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Fast Charge Translocations Associated with Partial Reactions of the Na,K-Pump: II. Microscopic Analysis of Transient Currents

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Summary. Nonstationary pump currents which have been observed in K^+-free Na^+ media after activation of the Na,K-ATPase by an ATP-concentration jump (*see* the preceding paper) are analyzed on the basis of microscopic reaction models. It is shown that the behavior of the current signal at short times is governed by electrically silent reactions preceding phosphorylation of the protein; accordingly, the main information on charge-translocating processes is contained in the declining phase of the pump current. The experimental results support the Albers-Post reaction scheme of the Na,K-pump, in which the translocation of Na^+ precedes translocation of K^+. The transient pump current is represented as the sum of contributions of the individual transitions in the reaction cycle. Each term in the sum is the product of a net transition rate times a "dielectric coefficient" describing the amount of charge translocated in a given reaction step. Charge translocation may result from the motion of ion-binding sites in the course of conformational changes, as well as from movement of ions in access channels connecting the binding sites to the aqueous media. A likely interpretation of the observed nonstationary currents consists in the assumption that the principal electrogenic step is the E_1-P/P-E_2 conformational transition of the protein, followed by a release of Na^+ to the extracellular side. This conclusion is supported by kinetic data from the literature, as well as on the finding that chymotrypsin treatment which is known to block the E_1-P/P-E_2 transition abolishes the current transient. By numerical simulation of the Albers-Post reaction cycle, the proposed mechanism of charge translocation has been shown to reproduce the experimentally observed time behavior of pump currents.

Key Words Na,K-ATPase · ion pumps · electrogenic transport · Albers-Post cycle · partial reactions

Introduction

In the experiments described in the preceding paper (Borlinghaus, Apell & Läuger, 1987) transient electrical signals have been recorded from a two-dimensional array of oriented Na,K-ATPase molecules after an ATP-concentration jump. In these experiments flat membrane fragments containing Na,K-ATPase were bound to a planar lipid bilayer membrane acting as a capacitive electrode. ATP was released within 1-10 msec from an inactive, photolabile derivative ("caged" ATP) by an intense light flash. This resulted in a transient charge-translocation within the membrane, which could be recorded as a current or voltage signal in the external measuring circuit. The electrical signals were dependent on Na^+ concentration in the medium but could be observed in the complete absence of K^+. This suggests that the observed charge displacements are associated with the movement of sodium ions in the ATPase molecule. From the recorded current signals, the intrinsic electric current $I_p(t)$ generated by the pump could be evaluated. In the following we analyze the time course of pump current I_p on the basis of microscopic reaction models.

Analysis of Nonstationary Pump Currents

Relation between Electric Current and Transition Rates

The analysis of transient pump currents may be based on the assumption that sudden release of ATP initiates a sequence of transitions between discrete states $P_1, P_2, \ldots, P_n$ of the pump molecule:

$$P_1 \underset{k_b^1}{\overset{k_f^1}{\rightleftharpoons}} P_2 \underset{k_b^2}{\overset{k_f^2}{\rightleftharpoons}} P_3 \rightleftharpoons \cdots \rightleftharpoons P_n. \qquad (1)$$

k_f^i and k_b^i are the rate constants of the i-th elementary reaction step in forward and backward direction. When the pump performs a closed cycle, state P_n is identical with the initial state P_1. Charge translocations associated with the individual transitions may be described by a set of (dimensionless) dielectric coefficients α_i (Läuger et al., 1981). In the transition $P_i \rightarrow P_{i+1}$ the electric charge $\alpha_i e_o$ is translo-

cated in the external measuring circuit (e_o is the elementary charge). The coefficients α_i are microscopic parameters describing the electrogenic properties of the ion pump in a hypothetical system in which the Na,K-ATPase membrane is on both sides in direct contact with an aqueous electrolyte solution. If in the transition $P_i \rightarrow P_{i+1}$ a charge $\gamma_i e_o$ moves over a distance a_i in a homogeneous dielectric layer of thickness d, the coefficient α_i has the form

$$\alpha_i = \gamma_i a_i / d. \tag{2}$$

Since in reality the protein layer is likely to be inhomogeneous with respect to its dielectric properties, the quantity a_i/d in Eq. (2) has to be replaced by an effective dielectric distance.

When x_i is the fraction of pump molecules in state P_i, the net rate Φ_i of transitions $P_i \rightarrow P_{i+1}$ (referred to a single pump molecule) is given by

$$\Phi_i = x_i k_f^i - x_{i+1} k_b^i. \tag{3}$$

This equation may be applied to the case that the system is perturbed at time $t = 0$ by a sudden change of an external parameter (such as ATP concentration) and thereafter relaxes toward a stationary state. Since $e_o N \alpha_i \Phi_i$ is the contribution of transitions $P_i \rightarrow P_{i+1}$ to the total charge translocation (N is the number of pump molecules in the membrane), the pump current may be represented by

$$I_p(t) = e_o N \sum_i \alpha_i \Phi_i(t). \tag{4}$$

Equation (4) connects the experimental quantity $I_p(t)$ with microscopic parameters (α_i, k_f^i, k_b^i) of the pump. When the pump goes through a closed cycle ($P_1 \equiv P_n$) in which ν elementary charges are translocated from one to the other aqueous solution, the relation

$$\sum_i \alpha_i = \nu \tag{5}$$

holds. This relation is obtained by applying Eq. (4) to the steady state ($t \rightarrow \infty$) in which all rates Φ_i become identical and equal to the stationary transport rate $\Phi = I_p(\infty)/\nu e_o$.

Transport Reactions of the Na,K-Pump in the Absence of Potassium

Before discussing possible microscopic interpretations of the observed current transients, we briefly summarize previous studies of the kinetic behavior

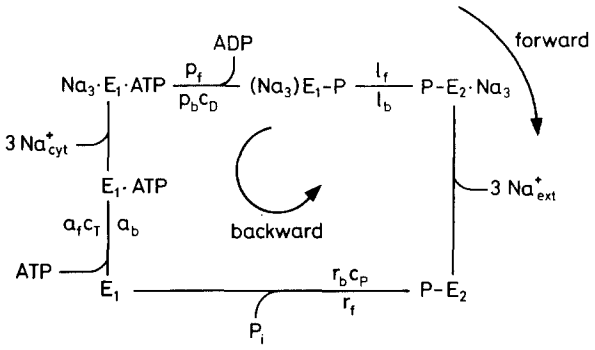

Fig. 1. Transport reactions of the Na,K-pump in the absence of potassium, based on the Albers-Post scheme of the pumping cycle (adapted from Cantley et al., 1984). E_1 and E_2 are conformations of the enzyme with ion binding sites exposed to the cytoplasmic and to the extracellular side, respectively. In the "occluded" state $(Na_3)E_1$-P the bound ions are unable to exchange with the aqueous solution. Dashes indicate covalent bonds and dots indicate noncovalent bonds. $a_f c_T$, p_f, l_f, r_f and a_b, $p_b c_D$, l_b, $r_b c_P$ are rate constants for transitions in forward and backward direction, respectively, c_T, c_D and c_P are the cytoplasmic concentrations of ATP, ADP and P_i (inorganic phosphate). In the presence of intra- and extracellular Na^+ an additional pathway (not shown in the Figure) exists involving transitions between P-$E_2 \cdot Na_n$ and $Na_n \cdot E_1$ ($n \leq 3$).

of the Na,K-pump in the absence of potassium ions. For this purpose we consider the transport cycle shown in Fig. 1, which is based on the Albers-Post reaction scheme of the Na,K-pump (Cantley et al., 1984; Glynn, 1985). In K^+-free media the enzyme is predominantly in conformation E_1, which has a high affinity for ATP; the equilibrium dissociation constant of ATP is in the range of 0.1–1 μM, virtually independent of Na^+ concentration (Hegyvary & Post, 1971; Nørby & Jensen, 1971). After binding of Na^+, the enzyme becomes phosphorylated, forming an "occluded" state $(Na_3)E_1$-P in which the bound Na^+ ions are unable to exchange with the aqueous phases (Glynn, 1985). After transition to state P-$E_2 \cdot Na_3$ in which the ion-binding sites are facing outward, Na^+ ions are released to the extracellular side. In the absence of K^+, the transition back to the original state involving dephosphorylation of the enzyme (P-$E_2 \rightarrow E_1$) is extremely slow (Post, Hegyvary & Kume, 1972; Mårdh & Post, 1977; Karlish & Yates, 1978; Kapakos & Steinberg, 1986; Schuurmans-Stekhoven et al., 1986). This means that in K^+-free media containing ATP, the enzyme is preferentially in state P-E_2 under stationary conditions.

