Volume 224, number 1, 83–88 FEB 05281 November 1987

Kinetics of pump currents generated by the Na^+,K^+-ATPase

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Received 17 August 1987

Purified Na^+,K^+-ATPase from pig kidney was attached to black lipid membranes. Pump currents of the enzyme could be measured with a time resolution of approx. 1 ms by releasing ATP from caged ATP with a UV laser flash. Analysis of the transient currents shows that a slow non-electrogenic step is followed by an electrogenic transition with a rate constant of 100 s^{-1} (22 °C). The exponential components found in the transient currents are compared to transitions in the Albers-Post scheme.

Na^+,K^+-ATPase; Black lipid membrane; Transient kinetics; Electrogenic property; Pump current

1. INTRODUCTION

The Na^+,K^+-ATPase from the plasma membrane is an electrogenic pump which exchanges three Na^+ for two K^+ per molecule of ATP hydrolysed. Although a considerable amount of work was done on structure, steady state kinetics and transient kinetics (review [1]), much less data are available concerning the electrical properties of the pump. Identification of the electrogenic steps and their kinetic investigation, however, is important for the understanding of the ion translocation mechanism.

The electrical properties of the Na^+,K^+-ATPase were investigated in whole cells (review [2]), reconstituted vesicles (e.g. [3,4]) and on black lipid membranes (BLM) [5,6]. Adsorption of membrane fragments containing the purified Na^+,K^+-ATPase to a BLM offers the possibility to monitor directly the charge translocation taking place during the Na^+,K^+-ATPase pumping cycle. By improving the method described before [5] the currents generated by the Na^+,K^+-ATPase after an ATP concentration jump could be measured with a time resolution of 1 ms. This provides at the same time information about the electrogenicity as well as the rate constants of partial reaction steps of the Na^+,K^+-ATPase.

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2. MATERIALS AND METHODS

2.1. *Preparation of caged ATP and Na^+,K^+-ATPase*

The triethylammonium salt of caged ATP (P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate) was synthesized as reported [5]. Na^+,K^+-ATPase was prepared from the red outer medulla of pig kidneys according to Jorgensen [7]. Membrane fragments in the form of cup shaped discs of 0.1–0.6 μm diameter were obtained. The suspension contained ~2 mg/ml protein with a typical specific activity of 30 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ at 37°C.

2.2. *Lipid bilayer setup*

Optically black lipid membranes with an area of 10^{-2} cm^2 were formed in a thermostated teflon cell with 1.5 ml of an appropriate electrolyte solution in each compartment. The temperature was kept at 22°C. The membrane forming solution contained

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00145793/87/$3.50

1.5% (w/v) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL, USA) and 0.025% (w/v) octadecylamine (Riedel de Haen, Hannover, FRG) in *n*-decane. The membrane was connected to an external measuring circuit via platinum electrodes. To avoid photoeffects, the electrodes were separated from the aqueous compartments of the teflon cell by agar-agar salt bridges. The signals were amplified with a current amplifier (gain, 10^7 V/A), followed by a voltage amplifier (gain, 100; bandwidth, 1 kHz) filtered at 500 Hz and recorded with a digital oscilloscope. For further details see [5].

The membrane bathing solution contained 3 mM $MgCl_2$, 25 mM imidazole-HCl, 130 mM NaCl and 20 mM KCl if required. 15 μl of the Na^+,K^+-ATPase containing suspension together with different amounts of caged ATP were added under stirring to one compartment of the cuvette. To photolyse the caged ATP light pulses of an excimer laser (Lambda Physik, EMG 100) of 10 ns duration and a wavelength of 308 nm were attenuated and focused onto the membrane. Care was taken that the membrane was uniformly illuminated, and that only the BLM itself and neither the cuvette nor the torus of the membrane were irradiated. The energy density of the membrane was 120 mJ/cm^2. Under these conditions each flash photolysed 10% of the caged ATP in the membrane plane.

3. RESULTS

Purified Na^+,K^+-ATPase was added together with caged ATP under stirring to one side of the BLM (see fig.1a) to allow adsorption of the enzyme containing discs to the BLM. After 15 min a transient current could be induced by a UV light pulse indicating adsorption of the discs to the BLM.

