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# Mechanisms Contributing to the Cardiac Inotropic Effect of Na Pump Inhibition and Reduction of Extracellular Na

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**ABSTRACT** Reduction of the transsarcolemmal [Na] gradient in rabbit cardiac muscle leads to an increase in the force of contraction. This has frequently been attributed to alteration of Ca movements via the sarcolemmal Na/Ca exchange system. However, the specific mechanisms that mediate the increased force at individual contractions have not been clearly established. In the present study, the [Na] gradient was decreased by reduction of extracellular [Na] or inhibition of the Na pump by either the cardioactive steroid acetylstrophanthidin or by reduction of extracellular [K]. Contractile performance and changes in extracellular Ca (sensed by double-barreled Ca-selective microelectrodes) were studied in order to elucidate the underlying basis for the increase in force. In the presence of agents that inhibit sarcoplasmic reticulum (SR) function (10 mM caffeine, 100–500 nM ryanodine), reduction of the [Na] gradient produced increases in contractile force similar to that observed in the absence of caffeine or ryanodine. It is concluded that an intact, functioning SR is not required for the inotropic effect of [Na] gradient reduction (at least in rabbit ventricle). However, this does not exclude a possible contribution of enhanced SR Ca release in the inotropic response to [Na] gradient reduction in the absence of caffeine or ryanodine. Acetylstrophanthidin (3–5  $\mu$ M) usually leads to an increase in the magnitude of extracellular Ca depletions associated with individual contractions. However, acetylstrophanthidin can also increase extracellular Ca accumulation during the contraction, especially at potentiated contractions. This extracellular Ca accumulation can be suppressed by ryanodine and it is suggested that this apparent enhancement of Ca efflux is secondary to an enhanced release of Ca from the SR. Under conditions where Ca efflux during contractions is minimized (after a rest interval in the presence of ryanodine), acetylstrophanthidin increased both the rate and the extent of extracellular Ca depletions. Thus, acetylstrophanthidin can increase both Ca influx and Ca efflux during the cardiac muscle contraction. These results can be explained by a simple model where the direction of net Ca flux via Na/Ca exchange during the action potential is determined by the changes in reversal potential of the Na/Ca exchange. Reduction of the [Na] gradient may well lead to net cellular Ca uptake (via Na/Ca exchange) and may also elevate the resting intracellular [Ca]. While net Ca uptake may occur with [Na] gradient reduction,

three independent mechanisms may contribute directly to the increase in contractile force observed at individual contractions in rabbit ventricular muscle: (a) enhanced Ca influx, (b) increased SR Ca release, and (c) increased resting intracellular [Ca].

#### INTRODUCTION

The exact role of Ca influx in cardiac muscle excitation-contraction coupling is unclear. It has been known since the experiments of Ringer (1883) that  $Ca_o$  is essential for myocardial contraction and there is general agreement that Ca influx plays an important role in the control of cardiac force development (e.g., Chapman, 1983; Fabiato, 1985). However, it is not known whether this Ca influx (a) directly activates the myofilaments, (b) controls the release of Ca from the sarcoplasmic reticulum (SR), or (c) determines the degree of Ca loading of the SR for subsequent release (e.g., Fabiato, 1983; Reiter et al., 1984; Bers, 1985). None of these precludes any of the others. It seems probable that some combination of these three mechanisms actually occurs and is condition dependent. In addition, different cardiac muscle preparations appear to vary in the degree to which contractile force depends on Ca release from the SR. That is, twitches in rabbit ventricle seem less dependent on SR Ca release than those in rabbit atrium or rat ventricle, but more dependent than twitches in frog ventricle (Bers, 1985). Thus, the use of rabbit ventricle constitutes an important aspect of the present study and one must exert caution in the extrapolation of results from excitation-contraction coupling studies like this from one cardiac preparation to another.

Ca influx is known to occur during the cardiac action potential through voltage-dependent Ca channels. This inward Ca current may be the main mechanism of Ca entry and has been estimated to be large enough to partially activate the myofilaments (K. S. Lee and Tsien, 1982; Isenberg, 1982; Hume and Giles, 1983; Fabiato, 1983). The extent to which sarcolemmal Na/Ca exchange contributes to Ca fluxes that occur during the various phases of the cardiac cycle is considerably less clear. This reversible exchange system is thought to extrude Ca from the cell using the energy provided by the influx of Na ions down the large Na electrochemical gradient. It has also been suggested that the Na/Ca exchange system could be responsible for Ca influx during depolarization and Ca efflux when the cells are repolarized (Mullins, 1979).

The positive inotropic effects of cardiac glycosides have often been explained as follows. Inhibition of the Na pump leads to an increase of intracellular Na activity ( $a_{Na}^i$ ), which in turn increases  $Ca_i$  via the Na/Ca exchange system (e.g., Ellis, 1977; C. O. Lee and Dagostino, 1982; Sheu and Fozzard, 1982; Bers and Ellis, 1982). This elevation of  $Ca_i$  could then lead to increased contractile force (e.g., Repke, 1964; Langer and Serena, 1970). A similar explanation could be used for the positive inotropic effects of decreased  $Na_o$ , except that the Na/Ca exchange is shifted in the direction of increased Ca uptake by decreased  $[Na]_o$  rather than increased  $[Na]_i$ . In the present study, we examine several possible ways in which these interventions can increase contractile force. The focus is on the mechanisms that may be immediately responsible for increased force at individual contractions.

Na pump inhibition (or reduced  $[Na]_o$ ) could lead to an increase of contractile force by increasing: (a) Ca influx, which occurs during each beat (which could be due to Na/Ca exchange or Ca current); (b) Ca available for release from the SR; (c) diastolic free [Ca] in the cell such that a constant increment in phasic Ca delivery to the myofilaments would lead to increased force. These mechanisms do not preclude one another and may even work in concert.

The present study has two specific aims within this context. The first is to determine whether increased SR Ca release is required in the inotropic response to [Na] gradient reduction. The second is to evaluate how transsarcolemmal Ca fluxes during individual contractions are altered by reduction of the [Na] gradient. The results indicate that an enhanced SR Ca release is not required, but probably does normally contribute to the inotropic effect of [Na] gradient reduction. The results also suggest that, during a contraction, both Ca influx and Ca efflux can be enhanced by [Na] gradient reduction. The potential influence of reported changes in resting free  $[Ca]_i$  to the inotropic response is also considered.

#### METHODS

Papillary muscles or ventricular trabeculae (0.1–0.7 mm diam) were obtained from the hearts of New Zealand white rabbits after intravenous administration of sodium pentobarbital (~75 mg/kg). The ends of the muscle were tied with fine suture. One end of the muscle was attached to a fixed hook and the other was attached to a transducer constructed from piezoresistive elements (AE-801, Aksjeselskapet Micro-Elektronikk, Horten, Norway) in a 0.15-ml superfusion chamber. The muscle was stimulated at 0.5 Hz by platinum wires and equilibrated for ~1 h. The superfusate was a normal Tyrode (NT) containing (millimolar): 140 NaCl, 6 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, and 5 HEPES, pH 7.40. All solutions were equilibrated with 100% O<sub>2</sub> and the bath temperature was maintained at 30°C. The flow rate in the chamber was ~2 ml/min. In low-Na solutions, NaCl was replaced isosmotically with Tris or LiCl. Acetylstrophanthidin (ACS; Sigma Chemical Co., St. Louis, MO) was added from a 5 mM ethanol stock solution, caffeine was added as a solid, and ryanodine (lot 704 RWP-2, Penick Corp., Lyndhurst, NJ) was added from a 1 mM aqueous stock solution. Muscles that showed any signs of aftercontractions or sustained increases in diastolic tension were excluded from this study. The concentrations of caffeine (10 mM) and ryanodine (100–500 nM) were sufficient to produce maximal effects of these agents in rabbit ventricular muscle at 30°C.

Double-barreled Ca-selective microelectrodes were fabricated from theta-style glass tubing (2 mm diam, R and D Optical Systems, Spencerville, MD) as previously described (Bers, 1983, 1985; Bers and MacLeod, 1986). Microelectrodes were pulled and broken to 2–12  $\mu$ M diam and the Ca barrel was silanized by exposure to *N,N*-dimethyltrimethylsilylamine vapor. The Ca barrel was filled with 10 mM CaCl<sub>2</sub> and 100 mM KCl, the reference barrel was filled with 140 mM NaCl, and a 50–500- $\mu$ m column of neutral Ca sensor cocktail (Fluka Chemical Co., Ronkonkoma, NY) was drawn into the Ca barrel. The potentials of both barrels and their difference were continuously recorded, but only the difference signal (indicative of [Ca]) is shown in the figures. In order to match the electrical time course of signals from the two barrels, an adjustable passive RC filter was placed in the reference barrel signal after the output stage of the high-input impedance preamplifier, as previously described (Bers, 1983). Inadequate matching of the time courses of response of the two barrels to square voltage pulses was the most common reason for rejection of a microelectrode. An electrode was considered satisfactorily

matched if the responses to a 50-mV, 100-ms voltage pulse in the two barrels were indistinguishable after the initial 5–10 ms (and closely matched before that). In order to obtain a better signal-to-noise ratio and a more stable microelectrode position, the experiments with Ca-selective microelectrodes were performed in the normal superfusate with reduced  $[Ca]$  (0.2–0.5 mM). Stimulus artifacts are usually apparent in the  $Ca_o$ -depletion traces, but after the first 5–15 ms (including the action potential upstroke), these records do not show any electrical artifacts (e.g., see Bers, 1983; Bers and Merrill, 1985). Mechanical artifacts also do not affect the  $Ca_o$ -depletion measurements. This has been previously addressed (Bers, 1983; Bers and Merrill, 1985; also see p. 491). Briefly, while the  $Ca_o$ -depletion amplitude and contractile force often change in a parallel fashion (e.g., with isoproterenol, Co, or ACS), under some conditions, the  $Ca_o$ -depletion signal decreases when tension increases (e.g., potentiated post-rest contractions and elevated  $[Ca]_o$ ).

