Biochimica et Biophysica Acta, 733 (1983) 133–141
Elsevier

BBA 71775

ATP-DEPENDENT Na^+ TRANSPORT IN CARDIAC SARCOLEMMAL VESICLES

KENNETH D. PHILIPSON and ANN Y. NISHIMOTO

Departments of Medicine and Physiology and the American Heart Association Greater Los Angeles Affiliate, Cardiovascular Research Laboratories, A3-381 CHS, University of California at Los Angeles, Los Angeles, CA 90024 (U.S.A.)

(Received January 14th, 1983)
(Revised manuscript received May 18th, 1983)

Key words: ($Na^+ + K^+$)-ATPase; Na^+ transport; Na^+-Ca^{2+} exchange; (Cardiac sarcolemma)

Although the enzyme ($Na^+ + K^+$)-ATPase has been extensively characterized, few studies of its major role, ATP-dependent Na^+ pumping, have been reported in vesicular preparations. This is because it is extremely difficult to determine fluxes of isotopic Na^+ accurately in most isolated membrane systems. Using highly purified cardiac sarcolemmal vesicles, we have developed a new technique to detect relative rates of ATP-dependent Na^+ transport sensitively. This technique relies on the presence of Na^+-Ca^{2+} exchange and ATP-driven Na^+ pump activities on the same inside-out sarcolemmal vesicles. ATP-dependent Na^+ uptake is monitored by a subsequent Na_i^+-dependent Ca^{2+} uptake reaction (Na^+-Ca^{2+} exchange) using $^{45}Ca^{2+}$. We present evidence that the Na^+-Ca^{2+} exchange will be linearly related to the prior active Na^+ uptake. Although this method is indirect, it is much more sensitive than a direct approach using Na^+ isotopes. Applying this method, we measure cardiac ATP-dependent Na^+ transport and ($Na^+ + K^+$)-ATPase activities in identical ionic media. We find that the ($Na^+ + K^+$)-ATPase and the Na^+ pump have identical dependencies on both Na^+ and ATP. The dependence on [Na^+] is sigmoidal, with a Hill coefficient of 2.8. Na^+ pumping is half-maximal at [Na^+] = 9 mM. The K_m for ATP is 0.21 mM. ADP competitively inhibits ATP-dependent Na^+ pumping. This approach should allow other new investigations on ATP-dependent Na^+ transport across cardiac sarcolemma.

Introduction

An ATP-dependent Na^+ pump maintains the low intracellular concentrations of Na^+ in cardiac muscle. Any disruption of the transsarcolemmal Na^+ gradient has profound effects on both the electrophysiologic and contractile properties of this tissue. For example, small increases in intracellular Na^+ have been associated with the positive inotropic response to glycoside [1]. In intact myocardium the Na^+ pump can be studied directly by isotopic [2] or ion-selective microelectrode [3] techniques under control conditions and when the Na^+ pump is inhibited (glycoside-poisoned). These procedures are difficult because of the need to distinguish accurately intra- and extracellular Na^+, because of time delays and diffusion limitations, because of the possibility of intracellular Na^+ compartmentation, and because of secondary effects (metabolic, ionic, electrophysiologic, osmotic) associated with Na^+ pump inhibition. Indirect information on the Na^+ pump in intact myocardium can also be obtained through electrophysiologic analyses [4,5].

In broken cell preparations, the enzymatic manifestation of the Na^+ pump, the ($Na^+ + K^+$)-ATPase, has been studied in great detail (for reviews, see Refs. 6,7). In studies using sarcolemma-enriched preparations, the dependencies of cardiac ($Na^+ + K^+$)-ATPase on its substrates, its inhibi-

tion by glycosides, and its reaction mechanism have attracted much attention. Although the ability of the $(Na^+ + K^+)$-ATPase to actively transport ions is its property of greatest biological interest, studies of ATP-dependent Na^+ pumping in sarcolemmal vesicles are noticeably absent. This is because isotopic movements of Na^+ are extremely difficult to quantitate in a vesicular preparation of cardiac sarcolemma. The measurements are hindered by the high surface-area-to-volume ratio of sarcolemmal vesicles, high passive Na^+ permeability, and low isotope specific activity when Na^+ is in the millimolar range.

In this report we describe a new technique for the rapid measurement and characterization of ATP-dependent Na^+ pumping in inside-out cardiac sarcolemmal vesicles. The technique takes advantage of the presence of a highly active Na^+-Ca^{2+} exchange mechanism on the same vesicles which possess $(Na^+ + K^+)$-ATPase activity [8]. Glycoside-sensitive, ATP-dependent Na^+ uptake is first activated; because of the asymmetric nature of active Na^+ transport this will occur only in the inside out subpopulation of vesicles [8,9]. The ATP-dependent Na^+ uptake is then detected by a Na_i^+-dependent Ca^{2+} uptake reaction (i.e., Na^+-Ca^{2+} exchange). Evidence is presented to demonstrate that the Ca^{2+} uptake is proportional to the amount of prior Na^+ pumping which has occurred. This is a sensitive technique which greatly amplifies the detected signal. Measurements of ATP-dependent Na^+ pumping (as detected by Na^+-Ca^{2+} exchange) are compared with $(Na^+ + K^+)$-ATPase activity measured in identical solutions. In a prior study [8], we used a similar experimental format to characterize Na^+-Ca^{2+} exchange in inside-out cardiac sarcolemmal vesicles.

