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COORDINATE INTERPLAY BETWEEN ($Na^+ + K^+$)-ATPase AND CREATINE PHOSPHOKINASE OPTIMIZES (Na^+/K^+)-ANTIPORT ACROSS THE MEMBRANE OF VESICLES FORMED FROM THE PLASMA MEMBRANE OF CARDIAC MUSCLE CELL *

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Summary

Starting from the discovery of Saks et al. (Saks, V.A., Lipina, N.V., Sharov, V.G., Smirnov, V.N., Chazov, E.I. and Grosse, R. (1977) Biochim. Biophys. Acta 465, 550—558) that the kinetic coupling of ($Na^+ + K^+$)-ATPase and creatine phosphokinase, both residing in the plasma membrane of cardiac muscle cell, renders possible the effective utilization of phosphocreatine for the phosphorylation of ADP produced in ($Na^+ + K^+$)-ATPase turnover, the present paper investigates and evidences the faculty of the coordinate interplay between ($Na^+ + K^+$)-ATPase and creatine phosphokinase to optimize the (Na^+/K^+)-antiport across the membrane of vesicles formed from the plasma membrane of cardiac muscle cell.

(1) The plasma membrane preparation used consists of 13% right-side-out vesicles and 87% inside-out vesicles of which the latter exhibit the catalytic centres of ($Na^+ + K^+$)-ATPase and associated creatine phosphokinase on the extravesicular face. The V values of the two enzymes are similar so that they may show a coordinate interplay under substrate-saturating conditions.

(2) The vesicles are impermeable to ATP and phosphocreatine, but show a relatively high permeability to Na^+ and K^+ so that an inward uphill transport of Na^+ and an outward uphill transport of K^+ can only be traced when their rates exceed the rates of the passive back diffusions of the cations through the vesicle membrane.

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* Dedicated in memory of Professor Karl Lohmann.

(3) With both a 'macroscopic' ATP-regenerating system consisting of phospho*enol*pyruvate and added pyruvate kinase and a 'microscopic' ATP-regenerating system consisting of phosphocreatine and membrane-associated creatine phosphokinase, ($Na^+ + K^+$)-ATPase is shown to effectuate uphill (Na^+/K^+)-antiport. The ratio of transported Na^+ to transported K^+ amounts to 2.8 : 1 under the ion conditions chosen (initially to 40 mM Na^+ and K^+ in the extra- and intravesicular solution).

(4) With both the macroscopic and the microscopic ATP-regenerating systems, the same V values of (Na^+/K^+)-antiport are reached, but 20 mM ATP or 0.3 mM ATP, respectively is required to maximize transport rates. This disparity is rationalized within the framework of present knowledge about low or high effectivities of sequentially working enzymes when they cooperate either physically separated as a bulk reaction-diffusion system or physically associated in an enzyme cluster as an integrated unit of catalytic activity.

Introduction

As generalized by Kedem and Kaplan [1], the efficiency of an energy-conversion device depends on the degree of coupling between energy-input and energy-output processes as well as on the conditions of operation, but the maximal efficiency and the efficiency at maximal output are uniquely determined by the degree of coupling. Reversible energy conversion with an efficiency of 100% is a limit towards which only fully coupled systems can tend at infinitesimal rates of the output process.

The energy interconversion in (Na^+/K^+)-antiport effectuated by ($Na^+ + K^+$)-ATPase is characterized by two interdependent features, high thermodynamic efficiency and reversibility, which enable the (Na^+/K^+)-antiporter system to interconvert Gibbs energy of ATP and Gibbs energies of Na^+ and K^+ gradients over the plasma membrane (reviewed in Refs. 2 and 3). The 80% efficiency found with the (Na^+/K^+)-antiporter system implies that the system works not far from the reverse driving region, and that the energy-input and energy-output processes are effectively coupled (cf. Refs. 1—3). In fact, in erythrocytes, the total Gibbs energy calculated from the concentrations of the five effectors of the (Na^+/K^+)-antiporter system appears to lie not far from thermodynamic equilibrium so that, besides net (Na^+/K^+)-antiport, also ATP synthesis should occur and the observed transport rate should be well below the theoretic maximum faculty [3].

The latter evaluation, however, was called into question by the discovery of Saks et al. [4] that the kinetic coupling of ($Na^+ + K^+$)-ATPase and creatine phosphokinase, both residing in the plasma membrane of cardiac muscle cell, is able to maintain a very low concentration of ADP produced in ($Na^+ + K^+$)-ATPase turnover. Apparently, were this effective utilization of phosphocreatine for immediate rephosphorylation of ADP to work also in the (Na^+/K^+)-antiporter system, this would optimize the transport function of ($Na^+ + K^+$)-ATPase via suppression of its reverse function as ATP synthase. The present paper shows that the coordinate interplay between ($Na^+ + K^+$)-ATPase and creatine phosphokinase accelerates considerably, at physiological ATP concen-

trations, the net rate of (Na^+/K^+)-antiport across the membranes of vesicles formed from the plasma membrane of cardiac muscle cell.

