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Electrogenic Properties of the Na^+,K^+-ATPase Probed by Presteady State and Relaxation Studies

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Electrical measurements on planar lipid bilayers, patch/voltage clamp experiments, and spectroscopic investigations involving a potential sensitive dye are reviewed. These experiments were performed to analyze the kinetics of charge translocation of the Na^+,K^+-ATPase. High time resolution was achieved by applying caged ATP, voltage-jump, and stopped-flow techniques, respectively. Kinetic parameters and the electrogenicity of the relevant transitions in the Na^+,K^+-ATPase reaction cycle are discussed.

KEY WORDS: BLM; patch clamp; rate constant; access channel; sodium pump; transport.

INTRODUCTION

The investigation of charge translocation of transporters or ion pumps is one of the most direct ways of obtaining insight into the transport mechanism of these proteins. Because of the small turnover of transporters ($\sim$100 s^{-1}) compared to that of ion channels ($\sim 10^6$ s^{-1}), the direct measurement of the current generated by the transporter using electrical or electrophysiological methods is a challenging task. For example, to obtain a current amplitude as generated by a single-channel molecule (typically in the order of 10^{-12}A), 10^4 ion pumps have to be activated. In particular, the determination of time-dependent currents with high resolution of amplitude and time requires optimal technical conditions. For the Na^+,K^+-ATPase, voltage or patch-clamp studies with high time resolution on intact cells (squid axon and heart cells) have been performed in the past (Nakao and Gadsby, 1986; Friedrich *et al.*, 1996; Holmgren *et al.*, 2000). Also, presteady state experiments using caged ATP on reconstituted membrane systems (black lipid membranes, solid supported membranes) and dissociated heart cells under giant patch-clamp conditions (Fendler *et al.*, 1985, 1987; Apell *et al.*, 1987; Borlinghaus *et al.*, 1987; Friedrich *et al.*, 1996) allowed a remarkable step forward in understanding the mechanism of Na^+,K^+-ATPase transport. In addition to the electrical and electrophysiological approach, the use of the voltage-sensitive dye RH421 combined with a rapid stopped-flow technique provided additional and complementary information on the pump mechanism (Forbush and Klodos, 1991; Kane *et al.*, 1997; Stürmer *et al.*, 1991; Fedosova *et al.*, 1995). In this minireview, we will restrict ourselves to a description of results obtained by these methods. We do this while bearing in mind that an overwhelming body of data has been obtained by other biochemical, molecular biological, and biophysical methods, which have yielded extremely useful information about the enzymic and structural aspects of the Na^+,K^+-ATPase (see e.g., Froehlich *et al.*, 1976; Glynn, 1993).

BLACK LIPID MEMBRANE EXPERIMENTS

Na^+,K^+-ATPase-enriched membrane fragments (Jørgensen, 1974) or Na^+,K^+-ATPase containing proteoliposomes can be adsorbed to black lipid membranes. As demonstrated earlier, the membrane fragments/proteoliposomes and the underlying black lipid membrane are capacitively coupled, so that after synchronized activation of the Na^+,K^+-ATPase, a capacitive pump

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current can be observed (Fendler *et al.*, 1985, 1987; Borlinghaus *et al.*, 1987). Synchronization was achieved by a light-induced concentration jump of ATP from caged ATP. The rate of light-induced release of ATP from caged ATP {if not indicated otherwise, the specific compound was P^3-[1-nitrophenyl)ethyl] ATP} is pH dependent, taking place in 2.5 ms at pH 6.4 and 25 ms at pH 7.4. Therefore, the choice of a moderately low pH is crucial for a sufficient time resolution (see Fendler *et al.*, 1987, 1993). Recently, improved caged ATP compounds have been introduced, which allow microsecond resolution even at neutral pH (Sokolov *et al.*, 1998b; Geibel *et al.*, 2000).

After the concentration jump, the pump currents can be recorded in a short-circuit experiment with extremely high signal-to-noise ratio due to the low conductance of the planar lipid bilayer. For kinetic investigations, the application of caged ATP is advantageous because in the absence of a hydrolyzable ATP, the Na^+,K^+-ATPase, depending on the ion conditions, is located in a defined intermediate of the transport cycle, i.e., E_1Na_3 or E_2K_2. This represents the ideal situation for the study of presteady state kinetics of the pump current.