Methods

Sarcolemmal isolation and characterization

Sarcolemmal vesicles were isolated from trimmed dog ventricles as described previously [8]. Most of the data reported here were obtained with sarcolemma having the following characteristics: specific activities of K^+-*p*-nitrophenylphosphatase and $(Na^+ + K^+)$-ATPase were 20.6 ± 1.4 and 28.7 ± 2.3 μmol/mg protein per h, respectively ($n = 7$). These activities were about 50-fold those of the crude tissue homogenate. The $(Na^+ + K^+)$-ATPase activity could be stimulated to 65.3 ± 4.6 μmol/mg protein per h by including alamethicin (12.5 μg/ml; donated by J.E. Grady, Upjohn Company) in the reaction medium [10]. These and other data [8,9] suggest that about 20–40% of the vesicles are inside-out. Less than 0.3% of homogenate succinate dehydrogenase activity was recovered in the sarcolemmal fraction.

ATP-dependent Na^+ uptake

To initiate active Na^+ transport, K^+ (140 mM)-loaded sarcolemmal vesicles (0.006 ml; 3–5 mg/ml) were added to a reaction medium (0.034 ml, final volume) with the following final concentrations (mM): KCl (25), NaCl (19), NaN_3 (5), choline chloride (74), Tris-ATP (2.4), $MgCl_2$ (3), Tris-maleate buffer (4, pH 7.4, 37°C). This was done in the presence and absence of 100 μM digitoxenin (or ethanol, 0.2%). After a fixed time (0.15 min unless otherwise stated), ATP-dependent Na^+ uptake was inhibited and Na_i^+-dependent Ca^{2+} uptake (Na^+-Ca^{2+} exchange) was initiated in order to quantitate intravesicular Na^+. This was done by diluting the reaction mixture 20-fold with Ca^{2+} uptake medium containing KCl (140 mM), $CaCl_2$ (0.010 mM), $^{45}CaCl_2$ (0.3 μCi), digitoxigenin (50 μM), and Tris-maleate buffer (5 mM, pH 7.4, 37°C). After 0.5 min, the Na^+-Ca^{2+} exchange reaction was stopped by the addition of 0.03 ml of 140 mM KCl/10 mM $LaCl_3$. The vesicles were collected by filtration (0.45 μm Millipore filters) and washed twice with 3 ml of 140 mM KCl/1 mM $LaCl_3$. This procedure measures only intravesicular Ca^{2+} and not superficially bound Ca^{2+}. The increase in Ca^{2+} uptake when digitoxigenin is not included in the initial Na^+ transport step is related to ATP-dependent Na^+ transport (see below). In initial experiments, the KCl-loaded vesicles were also preincubated with and without digitoxigenin. However, it was found that full Na^+ pump inhibition occurred without this step and vesicle pretreatment was eliminated. That is, digitoxigenin (a membrane-permeable aglycone) reached its inhibitory site at the inner surface of inside-out vesicles more rapidly than could be detected by any of our measurements. This technique is similar to that described in a previous report [8]. Our techniques for measuring

Na^+-Ca^{2+} exchange have been presented in detail [8,9,11,12]. All uptake measurements were performed at 37°C.

($Na^+ + K^+$)-ATPase

The ($Na^+ + K^+$)-ATPase reaction was carried out under ionic conditions identical to those used for ATP-dependent Na^+ pumping (see above) except that the final reaction volume was increased to 0.068 ml (0.006 ml of KCl-loaded vesicles were added to 0.062 ml). After 0.4 min (unless otherwise stated) the reaction was stopped by the addition of 0.1 ml of cold 5% trichloroacetic acid and inorganic phosphate was assayed by the method of Stewart [14] using citrate to stabilize color development [15]. Agents to unmask latent activity were not used.

Data are expressed as the mean ± S.E.

Results

Control experiments

In most of the experiments described below active Na^+ pumping was initiated by the addition of KCl-loaded sarcolemmal vesicles to a medium containing the substrates necessary for Na^+ pump activity. To detect the active Na^+ uptake, the reaction mixture was diluted into a KCl medium containing $^{45}Ca^{2+}$ (see Methods). Since the ensuing Ca^{2+} uptake (by Na^+-Ca^{2+} exchange) was used to monitor vesicular Na^+ content, it was important to ascertain the relationship between Na_i^+-dependent Ca^{2+} uptake and internal Na^+ levels. To examine this relationship we first equilibrated sarcolemmal vesicles with various levels of Na^+ by passive diffusion. As shown in Fig. 1, the Na_i^+-dependent Ca^{2+} uptake (measured at 0.5 min) was linearly related to internal [Na^+].

