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The Na,K-ATPase

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The energy dependent exchange of cytoplasmic Na^+ for extracellular K^+ in mammalian cells is due to a membrane bound enzyme system, the Na,K-ATPase. The exchange sustains a gradient for Na^+ into and for K^+ out of the cell, and this is used as an energy source for creation of the membrane potential, for its de- and repolarisation, for regulation of cytoplasmic ionic composition and for transepithelial transport. The Na,K-ATPase consists of two membrane spanning polypeptides, an α-subunit of 112-kD and a β-subunit, which is a glycoprotein of 35-kD. The catalytic properties are associated with the α-subunit, which has the binding domain for ATP and the cations. In the review, attention will be given to the biochemical characterization of the reaction mechanism underlying the coupling between hydrolysis of the substate ATP and transport of Na^+ and K^+.

KEY WORDS: Cation transport; Na,K-pump; Na,K-ATPase; ouabain; phosphorylation; occlusion; coupling between ATP hydrolysis and transport.

THE Na,K-ATPase—A SHORT HISTORICAL BACKGROUND

Active Transport and the Use of Isotopes

In the 1930s it was a general view that the cell membrane is impermeable to Na^+. This view had to be revised thoroughly when radioactive isotopes of Na^+ and K^+ were introduced in biology at the end of the decade (Hevesy, 1938), where it became possible to monitor ion fluxes across cell membranes. With the membrane being permeable to both K^+ and Na^+—and with the ions present predominantly as freely dissociable ions in the protoplasm—it was necessary to assume an active extrusion of Na^+ in order to explain that Na^+ is at a lower electrochemical potential inside than outside the cell. This led Dean in 1941 to introduce the idea of a pump in the cell membrane (Dean, 1941). On the basis of experiments on the exchange of Na^+ and K^+ in muscle (Heppel and Schmidt, 1938; Heppel, 1939; Steinbach, 1940) Dean concluded, "that the muscle can actively move potassium and sodium against concentration gradients . . . , this requires work. Therefore there must be some sort of a pump, possibly located in the fiber membrane, which can pump out the sodium or, what is equivalent, pump in the potassium" (Dean, 1941).

But what is the purpose of having a leaky cell membrane—permeable to Na^+ as well as to K^+—and then spend energy to keep Na^+ out of and K^+ inside the cell? From our present knowledge the answer is that the Na,K pump utilizes the energy from ATP to sustain gradients across the membrane for both Na^+ and K^+. These gradients are used as an energy source both for formation of, and for re- and depolarization of the membrane potential, for cell volume regulation, for secondary active transport of glucose and amino acids into cells against their concentration gradients, and for co- and countertransport of ions across the cell membrane. Active transport of Na^+ and K^+ is thus not merely a compensation for a leak in the cell membrane, but has a key function in transmission of information, in regulation of the exchange of substances between the cell and its surroundings, and in transepithelial transport.

The concept of active transport developed during

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the forties and fifties from experiments on muscle, frog skin, red blood cells, and nerves (Ussing, 1960). It was uncertain whether Na^+ or K^+ is the actively transported cation. By introducing the technique of short-circuiting the frog skin, Ussing and Zerahn in 1951 showed that in the frog skin the current is carried by an active transport of Na^+ (Ussing and Zerahn, 1951). However, subsequent experiments on giant axons (Hodgkin and Keynes, 1955) and on red blood cells showed that the efflux of Na^+ is dependent on an effect of extracellular K^+ (Harris and Maizels, 1951; Shaw, 1955; Glynn, 1956). A linkage between an active efflux of Na^+ and an active influx of K^+ was suggested from the observation that the concentration dependence of extracellular K^+ for the saturable influx of K^+ and for efflux of Na^+ is the same (Glynn, 1956).

Identification of ATP as Substrate

The effect of metabolic inhibitors on the active transport in red blood cells and in giant axons suggested that the substrates for the transport are energy-rich triphosphate esters (Maizels, 1954). By using a method to reversibly open the red blood cells, Gardos in 1954 was able to introduce ATP into the red blood cell, and showed that ATP can support the influx of K^+ (Gardos, 1954). Experiments by Hoffman showed that it is only ATP which can support the active transport in red blood cells, i.e., ATP cannot be replaced by other triphosphates such as ITP, UTP, or GTP (Hoffman, 1962).

Stoichiometry between Ion Transport and Hydrolysis

The Na^+/K^+ stoichiometry in red blood cells was found to be $1.5\,Na^+$ transported into the cell for each K^+ transported out (Post and Jolly, 1957). The same stoichiometry is found in nerve, in frog skin, and in other tissues, and is the coupling ratio under physiological conditions (see Clarke *et al.*, 1989). Using the short-circuited frog skin, Zerahn found that 18 moles of Na^+ is transported per mole of oxygen consumed above the resting level (Zerahn, 1956). With a P/O ratio of 3 for oxidative phosphorylation, this gives $3\,Na^+$ transported per ATP hydrolyzed.

This stoichiometry was later determined directly by Sen and Post in experiments on red blood cells, where it was shown that 1.16 moles of ATP was hydrolyzed when 3 moles of Na^+ and $2\,K^+$ are transported (Sen and Post, 1964). The same stoichiometry has been found by others on red blood cells and with purified reconstituted system.

Cardiac Glycosides

The important observation that cardiac glycosides inhibit the active transport of Na^+ and K^+ in red blood cells was made by Schatzmann in 1953. This inhibitory effect, which since has been found to be specific for the Na,K pump, has been very important for all subsequent work, both for localization, identification, and characterization of the pump.

Electrogenecity

In the mid sixties it was shown by Kernan (1962), and later by Thomas (1969), that the Na,K pump is electrogenic, i.e., that the transport ratio of $3\,Na^+$ to $2\,K^+$ generates a potential across the membrane, positive to the outside. The electrogenic effect of the pumping has been confirmed from experiments on a number of tissues and with purified reconstituted pumps (see Slayman, 1982; Clarke *et al.*, 1989). In the normal cell with its high permeability to Cl^-, the pumping of Na^+ and K^+ only adds a few millivolts to the membrane potential.