Methods

Preparation of plasma membrane vesicles

Plasma membranes of guinea-pig heart muscle cells were isolated by a modification of the method described by Kidway et al. [5]. All steps were carried out between 0 and 5°C. The preparation medium was composed of 0.4 μM tetrodotoxin, 30 mM sodium acetate, 30 mM potassium acetate, 0.3 M sucrose and 20 mM imidazole/acetic acid buffer, pH 7.4. The myocardium was cut into small pieces, placed into the preparation medium and homogenized, first with a Teflon-glass homogenizer at 1450 rev./min until no further breakup could be reached, and second with an Ultraturrax (type 18/2N) at 20 000 rev./min for 5 s. The final homogenate was filtered through gauze cloth and centrifuged at 100 000 × *g* for 30 min. The suspension of the sediment in the preparation medium was layered on the top of a sucrose density gradient and centrifuged exactly as described in Ref. 5. The yellowish band lying at the interface between the loading medium and the sucrose gradient (density 1.13 g/cm^3) was collected by slow aspiration of the material. After dilution with preparation medium, this material was centrifuged at 13 000 × *g* for 15 min to sediment trapped mitochondria. The supernatant was centrifuged at 100 000 × *g* for 30 min. The pellet designated as membrane fraction FI was suspended in a medium composed of 0.8 M sucrose, 20 mM imidazole/acetic acid buffer, pH 7.4, 30 mM sodium acetate and 30 mM potassium acetate.

The FI suspension was layered over a linear Ficoll gradient which was prepared in 0.9 M sucrose by mixing 3.85% Ficoll (d = 1.115 g/cm^3) and 10.4% Ficoll (d = 1.151 g/cm^3) in a 11 300 Ultrograd Gradient Mixer/LKB and centrifuged at 100 000 × *g* for 1 h. The material of the colourless band lying at the top of the gradient was collected, diluted with preparation medium to a final sucrose concentration of 0.6 M, and centrifuged at 100 000 × *g* for 45 min. The sediment, designated as membrane fraction FII, was suspended in 0.6 M sucrose, 20 mM imidazole/acetic acid buffer, pH 7.4, 30 mM sodium acetate, 30 mM potassium acetate, 0.2 mM EDTA, 0.3 mM dithiothreitol and 1 μM tetrodotoxin. The described solution, termed incubation medium, was used for the determination of enzymatic activities, and in the studies on cation movements. Fraction FII will be shown in Results to consist of vesicles and will thus also be designated as vesicle preparation.

Determination of enzymatic activities

The ATPase activities were determined at 30°C by means of the coupled-enzyme test system described by Schwartz [6]. The reaction medium (final volume 1 ml) was composed of the incubation medium (isotonic condition) or of the incubation medium without 0.6 M sucrose (hypotonic condition) plus additionally 0.14 mM NADH, 3 mM ATP, 5 mM $MgCl_2$, 0.8 mM phospho*enol*-pyruvate, 2 I.U. pyruvate kinase and 2 I.U. lactate dehydrogenase. The reaction was started by the addition of the FI or FII suspension (8—12 μg protein/ml). The rate of NADH dehydrogenation reflecting the rate of ATP hydrolysis

to ADP and P_i was followed by recording the change of absorbance at 340 nm in a Beckman DK2A spectrophotometer. The activities of fractions FI and FII were concurrently measured in the absence of specific inhibitors of $(Na^+ + K^+)$-ATPase yielding total ATPase activity, and in the presence of either 0.5 mM digitoxigenin (for the isotonic condition) or 0.5 mM ouabain (for the hypotonic condition) yielding the inhibitor-insensitive, Mg^{2+}-dependent ATPase activity. The difference between the two activities showed the $(Na^+ + K^+)$-ATPase activity.

The eventual presence of a Ca^{2+}-dependent ATPase was checked under otherwise similar conditions by looking for a difference in the rates of ATP hydrolysis in the additional presence of either 100 μM $CaCl_2$ or 0.5 mM EGTA.

The creatine phosphokinase activities were assessed at 30° C by the reverse reaction as described by Saks et al. [4]. The reaction medium (final volume 1 ml) was composed of the incubation medium (with or without 0.6 M sucrose) plus additionally 0.6 mM NADP, 20 mM glucose, 3.3 mM $MgCl_2$, 0.3 mM dithiothreitol, 5 mM AMP, 2 I.U. hexokinase, 2 I.U. glucose-6-phosphate dehydrogenase, 1 mM ADP, 15 mM phosphocreatine and 20—40 μg FI or FII protein. The reaction was started by the addition of phosphocreatine and followed by recording the absorbance change at 340 nm. The rate of phosphocreatine splitting was measured, in principle, as described earlier [4] but using the incubation medium to dissolve the various ingredients of the determination procedure.

Determination of cation movements

The movements of Na^+ and K^+ between the extravesicular and intravesicular space were followed by means of the tracer method using $^{22}Na^+$ or $^{86}Rb^+$ of which the latter is known to behave like $^{42}K^+$ [7]. Throughout the changes in the radioactive isotope, contents of the vesicles were determined. The standard procedure involved the following steps.

Aliquots of 8 μCi of carrier-free $^{22}NaCl$ dissolved in HCl, and $^{86}RbCl$ dissolved in H_2O were added to conical tubes and taken to dryness by maintaining them at 90° C for several hours. The tubes were put on crushed ice and fed with 2.1 ml ice-cold incubation medium containing the vesicle preparation (0.3 mg protein/ml). The tubes were immediately vortex mixed to dissolve $^{22}Na^+$ or $^{86}Rb^+$, thus initially yielding an isotope gradient from extra- to intravesicular space. After 30 min incubation near 0° C to reach isotope equilibrium, the tubes were transferred to a water bath to warm up the vesicle suspension to 30° C within 2 min. The reaction medium was then completed by adding the ingredients to be tested for their faculty to change the radioactive isotope contents of the vesicles or to drive uphill transports of Na^+ and K^+ across the vesicle membrane.