In the present series of experiments,  $Ca_o$  depletions were observed ~80% of the time. It is not clear why  $Ca_o$  depletions were sometimes not observed, but there are several possibilities that cannot be ruled out. The electrode tip may sometimes create an enlarged extracellular space, which would tend to reduce the apparent magnitude of the  $Ca_o$  depletions. The relatively large, blunt electrode might also destroy the cells in the immediate vicinity of the electrode tip; it also may not penetrate the sheath surrounding some trabeculae, or it may become blocked. All of these would reduce the apparent magnitude of the  $Ca_o$  depletions. The influence on the Ca microelectrode of each experimental solution was evaluated at the end of each experiment. Neither ryanodine nor ACS significantly altered the responses of the Ca microelectrodes used in these experiments.

## RESULTS

### *The Influence of Caffeine and Ryanodine on the Inotropy Induced by Reduction of the Transmembrane $[Na]$ Gradient*

It is possible that the degree of SR Ca loading (and release) is entirely responsible for the increase of contractile force observed when the transsarcolemmal  $[Na]$  gradient is reduced (e.g., by cardioactive steroids, low  $[K]_o$ , or low  $[Na]_o$ ). If this is the case, the inotropic effect of the transsarcolemmal  $[Na]$  gradient reduction by these means should be inhibited by agents that inhibit SR Ca accumulation or release (e.g., caffeine and ryanodine). This possibility is explored in the first series of experiments.

Fig. 1A shows a muscle that was exposed to 4  $\mu$ M ACS under control conditions. After 15–20 min, a new steady state was reached, with a substantial increase in contractile force. This effect of ACS was reversible and reproducible. In the experiment shown in Fig. 1A, ACS was washed out for 30 min and twitch tension returned to 95% of the pre-ACS level. Caffeine (10 mM) was added and, after a transient positive inotropy, twitch tension returned to approximately the control level again. After 30 min in caffeine, the muscle was again exposed to 4  $\mu$ M ACS and an increase in contractile force similar to that seen in the absence of caffeine was observed. The recovery of twitch tension after a 30-s rest interval was routinely assessed in these experiments under the different experimental conditions. The post-rest recovery patterns in the caffeine experiments were very similar to those described in the ryanodine experiments below (e.g., Fig. 2)

and the first three post-rest contractions are shown in the lower panel of Fig. 1A. Fig. 1B shows pooled results from five experiments like that shown in Fig. 1A. Caffeine (10 mM) by itself produced a small increase of force (Fig. 1B, middle bar). ACS also increased contractile force by a similar percent whether caffeine was present or absent (Fig. 1B, left and right bars). In absolute terms, without adjusting for caffeine effects, the developed tension in the presence of caffeine plus ACS was 106% of that with ACS alone.

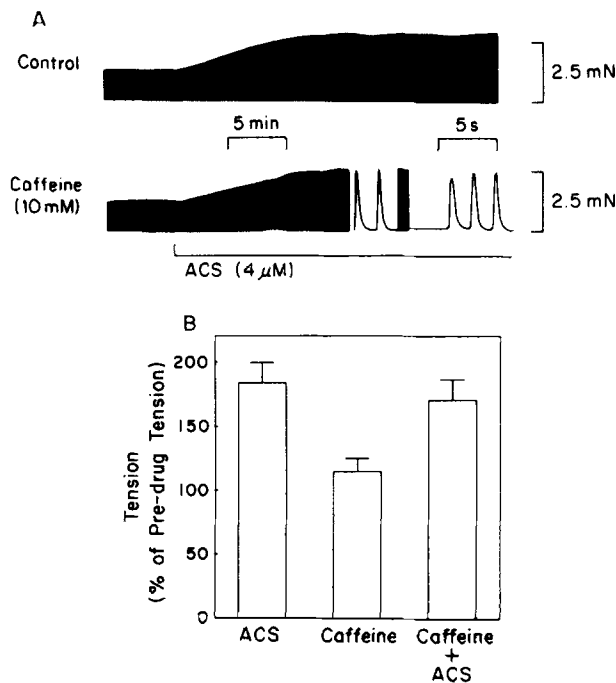


FIGURE 1. The effect on tension development of  $4 \mu\text{M}$  ACS applied to a rabbit ventricular muscle in the absence of, and after equilibration with, 10 mM caffeine. (A) After the top trace was obtained, ACS was washed out for 30 min before addition of and equilibration with caffeine. Two recording speeds are indicated. In the lower trace, several contractions recorded at high speed were omitted at the break and the last three contractions followed a 30-s rest interval. (B) The results are means  $\pm$  SEM from five experiments like the one shown in A. The results are expressed as percents of developed tension before addition of the drug. The column labeled "caffeine + ACS" is the percent of steady state developed tension in 10 mM caffeine when  $4 \mu\text{M}$  ACS was added to the caffeine-containing medium.

Fig. 2A shows an experiment that was similar to that shown in Fig. 1A, except that the muscle was equilibrated with 500 nM ryanodine (rather than 10 mM caffeine) after washout of the first ACS exposure. Again it can be seen that ACS produced a similar increase in contractile force in the absence and presence of ryanodine. Pooled results from 12 experiments like that in Fig. 2A are shown in Fig. 2B. By itself, ryanodine produced a modest depression of contractile force

in rabbit ventricular muscle (as reported by Sutko and Willerson, 1980, and Bers, 1985). ACS induced approximately the same percent of increase in contractile force when ryanodine was absent or present. The maximum rates of contraction ( $+dT/dt$ ) and relaxation ( $-dT/dt$ ) changed in a fashion similar to developed tension.

Fig. 2A also illustrates the influence of ACS and ryanodine on post-rest recovery. Both traces in Fig. 2A begin at the end of a 30-s rest interval and an

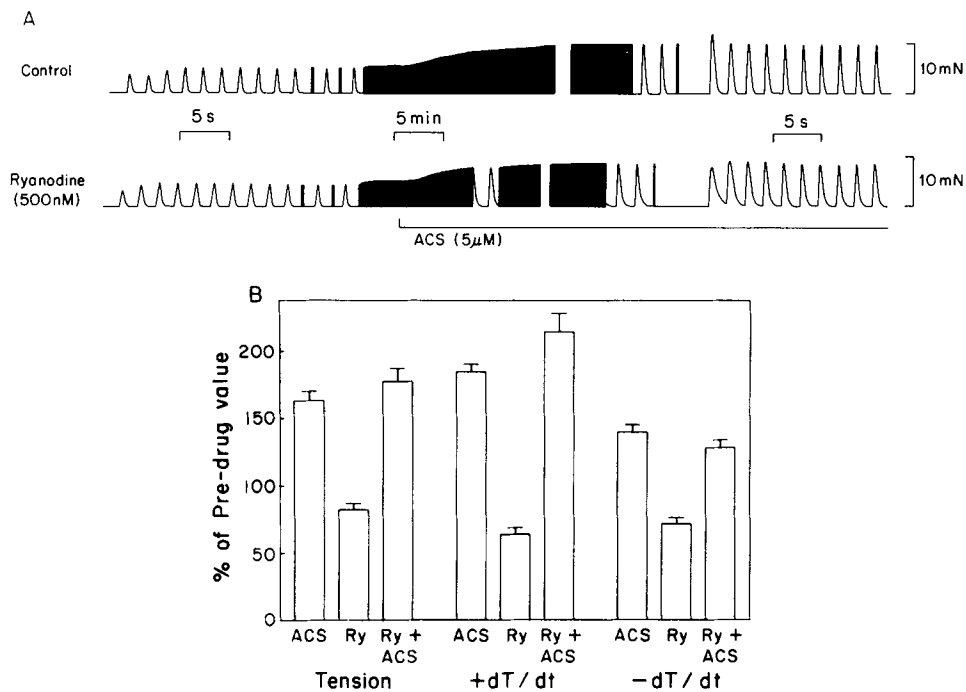


FIGURE 2. The effect of 5  $\mu$ M ACS in the absence and presence of 500 nM ryanodine on twitch tension and post-rest recovery of tension. (A) ACS was washed out and the muscle was equilibrated with 500 nM ryanodine between the two traces. Each trace begins at the end of a 30-s rest interval and ends with the first 10 beats after a 30-s rest interval in the presence of ACS. The results are means  $\pm$  SEM from 12 experiments like the one shown in A. Values are expressed as a percent of the value before drug addition. The "Ry + ACS" columns are the percent of the value in ryanodine alone when 5  $\mu$ M ACS is added. In absolute terms, the tension,  $+dT/dt$ , and  $-dT/dt$  with both ryanodine and ACS are, respectively, 88, 70, and 61% of the value with ACS alone.

additional 30-s rest is interposed after the muscle has nearly equilibrated with ACS (near the end of each trace). In control, the first post-rest beat (beat 1) is larger than the second (beat 2), but smaller than the steady state twitch (typical of rabbit ventricle; see Bers, 1985). After exposure to ACS, beat 1 was higher than the steady state twitch; this may reflect an increased level of SR Ca loading. In the presence of ryanodine, beat 1 was less than steady state, and tension recovery in subsequent beats was largely monotonic in both the absence or

presence of ACS. Post-rest recovery and the influence of ACS and ryanodine on post-rest contractions have been discussed in detail by Bers (1985) and Sutko et al. (1986a).

