

Contribution of Sarcolemmal Sodium–Calcium Exchange and Intracellular Calcium Release to Force Development in Isolated Canine Ventricular Muscle

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ABSTRACT The aim of this work was to determine the relationship between peak twitch amplitude and sarcoplasmic reticulum (SR) Ca^{2+} content during changes of stimulation frequency in isolated canine ventricle, and to estimate the extent to which these changes were dependent upon sarcolemmal Na^+ - Ca^{2+} exchange. In physiological $[\text{Na}^+]_o$, increased stimulation frequency in the 0.2–2-Hz range resulted in a positive inotropic effect characterized by an increase in peak twitch amplitude and a decrease in the duration of contraction, measured as changes in isometric force development or unloaded cell shortening in intact muscle and isolated single cells, respectively. Action potentials recorded from single cells indicated that the inotropic effect was associated with a progressive decrease of action potential duration and a marked reduction in average time spent by the cell near the resting potential during the stimulus train. The frequency-dependent increase of peak twitch force was correlated with an increase of Ca^{2+} uptake into and release from the SR. This was estimated indirectly using the phasic contractile response to rapid (< 1 s) lowering of perfusate temperature from 37°C to 0 – 2°C and changes of twitch amplitude resulting from perturbations in the pattern of electrical stimulation. Lowering $[\text{Na}^+]_o$ from 140 to 70 mM resulted in an increase of contractile strength, which was accompanied by a similar increase of apparent SR Ca^{2+} content, both of which could be abolished by exposure to ryanodine (1×10^{-8} M), caffeine (3×10^{-3} M), or nifedipine (2×10^{-6} M). Increased stimulation frequency in 70 mM $[\text{Na}^+]_o$ resulted in a negative contractile staircase, characterized by a graded decrease of peak isometric force development or unloaded cell shortening. SR Ca^{2+} content estimated under identical conditions remained unaltered. Rate constants derived from mechanical restitution studies implied that the

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depressant effect of increased stimulation frequency in 70 mM $[\text{Na}^+]_o$ was not a consequence of a decreased rate of refilling of a releasable pool of Ca^{2+} within the cell. These results demonstrate that frequency-dependent changes of contractile strength and intracellular Ca^{2+} loading in 140 mM $[\text{Na}^+]_o$ require the presence of a functional sarcolemmal Na^+ - Ca^{2+} exchange process. The possibility that the negative staircase in 70 mM $[\text{Na}^+]_o$ is related to inhibition of Ca^{2+} -induced release of Ca^{2+} from the SR by various cellular mechanisms is discussed.

INTRODUCTION

An important characteristic of the cardiac sarcolemmal Na^+ - Ca^{2+} exchange protein (Nicoll, Longoni, and Phillipson, 1990) is its ability to transport Ca^{2+} ions across the plasma membrane in either a forward or reverse direction during changes in membrane potential associated with various phases of the cardiac cycle (Mullins, 1981; Eisner and Lederer, 1985; Hilgemann and Noble, 1987). The extent to which Ca^{2+} can enter or leave the cell via this mechanism appears to be due to the physiochemical properties of the exchanger, including its stoichiometry (Phillipson and Nishimoto, 1982; Phillipson, 1985; Reeves, 1985), kinetics (Miura and Kimura, 1989; Crespo, Grantham, and Cannell, 1990; Li and Kimura, 1990), binding affinities for intracellular and extracellular Na^+ and Ca^{2+} ions (Miura and Kimura, 1989; Hilgemann, 1990; Li and Kimura, 1990), and the voltage across the sarcolemma at a given time in the cycle of contraction to relaxation. Accordingly, quantitative relationships have been described between the inotropic state of mammalian cardiac muscle and the concentration of Na^+ ions in the perfusate, $[\text{Na}^+]_o$ (Reuter and Sietz, 1968; Reuter, 1974; Bers, 1987; Watanabe, Ishide, and Takishima, 1987), intracellular Na^+ activity, $a_{\text{Na}^+}^i$ (Cohen, Fozzard, and Sheu, 1982; Eisner, Lederer, and Vaughan-Jones, 1984; Boyett, Hart, Levi, and Roberts, 1987; Brill, Fozzard, Makielski, and Wasserstrom, 1987; Wang, Chae, Gong, and Lee, 1988), and various time-, voltage-, and stimulation-dependent ionic currents attributed to an electrogenic Na^+ - Ca^{2+} exchange process (Hume and Uehara, 1986; Kimura, Miyamae, and Noma, 1987; Fedida, Noble, Shimoni, and Spindler, 1987; Beuckelmann and Wier, 1989; Egan, Noble, Powell, Spindler, and Twist, 1989; Terrar and White, 1989; Bridge, Smolley, and Spitzer, 1990; Hilgemann, 1990). However, despite recent data which describe modulation of myoplasmic Ca^{2+} levels by the Na^+ - Ca^{2+} exchanger during a single cardiac cycle, it remains uncertain how sarcolemmal Ca^{2+} fluxes mediated by the exchanger influence phasic contractile activity on a beat-to-beat basis, and whether or not such effects occur primarily through an intermediate pool of Ca^{2+} , such as the sarcoplasmic reticulum (SR) (Wood, Heppner, and Weidmann, 1969; Allen, Jewel, and Wood, 1976; Fabiato, 1985*a, b*; London and Krueger, 1986; Beuckelmann and Wier, 1988; Nabauer, Callewaert, Cleeman, and Morad, 1989), or by influencing Ca^{2+} binding to the myofilaments in a more direct fashion (Bers, 1987; Lewartowski and Pytkowski, 1988).

The purpose of this study was to examine whether external Na^+ ions were obligatory for the interval dependence of twitch force and SR Ca^{2+} loading in an intact preparation of mammalian ventricular muscle. A second purpose was to study in some detail the interrelationship between frequency-dependent changes of contractile strength and intracellular Ca^{2+} loading under a wide range of inotropic

conditions. Our principal approach was to describe the quantitative relationship between SR Ca^{2+} availability during different patterns of electrical stimulation and peak contraction amplitude in thin bundles of isolated ventricular muscle. The possibility that frequency-dependent alterations of twitch amplitude were related to sarcolemmal Na^+ - Ca^{2+} exchange was tested by repeating various stimulation protocols after altering the transmembrane driving force for the exchange process. The results obtained provided evidence in support of a role for sarcolemmal Na^+ - Ca^{2+} exchange in mediating the rate-dependent increase of contractile strength under physiological conditions.

Preliminary results from part of this work have been reported previously (Bouchard and Bose, 1990).

MATERIALS AND METHODS

General Preparation

The methods used in this study have been described elsewhere (Bouchard and Bose, 1989; Bouchard, Hryshko, Saha, and Bose, 1989). To summarize, thin, free-running, right ventricular trabeculae were dissected from the hearts of mongrel dogs weighing 6–12 kg. The length of muscles used in this study ranged from 3 to 5 mm and the diameter ranged from 0.2 to 0.5 mm. The normal physiological solution used to perfuse the preparations contained the following (mM): 140 NaCl, 4.7 KCl, 1 MgCl_2 , 2.5 CaCl_2 , 5 HEPES, and 10 glucose. Solutions were titrated to pH 7.4 at 37°C with NaOH. The muscles were suspended in a 2-ml horizontal chamber which formed part of a two-circuit parallel bypass recirculation system in which two sets of physiological solutions were maintained at 35–37 and 0–2°C, respectively. One end of the preparation was tied to a fixed stainless steel post and the other end was attached to a force transducer (model FT O3C; Grass Instrument Co., Quincy, MA).

Mechanical Measurements in Multicellular Tissues

Active force was measured in response to variation of the interval between beats or rapid changes of perfusate temperature. Frequency-force relations were obtained by comparing the amplitude of steady-state contraction at different drive rates, and restitution curves were constructed from the recovery kinetics of contraction to premature or postrest stimulation at these frequencies. The phasic contractile response to rapid lowering of perfusate temperature was used as an indirect assay to probe for the amount of Ca^{2+} located within pooled SR stores. We have used a protocol similar to that described originally for cardiac muscle by Kurihara and Sakai (1985) and Bridge (1986), whereby rapid cooling contractures are elicited by diverting a stream of warmed solution normally used to perfuse the muscle into a parallel circuit and allowing a stream of cooled solution (0–2°C) to flow through the muscle chamber in the absence of electrical stimulation. Manual switching of solutions from one parallel circuit to the next permitted very rapid (<1 s) switching of bathing media at a flow rate of ~35 ml/min, without appreciable mechanical artifact or perturbation of resting muscle length. The resulting contractures have been attributed to Ca^{2+} release from SR stores, as they are correlated under various inotropic conditions with SR Ca^{2+} content estimated with atomic absorption spectroscopy (Bridge, 1986), are reduced or abolished by moderate doses of ryanodine or caffeine (Bridge, 1986; Bers, Bridge, and Macleod, 1987; Bouchard et al., 1989; Hryshko, Stiffel, and Bers, 1989b), remain unaffected during blockade of sarcolemmal Ca^{2+} channels (Kurihara and Sakai, 1985), and exhibit a species dependence similar to Ca^{2+} -induced release of Ca^{2+} (Fabiato, 1983, 1985a; Bers, 1985, 1989; Bouchard and Bose, 1989). Sitsapesan, Montgomery,

MacLeod, and Williams (1991) have recently reported that a transient decrease in temperature from 32 to 5°C increased the probability of opening of sheep cardiac Ca^{2+} release channels in the presence of 1×10^{-7} – 1×10^{-6} M *cis*- Ca^{2+} . Within diffusional limitations, this result suggests that under our conditions rapid cooling may be expected to release the entire load of Ca^{2+} within the SR during the 20–30-s period of cooling (Kitazawa, 1984; Bers, Bridge, and Sptitzer, 1989), and hence does not discriminate between various functional “compartments” of Ca^{2+} within the SR. Cooling contractures were obtained both in the place of a regularly driven beat (e.g., steady-state cooling contracture) and after rest intervals identical to those described above for postrest contraction.