After various time intervals, 0.4 ml aliquots were removed from the tubes and the cation movements terminated by rapid separation of the vesicles from the extravesicular medium by means of vacuum filtration through Millipore filters, type 6S (0.22 μm pore size) or Gelman filters, type 6A-8 (0.2 μm pore size) with the aid of a Millipore filter holder. The filters were sucked dry and washed with 0.2 ml of an ice-cold solution composed of 0.6 M sucrose, 20 mM imidazole/acetic acid buffer, pH 7.4, 30 mM sodium acetate and 30 mM

potassium acetate. They were then transferred into scintillation vessels. After adding 1 ml H_2O and 10 ml scintillation fluid, the radioactivity trapped in the vesicles was measured by means of an LKB liquid scintillation spectrophotometer. Although the filters had been pretreated with aqueous solutions of NaCl or RbCl (100 mM each) to minimize and equalize the radioactive isotope binding to the filter materials, there remained a filter blank varying by ±20% which was subtracted from the measured total radioactivity.

The modifications of the described standard procedure and the details of various control experiments are specified in the legends to Figs. 2—6 and in Results.

General information

The protein contents of the vesicle suspensions were determined as described in Ref. [8]. Altogether, 80 vesicle preparations were isolated and tested in studies on cation movements across the vesicle membrane. All measurements were carried out in duplicate or triplicate. Figs. 2—5 show representative results.

Characteristics and sources of materials

^{86}RbCl (1—12 mCi/mg) was obtained from Amersham (U.K.); ^{22}NaCl (54 mCi/mg) from Rotop (Dresden, G.D.R.); suprapure NaCl and KCl from Merck (Darmstadt, F.R.G.); ATP (imidazole salt), ADP and phosphocreatine (sodium salts) from Reanal (Budapest, Hungary); NADP, phospho*enol*pyruvate (sodium salt) and hexokinase from Sigma (St. Louis, U.S.A.); tetrodotoxin from Sankyo (Tokyo, Japan); NADH from Calbiochem (U.S.A.); AMP, pyruvate kinase and lactate dehydrogenase from Boehringer (Mannheim, F.R.G.).

Results

Characterization of membrane preparation

Electron-microscopic examination of thin sections of plasma membrane preparations shows that the membrane preparation of cardiac muscle cell essentially consists of membrane-enveloped vesicles with diameters of 1000—2000 Å (Fig. 1). Mitochondria appear to be absent. The intactness and tightness of the vesicles preserved under isotonic conditions are evidenced by the demonstration of uphill cation transports shown below. A significant admixture of vesicles formed from sarcoplasmic reticulum seems unlikely because a Ca^{2+}-dependent ATPase activity cannot be traced. Rather, the vesicles appear to be formed from the plasma membrane, since they hold the plasma membrane marker enzyme, ($Na^+ + K^+$)-ATPase (Table I), and effectuate (Na^+/K^+)-antiport (Figs. 3 and 4), known to involve the plasma membrane of cardiac muscle cell.

The vesicle fraction obtained after Ficoll gradient centrifugation (FII) shows higher activities of both ($Na^+ + K^+$)-ATPase and creatine phosphokinase (Table I); therefore, this fraction was used for all further studies. The rates of these two reactions are similar so that ($Na^+ + K^+$)-ATPase and creatine phosphokinase may show, under substrate-saturating conditions, a coordinate interplay in driving (Na^+/K^+)-antiport (cf. below).

In principle, the preparation may consist of a mixture of inside-out vesicles

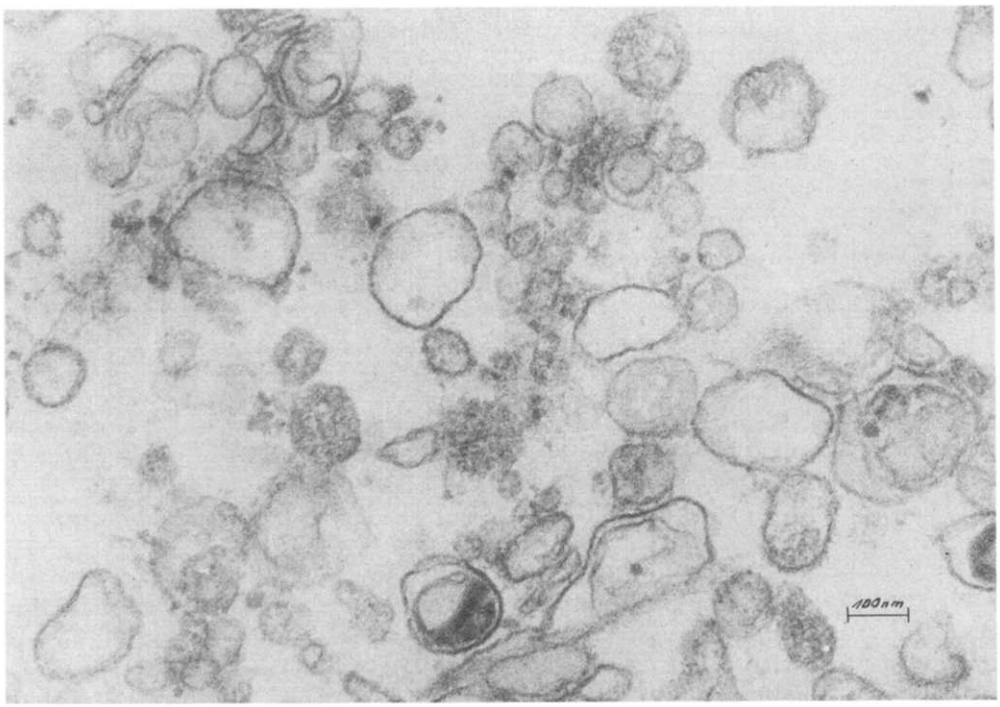

Fig. 1. Electron-microscopic picture of a thin section of plasma membrane fraction FII obtained from guinea-pig heart muscle after Ficoll gradient sedimentation. The FII pellet was fixed in glutaraldehyde, post-fixed in OsO_4, and embedded in Epon [9]. Thin sections were cut with an LKB Ultrotom. After staining with uranyl acetate and lead citrate, the section was examined in a Jeol JEM-100B electron microscope. Magnification, ×63 000.