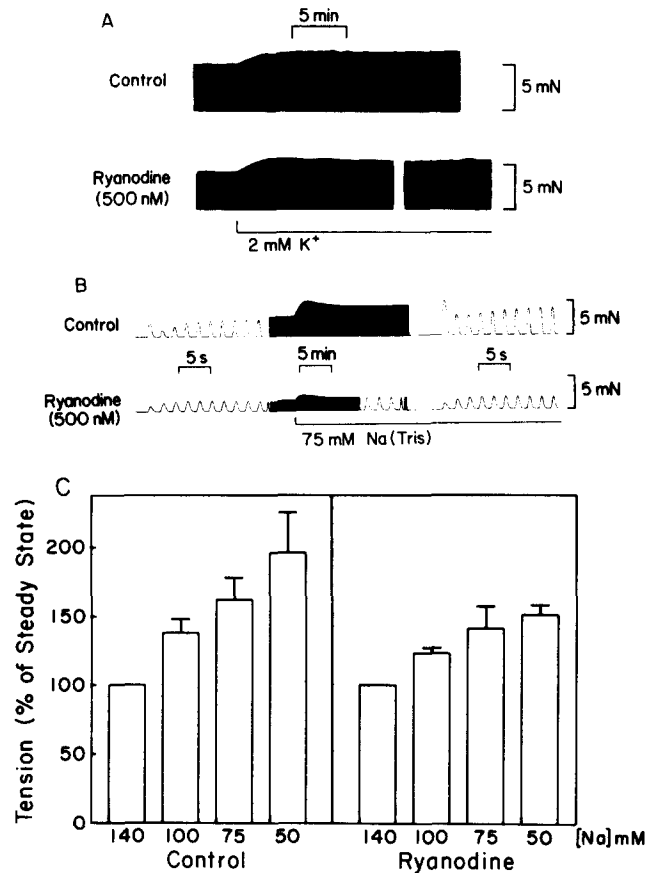


FIGURE 3. (A) The effect of reduction of  $[K]_o$  on tension development in the absence (top) and presence (bottom) of 500 nM ryanodine. Between the two traces,  $[K]_o$  was increased to 6 mM and the muscle was equilibrated with 500 nM ryanodine. (B) The effect of reduced  $[Na]_o$  on tension development in the absence and presence of 500 nM ryanodine.  $[Na]_o$  was reduced to 75 mM. The experimental traces begin at the end of a 30-s rest interval and end with the first 10 contractions after a 30-s rest interval in the presence of 75 mM  $Na_o$ . (C) Pooled results are means  $\pm$  SEM from eight experiments similar to that in B. Tension is expressed as a percent of steady state twitch tension in normal  $[Na]_o$  (140 mM). In this series of muscles, 500 nM ryanodine reduced twitch tension to  $68 \pm 6\%$  of control.

Fig. 3A illustrates that if the Na pump is inhibited by reduction of  $[K]_o$  from 6 to 2 mM, there is an increase in contractile force. After equilibration with 500 nM ryanodine (which decreased contractile force by 22%), the same  $[K]_o$  reduction induced an increase similar to that observed under control conditions (28



vs. 27%). Similar results were observed in four other experiments at different levels of reduced  $[K]_o$  (0.5–2 mM).

In addition to inhibition of the Na pump, the transsarcolemmal  $[Na]$  gradient can also be reduced by simply decreasing  $[Na]_o$ . Fig. 3B shows an experiment where  $[Na]_o$  was reduced in the absence and presence of 500 nM ryanodine. Fig. 3C summarizes results from eight experiments like those shown in Fig. 3B. This figure demonstrates that reduction of  $[Na]_o$  increases contractile force in both the absence and presence of 500 nM ryanodine (although the increases are a somewhat smaller percent after ryanodine treatment). Similar results were observed when 10 mM caffeine was used instead of ryanodine in these reduced- $[Na]_o$  experiments (not shown).

These results suggest that when normal SR function is depressed (by caffeine or ryanodine), substantial inotropic responses are still observed in response to ACS, low  $[Na]_o$ , and low  $[K]_o$ . This implies that normal SR function is not required for these inotropic effects. However, this does not preclude an important contribution of enhanced SR Ca release to the inotropy observed in the absence of caffeine or ryanodine (see Discussion).

#### *The Influence of ACS on $Ca_o$ Depletions*

Changes in  $[Ca]_o$  in response to excitation were measured with double-barreled Ca-selective microelectrodes. This technique has been previously evaluated and characterized, and these activation-dependent  $Ca_o$  depletions are indicative of cellular Ca uptake (Bers, 1983, 1985; Dresdner and Kline, 1985; Bers and MacLeod, 1986). During steady state stimulation, there are transient depletions of  $Ca_o$  during individual contractions that are related to cellular Ca influx and Ca efflux (Bers, 1983, 1985). If ACS either directly or indirectly alters transsarcolemmal Ca fluxes, the extent and/or time course of  $Ca_o$  depletion should change on application of the drug.

Application of ACS alters both the extent (Fig. 4) and the time course (Fig. 5) of  $Ca_o$  depletions. Fig. 4 shows  $Ca_o$ -depletion signals (16 sweeps averaged) for control contractions, contractions after exposure to 4  $\mu$ M ACS for 2, 5, and 10 min, and contractions 40 min after removal of ACS from the superfusate. The magnitude of the maximum  $Ca_o$  depletion under control conditions was 0.20 mV, or 4.5  $\mu$ M in an ambient  $[Ca]$  of 300  $\mu$ M. The maximum depletion increased to 7.1  $\mu$ M after 10 min in ACS and returned to 4.3  $\mu$ M after washout of the drug. Tension also increased to 263% of control after 10 min in ACS and returned to 97% after washout.

During steady state contractions,  $[Ca]_o$  returned to the bath level before the next excitation. The traces in the figure generally do not show the entire time course of this recovery. However,  $[Ca]_o$  does effectively recover for all the traces in Figs. 4–7 (even for Fig. 6C, which is not a steady state contraction). In contrast, the post-rest contractions in Fig. 8 are not near steady state, and net or cumulative  $Ca_o$  depletions occur.

An example of the way ACS alters the time course of  $Ca_o$  depletions associated with individual contractions is shown in a similar experiment with another muscle in Fig. 5. The control  $Ca_o$  depletion is similar in magnitude (4.7  $\mu$ M) to the

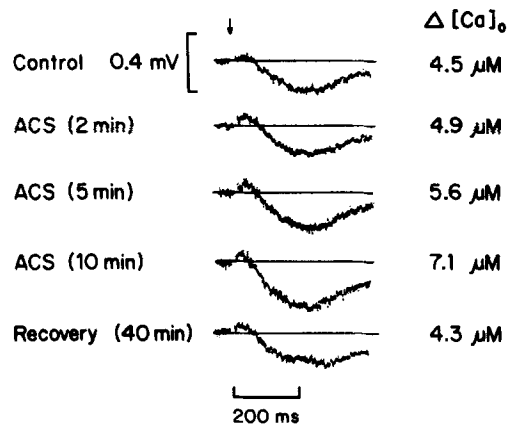


FIGURE 4. Changes in transient  $Ca_o$  depletion associated with single contractions before, during, and after exposure to  $4 \mu M$  ACS in a rabbit ventricular muscle. Each trace is the average from 16 consecutive depletions. These traces are the difference of the Ca and reference barrel signals of a double-barreled Ca-selective microelectrode on a linear millivolt scale at the left. The maximum extent of the decrease in  $[Ca]_o$  from the extended baseline is also listed at the right of each trace. The bath  $[Ca]$  in this experiment was  $300 \mu M$ . The arrow indicates when the stimulus was applied.

control  $Ca_o$  depletion in Fig. 4 (although the maximum rate of depletion is faster). After the muscle was exposed to  $4 \mu M$  ACS for 6 min (Fig. 5B), the initial rate of  $Ca_o$  depletion was greatly increased and was partially obscured by the stimulus artifact. However, the maximum extent of  $Ca_o$  depletion was in fact smaller, since after  $\sim 50$  ms,  $[Ca]_o$  began to increase. By 150 ms,  $[Ca]_o$  exceeded

