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Role of the Sodium Pump and the Background K^+ Channel in Passive $K^+(Rb^+)$ Uptake by Isolated Cardiac Sarcolemmal Vesicles

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Summary. A simple procedure was developed for the isolation of a sarcolemma-enriched membrane preparation from homogenates of bullfrog (*Rana catesbeiana*) heart. Crude microsomes obtained by differential centrifugation were fractionated in Hypaque density gradients. The fraction enriched in surface membrane markers consisted of 87% tightly sealed vesicles. The uptake of $^{86}Rb^+$ by the preparation was measured in the presence of an opposing K^+ gradient using a rapid ion exchange technique. At low extravesicular Rb^+ concentrations, at least 50% of the uptake was blocked by addition of 1 mM ouabain to the assay medium. Orthovanadate (50 μM), ADP (2.5 mM) or Mg (1 mM) were also partial inhibitors of Rb^+ uptake under these conditions, and produced a complete block of Rb^+ influx in the presence of 1 mM ouabain. When $^{86}Rb^+$ was used as a tracer of extravesicular K^+ ($Rb_o^+ \leq 40$ μM, $K_o^+ = 0.1$–5 mM) a distinct uptake pathway emerged, as detected by its inhibition by 1 mM Ba^{2+} ($K_{0.5} = 20$ μM). At a constant internal K^+ concentration ($K_{in}^+ = 50$ mM), the magnitude of the Ba^{2+}-sensitive K^+ uptake was found to depend on K_o^+ in a manner that closely resembles the K^+ concentration dependence of the background K^+ conductance (I_{K_1}) observed electrophysiologically in intact cardiac cells. We conclude that K^+ permeates passively this preparation through two distinct pathways, the sodium pump and a system identifiable as the background potassium channel.

Key Words K^+ transport · cardiac sarcolemma · ouabain · Ba^{2+} inhibition

Introduction

In intact cells, both passive and active K^+ fluxes across the plasma membrane contribute to the maintenance of a negative resting membrane potential that is essential for normal heart function (Carmeliet & Vereecke, 1979). Investigation of these K^+ transport pathways at the molecular level, however, requires the purification and reconstitution of individual proteins in a functional state. The ideal source of material for these procedures are preparations of cardiac sarcolemmal vesicles, provided that the transport systems observed in purified membranes display properties consistent with measurements of K^+ permeability "in vivo." In the present report, we characterized $K^+(Rb^+)$ uptake into cardiac sarcolemmal vesicles isolated from bullfrog heart. Passive K^+ transport in these membranes is mediated by two distinct systems: a ouabain-sensitive pathway identified as the Na^+,K^+-pump, and a Ba^{2+}-sensitive pathway which we postulate to be the inwardly rectifying background K^+ channel, I_{K_1}. The experimental approaches utilized here for detection of these two components rely on their specific transport properties and are potentially useful for monitoring their activity in future purification and reconstitution procedures.

Materials and Methods

Materials

$^{86}RbCl$, [^{3}H]-QNB,[1] ^3H_2O and [^{14}C]-sucrose were obtained from Amersham Corporation. Hypaque meglumine was obtained as a 60% (wt/vol) solution from Winthrop Laboratories. Dowex 50W-X8 (50–100 mesh), alamethicin, orthovanadate, protease inhibitors, enzymes and chemicals were purchased from Sigma. Monensin was from Calbiochem. Lubrol-WX was from Supelco. Bullfrogs were obtained from West Jersey Biological Supplies (Winona, NJ).

Membrane Preparation

Frogs were pithed and the hearts were immediately removed and rinsed with amphibian Ringer's solution (in mM: NaCl 90, HEPES 20, $MgCl_2$ 5, $CaCl_2$ 2.5, KCl 2.5; pH 7.4 with NaOH). The beating hearts were kept in Ringer's, at room temperature, and microdissected in order to remove the external layer of loose connective tissue and the *conus arteriosus*. All subsequent pro-

[1] Abbreviations used are: QNB, quinuclidinyl benzilate; NMG, N-methyl-D-glucamine; EGTA [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cedures were performed at 4°C. After removal of the *sinus venosus* the tissue was cut open and shredded by scraping with microscope slides. In most preparations, six animals were used (5–8 g of dissected tissue). The mince was suspended in 4 volumes of SKT buffer (in mM: sucrose 90, KCl 50, Tris-HCl 10, pH 7.4) containing protease inhibitors (pepstatin 1 μg/ml, aprotinin 5 μg/ml, and leupeptin 5 μg/ml). In three preparations, the KCl present in homogenization and gradient media was substituted for N-methyl-D-glucamine chloride (NMG · Cl). The suspension was further disrupted in a Waring blender equipped with an Eberbach microcontainer (15-sec burst, low speed). One volume of SKT was added and the suspension was homogenized with 10 passes of the pestle in a Teflon-glass homogenizer at 750 rpm. The homogenate was diluted fivefold and centrifuged at 650 × *g* for 10 min (all *g* values are g_{av}). The supernatant was reserved, while the pellet was resuspended and rehomogenized in 10 volumes of SKT. After a second centrifugation at 650 × *g* for 10 min the two supernatants were combined and centrifuged at 70,000 × *g* for 45 min. The pellets were suspended in a small volume of SKT and dispersed using a Teflon-glass homogenizer as described above. Portions (5 ml) of the suspension were layered over 20 ml of 18% (wt/vol) Hypaque in 50 mM KCl, 5 mM Tris · HCl, pH 7.4 and spun in a Beckman 60 Ti rotor at 73,000 × *g* for one hour. The white interfacial band and the top layer (sarcolemma-enriched fraction) were harvested with a Pasteur pipette and diluted at least 2.5-fold with SKT. The Hypaque layer and the dark brown pellet were discarded. The suspension was centrifuged at 114,000 × *g* for 30 min and the pellets were resuspended in SKT at a final concentration of 2.5–3.0 mg/ml. The vesicles were divided in 0.5 ml aliquots and stored in liquid nitrogen. Aliquots of the vesicles were quickly thawed at 37°C prior to use, and then kept on ice. In preliminary experiments, a continuous Hypaque gradient was used instead of a cushion of fixed concentration. Gradients were formed from 8 and 24% (wt/vol) Hypaque in 50 mM KCl, 5 mM Tris · HCl, pH 7.4 and centrifuged in an SW 27.1 rotor at 25,000 rpm for 19 hr. Fractions of 1.0 ml were collected from the top using an automatic fractionator (ISCO) and assayed for protein and muscarinic cholinergic binding sites, in order to locate the peak enriched in sarcolemma.

