenishment and that already, after a 3-s rest period (number 2), the contractile response was negligible.

signals, is lost still more rapidly than the reappearance of calcium in the extracellular space. See the figure legend for the complete stimulation protocol. In five of five observations, it was not possible to abolish the potentiated contraction just after the burst of rapid stimulation even with 10 μM continuous ryanodine superfusion in rabbit atrium (this was not the case in rat heart); at still higher concentrations of ryanodine (100 μM), the magnitude of depletion decreased as with caffeine (Hilgemann et al., 1983). It should be pointed out further, in the way of contrast, that caffeine does not clearly accelerate the re-equilibration of calcium in depletion/replenishment responses.

Calcium-dependent Diastolic Depolarization Induced by Ryanodine

The very rapid replenishment of large extracellular calcium depletions in the ryanodine state raises the question as to whether such a substantial charge

movement during quiescence might have electrophysiological implications. Although the diastolic membrane potential remained stable during the depletion/replenishment responses at low calcium (see Fig. 13), at higher calcium concentrations a cumulative depolarization developed during post-rest stimulation and is described in Fig. 12. The figure follows the baseline membrane potential during 2 Hz stimulation for 12 excitations after 2-min rest periods. The upper
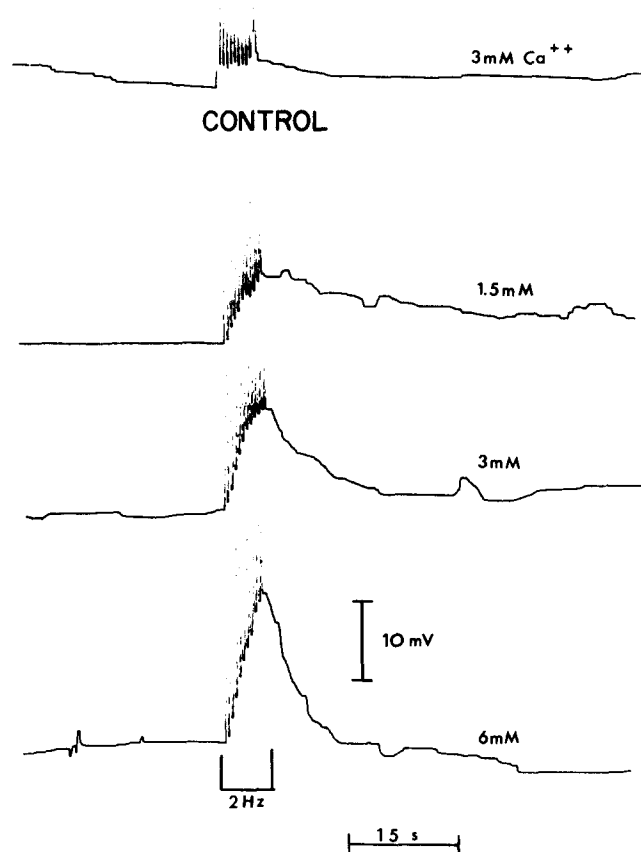

FIGURE 12.   Baseline membrane potential shifts caused by 2 Hz stimulation for 12 excitations. The upper record is the control response at 3 mM calcium; the lower three records are after ryanodine treatment with 1.5, 3, and 6 mM calcium as indicated. See text for details.

response is with 3 mM calcium under control conditions; the lower three responses are after a 20-min, 0.2-μM ryanodine incubation at 3 mM external calcium (0.5 Hz stimulation) followed by washout. Records are given for three calcium concentrations in the ryanodine state, made in the following order: 3, 1.5, and 6 mM. At 6 mM calcium, transient increases of "rest tension" followed the potential changes quite closely (not shown). This experiment was done entirely in the presence of 1 mM 4-AP to minimize the possible influence of

extracellular potassium accumulation; virtually identical results have been obtained without this precaution. The resting membrane potential was -75 mV, and did not change by more than 5 mV in the course of the experiment.

