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Voltage-dependent inhibition of the sodium pump by external sodium: Species differences and possible role of the N-terminus of the α-subunit

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Abstract. Currents generated by the Na^+/K^+ ATPase were measured under voltage clamp in oocytes of *Xenopus laevis*. The dependence of pump current on external $[Na^+]$ was investigated for the endogenous *Xenopus* pump as well as for wild-type and mutated pumps of electroplax of *Torpedo californica* expressed in the oocytes. The mutants had α-subunits truncated before position Lys^{28} (αΔK28) or Thr^{29} (αΔT29) of the N-terminus. The currents generated by all variants of pump molecules in the presence of 5 mM K^+ show voltage-dependent inhibition by external $[Na^+]$. The apparent K_I values increase with membrane depolarisation, and the potential dependence can be described by the movement of effective charges in the electrical potential gradient across the membrane. Taking into account Na^+-K^+ competition for external binding to the E_2P form, apparent K_I values and effective charges for the interaction of the Na^+ ions with the E_2P form can be estimated. For the *Xenopus* pump the effective charge amounts to 1.1 of an elementary charge and the K_I value at 0 mV to 44 mM. For the wild-type *Torpedo* pump, the analysis yields values of 0.73 of an elementary charge and 133 mM, respectively. Truncation at the N-terminus removing a lysine-rich cluster of the α-subunit of the *Torpedo* pump leads to an increase of the effective charge and decrease of the K_I value. For αΔK28, values of 0.83 of an elementary charge and 117 mM are obtained, respectively. If Lys^{28} is included in the truncation (αΔT29), the effective charge increases to 1.5 of an elementary charge and the apparent K_I value is reduced to 107 mM. The K_I values for pump inhibition by external Na^+, calculated by taking into account Na^+-K^+ competition, are smaller than the $K_{1/2}$ values determined in the presence of 5 mM $[K^+]$. The difference is more pronounced for those pump variants that have higher K_m values. The variations of the parameters describing inhibition by external $[Na^+]$ are qualitatively similar to those described for the stimulation of the pumps by external $[K^+]$ in the absence of extracellular $[Na^+]$. The observations may be explained by an acess channel within the membrane dielectric that has to be passed by the external Na^+ and K^+ ions to reach or leave their binding sites. The potential-dependent access and/or the interaction with the binding sites shows species differences and is affected by cytoplasmic lysine residues in the N-terminus.

Key words: Na-Pump current-voltage relationship – Na dependence – Access channel – N-Terminus truncation – (*Xenopus* oocyte)

Introduction

During enzyme activity the Na^+/K^+ ATPase cycles through at least two distinct conformations, a Na form (E_1) and a *K* form (E_2). In the physiological mode of operation, the Na^+/K^+ pump transports 3 Na^+ ions out of the cell and 2 K^+ ions into the cell per ATP molecule that is hydrolysed. For the Na^+/K^+ pump in *Xenopus* oocytes this stoichiometry is fixed under conditions of physiological cation concentrations (Schwarz and Gu 1988) and is even maintained in solutions without $[Na^+]$ and reduced $[K^+]$ (Vasilets and Schwarz 1992). Since one net charge is transported across the membrane during the reaction cycle, transport activity has been determined by measurements of the current generated by the pump in voltage-clamp experiments in these cells (Lafaire and Schwarz 1986; Eisner et al. 1987; Rakowski and Paxson 1988). The current-voltage (I-V) relationships of endogenous pumps in oocytes of *Xenopus laevis* as well as pumps of *Torpedo* electroplax expressed in oocytes suggest that the reaction cycle is regulated by at least two voltage-dependent steps (Lafaire and Schwarz 1986; Schwarz and Gu 1988). A positive slope at negative membrane poten-

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tials, that has also been detected in most other cells (see e.g., DeWeer et al. (1988)), may be attributed to a voltage-dependent step that involves the release of the Na^+ to the external medium (Nakao and Gadsby 1986; Nako and Gadsby 1989; Rephaeli et al. 1986; Goldshleger et al. 1987; Stürmer et al. 1991). A negative slope at positive potentials may be attributed to a voltage-dependent step that involves external K^+ binding (Rakowski et al. 1991; Stürmer et al. 1991; Bielen et al. 1991) and has been described by an access channel within the dielectric of the cell membrane. The voltage-dependent stimulation by external $[K^+]$ is qualitatively different for the endogenous *Xenopus* pump and the *Torpedo* pump expressed in the oocytes (Schwarz and Vasilets 1991). In addition, the voltage dependencies can be modulated by activation of protein kinases (Vasilets and Schwarz 1992), which phosphorylate the α-subunit of the Na^+/K^+ ATPase (Chibalin et al. 1991; 1992; 1993). Also truncation of the lysine-rich cluster at the N-terminus of the α-subunit changes the voltage dependencies of pump current (Vasilets et al. 1991).

It is generally accepted that the phosphorylated E_2 form (E_2P) binds extracellular K^+; from the same conformation Na^+ is released extracellularly (Yoda and Yoda 1987). Recently it was demonstrated that Na^+ and K^+ ions combine with the same 19 kDa C-terminal transmembrane domain (Karlish et al. 1990) and most likely interact with carboxyl groups of the same amino acid residues (Shani Sekler et al. 1988). Therefore, one may stipulate that Na^+ ions, like K^+ ions, have access to their binding sites via the same access channel. Voltage-dependent release of Na^+ has been deduced from fluorescence signals with photometric dyes in Na^+/K^+-ATPase membrane fragments (Stürmer et al. 1991). In the *Xenopus* pump, evidence for an access channel for external Na^+ has recently been proposed on the basis of measurements of transient pump current generated in the otherwise electrically silent Na^+/Na^+ exchange mode (Rakowski 1992). For the present investigation, we analysed the voltage dependence of Na^+-dependent inhibition of steady-state current generated by the 3 Na^+/2 K^+ pump mode. The results obtained for the endogeneous *Xenopus* pump as well as the wild-type *Torpedo* pump and the mutants with truncated *α-subunits* are interpreted in terms of an access channel in which Na^+ and K^+ ions compete for their binding sites.

Materials and methods

The methods of oocyte preparation, voltage clamp, and data analysis were identical to those described previously (see (Vasilets and Schwarz 1992)) and are briefly summarised.

Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetised with m-aminobenzoic acid ethylesther methane sulfonate (MS222, Sandoz, Basel (Switzerland)). Parts of the ovary were removed and treated with collagenase to remove enveloping tissue. Experiments were performed with the full-grown prophase-I arrested oocytes (stage V and VI, after Dumont (1972)) at room temperature (21 °C).