Fig.2 shows the current induced by a laser flash in the absence and presence of 20 mM K^+. Also the results of a fit with the function:

$$I(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} - (A_1 + A_2) e^{-t/\tau_3} \qquad (1)$$

were included in fig.2. The narrow peak at $t = 0$ is an artefact produced by the laser. It is independent of the presence of the protein and of caged ATP. To avoid distortion of the results by the artefact only data points with $t > 10$ ms were used for evaluation.

In the absence and presence of K^+ (fig.2I and 2II), the transient current rises with the same time constant ($\tau_1 = 16$ ms). In both cases the decay is characterized by two time constants τ_2 and τ_3, which become larger by a factor of 2 and 4, respectively, when K^+ is added to the electrolyte. Note that the amplitude of τ_3 is negative in the absence of K^+.

A schematic representation of the disc-BLM configuration is given in fig.1b. The ion pumps located in the discs are capacitively coupled to the measuring circuit via the capacitance of the BLM. The equivalent circuit of this arrangement is shown in fig.1c. A current is measured if a relaxation process of the ion pump is accompanied with a net charge movement perpendicular to the membrane surface. The current $I_p(t)$ of the pump, however, is distorted by the network formed by the disc-BLM compound membrane (fig.1c). In particular the characteristic time constant $\tau_0 = (C_m + C_p)/(G_m + G_p)$ of the network is introduced in the measured signal [8]. For purple membrane adsorbed to a BLM τ_0 is approx. 200 ms [9].

In the absence of K^+, dephosphorylation is slow [10] and the pump is virtually stopped after the Na^+-dependent steps. In contrast, rapid turnover is possible if K^+ is present. This is clearly demonstrated by the stationary currents generated by the Na^+,K^+-ATPase in the presence of ionophores [6]. On a time scale longer than the relaxation times due to the enzyme the current $I_p(t)$ can therefore be approximated by a short current pulse (mathematically described by a δ-function) if K^+ is absent and by a step-function in the presence of K^+. Analysis of the equivalent circuit (fig.1c) shows that the response of the system to a δ-function is a fast current pulse followed by a negative current, which decays with τ_0. A step function yields a positive current decaying with a time constant $\tau \leq \tau_0$ [9], where $\tau = \tau_0$ applies if the pump current $I_p(t)$ is voltage independent and $\tau < \tau_0$ if the current decreases with increasing voltage across the pump.

Inspection of the transient currents with and without K^+ (fig.2I and II) shows that the experimental results are in qualitative agreement with the behaviour expected from circuit analysis if the fast events (τ_1 and τ_2) are neglected and τ_3 is as-

