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Fast Charge Translocations Associated with Partial Reactions of the Na,K-Pump: I. Current and Voltage Transients after Photochemical Release of ATP

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Summary. Nonstationary electric currents are described which are generated by the Na,K-pump. Flat membrane sheets 0.2–1 μm in diameter containing a high density of oriented Na,K-ATPase molecules are bound to a planar lipid bilayer acting as a capacitive electrode. In the aqueous phase adjacent to the bound membrane sheets, ATP is released within milliseconds from an inactive, photolabile precursor ("caged" ATP) by an intense flash of light. After the ATP-concentration jump, transient current and voltage signals can be recorded in the external circuit corresponding to a translocation of positive charge across the pump protein from the cytoplasmic to the extracellular side. These electrical signals which can be suppressed by inhibitors of the Na,K-ATPase require the presence of Na^+ but not of K^+ in the aqueous medium. The intrinsic pump current $I_p(t)$ can be evaluated from the recorded current signal, using estimated values of the circuit parameters of the compound membrane system. $I_p(t)$ exhibits a biphasic behavior with a fast rising period, followed by a slower decline towards a small quasistationary current. The time constant of the rising phase of $I_p(t)$ is found to depend on the rate of photochemical ATP release. Further information on the microscopic origin of the current transient can be obtained by double-flash experiments and by chymotrypsin modification of the protein. These and other experiments indicate that the observed charge-translocation is associated with early events in the normal transport cycle. After activation by ATP, the pump goes through the first steps of the cycle and then enters a long-lived state from which return to the initial state is slow.

Key Words Na,K-ATPase · ion pumps · electrogenic transport · concentration jump · "caged" ATP

Introduction

The Na,K-pump in the plasma membrane of animal cells transports sodium ions outward and potassium ions inward, using ATP hydrolysis as an energy source (Skou, 1975; Robinson & Flashner, 1979; Cantley, 1981; Schuurmans-Stekhoven & Bonting, 1981; Jørgensen, 1982; Glynn, 1985; Kaplan, 1985). The pump molecule goes through a cycle of phosphorylation-dephosphorylation reactions and conformational changes during which ions are bound on one side and released to the other side. Since (under normal conditions) outward sodium flux is larger than inward potassium flux, the pump is electrogenic, i.e., it translocates net charge across the membrane. An important aspect of the electrogenic nature of the pump is the possibility of obtaining mechanistic information by studying the electric current generated by the pump (Hansen et al. 1981; Chapman, Johnson & Kootsey, 1983; De Weer, 1984; Glynn, 1984; Reynolds, Johnson & Tanford, 1985; Läuger & Apell, 1986). Currents associated with active transport of Na^+ and K^+ have been investigated so far mainly in the steady state of the pump (Nakashima & Takahashi, 1966; Meunier & Tauc, 1970; Isenberg & Trautwein, 1974; Abercrombie & De Weer, 1978; Eisner & Lederer, 1980; Glitsch et al. 1982; De Weer & Rakowski, 1984; Gadsby, 1984; Lederer & Nelson, 1984; Gadsby, Kimura & Noma, 1985; Hasuo & Koketsu, 1985; De Weer, Gadsby & Rakowski, 1986; Lafaire & Schwarz, 1986). Independent information on the kinetic parameters of the pumping cycle can be obtained by studying ion transport or pump current in the nonstationary state after a perturbation of the transport system (Forbush, 1984, 1985; Fendler et al., 1985; Karlish & Kaplan, 1985; Nakao & Gadsby, 1986).

In the following we describe experiments in which nonstationary pump currents are induced by a sudden change of ATP concentration. For the measurement of the transient current signals, flat membrane fragments rich in Na,K-ATPase are bound to a planar lipid bilayer acting as a capacitive electrode. The method of capacitive coupling has been widely used for studying fast charge translocations in membranes (Drachev et al., 1974; Herrmann & Rayfield, 1978; Hong & Montal, 1979; Keszthelyi & Ormos, 1980; Fahr, Läuger & Bamber, 1981; Trissl, 1985). The membrane preparation, which is obtained by dodecylsulfate extraction of kidney microsomes (Jørgensen, 1974), consists of

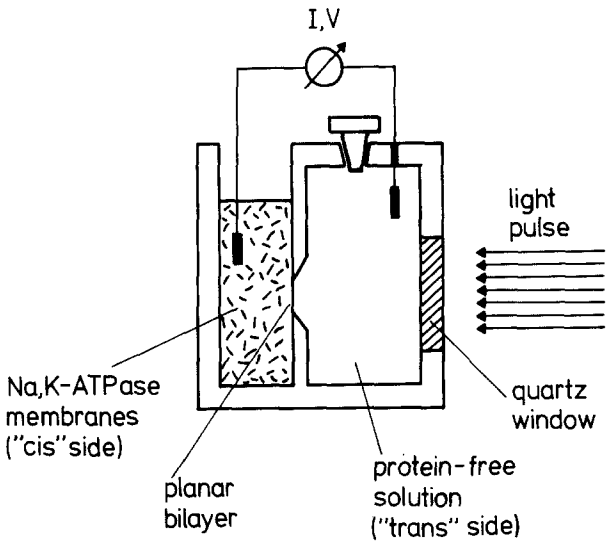


Fig. 1. Cell for the measurement of current and voltage signals from optically black lipid films with adsorbed Na,K-ATPase membrane fragments. The *trans* compartment was closed after filling with the solution. The solution volumes of the open and the closed compartment were 0.3 and 5 ml, respectively; the diameter of the circular membrane was approximately 1.3 mm. A magnetic stirrer (*not shown*) was present in the *cis* compartment. The solutions were connected to the external measuring circuit via agar bridges and silver/silver-chloride electrodes. In order to minimize stray-light artifacts from the electrodes, a suspension of carbon black was added to the agar

flat membrane sheets 0.2–1 μm in diameter containing oriented Na,K-ATPase molecules with a density of several thousand per μm^2 (Deguchi, Jørgensen & Maunsbach, 1977; Skriver, Maunsbach & Jørgensen, 1981; Hebert et al., 1982; Zampighi et al., 1986). In the aqueous phase which is in contact with the bound membrane sheets, ATP is released within milliseconds from an inactive, photolabile derivative ("caged" ATP) by an intense flash of light (Kaplan, Forbush & Hoffman, 1978; McCray et al., 1980). After the ATP-concentration jump which leads to a (nearly) simultaneous activation of many pump molecules, transient current and voltage signals can be recorded in the electrical circuit connecting the aqueous phases adjacent to the lipid bilayer. These electrical signals can be suppressed by inhibitors of the Na,K-ATPase and require the presence of Na^+ but not of K^+ in the aqueous medium; they are thought to reflect early electrogenic events in the pumping cycle.

Materials and Methods

Materials

L-1,2-diphytanoyl-3-phosphatidylcholine was obtained from Avanti Polar Lipids, Birmingham, Ala.; sodium dodecylsulfate (SDS) from Pierce Chemical Co., Rockford, Ill.; and sodium cholate from Merck, Darmstadt. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, luciferin, luciferase, NADH and ATP (disodium salt, Sonderqualität) were from Boehringer, Mannheim. α-chymotrypsin (type II), apyrase VI, ouabain, monensin and dithiothreitol (DTT) were purchased from Sigma, and carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP) from Serva (Heidelberg). NaCl was used in Suprapur quality (Merck). All other reagents were analytical grade.

P^3 - 1 - (2 - nitro)phenylethyladenosine - 5' - triphosphate ("caged" ATP) was synthetized by K. Janko using a modified version of the method of Kaplan et al. (1978). The purity of the product was checked by HPLC. The compound was stored as a tetramethylammonium salt in the dark at −40°C.

Enzyme Preparation

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure *C* of Jørgensen (1974). This method yields purified enzyme in the form of membrane fragments containing about 0.6 mg phospholipid and 0.2 mg cholesterol per mg protein (Jørgensen, 1974; 1982). The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971) and the protein concentration by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. For most preparations the specific activity was in the range between 1500 and 2200 μmol P_i per hr and mg protein at 37°C, corresponding to a turnover rate of 120–170 sec^{-1} (based on a molar mass of 280,000 g/mol). The suspension of Na,K-ATPase-rich membrane fragments (about 3 mg protein per ml) in buffer (25 mM imidazole, pH 7.5, 1 mM EDTA, 10 mg/ml saccharose) was frozen in samples of 100 μl; in this form the preparation could be stored for several months at −70°C without significant loss of activity.

Recording of Current and Voltage Signals

Optically black films were formed from a solution of 10 mg/ml diphytanoylphosphatidylcholine in *n*-decane (Läuger et al., 1967). The membrane cell (Fig. 1), which was made of Teflon, consisted of two compartments separated by a thin Teflon septum with a circular hole of 1.3 mm diameter. One compartment could be completely closed after filling with the aqueous solution; this increased the mechanical stability of the membrane and allowed efficient stirring in the open compartment (*cis* side) to which Na,K-ATPase membrane fragments were added. The solution volumes of the closed and the open compartment were 5 and 0.3 ml, respectively. The Teflon cell was enclosed in a thermostated metal block with openings for the entry of the UV-light beam and for the visual observation of the membrane. The area of the black film was measured with an eye-piece micrometer.

The solutions on either side of the lipid membrane were connected to the external measuring circuit via agar bridges and silver/silver-chloride electrodes. In order to minimize stray-light artifacts from the electrodes, a suspension of carbon-black was added to the agar. For current measurements under short-circuit conditions, the signal was amplified with a Keithley Mod. 427 current amplifier, with the rise-time usually set at 3 msec. For measuring voltage signals, a Mod. 3528 Burr-Brown amplifier with an input impedance of 10^{12} Ω was used. The signals were recorded with a transient recorder (Tracor, Mod. TN 1710) and stored on tape. The membrane cell and the electrodes were enclosed in a Faraday shield.