Measurements of ATP-dependent Na^+ pumping using $^{22}Na^+$ were compared with measurements made using Na^+-Ca^{2+} exchange as a probe for active Na^+ transport (Table I). That is, ATP-dependent Na^+ pumping was allowed to occur in the presence or absence of $^{22}Na^+$ (at [Na^+] = 16.5 mM). If $^{22}Na^+$ was present, the vesicles were filtered after 0.3 min. If no $^{22}Na^+$ tracer was present, the reaction was terminated (by digitoxigenin) at 0.3 min and Na^+-Ca^{2+} exchange was initiated. If the ATP level was reduced from 2.4 to

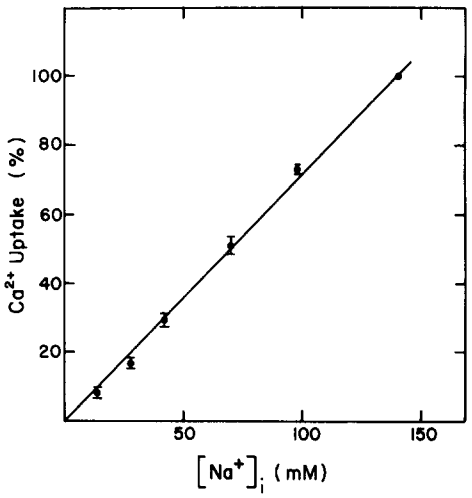

Fig. 1. Dependence of Na_i^+-dependent Ca^{2+} uptake on internal Na^+. Na^+ (140 mM)- and K^+ (140 mM)-loaded sarcolemmal vesicles were mixed in the appropriate proportion and incubated at 37°C for 20 min to obtain the desired internal Na^+ level. Control experiments indicated that this was sufficient time for passive equilibration of Na^+ and K^+ to occur. Na_i^+-dependent Ca^{2+} uptake was initiated by the addition of 0.005 ml of vesicles into 0.25 ml of medium containing 140 mM KCl, 10 μM $^{45}CaCl_2$, 5 mM Tris-maleate (pH 7.4, 37°C) and proceeded for 0.5 min. Ca^{2+} uptake into Na^+-free vesicles was subtracted as a blank. Ca^{2+} uptake when Na_i^+ equalled 140 mM was 20.3 ± 2.0 nmol/mg protein. $n = 3$.

0.2 mM, the magnitude of digitoxigenin-sensitive Na^+ transport was reduced by proportionately similar amounts as detected by the two techniques (Table I). The reasons why the indirect approach

TABLE I

ATP-DEPENDENT Na^+ PUMPING DETECTED BY $^{22}Na^+$ UPTAKE AND $^{45}Ca^{2+}$ EXCHANGE

[Na^+] during the active Na^+ transport reaction was 16.5 mM. $t = 0.3$ min for this reaction. The Mg^{2+}/ATP ratio was kept constant at 1.25. The data represent only the digitoxigenin-sensitive component of Na^+ transport. All other conditions are as described in Methods. $n = 3$.

[ATP] (mM)	A $^{22}Na^+$ pumped (nmol/mg)	B $^{45}Ca^{2+}$ exchanged for Na^+ pumped (nmol/mg)	$\frac{A}{B}$
2.4	97.8 ± 15.3	15.7 ± 2.1	6.2
0.2	39.7 ± 6.9	7.8 ± 1.3	5.1

(following $^{45}Ca^{2+}$ rather than $^{22}Na^{+}$ movements) gives much higher sensitivity will be addressed below. All of the internal Na^{+} will not participate in Na^{+}-Ca^{2+} exchange during our measurements and the data in Table I cannot be used to obtain the stoichiometry for this process.

We examined ATP-dependent Na^{+} pumping in cardiac sarcolemmal vesicles as a function of a variety of parameters (e.g., [Na^{+}], [ATP]). Thus, it was necessary to verify that none of these variables interfered with our detection system (i.e., Na^{+}-Ca^{2+} exchange). Since the ATP-dependent Na^{+} uptake reaction medium was diluted 20-fold into the Na^{+}-Ca^{2+} exchange uptake medium, modification of Na^{+} pump substrate levels did not cause large concentration changes in the Ca^{2+} uptake medium. In Table II we show that when vesicles were Na^{+} loaded by passive equilibration (rather than by active transport), Na^{+}-Ca^{2+} exchange was unaffected by low levels of a variety of substances in the Ca^{2+} uptake medium. These are the approximate levels which were present during Na^{+}-Ca^{2+} exchange following active Na^{+} pumping in the experiments below. Although ATP has been reported to modulate Na^{+}-Ca^{2+} exchange in the squid axon [16], we find no effect under our conditions. We have previously presented evidence eliminating the possibility that the sarcolemmal ATP-dependent Ca^{2+} pump interfered with any of our measurements [8].