Electrophysiological measurements

I-V dependencies were determined by two-microelectrode techniques. From a constant holding potential of −60 mV, rectangular voltage pulses of 500 ms duration and varying amplitude from negative to positive potentials were applied every 3 to 4 s, and steady-state currents were averaged during the last 100 ms. To reduce non-pump related K^+-sensitive currents, all solutions contained 5 or 10 mM $BaCl_2$ and 20 mM tetraethylammonium chloride (TEA-Cl); in addition 5 mM $NiCl_2$ were added to block possible electrogenic contributions by Na^+/Ca^{2+} exchange that can be detected in not completely defolliculated oocytes (Supplisson et al. 1991) and could be modulated by the changes in extracellular $[Na^+]$. Under these conditions, the current generated by the electrogenic Na^+/K^+ pump can usually be determined as the difference between total membrane current in solutions containing 5 mM KCl and that in Na^+- and K^+-free solution (see Rakowski et al. 1991). Occasionally, a Na^+-dependent and non-pump generated current could be detected. To avoid any contribution from a Na^+-dependent current not generated by the pump, on these occasions the pump current was determined as the difference between the total membrane current in a solution containing 5 mM KCl and that in a K^+-free solution plus the appropriate Na^+ concentration. Pump activity was expressed as density of pump-generated current assuming a surface area of 0.18 cm^2, which was calculated from the membrane capacitance averaged from different batches of oocytes (Vasilets et al. 1990; Vasilets and Schwarz 1992).

Experiments with expressed pumps

cRNAs for the α- and β-subunits of the Na^+/K^+ ATPase of electroplax of *Torpedo californica* were obtained as described previously (Noguchi et al. 1987; Ohta et al. 1991; Vasilets et al. 1991). cRNA for the β-subunit was coinjected with cRNA for either wild-type α-subunits or mutants truncated at the N-terminus leaving Lys^{28} ($\alpha\Delta K28$) or Thr^{29} ($\alpha\Delta T29$). Two to three days before an experiment oocytes were injected with about 10 to 20 ng of cRNA for each the α- and β-subunit. The number of pump molecules was determined by measurements of [^{3}H]ouabain binding (see Schmalzing et al. (1991)). The increase in the number of pump molecules roughly equals the increase in pump current.

Solutions

The composition of the K^+-free control solutions was (in mM): 100 tetramethylammonium chloride (TMA-Cl),

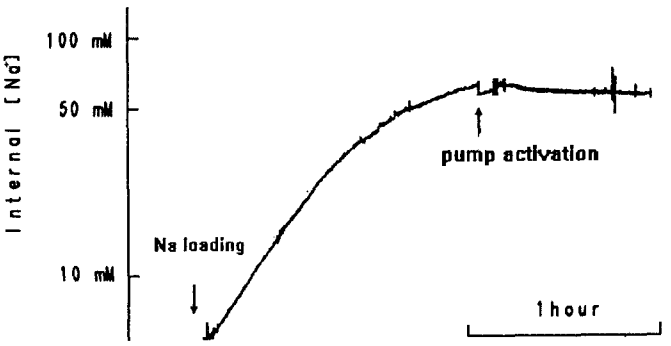
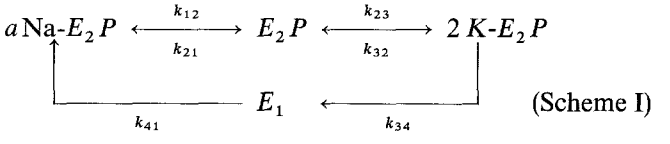


Fig. 1. Time course of intracellular [Na^+] as measured by Na^+-selective microelectrodes. At the time indicated by the downward-directed arrow, the oocyte was superfused with the Na^+-loading solution; at the time indicated by the upward-directed arrow, Na^+-free test solution with 5 mM KCl was applied to maximally stimulate pump activity

2 $CaCl_2$ and 5 $BaCl_2$ (or 0 $CaCl_2$ and 10 $BaCl_2$), 20 tetraethylammonium chloride (TEA-Cl), 5 $NiCl_2$, and 5 morpholinopropane sulfonic acid (MOPS, adjusted to pH 7.2 (or 7.8)). With the two types of solutions, identical results were obtained. In the nominally K^+-free solutions the actual concentration of K^+ was determined by flame photometry; the contaminating level was below 5 μM. The test solutions with varying concentrations of NaCl always contained 5 mM KCl to activate the Na^+/K^+ pump and the TMA-Cl was replaced by the corresponding concentration of NaCl. To increase pump activity, oocytes were preloaded with Na^+ by incubating the cells for at least one hour in a solution that had the following composition (in mM): 110 NaCl, 2.5 sodium citrate, 5 MOPS (adjusted to pH 7.6) (Rakowski et al. 1991). In the loaded oocytes, intracellular activity of Na^+ was about 80 mM after two hours of incubation as measured by Na^+-selective microelectrodes (Schmalzing et al. 1991). After changing back to Ca^{2+}- (or Ba^{2+}-) containing solution, the intracellular Na^+ content stays elevated if the Na^+ pump is stimulated (see Fig. 1). Even after several hours the value is well above the $K_{1/2}$ value of about 15 mM for pump stimulation by intracellular [Na^+] (Lafaire and Schwarz 1986).

Theory

For the description of voltage-dependent pump modulation by external Na^+ and K^+ we used a reduced version of an Albers-Post reaction diagram (Scheme I) that considers explicitly the interactions of external Na^+ and K^+ with the E_2P form:



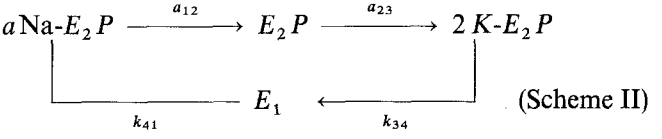
(Scheme I)

where k_{34} and k_{41} combine all rates of the forward-running cycle passing through E_1 conformations which are not involved in extracellular Na^+ and K^+ binding or release. For the analysis of our results, we assume that these rates do not contribute to voltage dependence of pump activity; intracellular cation interactions and conformational changes are believed not to contribute, at least not significantly (Goldshleger et al. 1987, 1990; Fendler et al. 1987; Borlinghaus et al. 1987; Stürmer et al. 1991). For a purely forward-running pump, the reverse rates k_{14} and k_{43} can be assumed to be zero. It is generally accepted that two external K^+ ions bind to the phosphorylated E_2P and then become occluded following the reaction cycle (Forbush 1988). The steps leading to the translocation of the three Na^+ ions from (3 Na)-E_1P to E_2P are not that well defined (see e.g. Nørby and Klodos (1988)) and in Scheme I the value of a may vary between 1 and 3. Voltage-dependent interactions of external K^+ and Na^+ with the pump molecule are described by the voltage-dependent rates

$$k_{21} = k_{21}^{\circ} \exp(-z_{Na}\, VF/RT)\,[Na^+]^n$$

$$k_{23} = k_{23}^{\circ} \exp(-z_K\, VF/RT)\,[K^+]^m$$

The z values represent relative effective charges moved during steps associated with extracellular Na^+ or K^+ binding, respectively. n and m represent Hill coefficients. Scheme I may be reduced to a simple cycle diagram following the rules elaborated by Stein (1976).