The analysis of the transient currents generated after photolytic ATP release on the black lipid membrane in the absence of K^+ yielded an electrical signal which could be assigned to the charge translocation activity of the Na^+,K^+-ATPase. The ATPase-related part of the signal consists of three exponential components: a lag phase, a fast rising phase, and a slow decay. The slow decay is ATP dependent and has a relaxation rate of about 30 to 60 s^{-1}. The fast rise is independent of ATP and can be fitted with a relaxation rate of 120 to 200 s^{-1}, depending on temperature (Fendler *et al.*, 1987, 1993). Because the transient current starts with a lag phase, it was concluded that the electrogenic step is preceded by at least two electroneutral steps. Interpretation of the early part of the signal (lag and rise) is complicated (see below). However the minimal conclusion that can be drawn from the transient currents is that an electroneutral ATP binding step with a relaxation rate of 30 to 60 s^{-1} is followed by an electrogenic transition with a relaxation rate equal or larger than 200 s^{-1} at 24°C (Fendler *et al.*, 1993).

A technique that directly addresses the electrogenic step is to apply a harmonic electrical perturbation to the enzyme, activate it by photolytic ATP release, and measure the response of the system using a phase-sensitive amplifier (Sokolov *et al.*, 1998a; Babes and Fendler, 2000). These experiments have confirmed the earlier results yielding relaxation rates for the $E_1P \rightarrow E_2P$ transition of 190 and 329 s^{-1} at pH 6.2 and 7.4, respectively (Babes and Fendler, 2000). Surprisingly, these measurements revealed that the E_1P/E_2P equilibrium is poised to the E_1P side (i.e., backward with respect to transport) and that Na^+ transport is driven by the low affinity of the extracellular Na^+ discharge site.

The interpretation of the transient currents is based on the reaction cycle of the Na^+K^+-ATPase—the Albers–Post model (Fig. 1). Since it has been shown that the electrogenic step is downstream from the phosphorylation reaction $E_1 \rightarrow E_1P$ (Borlinghaus *et al.*, 1987) and since, in the absence of K^+, the decay of E_2P is slow (6 s^{-1}, Hobbs *et al.*, 1985), the $E_1P \rightarrow E_2P$ transition is a plausible candidate for the observed electrogenic reaction. However, the assignment of a rate constant to this transition is not trivial since a number of different processes contribute to the lag and the rising phase: electronic filtering, release of ATP from caged ATP (ca. 2.5 ms at pH 6.2), and the phosphorylation reaction. In addition, the inhibitory effect of caged ATP has to be taken into account (Forbush, 1984; Fendler *et al.*, 1993, Clarke *et al.*, 1998). Some of these effects can be overcome by determining the rate of phosphorylation and by fitting the data with the complete reaction model (Fendler *et al.*, 1984). Also, a fast-releasing caged ATP can be used (Geibel *et al.*, 2000; Sokolov *et al.*, 1998b). Using these approaches, rate constants for the $E_1P \rightarrow E_2P$ transition of 200 to 1000 s^{-1} were obtained. However, the most direct access to this rate probably is the periodic relaxation technique (Babes and Fendler, 2000) that yields

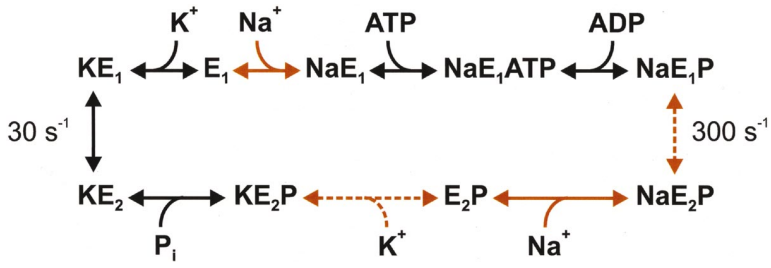

Fig. 1. Reaction cycle of the Na^+,K^+-ATPase (Albers–Post model). Na and K stoichiometry are not taken into account. Electrogenic steps are indicated by and asterisk, strongly electrogenic ones by a solid arrow and weakly electrogenic ones by a dashed arrow.

a relaxation rate of 190 s^{-1} (pH 6.2) and 320 s^{-1} (pH 7.4) at 24°C for the electrogenic $E_1P \rightarrow E_2P$ transition.