If not otherwise indicated, the aqueous solutions contained

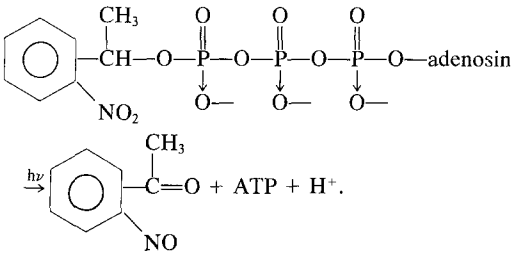
50 mM Tris, pH 7.0, 20 mM dithiothreitol (DTT), 20 mM $MgCl_2$ and various concentrations of NaCl. In the absence of DTT the photoresponse of the membrane slowly decreased with time (over a period of 15–30 min), presumably as a result of autoxidation of SH-groups of the protein. "Caged" ATP and membrane fragments (about 40 μg protein/ml) were added together to the open compartment (*cis* side) under stirring. The stirring was continued for about 1 min. Within a time period of about 10–20 min after addition of the membrane fragments, the lipid film assumed a silvery white appearance. This increase in reflectivity is likely to result from the adsorption of membrane fragments to the lipid bilayer. If not otherwise indicated, the temperature was 20 ± 1°C.

Photochemical Release of ATP

Light flashes of 40 μsec duration and a total energy of 5 J were generated with a xenon flash-tube (Mod. 5M-3) from EG&G Electro-Optics (Salem, MA). The emitted light passed through a bandpass filter with >50% transparency between 260 and 380 nm (Schott, Mainz, Mod. UG11) and was focussed onto the membrane.

In most experiments the concentration of "caged" ATP prior to the flash was 0.5 mM. At the absorption peak at 260 nm the molar extinction coefficient ε of caged ATP is about 2×10^4 M^{-1} cm^{-1} (Kaplan et al., 1978). This corresponds, at a concentration of $c = 0.5$ mM, to a length constant of $1/\varepsilon c \approx 1$ mm ($1/\varepsilon c$ is the distance over which the light intensity decreases e-fold).

At pH 7.0, ATP is liberated from "caged" ATP with a time constant of 4.6 msec (McCray et al., 1980) according to the reaction



The concentration of released ATP was estimated in the following way. The membrane cell was substituted by a quartz cuvette (optical path length 0.1 mm) which was filled with the standard solution normally present on the *cis* side of the membrane, including 0.5 mM caged ATP. The location of the quartz cuvette coincided with the usual location of the membrane. The amount of liberated ATP was determined by the luciferin/luciferase test, which was calibrated using solutions of known ATP concentrations (De Luca & McElroy, 1978; Ernst, Böhme & Böger, 1983). Accounting for the solution volume in the quartz cuvette and for the cross section of the light beam, the concentration of released ATP in the pathway of the beam was estimated to be about 50 μM, corresponding to a conversion efficiency of approximately 10%.

Circuit Properties of the Membrane System

The compound membrane system, consisting of a black lipid film with adsorbed Na,K-ATPase membrane fragments, may be represented by the equivalent circuit shown in Fig. 2. G_p and C_p are the conductance and the capacitance per unit area of the membrane fragments; G_f and C_f refer to the black film (G_p also accounts for conductive pathways parallel to the bilayer in the space between bilayer and membrane fragment). $A°$ and $A*$ are the areas of the uncovered and of the covered part of the film and $1/G_s$ is the series resistance of the circuit (aqueous solutions plus electrodes plus input resistance of the amplifier).

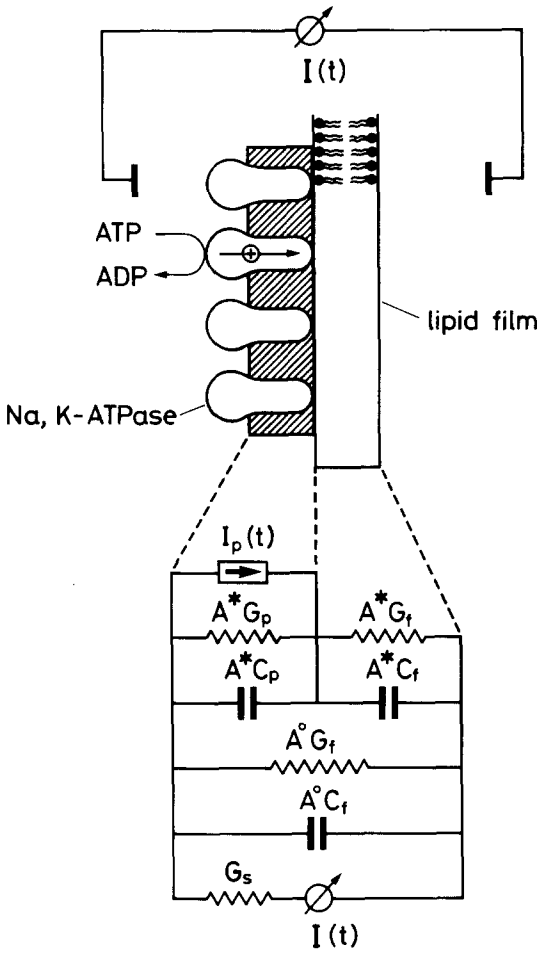


Fig. 2. Equivalent circuit of the compound membrane system consisting of a "black" lipid film with adsorbed Na,K-ATPase membrane fragments. Photochemical release of ATP leads to a transient pump current $I_p(t)$. In the external measuring circuit a time-dependent current $I(t)$ is recorded. G_p and C_p are the specific conductance and the specific capacitance of the membrane fragments (referred to unit area); G_f and C_f are the corresponding values of the black film. A° and A* are the areas of the uncovered and the covered part of the black film. $1/G_s$ is the series resistance of the circuit (aqueous solutions plus electrodes plus input resistance of the amplifier)

We assume that flash excitation at time $t = 0$ elicits a time-dependent (intrinsic) pump current $I_p(t)$; $I_p(t)$ is the hypothetical short-circuit current which would be recorded in the external measuring circuit if a continuous layer of oriented membrane fragments, but no lipid film, were present. Under the conditions of a real experiment (Fig. 2), the measurable short-circuit current $I(t)$ is influenced by the electric circuit parameters of the membrane system. If the conductance G_f of the black film and the series resistance $1/G_s$ are negligible, $I(t)$ and $I_p(t)$ are related in the following way (Appendix A):

$$I(t) = \frac{C_f}{C_f + C_p}\left[I_p(t) - \exp(-t/\tau_I)\frac{1}{\tau_I}\int_o^t I_p(t)\exp(t/\tau_I)dt\right] \quad (1)$$

$$I_p(t) = \left(1 + \frac{C_p}{C_f}\right)\left[I(t) + \frac{1}{\tau_I}\int_o^t I(t)dt\right] \quad (2)$$

$$\tau_I = \frac{C_f + C_p}{G_p}. \quad (3)$$

Using Eq. (2), the pump current $I_p(t)$ may be computed from the current signal $I(t)$.

From the decay time of voltage signals, a value of $G_p \approx 3$ μS/cm^2 may be estimated for the specific conductance of the membrane fragments. Assuming that the membrane fragments have a similar specific capacitance as a cell membrane ($C_p \approx 1$ μF/cm^2) and using a value of $C_f \approx 0.37$ μF/cm^2 for the specific capacitance of the diphytanoylphosphatidylcholine bilayer (Benz & Janko, 1976), the quantity $(C_f + C_p)$ may be estimated to be about 1–2 μF/cm^2; this yields a value of τ_I of the order of 0.5 sec.

Under the condition of ideal capacitive coupling ($G_p = 0$, $\tau_I \to \infty$), Eq. (1) reduces to

$$I(t) = \frac{C_f}{C_f + C_p} I_p(t). \quad (4)$$

Thus, for ideal capacitive coupling, the intrinsic pump current $I_p(t)$ is merely attenuated by the factor $\rho \equiv C_f/(C_f + C_p)$, whereas the time-course of the current remains unchanged. With $C_f \approx 0.37$ μF/cm^2 and $C_p \approx 1$ μF/cm^2, ρ may be estimated to be about 0.3. It should be noted that Eq. (1) reduces to Eq. (4) in the limit $t \to 0$, meaning that ideal capacitive coupling is always given at short times.

When the pump generates a time-independent current $I_p(t) \equiv I_{po}$, Eq. (1) yields

$$I(t) = \frac{C_f}{C_f + C_p} I_{po}\exp(-t/\tau_I). \quad (5)$$

In this case the externally measurable current $I(t)$ declines to zero with the circuit time constant τ_I; at long times the entire pump current I_p flows back through the conductance A^*G_p (Fig. 2).

It will be shown later that $I_p(t)$ can be represented by a sum of exponentials plus a stationary current I_p^∞:

$$I_p(t) = \sum_i a_i \exp(-t/\tau_i) + I_p^\infty. \quad (6)$$

In this case Eq. (1) yields

$$I(t) = \frac{C_f}{C_f + C_p}\left\{\sum_i \frac{a_i}{\tau_I - \tau_i}[\tau_I \exp(-t/\tau_i) - \tau_i \exp(-t/\tau_I)] + I_p^\infty \exp(-t/\tau_I)\right\}. \quad (7)$$

When τ_I is much larger than the largest of the τ_i, Eq. (7) assumes the form of Eq. (5), corresponding to ideal capacitive coupling.

In the presence of a series resistance $R_s \equiv 1/G_s$ (Fig. 2), current flow leads to a small voltage drop between the aqueous phases; furthermore, the rise-time of the current is limited by an additional time constant $\tau_s = R_s C_m$, where C_m is the total capacitance of the compound membrane. The series resistance R_s, which is mainly determined by the input resistance of the current amplifier, was found to be approximately 40 kΩ. This gives, together with the experimental value $C_m \approx 2$ nF (at a total membrane area $A \approx 0.7$ mm^2), a time constant $\tau_s \approx 80$ μsec, which is outside the experimental time range. At the experimentally observed current amplitudes ($I < 1$ nA), the voltage drop IR_s may be estimated to be <40 μV.

