Intact Vesicles of Canine Cardiac Sarcolemma

Evidence from Vectorial Properties of \( \text{Na}^+\text{,K}^+\text{-ATPase} \)

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SUMMARY Most biological membranes are functionally asymmetric. To study biochemical control of cardiac transsarcolemmal ion fluxes, it would be of obvious advantage to use isolated vesicles of sarcolemma which retains the low passive permeability characteristics of intact sarcolemma because in such vesicles the membrane should exhibit its normal asymmetric character with respect to enzymic activities. The purpose of this investigation was to attempt to identify such vesicles in a cardiac microsomal (membrane vesicular) preparation. We studied activation by \( \text{Na}^+ \) and \( \text{K}^+ \) of \( \text{Na}^+\text{,K}^+\text{-ATPase} \) and its associated \( \text{K}^+\text{-phosphatase} \) activities, using as substrates ATP or \( \text{p-nitrophenylphosphatase} \) (pNPP) in the presence of \( \text{Mg}^{2+} \). Optimal concentrations of \( \text{K}^+ \) alone (10 mM) stimulated \( \text{p-nitrophenylphosphatase} \) (pNPPase) activity 1.8-fold, and over 80% of the increase could be inhibited by ouabain. Optimal \( \text{Na}^+ \) plus \( \text{K}^+ \) concentrations (100 mM and 10 mM, respectively) stimulated the rate of ATP hydrolysis 2 fold, but only 11 ± 1.1% of the increased activity was ouabain-sensitive. Optimal pretreatment with sodium dodecyl sulfate (SDS) (0.3 mg/ml) rendered both activities completely sensitive to inhibition by ouabain and reduced the basal \( \text{Mg}^{2+}\text{-ATPase} \) activity by 70–90%. The \( \text{K}^+\text{-stimulated} \) pNPPase activity doubled after preincubation in SDS, but the ATPase activity stimulated by \( \text{Na}^+ \) plus \( \text{K}^+ \) fell by 50% under these conditions. A similar pattern of apparent activation was produced by preincubation with deoxycholate (DOC), except that basal \( \text{Mg}^{2+}\text{-dependent activities} \) were resistant to destruction by this detergent. The incremental responses to activation by ions and substrates, and inhibition by ouabain, are consistent with the hypothesis that permeability-intact vesicles of sarcolemma are present in the isolated preparation, and that detergent activation renders the vesicles highly permeable to the ions, substrates, and ouabain.

BIOCHEMICAL studies of the regulation of intracellular ion activities usually have involved two dissimilar approaches. For \( \text{Ca}^{2+} \) ions, for example, many investigators have used relatively purified vesicles of sarcoplasmic reticulum in studies to determine the physiological role of intact sarcoplasmic reticulum within muscle. Alternatively, others have recently followed the more traditional biochemical approach of first purifying the individual protein components of the sarcoplasmic reticulum and separately analyzing the properties of each. To achieve complete understanding of sarcoplasmic reticular function, both approaches are necessary, and are complementary.

Studies of the transsarcolemmal regulation of intracellular \( \text{Na}^+ \) and \( \text{K}^+ \) ion activities have relied principally on the latter approach and have used relatively highly purified preparations of \( \text{Na}^+\text{,K}^+\text{-ATPase} \) (EC 3.6.1.3) from cardiac and other tissues. The work to be described in the present paper represents an attempt to initiate studies of the former kind, namely, isolation of vesicles of sarcolemma, the membrane permeability of which may resemble that of intact sarcolemma. For convenience of discussion, we term these intact vesicles. Several procedures have recently been described for the partial purification of sarcolemma from myocardium. Subcellular fractions prepared by these methods yield two general morphological forms: large membrane sheets (broken cell ghosts) and small membrane vesicles. It seems logical to expect that enzymic activities of the same membrane in the two morphological forms may differ because of differences in the accessibility of activators, substrates, and inhibitors to their sites on the enzymes. With intact vesicles of sarcoplasmic reticulum, for example, \( \text{Ca}^{2+}\text{-stimulated ATPase activity is low and Ca}^{2+} \) uptake is high compared to that found after treatments which render the vesicles leaky to calcium ions. By analogy, \( \text{Na}^+\text{,K}^+\text{-ATPase activity of intact vesicles of sarcolemma might be expected to be low compared to that found after treatments with chaotropic agents such as high salt concentrations or with various detergents. Judiciously applied, such treatments have in fact been used by several investigators in attempts to purify \( \text{Na}^+\text{,K}^+\text{-ATPase}, and such treatments have often been said to unmask latent Na}^+\text{,K}^+\text{-ATPase activity.} \) It occurred to us that such unmasking may reflect simple destruction of sufficient membrane integrity to render sarcolemmal vesicles passively permeable (leaky) to \( \text{Na}^+, \text{ATP}, \) and ouabain.