(Scheme II)

According to the rule II, a bidirectional partial reaction that is followed by a unidirectional step (here k_{23}/k_{32} followed by k_{34}) can be replaced by a unidirectional step (here a_{23}); a_{23} is obtained by multiplying the forward rate (k_{23}) by the distal unidirectional rate (k_{34}) and by dividing by the sum of the unidirectional rate (k_{34}) and the backward rate (k_{32}):

$$a_{23} = \frac{k_{23}\, k_{34}}{k_{32} + k_{34}}$$

Correspondingly one obtains:

$$a_{12} = \frac{k_{12}\, a_{23}}{k_{21} + a_{23}}$$

Transport I can be described by:

$$I = P_{E_1}\, k_{41}$$

The probability P_{E_1} of the molecule being in state E_1 can be calculated by using the diagram method (Kirchhoff 1847; King and Altman 1956; Hill 1966):

$$P_{E_1} = \frac{a_{12}\, a_{23}\, k_{34}}{a_{23}\, k_{34}\, k_{41} + a_{12}\, k_{34}\, k_{41} + a_{12}\, a_{23}\, k_{34} + a_{12}\, a_{23}\, k_{41}}$$

I is then described by:

$$I = \frac{k_{34}\, k_{41}}{k_{34}\, k_{41}/a_{12} + k_{34}\, k_{41}/a_{23} + k_{34} + k_{41}},$$

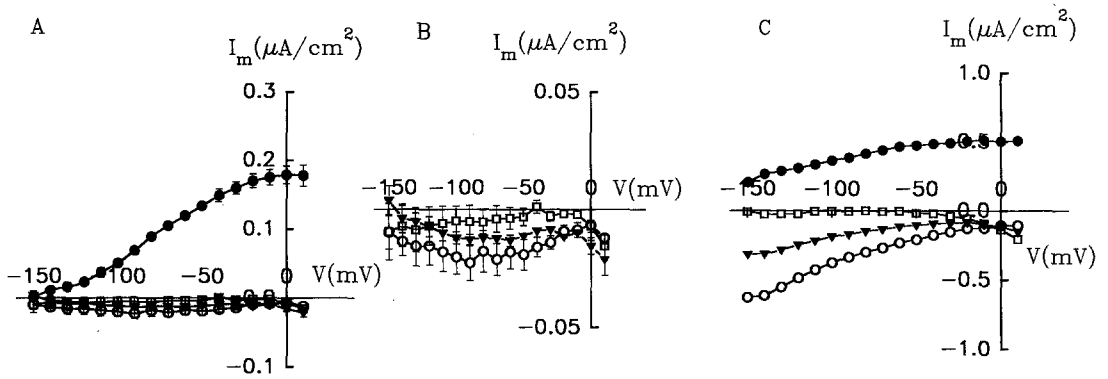


Fig. 2 A–C. **A** Difference currents obtained by subtracting the current measured in Na^+- and K^+-free solution from membrane current measured in solution containing (in mM) 100 NaCl, 5 KCl (filled circles), 100 NaCl, 0 KCl (*open circles*), 50 NaCl, 0 KCl (*filled triangles*), 5 NaCl, 0 KCl (*open squares*). **B** Difference curves in absence of KCl as in **A** but at an enlarged scale of current density. The data represent average values from 4 non-injected 1991 oocytes (±SEM). **C** Corresponding data as in **A**, but from a 1992 oocyte injected with cRNAs for wild-type *Torpedo* pump

which leads to:

$$I = \frac{k_{12}\,k_{23}\,k_{34}\,k_{41}}{(k_{12}+k_{21})(k_{32}+k_{34})\,k_{41}+k_{23}(k_{41}\,k_{34}+k_{41}\,k_{12}+k_{34}\,k_{12})} \quad \text{(A1)}$$

In the absence of extracellular $[Na^+]$ it can be written in the form:

$$I_{Na=0} = I_{max}\frac{[K^+]^m}{K_m^m+[K^+]^m} \quad \text{(A2)}$$

with

$$I_{max} = \frac{k_{12}\,k_{34}\,k_{41}}{k_{41}k_{34}+k_{41}\,k_{12}+k_{34}\,k_{12}}$$

and

$$K_m^m = \frac{k_{12}\,k_{41}\,(k_{32}+k_{34})}{k_{23}^{\circ}\,(k_{41}\,k_{34}+k_{41}\,k_{12}+k_{34}\,k_{12})}\exp(z_K\,VF/RT)$$

(Note: in case of the *Torpedo* pumps and their mutants, K_m was described by the sum of two exponentials (Vasilets et al. 1991; Vasilets and Schwarz 1992)).

In the presence of $[Na^+]$, transport is then represented by:

$$I = I_{Na=0}\frac{K_{1/2}^n}{K_{1/2}^n+[Na^+]^n} \quad \text{(A3)}$$

with

$$K_{1/2}^n = (1+([K^+]^m/K_m^m))\,(k_{12}/k_{21}^{\circ})\exp(z_{Na}\,VF/RT). \quad \text{(A4)}$$

