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EFFECT OF INOTROPIC AGENTS ON THE CALCIUM BINDING TO ISOLATED CARDIAC SARCOLEMMA

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Summary

Ca^{2+} binding to fragmented sarcolemma isolated from canine heart was measured by an ultracentrifugation technique. Two classes of binding site with dissociation constants of $2.0 \cdot 10^{-5}$ and $1.2 \cdot 10^{-3}$ M were identified. The capacities of the high- and low-affinity sites were 15 and 452 nmol/mg, respectively. These sites were not affected by treatment with neuraminidase. The effects of various cations and drugs on Ca^{2+} binding were studied. All cations tested inhibited Ca^{2+} binding with the following order of potency: trivalent > divalent > monovalent cations. The order of potency for the monovalent ions was: $Na^+ > K^+ > Li^+ \geqslant Cs^+$ and for the divalent and trivalent ions: $La^{3+} \geqslant Mn^{2+} > Sr^{2+} \geqslant Ba^{2+} > Mg^{2+}$. $1 \cdot 10^{-3}$ M caffeine and $1 \cdot 10^{-8}$ M ouabain increased the capacity of the low-affinity sites to 1531 and 837 nmol/mg, respectively. $1 \cdot 10^{-7}$ M verapamil, acidosis (pH 6.4), $1^{-}10^{-5}$ M Mn^{2+} and $1 \cdot 10^{-4}$ M ouabain depressed the capacity of the low-affinity sites to a range of 154—291 nmol/mg. The dissociation constants of the high- and low-affinity sites and the capacity of the high-affinity sites were not affected by these agents.

Introduction

The presence of extracellular Ca^{2+} is necessary for contraction in mammalian cardiac muscle [1,2]. This extracellular Ca^{2+} could either interact directly with the myofilaments [2], or induce further Ca^{2+} release from the sarcoplasmic reticulum [1]. It is generally accepted that there is a slow inward Ca^{2+} current during the plateau of the action potential, resulting in a transsarcolemmal Ca^{2+} influx [3]. Langer [2] has proposed that Ca^{2+} could, in addition, penetrate across the sarcolemma through an electroneutral Na^+/Ca^{2+} exchange. Finally,

there is indirect evidence for a superficial site of Ca^{2+} storage or binding which is in rapid equilibrium with Ca^{2+} in the interstitial space [4—6].

The present study was initiated in order to provide a direct measurement of the Ca^{2+} binding to the sarcolemma and to assess the role of this Ca^{2+} binding in cardiac excitation-contraction coupling. This study characterizes the Ca^{2+}-binding properties of a sarcolemma fraction from canine cardiac muscle [7]. Specifically, the experiments had the following goals: (1) to identify the classes of binding site on the isolated sarcolemma preparation and to measure their capacities and affinities for Ca^{2+}; (2) to test the purity of this preparation (i.e., the degree of contamination by sarcoplasmic reticulum or mitochondrial fragments); (3) to determine whether the Ca^{2+}-binding sites are on the sarcolemma and/or glycocalyx; (4) to assess whether the number of Ca^{2+} sites is sufficient for storing the Ca^{2+} necessary for the activation of the myofilaments, and finally (5) to assess whether modifications of Ca^{2+} binding by some inotropic agents are consistent with the known modifications which these agents produce on the slow inward Ca^{2+} current and the contractility of the intact heart [5,8].

Methods

Preparation of isolated cardiac sarcolemmal fraction. The heart was removed from dogs anesthetized with 30 mg pentobarbital per kg body wt. and was immediately washed with 0.9% NaCl at 4°C. Ventricular tissue was excised, blotted dry and weighed (within 5 min). The method for preparing isolated sarcolemma was that of Franson et al. [7]. All the solutions used for preparing the sarcolemma and the binding study were buffered by imidazole, and the pH was 7.0 unless specified otherwise. The tissue was minced with a razor blade, placed in 14 vols. (1 g tissue/14 ml solution) of an extraction solution containing 0.6 M KCl, 0.25 M sucrose and 10 mM imidazole, and homogenized with a Potter-Elvehjem homogenizer (ten passes of the pestle). The tissue was further disrupted for 5 s at full speed in a Sorvall Omnimixer (DuPont Co., Newton, CT). The homogenate was centrifuged at $1600 \times g$ for 20 min. The pellets were washed three times by resuspension in the original volume of extraction solution, filtration through four layers of cheesecloth, and centrifugation at $1600 \times g$ for 20 min. In order to remove excess KCl, the pellets were washed once with a solution containing 0.25 M sucrose and 10 mM imidazole. This preparation of crude sarcolemma was resuspended in 30% sucrose (w/w) with 10 mM imidazole. A continuous sucrose density gradient was formed with an equal volume (18 ml) of 55% sucrose (w/w) containing 10 mM imidazole and 30% sucrose (w/w) containing 10 mM imidazole and the crude sarcolemma. The gradient was centrifuged at $131\,000 \times g$ for 2 h in an SW-27 rotor (Beckman Instruments, Inc., Palo Alto, CA). Fractions of 2.5 ml were collected from the gradient.