In the absence of K^+ and in the presence of intra- and extracellular Na^+, the pump promotes electroneutral, one-for-one exchange of sodium iso-

topes (Garrahan & Glynn, 1967*a,c*; Sachs, 1970; Beaugé & Ortiz, 1973; Kennedy & De Weer, 1976). This exchange reaction, which requires the simultaneous presence of intracellular ATP and ADP, is assumed to proceed via transitions in the upper part of the reaction scheme of Fig. 1.

When erythrocytes are suspended in Na^+- and K^+-free media, ATP hydrolysis drives uncoupled, ouabain-sensitive Na^+-efflux (Garrahan & Glynn, 1967*a,b*; Beaugé & Ortiz, 1973; Lew, Hardy & Ellory, 1973; Karlish & Glynn, 1974; Glynn & Karlish, 1976). Similar results were obtained with Na,K-ATPase incorporated into artificial lipid vesicles (Forgac & Chin, 1982; Karlish & Kaplan, 1985). This transport mode of the Na,K-pump, which is poorly understood so far, appears to be associated with cotransport of anions such as Cl^- or SO_4^{2-} (Forgac & Chin, 1982; Dissing & Hoffman, 1983). The overall rate of uncoupled Na^+-efflux is probably limited by the rate of the dephosphorylation reaction $P\text{-}E_2 \rightarrow E_1$.

When millimolar concentrations of Na^+ are present in the extracellular medium, uncoupled Na efflux is inhibited (presumably by binding of sodium to a regulatory binding site), but under these conditions a small Na^+-Na^+ exchange persists which is driven by ATP hydrolysis (Glynn & Karlish, 1976; Lee & Blostein, 1980; Forgac & Chin, 1981, 1982; Cornelius & Skou, 1985). This exchange reaction which proceeds at a rate that is only a small fraction of the normal turnover rate of the pump may result from a slight K^+-like effect of Na^+ at the extracellular side. In this way three Na^+ ions may move outward (as in the normal pumping mode) and one to three Na^+ ions occupying the K^+ sites may move inward via transitions $P\text{-}E_2 \cdot Na_n \rightarrow Na_n \cdot E_1$ (representing a pathway parallel to the lower limb of the reaction cycle in Fig. 1).

In experiments with right-side-out membrane vesicles derived from the outer medulla of dog kidney, Forbush (1984, 1985) has studied transient Na efflux stimulated by photolytic release of intravesicular (cytoplasmic) ATP. In these experiments which were carried out under single-turnover conditions, the Na efflux was found to be independent of extravesicular Na^+ or K^+, indicating that sodium extrusion is an early step in the normal transport cycle.

Transient, strophantidin-sensitive charge movements have recently been observed by Nakao and Gadsby (1986) in voltage-jump experiments with heart cells in the absence of extracellular K^+. These charge movements, which required the presence of intra- and extracellular sodium and of intracellular ATP, are likely to be connected with a voltage-dependent step of Na-Na exchange.

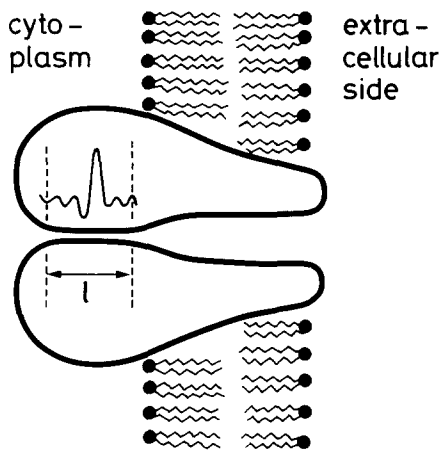

Fig. 2. Na,K-ATPase molecule represented as a transmembrane protein with a water-filled access channel (or vestibule) having low electrical resistance. The length of the long axis of the molecule perpendicular to the membrane has been estimated to be about 11 nm (Nicolas, 1984). The inset depicts a hypothetical potential-energy profile for Na^+ along the high-resistance part of the ion pathway in conformation E_1 of the enzyme

Microscopic Description of Transient Currents Generated by the Na,K-Pump

According to Eq. (4) the transient pump current $I_p(t)$ is determined by the dielectric coefficients α_i of the individual reaction steps. In principle, any of the transitions in the reaction scheme of Fig. 1 can be associated with charge translocation, corresponding to a nonzero value of α_i. Obvious candidates for charge displacements are the reactions $Na_3 \cdot E_1 \cdot ATP \leftrightarrow (Na_3)E_1\text{-}P \leftrightarrow P\text{-}E_2 \cdot Na_3$ involving transitions of Na^+-binding sites between inward-facing (E_1) and outward-facing (E_2) configurations. Since it is unlikely that binding sites move over the entire transmembrane length of the protein in the E_1/E_2 transition, it is usually assumed that the protein contains access channels connecting the binding sites with the respective aqueous phases. Part of the access channel may consist of a wide, water-filled pore having a low electric resistance (Fig. 2). Ion movement within the low-resistance pore does not result in charge displacement in the external circuit. The gating part of the pump molecule may be represented by a narrow, high-resistance pathway consisting of a series of energy barriers and wells (Fig. 3). In state $Na_3 \cdot E_1 \cdot ATP$ the ion-binding site is connected with the cytoplasmic phase via a series of low barriers but separated from the extracellular side by a high barrier. In the "occluded" state $(Na_3)E_1\text{-}P$ the energy barriers on either side are high. In state $P\text{-}E_2 \cdot Na_3$ the binding sites are easily accessible from the extracellular phase.

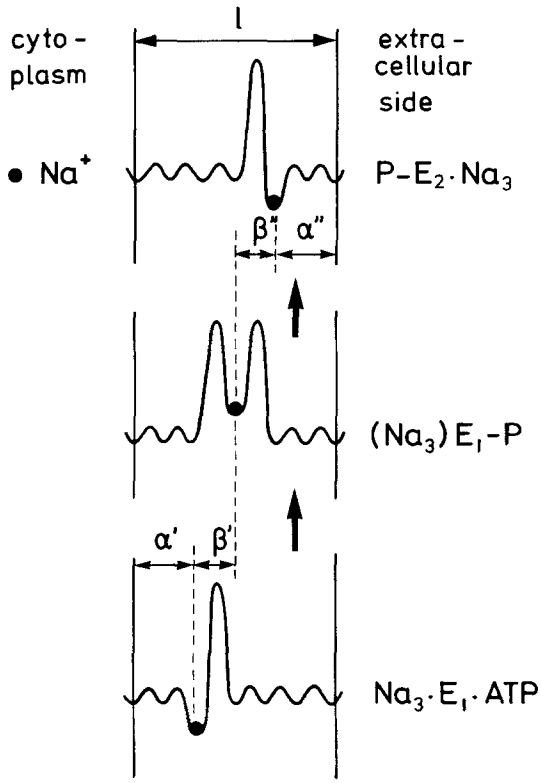

Fig. 3. Hypothetical energy profile of a sodium ion along the transport pathway. The ion binding sites in state $Na_3 \cdot E_1 \cdot ATP$ are connected with the cytoplasmic side by a series of low barriers, but separated from the extracellular medium by a high barrier. In the "occluded" state $(Na_3)E_1$-P the energy barriers on either side are high. In state P-$E_2 \cdot Na_3$ the binding sites are easily accessible from the extracellular phase. α', α'', β' and β'' are dielectric distances [Eqs. (6)-(8)], depending on the location of the ion binding site in the protein and on the dielectric properties of the protein and the surrounding medium

Since a Na^+ ion migrating from the cytoplasm to the binding site has, in general, to traverse part of the dielectric (Figs. 2 and 3), the binding step may be associated with charge displacement in the external circuit. The corresponding dielectric distance, α', is assumed to be the same for the three sodium-binding sites. In a completely analogous way the release of Na^+ to the extracellular side is described by a dielectric distance α'' (Fig. 3).

