Sodium-Sensitive Calcium Binding to Sarcolemma-Enriched Preparations from Canine Ventricle

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SUMMARY. Calcium binding to sarcolemma-enriched preparations from canine ventricle was evaluated. The preparation was exposed to calcium and \(^{45}\)Ca at physiological ionic strength, pH 7.4, for 15–18 hours at 5°C. Bound calcium was separated from free by filtration and washing of the filter with solutions containing calcium and LaCl\(_3\). After equilibration at 5°C, exposure to 37°C caused an irreversible loss of binding. Monovalent cations (157 mM) reduced calcium binding: Na\(^+\) \(\gg\) Li\(^+\) \(>\) Cs\(^+\) \(>\) K\(^+\) \(>\) Rb\(^+\) \(\approx\) choline. In 1 μM calcium, divalent cations (3 mM) reduced binding: Sr\(^{2+}\) \(>\) Ba\(^{2+}\) \(>\) Mg\(^{2+}\) \(\approx\) Mn\(^{2+}\). At 1–300 μM calcium, inhibition of the sodium-sensitive component of binding was characterized by \(K_d = 3.2–9.5\) mM sodium. Comparison of binding by centrifugation versus filtration suggested that the sodium-sensitive component resided on constituents within the membrane vesicles. Calcium binding in 1 mM ethyleneglycol-bis(\(\beta\)-aminoethylether)N,N'-tetraacetic acid at pH 7.1 and 5°C, revealed a single species of sodium-sensitive calcium-binding sites: \(K_d = 0.052\) μM and \(B_{max} = 6.73\) nmol/mg. In 3 mM magnesium, the \(K_d = 0.205\) μM and the \(B_{max}\) was 9.03 nmol/mg. Nearly complete inhibition of binding was observed as sodium was raised from 1 to 10 mM. Thus, a substantial number of calcium-binding sites were detected at 5°C in 3 mM magnesium at physiological ionic strength and pH 7.1. The affinity of these sites was in the range necessary to modulate intracellular free calcium. The sensitivity to sodium was at the lower end of the range estimated for intracellular sodium (Circ Res 55: 676–688, 1984).

INTRACELLULAR free calcium rises and falls over the cardiac cycle, and this fluctuation plays a critical role in contractility of the heart (Wolhfart and Noble, 1982; Chapman, 1983). In addition, myocardial contractility is a function of the extracellular (von Willbrandt and Koller, 1948; Lüttgau and Niedergerke, 1958) and intracellular (Eisner et al., 1983; Wassermann et al., 1983) concentrations (activities) of sodium. The influence of sodium on contractility is usually attributed to the operation of a sodium/calcium exchange system which couples the movement of sodium and calcium across the sarcolemma in opposite directions (Langer, 1972; Reuter, 1974; Blaustein, 1974; Mullins, 1981; Langer, 1982). Recent studies, however, suggest that a second mechanism may be involved. Bers and Langer (1979) reported a correlation between calcium bound to isolated sarcolemma and tension development by rat papillary muscle preparations. Subsequently, Philipson et al. (1980a) reported that sodium and calcium appeared to compete for sites on the external surface of the sarcolemma, and that calcium binding to these sites correlated, over a range of sodium concentrations, with the contractile strength of perfused rabbit interventricular septum preparations.

It is also apparent that sodium-sensitive calcium binding sites within the cell (Langer, 1968), perhaps associated with the internal surface of the sarcolemma (Besch and Schwartz, 1970; Lüllmann and Peters, 1979), could have major effects on myocardial contractility. To date, the affinity, capacity, and other characteristics of calcium-binding sites on the internal surface of the sarcolemma have not been well defined. These sites might act to influence contractility (1) by serving to release free calcium directly into the cytoplasm (Lüllmann et al., 1983), (2) by binding calcium, which has been released into the cytoplasm, thus decreasing the effective concentration of free calcium around the myofilaments (Fabiato, 1983), and/or (3) by modifying fluxes of calcium or other ligands through the sarcolemma. It is also possible that sodium modifies the binding of calcium to these sites. Will et al. (1976) reported a component of calcium binding to a partially purified Na\(^+\),K\(^-\)-ATPase preparation from porcine myocardium that had a high affinity for calcium and a high sensitivity to sodium. Pang (1980) found that calcium binding to a sarcolemma preparation from canine heart, measured by a centrifugation technique, was inhibited more by sodium than by other monovalent cations. More recently, Slaught et al. (1983) and Reeves and Hale (1984) reported that sodium reduced the equilibrium level of vesicular calcium in sarcolemma preparations from bovine heart. This was ascribed to a competition between sodium and calcium for binding sites located in the interior of the vesicles, presumably on the internal surface of the vesicular membrane.
The objective of this study was to test for the presence of sodium-sensitive calcium-binding sites on the intracellular surface of the myocardial cell's sarcolemma. Vesicles in a highly enriched sarcolemma preparation from canine ventricle (Van Alstyne et al., 1980) were found to bind calcium in a concentration- and protein-dependent manner at a physiological ionic strength in the presence of 3 mM magnesium. These sites appeared to be associated either with the internal surface of the sarcolemma or with a soluble constituent trapped within the vesicles. The binding sites exhibited a sensitivity to calcium and sodium such that an increase in sodium from 1 to 10 mM decreased the ability of calcium to bind at free calcium concentrations of 0.01–10 \( \mu \text{M} \).