Mechanical Measurements in Single Cells

Single cells from the canine right ventricle were obtained by enzymatic dissociation using a modification of the method reported by Tseng, Robinson, and Hoffman (1987). Briefly, the right coronary artery of male dogs (10–15 kg) was cannulated *in vivo* under general anesthesia and the right ventricle perfused with nominally Ca^{2+} -free normal Tyrode's solution at 34°C for 5 min. The right ventricle was then dissected out and perfused with the same solution supplemented with 0.2 mg/ml collagenase (Yakult, Honsha Co., Tokyo, Japan), 0.032 mg/ml protease (Sigma Chemical Co., St. Louis, MO), and 45 μM CaCl_2 for 60 min. After a 15-min stabilization period, ~1-mm-thick transverse slices of endocardium were shaved off with a razor blade every 2 min and cell aggregates were dispersed into 4-ml aliquots of Tyrode's solution containing 10 mg/ml albumin and 100 μM CaCl_2 . Cells were stored at room temperature (22–23°C) until use. Electrophysiological measurements were made with the whole cell voltage clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) using a List EP-7 amplifier (List Biological Laboratories, Inc., Campbell, CA). Data were filtered at 3.0 kHz using a 4-pole Bessel filter and digitized at 1–10 kHz for on-line storage on a microcomputer. Simultaneous measurements of unloaded cell shortening were made on-line with a video edge detection device (Crescent Electronics, Salt Lake City, UT) and stored for later analysis. Cell shortening records were compensated for a video delay of ~40 ms due to the average sampling frequency of the photodetector (Spurgeon, Stern, Baartz, Raffelli, Hansford, Talo, Lakatta, and Capogrossi, 1990). Due to changes in the radial position of the cell on the recording pipette over time, small changes of resting cell length during the course of an experiment could not be measured accurately. All single cell experiments were conducted at 22–23°C unless otherwise stated. Cells were superfused with a HEPES-containing solution similar to that used in experiments on multicellular tissues, except that external Ca^{2+} was lowered to 2.0 mM. Recording pipettes were pulled with a horizontal electrode puller (Sutter Instrument Co., Novato, CA) and had tip resistances of 1.5–2.5 M Ω when filled with the following solution (mM): 120 potassium aspartate, 30 KCl, 1.2 MgCl_2 , 5 HEPES, and 5 Na_2ATP (pH 7.10 with KOH). A liquid junction potential of ~10 mV (pipette solution negative) arose from the use of potassium aspartate in the recording pipette and the results have been corrected for by this amount.

Reduction of $[\text{Na}^+]_o$

Reduction of $[\text{Na}^+]_o$ from 140 to 70 mM was accomplished in the majority of experiments with equimolar replacement of Na^+ in the bathing solution with Li^+ , although in some experiments sucrose was used. Li^+ was chosen as the main substituent for Na^+ due to its reported ability to support the Na^+ current during the cardiac action potential (Mitchell, Powell, Terrar, and Twist, 1984; Schouten and ter Keurs, 1985) and reduce the magnitude of membrane currents attributed to sarcolemmal Na^+ - Ca^{2+} exchange (Mitchell et al., 1984; Fedida et al., 1987; Bridge et al., 1990). Furthermore, Li^+ can also support Na^+ - H^+ exchange by substituting for Na^+ in a

variety of tissues, including Purkinje fibers (Ellis and MacLeod, 1985), renal tubules (Ives, Yee, and Warrock, 1983), and red blood cells (Parker, 1986). Although the values reported for the apparent K_m and V_{max} for $\text{Li}^+\text{-H}^+$ exchange are smaller than those demonstrated for $\text{Na}^+\text{-H}^+$ exchange (Montrose and Murer, 1988), this property of Li^+ was beneficial for our purposes because the increased intracellular free Ca^{2+} concentration and force of contraction accompanying such a large reduction of $[\text{Na}^+]_o$ would be expected to result in acidification of the cell interior due either to the effect of reduced extracellular Na^+ on $\text{Na}^+\text{-H}^+$ exchange or displacement of protons from intracellular binding sites (Deitmer and Ellis, 1980; Bountra, Kaila, and Vaughan-Jones, 1988). The possibility also exists that excessive removal of $[\text{Na}^+]_o$ may result in a significant reduction in the capacity of the exchange process to shift from a thermodynamic mode which favors cellular Ca^{2+} efflux during diastole to one that favors Ca^{2+} influx (or decreased efflux) during the action potential, due to differences in values reported for the $K_m[\text{Na}^+]_o$ and $K_m[\text{Na}^+]_i$ of the exchange process (Miura and Kimura, 1989) or competition for the binding site at the external face of the membrane by external Ca^{2+} ions (Phillipson and Nishimoto, 1982; Phillipson, 1985). Thus, 50% reduction of $[\text{Na}^+]_o$ was chosen over more complete substitution to leave the exchange process functioning with enough residual activity to permit shifts in modal activity during the change in E_{NaCa} accompanying the action potential, such as that described by Mullins (1981) and Noble and co-workers (Di-Francesco and Noble, 1985; Noble, 1986; Hilgemann and Noble, 1987).

Whenever possible, self-controlled experiments were designed such that paired *t* tests could be conducted to determine the effects of a given intervention on muscle performance. On occasions when this was not possible, data analysis was performed using a repeated measures analysis of variance (ANOVA). The level of significance chosen for all blocked experiments was $P < 0.05$. Data are presented throughout as the mean \pm SE.

RESULTS

Frequency-Force Relationship in 140 mM $[\text{Na}^+]_o$

Fig. 1 illustrates the typical inotropic response of isolated canine ventricular muscle to an increase in the rate of stimulation from 0.5 to 1.0 and 1.5 Hz, respectively. The positive force staircase of steady-state contraction is shown in Fig. 1A, which also depicts the effect of stimulus frequency on the time course of isometric contraction. This increase of contractile strength was accompanied by a similar increase of SR Ca^{2+} content, which was estimated independently with the rapid cooling technique (Fig. 1B) and postrest electrical stimulation (Fig. 1C). Contractions elicited after brief periods of rest were taken to reflect the amount of Ca^{2+} available for release from SR stores, as they are preferentially blocked by maneuvers that deplete or inhibit the release of Ca^{2+} from the SR (Hilgemann, 1986a; Bers et al., 1987; Kort and Lakatta, 1988; Bouchard et al., 1989) and are insensitive to extracellular application of TTX, cesium, lanthanum, or cobalt (Bers, 1985; Boyett et al., 1987). Analysis of variance indicated that the frequency-dependent increase of peak twitch force was significantly greater than the accompanying increase of either cooling contracture amplitude or postrest contraction. In addition to the effects demonstrated on peak twitch force, alterations in the frequency of stimulation reduced the time to peak force from 161 ± 14 ms at 0.5 Hz to 145 ± 5 ms at 1.5 Hz, and decreased relaxation time from 220 ± 25 ms at 0.5 Hz to 198 ± 11 ms at 1.5 Hz.

The parallel increase of peak twitch force with apparent SR Ca^{2+} content observed here is compatible with previous reports describing the positive inotropic effects of

stimulation frequency on contractures induced by rapid cooling (Kurihara and Sakai, 1985) or high K^+ /low Na^+ (Gibbons and Fozzard, 1971) in mammalian heart, and probably reflects differences in time-averaged Ca^{2+} influx across the sarcolemma under these conditions. To obtain an estimate of the extent to which the rate-dependent changes of contraction and/or SR Ca^{2+} stores were dependent on sarcolemmal Na^+ - Ca^{2+} exchange, the effects of altering the transmembrane Na^+ gradient on the general contractile properties of the preparation and their frequency dependence were investigated.

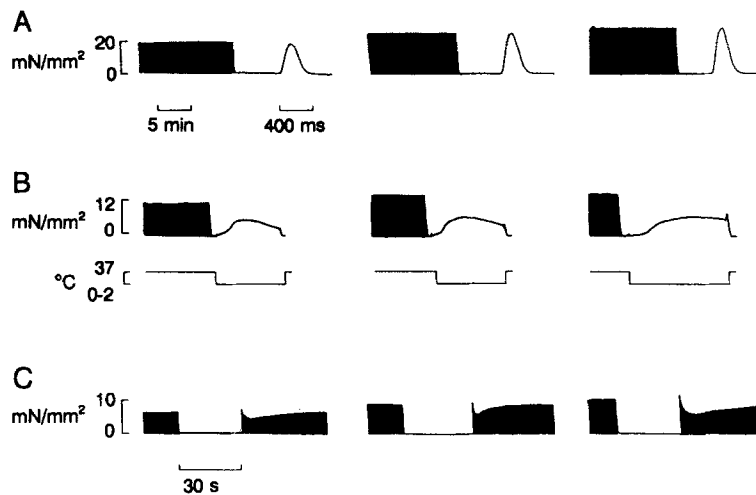


FIGURE 1. Frequency dependence of steady-state contraction, rapid cooling contracture, and postrest contraction. (A) Segments of chart recording illustrating the effect on peak and time course of isometric contraction of a stepwise increase in the rate of rhythmic stimulation. Peak twitch force was 16 ± 0.8 , 19 ± 0.8 , and 23 ± 2 mN/mm² at 0.5, 1.0, and 1.5 Hz. (B) Rate dependence of rapid cooling contractures elicited immediately after terminating rhythmic stimulation. Peak cooling contracture was increased from 15 ± 0.6 mN/mm² at 0.5 Hz to 17 ± 0.5 and 20 ± 1 mN/mm² at 1.0 and 1.5 Hz. (C) Rate dependence of contraction profile following resumption of stimulation after 30 s rest. Force production associated with the first postrest beat was increased from 22 ± 1 mN/mm² at 0.5 Hz to 25 ± 1.2 and 28 ± 0.6 mN/mm² at 1.0 and 1.5 Hz. $[Na^+]_o$ is 140 mM and $[Ca^{2+}]_o$ is 2.5 mM unless otherwise stated. Stimulation frequencies were 0.5 (left), 1.0 (middle), and 1.5 Hz (right), respectively. Calibration bars represent 5 min and 400 ms where indicated in A, and 30 s in B and C.

Effect of $[Na^+]_o$ on Steady-State Contractility

Muscles were re-equilibrated in solutions in which 50% of the bathing Na^+ was replaced with Li^+ (Fig. 2). The times at which the recirculating solution was exchanged with fresh Li^+ -substituted solution are indicated by the arrows in Fig. 2A and are typical for most of the preparations studied. After the second wash, the muscle was stimulated rhythmically at 0.5 Hz until peak contraction reached a new steady-state level. This usually took place within 30 min, during which time no

change in resting tension was observed in any of the experiments. In a total of 17 preparations studied with various protocols, reduction of $[\text{Na}^+]_o$ from 140 to 70 mM increased peak twitch force by $91.5 \pm 4\%$.

