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Voltage-dependent stimulation of Na^+/K^+-pump current by external cations: selectivity of different K^+ congeners

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Currents generated by the endogenous Na^+/K^+ pump in the oocytes of *Xenopus laevis* were determined under voltage-clamp as currents activated by different K^+ congeners. The voltage dependence of the pump current reflects voltage-dependent steps in the reaction cycle. The decrease of K^+-activated pump current at positive potentials has been attributed to voltage-dependent stimulation by the external K^+ (Rakowski, Vasilets, LaTona and Schwarz (1991) J. Membr. Biol. 121, 177–187). In Na^+-free solution, activation of the pump by external cations seems to be the dominating voltage-dependent and rate-determining step in the reaction cycle. Under these conditions, the voltage dependence of apparent K_m values for pump activation can be analyzed. The dependence suggests voltage-dependent binding of extracellular cations assuming that an effective charge of about 0.4 of an elementary charge is moved in the electrical field during a step associated with the cation binding. The apparent K_m values at 0 mV differ for various cations that stimulate pump activity. The values are in mM: 0.10 for Tl^+, 0.83 for K^+, 0.71 for Rb^+, 9.3 for NH_4^+, and 12.9 for Cs^+. The corresponding apparent affinities follow the same sequence as the cation permeability of the K^+-selective delayed rectifier channel of nerve cells. The results are compatible with the interpretation that the cations have to pass an ion-selective access channel to reach their binding sites in the pump molecule.

Introduction

Under physiological conditions, the Na^+/K^+-ATPase transports 3 Na^+ ions out of the cell and 2 K^+ ions into the cell for each ATP molecule hydrolysed. The ensuing net current is a measure of pump activity provided the stoichiometry is fixed. Combined flux and current measurements have shown this at least in squid giant axon [1] and *Xenopus* oocytes [2]. This is true even under conditions where the external solution is free of $[Na^+]$ and contains $[K^+]$ at unphysiologically low concentrations [3,4]. The voltage dependence of the pump current (I_p-V relationship) of the endogenous pump [5] and of the pump of *Torpedo* electroplax expressed in the oocytes [2] exhibit in media of physiological composition a positive slope at negative potentials and a negative slope at positive potentials. Stimulation of pump current by membrane depolarization has been observed also in other types of cells as well as in reconstituted systems (see e.g. Ref. 6). It has been suggested that a step involved in external liberation of Na^+ is associated with movements of charges [7–9]. The negative slope seen in the *Xenopus* oocytes can be attributed to a voltage-dependent apparent K_m value for pump activation by external $[K^+]$ [3,10]. Pump current in cardiac Purkinje cells can also be reduced by positive potentials. The magnitude of the effect depends on the nature and concentration of the activating cation species [11]. Different degrees of effectiveness of different K^+ congeners with the sequence $Tl^+ > K^+ = Rb^+ > NH_4^+ > Cs^+$ have also been demonstrated for protection of the pump against inhibition by removal of K^+ in cardiac cells [12] as well as for activation of the enzyme activity [13,14]. In the present study, we investigated the voltage dependence of the apparent K_m values for stimulation of the endogenous pump current by different external K^+ congeners in *Xenopus* oocytes. To avoid interference with the voltage- and Na^+-dependent step, all experiments were performed in Na^+-free media [10].

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Materials and Methods

The methods of oocyte preparation and voltage clamp were identical to those described previously (see Vasilets et al. (1990), Ref. 15) and are briefly summarized.

Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetized with *m*-aminobenzoic acid ethyl esther 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, [16]) at room temperature (21°C).

Electrophysiological measurements

Current-voltage (*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 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 mM $BaCl_2$ and 20 mM tetraethylammonium chloride (TEA-Cl). Under these conditions, the current generated by the electrogenic Na^+/K^+ pump can be determined as the difference between total membrane current in solutions containing a given concentration of K^+ and that in K^+-free solution (see Ref. 10). 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 [4].

Solutions

The composition of the test solutions was (in mM): 90 tetramethylammonium chloride (TMA-Cl), 2 $CaCl_2$, 5 $BaCl_2$, 20 tetraethylammonium chloride (TEA-Cl), 5 morpholinopropane sulfonic acid (Mops, adjusted to pH 7.4). The solutions with varying concentrations of either TlCl, RbCl, CsCl, or NH_4Cl had the same osmolarity which was achieved by substituting TMA-Cl. In the nominally K^+-free solutions the actual concentration of K^+ was determined by flame photometry; the contaminating level was below 5 μM. To increase pump activity, oocytes were usually preloaded with Na^+ by incubating the cells for at least one hour in solution that had the following composition (in mM): 110 NaCl, 2.5 sodium citrate, 5 Mops (adjusted to pH 7.6) [10]. In the loaded oocytes, intracellular activity of Na^+ was about 80 mM after 2 h of incubation as measured by Na^+-selective microelectrodes (Schmalzing et al. (1991), Ref. 17).