**Methods**

**Sarcolemma-Enriched Preparation**

Sarcolemma-enriched membrane preparations were isolated from canine ventricle at 4–5°C by the method of Van Alstyne et al. (1980) with minor modifications: (1) a Cuisinart processor was used to mince the tissue, and (2) the initial isolation medium contained 15 mM NaHCO\(_3\), 5 mM MgCl\(_2\), 5 mM Na\(_2\)ATP (brought to pH 7.4 with Tris base), and 10 mM Tris-Cl, pH 7.4. In some assays, 5 mM sodium azide was present. This preparation manifested a ouabain-sensitive, Na\(_+\),K\(_-\)-ATPase activity apparent to be manifested by leaky vesicles in the preparation, and the freeze-thaw treatment at low ionic strength, which causes loss of osmotic responsiveness (Van Alstyne et al., 1980), was assumed to make all vesicles leaky. Thus, the ratio of the two activities for before and after freeze-thaw yielded the fraction of leaky vesicles. The number of leaky vesicles, therefore, the sum of leaky and sealed I/O vesicles in the preparation which equated to 18 ± 4%. The remaining 82% were assumed to be sealed vesicles distributed in the right-side out (R/O) and inside out (I/O) configurations.

\[ [\text{Na}] + [\text{K}] + \text{ATP} \text{ase activity of 20.9 ± 2.8 and 135.8 ± 33.0 nmoL/mg per hr (means ± SE; } n = 5) \text{ before and after 15 freeze-thaw cycles.} \]

Ouabain-sensitive, Na\(_+\),K\(_-\)-ATPase activity appears to be manifested by leaky vesicles in the preparation, and the freeze-thaw treatment at low ionic strength, which causes loss of osmotic responsiveness (Van Alstyne et al., 1980), was assumed to make all vesicles leaky. Thus, the ratio of the two activities for before and after freeze-thaw yielded the fraction of leaky vesicles in the preparation, which equated to 18 ± 4%. The remaining 82% were assumed to be sealed vesicles distributed in the right-side out (R/O) and inside out (I/O) configurations.

**Calcium-Binding Assays**

**Filtration**

Aliquots of the sarcolemma-enriched preparation were exposed for 15–18 hours at 5°C to media that contained various salts and CaCl\(_2\) with \( ^{45}\text{Ca} \) (specific activity = 1600–2200 counts/min per pmol). In the initial set of experiments, 10 mM Tris-Cl, pH 7.4, was used, and no attempt was made to buffer free calcium (see below). Total ionic strength of the reaction media was between 167 and 170 mM. After a 15- to 18-hour incubation, an aliquot of the membrane preparation was diluted with an ice cold "stop/wash" solution, filtered (Millipore filters; type HA, 0.45 \( \mu \text{m} \)) and washed with 25–35 ml of the stop/wash solution. This solution consisted of the same ionic composition as the initial reaction medium, except that 1 mM unlabeled CaCl\(_2\) and 100 \( \mu \text{M} \) LaCl\(_3\) were present, and \( ^{45}\text{Ca} \) was absent (Bartschat and Lindenmayer, 1980). The washed filters were removed, dissolved in a scintillation cocktail, and assayed for \( ^{45}\text{Ca} \) by liquid scintillation spectroscopy. Protein assays were carried out by the method of Lowry et al. (1951).

In a subsequent set of experiments, EGTA* was included in the various reaction media in order (1) to define more optimally free calcium in the presence of calcium endogenous to the membrane preparation and (2) to minimize changes in free calcium which might have occurred as a result of calcium binding. The affinity and capacity of EGTA for calcium were determined for solutions containing 1 mM EGTA, 10 mM HEPES, and 157 mM potassium or sodium. This was accomplished by the use of a calcium-sensitive electrode (Orion 93-20) as described by Bers (1982). All solutions were adjusted to pH 7.1 at 5°C after the addition of the calcium with either KOH or NaOH. Thus, the monovalent cations were added as chloride and hydroxide salts to achieve the final concentrations desired. The dissociation constant was the same for solutions containing 157 mM potassium or 157 mM sodium and equaled 224 ± 15 mM (mean ± SE). With the calculated affinity and capacity of EGTA for calcium, reaction media were formulated to give a range of free calcium concentra-

* EGTA: ethyleneglycol-bis-(\( \beta \)-aminoethyl)ether)N,N'-tetraacetic acid.
trations in 10 mM HEPES, 1 or 5 mM EGTA, ± 3 mM MgCl₂, and either 157 mM potassium, 157 mM sodium, or a mixture of the two cations, so that potassium plus sodium equaled 157 mM (pH 7.1 at 5°C). Specific activity of ⁴⁵Ca was 18-22 counts/min per pmol. Aliquots of the sarcolemma preparation were exposed to these media. After 15-18 hours, calcium associated with the membrane vesicles was determined as described above. Protein concentration was constant for these assays at 250 μg/ml.

