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Effect of Caffeine on Calcium Flux across the Sarcolemma of Guinea Pig Atrial Trabeculae during Contracture

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The changes in extracellular calcium activities during contractures of guinea pig atrial trabecular muscles were measured with Ca^{2+}-selective electrodes. When the tissue was superfused with Na^+-free Tyrode solution, extracellular Ca^{2+} activities were decreased and contractures were induced with some delay. When the contracture was relaxed with Na^+-containing Tyróde solution, extracellular Ca^{2+} activities were increased transiently and recovered in a Na^+-dependent manner. The magnitude of extracellular Ca^{2+} activity decreased was proportional to the maximum magnitude of contracture induced by Na^+-free solution. Addition of caffeine (10 mM) to Na^+-free solution induced transient contracture following slow development of contracture and an increase in extracellular Ca^{2+} activity. Removal of caffeine from Na^+-free solution caused a slow relaxation of contracture and a decrease in extracellular Ca^{2+} activity. These results confirm that caffeine blocks Ca^{2+} uptake by the sarcoplasmic reticulum (SR) resulting in an increase in sarcoplasmic Ca^{2+} activity. Ca^{2+} activity in the extracellular space, the amount of Ca^{2+} transported into the cell (Ca^{2+} depletion in the extracellular space), and the magnitude of contracture are well correlated. Present experiments suggest that extracellular use of Ca^{2+}-selective electrodes provides continuous and quantitative monitoring of Na^+-dependent Ca^{2+} flux across the cardiac cell membrane.

Key Words: Contracture, Na^+-Ca^{2+} exchange transport, caffeine, Ca^{2+}-selective electrodes, guinea pig atrial trabeculae

It has been known that Na^+ has an important role in cardiac muscle contractility (Sulakhe & St. Louis 1980; Mullins 1981; Sheu & Blaustein 1986). Ca^{2+} is moved across the membrane coupled with a Na^+ influx, in an electrogenic fashion (normally 3 Na^+ versus 1 Ca^{2+}), depending on the prevailing Na^+ electrochemical gradient which is maintained through the active transport of Na^+ (Na^+-Ca^{2+} exchange transport). The current generated by electrogenic Na^+-Ca^{2+} exchange has been recorded from single myocytes (Kimura *et al.* 1986; Mechmann & Pott 1986, Kimura *et al.* 1987).

The Na^+-Ca^{2+} exchange mechanism has been implicated in Ca^{2+} efflux and maintenance of low resting levels of intracellular Ca^{2+}. Alternatively, an elevation of intracellular Na^+ is believed to result in an augmentation of intracellular Ca^{2+} via a sarcolemmal Na^+-Ca^{2+} exchange transport. The ability of such a countertransport carrier to produce both decreases as well as increases in intracellular Ca^{2+} has been explained within the context of different modes of electrogenic operation: an inward (forward) mode that is stimulated by increases in intracellular Ca^{2+} and results in enhanced Ca^{2+} eflux, and an outward (reverse) mode that is stimulated by increases in intracellular Na^+ and results in enhanced Ca^{2+} influx. The major factor that determines the direction of Ca^{2+} flux is believed to be a combination of transmembrane concentration gradients for Na^+ and Ca^{2+} and membrane potential (for review see Mullins 1981).

The application of caffeine to skeletal muscle produces a phasic contracture which has been shown to result from a release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Weber & Herz 1968). In cardiac muscle, the action of caffeine is more complicated and is considered to reduce the amount of accumulated Ca^{2+} in intracellular storage site(s) by Ca^{2+} release

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and/or by inhibition of Ca^{2+} sequestration (Bodem & Sonnenblick 1975; Chapman & Leoty 1976). In addition to the effects on the SR and mitochondria (Blayney *et al.* 1978), caffeine has direct or indirect effects on membrane currents. Ca^{2+} influx through the cell membrane seems to be potentiated (Kimoto 1972; Niedergerke & Page 1981) or inhibited (Eisner *et al.* 1979) or unchanged (Ohba 1973) by caffeine, probably depending on the preparation. Caffeine modulation of the Ca^{2+} sensitivity of a contractile system has also been suggested by results obtained from skinned or hyperpermeable preparations (Fabiato & Fabiato 1973; Endo & Kitazawa 1978; McClellan & Winegrad 1978; Wendt & Stephenson 1983; Eisner & Valdeolmillos 1985).