TABLE II

EFFECTS OF VARIOUS SUBSTANCES ON Na^{+}-Ca^{2+} EXCHANGE

Sarcolemmal vesicles (0.005 ml) preloaded by passive diffusion with 70 mM NaCl were diluted into 0.25 or 0.50 ml of 140 mM KCl, 10 μM $^{45}CaCl_2$, 5 mM Tris-maleate (pH 7.4, 37°C) and Na_i^{+}-dependent Ca^{2+} uptake was allowed to proceed for 0.5 mins. The listed substances were added to this Ca^{2+} uptake medium and were not present prior to initiation of Na^{+}-Ca^{2+} exchange. Control Ca^{2+} uptake activity was 14.4 ± 3.0 nmol/mg. $n = 3$ or 4.

	Na_i^{+}-dependent Ca^{2+} uptake (% of control activity)
Digitoxigenin (50 μM)	99.5 ± 3.0
Mg^{2+} (200 μM)	101.3 ± 3.0
Mg^{2+}ADP (100 μM)	100.7 ± 1.7
Mg^{2+}ATP (160 μM)	100.0 ± 3.3
Na^{+} (3 mM)	100.3 ± 2.9

ATP-dependent Na^{+} pumping and (Na^{+} + K^{+})-ATPase activity in cardiac sarcolemmal vesicles

Time courses for ATP-dependent Na^{+} uptake and (Na^{+} + K^{+})-ATPase activity in cardiac sarcolemmal vesicles are shown in Fig. 2. For these and all subsequent results Na^{+} transport was detected by Na^{+}-Ca^{2+} exchange. Only digitoxigenin-sensitive components of activity are shown. Na^{+} uptake was linear to 0.15 min, was maximal at about 0.5 min, and then slowly declined. In contrast, (Na^{+} + K^{+})-ATPase activity was approximately linear for at least 1.0 min. The major source for this discrepancy in behavior between two closely related activities is probably that passive Na^{+} efflux from the vesicles increased as the Na^{+} uptake reaction proceeded. As the rate of Na^{+} efflux approached the influx rate, a plateau of intravesicular Na^{+} content would be obtained. The rapid deviation of Na^{+} uptake from linearity demonstrates the relatively high Na^{+} permeability of the sarcolemmal vesicles. At longer times, the small decline in Na^{+}

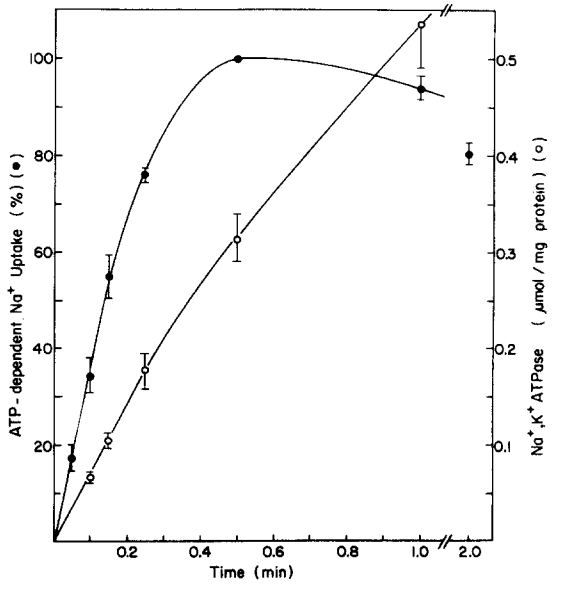

Fig. 2. Time-dependence of ATP-dependent Na^{+} uptake and (Na^{+} + K^{+})-ATPase activity. Active Na^{+} pumping (detected by Na^{+}-Ca^{2+} exchange) and (Na^{+} + K^{+})-ATPase activity were measured as described in Methods. The Na_i^{+}-dependent Ca^{2+} uptake at $t = 0.5$ min (taken as 100% Na^{+} uptake) was 5.4 ± 1.0 nmol Ca^{2+}/mg protein. $n = 4$ (Na^{+} uptake) or 5 ((Na^{+} + K^{+})-ATPase).

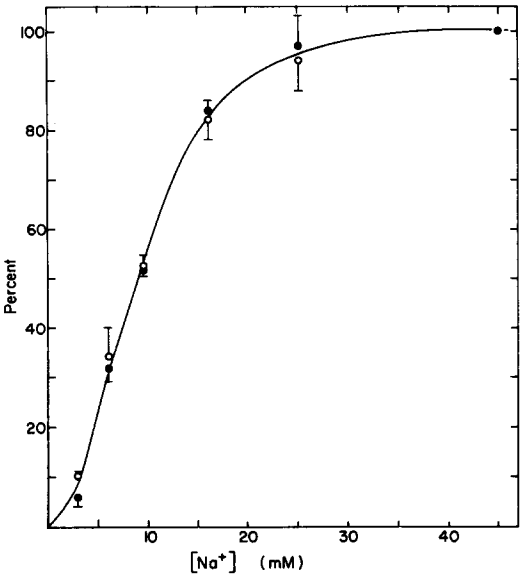

Fig. 3. Na^+ dependence of ATP-dependent Na^+ influx and $(Na^+ + K^+)$-ATPase activity. Choline chloride concentration was adjusted as NaCl was varied to maintain osmolarity. ATP-dependent Na^+ influx (●) and $(Na^+ + K^+)$-ATPase activity (○) were measured at 0.15 and 0.4 min, respectively. See methods for other details. Na_i^+-dependent Ca^{2+} uptake after maximal active Na^+ pumping (at $[Na^+] = 45$ mM) was 6.0 ± 0.6 nmol Ca^{2+}/mg protein. $(Na^+ + K^+)$-ATPase activity at $[Na^+] = 45$ mM was 59.3 ± 11.9 μmol/mg protein/h. $n = 4$.