**Filtration vs. Centrifugation Experiments**

Aliquots of the sarcolemma preparation were exposed to either 157 mM KCl or 157 mM NaCl, 10 mM Tris (pH 7.4 at 5°C), 3 mM MgCl₂, and either 1 or 15 μM added CaCl₂ (EGTA was not present in these experiments.) After 15-18 hours at 5°C, the reaction in one aliquot of the suspension was terminated by filtration, as described above, and in a second aliquot, by centrifugation. For the latter, tubes containing the membrane suspensions were centrifuged at 200,000 g for 10 minutes. The supernatant was decanted, the tube around the pellet was wiped dry, and the pellet was exposed to 0.3 ml of 0.2 N NaOH at 70°C. The suspension was collected, the tube was rinsed with 0.3 ml H₂O, and the suspension and wash were assayed for ⁴⁵Ca content. Total radioactivity in the pellet was bound and free inside the vesicles, and 3 bound to the extravesicular membrane surface. To correct for ⁴⁵Ca trapped in the extravesicular space, a parallel series of experiments (in the absence of ⁴⁵Ca) were employed wherein ¹⁴C]sucrose and sucrose were added to the membrane suspension immediately before centrifugation at a final concentration of 5 × 10⁻⁴M. Sucrose was assumed to be excluded from the intravesicular space of the vesicles during the brief period of centrifugation. The ¹⁴C]sucrose content of the pellets was used to calculate an extravesicular water space. Total radioactivity per pellet, determined in the presence of ⁴⁵Ca, was then corrected to eliminate the component of ⁴⁵Ca in the extravesicular space. In a separate series of experiments, the recovery of specifically bound [³H]ouabain after filtration and centrifugation was measured to assess recovery of sarcolemma by the two procedures. The preparation was exposed to the drug for 30 minutes at 37°C, as described above. Subsequently, the temperature was decreased to 5°C, and the preparation was loaded with 157 mM KCl and 10 mM Tris-Cl, pH 7.4, for 16 hours. After loading, an aliquot of the suspension was removed for filtration. The remainder was centrifuged, and the filter and pellet were assayed for radioactivity.

**Materials**

⁴⁵Ca and ¹⁴C]sucrose were obtained from New England Nuclear. “Gold-label” RBCl was obtained from Aldrich Chemical Co. A calcium standard (Orion 92-20-06) was obtained from Fisher Scientific Co. All other chemicals were of reagent grade.

**Results**

As stated above (Methods), the sarcolemma preparation employed for the following experiments appeared to consist of 18 ± 4% leaky vesicles, 14 ± 2% sealed I/O vesicles, and 68 ± 4% sealed R/O vesicles. Thus, the internal surface of the sarcolemma would be on the internal surface of approximately 83% of the sealed vesicles in the preparation. The following protocol was used in an attempt to detect calcium binding sites associated with the internal surface of sealed vesicles. First, the preparation was exposed to calcium with ⁴⁵Ca for 15-18 hours at 5°C. Longer exposures (out to 41 hours) showed that 15-18 hours were sufficient for the equilibration of calcium between the extravesicular space and the various compartments of the vesicular preparation. Second, the suspension was diluted 1:250 with an ice-cold stop/wash solution and filtered. Prior to removal for assay of ⁴⁵Ca content, the filter was washed with an additional 25-35 ml of the stop/wash solution which contained 1 mM unlabeled calcium and 0.1 mM LaCl₃. The rationale for these maneuvers was that unlabeled calcium and lanthanum in the stop/wash solution should displace ⁴⁵Ca bound to the external surface of sealed vesicles and to both surfaces of leaky vesicles. In addition, the unlabeled calcium should dilute the specific activity of ⁴⁵Ca in the extravesicular space, and lanthanum should stop any movement of calcium across the membrane of sealed vesicles (Bartschat and Lindenmayer, 1980). Parallel assays were always carried out in the absence of the preparation to yield values for ⁴⁵Ca remaining on the filter per se after the wash procedure. The difference between assays in the presence and absence of the preparation was defined as calcium "bound" to the preparation. The amount bound was linear with assay protein up to 6 μg protein/assay, which equated to 300 μg/ml (Fig. 1).

![Figure 1. Calcium binding vs. protein in assays. Aliquots of the sarcolemma preparation were exposed to CaCl₂/⁴⁵Ca (as shown) for 15-18 hours at 5°C in a medium containing 157 mM KCl and 10 mM Tris-Cl, pH 7.4. Final volume of the assays was 0.26 ml. Values are means ± se (n = 4).](image-url)
Calcium binding detected by this protocol was labile at higher temperatures, particularly at lower calcium concentrations (Fig. 2A). In this experiment, the preparation was exposed to 37°C after prior exposure to calcium with $^{45}$Ca for 15–18 hours at 5°C. The loss of binding appeared to occur through at least two processes, the faster of which was apparent at 1 and 10 mM calcium, but not at 300 mM. For the initial experiments reported here, we did not take into account the contribution of endogenous calcium to the calcium concentrations cited (see below). The in vitro loss of binding appeared to be irreversible. Exposure of the preparation to calcium at 5°C for 15–18 hours, followed by a 5- or 30-minute incubation at 37°C (with loss of binding) and then a return to 5°C for 2 hours (Fig. 2B) or 24 hours (data not shown), did not result in any increase in binding. A possible explanation for the lability involved a change in pH as the temperature was changed. Tris was used as the buffer in these experiments and was adjusted to pH 7.4 at 5°C which equated to pH 6.5 at 37°C. When the buffer was adjusted to pH 7.4 for 37°C, the same profile for loss of binding was observed (Fig. 2C). Thus, the lability was not due to a change in pH. Since the binding sites were labile in vitro, all of the experiments reported below were carried out at 5°C.

The experiments described in Figures 1 and 2 were carried out in 157 mM KCl to set the ionic strength of the assays at a physiological level. The next experiment was designed to determine how a series of monovalent salts affected calcium binding. Chloride salts of the alkali metals were compared with choline chloride and sucrose. The salts inhibited binding according to the sequence: $\text{Na}^+ > \text{Li}^+ > \text{Cs}^+ > \text{K}^+ > \text{Rb}^+ \approx \text{choline} \approx \text{sucrose}$ (Fig. 3A). In addition to the other constituents of the assay media, these experiments included 3 mM magnesium which may be in the range of intracellular free magnesium (Tsien, 1983). This concentration of magnesium or manganous ion did reduce calcium binding to some extent in the presence of 1 mM calcium, but marked inhibition was observed with 3 mM strontium (Fig. 3B). Inhibition by barium was between that observed for magnesium and strontium. Thus, a sodium-sensitive component of calcium binding could be detected at physiological ionic strength in the presence of 3 mM magnesium.