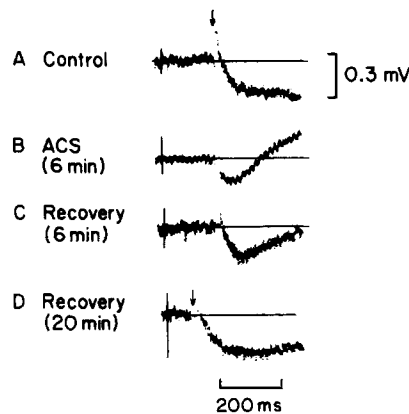


FIGURE 5. Changes in  $[Ca]_o$  associated with single contractions (16 signals averaged) before (A), during (B), and after (C and D) exposure to  $4 \mu M$  ACS. These traces are the difference signals from a double-barreled Ca microelectrode. A decrease of  $0.3$  mV corresponds to a decrease in  $[Ca]_o$  of  $6.8 \mu M$  from the bath level of  $300 \mu M$ . The stimuli are indicated by arrows. Between C and D, the delay was decreased. The short initial upward deflections may be due to imperfect barrel matching during the time that the potential was rapidly changing.

the bath level, which indicates that a net loss of cellular Ca was occurring after an initial cellular Ca uptake. The difference between the effects of ACS on  $\text{Ca}_o$  depletion in Figs. 4 and 5 probably represents an important variability in the response of rabbit ventricular muscle to ACS. It might be that in the experiment in Fig. 5, ACS led to a large SR Ca release and subsequent Ca extrusion. This possibility is discussed below (pp. 488–489 and 497–499). Fig. 5C was obtained after ACS had been removed from the superfusate for 6 min. At that time, the initial  $\text{Ca}_o$  depletion was more like the control and reached about the same minimum  $[\text{Ca}]_o$ . Although  $[\text{Ca}]_o$  began to rise after  $\sim 75$  ms, it did not exceed the bath level by the end of the trace (250 ms). Fig. 5D was obtained 20 min after removal of ACS. When the  $\text{Ca}_o$ -depletion signals in Fig. 5, B, C, and D, were recorded, twitch tension was 225, 93, and 86%, respectively, of that under control conditions (Fig. 5A).

Fig. 6 shows  $\text{Ca}_o$ -depletion and tension traces from single contractions (i.e., not signal-averaged). Panel A shows a control contraction and the small associated

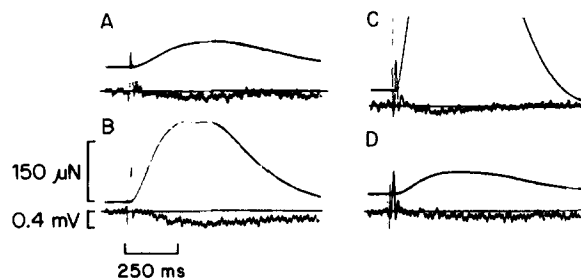


FIGURE 6. The influence of  $4 \mu\text{M}$  ACS on tension and  $[\text{Ca}]_o$  (top and bottom trace in each panel, respectively) during single contractions (not averaged). (A) Steady state contraction in control  $0.3 \text{ mM}$  Ca superfusate. (B) Steady state contraction with  $4 \mu\text{M}$  ACS. (C) First post-rest contraction after a 1-min rest interval with  $4 \mu\text{M}$  ACS (1.5 min after the record in panel B). (D) Steady state contraction 15 min after removal of ACS from the superfusate. A decrease of  $0.4 \text{ mV}$  corresponds to a decrease in  $[\text{Ca}]_o$  of  $9.1 \mu\text{M}$ .

transient depletion ( $0.15 \text{ mV}$ , or  $3.5 \mu\text{M}$  in an ambient  $[\text{Ca}]$  of  $300 \mu\text{M}$ ). Panel B shows that 12 min in  $4 \mu\text{M}$  ACS increased  $\text{Ca}_o$  depletion and twitch tension (to 197 and 320% of control, respectively). Fig. 6C shows the first beat after a 1-min rest interval (1.5 min after Fig. 6B), still in the presence of  $4 \mu\text{M}$  ACS. The initial 200 ms of the  $\text{Ca}_o$  depletion in C is similar to that in B, but  $[\text{Ca}]_o$  reached a minimum at about that time and then increased; this indicates a small net cellular Ca loss by the end of the trace. Developed force at this contraction was 540% of control. After 15 min washout of ACS, the traces in Fig. 6D were obtained. The maximum extent of  $\text{Ca}_o$  depletion and the developed tension at this contraction were 92 and 86% of the initial control, respectively. These results suggest that ACS can increase both  $\text{Ca}_o$  depletion and  $\text{Ca}_o$  accumulation.

#### *Ca<sub>o</sub> Depletions in the Presence of Ryanodine*

It is possible that the reversal of  $\text{Ca}_o$  depletion and the net Ca loss observed in the presence of ACS in Figs. 5B and 6C are attributable to a large release of Ca from the SR and consequent stimulation of Ca efflux (see Discussion). That is,

SR Ca release may be greatly increased with ACS, particularly at potentiated contractions (Fig. 6C), but also at steady state contractions in some muscles (Fig. 5B). This may elevate  $[Ca]_i$  sufficiently to increase Ca efflux, simply considering the principle of mass action. To obtain a clearer picture of how ACS affects Ca influx during individual contractions, it may be useful to examine the influence of ACS on  $Ca_o$  depletions under conditions where release of Ca from the SR is inhibited. This may reduce the effect of a large Ca efflux that begins before Ca influx has exerted its maximum effect to decrease  $[Ca]_o$ . Pretreatment of the muscle with ryanodine may inhibit SR Ca release (see Discussion) and this approach was pursued. Therefore, muscles were pretreated with ryanodine in order to inhibit both SR Ca release and the changes in Ca efflux that are driven by this Ca release.

Before ryanodine exposure, the muscle in Fig. 7 exhibited  $Ca_o$  accumulation during twitches in the presence of ACS (i.e., compare A and B). Fig. 7C shows

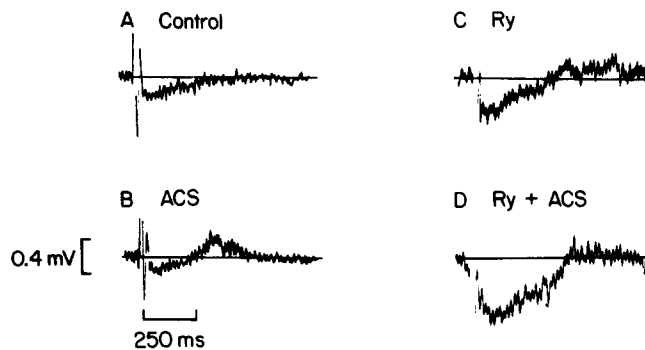


FIGURE 7. The influence of 5  $\mu$ M ACS on transient  $Ca_o$  depletions in absence (A and B) and presence (C and D) of 100 nM ryanodine. The muscle was equilibrated with ryanodine for 30 min before the recording in panel C. These single sweeps were taken before (A and C), 5 min after (B), and 4 min after (D) inclusion of 5  $\mu$ M ACS in the superfusate. Ambient  $[Ca]_o$  was 300  $\mu$ M and a decrease of 0.4 mV corresponds to a 9.1  $\mu$ M decrease in  $[Ca]_o$ .

an individual (not averaged)  $Ca_o$ -depletion trace from this muscle after exposure to 100 nM ryanodine for 30 min (which is adequate for a maximum steady state effect). When 5  $\mu$ M ACS was added to the superfusate for 4 min, the  $Ca_o$  depletion and twitch tension were increased by 58 and 54%, respectively (Fig. 7, C and D). In the presence of ryanodine, this muscle no longer showed  $Ca_o$  accumulation during twitches in the presence of ACS. These transient  $Ca_o$  depletions (where  $[Ca]_o$  returns to the bath level before the subsequent beat) are seen during steady state stimulation and indicate Ca influx and efflux associated with individual excitations (e.g., Bers, 1983, 1985). Cumulative  $Ca_o$  depletions are also observed under non-steady state conditions and reflect net uptake of Ca by the cells (such as that required to refill the SR, which becomes Ca-depleted during rest; see Bers and MacLeod, 1986; MacLeod and Bers, 1987).