Results

Under physiological conditions of high extracellular $[Na^+]$ as well as in Na^+-free medium, current generated by Na^+/K^+ pumps in *Xenopus* oocytes can usually be determined as current activated by extracellular application of K^+ (Rakowski et al. 1991). Maximum stimulation is achieved by 5 mM K^+ for the endogenous *Xenopus* pump (Rakowski et al. 1991) and for the pump of *Torpedo* electroplax (Vasilets and Schwarz 1992) and their truncated mutants (Vasilets et al. 1991) expressed in the oocytes. Also in the present investigation using a range of different concentrations of extracellular $[Na^+]$, pump current was determined as K^+-sensitive current. In the absence of extracellular $[K^+]$, variations of $[Na^+]$ were without significant effect on membrane currents in those experiments we performed during the summer and autumn of 1991 (1991 oocytes). This is illustrated for averaged data in Fig. 2 A by raising the extracellular $[Na^+]$ from 0 to 5, 50, or 100 mM. For comparison, the current generated by the endogenous pump in the presence of 100 mM Na^+ and 5 mM K^+ is shown. Figure 2 B shows the small Na^+-sensitive current at higher resolution; correction for the small, non-pump and Na^+-sensitive current did not influence the results described in this investigation. In these experiments, the K^+-free control solutions contained either no or 100 mM NaCl which was without effect on the results. On the other hand, in experiments performed during winter of 1991/92 and spring of 1992 (1992 oocytes), a pronounced Na^+-dependent current could often be detected. In the example shown in Fig. 2 C this inward-rectifying current reaches magnitudes which are more than one order of magnitude larger that those observed in the 1991 oocytes. In 100 mM Na^+, the Na^+-dependent current can even exceed the pump current of oocytes with additionally expressed *Torpedo* pumps. Preliminary experiments gave no indications for sensitivity of the currents to 1 mM tetrodotoxin (J. Rettinger, umpubl.), but a small component of this current may be inhibited by amiloride (L. A. Vasilets, H. S. Omay, W. Schwarz, unpubl.). Therefore, in the experiments with 1992 oocytes, the K^+-free control solutions contained the appropriate Na^+ concentration, if necessary.

Experiments with the endogenous Xenopus pump

The protocol of a typical experiment where Na^+-free control solution could be used is illustrated in Fig. 3 A by a chart record of holding current. The example shows the results for the *Xenopus* pump obtained from an oocyte not injected with cRNAs. During the experiment the holding potential was set to −60 mV, and the oocyte was superfused for several minutes with different solutions. Before and after superfusion with the test solutions con-

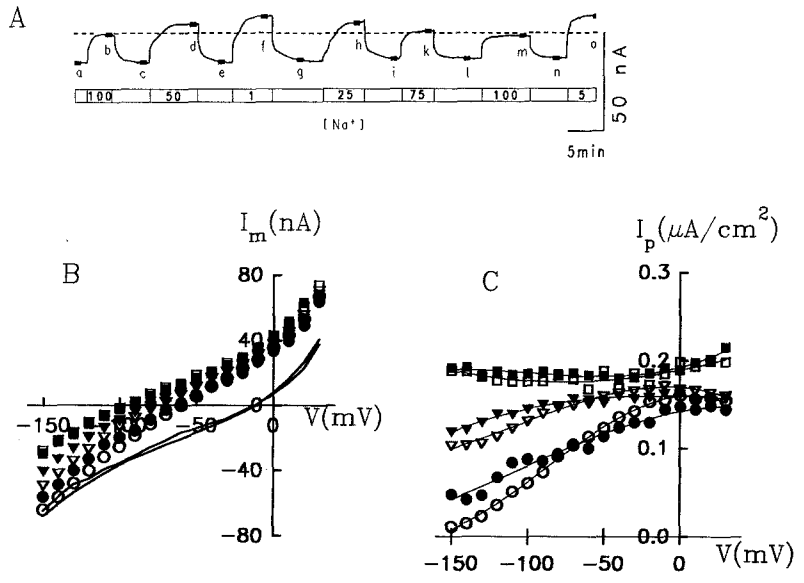


Fig. 3A–C. B Chart record of holding current of a typical voltage-clamp experiment where Na^+- and K^+-free control solution was used. The holding potential was set to −60 mV. The letters a to o indicate where I-V measurements were performed. The experiment was performed on an uninjected oocyte (130991/4) containing only enodgenous *Xenopus* pumps. During the upward deflections of holding current (representing pump-generated current) the chamber was perfused with solution containing in the presence of 5 mM KCl different concentrations of NaCl as indicated by the numbers in mM. **B** I-V curves of total membrane current in presence of 5 mM KCl and different concentrations of NaCl measured at *b* (*open circles*, 100 mM), *k* (*filled* circles, 75 mM), *d* (*open triangles*, 50 mM) *h* (*filled triangles*, 25 mM), *o* (*open squares*, 5 mM), *f* (*filled squares*, 1 mM). The two solid lines represent control I-V curves in absences of external $[K^+]$ measured at *c* and *n*. **C** Pump I-V curves determined as difference curves as described in text for different NaCl concentrations (*symbols as in* **B**, lines are polynomial fits to the data points)

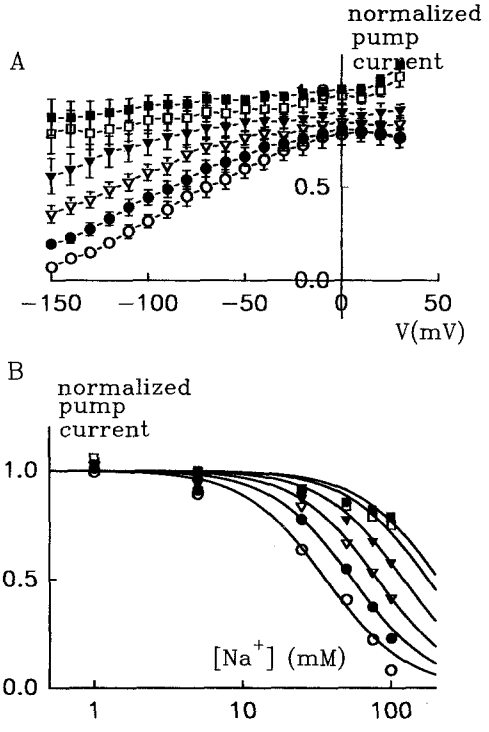


Fig. 4 A, B. A Potential dependence of *Xenopus* pump current at 5 mM KCl and different concentrations of NaCl in the external medium; symbols as in Fig. 3C. Data represent average values ± SEM from 6 experiments. The data were normalised to the value at 0 mV and 1 mM NaCl. **B** Dependence of pump current on Na^+ concentration for 6 different membrane potentials (*same data as in* **A**); (*open circles*, −150 mV) (*filled circles*, −120 mV), (*open triangles*, −90 mV), (*filled triangles*, −60 mV), (*open squares*, −30 mV), (*filled squares*, 0 mV). *Solid lines* represent fits of (1) to the data with $n=1.6$. The fitted $K_{1/2}$ values are shown in Fig. 8 (*open circles*)