Enzyme Assays and Protein Determination

Na^+,K^+-ATPase activity was assayed spectrophotometrically, at 22°C and pH 7.4. The medium contained, in mM: 80 NaCl, 15 KCl, 3 $MgCl_2$, 1 K · EGTA, 3 MgATP, 30 Tris · SO_4, 3 $MgSO_4$, 20 HEPES · Na, 0.2 NADH and 3 phospho(enol)pyruvate, in addition to 12 μg/ml of pyruvate kinase and 10 μg/ml of lactate dehydrogenase. The protein concentrations were 5–6 μg/ml for the sarcolemma-enriched fraction and 30–35 μg/ml for homogenates. Rates of ATP hydrolysis were also determined in media containing 0.5 mM ouabain. Unmasking of latent Na^+,K^+-ATPase activity was performed either by inclusion of 0.02% (wt/vol) Lubrol WX in the assay medium or by preincubating the membranes (1.5 mg/ml protein) with 0.8 mg/ml saponin for 20 min at room temperature, with similar results. The Na^+,K^+-ATPase activity was defined as the ouabain-sensitive fraction of the total (that is, patent plus latent) ATPase activity. The opposite sidedness of interaction of the impermeant ligands (ouabain, ATP) with the pump was then employed in order to estimate the orientation of the sealed vesicles (Boumendil-Podevin & Podevin, 1983). For one preparation, the assay was also performed with sarcolemmal membranes preincubated for 20 min at 22°C with alamethicin (1 mg/mg protein) or 5 μM monensin. These samples were then assayed in the presence of 0.5 mM ouabain, 0.5 mM strophanthidin or in the absence of both. Ionophores and strophanthidin were delivered from stock solutions in ethanol; appropriate controls using equal volumes of solvent were used to correct the results. Succinic dehydrogenase activity was assayed as a marker for mitochondria (King, 1967). Protein was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

$[^3H]$-QNB Binding

Specific binding of $[^3H]$-QNB (34.7 Ci/mmol) to muscarinic cholinergic receptors was used as an additional surface membrane marker. Aliquots of the purified membranes (30 μg of protein) or homogenates (100 μg of protein) were added to 5 ml of Ringer's containing 5 nM $[^3H]$-QNB. After 90 min of incubation at room temperature, the bound $[^3H]$-QNB was separated from the free ligand by filtration through Whatman GF/F filters, followed by three washes with 5 ml of ice-cold Ringer's. Nonspecific binding was determined in the presence of 4 μM atropine. Experiments were performed in duplicate. The data are reported as specific (atropine-displaceable) binding.

Uptake Measurements

Uptake of $^{86}Rb^+$ by the sarcolemma-enriched fraction was measured in the presence of an opposing potassium gradient (Garty, Rudy & Karlish, 1983). Small columns of Dowex 50W-X8 were used both to exchange extravesicular cations for N-methyl-D-glucamine (NMG) immediately before the assay (Dowex-NMG form), and to stop the uptake by removing free $^{86}Rb^+$ from the reaction medium (Dowex-Tris form), at the times indicated in Results. Both ion exchange steps were performed under negative pressure (100 mm Hg) (Langridge-Smith & Dubinsky, 1986). The use of negative pressure had two important advantages: first, the vesicles were in contact with the ion exchange column for very short periods of time (elution was complete in 5–10 sec), and second, the recovery of membranes was high and reproducible, so it was not necessary to pretreat the resin with bovine serum albumin. Moreover, the stop columns could be used twice without measurable increases in background counts.

After the resin was washed and converted to the desired form, it was poured into Pasteur pipettes, and equilibrated with the appropriate elution buffer (*see below*). Prior to use, each column was drained under vacuum for 15 sec. For the uptake experiments, membranes (50–100 μl) were diluted with one volume of SKT and kept at room temperature for 10 min. The suspension was aspirated down a Dowex (NMG form) column to remove extravesicular cations, and eluted with 4 volumes of 180 mM sucrose, 10 mM HEPES · NMG, pH 7.4. When required, ouabain was included in this elution buffer. The diluted suspension was mixed with any additional reagents (e.g. KCl, $BaCl_2$, etc.) and the assay was started by addition of $^{86}Rb^+$ (1–5 μCi/ml). At appropriate times, 100 μl aliquots of the mixture were removed, applied to Dowex (Tris form) columns that were placed 5 sec beforehand in a Millipore manifold connected to a vacuum pump, and eluted immediately with 0.9 ml of ice-cold 195 mM sucrose. The columns were removed from the support 20 sec later. The eluate was mixed with 8 ml of scintillation fluid and the amounts of $^{86}Rb^+$ associated with the vesicles were determined. Assays were performed at 22°C. Background values were estimated by replacing vesicles with an equal volume of SKT buffer,

Table 1. Biochemical markers in isolated cardiac sarcolemma[a]

	Specific activity (μmol/mg/hr) or specific binding (pmol/mg)	Purification factor (-fold)	Recovery (%)
Na^+,K^+-ATPase ($n = 6$)	64.9 ± 10.6	18.2 ± 2.5	24.7 ± 3.4
Succinic dehydrogenase ($n = 3$)	6.2 ± 2.9	1.0 ± 0.5	1.4 ± 0.6
[^{3}H]-QNB binding ($n = 4$)	7.1 ± 1.3	23.7 ± 5.7	33.8 ± 7.3
Protein ($n = 6$)	—	—	1.36 ± 0.18

[a] Membrane isolation, determination of enzyme activities and binding of QNB to muscarinic cholinergic sites were performed as described under Materials and Methods. The values for Na^+,K^+-ATPase activity were obtained after permeabilization of sealed vesicles with detergent. The purification factors and recoveries were calculated using the corresponding values for homogenates as reference.

and ranged from 0.004 to 0.01% of the total counts applied to the columns.