Fig. 1 shows a typical gradient separation of the cardiac sarcolemma. A large protein peak followed by a small one (Fig. 1A), as well as a large $(Na^+ + K^+)$-ATPase peak and a small one, are observed (Fig. 1B). Fig. 1B demonstrates the distribution of the $(Na^+ + K^+)$-ATPase (sarcolemma marker) and cytochrome oxidase (mitochondrial marker) in the gradient. It is apparent that the sarcolemma fraction with the highest $(Na^+ + K^+)$-ATPase and the lowest cytochrome

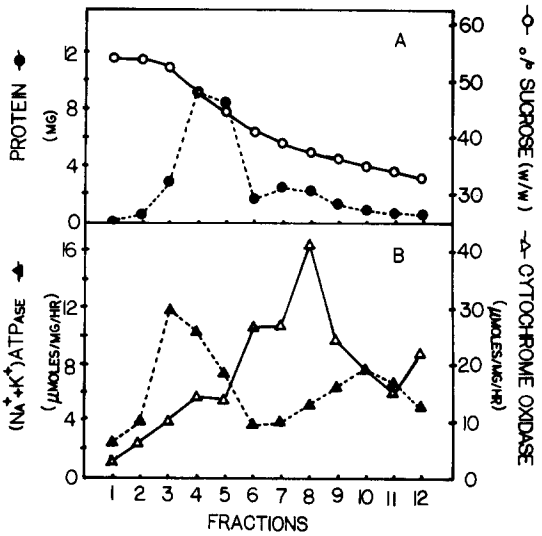

Fig. 1. Distribution of protein, $(Na^+ + K^+)$-ATPase and cytochrome oxidase after sucrose density gradient centrifugation. Crude sarcolemma was centrifuged in a continuous gradient (30—55% sucrose, w/w) for 2 h at 131 000 × *g*. Fractions were assayed for protein (●) and sucrose (○) concentrations. The activities of $(Na^+ + K^+)$-ATPase (▲) and cytochrome oxidase (△) were also determined.

oxidase activity resides in a sucrose range of 48—52% (w/w), a similar range adopted by St. Louis and Sulakhe [9] for their cardiac sarcolemma fraction. Accordingly, the fraction corresponding to this range was selected as the sarcolemma fraction.

Calcium binding and accumulation by the sarcolemma. ATP-dependent Ca^{2+} accumulation by the sarcolemma was determined according to the method of Sulakhe et al. [10]. Cardiac sarcolemma (0.1 mg protein/ml) was incubated with 50 μM $^{45}CaCl_2$ (New England Nuclear Co., Boston, MA), 5 mM MgATP, 20 mM imidazole, and 100 mM KCl at 37°C. At desired times, aliquots of incubation mixture were filtered through Millipore disks (0.45 μm). To account for nonspecific binding to the Millipore disks, blanks were treated the same way with an incubation mixture without sarcolemma. Counts from the blanks were subtracted from the values obtained in the presence of sarcolemma.

For the determination of passive Ca^{2+} binding, the sarcolemma (0.1 mg/ml) was incubated with varying concentrations of $^{45}CaCl_2$ in the presence of 10 mM imidazole (final volume of 5 ml) at room temperature for 10 min. The bound Ca^{2+} was then separated from the free Ca^{2+} by centrifugation at 105 000 × *g* for 30 min. The amount of Ca^{2+} bound was calculated from the decrease in radioactivity of the unbound fraction.

For neuraminidase pretreatment, sarcolemma (1.0 mg/ml) was incubated with 0.25 units/ml purified neuraminidase from Worthington Biochemicals (1 unit released 1 μmol sialic acid/min from bovine submaxillary mucin) at 37°C for 30 min in the presence of 0.25 M sucrose and 10 mM imidazole [11]. The neuraminidase-treated sarcolemma was then used for Ca^{2+}-binding determination, and the result compared to untreated controls.

The following drugs were used in some experiments: verapamil (isoptin hydrochloride) was obtained from Knoll AG, Ludwigshafen am Rhein, F.R.G., caffeine, ±-epinephrine, and ouabain (strophanthin G) were obtained from Sigma Chemical Co., St. Louis, MO.

Calcium binding and accumulation by the sarcoplasmic reticulum. Cardiac sarcoplasmic reticulum was prepared according to the method of Briggs et al. [12] with the modification that the sarcoplasmic reticulum was further purified with isopynic sucrose centrifugation, and the membrane fraction was collected at 27.5% sucrose (w/w). Ca^{2+} binding (in the absence of oxalate) and accumulation (in the presence of oxalate) were determined by the method of Solaro et al. [13].

Other assays. $(Na^+ + K^+)$-ATPase activity was measured by continuously monitoring the oxidation of NADH in a Beckman Model 25 spectrophotometer equipped with a constant temperature bath (37° C), at a wavelength of 340 nm, employing linked enzyme reactions [14]. The cytochrome oxidase activity was determined spectrophotometrically by observing the rate of oxidation of ferrocytochrome *c* at 550 nm at room temperature [15].

Gel electrophoresis was performed according to the improved method of Porzio and Pearson [16] to give more effective separation and resolution. The running gel consisted of 10% acrylamide with 0.1% bisacrylamide cross-linker (100 : 1) incorporating 400 mM Tris-glycine (pH 8.8), 0.1 mM EDTA, 5% glycerol and 0.1% sodium dodecyl sulfate.

Results were given as mean ± S.E., compared with Student's *t*-test for paired data and considered as statistically different where $P < 0.05$. To ascertain the classes of binding site in the sarcolemma, data were plotted according to the method of Scatchard et al. [17]. Theoretical curves were then calculated on the assumption that the binding to the sarcolemma had taken place in two classes of site according to the formula

$$\sum\left(\frac{n_i K_i\,[Ca^{2+}]}{1 + K_i\,[Ca^{2+}]}\right)$$

where $i = 1, 2$; K, dissociation constant, and n, capacity of binding.