Hereafter, calcium binding observed in 157 mM potassium minus binding observed in 157 mM sodium will be referred to as "sodium-sensitive calcium binding." It should be noted that the "signal-to-noise" ratio of the binding was quite favorable for detection of changes in the sodium-sensitive component. For a typical experiment in 1 mM calcium and 3 mM magnesium, calcium binding was: (1) 4439.3 counts/min in 157 mM potassium; (2) 383.9 counts/min in 157 mM sodium; and (3) 66.0 counts/min for filter blanks (values are averages of duplicates). We again emphasize that the results of experiments carried out in the absence of a calcium buffer are expressed as counts/min per mg protein. This was necessary because of contamination of solutions/preparations with calcium which elevated the total calcium concentrations over what is stated to be present and lowered the specific activity of the $^{45}$Ca added to the assays. Experiments with EGTA as a calcium buffer will be presented below.
As argued above, sites responsible for the calcium binding were thought to reside in sealed R/O vesicles because of the composition of the preparation and the protocol employed to study binding. Two experiments were carried out to test this argument. In the first experiment, 1 mM EGTA with or without the calcium ionophore, A23187, was added after exposure of the preparation to 15 μM CaCl₂ with ⁴⁰Ca for 15–18 hours. The presence of EGTA alone for 2–2.5 minutes before filtration and washing had minimal effect on vesicular calcium despite the presence of a presumably large outwardly directed calcium gradient (Table 1). Subsequent addition or co-administration of A23187 caused a substantial loss of the sodium-sensitive and sodium-insensitive components within 2 minutes. These results were consistent with the argument that at least most of the calcium, which remained after filtration and washing, did reside in sealed vesicles.

The second of these two experiments was designed to compare bound calcium, remaining after filtration and washing, to total calcium bound to the preparation. The latter was obtained by sedimenting an aliquot of the preparation after exposure to calcium.

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Salt</th>
<th>Control</th>
<th>EGTA</th>
<th>EGTA + solvent</th>
<th>EGTA + A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>KCl</td>
<td>10.90</td>
<td>8.62</td>
<td>6.55</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>2.11</td>
<td>1.31</td>
<td>1.24</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>(K⁺⁻⁻Na⁺)</td>
<td>8.81</td>
<td>7.31</td>
<td>5.31</td>
<td>0.44</td>
</tr>
<tr>
<td>B</td>
<td>KCl</td>
<td>10.37 ± 2.39</td>
<td>10.30 ± 2.28</td>
<td>9.36 ± 1.81</td>
<td>1.31 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>1.65 ± 0.18</td>
<td>1.12 ± 0.12</td>
<td>1.22 ± 0.12</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(K⁺⁻⁻Na⁺)</td>
<td>8.73 ± 2.23</td>
<td>9.19 ± 2.23</td>
<td>8.14 ± 1.70</td>
<td>1.03 ± 0.16</td>
</tr>
</tbody>
</table>

Aliquots of the sarcolemma preparation (50 μg/ml final concentration) were exposed to 15 μM CaCl₂ with ⁴⁰Ca for 15–18 hours at 5°C in media containing 157 mM KCl or NaCl, 3.16 mM MgCl₂ and 10 mM Tris-Cl, pH 7.4. Subsequently, the following additions were made as indicated (final concentrations given). Experiment A: 1 mM EGTA followed in some assays 0.5 minute later by 1% (vol/vol) ethanol or 10 μM A23187 in ethanol; samples were filtered after an additional 2 minutes (values are averages; n = 2). Experiment B: 1 mM EGTA, 1 mM EGTA plus 0.068% dimethylsulfoxide or 1 mM EGTA plus 11.8 μM A23187 in dimethylsulfoxide with samples filtered 2 minutes later (means ± SE; n = 3). All values are normalized to a constant specific activity of 1770 counts/min per pmol.
The results of the study were consistent with localization of the sodium-sensitive calcium-binding sites on a soluble constituent trapped in the membrane vesicles or on the internal surface of the sarcolemma (Experimental Observations, Table 2). We again emphasize the uncertainty which must be placed on the estimate of sealed I/O vesicles (and, consequently, of sealed R/O vesicles) in the preparation. If the fraction of sealed I/O vesicles were higher than the estimated 14%, the ratio for the case of binding located on the internal sarcolemma surface (Predictions, Table 2) would be higher than 1.47 and, therefore, would deviate to a greater extent from the observed ratios (i.e., 1.28 and 1.26 uncorrected; 1.42 and 1.40, corrected; Experimental Observations, Table 2). Conversely, if the fraction of sealed I/O vesicles were less than 14%, the ratio predicted for this case would decrease.