It is of interest to know whether the electrogenic effect of the pump has a physiological function. Although the pump only adds a few millivolts to the membrane potential, this may have a critical effect on the excitability in synapses in the central nervous system, which are very sensitive to changes in the membrane potential. Another possibility is that the 3/2 ratio of the transported cations is of importance for the "construction" of an efficient pump.

Identification of the Active Transport System (the Na,K-ATPase)

Lundegårdh had in 1940—a year before Dean's proposal of a cation pump—suggested a model for active transport based on the Danielli–Davson membrane model. In their model of the cell membrane (Danielli and Davson, 1935), the membrane is formed by a bilayer of lipids, and proteins are adsorbed on the bilayer (but not traversing the membrane). Lundegårdh had given good evidence that anions are actively transported into the protoplasm of plant roots. He suggested that the ions, which combine with the lipids in the monolayer, are transferred across the membrane by a flip-flop of the lipids. The release of the anions is dependent on the expenditure of energy, while the

release of the cations which are not actively transported is dependent on electrostatic forces (Lundegårdh, 1940).

However, the idea of a pump across the cell membrane was incompatible with the Danielli–Davson model of the membrane, which was commonly accepted in the forties. There was no structural element which could be related to the cation pump proposed by Dean. There were in the forties and early fifties apparently no response to Dean's proposal of a cation pump, in the sense that no attempts were made to identify a pump element in the membrane.

It was not until 1957 that a suggestion was made on the nature of the pump as a membrane-bound protein with catalytic properties, an ATPase (Skou, 1957). The experiments were initiated in 1954 by an observation by Libet (1948) that there is an ATPase in the sheath part of the giant axon. The test object was crab nerve membranes, and it was shown that the membranes have a Mg^{++}-activated ATPase activity, and that it was characteristic for the ATPase activity that it was activated by a combined effect of Na^+ and K^+. Based on this and on the observations by Hodgkin and Keynes that triphosphates are the substrates for the active transport of Na^+ and K^+ in giant axons (Hodgkin and Keynes, 1955), it was suggested that the ATPase was involved in the active transport of Na^+.

Results of subsequent experiments from many different laboratories supported the view, and showed that the Na,K-ATPase, as it was named, fulfilled the requirements of a system responsible for the active transport of Na^+ and K^+ across the cell membrane (see Skou, 1965).

The models of the cell membrane structure were in the fifties and sixties difficult to reconcile with the structure of a cation-pumping ATPase. In 1959, the Danielli–Davson model was replaced by Robertson's unit membrane, a bilayer of lipids with proteins arranged asymmetrically on the two sides of the membrane, but still with no proteins spanning the bilayer (Robertson, 1959). The transport system, which is a protein, must, however, have access to both sides of the membrane. A suitable model was introduced as the fluid mosaic membrane model, as formulated by Singer and Nicolson (1972). In this model the nonpolar parts of globular proteins are embedded in the fluid bilayer, and the polar parts face the two sides of the membrane. The proteins have lateral mobility but do not flip flop, and they form the pathway for the transport of hydrophilic substances (including cations) across the cell membrane.

STRUCTURE OF THE Na,K-ATPase[2]

The Na,K-ATPase is a complex of two polypeptides, α and β, and a number of lipid molecules incorporated into the lipid bilayer of the plasma membrane.

Preparative Aspects

Preparation of the enzyme in amounts sufficient for biochemical characterization has taken advantage of the great variability in the number of Na,K-ATPase molecules per cell. The numbers vary from, for example, about 200 in a red cell membrane to several millions in cells in the thick ascending limb of Henle in the kidney (De Weer, 1985). By using cell membranes from tissue with a high capacity for active Na-K transport, like the outer medulla of kidney, rectal glands from shark, electric eel tissue, or salt glands from marine birds it is possible to purify the enzyme to homogeneity in the sense that the protein content is more than 90% due to α- and β-subunits (reviewed by Jørgensen, 1975a; Glynn, 1985). One approach has been to solubilize the enzyme in detergent and to reconstitute the enzyme in the membranous form after several purification steps (Hokin *et al.*, 1973; Lane *et al.*, 1973). Other procedures employed are treatment with ionic detergents (such as DOC or SDS) under suitable conditions to remove contaminating proteins from the membranes by selective extraction, followed by purification by centrifugation techniques (Jørgensen and Skou, 1968; Kyte, 1971; Jørgensen, 1974; Skou and Esmann, 1979). The detergent $C_{12}E_8$ has also been used extensively to characterize the properties of soluble preparations of Na,K-ATPase (Esmann *et al.*, 1980). This detergent selectively solubilizes Na,K-ATPase from partially purified enzyme preparations, and the enzymatic activity is retained for long periods of time, allowing for detailed analysis of the properties of single ATPase molecules [see Hayashi *et al.* (1991) for a review].

[2]For more detailed information on the Na,K pump than given in this short review, see the extensive review by Glynn (1985), and the proceedings from the 6th international conference on the Na,K pump: Vol. 1. *The Sodium Pump: Structure, Mechanism, and Regulation.* Vol. 2. *The Sodium Pump: Recent Developments.* Edited by J. H. Kaplan and P. De Weer, Society of General Physiologists Series, Vol. 46. Rockefeller University Press, New York, 1991.

Molecular Components

The α-Subunit

The α-subunit contains about 1012 amino acids, the first mammalian species to be determined from *c*DNA being the sheep kidney α-subunit in 1985 (Shull *et al.*, 1985). The sequences of α-subunits from a number of species and different tissues are almost identical. Three isoforms of the α-subunit have been identified in vertebrates. They are found in different proportions in cells from different tissues. Isoform α_1 is found in practically all cells, α_2 predominates in skeletal muscle, and α_3 is, first of all, found in nervous tissue together with α_1 and α_2. The three isoforms have the same molecular weight, but there are differences in their amino acid composition and sequence (reviewed by Lingrel *et al.*, 1991). The sequence identity is about 82%, with the major differences in the N-terminal part. They differ in their sensitivity towards cardiac glycosides. In rat brain tissue, α_2 and α_3 are more sensitive than α_1. The homology between the three isoforms in brain is about 90%, about the same as the homology between α-subunits from such diverse species as pig and torpedo. Mammalian α_1-isoforms are more than 98% identical (see Jørgensen and Andersen, 1988).