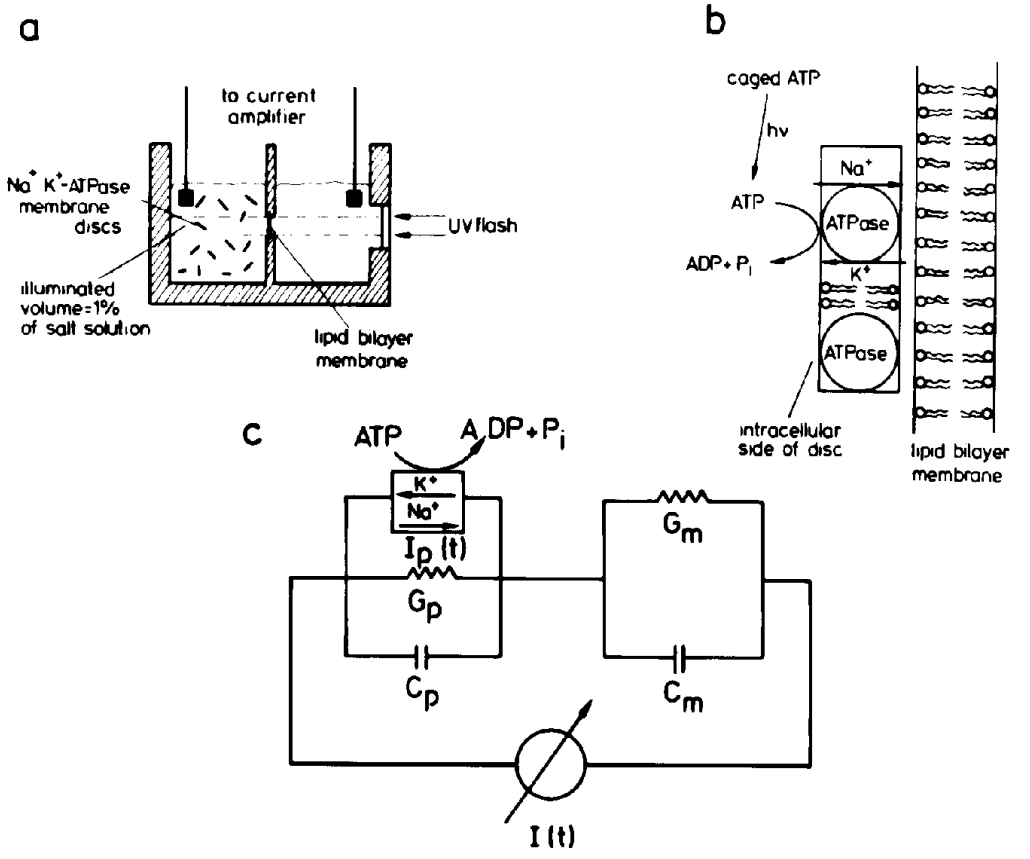

Fig.1. Schematic representation of the bilayer setup. (a) Teflon chamber with black lipid membrane (BLM) and adsorbed Na^+,K^+-ATPase discs. (b) Proposed sandwich-like arrangement of discs and underlying lipid membrane. The two membranes are capacitively coupled. (c) Equivalent circuit diagram of the two membranes in series. G_m and G_p refer to the conductance of the BLM and the disc membrane, respectively, C_m and C_p to the capacitance of the underlying BLM and of the discs, respectively. I_p designates the current generator.

signed to the characteristic time constant of the network. Following the arguments given above the slow decay time ($\tau_3 = 339$ ms without K^+) should remain constant or even decrease after addition of K^+. This is not observed ($\tau_3 = 1440$ ms with K^+). This inconsistency is not understood. It may be related to the fact that the conductivities involved in the equivalent circuit are dependent on the composition of the electrolyte.

Comparison of the time constants summarized in fig.2 with data available in the literature gives additional evidence for the assignment of τ_3. The turnover of the Na^+,K^+-ATPase under conditions comparable to those of fig.2II (i.e. in presence of Na^+ and K^+) is approx. 7 s^{-1} [11]. Therefore only time constants up to roughly 150 ms can be assigned to the characteristic time constant of the network. Following the arguments given above the slow decay time ($\tau_3 = 339$ ms without K^+) should remain constant or even decrease after addition of K^+. This is not observed ($\tau_3 = 1440$ ms with K^+). This inconsistency is not understood. It may be related to the fact that the conductivities involved in the equivalent circuit are dependent on the composition of the electrolyte.

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Because of the slow dephosphorylation in the absence of K^+ the time constant $\tau_3 = 339$ ms may be alternatively explained by a slow electrogenic transport of Na^+ to the intracellular side of the Na^+,K^+-ATPase. However, the addition of ionophores completely abolishes component 3 of the transient current [6]. This rules out that τ_3 is an intrinsic time constant of the enzyme.

It was shown that both in the absence and presence of K^+ τ_3 has to be assigned to the characteristic time τ_0 of the equivalent circuit. Because τ_3 is much larger than τ_1 and τ_2 it has a