In contrast to caffeine-induced transient contractures (Champan & Leoty 1976), other studies have found that caffeine does not produce a contracture when applied under normal conditions (Blinks *et al.* 1972; Champan & Miller 1974; Jundt *et al.* 1975; Busselen & van Kerkhove 1978). However, if external Na^+ is removed or the membrane is depolarized, the subsequent addition of caffeine produces a contracture (Chapman & Miller 1974; Jundt *et al.* 1975; Busselen & van Kerkhove 1978). This result has been explained by suggesting that, under normal conditions, any Ca^{2+} released from the SR by caffeine can be transported out of the cell via a sarcolemmal Na^+-Ca^{2+} exchange. If the preparation is depolarized or exposed to a Na^+-free solution, this Ca^{2+} efflux is decreased and the Ca^{2+} released by caffeine will therefore activate contraction. Similarly, when the ability of the Na^+-Ca^{2+} exchange to extrude Ca^{2+} is reduced by increasing the intracellular Na^+ concentartion, depolarization increases the resting or tonic force (Eisner & Lederer 1979). The addition of caffeine markedly increases this component of force (Eisner & Lederer 1982; Karagueuzian & Katzung 1982).

In view of the foregoing ideas, direct measurements of Ca^{2+} flux across the sarcolemma via Na^+-Ca^{2+} exchange and examination of the contribution of Ca^{2+} flux to contracture are worthwhile. The development of Ca^{2+}-selective electrodes has made it possible to continuously measure the Ca^{2+} flux across the sarcolemma (Suh & Sleator 1982; Bers & MacLeod 1986; Suh *et al.* 1987).

In this study, extracellular Ca^{2+} activities of guinea pig atrial trabeculae were measured with Ca^{2+}-selective electrodes and the changes in extracellular Ca^{2+} were correlated with contractures induced by Na^+-deficient solutions. The effect of caffeine on contractile strength along with Ca^{2+} movements across the sarcolemma during the contracture was investigated.

METHODS

Guinea pigs of either sex ranging in size from 200 to 500 grams were sacrificed with a blow to the head. The heart was rapidly excised and placed in oxygenated (100% O_2) Tyrode solution. To reduce diffusion delay, which seems to be essential to observe caffeine contracture in cardiac preparation (Chapman & Leoty 1976), thin trabecular muscles of the left atrium were dissected under a microscope and tied with surgical silk threads (7/0 Mersilk, Ethicon) and then transferred to a recording chamber which was continuously perfused with the oxygenated Tyrode solution. Tension was monitored by a Model 400A Force Transducer System (Cambridge Technology, Inc.) and displayed on a Philips PM 3305 digital storage oscilloscope and on a Gould Brush 220 recorder along with the signal from the differential electrometer for the Ca^{2+}-selective electrode (E^e_{Ca}). The temperature in the recording chamber was maintained at 27.0±0.2°C. The Tyrode solution had the following composition in millimoles per liter (mM): NaCl 133.5; KCl 4.0; KH_2PO_4 0.3; $MgSO_4 \cdot 7H_2O$ 1.2; $CaCl_2$ 1.8; Tris 10; dextrose 10. For Na^+-deficient solutions, NaCl was isotonically replaced with tetramethylammonium (TMA) chloride since the ability of the cardiac tissue to withstand multiple exposure to Na^+-deficient solutions was greatest among Na^+ substitutes (Chapman *et al.* 1983). The flow rate of superfusion solutions was kept constant (about 5 ml/minute) in such a way that the solution levels of the solution containers were maintained constant with spring-aided small supports.

Ca^{2+}-selective electrodes and reference electrodes were manufactured as described previously (Suh, *et al.* 1987) with some modifications for extracellular recordings. The tip of the microelectrode, which was beveled with a Narishige EG-3 microgrinder and a Narishige MF-83 microforge, had an opening of 2 to 5 μm in diameter. The resin used for the Ca^{2+}-selective electrodes was provided by Professor W. Simon. The mixture was that of Oehme *et al.* (Oehme *et al.* 1976). Polyvinylchloride (PVC), approximately 10% by weight, was added to the mixture for better stability of the Ca^{2+}-selective electrodes.

The e.m.f. from the Ca^{2+}-selective electrode was measured with an electrometer (Analog Devices 311 J or AD515 operational amplifier, Norwood, MA U.S.A.), and the membrane potential measured by the reference electrode was electronically subtracted from the e.m.f. measured with the Ca^{2+}-selective electrode.