For the measurement of transmembrane voltage $V(t)$, the current amplifier is replaced by a voltage amplifier of virtually infinite impedance. Assuming that the conductance of the black film is negligible ($G_f \approx 0$), analysis of the equivalent circuit of Fig. 2 leads to the following expression for $V(t)$:

$$V(t) = -\frac{\exp(-t/\tau_V)}{AC_p + A^\circ C_f}\int_o^t I_p(t)\exp(t/\tau_V)dt \quad (8)$$

$$V(\infty) = -I_p(\infty)/AG_p \quad (9)$$

$$\tau_V \equiv [C_p + (A^\circ/A)C_f]/G_p \quad (10)$$

(Appendix A). Comparison with Eq. (1) shows that the following relation exists between the short-circuit current and the time derivative of the voltage signal:

$$\frac{I}{dV/dt} = -AC_f\frac{\tau_V}{\tau_I}\cdot\frac{I_p - \exp(-t/\tau_I)\frac{1}{\tau_I}\int_o^t I_p\exp(t/\tau_I)dt}{I_p - \exp(-t/\tau_V)\frac{1}{\tau_V}\int_o^t I_p\exp(t/\tau_V)dt}. \quad (11)$$

According to Eqs. (3) and (10), τ_V/τ_I is limited by $\chi < \tau_V/\tau_I < 1$, with $\chi \equiv C_p/(C_p + C_f) \approx 0.7$. Thus, τ_V and τ_I are nearly equal, meaning that $I(t)$ should be approximately proportional to dV/dt:

$$I(t) \approx -AC_f\frac{dV}{dt}. \quad (12)$$

This prediction can be used as a test for the consistency of the circuit analysis, as will be discussed later.

When $I_p(t)$ can be represented by a sum of exponentials (Eq. 6), $V(t)$ becomes

$$V(t) = -\frac{\tau_V}{AC_p + A^\circ C_f}\left\{\sum_i \frac{a_i\tau_i}{\tau_V - \tau_i}[\exp(-t/\tau_V) - \exp(-t/\tau_i)] + I_p^\infty[1 - \exp(-t/\tau_V)]\right\}. \quad (13)$$

A simple situation is given when the intrinsic time constants τ_i of the pump are much smaller than the time constant τ_V; the voltage signal is then approximately described by:

$$V(t) = V_{max}\frac{\sum_i a_i\tau_i[1 - \exp(-t/\tau_i)]}{\sum_i a_i\tau_i} \qquad (t \ll \tau_V) \quad (14)$$

$$V(t) = V_\infty + (V_{max} - V_\infty)\exp(-t/\tau_V) \qquad (t \gg \tau_i) \quad (15)$$

$$V_{max} = -\frac{\sum_i a_i\tau_i}{AC_p + A^\circ C_f}; \qquad V_\infty = -\frac{I_p^\infty}{AG_p}. \quad (16)$$

V rises to a maximum value V_{max} and thereafter declines toward a stationary value V_∞. If the value of $C_p + (A^\circ/A)C_f \equiv C_x$ is known, the specific conductance G_p of the membrane fragments may be evaluated by measuring τ_V (Eq. (10)). C_x may be estimated from the relation $C_p < C_x < C_p + C_f$ to be approximately 1–2 μF/cm^2.

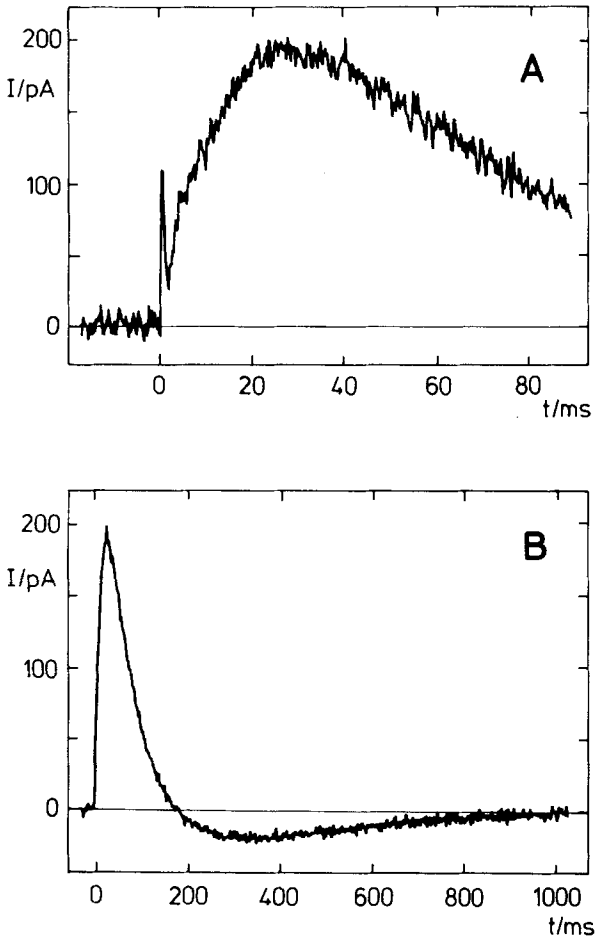


Fig. 3. Current transients in the absence of K^+ after a 40 μsec light flash given at time $t = 0$, recorded at two different time scales. The aqueous solutions contained 150 mM NaCl, 50 mM Tris chloride, pH 7.0, 20 mM dithiothreitol (DTT) and 2 mM $MgCl_2$. NaCl was Suprapur quality (Merck); deliberate addition of K^+ up to 50 μM had no effect on the current signal. The temperature was 20°C. 0.5 mM "caged" ATP and 40 μg/ml Na,K-ATPase in the form of membrane fragments were added to the *cis* compartment 20 min prior to the flash experiment. The positive sign of the current corresponds to a translocation of positive charge from the membrane fragment towards the black film (Fig. 2). The area of the black film was 0.64 mm^2. The current spike at time $t = 0$ is also seen in the presence of "caged" ATP alone, without addition of protein. The current signals were recorded at a bandwidth of 1 kHz

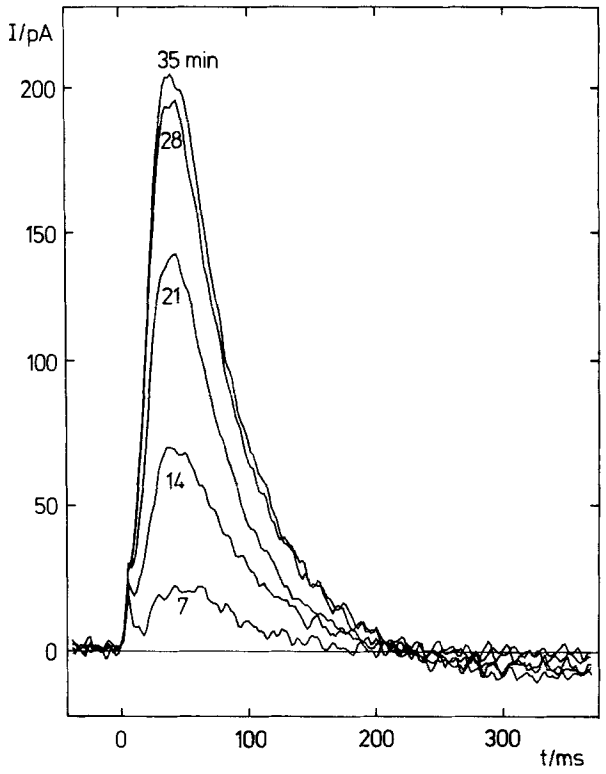


Fig. 4. Current response to a light flash, recorded 7, 14, 21, 28 and 35 min after addition of Na,K-ATPase membrane fragments to the *cis* compartment. The experimental conditions were the same as in Fig. 3. After 30 min the signal amplitude remained more or less constant when 20 mM dithiothreitol was present in the aqueous solutions. In the absence of dithiothreitol the signal amplitudes slowly declined after repeated flashes, presumably as a result of reactions of photoproducts with the protein (Goldman, Hibberd & Trentham, 1984)

Results

Transient Current Signals in the Absence of K^+

In the experiment represented in Fig. 3 the aqueous solutions contained 150 mM Na^+ and 2 mM Mg^{2+}, but no K^+. When 0.5 mM "caged" ATP and 40 μg/ml Na,K-ATPase in the form of membrane fragments were added to the *cis* side, transient current signals could be recorded after a 40 μsec light flash given at time $t = 0$. The current rose within about 30 msec toward a maximum and thereafter declined, reversing its sign at $t \approx 180$ msec. The direction of the current during the first 180 msec corresponds to a translocation of positive charge from the membrane fragment towards the black film (Fig. 2). As will be discussed later, the negative phase of the signal is likely to result from a backflow of charge through the membrane fragments.

The current response to the light flash developed within 20–30 min after addition of the membrane fragments (Fig. 4). This slow increase of photosensitivity is likely to reflect the time course of the adsorption of membrane fragments to the black film. This assumption is supported by the observation that the reflectivity of the lipid film markedly increased within the first 20–30 min after protein

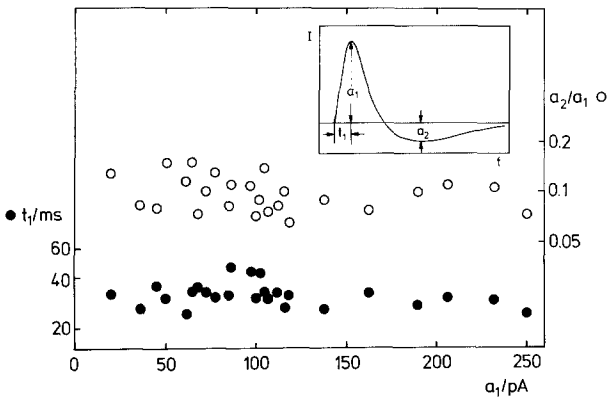


Fig. 5. Range of variation of time constants and current amplitudes recorded from different membranes. For each experiment the time to peak, t_1, and the amplitude ratio a_1/a_2 (*see* inset) have been plotted as a function of the amplitude a_1 of the peak current. The current signal has been recorded 20 min after addition of membrane fragments to the *cis* compartment. The other conditions were the same as described in the legend of Fig. 3

addition. The amplitude of the fully developed photoresponse varied up to threefold from experiment to experiment under otherwise identical conditions. The largest values of the peak current (referred to unit area of the black film) under the experimental conditions of Fig. 3 were about 70 nA/cm^2. In contrast to the large variations of current amplitude, the time course of the signal was more reproducible from experiment to experiment. This may be seen from Fig. 4 in which current signals recorded at different times after addition of membrane fragments are represented. The invariance of signal shape is further illustrated in Fig. 5 in which the time constant t_1 and the amplitude ratio a_1/a_2 of current signals from different membranes and for different waiting times after protein addition are compared (*see* inset of Fig. 5 for the definition of t_1, a_1 and a_2). Apart from the scatter of the experimental values, no systematic dependence of t_1 and a_1/a_2 on signal amplitude a_1 can be detected.

