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Transient behaviour of the Na^+/K^+-pump: Microscopic analysis of nonstationary ion-translocation

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In recent years fast perturbation techniques have been applied for investigating the mechanism of the Na^+/K^+-pump. Experiments in which nonstationary pump-currents and ion fluxes are measured after a voltage or ATP-concentration jump yield kinetic information which cannot be obtained from ordinary steady-state experiments. In this paper a theoretical treatment is described by which transient pump-currents and ion fluxes can be analyzed in a unified way. The method is based on the assumption that the operation of the pump involves a sequence of conformational transitions and ion-binding and -release steps. The charge displacements associated with the individual reaction steps are described by a set of dielectric coefficients. The nonstationary behaviour of the Na^+/K^+-pump is analyzed on the basis of the Albers-Post reaction cycle. It is shown that the different studies of transient pump-currents and ion fluxes carried out so far lead to internally consistent conclusions with respect to the nature of the electrogenic steps of the transport cycle.

Introduction

Ion transport by the Na,K-pump has been studied in the past mainly under steady-state conditions. Insight into the mode of operation of the pump has been obtained by measuring enzymatic activity and transport rates as a function of ion and nucleotide concentrations in the stationary state [1–7]. Such steady-state experiments are not sufficient, however, for a complete kinetic analysis of the pumping cycle. Additional information may be obtained by studying the time-dependent behaviour of the system after a sudden perturbation. The first experiments of this kind were concerned with the kinetics of phosphorylation and dephosphorylation of the enzyme [8–14] and with the rates of the E_1/E_2 conformational transitions [15–21].

Only recently it has become possible to investigate ion translocation by the Na,K-pump under nonstationary conditions [22–35]. In one type of experiment, right-side out plasma-membrane vesicles derived from kidney cells are loaded with $^{22}Na^+$ and with a photolabile, inactive ATP derivative ('caged' ATP) from which ATP can be set free by a light flash. After the flash-induced ATP concentration jump, the release of $^{22}Na^+$ to the extravesicular medium can be followed in the millisecond to second time-range by a rapid filtration technique [23–26].

Another possibility consists in measuring the transient electric current associated with pump activity after an ATP concentration jump. In these experiments flat membrane fragments containing densely packed arrays of oriented Na,K-ATPase molecules are bound to a planar lipid bilayer acting as a capacitive electrode, and ATP is released from caged ATP in the aqueous solution adjacent to the membrane [29–34].

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Time-dependent pump currents in isolated heart cells can be studied using the technique of whole-cell recording [35]. After depolarizing or hyperpolarizing voltage-jumps, pump-specific current relaxations are observed which depend on the presence of intracellular sodium and ATP.

As will be discussed in the following, these different groups of experiments can be analyzed in an unified way, using methods previously applied to the description of relaxation processes in membranes [36,37]. By numerical simulation of the Albers-Post cycle [6], predictions on time-dependent properties of the pump may be derived. Information on the pumping mechanism may be obtained by comparing the predicted time behaviour with experimental results.

General method for the description of nonstationary behaviour of ion pumps

The operation of an ion pump may be described by the reaction scheme of Fig. 1 in which it is assumed that the pump goes through a cycle of conformational transitions and ion-binding and -release steps. The reaction cycle of Fig. 1 represents a minimal model, which may be easily modified, if necessary, to account for branched pathways. The transition rate constants k_i' and k_i'' are, in general, pseudo-monomolecular rate constants which may contain concentrations, such as ion or nucleotide concentrations. We consider an ensemble of pump molecules and denote the fraction of pumps which are in state P_i at time t by $x_i(t)$:

$$\sum_{i=1}^{n} x_i(t) = 1 \tag{1}$$

In a relaxation experiment, the system at time zero

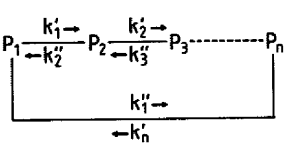


Fig. 1. Reaction cycle of an ion pump. $P_1, P_2, \ldots, P_n$ are different states of the pump molecule. Rate constants of transitions in forward and backward direction are denoted by k_i' and k_i'', respectively ($i = 1, 2, \ldots, n$). The k_i' and k_i'' are pseudomonomolecular rate constants which may contain concentrations.

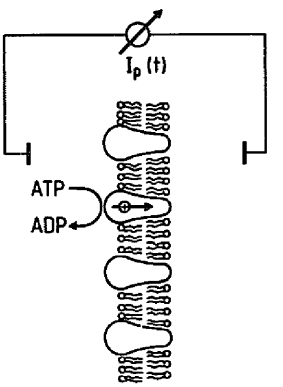


Fig. 2. Membrane with embedded ion pumps interposed between aqueous electrolyte solutions. A fast perturbation, such as a stepwise change of voltage or an ATP-concentration jump results in a time-dependent current $I_p(t)$ in the external measuring circuit.

is present in a steady state which is described by rate constants $\bar{k}_i'$ $\bar{k}_i''$ and by initial conditions $x_i = x_i(0)$. By an external perturbation, some of the rate constants are instantaneously shifted to new values k_i', k_i''. Thereafter, the system evolves toward a new stationary state with $x_i = x_i(\infty)$. The net forward rate of transitions between states P_i and P_{i+1}, referred to a single pump molecule, is given by:

$$\Phi_i = k_i' x_i - k_{i+1}'' x_{i+1} \tag{2}$$

The rate of change of x_i may then be written as:

$$\frac{dx_i}{dt} = \Phi_{i-1} - \Phi_i \tag{3}$$

Eqns. 1–3 have the solution [36]:

$$x_i(t) = \sum_{j=1}^{n-1} a_{ij} \exp(-t/\tau_j) + x_i(\infty) \tag{4}$$

The time constants τ_j and the 'amplitudes' a_{ij} are functions of the rate constants k_i' and k_i''.

In the following we consider experiments in which a membrane with embedded ion pumps is interposed between two aqueous electrolyte solutions (Fig. 2). Pump activation by a sudden perturbation such as a voltage or concentration jump leads, in general, to a time-dependent current $I_p(t)$ in the external measuring circuit. I_p is an average current which results from the superposition of

individual charge translocations in the pump molecules associated with transitions between states of the reaction cycle. The contribution of transition $P_i \rightarrow P_{i+1}$ is given by $\alpha_i e_0$, where e_0 is the elementary charge and α_i the so-called dielectric coefficient [36] of the transition. The meaning of α_i is simple in the limiting case of a membrane consisting of a homogeneous dielectric layer of thickness d. When in a given transition ν elementary charges are translocated over a distance a_i, the dielectric coefficient α_i is equal to $\nu a_i/d$. In the case of a membrane with embedded transport proteins, however, the coefficients α_i depend on the geometry and the dielectric properties of the protein and the surrounding medium.