The overall homology of the α-subunit with other P-type ATPases is about 62% with the H,K-ATPase from gastric mucosa (Sweadner, 1991) and about 24% with the Ca^{2+} ATPase from sarcoplasmic reticulum. The homologies for specified ligand-binding domains such as the nucleotide site is much higher (Shull *et al.*, 1988). From the about 70% identity and homology of the H,K-ATPase sequence with that of the Na,K-ATPase, and the finding that both enzymes have a β-subunit, Sweadner (1991) has suggested that the H,K-ATPase is a fourth isoform of the Na,K-ATPase.

The location of the N-terminal amino acid of the α-subunit is on the intracellular side of the membrane, and recent studies suggest that the C-terminus also is on the intracellular side (Antolovic *et al.*, 1991; see also Bayer, 1990). The number of membrane-spanning segments deduced from hydropathy profiles is 7 or 8. With both ends of the protein on the same side of the membrane, the most probable number of helices is 8.

Structural studies on the α-subunit suggest that the membrane-spanning segments are of α-helical structure. The large intracellular loop, which contains among other things the nucleotide binding site, is predominantly of β-sheet structure (reviewed by Modyanov *et al.*, 1991). The α-subunit has the site for phosphorylation (ASP 369) on the cytoplasmic side. The binding of ouabain takes place from the extracellular side. The extracellular junction between the first and second transmembrane segments of the α-subunit is of importance for the sensitivity toward the cardiac glycosides. Substitution of the neutral glutamine and asparagine in this junction with charged amino acids decreases the affinity for the cardiac glycosides (residues 111 and 122; see Price and Lingrel, 1988). Cation binding sites (see also below) have not yet been identified, but occlusion of Rb^+ and Na^+ seems to be related to the structure of a 19-kD tryptic fragment with an amino-terminal end at residue 831 (Karlish *et al.*, 1990), possibly in conjunction with other peptides (see below).

Recent experiments suggest that the α-subunit is glycosylated on the intracellular side, but the amount of sugar bound is apparently low and has not yet been characterized in detail (Pedemonte and Kaplan, 1991).

The β-Subunit

The β-subunit contains about 300 amino acids, determined from *c*DNA from sheep kidney in 1986 (Shull *et al.*, 1986). The sequences of β-subunits from a number of species and different tissues reveal a low homology between the isoforms, about 40% between β_1 (the predominant form in mammalian kidney) and β_2, which is isolated from brain. Isoform β_2 has been shown to play a role in cell–cell interaction. A third isoform, β_3, as been isolated from *Xenopus*, and finally the H,K-ATPase β_4-subunit has been isolated and cloned from rabbit and rat (Sweadner, 1991; Canfield *et al.*, 1991).

There is one membrane-spanning segment near the N-terminal part of the protein, and the C-terminus is located on the extracellular side of the membrane. The β-subunit is heavily glycosylated on three ASP residues on the extracellular side, with a mass of sugars of about 10 kD (Esmann *et al.*, 1980).

The β-subunit appears to be of importance for the insertion of the αβ complex in the membrane. It has not been possible to separate the β-subunit from the α-subunit without loss of enzymatic activity. It has also been shown that reduction of disulfide bonds in the β-subunit leads to inactivation of enzyme activity. These observations suggest a structural role for β.

Phospholipids and Cholesterol

Besides the proteins, phospholipids are necessary

for enzymatic activity (Ottolenghi, 1979). Among the lipids there seems to be a requirement for negatively charged phospholipids for full enzymatic activity as well as for activity of enzyme reconstituted in liposomes (Roelofsen and Van Deenen, 1973; Cornelius and Skou, 1988). At present, it has not been possible to exchange all the lipids for detergent and retain activity, in contrast to the properties of the Ca^{2+} ATPase (see Andersen *et al.*, 1986). It is not clear why lipids are necessary for the activity. The bilayer lipids act as a solvent for the proteins, and may thus have a structural effect on the protein, perhaps giving the optimal milieu for the protein to perform the necessary conformational changes. A more specific (regulatory) effect of phospholipids has not been demonstrated.

It should be noted that the detergent-solubilized fully active $(\alpha\beta)_2$ form of the enzyme has bound about 50 molecules of phospholipid molecules and about 40 molecules of cholesterol in the protein/lipid/detergent mixed micelle (Esmann *et al.*, 1980). This number of lipids is just enough to form an annulus around the protein, presumably shielding it against the detergent. Of the phospholipids present in these micelles, less than 2% are negatively charged. A protective effect of negatively charged lipids has also been observed in chromatography experiments, where delipidation is a cause of inactivation (Hayashi *et al.*, 1989).

Oligomeric Structure

The oligomeric structure of the Na,K-ATPase in the membrane is a subject of controversy. It is generally assumed tht the ratio between the two polypeptide chains in the complex on a mole basis is 1 : 1, i.e., that the aβ-unit is the protomer of the enzyme. This unit carries one nucleotide binding site, and it is of great importance to analysis of enzyme kinetic data to know whether this unit can perform all the necessary reactions of the enzyme, or whether a higher oligomer is necessary for function, for example for interaction between nucleotide binding sites.

Studies of Detergent-Solubilized Enzymes

The nonionic detergent $C_{12}E_8$ has been used extensively to study the properties of solubilized Na,K-ATPase. At low detergent/protein ratios the predominant structure of shark enzyme is $(\alpha\beta)_2$ (or higher oligomers), and at higher ratios $(\alpha\beta)_2$ dissociates to $(\alpha\beta)$ (Esmann, 1984). There is evidence that the aβ-structure can perform the elementary steps of the catalytic cycle but is considerably more labile than $(\alpha\beta)_2$ (Brotherus *et al.*, 1981; Craig, 1982; Vilsen *et al.*, 1987). The work of, among others, Hayashi and coworkers (1989) addresses this point in detail.

Radiation Inactivation Studies

One technique, which allows for an analysis of the properties of membrane-bound proteins *in situ*, is radiation inactivation analysis. A major advantage is that the molecular size is determined from enzymatic properties of the enzyme, which gives an opportunity to study also impure preparations of Na,K-ATPase (Kepner and Macey, 1968), and the technique has been used in a number of laboratories (Ottolenghi and Ellory, 1983; Karlish and Kempner, 1984). Recent experiments suggest that catalytic activity associated with Na^+ and K^+ activated ATP hydrolysis requires structural contact between two units of $(\alpha\beta)$, i.e., an $(\alpha\beta)_2$ structure. The experiments also suggest that a single $(\alpha\beta)$-unit has normal activity even if the other $(\alpha\beta)$ is partly denatured. Partial reactions of the enzyme seem to require less structural integrity, with most elementary reactions just requiring an intact single α-subunit (Nørby and Jensen, 1989).