Measurement of Internal Volume

Vesicles (80–120 μg of protein per determination) were incubated with 2 μCi of ^3H_2O and 0.3 μCi of [^{14}C]-sucrose for 5 min at 20°C. After centrifugation in a Beckman Airfuge (5 min at 30 psi) the tubes were placed on ice. The supernatants were carefully removed and aliquots taken for scintillation counting. The pellets were dissolved in 1% SDS. Aliquots of the resuspended pellets were taken for scintillation counting and protein determination. Measurements were performed in duplicate and the internal volume was calculated using the distribution volumes of H_2O and sucrose (Forbush, 1982).

Results

Characterization of the Sarcolemma-Enriched Fraction

The protocol developed for isolating sarcolemma from bullfrog heart is simple and can be performed in a relatively short period of time. Table 1 summarizes the characteristics of the preparation. The fraction enriched in surface membrane markers was obtained routinely at high yield, 1.1 ± 0.25 mg of protein per gram of dissected tissue ($n = 13$). Recovery values for the two sarcolemmal markers, namely 33.8% for muscarinic cholinergic binding sites and 24.7% for Na^+,K^+-ATPase activity, are superior to those reported for other methods (Barr et al., 1974; Bers, 1979; Van Alstyne et al., 1980; Phillipson & Nishimoto, 1982). The purification factors are quite high, considering that the procedure consists of five short centrifugation steps with no special purification strategies included. Moreover, the sarcolemmal preparation displays Na^+,K^+-ATPase activity values comparable to those found in preparations characterized by considerably higher purification factors (Jones, Maddock & Besch, 1980; Van Alstyne et al., 1980; Phillipson & Nishimoto, 1982). This may result from the fact that the ratio of nonmuscle cells to myocytes in frog heart tissue is far smaller than in mammalian heart (Barr et al., 1974), and this ratio is presumably preserved in the purified surface membrane fraction as well. This results in higher Na^+,K^+-ATPase activities, since the density of Na^+,K^+-pump sites in the plasma membrane of muscle cells is usually higher than in other cell types (Jorgensen, 1980). The contamination by membranes of mitochondrial origin is minimal, as reflected by the low recovery and purification factor for succinic dehydrogenase activity (Table 1). Because the sarcoplasmic reticulum of frog heart muscle cells is sparse (Sommer & Johnson, 1969; Barr et al., 1974), no attempts were made to quantify enrichment of membranes from this system in the preparation.

Of the total ATPase activity, 51.0 ± 5.1% ($n = 7$) was sensitive to ouabain. The Na^+,K^+-ATPase activity was increased 7.9 ± 2.7-fold ($n = 7$) by detergent permeabilization, implying that, on the average, 87% of the surface membrane vesicles are sealed. This high yield of sealed vesicles may result, in part, from performing the isolation of plasma membranes, including the density gradient separation step, under nearly isotonic conditions. Moreover, we employed Hypaque as a density gradient medium in order to increase the efficacy of separa-

tion of sealed vesicles from leaky fragments (Forbush, 1982). Determinations of the intravesicular volume in terms of differential accessibility to water and sucrose yield a value of 9.85 ± 0.75 μl/mg of protein (n = 3). This value is within the range reported for cardiac sarcolemmal vesicles of mammalian origin using the method employed here, 4.7–18.8 μl/mg (Reeves & Sutko, 1979; Bers, Phillipson & Nishimoto, 1980; Slaughter, Sutko & Reeves, 1983) or by measuring the intravesicular volume of distribution of chloride, 7.5 μl/mg (Schilling et al., 1984).

The sidedness of the sealed vesicles was investigated in seven membrane preparations. The method was based on detergent permeabilization of vesicles and its effects on the total and ouabain-sensitive ATPase activity (*see* Materials and Methods). The data obtained are consistent with 79.6% ± 5.9% of the sealed vesicles being right-side out. This predominance of right-side-out vesicles is commonly observed in cardiac sarcolemma isolated by a variety of procedures (Phillipson & Nishimoto, 1982). For one preparation, the orientation of sealed vesicles was determined by a second, independent method (Caroni & Carafoli, 1983; Frank, Phillipson & Beydler, 1984) which employs monensin and a permeant cardiac glycoside (in this case, strophanthidin) to estimate the proportion of inside-out vesicles. The vesicles were permeabilized with alamethicin instead of detergent to obtain the total activity (Jones et al., 1980). There was no significant difference between the results obtained with the two approaches.

K^+-Gradient-Dependent $^{86}Rb^+$ Uptake into Sarcolemmal Vesicles

In the experiments described below, the uptake of $^{86}Rb^+$ was measured against a large, outwardly directed K^+ gradient. These experimental conditions allow vesicles that are selectively K^+ permeable to accumulate the tracer, thereby magnifying the ratio of transport-mediated uptake to nonspecific binding. Moreover, due to the high intravesicular K^+ concentration, tracer equilibration occurs with a slower time course, permitting manual monitoring of isotope fluxes. The method was originally proposed for measurements of fluxes through ion channels (Garty et al., 1983). However, it is also applicable to the study of fluxes mediated by transporters able to exchange the internal ion for the extravesicular tracer, such as the Na^+,K^+-ATPase (Karlish & Stein, 1982*a*) and the Na^+,K^+,Cl^--transporter (Burnham, Karlish & Jorgensen, 1985). The only modification introduced here is the use of a rapid ion exchange technique to stop the uptake (*see* Materials and Methods).