Since Φ_i is the net transition rate, the contribution of the reaction $P_i \rightleftharpoons P_{i+1}$ to the total current is equal to $\alpha_i e_0 \Phi_i$. For an ensemble of N pump molecules the total current is thus given by:

$$I_p(t) = e_0 N \sum_{i=1}^{n} \alpha_i \Phi_i(t) \tag{5}$$

This relation represents the basis for the microscopic interpretation of transient currents; it is valid not only for the cyclic process of Fig. 1, but for any (arbitrarily branched) reaction scheme, provided that the summation is carried out over all individual transitions.

In the steady state, all rates Φ_i are identical and equal to the stationary turnover rate Φ_s, so that the stationary current assumes the form:

$$I_{p,s} = e_0 m N \Phi_s \tag{6}$$

m is the number of elementary charges translocated across the membrane in a single turnover of the pump. This means that the following relation must hold:

$$\sum_{i=1}^{n} \alpha_i = m \tag{7}$$

According to Eqns. 2, 4 and 5, the current I_p is given by the sum of $n-1$ exponential terms plus a stationary current $I_p(\infty)$:

$$I_p(t) = e_0 N \sum_{i=1}^{n} \left[\alpha_i \sum_{j=1}^{n-1} A_{ij} \exp(-t/\tau_j) \right] + I_p(\infty) \tag{8}$$

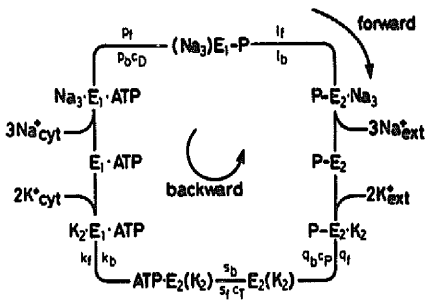


Fig. 3. Albers-Post scheme for the pumping cycle of Na,K-ATPase [38]. E_1 and E_2 are conformations of the enzyme with ion-binding sites exposed to the cytoplasm and the extracellular medium, respectively. In the 'occluded' states $(Na_3)E_1$ and $E_2(K_2)$ the bound ions are unable to exchange with the aqueous phase. Dashes indicate covalent bonds and dots indicate noncovalent bonds. p_f, l_f, q_f, $s_f c_T$, k_f and $p_b c_D$, k_b, s_b, $q_b c_P$, l_b 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).

Since the quantities τ_j and A_{ij} are, in general, complicated functions of the rate constants, an explicit solution for $I_p(t)$ is difficult to obtain, and in practice, I_p has to be evaluated from Eqn. 5 by numerical simulation of the reaction cycle.

The Na,K-pump under nonstationary conditions

Kinetic scheme of the pumping cycle

The analysis given below is based on the Albers-Post cycle of the Na,K-pump [6,38] which is represented in Fig. 3. According to the Albers-Post scheme, the enzyme can assume two conformations, E_1 and E_2. Form E_1 which has the ion-binding sites exposed to the cytoplasmic side of the membrane can be phosphorylated after binding of three Na^+ (forward direction of the pumping cycle in Fig. 3). In the phosphorylated state, E_1-P, the Na^+ are 'occluded', i.e., they are unable to exchange with either aqueous phase. The occluded form, $(Na_3)E_1$-P, undergoes a conformational transition to state $P\text{-}E_2 \cdot Na_3$ from which Na^+ is released to the extracellular medium. After binding of K^+ and formation of state $P\text{-}E_2 \cdot K_2$, the protein becomes dephosphorylated. The cycle is completed by the transition from state $ATP \cdot E_2(K_2)$ with occluded potassium to state $ATP \cdot E_1$

$\cdot K_2$, followed by release of K^+ to the cytoplasm and rebinding of Na^+.

As indicated in Fig. 3, the reaction rates are described by rate constants p_f, l_f, q_f, $s_f c_T$, k_f for transitions in forward direction and by $p_b c_D$, k_b, s_b, $q_b c_P$, l_b for transitions in backward direction; c_T, c_D and c_P are the cytoplasmic concentrations of ATP, ADP and inorganic phosphate (P_i), respectively. In the notation adopted here, the bimolecular reaction $(Na_3)E_1\text{-}P + ADP \rightarrow Na_3 \cdot E_1 \cdot ATP$ is described by a pseudo-monomolecular rate constant $p_b c_D$; the rate constants $q_b c_P$ and $s_f c_T$ are defined in an analogous way.

We assume that the rate constants of binding and release of Na^+ and K^+ are large, so that these reactions are always in equilibrium. This assumption is justified when binding from the aqueous phase is diffusion-controlled (rate constant k_{on} of ion binding of the order of $10^9\ M^{-1} \cdot s^{-1}$). In this case the binding rate $c \cdot k_{on}$ is at least $10^6\ s^{-1}$ for ion concentrations $c > 1$ mM, and the dissociation rate constant k_{off} is at least $10^5\ s^{-1}$, assuming a lower limit for the equilibrium dissociation constant k_{off}/k_{on} of 0.1 mM. While binding rates of alkali ions to oxygen ligands are usually very large and close to the limit of diffusion-controlled reactions [39], direct experimental information on binding rate constants in the case of the Na,K-pump are lacking. For this reason the assumption of fast binding and dissociation should be considered as tentative. If c'_N is the Na^+ concentration on the cytoplasmic side and $x[A]$ the fraction of Na,K-ATPase present in form A, sodium binding at the cytoplasmic side is then described by:

$$\frac{x[Na_{i-1} \cdot E_1]}{x[Na_i \cdot E_1]} = \frac{\sigma'_i}{\rho'_i c'_N} = \frac{K'_{Ni}}{c'_N} \equiv \frac{1}{n'_i} \qquad (9)$$

$(i = 1, 2, 3)$

σ'_i and ρ'_i are the rate constants for dissociation and association, and K'_{Ni} is the equilibrium dissociation constant. Analogous equations hold for the other binding equilibria.

Dielectric coefficients and voltage dependence of kinetic parameters

To evaluate the dielectric coefficients of the different reaction steps, we introduce the energy profile of the ion along the transport pathway [40].

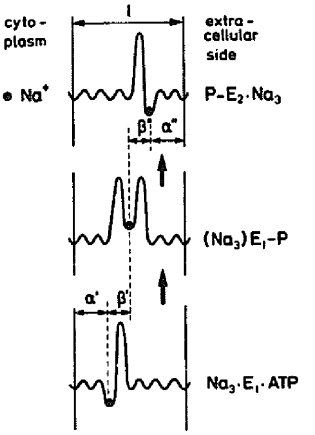


Fig. 4. Hypothetical energy profile of a sodium ion along the transport pathway. The ion-binding sites in state $Na_3 \cdot E_1 \cdot ATP$ are connected with the cytoplasmic side by a series of low barriers, but separated from the extracellular medium by a high barrier. In the 'occluded' state $(Na_3)E_1\text{-}P$ 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. α', α'', β' and β'' are dielectric distances, depending on the location of the ion-binding site in the protein and on the dielectric properties of the protein and the surrounding medium.