In order to examine the possible origin of the observed current signals, a number of control experiments was carried out:

1) In the absence of "caged" ATP no photoresponse was observed (Fig. 6).
2) In the presence of "caged" ATP but without addition of protein only a brief current spike (which is also seen in Fig. 3) was recorded. This current spike, which was always observed when "caged" ATP was present in the open compartment, may result from the creation of an interfacial potential by the photochemical reaction of "caged" ATP adsorbed to the lipid membrane.
3) In the absence of Mg^{2+} (which is required as a cofactor in the phosphorylation of the protein) no flash-induced signal was observed apart from the initial current spike; subsequent addition of 10 mM Mg^{2+} to the open compartment restored the normal photoresponse (Fig. 6).
4) Addition of 100 μM ATP prior to flash excitation completely inhibited the photoresponse. A likely explanation of this finding consists in the assumption that in the presence of ATP the pump spends most of its time in phosphorylated states and thus is unavailable for phosphorylation following photo-release of ATP.
5) When the membrane fragments were preincubated for 10 min with 4 mM ouabain prior to addition to the membrane cell, neither the first nor subsequent light flashes elicited a photoresponse. When thereafter 80 mM K^+ was added, the photoresponse was restored within minutes. This finding is consistent with the known antagonism between ouabain and K^+ (Akera, 1981). Addition of ouabain to the open compartment after binding of the membrane fragments resulted in an inhibition which developed over a period of about 10–20 min.
6) In the presence of 50 mM K^+ on both sides, but in the absence of Na^+, no flash-induced photosignal was detected. Experiments in which Na^+ and K^+ were simultaneously present will be described later.
7) 250 μM ortho-vanadate in the presence of 150 mM Na^+ and 50 mM K^+ reduced the photosignal to less than 10% of the control value within 20 min. In the absence of K^+, vanadate had virtually no effect, as expected from the known action of K^+ on inhibition by vanadate (Glynn, 1985).
8) In experiments with reconstituted vesicles (Apell et al., 1985) "caged" ATP in a concentration of 0.8 mM did not inhibit ATP-driven ion transport when the pump was activated by addition of 2.5 mM ATP to the vesicle suspension.

The results presented so far are consistent with the notion that membrane fragments containing Na,K-ATPase bind with their extracellular side to the lipid film, as schematically depicted in Fig. 2. Photochemically released ATP activates the pump from the cytoplasmic side, leading to a movement of Na^+ towards the extracellular side.

It cannot be excluded that part of the membrane fragments is bound to the lipid film with the inverse orientation, i.e., with the cytoplasmic side facing the bilayer. A contribution to the current signal from inversely oriented pump molecules is unlikely, however, since ouabain (which is known to act from the extracellular side) has no immediate effect on the current when it is added to the medium after adsorption of membrane fragments. Activation of

inversely oriented pumps by ATP from the solution would be slow since in this case the ATP binding sites are not directly accessible from the medium. The extremely slow effect of ouabain indicates that the space between bound membrane fragments and lipid film is narrow, so that diffusional access of ouabain to the extracellular binding sites is severely restricted.

When membrane fragments were bound to the lipid bilayer in the absence of Na^+, no photosignals could be elicited, as mentioned above. When then Na^+ was added to the solution under stirring, the full photoresponse could be observed within a few seconds after mixing of the solution was complete. From the ouabain experiments discussed above and from the effects of K^+ addition to be described later, it may be expected that the equilibration time of Na^+ with the intermembrane space is of the order of minutes. The nearly immediate effect of Na^+ addition therefore indicates that Na^+ acts exclusively on the cytoplasmic side of the pump.

Factors Influencing the Rise-Time of the Current Signal

As seen from Fig. 3, the current signal after the light flash rises within a finite time (about 30 msec under the given experimental conditions) towards a maximum. This finite rise-time may have different origins. One possibility, that the rise-time is limited by diffusion of the flash-generated ATP in the aqueous phase, is easily excluded: In order to estimate the diffusion time, we assume that the membrane surface acts as an ideally absorbing wall. If c_T is the ATP concentration in front of the membrane at time $t = 0$ (immediately after the light flash), the number of bound ATP molecules per unit area, $n(t)$ is given by

$$n(t) = 2c_T\sqrt{Dt/\pi} \tag{17}$$

(Frank & von Mises, 1961). D is the diffusion coefficient of ATP in water. The characteristic diffusion time τ_d may be taken to be the time at which $n(t)$ is equal to the density of ATPase molecules in the membrane fragments (about 10^3–10^4 μm^{-2}). With $c_T \approx 50$ μM (*see* Materials and Methods) and $D \approx 10^{-6}$ cm^2/sec, the diffusion time τ_d becomes of the order of 0.1–1 msec, much less than the rise time of the current.

Since diffusion in the aqueous phase is fast within the time scale of the experiment, the rise-time of the current must be limited by the finite rate of at least one of the reaction steps preceding

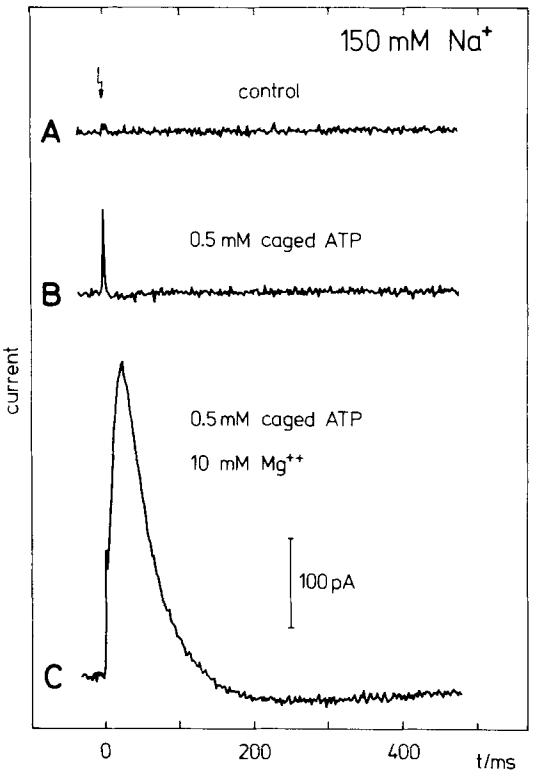


Fig. 6. Current signals in the absence and in the presence of Mg^{2+} and "caged" ATP after a light-flash of 40 μsec duration at time $t = 0$. Trace *A* was recorded in the absence of "caged" ATP and of Mg^{2+}. Trace *B*: after addition of 0.5 mM "caged" ATP to the *cis* compartment. Trace *C*: after addition of 0.5 mM "caged" ATP and of 10 mM $MgCl_2$ to the *cis* compartment. Both aqueous solutions contained 150 mM NaCl, 50 mM TrisCl, pH 7.0, and 20 mM dithiothreitol; $T = 20$°C. The area of the black film was 0.72 mm^2. All signals were recorded from the same membrane

charge translocation. From experiments with microsomal Na,K-ATPase from dog kidney, Forbush (1984) concluded that "caged" ATP (cgATP) binds to the enzyme with an equilibrium dissociation constant of $K_D \approx 43$ μM (under the same conditions, K_D for ATP is about 0.5 μM). This means that at a "caged" ATP concentration of 0.5 mM (as normally used in our experiments) the ATP-binding sites are almost completely occupied by "caged" ATP. Formation of the enzyme-ATP complex from which phosphorylation starts may occur by photogeneration of ATP in situ (via the photochemical intermediate "caged" ATP′ from which ATP is set free by hydrolysis):

$$E \cdot \text{cgATP} \xrightarrow{h\nu} E \cdot \text{cgATP}' \rightarrow E \cdot \text{ATP} \tag{18}$$

or by replacement of bound cgATP by ATP generated in aqueous solution:

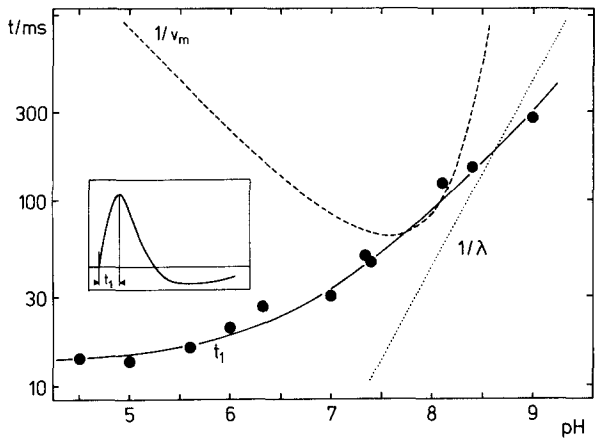


Fig. 7. Dependence of time t_1 on the pH of the aqueous solution in the *cis* compartment at 20°C. t_1 is the time between the light flash and the peak of the current signal (*see* inset). For comparison, the photochemical release-time $1/\lambda$ of ATP (McCray et al., 1980) as well as the cycle time $1/v_m$ are plotted as functions of pH. v_m is the maximum turnover rate of the Na,K pump at 20°C, as determined from K^+-flux experiments with kidney enzyme at saturating cytoplasmic concentrations of ATP and Na^+ and saturating extracellular K^+ concentration (Apell & Marcus, 1986). v_m was corrected for a temperature of 20°C using an activation energy of 115 kJ/mol (Apell et al., 1985). The pH of the solution was adjusted by addition of HCl or NaOH. The other experimental conditions were the same as described in Fig. 3. The experimental points represent averages from different membranes

$$\text{cgATP} \xrightarrow{h\nu} \text{cgATP}' \rightarrow \text{ATP} \qquad (19)$$

$$E \cdot \text{cgATP} + \text{ATP} \rightleftarrows E \cdot \text{ATP} + \text{cgATP}. \qquad (20)$$

At 20–25°C the dissociation rate-constant of $E \cdot$ ATP is about 20–30 sec^{-1} (Mårdh & Post, 1977; Karlish, Yates & Glynn, 1978).

From the studies of McCray et al. (1980) it is known that the rate-limiting step in the photochemical release of ATP [Eq. (19)] is catalyzed by H^+ with a rate constant $\lambda \approx (2.2 \times 10^9\ \text{M}^{-1}\ \text{sec}^{-1}) \cdot [H^+]$ at 22°C. Thus at pH 7 the release time $1/\lambda$ is about 4.5 msec and at pH 6 about 0.45 msec. McCray et al. (1980) reported that λ was independent of Mg^{2+} concentration up to $[Mg^{2+}] = 5$ mM and that the yield of ATP was not affected by pH in the pH range from 6 to 8.