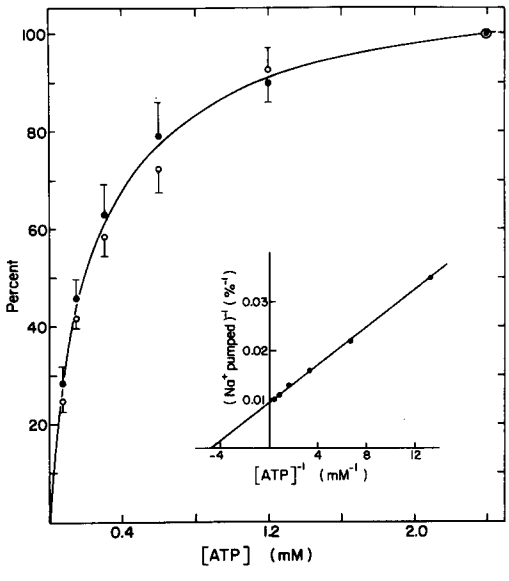

Fig. 4. ATP dependence of ATP-dependent Na^+ influx (●) and $(Na^+ + K^+)$-ATPase activity (○). The $[Mg^{2+}]/[ATP]$ ratio was kept constant at 1.25. See Methods for other details. Inset: Lineweaver-Burk plot of the Na^+ influx data as a function of [ATP]. Na_i^+-dependent Ca^{2+} uptake after maximal active Na^+ pumping (at $[ATP] = 2.4$ mM) was 5.4 ± 0.9 nmol Ca^{2+}/mg protein. $(Na^+ + K^+)$-ATPase activity at $[ATP] = 2.4$ mM was 47.3 ± 7.6 μmol/mg protein/h. $n = 6$.

content may be due to a decrease in Na^+ influx as the ATP level falls. (ATP concentration would have declined by about 20%/min during active Na^+ transport and by about 10%/min under the conditions used to measure $(Na^+ + K^+)$-ATPase activity.) In contrast, the reverse reaction of $(Na^+ + K^+)$-ATPase activity (resynthesis of ATP from $ADP + P_i$) would not occur to an appreciable extent under our conditions and linearity would be maintained for $(Na^+ + K^+)$-ATPase activity. In all experiments presented below, Na^+ pumping and $(Na^+ + K^+)$-ATPase activity were measured during their initial linear phases at 0.15 and 0.4 min, respectively. Note that identical reaction media (except for a difference in volume) were used for the Na^+ pump and $(Na^+ + K^+)$-ATPase reactions.

ATP-dependent Na^+ influx and $(Na^+ + K^+)$-ATPase activity were determined as functions of the external Na^+ concentration (Fig. 3). Activities were half-maximal at about 9 mM Na^+ and a marked sigmoidicity was evident. On a Hill plot, the slope of the ATP-dependent Na^+ influx data is 2.8 (correlation coefficient, r, equals 0.99).

Fig. 4 displays ATP-dependent Na^+ influx and $(Na^+ + K^+)$-ATPase activity as functions of the medium ATP concentration. Again, close agreement between the behavior of the two activities is observed. A Lineweaver-Burk plot of the Na^+

TABLE III

ATP-DEPENDENT Na^+ PUMPING AS A FUNCTION OF Mg^{2+} CONCENTRATION

[ATP] = 2.4 mM. See Methods. Na_i^+-dependent Ca^{2+} uptake after maximal active Na^+ pumping (at $[Mg^{2+}] = 3.0$ mM) was 3.7 ± 1.0 nmol Ca^{2+}/mg protein. $n = 3$.

$[Mg^{2+}]$(mM)	$\frac{[Mg^{2+}]}{[ATP]}$	Na^+ pumped (%)
0	0	2.7 ± 1.7
1.5	0.63	80.3 ± 3.3
3.0	1.25	100
6.0	2.50	93.3 ± 3.3

TABLE IV

ATP-DEPENDENT Na^+ PUMPING AS A FUNCTION OF ADP CONCENTRATION

[ATP] = 2.4 mM. ADP was added as MgADP. See Methods. Na_i^+-dependent Ca^{2+} uptake after maximal active Na^+ pumping (at zero [ADP]) was 3.9 ± 1.0 nmol Ca^{2+}/mg protein. $n = 3$.