The energy profile consists of a series of barriers and wells (Fig. 4); it reflects the interaction of the ion with the protein in a particular conformation [41]. According to the assumption of a fast association–dissociation equilibrium, the ion-binding site in state $Na_3 \cdot E_1 \cdot ATP$ is connected with the cytoplasmic side by a series of low barriers (Fig. 4); the site is separated from the extracellular medium by a high barrier.

Since a Na^+ ion migrating from the cytoplasm to the binding site has, in general, to traverse part of the dielectric (Fig. 4), the binding step may be electrogenic. For simplicity, we assume that the corresponding dielectric distance α' is the same for the three sodium-binding sites. In a complete analogous way the release of Na^+ to the extracellular side is described by a dielectric distance α'' (Fig. 4). The introduction of α' and α'' corresponds to the notion that an ion-binding site may be connected with the adjacent aqueous phase via a high-field access channel (or 'ion well') over which part of an externally applied voltage drops [42].

In the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ the loaded binding sites move over a dielectric distance β' (Fig. 4). If $z_L e_0$ is the charge of the ligand groups, the dielectric coefficient associated with the reaction $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ (rate constant p_f) is given by:

$$\alpha_p = (3 + z_L)\beta' + \eta' \tag{10}$$

The parameter η' accounts for rotation of dipolar groups and translocation of intrinsic charges of the protein other than charged ligands [43]. In an analogous way the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ (rate constant l_f) is described by a dielectric coefficient α_l:

$$\alpha_l = (3 + z_L)\beta'' + \eta'' \tag{11}$$

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

$$\alpha' + \alpha'' + \beta' + \beta'' = 1 \tag{12}$$

The dielectric coefficients of the other reaction steps are obtained in a completely analogous way.

If an electrical potential difference, $V = \psi' - \psi''$, exists across the membrane, a fraction $\alpha' V$ drops between the cytoplasm and the sodium binding sites (Fig. 4). As the potential energy of an ion in the binding site is modified by the presence of a voltage V, the equilibrium dissociation constants K'_{Ni} become voltage-dependent:

$$K'_{Ni} = \tilde{K}'_{Ni} \exp(-\alpha' u) \tag{13}$$

$$u \equiv \frac{V}{kT/e_0} \equiv \frac{\psi' - \psi''}{kT/e_0} \tag{14}$$

ψ' and ψ'' are the electric potentials at the cytoplasmic and at the extracellular side, respectively. $\tilde{K}'_{Ni}$ is the value of K'_{Ni} at zero voltage, k is the Bolzmann constant, and T the absolute temperature. If $\alpha'' V$ is the potential at the binding site in state E_2 with respect to the extracellular medium (Fig. 4), the voltage dependence of K''_{Ni} is given by

$$K''_{Ni} = \tilde{K}''_{Ni} \exp(\alpha'' u) \tag{15}$$

Analogous expressions may be obtained for the equilibrium dissociation constants of K^+.

To evaluate the voltage dependence of the rate constants, we assume that the reaction $P_i \rightarrow P_{i+1}$ (Fig. 1) can be approximately described as a transition over a symmetrical, narrow activation barrier [44]. According to the theory of absolute reaction rates [45], the voltage dependence of p_f and p_b is determined by the electrostatic contribution, $\alpha_p V$, to the total energy difference between states $(Na_3)E_1\text{-}P$ and $Na_3 \cdot E_1 \cdot ATP$ (Eqn. 10). This gives [44]:

$$p_f = \tilde{p}_f \exp(\alpha_p u/2) \tag{16}$$

$$p_b = \tilde{p}_b \exp(-\alpha_p u/2) \tag{17}$$

Similarly, for the transition between $(Na_3)E_1\text{-}P$ and $P\text{-}E_2 \cdot Na_3$:

$$l_f = \tilde{l}_f \exp(\alpha_l u/2) \tag{18}$$

$$l_b = \tilde{l}_b \exp(-\alpha_l u/2) \tag{19}$$

According to the principle of detailed balance, the rate constants and equilibrium constants of the reaction cycle are not independent of each other, but fulfill the following relation:

$$\frac{a_f p_f l_f r_f}{a_b p_b l_b r_b} \cdot \frac{K''_{N1} K''_{N2} K''_{N3}}{K'_{N1} K'_{N2} K'_{N3}} \cdot \frac{1}{K} = \exp(3u) \tag{20}$$

$K \equiv \bar{c}_D \bar{c}_P / \bar{c}_T$ is the equilibrium constant of ATP hydrolysis ($\bar{c}_T$, $\bar{c}_D$ and $\bar{c}_P$ are equilibrium concentrations of ATP, ADP and P_i, respectively). For a derivation of Eqn. 20, see Ref. 43.

Kinetic behaviour in the absence of K^+

Several studies of transient behaviour of the Na,K-pump have been carried out in the presence of Na^+ and in the absence of K^+ [22–26,29–35]. Under this condition, the Albers-Post reaction cycle (Fig. 1) reduces to the reaction scheme shown in Fig. 5A and B. Phosphorylation in the presence of Na^+ induces a transition to state $P\text{-}E_2 \cdot Na_3$, followed by release of Na^+ to the extracellular medium. In the absence of K^+, dephosphorylation of the protein in state $P\text{-}E_2$ and return to state E_1 is known to be extremely slow [12,46–49].

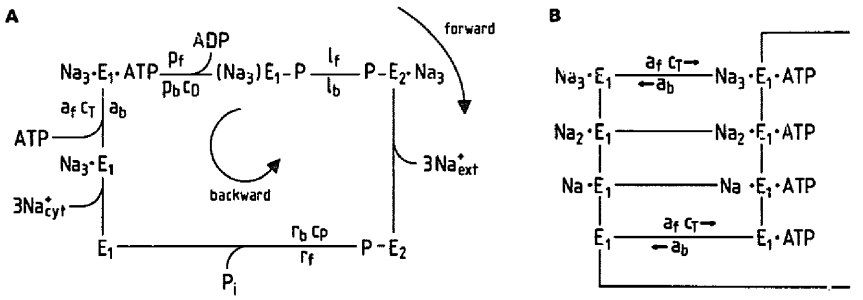


Fig. 5. Transport reactions of the Na,K-pump in the absence of K^+, based on the Albers-Post reaction scheme (Fig. 3). (A) Overall reaction cycle. (B) Association-dissociation reactions in state E_1. The rate constants a_f and a_b of association and dissociation of ATP are assumed to be independent of the number of Na^+ ions bound to E_1.

For the analysis of the reaction scheme of Fig. 5 the following simplifying assumptions are introduced:

(1) Phosphorylation by ATP and transitions between states E_1-P and P-E_2 are only possible with fully occupied sodium-binding sites [1–7]. Furthermore, the existence of additional forms of the phosphoenzyme [50,51] is neglected.

(2) The three sodium-binding sites are equivalent, so that states differing in the position of bound sodium ions are kinetically indistinguishable.

(3) Binding and release of Na^+ are not rate-limiting, meaning that states E_j, $Na \cdot E_j$ and $Na_2 \cdot E_j$ are always in equilibrium with each other. (This assumption has already been used for the derivation of Eqn. 9).