The increase of peak twitch force resulting from $[\text{Na}^+]_o$ reduction was correlated with a similar increase of SR Ca^{2+} content at all times during exposure to low $[\text{Na}^+]_o$. Fig. 2 *B* illustrates that both contraction and cooling contracture amplitudes were nearly doubled after stabilization of steady-state contraction in 70 mM $[\text{Na}^+]_o$. As indicated by the chart recordings in Fig. 2 *C*, similar observations were made with respect to the $[\text{Na}^+]_o$ sensitivity of postrest contraction. Analysis of variance indicated that the $[\text{Na}^+]_o$ -dependent increase of steady-state contraction ($91.5 \pm 4\%$), steady-state cooling contracture ($90 \pm 7\%$), and postrest contraction ($110 \pm 8\%$) were not statistically different, although the trend was such that the inotropic effect of lowering

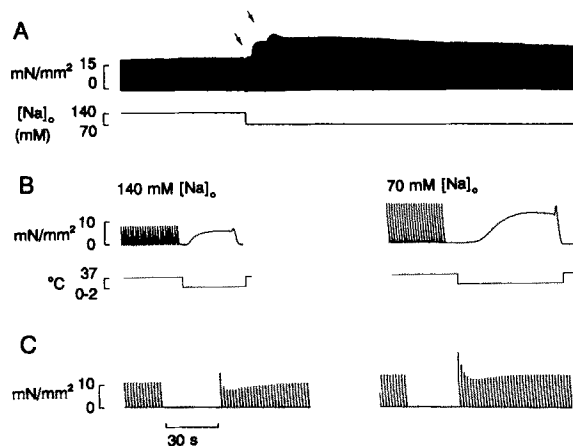


FIGURE 2. Effect of reducing $[\text{Na}^+]_o$ on developed tension in response to electrical stimulation and rapid cooling. *A* illustrates the effect of reducing $[\text{Na}^+]_o$ from 140 to 70 mM on peak developed and resting tension in response to rhythmic stimulation for 30 min at 0.5 Hz. Arrows indicate the time at which the recirculating perfusion solution was replaced with fresh Li^+ -containing solution. *B* and *C* illustrate the influence of $[\text{Na}^+]_o$ depletion on steady-state rapid cooling contracture

amplitude and postrest contraction after 30 s rest, respectively. Mean \pm SE increase of steady-state contraction, cooling contracture, and postrest contraction were 91 ± 4 , 90 ± 7 , and $110 \pm 8\%$ ($n = 17$). $[\text{Na}^+]_o$ was lowered in all experiments with equimolar Li^+ substitution. Calibration bar represents 30 s in *B* and *C*.

$[\text{Na}^+]_o$ on postrest contraction was greater than that associated with changes of steady-state contraction or cooling contracture. When the amplitudes of steady-state cooling contractures were calculated as a percentage of preceding steady-state contractions, lowering $[\text{Na}^+]_o$ had no effect on the ratio of peak contraction to peak cooling contracture: the values were 85 ± 4 and $79.3 \pm 5.6\%$ for 140 and 70 mM $[\text{Na}^+]_o$, respectively.

Addition of either caffeine or ryanodine to the low $[\text{Na}^+]_o$ perfusate markedly depressed contractile strength in response to 0.5-Hz stimulation. Caffeine (3×10^{-3} M) decreased peak contraction to $40 \pm 15\%$ control ($n = 8$), whereas ryanodine (1×10^{-8} M) reduced contraction to $25 \pm 15\%$ of the pretreatment values ($n = 8$). Similarly, blockade of sarcolemmal Ca^{2+} channels with nifedipine (2×10^{-6} M) in 70 mM $[\text{Na}^+]_o$ decreased contraction amplitude by $72 \pm 5\%$, suggesting a requirement

of the positive inotropy for sustained trans-sarcolemmal Ca^{2+} entry. The reversal of the positive inotropic response in low $[\text{Na}^+]_o$ after elimination of SR Ca^{2+} stores suggests that the majority of twitch potentiation under these conditions is related to enhanced uptake and release of Ca^{2+} from the SR. These results contrast with those obtained in species with poorly developed sarcotubular networks, such as rabbit ventricle, where greater concentrations of caffeine (1×10^{-2} M) or ryanodine ($1-5 \times 10^{-7}$ M) do not prevent the inotropic response to a stepwise decrease of $[\text{Na}^+]_o$ from 140 to 50 mM (Bers, 1987).

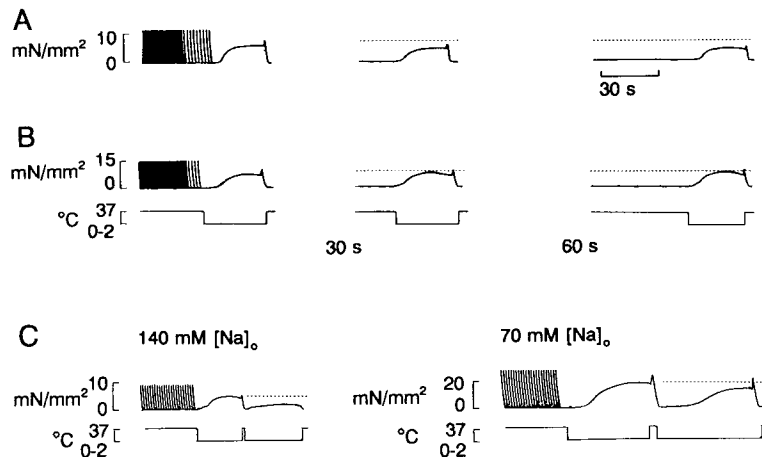


FIGURE 3. Effect of $[\text{Na}^+]_o$ reduction on the time dependence of rapid cooling contracture amplitude in the absence of electrical stimulation. (A) Time-dependent decay of peak cooling contracture amplitude in 140 mM $[\text{Na}^+]_o$ of 20 ± 1.5 and $31 \pm 3\%$ following rest for 30 and 60 s, respectively. Stimulation frequency was 0.5 Hz. (B) Inhibition of cooling contracture decay after reduction of $[\text{Na}^+]_o$ to 70 mM. Peak cooling contracture declined by only 3 ± 0.12 and $9.7 \pm 4\%$ after 30 and 60 s rest. (C) Paired cooling protocol in which a second rapid cooling contracture (P2) is elicited immediately after termination of the rewarming phase of the preceding contracture (P1) at 0.5 Hz. Lowering $[\text{Na}^+]_o$ resulted in a significant increase ($P < 0.05$) of the P2/P1 ratio from 0.5 ± 0.05 in 140 mM $[\text{Na}^+]_o$ (left recording) to 0.63 ± 0.05 after the decrease to 70 mM (right recording). Note the slowing of cooling contracture relaxation in low $[\text{Na}^+]_o$. Dashed lines above each segment of chart recording indicate the peak of the preceding steady-state contracture. Calibration bar represents 30 s in A–C.

Effect of $[\text{Na}^+]_o$ on Cellular Ca^{2+} Efflux

Previous studies have suggested the presence of a diastolic “leak” of Ca^{2+} from ryanodine-sensitive sites within the SR (guinea pig ventricle, Kurihara and Sakai, 1985; rabbit atrium and ventricle, Bridge, 1986; Hilgemann, 1986a; Sutko, Bers, and Reeves, 1986; Bers et al., 1987; dog ventricle, Bouchard et al., 1989; Hryshko, Kobayashi, and Bose, 1989a), and extrusion of Ca^{2+} from the cell during this process appears to be dependent primarily upon sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchange (Bridge, 1986; Hilgemann, 1986a, b; Lewartowski and Pytkowski, 1988; Bers et al., 1989). We observed a similar response under the present conditions, as shown in Fig. 3A. In

these experiments, rhythmic stimulation was terminated and rest intervals of either 30 or 60 s were interpolated before the onset of cooling. The dashed lines above the individual recordings are for reference and represent the peak amplitude of steady-state cooling contractures. In these experiments, a gradual increase in the period of time preceding the onset of cooling resulted in a proportional decrease of peak cooling contracture amplitude. As illustrated in the panel below (Fig. 3 B), reduction of $[\text{Na}^+]_o$ almost abolished this time-dependent loss of cell Ca^{2+} , as the amplitude of cooling contractures after 30 and 60 s rest were essentially unchanged from those recorded during rhythmic stimulation. Similar results were obtained in nine other experiments.

In a second set of experiments rapid cooling contractures were elicited in a serial fashion to probe the ability of the cell to extrude Ca^{2+} during the 0.5–1.0-s period of rewarming (cf. Hryshko et al., 1989b). As shown in Fig. 3 C, the peak of the second cooling contracture (P2) in canine ventricle perfused with 140 mM $[\text{Na}^+]_o$ was $50 \pm 5\%$ that of the first contracture (P1) after termination of stimulation at 0.5 Hz. As illustrated in the recording on the right, reduction of $[\text{Na}^+]_o$ to 70 mM increased the amplitude of P2 to $63 \pm 5\%$ that of P1 ($P < 0.05$) and slowed the rate of relaxation of both cooling contractures. In addition to its effects on peak contraction, reduction of $[\text{Na}^+]_o$ also prolonged the duration of contraction by 16 ± 0.9 , 21 ± 1.2 , and $21.5 \pm 1.3\%$ at 0.5, 1.0, and 1.5 Hz, respectively.

Frequency–Force Relationship in 70 mM $[\text{Na}^+]_o$

In marked contrast to observations made in physiological $[\text{Na}^+]_o$, increased stimulation frequency exerted a negative inotropic effect on peak twitch force in 70 mM $[\text{Na}^+]_o$ (Fig. 4 A). This depressant effect on contraction occurred despite the demonstration that increasing the rate of stimulation had little effect on the amplitude of steady-state cooling contractures. An example of this “uncoupling” of peak developed tension and apparent intracellular Ca^{2+} content is shown in Fig. 4 B, which illustrates that while an increase in the rate of stimulation from 0.5 to 1.0 and 1.5 Hz depressed steady-state contraction by 15 and 35%, cooling contracture amplitude was reduced by only 3 and 5%. Similar observations were made with respect to postrest contraction (Fig. 4 C), which was slightly augmented in response to increased stimulation frequency. This effect was not due to metabolic “run down” of the preparation, as the negative staircase was stable over the course of a 1-h test period and completely reversible after the subsequent return to 140 mM $[\text{Na}^+]_o$ ($n = 3$).

Fig. 5 shows the frequency dependence of contraction under conditions where SR Ca^{2+} content was varied in a graded fashion. Lowering $[\text{Ca}^{2+}]_o$ from 2.5 to 1.25 mM reduced peak twitch strength and rapid cooling contracture amplitude to 44 ± 3 and $64 \pm 5\%$ of the equivalent values in 2.5 mM $[\text{Ca}^{2+}]_o$. Increased stimulation frequency in this situation resulted in a large increase of peak twitch force, the extent of which exceeded that demonstrated in 2.5 mM $[\text{Ca}^{2+}]_o$ (Fig. 5 A). Conversely, lowering $[\text{Na}^+]_o$ with either isosmotic sucrose or equimolar Li^+ substitution reversed the inotropic response to rate changes, despite increasing twitch force in response to rhythmic stimulation at 0.5 Hz by 92 and 106%, respectively. Fig. 5 B illustrates the effect of caffeine (3×10^{-3} M) and ryanodine (1×10^{-8} M) on the frequency

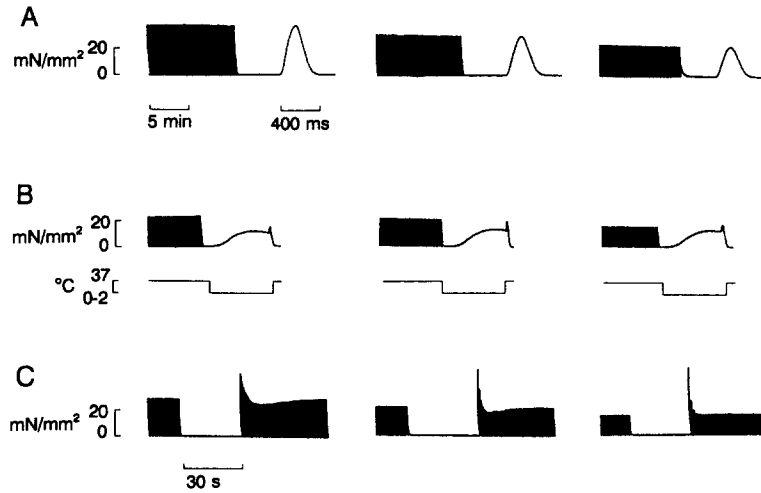


FIGURE 4. Frequency dependence of steady-state contraction, rapid cooling contracture, and postrest contraction in 70 mM $[\text{Na}^+]_o$. (A) Negative staircase. Peak twitch force was reduced from 30 ± 2 mN/mm 2 at 0.5 Hz to 26 ± 0.2 and 19 ± 0.7 mN/mm 2 at 1.0 and 1.5 Hz. (B) Corresponding cooling contracture amplitude remained relatively unchanged, and was decreased from 29 ± 0.6 mN/mm 2 at 0.5 Hz to 28 ± 0.8 and 27 ± 2 mN/mm 2 at 1.0 and 1.5 Hz. (C) Postrest contraction was increased at similar rates from 45.4 ± 5 mN/mm 2 to 50 ± 4 and 54 ± 3 mN/mm 2 . Stimulation protocol was identical to that described in Fig. 1. Calibration bars represent 5 min and 400 ms where indicated in A, and 30 s in B and C.