In the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-$P$ the loaded binding sites move over a dielectric distance β' (Fig. 3). If $z_L e_o$ is the charge of the ligand groups, the dielectric coefficient associated with $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-$P$ (rate constant p_f) is given by

$$\alpha_p = (3 + z_L)\beta' + \eta'. \tag{6}$$

The parameter η' accounts for rotation of dipolar groups and translocation of intrinsic charges of the protein other than charged ligands (Läuger & Apell, 1986). In an analogous way the transition $(Na_3)E_1$-$P \rightarrow P$-$E_2 \cdot Na_3$ (rate constant l_f) is described by a dielectric coefficient α_l:

$$\alpha_l = (3 + z_L)\beta'' + \eta''. \tag{7}$$

In the (hypothetical) process $Na^+_{cyt} + E_1 \cdot ATP \rightarrow \ldots \rightarrow P$-$E_2 + Na^+_{ext}$ (Fig. 1) a sodium ion is translocated from the cytoplasm to the extracellular medium. This means that the following relation must hold:

$$\alpha' + \alpha'' + \beta' + \beta'' = 1. \tag{8}$$

Furthermore, combining Eqs. (5)-(8) yields:

$$\alpha_a + z_L(\beta' + \beta'') + \eta' + \eta'' + \alpha_r = 0. \tag{9}$$

α_a and α_r are the dielectric coefficients of reactions $E_1 + ATP \rightarrow E_1 \cdot ATP$ (rate constant a_f) and P-$E_2 \rightarrow E_1 + P_i$ (rate constants r_f), respectively.

According to Eq. (4) the transient pump current $I_p(t)$ is given by the sum of the transition rates Φ_i multiplied by the corresponding dielectric coefficients α_i. Denoting the fraction of pump molecules in state P_j by $x[P_j]$, the net forward rates in the reaction cycle of Fig. 1 may be written as:

$$\Phi_a = a_f c_T x[E_1] - a_b x[E_1 \cdot ATP] \tag{10}$$

$$\Phi_i' = \rho_i' c_N' x[Na_{i-1} \cdot E_1 \cdot ATP] - \sigma_i' x[Na_i \cdot E_1 \cdot ATP] \quad (i = 1,2,3) \tag{11}$$

$$\Phi_p = p_f x[Na_3 \cdot E_1 \cdot ATP] - p_b c_D x[(Na)_3 E_1 \cdot ATP]. \tag{12}$$

c_T and c_D are the concentrations of ATP and ADP, respectively, c_N' is the cytoplasmic concentration of Na^+, and ρ_i' and σ_i' are concentration-independent rate constants. The net forward rates Φ_l, Φ_1'', Φ_2'', Φ_3'' and Φ_r are obtained in a completely analogous way. The pump current $I_p(t)$ can thus be represented by

$$I_p/e_o N = \alpha_a\Phi_a + \alpha_1'\Phi_1' + \alpha_2'\Phi_2' + \alpha_3'\Phi_3' + \alpha_p\Phi_p + \alpha_l\Phi_l + \alpha_1''\Phi_1'' + \alpha_2''\Phi_2'' + \alpha_3''\Phi_3'' + \alpha_r\Phi_r. \tag{13}$$

In the following we assume that the three sodium binding sites are equivalent ($\alpha_i' \equiv \alpha'$, $\alpha_i'' \equiv \alpha''$) and that the rate constants ρ_i', ρ_i'', σ_i' and σ_i'' are large, so that the association and dissociation reactions of Na^+ are always in equilibrium. This means that the mole fractions $x[Na_i \cdot E_1 \cdot ATP]$ are given by the equilibrium dissociation constants K_{Ni}':

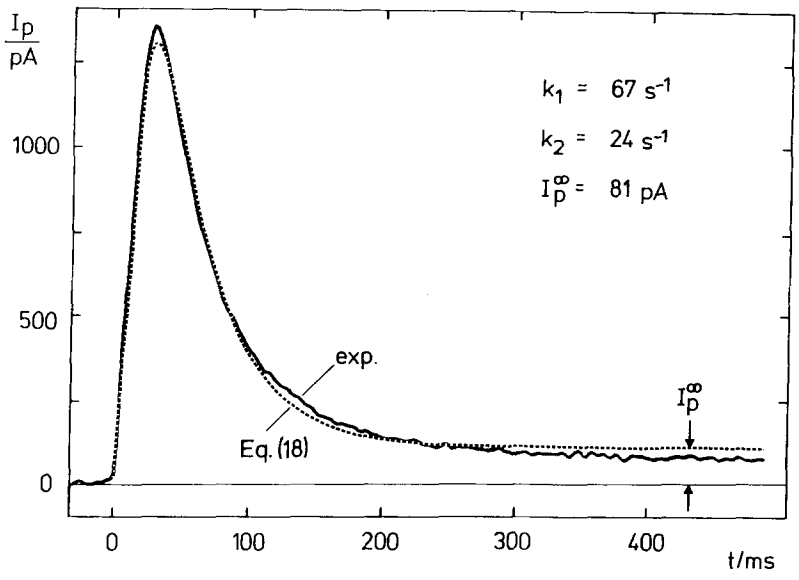

Fig. 4. Fit of Eq. (18) to the experimentally observed pump current $I_p(t)$. The experimental curve (solid line) has been taken from Fig. 11 of part I. The fitting curve (dashed line) has been calculated with k_1 = 67.3 sec^{-1}, k_2 = 24.2 sec^{-1}, I_1 = −2.54 nA, I_2 = 2.86 nA, I_p^∞ = 81 pA. The area of the black film was A = 0.75 mm^2

$$\frac{x[\mathrm{Na}_{i-1} \cdot E_1 \cdot \mathrm{ATP}]}{x[\mathrm{Na}_i \cdot E_1 \cdot \mathrm{ATP}]} = \frac{\sigma_i'}{\rho_i' c_N'} = \frac{K_{Ni}'}{c_N'} \equiv \frac{1}{n_i'} \quad (i = 1,2,3). \tag{14}$$

Analogous relations hold for the extracellular side (equilibrium constants K_{Ni}''). The rate constants and equilibrium constants are connected (at zero transmembrane voltage) by the relation

$$\frac{a_f p_f l_f r_f}{a_b p_b l_b r_b} \cdot \frac{K_{N1}'' K_{N2}'' K_{N3}''}{K_{N1}' K_{N2}' K_{N3}'} = K \tag{15}$$

which follows from the principle of microscopic reversibility (Läuger & Apell, 1986). $K \equiv \bar{c}_D \bar{c}_P / \bar{c}_T$ is the equilibrium constant of ATP hydrolysis ($\bar{c}_T$, $\bar{c}_D$ and $\bar{c}_P$ are the equilibrium concentrations of ATP, ADP and P_i).

Semiempirical Representation of the Experimental Pump Current $I_p(t)$

As shown in the preceding paper, the pump current $I_p(t)$ in response to a light flash that releases ATP exhibits a biphasic behavior consisting of a fast rising phase and a slow decline towards a small quasi-stationary current $I_p(\infty)$. The shape of $I_p(t)$ could be approximately described by a sum of two exponential functions. Such a time behavior of I_p may be expected when an electrogenic reaction step is preceded by an electrically silent transition:

$$P_1 \xrightarrow[\alpha_1 = 0]{k_1} P_2 \xrightarrow[\alpha_2 > 0]{k_2} P_3. \tag{16}$$

In this case the current is given by $I_p = \alpha_2 e_o k_2 N_2$, where e_o is the elementary charge, α_2 the dielectric coefficient of the transition $P_2 \rightarrow P_3$, k_2 the transition frequency and $N_2(t)$ the number of pump molecules in state P_2. By integration of the rate equations corresponding to the reaction sequence (16) the current is obtained in the form

$$I_p(t) = \alpha_2 e_o N \frac{k_1 k_2}{k_2 - k_1} [\exp(-k_1 t) - \exp(-k_2 t)]. \tag{17}$$

N is the number of pump molecules which are in state P_1 prior to the flash.