The time course of the flash-induced current signal was found to depend on the pH of the solution. This is shown in Fig. 7 in which the time constant t_1 (*compare* inset in Fig. 7) is plotted as a function of pH. t_1 is pH insensitive below pH 6 but strongly increases with pH above pH 6.5. For comparison, the photochemical release-time of ATP, $1/\lambda$, is also plotted in Fig. 7, as well as the cycle time $1/v_m$ of the Na,K-pump. v_m is the maximum turnover rate as determined from potassium-flux experiments at saturating concentrations of ATP, Na^+ and K^+ (Apell & Marcus, 1986). As seen from Fig. 7, t_1 is considerably larger than $1/\lambda$ below pH 7, but becomes comparable to $1/\lambda$ at pH > 8. This indicates that at high pH the rise-time of the current signal is limited by the rate of ATP release. Furthermore, it is obvious from Fig. 7 that (at pH < 7) the time for the current signal to reach its peak value is much shorter than the steady-state cycle time of the pump under optimal conditions.

Double-Flash Experiments; Effects of Apyrase VI

The factors influencing the time course of the current signal were further investigated by double-flash experiments. Figure 8 represents the results of experiments in which the first light-flash is followed by a second flash after a waiting time Δt. When the second flash is given before or shortly after the current has reached the peak, a substantial increase in current amplitude is observed. This agrees with the observation from separate experiments (*not shown*) that the photoresponse is below saturation at the attainable flash intensities. As discussed in Materials and Methods, only about 10% of "caged" ATP present in the path of the light beam in front of the membrane is photolyzed in a single flash, and therefore an additional photoresponse may be elicited by a second flash. When the time interval Δt is increased, the current increment becomes smaller and smaller; at $\Delta t \approx 1$ sec the second flash has virtually no effect at all. This refractory state persists for time periods between one second and several minutes depending on the rate by which ATP is eliminated from the solution (*see below*).

Since the rate of phosphorylation of the protein is known to be nearly maximal at ATP concentrations as low as 1 μM (Glynn & Karlish, 1976), the question arises why a single flash, which liberates about 50 μM ATP, does not saturate the photoresponse. A likely explanation is the competition between "caged" ATP and ATP at the ATP-binding site (*see above*). Increasing the ATP concentration leads to an increase of the rate by which "caged" ATP is replaced by ATP and to a concomitant increase in current amplitude. This prediction is confirmed by numerical simulation of reactions (18)–(20), as described in the following paper (Apell, Borlinghaus & Läuger, 1987).

The results of the double-flash experiments at long waiting times ($\Delta t > 1$ sec) can be influenced by addition of apyrase VI, an enzyme mixture with ATPase (and ADPase) activity (Liébecq, Lallemand & Degueldre-Guillaume, 1963). This is shown in Fig. 9 in which the amplitude ratio a_1/a_{10} is plotted as a function of waiting time Δt for different apyrase activities in the solution. As indicated in Fig. 8, a_{10} is the maximum amplitude of the current elicited by the first flash and a_1 is the (incremental)

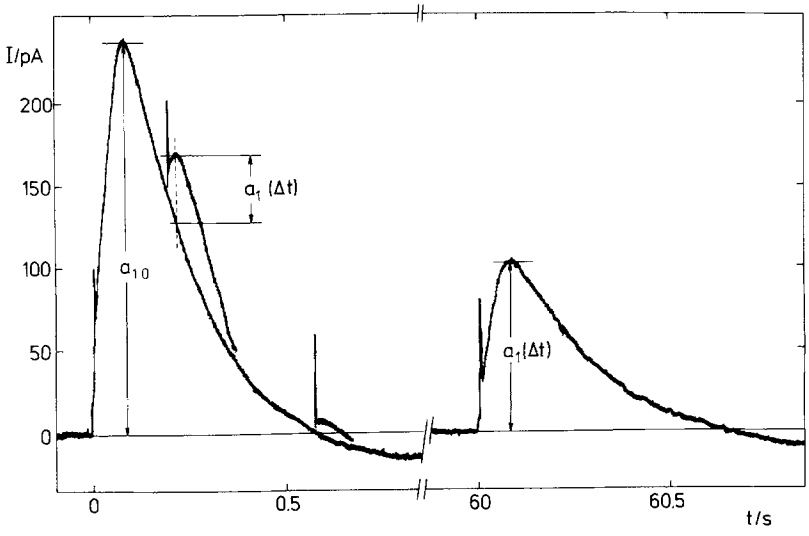


Fig. 8. Current signals in double-flash experiments. Following the first light flash at time $t = 0$, a second flash is given after a variable time interval Δt. The continuous (lower) curve represents the normal signal in a single-flash experiment. The current increments elicited by the second flash are indicated by the superimposed curves. The increment decreases for increasing Δt; after $\Delta t \approx 1$ sec the system remains refractory for a prolonged period. The length of the refractory period has been reduced by addition of 1.0 units/ml apyrase VI to the solution, an enzyme preparation with ATPase and ADPase activity. In the absence of apyrase VI, a half-maximal photoresponse is observed after $\Delta t \approx 200$ sec. The other experimental conditions were the same as in Fig. 3

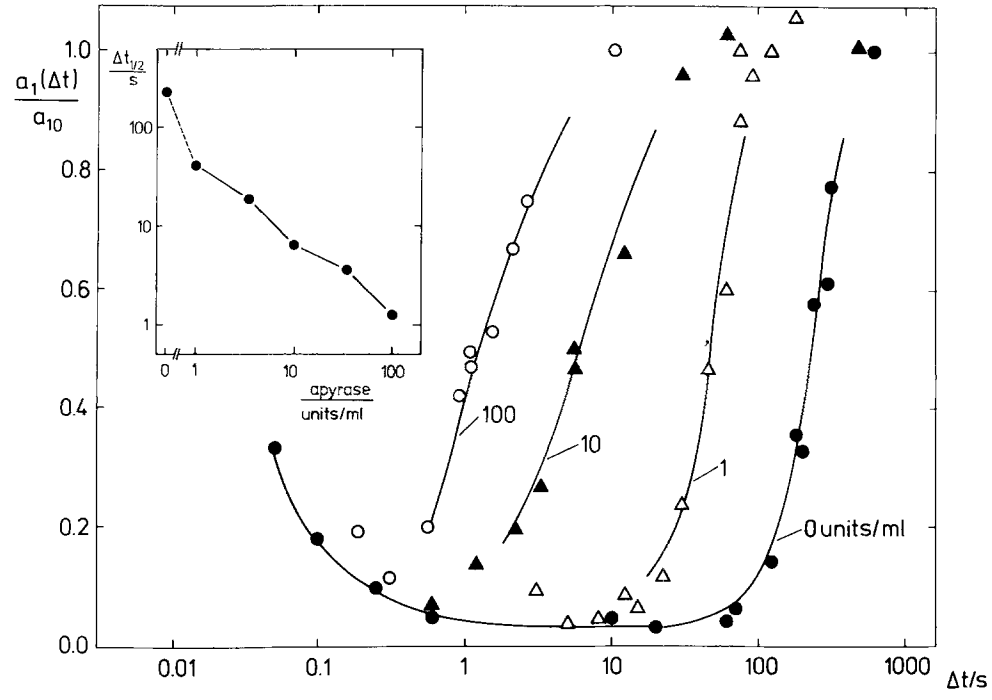


Fig. 9. Recovery of the photoresponse in double-flash experiments for different activities of apyrase VI in the solution. a_{10} is the peak amplitude of the current signal after the first flash, and a_1 is the (incremental) peak amplitude after the second flash given Δt seconds later (*compare* Fig. 8). Values of a_1/a_{10} at small waiting times Δt on curve labeled *0 units/ml* correspond to the left part of Fig. 8 and reflect the fact that the photoresponse is below saturation at the experimental light intensities. Apart from the addition of apyrase VI, the experimental conditions were the same as in Fig. 3. *Inset:* waiting time $\Delta t_{1/2}$ at which the signal amplitude is half-maximal ($a_1/a_{10} = 0.5$), as a function of apyrase activity. Since the commercial preparation of apyrase VI is known to contain traces of K^+, a number of control experiments was carried out after dialyzing 0.1 ml of the apyrase solution for 20 hr against 300 ml of a solution containing 50 mM TrisCl, 150 mM NaCl and 20 mM $MgCl_2$, pH 7.0

amplitude at the second flash. In the inset of Fig. 9 the waiting time $\Delta t_{1/2}$ after which the second flash elicits a half-maximal current signal is plotted as a function of apyrase activity. It is seen that the refractory time $\Delta t_{1/2}$ is about 200 sec in the absence of apyrase VI and decreases to $\approx$1 sec in the limit of high apyrase activity. The time course of the current signal at short times ($t \leq 1$ sec) was found to be independent of the presence of apyrase VI. The observation that apyrase VI speeds up the recovery time may be explained by the assumption that at finite ATP concentration the pump is present pre-

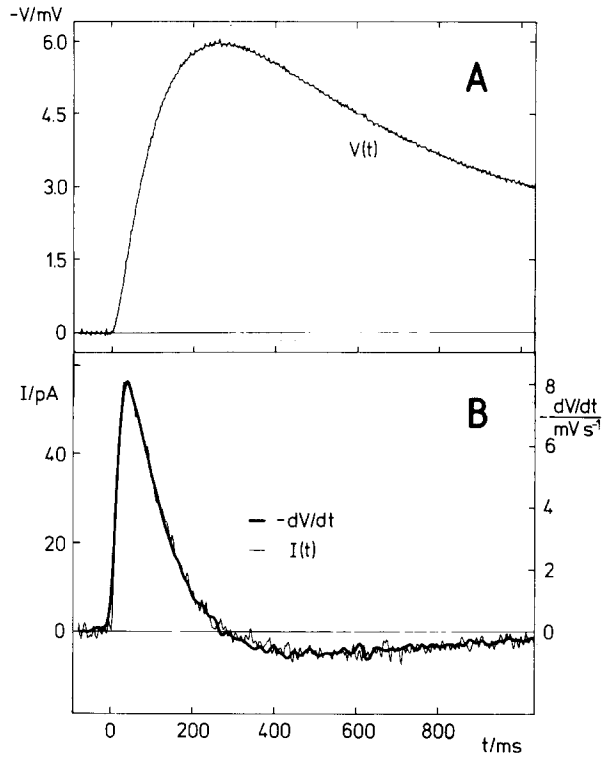


Fig. 10. (*A*) Transmembrane voltage $-V(t)$ after a light flash at time $t = 0$. V is the potential of the *cis* side referred to the potential of the *trans* side (Fig. 1). For the measurement of $V(t)$ the electrodes were connected to a voltage amplifier with an input resistance of about 10^{12} Ω. The other conditions were the same as described in the legend of Fig. 3. (*B*) Comparison of the time derivative $-dV/dt$ with the short-circuit current $I(t)$; both curves were normalized to the same peak value by suitable choice of the ordinate scale. dV/dt was computed from the (digitally smoothed) voltage signal given in part *A* of the Figure. $I(t)$ was recorded from the same membrane about 7 min after measuring $V(t)$. Repeating the measurement of $V(t)$ again 7 min later yielded a signal which was indistinguishable from $V(t)$ given in *A*.

dominantly in phosphorylated form and thus is unavailable for further phosphorylation.