Isolated preparations of cardiac sarcoplasmic reticulum must retain the relatively low passive \( \text{Ca}^{2+}\)-permeability of intact sarcoplasmic reticulum, in order to exhibit high \( \text{Ca}^{2+}\)-binding and uptake activities. Since we wished to preserve membrane integrity, we began with the method for preparation of cardiac sarcoplasmic reticulum devised by Harigaya and Schwartz to prepare membrane vesicles because this preparation exhibits the highest \( \text{Ca}^{2+}\)-binding activity yet published. If the sarcolemmal vesicles formed during homogenization are not substantially different in size from sarcoplasmic reticular vesicles formed at the same
time, many of the former should sediment with the latter and they might retain the relatively low, passive permeability to Na⁺, ATP, and ouabain characteristic of intact sarcolemma. Such intact sarcolemmal vesicles would not be expected to exhibit high Na⁺,K⁺-ATPase activity, even if they were present in substantial quantity. However, their presence could be revealed by judicious treatments designed to render them leaky, without substantially altering the functional enzymic activity.

The present paper describes results strongly suggesting the presence of intact sarcolemmal vesicles in a subcellular membrane fraction prepared from canine myocardium. We have used the known vectorial properties of the putatively sarcolemmal enzyme Na⁺,K⁺-ATPase (and its associated K⁺-phosphatase activity) to demonstrate the presence of sarcolemma. In addition, our results suggest the presence of both right-side-out and inside-out intact sarcolemmal vesicles. The preparation containing these activities has been referred to in the literature as membrane vesicles, cardiac relaxing factor, sarcoplasmic reticulum fragments, and H₂O-microsomes. We prefer the noncommittal term membrane vesicles. In describing the orientation of the membrane comprising the vesicles, the abbreviations RO and IO will be used to denote right-side-out and inside-out vesicles, respectively. Such sidedness is referenced to the intact cell.

Methods

The plan of the experiments was to compare Na⁺,K⁺-ATPase and K⁺-phosphatase activities of intact membrane vesicles with those of the vesicles treated in such a way as to increase membrane permeability. The difference in activity, if any, should reflect sidedness of the membrane comprising the vesicles in the pattern of stimulation by activators, and inhibition by the cardiac glycoside ouabain.

PREPARATION OF MEMBRANE VESICLES

Membrane vesicles were prepared from dog hearts homogenized in dilute bicarbonate buffer by the method of Harigaya and Schwartz as previously described. The hearts from two mongrel dogs (10-15 kg) were quickly removed and placed in ice-cold isotonic saline after the dogs had been anesthetized with methohexital (10 mg/kg) given intravenously. The epicardium, endocardium, and fatty tissue were stripped away, and the ventricles (≈150-200 g) were then suspended in 4 vol of distilled water containing 10 mM sodium bicarbonate plus 5 mM sodium azide and homogenized with a Polytron PT-20 (Brinkman Instruments) for three 5-second periods at half-maximal speed. The homogenate (600-800 ml) was next centrifuged at 11,600 g (max) for 20 minutes in a JA-14 rotor using a Beckman model J-21B preparative centrifuge. The supernatant fraction was collected and recentrifuged at 14,300 g (max) for 20 minutes in a JA-14 rotor. The second supernatant fraction was then centrifuged at 43,600 g (max) for 30 minutes in a JA-20 rotor and the pellets were resuspended in 20 mM imidazole-maleic buffer (pH 6.8) containing 0.6 M KCl. This suspension was resedimented at 43,600 g (max) for 30 minutes and the resulting pellet was resuspended in 0.2 M sucrose, 30 mM DL-histidine buffer (pH 7.5) to give a final protein concentration of 8-10 mg/ml. The final yield of protein was about 0.15 mg/g wet weight of heart. Samples used in calcium-binding experiments were assayed within 5 hours after preparation. The remainder of the preparation was frozen at -20°C in small samples and used for the various enzyme assays within 1-3 weeks.

ENZYME ASSAYS AND PROTEIN CONTENT

All enzyme assays were performed with samples which had been frozen and thawed no more than once. The activities were stable for periods up to 3 weeks after freezing, and all reactions were linear up to 40 minutes after the onset of incubation. Generally, 15-25 μg of vesicle protein was assayed in 1 ml of reaction mixture. ATPase activity was measured by recording the rate of inorganic phosphorus (P₃) release from ATP. Incubation periods were 15-30 minutes. The P₃ was determined by a modified Fiske-Subbarow assay, using p-methylaminophenol sulfate (Elon) to stabilize the color. Basal Mg²⁺-ATPase activity was defined as the rate of P₃ release at 37°C in basal buffer consisting of 3 mM MgCl₂, 50 mM DL-histidine (pH 7.5), and 3 mM tris(hydroxymethyl)aminomethane (Tris) ATP. The azide-sensitive ATPase activity was the difference between basal activity and that in the presence of 5.0 mM NaN₃. The Ca²⁺-ATPase was measured by subtracting the activity in the basal buffer supplemented with 0.2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) from that in the basal buffer to which 0.1 mM CaCl₂ had been added. The Na⁺,K⁺-ATPase activity was defined as the difference between the basal activity and that in the presence of 100 mM NaCl plus 10 mM KCl. Ouabain sensitivity of the Na⁺,K⁺-ATPase activity was measured in the presence of 1.0 mM ouabain.

K⁺-stimulated p-nitrophenylphosphatase (K⁺-pNPPase) was measured as described by Skou. The rate of hydrolysis of 3 mM Tris p-nitrophenolphosphate (pNPP) was measured at 37°C in the presence of 50 mM Tris-HCl (pH 7.8) and 3 mM MgCl₂ plus varying concentrations of KCl. Sensitivity to ouabain was measured in the presence of 1.0 mM ouabain and 10 mM KCl. The reactions were stopped by addition of 0.1 ml of 50% trichloroacetic acid to 1.0 ml of reaction mixture, and the color was developed by the addition of 2.0 ml of 0.5 M Tris base. The production of p-nitrophenol (NP) was then measured by recording the absorbance at 410 nm.