In the experiments $I_p(t)$ is found to approach a small quasi-stationary current I_p^∞ at long times. This is accounted for by replacing Eq. (17) by the semiempirical relation

$$I_p(t) = I_1 \exp(-k_1 t) + I_2 \exp(-k_2 t) + I_p^\infty \tag{18}$$

where I_1 and I_2 are time-independent constants.

Since I_p/α_2 in Eq. (17) is invariant against an exchange of k_1 and k_2, it is impossible to decide from the form of $I_p(t)$ whether the nonelectrogenic reaction $P_1 \rightarrow P_2$ represents the fast or the slow process. However, from the finding that a change of the release rate of ATP affects only the larger of the two rate constants, the nonelectrogenic reaction may be assigned to the fast process.

An example of a fit of Eq. (18) to the experimentally determined pump current $I_p(t)$ is given in Fig. 4. It is seen that $I_p(t)$ can be adequately described by Eq. (18) when the rate constants are chosen to be

$k_1 \approx 67\ \text{sec}^{-1}$ and $k_2 \approx 24\ \text{sec}^{-1}$. Average values of k_1 and k_2, taken from 22 different experiments at $T = 20°C$ are $k_1 \approx 58.4\ \text{sec}^{-1}$ and $k_2 \approx 23.8\ \text{sec}^{-1}$. The rate constant for the decay of the current signal, $k_2 \approx 24\ \text{sec}^{-1}$, may be compared with the steady-state turnover rate of the pump. In the absence of K^+, the maximum turnover rate v_m of Na,K-ATPase from guinea-pig kidney in a Na^+ medium is about $3\ \text{sec}^{-1}$ at 25°C, which is about 5% of the maximum rate in the presence of both Na^+ and K^+ (Mårdh & Post, 1977). A similar value of the maximum turnover rate, $v_m \approx 5\ \text{sec}^{-1}$, may be estimated for ATP-driven sodium extrusion from erythrocytes into Na^+- and K^+-free media (Glynn & Karlish, 1976). The finding that k_2 is larger than v_m means that the pump current in the ATP concentration-jump experiment decays to nearly zero before the pump has completed a full transport cycle. This is consistent with the view that the electrogenic processes giving rise to the observed current are early steps in the transport cycle.

From the value of $I_p^\infty \approx 10\ \text{nA/cm}^2$ a rough estimate of the quasistationary turnover rate v_∞ may be obtained in the following way. In part I of the paper the total charge which is translocated in the course of the transient process has been determined to be

$$Q = \int_o^\infty (I_p - I_p^\infty)dt \approx 15\ \text{nC/cm}^2.$$

Assuming that this charge translocation results from the transfer of three sodium ions per pump, the average density of functionally oriented pump molecules attached to the planar bilayer becomes $n = Q/3e_o \approx 3 \times 10^{10}\ \text{cm}^{-2}$. Assuming further that in the stationary mode of the pump a single net charge is transported per cycle (corresponding to a back-transport of two Na^+ ions), the quasistationary turnover rate becomes $v_\infty = I_p^\infty/ne_o \approx 2\ \text{sec}^{-1}$. This value approximately agrees with the maximum turnover rate $v_m \approx 3\ \text{sec}^{-1}$ of the Na,K-ATPase in K^+- free media (*see above*).

While the agreement between Eq. (18) and the experimentally determined $I_p(t)$ is consistent with reaction sequence (16), it does not exclude other possibilities. In particular, it is feasible that the rate-limiting reaction $P_2 \rightarrow P_3$ is electrically silent ($\alpha_2 = 0$), but is followed by a fast electrogenic process $P_3 \rightarrow P_4$:

$$P_2 \xrightarrow[\alpha_2 = 0]{\text{slow}} P_3 \xrightarrow[\alpha_3 > 0]{\text{fast}} P_4. \qquad (19)$$

For instance, process $P_2 \rightarrow P_3$ could represent the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ and process $P_3 \rightarrow P_4$ a fast movement of Na^+ ions from the binding sites across the access channel to the extracellular medium ($P\text{-}E_2 \cdot Na_3 \rightarrow P\text{-}E_2 + 3Na_{ext}^+$). In this case the rates of processes $P_2 \rightarrow P_3$ and $P_3 \rightarrow P_4$ become equal. This means that $I_p(t)$ is again given by Eq. (17) if α_2 is replaced by α_3. A more detailed discussion of the microscopic parameters influencing $I_p(t)$ is possible on the basis of a numerical simulation of the reaction cycle of Fig. 1 (*see below*).

Kinetic Interpretation of the Shape of $I_p(t)$

Behavior at Short Times

As mentioned above, a likely interpretation of the behavior of pump current $I_p(t)$ at short times consists in the assumption that the rising phase of $I_p(t)$ is governed by electrically silent processes, including photochemical release and binding of ATP to the protein. Consistent with this interpretation is the observation that the risetime of I_p decreases with decreasing pH of the medium, as predicted from the pH dependence of photolysis rate of "caged" ATP (McCray et al., 1980). An analysis of the reactions preceding phosphorylation of the protein has to account for the possibility that ATP which is released in the aqueous solution competes with caged ATP bound at the ATP site of the enzyme (Forbush, 1984; 1985). Furthermore, bound caged-ATP may be transformed into ATP in situ.

The kinetic properties of the reaction system consisting of ATP, caged ATP and enzyme were studied by numerical analysis of the rate equations, using values of the kinetic parameters from the literature, as described in Appendix A. The result is represented in Fig. 5 in which the half-time $t_{1/2}$ for the formation of the enzyme-ATP complex (from which phosphorylation starts) is plotted as a function of pH. The numerical simulation was carried out for an initial concentration, $c_X^o = 0.5$ mM, of caged ATP (Borlinghaus et al., 1987) and for different values of the fraction θ of caged ATP which is converted to ATP by the light flash. The dashed line in Fig. 5 represents the pH-dependent time constant $(\ln 2)/\lambda$ of photochemical ATP release (McCray et al., 1980). It is seen that at low pH where ATP release is fast, the half-time $t_{1/2}$ is larger than $(\ln 2)/\lambda$ (except for $\theta = 1$); in this pH range the rate of formation of $E \cdot$ ATP is limited by the rate of ATP binding. On the other hand, at high pH where ATP release is slow, $t_{1/2}$ may be shorter than the photochemical time constant $(\ln 2)/\lambda$. This results from the high rate and the high affinity of ATP binding.

The dotted line in Fig. 5 represents the experimentally observed pH dependence of $t_1/2$ where t_1 is the time required for the current signal to reach peak amplitude. The pH dependence of t_1 approxi-