Results

Characterization of the cardiac sarcolemmal preparation

Cardiac sarcolemma showed activities of $(Na^+ + K^+)$-ATPase in the range of 9.5—14.0 μmol/mg per h, values which are 7—8-fold higher than those measured in the homogenate (1.2—2.0 μmol/mg per h). To test the stability of the sarcolemma fractions, the $(Na^+ + K^+)$-ATPase activities were also determined at various periods after isolation. It was found that the $(Na^+ + K^+)$-ATPase activities of the sarcolemma fraction decreased significantly after isolation from a value of 11.8 ± 0.9 μmol/mg per h within the first 12 h to 8.2 ± 1.1 μmol/mg per h at 24—36 h and 6.1 ± 0.2 μmol/mg per h at 80—96 h.

To further characterize the sarcolemma fraction, gel electrophoresis was performed. The electrophoretic pattern of the cardiac sarcolemma (Fig. 2A) showed the presence of five major protein components, namely, the 16 000 band, 30 500 band, 52 000—62 000 bands, 102 000 band and 138 000—180 000 bands. The 52 000—62 000 bands accounted for nearly 30% of the total pro-

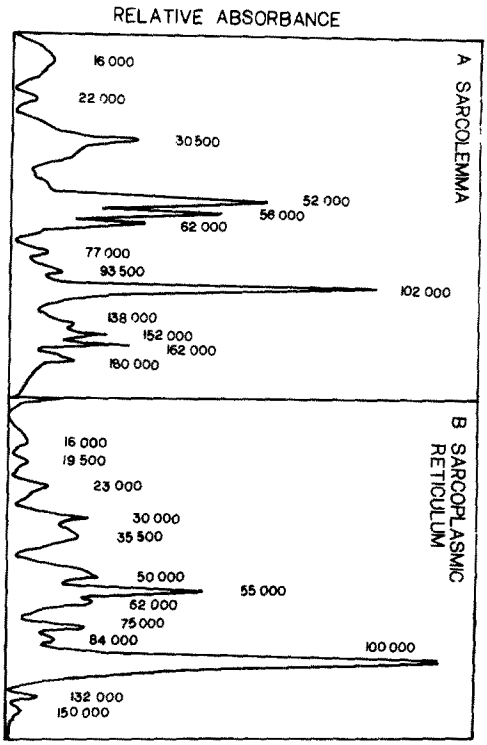

Fig. 2. Electrophoretic gel patterns of sarcolemma and sarcoplasmic reticulum from cardiac muscle.

tein. The order of decreasing concentrations of the protein components were 52 000—62 000 bands > 102 000 band > 138 000—180 000 bands > 30 500 band > 16 000 band (concentration ratios of 4.2 : 3.0 : 2.4 : 2.3 : 1, respectively).

Assessment of the degree of purity of the sarcolemmal fraction

In order to evaluate the degree of contamination of the sarcolemma preparation by sarcoplasmic reticulum fragments, a comparative gel electrophoresis was performed on the cardiac sarcoplastic reticulum preparation (Fig. 2B). There were only three major protein components present in this preparation (Fig. 2B) instead of the five observed in the sarcolemma (Fig. 2A). In addition, the distribution of the percentage of the total protein was completely different in the two profiles. In the sarcoplasmic reticulum, the 100 000 band was more concentrated and accounted for nearly one-half of the total protein. The order of decreasing concentrations of the protein components in the sarcoplasmic reticulum was 100 000 band > 50 000—62 000 bands > 30 000—35 000 bands (concentration ratios of 3.6 : 1.9 : 1, respectively). Thus, there is a major qualitative difference in the electrophoretic profiles of the sarcolemma and the sarcoplasmic reticulum.

Table I demonstrates the Ca^{2+}-accumulating abilities of the cardiac sarcolemma and sarcoplasmic reticulum in the presence of ATP. Cardiac sarcolemma bound 4.5 nmol Ca^{2+}/mg in 5 min (prolonged incubation did not result in

TABLE I

ATP-DEPENDENT CALCIUM ACCUMULATION BY CARDIAC SARCOLEMMA AND SARCOPLASMIC RETICULUM

	Ca^{2+} accumulation (nmol/mg)	
	2 min	5 min
A Sarcolemma		
control	1.8 ± 0.2	4.5 ± 0.4
oxalate (5 mM)	0.2 ± 0.2	1.0 ± 0.6
B Sarcoplasmic reticulum		
rate of Ca^{2+} accumulation (5 mM oxalate)		1.8 μmol/mg per min
capacity of Ca^{2+} binding (no oxalate)		30.0 nmol/mg

greater accumulation of Ca^{2+}). Addition of oxalate did not enhance the energy-dependent Ca^{2+} accumulation by the sarcolemma. Comparatively, the rate of Ca^{2+} accumulation of the sarcoplasmic reticulum (in the presence of oxalate) was 1.8 μmol/mg per min and the capacity of Ca^{2+} binding was 30 nmol/mg. The lack of oxalate stimulation in the sarcolemma suggests that the sarcolemma is free of any functional sarcoplasmic reticulum.