Under control conditions (upper record; 3 mM calcium), the 2-Hz stimulation burst results in a slight depolarization of <3 mV over the 12 excitations; after the ryanodine treatment (see the 3-mM calcium response), a cumulative depolarization of 12 mV develops during rapid stimulation; the membrane potential returns to the prestimulatory potential over the course of 20 s during quiescence. The magnitude of the cumulative depolarization is decreased to 7 mV by decreasing calcium to 1.5 mM, and increased to 15 mV by increasing extracellular calcium to 6 mM. The cumulative depolarization can be essentially eliminated by lowering calcium to 0.1 mM, by 5 mM cobalt or by 1 μM nifedipine (not shown). The time course of re-equilibration is not unlike the replenishment of extracellular calcium found at low calcium ($t_{1/2}$ of 5–12 s); this time course often becomes faster with increasing calcium, as is the case here.

Facilitation of Calcium Influx in Ryanodine-pretreated Atria

As described in Fig. 3, ryanodine pretreatment dissociates the positive action potential and contractile staircases of this preparation from the negative staircases of the late action potential and contraction. Figs. 13–15 describe extracellular calcium transients related to the positive action potential staircase in ryanodine-pretreated atria; all results were obtained in atria pretreated with 0.1 μM ryanodine for 15 min at 0.5 Hz during the equilibration period with 2 mM calcium (no ryanodine was present in the subsequent superfusates). Fig. 13 shows action potentials (A) and light signals (B) for post-rest stimulation at 1 Hz after a 2-min rest period (250 μM total calcium). In part A, note the smooth repolarization at the first excitation; the action potential staircase is complete within $\sim$12 excitations. In the cumulative depletion response in part B, note the small magnitude of contraction artifacts (see 680-nm signal). The dots in the light signals correspond to the first excitation; the steps in the light signals correspond to the effect of individual excitations. The depletion response has an S-shape, corresponding to an increasing net calcium depletion per beat during the first eight excitations, and then a decrease of the net depletion per beat as the depletion approaches a maximum. Total absorbance shifts ($-0.013\ A$ at 470 nm, $0.008\ A$ at 580 nm) correspond to a mean reduction by 98 and 91 μM (0.7 mm muscle), respectively, of the total 250 μM extracellular calcium ($>30\%$ of total). Note that extracellular calcium re-equilibrated almost completely within 30 s during quiescence.

The positive inotropic effect of adrenergic stimulation in atrial muscle is frequency dependent (Koch-Weser et al., 1964), and on the basis of a pharmacological analysis it was speculated that the underlying mechanism could be a partial voltage dependence of a sarcolemmal phosphorylation step in the cyclic AMP system (Mensing and Hilgemann, 1981). For this reason, the possible cAMP dependence of the staircase responses was examined. Briefly, it was found that isoproterenol (and the cAMP derivative chlorphenylthio-cAMP) greatly reduced the number of excitations needed to produce facilitation of both action potential and net calcium depletion per beat in a concentration-dependent
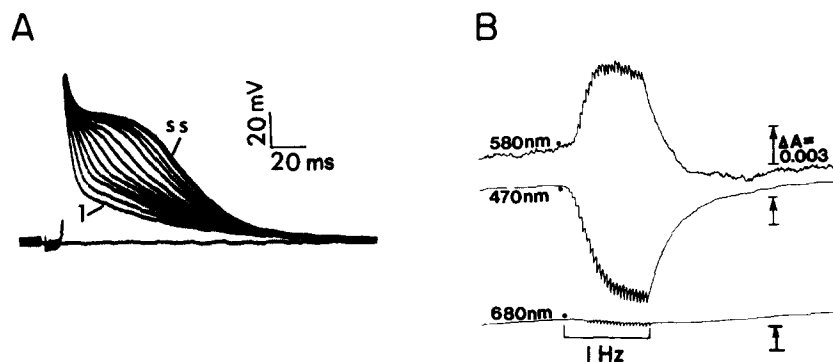
FIGURE 13.   Action potential staircase (A) and extracellular calcium transients (B) after a 15-min pretreatment with 10^{-7} M ryanodine, 0.25 mM total calcium. 1 Hz stimulation after a 2-min rest period. Note the smooth repolarization of the first action potential. Steps in light signals correspond to single excitations. Dots mark the first stimulation.