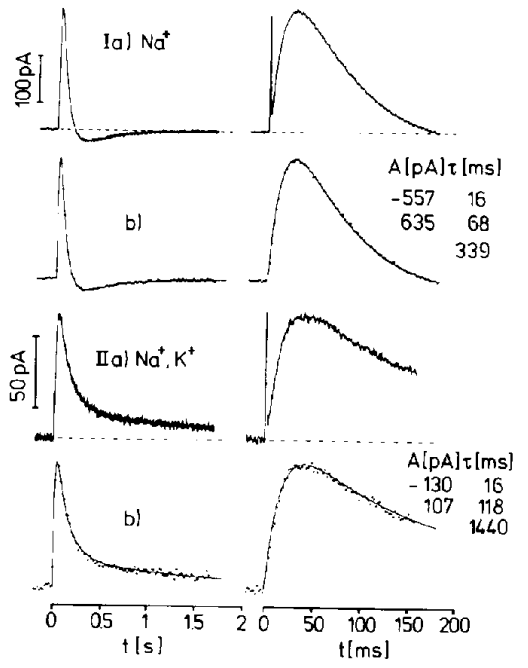

Fig.2. Short circuit currents of the disk/BLM system in the absence (I) and presence (II) of K^+. At $t = 0$ the system was illuminated with a UV flash. The signal was recorded simultaneously with two different sample rates (left hand side and right hand side of the figure). Both traces were simultaneously fitted using eqn 1. Fitted curve (solid line) and data (dots) are compared in part (b) for each signal. For clarity only a reduced number of data points is displayed. Also the resulting fit parameters are included.

comparatively small amplitude and only slightly affects the amplitudes of the fast compononents τ_1 and τ_2. In the following τ_3 will therefore be neglected.

The dependence of the time constants on the ATP concentration in the presence of Na^+ and K^+ was measured at pH 6.2 and pH 7.7 by adding appropriate amounts of caged ATP to the enzyme containing compartment of the cuvette. As discussed above only τ_1 and τ_2 have to be taken into account. This was achieved by selecting an appropriate time range 10 ms $< t >$ 400 ms for fitting of the data.

The two intrinsic time constants of the Na^+,K^+-ATPase found in the photoinduced current suggest a two-step first-order reaction sequence with an electrosilent transition followed by an electrogenic one. The latter is concluded from the fact that the current rises from zero with a time constant much slower than the rise time of the measuring circuit or the release of ATP (0.7 ms [12]). In addition it was assumed that at different ATP concentrations the same charge is translocated during the transitions described by τ_1 and τ_2 and that the second step is irreversible. Under these conditions the current transient is described by:

$$I(t) = \frac{A}{\tau_2 - \tau_1} \cdot (e^{-(t-D)\tau_2} - e^{-(t-D)/\tau_1}) \qquad (2)$$

where A has to be constant at different ATP concentrations. In addition the delay time, D, was introduced to account for the kinetics of ATP release from caged ATP.

The dependence of the resulting fit parameters τ_1, τ_2 and D on the ATP concentration is shown in fig.3. The delay D and the inverse of the relaxation time $1/\tau_1$ are approximately constant, while $1/\tau_2$

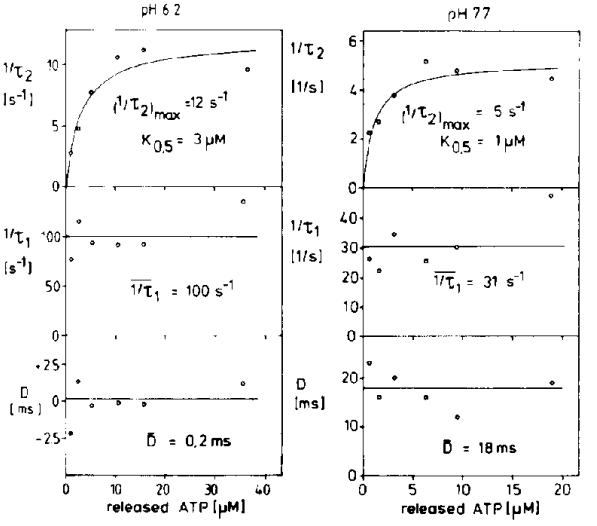

Fig.3. ATP dependence of the inverse time constants $1/\tau_1$ and $1/\tau_2$ and of the delay D (see eqn 2) of the UV flash induced current at pH 6.2 and pH 7.7. Different amounts of caged ATP were added. Each flash converted 10% of the caged ATP to ATP resulting in concentrations of released ATP as given in the figure. For $1/\tau_2$ the solid line represents a fit of a Michaelis-Menten type concentration dependence $1/\tau_2 = (1/\tau_2)_{max} \cdot ATP/(ATP + K_{0.5})$ to the data. For $1/\tau_1$ and D the solid line is a horizontal line at the average value $1/\tau_1$ and D, respectively.

saturates with a $K_{0.5}$ of 1 μM (pH 7.7) and 3 μM (pH 6.2) ATP.