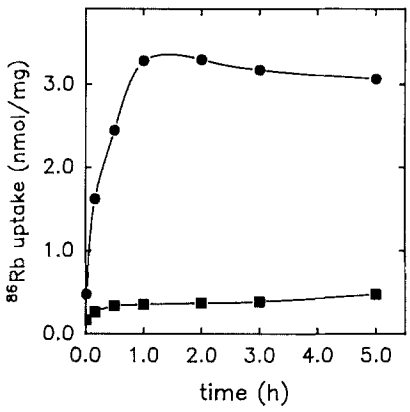

Fig. 1. Time course of $^{86}Rb^+$ uptake by cardiac sarcolemmal vesicles. Vesicles were prepared as described under Materials and Methods, with buffers containing either 50 mM KCl (●) or 50 mM NMG · Cl (■). $^{86}Rb^+$ uptake was monitored at an extravesicular Rb^+ concentration of 30 μM. Data are averages from two experiments

Figure 1 shows that cardiac sarcolemmal vesicles are able to accumulate $^{86}Rb^+$ passively; also illustrated is the effect of the intravesicular K^+ concentration on the time course and extent of isotope uptake. With vesicles prepared to contain 50 mM KCl the tracer uptake is relatively rapid at first, and then slowly approaches a maximum after 1 hr. The tracer associated with the vesicles decays from this maximal level at a very slow rate; this result indicates that collapse of the transmembrane gradient through exit of Cl^- or KCl does not occur at significant rates. This is in agreement with the low Cl^- permeability reported for cardiac sarcolemma (Fozzard & Lee, 1976). In contrast, when the internal K^+ is replaced by NMG^+, the extent of $^{86}Rb^+$ uptake is reduced to very small values. Hence, as expected, cardiac sarcolemmal vesicles respond to the establishment of an outwardly directed K^+ gradient by markedly increasing passive $^{86}Rb^+$ accumulation.

The Effects of Inhibitors of K^+ Transport on $^{86}Rb^+$ Uptake

In intact heart cells, potassium permeates the plasma membrane either passively, through selective ion channels or actively, via the Na^+,K^+-pump. However, Na^+,K^+-ATPase can also operate as a passive Rb^+-Rb^+ exchanger, under conditions similar to those of the experiment displayed in Fig. 1 (Karlish & Stein, 1982*a*). The identity of the transport system(s) responsible for the $^{86}Rb^+$ influx observed was investigated with the help of specific

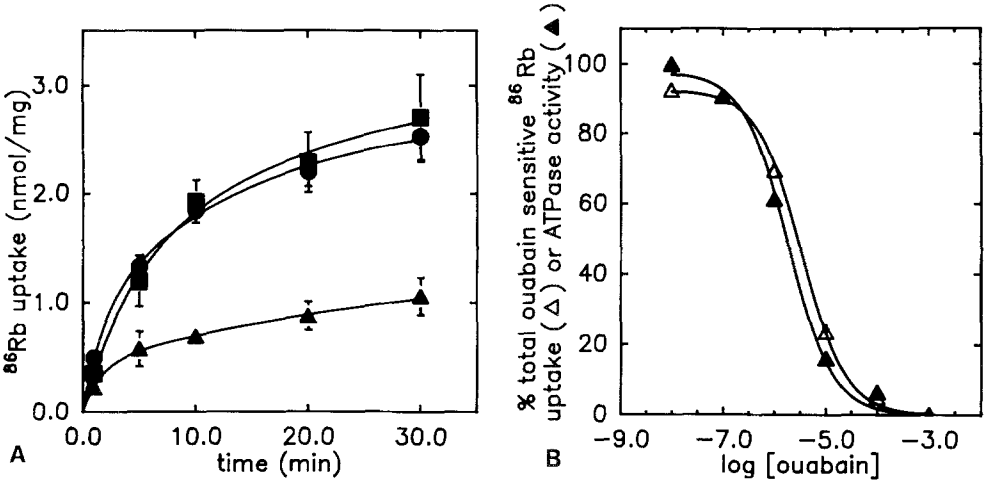

Fig. 2. Effect of Ba^{2+} and ouabain on $^{86}Rb^+$ uptake. (*A*) $^{86}Rb^+$ uptake was measured in the presence of 1 mM $BaCl_2$ (■), 1 mM ouabain (▲) or under control conditions (●). Values are means ± SE from three experiments in two preparations. $[Rb^+]_o$ was 20 μM. (*B*) dose-response curve for ouabain inhibition of Na^+,K^+-ATPase activity (▲) and Rb^+ uptake (△). Na^+,K^+-ATPase was determined as described (Materials and Methods), and $^{86}Rb^+$ uptake was measured after 10 min of incubation. Data are from three experiments where each point was determined in duplicate. In uptake experiments, $[Rb^+]_o$ was 8 μM. Results are expressed as percent of the maximal ouabain-sensitive ATPase activity or Rb^+ uptake, determined in the presence of 2 mM ouabain

blockers of K ion transport pathways. We used Ba ions—which do not affect Na^+,K^+-pump function (Glitsch et al., 1982; Nakao & Gadsby, 1986)—to selectively block potassium channels, and the cardiac glycoside ouabain to selectively inhibit transport through the pump (Schwartz, Lindenmayer & Allen, 1975). Figure 2*A* shows the time course of $^{86}Rb^+$ uptake into cardiac sarcolemmal vesicles in the presence of either 1 mM Ba^{2+} or 1 mM ouabain. While Ba^{2+} does not measurably affect $^{86}Rb^+$ accumulation, both the rate and maximal extent of uptake are greatly reduced by ouabain. The lack of a Ba^{2+} effect suggests that under this particular set of experimental conditions there is no detectable $^{86}Rb^+$ flux through K^+ channels. In contrast, the large inhibition of $^{86}Rb^+$ uptake by ouabain indicates that part of the uptake is mediated by Na^+,K^+-ATPase present in right-side-out sarcolemmal vesicles. Half-maximal block of $^{86}Rb^+$ uptake occurs at a ouabain concentration of 3 μM, a value similar to the half-maximal inhibition constant determined in assays of Na^+,K^+-ATPase activity in this preparation, 1.8 μM (Fig. 2*B*), indicating that the site of cardiac glycoside action is the same in these two instances. Note that ouabain blocks the pump with relatively low affinity, a characteristic of frog heart tissue (Akera, 1984). Also, the inhibitory effects of 1 mM ouabain are not due to decreases in $^{86}Rb^+$ binding, since ouabain does not affect significantly the residual uptake observed in vesicles preincubated with 0.8 mg/ml saponin (Fig. 3) or 1 mg alamethicin/mg protein (*not shown*).