Each Ca^{2+}-selective electrode was tested for response before and after the experiments, and was equilibrated in the Tyrode solution for at least one hour before being used. The Ca^{2+} activity coefficient of 0.32 was used to calculate Ca^{2+} activity in the solution (Lee 1981)

RESULTS

Prior to testing the effect of caffeine, the competence of the experimental technique to detect the changes in extracellular Ca^{2+} by Ca^{2+}-selective electrodes was determined by measuring extracellular Ca^{2+} activities (a^{e}_{Ca}) and contractile strength of the left atrial trabecular muscle stimulated at rate of 2 contractions per second (Fig. 1). When the rest intervals were given, extracellular Ca^{2+} activities started to increase and reached a maximum level in approximately 30 seconds (about 1.7 mV, which is equivalent to 330 μM Ca^{2+} activity change). As the rest interval lasted longer, Ca^{2+} activity slowly decreased to the basal level. When the tissue was stimulated again, the contractile strength increased to that of the steady-state contractions, while extracellular Ca^{2+} activities decreased. These measurements were done before the main (contracture) experiments, which assured the position of the Ca^{2+}-selective electrodes in the extracellular space (Suh *et al.* 1987).

The effects of Na^+ concentration in the superfusion solutions on muscle contractility and extracellular Ca^{2+} activity were measured. Fig. 2 shows a series of recordings of extracellular Ca^{2+} activities and contractility induced by different concentrations of Na^+ in the superfusion solution, which also contained 10 mM caffeine to induce strong contraction. When the tissue was superfused with Na^+-free, caffeine (10mM) containing Tyrode solution, Ca^{2+} activity in the extracellular space started to decline and with some delay the contracture was induced. As the superfusion of Na^+-free solution continued, a^{e}_{Ca} remaied decreased (3.9mV; 690μM) and the contracture reached the maximum contractility (2.6mN) and spontaneously relaxed on occasion. When the tissue was superfused with 100% Na^+ solution, the contracture was relaxed accompanying an increase in a^{e}_{Ca} (4.1 mV; 760 μM) which was decreased to the basal level with about 1 minute's delay, as the superfusion of 100% Na^+ solution continued. When 50% Na^+-solution was superfused following the contracture induced with Na^+-free solution, a^{e}_{Ca} did not increase as much as when superfused with 100% Na^+ (2.4 mV; 420 μM), although the contracture was relaxed. When an additional superfusion of 100% Na^+ solution was followed, a^{e}_{Ca} was increased back to the level (0.9 mV; 220 μM) which was reached with 100% Na^+-solution alone.

On the other hand, when the tissue was superfused with 50% Na^+-solution following the relaxation with 100% Na^+-solution, a^{e}_{Ca} was decreased (2.5 mV; 250 μM) but not as much as when superfused with Na^+-free solution and no visible contracture was recorded. However, when the tissue was again superfused with

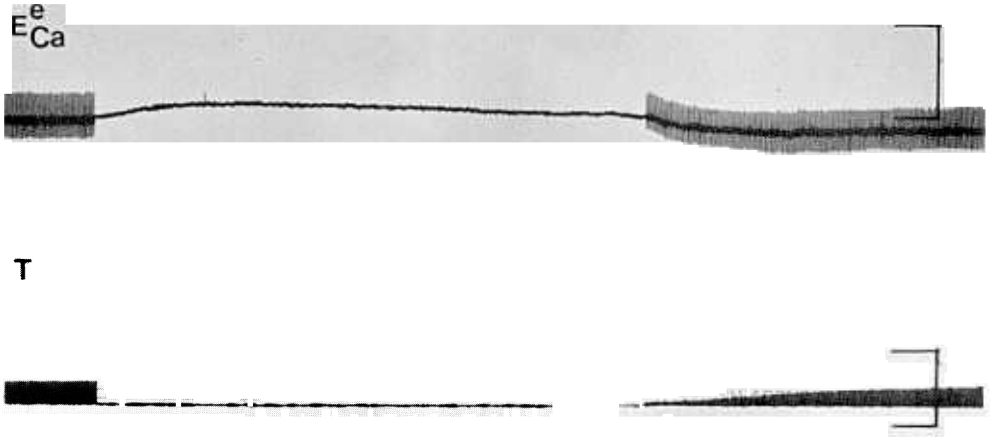

Fig. 1. Changes in extracellular Ca^{2+} activity during rest period.
Horizontal scale = 10 seconds. Vertical scale = 5 mV for E^{e}_{Ca} (upper trace); 10 mN for tension (lower trace). The lower bar of the horizontal scale also represents the level of Ca^{2+} activity in the superfusion solution.