(4) The affinities and rate constants for ATP binding in states E_1, $Na \cdot E_1$, $Na_2 \cdot E_1$ and $Na_3 \cdot E_1$ are the same. This further means that also the sodium affinities of E_1 and $E_1 \cdot ATP$ are identical [52,53].

(5) Binding of ATP to the protein in state E_1 is nonelectrogenic. This assumption is based on the notion that in the enzyme-ATP complex, the charged phosphate residues of ATP are located at the cytoplasmic surface of the protein. Under this condition, Eqns. 7 and 10–12 yield:

$$z_L(\beta' + \beta'') + \eta' + \eta'' + \alpha_r = 0 \tag{21}$$

α_r is the dielectric coefficient of the reaction P-E_2 $\rightarrow E_1 + P_i$ (rate constant r_f).

Denoting the fraction of pump molecules in state P_j by $x[P_j]$, the net forward rates in the reaction cycle of Fig. 5 may be written as:

$$\Phi_i' = \rho_i' c_N' (x[Na_{i-1} \cdot E_1] + x[Na_{i-1} \cdot E_1 \cdot ATP]) - \sigma_i' (x[Na_i \cdot E_1] + x[Na_i \cdot E_1 \cdot ATP]) \tag{22}$$

$(i = 1, 2, 3)$

$$\Phi_p = p_f x[Na_3 \cdot E_1 \cdot ATP] - p_b c_D x[(Na_3)E_1 - P] \tag{23}$$

c_N' is the cytoplasmic concentration of Na^+, c_D the concentration of ADP, and ρ_i' and σ_i' are concentration-independent rate constants introduced above (Eqn. 9). The net forward rates Φ_f, Φ_1'', Φ_2'', Φ_3'' and Φ_r are obtained in a completely analogous way.

Experimentally observable quantities are the transient pump-current $I_p(t)$ and the rate of sodium release to the extracellular medium, $J_{Na}(t)$. The time-course of J_{Na} has been studied with $^{22}Na^+$-loaded membrane vesicles after an ATP concentration jump [23–26]. According to Eqn. 5, the pump current is obtained as

$$I_p/e_0 N = \alpha'(\Phi_1' + \Phi_2' + \Phi_3') + \alpha_p \Phi_p + \alpha_f \Phi_f + \alpha''(\Phi_1'' + \Phi_2'' + \Phi_3'') + \alpha_r \Phi_r \tag{24}$$

Since, according to assumption 3, dissociation of Na^+ from the binding sites is not rate-limiting,

the rate J_{Na} of (unidirectional) sodium release is simply given by

$$J_{Na} = 3Nl_f x[(Na_3)E_1\text{-}P] \tag{25}$$

Numerical simulations

A kinetic interpretation of nonstationary experiments becomes possible by numerical simulation of the reaction cycle of Fig. 5. In all calculations the following additional assumptions have been used: (a) Sodium ions bind independently to three sites of equal intrinsic affinity; this means that $K'_{N1} = K'_N/3$, $K'_{N2} = K'_N$, $K'_{N3} = 3K'_N$; $K''_{N1} = K''_N/3$, $K''_{N2} = K''_N$, $K''_{N3} = 3K''_N$, where K'_N and K''_N are the equilibrium dissociation constants in states E_1 and E_2, respectively [43]. This assumption is introduced here chiefly in order to keep the number of adjustable parameters as small as possible; it may replaced by a more refined treatment as soon as more experimental data on Na^+ binding affinities become available. For further discussion of this point, see Refs. 54 and 55. (b) Intrinsic charge-displacements (other than movements of the sodium binding sites) are neglected ($\eta' = \eta'' = 0$). Under this condition the relation $\alpha_p + \alpha_l = (3 + z_L)(1 - \alpha' - \alpha'')$ holds.

As described in Appendix A, the time course of I_p and of J_{Na} can be evaluated by numerical integration of the rate equations derived from the reaction scheme of Fig. 5.

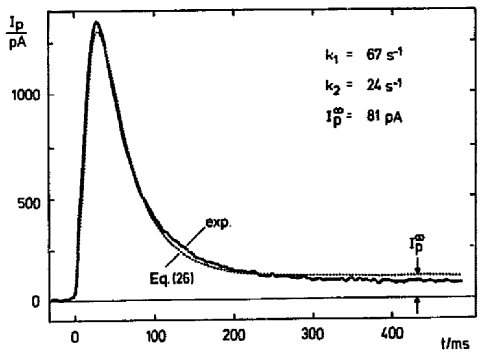


Fig. 6. Transient pump-current $I_p(t)$ after an ATP-concentration jump (compare Fig. 2). At time zero about 50 μM ATP were released from 'caged ATP' by a brief light-flash. Solid line: experimental curve ($T = 20\,°C$). Dashed line: fitting curve calculated from Eqn. 26. Taken from Ref. 31.

Comparison between pump current $I_p(t)$ and rate of sodium-release, $J_{Na}(t)$, after an ATP-concentration jump

Nonstationary pump currents after an ATP-concentration jump have been recorded in a compound membrane system consisting of flat Na,K-ATPase membrane fragments bound to a planar lipid bilayer [29,30]. ATP was released from a photolabile precursor ('caged' ATP) by a light flash in the absence of K^+. From the observed current signal the intrinsic pump-current $I_p(t)$ corresponding to the idealized arrangement of Fig. 2 can be evaluated. The pump current which is obtained in this way exhibits a biphasic shape (Fig. 6) and can be approximately described by the empirical relation:

$$I_p(t) = I_1 \exp(-k_1 t) + I_2 \exp(-k_2 t) + I_p^\infty \tag{26}$$

where k_1, k_2, I_1, I_2 and I_p^∞ are time-independent constants [31].

The measurement of $I_p(t)$ can be compared with studies of transient sodium fluxes in plasma membrane vesicles containing Na,K-ATPase [24,25]. In these experiments 'caged' ATP together with $^{22}Na^+$ was included in the intravesicular aqueous space, and the rate J_{Na} of $^{22}Na^+$ extrusion after flash-induced release of ATP was recorded under K^+-free conditions. The time-course of J_{Na} was found to be of the form of Eqn. 26 with approximately the same values of the parameters k_1 and k_2 ($k_1 \approx 100\ s^{-1}$, $k_2 \approx 35\ s^{-1}$ at 20 °C).