From the results of the double-flash experiments in the absence of apyrase it may be concluded that the decline of the current signal is not caused by depletion of ATP (which could result from hydrolysis or from convective and diffusive exchange with nonilluminated parts of the solution); otherwise a second flash given immediately after the decline of the signal would elicit a new photoresponse.

It may also be excluded that the decline of current is caused by the finite conductance of the membrane fragments. According to Eq. (5) an exponentially decaying current signal in the external circuit could result from a pump operating at constant rate, since at long times a steady pump current is canceled by an equal and opposite current flowing back through the conductance A^*G_p of the membrane fragment (Fig. 2). The possibility that a substantial pump current persists at long times ($t \approx 1$ sec) is unlikely, however, since in this case a second flash at $t \approx 1$ sec should give rise to a new current signal, as long as the photoresponse is not saturated. Further evidence for the transient nature of the intrinsic pump current is given later.

Voltage Transients under Open-Circuit Conditions

In a further series of experiments the external voltage $V(t)$ was measured at virtually infinite impedance of the external circuit. The voltage transient which is shown in Fig. 10*A* has been recorded under otherwise identical conditions as the current transient of Fig. 3. As seen from Fig. 10*A*, the voltage signal which is elicited by the light flash passes through a maximum and thereafter declines, approaching a finite value V_∞ at long times.

According to Eq. (12) the time derivative dV/dt of the voltage signal should be approximately equal to the short-circuit current I (apart from a scaling factor $-AC_f$), provided that the values of τ_I and τ_V are not significantly different. This expectation is tested in Fig. 10*B* in which dV/dt is plotted together with the current signal $I(t)$, which has been recorded from the same membrane. It is seen that the shape of dV/dt is very similar to the shape of $I(t)$. In particular, the negative phase of $I(t)$ at long times is found to correspond to the negative slope of $V(t)$. The nearly exact proportionality of $I(t)$ and dV/dt indicates, according to Eq. (11), that τ_I and τ_V are approximately equal, meaning that

$$\frac{\tau_V}{\tau_I} = \frac{C_p + (A^\circ/A)C_f}{C_p + C_f} \approx 1. \tag{21}$$

Since C_p may be estimated to be about 1 μF/cm² whereas C_f is only 0.37 μF/cm² (Benz & Janko, 1976), Eq. (21) is approximately fulfilled even for values of A°/A considerably smaller than unity. From Fig. 10 the ratio $I/(dV/dt)$ may be determined to be about 3 nF. With $\tau_V \approx \tau_I$, this ratio should be approximately equal to the capacitance AC_f of the black film [Eq. (11)]. With $A \approx 7 \times 10^{-3}$ cm², the specific capacitance C_f is estimated to be ≈0.43 μF/cm², which approximately agrees with the directly determined value $C_f \approx 0.37$ μF/cm².

An estimate of the voltage V_p that is built up across the membrane fragment after activation of the pump under the condition $I = 0$ may be obtained from the relation $V_p = (A/A^*)V$ derived in Appendix A. Experimental values of V are of the order of 10

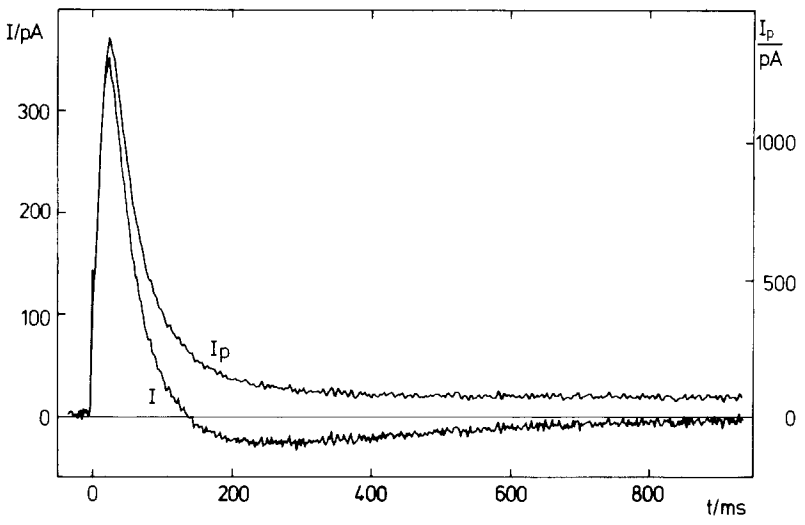


Fig. 11. Intrinsic pump current $I_p(t)$, calculated from the experimental current signal $I(t)$ according to Eq. (2). The left ordinate scale refers to $I(t)$ and the right ordinate scale to $I_p(t)$. The time constant $\tau_I \approx \tau_V$ in Eq. (2) was estimated from the decay time of the open-circuit voltage (measured on the same membrane) to be about 400 msec. For the scaling factor $(1 + C_p/C_f)$ a value of 3.7 was used (*see* text). $I(t)$ was measured under the experimental conditions described in the legend of Fig. 3; the membrane area was 0.75 mm². $I_p(t)$ can be approximately fitted by Eq. (22) with $k_1 = 67.3$ sec^{-1}, $k_2 = 24.2$ sec^{-1}, $I_1 = -2.54$ nA, $I_2 = 2.86$ nA, $I_p^\infty = 81$ pA

mV; however, since A^* can be much smaller than A, V_p may be considerably larger than V.

From the biphasic shape of $V(t)$ the possibility that the pump generates a time-independent current I_p can be excluded. If I_p were constant, $V(t)$ would monotonically rise to a limiting value. The form of the observed current and voltage signals can be explained by the assumption that the intrinsic pump current I_p is time dependent, rising toward a maximum and thereafter declining to a much smaller quasistationary value. When the pump current ceases, the voltage that has been built up across the membrane fragment decays by conduction via G_p, giving rise to a negative current in the external circuit. On the basis of this assumption, the specific conductance G_p of the membrane fragments may be estimated from the time constant τ_V of voltage decay, using Eqs. (10) and (15). Observed time constants τ_V varied between 300 msec and 2 sec in experiments with different films; the variation of τ_V probably reflects variations in G_p. From the numerical estimate $C_p + (A°/A)C_f \approx 1$–$2$ μF/cm² (*see above*), G_p is found to lie in the range of 1–5 μS/cm².

In all measurements of $V(t)$ a finite limiting voltage $V(\infty) \equiv V_\infty$ has been observed at long times (Fig. 10). In order to test whether V_∞ is associated with the activity of the pump, a number of control experiments was performed. In one series of experiments Mg^{2+} was omitted from the medium. A second series of experiments was carried out in the presence of 0.8 mM ouabain added to the medium. In both cases no voltage signal was observed after the flash even at long times ($t \approx 1$ sec), apart from the usual erratic baseline drift which was less than 20 μV/sec. These findings indicate that V_∞ is connected with pump activity. As discussed in the following section, the pump current $I_p(t)$ does not completely vanish for $t \to \infty$ but approaches a quasistationary value $I_p(\infty) = AG_pV(\infty)$.

Evaluation of the Intrinsic Pump Current $I_p(t)$

According to Eq. (2), the intrinsic pump current $I_p(t)$ can be calculated from the current signal $I(t)$ when the circuit time constant $\tau_I = (C_f + C_p)/G_p$ as well as the ratio C_p/C_f are known. τ_I may be estimated from the decay time τ_V of the voltage signal, since τ_I and τ_V are nearly equal [Eq. (21)]. As discussed above, the ratio C_p/C_f is only approximately known ($C_p/C_f \approx 2.7$); however, errors in the quantity $(1 + C_p/C_f)$, which appears only as a scaling factor in Eq. (2), do not affect the time dependence of I_p.

An example for the evaluation of $I_p(t)$ from $I(t)$ is shown in Fig. 11. $I_p(t)$ was computed from $I(t)$ on the basis of Eq. (2) using the value of $\tau_V \approx \tau_I$ determined by recording $V(t)$ from the same membrane [*compare* Eq. (15)]. It is seen that the time course of I_p and I is identical at short times, corresponding to ideal capacitive coupling. The negative phase of $I(t)$ at large t, however, is no longer present in $I_p(t)$, which monotonously decreases to a small quasistationary current I_p^∞. The biphasic shape of $I_p(t)$ can be approximately represented by:

$$I_p(t) = I_1 \exp(-k_1 t) + I_2 \exp(-k_2 t) + I_p^\infty \qquad (22)$$

I_1, I_2 and I_p^∞ are time-independent constants. For the I_p curve represented in Fig. 11 an optimum fit was obtained with $k_1 = 67.3$ sec^{-1} and $k_2 = 24.2$ sec^{-1}. From 22 experiments with different mem-

branes, the following average values and standard deviations were evaluated:

$k_1 = (58.4 \pm 13.1)\ \text{sec}^{-1}$

$k_2 = (23.8 \pm 8.2)\ \text{sec}^{-1}$.

The meaning of Eq. (22) in terms of kinetic models is discussed in the following paper (Apell et al., 1987).