Adenylate cyclase activity and calcium-binding activity were measured as described previously. Briefly, adenylate cyclase activity was determined by measuring cyclic AMP production from ATP with a competitive protein-binding assay using binding protein derived from beef adrenal glands. Calcium binding was measured with a dual beam spectrophotometric procedure utilizing the Ca²⁺-binding dyes, murexide or arsenazo III.

For experiments in which membrane vesicles were treated with detergents, the method of Jørgensen was followed. Freshly thawed vesicles were diluted 1:10 in imidazole-HCl buffer (pH 7.1) containing varying concentrations of sodium dodecyl sulfate (SDS) or sodium deoxycholic acid (DOC). After preincubation for 20 minutes at room temperature, 25 μl (≈25 μg of protein) of the diluted suspension was added to previously prepared reaction tubes containing 1 ml of
incubation medium. This resulted in a 1:40 dilution of the original concentrations of detergents. Dilution was essential because no activity was observed when the membranes were assayed in incubation buffer containing the original concentration of detergent. For experiments with detergents, Na⁺,K⁺-ATPase and K⁺-activated phosphatase activity were determined as described above except that in some cases 130 mM NaCl and 20 mM KCl were used to measure Na⁺ plus K⁺ stimulation of ATPase activity. No apparent difference in stimulation was seen at this slightly different concentration of monovalent cations. Assays were carried out in triplicate or quadruplicate. Control tubes were run in parallel with each assay.

For experiments in which the recovery of enzymic activity was assessed, after preincubation in imidazole-HCl buffer with or without SDS (0.3 mg/ml), samples were centrifuged for 60 minutes at 108,000 g (max) in a Beckman model L centrifuge using a 40 rotor. Protein and enzyme assays were immediately performed on the resuspended particulate and the soluble fractions. A freshly thawed control sample not subjected to recentrifugation was analyzed concurrently.

The protein content of the preparations was estimated by the procedure of Lowry et al.³³

**REAGENTS**

DL-Histidine, Tris-ATP, L-isoproterenol HCl, ouabain, murexide, Tris-pNPP, and Elon were purchased from Sigma; SDS was purchased from BioRad; DOC from Pfaltz and Bauer; and arsenazo III from Aldrich. All other reagents were of the highest commercial grade available.

**Results**

**INTACT MEMBRANE VESICLES**

**Calcium Binding and Adenylate Cyclase Activity**

Calcium binding was routinely monitored for freshly prepared membrane vesicles and was found to range consistently between 50 and 80 nmol Ca⁺⁺/mg protein. This level of activity agrees well with our previous results¹⁵, ¹⁷, ²² and with the results of others,¹⁵, ¹⁷, ²² and suggests that this fraction is enriched in sarcoplasmic reticulum vesicles. Preincubation with detergent to increase membrane permeability (see below) completely prevented Ca⁺⁺ binding (data not shown).

The preparation also contained high levels of adenylate cyclase activity which was stimulated by isoproterenol (10⁻⁴ M) and by NaF (8 × 10⁻⁴ M). The average basal activity from four separate preparations was 99.6 ± 4.8 (X ± se) pmol/mg protein per min, with 48.0 ± 8.8% stimulation by isoproterenol and 535 ± 102% stimulation by NaF. Although not unequivocal, the adenylate cyclase activity suggests the presence of sarcolemma in this membrane fraction.

**ATPase Activities**

Various ATPase activities were measured for the membrane vesicles in an attempt to partially identify the subcellular particles in this fraction. Azide-sensitive ATPase, Ca⁺⁺-ATPase, and Na⁺,K⁺-ATPase were used as enzyme markers for the presence of mitochondria, sarcoplasmic reticulum, and sarcolemmal vesicles, respectively.¹⁹

The results from two representative preparations are shown in Table 1. The data confirm that the membrane vesicles are a heterogeneous mixture of cell membranes.¹⁰ Basal Mg⁺⁺-ATPase was inhibited by slightly more than 40% by 5.0 mM NaNO₃, which suggests the presence of mitochondria in this fraction. The specific activity of the Ca⁺⁺-ATPase was 5–10 μmol P₄/mg protein per hour, and mitochondrial ATPase activity was of the same order of magnitude. The presence of a Ca⁺⁺-stimulated ATPase in the membrane vesicle is consistent with the Ca⁺⁺-binding studies above. The basal Mg⁺⁺-ATPase activity of 15–25 μmol P₄/mg protein per hour was approximately doubled by optimal concentrations of Na⁺ (100 mM) plus K⁺ (10 mM) which are commonly used to measure ouabain-sensitive Na⁺,K⁺-ATPase activity. Thus, all of the ATPase activities measured in the membrane vesicles, the activity stimulated by Na⁺ plus K⁺ was the highest. This latter result suggests the presence of a substantial amount of sarcosomal vesicles in this subcellular fraction.