If the sodium-sensitive calcium-binding sites re-

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**TABLE 2**

**Sodium-Sensitive Calcium Binding Centrifugation vs. Filtration**

<table>
<thead>
<tr>
<th>Location of binding sites</th>
<th>Predictions</th>
<th>Centrifugation</th>
<th>Filtration</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>On internal surface in situ</td>
<td>([R/O + I/O + L] + [R/O])</td>
<td>1.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>On external surface in situ</td>
<td>([R/O + I/O + L] + [I/O])</td>
<td>7.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symmetrical, on internal and external surfaces in situ</td>
<td>2x([R/O + I/O + L] + [R/O + I/O])</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble, trapped in vesicles</td>
<td>([R/O + I/O + L] + [R/O + I/O])</td>
<td>1.22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Experimental observations**

<table>
<thead>
<tr>
<th>[Ca⁺²] Method of separation</th>
<th>Calcium bound</th>
<th>Cent./filt. corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Centrifugation</td>
<td>3.38 ± 0.19</td>
</tr>
<tr>
<td>Filtration</td>
<td>2.63 ± 0.12</td>
<td>2.39 ± 0.12</td>
</tr>
<tr>
<td>15</td>
<td>Centrifugation</td>
<td>17.17</td>
</tr>
<tr>
<td>Filtration</td>
<td>12.59</td>
<td>10.14</td>
</tr>
</tbody>
</table>

*Predictions (upper): ratios for sodium-sensitive calcium binding obtained by centrifugation and filtration as a function of the location of the sites for a sarcolemma preparation consisting of 68% sealed R/O vesicles, 14% sealed I/O vesicles, and 18% leaky vesicles (L). "Leaky vesicles" were defined as vesicles leaky to calcium and other salts but not to any soluble calcium-binding constituents (e.g., proteins) in the vesicles. [It should be noted that if these vesicles were leaky to salts and a soluble binding constituent, the last case would yield a ratio of 1.00 instead of 1.23 (i.e., "L" drops out in the numerator of the ratio.)] The centrifugation protocol was assumed to detect sodium-sensitive, high affinity calcium binding to all sites in the preparation regardless of their location (i.e., extravesicular surface, intravesicular surface or space). The filtration protocol was assumed to detect binding only to these sites in sealed vesicles. Experimental Observations (lower): Aliquots of the preparation (50 µg/ml final concentration) were exposed to 1 or 15 µM CaCl₂ with ⁴⁵Ca for 15-18 hours at 5°C in the presence of 157 mM KCl or NaCl, 3 mM MgCl₂ and 10 mM Tris-Cl, pH 7.4. Reactions were terminated by centrifugation or filtration (see Methods). Total pellet ⁴⁵Ca was corrected for ⁴⁵Ca trapped in extravesicular water space of pellets, which was determined in parallel studies with ¹⁴C]sucrose. This space was 13.0 ± 0.5 µl/mg for potassium = 157 mM and 12.8 ± 0.8 for sodium = 157 mM (n = 6). Values for binding were normalized to a constant specific activity of 1931 counts/min per pmol. Values for binding and ratios are means ± SE (n = 4) for 1 µM Ca⁺² and averages (n = 2) for 15 µM Ca⁺². In a separate set of experiments, the recovery of specifically bound [³⁴H]ouabain was 180.9 ± 7.9 and 165.3 ± 10.6 pmol/mg (means ± SE, n = 4) after filtration and centrifugation, respectively. This yielded a ratio of filtration/centrifugation 1.11 ± 0.07 for recovery of this sarcolemma marker. The values for "Cent./filt. for (K⁺-Na⁺)" were multiplied by 1.11 to obtain "Cent./filt. corrected."
side on the internal surface of the sarcolemma, their sensitivity to sodium is important with respect to possible roles in the excitation-contraction-relaxation sequence of the myocardial cell. In other words, could sodium affect calcium binding in the range estimated for physiological concentrations of intracellular sodium? Titrations revealed that the sodium required for 50% inhibition of calcium binding to the sodium-sensitive sites ranged from 3.2 mM at 1 \( \mu \text{M} \) calcium to 9.5 mM at 300 \( \mu \text{M} \) calcium (Fig. 4). It should be noted that the sum of potassium plus sodium was held constant at 157 mM. This meant that the \( I_{50} \) values for sodium of 3.2 and 9.5 mM would pertain to assay media containing 153.8 and 147.5 mM potassium, respectively. This reflected small changes in potassium from the control case where potassium was 157 mM. Thus, it seemed unlikely that the drop in potassium per se caused inhibition. This conclusion was reinforced by the observation that potassium, in fact, inhibited binding relative to rubidium or choline, but was a much weaker inhibitor than sodium (Fig. 3). It should be noted that the profiles in Figure 4 are similar to that presented by Reeves and Hale (1984; their Fig. 5) for the effects of sodium on intravesicular calcium levels at equilibrium measured at 15 \( \mu \text{M} \) calcium.

The final set of experiments addressed three issues: (1) the number of sodium-sensitive binding sites in the sarcolemma preparation, (2) the affinity of the sites for calcium, and (3) the effects of magnesium and sodium on the number and affinity of the sites. Given that 6.2 ± 1.0 nmol endogenous calcium/mg protein was previously found for the sarcolemma preparation (Bartschat and Lindenmayer, 1980), the experimental protocol was modified to include EGTA in the reaction media as a calcium buffer. Titrations with a calcium electrode yielded a dissociation constant for interaction of calcium and EGTA equal to 0.224 nM for the conditions employed (Methods). The calcium range for the titrations was from 0.5 to 100 \( \mu \text{M} \). It proved necessary to analyze binding at free calcium concentrations below resolution by the electrode. Thus, the free calcium concentrations cited below were calculated from the equilibrium equation for the chelation of calcium by EGTA. Where magnesium was present, the calculated free calcium was adjusted for chelation of magnesium by EGTA. A value of 18.8 mM was used for the dissociation constant of the latter reaction (Fabiato and Fabiato, 1979).