manner. This effect was not duplicated by increasing calcium, and even with 6 mM calcium the positive action potential staircases took four to seven excitations to equilibrate. Fig. 14 shows the extreme case reached with strong β-adrenergic stimulation (10^{-7} M isoproterenol). Part A shows the action potential staircase with 1 Hz stimulation after 2 min rest; repolarization was rapid at the first excitation, the action potential plateau was fully developed at the second excitation, and further excitations resulted in little change. Note that the initial peak of the action potential increased by a few millivolts. Extracellular calcium transients also showed a calcium influx staircase over just two excitations (not presented for brevity). In part B, the time dependence of the decay of facilitation is presented. Two stimuli were made at a variable interval, given above the 680-nm motion record, with a 40-s pause between stimulus pairs; each dot corresponds to one stimulation. The intervals between the stimulus pairs are, consecutively, 6, 1, 20, 3, and 15 s. The first excitation after a 40-s pause resulted in negligible calcium depletion at the present resolution; a 20-s pause was long
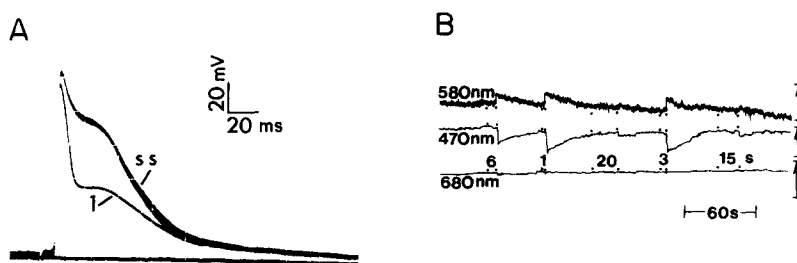

FIGURE 14.   Same conditions as in Fig. 13 but with 10^{-7} M isoproterenol. Part A shows action potential staircase for 1 Hz stimulation after a 2-min rest period. Note that the action potentials showed little change after the second excitation. Part B shows the decay of facilitation. Each dot corresponds to one stimulation. Times between excitations are given above the 680-nm signal. See text for further details.

enough for the effect of the first excitation on a subsequent excitation to decay for the most part.

A transient outward current has been described in sheep Purkinje fibers that is apparently not calcium sensitive and can decrease cumulatively during repeti-
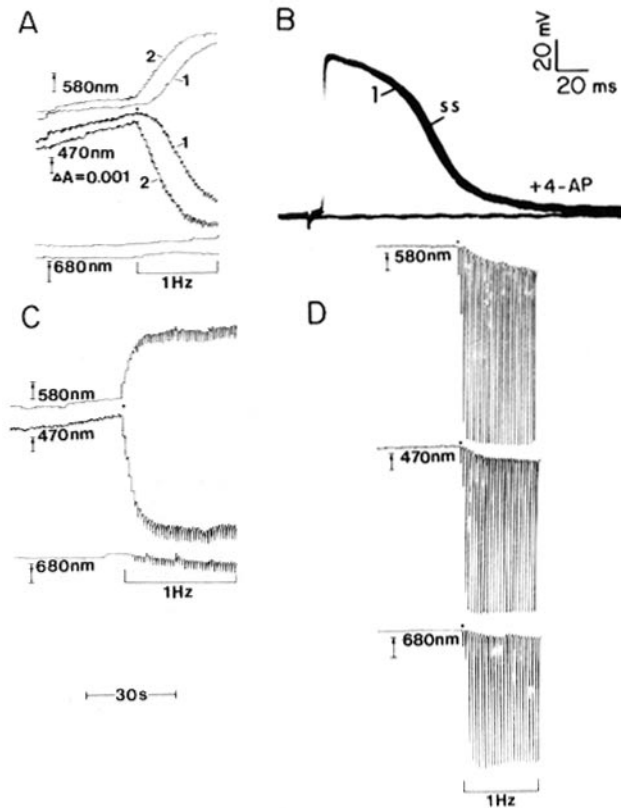