It may be noted, however, that the increment of activity produced by optimal concentrations of Na⁺ plus K⁺ was inhibited by only 11 ± 1.1% (X ± se; n = four separate determinations from two preparations) by 1.0 mM ouabain, a concentration which is several orders of magnitude higher than that typically used to inhibit this enzyme completely in other preparations.¹¹ Thus, if the amount of Na⁺,K⁺-ATPase activity is defined from the degree of inhibition by ouabain (as is most often the case in the literature), the value is 1.8–2.2 μmol P₄/mg protein per hour. This is consistent with the low values previously reported for this membrane fraction,¹¹, ²³, ²⁴ On the other hand, the amount of optimal

**Table 1 Mitochondrial, Ca⁺⁺-, and Na⁺,K⁺-ATPase Activities in Membrane Vesicles**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Prep A</th>
<th>Prep B</th>
<th>Specific ATPase</th>
<th>Prep A</th>
<th>Prep B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg⁺⁺</td>
<td>16.4 ± 0.12</td>
<td>24.6 ± 0.37</td>
<td>Mitochondrial</td>
<td>6.9</td>
<td>11.0</td>
</tr>
<tr>
<td>+ NaNO₃</td>
<td>9.46 ± 0.10</td>
<td>13.6 ± 0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ EGTA</td>
<td>14.4 ± 0.09</td>
<td>20.1 ± 0.13</td>
<td>Ca⁺⁺-ATPase</td>
<td>5.1</td>
<td>9.0</td>
</tr>
<tr>
<td>+ CaCl₂</td>
<td>19.5 ± 0.15</td>
<td>29.1 ± 0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Na⁺,K⁺</td>
<td>32.1 ± 0.29</td>
<td>46.0 ± 0.46</td>
<td>Na⁺,K⁺-ATPase</td>
<td>15.7</td>
<td>21.4</td>
</tr>
<tr>
<td>+ Na⁺,K⁺</td>
<td>30.3 ± 0.24</td>
<td>43.8 ± 0.31</td>
<td>Ouabain-sensitivity</td>
<td>1.8</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Results from two typical preparations, expressed as μmol P₄ liberated/mg protein per hour (X ± se, n = three determinations). The concentration of the various agents added and the determination of the specific ATPase activities are described in Methods. All activities were determined at 37°C.
INTACT SARCOLEMMLAL VESICLES/Besch et al.

**K⁺-p-nitrophenylphosphatase (K⁺-pNPPase) activity in membrane vesicles.** Activities were obtained at various concentrations of KCl (○) or NaCl (△), which were added to the basal buffer containing Mg²⁺ (dashed line). Sensitivity to ouabain in 10 mM KCl (△) is the averaged results of two separate preparations (X ± SE, n = five determinations). NP = p-nitrophenol.

**Figure 1**

**DETERGENT-TREATED MEMBRANE VESICLES**

**SDS Enhancement of Ouabain-Sensitivity of Na⁺,K⁺-ATPase and K⁺-pNPPase Activities**

Sensitivity to ouabain of that portion of ATPase activity which was stimulated by Na⁺ plus K⁺ was measured in membrane vesicles after they had been preincubated in SDS (Fig. 2). With optimal concentrations of SDS, ouabain-sensitivity increased about 6-fold over control. For the preparation illustrated in Figure 2, the activity increased from a low of 1.8 μmol P₁/mg protein per hour in the absence of SDS to a maximum of 10.5 μmol P₁/mg protein per hour after preincubation at an SDS concentration of 0.3 mg/ml. Na⁺ plus K⁺ stimulation of ATPase activity was slightly increased, then progressively inhibited with increasing concent-

**Figure 2**

Effect of preincubation in sodium dodecyl sulfate (SDS) on ATPase activities in membrane vesicles. Basal Mg²⁺ activity (○), stimulation of basal activity by Na⁺ plus K⁺ (△), and ouabain-sensitive stimulation of activity (□) were calculated from results with duplicate samples.
Sodium dodecyl sulfate (SDS)-induced changes in ouabain-sensitivity of Na\(^+\) plus K\(^+\) stimulation of ATPase activity. The data from several preparations were used to calculate the percent of Na\(^+\), K\(^+\) stimulation which was ouabain-sensitive and the percent of original Na\(^+\) plus K\(^+\)-stimulated activity which remained after pretreatment with SDS. The numbers in parentheses indicate the number of separate preparations. The values are expressed as the mean ± SE.

The ouabain-sensitivity of K\(^+\)-pNPPase activity was also increased by SDS preincubation. The pattern of activation of K\(^+\)-pNPPase, however, was somewhat different from that of Na\(^+\), K\(^+\)-ATPase. First, ouabain-sensitivity increased only about 2-fold, from 0.85 μmol NP/mg protein per hour in the absence of SDS preincubation to 1.8 μmol NP/mg protein per hour after preincubation in SDS at a concentration of 0.3 mg/ml. This difference in activation of K\(^+\)-pNPPase compared to Na\(^+\), K\(^+\)-ATPase suggests that more vesicles are oriented inside-out than right-side-out. Second, whereas SDS preincubation decreased retention of Na\(^+\) plus K\(^+\) stimulation of ATPase activity, it increased K\(^+\) stimulation of pNPPase activity at all concentrations used. Increasing K\(^+\) stimulation thus parallels increasing ouabain-sensitivity. Basal Mg\(^2+\)-pNPPase activity was found to be inhibited by SDS preincubation, much as basal Mg\(^2+\)-ATPase activity was. For example, the activity was reduced about 70% by SDS preincubation at a concentration of 0.3 mg/ml, analogous to the degree of inhibition of basal Mg\(^2+\)-ATPase activity observed after a similar SDS preincubation (Fig. 2). In some cases, after detergent treatment, ouabain-sensitive activity measured with either substrate slightly exceeded cation-stimulated activity. This is commonly observed in Na\(^+\), K\(^+\)-ATPase preparations and is explained by the presence of contaminating monovalent cations which slightly stimulate the basal activity.