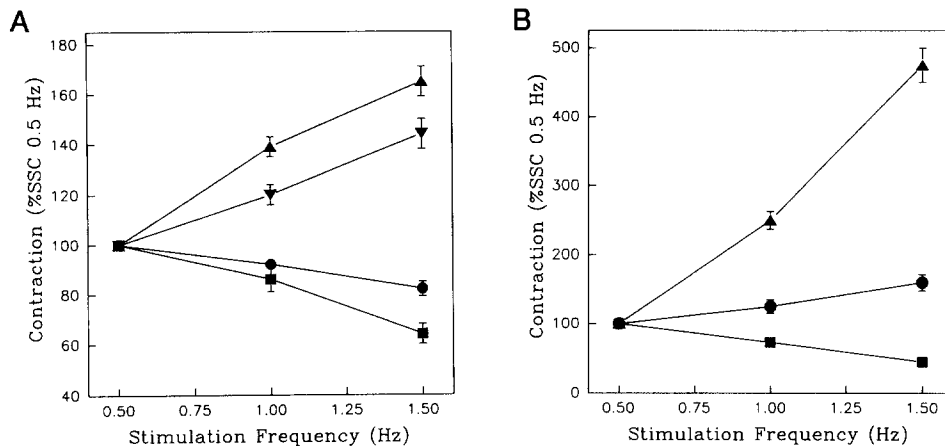


FIGURE 5. Dependence of the frequency-force relationship on the level of cell Ca^{2+} loading. (A) Frequency dependence of peak twitch force in 1.25 $[\text{Ca}^{2+}]_o/140$ $[\text{Na}^+]_o$ (closed triangles), 2.5 $[\text{Ca}^{2+}]_o/140$ $[\text{Na}^+]_o$ (inverted triangles), 2.5 $[\text{Ca}^{2+}]_o/70$ $[\text{Na}^+]_o$ -sucrose substitution (closed circles), and 2.5 $[\text{Ca}^{2+}]_o/70$ $[\text{Na}^+]_o$ - Li^+ substitution (closed squares). Note the blunting and then reversal of the positive staircase as SR Ca^{2+} was gradually increased. (B) Frequency dependence of contraction in 70 mM $[\text{Na}^+]_o$ after unloading of SR Ca^{2+} stores with ryanodine (1×10^{-8} M, closed triangles), caffeine (1×10^{-2} M, closed circles), or nifedipine (2×10^{-6} M, closed squares). Contraction amplitudes are expressed throughout as percent of peak twitch amplitude at 0.5 Hz stimulation within a given inotropic intervention.

dependence of contraction in 70 mM $[\text{Na}^+]_o$. As discussed above (Fig. 3), both ryanodine and caffeine reversed the positive inotropic effect of $[\text{Na}^+]_o$ withdrawal. Exposure of muscles to caffeine restored the positive staircase of twitch force and had the additional effect of increasing diastolic tension during high frequency stimulation, which could be reversed after an abrupt decrease to lower frequencies (data not shown). Similar results were obtained with ryanodine, which depressed twitch force to a larger extent and dramatically increased the sensitivity of contraction to stimulation frequency without producing a comparable change in the level of resting tension. Conversely, inhibition of Ca^{2+} entry with nifedipine (2×10^{-6} M) in 70 mM $[\text{Na}^+]_o$ resulted in a frequency-dependent decrease in peak twitch force.

Data presented thus far indicate that external Na^+ is required for the positive staircase of twitch force and rapid cooling contracture amplitude observed under the present experimental conditions. The inotropic effect associated with changes of stimulation frequency appears to depend strongly on the level of cell Ca^{2+} loading immediately preceding the rate change; once a certain threshold level of SR Ca^{2+} is achieved, further reductions in basic cycle length exert a marked depressant effect on contraction which appears to occur despite adequate loading of the SR with Ca^{2+} . The remainder of experiments were thus aimed at addressing the mechanism of the negative staircase and determining whether the $[\text{Na}^+]_o$ dependence of the staircase could also be demonstrated at the level of the single cell.

Effect of $[\text{Na}^+]_o$ on SR Ca^{2+} Transport Kinetics

It has been hypothesized that the kinetics of diastolic Ca^{2+} transport within the SR may influence the amount of Ca^{2+} available for release in several mammalian species, including humans (Allen et al., 1976; Bers, 1985; Fabiato, 1985b; Capogrossi, Stern, Spurgeon, and Lakatta, 1988; Bouchard and Bose, 1989; Hryshko et al., 1989b; Morgan, Erny, Allen, Grossman, and Gwathmey, 1990). To study the possible involvement of this mechanism in the negative staircase described in Fig. 4, we extended the range of rest intervals to include those close to the interval between rhythmically stimulated contractions at different basic cycle lengths to permit construction of postrest mechanical restitution curves.

Fig. 6 shows the protocol used to investigate the sensitivity of mechanical restitution to changes of $[\text{Na}^+]_o$. Segments of chart recording obtained at 1.0-Hz stimulation are shown which illustrate the effect on contractile strength of imposing rest intervals ranging from 5 to 120 s before (Fig. 6A) and after (Fig. 6B) lowering $[\text{Na}^+]_o$ from 140 to 70 mM. The dashed lines above each set of recordings are for reference and indicate the peak of the restitution process for postrest contraction at 0.5 Hz in each situation. As shown in Fig. 6A, a gradual increase in the period of time preceding resumption of stimulation at 1.0 Hz in physiological $[\text{Na}^+]_o$ resulted in a time-dependent increase in the amplitude of the first postrest contraction. A comparison of the dashed line with the observed potentiation of contractile strength shows that increasing the rate of stimulation results in an increase in both the peak and rate of mechanical restitution. Similar changes in the pattern of restitution have been observed previously in Purkinje fibers (Lipsius, Fozzard, and Gibbons, 1982; Boyett et al., 1987), ventricular and atrial muscle (Koch-Weser and Blinks, 1963; Allen et al., 1976; Fabiato, 1985a), and myocytes (Capogrossi, Kort, Spurgeon, and Lakatta, 1986), where they have been interpreted to result from replenishment of a

releasable pool of Ca^{2+} within the SR during diastole and/or reactivation of the SR Ca^{2+} -release channel. The corresponding response of the preparation to rapid cooling (Fig. 6A; lower recording) suggests that this shift in the pattern of contractile restitution appears to be correlated with an increase in the apparent rate of Ca^{2+} loss from the SR. Comparison of the horizontal position of the arrow with the rapid cooling profile obtained at 1.0 Hz shows that despite a 20% increase in steady-state cooling contracture at 1.0 Hz, contractures elicited subsequent to 120 s rest at 0.5 and 1.0 Hz stimulation were nearly identical. The apparent rate of loss of SR Ca^{2+} over a 120-s period of rest estimated by the slope of the time-dependent decay of cooling contracture amplitude was increased from $k = 0.517$ at 0.5 Hz to 0.68 and 1.1 at 1.0 and 1.5 Hz, respectively, while the half-time for decay of contracture

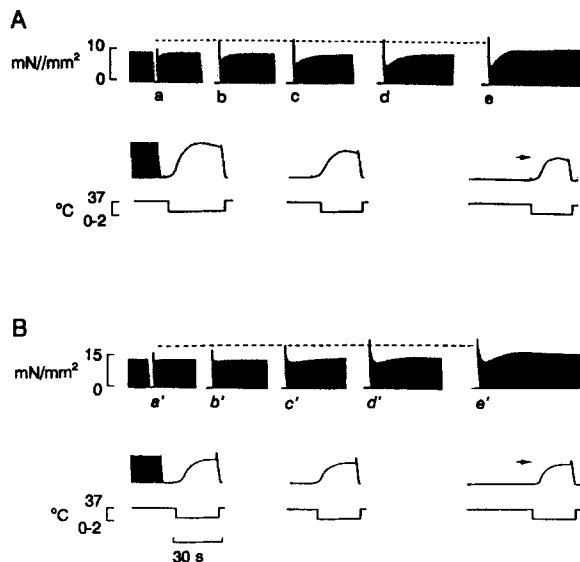


FIGURE 6. Effect of $[\text{Na}^+]_o$ reduction on the time and frequency dependence of postrest contraction and rapid cooling contracture amplitude. (A) *Upper recordings*, segments of chart recording illustrating the effect in 140 mM $[\text{Na}^+]_o$ on the first 20–30 contractions of gradually extending the duration of rest before resuming stimulation. Test intervals were 5, 10, 30, 60, and 120 s (a–e). Stimulation frequency was 1.0 Hz. *Lower recordings*, effect of 30 or 60 s rest on peak rapid cooling contracture amplitude. Dashed lines in each set of recordings are for comparison and indicate peak restitution for post-rest contraction obtained at 0.5 Hz in the same preparation. Arrow represents peak cooling contracture after 120 s rest at 0.5 Hz. (B) Corresponding response in the same preparation to postrest electrical stimulation or rapid cooling after lowering $[\text{Na}^+]_o$ to 70 mM. Test intervals were 5, 10, 30, 60, and 120 s and are denoted a'–e'. Calibration bars represent 30 s in A and B.

amplitude ($t_{1/2}$) remained unchanged from the value calculated at 0.5 Hz of 61 s (see also Kitazawa, 1984; Bridge, 1986).