Fig. 8 shows  $Ca_o$ -depletion and tension traces during and after a 1-min rest interval under various conditions. Both transient and cumulative  $Ca_o$  depletions can be seen. In control conditions when stimulation was stopped (Fig. 8A),

[Ca]<sub>o</sub> increased above the bath level. If the rest interval had been allowed to proceed, the excess Ca<sub>o</sub> would have been slowly washed away and the measured [Ca]<sub>o</sub> would have returned to the bath level in 4–5 min in this muscle (not shown). When stimulation was resumed, a net Ca<sub>o</sub> depletion developed over the first 5–10 beats (1.1 mV, or 24.3 μM in ambient 300 μM Ca). With continued stimulation, the [Ca]<sub>o</sub> returned to the bath level in ~3–4 min. These cumulative changes in [Ca]<sub>o</sub> have been previously described and probably represent the slow

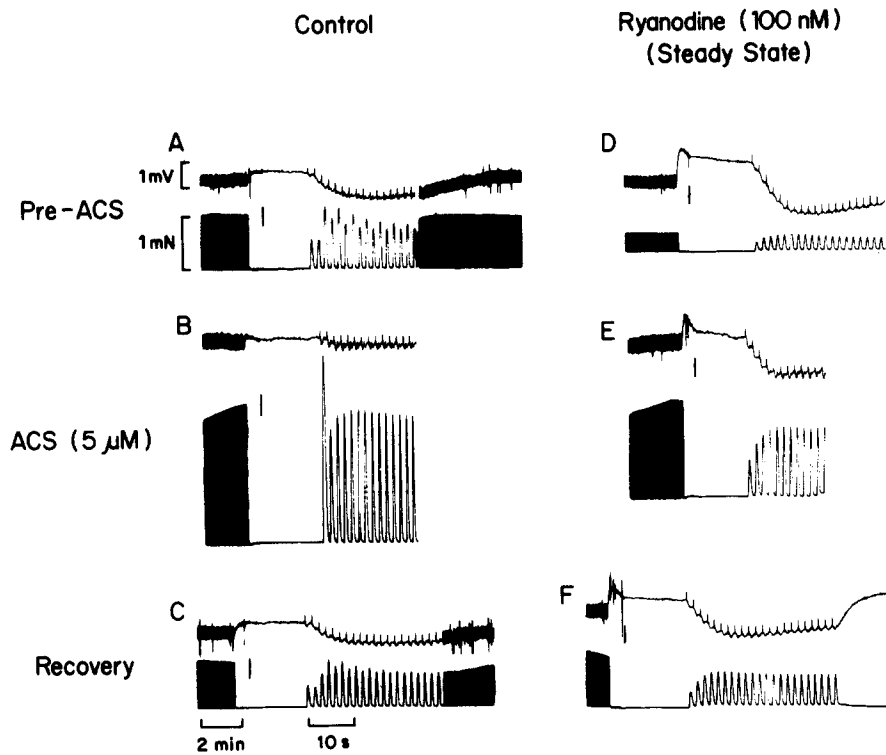


FIGURE 8. The effect of 5 μM ACS on [Ca]<sub>o</sub> changes and tension development in the absence (left) and during steady state in the presence (right) of 100 nM ryanodine. The recordings were made before exposure to ACS (pre-ACS, A and D), during ACS treatment (B and E), and during (F) or after (C) washout of ACS. Small vertical bars indicate changes in the recording rate. In each panel, stimulation (0.5 Hz) was stopped for 1 min and then resumed. A 1-mV change in the Ca signal corresponds to a 22-μM decrease (or a 24-μM increase) in [Ca]<sub>o</sub>.

loss of SR Ca from the rabbit ventricular cells during rest and refilling of the SR with Ca upon subsequent stimulation (Bers and MacLeod, 1986; MacLeod and Bers, 1987). Depletions associated with individual beats are also just discernible in this trace. The Ca<sub>o</sub> depletion associated with the first post-rest contraction is smaller than that associated with the next few contractions.

The same protocol was performed after a muscle was exposed to 5 μM ACS for 7 min (Fig. 8B). Two effects of ACS on [Ca]<sub>o</sub> changes are most apparent.

First, both the  $Ca_o$  accumulation during rest and the cumulative depletion upon restimulation are reduced. These cumulative depletions are believed to represent refilling of  $Ca_i$  stores that lose Ca during rest (Bers and MacLeod, 1986; MacLeod and Bers, 1987). ACS slows or even reverses the rest decay of contractile force, probably because the SR retains Ca more effectively during rest with ACS (Sutko et al., 1986a; Bers and MacLeod, 1986). Thus, little cumulative  $Ca_o$  depletion is associated with refilling the SR to its pre-rest level. The second notable effect of ACS is that the  $Ca_o$  depletions associated with individual contractions are increased. It should be emphasized that these  $[Ca]$  records do not show any contraction artifacts. This can be appreciated by considering the first two contractions in Fig. 8B (and also Fig. 6, B and C). The first post-rest beat (beat 1) exhibits a much stronger contraction, but a smaller transient  $Ca_o$  depletion than the second post-rest beat (or steady state beat, Fig. 6B). Thus, the increase in transient  $Ca_o$  depletion observed with ACS cannot be attributed to any influence of movement on the electrode signals. Fig. 8C was obtained 27 min after removal of ACS from the superfusate. The effects of ACS were almost completely reversed. This muscle was then exposed to 100 nM ryanodine for 20 min and the traces in Fig. 8D were obtained. In the presence of ryanodine, the loss of  $Ca_i$  during rest leads to a larger and more rapid accumulation of  $Ca_o$  than in control. When stimulation is resumed, the cumulative  $Ca_o$  depletion is also larger in the presence of ryanodine than in control conditions. At the first few contractions, only  $Ca_o$  depletion occurs. There is no evidence of  $Ca_o$  repletion between the contractions (compare with Fig. 8, A–C, and see in Fig. 9A). It is possible that ryanodine inhibits the Ca efflux that may be associated with  $Ca_i$  release.

When 5  $\mu$ M ACS was added to the ryanodine-containing superfusate for 9 min, the traces in Fig. 8E were obtained. ACS decreased the magnitude of the  $Ca_o$  accumulation during rest and the extent of cumulative  $Ca_o$  depletion, with resumption of stimulation (by 17 and 30%, respectively). In the presence of ryanodine, the SR may still lose Ca rapidly, even in the presence of ACS (MacLeod and Bers, 1987). This may account for the smaller reduction of the cumulative  $Ca_o$  depletions by ACS in the presence of ryanodine.

In ACS, the  $Ca_o$  depletions associated with individual contractions are notably increased. The first post-rest contraction in the presence of ryanodine may be the closest one might hope to get to estimation of the unidirectional Ca influx with this approach. Under these conditions, the SR is probably virtually empty, does not release Ca, and may sequester (at least temporarily) much of the Ca that enters the cell at that contraction (Bers and MacLeod, 1986; Bers et al., 1987). Thus, there is likely to be little Ca efflux contributing to the  $[Ca]_o$  changes observed. As stimulation continues, approaching steady state, Ca influx and efflux must be the same and will undoubtedly overlap. These aspects can be best appreciated in Fig. 9, which shows a closer view of the initial post-rest segments of the  $[Ca]$  traces in Fig. 8, D and E. At the first post-rest contraction (Fig. 9A), the extent of  $Ca_o$  depletion is 7.3  $\mu$ M, but takes nearly an entire cycle length (2 s) to reach this value in ryanodine, where, in the presence of ACS, the  $Ca_o$  depletion reaches 10  $\mu$ M in less than half that time (Fig. 9B). At the 10th

contraction, the transient  $Ca_o$  depletion in the presence of ACS was  $\sim 2.5$  times that in ryanodine alone. While there was surely temporal overlap between Ca influx and efflux at this point, both Ca influx and efflux may have been enhanced.

Fig. 8F was obtained 10 min after ACS removal from the ryanodine-containing superfusate. There was partial, but not complete, reversal of the ACS effect at that time. Fig. 8F also demonstrates, at faster recording rate, the rapid Ca loss that occurs upon termination of stimulation in the presence of ryanodine described previously (Bers and MacLeod, 1986; MacLeod and Bers, 1987; Bers et al., 1987). The experiments with  $Ca_o$  microelectrodes (Figs. 4–9) were performed with a lower  $[Ca]_o$  than the experiments in Figs. 1–3. However, Fig. 8 shows that the same qualitative results were obtained at low  $[Ca]_o$ . That is, ACS produced a substantial inotropic effect in both the absence and presence of ryanodine.

The results in this section indicate that when SR Ca release is minimized, the increase in Ca influx induced by ACS is most apparent. Furthermore, these results suggest that both Ca influx and efflux are increased in the presence of ACS at steady state.

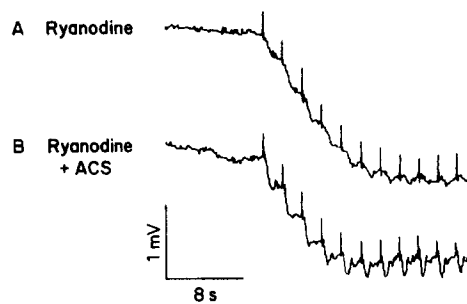


FIGURE 9. A closer view of the  $[Ca]_o$  changes shown in Fig. 8, D and E, after resumption of stimulation in the presence of 100 nM ryanodine (A) or in the presence of 100 nM ryanodine and 5  $\mu$ M ACS (B).

#### DISCUSSION

It is now generally accepted that the mechanism of inotropic action of cardioactive steroids depends on sarcolemmal Na pump inhibition and Na/Ca exchange (e.g., Langer and Serena, 1970; Langer, 1981; Lee, 1985). There is a very steep relationship between intracellular Na activity ( $a_{Na}^i$ ) and contraction force (C. O. Lee et al., 1980; Wasserstrom et al., 1983; Im and Lee, 1984; Eisner et al., 1984), and the elevation of  $a_{Na}^i$  has been associated with increased resting free  $[Ca]_i$  (Bers and Ellis, 1982; C. O. Lee and Dagostino, 1982). Inhibition of the Na pump by reducing  $[K]_o$  also results in elevated  $a_{Na}^i$  and free  $[Ca]_i$  (Bers and Ellis, 1982; Sheu and Fozzard, 1982; Eisner et al., 1984), and reduction of  $[Na]_o$  leads to increased free  $[Ca]_i$  (Marban et al., 1980; Bers and Ellis, 1982; Sheu and Fozzard, 1982). At this point, it seems clear that a shift in the Na/Ca exchange system is crucial in the inotropic effect of Na pump inhibition (by cardioactive steroids or  $[K]_o$  reduction) or reduction of  $[Na]_o$ . In each case, the transsarcolemmal  $[Na]$  gradient is reduced, such that the Na/Ca exchange is shifted more in favor of Ca entry and less in favor of Ca efflux.