DOC Enhancement of Ouabain-Sensitivity of Na\(^+\), K\(^+\)-ATPase and K\(^+\)-pNPPase Activities

The effects of DOC pretreatment on Na\(^+\), K\(^+\)-ATPase and K\(^+\)-pNPPase activities of membrane vesicles were similar to those of SDS pretreatment. Na\(^+\) plus K\(^+\) stimulation of ATPase activity became progressively more ouabain-sensitive with increasing concentrations of DOC. The ouabain-sensitive activity was maximally stimulated 4.3-fold after preincubation in DOC at a concentration of 1.4 mg/ml (Fig. 5). The effects of DOC on K\(^+\)-pNPPase activity were nearly identical to those obtained with SDS (Fig. 6). However, DOC preincubation did not inhibit basal Mg\(^2+\) hydrolytic activity obtained with either substrate to the degree observed after SDS preincubation. After treatment with a DOC concentration of 1.4 mg/ml, Na\(^+\) plus K\(^+\) stimulation of ATPase activity was ouabain-sensitive, but basal Mg\(^2+\) activity was reduced by only 20% and 3%, using ATP and pNPP as the substrates, respectively. Therefore, contrary to previous suggestions, detergent activation of ouabain-sensitive activities apparently does not necessarily involve interconversion of ouabain-insensitive Mg\(^2+\) activities to ouabain-sensitive, monovalent cation-stimulated activities. A similar conclusion has been reached by others. It is interesting that the net effect of treatment with SDS (but not DOC) is similar to that obtained by...
INTACT SARCOLEMNAL VESICLES/Besch et al.

Figure 5: Effect of preincubation in deoxycholate (DOC) on ATPase activities in membrane vesicles. Shown are basal Mg²⁺ activity (•), stimulation of ATPase activity by Na⁺ plus K⁺ (Δ), and ouabain-sensitivity of the activity (O). The inset shows the percent of the stimulation by Na⁺ plus K⁺ which was inhibitable by ouabain.

Careful Na⁺ treatments, in that the resultant activities in the presence of Mg²⁺ alone are preferentially inactivated, whereas the ouabain-sensitive activity is increased.

Effect of Detergent Pretreatment on Stimulation of ATPase Activities by Na⁺ plus K⁺

If intact IO sarcolemmal vesicles are the source of the ouabain-insensitive, monovalent cation-stimulated ATPase activity found in the membrane vesicles, Na⁺ ions alone added to the medium should stimulate the ATPase activity of these vesicles, because in the intact cell the inner membrane surface contains the site for activation by this ion. Activation by Na⁺ alone would require the presence of K⁺ ions inside the intact IO vesicles, a condition which should have been obtained, because the final step in the preparation of the vesicles involves sedimentation from 600 mM KCl and because sarcolemmal permeability to potassium is relatively high. Table 2 shows the monovalent cation requirements of the ATPase activity before and after preincubation in an optimal concentration of SDS. Before SDS preincubation, Na⁺ ions alone were found to be adequate for stimulation of nearly all of the realized activity. Only a small additional increment of activity (12%) was obtained by the addition of K⁺ ions in the presence of optimal Na⁺ ion concentrations, suggesting the presence of a small amount of leaky vesicles. The increased activity was completely ouabain-sensitive. After optimal preincubation in SDS, however, K⁺ ions stimulated nearly 4-fold that activity which was obtained in the presence of Na⁺ ions alone, and all of the activity stimulated by either ion was completely inhibitable by ouabain. Thus, after treatment with SDS, the preparation demonstrated the known dependence on both Na⁺ and K⁺ ions for optimal stimulation of ATPase activity, and at the same time this ATPase activity became 100% inhibitable by ouabain. Table 3 shows similar results obtained after DOC pretreatment. With pretreat-

Table 2: Effect of Pretreatment with Sodium Dodecyl Sulfate (SDS) on Sodium and Potassium Stimulation of ATPase Activity