A similar response was observed after reduction of $[\text{Na}^+]_o$ to 70 mM, despite the observation that postrest cooling contractures remained relatively unchanged from those associated with rhythmic stimulation. As illustrated in Fig. 6B, a gradual increase in the duration of rest preceding stimulation at 1.0 Hz resulted in a time-dependent increase in the amplitude of the first postrest beat. At all frequencies studied, postrest contraction recovered to a peak level which exceeded that associated with the corresponding response before lowering $[\text{Na}^+]_o$. However, despite similarities in the pattern of twitch restitution in 70 and 140 mM $[\text{Na}^+]_o$, a number of differences in the quantitative relationship between peak twitch amplitude and

duration of the preceding stimulus interval were noted. One such difference was for contractions elicited at test intervals between 2.5 and 10 s, which were closest to diastolic intervals associated with rhythmic stimulation at 0.5–1.5 Hz (0.67–2.0 s). As illustrated in Fig. 7, A–C (closed circles) these were inhibited in a frequency-

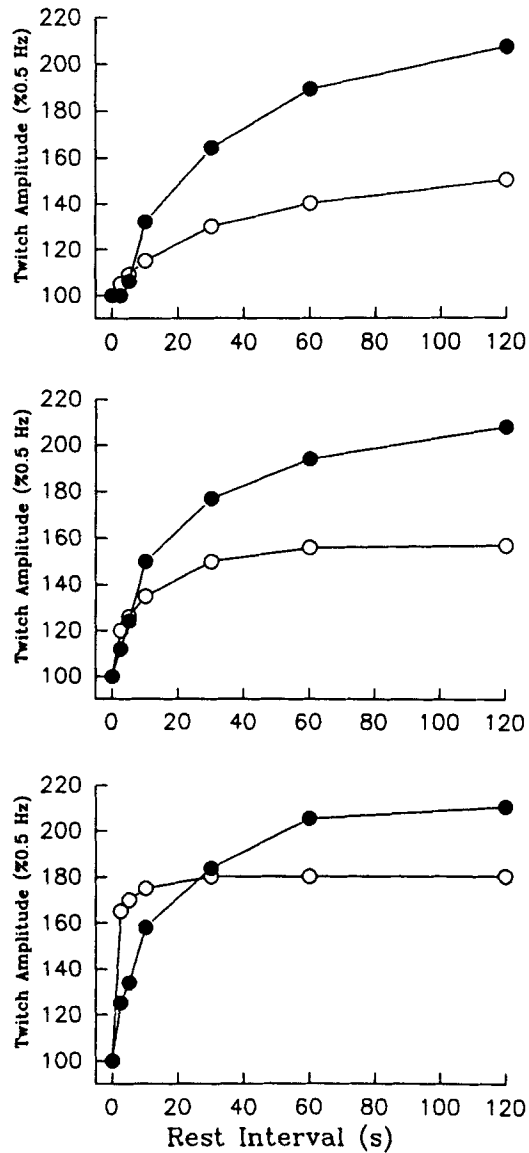


FIGURE 7. Effect of $[\text{Na}^+]_o$ reduction on restitution curves for postrest contraction. Time- and frequency-dependent changes of peak twitch amplitude after rest for 2.5, 5, 10, 30, 60, and 120 s are compared before (*open circles*) and after (*closed circles*) lowering $[\text{Na}^+]_o$ from 140 to 70 mM. Stimulation frequencies were 0.5, 1.0, and 1.5 Hz, and are arranged from top to bottom. All data points have been normalized to peak twitch amplitude at 0.5 Hz steady-state stimulation in each $[\text{Na}^+]_o$ tested, and represent the mean of nine paired experiments. Standard error bars have been omitted for clarity.

dependent manner when compared directly with the responses in 140 mM $[\text{Na}^+]_o$ (*open circles*). This is particularly evident in Fig. 7 C, where contraction amplitude after rest for 2.5, 5, 10, and 30 s at 1.5 Hz was markedly depressed, even though contraction recovered to a greater peak during later test intervals. Second, despite

the gradual inhibition of postrest twitch amplitude at rest intervals <10 s, the frequency-dependent increase of postrest contraction amplitude of the type observed in 140 mM $[Na^+]_o$ was maintained after the decrease of $[Na^+]_o$ to 70 mM, and contraction recovered to a common peak (207–210%) at all three frequencies investigated. This modest increase of contractile force at test intervals between 2.5 and 60 s was observed consistently despite the absence of accompanying changes in total SR Ca^{2+} content estimated with the cooling technique (6 B, lower panel). This result implies that the rate of replenishment of a releasable store of Ca^{2+} may not be the limiting factor for the negative staircase observed in Na^+ -depleted solutions and/or that the release of Ca^{2+} from a fully loaded store may be inhibited at higher rates of stimulation.

Effect of $[Na^+]_o$ on Apparent Recovery of the SR Ca^{2+} Release Process

Removal of external Na^+ from the perfusate or inhibition of the Na^+ pump has been shown previously to increase microscopic diastolic myofilament oscillations (Stern, Kort, Bhatnagar, and Lakatta, 1983; Ishide, Watanabe, and Takishima, 1984; Bose, Kobayashi, Bouchard, and Hryshko, 1988), presumably due to enhanced spontaneous release of Ca^{2+} from the SR. The observation that elevated myoplasmic Ca^{2+} inhibits SR Ca^{2+} release in skinned cardiac (Fabiato, 1985a) or intact voltage-clamped skeletal muscle fibers (Schneider and Simon, 1988; Simon, Klein, and Schneider, 1991), raises the possibility that the inhibition of cellular Ca^{2+} efflux in the presence of lowered $[Na^+]_o$ (Fig. 3) may act in a similar fashion to inhibit SR Ca^{2+} release during high frequency stimulation. This would be consistent with the gradual increase in rest potentiation as the stimulation rate is enhanced (Figs. 4, 5, and 7), as sufficient time would have elapsed during the 30-s rest period for the majority of SR Ca^{2+} release channels to pass through their 0.8–3.5-s refractory phase(s) with a $Q_{10} > 4.0$ (Fabiato, 1985a). We attempted to test this possibility in the intact muscle preparation by interpolating premature stimuli at coupling intervals within this test range (0.1–1s) and observing the rate and extent of recovery of extrasystolic and post-extrasystolic contraction.

140 mM $[Na^+]_o$. Fig. 8 illustrates the typical recovery of extrasystolic and post-extrasystolic contractions elicited in response to premature stimuli delivered at coupling intervals ranging from 200 to 800 ms. The control solution contained 140 mM $[Na^+]_o$ and 2.5 mM $[Ca^{2+}]_o$. As depicted in the schematic diagram in the top panel, delivery of each premature stimulus was preceded by 100 steady-state contractions at each test frequency. The following post-extrasystolic contraction was elicited at a coupling interval equivalent to the basic cycle length preceding delivery of the premature stimuli, thus ensuring a constant interval during which a releasable store of Ca^{2+} could be replenished at a given stimulation frequency. For the superimposed control recordings shown in Fig. 8 A, the basic cycle length was 2 s (0.5 Hz) and the extrasystolic contraction recovered to the level of the preceding steady-state contraction by 900 ms, with a time constant of recovery of 450 ms. Peak post-extrasystolic potentiation occurred at 200 ms, which corresponded to the first coupling interval at which a significant premature contraction could be elicited,

following which the amplitude of contraction decayed exponentially back to the level of the preceding steady-state contraction (Yue, Burkoff, Franz, Hunter, and Sagawa, 1985; Wier and Yue, 1986).

Data points for recovery of extrasystolic and post-extrasystolic contractions were well fitted by a single exponential function of the form $F(t) = A_0 + (1 - A_0)[1 - \exp(-t/\tau)]$, where $F(t)$ is the fractional recovery of contraction at time t , and A_0 and τ are the time-independent base factor and time constant of recovery, respectively. In the control solution containing 140 mM $[\text{Na}^+]_o$ and 2.5 mM $[\text{Ca}^{2+}]_o$, increased stimulation frequency resulted in a graded increase in the rate of recovery

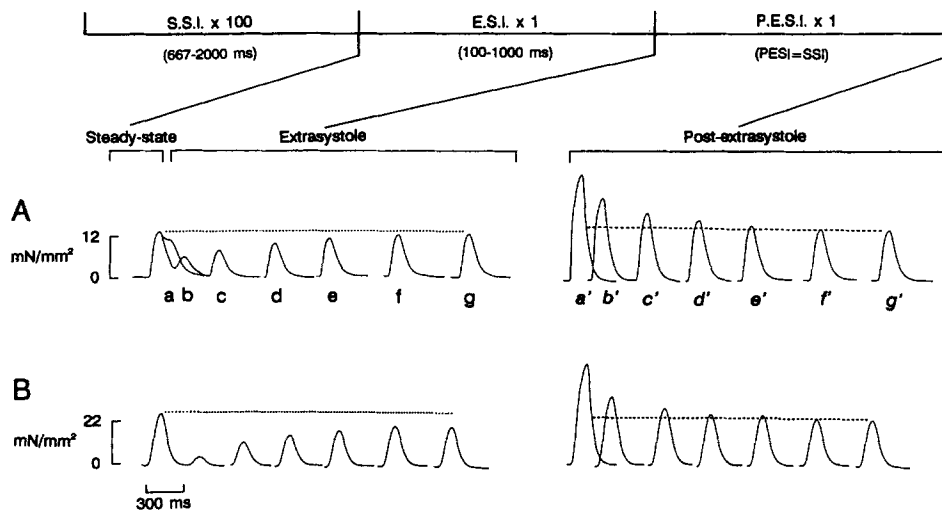


FIGURE 8. Effect of $[\text{Na}^+]_o$ depletion on the peak and time course of recovery of extrasystolic and post-extrasystolic contraction. Stimulation protocol used to construct families of recovery curves during stimulation at basic cycle lengths of 2,000, 1,000, or 667 ms is shown in the inset. (A) Superimposed extrasystolic ($a-g$) and post-extrasystolic contraction ($a'-g'$) in response to premature stimuli delivered at coupling intervals of 200, 300, 400, 500, 600, 700, and 800 ms at a basic cycle length of 2,000 ms (0.5 Hz). Dashed lines in each set of recordings represent the amplitude of peak steady-state contraction. B shows the equivalent response after reduction of $[\text{Na}^+]_o$ from 140 to 70 mM with equimolar Li^+ substitution. Calibration bar represents 300 ms in A and B.

for premature contraction (Fig. 9, A and B, open circles). Analysis of the recovery kinetics for contraction yielded time constants of 438 ± 110 , 253 ± 50 , and 184 ± 23 ms at basic cycle lengths of 2, 1, and 0.67 s, respectively.