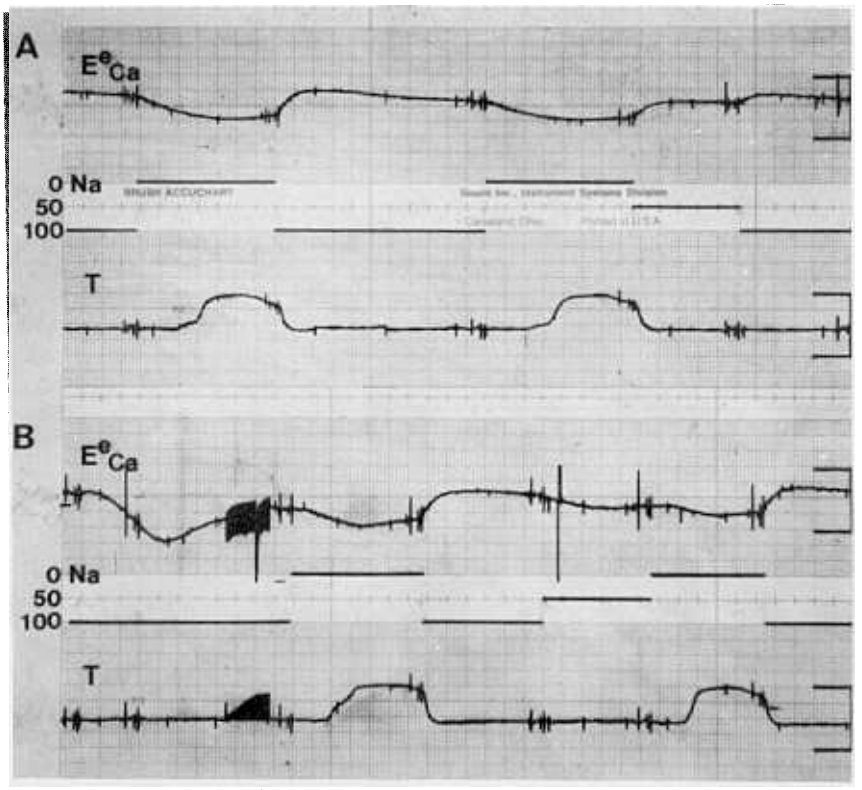

Fig. 2. *Changes in extracellular Ca^{2+} activity during contractures induced with different concentrations of Na^+ Horizontal scale=2 minutes. Vertical scale = 5 mV for E^e_{Ca} (upper trace); 5 mN for tension (lower trace). The horizontal bars (between upper and lower traces in both A and B) represent the level of Na^+ activity (0, 50, 100% from the top) in the superfusion solutions.*

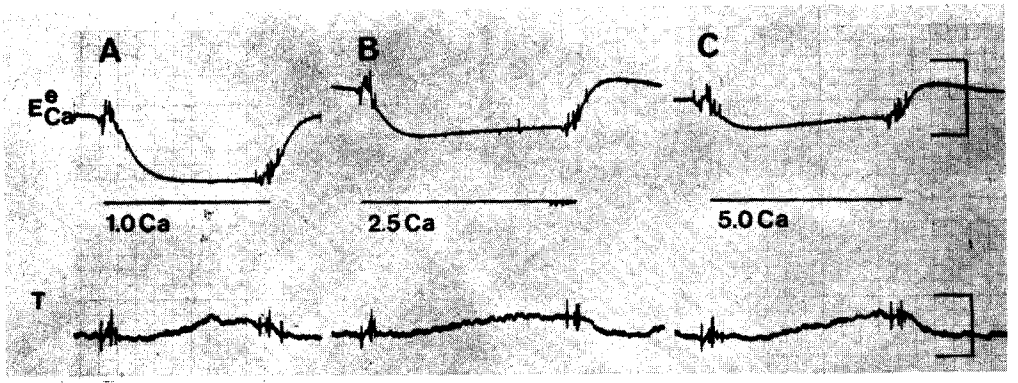

Fig. 3. *Ca^{2+}-dependence of changes in extracellular Ca^{2+} activity during contracture. Horizontal scale = 2 minutes. Vertical scale = 5 mV for E^e_{Ca} (upper trace); 5 mN for tension (lower trace). The horizontal bars (between upper and lower traces) represent the periods when Na^+-free solutions with different concentrations of Ca^{2+} (A, 1.0 mM; B, 2.5 mM; C, 5.0 mM Ca^{2+}) were superfused. The superfusion with 1.0 mM Ca^{2+} solution induced greater E^e_{Ca} changes than with higher concentrations of Ca^{2+}.*