<table>
<thead>
<tr>
<th>SDS (mg/ml)</th>
<th>Mg</th>
<th>MgNa</th>
<th>MgNaK</th>
<th>Na⁺ stimulation (A)</th>
<th>Na⁺K⁺ stimulation (B)</th>
<th>% (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>23.4 ± 0.20</td>
<td>38.0 ± 0.19</td>
<td>39.7 ± 0.50</td>
<td>14.6</td>
<td>16.3</td>
<td>112</td>
</tr>
<tr>
<td>+ ouabain (1 mM)</td>
<td>37.7 ± 0.35</td>
<td>38.2 ± 0.32</td>
<td>14.3</td>
<td>14.8</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>5.52 ± 0.10</td>
<td>7.97 ± 0.05</td>
<td>14.8 ± 0.10</td>
<td>2.45</td>
<td>9.3</td>
<td>379</td>
</tr>
<tr>
<td>+ ouabain (1 mM)</td>
<td>5.59 ± 0.15</td>
<td>5.15 ± 0.15</td>
<td>0.07</td>
<td>-0.37</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Results from a typical preparation of membrane vesicles preincubated in the presence and absence of SDS as described in Methods. The assays were conducted in the presence of NaCl alone (130 mM) or NaCl (130 mM) plus KCl (20 mM). Activities are expressed as μmol P/mg protein per hour (X ± se, n = four determinations).
ment with the latter detergent, however, the increment in activity produced by addition of K⁺ ions was somewhat less than that observed in membrane vesicles treated with SDS.

Recovery of Particulate Enzyme Activity after Pretreatment of Vesicles in SDS

Pretreatment of the vesicles with SDS at a concentration of 0.3 mg/ml released to the soluble fraction approximately 50% of the total protein of the membrane vesicles (Table 4). In contrast, all enzyme activity was retained in the particulate fraction, suggesting that integral membrane proteins were not solubilized with this concentration of SDS. The final ouabain-sensitive ATPase activity in the pellet was 19.2 μmol Pj/mg protein per hour (12-fold stimulation over control). Treatment with SDS thus expediently produced a Na⁺,K⁺-ATPase preparation with activities which rival or exceed those of relatively purified Na⁺,K⁺-ATPase prepared from heart by far more elaborate techniques. The final ouabain-sensitive ATPase activity was 3.66 μmol NP/mg protein per hour (≈4-fold increase over control activity). A second putative sarcolemmal integral enzyme, 5'-nucleotidase, was also retained entirely in the particulate fraction (data not shown).

Discussion

In the present studies, ATPase activity stimulated by sodium plus potassium was identified in high yield in membrane vesicles prepared from dog heart homogenates. Potassium-stimulated pNPPase activity was also present, probably as a partial expression of Na⁺,K⁺-ATPase activity. As isolated, the membrane vesicle fraction contained compartments functionally inaccessible to ouabain, to Na⁺ and ATP and to K⁺ and pNPP. But after judicious pretreatment with detergents, sites for interaction of these agents were exposed. These results are consistent with the hypothesis that the apparent compartments are expressions of vesicles of relatively normal (low) membrane permeability, some of which are oriented right-side-out with respect to the intact cells while others are inside-out.

Although some of our findings could be accommodated by the notion that SDS selectively solubilizes inhibitors of the enzymic activities, the suggested hypothesis seems to be the simplest one consistent with the bulk of the data. Partial stimulation of substrate hydrolysis by individual monovalent cations is explained, as is the variable ratio between ATPase and phosphatase activities before activation. Insensitivity to ouabain before activation is accounted for. Latency of both Na⁺,K⁺-ATPase and K⁺-phosphatase activities is explicable by the hypothesis. That full activities after activation are equal to the sum of the estimated latent activities irrespective of the substrate used required only the single reasonable assumption of partial enzyme destruction during detergent activation (see Table 5). We have been unable to find alternative explanations of all the data without invoking additional assumptions.

That some Na⁺,K⁺-ATPase activity may be latent in subcellular fractions of mammalian tissues has been previously recognized. The specific activity of this enzyme (frequently defined on the basis of inhibition by ouabain rather than stimulation by Na⁺ plus K⁺) can be increased by aging, freezing, and thawing in a hypotonic medium or treatment with detergents or various chaotropic agents. These treatments have been said to activate or unmask latent activity and increase the full activity many-

<table>
<thead>
<tr>
<th>Sample</th>
<th>% protein in sediment</th>
<th>Stimulation (A)</th>
<th>Inhibition (B)</th>
<th>% (B/A)</th>
<th>Stimulation (A)</th>
<th>Inhibition (B)</th>
<th>% (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uncentrifuged)</td>
<td>—</td>
<td>26.1</td>
<td>1.6</td>
<td>6</td>
<td>1.24</td>
<td>0.94</td>
<td>76</td>
</tr>
<tr>
<td>(-) SDS</td>
<td>94</td>
<td>25.5</td>
<td>2.7</td>
<td>11</td>
<td>1.21</td>
<td>0.93</td>
<td>77</td>
</tr>
<tr>
<td>(+) SDS</td>
<td>54</td>
<td>21.3</td>
<td>19.2</td>
<td>90</td>
<td>3.66</td>
<td>3.66</td>
<td>100</td>
</tr>
</tbody>
</table>

pNPPase = p-nitrophenylphosphatase.