There are several levels at which such an ionic shift could lead to a change in tension development: (a) Ca influx associated with individual contractions could be increased; (b) the amount of Ca released by the SR could be increased; (c) resting free  $[Ca]_i$  could be elevated. These three possibilities will be discussed in detail below in reverse order. It is unlikely that the inotropic effects are due to alteration of myofilament Ca sensitivity or mitochondrial Ca shifts. Similar effects on tension are seen with ACS, reduced  $[Na]_o$ , or Na pump inhibition by low  $[K]_o$ . Reduction of  $[Na]_o$  decreases  $a_{Na}^i$ , while ACS increases  $a_{Na}^i$ . On this basis, these two interventions might be expected to produce opposite effects on mitochondrial Ca content (Crompton et al., 1976). There is also no evidence to suggest that myofilament characteristics are altered by cardioactive steroids or small monovalent cationic changes (Fabiato and Fabiato, 1973; Nayler, 1973; Kentish, 1984). While other mechanisms may be involved, the remainder of the Discussion will focus on whether increases of resting free  $[Ca]_i$ , SR Ca release, or Ca influx can contribute to the inotropic effect of the reduced  $[Na]$  gradient.

#### *Increased Resting Free $[Ca]_i$ ?*

Cardioactive steroids, low  $[K]_o$ , and low  $[Na]_o$  have all been shown to increase the resting free  $[Ca]_i$  in cardiac tissues (Dahl and Isenberg, 1980; Lee et al., 1980; Marban et al., 1980; Bers and Ellis, 1982; Lee and Dagostino, 1982; Sheu and Fozzard, 1982; Allen et al., 1983; Weingart and Hess, 1984; Wier and Hess, 1985). The increases in the resting free  $[Ca]_i$  under conditions that might be considered to be relevant to an inotropic situation are variable, and the resting  $[Ca]_i$  is near the detection limits of both  $Ca_i$  microelectrodes and aequorin. To estimate the effect that these changes in the resting  $[Ca]_i$  may have on contractions, I will use the model of cellular Ca buffering described by Fabiato (1983) and an educated guess as to the true resting and increase in free  $[Ca]_i$ .

Fig. 10 shows Fabiato's calculated relationship between total  $Ca_i$  and tension. Three different diastolic free  $[Ca]_i$  values are indicated: 125 (A), 300 (B), and 480 (C) nM. These values correspond to total Ca of 5, 10, and 15  $\mu\text{mol/kg}$  wet weight and are below or just at the threshold of contractile activation. These values are in the range of resting and elevated free  $[Ca]_i$  associated with inotropic cardioactive steroids or a reduction of  $[Na]_o$  to  $\geq 50\%$  of normal (see above). If a constant increment of cellular Ca is added to these three diastolic levels (20  $\mu\text{mol/kg}$  wet weight), the peak tensions at A', B', and C' are predicted (corresponding to 1–2  $\mu\text{M}$  peak  $[Ca]_i$ ). Going from A' to B' and A' to C', respectively, represents 57 and 114% increases in contractile force. Thus, it seems possible that changes in resting free  $[Ca]_i$  can contribute quantitatively to the inotropic effect of transsarcolemmal  $[Na]$  gradient reduction, even with a constant phasic supply of activating Ca.

#### *Increased SR Ca Release?*

The following is probably the most widely held explanation of this inotropy. That is, reduction of the  $[Na]$  gradient limits Ca extrusion by the Na/Ca exchange and this results in an increased SR Ca load and release in response to muscle activation. The results of the present study (Figs. 1–3 and 8) illustrate that



pretreatment of rabbit ventricular muscle with caffeine or ryanodine does not abolish the increase of contractile force induced by ACS, reduced  $[Na]_o$ , or reduced  $[K]_o$ . On the contrary, the increases in contractile force are very similar in terms of a percent increase (and also in absolute terms). If it is assumed that caffeine and ryanodine inhibit SR Ca release (see below), these results suggest that a normally functioning SR is not required for the observed inotropic effects of ACS, low  $[Na]_o$ , and low  $[K]_o$ . Furthermore, these results imply that some other mechanism may be responsible for a substantial component of the increases of contractile force.

On the other hand, it is not to be concluded that increased SR Ca loading and release do not contribute to the inotropic effects of ACS, low  $[Na]_o$ , and low  $[K]_o$  under normal conditions (i.e., in the absence of caffeine or ryanodine). It is likely that ACS and low  $[Na]_o$  do increase the SR Ca content and allow the SR

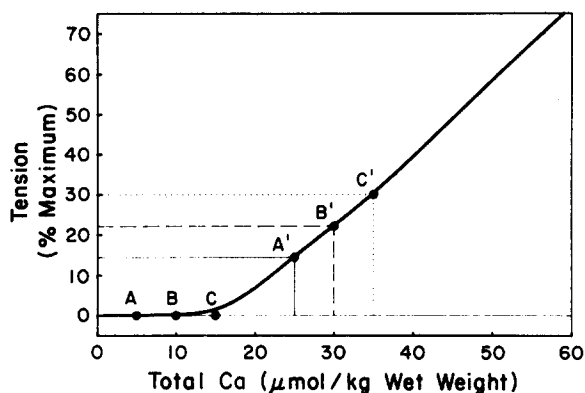


FIGURE 10. The relationship between total cytoplasmic Ca and percent maximum tension using values calculated by Fabiato (1983) and using his conversion factor 0.399 liter cell water/kg wet weight. These values were fit by a least-squares fitting procedure by the following arbitrarily chosen equation,  $y = 127 / \{1 + [1 / (1.42 \times 10^{-6})(x^{2.8})]\}$ , to generate the curve. The points along the tension = 0 line (A-C) indicate three possible values of diastolic  $[Ca]_i$ . The other points (A', B', C') indicate the corresponding tension, which would be reached by increasing cytoplasmic Ca by 20  $\mu\text{mol/kg}$  wet weight.

to retain this Ca longer in this preparation (Sutko et al., 1986a; Bridge, 1986; Bers and Bridge, 1987). Ca transients sensed by aequorin, which are believed to be due to SR Ca release, are also increased by ACS (Wier and Hess, 1984; Allen et al., 1985). While the aequorin transients may not be exclusively SR dependent, it appears likely that under control conditions, increased SR Ca uptake and release may be partly, but certainly not wholly, responsible for the increase of force produced by ACS, low  $[K]_o$ , and low  $[Na]_o$ .

The conclusions in the foregoing section are dependent upon the ability of 10 mM caffeine and 100–500 nM ryanodine to inhibit SR Ca accumulation and/or Ca release. These are concentrations of these agents that we have found sufficient to produce apparently full depression of SR function. Caffeine is a classic probe of SR function, and at 10 mM it appears to render the SR permeable to Ca, such that the SR is unable to accumulate Ca and hence unable to release Ca in

response to excitation (Weber and Herz, 1968; Blaney et al., 1978). Both caffeine and ryanodine strongly inhibit Ca transients sensed by aequorin (Hess and Wier, 1984; Wier et al., 1985) and contractions thought to be principally dependent upon Ca release from the SR (e.g., post-rest contractions and rapid cooling contractures; Figs. 2A, 3B, and 8) (Bers, 1985; Sutko et al., 1986a, b; Bridge, 1986; Bers et al., 1987). In addition to preventing SR Ca uptake and release, caffeine exerts several other cellular effects at this concentration that complicate conclusions (e.g., it increases myofilament Ca sensitivity [Fabiato, 1981; Wendt and Stephenson, 1983], it increases Ca influx [Blinks et al., 1972; Bers, 1985], and it is a phosphodiesterase inhibitor). While ryanodine appears to be more specific, its action on the SR is more complex (Hilgemann et al., 1983; Sutko et al., 1985, 1986b; Bers and MacLeod, 1986; Bers et al., 1987). In the presence of ryanodine, the SR can still accumulate Ca, but it does not appear to release the Ca normally in response to activation, losing the Ca more slowly during the few seconds after activation (Bers et al., 1987). In effect, both of these agents can be expected to inhibit SR Ca release.

It may be noted that, under these conditions, developed tension in rabbit ventricular muscle is not greatly depressed by either caffeine or ryanodine, especially when compared with certain other cardiac tissues (e.g., rat ventricle, Purkinje fibers, or even rabbit atrium; see Sutko and Willerson, 1980; Bers, 1985). These results were interpreted to suggest that in the absence of normal SR function, Ca influx can activate tension development fairly well in rabbit ventricle, but only minimally in rat ventricle. We have also recently compared the ryanodine sensitivity of rat and rabbit ventricle at several temperatures (Shattock and Bers, 1987). While tension development was depressed to a much greater extent in rat ventricle, the ryanodine concentrations required for a half-maximal effect in the two tissues were almost identical (0.5 nM at 37°C, 5 nM at 29°C, and 20–30 nM at 23°C). The limited ability of caffeine and ryanodine to decrease developed tension in rabbit ventricle is probably not due to lower SR susceptibility, but rather to a fundamental difference in dependence on SR Ca release (e.g., compared with rat ventricle).