To evaluate the degree of contamination by mitochondrial fragments, cytochrome oxidase was chosen as the marker. The cytochrome oxidase activities of the sarcolemma fractions averaged 9.4 ± 0.9 μmol/mg per h. This compares with cytochrome oxidase activity of a mitochondrial fraction from canine heart of 281 ± 25 μmol/mg per h [18]. Accordingly, one can estimate that the level of contamination by mitochondrial fragments in the present sarcolemma fraction was less than 3.5%. The homogenate contained a cytochrome oxidase activity of 43.4 ± 3.2 μmol/mg per h, which is about five times higher than that of the sarcolemma fraction.

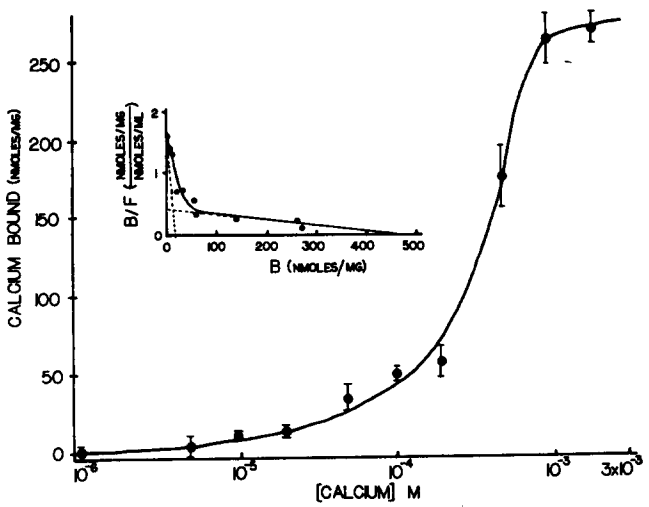

Fig. 3. Calcium binding to cardiac sarcolemma. Cardiac sarcolemma (0.1 mg/ml) was incubated with $^{45}CaCl_2$, 10 mM imidazole (pH 7.0) at room temperature for 10 min. the bound $^{45}Ca^{2+}$ was separated from free Ca^{2+} by centrifugation.

Passive calcium binding to the sarcolemma

To measure passive Ca^{2+} binding, experiments were carried out in the presence of imidazole and in the absence of ATP or any cations other than Ca^{2+}. Under these conditions, binding to the sarcolemma was found to increase on increasing the concentration of Ca^{2+} (Fig. 3). A Scatchard plot [17] of these data revealed two classes of binding site over the concentration of Ca^{2+} studied (1 μM—2 mM): the high-affinity sites had a dissociation constant of $2.0 \cdot 10^{-5}$ M and capacity of 15 nmol/mg; the low-affinity sites had a dissociation constant of $1.2 \cdot 10^{-3}$ M and a capacity of 452 nmol/mg.

Effect of neuraminidase on calcium binding to the sarcolemma

To determine if sialic acid residues are involved in the Ca^{2+} binding, cardiac sarcolemma was incubated in the presence and absence of 0.25 units of highly purified neuraminidase/mg of sarcolemma at 37°C for 30 min [11]. Consistent with the findings of Limas [19], this treatment did not alter significantly the Ca^{2+}-binding properties of the sarcolemma (Fig. 4). This result suggests that Ca^{2+}-binding sites are present on the sarcolemma membrane itself, and not on the glycocalyx [11]. However, the present study cannot rule out that there are also Ca^{2+}-binding sites on the glycocalyx, if it is assumed that glycocalyx is being removed during the isolation of the sarcolemma fraction [11,20]. It is also interesting to note that neuraminidase does not affect the Ca^{2+}-dependent slow action potential in the myocardial cells (Vogel, S. and Sperelakis, N., personal communication).

Effect of cations on calcium binding to the sarcolemma

The effects of monovalent, divalent and trivalent cations on Ca^{2+} binding to the sarcolemma are shown in Table II. All cations tested inhibited Ca^{2+} binding to the sarcolemma. The order of potency for the inhibitory effect was trivalent > divalent > monovalent cations, with the exception of Na^+ at the concentration ratio of 1.0 mM Na^+/0.1 mM Ca^{2+}. For the monovalent cations, Na^+ was the

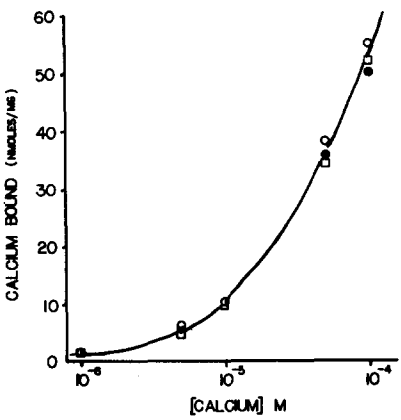

Fig. 4. Effect of neuraminidase on calcium binding to sarcolemma. Sarcolemma (1.0 mg/ml) was incubated with purified neuraminidase (0.25 units/ml) at 37°C for 30 min. Aliquots were then used for Ca^{2+}-binding assays: •, control at 0°C; ○, control at 37°C; □, neuraminidase-treated at 37°C.

TABLE II

EFFECTS OF CATIONS ON CALCIUM BINDING TO CARDIAC SARCOLEMMA

Cardiac sarcolemma (0.1 mg/ml) was incubated in 0.1 mM $^{45}CaCl_2$ and 10 mM imidazole (pH 7.0) for 10 min at room temperature. All cations used were chloride salts. The average value of Ca^{2+} binding on the sarcolemma in the absence of added cations was 31.6 nmol/mg.