Results from a typical preparation, preincubated in imidazole buffer + SDS as described in Methods. Enzyme rates are expressed as μmol product/mg protein per hour; SD of replicates was less than 5% of the mean. No detectable enzyme activity remained in the soluble fraction. Monovalent cation-stimulated activity (A) and ouabain-inhibitable activity (B) were calculated as described in Methods. Similar results have been obtained from subsequent preparations.
TABLE 5  Estimation of Ratio of Inside-Out (IO) to Right-Side-Out (RO) Vesicles

<table>
<thead>
<tr>
<th>Activity (μmol Pi/mg protein/hr)</th>
<th>Manifest</th>
<th>Full</th>
<th>Latent</th>
<th>IO/RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>14.8‡</td>
<td>21.2</td>
<td>6.4</td>
<td>2.3</td>
</tr>
<tr>
<td>K⁺-pNPPase</td>
<td>0.851</td>
<td>3.60</td>
<td>2.75‡</td>
<td>3.2</td>
</tr>
</tbody>
</table>

p-NPPase = p-nitrophenylphosphatase.
* Full activities were calculated assuming 50% enzyme destruction by sodium dodecyl sulfate (SDS). If 46.5% destruction occurred, IO/RO calculated from either is 2.95. Data are comparable to those of Figures 2 and 3.
† Activity of IO vesicles.
‡ Activity of RO vesicles.

That such apparent activation may result from increased access of substrates has largely been ignored, though strong evidence in favor of this view has been published. Taken together, these studies provide compelling evidence supporting a relationship between latent enzymic activities and sealed membrane vesicles.

Because Na⁺,K⁺-ATPase is asymmetric with respect to activation and inhibition sites, an estimate of the relative proportions of RO and IO intact membrane vesicles can be made from the magnitudes of measured Na⁺,K⁺-ATPase and K⁺-pNPPase activities (Table 5). The measured (manifest) Na⁺,K⁺-ATPase activity of about 15 μmol Pi/mg protein per hour in our intact preparation implies a latent activity of about 2.5 μmol NP/mg protein per hour for the K⁺-stimulated pNPPase of IO vesicles (16.6%; also found for ratio of the two activities after detergent treatment in the present study). This is very close to the value of 2.75 μmol/mg protein per hour actually found. Similarly, the value of about 0.9 μmol NP/mg protein per hour for pNPPase activity in the intact vesicles suggest a latent activity of about 5.5 μmol Pi/mg protein per hour for the Na⁺ plus K⁺-stimulated ATPase of RO vesicles, again close to that actually found. These values suggest that the ratio of IO to RO vesicles in the intact membrane preparation is about 2.5. This ratio predicts that full pNPPase activity should be 3.5 μmol Pi/mg protein per hour, and that Na⁺,K⁺-ATPase activity should be 21 μmol Pi/mg protein per hour after detergent activation. When determined on SDS-pretreated vesicles, however, the K⁺-pNPPase activity measured was about 1.8 μmol Pi/mg protein per hour and that of Na⁺,K⁺-ATPase was about 10.6 μmol Pi/mg protein per hour. Destruction of a portion of the activities is indicated by the lower amount of Na⁺ plus K⁺ stimulation of ATPase in the vesicles after treatment with the detergents. If destruction of 50% is arbitrarily assumed, the results found correspond well with the above predictions. If the mean value (53.5%, Fig. 3) for retention of Na⁺ plus K⁺ stimulation after optimal SDS preincubation is used, the IO/RO ratio becomes 2.95, whether calculated exclusively from Na⁺ plus K⁺-stimulated ATPase activities or from K⁺-stimulated pNPPase activities. This analysis, presented graphically in Figure 7, provides an exact correspondence of predicted and measured activities, but it may nevertheless be only fortuitous. We have not included a possible contribution of leaky vesicles to our results. Neither have we accounted for the reported greater resistance of K⁺-phosphatase (over Na⁺,K⁺-ATPase) against destruction by detergents. Thus, our solution that intact IO sarcolemmal vesicles represent a large proportion of the total vesicles present is not exclusive; alternative explanations are possible, given additional assumptions. For example, latent membrane activities may arise from adherent portions of transverse tubular elements in our preparation. Nevertheless, the interpretation that this subcellular fraction contains intact vesicles of sarcolemmal membrane (regardless of orientation) is strongly supported. Purification and further characterization of the intact vesicles are in progress.

The interpretation that more IO than RO vesicles are present in the preparation explains an apparent discrepancy...
in our results, namely, that full activation of K+-phosphatase results in an increased rate of substrate hydrolysis (170% of control activity), whereas it produces a decreased rate of substrate hydrolysis by Na+,K+-ATPase (50–55% of control activity). If our analysis is correct, both expressions of activity are diminished by about half, but the latent K+-pNPPase of IO vesicles is sufficiently large (due to the greater number of IO vesicles present) that the destruction is obscured under conditions that allow both populations of vesicles to simultaneously contribute to the hydrolysis rate.