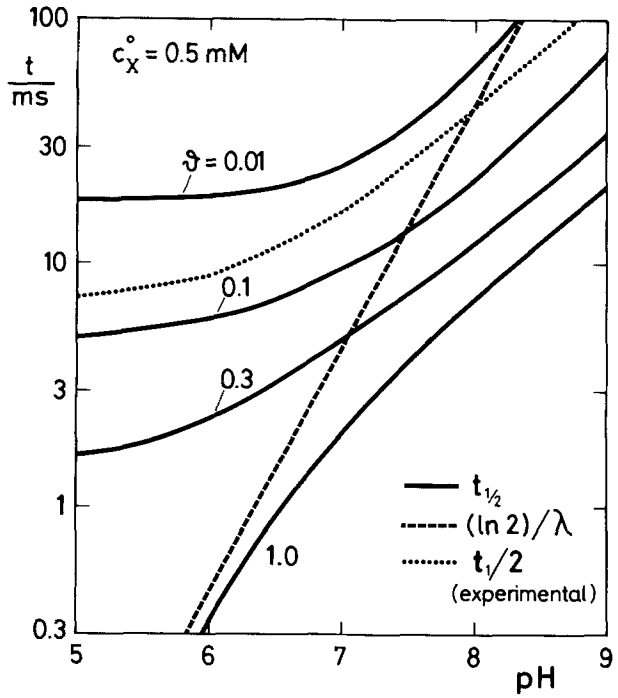

Fig. 5. Comparison between experimentally-observed rise time of the current signal and theoretical predictions. The experimental curve (dotted line) represents the pH dependence of $t_1/2$, where t_1 is the time required for the current signal to reach the peak value (taken from Fig. 7 of part I). The theoretical curves (solid lines) have been obtained by numerical simulation of the reactions preceding phosphorylation of the protein, as described in Appendix A. By integration of the rate equations corresponding to reactions (A1)-(A5), the concentration c_{ET} of the enzyme-ATP complex has been calculated as a function of time t after the light flash. $t_{1/2}$ is the time at which the half-maximal concentration $c_{ET}(\infty)/2$ is reached. c_X^0 is the concentration of caged ATP prior to the flash, and θ is the fraction of caged ATP which, at time $t = 0$, is converted to the photochemical intermediate Y from which ATP release starts. (ln2)/λ (dashed line) is the half-time of decay of Y into ATP. The following values of the rate constants were used for the simulation (*see* Appendix A): $a_T = 5 \times 10^7$ M^{-1} sec^{-1}, $b_T = 20$ sec^{-1}, $a_X = a_Y = 5 \times 10^6$ M^{-1} sec^{-1}, $b_X = b_Y = 200$ sec^{-1}, $\lambda = \lambda_E = (2.2 \times 10^9$ M^{-1} sec$^{-1}) \cdot [H^+]$. a_X and b_X were chosen such that the ratio b_X/a_x agreed with the experimental value (≈ 40 μM), assuming that b_X is ten times larger than b_T.

mately agrees with the theoretical curve ($t_{1/2}$) for $\theta = 0.1$; this value of θ corresponds to the estimated photochemical conversion efficiency of ~10% in the flash experiments. The qualitative agreement between the experimental and the predicted pH dependence of t_1 supports the notion that the rise time of the current signal is determined by electrically silent processes preceding phosphorylation.

Electrogenic Transitions

According to the kinetic scheme of Fig. 1, formation of the enzyme-ATP complex $Na_3 \cdot E_1 \cdot ATP$ initiates a sequence of reactions, viz., phosphorylation, occlusion of Na^+, transition to state $P\text{-}E_2 \cdot Na_3$, and release of Na^+ to the extracellular side. In principle, any of these reaction steps can be associated with charge translocation.

The possibility that the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ is an electrogenic step may be excluded on the basis of the experiments with chymotrypsin-treated enzyme described in the preceding paper (Borlinghaus et al., 1987). Under the conditions of these experiments, chymotrypsin cleaves a single peptide bond in the α-subunit of the protein, between Leu-266 and Ala-267 (Jørgensen & Collins, 1986). In the chymotrypsin-modified enzyme the phosphorylation reaction and the formation of the "occluded" state $(Na_3)E_1\text{-}P$ is preserved, but the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ is blocked (Glynn, Hara & Richards, 1984; Jørgensen & Petersen, 1985). The finding (Borlinghaus et al., 1987) that in chymotrypsin-treated Na,K-ATPase membranes the photoresponse was abolished therefore indicates that the reactions preceding the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ are electrically silent.

It is pertinent to note that the experiments described in the preceding paper (Borlinghaus et al., 1987) have been carried out at high sodium concentration (150 mM) at which Na^+ is already bound to the cytoplasmic sites prior to ATP release. This means that charge translocation associated with binding of Na^+ would not contribute to the observed current transient. Such a "sodium-well" effect (if present) could contribute, however, to the quasistationary current.

The rate constant p_f of the phosphorylation reaction $(Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P)$ was found to be ~220 sec^{-1} for enzyme from guinea-pig kidney at 25°C (Mårdh & Post, 1977) and ~180 sec^{-1} for bovine-brain enzyme at 21°C (Mårdh & Zetterquist, 1974; Mårdh & Lindahl, 1977). Thus, phosphorylation and formation of the occluded state are fast processes within the time scale of the current transient (Fig. 4).

A possible candidate for the reaction governing the slow decay of the current signal (rate constant $k_2 \approx 20$–30 sec^{-1}) is the E_1/E_2 transition that is associated with the deocclusion of sodium $[(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3]$. From experiments with bovine-brain enzyme, Mårdh (1975) reported a value of $1_f \approx 80$ sec^{-1} at 21°C for the rate constant of the E_1/E_2 transition. Nakao and Gadsby (1986) recently described voltage-jump studies of ouabain-dependent Na^+ currents in heart cells under K^+-free conditions and proposed that the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ is a rate-limiting electrogenic step. Similarly, Karlish and Kaplan (1985) concluded that Na^+ deocclusion is a slow step which limits the rate of the tran-

sient (pre-steady state) sodium flux in uncoupled ATP-driven Na^+-transport.

The following reaction step, the release of Na^+ from the outside-facing binding sites ($P\text{-}E_2 \cdot Na_3 \rightarrow P\text{-}E_2 + 3Na^+_{ext}$) is likely to be much faster. This assumption is based on the low affinity of the extracellular sites for sodium binding ($K_D \approx 0.1$ M) and on the fact that association-dissociation reactions of alkali ions with ligands are generally very fast (Eigen & Maass, 1966). It is feasible, however, that a fraction of the observed charge displacement is associated with the release of Na^+, if Na^+ ions have to migrate through part of the membrane dielectric on their way from the binding sites to the extracellular medium (Fig. 3; $\alpha'' > 0$). This is the situation described by reaction scheme (19) in which a rate-determining step is followed by a fast electrogenic process.

Return to the Initial State

The small quasistationary current I_p^∞ which is observed after the decline of the current transient is likely the result from pump molecules which return to the initial state E_1, bind ATP and enter the cycle again. The reaction from $P\text{-}E_2$ to E_1 may take place with unloaded ion-binding sites. This would correspond to "uncoupled Na^+ efflux" which was observed in experiments with erythrocytes when the extracellular medium was free of Na^+ and K^+ (Glynn, 1985). It cannot be excluded, however, that Na^+ ions are already present in the gap between the membrane fragment and the planar bilayer at the start of the experiment. Extracellular Na^+ is known to inhibit ATP-driven sodium-efflux in the absence of K^+ (Glynn, 1985). A small Na^+ efflux, however, persists which is coupled to an influx of Na^+. In this transport mode (commonly referred to as "ATP-driven Na^+-Na^+ exchange) one to three Na^+ ions presumably bind to the potassium sites and are translocated as if they were K^+ ions. It cannot be excluded that ATP-driven Na^+-Na^+ exchange which is known to be electrogenic (Cornelius & Skou, 1985) is responsible for the quasistationary pump current I_p^∞.

Comparison with Na^+-Flux Studies under Nonstationary Conditions

The interpretation of $I_p(t)$ discussed above is supported by recent studies of transient, ATP-driven sodium fluxes in membrane vesicles derived from canine kidney (Forbush, 1984; 1985). After photolytic release of ATP in the internal space of the right-side-out vesicles, an efflux of $^{22}Na^+$ was observed which was independent of the presence of extravesicular K^+ or Na^+. The efflux rate exhibited a biphasic behavior with a fast rising phase and a slower decline. The rate constants of the rising and falling phase ($k_1 \approx 100$ sec^{-1}, $k_2 \approx 35$ sec^{-1} at 20°C) may be compared with the rate constants describing the time course of I_p($K_1 \approx 60$ sec^{-1}, $k_2 \approx 24$ sec^{-1} at 20°C). The similarity in the time course of isotope-flux rate and pump currents is consistent with the assumption that the observed charge-translocation is associated with the transfer of sodium ions across the membrane dielectric. This means that late steps in the transport cycle, i.e., transitions following the release of Na^+ to the extracellular side, are unlikely to contribute significantly to the current transient.