The effect of varying the coupling interval for premature stimulation on the recovery of post-extrasystolic contraction is illustrated in the recordings of Fig. 8 A, and plotted against the preceding extrasystolic interval in Fig. 9, C and D at 0.5 and 1.5 Hz, respectively. At all three cycle lengths studied, peak post-extrasystolic potentiation was observed at 200 ms, after which contraction decayed exponentially to basal levels with a time constant that was not statistically different from those

calculated for extrasystolic contractions at each frequency tested. Along with the faster rate of recovery, a significant increase in the degree of post-extrasystolic potentiation was noted. The ratio of post-extrasystolic to steady-state contraction was increased from 2.4 at 0.5 Hz to 2.9 at 1.5 Hz stimulation. This rate-dependent

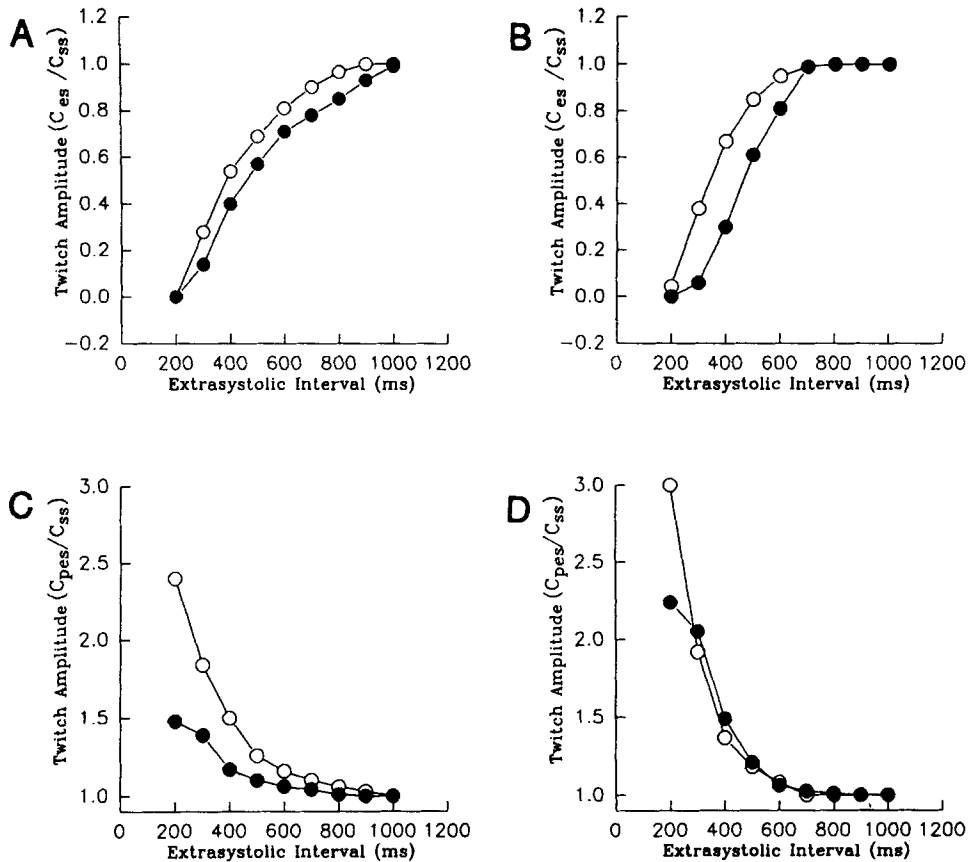


FIGURE 9. Effect of Li^+ substitution on the recovery of extrasystolic and post-extrasystolic contraction. (A and B) Slowed recovery of premature contraction during 0.5- and 1.5-Hz stimulation after reduction of $[Na^+]_o$ from 140 (open circles) to 70 mM (closed circles). Rate constants for exponential recovery back to steady state were calculated by the fit of data to the equation $F(t) = A_0 + (1 - A_0)[1 - \exp(-t/\tau)]$ for a single exponential or $F(t) = 1 + [A_1 \exp(-t/\tau_1)] + [A_2 \exp(-t/\tau_2)]$ to a twin exponential. See text for details of curve fitting procedure. (C and D) Effects of lowering $[Na^+]_o$ on peak potentiation and rate of recovery for post-extrasystolic contraction at 0.5- and 1.5-Hz stimulation, respectively. Note the differences in recovery kinetics for extrasystolic and post-extrasystolic contraction in low $[Na^+]_o$. Data points in all plots represent the mean of 11 paired experiments.

increase of post-extrasystolic potentiation corresponds well to the equivalent increase of steady-state contraction and rapid cooling contracture amplitude (Fig. 1), the peak and rate of restitution for postrest contraction (Figs. 6 and 7), and the slope of cooling contracture decay during extended rest (Fig. 6), suggesting that the rate of

replenishment of a releasable pool of Ca^{2+} may be accelerated as the gradient of Ca^{2+} within the SR is enhanced.

70 mM $[\text{Na}^+]_o$. 50% substitution of external Na^+ with Li^+ exerted a profound inhibitory effect on the rate of recovery for extrasystolic contraction at all frequencies tested. As illustrated in Fig. 8 B, reduction of $[\text{Na}^+]_o$ with Li^+ rendered the preparation insensitive to premature stimulation at coupling intervals < 300 ms and greatly slowed the recovery of contraction as the coupling interval was gradually extended over the 1,000-ms test range. The time course of restitution for extrasystolic contraction in the presence of Li^+ could not be fitted consistently with either a single or double exponential function. Only 5 of 11 muscles yielded exponential recovery curves at 0.5-Hz stimulation, and these were abolished by reducing the basic cycle length to 1 or 0.67 s. This is the reason for the calculated time constant of 618 ± 132 ms at 0.5-Hz stimulation, and the ceiling values set arbitrarily at 1,000 ms at the two faster rates of stimulation. Analysis of variance on force production during the recovery process in low $[\text{Na}^+]_o$ revealed that decreasing the basic cycle length from 2 to 0.67 s significantly ($P < 0.05$) depressed the peak twitch force associated with premature stimulation at coupling intervals < 500 ms.

The frequency dependence of post-extrasystolic contraction in Li^+ -substituted solution is shown in Fig. 8 B and plotted against the preceding extrasystolic interval at 0.5 and 1.5 Hz stimulation in Fig. 9, C and D, respectively. As was the case before lowering $[\text{Na}^+]_o$, the degree of post-extrasystolic potentiation was dependent on the preceding frequency of stimulation. However, once the depressant effect of stimulation frequency on twitch force associated with rhythmic stimulation was taken into account, the absolute magnitude of the post-extrasystolic contraction remained unaffected by stimulation frequency. This result is very similar to the rate-dependent dissociation of peak twitch force from rapid cooling contracture amplitude in low $[\text{Na}^+]_o$ (Fig. 4) and illustrates that the depressant effects of high frequency stimulation can be completely reversed within a single diastolic interval > 667 ms. Interestingly, changes of stimulation frequency also had marked effects on the recovery kinetics of post-extrasystolic contraction in 70 mM $[\text{Na}^+]_o$. As illustrated in Fig. 9, C and D, increasing stimulation frequency from 0.5 to 1.0 and 1.5 Hz enhanced the recovery rate for post-extrasystolic contraction. This is in contrast to effects observed on preceding extrasystolic contractions, which were depressed in a frequency-dependent manner. The results of this experiment are thus similar to postrest experiments in which contractions elicited at test intervals between 2.5 and 10 s were depressed in a frequency-dependent manner, despite indirect evidence suggesting that total SR Ca^{2+} stores remain unaltered.

1.25 mM $[\text{Ca}^{2+}]_o$. To test the hypothesis that the rate of recovery for premature contraction was related to the level of cell Ca^{2+} loading, SR Ca^{2+} stores were depleted by reducing external Ca^{2+} . As detailed above, lowering $[\text{Ca}^{2+}]_o$ from 2.5 to 1.25 mM decreased peak twitch force and apparent SR Ca^{2+} content by 55 and 35%, respectively. In addition, the time constant for recovery of premature contraction during stimulation at 0.5 Hz was prolonged from 402 ± 48 ms in 2.5 mM $[\text{Ca}^{2+}]_o$ to 600 ± 40 ms in 1.25 mM $[\text{Ca}^{2+}]_o$ ($n = 10$). As illustrated in Fig. 10 B, increased stimulation frequency markedly accelerated the recovery of premature contraction in the 100–1,000-ms range, the time constant for which was reduced to 424 ± 21 and 361 ± 31 ms at 1.0 and 1.5 Hz, respectively. This effect was similar to that

demonstrated for steady-state contraction, which had a greater sensitivity to frequency increments than the corresponding response in 2.5 mM $[Ca^{2+}]_o$ (cf. Fig. 5A). The relationship between rapid cooling contracture amplitude, twitch force, and the time constant of recovery for extrasystolic contraction under a range of inotropic conditions is summarized in Fig. 10. It is apparent that both steady-state contraction and the time constant for premature contraction were increased proportionally with SR Ca^{2+} content up until a certain threshold level, after which a further reduction of basic cycle length depressed rather than enhanced twitch force and markedly prolonged recovery of premature contraction. These results are similar to those noted for the dependence of the frequency–force relationship on cell Ca^{2+} loading (Fig. 5).

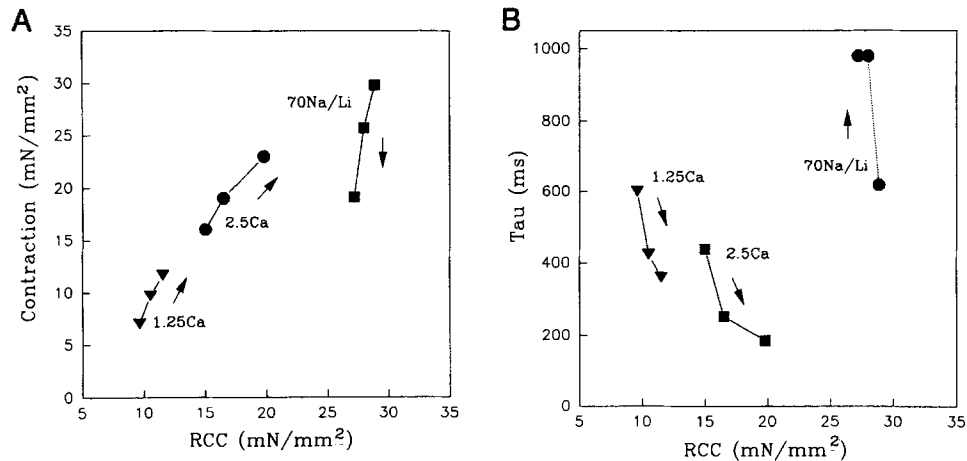


FIGURE 10. Relationship between rapid cooling contracture amplitude, steady-state contraction, and the rate of recovery for extrasystolic contraction. (A) Dependence of peak twitch force on apparent SR Ca^{2+} content during stimulation at 0.5, 1.0, and 1.5 Hz at varying levels of cell Ca^{2+} loading. Arrows indicate the direction of inotropic change in response to frequency increments. (B) Relation between apparent SR Ca^{2+} content and time constant of recovery for premature contraction under conditions identical to those in A. Note that the positive correlation between SR Ca^{2+} loading and peak contraction or recovery rate for premature contraction reverses once the maximal level of cell Ca^{2+} loading was achieved. $[Na^+]_o$ was lowered in all experiments with equimolar Li^+ substitution.