taining 5 mM KCl and different concentrations of NaCl, K^+-free control solution was applied. The corresponding control I-V curves before and after application of the test solutions were used for corrections of linear drift with time. At the beginning and close to the end of an experiment, pump I-V curves were determined under identical conditions (here 100 mM Na^+) and used for correction of run-down if necessary by assuming exponential decline of pump current with time. Figure 3B shows the test I-V curves (symbols) and two control I-V curves (lines). The differences I-V curves representing pump currents are plotted in Fig. 3C. Figure 4A shows normalised I-V curves for the *Xenopus* pump averaged from 6 experiments of this type. In 100 mM NaCl, the typical voltage dependence of pump current at high external $[K^+]$ is seen with a positive slope in the I-V relationship at negative membrane potentials and saturation at potentials beyond 0 mV. Reduction of external $[Na^+]$ reduces the voltage dependence, and at 1 mM there is little voltage dependence left. The dependence on $[Na^+]$ can be described by voltage-dependent inhibition of the pump cycle. For further analysis, the concentration dependence of pump current was plotted for different membrane potentials (Fig. 4B), and $K_{1/2}$ values for pump inhibition were determined by fitting the following equation to the data (compare Theory section (A3)):

$$I_p = I_{max} \cdot K_{1/2}^n / (K_{1/2}^n + [Na^+]^n) \quad (1)$$

Separate fits for the different membrane potentials yielded an average value for the Hill coefficient n of about 1.6. Though there was slight tendency of decreasing n with less negative potentials, the average value was used for all fits. A slight decrease of n with less negative potentials has also been observed in similar experiments on ventricular myocytes (Nakao and Gadsby 1989). In Fig. 8, the voltage dependence of the $K_{1/2}$ values for the *Xenopus* pump is shown by open circles; the presented data are averaged values obtained from the data shown in Fig. 4 and from those obtained in experiments using Na^+-containing control solutions. Separate analyses yielded nearly identical results.

Experiments with wild-type Torpedo pumps

The protocol of a typical experiment using Na^+-containing control solutions is illustrated in Fig. 5A again by a

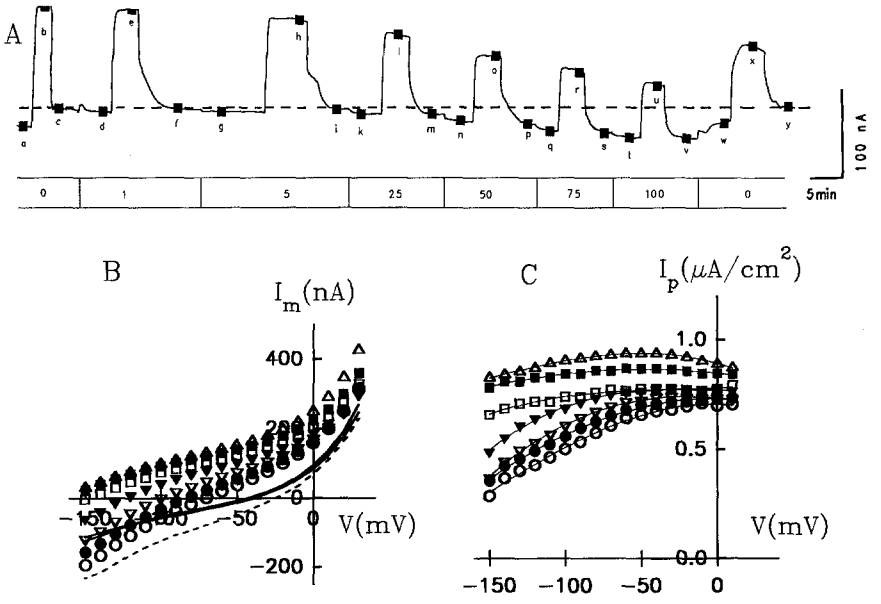


Fig. 5A–C. Chart record of holding current of a typical voltage-clamp experiment where K^+-free but Na^+-containing control solutions were used. The holding potential was set to −60 mV. The letters *a* to *y* indicate where I-V measurements were performed. The experiment was performed on an oocyte injected with cRNAs for the wild-type *Torpedo* pump (150592/1). During the upward deflections of holding current (representing pump-generated current) the chamber was perfused with solution containing in the presence of 5 mM KCl different concentrations of NaCl as indicated by the numbers in mM. **B** I-V curves of total membrane current in presence of 5 mM KCl and different concentrations of NaCl measured at *b* (*open circles*, 100 mM), *e* (*filled circles*, 75 mM), *h* (*open triangles down*, 50 mM), *I* (*filled triangles down*, 25 mM), *o* (*open squares*, 5 mM), *r* (*filled squares*, 1 mM) *u* (*open triangles up*, 0 mM). The two *solid lines* represent control I-V curves in absences of [Na^+] measured at *c* and *y*, and in the presence of 100 mM Na^+ at *v*. **C** Pump I-V curves determined as difference curves as described in text for different NaCl concentrations (*symbols as in* **B**). Pump I-V curves determined at *b* and *x* were used for correction of run down

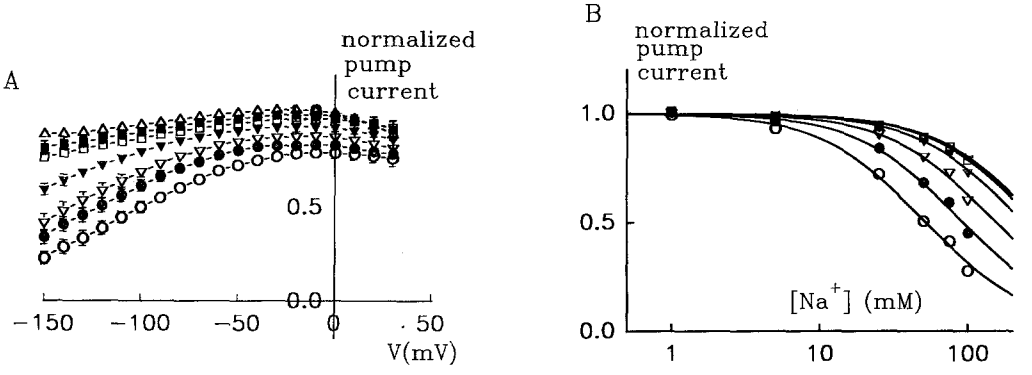


Fig. 6 A, B. **A** Potential dependence of pump current at 5 mM KCl and different concentrations of NaCl in the external medium (as indicated by the numbers in mM). Data were obtained from oocytes that were injected with cRNA for the wild-type α-subunit and the β-subunit and represent average values ± SEM from 7 experiments. The data were normalised to the value at 0 mV and 0 mM NaCl. **B** Dependence of pump current on Na^+ concentration for 6 different membrane potentials (*same data as in* **A**); *symbols as in Fig. 4 B*. *Solid lines* represent fits of (2) to the data with $n=1.2$ and $r=0.15$. The fitted $K^T_{1/2}$ values are shown in Fig. 8 (*filled circles*)