Results

In solutions without $[Na^+]$, pump stimulation by external $[K^+]$ seems to be a dominating step in the reaction cycle that is voltage-dependent and rate-determining. To investigate the voltage dependence of the apparent K_m value for pump stimulation by K^+ congeners, experiments were performed in Na^+-free solutions containing different activating cation species at a range of different concentrations. In the presence of $BaCl_2$ and TEA-Cl, effectively all current activated by application of the cations can be attributed to pump current; as has been shown for K^+-activated currents previously [10], also current activated by the K^+ congeners Tl^+, Rb^+, Cs^+ and NH_4^+ under these conditions can be attributed to pump current. In the presence of ouabain, cation-sensitive currents amount to no more than a few percent of pump current over the potential range from −140 to 0 mV, and only at positive potential this component may reach values of up to 10%. The voltage-dependence of pump current determined as ouabain-sensitive current is not affected by the presence of 5 mM $BaCl_2$ (see Ref. 18).

A typical voltage-clamp experiment using Cs^+ at a range of different concentrations as activating cation is illustrated in Fig. 1. After the holding current has stabilized, an *I-V* curve (a) was recorded (see chart record in Fig. 1A). The cell was then exposed to solutions with different Cs^+ concentrations and for each concentration an *I-V* curve was measured (b, d, f, h). After each change to a new Cs^+ concentration, the chamber with the oocyte was perfused again with Cs^+-free solution to obtain reference *I-V* curves (c, e, g, i) that were also used for corrections of linear drifts with time. A selection of *I-V* curves from this experiment is shown in Fig. 1B. In Fig. 1C the voltage dependencies of pump currents activated by the different Cs^+ concentrations are shown; activity of the pump is expressed as density of pump current. While pump current at 50 mM $[Cs^+]$ shows little voltage dependence over a wide range of potentials, at Cs^+ concentrations of 10 mM or less, a pronounced negative slope is obtained for the entire potential range. Fig. 2A shows averaged *I-V* curves from a series of such experiments using Cs^+ as pump-activating cation. To average data from different oocytes, pump currents were normalized with respect to the pump current obtained in each experiment at −120 mV and the highest cation concentration used. Under these conditions pump activity is nearly maximum. The average values of pump current densities are given in the figure legends.

For further analysis of pump stimulation by different cations, we followed the same protocol we used previously to describe the potential-dependent stimulation by $[K^+]$ [10]. The dependence of pump current on cation concentration was plotted for different mem-

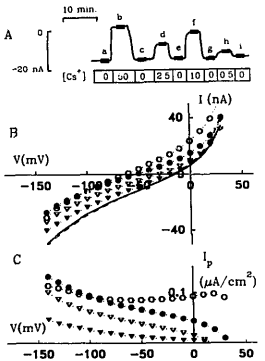

Fig. 1. (A) Chart record of holding current of a typical voltage-clamp experiment in Na^+-free solution; the holding potential was set to −60 mV. During the experiment the test chamber was continuously perfused with different solutions. The experiment was started in solution without any pump-activating cation. After the holding current had stabilized, *I-V* curves were measured at the times as indicated by brief black bars and by the letters (a–i). During upward deflections of holding current (lasting a few minutes and representing pump-generated current), the chamber was perfused with solutions containing pump-activating cations (here Cs^+) at concentrations in mM as given by the numbers. (B) *I-V* curves of total membrane current at different Cs^+ concentrations: open circles (50 mM), filled circles (10 mM), open triangles (2.5 mM), filled triangles (0.5 mM). The two lines represent *I-V* curves measured in Cs^+-free solution at the beginning (a) and the end (i) of the experiment. (C) Potential dependence of pump currents at different Cs^+ concentrations (symbols as in (B)).

brane potentials; a selection of potentials taken from the data in Fig. 2A is shown in Fig. 2B. Assuming saturation kinetics for the dependence on cation concentration [X] the following equation was fitted to the data:

$$I_p = [X]^n / (K_m^n + [X]^n) \qquad (1)$$

K_m represents an apparent, voltage-dependent half-saturation constant for pump stimulation by the external cation. The data show that K_m increases with membrane depolarization. For the Hill coefficient n an average value of 1.14 + 0.11 was obtained for the different pump-activating cations. Previously, we obtained from detailed analysis of pump activation by $[K^+]$ a value of $n = 1.3$ [10]. Though slight variability of n with membrane potential and cation species cannot be excluded, a constant value of $n = 1.3$ was used for further analysis. The voltage dependence of the calculated K_m values for pump stimulation by $[Cs^+]$ is shown by the open circles in Fig. 7.