Frequency Dependence of Contraction in Single Cells

Fig. 11 shows changes in action potential shape in single cells from canine right ventricle recorded at 34 and 22°C in response to increased stimulation frequency. Care was taken in these experiments to ensure that cells were isolated from the same area of the ventricle from which the free running trabeculae used for cooling contracture experiments were obtained. In this respect it is important to note that action potentials recorded at these two temperatures were similar in shape (Fig. 12) and their interval (Figs. 11 and 12) dependence to those reported previously in

canine right ventricular trabeculae and Purkinje fibers under similar experimental conditions (Boyett and Fedida, 1984; Hryshko, Kobayashi, and Bose, 1989a; Bouchard et al., 1989), and isolated Purkinje and ventricular cells (Robinson, Boyden, Hoffman, and Hewett, 1987; Tseng et al., 1987; Tseng and Hoffman, 1989).

At 35°C, action potential duration gradually decreased following an increase in the rate of stimulation, as described earlier for intact canine ventricular muscle (Miller, Wallace, and Feezor, 1971). The duration of the action potential at 95% repolarization (APD₉₅) was reduced from 442 ± 40 ms at 0.1 Hz to 242 ± 12 ms at 2.0 Hz

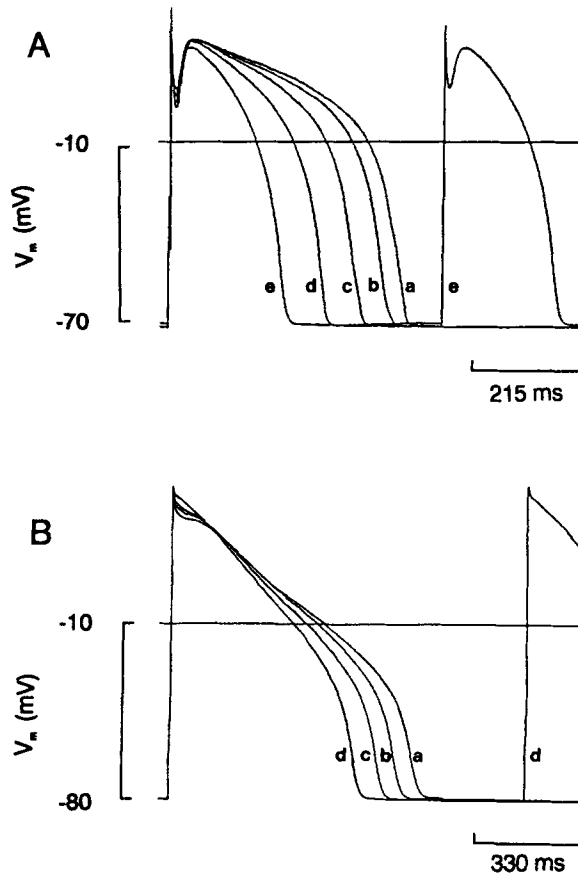


FIGURE 11. Action potential shape changes in canine right ventricular myocytes recorded in response to increased stimulation frequency. (A and B) Reduction of action potential duration after increased stimulation frequency from a basal rate of 0.2 Hz to 0.33, 0.5, 1.0, and 2.0 Hz (a-e). Temperature was 34 and 22°C in A and B, respectively. Recordings shown are averages of five sweeps at each individual frequency. Action potential duration at 95% repolarization at 35°C was reduced from 442 ± 40 ms at 0.1 Hz to 242 ± 12 ms at 2.0 Hz, and from 650 ± 49 ms at 0.2 Hz to 415 ± 21 ms at 2.0 Hz at 22°C. Average values for R_{seal} and R_{series} were 5.2 ± 1 GΩ and 6.4 ± 2 MΩ, cell capacitance was 122 ± 32 pF, and cell dimensions were 118 ± 9 × 21 ± 4 μm ($n = 8$). Calibration bar represents 215 and 330 ms for A and B, respectively.

($n = 3$). Consequently, the time spent by the cell near the resting membrane potential was reduced from 96% of the cardiac cycle at 0.1 Hz to 52% at 2.0 Hz. No change in the rate of membrane repolarization negative to -10 mV or resting membrane potential ($E_m = -75 ± 2$ mV) was observed during changes of stimulation frequency, except for a transient depolarization at 2.0 Hz (Fig. 11 A, trace e). The resting membrane potential was $-77.3 ± 1.5$ mV at 22°C (Fig. 12 B), and increased stimulation frequency shortened APD₉₅ from 650 ± 49 ms at 0.2 Hz to 415 ± 21 ms at 2.0 Hz ($n = 5$). The integral of the diastolic interval per minute was decreased

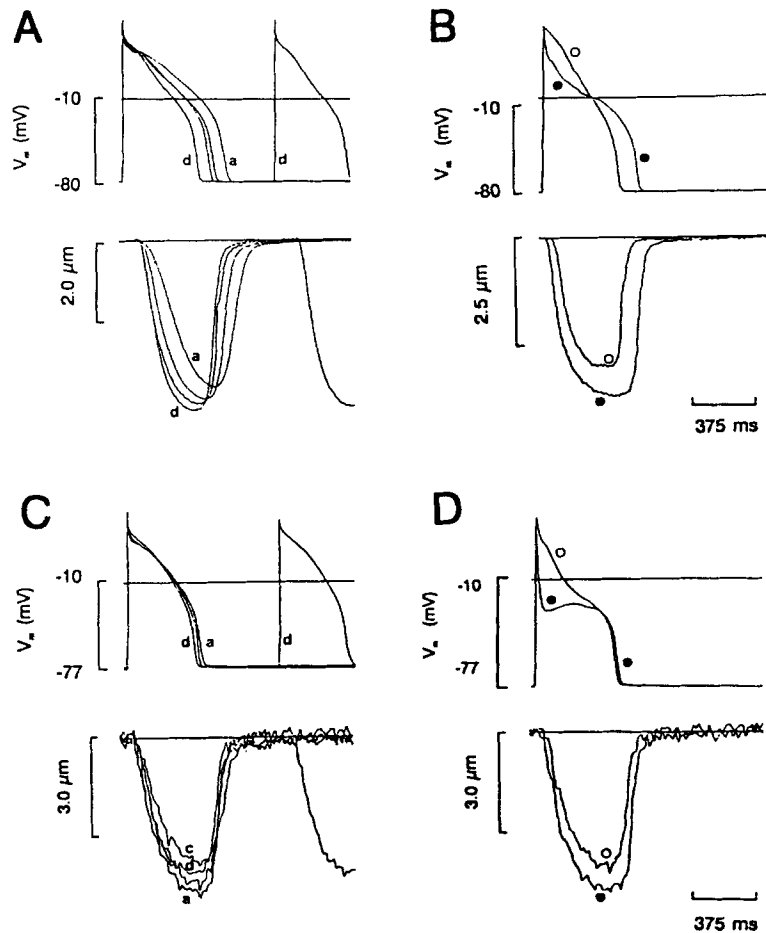


FIGURE 12. Simultaneous recordings of action potentials and unloaded cell shortening in response to changes in the rate and rhythm of stimulation. (A) Increased stimulation frequency from 0.2 Hz to 0.33, 0.5, and 1.0 Hz (*a-d*) in 140 mM $[\text{Na}^+]_o$ resulted in a graded decrease of action potential duration and a positive inotropic effect characterized by an increase in peak cell shortening and a decrease in the duration of contraction. Each trace is the average of five sweeps. (B) Single sweep recordings illustrating potentiation of contraction in response to a 10-s rest period (*closed circles*). Stimulation frequency was 0.5 Hz. (C) Negative staircase of unloaded cell shortening in 70 mM $[\text{Na}^+]_o$. Same cell as in A. (D) Reversal of the depressant effect of stimulation frequency in response to brief (10 s) rest in low $[\text{Na}^+]_o$ (*closed circles*). Same cell as in B. Note the similarities in the pattern of cell shortening in response to variation of frequency or $[\text{Na}^+]_o$ compared with the equivalent responses described earlier for isometric force production in the intact muscle (Figs. 1 and 4). All experiments were conducted at room temperature (22–23°C). Calibration bar represents 375 ms in A–D. $[\text{Ca}^{2+}]_o$ was 2.0 mM.

from 87 to 17% of the cardiac cycle, suggesting that diastolic Ca^{2+} extrusion via $\text{Na}^+-\text{Ca}^{2+}$ exchange may be significantly reduced at both temperatures tested.

Simultaneous recordings of action potentials and unloaded cell shortening in 140 mM $[\text{Na}^+]_o$ are shown in Fig. 12 A, which illustrates that a graded increase of

stimulation frequency resulted in a positive inotropic effect, characterized by an increase of peak shortening and a decrease in the duration of contraction. Time to peak shortening was reduced from 393 ms at 0.2 Hz to 279 ms at 1.0 Hz, while time required for 90% cell relaxation was decreased from 198 ms at 0.2 Hz to 168 ms at 1.0 Hz. As in the intact preparation (Figs. 1 and 6), resumption of stimulation after a brief rest resulted in potentiation of contraction in the single cell (Fig. 12 B). Action potentials associated with postrest stimulation were prolonged and had decreased plateau voltages, as reported previously for canine ventricular muscle (Bouchard et al., 1989; Hryshko et al., 1989a) and Purkinje fibers (Boyett and Fedida, 1984), and single cells (Robinson et al., 1987).

The effect on action potentials and accompanying contractions of lowering $[\text{Na}^+]_o$ to 70 mM is shown in Fig. 12, C and D. As expected from previous reports in intact ventricular tissues (Schouten and ter Keurs, 1985) and single cells (Mitchell et al., 1984) partial replacement of $[\text{Na}^+]_o$ with Li^+ decreased action potential duration despite its potentiating effect on twitch amplitude and duration. Peak unloaded cell shortening was increased by 10%, and time to peak shortening and that required for 90% relaxation were prolonged to 452 ± 20 and 419 ± 30 ms, respectively. As was observed in the multicellular preparation (Fig. 4), reduction of $[\text{Na}^+]_o$ gave rise to a negative frequency-shortening relationship (Fig. 12 C). In addition, the duration of contraction remained relatively insensitive to stimulation frequency when compared with the corresponding response in 140 mM $[\text{Na}^+]_o$, which was reflected by the rather small changes in duration of accompanying action potentials. Fig. 12 D shows that the depressant effects of high frequency stimulation in low $[\text{Na}^+]_o$ could be transiently reversed by interpolation of a brief period of rest. The results show that the frequency-dependent contractile properties of intact canine ventricle in 70 and 140 mM $[\text{Na}^+]_o$ are similar to those observed at the level of the single cell, as has been previously demonstrated for isolated guinea pig, rabbit, and rat ventricular cells (Capogrossi et al., 1986; London and Krueger, 1986; Frampton, Harrison, Boyett, and Orchard, 1991).