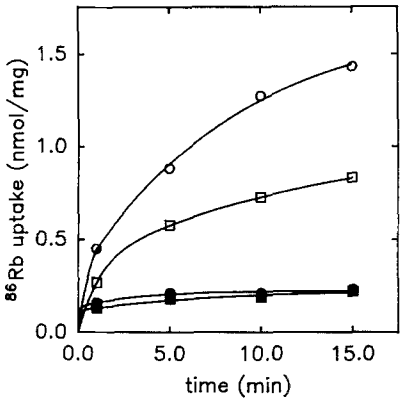

Fig. 3. $^{86}Rb^+$ uptake by permeabilized vesicles. Two aliquots of vesicles (1.5 mg/ml) were incubated for 20 min at room temperature; for one of these the preincubation mixture contained 0.8 mg/ml saponin (filled symbols). Uptake measurements were performed in both sets in the presence (□, ■) or absence (○, ●) of 1 mM ouabain, with an extravesicular Rb^+ concentration of 30 μM

The vesicle preparations exhibited significant ouabain-insensitive fluxes as well. This residual influx might result either from the activity of pumps present in inside-out veiscles or from operation of a different transport system. Since ouabain is impermeant, and binds only to an extracellular site on the enzyme, it would fail to inhibit influx in everted vesicles. Note that permeable cardiac glycosides would not be effective under the conditions of our uptake experiments, since the high intravesicular K^+ concentration precludes their interaction with

Table 2. Inhibition of $^{86}Rb^+$ uptake by orthovanadate[a]

Preincubation conditions	Assay	$^{86}Rb^+$ uptake (nmol · mg^{-1} · min^{-1})	n
1 mM $MgCl_2$	no ouabain	0.185 ± 0.037	8
1 mM $MgCl_2$, 50 μM Na_3VO_4	no ouabain	0.138 ± 0.017	6
1 mM $MgCl_2$	1 mM ouabain	0.032 ± 0.007	6
1 mM $MgCl_2$, 50 μM Na_3VO_4	1 mM ouabain	0.002 ± 0.004	6

[a] Vesicles (1.5 mg/ml) were preincubated for 30 min at 22°C with either 1 mM $MgCl_2$ or with 1 mM $MgCl_2$ and 50 μM Na_3VO_4. Vanadate was added from a 10 mM stock solution made in 300 mM TES (pH 7.4 with KOH); controls contained an equal volume of this buffer. Rb^+ uptake was monitored as described in Materials and Methods with 30 μM $^{86}Rb^+$, in the presence or absence of 1 mM ouabain. Initial rates were determined from uptake values at 0.5, 1.5 and 2.5 min. Values obtained with vesicles permeabilized with saponin have been subtracted. Data are averages from experiments performed with two different membranes preparations (n = number of determinations).

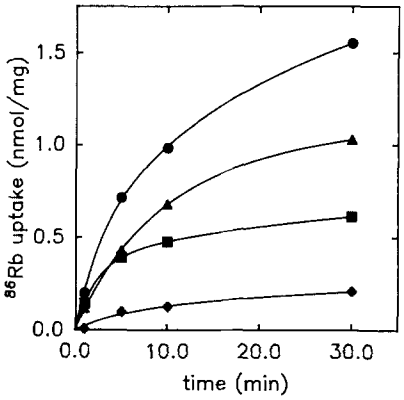

Fig. 4. Inhibition of $^{86}Rb^+$ uptake by Mg ions. Uptake was measured in media containing 1 mM $MgCl_2$ (▲), 1 mM ouabain (■), 1 mM ouabain plus 1 mM $MgCl_2$ (◆) or under control conditions, (●). Data points are means from two experiments in two different preparations. $[Rb^+]_o$ was 15 μM. Uptake measured in vesicles treated with saponin has been subtracted

the binding site (Forbush, 1983). An alternative pump inhibitor is orthovanadate, which acts intracellularly with high affinity in the presence of Mg^{2+} and K^+ (Smith, Zinn & Cantley, 1980). The disadvantages of using vanadate under our assay conditions are that formation of the enzyme-vanadate complex is slow, and it is impaired at the low cytoplasmic Rb^+ concentrations used here, 15–50 μM (Karlish & Stein, 1982*b*). These difficulties were circumvented by preincubating the vesicle suspension, which contains 50 mM K^+, for 30 min with a relatively high concentration (50 μM) of vanadate, in the presence of 1 mM Mg^{2+}. Although prior to the assay the external Mg^{2+} and K^+ are removed and the vanadate is diluted by a factor of 5, the pump-vanadate complex dissociates slowly (Smith et al., 1980; Karlish & Pick, 1981), so that a full inhibition of inside-out pumps should be observable at least during the first minutes of uptake. Results of experiments of this kind are summarized in Table 2, which shows the initial rates of $^{86}Rb^+$ uptake in the presence and absence of ouabain, in vesicles preincubated with Mg^{2+} alone or with Mg^{2+} and vanadate. Treatment with vanadate or ouabain separately leads to partial decreases in the initial rate of ^{86}Rb influx, but the combination of ouabain and vanadate produces complete (99%) inhibition. These results suggest that the portion of the ^{86}Rb influx that is insensitive to ouabain is mediated by Na^+,K^+-pumps present in inside-out cardiac sarcolemmal vesicles.