The observed similarity in the time dependence of transient charge-movement and of transient sodium-release after pump activation has interesting implications on the transport mechanism. This is illustrated by the examples given below in which $I_p(t)$ and $J_{Na}(t)$ have been numerically simulated, using different sets of assumptions on the microscopic parameters of the reaction cycle. The principal aim of the simulation was a qualitative comparison of $I_p(t)$ and $J_{Na}(t)$, without attempting to achieve an optimal fit of the experimental time-course of I_p and J_{Na}. For this purpose the following values of kinetic parameters have been (more or less arbitrarily) chosen: $a_f c_T = 300\ s^{-1}$, $p_f = 100\ s^{-1}$, $l_f = 30\ s^{-1}$, $l_b = 2\ s^{-1}$, $r_f = 1\ s^{-1}$, $K'_N = 4$ mM and $a_b = 20\ s^{-1}$. Since the ATP concentra-

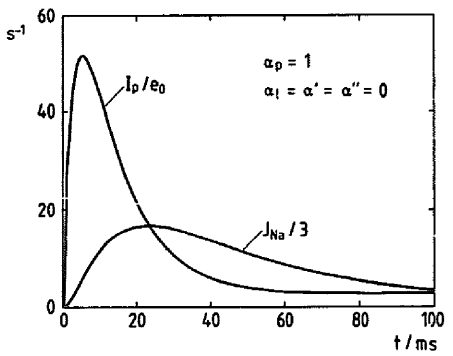


Fig. 7. Pump current I_p and rate of sodium release, J_{Na}, after an ATP-concentration jump. I_p is expressed as a translocation rate I_p/e_0 (e_0 is the elementary charge). The factor 1/3 in J_{Na} corresponds to the assumption that three Na^+ are released in a single transport cycle. $I_p(t)$ and $J_{Na}(t)$ have been obtained by numerical evaluation of Eqns. 24 and 25 and are referred to a single pump molecule ($N=1$). The numerical simulations have been carried out using the following parameter values: $a_f a_T = 300\ s^{-1}$, $a_b = 20\ s^{-1}$, $p_f = 100\ s^{-1}$, $p_b c_D = 0$, $l_f = 30\ s^{-1}$, $l_b = 2\ s^{-1}$, $r_f = 1\ s^{-1}$, $r_b c_P = 0$, $c'_N = 100$ mM, $3\,K'_{N1} = K'_{N2} = K'_{N3}/3 = K'_N = 4$ mM, $c''_N = 0$, $\alpha_l = \alpha' = \alpha'' = \eta' = \eta'' = 0$, $\alpha_p = 1$. The rising phase of $I_p(t)$ is mainly determined by the rate of ATP binding ($a_f c_T = 300\ s^{-1}$), the decay phase by the phosphorylation rate ($p_f = 100\ s^{-1}$); the rising phase of $J_{Na}(t)$ is determined by p_f, the decay phase by $l_f = 30\ s^{-1}$.

tion-jump experiments have been carried out in the absence of ADP, inorganic phosphate and extracellular sodium, the condition $c_D = c_P = c''_N = 0$ has been used in the simulation.

In Fig. 7 the time-course of the pump current, I_p, and of the rate of sodium release, J_{Na}, is compared under the assumption that the only electrogenic step in the pumping cycle is phosphorylation by ATP and occlusion of sodium ($\alpha_p = 1$, $\alpha' = \alpha'' = \alpha_l = 0$). Under the conditions of Fig. 7, $I_p(t)$ is governed by the fast electrogenic transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ ($p_f = 100\ s^{-1}$), whereas $J_{Na}(t)$ is governed by the slow deocclusion of sodium ($l_f = 30\ s^{-1}$). The predictions of Fig. 7 are clearly at variance with the experimental finding that the time-course of I_p parallels the time-course of J_{Na}. This means that a mechanism in which a fast electrogenic phosphorylation step is followed by slow electroneutral sodium-release may be excluded.

Fig. 8 represents the time course of I_p and J_{Na} calculated under the assumption that occlusion of Na^+ is electroneutral ($\alpha_p = 0$), but that deocclusion and dissociation of Na^+ are electrogenic ($\alpha_l = 0.6$, $\alpha'' = 0.4$). All other kinetic parameters are the same as in Fig. 7. It is seen that in this case I_p and J_{Na} have the same time dependence. A qualitatively similar result, but with a different amplitude of I_p would have been obtained for other values of α_l and α'', as long as α_p is negligibly small. (Note that the rate of Na^+ translocation through the extracellular access-channel is always limited by the rate of deocclusion, since dissociation has been assumed to be very fast). According to Fig. 8, the experimental results of the ATP concentration-jump experiments are consistent with the assumption that a major electrogenic step in the pumping cycle is the deocclusion of Na^+, followed by release to the extracellular medium. Direct evidence that phosphorylation by ATP and occlusion of Na^+ is an electrically silent process has been obtained from experiments in which the deocclusion step has been blocked by chymotrypsin modification of the enzyme [30].

Little information is available so far on whether an ion well at the cytoplasmic side is present (corresponding to $\alpha' > 0$). Experiments with chymotrypsin-modified enzyme have been done so far only at high cytoplasmic sodium concentration ($c'_N = 150$ mM). Under this condition the cytoplasmic binding sites are nearly saturated, so that

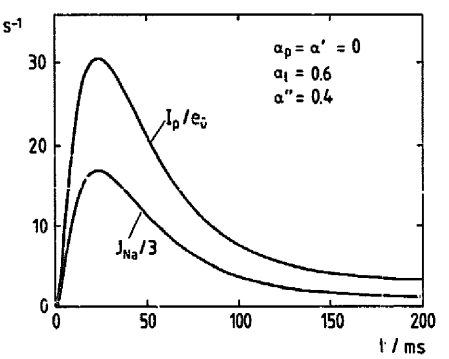


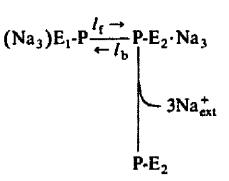
Fig. 8. Pump current I_p and rate of sodium release, J_{Na}, as a function of time t. The same parameter values have been used as in Fig. 7, except for $\alpha_p = \alpha' = 0$, $\alpha_l = 0.6$ and $\alpha'' = 0.4$. $J_{Na}(t)$ is the same as in Fig. 7.

sodium translocation from the aqueous phase to the binding sites after the ATP concentration-jump is negligible.

Voltage-jump current-relaxation experiments

Nakao and Gadsby [35] recently described experiments with cardiac cells in which transient pump-currents were elicited by a sudden displacement of transmembrane voltage. The ouabain-sensitive component of the current was identified with the current I_p generated by the Na,K-pump. The transient pump-current was observed in the absence of extracellular potassium, but required the presence of intracellular ATP and both the presence of intra- and extracellular Na^+. A voltage jump from a holding potential of -40 mV to $+60$ mV resulted in an outward-directed transient current which nearly exponentially decayed to zero with a time constant of about 5 ms. Voltage jumps to more negative potentials gave rise to transient inward currents.