The amount of calcium bound to the sarcolemma in 157 mM potassium or sodium was clearly a complicated function of free calcium (Fig. 5A). The "signal-to-noise" ratio of these assays were as follows for typical experiments carried out at 0.00375 \( \mu \text{M} \) free calcium (lowest concentration used) and at 50 \( \mu \text{M} \) free calcium, respectively: (1) 1039.8 and 16,369.3 counts/min in 157 mM potassium, (2) 212.2...
and 8170.8 counts/min in 157 mM sodium, and (3) 37.2 and 230.8 counts/min for filter blanks (values are averages of duplicates). After filtration and washing, calcium with $^{45}$Ca in the vesicles presumably existed in three states: (1) calcium associated with sites in the vesicles, (2) free calcium in the intravesicular water space in equilibrium with free calcium in the extravesicular space, and (3) calcium chelated to EGTA in the intravesicular water space. In an attempt to separate these components, we transformed the data in Figure 5A to volumes of distribution ($\mu$L/mg protein) by the equation:

\[
\text{Volume of distribution} = \frac{\text{nmol Ca}^{++} \text{ bound/mg protein}}{[\text{Ca}^{++} \text{ (nmol/}\mu\text{L})]} \tag{1}
\]

where the denominator refers to free calcium in the assay medium. These volumes of distribution were compared to values for the volume of distribution of sodium, potassium, and chloride [measured in the absence of added calcium and the absence of EGTA (Schilling et al., 1984)]. It was clear that the volume of distribution for calcium was very large at lower free calcium compared to the volume of distribution for the monovalent cations and chloride (Fig. 5B). As free calcium was raised, the volume of distribution declined to levels approaching that for sodium, potassium, and chloride. This presumably reflected a greater contribution of free intravesicular calcium to total vesicular calcium. At 1 mM free calcium, the volume of distribution for calcium (taken from the curve) was 35.5 $\mu$L/mg. This value agrees closely with the previously published value of 32 $\mu$L/mg which was determined in the absence of EGTA (Bartschat and Lindenmayer, 1980).

It was possible that EGTA did not completely equilibrate across the membrane in 15–18 hours, and that the amount of intravesicular EGTA after 15–18 hours varied with free calcium (e.g., due to charge differences between EGTA and the Ca-EGTA complex). Given that radiolabeled EGTA was not commercially available, the validity of these concerns was tested by calculating the volume of distribution for the Ca-EGTA complex. The experiments shown in Figure 5, carried out in 1 mM EGTA, were repeated at six concentrations of calcium (free calcium ranged from 0.0079 to 5 mM) in the presence of 5 mM EGTA. For each concentration of calcium, simultaneous equations were set up as follows:

For 1 mM EGTA: \[X + Y = \text{Ca}^{++} \text{ in vesicles} \tag{2}\]

For 5 mM EGTA: \[5X + Y = \text{Ca}^{++} \text{ in vesicles} \tag{3}\]

where \(X = \text{Ca} \cdot \text{EGTA in vesicles}\), \(Y = \text{calcium bound}\).

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**Figure 5.** Calcium binding and volume of distribution vs. free calcium. Panel A: aliquots of the sarcolemma preparation were exposed at 5°C for 15–18 hours to various concentrations of CaCl$_2$/$^{45}$Ca in the presence of 1 mM EGTA, 10 mM HEPES (pH = 7.1 at 5°C, and 157 mM potassium or sodium (chloride and hydroxide salts). Values for free calcium (abscissa) were calculated based on measured affinity and capacity constants for the stock EGTA solution (Methods). Inset: The difference in calcium binding measured in the presence of 157 mM potassium and 157 mM sodium vs. free calcium over the range of 0.0038 to 50 mM. Panel B: data from panel A recalculated as volume of distribution for calcium associated with the membrane preparation. Volume of distribution for the Ca-EGTA complex was calculated as described in the text for data obtained in 157 mM sodium. Values for the volumes of distribution for chloride, sodium, and potassium [concentration of ligand = 150 mM; assays at 5°C, pH 7.4 (from Schilling et al., 1984)] are shown for comparative purposes. We emphasize that the values from Schilling et al., 1984, were measured in the absence of exogenous calcium or EGTA. Thus, values for free calcium (abscissa) are irrelevant for the volumes of distribution of chloride, sodium, and potassium.
to sites in vesicles and calcium free in the intravesicular water space, and "Ca++ in vesicles" (right side of equations) was measured in the presence of 157 mm sodium by the filtration and washing procedure. The rationale for the coefficient of 5 in the left side of Equation 3 was that the concentration of the Ca-EGTA complex was for practical purposes five times greater in 5 mM EGTA than in 1 mM EGTA. The equations were solved for the two unknowns and "X" (nmol/mg) was converted to volume of distribution for the Ca-EGTA complex (i.e., by Equation 1; denominator equal to concentration of Ca-EGTA complex in assay medium). The results showed that the volume of distribution for Ca-EGTA did not change over a range of free calcium from 0.0079 to 5 µM (Fig. 5B).

The fact that constant solutions could be obtained for "X" was consistent with the assumptions inherent in Equations 2 and 3: (1) that intravesicular Ca-EGTA was directly proportional to the concentration of Ca-EGTA in the assay medium, and (2) that "Y" was the same in the presence of 1 and 5 mM EGTA. It should be noted that the latter holds only for binding measured in 157 mM sodium. Binding in 157 mM potassium was different for the two concentrations of EGTA which equated to an effect of EGTA on the sodium-sensitive component of calcium binding (see below).