If K+-stimulated phosphatase activity is simply an alternate expression of Na+,K+-ATPase activity, the ratio of the two activities would be expected to remain constant under a variety of conditions. This has been found to be the case in several experiments in which Na+,K+-ATPase has been purified to high specific activities. In general, K+-phosphatase activity is about 3 to 5% of the Na+,K+-ATPase activity, when p-nitrophenylphosphate is used to determine K+-phosphatase activity. In the present study, however, before preincubation in SDS the ratio varied from as low as 1:21 (data from Table 4) to as high as 1:14 (data from Figs. 2 and 4). After SDS preincubation, K'-pNPPase activity was constant at 3% of measured Na+,K'-ATPase activity. Illustrative data supporting the concept of intact IO and RO vesicles as the explanation for the discrepant ratios are presented in Figure 8. If the Na+ plus K+ stimulation of ATPase activity occurred in a preparation which allowed both faces of the enzyme to be exposed to components added to the incubation medium, stimulation with Na+ plus K+ should be numerically equal to inhibition by ouabain. Before SDS preincubation, it clearly was not. Ouabain inhibition was only 10% of Na+ plus K+ stimulation. Furthermore, in an exposed preparation, Na+ plus K+-stimulated ATPase activity should correlate with K'-stimulated phosphatase activity. Before SDS preincubation, it clearly did not. Based on a Na+ plus K+ stimulation of 17.5 μmol Pi/mg protein per hour, K'-pNPPase should have measured 1.75 to 2.92 μmol NP/mg protein per hour. On the other hand, if ouabain-inhibitable Na+,K'-ATPase activity is taken as the basis for calculation of the activity of K'-pNPPase (as in Fig. 8), the latter should have measured 0.18 to 0.29 μmol NP/mg protein per hour. Since the value found was outside both the ranges, some other contributing K'-pNPPase must have been present. An alternative argument based on measured K'-pNPPase activities and predicted Na+,K'-ATPase activities also holds. Since the ratio of predicted to found activities approximate 1 after preincubation with optimal detergent concentrations, it seems likely that SDS acted to increase accessibility of both enzyme faces to agents added to the incubation medium. This is tantamount to increasing passive membrane permeability of intact sarcolemmal vesicles.

Half, but only half, of our evidence for sidedness of the intact membrane vesicles rests on the assertion that p-nitrophenophosphate interacts with Na+,K'-ATPase from the exterior face of the enzyme. Although Askari and Rao have provided good evidence for the validity of this assertion, at least in red blood cells, this point is contestable.4 That our estimate of the IO/RO vesicle ratio based only on Na+,K'-ATPase activities closely corresponds to that based only on K'-pNPPase activities provides further evidence in favor of the assertion. If either measured activity (or both) were incorrect, it is unlikely that independent measurements would produce identical ratios.

That substantial quantities of sarcolemma are present in this preparation is documented by our measurements of high Na+,K'-ATPase activity. Previous assessments of the amount of this enzyme present, including our own, have essentially been negative. This is true whether the amount is measured from activity or from binding of H-ouabain. However, in each case only manifest Na+,K'-ATPase activity has been measured, either as ouabain-sensitive ATP hydrolysis or as the small increment of additional hydrolysis stimulated by addition of K+ ions to an incubation medium containing sodium.11 The results of the present study, however, demonstrate that any of these methods can underestimate the amount of Na+,K'-ATPase present. Before activation, the Na+,K'-ATPase activity is entirely latent, expressing neither ATP hydrolysis nor H-ouabain binding.

The demonstration of substantial quantities of sarcolemma in membrane vesicles which are considered the best available preparation of isolated cardiac sarcoplasmic reticulum suggests that considerable caution is warranted in interpretation of results using it. Even though this cardiac sarcoplasmic reticulum preparation exhibits high amounts of Ca2+ binding and Ca2+ uptake, and is capable of sufficiently rapid Ca2+ binding to quantitatively account for cardiac relaxation, the presence of sarcolemma cannot be ignored. For example, adenylate cyclase activity of the preparation, previously found by others and documented herein, may simply reflect the presence of sarcolemma and may not be conclusively used to assign subcellular location of adenylate cyclase to either sarcoplasmic reticulum or sarcolemma. Separation of manifest Na+,K'-ATPase activity from Ca2+-binding or Ca2+ uptake activities cannot be taken as proof of separation of sarcolemma from sarcoplasmic reticulum. Unequivocal subcellular localization of ade-
nlylate cyclase must await purer preparations of subcellular membrane systems. Nevertheless, our previous results suggesting that Na\(^+\),K\(^+-\)ATPase may be a pharmacological receptor for cardiac glycosides are in no way invalidated by the present study. In fact, we have recently found, in membrane vesicles from the hearts of two dogs given lethal doses of ouabain, that it is not possible to unmask Na\(^+\),K\(^+-\)ATPase activity (data not shown).

The goal of these studies was to demonstrate the presence of permeability-intact sarcolemma in cardiac membrane vesicles because vectorial transport can be demonstrated only when passive permeability barriers persist. As previously mentioned, our conclusion that sarcolemma is present in the membrane vesicle fraction and exhibits sidedness of activation and inhibition rests on the reasonable notion that Na\(^+\),K\(^+-\)ATPase is, in fact, a sarcolemmal enzyme. This conclusion must be tempered by the fact that Na\(^+\),K\(^+-\)ATPase has not yet been definitively proved to reside exclusively in sarcolemma.

Addendum

We have recently reproduced these same activation results on a sarcoplasmic reticulum preparation sent to us by Drs. A. Schwartz, B. Pitts, and M. Entman, Baylor College of Medicine. In addition, they have been able to reproduce qualitatively the activation of Na\(^+\),K\(^+-\)ATPase in our preparation of membrane vesicles.

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References

12. Jarnecil J: Conversion of the Na\(^+\) and K\(^+\) – independent part of the brain microsomal ATPase to a form requiring added Na\(^+\) and K\(^+\). Biochim Biophys Acta 17: 330–334, 1964
Intact vesicles of canine cardiac sarcolemma: evidence from vectorial properties of Na+, K+-ATPase.
H R Besch, Jr, L R Jones and A M Watanabe

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