chart record of holding current. This example shows results obtained from a 1992 oocyte injected with cRNAs for the wild-type *Torpedo* pump. Evaluation of the data was similar to that described above. Control I-V curves were recorded before and after application of each test solution with 5 mM K^+. The control solutions contained the same concentration of Na^+ as the test solution, and the corresponding control I-V curves were used for correction of linear drift with time. Pump I-V curves obtained under identical conditions at the beginning and end of an experiment were used for run-down correction, if necessary. Figure 5B shows the test I-V curves (symbols) and two control I-V curves without [Na^+] (solid lines) that were recorded at the beginning and end of the experiment. In addition, a control I-V curve recorded at 100 mM [Na^+] is shown (broken line) demonstrating the existence of a Na^+-dependent current in absence of external [K^+]. The difference I-V curves representing pump currents are plotted in Fig. 5C. Figure 6A shows averaged pump currents from 1992 oocytes with the wild type *Torpedo* pump obtained in experiments of this type. For the analysis of the dependence on Na^+ concentration of the *Torpedo* pump current (I_p^T), the contribution of the endogenous *Xenopus* pump current (I_p^X) to total pump

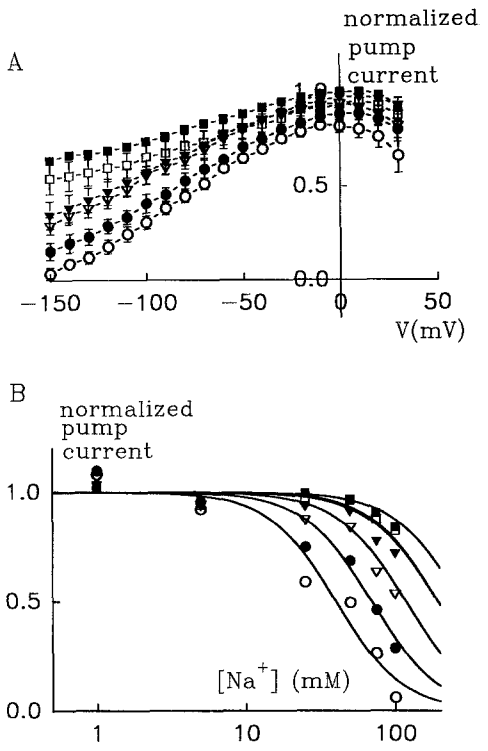


Fig. 7 A, B. **A** Potential dependence of pump current at 5 mM KCl and different concentrations of NaCl in the external medium. Data were obtained from 1991 oocytes that were injected with cRNA for the $\alpha\Delta T29$-subunit and the β-subunit and represent average values ±SEM from 4 experiments. The data were normalised to the value at 0 mV and 1 mM NaCl. **B** Dependence of pump current on Na^+ concentration for 6 different membrane potentials (*same data as in* **A**); *symbols as* in Fig. 4B *Solid lines* represent fits of Eq. (2) to the data with $n=2$ and $r=0.37$. The fitted $K^T_{1/2}$ values are shown in Fig. 8 (*filled triangles*)

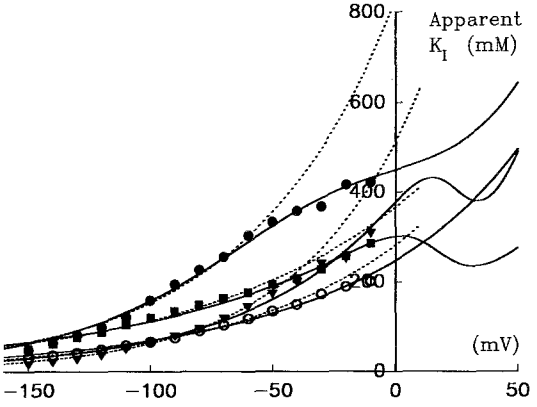


Fig. 8. Voltage dependence of the apparent K_I values for pump inhibition by external [Na^+] in solution with 5 mM KCl. The values were obtained from the data shown in Figs. 4B, 6B, 7B and from two experiments with the $\alpha\Delta K28$ mutant fitting (1) or (2) to the data, respectively. Open circles represent the values for the endogenous *Xenopus* pump, filled symbols for the various *Torpedo* pumps (*circles with wild-type, squares with* $\alpha\Delta K28$-, *triangles with* $\alpha\Delta T29$-subunit). The voltage dependence up to −50 mV was fitted by a single exponential ((3), *broken lines*). Equation 4 was fitted for the entire potential range (*solid lines* represent fits of this Eq. to the data). The fitted parameters are listed in Table 1

current (I_p) had to be subtracted. Corresponding to our previous procedure for the separation of the two components (Vasilets et al. 1991; Vasilets and Schwarz 1992), the concentration dependence was fitted by:

$$I_p = I_p^T + I_p^X \qquad (2)$$
$$= I_{max}[(1-r).K^{T}_{1/2}{}^{n}/(K^{T}_{1/2}{}^{n}+[Na^+]^n) + r.K^{X}_{1/2}{}^{1.6}/(K^{X}_{1/2}{}^{1.6}+[Na^+]^{1.6})]$$

where r represents the relative contribution of the *Xenopus* pump to total pump current. This parameter was determined for the different sets of experiments from the difference in ouabain binding (see Vasilets and Schwarz (1992)) or in pump currents obtained in Na^+-free solution at 0 mV as measured in non-injected and cRNA-injected oocytes. For the Hill coefficient n of the [Na^+] dependence for the *Torpedo* pump a value of about 1.2 turned out to give reasonable fits for all potentials. As for the *Xenopus* pump, n showed a tendency of decreasing values with less negative potentials. The values for $K^X_{1/2}$ were taken from data obtained from non-injected oocytes that were always investigated in parallel. The solid lines in Fig. 6B are fitted curves of (2) with $r=0.15$. The voltage dependence of the $K^T_{1/2}$ values is plotted by the filled circles in Fig. 8. These data are averages of $K^T_{1/2}$ values obtained from experiments using the two types of protocols, with either Na^+-free or Na^+-containing control solutions.