Whereas the volume of distribution for Ca-EGTA was small and constant, the concentration of Ca-EGTA in the assay media was large compared to free calcium, and increased as free calcium was raised. Solution of Equations 2 and 3 for "X" showed that Ca-EGTA contributed to 81% of total calcium "bound" in 157 mM sodium and (assuming that intravesicular Ca-EGTA was the same for the sodium and potassium media; see below) from 9 to 24% of total calcium "bound" in 157 mM potassium (Fig. 5A) over the range of 0.0079 to 5 µM free calcium.

Subtraction of calcium bound in 157 mM sodium from calcium bound in 157 mM potassium, between 0.0038 and 50 µM free calcium in 1 mM EGTA, revealed a sodium-sensitive component that saturated at higher calcium and that was half-maximally saturated between 0.01 and 0.1 µM free calcium (inset, Fig. 5A). Scatchard plots, carried out for free calcium up to 10 µM, were generally consistent with the presence of a single species of sodium-sensitive calcium-binding sites with an apparent dissociation constant, Kd, of 0.052 µM and maximal binding of 6.73 nmol/mg (Fig. 6). A similar Kd (0.044 µM) was obtained in the presence of 5 mM EGTA, but maximum binding was somewhat reduced. The latter suggests that EGTA reduces the number of binding sites. In the presence of 3 µM magnesium and 1 mM EGTA, the Kd was 0.205 µM and maximal binding was 9.03 nmol/mg (Fig. 6).

Sodium at 1 mM had no apparent effect on calcium binding to the sodium-sensitive sites in the presence of 1 mM EGTA, 0.01-10 µM free calcium, and 3 mM magnesium, but 2-10 mM sodium caused a progressive inhibition of binding (Fig. 7). These effects were further analyzed by Scatchard plots which revealed

![Figure 6. Scatchard plots for the sodium-sensitive component of calcium binding (i.e., difference in binding measured in the presence of 157 mM potassium and 157 mM sodium). Experiments were carried out as described in the legend for Figure 5A for free calcium concentrations of 0.01-10 µM and EGTA and magnesium concentrations as shown. For each membrane preparation, the data were converted to percentage of the binding observed in 157 mM KCl, 10 mM HEPES, pH 7.1, 1 mM EGTA, and 5.0 µM calcium with 45Ca. The mean value for this condition was 10.48 ± 0.48 nmol/mg for 17 preparations, which was used to convert the mean values (n = 3) for sodium-sensitive calcium binding (in percentages) back to units of bound calcium. This was done to minimize the effect of variation between preparations. Least squares analysis was used to obtain values for the lines drawn through the data and for the dissociation constant, Kd, and maximal number of sites, Bmax (inset).](http://circres.ahajournals.org/content/suppl/1984/11/5/684.DC1.html)
that 2–5 mM sodium caused a progressive increase in the $K_d$ and decrease in maximal binding (inset, Fig. 7).

**Discussion**

This study demonstrated that sealed vesicles in a sarcolemma-enriched preparation from canine ventricle appear to have an enormous ability to concentrate calcium in the absence of a concentration gradient for calcium across the membrane. This ability can best be appreciated by examining the sodium-sensitive component of calcium binding. This component was obtained by subtracting calcium binding in 157 mM potassium from binding in 157 mM sodium. If the intravesicular concentrations of Ca-EGTA and free calcium were the same for the potassium and sodium media, then the subtraction eliminated any contribution of intravesicular Ca-EGTA and free calcium to sodium-sensitive calcium binding. Whereas it was not possible to measure intravesicular Ca-EGTA and free calcium for the sodium and potassium media, Schilling et al. (1984) [using the Van Alstyne et al. (1980) preparation] did find that the volume of distribution for chloride was the same for 150 mM NaCl and 150 mM KCl at 5°C. Thus, it can be concluded that the intravesicular water space was the same for vesicles suspended in the sodium and potassium media. Over the range of 0.00792 to 5.0 $\mu$M free calcium, sodium-sensitive calcium binding ranged from 0.87 to 7.36 nmol/mg (Fig. 5). If one takes the volume of distribution for intravesicular chloride (7.5 $\mu$l/mg; Schilling et al., 1984) as the intravesicular water space, free calcium in the vesicles ranged from $5.94 \times 10^{-5}$ to $3.75 \times 10^{-1}$ nmol/mg over the range of free calcium in the assay media. Dividing the latter into the values for sodium-sensitive calcium binding showed that the sodium-sensitive binding sites concentrated calcium by 14,646–to 196-fold over the range of 0.00792 to 5.0 $\mu$M free calcium. The concentrating power of the preparation was observed under several conditions thought to mimic those existing in the cardiac cell: (1) ionic strength (167–170 mM), (2) pH 7.1, and (3) 3 mM magnesium.

The results from the experiment, comparing binding by the centrifugation technique to that by the filtration/wash technique, suggest that the sodium-sensitive calcium-binding sites were associated either with a constituent trapped in the vesicles or with the internal surface of the sarcolemma. We did not eliminate either of these possibilities, but can state that treatment of the preparation with five freeze-thaw cycles eliminated the sodium-sensitive component of calcium binding (unpublished). Since freeze-thaw cycles at physiological ionic strength appear to render the vesicles transiently leaky (Schilling and Lindenmayer, 1984), it was possible that a soluble constituent or a constituent loosely associated with the internal vesicular membrane surface escaped from the vesicles during this treatment.