($(Na^+ + K^+)$-ATPase projects its catalytic centre for MgATP and its combining site for the inhibitor, ouabain, to the extravesicular and intravesicular space, respectively) and of right-side-out vesicles (the mentioned sites face the reverse spaces). Due to high polarity, ATP, phosphocreatine and ouabain cannot penetrate the vesicle membrane within the short experimental period chosen. Hence, the easiest way to determine the sidedness of the vesicles and to obtain MgATP-supported, unidirectional antiport of Na^+ and K^+ across the vesicle membrane is to add MgATP or MgATP plus phosphocreatine, and if necessary ouabain, to the isotonic incubation medium in which the vesicles are suspended.

The barrier properties and ATPase activities of the vesicles are characterized by the following findings. Under isotonic conditions, the presence of 0.25 mM ouabain, sufficient to suppress the $(Na^+ + K^+)$-ATPase activity of non-vesicular preparations of the same source, does not reduce total ATPase activity (sum of Mg^{2+}-dependent ATPase and $(Na^+ + K^+)$-ATPase activities) which shows that the vesicles are not permeable to ATP and ouabain as well as showing that non-vesicular plasma membrane fragments holding $(Na^+ + K^+)$-ATPase molecules are absent. However, the presence of 0.25 mM digitoxigenin rapidly penetrating the vesicle membrane reduces the total ATPase activity by about 40—50%

TABLE I

ATPase AND CREATINE PHOSPHOKINASE ACTIVITIES IN VESICULAR PLASMA MEMBRANE FRACTIONS OF GUINEA-PIG HEART MUSCLE CELL

The activities of the fractions obtained without (FI) or after Ficoll gradient sedimentation (FII) were measured under the standard conditions described in Methods. Hypotonicity was secured by omission of sucrose from the buffered incubation medium. The numbers refer to the arithmetical mean, the standard deviation, and the number of determinations (in parentheses). Activities are expressed as μmol P_i/mg protein per h.

Fractions and conditions	Total ATPase	Activities		
		Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-ATPase	Creatine phospho-kinase
FI isotonic	92 ± 13 (10)	62 ± 2 (10)	30 ± 2 (10)	24 ± 8 (10)
FII isotonic	101 ± 20 (22)	56 ± 14 (20)	45 ± 14 (20)	47 ± 16 (15)
FII hypotonic	121 ± 40 (5)	70 ± 19 (5)	51 ± 19 (5)	53 ± 20 (3)

corresponding, to the inhibitor-sensitive $(Na^+ + K^+)$-ATPase activity (Table I). After disruption of the continuity of vesicle membrane by exposure to the hypotonic suspension medium (Table I), or to sodium cholate (0.2 mg/0.03 mg protein per ml), also ouabain reduces total ATPase activity by about 40—50%, as found with digitoxigenin under isotonic and hypotonic conditions. In other words, 50—60% of the total ATPase activity is derived from the inhibitor-insensitive, Mg^{2+}-dependent ATPase which is not involved in driving uphill (Na^+/K^+)-antiport as will be shown below. This makes it unreasonable to refer the stoichiometries of transported cations to the molecules of hydrolyzed ATP.

The sidedness of the vesicles is quantified in the following way. The change of the vesicle suspension medium from isotonicity to hypotonicity increases the activities of Mg^{2+}-dependent ATPase, $(Na^+ + K^+)$-ATPase and creatine phosphokinase on average by 13% (Table I). This indicates that the percentages of right-side-out or inside-out vesicles — with catalytic centres of $(Na^+ + K^+)$-ATPase and creatine phosphokinase on the intravesicular or extravesicular face — are approx. 13 and 87%, respectively. In line with these deductions, MgATP or MgATP plus phosphocreatine added to the extravesicular space will be shown to promote uphill transport of Na^+ from the extra- to the intravesicular space and the uphill transport of K^+ from the intra- to the extravesicular space. This is the opposite of the physiological situation in which MgATP and phosphocreatine are in the intracellular space, and uphill transports of Na^+ and K^+ proceed from inside to outside and outside to inside of the plasma membrane, respectively.

Cation movements through vesicle membrane

The movements of Na^+ and K^+ between extra- and intravesicular space are followed by means of $^{22}Na^+$ and $^{86}Rb^+$ as tracers. $^{86}Rb^+$ is known to behave like $^{42}K^+$ in similar studies [7] but it lives longer than $^{42}K^+$ and thus is more convenient to use. The experiments are carried out at 30°C using always a single vesicle preparation for the comparative exploration of a whole set of experimental variables so that, in parallel in separate batches, Na^+ movements,

MgATP- or MgATP plus phosphocreatine-supported Na^+ accumulation in the vesicles, and K^+ movements, and MgATP- or MgATP plus phosphocreatine-supported K^+ extrusion from the vesicles are measured. The uphill Na^+ or K^+ transports are referred to as (Na^+/K^+)-antiport, since they are known to be coupled (cf. Ref. [7]).

The correct demonstration of uphill transports requires the start to occur at radioactive isotope equilibrium. At 0°C, the equilibration of extravesicularly added $^{22}Na^+$ or $^{86}Rb^+$ with the intravesicular space by passive diffusion through the membrane needs about 10 and 15 min, respectively (Fig. 2). Under standard conditions, the isotope equilibration period is extended to 30 min for reasons of certainty. The isotope equilibrium remains unchanged during the 2 min period of warming up the vesicle batch from 0 to 30°C (cf. the data points at zero time and at 2 min in Fig. 3), although the permeability of the vesicle membrane for Na^+ and K^+ dramatically increases at elevated temperatures. Indeed, at 30°C, the isotope equilibrium is reached before any measurement can be carried out, i.e., within less than 10 s (not shown).