#### *Increased Ca Influx?*

The results with Ca<sub>o</sub>-selective microelectrodes (Figs. 4–9) illustrate that Ca<sub>o</sub> depletions associated with individual contractions are increased by ACS, at least early in the contraction. Ca<sub>o</sub> depletions in cardiac muscle assessed with Ca-selective microelectrodes have been extensively characterized (Bers, 1983, 1985; Dresdner and Kline, 1985; Bers and MacLeod, 1986; MacLeod and Bers, 1987). Ca<sub>o</sub> depletions in cardiac muscle have also been studied using Ca<sub>o</sub>-sensitive dyes (Hilgemann et al., 1983; Hilgemann and Langer, 1984; Cleeman et al., 1984; Pizzaro et al., 1985; Hilgemann, 1986a, b). The results with the two techniques have in general been in excellent agreement. The only relevant discrepancy in the results reported with these two approaches is that Pizzaro et al. (1985) reported that 1 μM strophanthidin did not appreciably alter the Ca<sub>o</sub> depletion in frog ventricular muscle, whereas the present study shows that ACS can appreciably alter Ca<sub>o</sub> depletions in rabbit ventricle.

The recorded changes of [Ca]<sub>o</sub> are indicative of cellular Ca uptake and loss.

However, they cannot be expected to provide unidirectional flux measurements. That is, both Ca influx and efflux occur during single contractions and only the net changes in  $[Ca]_o$  can be detected by the Ca-sensitive microelectrode. Thus, if Ca influx is increased in ACS, but Ca efflux is also increased (e.g., owing to a large SR Ca release), the  $[Ca]_o$  may increase or decrease, or do neither or both. The extracellular Ca microelectrode records the net result of Ca influx and efflux, which may be more relevant to contraction or overall Ca movements than any single route of Ca movement (e.g., current through Ca channels). However, to obtain information about unidirectional Ca influx with this technique, it is useful to depress Ca efflux preferentially. It is possible that the rise of  $[Ca]_i$  associated with SR Ca release is a major stimulator of Ca efflux during a contraction. If so, inhibition of SR Ca release should diminish the influence of Ca efflux on the  $[Ca]_o$  change so that it is more representative of Ca influx. This was the rationale for using ryanodine in the experiments in Figs. 7–9 and especially for examining the first post-rest beat when the SR and the cell were relatively depleted of Ca (Figs. 8, *D–F*, and 9). Under these conditions, the increase of Ca uptake induced by ACS is most apparent.

The changes of  $[Ca]_o$  sensed by the Ca microelectrode are also complicated by diffusional replenishment and  $Ca_o$  buffering. The halftimes for washout or replenishment of  $[Ca]_o$  changes by the superfusate vary, but are ~1–4 min (e.g., Fig. 8A). This is very slow compared with the rapid  $Ca_o$  depletions at single excitations, but can complicate the slower cumulative depletions of  $Ca_o$ . Extracellular sites that bind Ca (e.g., the sarcolemma and glycocalyx) will buffer changes in  $[Ca]_o$  and would tend to diminish the measured changes in  $[Ca]_o$  produced by any transsarcolemmal Ca movement.

The exact location of the Ca-selective microelectrode tip in the extracellular space is unknown and is probably variable. This places some limitations on quantitative extrapolations to intact tissue Ca fluxes (as discussed by Bers, 1983; Bers and Merrill, 1985; Bers and MacLeod, 1986). The  $Ca_o$  depletions associated with individual contractions are fairly consistent in amplitude ( $4.66 \pm 0.34 \mu\text{M}$  under control conditions in superfusate containing  $300 \mu\text{M}$  Ca,  $n = 8$ ), but are more variable in the time course of  $Ca_o$  depletion (Figs. 4–8; Bers, 1983). This sort of variation would be expected if the tip of the microelectrode is at a variable distance from the site of transmembrane Ca movement and if the maximum depletion is reflective of the mean extracellular space  $Ca_o$  depletion. The quantitative agreement between  $Ca_o$  depletions measured by Ca-sensitive dyes and microelectrodes also supports this conclusion (Bers, 1983; Hilgemann and Langer, 1984; Hilgemann, 1986*b*; Bers and MacLeod, 1986; MacLeod and Bers, 1987).

Inward Ca current in cardiac muscle has been reported to be increased (Dramane et al., 1971; Weingart et al., 1978; Lederer and Eisner, 1982; Marban and Tsien, 1983) or unaffected by cardioactive steroids (Greenspan and Morad, 1975; McDonald et al., 1975). It is, however, certain that Na pump inhibition alters Ca movements mediated by Na/Ca exchange. It is useful to consider the impact that ACS may have on Ca movements via Na/Ca exchange during the rabbit ventricular action potential. In a manner similar to that described by

Mullins (1979), a simple, symmetrical, reversible Na/Ca exchange system with a coupling ratio of 3 Na:1 Ca (Reeves and Hale, 1984) will be considered.

Fig. 11 shows a typical rabbit ventricular action potential (recorded at 30°C) and also shows how the reversal potential of the Na/Ca exchanger would be

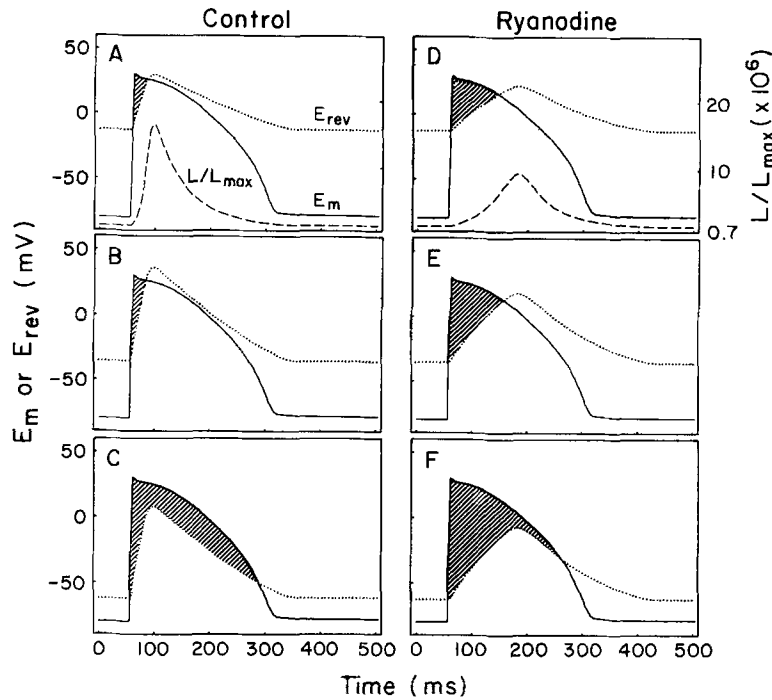


FIGURE 11. Rabbit ventricular action potential ( $E_m$ ) and estimated changes in the reversal potential of the Na-Ca exchange ( $E_{rev}$ ) in the absence (left) and presence (right) of ryanodine. The  $a_{Na}^i$  was assumed to be 6 (A and D), 8 (B and E), and 12 (C and F) mM. Resting free  $[Ca]_i$  was assumed to be 200 nM (A, B, D, and E) or 250 nM (C and F) and increased during contractions to 1 (A), 3 (B), 3.5 (C), 0.6 (D), 1.5 (E), and 3 (F)  $\mu$ M. The Ca transient and  $E_{rev}$  were assumed to reach a maximum in 40 and 120 ms in the absence and presence of ryanodine, respectively. The change in  $[Ca]_i$  that causes the change in  $E_{rev}$  could also produce an aequorin light transient. The aequorin light transient that would be associated with the  $E_{rev}$  changes in A and D were calculated from the Nernst equation and the following equation from Wier and Hess (1984):  $L/L_{max} = \{(1 + K_R[Ca]) / (1 + K_{TR} + K_R[Ca])\}^3$ , where  $K_R = 2.6 \times 10^6 \text{ M}^{-1}$  and  $K_{TR} = 126$ . The halftimes of decline of the  $E_{rev}$  signal are  $\sim 100$  ms and of the aequorin transient are 40 and 50 ms in the absence and presence of ryanodine, respectively.

affected by both changes of free  $[Ca]_i$  associated with contraction (in each panel) and increases in  $a_{Na}^i$  due to Na pump inhibition (going from top to bottom). For simplicity, the same action potential is used throughout the figure (although ACS does decrease action potential duration and overshoot and ryanodine does

prolong the action potential). The reversal potential for Na/Ca exchange ( $E_{rev}$ ) was calculated from  $E_{rev} = 3 E_{Na} - 2 E_{Ca}$  (Mullins, 1979). The time courses of the  $E_{Ca}$  changes were estimated from reported aequorin light transients (Allen and Orchard, 1984; Wier and Hess, 1984; Morgan, 1985; Wier et al., 1985). The aequorin light transients that would correspond to the  $E_{rev}$  changes in panels *A* and *D* are also shown for the purpose of comparison. When the membrane potential ( $E_m$ ) exceeds  $E_{rev}$ , Ca will enter the cell via Na/Ca exchange. The periods when this occurs are shaded in Fig. 11 and the driving force is proportional to the potential difference ( $E_m - E_{rev}$ ). When  $E_{rev}$  is greater than  $E_m$ , the Na/Ca exchange will engage in net Ca efflux.