DISCUSSION

The aim of this study was to examine the contribution of sarcolemmal Na^+ - Ca^{2+} exchange and the release of Ca^{2+} from intracellular stores to the interval dependence of contraction in an intact preparation of mammalian ventricular muscle. The covariation of steady-state contraction, rapid cooling contracture amplitude, and postrest contraction after reduction of $[\text{Na}^+]_o$ (Figs. 2 and 6) and the sensitivity of these measurements to ryanodine or caffeine (Fig. 5) suggest that the inotropic effect of low $[\text{Na}^+]_o$ perfusion is due to enhanced availability and release of Ca^{2+} from the SR. Differences in the relationship between peak twitch force and apparent SR Ca^{2+} content during changes of stimulation frequency in 140 and 70 mM $[\text{Na}^+]_o$ (Fig. 10) suggest that a normal electrochemical gradient for Na^+ is obligatory for the rate-dependent increase in twitch force and SR Ca^{2+} availability, and thus provide evidence in support of a functional role for Na^+ - Ca^{2+} exchange in the positive staircase observed in physiological $[\text{Na}^+]_o$.

Frequency–Force Relationship in Physiological $[Na^+]_o$

In isolated cardiac preparations that show a sustained positive inotropy, increased stimulation frequency results in an increase of net Ca^{2+} influx measured by ^{45}Ca uptake (Langer, 1965) or cumulative extracellular Ca^{2+} depletions (Hilgemann, 1986b). This increase in Ca^{2+} uptake is accompanied by a gradual rise of intracellular Na^+ activity (Cohen et al., 1982; Boyett et al., 1987; Wang et al., 1988), leading to the hypothesis that the changes of peak twitch force are dependent on sarcolemmal Na^+ - Ca^{2+} exchange (Langer, 1965, 1973). An important observation in this study was that the positive staircase of contraction depended on the accompanying changes of SR Ca^{2+} content (Figs. 1 and 5), and that both were abolished after inhibition of Na^+ - Ca^{2+} exchange activity (Figs. 4 and 10). While confirming the involvement of the Na^+ - Ca^{2+} exchanger in the force staircase, our results do not allow us to conclude whether the predominant inotropic effect is related to exchange activity during the action potential (cf. Hilgemann and Noble, 1987; Leblanc and Hume, 1990) or during the diastolic interval, though the marked decrease in time spent near resting potentials at higher rates (Fig. 11) supports the latter possibility. Transport of Ca^{2+} between anatomical, or functionally distinct, compartments within the SR during diastole is a feature of many models of excitation–contraction coupling in the heart (Wood et al., 1969; Allen et al., 1976; Morad and Cleeman, 1987; Schouten, van Deen, de Tombo, and Verveen, 1987). Although there is no direct evidence to support passive recycling of Ca^{2+} from an “uptake” to a “release” site, the increased rate of the decline in cooling contracture amplitude at 1.5 compared with 0.5 Hz (Fig. 6) suggests that Ca^{2+} may be either transported within the SR or lost from ryanodine-sensitive stores at a rate faster than that which occurs at lower rates of stimulation. In either case the effect will be to enhance the rate of replenishment of a releasable pool of Ca^{2+} during diastole, consistent with the increased rate of mechanical restitution observed in this (Figs. 6 and 9) and other studies on mammalian cardiac tissues (Koch-Weser and Blinks, 1963; Allen et al., 1976; Lipsius et al., 1982; Schouten et al., 1987).

We also observed that the sensitivity of contraction to changes of stimulation frequency was strongly related to the level of cell Ca^{2+} loading (Figs. 5 and 10). The positive inotropic response to stimulus frequency was greatest at low levels of SR Ca^{2+} loading; e.g., 1.25 mM $[Ca^{2+}]_o$ or treatment with ryanodine in low $[Na^+]_o$. Maneuvers that further increased SR Ca^{2+} content first blunted the frequency response (2.5 mM $[Ca^{2+}]_o$) and then reversed it (low $[Na^+]_o$) as the maximal level of SR Ca^{2+} loading was achieved. This spectrum of frequency dependence is similar to that described in rabbit papillary muscle bathed in varying $[Ca^{2+}]_o$ (Kort and Lakatta, 1988) or in rat ventricular muscle and myocytes (Mitchell, Powell, Terrar, and Twist, 1985; Kort and Lakatta, 1988) after a decrease in cell Ca^{2+} loading with ryanodine or caffeine. Reduction of twitch force under conditions of high cell Ca^{2+} loading implies that there may be a negative feedback mechanism modulating the release of Ca^{2+} from the SR, such as that proposed by Fabiato (1985a, b) or Capogrossi et al. (1988). Alternatively, some other mechanism may reduce developed tension at a given level of myoplasmic Ca^{2+} , e.g., intracellular acidosis (Vaughan-Jones, Lederer, and Eisner,

1983), increased inorganic phosphate levels (Kentish, 1986), and/or decreased myofilament Ca^{2+} sensitivity (Eisner et al., 1984).

Frequency-Force Relationship in Low $[\text{Na}^+]_o$

We found in earlier work that the negative staircase characteristic of rat ventricular muscle was not accompanied by a parallel decrease in SR Ca^{2+} content (Bouchard and Bose, 1989), a result recently confirmed in fura-2-loaded rat ventricular cells (Frampton et al., 1991). A limitation of our earlier study, however, was the lack of information pertaining to the restitution process at physiologically relevant diastolic intervals. Data in Figs. 6–10 indicate that the negative staircase in Ca^{2+} -loaded canine ventricular muscle is not correlated with SR Ca^{2+} content estimated with the rapid cooling technique or postrest stimulation, and appears to occur despite adequate filling of a releasable pool of Ca^{2+} . Nonlinearities in the relationship between peak twitch force and the level of SR Ca^{2+} loading are typically observed in situations where intracellular stores have been saturated with Ca^{2+} (Orchard, Eisner, and Allen, 1983; Allen, Eisner, Pirolo, and Smith, 1985; Kotake and Vassalle, 1986; Wier, Cannell, Berlin, Marban, and Lederer, 1987; Bers and Bridge, 1988; Capogrossi et al., 1988; Kort and Lakatta, 1988) and the reduction of twitch strength under these conditions is generally attributed to a state of “intracellular Ca^{2+} overload.”

Due to the indirect nature of the present experiments, our results do not allow us to determine the cellular mechanisms underlying the negative staircase observed in low $[\text{Na}^+]_o$ solution. Under more controlled conditions, Fabiato has shown that Ca^{2+} -induced release of Ca^{2+} in skinned cardiac muscle can be inactivated by supraoptimum triggering pulses greater than pCa 5.5 (Fabiato, 1985b). Similar observations have been made in skinned (Kwok and Best, 1991) and intact voltage-clamped skeletal muscle fibers (Schneider and Simon, 1988; Simon, Klein, and Schneider, 1991), where the fast inactivating component of Ca^{2+} release is inhibited by increased myoplasmic Ca^{2+} , with half-maximal inactivation at $\sim 0.3 \mu\text{M}$ free Ca^{2+} (Simon et al., 1991). These results contrast with observations made in cardiac or skeletal muscle, where the rate of ^{45}Ca efflux in heavy SR vesicles (Meissner, Darling, and Eveleth, 1986) or open probability of Ca^{2+} release channels incorporated into planar lipid bilayers (Smith, Coronado, and Meissner, 1986; Anderson, Lai, Rousseau, Erickson, and Meissner, 1989; Ashley and Williams, 1990) remain insensitive to cytoplasmic Ca^{2+} or are decreased only in response to unphysiologically high levels of Ca^{2+} (0.1–1 mM). It is possible, however, that inactivation of the release mechanism under the conditions used in vesicle or bilayer experiments may have been either disrupted by the isolation procedure, or developed to a near maximal extent, and thus overcome by the lower affinity activation process (Simon et al., 1991; but see Fabiato, 1985a, 1989).

A second possibility is that raised SR Ca^{2+} levels after inhibition of Na^+ - Ca^{2+} exchange results in enhanced spontaneous release of Ca^{2+} , as demonstrated previously in intact ventricular muscle (Stern et al., 1983; Kort and Lakatta, 1988) and single cells (Capogrossi et al., 1988). This could limit maximal force production or cell shortening due to the temporal relationship between Ca^{2+} -induced release of

Ca^{2+} and the preceding spontaneous release of Ca^{2+} (Capogrossi et al., 1988) and/or increased series compliance resulting from spatial inhomogeneities of myoplasmic Ca^{2+} (Orchard et al., 1983; Allen et al., 1985; Wier et al., 1987). It is conceivable that the effects of asynchronous Ca^{2+} release on peak twitch force were minimized during our experiments due to the relatively high stimulation rates used. Previous data indicated that microscopic tension fluctuations in intact ventricular muscle (Kort and Lakatta, 1988) or the occurrence of spontaneous contractile waves in single cells (Capogrossi et al., 1986, 1988) are depressed at higher stimulation frequencies for a given level of cell Ca^{2+} loading. The demonstration that the depressant effect of low $[\text{Na}^+]_o$ perfusion was greatest at basic cycle lengths between 0.67 and 1 s argues against a significant role for spontaneous release of Ca^{2+} , although it does not necessarily exclude it. Moreover, removal of inhibition of peak twitch force was time dependent, taking ~ 60 s to recover fully (Fig. 7), and could be demonstrated after a single diastolic interval as short as 667 ms (Fig. 9). This observation also argues against a role for altered myofilament Ca^{2+} sensitivity or intracellular pH changes in the negative staircase, as these would probably require >700 ms for complete reversal (Vaughan-Jones et al., 1983; Kentish, 1986; Bountra et al., 1988). Despite these observations, the possibility that spontaneous Ca^{2+} release can account for some of our results cannot be discounted, as a thorough investigation of this phenomenon was not undertaken in our single cell experiments.

In summary, we have shown that the positive staircase of peak twitch force and SR Ca^{2+} content requires the presence of a functional Na^+ - Ca^{2+} exchange mechanism. The sensitivity of contraction to changes in the stimulation rate appears to be correlated with the level of SR Ca^{2+} loading immediately preceding the rate change until the maximal level of loading is achieved, after which reduction of the basic cycle length depresses rather than enhances twitch strength. The negative staircase reported here in low $[\text{Na}^+]_o$ is similar to that demonstrated previously for rat ventricle (Bouchard and Bose, 1989; Frampton et al., 1991) or for rabbit (Kort and Lakatta, 1988) or dog ventricle (Kotake and Vassalle, 1986) under conditions of maximal SR Ca^{2+} loading. The depressant effect of high frequency stimulation may be related to either Ca^{2+} -dependent inactivation of SR Ca^{2+} release (Fabiato, 1985a) or enhanced spontaneous release of Ca^{2+} from SR stores (Capogrossi et al., 1988), or some other Ca^{2+} -dependent mechanism such as that reported for inactivation of plasma membrane Ca^{2+} channels in mammalian heart (Lee, Marban, and Tsien, 1985) or *Helix* neurons (Chad and Eckert, 1986).

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