As another K^+ congener NH_4^+ was tested. NH_4^+ is taken up by the oocytes by an electrogenic mechanism [19] distinct from the pump; if the oocytes are preincubated for 20 min in solution containing 25 mM NH_4Cl, this transport does no longer contribute to membrane current. On the other hand, this treatment will result in a reduction of the intracellular pH [19,20]. To test whether the change in pH effects pump current, experiments with untreated and NH_4Cl-pretreated oocytes were performed using Cs^+ as an activator. The reduced pH leads to a reduction of maximum pump current by a factor of 1.6 ± 0.2 ($n = 5$), but similar results are obtained with respect to the voltage dependence of pump stimulation; Fig. 3 demonstrates for the entire potential range nearly identical K_m values independent of whether or not the oocytes were preincubated in NH_4Cl-containing solution. The reduced intracellular pH is obviously without significant relevance for the present investigation. Fig. 4 shows the

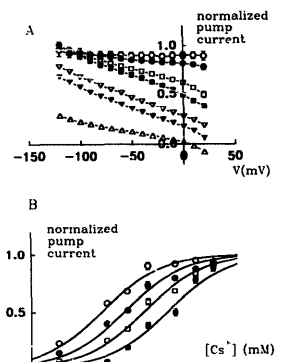

Fig. 2. (A) Potential dependence of pump currents at different Cs^+ concentrations: open circles (90 mM), filled circles (50 mM), open squares (25 mM), filled squares (10 mM), open triangles down (5 mM), filled triangles down (2.5 mM), open triangles up (0.5 mM). Data represent average values ± S.E. of 3–10 experiments depending of the concentration. For each experiment, pump currents were normalized to the value at −120 mV in 50 mM $[Cs^+]$; the averaged current density under these conditions is 0.18 ± 0.01 μA/cm². (B) Dependence of pump current on Cs^+ concentration for different membrane potentials (same data as in (A)); open circles (−100 mV), filled circles (−60 mV), open squares (−20 mV), filled squares (+20 mV). Solid lines represent fits of Eqn. 1 to the data. The fitted apparent K_m values are shown in Fig. 7.

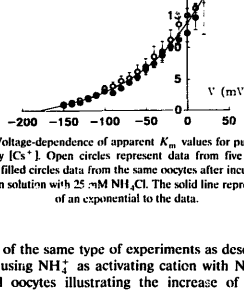

Fig. 3. Voltage-dependence of apparent K_m values for pump stimulation by [Cs^+]. Open circles represent data from five untreated oocytes, filled circles data from the same oocytes after incubation for 20 min in solution with 25 mM NH_4Cl. The solid line represents a fit of an exponential to the data.

results of the same type of experiments as described in Fig. 2 using NH_4^+ as activating cation with NH_4^+-pretreated oocytes illustrating the increase of the K_m value with increasing membrane potential (see also open triangles in Fig. 7).

Fig. 4. (A) Potential dependence of pump currents at different NH_4^+ concentrations: open circles (25 mM), filled circles (5 mM), open squares (1 mM), filled squares (0.5 mM), open triangles (0.1 mM). Data represent average values ± S.E. of four experiments. For each experiment, pump currents were normalized to the value at −120 mV in 25 mM [NH_4^+]; the averaged current density under these conditions is 0.15 ± 0.02 $\mu A/cm^2$. (B) Dependence of pump current on NH_4^+ concentration for different membrane potentials (same data as in (A)). Symbols represent the same potentials as in Fig. 2B. Solid lines represent fits of Eqn. 1 to the data. The fitted apparent K_m values are shown in Fig. 7.