FIGURE 15. Abolition of calcium depletion staircase with 4-AP. Part *A* shows the effect of 1 mM 4-AP on the cumulative depletion response. The curves labeled 1 are before and curves labeled 2 are 20 min after application. Note that curves 2 show steps corresponding to each excitation beginning at the first excitation. Part *B* shows the first 10 action potentials at 1 Hz after 2 min rest with 1 mM 4-AP. The action potentials are almost superimposable. Part *C* was made after the addition of 10^{-7} M isoproterenol, which shows that isoproterenol cannot induce a staircase of calcium depletion after 4-AP. Note that the total depletion response is not increased in comparison to part *A*. Between parts *C* and *D*, total calcium was increased to 3 mM. Note in *D* the pronounced staircase of contraction artifacts over four excitations; opposite baseline absorbance shifts at 580 and 470 nm are suppressed. See text for further details.

tive stimulation (Boyett, 1981*a*). Since a similar mechanism could underly these action potential staircases, the effect of 4-AP (Boyett, 1981*b*) was examined; if a facilitation of calcium channel conductance were involved (Hoshi et al., 1984), the calcium depletion staircases should not have been principally affected. Fig. 15*A* shows cumulative calcium depletion responses before (1) and after (2)

application of 1 mM 4-AP. The net depletion at the first excitation with 1 mM 4-AP was increased to roughly the same magnitude as the maximal depletion per beat attained in the control response, which was typical for six similar observations. Part B shows the first 10 action potentials in the presence of 1 mM 4-AP. There was essentially no staircase of the action potential plateau over the first 10 action potentials; this was typical for five similar observations. Part C shows further that isoproterenol (10^{-7} M) does not induce a staircase of net depletion per beat after 4-AP; net calcium depletion at the first excitation was increased roughly fourfold in comparison with part A and did not increase further during subsequent excitations.

Another possible control for this method is given in Fig. 15D, which illustrates the abolition of calcium-dependent light signals by an increase in calcium concentration to 3.5 mM (these records were made immediately after part C). At this calcium concentration, the slope of the dye calibration curve was reduced by a factor of ~5. Motion artifacts increased greatly over the first four excitations to a magnitude >10 times greater than in part C. The baseline absorbance shifted downward slightly at all wavelengths. The opposite absorbance shifts at 580 and 470 nm were completely abolished. It is pointed out from more than 10 observations that the calcium-dependent absorbance shifts decreased more markedly with increasing calcium concentrations than would be expected from dye calibrations and the assumption that calcium depletion is proportional to extracellular calcium concentration.

Finally, two relevant findings are mentioned but not illustrated, in the interest of brevity. (a) Calcium depletions under all conditions studied were decreased to ~20% of control by 1 μM nifedipine. (b) At normal calcium, positive staircases of the action potential correlated with positive contractile staircases, taking a few more excitations to accumulate than the action potential staircases; also, the contractile staircases accumulated in just a few excitations with the cAMP-dependent interventions, and were effectively abolished by 4-AP with a much larger positive inotropic effect at low frequencies than at high frequencies.