Effects of Mg^{2+} and ADP

The overall characteristics of the ouabain and vanadate-sensitive $^{86}Rb^+$ influx are consistent with the view that under our conditions the pumps in both everted and right-side-out vesicles are operating as Rb^+-K^+ exchangers. However, vanadate is not a specific inhibitor of the Na^+,K^+-ATPase, so that vanadate inhibition alone is not sufficient to identify flux through inside-out sodium pumps. Fortunately, the general properties of exchange fluxes through Na^+,K^+-ATPase in inside-out vesicles have been studied in detail (Karlish & Stein, 1982*a*,*b*, 1985) and extensively characterized in terms of the effects of ligands such as Mg^{2+}, ATP and phosphate. These findings allow some predictions to be made and tested experimentally in order to confirm the origin of vanadate-sensitive fluxes.

Firstly, Mg ions at the cytoplasmic aspect of the enzyme should inhibit Rb^+-K^+ exchange, an effect that is antagonized by cytoplasmic Rb^+ or K^+ (Karlish & Stein, 1982*a*). Moreover, at millimolar concentrations of Mg^{2+}, phosphate should have no effect on these exchange fluxes (Karlish & Stein, 1982*b*). Confirming these expectations, Fig. 4 shows that 1 mM $MgCl_2$ does indeed reduce $^{86}Rb^+$ uptake in cardiac sarcolemmal vesicles when $[Rb^+]_o$ = 15 μM, and that its effects are additive to those of 1 mM ouabain. Conversely, Mg^{2+} does not affect uptake when the extravesicular K^+ concentration is raised (*see* Fig. 8). Addition of 1 mM inorganic

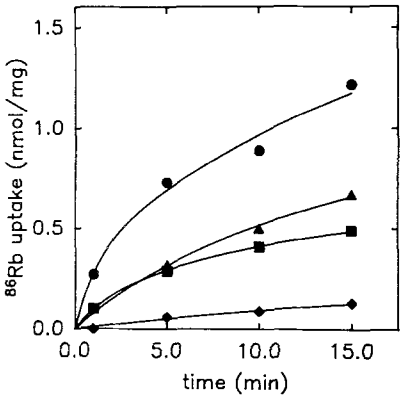

FIg. 5. Inhibition of $^{86}Rb^+$ uptake by ADP. $^{86}Rb^+$ uptake was measured under control conditions (●), or in the presence of 2.5 mM ADP (▲), 1 mM ouabain (■) or 2.5 mM ADP + 1 mM ouabain (◆). $[Rb^+]_o$ was 30 μM. Corresponding values obtained with permeabilized vesicles were subtracted. ADP (K-form) was converted to the NMG form using a Dowex · NMG column. The actual ADP concentration of stock solutions was determined spectrophotometrically

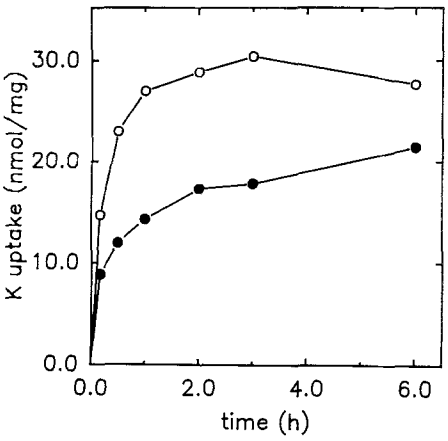

Fig. 6. Ba^{2+} inhibits K^+ uptake at 1 mM K_o^+. Uptake was measured in media containing 1 mM KCl, with $^{86}Rb^+$ as a tracer, in the presence (●) or absence (○) of 1 mM $BaCl_2$

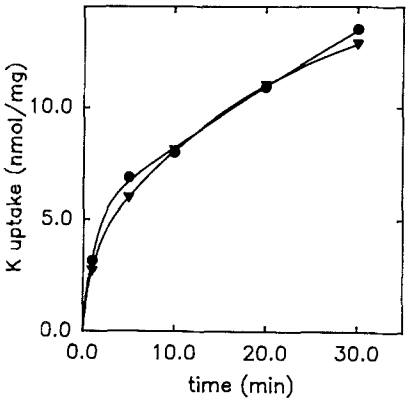

Fig. 7. Effect of Mg^{2+} on K^+ uptake. Uptake was measured in the presence of 1 mM KCl, with $^{86}Rb^+$ as a tracer, in the presence (▼) or absence (●) of 1 mM $MgCl_2$

phosphate had no effect on $^{86}Rb^+$ uptake either in the presence or absence of Mg^{2+} (*not shown*).

Secondly, high concentrations of cytoplasmic ATP (Karlish & Stein, 1982*b*) or ADP (Karlish & Stein, 1985) should inhibit Rb^+-K^+ exchange independently of Mg^{2+}, and this effect should be more pronounced at low cytoplasmic Rb^+ concentrations. Figure 5 shows that 2.5 mM ADP not only partially inhibits $^{86}Rb^+$ flux, but in the presence of 1 mM ouabain virtually abolishes $^{86}Rb^+$ uptake by cardiac sarcolemmal vesicles.

Taken together, the inhibition of $^{86}Rb^+$ uptake by ADP and Mg^{2+}, the absence of phosphate effects, and the sensitivity of uptake to vanadate provide strong support for the hypothesis that at tracer concentrations of Rb_o^+ ouabain-resistant $^{86}Rb^+$ uptake reflects exchange fluxes mediated by Na^+,K^+-pumps located in everted surface membrane vesicles.

Pump-Independent $K^+(Rb^+)$ Uptake Induced by High K_o^+

Figure 6 shows potassium uptake by vesicles containing 50 mM KCl when $[K^+]_o$ = 1 mM. The results are expressed in terms of nmol of K^+ per mg of protein, since in these experiments $^{86}Rb^+$ serves as a K^+ tracer. In contrast to the low $[K^+]_o$ situation (Fig. 2), addition of 1 mM $BaCl_2$ now markedly inhibits K^+ uptake. Note that Ba^{2+} does not affect the uptake measured in vesicles permeabilized with saponin (*not shown*) and therefore its effect can not be ascribed to effects on K^+ binding. Half-maximal block of the uptake occurs at 20 μM $BaCl_2$ (*not shown*). Addition of 1 mM Mg^{2+} has no effect on uptake at 1 mM K_o^+, indicating that the action of Ba^{2+} is specific (Fig. 7).