Cation	% of Ca^{2+} bound	
	1.0 mM cation/ 0.1 mM Ca^{2+}	0.1 mM cation/ 0.1 mM Ca^{2+}
No addition	100 ± 5	100 ± 5
Choline$^+$	100 ± 5	—
Cs^+	80 ± 15	89 ± 10
Li^+	73 ± 7	90 ± 14
K^+	65 ± 12	76 ± 5
Na^+	29 ± 1	61 ± 8
Mg^{2+}	35 ± 3	57 ± 9
Ba^{2+}	21 ± 9	52 ± 8
Sr^{2+}	17 ± 11	55 ± 4
Mn^{2+}	0 ± 0	43 ± 4
La^{3+}	0 ± 0	39 ± 8

most potent and Cs^+ the least. These cations inhibited Ca^{2+} binding to the sarcolemma with the order of potency of $Na^+ > K^+ > Li^+ \geqslant Cs^+$ (Table II). For the divalent cations, Mn^{2+} was the most potent and Mg^{2+} the least ($Mn^{2+} > Sr^{2+} \geqslant Ba^{2+} > Mg^{2+}$). The only trivalent cation tested, La^{3+}, inhibited the Ca^{2+} binding completely at the concentration ratio of 1.0 mM La^{3+}/0.1 mM Ca^{2+}.

Because of the demonstrated participation of an Na^+/Ca^{2+} exchange in cardiac excitation-contraction coupling [3,5], the Na^+ effect on the Ca^{2+} binding was further studied (Fig. 5). Unexpectedly, Na^+ elicited a biphasic response in the Ca^{2+} binding to the sarcolemma. At a concentration of $5 \cdot 10^{-5}$ M, Na^+ stimulated Ca^{2+} binding regardless of the concentration of Ca^{2+} used. On increasing the Na^+ concentration from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-2}$ M, Ca^{2+}

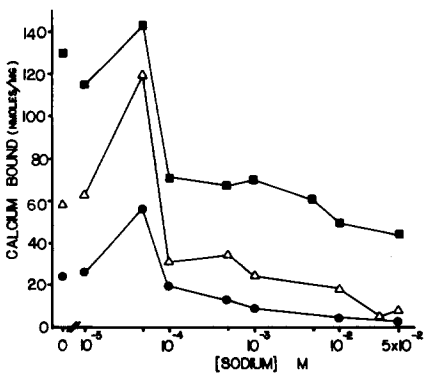

Fig. 5. Effect of Na^+ on calcium binding to the sarcolemma. Sarcolemma (0.1 mg/ml) was incubated with NaCl in the presence of 0.5 mM (■), or 0.1 mM (△), or 0.05 mM (●) $^{45}CaCl_2$ and 10 mM imidazole (pH 7.0).

binding was decreased progressively. The observation that decreasing external Na^+ concentration increased the amount of Ca^{2+} bound is especially interesting, since it has been demonstrated that decreasing external Na^+ concentration also increases cardiac contractility [3]. If the Ca^{2+} bound to the sarcolemma is involved in the Na^+/Ca^{2+} exchange as well as Ca^{2+} influx through the slow inward Ca^{2+} current, the increase in Ca^{2+} binding to the sarcolemma could be equated to an increase in the available Ca^{2+} store, which could be the cause of enhanced Ca^{2+} influx, leading to increase in tissue Ca^{2+} and finally to elevation in the contractile force.

Effect of pH on calcium binding to the sarcolemma

Fig. 6 shows the effect of alteration of pH from 6.0 to 7.6 on the Ca^{2+} binding to the sarcolemma. On increasing the pH from 7.0 to 7.6, the amount of Ca^{2+} bound to the sarcolemma was increased irrespective of the concentration of Ca^{2+} present. Conversely, decreasing the pH from 7.0 to 6.0 drastically depressed the amount of Ca^{2+} bound. The inhibitory effect was much more pronounced as the concentration of Ca^{2+} in the medium increased from 10 to 100 μM. The action of pH on Ca^{2+} binding agrees well with the dependence of contractile force and the slow inward Ca^{2+} current upon the external H^+ concentration [21,22].

Effect of inotropic agents on calcium binding to the sarcolemma

Fig. 7 shows that increasing the concentration of caffeine from $1 \cdot 10^{-6}$ M to $1 \cdot 10^{-3}$ M resulted in a large increase in the amount of Ca^{2+} bound. At $1 \cdot 10^{-3}$ M, caffeine enhanced Ca^{2+} binding to nearly 150% of the control. This lends support to the conclusion of Kavaler et al. [23] from experiments in the intact frog heart that the site of caffeine action on this tissue is the sarcolemma. There is also evidence that at least part of the action of caffeine in the mammalian heart is also on the sarcolemma [24,25].

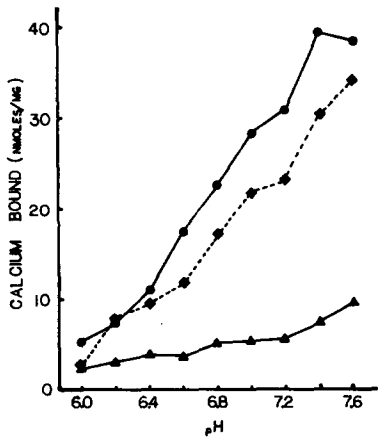

Fig. 6. Effect of pH on calcium binding to the sarcolemma. Sarcolemma (0.1 mg/ml) was incubated in 0.01 mM (▲), or 0.05 mM (♦), or 0.10 mM (●) $^{45}CaCl_2$ and 10 mM imidazole. The pH of the imidazole was adjusted with either Tris-HCl or hydrochloric acid.