Numerical Simulation of the Transport Cycle

The mechanistic interpretation of the pump current $I_p(t)$ discussed above may be substantiated by numerical simulation of the transport cycle of Fig. 1, using Eq. (13), as described in Appendix B. For this purpose the following assumptions are introduced:

a) At time zero a fraction θ of the initially present caged ATP (concentration c_X^0) is transformed into ATP. For the pseudo-first-order rate constant $a_f c_T$ an effective value of 100 sec^{-1} is used that includes the finite rate of photolysis and the competition between ATP and caged ATP for the binding sites (Forbush, 1984). $a_f c_T$ has been estimated by numerical simulation of the reaction scheme (A1)-(A5) of Appendix A under the condition $c_X^0 = 0.5$ mM, $\theta = 0.1$, pH = 7.0 (Fig. 5). The dissociation constant of ATP is taken to be $a_b \equiv b_T = 20$ sec^{-1} (Forbush, 1984).

b) The equilibrium dissociation constants for Na^+ at the cytoplasmic side [Eq. (14)] are chosen to be $K'_{N1} = K'_N/3$, $K'_{N2} = K'_N$, $K'_{N3} = 3K'_N$; $K'_N = 4$ mM (Läuger & Apell, 1986).

c) For the rate constant of phosphorylation a value of $p_f = 200$ sec^{-1} is used (Mårdh & Post, 1977). Furthermore, since the concentrations of ADP and P_i are extremely small under the given experimental conditions, the rate constants $p_b c_D$ and $r_b c_P$ are set equal to zero.

d) The sodium concentration at the extracellular side is assumed to be much smaller than the equilibrium dissociation constant of sodium at the release sites ($c''_N \ll K''_N \approx 0.1$ M).

e) The values of l_f and r_f are chosen such that an approximate fit to the decay phase of $I_p(t)$ is obtained ($k_2 \approx 20$ sec^{-1}, $I_p^\infty/I_{p,\max} \approx 0.08$). This yields $l_f = 19$ sec^{-1} and $r_f = 0.9$ sec^{-1}. The backward-rate constant l_b is estimated from $l_f/l_b \approx 12$ (Läuger & Apell, 1986).

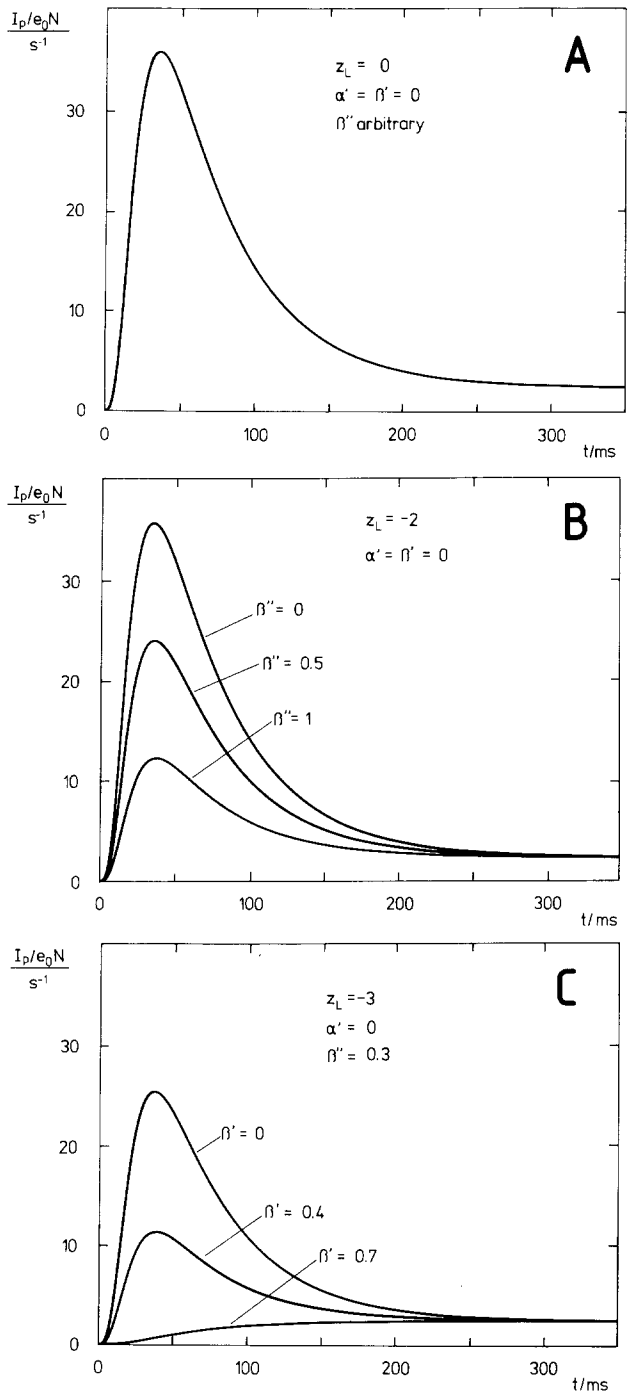

Fig. 6. Numerical simulation of the transport cycle represented in Fig. 1, using Eq. (13). The transient pump current I_p is plotted as a function of time t. I_p is referred to a single pump molecule and is given in sec^{-1}; e_o is the elementary charge and N the number of pump molecules which are activated at $t = 0$. $z_L e_o$ is the charge of the ligand system, and α', β', and β'' are dielectric coefficients defined by Fig. 3. The simulation has been carried out using the following values of the kinetic parameters (*see* text): $a_f c_T = 100\ sec^{-1}$, $a_b = 20\ sec^{-1}$, $p_f = 200\ sec^{-1}$, $p_b c_D = 0$, $l_f = 19\ sec^{-1}$, $l_b = 1.6\ sec^{-1}$, $r_f = 0.9\ sec^{-1}$, $r_b c_P = 0$, $K_N' = 4$ mM, $c_N' = 100$ mM, $c_N'' \ll K_N''$, $\alpha_a = \eta' = \eta'' = 0$. These values were chosen to give an approximate fit to the observed time behavior of the current signal

f) The effect of voltage on the rate constants is neglected.

g) The three sodium binding sites are assumed to be equivalent ($\alpha_1' = \alpha_2' = \alpha_3' \equiv \alpha'$; $\alpha_1'' = \alpha_2'' = \alpha_3'' \equiv \alpha''$).

h) Binding and release of ATP at the cytoplasmic side are assumed to be electrically silent ($\alpha_a = 0$). Furthermore, intrinsic charge displacements of the protein (other than movements of the sodium-binding sites) are neglected ($\eta' = \eta'' = 0$).

According to Eqs. (6), (7) and (13), together with assumptions (g) and (h), the pump current $I_p(t)$ is determined by the dielectric coefficients α', $\alpha_p = (3 + z_L)\beta'$ and $\alpha_1 = (3 + z_L)\beta''$ which can be fitted to the experimental results by varying z_L, β' and β'' (Fig. 6). The coefficients α'' and α_r are then obtained from Eqs. (8) and (9) as $\alpha'' = 1\text{-}\alpha'\text{-}\beta'\text{-}\beta''$ and $\alpha_r = -z_L(\beta' + \beta'')$. Since the experiments with chymotrypsin-modified enzyme have shown that reactions preceding deocclusion of Na^+ are electrically silent, the dielectric coefficient $\alpha_p = (3 + z_L)\beta'$ must be very small. This means that either $\beta' \approx 0$ or $z_L = -3$ (or both).

According to these considerations, numerical simulations were carried out in order to study the influence of the dielectric parameters z_L, α', β' and β'' on the transient pump current. In a first series of simulations, the charge of the ligand system was assumed to be zero ($z_L = 0$). According to the relation $\alpha_p = (3 + z_L)\beta' = 0$, the coefficient β' must then be zero. The results of the simulation for $\alpha' = 0$ and different values of β'' are shown in Fig. 6*A*. It is seen that the simulated pump current qualitatively agrees with the experimentally observed time course of I_p. Current signals calculated for different values of β'' exactly coincide. This is a consequence of the relation $\alpha'' + \beta'' = 1$ (which follows from $\alpha' = \beta' = 0$) and of the assumption that release of Na^+ in state $P\text{-}E_2 \cdot Na_3$ is a fast process following the rate-limiting transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$. The finding that for $z_L = 0$ the current transient is insensitive to the choice of α'' and β'' means that in this case it cannot be distinguished whether the main electrogenic event is the translocation of the positively-charged binding sites or the release of Na^+ from the binding sites to the extracellular medium.