Saturation Transfer ESR Spectroscopy

A novel method for analysis of protein rotational diffusion—and thus molecular size—is the saturation transfer ESR (STESR) method (reviewed in Marsh, 1989). This technique is sensitive to motions in the microsecond regime, which is ideally suited for analysis of integral membrane proteins. Experiments employing covalent labelling of sulfhydryls in shark Na,K-ATPase with maleimide nitroxide derivatives give rotational correlation times in the 25–50 μsec range (Esmann *et al.*, 1987), which suggests the presence of $(\alpha\beta)_2$-diprotomers or higher oligomers in the membrane phase (note that this determines a *structural* minimal unit—the *functional* minimal unit can always be smaller than the structural unit, but not larger). Binding of spin-labelled ouabain to Na,K-ATPase from kidney reveals the presence of large clusters of proteins within the membranes (Mahaney *et al.*, 1990).

Analysis of Two-Dimensional Crystals

Ordering of Na,K-ATPase molecules in the membrane in two-dimensional crystals was first observed with enzyme treated with vanadate (Skriver *et al.*, 1981). The crystals were of $p1$ symmetry with

only a single $\alpha\beta$-unit per unit cell, which suggested that the minimal functional unit is $\alpha\beta$. Subsequently it was possible to induce several other crystal forms of the enzyme, with symmetries of $p21$ (with the $(\alpha\beta)_2$-structure induced by vanadate in the presence of Mg) and $p4$ (with an $(\alpha\beta)_4$-structure induced by cobalt-tetrammine-ATP) which implies that the enzyme has several different modes of organization within the membrane (reviewed by Maunsbach *et al.*, 1991).

From an analysis of tilted, negatively stained two-dimensional crystals, a three-dimensional model of the Na,K-ATPase has been constructed (see Maunsbach *et al.*, 1991; Modyanov *et al.*, 1991). The $p1$ crystal, which has one protomer in the unit cell, has a height of about 120 Å. It protrudes about 60 Å on the cytoplasmic side of the membrane and about 20 Å extracellularly. In the $p21$ crystals the two protomers are rod-shaped and with an about 20 Å high contact area asymmetrically located relative to the center of the rods. The diprotomer protrudes about 20 Å outside the bilayer on the extracellular side, probably due to the β-subunit, while the 40 Å protrusion on the cytoplasmic side is due to the α-subunit.

REACTIONS OF THE Na,K-ATPase

Properties of Cation Activation

A characteristic feature of the Na,K-ATPase is that in the presence of Mg^{2+} the hydrolysis of ATP is activated by a combined effect of Na^+ on the cytoplasmic side and K^+ on the extracellular side. In the test tube with Na,K-ATPase in broken membrane fragments no transport of the cations is measured, only the ouabain-dependent hydrolysis of ATP. However, experiments on purified systems reconstituted into liposomes show that the Na,K-ATPase is not just the catalytic part of the pump but also contains the cation translocating parts (Goldin, 1977; Cornelius and Skou, 1988). This suggests that the cations activating ATP hydrolysis also are the transported cations. It also implies that the catalytic activity measured in the test tube is an equivalent of cation transport through the membrane.

K^+ acts as an inhibitor at the cytoplasmic sites, and Na^+ similarly inhibits at the extracellular sites. The inhibitory potencies are, however, quite different. The apparent affinity for Na^+ relative to K^+ on the cytoplasmic side increases with an increase in the ATP concentration. With saturating ATP, the apparent affinity for Na^+ is about 3 times that of K^+ at the

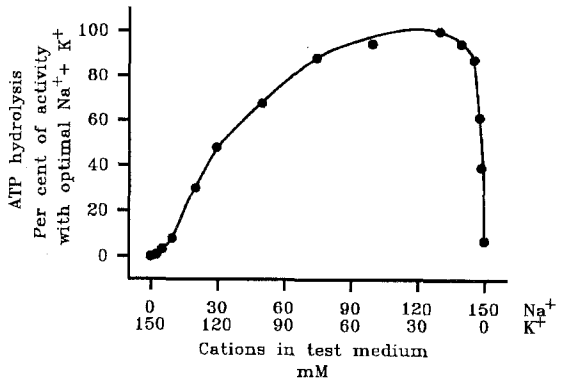

Fig. 1. ATP hydrolysis as a function of $Na^+ + K^+$ concentrations by the Na,K-ATPase. The enzyme-containing plasma membrane pieces are isolated from ox brain, and the activity is tested in 30 mM histidine (pH 7.4) at 37°C with 3 mM ATP and 3 mM Mg^{2+} present.

cytoplasmic site, whereas the affinity for K^+ is about 100 times higher that of Na^+ on the extracellular site. Due to this large difference in affinity ratios, it is possible—in the test tube with broken membrane pieces where the Na^+ and K^+ concentrations are the same on the two sides of the membrane—to observe the combined activating effect of cytoplasmic Na^+ and extracellular K^+. It is also possible to study the Na^+/K^+ competition on the cytoplasmic sites with the extracellular sites saturated with K^+, the left part of the curve in Fig. 1, and the extracellular K^+/Na^+ competition with the cytoplasmic sites saturated with Na^+, the right part of the curve in Fig. 1.

On the extracellular sites a number of the monovalent cations can replace extracelular K^+ for activation, but with decreasing affinity in the order $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+ > Na^+$. With Na^+ alone, i.e. with Na^+ activating both on cytoplasmic and extracellular sites, the activity is only about 5% of the activity with optimal Na^+ plus K^+ concentrations (Fig. 1).