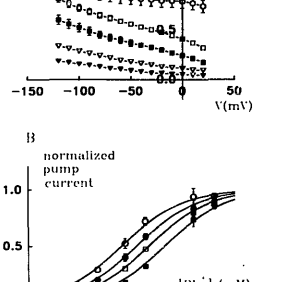

Fig. 5. (A) Potential dependence of pump currents at different Rb^+ concentrations: open circles (5 mM), filled circles (2.5 mM), open squares (0.5 mM), filled squares (0.25 mM), open triangles (0.1 mM), filled triangles (0.05 mM). Data represent average values ± S.E. of nine experiments; the averaged current density under these conditions is 0.11 ± 0.01 $\mu A/cm^2$. For each experiment, pump currents were normalized to the value at −120 mV in 5 mM [Rb^+]. (B) Dependence of pump current on Rb^+ concentration for different selected membrane potentials (same data as in (A)). Symbols represent the same potentials as in Fig. 2B. Solid lines represent fits of Eqn. 1 to the data. The fitted apparent K_m values are shown in Fig. 7.

Analogous results were obtained for pump stimulation by Tl^+ and Rb^+ (see Figs. 5 and 6, respectively). Inspection of the concentration dependencies in Figs. 2B and 4B–6B reveals, in addition to the voltage dependence, a cation-dependent variability of the apparent K_m values over more than one order of magnitude. While Cs^+ and NH_4^+ give half maximum activation in the millimolar range, Rb^+ activates in the 100-micromolar range, and Tl^+ even at concentrations below 100 μM. The voltage dependencies of the respective K_m values are summarized in Fig. 7. For comparison, the data for K^+ as stimulating cation [10] are included. Since the K_m values for Tl^+, K^+, and Rb^+ are at least an order of magnitude smaller than those for Cs^+ and NH_4^+, their dependencies were replotted at higher resolution in the inset. It has been shown previously that the dependence on membrane potential (V) of the K_m value for stimulation of the endogenous Na^+/K^+ pump by external [K^+] can be described by a single exponential

$$K_m(V) = K_m(V = 0\,\text{mV}) \cdot \exp(z^* VF/RT) \qquad (2)$$

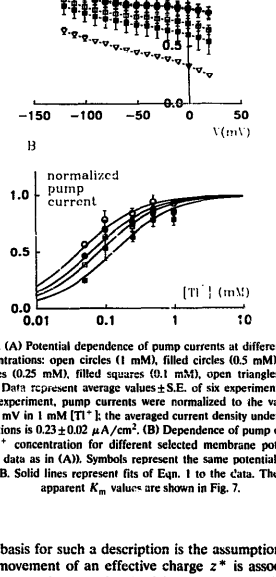

Fig. 6. (A) Potential dependence of pump currents at different Tl^+ concentrations: open circles (1 mM), filled circles (0.5 mM), open squares (0.25 mM), filled squares (0.1 mM), open triangles (0.05 mM). Data represent average values ± S.E. of six experiments. For each experiment, pump currents were normalized to the value at −120 mV in 1 mM $[Tl^+]$; the averaged current density under these conditions is $0.23 \pm 0.02\ \mu A/cm^2$. (B) Dependence of pump current on Tl^+ concentration for different selected membrane potentials (same data as in (A)). Symbols represent the same potentials as in Fig. 2B. Solid lines represent fits of Eqn. 1 to the data. The fitted apparent K_m values are shown in Fig. 7.

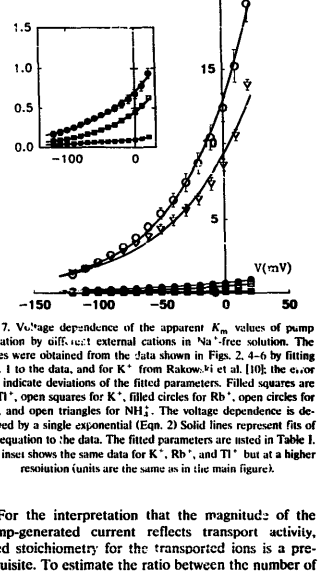

Fig. 7. Voltage dependence of the apparent K_m values of pump activation by different external cations in Na^+-free solution. The values were obtained from the data shown in Figs. 2, 4–6 by fitting Eqn. 1 to the data, and for K^+ from Rakowski et al. [10]; the error bars indicate deviations of the fitted parameters. Filled squares are for Tl^+, open squares for K^+, filled circles for Rb^+, open circles for Cs^+, and open triangles for NH_4^+. The voltage dependence is described by a single exponential (Eqn. 2) Solid lines represent fits of this equation to the data. The fitted parameters are listed in Table I. The inset shows the same data for K^+, Rb^+, and Tl^+ but at a higher resolution (units are the same as in the main figure).