The case $z_L = -2$ is analyzed in Fig. 6*B*. A model with a ligand system bearing two negative charges has been recently proposed by Karlish, Raphaeli and Stein (1985) as an explanation for the effect of transmembrane voltage on pumping rates. As seen from Fig. 6*B*, the amplitude of the current transient decreases with increasing values of the dielectric coefficient β''. This results from the fact that for $z_L = -2$ the E_1/E_2 transition is associated with translocation of a single net (positive) charge, whereas in the process $P\text{-}E_2 \cdot Na_3 \rightarrow P\text{-}E_2 + 3Na_{ext}^+$ which is described by the coefficient $\alpha'' = 1\text{-}\beta''$ three

charges are translocated. When β'' is increased, the relative contribution of the second process (release of 3 Na^+) decreases. A qualitatively similar behavior may be expected under the assumption $z_L = -1$ (Lafaire & Schwarz, 1986).

The signals shown in Fig. 6*C* have been calculated for a ligand system bearing three negative charges ($z_L = -3$). In this case the loaded binding sites are electrically neutral, meaning that the only electrogenic step is the release of Na^+ to the extracellular side. Furthermore, the relation $\alpha_p = (3 + z_L)\beta' = 0$ can now be satisfied for arbitrary values of β'. The simulation that has been carried out for a fixed value of β'' ($\beta'' = 0.3$) shows that the amplitude of the current signal decreases with increasing β', as may be expected from the relation $\alpha'' = 1\text{-}\alpha'\text{-}\beta'\text{-}\beta''$.

The results presented in Fig. 6 show that the observed time course of I_p is consistent with the proposal (Karlish et al., 1985; Nakao & Gadsby, 1986) that the ligand system for Na^+ bears two negative charges ($z_L = -2$). However, as a comparison of Fig. 6*A*, *B* and *C* shows, other values of z_L, such as $z_L = 0$ or $z_L = -3$, cannot be excluded from our experiments so far.

Discussion

In this paper the nonstationary pump currents which are observed in K^+-free Na^+ media after activation of the Na,K-ATPase by a ATP-concentration jump have been analyzed in terms of microscopic reaction models. Evidence has been presented that the rise time of the current signal is determined by the rate of nonelectrogenic processes preceding phosphorylation of the enzyme. Accordingly, the main information on charge-translocating processes is contained in the declining phase of the pump current. The analysis has been based on the model shown in Fig. 1 which is derived from the Albers-Post reaction-scheme of the Na,K-pump. The overall pump-current may be represented as a sum of contributions of the individual transitions in the reaction cycle [Eq. (13)]. Each term in the sum is the product of a net transition rate times a "dielectric coefficient" describing the magnitude of charge translocated in the given reaction step. Charge translocation may result from the motion of ion binding sites in the course of conformational changes of the protein, as well as from movement of ions in narrow access channels connecting the binding sites with the aqueous media.

The observation that the transient pump current requires sodium but not potassium indicates that charge movement is associated with Na^+ translocation and is thus in agreement with the Albers-Post reaction scheme in which translocation of Na^+ precedes translocation of K^+. A "simultaneous" model in which Na^+ and K^+ are translocated in the same step, would be difficult to reconcile with the experimental findings.

From the kinetic scheme of Fig. 1 it is obvious that several transitions are potential candidates for charge-translocating steps. The number of possibilities is reduced, however, by the finding that chymotrypsin treatment of the enzyme, which is known to block the $P\text{-}E_1/P\text{-}E_2$ conformational change, abolishes the current transient. This indicates that phosphorylation of the protein and occlusion of Na^+ are nonelectrogenic processes. An explanation of this finding could be that the total charge of the loaded Na^+-binding sites is zero so that movement of the sites in the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ is electrically silent. An alternative possibility would be that the loaded sites are charged but that the amplitude of motion is negligible. These two possibilities cannot be distinguished so far.

From kinetic studies of the Na,K pump in erythrocytes it is known that the rate of the transition $P\text{-}E_2 \rightarrow E_1$ is extremely low in potassium-free sodium-media, of the order of 5 sec^{-1} at 20°C (Glynn & Karlish, 1976). This rate is much smaller than the decay rate, $k_2 \approx 20\text{–}30$ sec^{-1}, of the nonstationary pump current in the ATP-concentration jump experiments. It is therefore unlikely that the current signal results from the transition $P\text{-}E_2 \rightarrow E_1$.

The most likely interpretation of the observed pump currents consists in the assumption that the principal electrogenic step is the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$, followed by release of Na^+ to the extracellular side. This interpretation agrees with recent proposals based on work with reconstituted vesicles (Karlish et al., 1985; Rephaeli, Richards & Karlish, 1986*a*,*b*; Goldschlegger et al., 1987) and with intact heart cells (Nakao & Gadsby, 1986). Since Na^+ ions migrating from the binding sites to the extracellular medium probably have to traverse part of the membrane dielectric, the release process may contribute to the overall charge translocation.

A question that requires further study is the nature of the space between ATPase membrane and lipid bilayer. Little is known so far about ion concentrations in the intermembrane space and about the change of concentration following activation of the pump. For this reason the analysis of the experiments has been restricted chiefly to the early events in the pumping cycle which are likely to be unaffected by the Na^+ (and K^+) concentration on the extracellular side.

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Appendix A

Kinetic Analysis of Reactions Preceding Phosphorylation: Photolytic ATP-Release and ATP Binding to the Protein

In part I of the paper it has been shown that the pump current $I_p(t)$ rises toward a peak within 10–30 msec after the light flash and thereafter declines to nearly zero. The finite rise-time of $I_p(t)$ indicates that charge translocation by the pump is preceded by slow, electrically silent steps. From the results presented in Fig. 7 of part I it may be concluded that at high pH (above pH 8.5) photochemical ATP release is rate determining. At low pH where ATP release is fast, the rise time of I_p is likely to be limited by the rate of binding of the flash-generated ATP to the enzyme. The initial binding rate of ATP should be proportional to the solution concentration c_T of released ATP. Increasing the concentration of caged ATP increases the concentration of flash-released ATP. The dependence of $I_p(t)$ on the concentration of caged ATP is complicated, however, by the fact that caged ATP competes with ATP for the ATP binding site involved in phosphorylation (Forbush, 1984).

In order to discuss the influence on $I_p(t)$ of the processes preceding phosphorylation more quantitatively, we consider the following reaction scheme in which E stands for the enzyme, T for ATP, X for caged ATP, and Y for the photochemical intermediate from which ATP is formed in a H^+-catalyzed hydrolysis step (McCray et al., 1980):

$$X \overset{\text{light}}{\rightleftharpoons} X^* \longrightarrow Y \xrightarrow{\lambda} T \tag{A1}$$

$$EX \overset{\text{light}}{\rightleftharpoons} EX^* \longrightarrow EY \xrightarrow{\lambda_E} ET \tag{A2}$$

$$E + T \underset{b_T}{\overset{a_T}{\rightleftharpoons}} ET \tag{A3}$$

$$E + X \underset{b_X}{\overset{a_X}{\rightleftharpoons}} EX \tag{A4}$$

$$E + Y \underset{b_Y}{\overset{a_Y}{\rightleftharpoons}} EY. \tag{A5}$$

Light absorption generates an excited species X^* which either decays back to the ground state X or forms Y. The yield of ATP in reaction (A1) has been found to be independent of pH (McCray et al., 1980). Implicit in the reaction sequence (A1)-(A5) is the assumption that T, X and Y compete for the same binding site on the enzyme E. Furthermore, it is assumed that caged ATP may be transformed to Y not only in solution ($X \rightarrow Y$) but also in bound form ($EX \rightarrow EY$), and that ET may be formed in situ from EY.