At the cytoplasmic sites Li^+ as the only cation can replace Na^+ for activation, but the apparent affinity is about 5 times lower than that of Na^+. At the optimal pH value, which is 6.0 with Li^+ alone and 6.8 with Na^+ alone (and pH 7.2–7.4 with Na^+ plus K^+), the activity with Li^+ is 4–5 times higher than with Na^+ (Skou, unpublished observations). Due to the unfavorable Li^+ relative to K^+ affinity on the cytoplasmic sites, it is difficult with broken membrane pieces in the test tube to see the activating effect of extracellular K^+ with Li^+ as the cytoplasmic activating cation.

In the intact cell the transport system operates with a fraction of its maximum capacity. With the

normal intracellular concentrations of 10–20 mM Na^+ and about 120 mM K^+, the Na^+ activation is only about 15–20% of maximum, and with the normal extracellular 4 mM K^+ and 140 mM Na^+, the K^+ activation is about 85% of maximum (cf. Fig. 1). The turnover with saturating ligand concentrations is about 10.000 per minute at 37°C.

Intermediary Steps and Conformational Transitions

It is characteristic for the Na,K-ATPase as well as for the other ion-transporting ATPases that they have two major conformations, denoted the E_1- and E_2-forms, respectively. In the absence of Na^+ and K^+ and with low concentrations of buffer cations, the Na,K-ATPase is in the E_2-form (Skou and Esmann, 1983). Na^+ with a $K_{0.5}$ of about 5 mM turns the enzyme into the E_1-form, E_1Na_3 (it is assumed that there are three Na^+ bound since three Na^+ are transported; see below). The effect is not specific for Na^+; organic cations such as Tris and histidine will do the same, as will also Mg^{++} and Li^+.

K^+ binds to E_2 with a low affinity (giving E_2K_2 with two K^+ bound) and turns the E_2-form into a form with K^+ occluded, $E_2'(K_2)$ (Glynn and Richards, 1982). In this form K^+ has a very low rate of exchange with K^+ from the surrounding medium. The equilibrium between E_2K_2 and $E_2'(K_2)$ is poised far toward $E_2'(K_2)$, which means that the apparent affinity of E_2 for K^+ is very high: $K_{0.5}$ is a few μM. These effects of Na^+ and of K^+ on the conformation are on the cytoplasmic sites (Karlish, 1980).

$$E_2'(K_2) \leftrightarrow E_2K_2 \leftrightarrow E_2 \leftrightarrow E_2Na_3 \leftrightarrow E_1Na_3$$

The E_1Na_3- and $E_2'(K_2)$-forms can be distinguished from their susceptibility to tryptic digestion (Jørgensen, 1975b), from their affinity for ATP (which is high for E_1Na_3 and is low for $E_2'(K_2)$, Nørby and Jensen, 1971; Hegyvary and Post, 1971), and from differences in the fluorescence of intrinsic (Karlish and Yates, 1978) as well as extrinsic probes (Karlish *et al.*, 1978; Karlish, 1980; Skou and Esmann, 1981; Kapakos and Steinberg, 1982; Taniguchi *et al.*, 1988). The rate of transition from E_1Na_3 to $E_2'(K_2)$ is high, $t_{1/2}$ is a few milliseconds at 22°C, while the rate of transition from $E_2'(K_2)$ to E_1Na_3 is low, with a $t_{1/2}$ of a fraction of a second, but with both rates influenced by the concentration of the cations (Karlish *et al.*, 1978; Skou and Esmann, 1983). The rate of transition from $E_2'(K_2)$ to E_1Na_3 is increased by ATP. E_2 (enzyme with neither Na^+ nor K^+ bound) can be distinguished from $E_2'(K_2)$ by a 100-fold higher rate of transition to E_1Na_3 (Skou and Esmann, 1983).

ATP with a low affinity increases the rate of deocclusion of K^+ from $E_2'(K_2)$ and thereby the rate of transition to E_1Na_3. In the presence of Mg^{2+}, Na^+ confers catalytic activity to the system and the phosphate from ATP is transferred to aspartic acid (residue 369) in the α-subunit. This leads to occlusion of Na^+, E_1-P(Na_3)ADP (Glynn *et al.*, 1984). Oligomycin, which occludes Na^+ in the E_1 form of the enzyme (giving $E_1(Na_3)$; Esmann and Skou, 1985), increases the rate of phosphorylation, suggesting that the slowest step in the phosphorylation reaction is the occlusion of Na^+, and that occlusion must accompany, or be prior to, phosphorylation (Skou, 1991). In following steps Na^+ is deoccluded and the E_1-P(Na_3) is converted to an E_2-P form, which has a low affinity for Na^+, but high for K^+. The reaction involves—besides E_1-P(Na_3)ADP—at least three phosphoenzymes (reviewed by Nørby *et al.*, 1983; Nørby and Klodos, 1988).

$$E_1Na_3MgATP \leftrightarrow E_1\text{-}P(Na_3)MgADP \leftrightarrow E_1'\text{-}PNa_3 \leftrightarrow E_1''\text{-}PNa_3 \leftrightarrow E_2\text{-}P \quad (2)$$

E_1'-PNa_3 is ADP-sensitive, and an increase in the Na^+ concentration also confers ADP sensitivity to E_1''-PNa_3 in the sense that at a high concentration of Na^+ both phosphoenzymes disappear rapidly in the backward reaction when ADP is added. E_2-P is ADP-insensitive, but K^+-sensitive and so is E_1''-PNa_3, while E_1'-PNa_3 is K^+-insensitive (Nørby *et al.*, 1983). Addition of K^+ thus increases the rate of disappearance of E_2-P as well as E_1''-PNa_3, and K^+ also seems to decrease the rate of transition of E_1''-PNa_3 to E_1'-PNa_3.