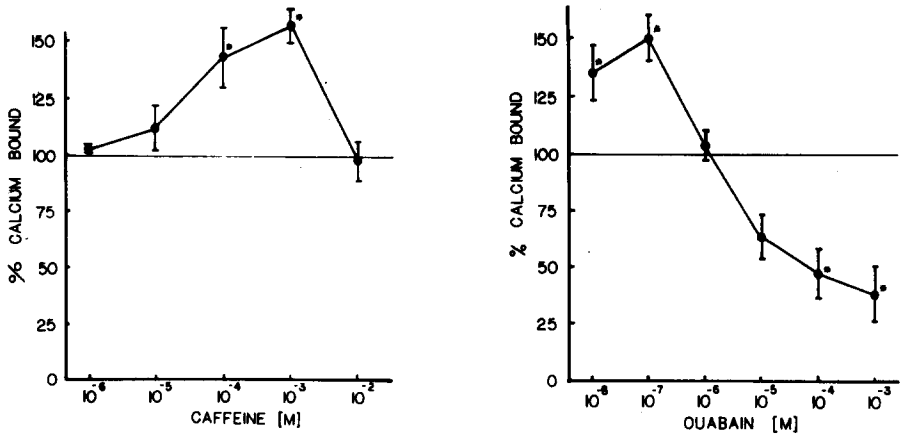

Fig. 7. Effect of caffeine on calcium binding to the sarcolemma. Sarcolemma (0.1 mg/ml) was incubated with caffeine in the presence of 0.1 mM $CaCl_2$ and 10 mM imidazole (pH 7.0). $^*P < 0.05$.

Fig. 8. Effect of ouabain on calcium binding to the sarcolemma. Conditions the same as in Fig. 7 except that ouabain was added instead of caffeine.

Fig. 8 shows that ouabain at concentrations of $1 \cdot 10^{-8}$—$1 \cdot 10^{-7}$ M stimulated Ca^{2+} binding to the sarcolemma. However, on increasing the concentration of ouabain from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ M, the glycoside depressed the amount of Ca^{2+} bound. At $1 \cdot 10^{-3}$ M, ouabain inhibited Ca^{2+} binding to 40% of the control. The stimulatory effect of ouabain ($1 \cdot 10^{-8}$—$1 \cdot 10^{-7}$ M) on Ca^{2+} binding is in agreement with the observation that ouabain enhanced the size of a fast-exchanging, superficially-located Ca^{2+} compartment in the mammalian heart [4,6]. At the range of $1 \cdot 10^{-6}$—$1 \cdot 10^{-4}$ M, ouabain also inhibited the slow inward Ca^{2+} current [26].

Addition of $1 \cdot 10^{-5}$ M epinephrine or cyclic AMP to the sarcolemma inhibited Ca^{2+} binding to 61 ± 5% and 64 ± 2% of the control. Since it has been shown that protein kinase activation is involved in the enhancement of contractile force by the epinephrine [27], the lack of stimulatory effect by epinephrine and cyclic AMP on the Ca^{2+} binding can be explained by the absence of ATP and protein kinase in the incubation medium.

Verapamil, an inhibitor of the slow inward Ca^{2+} current [8,28], at $1 \cdot 10^{-7}$ M, depressed Ca^{2+} binding to the sarcolemma to 54% of the control (Fig. 9). On increasing the concentration to $1 \cdot 10^{-3}$ M, verapamil completely inhibited Ca^{2+} binding. Verapamil, at $1 \cdot 10^{-7}$ M, also inhibits 50% of the contraction frequency and Na^+ influx of myocardial cells in monolayer of tissue culture [29], even though the relationship between Ca^{2+} binding, contraction frequency and Na^+ influx is not clear.

Since the inhibitory effect of pH was much more pronounced with increased Ca^{2+} concentration, the agents may be also affecting the two Ca^{2+} sites in the sarcolemma differently.

Thus, the effect of the inotropic agents on the Ca^{2+} binding were repeated with a series of Ca^{2+} concentrations ($1 \cdot 10^{-6}$—$1 \cdot 10^{-3}$ M). The data were analyzed with the Scatchard plot and shown in Table III. At optimal concentra-

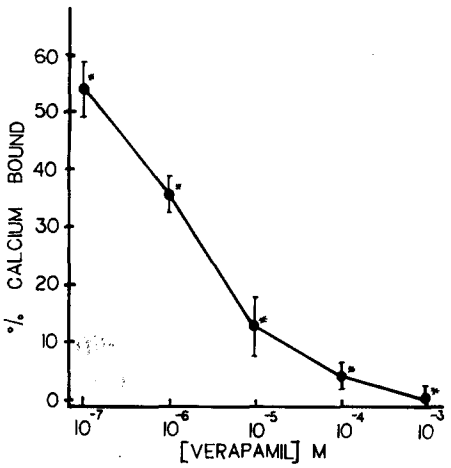

Fig. 9. Effect of verapamil on calcium binding to the sarcolemma. Conditions the same as in Fig. 7 except that verapamil was added instead of caffeine.

tions chosen from Figs. 6—9, all the inotropic agents specifically modified the capacity of the low-affinity sites in the cardiac sarcolemma. $1 \cdot 10^{-3}$ M caffeine and $1 \cdot 10^{-8}$ M ouabain increased the capacity of the low-affinity sites from a control value of 452 nmol/mg to 1531 and 837 nmol/mg, respectively. On the other hand, negative inotropic agents like $1 \cdot 10^{-7}$ M verapamil, acidosis (pH 6.4), $1 \cdot 10^{-5}$ M Mn^{2+} and $1 \cdot 10^{-4}$ M ouabain depressed the capacity of the low-affinity sites to a range of 154—291 nmol/mg: (all the capacity values for the low-affinity sites in the presence of inotropic agents were significantly different from the control with $P < 0.001$.) The dissociation constants of the high and low-affinity sites and the capacity of the high-affinity sites were not changed by these inotropic agents (Table III).