[ADP](mM)	Na^+ pumped (%)
0	100
0.12	96.0 ± 1.6
0.36	79.7 ± 1.2
1.08	49.3 ± 2.9

influx data (Fig. 4, inset) gave a K_m for ATP of 0.21 mM. Similar K_m values for ATP for cardiac muscle $(Na^+ + K^+)$-ATPase have been reported [17,18].

The dependence on Mg^{2+} of active Na^+ transport in cardiac sarcolemmal vesicles is demonstrated in Table III. Na^+ pumping rapidly increased as the $[Mg^{2+}]$ approached the [ATP] and was slightly inhibited in the presence of excess Mg^{2+}.

The effects of added ADP in the Na^+ pump reaction medium is shown in Table IV. In the presence of 2.4 mM ATP, low concentrations (under 0.12 mM) of ADP had little effect on Na^+ transport. In these experiments, 1.08 mM ADP inhibited active Na^+ transport by about 50%. Since ADP may compete with ATP for the same binding site, we also examined the inhibition of Na^+ transport by ADP at a reduced ATP level. As shown in Table V, the inhibiting influence of ADP increased when the [ATP] was reduced from 2.4 to 0.3 mM.

TABLE V

INHIBITION OF ATP-DEPENDENT Na^+ PUMPING BY ADP AS A FUNCTION OF [ATP]

See Methods for details. $n = 3$.

ATP (mM)	ADP (mM)	Na^+ pumped (%)	Inhibition (%)
2.4	0	100	
2.4	1.1	62.8 ± 3.0	37.2
0.3	0	77.8 ± 7.6	
0.3	1.1	31.8 ± 5.3	59.1

Discussion

The active Na^+ pump is intimately involved in the regulation of myocardial contractile and electrophysiologic events. Despite this importance, detailed characterization of myocardial ATP-dependent Na^+ transport has been elusive. We describe a sensitive new technique for measuring relative rates of ATP-dependent Na^+-transport in the subpopulation of inside out cardiac sarcolemmal vesicles. The technique relies on the simultaneous presence of Na^+ pump and Na^+-Ca^{2+} exchange activities in the same sarcolemmal vesicles. The procedure is first to allow ATP-dependent Na^+ transport to occur; the active Na^+ uptake is subsequently assayed by a Na_i^+-dependent Ca^{2+} uptake reaction (Na^+-Ca^{2+} exchange) using $^{45}Ca^{2+}$. Applying this technique, we characterize the ATP-dependent Na^+ pump in cardiac sarcolemmal vesicles. We discuss now in order the accuracy, sensitivity, advantages, disadvantages and usefulness of this method.

The use of Na^+-Ca^{2+} exchange as an assay for active Na^+ transport is indirect; accuracy of the technique depends on the relationship between the magnitude of ATP-dependent Na^+ uptake and the Na^+-Ca^{2+} exchange measurement. There are three arguments which support a linear relationship. (1) When the vesicles are Na^+ loaded by passive equilibration (rather than by ATP-dependent Na^+ transport), Na_i^+-dependent Ca^{2+} uptake is proportional to the initial Na^+ load (Fig. 1). This relationship suggests that Na^+-Ca^{2+} exchange should also be proportional to internal Na^+ when the vesicles are loaded with Na^+ by the ATP-dependent Na^+ pump. However, Na^+ uptake by active pumping will occur only in inside-out vesicles (due to the asymmetric orientation of the $(Na^+ + K^+)$-ATPase) while Na^+ uptake by passive diffusion occurs in all sarcolemmal vesicles. Thus the relationship obtained above (Fig. 1) may not be valid for inside out vesicles. We have previously presented evidence [8] that Na^+-Ca^{2+} exchange in inside-out vesicles is very similar to that in the entire population of vesicles. Thus, the data suggest that Na^+-Ca^{2+} exchange should be a useful monitor for ATP-dependent Na^+ uptake in cardiac sarcolemmal vesicles. (2) In the same experiments, ATP-dependent Na^+ transport was measured di-

rectly, using $^{22}Na^+$, and indirectly, using Na^+-Ca^{2+} exchange (Table I). When [ATP] was reduced, the rate of Na^+ transport declined in a similar manner as measured by both techniques. The difficulties of using $^{22}Na^+$ to study transport in sarcolemmal vesicles (see below) make more detailed comparisons of this type impractical. Nevertheless, the qualitative results again suggest that Na^+-Ca^{2+} exchange can be used as a valid probe for ATP-dependent Na^+ pumping. (3) It is generally assumed, although demonstrated qualitatively only in a few specific cases (e.g., the red blood cell), that the substrate dependencies of the ($Na^+ + K^+$)-ATPase and the Na^+ pump are identical. We obtain excellent correlations between the the dependencies of ATP-dependent Na^+ pumping and ($Na^+ + K^+$)-ATPase on both Na^+ and ATP levels (Figs. 3 and 4). Although these correlations may be fortuitous (see below), they support the contention that our indirect measurements are an accurate reflection of ATP-dependent Na^+ transport.