As we have found that Ba^{2+} does not affect Na^+-K^+ pump-mediated cation uptake, increases in extravesicular K^+ appear to unmask an additional pathway of K^+ uptake into the vesicles. The Ba^{2+}-sensitive inwardly rectifying K^+ channel, known to be present in cardiac sarcolemma (Hume & Giles, 1983; Momose, Giles & Szabo, 1983; Sakmann & Trube, 1984), is the most likely source of this flux component. In electrophysiological experiments, the relationship between the conductance (g) of the

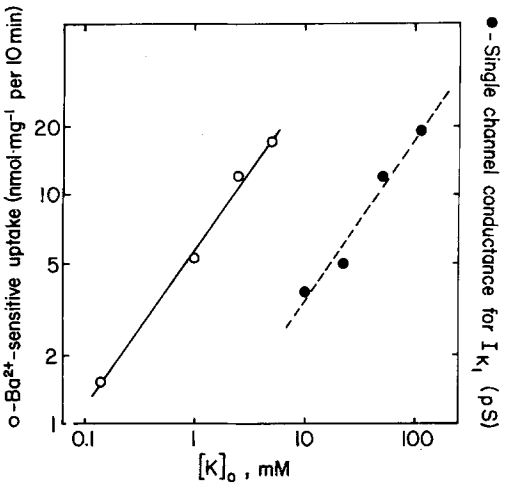

Fig. 8. Dependence of Ba^{2+}-sensitive uptake on $[K^+]_o$. Double-logarithmic plot of Ba^{2+}-sensitive uptake (each point represents the mean of 3–7 experiments) versus $[K^+]_o$ (○) $[BaCl_2]$ = 1 mM. The regression line fitted to the points has a slope of 0.690 (r = 0.997). The data fitted by the dotted regression line (slope = 0.706, r = 0.981) are the single-channel conductances (●) of the inward rectifier of bullfrog atrial cells, determined at various $[K^+]_o$

inwardly rectifying channels and $[K^+]_o$ (at a constant intracellular $[K^+]$) was found to be given by:

$$g = \text{constant} \cdot [K_o]^n \quad (1)$$

where n is approximately 0.5 (Sakmann & Trube, 1984). If Ba^{2+} decreases the amount of K^+ associated with the vesicles by blocking flux through the background K^+ channels, then the Ba^+-sensitive portion of the uptake should show this so-called square-root dependence on the external K^+ concentration. Figure 8 shows a double-logarithmic plot of Ba^{2+}-sensitive K^+ uptake at different $[K^+]_o$. Values for the single-channel conductance of the inwardly rectifying K^+ channel in isolated bullfrog atrial cells (Y. Momose, W. Giles & G. Szabo, *unpublished experiments*) are also shown for comparison. The uptake data points can be fitted to a straight line which has a slope of 0.69, while the slope for the regression line obtained for the single-channel data is 0.706. Thus, the dependence of the Ba^{2+}-sensitive uptake on $[K^+]_o$ supports the hypothesis that this uptake pathway is the inwardly rectifying K^+ channel of cardiac sarcolemma.

Discussion

In this study, we sought to identify the pathways responsible for passive K^+ transport across isolated cardiac sarcolemma. The method used for the flux measurements is extremely sensitive, and permits selective detection of ionic fluxes even when the transport system under study is present in only a small fraction of the vesicle population (Garty et al., 1983). Moreover, the rapid ion exchange technique used to stop the uptake further enhances the resolution of the method. The choice of amphibian heart as a source of tissue is a important feature of this report. Frog heart contains no capillaries or other internal blood vessels and consists predominantly of muscle cells, so contamination of isolated sarcolemma with plasmalemma from other cell types is likely to be smaller than in preparations of mammalian heart. The paucity of sarcoplasmic reticular membranes, as well as the absence of T-tubules in frog cardiac myocytes greatly simplify the isolation procedure, since there is no need to include additional steps to minimize co-purification of these contaminants. The end result of using this particular tissue for sarcolemmal preparations is that highly purified surface membranes can be obtained with a high yield in a short period of time.

Role of the Sodium Pump in Potassium Transport

The present work demonstrates that a large, outwardly directed K^+ gradient promotes $^{86}Rb^+$ uptake into cardiac sarcolemmal vesicles. The finding that ouabain partially blocks $^{86}Rb^+$ influx with a $K_{0.5}$ equal to that observed for ouabain inhibition of Na^+,K^+-ATPase activity in the same preparation implies that the ouabain-sensitive influx is mediated by this enzyme. The sidedness of ouabain binding to Na^+,K^+-ATPase, and its inability to cross membranes indicate that this portion of the influx takes place in right-side-out vesicles. The maximal extent of ouabain-sensitive influx varies between preparations (as seen by comparing experiments shown in Table 2 and Figs. 2–5), but is roughly in agreement with the estimate of 80% right-side-out vesicles.

ATP or phosphate were not included in the membrane isolation media and no efforts were made to inhibit nucleotide degradation or phosphate efflux, so these ligands are not likely to be required for operation of the Na^+,K^+-pump or for its inhibition by ouabain in the experiments described here. In fact, ouabain is able to bind to its site under rather unfavorable conditions, that is, when no specific ligands are added to assay solutions (Forbush, 1983). Furthermore, our results are in agreement with previous observations of significant binding of labeled ouabain to sarcolemmal vesicles isolated from dog heart in the absence of supporting ligands (Wellsmith & Lindenmayer, 1980; Schilling &

Drewe, 1986). Thus, the ouabain-sensitive $^{86}Rb^+$ influx observed probably reflects slow passive Rb^+-K^+ exchange fluxes through the sodium pump, first described for purified enzyme reconstituted in phospholipid vesicles (Karlish & Stein, 1982*a*), and subsequently identified in resealed erythrocyte ghosts (Kenney & Kaplan, 1986; Sachs, 1986).