Since dV/dt and $I(t)$ have been found to be nearly proportional (Fig. 10*B*), τ_V can be directly obtained from $I(t)$ without a separate measurement of $V(t)$. For this purpose $I(t)$ is integrated in time to give $-AC_f V(t)$, according to Eq. (12).

Amount of Translocated Charge

From the intrinsic pump current $I_p(t)$ the total charge Q may be estimated which is translocated in the course of the nonstationary process:

$$Q = \int_o^\infty (I_p - I_p^\infty)dt. \tag{23}$$

By numerical integration of $I_p - I_p^\infty$, the translocated charge (referred to unit area of the black film) is estimated to be $Q < 20$ nC/cm^2 (20 nC/cm^2 is the largest value of Q obtained in about 20 different experiments). This value may be compared with the upper limit Q_o of translocated charge corresponding to a monolayer of adsorbed Na,K-ATPase membranes. From electron-microscopic studies (Deguchi et al., 1977) it is known that the membrane fragments contain Na,K-ATPase dimers ($\alpha_2\beta_2$) with a density of up to 6000 μm^{-2}. Translocation of three Na^+ ions per dimer would correspond to a charge translocation of $Q_o = 290$ nC/cm^2, which is about 15 times higher than the experimental value $Q = 20$ nC/cm^2. A possible reason for the difference between Q and Q_o may be that far less than 6000 active pump molecules (cytoplasmic side facing the aqueous solution) are bound per μm^2 of the lipid film. Another possibility is that the dielectric distance over which charge is translocated is much smaller than the dielectric length of the transport pathway. This point is discussed in more detail in the accompanying paper.

Current Transients in the Presence of Monensin

For an explanation of the decline of $I_p(t)$ (Fig. 11) at least two possibilities exist. The pump may enter a long-lived state in the pumping cycle from which return to the initial state is slow. Or the accumulation of sodium ions in the gap between the membrane fragment and the black film leads to a reduction in net pumping rate. (The further possibility, that the gradient of electrical potential built up across the membrane fragment inhibits the pump, is unlikely in view of the observed shape of $V(t)$, as will be discussed later).

In order to distinguish between these possibilities, short-circuit measurements were carried out in the presence of monensin and carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). Monensin is known to promote electroneutral (one-for-one) exchange of Na^+ and H^+ across lipid bilayers, whereas FCCP acts as an electrogenic carrier of H^+ (Sandeaux et al., 1978; McLaughlin & Dilger, 1979). The simultaneous addition of monensin and FCCP may be expected to lead to an elimination of Na^+ and H^+ concentration gradients (as well as of potential gradients). In the presence of 1 μM monensin and 1 μM FCCP on both sides of the lipid film at pH 7.0, the time course of the flash-induced current signals was virtually the same as in the control experiment. The same result was obtained when the concentration of FCCP was increased fivefold.

Since it has not been possible to test independently whether the combination of monensin and FCCP acted in the expected way in the lipid bilayer covered by membrane fragments, these experiments are not strictly conclusive. However, the lack of an effect of monensin plus FCCP on the time-course of the current signal makes it unlikely that the pump is blocked by a potential gradient or by a high Na^+ concentration in the gap. This conclusion is further supported by experiments with chymotrypsin-modified enzyme (*see below*).

Chymotrypsin-Modified Enzyme

Treatment of Na,K-ATPase with α-chymotrypsin in the presence of Na^+ at low ionic strength leads to cleavage of a single peptide bond in the α-subunit; the split is located in the cytoplasmic portion of the protein between Leu-266 and Ala-267 (Jørgensen & Collins, 1986). Under suitable reaction conditions secondary cleavage is negligible. In the chymotrypsin-treated enzyme, phosphorylation by ATP, occlusion of Na^+ and ADP/ATP exchange are preserved, while Na^+,K^+ pumping and Na^+,Na^+ exchange are abolished (Glynn, Hara & Richards, 1984; Jørgensen & Petersen, 1985). These findings indicate that modification by chymotrypsin stabilizes the enzyme in the E_1 conformation by preventing the $E_1 \rightarrow E_2$ transition.

Experiments with chymotrypsin-treated mem-

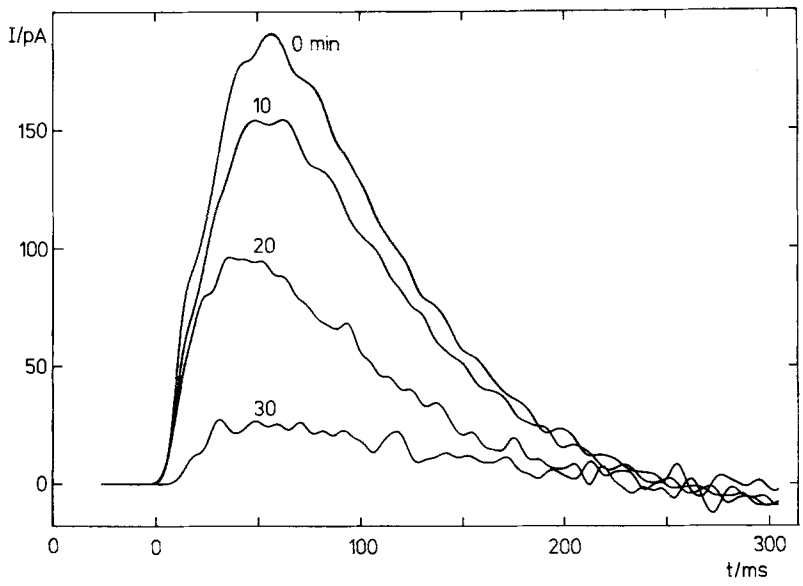


Fig. 12. Modification of bound Na,K-ATPase membranes by chymotrypsin in situ. Membrane fragments were added to the *cis* compartment in the presence of 10 mM NaCl, 50 mM TrisCl, 5 mM $MgCl_2$, 20 mM dithiothreitol and 0.5 mM "caged" ATP, pH 7.0. The temperature was 20°C. After a waiting time of 30 min, the short-circuit current signal was measured after a 40 μsec light flash (curve labeled *0 min*). Thereafter, 5 mg/ml α-chymotrypsin were added to the *cis* compartment, and the photoresponse was recorded again after 10, 20, and 30 min. In control experiments without chymotrypsin, the amplitude of the current signal was found to remain nearly constant in the same time period

brane fragments may thus provide information on the nature of the charge-translocating steps. In a first series of experiments, Na,K-ATPase membranes were preincubated for 10–30 min at 30°C in a medium containing 160 μg/ml α-chymotrypsin, 2 μg/ml Na,K-ATPase, 10 mM NaCl and 15 mM TrisCl, pH 8.1. Under these conditions the proteolytic reaction may be expected to be selective, consisting in the cleavage of a single peptide bond, as discussed above. After addition of the modified membrane fragments to the *cis* compartment, no photoresponse was observed in the usual flash experiment. (The same result was obtained when the solution of "caged" ATP was preincubated with 300 units/ml apyrase VI for 30 min at 20°C prior to the addition to the membrane cell, in order to remove traces of ATP which may be present in the solution.) In order to test for the possibility that the modified membrane fragments no longer bind to the planar bilayer, unmodified membranes were added to the *cis* compartment at the end of the experiment. This did not result in a restoration of the photoresponse, indicating that the surface of the planar bilayer was already saturated with chymotrypsin-modified membrane fragments.

The observation that chymotrypsin treatment abolishes the transient current, which is normally seen after the ATP-concentration jump, indicates that phosphorylation by ATP and occlusion of Na^+ are electrically-silent reaction steps. The mechanistic implications of this finding will be discussed in more detail in part II of the paper.

In a second series of experiments, bound Na,K-ATPase membranes were treated with chymotrypsin in situ. Membrane fragments were added to the *cis* compartment in the presence of 10 mM Na^+ and 50 mM Tris, pH 7.0 (total ionic strength about 260 mM). After a waiting time of about 30 min, the photoresponse was measured as usual (curve labeled *0 min* in Fig. 12). Thereafter a high concentration of α-chymotrypsin (5 mg/ml) was added to the *cis* compartment, and the photoresponse was recorded again after certain time intervals. As seen from Fig. 12, the amplitude of the current signal progressively decreased within about 30 min, whereas the time course of the signal remained essentially unchanged.[1]

The finding that with partially inactivated membranes the time course of the current signal is the same as with untreated membranes has important implications for the interpretation of the current transients. From previous studies it is known that, at the level of the single pump molecule, inactivation by chymotrypsin is an all-or-none process (Jørgensen & Collins, 1986); one may therefore assume that partially inactivated membranes represent a mixture of completely blocked and fully active ATPase molecules. When pumps are activated by ATP and translocate Na^+, a voltage V_p (and

[1] Similar results were obtained when membrane fragments were preincubated with chymotrypsin for shorter periods than required for the reaction to go to completeness. After adding these membrane fragments to the *cis* compartment, current signals of reduced amplitude were observed which exhibited the same time course as observed without chymotrypsin treatment. The result of such experiments is less conclusive, however, in view of the previously mentioned variations in current amplitude, which are observed in control experiments with untreated membranes.