DISCUSSION

Qualitative and Quantitative Relationships Between Extracellular Calcium Transients and Post-Stimulatory Potentiation

This study has circumstantially correlated cumulative depletion and replenishment of extracellular calcium with the production and decay of post-stimulatory potentiation under several experimental conditions. (a) Rapid stimulation with low contractile activity is associated with a net loss of extracellular calcium; subsequent excitations with much greater contractile activity are associated with net increments of extracellular calcium. (b) When the loss of contractile potentiation takes more than one contraction cycle, as with isoproterenol (Fig. 5) or with 4-AP (Fig. 8), there is also a replenishment of extracellular calcium at subsequent post-stimulatory excitations. (c) When rapid stimulation does not result in a cumulative extracellular calcium depletion, as with a strong external

sodium reduction, post-stimulatory beats are not potentiated and do not result in an increment of extracellular calcium (Fig. 10). (*d*) When the rested-state contraction is large and the subsequent contraction is small, analogous to the loss of post-stimulatory potentiation, the rested-state contraction is accompanied by a net increment of extracellular calcium; the slow recovery of contractility (Fig. 9) during rest correlates with a slow net decrease of extracellular calcium. (*e*) Ryanodine induces a very rapid loss of post-stimulatory potentiation during quiescence, and also induces a rapid replenishment of the extracellular calcium depletion (Fig. 11). All of these correlations are consistent with the idea that the extracellular calcium transients reflect primarily the filling and depletion of an internal calcium store; release of the store would in some way be inhibited during rapid stimulation, whereas the uptake function remains powerful. At the subsequent potentiated contractions, large portions of the calcium released would be extruded across the sarcolemma.

Although these qualitative correlations are consistent with simple interpretations of the post-stimulatory potentiation sequences (e.g., Morad and Goldman, 1973), the quantitation of calcium movements does not definitively account for the loss of potentiation by sarcolemmal calcium extrusion with subsequent calcium store depletion. Factors that add an uncertainty to the quantitative treatment are the exact magnitude of extracellular calcium buffering (Bers and Langer, 1979), the possibility that intact muscle cannot use the full pCa-tension relationship found in skinned preparations (Fabiato, 1981), and many unknowns about kinetic determinants of calcium requirements for activation (e.g., competition between myofilament binding and uptake by the sarcoplasmic reticulum). The almost complete loss of potentiation was associated with a $4-7-\mu M$ increment of extracellular calcium or $2-3$ $\mu mol/kg$ wet weight. From the calcium binding measurements of Solaro et al. (1974) in skinned fibers, which probably reflect minimum calcium requirements, that amount of calcium would account for a change of steady state contraction force on the order of 15% of maximum developed tension. The contractility changes occurring in this study at 130 μM free calcium are in just this range of maximal contractions; the inferred net calcium efflux is not excessive.

By the more recent calculations of Fabiato (1983), the measured calcium movements would alone be insufficient to explain the loss of contractile potentiation. In this context, it can be pointed out that total calcium exchange is definitely larger than the amount exchanged during repeated post-stimulatory potentiation sequences (e.g., multiple depletion responses before attaining post-stimulatory potentiation responses; Fig. 5). One possibility is that calcium release is inhibited until the filling of a critical store supports positive feedback upon initiation of release (all-or-none regenerative release). A conservative possibility would be that a large portion of the calcium taken up initially fills a store (or stores) to a level at which subsequently loaded calcium can be rapidly released. For the time being, therefore, it can be concluded with confidence only that calcium extrusion at activation contributes significantly to the loss of potentiated contractility. An involvement of internal calcium redistributions and/or the function of a release mechanism is not excluded.