Before starting the transport experiments, 30 mM Na^+ and 30 mM K^+ are present in both the extra- and intravesicular space. The addition of phospho*enol*pyruvate or phosphocreatine, used as sodium salts, increases the Na^+ concentration. However, the isotope equilibrium is not changed after elevation of the Na^+ concentration from 30 to 40 mM. To equalize the conditions in the transport studies, all additions after the 2 min warming up are designed to adjust the Na^+ concentration to 40 mM. By virtue of the mentioned rapid cation equilibration at 30°C, the initial concentrations in the extra- and intravesicular space are taken to be 40 mM Na^+ and 30 mM K^+. Calculated on the basis of the specific radioactivities of the tracers, the intravesicular Na^+ and K^+ contents of the vesicles amount to 120 nmol Na^+ and 104 nmol K^+ per mg vesicle protein.

The rapid cation equilibration at 30°C implies that the MgATP-supported, uphill cation transports across the vesicle membrane can only be traced when

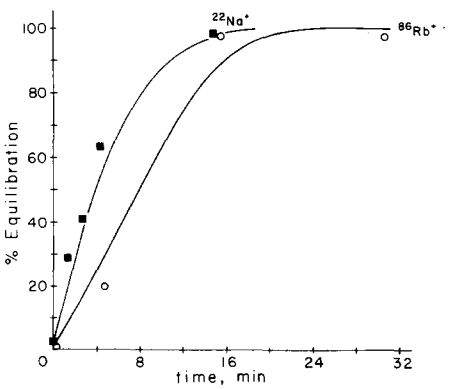

Fig. 2. Time course of passive equilibration (absence of ATP and phosphocreatine) of $^{22}Na^+$ (■———■) and $^{86}Rb^+$ (○———○) between suspension medium and the space enclosed by plasma membrane at 0°C. 30 mM Na^+ and 30 mM K^+ were present in the intravesicular space and the extravesicular space to which the isotopes were added.

their rates exceed the rates of the passive back diffusions of the cations through vesicle membrane. This statement forms the basis for the interpretation of the experimental data on the cation transport faculties of the vesicles to be described in the next section.

Uphill cation transports across vesicle membrane

The intravesicular Na^+ and K^+ contents, present in cation equilibrium with the extravesicular space, remain unchanged in the additional presence of Mg^{2+}, ATP and phosphocreatine when added separately (not demonstrated). The presence of 0.3 mM MgATP is also insufficient to change the Na^+ and K^+ contents of the vesicles (cf. the data points at zero time and 2 min in Fig. 4). Hence, we decided to maximize the rates of ATP-driven cation transports by maximizing the ATP supply in the following two ways.

First, 0.3 mM MgATP is applied together with 5 mM phosphocreatine, yielding in combination with the membrane-associated creatine phosphokinase (cf. Table I), the 'microscopic' ATP-regenerating system. The indicated concentrations of ATP and phosphocreatine were chosen, since they are known to afford the maximum rate of creatine formation in the coupled reactions of $(Na^+ + K^+)$-ATPase and creatine phosphokinase [4]. Second, higher MgATP concentrations are applied together with 5 mM phospho*enol*pyruvate and 32 I.U. per ml pyruvate kinase, yielding the 'macroscopic' ATP-regenerating system. The activity of added pyruvate kinase is 30-fold in excess over total ATPase activity so that rapid phosphorylation of ADP released from the ATPases into the extravesicular medium should be warranted.

Na^+ accumulation in the vesicles is obtained with both the microscopic and the macroscopic ATP-regenerating systems, but they differ remarkably in the MgATP concentrations needed to maximize Na^+ uptake (Fig. 3). With the microscopic ATP-regenerating system, already 0.3 MgATP produces maximum rates and peak levels of Na^+ accumulation. The finding that the increase in MgATP concentrations from 0.3 to 2 mM does not increase the net changes of intravesicular Na^+ and K^+ contents (not demonstrated) is in line with the mentioned kinetic characteristics of the coupled reactions of $(Na^+ + K^+)$-ATPase and creatine phosphokinase [4]. The MgATP concentration saturating $(Na^+ + K^+)$-ATPase when coupled with creatine phosphokinase in driving cation transports (0.3 mM) is insufficient to saturate non-coupled, non-vesicular and thus not uphill-transporting $(Na^+ + K^+)$-ATPase, of which the K_m (MgATP) value lies near 0.3 mM [10]. In contrast to the linear rate of creatine formation observed in the coupled reactions of $(Na^+ + K^+)$-ATPase and creatine phosphokinase (inset to Fig. 3), the net Na^+ uptake of the vesicles increasingly levels off (Fig. 3). This implies that the inward transport of Na^+ into vesicles becomes more and more compensated for by the outward passive back diffusion of Na^+ into the extravesicular space through the leaky vesicle membrane.

With the macroscopic ATP-regenerating system, 2 mM MgATP although affording maximum activity of non-coupled, non-vesicular $(Na^+ + K^+)$-ATPase effects but moderate Na^+ uptake in the vesicles. Only when 20 mM MgATP is applied, do the rates and amplitudes of intravesicular Na^+ accumulation become similar to those reached with 0.3 mM MgATP and the microscopic ATP-regenerating system (Fig. 3).