Although the preparation employed is highly enriched with putative sarcolemma markers (Van Alstyne et al., 1980), it is not pure sarcolemma. Thus, it remains possible that the sites were associated with membrane contaminants from the myocardial cell or from other cell types in ventricular tissue (e.g., nerve endings, endothelial cells). It is also possible that the sites were on the sarcolemma and other membrane systems of the myocardial cell (e.g.,
sarcoplasmic reticulum, mitochondria) and/or existed in soluble form in the cytoplasm. Other investigators have ascribed calcium-binding sites on sarcolemma to various membrane constituents or combination of constituents including protein, phospholipid, lipoprotein, and sialic acid residues (Feldman and Weinhold, 1977; Lüllmann and Peters, 1977; Limas, 1977; Philipson et al., 1980b; Matsukubo et al., 1981; Burt and Langer, 1983). The number of sodium-sensitive calcium-binding sites reported here was high (i.e., nmol/mg vs. pmol/mg), which suggests that the sites were phospholipids or sialic acid residues. However, the high selectivity of the sites for calcium over magnesium and for sodium over potassium suggests that the sites were either on proteins or associated with proteins which render the selectivity observed.

Sodium-sensitive calcium binding appeared to involve a single class of sites which saturated at approximately 10 \( \mu \text{M} \) free calcium in the absence of magnesium. Calcium binding to these sites occurred over a range of free calcium that is thought to exist in the myocardial cell during diastole and systole (Solaro et al., 1974; Sheu and Fozzard, 1982; Lado et al., 1982; Fabiato, 1983). Calcium binding was inhibited by sodium at concentrations ranging from 2 to 10 mM, and the inhibition appeared to reflect a decrease in both the maximal number of calcium-binding sites and the apparent affinity of calcium for these sites. Thus, sodium-sensitive calcium binding was inhibited by sodium over the lower part of the range estimated for bulk intracellular sodium activity (Lee and Fozzard, 1975; Ellis, 1977; Eisner et al., 1981). The affinity of the sodium-sensitive sites for calcium and their sensitivity to sodium were such that these sites might be expected to participate in the regulation of intracellular free calcium. However, the affinities for calcium and sodium observed at 5°C will differ to some extent from those which exist in situ (at 37°C). This difference arises in part because of the temperature dependence of equilibrium constants for chemical reactions. As stated above, Will et al. (1976) reported a species of sodium-sensitive calcium-binding sites in a partially purified Na\(^{+}\),K\(^{+}\)-ATPase preparation from porcine myocardium. The affinity of these sites for calcium and their sensitivity to sodium, measured at 20°C and pH 6.8, were essentially identical to those reported here. A major difference, however, is that the number of high-affinity, sodium-sensitive binding sites was 20 to 30 times greater for the sarcolemma preparation employed in the present study. This may be due in part to the higher temperature used by Will et al. (1976), but also could possibly have resulted from the use of high salt and detergent to obtain the Na\(^{+}\),K\(^{+}\)-ATPase preparation.

We were unable to conduct binding experiments at 37°C because of the lability of the binding sites at higher temperatures. There are a number of possibilities which could account for the lability. First, the ordering of lipids (i.e., tighter packing) at lower temperatures might be expected to have resulted in a change in affinity for calcium if the binding sites were closely associated with or influenced by the lipid domain of the membrane. This explanation, however, seemed inadequate to explain the irreversibility of the temperature change. Second, the activity of proteases, phosphatases, or lipases present in the sarcolemma preparation would have increased at the higher temperatures. Third, calcium-binding sites, loosely associated with the internal surface of the membrane at lower temperatures, could have dissociated from the membrane surface at higher temperatures in a way that decreased the affinity or capacity for calcium. Since these sites are labile at higher temperatures, it also seemed likely that a portion of the sodium-sensitive calcium-binding sites were lost between removal of the heart and homogenization of the tissue. Furthermore, exposure of the preparation to EGTA may have reduced the number of binding sites (Fig. 6). Thus, the capacity of these sites reported above may have been greater in situ.

If one assumes that the sites were derived from the myocardial cell and have fairly similar affinities and capacity for calcium and sodium at 5°C and 37°C, the sensitivity of the sites to calcium and sodium suggest that they could participate in myocardial cell function in several ways. They may serve as a sodium-sensitive buffer of free intracellular calcium. If so, the sensitivity to sodium distinguishes these sites from the other major calcium buffering components which have been postulated to influence intracellular free calcium (Fabiato, 1982). If hydrogen ions compete with calcium for the sites, changes in free calcium or sodium around the sites could help explain the influence of sodium and calcium on intracellular pH (Vaughan-Jones et al., 1983). Interest in these sites becomes even greater if, in fact, they are located on the internal surface of the sarcolemma. Swings in the concentration of free sodium would be expected to be greater at the internal surface than in "bulk" cytoplasm. This is so since a concentration gradient exists across the sarcolemma, and it is through channels and pumps in this membrane that sodium, at least for the most part, is believed to enter and leave the cell. Such a cyclic change in sodium concentration in the vicinity of the sodium-sensitive calcium-binding sites might result in a release followed by a rebinding of calcium as sodium is pulsed in and pumped out (Akera and Brody, 1978). Finally, binding sites with high affinities for calcium and sodium on the internal surface of the membrane might modify the fluxes of various ligands across the sarcolemma. For example, elevations in intracellular sodium and, perhaps calcium, as a consequence, have been reported to enhance the slow inward current (Marban and Tsien, 1982; Lederer and Eisner, 1982). It is reasonable to assume that sodium and/or calcium elicit this effect by in-
tering with constituents on the internal surface of the sarcolemma. Thus, the sensitivity of these sites to sodium, coupled with their high affinity for calcium, make them likely candidates for a role in the excitation-contraction-relaxation sequence of the myocardial cell.

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