The basis for such a description is the assumption that the movement of an effective charge z^* is associated with a reaction step involved in pump stimulation by external $[K^+]$ [10]; an explanation for this voltage dependence would be that local concentrations at the binding sites vary according to a Boltzmann distribution $[K^+] = [K^+]_0 \cdot \exp(z^*VF/RT)$. Also for the other pump-activating cations used in this investigation, a single exponential fits the voltage dependencies (see Fig. 7). The fitted values for z^* and K_m at 0 mV are summarized in Table I. The calculated effective charges vary between 0.20 and 0.47 of an elementary charge with an average of 0.39 ± 0.05. The differences between z^* values for the different cation species possibly reflect variabilities among different batches of oocytes (see Ref. 4), but effects on the effective charges that depend on the properties of the individual cation species can of course not be excluded. In contrast to the effective charges, the K_m values at 0 mV clearly show cation-specific differences which cover a range of more than two orders of magnitude between 0.1 mM for Tl^+ and about 13 mM for Cs^+.

For the interpretation that the magnitude of the pump-generated current reflects transport activity, fixed stoichiometry for the transported ions is a prerequisite. To estimate the ratio between the number of

TABLE I

Fitted parameters of the effective charge z^* and apparent K_m value at 0 mV (see Eqn. 2) for the data shown in Fig. 7

Data for K^+ are averages from the values reported previously [4,10]. Relative apparent affinities A are expressed as the reciprocal of K_m. P_X are relative permeabilities of the delayed rectifier channel of the frog node (see Ref. 33). A^* are relative apparent affinities determined from data reported for cardiac Purkinje cells at −20 mV and in presence of 150 mM $[Na^+]$ [11].

Parameter	Tl^+	K^+	Rb^+	NH_4^+	Cs^+
z^* (elementary charge)	0.2	0.47	0.29	0.41	0.46
K_m ($V = 0$ mV) (mM)	0.10	0.65	0.71	9.3	12.9
A ($V = 0$ mV) (normalized)	6.3	1.0	0.89	0.07	0.05
P_X	2.3	1.0	0.91	0.13	0.08
A^* ($V = -20$ mV) (normalized)	4.8	1.0	–	0.35	–

Na^+ ions transported out of the cell and of pump-activating cations into the cell, efflux of $^{22}Na^+$ and pump-generated currents were determined on the same oocyte as has been described previously for K^+ as activating cation [2,4]. In the present investigation we used as an extreme Cs^+ for pump activation which has the highest K_m value. From the rate of $^{22}Na^+$ efflux and intracellular Na^+ activity the number of Na^+ ions and from the pump current the number of charges translocated by the pump per second and oocyte were calculated to about $140 \cdot 10^{10}$ and $50 \cdot 10^{10}$, respectively, with a ratio of 2.63 ± 0.32 ($n = 5$). This value is practically identical to the value of 2.7 we obtained previously for K^+ [4] and is compatible with a $3Na^+ : 2Cs^+$ stoichiometry.

Discussion

During physiological operation of the Na^+/K^+ pump, 3 Na^+ ions are transported out of the cell and 2 K^+ ions into the cell for 1 ATP molecule that is hydrolysed during a pump cycle. Hence, the presence of extracellular $[K^+]$ is essential for normal pump operation. The pump-generated current can be calculated from total membrane current by subtracting the current remaining in absence of $[K^+]$ from current determined in presence of $[K^+]$. The validity of the difference formation requires that non-pump related K^+-sensitive currents can completely be blocked. In the *Xenopus* oocytes, this has been demonstrated for the endogenous pump [10] as well as for the pump of *Torpedo* electroplax expressed in the oocytes [3] if K^+ is used as activating cation. The same results we obtained when Cs^+ was used as K^+ congener. Also in cardiac Purkinje fibres, it has been shown that pump current can be determined as K^+-activated current [21]. The application of external $[K^+]$ stimulates the Na^+/K^+ pump in the oocytes not only in a concentration-dependent but also in a voltage-dependent manner that can be described by a potential-dependent apparent K_m value [3,10]. As a possible interpretation, it has been suggested that the K^+ ions have to pass an access channel under the influence of the electrical field to reach their binding sites. The membrane potential should, therefore, affect the degree of occupancy of the binding sites. In the absence of extracellular Na^+, this will lead to a monotonic increase in the apparent K_m value with membrane depolarization [22]. The assumption of an access channel within the electrical field is also compatible with observations obtained on cardiac Purkinje cells [11] and open membrane fragments of rabbit kidney coupled to planar lipid bilayers [23].