Table 1. Extracellular Ca^{2+} depletion and magnitude of contracture

Na^+-free solutions	$E_{\xi_{Ca}}$ (mV)	Ca^{2+} depletion (μM)	Contracture (mN)
A. Ca^{2+}			
1.0 mM	4.3±0.1	345±2	1.8±0.1
2.5 mM	2.8±0.2	465±34	2.5±0.3
5.0 mM	1.9±0.1	593±32	2.6±0.1
B. Caffeine			
0 mM	7.8±0.6	464±42	1.1±0.1
10 mM	8.9±0.1	446±37	2.2±0.2

* Due to valiant and deteriorating conditions of the tissue during the contracture-relaxation cycles, the number of data collected from the single preparation was limited to n=4. Numbers represent mean±S.D.

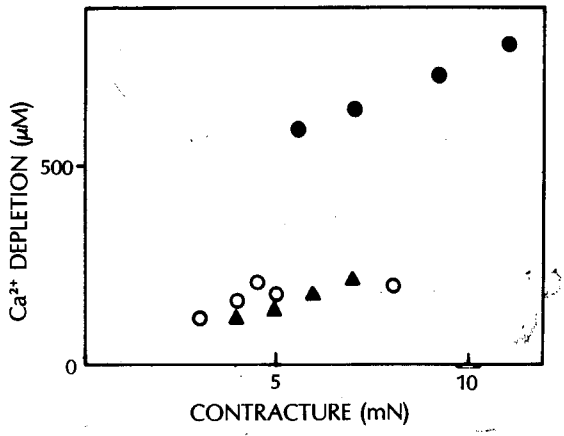

Fig. 4. *Relation between changes in extracellular Ca^{2+} activity and magnitudes of contractures.*
Data (O, •, Δ) were collected from 3 different preparations, whose various contractile strengths were induced by different concentrations of Ca^{2+} in the superfusion solution. Note that there is a good linearity between two parameters.

Na^+-free solution, a^{ξ}_{Ca} was further decreased (1.1 mV; 170 μM) and the contracture (2.6 mN) was induced. The magnitude of contracture induced with Na^+-free solution following 50% Na^+ solution was the same as that induced with Na^+-free solution alone and the rising phase of contracture was faster with the pretreatment of 50% Na^+ solution.

Na^+-dependent changes in a^{ξ}_{Ca} were further studied by changing the Ca^{2+} concentration of the superfusion solutions (Fig. 3 and Table 1). When the tissue was superfused with Na^+-free, 2.5 mM Ca^{2+} solution, the magnitude of decrease in $E_{\xi_{Ca}}$ was 2.8±0.2 mV, which was equivalent to about 465±34 μM, and the magnitude of contracture induced was 2.5±0.3 mN. When the Ca^{2+} concentration in the superfusion solutions was raised to 5.0 mM, the change in $E_{\xi_{Ca}}$ was only 1.9±0.1 mV, which is much smaller than that with 2.5 mM Ca^{2+}. However, this recorded electrical signal was equivalent to a change in a^{ξ}_{Ca} of 593.0±32 μM, and the magnitude of contracture was 2.6±0.1 mN. When superfused with Na^+-free solutions containing 1.0 mM Ca^{2+}, the change in $E_{\xi_{Ca}}$ was 4.3±0.1 mV, which is equivalent to 345±2 μM and the magnitude of induced contracture was 1.8±0.1 mN.

The maximal strengths of contractures from different preparations, which were induced by Na^+-free solution with various Ca^{2+} concentrations, were plotted against the accompanying changes in a^{ξ}_{Ca} (Fig. 4). The contractile strengths were increased as the decrease in a^{ξ}_{Ca} got larger. As the more extracellular Ca^{2+} was depleted, the strength of the corresponding contracture got stronger in a proportionate manner.

In order to look into the effect of caffeine on extracellular Ca^{2+} depletion and tension, the contracture and accompanying extracellular Ca^{2+} depletion induced by Na^+-free solution alone were compared with those induced with caffeine-containing Na^+-free solution (Table 1). The magnitude of contracture induced without caffeine was about 50% of that induced with caffeine (1.1±0.1 mN vs. 2.2±0.2 mN) and the extracellular Ca^{2+} depletion was larger without caffeine (464±42 μM vs. 446±37 μM), but not significantly. The effect of caffeine during contracture was further measured with successive superfusion of Na^+-free solution and caffeine-containing solution (Fig. 5). When superfused with Na^+-free caffeine (10 mM) solution, a strong contracture was induced as normally seen. While the contracture was sustained, an additional superfusion of Na^+-free solution without caffeine induced slow relaxation of contracture along with an additional depletion of a^{ξ}_{Ca} (Fig. 5-A). When the tissue was superfused with Na^+-free solution without caffeine, a much weaker contracture was slowly developed and depletion of a^{ξ}_{Ca} was sustained for longer than 5 min, compared to about 1 min with caffeine containing solution (Fig. 5-B). When caffeine (10 mM) was added into the Na^+-free solution, fast and strong contracture was developed in 1 min and relaxed spontaneously. The addition of caffeine also caused a slow but significant increase in a^{ξ}_{Ca} until the contracture already started to relax (Fig. 5-B).