Table 1. Fitted parameters for the data shown in Fig. 8. Relative effective charges z^* and z_{Na}, and the $K_{1/2}$ and K_I values at 0 mV were determined according to (3) and (4), respectively. The values z_K and K_m (0 mV) for pump stimulation by external K^+ were obtained from data published previously: for the *Xenopus* pump, data for K^m_m (Rakowski et al. 1991; Vasilets and Schwarz 1992) were fitted by a single exponential for the potential range $V<0$ mV; for the *Torpedo* pump parameters for the component with the lower effective charge of the two-exponential fit (Vasilets et al. 1991; Vasilets and Schwarz 1992) are listed

	fitted by (3)		fitted by (4)			
	z^*	$K_{1/2}$(0 mV) (mM)	z_{Na}	K_I (0 mV) (mM)	z_K	K_m (0 mV) (mM)
Xenopus	0.36	286	1.10	44	0.61	0.63
Torpedo αWT	0.42	826	0.73	133	0.16	1.51
Torpedo $\alpha\Delta K28$	0.29	365	0.84	117	0.33	0.89
Torpedo $\alpha\Delta T29$	0.52	512	1.50	107	0.66	0.44

Experiments with mutated Torpedo pumps

Figures 7A shows averaged results of the same type of experiments as described above but for the mutated pumps with $\alpha\Delta T29$, where 28 amino acid residues were truncated at the N-terminus, including a lysine-rich cluster. The dependencies of total pump current on external [Na^+] (Fig. 7B) was again fitted by (2) for different membrane potentials. An averaged Hill coefficient of $n=2$ was

used for all potentials. The calculated $K^T_{1/2}$ values are plotted in Fig. 8 as the filled triangles. For investigation of apparent K_m values for pump stimulation by external $[K^+]$ we formerly analysed, in addition, the truncated mutant $\alpha\Delta K28$ (Vasilets et al. 1991). The modification in voltage dependence of $K_{1/2}$ was qualitatively similar to that with $\alpha\Delta T29$, though quantitatively less pronounced. Therefore, we did detailed analysis only with the $\alpha\Delta T29$ mutant, but Fig. 8 also shows $K_{1/2}$ values for the $\alpha\Delta K28$ mutant (filled squares) that were obtained from two experiments where a Hill coefficient of $n=1.8$ was used.

Discussion

The reaction cycle of the Na^+/K^+ pump can be influenced by changes in membrane potential; at least two voltage-dependent steps determine the turnover rate in *Xenopus* oocytes under physiological conditions (Lafaire and Schwarz 1986), one that depends on extracellular $[Na^+]$ (see also Nakao and Gadsby (1989)), the second one on extracellular $[K^+]$ (Rakowski et al. 1991). Dependence of the voltage dependence on extracellular $[Na^+]$ at negative membrane potentials has been described for several other preparations, including squid giant axon (Rakowski et al. 1989), heart muscle (Gadsby and Nakao 1989; Nakao and Gadsby 1989; Glitsch et al. 1989), reconstituted systems (Goldshleger et al. 1987, 1990), as well as membrane fragments attached to lipid bilayers (Apell et al. 1987; Borlinghaus et al. 1987). Modulation of the voltage dependence at positive membrane potentials by changes in extracellular $[K^+]$ was first detected in the oocytes (Rakowski et al. 1991; Schwarz and Vasilets 1991), but has recently also been demonstrated in cardiac Purkinje cells (Bielen et al. 1991). For the interpretation of the voltage and concentration dependencies the existence of an access channel within the electrical field of the membrane has been suggested, a channel that has to be passed by the ions to reach their binding sites (Nakao and Gadsby 1986; Goldshleger et al. 1987; Läuger and Apell 1988; Rakowski et al. 1991; Bielen et al. 1991; Stürmer et al. 1991).

Voltage dependence of $K_{1/2}$

Since the oocytes of *Xenopus* allow functional expression of foreign pump molecules by injection of cRNAs for the α- and β-subunit (Noguchi et al. 1987; Schwarz and Gu 1988), differences in transport characteristics can be investigated under identical environmental conditions. Comparison of the different types of pump molecules analysed in the present investigation shows that increasing extracellular $[Na^+]$ leads, in all cases, to more pronounced voltage dependence of pump current with a positive slope (see Figs. 4A, 6A, and 7A), which can be described by voltage-dependent inhibition of pump current by external Na^+ ions. Already the concentration dependencies (see Figs. 4B, 6B, 7B) suggest quantitative differences in pump inhibition. Separation of the currents generated by *Xenopus* and *Torpedo* pumps and more detailed analysis of the corresponding apparent $K_{1/2}$ values clearly exhibits differences and yields exponential dependence over the negative potential range at least up to -50 mV (compare broken lines and symbols in Fig. 8).

In our previous work on potential-dependent stimulation of the pump by external $[K^+]$ we interpreted the exponential dependence of apparent K_m values by assuming an access channel within the electrical field that has to be passed by the K^+ ions to reach their binding sites within the pump protein (Rakowski et al. 1991; Schwarz and Vasilets 1991; Vasilets and Schwarz 1992). Since Na^+ ions are assumed to interact with the same amino acid residues as the K^+ ions (Yoda and Yoda 1987; Shani Sekler et al. 1988; Karlish et al. 1990), the existence of the access channel seems to be likely also for Na^+ ions. As for voltage-dependent stimulation of the *Xenopus* pump by $[K^+]$, we tried to fit the voltage dependence of the $K_{1/2}$ value for pump inhibition by $[Na^+]$ by an exponential:

$$K_{1/2}(V) = K_{1/2}(0\text{ mV}) \exp(z^* VF/RT) \quad (3)$$

z^* represents a relative fraction of an elementary charge that can be assumed to move in the electrical field during steps involved in binding of external cations to the pump molecule. Reasonable fits are only obtained for the more negative potentials, and the broken lines in Fig. 8 represent the fits to the data for potentials more negative than -50 mV. The fitted parameters are listed in the table. Similar values were obtained for the Na^+ pump in ventricular myocytes; Nakao and Gadsby (1989) reported $K_{1/2}$ values of 91, 170, and 357 mM in presence of 5.4 mM external $[K^+]$ for the potentials -120, -80 and -40 mV, respectively. These values are comparable with our values for the wild-type *Torpedo* pump. For the voltage dependence of $K_{1/2}$ values the authors report an e-fold change in roughly 60 mV for the potential range more negative than -40 mV, which is also close to our observations for the *Torpedo* pump with an effective charge of about 0.4 of an elementary charge calculated from (3).