Ryanodine Action

In the present work, ryanodine has been used as a tool to accelerate the decay of post-stimulatory potentiation and accumulation of extracellular calcium during quiescence. In this context, it is not essential to define the mechanism of action, and the basic effect of ryanodine on extracellular calcium transients was discussed previously (Hilgemann et al., 1983). Since that writing, stimulation of net calcium uptake by high concentrations of ryanodine (Jones and Cala, 1981) has been described in an isolated sarcoplasmic reticulum fraction from skeletal muscle (Seiler et al., 1984), where ryanodine produces contracture (see Jenden and Fairhurst, 1969, for review). It is now also known that mechanically skinned cardiac fibers are quite insensitive to ryanodine; calcium-induced calcium release is inhibited by high concentrations of ryanodine, whereas caffeine-induced contractions are not (Fabiato, 1985). Finally, very high concentrations of ryanodine have been shown to inhibit a calcium-induced calcium release process in isolated cardiac sarcoplasmic reticulum (Chamberlain et al., 1984). Thus, almost all recent findings in isolated systems are consistent with inhibition of release as the mechanism of action (Sutko and Kenyon, 1983), and are not accounted for by a calcium leak hypothesis. It remains unclear how and if an inhibition of release could account for the rapid replenishments of extracellular calcium in the present experiments. Another problematic result in the present work is that high concentrations of ryanodine ($1-10$ μM) did not abolish the potentiated contraction, elicited at the peak of a large depletion response in rabbit atrium. Since the potentiated contraction is almost certainly not activated by a direct influx of calcium, this finding further negates the possibility of dissecting out the relative roles of sarcolemmal influx and internal calcium release in any simple fashion with this agent (e.g., Hajdu, 1969; Bers, 1985). It remains a fascinating mystery why removal of the sarcolemma or isolation of sarcoplasmic reticulum should result in a loss of ryanodine sensitivity by 1,000-fold (assuming that actions in isolated systems are somehow related to those in intact muscle), and why high-affinity ryanidine receptors appear to exist in frog heart, where inotropic actions are absent (Ciofalo, 1973).

Positive Action Potential Staircase and Facilitation of Calcium Influx

In the ryanodine state, prolongation of the action potential plateau during post-rest stimulation has been associated with a facilitation of calcium influx. Since the staircases of both action potential and calcium influx are abolished (or superseded) by a high concentration of 4-AP, the underlying mechanism is probably a decreasing outward current similar to that described in sheep Purkinje fibers (Boyett, 1981a, b). It is noteworthy that agents that stimulate cAMP-dependent protein kinase activity decrease greatly the number of excitations over which staircases accumulate, whereas even large increments of the extracellular calcium concentration have at most a modest effect. Therefore, an interaction of a decaying potassium conductance against an inward current of variable magnitude might not be an adequate explanation of staircase kinetics. In rabbit ventricle, similar action potential staircases have been described, although they are less pronounced than in atrium, and a similar pharmacological

study yielded very similar results (Saxon and Safronova, 1982). A possible relation of the present results to action potential staircases in neurons is also given (e.g., Aldrich et al., 1979). Although the staircases described in the present work accumulate over 2–12 excitations, current staircases thought to reflect an increasing inward current in the rabbit sinus node accumulate over the course of 50–70 voltage-clamp pulses (Brown et al., 1984).

Calcium Efflux

In cardiac muscle, experimental evidence for the existence of both an electrogenic sodium-calcium exchanger and a nonspecific cation channel activated by internal calcium has led to conflicting interpretations of ionic currents with little hope of definitive resolution by voltage-clamp techniques under any reasonably physiological condition (e.g., Reuter, 1984; Noble, 1984). Staircases of the late action potential described here are prime candidates for both mechanisms: (a) under control conditions, the late action potential decreases in parallel with the loss of contractility; (b) a late phase of the post-rest action potential is completely abolished by ryanodine,[1] as is the post-rest contraction; (c) low external sodium suppresses the late action potential as expected for both mechanisms. The finding of net extracellular calcium accumulation under each of the conditions expected to support calcium efflux by electrogenic sodium-calcium exchange clearly favors this mechanism.

Other details of the signals described can also be derived from the expected behavior of a fast electrogenic sodium-calcium exchanger. On the one hand, calcium extrusion appears to be very powerful at potentiated contractions; even with adrenergic stimulation, which enhances calcium uptake by the sarcoplasmic reticulum (Tada et al., 1974), potentiation decays in two or three excitations accompanied by net extracellular calcium accumulation. On the other hand, calcium efflux appears to be completely inactive at excitations with little or no contractile response and presumably low internal calcium release (e.g., cumulative depletions in the ryanodine state). In that case, an electrogenic exchanger would not favor extrusion, but rather would favor calcium influx. It is at least difficult to explain these characteristics by a sarcolemmal calcium pump or an electroneutral exchange process.