4. DISCUSSION

It was previously shown that the current transient generated by the Na^+,K^+-ATPase after an ATP concentration jump corresponds to early steps of the enzymatic reaction cycle were Na^+ is bound and probably transported [5,6]. The measurements shown in fig.2. support this idea because the time constants τ_1 and τ_2 of the enzyme are very similar in the presence and absence of K^+.

Additional information can be drawn from the ATP dependence of the time constants. The saturating behaviour of $1/\tau_2$ shows that substrate binding is involved in this step and that it consequently has to precede the reaction characterized by τ_1. The ATP dissociation rate constant of 20 s^{-1} [18] is of the same order of magnitude as $(1/\tau_2)_{max}$. Fitting the ATP dependence of $1/\tau_2$ with a Michaelis-Menten type substrate dependence (fig.3) yields half saturation concentrations of $K_{0.5}$ = 3 μM (pH 6.2) and $K_{0.5}$ = 1 μM (pH 7.7). They agree well with the ATP binding constants of 2 μM as obtained previously from the concentration dependence of the peak currents [6]. The situation is, however, complicated by the fact that caged ATP is a competitive inhibitor of the Na^+,K^+-ATPase [19,6]. Since the dissociation rate constant of caged ATP is not known binding and competition of ATP and caged ATP cannot be discussed quantitatively.

The arguments given above support a kinetic model consisting of a fast electrogenic step with rate constant 100 s^{-1} (pH 6.2) which is preceded by a non-electrogenic slow process which takes place in approximately 80 ms (at high ATP concentrations and pH 6.2) and involves binding of the substrate. The speed of both reactions is decreased when increasing the pH to 7.4. It is tempting to compare these findings with the current model of the ATPase reaction cycle, as first formulated by Albers and Post et al. [13,14]. The Na^+ dependent part of this reaction scheme is:

$$E_1ATP \rightarrow E_1 \cdot ATP \rightarrow E_1P \rightarrow E_2P. \quad (3)$$

Rate constants for the different steps obtained by quenched flow experiments were reported previously. For the phosphorylation step $E_1 \rightarrow E_1P$ a rate constant of 180 s^{-1} was found [16] while for the conformational transition $E_1P \rightarrow E_2P$ a value of 77 s^{-1} was reported [15].

As already discussed τ_2 is attributed to substrate binding. τ_1 must then be due to reactions of the phosphoenzymes which agrees with the recently reported electrogenicity of $E_1P \rightarrow E_2P$ [17]. No indication for a third reaction step of the enzyme was found in the transient currents (fig.2). If, however, one of the phosphoenzyme reactions is fast the two-step process would essentially look like a single transition. Simulations of the transient current on the basis of eqn 3 using the rate constants given in the literature (20 s^{-1}, 180 s^{-1} and 77 s^{-1}) were indeed consistent with the data presented in fig.2. Even so assignment of the observed transition by comparison with the rate constants reported in [15,16] is not possible because of differences of in enzyme provenience and electrolyte composition. The decision of which of the phosphoenzyme transitions is described by τ_1 has therefore to remain open.

D was introduced to allow for the delayed release of ATP from caged ATP. As shown in fig.3, D is approximately constant at all ATP concentrations. The average value of D was 18 ms at pH 7.7 and 0.2 ms at pH 6.2. This is in agreement within the experimental error with the relaxation time for the release of caged ATP determined in [12] (23 ms at pH 7.7 and 0.9 ms at pH 6.2).

It was shown that quantitative kinetic information about individual steps of the Na^+,K^+-ATPase reaction cycle may be obtained by measuring the electrical current generated by a purified enzyme preparation adsorbed to a BLM. The experiments demonstrate that the method which was developed for light driven ion pumps like bacteriorhodopsin may be applied to transport proteins driven by chemical energy.

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