A separate problem is at which step Na^+ is deoccluded. In sequence (2) it is the release of Na^+ which allows the transition to the K^+-sensitive form E_2-P. Another possibility is that one of the three Na^+ is released from the occluded form, and the phosphoenzyme with two occluded Na^+ undergo the conformational change to the deoccluded E_2-P form (Yoda and Yoda, 1987).

$$E_1Na_3MgATP \leftrightarrow E_1\text{-}P(Na_3)MgADP \leftrightarrow E_1\text{-}P(Na_3) \leftrightarrow E_1'\text{-}P(Na_2) \leftrightarrow E_2\text{-}PNa_2 \quad (3)$$

The rate of interconversion of the phosphoenzymes is rapid enough to account for the overall rate of hydrolysis of ATP in the presence of Na^+ alone. A serious problem is, however, that this is not the case when K^+

is added to phosphoenzyme preformed in the presence of Na^+. The rate of the conversion of the ADP-sensitive phosphoenzyme to the K^+-sensitive phosphoenzyme is too small (see Glynn, 1988). This may be a methodological problem, due to the perturbation of the system when phosphorylation is stopped by addition of either excess nonradioactive ATP or Mg^{2+} chelating agents in order to measure the rate of dephosphorylation of the ^{32}P-labelled phosphoenzyme. It may also mean that the phosphoenzymes formed in the presence of both Na^+ and K^+ are different from those formed in the presence of Na^+ alone (Plesner *et al.*, 1981). Another complicating factor is the effect of anions on the distribution of phosphoenzymes (see Post and Suzuki, 1991).

E_2-P has a high affinity for K^+ and dephosphorylates rapidly in the presence of K^+. The dephosphorylation leads to an occlusion of K^+ [giving $E_2'(K_2)$]. It was the observation by Post *et al.* (1972) that K^+ has a low rate of release from the enzyme after K^+-induced dephosporylation, which led to the concept of occlusion of the cations.

$$E_2\text{-}P \leftrightarrow E_2\text{-}PK_2 \leftrightarrow E_2'(K_2) \qquad (4)$$

ATP with a low affinity increases the rate of deocclusion and in the presence of Na^+ increases the rate of transfer to E_1Na_3MgATP

$$E_2'(K_2) \leftrightarrow E_2'(K_2)MgATP \leftrightarrow E_2K_2MgATP$$
$$\leftrightarrow E_2MgATP \leftrightarrow E_1Na_3MgATP \qquad (5)$$

A combination of (2) or (3) with (4) and (5) gives a scheme for the Na^+- and K^+-dependent hydrolysis of ATP

$$E_1Na_3MgATP \leftrightarrow E_1\text{-}P(Na_3)MgADP \leftrightarrow E_1\text{-}P(Na_3)$$
$$\leftrightarrow E_1'\text{-}P(Na_2) \leftrightarrow E_2\text{-}PNa_2 \leftrightarrow E_2\text{-}PK_2 \leftrightarrow E_2'(K_2)$$
$$\leftrightarrow E_2'(K_2)MgATP \leftrightarrow E_2K_2MgATP$$
$$\leftrightarrow E_2MgATP \leftrightarrow E_1Na_3MgATP \qquad (6)$$

Coupling of ATP Hydrolysis and Transport

In Fig. 2 sidedness is added to the reaction with ligands (Mg^{2+} is omitted), which gives a model for the coupling between the chemical reaction and the translocation of the cations. This is basically the Albers–Post scheme, in which the reactions with Na^+ and K^+ are consecutive with a Na^+-dependent phosphorylation followed by a K^+-dependent dephosphorylation. Included are also modifications based on information on phosphorylation–dephosphorylation

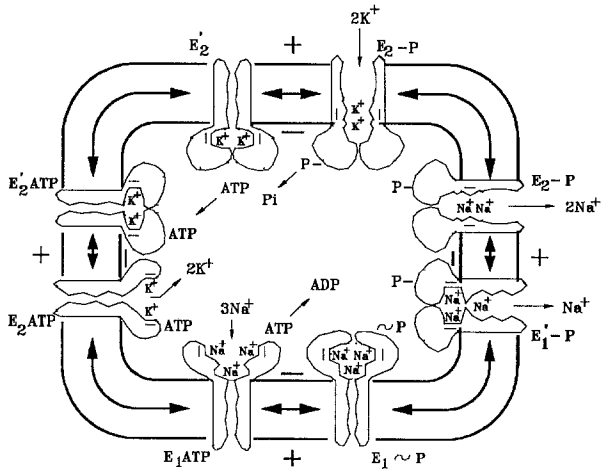

Fig. 2. A scheme for the Na^+–K^+ exchange across the cell membrane by the Na,K-ATPase. The reaction with the cations is consecutive and the scheme is based on the Albers–Post scheme (Albers, 1967; Post *et al.*, 1969), modifications of this scheme by Karlish *et al.* (1978), on the scheme for formation of the phosphoenzymes proposed by Nørby *et al.* (1983), and one the electrostatic model for transport by Stürmer *et al.* (1991).

reactions, on occlusion–deocclusion properties of the cations, and on the electrogenic properties of the transport. The main principle behind the scheme is a shift in affinities for the cations and an occlusion-deocclusion of the cations governed by the stepwise degradation of ATP. Although as discussed there are steps in the reaction sequence which cannot account for the rate of the overall hydrolysis—and the scheme therefore must be considered incomplete—the Albers–Post scheme has been useful as a working hypothesis.

Electrogenicity of the Pump

From experiments on intact cells, on enzyme reconstituted into liposomes, on enzyme adsorbed to or incorporated into a lipid bilayer, it is shown that the electrogenic effect of the pumping is due to a net positive charge carried in the Na^+ translocation step, while the K^+ translocation step is electroneutral (for references see De Weer *et al.*, 1988; Eisenrauch *et al.*, 1991; Schwarz and Vasilets, 1991; Rakowski, 1991; Gadsby *et al.*, 1991). There is no charge translocation in the Na^+ binding and the following phosphorylation and occlusion step, E_1 to E_1Na_3MgATP to $E_1\text{-}P(Na_3)$, suggesting that the charge translocation is in the deocclusion step or one of the following steps to E_2-P (Borlinghaus *et al.*, 1987). It is suggested that the transport system has two negative charges in the cation-binding domain, which neutralizes the $2\,K^+$ or

$2\,Na^+$, respectively (Goldshleger *et al.*, 1987), with the third Na^+ transported not being neutralized. The current is voltage dependent. A hyperpolarization decreases, while a depolarization increases, the current carried by the pumping, i.e., the current–voltage curve (*I–V* curve) has a positive slope and saturates at a potential positive to the inside. As the $3\,Na^+$ to $2\,K^+$ stoichiometry is not influenced by a change in voltage across the membrane (see Rakowski, 1991; Schwarz and Vasilets, 1991), the voltage effect on the current indicates a change in translocation rate.