In ryanodine-pretreated atria, it is not only striking that net calcium influx can be of large magnitude in the absence of contraction, but also that the replenishment of depletions during quiescence can be very rapid in the absence of contractile activity. These findings would both be explained by powerful internal uptake and sarcolemmal extrusion processes, which prevent cytosolic calcium accumulation except by a very rapid release of calcium. At calcium concentrations higher than those used for the extracellular calcium transients, cumulative depolarizations in the ryanodine state (Fig. 12) are consistent with electrogenic extrusion at diastolic potentials. A mechanism capable of powerful competition with the sarcoplasmic reticulum for calcium at activation should be able to

[1] This effect is only possible in preparations with rapid early repolarization, since otherwise ryanodine prolongs the action potential in a positive potential range (compare, for example, the present results, Frank and Sleator [1975], and Mitchell et al. [1984b]).

prevent contracture with even a rapid continuous loss of calcium from the sarcoplasmic reticulum between beats.

Although the effects of lowering external sodium are complex, relatively simple explanations are possible. In the case of a moderate reduction, the higher resting calcium concentration determined by the exchanger would result in calcium store loading and, therefore, a relatively large rested-state contraction (in atrial muscle) even with low external calcium. With the rapid repolarization of a post-rest action potential, the exchange mechanism would still be fast enough to extrude a substantial amount of calcium at the rested-state contraction. During subsequent quiescence, the sarcoplasmic reticulum would reload slowly with calcium and the movement of calcium from the cytosol to the sarcoplasmic reticulum would be compensated for by the exchanger and reflected in the extracellular space. The time course of these processes would correspond to the slow phase of recovery of atrial contractility after a rested-state contraction (slow phase of decay of the "negative inotropic effect of activation"; Blinks and Koch-Weser, 1961). It is striking that recovery of "slow inward current" in rabbit sinus node follows a similarly slow time course after single post-rest voltage clamps (Brown et al., 1984); however, relationships to contractility are not available.

With a strong reduction of external sodium to 60 mM, calcium exchange with the extracellular space appears to be inhibited (Fig. 9). The high rested-state contractility is not lost during repetitive stimulations, and it is not possible to evoke net calcium efflux at a post-rest contraction, which suggests that either the mechanism of calcium efflux is inhibited or the equilibrium of exchange does not favor efflux. Net extracellular calcium depletions are also inhibited, and this correlates with the finding of very marked inhibition of myocyte calcium currents, but not strontium currents, with the reduction of extracellular sodium (Mitchell et al., 1983). The likely mechanism would be calcium-dependent inactivation of the calcium current. Inhibition of both calcium efflux and influx would together make contractility much more dependent on an internal recycling of calcium than under control conditions.

Although the interpretations given in this discussion are compatible with current concepts about the role of sodium-calcium exchange in cardiac muscle, quantitative discrepancies with other data must be mentioned. Reeves and Hale (1984) concluded in work with cardiac vesicles that the exchanger would be quite inactive at resting internal calcium concentrations; this conflicts with the finding of rapid calcium extrusion during quiescence in the ryanodine state. The calcium extrusion predicted by the present work is almost an order of magnitude greater than can be projected from the work with isolated cardiac vesicles (Philipson, K., personal communication of relevant calculations). In simulations of the post-stimulatory potentiation sequence (Hilgemann and Noble, 1986), with modifications of the DiFrancesco-Noble (1984) model, it appeared almost impossible to maintain resting cytosolic calcium at nearly an order of magnitude above the exchange equilibrium (e.g., Sheu and Fozzard, 1982) with the exchange rates needed to account for efflux. Finally, it is relevant that several calcium flux studies in cardiac muscle have not supported the sodium dependence of calcium efflux (Wendt and Langer, 1977; Langer et al., 1976; Barry and

Smith, 1982), which is necessarily monitored over seconds and minutes by those techniques.

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