The increased sensitivity obtained by using Na^+-Ca^{2+} exchange as a monitor for ATP-dependent Na^+ pumping is primarily due to isotopic specific activity considerations. This is exemplified by the data in Table I. At [ATP] = 2.4 mM, 6.2-times as much Na^+ is transported (by ATP-dependent pumping) than Ca^{2+} (in exchange for Na^+ pumped). However, the Na^+ pumping occurs at $[Na^+] = 16.5$ mM, whereas the Ca^{2+} transport step proceeds at $[Ca^{2+}] = 0.01$ mM. If equal concentrations of isotope ($^{22}Na^+$ or $^{45}Ca^{2+}$) are used, the specific activity will be 1650-times higher using $^{45}Ca^{2+}$. In this example, a signal amplification of about 270 (obtained by dividing 1650 by 6.2) is achieved. It should be noted that Na^+ pumping experiments using $^{22}Na^+$ (Table I) were designed to obtain a maximal $^{22}Na^+$ uptake signal. The reaction proceeded for 0.3 min at a Na^+ concentration of 16.5 mM. This was twice the reaction period (0.15 min) used in most of our experiments and was past the linear portion of the time-course of Na^+ uptake (Fig. 2). At $[Na^+] = 16.5$ mM, Na^+ pumping would be near maximal (Fig. 3); higher concentrations would not increase Na^+ uptake but would lower isotope specific activity. Measurements of Na^+ pumping using $^{22}Na^+$ under more stringent conditions were not feasible due to the ^{22}Na signal approaching background levels. Thus we were able to obtain detailed results (Figs. 2–4) using our indirect approach ($^{45}Ca^{2+}$) when the direct approach ($^{22}Na^+$) was inappropriate.

There are other advantages to converting the determination of ATP-dependent Na^+ pumping from a measurement of Na^+ to a measurement of $^{45}Ca^{2+}$. (1) Techniques for measuring Ca^{2+} transport are more reliable than those for Na^+ because various agents (e.g., EGTA, La^{3+}, ionophore A23187) are usable in Ca^{2+} transport studies. These agents help distinguish between intra- and extravesicular Ca^{2+} or block Ca^{2+} fluxes. Thus, transmembrane Ca^{2+} movements can be ascertained with confidence. Comparably specific agents for Na^+ are not in use. (2) Sarcolemmal membranes have a much greater permeability to Na^+ than to Ca^{2+}. During measurements of $^{22}Na^+$ transport, this introduces uncertainties owing to the possible loss of intravesicular Na^+ during experimental manipulations such as filtration (3) Na^+ isotopes are costly and necessitate exposure to high-energy radiation.

The disadvantage of the use of Na^+-Ca^{2+} exchange as a monitor of active Na^+ transport is that it does not permit absolute quantitation of Na^+ pumping; only relative changes in pump rates can be measured. In many experiments this is sufficient: for example, determination of substrate dependencies (Figs. 3 and 4); in these experiments small changes (20%) in Na^+ pump rate could be easily and reproducibly detected. In addition, care must be taken to ensure that a variation in the conditions of the Na^+ pump reaction will not affect the subsequent Na^+-Ca^{2+} exchange reaction. Control experiments (e.g., Table II) determined that this was true for all Na^+ pump results reported here. Fortunately, Na^+-Ca^{2+} exchange is relatively insensitive to many interventions. This is especially true when the Na^+-Ca^{2+} exchange reaction proceeds for as long as 0.5 min as used here. Nevertheless, it is essential to be cautious when using this technique and to evaluate possible artifacts which may arise with different interventions.

We have begun to characterize the cardiac Na^+ pump and find that if sarcolemmal ($Na^+ + K^+$)-ATPase and ATP-dependent Na^+ pumping are measured in identical media, they have identical

dependencies on Na^+ (Fig. 3) and ATP (Fig. 4). This confirms the identity of the $(Na^+ + K^+)$-ATPase as the Na^+ pump in this system. However, this conclusion must be qualified. Whereas active Na^+ uptake will occur only in sealed inside out sarcolemmal vesicles, $(Na^+ + K^+)$-ATPase activity will be a complex function of inside-out, right-side-out, and broken vesicles, and of substrate permeabilities. Thus, although the Na^+ pump and $(Na^+ + K^+)$-ATPase measurements were made in identical ionic media, the concentrations of substrates at the active sites would not necessarily be identical for the two measurements. For example, leaky vesicles which would make a major contribution to $(Na^+ + K^+)$-ATPase measurements would have both membrane surfaces exposed to the reaction medium; the inside-out vesicles responsible for Na^+ uptake, however, would be exposed to different media at their internal and external surfaces. Thus, the direct comparisons of Na^+ pump and $(Na^+ + K^+)$-ATPase activities must be viewed cautiously. These considerations are exemplified by the high ratio of ATP hydrolyzed to Na^+ transported in this system. Using the Na^+ pump and $(Na^+ + K^+)$-ATPase data of Figs. 2, 3 and 4 and a ratio of 6 Na^+ pumped/Ca^{2+} exchanged (Table I), we calculate an ATP/Na^+ of about 5. The efficiency of the Na^+ pump is unlikely to be so low. A large part of the high ratio is probably due to unsealed vesicles in the preparation; these vesicles would contribute to $(Na^+ + K^+)$-ATPase activity but not to Na^+ pumping.