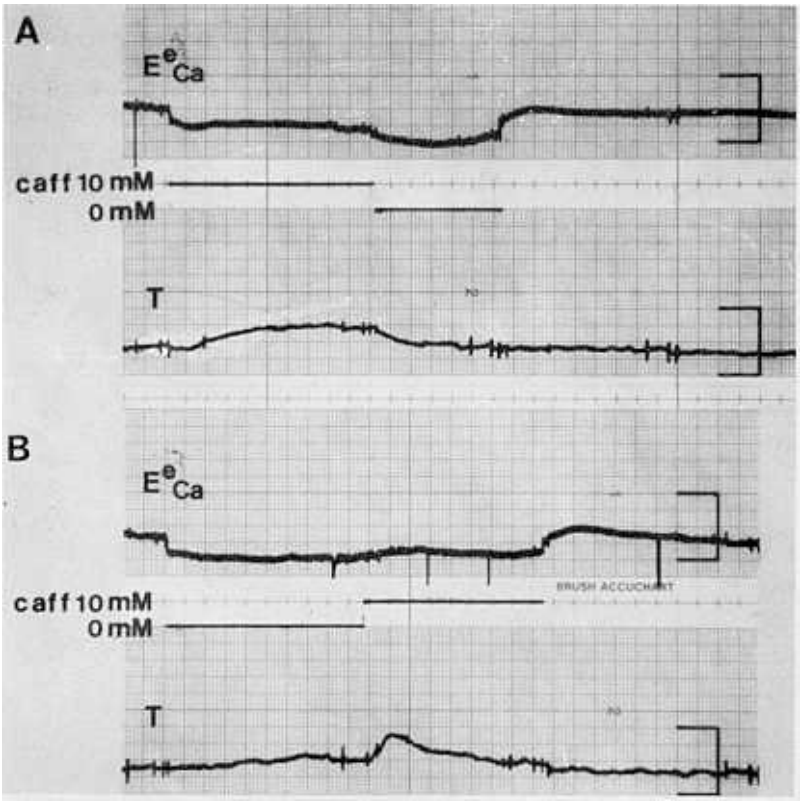

Fig. 5. *Effect of caffeine on extracellular Ca^{2+} activity and contracture.*
Horizontal scale = 2 minutes. Vertical scale = 5 mV for E^e_{Ca} (upper trace); 5mN for tension (lower trace). The horizontal bars (between upper and lower traces) represent the level of caffeine (10 mM, upper; 0 mM, lower) concentration in the superfusion solutions. A: When superfused with Na^+-free caffeine (10mM) solution, strong contracture was induced as normally seen. While the contracture was sustained, an additional superfusion of Na^+-free solution induced slow relaxation of contracture along with an additional depletion of a^e_{Ca}. B: When the tissue was superfused with Na^+-free solution without caffeine, a much weaker contracture was slowly developed and depletion of a^e_{Ca} was sustained for longer than 5 min, compared to about 1 min with the caffeine containing solution. When caffeine (10 mM) was added into the Na^+-free solution, fast and strong contracture was developed in 1 min and relaxed spontaneouisly. Addition of caffeine also caused a slow but significant increase in a^e_{Ca} until the contracture already started to relax.

DISCUSSION

There is a universal agreement that the excitation-contraction coupling of cardiac muscle is completed by the release of Ca^{2+} into the sarcoplasm of cardiac cells, and that Ca^{2+} reacts with the contractile proteins to produce contraction.

In mammalian cardiac muscle, a small but significant amount of Ca^{2+} enters the myocardial cells from the extracellular space via a slow inward current and outward (reverse) mode of Na^+-Ca^{2+} exchange during the plateau phase of the action potential (Reuter 1979; Noble 1984; Fedida *et al.* 1987). This Ca^{2+} is not immediately available for contraction, but can replenish or augment the amount of Ca^{2+} in intracellular stores (Winegrad 1979). As the depolarization occurs, mammalian cardiac muscle is activated primarily by Ca^{2+} released from the SR (Chapman 1983; Winegrad 1979). The mechanism by which depolarization activates Ca^{2+} release from the SR is not well known, but there is evidence that a Ca^{2+}-induced Ca^{2+} release mechanism may be involved in cardiac muscle

(Fabiato 1983; Fabiato 1985ab).