Under the conditions of the experiments of Nakao and Gadsby, i.e., in the (nominal) absence of ADP, the reaction $Na_3 \cdot E_1 \cdot ATP \rightleftharpoons (Na_3)E_1\text{-}P$ (Fig. 5A) is shifted far to the right. Furthermore, since the pump current after the voltage jump decayed to virtually zero, one may infer that the rate of the transition $P\text{-}E_2 \rightarrow E_1$ was negligibly small. Under these circumstances the reaction scheme of Fig. 5A reduces to the following transitions:



(27)

This means that under the given experimental conditions (absence of extracellular K^+ and of intracellular ADP), a quasi-equilibrium exists between states $(Na_3)E_1\text{-}P$, $P\text{-}E_2 \cdot Na_3$, $P\text{-}E_2 \cdot Na_2$, $P\text{-}E_2 \cdot Na$ and $P\text{-}E_2$ of the pump. Depending on the magnitude of the dielectric coefficients α_l and α'', a voltage jump leads to a shift of this equilibrium and to a concomitant transient current $I_p(t)$. The time-dependent pump current after a jump from the holding potential, V_0, to the final voltage, V_∞, is given by (compare Appendix B):

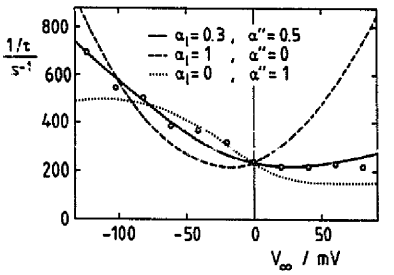


Fig. 9. Relaxation time τ of pump current after a voltage jump to V_∞. The experimental results of Nakao and Gadsby (Ref. 35, circles) are compared with numerical predictions from Eqn. 32. The theoretical curves have been calculated for different combinations of the dielectric coefficients α_l and α'' using the following fixed values of kinetic parameters: $\tilde{l}_f = 160\ s^{-1}$, $\tilde{l}_b = 348\ s^{-1}$, 3 $\tilde{K}''_{N1} = \tilde{K}''_{N2} = \tilde{K}''_{N3}/3 = \tilde{K}''_N = 100$ mM, $c''_N = 150$ mM.

$$I_p(t) = e_0 N l_b^\infty (\alpha_l + \alpha'' p_\infty) \cdot \frac{\rho_0 K_l^\infty - \rho_\infty K_l^0}{\rho_0 + K_l^0} \exp\left(-\frac{t}{\tau}\right) \quad (28)$$

$$n_i'' \equiv c_N''/K_{Ni}''; \quad P'' = 1 + n_1'' + n_1'' n_2'' + n_1'' n_2'' n_3'' \quad (29)$$

$$p_\infty \equiv [(3 + 2n_1'' + n_1'' n_2'')/P'']_\infty \quad (30)$$

$$\rho_0 \equiv [n_1'' n_2'' n_3''/P'']_0; \quad \rho_\infty \equiv [n_1'' n_2'' n_3''/P'']_\infty \quad (31)$$

$$K_l^0 = l_f^0/l_b^0; \quad K_l^\infty = l_f^\infty/l_b^\infty \quad (32)$$

$$1/\tau = l_f^\infty + \rho_\infty l_b^\infty \quad (33)$$

The subscripts 0 and ∞ indicate that the quantities n_i'' have to be taken at voltages V_0 or V_∞, respectively. l_f^0, l_f^∞, l_b^0 and l_b^∞ denote the values of the rate constants l_f and l_b at voltages V_0 and V_∞, the voltage dependence of l_f, l_b and K''_{Ni} being given by Eqns. 15, 18 and 19.

Eqn. 28 predicts, in agreement with the experimental findings [35], a current transient which is described by a single relaxation time constant τ. According to Eqn. 32, τ depends on voltage and (through ρ_∞) on extracellular sodium concentration. $1/\tau$ is represented in Fig. 9 as a function of voltage V_∞ for different values of α_l and α'' and fixed values of $\tilde{l}_f$, $\tilde{l}_b$ and $\tilde{K}''_{Ni}$. A reasonable fit of the experimental data is obtained for $\alpha_l = 0.3$ and $\alpha'' = 0.5$. No fit was possible when α'' was set

equal to zero and all other parameters were varied. This finding provides evidence for the existence of a high-field access channel (or 'ion well') at the extracellular side. The fact that α_i as well as α'' have to be chosen to be non-zero in order to fit the experimental values of $1/\tau$ means that both deocclusion as well as release of Na^+ from the binding sites contribute to the observed current transient.

From the numerical fit represented in Fig. 9, values of the rate constants of the reaction $(Na_3)E_1\text{-}P \rightleftarrows P\text{-}E_2 \cdot Na_3$ may be estimated. The values obtained in this way for cardiac cells at 35°C, $\tilde{l}_f \approx 160\ s^{-1}$ and $\tilde{l}_b \approx 350\ s^{-1}$, may be compared with estimates derived from the ATP-concentration jump experiments discussed in the previous section, $\tilde{l}_f \approx 30\ s^{-1}$, $\tilde{l}_b \approx 2\ s^{-1}$ (kidney enzyme at 20°C). Studies with bovine brain enzyme at 21°C yielded values of $\tilde{l}_f \approx 80\ s^{-1}$ and $\tilde{l}_b \approx 8\ s^{-1}$ [9,60]. The large differences between the various estimates for $\tilde{l}_f$ and $\tilde{l}_b$ may result from differences in experimental conditions and sources of the enzyme.

A further experimental quantity of interest is the electric charge which is translocated after the voltage jump. The total translocated charge may be represented as the sum of two contributions, Q^* and Q, where Q^* describes the virtually instantaneous charge-displacement resulting (for $\alpha'' \neq 0$) from fast binding or release of Na^+ at the extracellular side *; Q is the time integral of the observed current transient $I_p(t)$:

$$Q = \int_0^\infty I_p dt = e_0 N(\alpha_i + \alpha'' \rho_\infty)\left[\frac{\rho_0}{\rho_0 + K_i^0} - \frac{\rho_\infty}{\rho_\infty + K_i^\infty}\right] \quad (34)$$

As may be seen from Fig. 10, the experimentally observed voltage-dependece of Q [35] could be fitted by Eqn. 34, using $\alpha_i = 1$ and $\alpha'' = 0$, but an acceptable fit with the values of α_i and α'' ($\alpha_i = 0.3$, $\alpha'' = 0.5$) which gave an optimum fit of $1/\tau = f(V_\infty)$ (Fig. 9) was not possible. The origin of this discrepancy is not clear so far. It could be that the transient current contains a component resulting

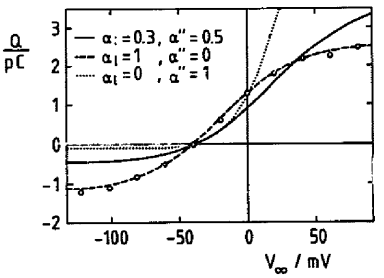
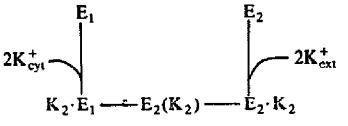


Fig. 10. Translocated charge Q after a voltage jump from −40 mV to V_∞. The experimental results of Nakao and Gadsby (Ref. 35, circles) are compared with numerical predictions from Eqn. 34. The theoretical curves have been calculated for different combinations of the dielectric coefficients α_i and α'' using fixed values of the other kinetic parameters as given in the legend of Fig. 9.

from slow translocation of Na^+ in the extracellular access channel which is not accounted for in Eqn. 34. Another possible source of the discrepancy could be our assumption that the cycle contains only two phosphorylated forms of the enzyme (E_1-P and P-E_2). Evidence has been presented that the transition from $(Na_3)E_1$-P to P-E_2 proceeds through an intermediate state E*-P which may have less than three Na^+ bound [50,51,61,62]. The postulated intermediate steps may contribute to the overall charge translocation in the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2$. Still another (less likely) explanation of the finding that different sets of dielectric coefficients are required to fit the voltage dependence of τ and of Q may consist in the assumption than the energy barrier associated with the transition between $(Na_3)E_1$-P and P-$E_2 \cdot Na_3$ is asymmetric.