Under control conditions,  $a_{Na}^i$  was assumed to be 6 mM with an  $a_{Na}^o$  of 110 mM (C. O. Lee and Fozzard, 1975; Ellis, 1977). The resting free  $[Ca]_i$  was assumed to be 200 nM and to increase to 1  $\mu$ M ~40 ms after the start of the action potential with  $[Ca]_o = 2$  mM. Under these conditions (Fig. 11*A*), Ca entry via Na/Ca exchange would occur only briefly at the beginning of the action potential. With exposure to ACS sufficient to raise  $a_{Na}^i$  from 6 to 8 mM and peak  $[Ca]_i$  to 3  $\mu$ M (Fig. 11*B*), Ca entry via Na/Ca exchange would initially be increased, but then Ca efflux will dominate as  $[Ca]_i$  rises and  $E_m$  falls (especially with a shorter action potential in ACS). This will be particularly true at the first post-rest contraction, where  $[Ca]_i$  probably reaches a higher peak (e.g., Figs. 6 and 8) and the action potential is also shorter. This is probably the situation where early  $Ca_o$  depletion is enhanced, but is followed by a net cellular Ca loss (Fig. 6). Sometimes this is also seen with ACS during regular stimulation (Fig. 5). Hilgemann (1986*b*) has also observed this pattern of  $Ca_o$  depletion in rabbit atrium at potentiated contractions using extracellular Ca dye to monitor  $[Ca]_o$ . The  $E_{rev}$  curve in Fig. 11*C* is projected if  $a_{Na}^i$  increases to 12 mM and resting and peak  $[Ca]_i$  increase to 0.25 and 3.5  $\mu$ M, respectively. Whether net  $Ca_o$  accumulation or depletion will actually occur in a particular cell depends critically on the degree of Na loading, the actual resting and peak free  $[Ca]_i$  that are achieved, and the action potential configuration. These three parameters may vary from muscle to muscle and this may readily explain the variability of  $Ca_o$  depletions seen with ACS (Figs. 4–8).

In the presence of ryanodine, the rise of  $[Ca]_i$  is slower and smaller (Wier et al., 1985) and Ca entry via Na/Ca exchange would be expected to be prolonged (Fig. 11, *D–F*). This was sometimes observed, but it was most apparent at post-rest contractions (e.g., Figs. 8*D* and 9). Hilgemann (1986*b*) has also made this observation in rabbit atrium with  $Ca_o$  dyes. Ryanodine also inhibits early repolarization and increases action potential duration in rabbit ventricle (Bers, 1985). This would also prolong Ca entry via Na/Ca exchange. Fig. 11, *E* and *F*, shows the expected changes of  $E_{rev}$  with progressive Na loading (to 8 and 12 mM, respectively).

The  $E_{rev}$  changes in Fig. 11 are those that might be expected under control conditions (at 2 mM  $Ca_o$ ). However, the  $Ca_o$  depletions in the present study were performed at reduced  $[Ca]_o$  (usually 0.3 mM). Fig. 12 shows estimated  $E_{rev}$  changes that might occur with 0.3 mM  $Ca_o$  under control conditions with progressive Na loading (Fig. 12, *A–C*) and with ryanodine and progressive Na

loading (Fig. 12, *D–E*). Reduced  $[Ca]_o$  increases  $a_{Na}^i$  (Ellis, 1977; Sheu and Fozzard, 1982). The resting  $a_{Na}^i$  in Fig. 12 is assumed to be 8 mM and the peak values of  $[Ca]_i$  reached during a contraction are also reduced, reflecting the decrease in force at 0.3 mM  $[Ca]_o$ . The reduction of  $[Ca]_o$  produces some quantitative changes (e.g., more positive  $E_{rev}$ ), but the qualitative results are not very different and are consistent with the experimental observations and preceding discussion.

Clearly, the foregoing projected  $E_{rev}$  changes are dependent upon the  $a_{Na}^i$ ,  $[Ca]_i$ , coupling ratio, and time course of the  $[Ca]_i$  change. While these may be reasonable values, it is not suggested that they are unique actual values. Regardless of the specific values chosen (save reduction of the Na/Ca coupling ratio to

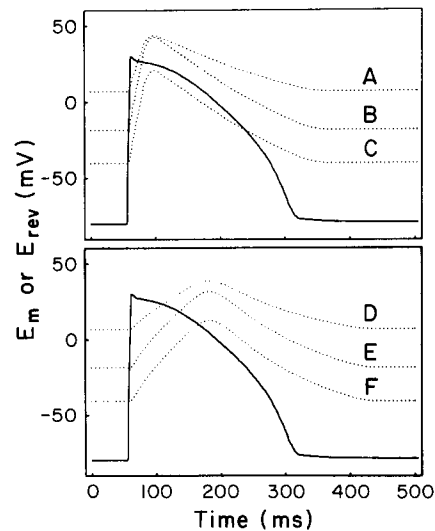


FIGURE 12. Rabbit ventricular action potential and estimated changes in  $E_{rev}$  of the Na/Ca exchange in the absence (top) and the presence of ryanodine (bottom) as in Fig. 11, but modified for  $[Ca]_o = 0.3$  mM. The  $a_{Na}^i$  is assumed to be 8 (A and D), 11 (B and E), and 16 (C and F) mM. Resting  $[Ca]_i$  was assumed to be 150 nM (A, B, D, and E) and 200 nM (C and F). Peak  $[Ca]_i$  was assumed to reach 0.6 (A), 1.5 (B), 2 (C), 0.5 (D), 1 (E), and 1.5 (F).

$\leq 2$ ), the trends predicted would be the same. It should also be emphasized that relatively modest changes in  $a_{Na}^i$  or  $a_{Ca}^i$  (resting or peak) can make important differences in whether early Ca loss like that in Fig. 5 is observed or not, as in Fig. 4 (e.g., compare B and C of Fig. 12). Thus, the variability illustrated in Figs. 4 and 5 may represent real biological variability in the way ACS alters cellular Na and Ca metabolism in rabbit ventricular muscle.

It is clear that Ca influx via the Na/Ca exchange will be most strongly favored in the early part of the action potential and that reduction in the transsarcolemmal  $[Na]$  gradient would increase this Ca influx. Ca influx via Ca current would also be expected to be large at early times. Ca current may also be increased by Na

pump inhibition (see above, p. 496), and this effect has been attributed to an increase of resting free  $[Ca]_i$  (via an effect on Ca channel availability; Marban and Tsien, 1982). Under control conditions of low  $[Ca]_o$ ,  $Ca_o$  depletions can be largely inhibited by verapamil or nifedipine (Bers, 1983; Bers and MacLeod, 1986). This is consistent with our observations that 1  $\mu$ M nifedipine almost eliminates tension development in these preparations and in the top curve in Fig. 12 (where Ca influx via Na/Ca exchange would be expected to be small). However, during steady state exposure to ACS, addition of nifedipine is less effective at reducing twitch tension than under control conditions (unpublished observations). The implication of these results is that, under control conditions, Ca current is probably responsible for most of Ca influx (and  $Ca_o$  depletion), but decreasing the transmembrane  $[Na]$  gradient can increase the contribution of Na/Ca exchange to Ca influx.

It should also be noted that Ca entry via a 3:1 Na/Ca exchange would produce an outward membrane current. Thus, if Ca current is enhanced by cardioactive steroids, the increased outward Na/Ca exchange current would lead to underestimation of the increased Ca current. As pointed out by Marban and Tsien (1982), the transient outward current could have complicated some previous experiments on the effects of cardioactive steroids on Ca current. The important issue at present is that even if Ca current were unaffected, Ca entry at early times in the action potential would probably be enhanced by ACS.

Reduction of  $[Na]_o$  has also been shown to increase transient  $Ca_o$  depletions and decrease cumulative  $Ca_o$  depletions (Bers, 1983; Bers and MacLeod, 1986) and it would be expected that  $[Na]_o$  reduction would have effects similar to  $Na_i$  loading (if the reduction of the  $[Na]$  gradient is the primary action). However, results with reduced  $[Na]_o$  are complicated, because  $a_{Na}^i$  also decreases and the  $[Na]$  gradient will tend to approach normal (Ellis, 1977).

### Conclusions

Under normal conditions, the Na/Ca exchange system would be expected to produce net Ca extrusion during most of the cardiac cycle. When the inward  $[Na]$  gradient is reduced, Ca influx via Na/Ca exchange would be expected to be increased (and Ca efflux reduced) during the cardiac cycle. This will result in both a net gain in cellular Ca and a shift in the balance of Ca influx/efflux mediated by the Na/Ca exchange during the cardiac cycle. While reduction of the  $[Na]$  gradient leads to net uptake of Ca by the cells, three independent mechanisms may contribute directly to the increase in contractile force observed during individual contractions: (a) enhanced Ca influx, (b) increased SR Ca release, and (c) increased resting  $[Ca]_i$ .

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