The Na^+ binding step at the intracellular side ($E_1 + Na \rightarrow NaE_1$) was also investigated using a planar lipid membrane technique. This electrical measurement proved to be complicated, since it required a Na^+ concentration jump, which is difficult to realize in a black lipid membrane measurement. However, the development of the solid-supported membrane allowed such an experiment and, indeed, an electrical signal associated with a Na^+ concentration jump was found (Pintschovius *et al.*, 1999). Since, under these conditions, the enzyme was in the E_1 conformation, this proves electrogenicity of intracellular Na^+ binding.

The electrical experiments described so far were done in the absence of K^+, where the dephosphorylation is slow (Hobbs *et al.*, 1985). Therefore, a second electrogenic step in the K^+-dependent limb, even if present, could not be detected. However, black lipid membrane experiments in the presence of K^+ failed to show an electrogenic reaction in the K^+-dependent limb of the reaction cycle (Gropp *et al.*, 1998). These authors give an upper limit for the charge translocation during K^+ transport of 20% of that during Na^+ transport. In contrast, Schwarz and colleagues (Lafaire and Schwarz, 1986; Rakowski *et al.*, 1991) showed that the shape of the current voltage dependence of the stationary pump currents of Na^+,K^+-ATPase in oocytes from *Xenopus laevis* requires a second electrogenic step during K^+ transport. Possibly the electrogenic contribution of K^+ transport is much smaller than that of Na^+ translocation and was not detected in the black lipid membrane experiments.

K^+ release at the cytoplasmic side was investigated using the solid-supported membrane. In contrast to Na^+, it was found that K^+ deocclusion and cytoplasmic K^+ release [$E_2(K) \rightarrow E_1K \rightarrow E_1 + K$] are probably electroneutral (Pintschovius *et al.*, 1999) or that the charge translocation compared to that during Na^+ transport is <10%.

PATCH-CLAMP AND VOLTAGE-CLAMP STUDIES

As early as 1986, Nakao and Gadsby reported an electrogenic process in the Na^+-dependent part of the Na^+,K^+-ATPase reaction cycle using voltage jumps on whole cell patches of dissociated heart cells. They determined a forward rate for the electrogenic event of about 63 s^{-1}. (Since the measurements we are comparing were performed at different temperatures, we give here and in the following only corrected values for 24°C using an activation energy of 80 kJ/mole; Friedrich and Nagel, 1997). Rakowski *et al.* (1991) found a forward rate constant of 225 s^{-1} in *Xenopus* oocytes. The introduction of the giant patch technique (patch diameter of 20 to 30 μm; Hilgemann, 1989) allowed the study of the electrical properties on heart cells with both the voltage-jump technique and the concentration-jump technique via the application of caged ATP (Friedrich and Nagel, 1997; Friedrich *et al.*, 1996). Both Hilgemann's data, as well as the data obtained on giant patches via voltage jump or concentration jump by Friedrich and colleagues, yielded rates of ~200 s^{-1}. The discrepancy between the results obtained with heart cells in the whole cell configuration and the oocyte or giant patch studies, respectively, might be due to the fact that under whole cell conditions the pump is downregulated by a yet unknown factor in the cytosol (Hilgemann, 1994; Friedrich *et al.*, 1996).

Interestingly, the relaxation rate in the voltage-jump experiments decreases with depolarization of the membrane from −200 to +50 mV (Nakao and Gadsby, 1986; Friedrich *et al.*, 1997) reaching a plateau at positive voltage. Since the observed relaxation rate is the sum of the forward and reaction rate constants, it was concluded that only the backward rate constant is voltage dependent and that the plateau reflects the potential independent forward rate constant (Nakao and Gadsby, 1986). A plausible kinetic explanation for these findings is that a rate-limiting electroneutral $E_1P \rightarrow E_2P$ transition is followed by a fast (too fast to be time resolved) electrogenic step. On the basis of further voltage-clamp studies on squid axon membranes, DeWeer and colleagues (Gadsby *et al.*, 1993, Holmgren *et al.*, 2000) in a series of papers, came to the conclusion that the main electrogenic step during the Na^+ transport is, indeed, the release of Na^+ via a high-field access channel. Further detailed studies with a high time-resolution voltage clamp technique allowed the authors to determine the sequential release process of the 3 Na^+ ion on the extracellular side, with rates for the electrogenic steps of 1000 s^{-1} and faster.