Since Ca^{2+} enters the cardiac cell during each action potential, to attain a steady-state there must be a mechanism to get Ca^{2+} out of the cell. Although a Ca^{2+}-activated ATPase in SR vesicles has been shown to be associated with the Ca^{2+} pump, this has not been clearly identified in cardiac sarcolemma. Thus, the Ca^{2+} efflux across the sarcolemma is believed to be accomplished by means of a Na^+-Ca^{2+} exchange transport (Mullins 1981; Sheu & Blaustein 1986).

The contribution of the Na^+-Ca^{2+} exchange transport to the maintenance of a low Ca^{2+} concentration in the sarcoplasm (and a relaxed state of the contractile system) was shown by measuring a net release of radioisotope $^{45}Ca^{2+}$ and resting tension from isolated guinea pig auricles under conditions known to change the intracellular free Ca^{2+} activity (Jundt *et al.* 1975). The rate of net efflux of Ca^{2+} from the cell, in the presence of caffeine, depends upon the Na^+ concentration of the bathing medium. Recent development of Ca^{2+}-selective electrodes has made it possible to continuously measure the Ca^{2+} flux across the sarcolemma (Suh & Sleator 1982; Bers & MacLeod 1986; Suh *et al.* 1987). Ca^{2+} sensitive dyes were also used to measure Ca^{2+} changes in extracellular space of mammalian cardiac muscle (Hilgemann *et al.* 1983; Hilgemann 1986). Both of these approaches have indicated that the cumulative Ca^{2+} depletions (and accumulations) observed can be accounted for by Ca^{2+} uptake (and release) by the SR. After a long rest period the SR is depleted of Ca^{2+} and resumption of stimulation will increase Ca^{2+} influx and progressively refill the SR. This will require net movement of Ca^{2+} from the extracellular space into the cell and will decrease extracellular Ca^{2+} activity (Ca^{2+} depletion) as shown in Fig. 1. When Ca^{2+} continuously leaks from the SR during the resting state (Kitazawa 1984; Bridge 1986), or the SR releases all of its Ca^{2+} to the sarcoplasm and cannot resequester this Ca^{2+} by pharmacological agents (Fabiato & Fabiato 1973), some of that Ca^{2+} will be extruded from the cell and increase extracellular Ca^{2+} activity (Ca^{2+} accumulation), in a Na^+-dependent fashion. This is clearly shown in Fig. 1 and 2.

Previously, the amount of Ca^{2+} transported across the sarcolemma into the cell was also estimated from the extracellular Ca^{2+} depletion caused by steady-state contractions (Bers 1983; Suh & Sleator 1983). However, the slow time response of Ca^{2+}-selective electrodes (compared to the time course of action potential) complicates the interpretation of extracellular Ca^{2+} changes during excitation. Measurements of Ca^{2+} flux across the sarcolemma during the resting state can minimize this problem and the Ca^{2+} flux during the resting state can be enhanced with greater efficiency of Na^+-Ca^{2+} exchange which is dependent on the Na^+ gradient across the sarcolemma. In this study, all of the Na^+ was removed from the superfusion solution to have Na^+-Ca^{2+} exchange work in the outward mode: Ca^{2+} influx coupled with Na^+ efflux. As Ca^{2+} is transported into the cell, extracellular Ca^{2+} is depleted and intracellular Ca^{2+} activity is raised to induce an increase in tonic tension (Na^+ withdrawal contracture). If some of the Ca^{2+} that enters the cell is taken up by the SR, as reported in mamalian trabeculae (Chapman *et al.* 1983), the blockage of SR sequestering activity with caffeine would enhance the Na^+ withdrawal contracture, as discussed later.

The magnitude of change in E^e_{Ca} is a function of Ca^{2+} activity in the extracellular space:

$$E^e_{Ca} = s \cdot \log (1 + \Delta a^e_{Ca}/a^e_{Ca})$$

where s is a calibration constant (about 30 mV), and a^e_{Ca} and Δa^e_{Ca} are Ca^{2+} activity and its change, respectively. Assuming the changes in extracellular Ca^{2+} activity (Δa^e_{Ca}) do not vary to a large extent in different background Ca^{2+} activity (a^e_{Ca}), lower Ca^{2+} activity in the superfusion solution yields larger recorded E^e_{Ca} than higher activity (Fig. 3). These results, along with the Na^+-dependence of a^e_{Ca} in Fig. 2 strongly support the empirical justification of this study: extracellular recordings of Ca^{2+} flux with Ca^{2+}-selective electrodes.