Our data indicate the participation of multiple sodium ions in the Na^+ pump reaction (slope of Hill plot = 2.8) [19,22]. It is notable that the dependence of the Na^+ pump on $[Na^+]$ is especially steep at physiological Na^+ concentrations in cardiac muscle [3]. The presence of Mg^{2+} is essential for ATP-dependent Na^+ pumping to occur (Table III); this reflects the fact that MgATP is the necessary substrate for the active Na^+ pump [7]. ADP inhibits ATP-dependent Na^+ pumping at relatively high concentrations (Table IV) and may compete with ATP (Table V). Competitive inhibition of brain $(Na^+ + K^+)$-ATPase by ADP has been reported [23]. This could be relevant in the ischemic heart where [ATP] falls and [ADP] rises. Thus, both the ATP and ADP levels must be considered when in vivo Na^+ pump activity is estimated.

The most detailed previous measurements of ATP-dependent Na^+ pumping have been made in systems such as red blood cells [19], squid axons [20], and reconstituted vesicles [21]. These are systems with relatively low Na^+ pump activity, large internal volume, or, importantly, low passive Na^+ permeability. Thus measurements can be made over the course of several minutes or even hours using Na^+ isotopes. These techniques do not work well with cardiac sarcolemmal vesicles and this emphasizes the utility of our techniques. An advantage of measurements of Na^+ pumping over measurements of $(Na^+ + K^+)$-ATPase is that vectorial active Na^+ uptake will occur only in sealed, inside-out sarcolemmal vesicles. In contrast to this well-defined system, $(Na^+ + K^+)$-ATPase activity will be determined by more complex factors (see above). The ability to measure active Na^+ transport in sarcolemmal vesicles may be useful in ascertaining whether various drugs and other regulatory interventions cause dissociation of Na^+ pump and $(Na^+ + K^+)$-ATPase activities. Our technique for measuring active Na^+ transport is limited only to certain tissues. Vesicles must be obtained which simultaneously display both high $(Na^+ + K^+)$-ATPase and Na^+-Ca^{2+} exchange activities. In addition to cardiac sarcolemma, brain membrane preparations may be appropriate.

Acknowledgments

We are grateful to Drs. D.M. Bers, M.M. Bersohn, T.L. Trosper and E.M. Wright for critical reading of this manuscript and to Mrs. L. Green for expert secretarial assistance. This research was supported by the United States Public Health Service (HL 27821) and by the Castera Foundation.

References

1 Akera, T. and Brody, T.M. (1978) Pharmacol. Rev. 29, 187–220
2 Bridge, J.H.B., Cabeen, W.R., Langer, G.A. and Reeder, S. (1981) J. Physiol. 316, 555–574
3 Ellis, D. (1977) J. Physiol. 273, 211–240
4 Glitsch, H.G. (1979) Am. J. Physiol. 236, H189–H199
5 Gadsby, D.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4035–4039
6 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3–134

7 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176
8 Philipson, K.D. and Nishimoto, A.Y. (1982) J. Biol. Chem. 257, 5111–5117
9 Bers, D.M., Philipson, K.D. and Nishimoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358–371
10 Jones, L.R., Maddock, S.W. and Besch, Jr., H.R. (1980) J. Biol. Chem. 255, 9971–9980
11 Philipson, K.D. and Nishimoto, A.Y. (1980) J. Biol. Chem. 255, 6880–6882
12 Philipson, K.D. and Nishimoto, A.Y. (1981) J. Biol. Chem. 256, 3698–3702
13 Philipson, K.D., Bersohn, M.M. and Nishimoto, A.Y. (1982) Circ. Res. 50, 287–293
14 Stewart, D.J. (1974) Anal. Biochem. 62, 349–364
15 Lanzetta, P.A., Alverez, L.J., Reinach, P.S. and Candia, O.A. (1979) Anal. Biochem. 100, 95–97
16 Blaustein, M.P. (1976) Fed. Proc. 35, 2574–2578, 1976
17 Matsui, H. and Schwartz, A. (1966) Biochim. Biophys. Acta 128, 380–390
18 Sperelakis, N. and Lee, E.C. (1971) Biochim. Biophys. Acta 233, 562–579
19 Garay, R.P. and Garrahan, P.J. (1973) J. Physiol. 231, 297–325
20 Beaugé, L.A. and DiPolo, R. (1979) Biochim. Biophys. Acta 553, 495–500
21 Racker, E. and Fisher, L.W. (1975) Biochem. Biophys. Res. Commun. 67, 1144–1150
22 Sen, A.K. and Post, R.L. (1964) J. Biol. Chem. 239, 345–352
23 Hexum, T., Samson, F.E., Jr. and Himes, R.H. (1970) Biochim. Biophys. Acta 212, 322–331