How can the relaxation rates determined in the black lipid membrane experiments (200–300 s^{-1}) be reconciled with the fast electrogenic reactions found in the squid axon measurements? If the time resolution of a technique is not sufficient to resolve the fast reaction, this compound reaction is kinetically equivalent to an electrogenic reaction with the rate constant of the limiting process. Therefore, black lipid membrane as well as patch-clamp and voltage-clamp measurements are compatible with an electroneutral $E_1P \rightarrow E_2P$ transition with a rate of ca. 200–300 s^{-1}, followed by an electrogenic Na^+ binding/release step. However, this does not rule out partial electrogenicity of the $E_1P \rightarrow E_2P$ transition itself (Ganea *et al.*, 1999).

In addition to the stationary measurements of Schwarz and colleagues (Lafaire and Schwarz, 1986;

Rakowski *et al.*, 1991) relaxation experiments also support the concept of an electrogenic reaction in the K^+-dependent part of the Na^+,K^+-ATPase reaction cycle. Using the whole cell patch-clamp technique on heart cells cells and Tl^+ instead of K^+, Peluffo and co-workers (Peluffo and Berlin, 1997) demonstrated relaxation currents after a voltage-jump protocol that could be assigned to extracellular K^+ binding.

FLUORESCENCE STUDIES USING RH421

The voltage-sensitive fluorescent dye RH421 has been used for detection of electrogenic events in the transport cycle of the Na^+,K^+-ATPase. It was initially proposed that this dye directly reports on the transmembrane potential or senses the translocation of the cations (Stürmer *et al.*, 1991). This view may need to undergo some revision, since it has been found that the dye interacts strongly with the protein and responds as well to its conformational state (Frank *et al.*, 1996; Fedosova *et al.*, 1995). However, as will be shown below, the combination of presteady state direct electrical measurements with complementary results using a potential sensitive dye allows a detailed characterization of single steps of the transport cycle in terms of electrogenicity and rate.

RH421 and its analog RH160 were originally applied to the Na^+,K^+-ATPase by Forbush and Klodos (1991) in order to study conformational changes of the Na^+ pump. An important requirement for detection of the RH421 fluorescent changes is a membrane preparation with a high protein concentration. Fortunately, this is available for the Na^+,K^+-ATPase (Jørgensen, 1974) and the dye was used extensively by various groups (Stürmer *et al.*, 1991; Pratap and Robinson, 1993; Fedosova *et al.*, 1995; Kane *et al.*, 1997). In combination with a rapid stopped-flow technique, it allowed the determination of the kinetic parameters of various partial reactions of the enzyme.

Using RH421, partial reactions throughout the reaction cycle were kinetically characterized. In particular, these measurements yielded rate constants for the $E_1P \rightarrow E_2P$ transition ($\geq$260 s^{-1}), dephosphorylation (280 s^{-1}), and the $E_2 \rightarrow E_1$ transition ($\leq$39 s^{-1}) on preparations of Na^+,K^+-ATPase from pig and rabbit kidney (Kane *et al.*, 1997; Clarke *et al.*, 1998). These data were compatible with the results of the electrical measurements and showed no significant species difference.

CONCLUSIONS

The kinetic and electrical data available so far suggest that at least two of the four possible cation binding/release steps are electrogenic: intracellular Na^+ binding and extracellular Na^+ release. As for the steps involving K^+ binding and release, the answer is much less clear-cut. Extracellular K^+ binding is probably electrogenic while intracellular release and the $E_2 \rightarrow E_1$ transition are not. Possibly these steps are rather weakly electrogenic, only 10–20% of the electrogenicity of the Na^+ translocation and release event. The obvious difference between Na^+ and K^+ suggests that the translocation pathways of the two cations in the protein are different. The conformational transitions that switch the binding sites from intracellular access to extracellular access and back, $E_1P \rightarrow E_2P$ with a rate of $\sim$300 s^{-1} and $E_2 \rightarrow E_1$ with a rate of $\sim$40 s^{-1}, are probably not strongly electrogenic. The proposed structural concept (Läuger, 1991; Gadsby *et al.*, 1993) for the electrogenic cation binding steps—a high-field access channel on the extracellular side—is plausible. However, the final proof of that concept can only come from structural data. It is hoped that the now-available structure of the Ca^{2+}-ATPase from sarcoplasmic reticulum, when solved for different conformations (E_1/E_2), can help to decide this question. Because of the homology between the Ca^{2+}-ATPase and the Na^+,K^+-ATPase, such an approach appears to be justified.

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