The current is not only decreased by a hyperpolarization, but also at a given voltage by an increase in the concentration of extracellular Na^+ with a low affinity (see Gadsby *et al.*, 1991; Rakowski, 1991). The effect is synergistic, an increase in the Na^+ concentration gives a shift to the right in the *I–V* curve, while hyperpolarization decreases $K_{0.5}$ for the inhibitory effect of extracellular Na^+.

There is no voltage effect on the K^+ translocation step with saturating extracellular K^+. However, in experiments with *Xenopus* oocytes and with Na,K pumps from *Torpedo* electroplax expressed in *Xenopus* oocytes, with no Na^+ extracellular and with nonsaturating concentrations of K^+ the current decreases with an increasing inside positive potential. The *I–V* curve has a negative slope and saturates at a negative potential. The voltage has an effect on $K_{0.5}$ for extracellular K^+, which increases with a shift in potential from negative inside to positive (see Schwarz and Vasilets, 1991; Rakowski, 1991). With Na^+ and nonsaturating concentrations of K^+ extracellular, the *I–V* curve becomes bell-shaped, i.e., a voltage effect is observed both on the Na^+ and on the K^+ translocation step. The effect of voltage on the K^+ step has so far not been observed in other tissues.

There is also a voltage effect on the Na^+–Na^+ exchange. This has been shown in experiments on giant axons. The Na^+ efflux–voltage curve has a negative slope, i.e., a hyperpolarization increases the Na^+ efflux and decreases K_m for extracellular Na^+, suggesting that hyperpolarization enhances rebinding of Na^+ to the extracellular sites (see Rakowski, 1991).

The results of the effect of voltage on the current carried by the pump suggest an effect of voltage on the binding and/or release of the extracellular cations, apparently with the major voltage effect on the reverse rate in the Na^+ translocation step and at least in some tissues an effect on the forward rate in the K^+ translocation step. An inside negative potential decreases the rate of Na^+ release extracellular and/or increases the rate of the reversal binding of extracellular Na^+, and increases the rate of binding of extracellular nonsaturating K^+. An inside positive potential has the opposite effect.

As the voltage effect can also be seen on the electroneutral K^+ translocation step, the voltage effect on the Na^+ translocation step apparently can be explained without including an effect on the charge translocation. The charge translocation step, which may be the deocclusion step, may be too fast compared with the voltage-sensitive release and binding step and an effect of voltage on the charge translocation cannot therefore be observed.

The fluorescence signal from electrochromic styryl dyes inserted in membranes responds to a change in membrane potential (Klodos and Forbush, 1988). Measurements with such dyes of the effect of phosphorylation from ATP in the presence of Na^+, and of the effect of Na^+ and of K^+ of the fluorescence response, support the view that the binding and/or release of the cations on the extracellular side is an electrogenic event (see Läuger, 1991). The explanation of these results and of the discussed results of the voltage effect is that the binding sites in the deoccluded phospho form are connected to the extracellular medium along a narrow channel which traverses a part of the membrane dielectric, a high-field access channel, or ion well (Läuger, 1991); see Fig. 2. The experiments with the electrochromic dyes suggest that the binding of cytoplasmic K^+ is nonelectrogenic, indicating a high conductance access to the two binding sites, which as discussed above is assumed to have two negative charges. There is a minor electrostatic effect of binding of cytoplasmic Na^+, suggesting that the two Na^+, like the two K^+, have a high conductance access to the binding sites, while the third Na^+ is bound to a site in a low-conductance well located close to the dielectric interface (see Fig. 2). As binding of Na^+ and occlusion as discussed above does not transfer charge, this suggests that Na^+ in the occluded form is on the cytoplasmic side of the membrane dielectric.

According to the above discussion it is the deocclusion which transfers charge into the dielectric of the membrane, to the bottom of the narrow well, meaning that the ions that are still inside part of the membrane dielectric and in the well must move against a potential gradient. A problem arises in this context: Where is the energy barrier in the protein, and how far does it span, a few angstroms? Or does the narrow channel mean that is is far wider? Could an alternative to

the narrow channel be that it is the deocclusion–reocclusion which is potential sensitive? And in the deocclusion step where charge is transferred into the dielectric, is it the charge which moves or is it the energy barrier?

Binding Sites for the Cations

An important question to answer in order to understand the transport reaction concerns the nature and the location of the cation binding sites. Are the binding sites for Na^+ and K^+ identical (see above) and what determines the specificity for the cations? What types of structure lead to the occlusion–deocclusion reactions? So far the information on the protein-chemistry side is sparse.

Recent evidence comes from extensive tryptic digestion of the membrane-bound enzyme (Karlish *et al.*, 1990), where removal of a major part of the cytoplasmic extramembranous part of the enzyme does not lead to loss of the ability to occlude K^+ or Na^+. The tryptic digestion leaves a 19-kD fragment of the α-subunit as well as some smaller 8- and 9-kD fragments in the membrane pieces. Inactivation experiments with the carboxylic reagent DCCD suggests that reaction of two carboxyl groups with DCCD leads to inactivation of occlusion. One of the carboxyls is located on the 19-kD fragment while another seems to be on a smaller 9-kD fragment, suggesting that both the 19- and the 9-kD fragments are necessary for occlusion of the cations. As discussed above, there seems to be two negative charges at the cation-binding domain, and from the involvement of the carboxyl groups in occlusion it is suggested that these are the carboxyl groups to which the $2\,K^+$ or $2\,Na^+$ are bound in the transport reaction. Furthermore, the experiments suggest that the occlusion and transport sites for K^+ and for Na^+ are identical, i.e., that the transport of K^+ and Na^+ is a sequential process.

As the trypsin-digested enzyme has lost the ATP binding domain, and thereby the ATPase as well as the phosphorylation activity, these protein structures are not necessary for occlusion. This does not mean that there is no interaction between the nucleotide-binding site and the occlusion reaction in the intact system. Occlusion of K^+ decreases the affinity for ATP, while binding of Na^+ to the sites without occlusion increases the affinity for ATP. Phosphorylation leads to occlusion of Na^+, while dephosphorylation leads to occlusion of K^+.