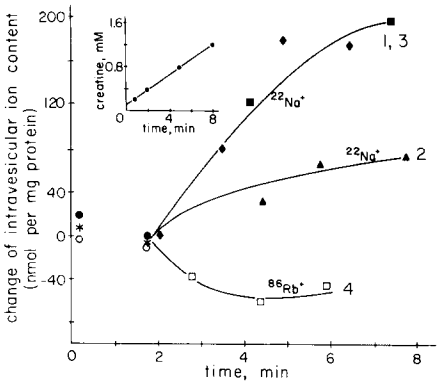

Fig. 3. MgATP- or MgATP plus phosphocreatine-effected changes in intravesicular contents of Na^+ and K^+ as traced by means of $^{22}Na^+$ and $^{86}Rb^+$. At the start of the reaction, the intravesicular cation contents were calculated to amount to 120 nmol Na^+ and 104 nmol K^+ per mg vesicle protein set to be zero in Figs. 3 and 5. The intravesicular Na^+ contents remained constant during the 2 min period of warming up the vesicle batch from 0 to 30°C (●,*) and during the following 4 min incubation at 30°C (not shown). They increased in the presence of 0.3 mM MgATP and 5 mM phosphocreatine (curve 1, ■———■); 2 mM MgATP, 32 I.U. pyrivate kinase and 5 mM phospho*enol*pyruvate (curve 2, ▲———▲); or 20 mM MgATP, 32 I.U. pyruvate kinase and 5 mM phospho*enol*pyruvate (curve 3, ♦———♦). The intravesicular K^+ contents remained constant during the 2 min warming up period (○) and during the following 4 min incubation at 30°C (not shown). They decreased in the presence of 0.3 mM MgATP and 4 mM phosphocreatine (curve 4, □———□), but remained unchanged in the presence of 2 mM MgATP, 32 I.U. pyruvate kinase and 5 mM phospho*enol*pyruvate (not shown). The concentration of magnesium acetate was always 2 mM in excess over the concentration of ATP so that no uncomplexed ATP was present. In this figure and in all similar experiments, the standard deviation of data points determined in duplicate or triplicate amounted to ±11% of the arithmetical mean. The differences between the MgATP- and the MgATP plus phosphocreatine-effected changes in the intravesicular contents of Na^+ and K^+ were statistically evident at the 5% level in all experiments carried out in parallel on the same vesicle preparation for which Fig. 3 is representative. Inset: linearity of creatine formation under conditions as applied in the transport studies with 0.3 mM MgATP and 5 mM phosphocreatine. Per unit time, the molar amounts of P_i produced (not shown) by ($Na^+ + K^+$)-ATPase, and of creatine produced by creatine phosphokinase were the same, evidencing tight kinetic coupling of both enzymes.

With both ATP-regenerating systems and suitable MgATP concentrations, the intravesicular Na^+ contents are raised from 120 to, on average, 320 nmol Na^+ per mg vesicle protein (Fig. 3), i.e., by a factor of 2.7. This implies that the intravesicular Na^+ concentration increases from initially 40 to 108 mM at the steady-state peak level resulting from inward Na^+ transport and outward Na^+ back diffusion. In other words, an uphill Na^+ transport is realized. Compared to Na^+ transport into the vesicles, the K^+ extrusion from the vesicles is more difficult to trace because of the small, rapidly exhaustible K^+ contents of the vesicles. Thus, with the macroscopic ATP-regenerating system and 2 mM MgATP, the equilibrium concentration of intravesicular K^+ remains unchanged (not demonstrated). However, with the microscopic ATP-regenerating system, 0.3 mM MgATP reduces the K^+ contents of the vesicles from 104 to, on average, 44 nmol K^+ per mg vesicle protein (Fig. 3). This implies that the K^+ concentration, being initially 30 mM in both the intra- and extravesicular space, is decreased to 13 mM in the vesicles, i.e., an uphill K^+ transport is realized.

Calculated from the mean values of the maximal increment of intravesicular Na^+ contents and of the maximal decrement of intravesicular K^+ contents reached with 0.3 mM MgATP and the microscopic ATP-regenerating system

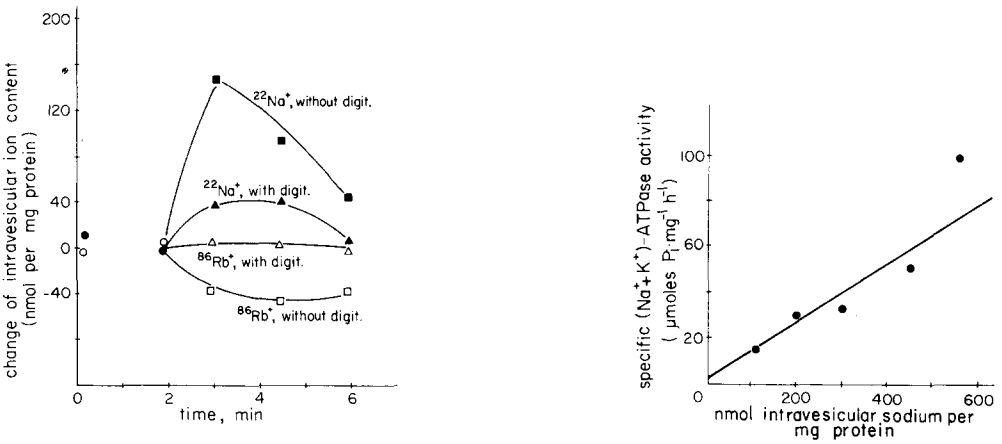

Fig. 4. Digitoxigenin inhibition of MgATP plus phosphocreatine-effected changes in intravesicular contents of Na^+ and K^+ as traced by $^{22}Na^+$ and $^{86}Rb^+$. The conditions for studying the ion transports were similar to those described in Fig. 3 except that the vesicle suspension before starting transport reactions was preincubated for 2 min at 30°C with 0.3 mM MgATP in the absence or presence of 0.5 mM digitoxigenin. This MgATP concentration was sufficient to promote inhibitory interaction of digitoxigenin (digit.) with $(Na^+ + K^+)$-ATPase but insufficient to produce net changes of intravesicular contents of Na^+ (●) and K^+ (○) both in the absence and presence of digitoxigenin. However, the presence of 0.3 mM MgATP plus 5 mM phosphocreatine effected both Na^+ uptake (■———■) which digitoxigenin strongly inhibited (▲———▲), and K^+ extrusion (□———□) which digitoxigenin annulled (△———△).