The low affinity of the frog heart Na^+-K^+-ATPase to cardiac glycosides is disadvantageous in some conditions. Thus, it would be of interest to calculate the kinetic parameters for ouabain-blockable uptake in cardiac SL vesicles. However, increases in the extravesicular Rb^+ (or K^+) concentration beyond 1 mM were found to interfere progressively with the inhibition by ouabain, to the point that no block could be observed at 10 mM Rb_o^+ (*not shown*). This marked antagonism between K^+ and ouabain appears to be a characteristic of forms of Na^+,K^+-ATPase that show low sensitivity to cardiac glycosides (Akera et al., 1985). At $[K^+]_o$ = 1 mM, however, when a blocking effect of Ba^{2+} is evident (Fig. 6), 20% of the K^+ influx is still inhibited by ouabain; as shown in Fig. 9, the effects of these blockers are additive as expected for two independent pathways of uptake. Also, most[2] of the uptake that is not sensitive to either Ba^{2+} or ouabain represents binding, since it is abolished by saponin.

The overall pattern of $^{86}Rb^+$ uptake block by different Na^+,K^+-ATPase ligands leads to the conclusion that, at low external Rb^+ concentrations practically all the influx into KCl-loaded cardiac sarcolemmal vesicles is mediated by this enzyme. Note that the absence of fluxes through other pathways is additional evidence for the high purity of our preparation.

Channel-Mediated Potassium Uptake

Resting heart cells exhibit a K^+-selective permeability resulting from the presence of an ion-conductive channel, the so-called inwardly rectifying or background K^+ channel, I_{K_1}, which should co-purify with sarcolemmal markers. However, our results indicate that when only tracer Rb^+ is present in the extravesicular solution, the fluxes observed are primarily attributable to the sodium pump. There are two features of the background K^+ channel that might explain why its relative contribution to the $^{86}Rb^+$ uptake at submillimolar $[Rb^+]_o$ is negligible. First, channel conductance decreases when extracellular K^+ is decreased (Hume & Giles, 1983; Mo-

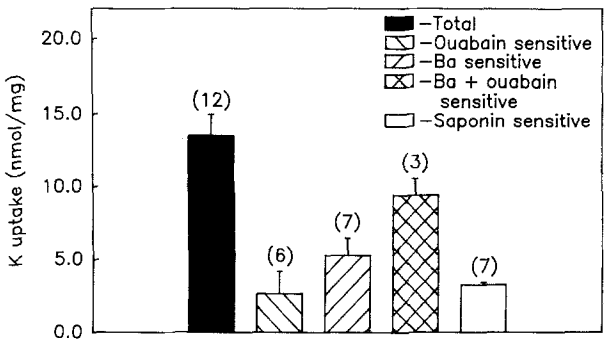

Fig. 9. Inhibition of K^+ uptake by Ba^{2+} and ouabain at K_o = 1 mM. Uptake at 10 min was measured under control conditions, in the presence of 1 mM $BaCl_2$, 1 mM ouabain, 1 mM $BaCl_2$ + 1 mM ouabain, or after saponin treatment (*see* legend to Fig. 3). $[K^+]_o$ was 1 mM. In parentheses are the number of experiments used to calculate averages ± SEM

mose et al., 1983; Sakmann & Trube, 1984). Second, Rb^+ appears to partially block the channel, as evidenced by a significant reduction of inwardly rectifying membrane currents upon total substitution of external K^+ for Rb^+ (2.5 mM) in voltage-clamped bullfrog atrial cells (G.E. Breitwieser, *personal communication*). Note, however, that while Rb^+ can act as a weak blocker for some types of K^+ channels under total substitution conditions, when used at low concentrations and in the presence of excess K^+, it is a less effective blocker (Cohen, Falk & Mulrine, 1983) and can be a convenient tracer of K^+ permeability (Hunter & Nathanson, 1985). As the extravesicular K^+ concentration is increased, the pump-mediated $^{86}Rb^+$ influx is expected to saturate, while the influx through the background K^+ channel should increase with K^+ concentration (up to at least 100 mM K^+). Thus, increasing the external concentration of K^+ (while maintaining $^{86}Rb^+$ at tracer levels), should reveal channel-mediated K^+ uptake traced by $^{86}Rb^+$, a component characterized by its sensitivity to Ba^{2+}, a well-accepted, specific K^+ channel blocker. In addition, Ba^{2+} does not affect the pump-mediated Rb^+-K^+ exchange (*see* Fig. 2) so that the effects of Ba ions observed at high K_o^+/Rb_o^+ ratios should not be tainted by a parallel block of pump fluxes.[3]

[2] The portion of K^+ uptake which is not sensitive to ouabain, Ba^{2+} or saponin amounts to 1.2 ± 0.7 nmol/mg (n = 6) and is probably due to pump fluxes through inside-out vesicles.

[3] Two other K channel blockers, tetraethylammonium chloride (TEA; 20 mM) and 4-amino-pyridine (4AP; 5 mM), were found to affect the pump-mediated $^{86}Rb^+$ uptake measured under the conditions of Fig. 2 (*data not shown*). The inhibitory effects of TEA on the Na^+,K^+-pump have been previously reported (Sachs & Conrad, 1968) and reflect competition between K^+ and TEA. Since antagonism between K^+ and ouabain makes it difficult to determine the extent of pump-related influx when extravesicular $[K^+]_o$ is raised, TEA and 4AP could not be used as specific blockers in order to distinguish channel fluxes from pump exchange fluxes in our preparation.

As seen in Results (Figs. 6–8), the characteristics of the Ba^{2+}-sensitive uptake seem to parallel basic electrophysiological properties of the inwardly rectifying K^+ channel of heart sarcolemma and are therefore interpretable as evidence that the Ba^{2+}-blockable uptake reflects K^+ influx through selective channels into vesicles of sarcolemmal origin.

In summary, the experiments described here demonstrate that potassium influx into cardiac sarcolemmal membrane vesicles can be dissected in two distinct components. Specifically, K^+ transport in this preparation is mediated by the Na^+,K^+-ATPase, and by a system whose properties are indistinguishable from those of the inwardly rectifying K^+ channel of heart cells.

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