In a more recent study, Bahinski, Nakao and Gadsby [63] investigated the transient behaviour of the Na,K-pump in cardiac cells in the presence of intra- and extracellular K^+, but in the complete absence of Na^+. Under this condition the pump is engaged in potassium-potassium exchange:



(35)

(for simplicity, bound ATP and phosphate have

* Under the conditions of a voltage-jump experiment, the current signal associated with Q^* is masked by the capacitive transient.

been omitted from the reaction scheme; compare Fig. 3). From the absence of an ouabain-inhibitable current-transient in the voltage-jump experiment, the authors concluded that potassium translocation is an electroneutral process.

The experimental finding of Bahinski et al. [64] is consistent with the assumption that the transitions $K_2 \cdot E_1 \rightleftarrows E_2(K_2)$ and $E_2(K_2) \rightleftarrows E_2 \cdot K_2$ are electroneutral. On the other hand, it does not exclude the possibility that binding and dissociation of K^+ at the extracellular site are electrogenic (corresponding to the presence of a 'potassium well'), since the experiments of Bahinski et al. [64] have been carried out at saturating extracellular K^+ concentration ($c''_K = 5.4$ mM). At high c''_K, the extracellular sites are predominantly in the potassium-loaded form ($E_2 \cdot K_2$), so that a voltage change is unable to shift the equilibrium between E_2 and $E_2 \cdot K_2$ appreciably, even if the equilibrium constant is voltage-dependent. However, a transient current should be observed at intermediate potassium concentrations ($c''_K \approx K''_K$), if an extracellular ion-well exists.

Discussion

In recent years fast perturbation techniques have been applied for investigating the mechanism of the Na,K-pump. In these experiments nonstationary pump-currents and ion fluxes are measured after a voltage or ATP-concentration jump. Such studies of transient pump states yield kinetic information which is complementary to the results of ordinary steady-state experiments. In this paper we have described a theoretical approach by which nonstationary pump-currents and ion fluxes can be analyzed in an unified way. The treatment is based on the assumption that operation of the pump involves a sequence of conformational transitions and ion-binding and -release steps. Accordingly, transitions between the states of the pumping cycle may be described by (pseudo)monomolecular rate constants.

Nonstationary pump currents yield information on the nature of the charge-carrying steps in the pumping cycle. The magnitude of charge displacement in the external measuring circuit associated with a given transition i is described by a dielectric coefficient α_i which depends both on the distance over which the charge moves, as well as on the position-dependent electric polarizability of the protein matrix.

The nonstationary behaviour of the Na,K-pump in the absence of potassium has been analyzed on the basis of the reaction scheme of Fig. 5 which is derived from the Albers-Post cycle. A particularly simple situation is given when neither extracellular K^+ nor intracellular ADP is present, as it was the case in the voltage-jump experiments of Nakao and Gadsby with cardiac cells. Under this condition the time-dependence of pump current after a voltage jump can be described by a simple analytical expression. The experimentally observed voltage dependence of the relaxation time indicates that both deocclusion of Na^+ as well as release of Na^+ to the extracellular medium contribute to the transient current. The proposal that the release step is electrogenic corresponds to the notion that an ion migrating from the binding site toward the aqueous medium moves through a narrow access channel within the protein matrix.

The results of the voltage-jump studies are consistent with conclusions drawn from the analysis of ATP-concentration jump experiments with kidney enzyme. The transient pump current $I_p(t)$ under short-circuit conditions was found to have essentially the same time dependence as the rate of sodium release, $J_{Na}(t)$, from $^{22}Na^+$-loaded vesicles. Such a similarity in the time course of I_p and J_{Na} is predicted when the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ is followed by a fast sodium-release step, and when one or both processes (deocclusion and release) are electrogenic. The alternative assumption, that the phosphorylation reaction $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ is an electrogenic step which is followed by a slow electrically-silent transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ would lead to a nonparallel time-course of I_p and J_{Na}.

Charge translocation in the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ occurs when deocclusion of Na^+ involves a movement of the sodium binding sites, and when the binding sites bear less than three negative charges ($z_L > -3$), so that the overall charge of the loaded sites is positive. The assumption that z_L is larger than -3 agrees with models discussed recently by other authors [35,56–59,63]. From the voltage dependence of pumping rate and from the voltage independence of Rb^+/Rb^+ ex-

change, Goldschlegger et al. [56] proposed that the ligand system bears a charge of $z_L = -2$. This conclusion is consistent with the observed monotonic shape of the current-voltage characteristic of the Na,K-pump in cardiac cells [57], squid giant axon [58] and reconstituted vesicles [64].

Acknowledgements

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Appendix A

Numerical simulation of the reaction cycle of Fig. 5

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

$$x_1 \equiv x[E_1] + x[Na \cdot E_1] + x[Na_2 \cdot E_1] + x[Na_3 \cdot E_1] \quad \text{(A-1)}$$

$$x_2 \equiv x[E_1 \cdot ATP] + x[Na \cdot E_1 \cdot ATP] + x[Na_2 \cdot E_1 \cdot ATP] + x[Na_3 \cdot E_1 \cdot ATP] \quad \text{(A-2)}$$

$$x_3 \equiv x[(Na_3)E_1\text{-}P] \quad \text{(A-3)}$$

$$x_4 \equiv x[P\text{-}E_2] + x[P\text{-}E_2 \cdot Na] + x[P\text{-}E_2 \cdot Na_2] + x[P\text{-}E_2 \cdot Na_3] \quad \text{(A-4)}$$

$$x_1 + x_2 + x_3 + x_4 = 1 \quad \text{(A-5)}$$

According to the assumption introduced above that Na^+ and ATP bind independently, the following relation holds (compare Eqn. 9):

$$\frac{x[Na_{i-1} \cdot E_1]}{x[Na_i \cdot E_1]} = \frac{x[Na_{i-1} \cdot E_1 \cdot ATP]}{x[Na_i \cdot E_1 \cdot ATP]} \equiv \frac{K'_{Ni}}{c'_N} \equiv \frac{1}{n'_i} \quad \text{(A-6)}$$