Fig. 5. Relationship between specific activities of $(Na^+ + K^+)$-ATPase and peak levels of Na^+ accumulation as found with five different vesicle preparations. The Na^+ transport was driven by 0.3 mM MgATP plus 5 mM phosphocreatine. The further experimental conditions were the same as detailed in the legend to Fig. 3.

(Fig. 3), the ratio of transported Na^+ to transported K^+ amounts to 2.1 : 1 *. Similar coupling ratios are known (reviewed in Ref. 11) for frog striated muscle (3 Na^+ : 1 K^+ or 5 Na^+ : 1 K^+) and squid axon (3 Na^+ : 1 K^+ at 40 mM intracellular Na^+). The coupling ratio found by us with plasma membrane vesicles of cardiac muscle cell suggests that the (Na^+/K^+)-antiport in this tissue is electrogenic. Indeed, there is extensive evidence that the pump in Na^+-loaded heart muscle cell is electrogenic [11].

The proper transport capacities of some vesicle preparations are not capable of being assessed. In such cases, the initial increase in intravesicular Na^+ contents is followed by more or less rapid decreases so that no steady-state level is reached (an example is depicted in Fig. 4). The change of intravesicular K^+ contents can also show biphasic kinetics (not demonstrated). Since with these vesicle preparations, also the creatine formation in the coupled reactions of $(Na^+ + K^+)$-ATPase and creatine phosphokinase proceeds with an unchanged rate during the whole experimental period, the observed decay of transport capacities cannot be caused by uncoupling or denaturation of the two enzymes, but must be produced by an increase in the cation permeabilities of the vesicle membrane.

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* In this estimate, the vesicle membrane is assumed to show a similar leakiness for Na^+ and K^+ (cf. Fig. 2).

All vesicle preparations contain besides ($Na^+ + K^+$)-ATPase also an inhibitor-insensitive, Mg^{2+}-dependent ATPase (Table I). The latter enzyme, however, is not involved in the MgATP-driven (Na^+/K^+)-antiport as evidenced by the following two independent arguments. First, the uphill Na^+ and K^+ transports become suppressed by digotoxigenin (Fig. 4), known to inhibit solely ($Na^+ + K^+$)-ATPase. Second, the Na^+ transport capacities found with the microscopic ATP-regenerating system, and the specific ($Na^+ + K^+$)-ATPase activities — both varying considerably with the different vesicle preparations — are linearly correlated (Fig. 5). Hence, (Na^+/K^+)-antiport is effectuated solely by ($Na^+ + K^+$)-ATPase.

Plasma membrane-enclosed vesicle preparations from cardiac muscle of guinea-pig [5,12] and dog [13] have been already described. Our present study, however, demonstrates for the first time the effectuation of (Na^+/K^+)-antiport in a vesicular preparation from this tissue and for the first time from an excitable tissue at all.

Discussion

An interesting result of our study is that the ATP concentration required to obtain maximum (Na^+/K^+)-antiport with the vesicular ($Na^+ + K^+$)-ATPase preparation and the macroscopic ATP-regenerating system is about 10-times higher than the ATP concentration to afford maximum ($Na^+ + K^+$)-ATPase activity with a non-vesicular preparation. This disparity may be accounted for by the following differences in the operational modes of the two preparations. In the preparation consisting of vesicles with a small internal volume, an Na^+ gradient is readily formed which energetically counteracts the (Na^+/K^+)-antiporter mode of ($Na^+ + K^+$)-ATPase action. The resulting reduction of the driving force for cations uphill movement — i.e., the difference between effective Gibbs energy of ATP and Gibbs energy of the transmembrane ion gradients — must be compensated for by a higher ATP concentration to maximize the transport rate. In the non-vesicular preparation, an electrochemical cation gradient cannot be formed so that ($Na^+ + K^+$)-ATPase effects unidirectionally ATP hydrolysis. Thus, a lower ATP concentration suffices to maximize the rate of ATP hydrolysis.

Our most remarkable observation is that maximum (Na^+/K^+)-antiport across cardiac plasma membrane requires with the macroscopic ATP-regenerating system an about 70-times higher ATP concentration than with the microscopic ATP-regenerating system, but that the V values reached are the same with both systems (cf. Fig. 3).

These findings can be understood within the framework of present knowledge about low or high effectivities of sequentially working enzymes when they cooperate either physically separated as a bulk reaction-diffusion system or physically associated in an organized enzyme cluster as an integrated unit of catalytic activity (for a review, cf. Ref. 14).

In a heterogeneous membrane system, the enzyme activity is potentially limited by diffusion constraints so that the Michaelis-Menten kinetics must be supplemented by diffusion-related terms to describe the spatio-temporal variation of substrate-product concentrations (cf. Ref. 14). In line with this

reasoning, our finding that maximum (Na^+/K^+)-antiport requires with the macroscopic ATP-regenerating system extremely high ATP concentrations points to the conclusion that this low effectivity is a consequence of diffusion constraints for ATP and ADP, of which the degradative influence on the locally effective Gibbs energy charge determined essentially by the [ATP] : [ADP] ratio must be compensated for by a respective increase in the bulk concentration of ATP.