TABLE III

EFFECTS OF INOTROPIC AGENTS ON CALCIUM-BINDING SITES IN CARDIAC SARCOLEMMA

n_1 and n_2, maximal number of binding sites (nmol/mg). K_1 and K_2, dissociation constants.

	Ca^{2+} binding sites			
	High-affinity sites		Low-affinity sites	
	n_1	K_1 (M)	n_2	K_2 (M)
Caffeine ($1 \cdot 10^{-3}$ M)	12	$3.5 \cdot 10^{-5}$	1531	$4.5 \cdot 10^{-3}$
Ouabain ($1 \cdot 10^{-8}$ M)	12	$3.6 \cdot 10^{-5}$	837	$3.1 \cdot 10^{-3}$
pH 7.4	15	$2.4 \cdot 10^{-5}$	555	$1.3 \cdot 10^{-3}$
Control (pH 7.0)	15	$2.0 \cdot 10^{-5}$	452	$1.2 \cdot 10^{-3}$
Acidosis (pH 6.4)	15	$5.3 \cdot 10^{-5}$	291	$1.1 \cdot 10^{-3}$
Verapamil ($1 \cdot 10^{-7}$ M)	12	$2.1 \cdot 10^{-5}$	252	$1.7 \cdot 10^{-3}$
Ouabain ($1 \cdot 10^{-4}$ M)	12	$2.5 \cdot 10^{-5}$	181	$1.0 \cdot 10^{-3}$
Mn^{2+} ($1 \cdot 10^{-5}$ M)	14	$2.9 \cdot 10^{-5}$	154	$1.0 \cdot 10^{-3}$

Discussion

The major new observation made in the present study is that the Ca^{2+} binding to the sarcolemma is highly sensitive to ionic interventions and drugs (Table II and Figs. 6—9) that modify the slow inward Ca^{2+} current [8] and the contraction in the intact heart [2,5]. Recently, Bers and Langer [30] have stressed the consistency between the effects of various cations on Ca^{2+} binding to the sarcolemma and cardiac contractility. The present study confirms and extends this conclusion by including Sr^{2+} and Ba^{2+}. Thus, the observed sequence ($Mn^{2+} > Sr^{2+} \geqslant Ba^{2+} > Mg^{2+}$) in the effectiveness of these ions in inhibiting Ca^{2+} binding to the sarcolemma is consistent with the relative efficiency of these cations in the depression of cardiac excitation-contraction coupling [31]. It is remarkable that the positive inotropic agents like caffeine and ouabain substantially increased the binding of Ca^{2+} to the sarcolemma (Figs. 7 and 8). Conversely, negative inotropic agents including verapamil, Mn^{2+} and acidosis depressed Ca^{2+} binding (Table II and Figs. 6 and 9). Surprisingly, these agents only affect the low-affinity sites without interfering with the capacity and the dissociation constant of the high-affinity sites (Table III).

The high-affinity sites with a dissociation constant of $2.0 \cdot 10^{-5}$ M and low-capacity value of 15 nmol/mg (as compared to that of the low-affinity sites) may not serve any known purpose. This inference is drawn from the nature of its dissociation constant and the fact that these sites are not affected by inotropic agents (Table III). The low-affinity sites, on the other hand, may serve a very important purpose of regulation of available Ca^{2+} for the process of excitation-contraction coupling. This conclusion is inferred from the observation that the low-affinity sites are affected the same way by the inotropic agents as that of cardiac contractility (Table IV).

TABLE IV

SUMMARY OF THE EFFECTS OF VARIOUS INOTROPIC AGENTS ON SARCOLEMMAL CALCIUM-BINDING SITES, THE LOW INWARD CALCIUM CURRENT AND CARDIAC CONTRACTILITY

0, no effect or control; +, stimulation, and —, inhibition.

Agent	Sarcolemmal Ca^{2+} binding sites		Slow inward Ca^{2+} current	Cardiac contractility
	High affinity	Low affinity		
Caffeine ($1 \cdot 10^{-3}$ M)	0	+	+	+
Ouabain ($1 \cdot 10^{-6}$ M)	0	+	0 *	+
Control (pH 7.4) **	0	0	0	0
Acidosis (pH 6.4)	0	—	—	—
Verapamil ($1 \cdot 10^{-7}$ M)	0	—	—	—
Mn^{2+} ***	0	—	—	—
Ouabain ($1 \cdot 10^{-4}$ M)	0	—	—	—

* In vascular smooth muscle, ouabain ($1 \cdot 10^{-9}$—$1 \cdot 10^{-6}$ M) did potentiate the slow inward Ca^{2+} current [40].

** Reference for contractility and the slow inward Ca^{2+} current is at pH 7.4.

*** Concentration of Mn^{2+} for contractility and the slow inward Ca^{2+} current is at $1 \cdot 10^{-3}$ M, and for Ca^{2+} binding at $1 \cdot 10^{-5}$ M.