$(i = 1, 2, 3)$

Using Eqns. A-1, A-2 and A-6, the mole fractions x[A] are obtained as:

$$x[E_1] = \frac{x_1}{P'}; \quad x[Na \cdot E_1] = n'_1 \frac{x_1}{P'} \quad \text{(A-7)}$$

$$x[Na_2 \cdot E_1] = n'_1 n'_2 \frac{x_1}{P'}; \quad x[Na_3 \cdot E_1] = n'_1 n'_2 n'_3 \frac{x_1}{P'} \quad \text{(A-8)}$$

$$x[E_1 \cdot ATP] = \frac{x_2}{P'}; \quad x[Na \cdot E_1 \cdot ATP] = n'_1 \frac{x_2}{P'} \quad \text{(A-9)}$$

$$x[Na_2 E_1 \cdot ATP] = n'_1 n'_2 \frac{x_2}{P'}; \quad x[Na_3 \cdot E_1 \cdot ATP] = n'_1 n'_2 n'_3 \frac{x_2}{P'} \quad \text{(A-10)}$$

$$P' \equiv 1 + n'_1 + n'_1 n'_2 + n'_1 n'_2 n'_3 \quad \text{(A-11)}$$

In an analogous way, the mole fractions x[P-E_2], x[P-$E_2 \cdot$ Na],... may be expressed by x_4, n''_i and P''.

According to the reaction scheme of Fig. 5, the time derivatives of the variables x_j are given by:

$$\dot{x}_1 = -\left[a_f c_T + \frac{r_b c_P}{P'}\right] x_1 + a_b x_2 + \frac{r_f}{P''} x_4 \quad \text{(A-12)}$$

$$\dot{x}_2 = a_f c_T x_1 - \left[a_b + p_f \frac{n'_1 n'_2 n'_3}{P'}\right] x_2 + p_b c_D x_3 \quad \text{(A-13)}$$

$$\dot{x}_3 = p_f \frac{n'_1 n'_2 n'_3}{P'} x_2 - (p_b c_D + l_f) x_3 + l_b \frac{n''_1 n''_2 n''_3}{P''} x_4 \quad \text{(A-14)}$$

x_4 is obtained from Eqn. A-5. After numerical integration of Eqns. A-12–A-14, the net rates Φ_p, Φ_l and Φ_r may be calculated from the relations:

$$\Phi_p = p_f n'_1 n'_2 n'_3 x_2 / P' - p_b c_D x_3 \quad \text{(A-15)}$$

$$\Phi_l = l_f x_3 - l_b n''_1 n''_2 n''_3 x_4 / P'' \quad \text{(A-16)}$$

$$\Phi_r = r_f x_4 / P'' - r_b c_P x_1 / P' \quad \text{(A-17)}$$

Furthermore, from the relations:

$$\Phi_r - \Phi'_1 = \dot{x}[E_1] + \dot{x}[E_1 \cdot ATP] = (\dot{x}_1 + \dot{x}_2)/P' \quad \text{(A-18)}$$

$$\Phi'_1 - \Phi'_2 = \dot{x}[Na \cdot E_1] + \dot{x}[Na \cdot E_1 \cdot ATP] = n'_1 (\dot{x}_1 + \dot{x}_2)/P' \quad \text{(A-19)}$$

$$\Phi'_2 - \Phi'_3 = \dot{x}[Na_2 \cdot E_1] + \dot{x}[Na_2 \cdot E_1 \cdot ATP] = n'_1 n'_2 (\dot{x}_1 + \dot{x}_2)/P' \quad \text{(A-20)}$$

$$\dot{x}_1 + \dot{x}_2 = \Phi_r - \Phi_p \quad \text{(A-21)}$$

one obtains:

$$\Phi'_1 = [(P' - 1)\Phi_r + \Phi_p]/P' \quad \text{(A-22)}$$

$$\Phi'_2 = [(P' - 1 - n'_1)\Phi_r + (1 + n'_1)\Phi_p]/P' \quad \text{(A-23)}$$

$$\Phi'_3 = [n'_1 n'_2 n'_3 \Phi_r + (1 + n'_1 + n'_1 n'_2)\Phi_p]/P' \quad \text{(A-24)}$$

In an analogous way one finds:

$$\Phi_1'' = [(P''-1)\Phi_r + \Phi_l]/P'' \quad \text{(A-25)}$$

$$\Phi_2'' = [(P''-1-n_1'')\Phi_r + (1+n_1'')\Phi_l]/P'' \quad \text{(A-26)}$$

$$\Phi_3'' = [n_1''n_2''n_3''\Phi_r + (1+n_1''+n_1''n_2'')\Phi_l]/P'' \quad \text{(A-27)}$$

Introduction of Eqns. A-15–A-17 and A-22–A-27 into Eqn. 24 yields the pump current $I_p(t)$. Furthermore, according to Eqn. 25, the rate of sodium release is obtained as $J_{Na}(t) = 3l_f x_3(t)$.

Appendix B

Derivation of Eqns. 28–32

Under conditions where the transport cycle (Fig. 5) reduces to reaction scheme 27, the relation:

$$\Phi_1' = \Phi_2' = \Phi_3' = \Phi_p = \Phi_r = 0 \quad \text{(B-1)}$$

holds. According to Eqn. 24, the pump current is then given by:

$$I_p/e_0 N = \alpha_l \Phi_l + \alpha''(\Phi_1'' + \Phi_2'' + \Psi_3'') = \Phi_l(\alpha_l + \alpha'' p) \quad \text{(B-2)}$$

$$p \equiv [3 + n_1''(2 + n_2'')]/P'' \quad \text{(B-3)}$$

(compare Eqns. A25–A27). Φ_l may be obtained from the following relations:

$$\Phi_l = l_f x[(Na_3)E_1\text{-}P] - l_b x[P\text{-}E_2 \cdot Na_3] = -\dot{x}_3 \quad \text{(B-4)}$$

$$\dot{x}_3 = -l_f x_3 + l_b \rho x_4; \quad \rho \equiv n_1'' n_2'' n_3''/P'' \quad \text{(B-5)}$$

$$x_3 + x_4 = 1 \quad \text{(B-6)}$$

Eqn. B-4 may be integrated with the following boundary conditions:

$$x_3(t=0) = \frac{\rho_0}{\rho_0 + K_l^0}; \quad x_3(t \to \infty) = \frac{\rho_\infty}{\rho_\infty + K_l^\infty} \quad \text{(B-7)}$$

$$K_l^0 \equiv l_f^0/l_b^0; \quad K_l^\infty \equiv l_f^\infty/l_b^\infty \quad \text{(B-8)}$$

ρ_0, l_f^0 and l_b^0 are the values of ρ, l_f and l_b at voltage V_0, and ρ_∞, l_f^∞ and l_b^∞ the corresponding values for V_∞. Eqns. B-4–B-7 yield:

$$\Phi_l = l_b^\infty \frac{\rho_0 K_l^0 - \rho_\infty K_l^\infty}{\rho_0 + K_l^0} e^{-t/\tau} \quad \text{(B-9)}$$

$$1/\tau = l_f^\infty + \rho_\infty l_b^\infty \quad \text{(B-10)}$$

Introducing Eqn. B-9 into Eqn. B-2 yields Eqn. 28.

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