As stated by Welch [14], the assembly of sequential enzymes in a organized cluster may increase the effectivity of the overall process even if the intrinsic catalytic activities of the component enzymes are not altered upon association; the advantages here result simply from the proximate juxtaposition of the constituent active centres within the enzyme system. This mechanism for optimizing effectivity appears to be true for ($Na^+ + K^+$)-ATPase when driving (Na^+/K^+)-antiport in the interplay with the microscopic ATP-regenerating system. As shown earlier [4] and corroborated here (cf. Table I), ($Na^+ + K^+$)-ATPase is physically associated with creatine phosphokinase via binding to the plasma membrane. When the ATP concentration is raised at saturating phosphocreatine concentration, the amplitude of ADP production by the activities of ($Na^+ + K^+$)-ATPase and contaminating Mg^{2+}-dependent ATPase remains low and constant with time, whereas the amplitude and rate of creatine production by the activity of creatine phosphokinase become greater and increase linearly with time [4]. Ouabain inhibits the activities of ($Na^+ + K^+$)-ATPase as well as of creatine phosphokinase in this coupled system [4], although only the former enzyme exhibits an inhibitory ouabain site. The demonstrated coordinate activation and inhibition of both ($Na^+ + K^+$)-ATPase and creatine phosphokinase evidence that the two enzymes constitute an integrated unit of catalytic action ('enzyme cluster').

The kinetic properties of the MM isoenzyme of creatine phosphokinase shown to be bound to cardiac plasma membrane are favourable for rapid ADP removal and ATP regeneration at the expense of phosphocreatine. The K_m (ADP) value is rather low (0.05 mM) and the V value for phosphorylation of ADP from phosphocreatine is 4.2-times higher than that for phosphorylation of creatine from ATP [4,15]. These kinetic properties of creatine phosphokinase are well suited to maintaining high [ATP] : [ADP] ratios and high ATP concentrations near the catalytic centre of ($Na^+ + K^+$)-ATPase even at high rates of ATP hydrolysis during maximum (Na^+/K^+)-antiport. The coordinate interplay between ($Na^+ + K^+$)-ATPase and creatine phosphokinase thus optimizes the effectivity of (Na^+/K^+)-antiport in two ways. First, the maintenance of high [ATP] : [ADP] ratios near the catalytic centre reduces or eliminates the faculty of ($Na^+ + K^+$)-ATPase to function as ATP synthase [3,16], and compels the enzyme to work essentially as an (Na^+/K^+)-antiporter. Hence, the coordinate interplay between ($Na^+ + K^+$)-ATPase and creatine phosphokinase realizes vectorial catalysis in effectuating vectorial transmembrane transport (for a review, cf. Ref. 17). Second, cyclic channelling of ATP and ADP between the catalytic centres of the two clustered enzymes results in a high local concentration of ATP even at a comparatively low global concentration of ATP. Under physiological conditions, this may guarantee that ($Na^+ + K^+$)-ATPase is always saturated with ATP for driving (Na^+/K^+)-antiport.

The argumentation put forward in favour of cyclic channelling of ATP and ADP between the catalytic centres of the enzyme cluster includes the existence of centre-bound ATP and ADP compartments in the cell for which some pieces of evidence are already available. Indirectly, the concept of adenine nucleotide compartmentation in heart cells is supported by the findings that the intracellular energy transport occurs mostly via the phosphocreatine pathway (cf. Ref. 18, for review). In living heart, studies on creatine phosphokinase kinetics by means of ^{31}P-NMR directly confirmed the compartmentation of spatially separated cellular ATP pools, of which the localization, however, still awaits clarification [19]. In frog skeletal muscle, a compartment near $(Na^+ + K^+)$-ATPase can be deduced from the finding that a large decrease in the bulk [ATP] : [ADP] ratio does not alter the amplitude of (Na^+/K^+)-antiport [20]. Apparently, the (Na^+/K^+)-antiport-effecting fraction of $(Na^+ + K^+)$-ATPase molecules is tightly coupled with creatine phosphokinase so that the microcompartment formed by their proximate catalytic centres is not sensitive to a heavy reduction of the cytosolic [ATP] : [ADP] ratio. In line with our reasoning, Proverbio and Hoffman [21] provided with red cell ghosts experimental support for a membrane compartment of ATP which links $(Na^+ + K^+)$-ATPase with membrane-bound phosphoglycerate kinase. Taken together, the available information suggests that $(Na^+ + K^+)$-ATPase in effecting (Na^+/K^+)-antiport works with high thermodynamic efficiency owing to Gibbs energy-conserving complementary couplings between all exergonic and endergonic events in enzyme turnover [2,3] as well as with a high rate at physiological ATP concentration owing to coordinate interplay between $(Na^+ + K^+)$-ATPase and creatine phosphokinase (present paper).

The Ca^{2+}-activated, Mg^{2+}-dependent ATPase of cardiac sarcoplasmic reticulum has also been shown to be coupled with membrane-associated creatine phosphokinase [22,23], and to effectuate as an integrated unit Ca^{2+} transport with a high rate at low ATP and saturating phosphocreatine concentrations [24]. Hence, in cardiac muscle, the interplay of transport ATPases with creatine phosphokinase appears to be of more general importance for optimizing their transport activities.

In connection with this report, it is worth recalling experiments in squid axon which showed that phosphoarginine restored Na^+ extrusion depressed by low [ATP] : [ADP] ratios after cyanide poisoning; therefore, the arginine phosphokinase system was assumed to play an important role in the energy supply for (Na^+/K^+)-antiport [25,26]. More recent studies confirmed that phosphoarginine becomes effective through increase in the [ATP] : [ADP] ratio [27,28], but whether $(Na^+ + K^+)$-ATPase cooperates in squid axon with arginine phosphokinase as a macroscopic or microscopic ATP-regenerating system still awaits experimental clarification.

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