It is certainly difficult at this point to describe more precisely the mechanism whereby Ca^{2+} binding to the sarcolemma may be related to cardiac contraction. It may be noted, however, that the order of potency of the divalent ions and of the drugs inhibiting Ca^{2+} binding to the sarcolemma is the same as that for the slow inward Ca^{2+} current (Table IV). In the hypothetical scheme for the regulation of Ca^{2+} channels in the cardiac muscle [32], one of the steps for trans-sarcolemmal Ca^{2+} influx could possibly be the binding of Ca^{2+} to the surface membrane. Should this hypothesis be confirmed, the similarity between the modifications by inotropic agents on Ca^{2+} binding, the slow inward Ca^{2+} current and contractility would be significant. Since a larger trans-sarcolemmal Ca^{2+} influx for positive inotropism could be interpreted as an increase in the available Ca^{2+} channels, which could in turn be supplied by the increased amount of Ca^{2+} bound the sarcolemma, and a smaller trans-sarcolemmal Ca^{2+} influx for negative inotropism could be interpreted as the result of decreased binding of Ca^{2+} to the sarcolemma, the Ca^{2+} sites on the sarcolemma would be involved in the beat-to-beat regulation of Ca^{2+} movements in the contraction of the mammalian heart.

Another major question is whether the Ca^{2+} bound to the sarcolemma could directly activate the myofilaments during excitation-contraction coupling. The Ca^{2+} binding sites on the sarcolemma are clearly abundant enough to account for force generation in the myocardial cells, as shown by the following simplified calculations. Assuming that the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-binding properties were not altered during isolation, the maximum amount of Ca^{2+} bound to the high-affinity sites could be expressed as:

Amount of Ca^{2+} bound = maximum number of binding sites × $(Na^+ + K^+)$-ATPase in homogenate/$(Na^+ + K^+)$-ATPase in sarcolemma × concentration of protein/kg wet wt. of muscle = μmol Ca^{2+}/kg wet wt.

Since it was found that the activities of $(Na^+ + K^+)$-ATPase in the homogenate and in the sarcolemma from one preparation were 1.23 μmol/mg per h and 9.55 μmol/mg per h, respectively, and the protein concentration of the homogenate was 157.2 mg/g wet wt., the high-affinity sites bound 303 μmol Ca^{2+}/kg wet wt. and the low-affinity sites bound 9151 μmol Ca^{2+}/kg wet wt. Compared with the total concentration of Ca^{2+} (92.8 μmol Ca^{2+}/kg wet wt.) necessary for the generation of maximum tension in canine cardiac muscle [33], the amount of Ca^{2+} in the high-affinity sites alone are more than enough to accomplish the task.

However, before pursuing the hypothesis that the Ca^{2+} sites on the sarcolemma are the candidates for being the superficial Ca^{2+} store [4—6], it is necessary to assess whether the Ca^{2+}-binding sites measured in this study are truly on the sarcolemma itself, and not on other contaminants like mitochondria and sarcoplasmic reticulum. The $(Na^+ + K^+)$-ATPase activities (Fig. 1) denote that the fractions are highly enriched in sarcolemma and their activities are similar to the sarcolemma fractions obtained in other laboratories [9,30,34]. In addition, two classes of Ca^{2+}-binding sites were found in the present study (Fig. 3), a finding consistent with those of previous investigators [19,30,35]. Mitochondrial contamination, as indicated by cytochrome oxidase activities (Fig. 1) is minimal and the level is also similar to that reported by other investigators [9,30,34].

The estimate of contamination by the sarcoplasmic reticulum in the sarcolemma fraction is more difficult as both membrane systems have been shown to accumulate Ca^{2+} in the presence of MgATP [10,13]. In addition, the sarcoplasmic reticulum from skeletal muscle [36] has also been shown to possess at room temperature, two classes of Ca^{2+}-binding site, with dissociation constants and capacities that are quite similar to that obtained for the cardiac sarcolemma (Fig. 3 and Table III). Yet, the following findings support the idea that even if there is some contamination by remaining sarcoplasmic reticulum fragments, the sarcoplasmic reticulum was not functional under the experimental conditions in the present study: (1) oxalate is unable to enhance the energy-dependent Ca^{2+} accumulation (Table I), a finding also observed by Sulakhe et al. [10] and used by Jones et al. [34] to separate cardiac sarcolemma from the sarcoplasmic reticulum; (2) the protein profiles in the electrophoretic gel patterns are different (Fig. 2); (3) the dose of verapamil required for the inhibition of Ca^{2+} accumulation is much higher for the sarcoplasmic reticulum. Verapamil does not show any inhibition of Ca^{2+} accumulation and release in the isolated sarcoplasmic reticulum [37] or the sarcoplasmic reticulum in the skinned cardiac cells until the concentration is well over $1 \cdot 10^{-4}$ M [38], and (4) caffeine enhances Ca^{2+} binding in the sarcolemma (Fig. 7) but stimulates Ca^{2+} release in the sarcoplasmic reticulum [39].

Understandably, the sarcolemma fractions used in this study are still considerably 'contaminated'. However, recent preliminary study in this laboratory indicates that the effects of the inotropic agents on Ca^{2+} binding are unique to the sarcolemma, since these same agents do not elicit similar responses from enriched fractions of sarcoplasmic reticulum, mitochondria and myofibrils from cardiac muscle. Thus, the Ca^{2+} sites measured in this study are indigenous to the sarcolemma and these sites may participate in the regulation of cardiac contractility in some yet undetermined fashion.

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