With Na^+-Ca^{2+} exchange transport maximized, the amount of Ca^{2+} transported is determined by the amount of Ca^{2+} available in the extracellular space. The amount of Ca^{2+} transported determines the magnitude of contracture, in a proportionate way (with the physiological range), if intracellular stores do not take up any fraction of the Ca^{2+} which entered. These three parameters, namely Ca^{2+} activity in the extracellular space, the amount of Ca^{2+} transported into the cell (Ca^{2+} depletion in the extracellular space), and the magnitude of contracture are well correlated as shown in Fig. 4.

It does not appear that caffeine has a direct effect on the Ca^{2+} flux across the sarcolemma, since a^e_{Ca} is increased when the addition of caffeine to Na^+-free solution induces strong transient contarcture (Fig. 5). The removal of Na^+ increases Ca^{2+} influx via Na^+-Ca^{2+} exchange and consequently increases sarcoplasmic Ca^{2+} activity which leads to the induction of contracture. Meanwhile, the SR also takes up Ca^{2+} from the sarcoplasm and lessens the amount of Ca^{2+} available for tension generation. Thus, Ca^{2+} keeps being transported into the cell depleting extracellular Ca^{2+} as shown in this study and the resting tension con-

tinuously increases. The blockage of the SR Ca^{2+} uptake by caffeine further increases the sarcoplasmic Ca^{2+} activity and enhances the available amount of Ca^{2+} for tension generation. Since the operating mode of Na^+-Ca^{2+} exchange is also dependent upon sarcoplasmic Ca^{2+} activity and Na^+ may not be completely removed from the extracellular space, it is possible that Ca^{2+} is transported out by Na^+-Ca^{2+} exchange under the condition that induces Na^+-free contracture. When caffeine is removed from the Na^+-free solution, extracellular Ca^{2+} activity transiently decreases under the condition of slow relaxation of contracture. It could happen if the SR sequesters and lowers sarcoplasmic Ca^{2+} activity to the level where Ca^{2+} is transported into the cell under Na^+-free conditions. This explanation is persuasive if Ca^{2+} kinetics among intracellular binding sites, eg. the SR and myofilaments and sarcoplasm are known. Direct recordings of intracellular Ca^{2+} activity during contractures are needed for further information.

The substitution of Na^+ with TMA might cause potential offsets of the difference signal from the Ca^{2+}-selective electrode and reference electrode, which originates from the different selectivity coefficients of Ca^{2+}-resin toward Na^+ and TMA. The magnitude of potential offsets is a few mV in negative and may cause overestimation of Ca^{2+} depletion and understimation of Ca^{2+} accumulation. To make the situation more complicated, the flow rate of superfusion solutions, which seems to be one of the important factors to induce contracture (Chapman 1983) determines the degree of Ca^{2+} activity changes such as Ca^{2+} accumulation following Ca^{2+} efflux. Thus the quantification of changes in Ca^{2+} activity needs careful measurements and interpretation of recorded data.

The present experiments do not provide direct information about the quantification of Na^+-Ca^{2+} exchange transport. If the changes in a^e_{Ca} brought about by the $[Na^+]$ and $[Ca^{2+}]$ in the superfusion solutions is to be interpreted as a movement of Ca^{2+} across the cell membrane, the net Ca^{2+} influx can be calculated from the following equation

$$\text{net } Ca^{2+} \text{ influx} = Ki \cdot [Ca^{2+}]_e \cdot V_e/A$$

where Ki is an influx rate constant, V_e is the volume of extracellular space, and A is the surface area of the cells. The influx rate constant Ki is the initial rate of E^e_{Ca} change assuming there is no Ca^{2+} efflux during the period, and with the information on extracellular space to cell surface area, the net Ca^{2+} influx can be estimated. However, it does appear that Ca^{2+} activity in the the extracellular space, the amount of Ca^{2+} transported into the cell (Ca^{2+} depletion in the extracellular space), and the magnitude of contracture are well correlated. At this point it is reasonable to say that the measurement of extracellular Ca^{2+} activity with Ca^{2+}-selective electrodes provides continuous and direct monitoring of Na^+-dependent Ca^{2+} flux across the cell membrane.

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