In the results, we have demonstrated that, similar to K^+, Tl^+, Rb^+, Cs^+, and NH_4^+ stimulate pump activity. For the analysis of the data, the magnitude of cation-activated current is assumed to represent transport activity; this is only allowed if the $3Na^+ : 2X^+$ stoichiometry is maintained also for the K^+ congeners X^+. Indications for fixed coupling ratio have been reported for Rb^+, Cs^+, Li^+, Na^+ and possibly also NH_4^+ at saturating cation concentrations for the reconstituted renal Na^+/K^+-ATPase [24] and for Tl^+, Rb^+, NH_4^+, and Cs^+ at a range of concentration for the pump in cardiac Purkinje cells [11,25]. In previous experiments, we have shown that the ratio between $^{22}Na^+$ efflux and pump current does not significantly vary with membrane potential or K^+ concentration, and that this ratio is compatible with a $3Na^+ : 2K^+$ stoichiometry [2,4]. Even if Cs^+ is used as activating cation, which has a K_m value more than an order of magnitude higher than that for K^+, a value of $2.6 + 0.3$ is obtained for the ratio between the number of Na^+ ions and charges translocated by the pump which is compatible with a $3Na^+ : 2Cs^+$ stoichiometry.

The pump stimulation by the different cations can be described by potential dependence of apparent K_m values (Fig. 7). In terms of the assumption of an access channel, the effective charge z^* is a measure for the depth of the binding site in the membrane dielectric. For the cations tested, the effective charges are similar and of the order of about 0.4 of an elementary charge. This is what one would expect if the presence of the cations would not influence the tertiary structure of the binding sites. An alternative to a fixed access channel will briefly be discussed below.

The apparent affinities for pump stimulation of the different activating cations clearly show a high degree of variability with a sequence of effectiveness of $Tl^+ > K^+ \geq Rb^+ > NH_4^+ > Cs^+$. This sequence is similar to that reported for stimulation of ATPase activity [13,14], block of release of occluded Rb^+ [26,27] and for protection of pump inhibition by removal of extracellular $[K^+]$ [12]. These observations suggest that the selectivity sequences reflect cation specific binding or access of the ions to their activation sites. In particular one could speculate that the access channel has similar characteristics to cation-selective pores where K^+ ions have to pass in single file a series of energy barriers and wells (see Ref. 28). This view is supported by the facts that K^+ ions are bound sequentially [27,29], that the pump stimulation can be described by the movement of two different effective charges for the Na^+/K^+ pump of *Torpedo* electroplax [4], and that the apparent affinities for the different cation species follow the same sequence as the cation permeabilities P_X for the delayed rectifier K^+ channels in nerve cells (see Table I). For comparison, relative apparent affinities are expressed as the reciprocal of the apparent K_m values at 0 mV normalized to the corresponding value for K^+. In cardiac Purkinje cells, a variety of monovalent cations has been demonstrated to produce concentration- and

potential-dependent activation of the pump [11] and apparent K_m values for pump stimulation by external $[K^+]$, $[Tl^+]$, and $[NH_4^+]$ were calculated. The corresponding relative apparent affinities at −20 mV we also included in Table I. The values again fall in the same sequence and are of similar size as the relative permeabilities for the K^+ channel.

As an alternative to the assumption of an ion-selective access channel, voltage-dependent and cation-selective affinities could result from voltage-dependent conformations that affect ion interaction with the binding site. Selectivity and voltage dependence could then be described in terms of energy profiles by modulations of energy barriers and ion wells [22,30,31]. From the differences of the z^* values for the different cation species in Table I, we of course can also not exclude the possibility of cation-specific effective charges which could reflect differences in the location or polarizability of the binding sites and the transported ions. In addition to voltage-dependent access, modulation of the apparent affinity has been suggested on the basis of observations in *Xenopus* oocytes that the effective charge and the K_m (0 mV) value can be modified from the intracellular side, that the effective charges may even exceed the value of two elementary charges which would be incompatible with the movement of two monovalent cations in an access channel, and that after stimulation of protein kinase A or C [4] the voltage dependence of pump stimulation by $[K^+]$ becomes more or less pronounced, respectively. Also mutants of α-subunits with truncations at the N-terminus of the pump of *Torpedo* electroplax expressed in the *Xenopus* oocytes exhibit modified K_m values and increased effective charges [32]. These data have been interpreted in terms of potential-dependent modulation of the access channel.

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