INHIBITION AND REGULATION OF PUMP ACTIVITY

Inhibitors of the Pump

Cardiac Glycosides

Cardiac glycosides are specific inhibitors of the Na,K-ATPase. They inhibit from the extracellular side of the membrane. Mg^{2+} is required, but binding and inhibition in the presence of Mg^{2+} alone is very slow. The rate of inhibition is much increased by phosphorylation of the enzyme either backwards from $Mg^{2+} + P_i$, or forward from $Mg^{2+} + Na^+ + Na^+ + ATP$ (with formation of E_2-P). When the phosphoenzyme is formed in the presence of Mg^{2+}, Na^+, and ATP, addition of K^+ protects against inhibition of cardiac glycosides, probably due to the dephosphorylating effect of K^+ (see Schwartz, 1983). Cardiac glycosides differ in their affinity. With a given cardiac glycoside and a given combination of ligands, the inhibitory effect varies for different species, and for a given species, for different tissues. As discussed above, this is related to the presence of isoforms of the enzyme with different affinities for cardiac glycosides. The most widely used cardiac glycoside, the water-soluble ouabain (*g*-strophanthin), has a $K_{0.5}$ on the order of 10^{-7}–10^{-5} M for enzyme from most tissues.

The Na,K-ATPase is the receptor for the inotropic effect of glycosides on the heart, as suggested by Repke (1963) from the correlation between the inotropic effect of cardiac glycosides and their inhibition of the Na,K-ATPase. This has since been substantiated, and an explanation of the link between inhibition of the sodium pump and an inotropic effect was given by Baker *et al.* (1969). An increase in intracellular Na^+ (which follows from partial inhibition of the pump) leads to an enhanced Ca^{2+} influx and/or a decreased Ca^{2+} efflux through the Na^+–Ca^{2+} exchange carrier in the cardiac muscle, and the increased intracellular Ca^{2+} concentration leads to increased contractility by the heart muscle.

Vanadate

Vanadate is a transition-state analogue of the phosphate bound to the phosphorylation site in P-type ATPases. The use of vanadate in characterizaton of, first, Na,K-ATPase and, later, other ATPases was initiated after it was discovered as a contaminant in certain commerical preparations of ATP (Cantley *et al.*, 1978). In the presence of Mg^{2+},

vanadate inhibits the Na,K-ATPase at nanomolar concentrations by binding to the phosphorylation site. K^+ on the cytoplasmic sites of the system promotes vanadate binding, while extracellular K^+ has no effect. Na^+ on the extracellular sites protects against vanadate binding, and the effect can be overcome by displacement of Na^+ from the sites by K^+.

Oligomycin

The antibiotic oligomycin inhibits the enzyme from the extracellular side, but in contrast to cardiac glycosides and vanadate, oligomycin does not give complete inhibition of enzyme activity. It decreases the rate of hydrolysis and of transport (see Glynn, 1985). Oligomycin decreases the rate of transition from E_1Na_3 to $E_2(K_2)$ due to an occlusion of Na^+ (Esmann and Skou, 1985), i.e., oligomycin turns the nonphosphorylated E_1Na_3 form into an $E_1(Na_3)$ form with a low rate of release of Na^+. This suggests that under turnover conditions oligomycin decreases the rate of hydrolysis by decreasing the rate of deocclusion of Na^+ from $E_1(Na_3)$-P and thereby decreases the rate of formation of E_2-P, the phosphoenzyme which becomes dephosphorylated by K^+.

Intestinal Peptides

Recent experiments by Araki *et al.* (1989) suggest that a class of peptides isolated from porcine intestine have a specific inhibitory action on the Na,K-ATPase. The 60-residue peptides have been characterized in detail (Araki *et al.*, 1991), but the mode of interaction with the pump remains to be elucidated.

Regulators of Pump Activity

Due to the combined effect of Na^+ on the cytoplasmic side and K^+ on the extracellular side of the membrane, and to the cytoplasmic K^+ and extracellular Na^+ inhibition, any change in passive fluxes of Na^+ and K^+ across the cell membrane will lead to compensatory changes in the active transport of the two cations. With the normal cytoplasmic and extracellular Na^+ and K^+ concentrations, the pump operates as discussed with an activity which is a low fraction of maximum. The large reserve power is important, for example, during exercise where the muscle gains Na^+ and loses K^+. During maximal exercise the net loss of K^+ to the extracellular space may amount to 40 mmol/min or more (Clausen, 1986). This will rapidly give a deleterious concentration of K^+ in plasma unless it is cleared by an increase in pumping. The maximal capacity for reabsorption of K^+ is, in the total muscle pool, about 125 mmol/min (Clausen, 1986).

Besides the cation effect on the activity, there is a stimulating effect of insulin, epinephrine, and norepinephrine, which may also play a role during exercise. These hormones have no direct effect on the isolated Na,K-ATPase, and mediation of their effect in the intact cell seems to involve cyclic AMP. They have an immediate effect on the pump activity (see Clausen, 1986). Thyroid hormones and corticosteroids also increase the activity of the pump, but the effect develops slowly and seems to be due to a *de novo* synthesis of enzyme molecules (see Clausen, 1986, for a discussion).

There is no other known effect of cardiac glycosides apart from their inhibitory effect on the transport system. This has raised the question whether there is an endogenous cardiac glycoside-like factor in plasma of importance for regulation of pump activity, a question which—since cardiac glycosides give vasoconstriction—has been extended to whether there is a circulating factor responsible for development of hypertension (Haupert, 1988). These questions have proved difficult to address experimentally. Plasma does inhibit the activity of the isolated Na,K-ATPase, but identification of the inhibitor has been equivocal. The vasoconstrictor effect may be partly due to pump inhibition in nerve terminals with an increased transmitter release and/or decreased reuptake.

CONCLUSIONS

More than 30 years of work on the Na,K-ATPase has given a wealth of information, but in spite of this there still seems to be a long way to go before it is understood at the molecular level how the hydrolysis of ATP is coupled to a transport of the cations.

New methods open up new possibilities of obtaining information, not the least gene technology. However, this does not mean that the currently used methods are obsolete; there is still much information which can be obtained only by classical biochemical and physiological methods. They are to a large extent necessary for the interpreation of the gene-technological experiments.

The methods used and the results obtained are, however, not enough. The slow progress may be due

to a lack of new ideas more than a lack of new information. It is, however, difficult to ignore the traditional way of interpretation, to know by intuition.

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