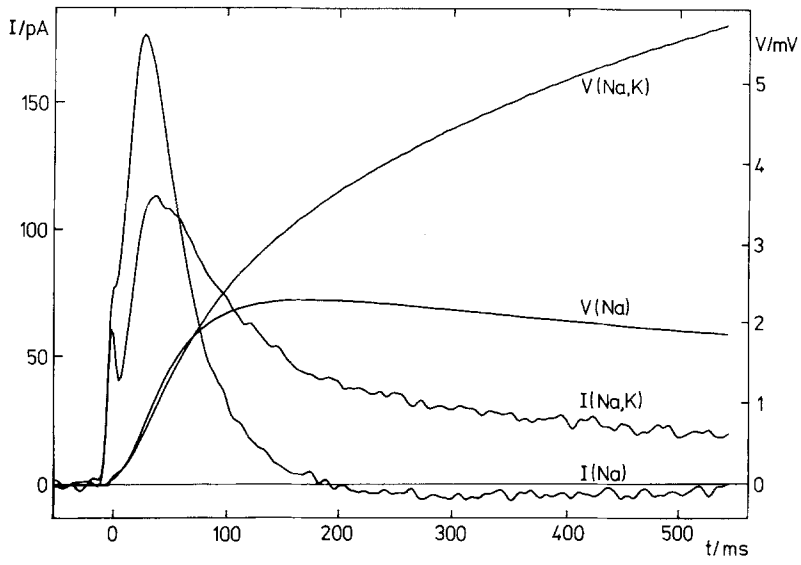


Fig. 13. Short-circuit current $I(t)$ and open-circuit voltage $V(t)$ in the presence and in the absence of K^+. At the beginning of the experiment $V(t)$ and $I(t)$ were measured in the presence of 150 mM NaCl, 20 mM DTT and 50 mM TrisCl, pH 7.0. Then, after addition of 50 mM KCl to the *cis* compartment, $I(t)$ and $V(t)$ were recorded again. The waiting time between recordings was 10 min. The area of the black film was 0.88 mm^2; $T = 20°C$

probably also a Na^+-concentration gradient) is generated across the membrane fragment, which tends to inhibit the transport reaction. The magnitude of the voltage built up in a given time after ATP release depends on the density of functionally active pumps in the membrane. If the decline of pumping current results from the inhibitory action of transmembrane voltage (or of a Na^+-concentration gradient), the decay of the current signal should become slower when part of the pump molecules are inactivated by chymotrypsin.[2] Since such a dependence of decay time on the degree of proteolytic inactivation is not found in the experiment, it is unlikely that the decline of the current results from concentration or potential gradients. A more probable explanation for the shape of $I_p(t)$ consists in the assumption that the pump, after activation by ATP, goes through the first steps of the transport cycle and then enters a kinetically stable state from which return to the initial state is slow.

Effects of K^+

When 50 mM K^+ are present in the solution in addition to 150 mM Na^+, the peak amplitude of the current signal $I(t)$ is reduced and the decay of $I(t)$ is prolonged (Fig. 13). The observed reduction of I_{max} is consistent with the known inhibitory effect of cytoplasmic K^+ on the Na,K-ATPase, which is explained by a competition between K^+ and Na^+ for cytoplasmic Na^+-binding sites (Glynn, 1985). It is also seen from Fig. 13 that the open-circuit voltage monotonously increases over a time period of at least 1 sec in the presence of K^+. The prolonged decay of $I(t)$ and the increase of $V(t)$ at long times indicate that in the presence of K^+ a quasistationary current I_p^∞ is maintained by the pump, which exceeds the value of I_p^∞ observed under K^+-free conditions. If K^+ is present in the gap between the membrane sheet and the lipid film, the pump may go through the normal transport cycle corresponding to the Na/K-exchange mode. When the compound membrane is formed in a K^+-free solution and K^+ is added afterwards to the *cis* side, the K^+-effect on $I(t)$ develops only slowly, in the course of minutes. This slow response probably results from the restricted accessibility of the gap between membrane sheet and black film.

Discussion

The results presented in this study demonstrate that the Na,K-pump is able to generate transient, Na^+-dependent currents in the absence of K^+; this confirms earlier observations of Fendler et al. (1985). The electrogenic activity of the pump was investigated in a compound membrane system obtained by binding Na,K-ATPase membranes to a planar lipid bilayer acting as a capacitive electrode. Fast activation of the Na,K-pump by photochemical release of ATP leads to a transient short-circuit current $I(t)$ in

[2] Implicit in this conclusion is the assumption that the ion mobility in the gap between membrane fragment and planar bilayer is sufficiently high, so that lateral gradients over a length equal to the average distance of pump molecules (less than 40 nm) dissipate within the decay time of the current signal (≈100 msec).

the external measuring circuit, corresponding to a translocation of positive charge across the pump protein from the cytoplasmic towards the extracellular side.

The intrinsic pump current $I_p(t)$ can be evaluated from the recorded current signal $I(t)$, using estimated values of the circuit parameters of the compound membrane. $I_p(t)$ exhibits a biphasic behavior with a fast rising period, followed by a slower decline towards a small quasistationary current I_p^∞. The rate constant k_1 of the rising phase depends on the experimental conditions of photochemical ATP release. As discussed in the following paper (Apell et al., 1987), k_1 is likely to be determined by non-electrogenic reactions preceding phosphorylation of the protein.

The declining phase of the pump current can be described, to a first approximation, by a single exponential process with a rate constant $k_2 \approx 20\ \text{sec}^{-1}$. This value is much larger than the maximum steady-state rate of ATP hydrolysis in the absence of extracellular K^+, which may be estimated to be about 3 sec^{-1} at 25°C (Mårdh & Post, 1977). This means that I_p decays to nearly zero before the pump has completed a full transport cycle.

For the origin of the decline of I_p at least two possibilities exist. Translocation of Na^+ ions may create gradients of electric potential and Na^+-concentration across the membrane sheet which tend to inhibit the pump. An effect of transmembrane voltage V_p as the sole origin of the decline of $I_p(t)$ can be excluded from the observed biphasic shape of $V(t)$, since inhibition of charge translocation by an opposing electric field should result in a monotonically increasing voltage signal. The influence of a sodium concentration gradient built up by the pump is more difficult to estimate. When the thickness of the gap is assumed to be 1 nm and when the pump density in the sheet is 6000 μm^{-2} (Deguchi et al., 1977), a single turnover of the pump, corresponding to a translocation of 3 Na^+ ions, would raise the Na^+ concentration in the gap by about 30 mM. The meaning of this estimate is doubtful, however, since the gap between the two membranes is unlikely to have the properties of a macroscopic aqueous phase. The long delay in the effect of an addition of K^+ to the aqueous phase indicates that diffusional exchange between gap and solution is strongly inhibited.

More direct information on the effects of potential and ion-concentration gradients is obtained from experiments in which the density of functionally active pump molecules has been reduced by chymotrypsin treatment. The observation that in situ modification by chymotrypsin merely reduced the signal amplitude, but left the time course of the current transient unchanged, indicates that effects of concentration and potential gradients on pumping current $I_p(t)$ are small. A similar conclusion can be drawn from experiments in which the permeability of the lipid film for Na^+ and H^+ has been increased by addition of monensin and FCCP. The finding that the time course of I_p remained virtually unchanged under these conditions again argues against an influence of concentration and potential gradients. While Na^+ ions are likely to be accumulated in the gap as a result of pump activity, the effect on the current transient may be small since the affinity of the extracellular sodium-release sites is very low ($K_D \approx 0.1$ M; *see* Glynn, 1985).

A likely interpretation of the time course of pumping current I_p consists in the assumption that the decline of $I_p(t)$ reflects an intrinsic property of the transport cycle. After activation by the ATP-concentration jump, the pump goes through the first steps of the cycle and then enters a long-lived state from which return to the initial state is slow. As will be discussed in part II of the paper, the rate-limiting step for the completion of the cycle may be the dephosphorylation of the protein, which is extremely slow in the absence of potassium. As long as ATP is present in the solution, pump molecules re-entering the initial state may become phosphorylated again, which give rise to a small quasistationary current I_p^∞.

The results reported here may be compared with recent studies of transient sodium fluxes in a vesicular preparation of Na,K-ATPase from canine kidney (Forbush, 1984, 1985). After photolytic release of ATP in the internal space of the right-side-out oriented vesicles, a time-dependent, biphasic efflux of $^{22}Na^+$ was observed with $k_1 \approx 100\ \text{sec}^{-1}$ and $k_2 \approx 35\ \text{sec}^{-1}$ (values extrapolated to 20°C). These values are similar to the rate constants k_1 and k_2 determined from $I_p(t)$ at 20°C ($k_1 \approx 58\ \text{sec}^{-1}$, $k_2 \approx 24\ \text{sec}^{-1}$). The ATP-driven efflux of $^{22}Na^+$ was independent of the presence of K^+ or Na^+ in the extravesicular solution. Under single-turnover conditions, i.e., when the amount of released ATP per vesicle was much smaller than the number of pump molecules in the vesicle membrane, the (ouabain-sensitive) Na-flux declined to zero, whereas at large ATP concentration a small quasistationary sodium efflux persisted at long times. From the similarity in the time course of Na^+-efflux rate and of pump current I_p it is likely to assume that essentially the same process is observed in both experiments. A simple situation in which $I_p(t)$ is proportional to the isotope-flux rate is given when the current results from the translocation of sodium ions across the entire membrane dielectric, corresponding to the transfer of $^{22}Na^+$ from the intravesicular to the extravesicular aqueous space in the flux experiment.

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Appendix A

Derivation of Eqs. (1), (2), (8), and (9)

We consider the equivalent circuit of Fig. 2 in the limit $G_f \to 0$, $G_s \to \infty$. If the current $I(t)$ is measured under short-circuit conditions, the sum of the voltages, V_p and V_f, across G_p and G_f vanishes:

$$V_p + V_f = 0. \tag{A1}$$

Furthermore, the following relations hold according to Fig. 2:

$$I = A^* C_f \frac{dV_f}{dt} \tag{A2}$$

$$\frac{I_p}{A^*} + G_p V_p + C_p \frac{dV_p}{dt} = C_f \frac{dV_f}{dt}. \tag{A3}$$

Equations (A1) and (A3) yield (with $\tau_I = (C_f + C_p)/G_p$):

$$\frac{dV_f}{dt} + \frac{V_f}{\tau_I} = \frac{I_p(t)}{A^*(C_f + C_p)}. \tag{A4}$$

The solution satisfying the condition $V_f(0) = 0$ reads:

$$V_f(t) = \frac{\exp(-t/\tau_I)}{A^*(C_f + C_p)} \int_o^t I_p(t) \exp(t/\tau_I) dt. \tag{A5}$$

Introducing Eq. (A5) into Eq. (A2) yields Eq. (1). Furthermore, Eq. (2) is obtained from Eqs. (A2) and (A4). Eq. (8) is derived in a completely analogous way, replacing Eq. (A1) by the relation $V_p + V_f = V$. From $A^*C_f dV_f/dt = -A^\circ C_f dV/dt$ it follows that (for $I = 0$) V_p and V are connected by

$$V_p = \frac{A}{A^*} V. \tag{A6}$$

Eq. (9) is obtained from Eqs. (8) and (10) using the general relation (E. Bohl, *personal communication*):

$$\lim_{t \to \infty} \left[\exp(-t/\tau) \int_o^t I_p(s) \exp(s/\tau) ds \right] = \tau I_p(\infty). \tag{A7}$$