The influence of reactions (A1)-(A5) on the time course of pump current I_p may be analyzed by numerical simulation of the reaction sequence, using kinetic parameters taken from the literature. The dissociation rate constant of ET at 20°C may be estimated to be $b_T \approx 20\ \text{sec}^{-1}$ (Mårdh & Post, 1977; Karlish, Yates & Glynn, 1978). With an equilibrium dissociation constant $b_T/a_T \approx 0.4\ \mu\text{M}$ (Robinson & Flashner, 1979; Forbush, 1984), the association rate constant becomes $a_T \approx 5 \times 10^7\ \text{M}^{-1}\ \text{sec}^{-1}$. The equilibrium dissociation constant of caged ATP has been determined by Forbush (1984) to be $b_X/a_X \approx 40\ \mu\text{M}$. This means that caged ATP binds about one hundred times weaker than ATP; on the other hand, at the normal concentration of caged ATP in the photolysis experiments ($c_X = 0.5$ mM), most of the nucleotide binding sites of the enzyme are occupied by caged ATP prior to the flash. According to McCray et al. (1980) the pH-dependent rate constant of ATP release is given by $\lambda \approx (2.2 \times 10^9\ \text{M}^{-1}\ \text{sec}^{-1}) \cdot [H^+]$ at 22°C. For the numerical analysis the following additional assumptions are introduced:

a) The conversion of X into Y and of EX into EY after the light flash is virtually instantaneous.

b) The rate of conversion of Y into T is the same in bound and in free form of Y ($\lambda_E \approx \lambda$).

c) The association and dissociation rates of X and Y are identical ($a_X \approx a_Y$, $b_X \approx b_Y$).

d) The concentration of ATP-binding sites (referred to total aqueous volume) is much smaller than the concentration of X prior to the flash.

e) Diffusion in the aqueous phase is not rate limiting.

By numerical integration of the rate equations [reactions (A1)-(A5)], the concentration c_{ET} of the enzyme-ATP complex was evaluated as a function of time t after the light flash. From $c_{ET}(t)$ the time $t_{1/2}$ was obtained at which the half-maximal concentration, $c_{ET}(\infty)/2$, is reached. The result is shown in Fig. 5 for an initial concentration of caged ATP of $c_X^o = 0.5$ mM and differ-

ent pH values; θ is the fraction of caged ATP which is converted into the photochemical intermediate Y by the light flash. The dashed line in Fig. 5 represents the pH-dependent time constant $(\ln 2)/\lambda$ of the decay of Y into ATP. It is seen that at high pH where the reaction $Y \rightarrow$ ATP is slow, the half-time $t_{1/2}$ for $E \cdot$ ATP formation may be shorter than the decay time of Y.

In the pH range in which photolysis of caged ATP is not rate limiting, a simple expression is obtained for the relaxation time τ of the ATP-binding reaction $E + T \rightleftarrows ET$, assuming that binding and dissociation of caged ATP is fast:

$$\frac{1}{\tau} = b_T + \frac{\alpha_T c_T}{1 + c_X/K_X}. \tag{A6}$$

$K_X \equiv b_X/a_X$ is the equilibrium dissociation constant of EX; $c_T = c_X^0\theta$ and $c_X = c_X^0(1 - \theta)$ are the aqueous concentrations of ATP and caged ATP after the flash. For $c_X \gg K_X \approx 40\ \mu$M, the relaxation time τ is independent of the initial concentration c_X^0 of caged ATP. As discussed above, this results from the competition between ATP and caged ATP for the binding site.

Appendix B

Numerical Simulation of the Reaction Cycle of Fig. 1

For the calculation of time-dependent net rates $\Phi_a(t)$, $\Phi_p(t)$, . . . [Eq. (3)] we denote the fraction of pump molecules in state A by $x[A]$ and introduce the following variables:

$$x_1 \equiv x[E_1] \tag{B1}$$

$$x_2 \equiv x[E_1 \cdot \mathrm{ATP}] + x[\mathrm{Na} \cdot E_1 \cdot \mathrm{ATP}] + x[\mathrm{Na_2} \cdot E_1 \cdot \mathrm{ATP}] + x[\mathrm{Na_3} \cdot \mathrm{E_1} \cdot \mathrm{ATP}] \tag{B2}$$

$$x_3 \equiv x[(\mathrm{Na_3}) \cdot E_1 \cdot \mathrm{ATP}] \tag{B3}$$

$$x_4 \equiv x[P\text{-}E_2] + x[P\text{-}E_2 \cdot \mathrm{Na}] + x[P\text{-}E_2 \cdot \mathrm{Na_2}] + x[P\text{-}E_2 \cdot \mathrm{Na_3}] \tag{B4}$$

$$x_1 + x_2 + x_3 + x_4 = 1. \tag{B5}$$

Using Eq. (14), the mole fractions $x[P_i]$ are obtained as

$$x[E_1 \cdot \mathrm{ATP}] = \frac{x_2}{P'}\,;\ x[\mathrm{Na} \cdot E_1 \cdot \mathrm{ATP}] = x_2\frac{n_1'}{P'} \tag{B6}$$

$$x[\mathrm{Na_2} \cdot E_1 \cdot \mathrm{ATP}] = x_2\frac{n_1'n_2'}{P'}\,;\ x[\mathrm{Na_3} \cdot E_1 \cdot \mathrm{ATP}] = x_2\frac{n_1'n_2'n_3'}{P'} \tag{B7}$$

$$P' = 1 + n_1' + n_1'n_2' + n_1'n_2'n_3'. \tag{B8}$$

In an analogous way, the mole fractions $x[P\text{-}E_2]$, $x[P\text{-}E_2 \cdot \mathrm{Na}]$, . . . may be expressed by x_4, n_i'' and P''. According to the reaction scheme of Fig. 1, the time derivatives of the variables x_j are given by:

$$\dot{x}_1 = -(a_f c_T + r_b c_P)x_1 + \frac{a_b}{P'}x_2 + \frac{r_f}{P''}x_4 \tag{B9}$$

$$\dot{x}^2 = a_f c_T x_1 - \frac{1}{P'}(a_b + p_f n_1'n_2'n_3')x_2 + p_b c_D x_3 \tag{B10}$$

$$\dot{x}^3 = \frac{p_f}{P'}n_1'n_2'n_3'x_2 - (p_b c_D + l_f)x_3 + \frac{l_b}{P''}n_1''n_2''n_3''x_4. \tag{B11}$$

x_4 is obtained from Eq. (B5). Eqs. (B9)-(B11) are numerically integrated with the initial condition $x_1(0) = 1$, $x_2(0) = x_3(0) = 0$. The net rates Φ_a, Φ_p, Φ_l, and Φ_r may then be calculated from the relations

$$\Phi_a = a_f c_T x_1 - a_b x_2/P' \tag{B12}$$

$$\Phi_p = p_f n_1'n_2'n_3'x_2/P' - p_b c_D x_3 \tag{B13}$$

$$\Phi_l = l_f x_3 - l_b n_1''n_2''n_3'' x_4/P'' \tag{B14}$$

$$\Phi_r = r_f x_4/P'' - r_b c_P x_1. \tag{B15}$$

Furthermore, from the relations

$$\Phi_a - \Phi_1' = x[E_1 \cdot \mathrm{ATP}] = x_2/P' = (\Phi_a - \Phi_p)/P' \tag{B16}$$

$$\Phi_1' - \Phi_2' = x[\mathrm{Na} \cdot E_1 \cdot \mathrm{ATP}] = x_2 n_1'/P' \tag{B17}$$

$$\Phi_2' - \Phi_3' = x[\mathrm{Na_2} \cdot E_1 \cdot \mathrm{ATP}] = x_2 n_1'n_2'/P' \tag{B18}$$

one obtains

$$\Phi_1' = [n_1'(1 + n_2' + n_2'n_3')\Phi_a + \Phi_p]/P' \tag{B19}$$

$$\Phi_2' = [n_1'n_2'(1 + n_3')\Phi_a + (1 + n_1')\Phi_p]/P' \tag{B20}$$

$$\Phi_3' = [n_1'n_2'n_3'\Phi_a + (1 + n_1' + n_1'n_2')\Phi_p]/P'. \tag{B21}$$

In an analogous way one finds

$$\Phi_1'' = [n_1''(1 + n_2'' + n_2''n_3'')\Phi_r + \Phi_l]/P'' \tag{B22}$$

$$\Phi_2'' = [n_1''n_2''(1 + n_3'')\Phi_r + (1 + n_1'')\Phi_l]/P'' \tag{B23}$$

$$\Phi_3'' = [n_1''n_2''n_3''\Phi_r + (1 + n_1'' + n_1''n_2'')\Phi_l]/P''. \tag{B24}$$

Introduction of Eqs. (B12)-(B15) and of Eqs. (B19)-(B24) into Eq. (13) yields the pump current $I_p(t)$.