Voltage-dependent Na^+_0-K^+_0 interaction

It is obvious, on the other hand, that the data for pump inhibition by Na^+ start to deviate from exponential dependence at potentials beyond -50 mV. This becomes particularly evident for the wild-type *Torpedo* pump, and may reflect Na^+/K^+ interaction for the K^+ transport site or voltage dependence of an additional step in the reaction cycle. To describe the potential dependence of the apparent K_I value more accurately, we tried to fit our data by a simple reaction cycle with voltage-dependent interaction of external Na^+ and K^+ with the E_2P form (see Scheme I). In this case the voltage and $[K^+]$ dependence of the apparent $K_{1/2}$ value may be described by (see Theory section, (A4)):

$$K_{1/2}(V, [K^+]) = [(1 + ([K^+]^m/K_m^m))\, K_I(0\text{ mV}) \exp(z_{Na} VF/RT)]^{1/n} \quad (4)$$

where m represents the appropriate Hill coefficient for pump stimulation by external $[K^+]$ which was deter-

mined previously (Rakowski et al. 1991; Vasilets and Schwarz 1992; Vasilets et al. 1991). n is the Hill coefficient for pump inhibition by external $[Na^+]$ as determined in the present investigation. In addition to the explicit voltage dependence due to the movement of the effective charge $z_{Na}e$ (z_{Na} = fraction of an elementary charge e), additional voltage dependence is also brought in by the voltage dependence of pump stimulation by external $[K^+]$ described by the apparent K_m value determined in the absence of $[Na^+]$. Using the values for K_m we obtained previously (Vasilets et al. 1991; Vasilets and Schwarz 1992), the potential dependence of the apparent $K_{1/2}$ value can be described for the entire potential range (see solid lines in Fig. 8). The fitted parameters are listed in the table. Owing to the voltage-dependent stimulation by K^+, the presence of 5 mM K^+ leads to a dramatic increase of the $K_{1/2}$ values. For the wild-type *Torpedo* pump, where the apparent K_m value for pump stimulation by external K^+ exceeds 1 mM, the deviation between K_I and $K_{1/2}$ is most prominent. This becomes particularly evident by comparing the calculated K_I (0 mV) values with the $K_{1/2}$ values for 0 mV calculated by fitting a simple exponential to the data (see Table). These latter values are larger by a factor of about 4.5. At positive potentials a complex potential dependence with maximum and minimum is predicted for the mutants. These characteristics could not be demonstrated since the accuracy of the data analysis at positive potentials is restricted.

Species differences

The potential dependence of K_m of the *Torpedo* pump by $[K^+]$ had to be described by the sum of two exponential components (Vasilets and Schwarz 1992), and was interpreted by sequential binding of the two K^+ ions. Therefore, one cannot exclude the possibility that a second exponential component is masked and Na^+ binding also occurs sequentially. Sequential release of Na^+ following

$$(3\,Na)\text{-}E_1P \rightarrow (2\,Na)\text{-}E_2P \rightarrow \rightarrow E_2P$$

has been suggested recently (Jørgensen and Andersen 1988; Yoda and Yoda 1987; Nørby and Klodos 1988). But the description of pump inhibition by Na^+ by a single exponential may also be an indication that release of only one Na^+ ion is voltage-dependent. In any case, the apparent K_I values for the wild-type *Torpedo* pump are larger than those for the *Xenopus* pump in the accessible potential range, though the voltage dependence of the K_i^n value (represented by z_{Na}) is less pronounced. Qualitatively similar differences were also obtained for the apparent K_m values for pump stimulation by $[K^+]$ (Vasilets and Schwarz 1992). The K_m values for the *Torpedo* pump are larger than those for the *Xenopus* pump, and if the voltage dependence of K_m^m is fitted by a single exponential for the negative potential range the corresponding z_K are smaller (see Table). The results are compatible with the idea of a common access channel for Na^+ and K^+ that on the other hand differs for *Xenopus* and *Torpedo* pumps.

Role of N-terminus in external cation binding

Parallel variations in K_I and K_m are also observed if the wild-type and mutated *Torpedo* pumps are compared. As has been demonstrated previously for pump stimulation by external K^+ (Vasilets et al. 1991), truncation of the N-terminus leads to a reduction of the K_m value and voltage-dependence becomes more pronounced (expressed by an increase in the effective charge z_K, see Table). The same variations were obtained for pump inhibition by external Na^+; K_I is reduced and z_{Na} increased after truncation, which removes a lysine-rich cluster. The N-terminus and, in particular, the lysine-rich region seem to play an important role during the conformational changes and the interactions of the cations with their binding sites (Jørgensen and Karlish 1980; Jørgensen and Collins 1986; Lingrel et al. 1990); interestingly, also external Na^+ and K^+ (Vasilets et al. 1991) binding are influenced by the cytoplasmic N-terminus. As for the K_m value, removal of the Lys^{28} has a particularly pronounced effect on K_I. All these similarities support the view that external Na^+ and K^+ ions reach their binding sites via the same access channel within the electrical field.

Though the variations of the K_m and K_I values and their respective effective charges z_K and z_{Na} are qualitatively similar, there are clear quantitative differences (see Table). This may be an indication that in addition to voltage-dependent Na^+ and K^+ binding, represented by the voltage-dependent rate constants k_{21} and k_{23} (Scheme I), further steps combined in the rates k_{34} and k_{41} contribute to voltage-dependent pump regulation. Voltage-dependent access for intracellular Na^+ has been suggested recently (Goldshleger et al. 1987; Stürmer et al. 1991; David et al. 1992) and would result in a voltage-dependent k_{34}. The existence of an additional voltage-dependent step is further supported by the finding that the second effective charge describing pump stimulation by external K^+ reaches a value of about 4 in the truncated mutant $\alpha\Delta T29$ (Vasilets et al. 1991), a value that is not compatible with the simple movement of two K^+ ions in the electrical field.

Conclusion

In conclusion, similar variations of K_I and K_m for different pump species support the view that interaction of external Na^+ and K^+ with the pump molecule occurs via the same access channel in the electrical field. In particular, the more pronounced voltage dependence of both K_m and K_I values after truncation of the lysine-rich cluster at the N-terminus of the α-subunit suggest that the N-terminus plays an important role in the external cation-protein interaction. Since the N-terminus shows the highest degree of sequence diversity among α-subunits of different species, the N-terminus may particularly account for species differences in the cation dependencies of Na^+/K^+ pumps.

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