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Regulation of Endogenous and Expressed Na^+/K^+ Pumps in *Xenopus* Oocytes by Membrane Potential and Stimulation of Protein Kinases

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Summary. Modulation of the current generated by the Na^+/K^+ pump by membrane potential and protein kinases was investigated in oocytes of *Xenopus laevis*. In addition to a positive slope region in the current-voltage (*I-V*) relationship of the Na^+/K^+ pump, a negative slope region has been described in these cells (Lafaire & Schwarz, 1986) and has been attributed to a voltage-dependent apparent K_m value for pump stimulation by external $[K^+]$ (Rakowski et al., 1991). To study this feature in more detail, *Xenopus* oocytes were used for comparative analysis of the negative slope of the *I-V* relationship of the endogenous Na^+/K^+ pump and of the Na^+/K^+ pump of the electric organ of *Torpedo californica* expressed in the oocytes. The effects of stimulation of protein kinases A and C on the negative slope were also analyzed. To investigate the negative slope over a wide potential range, experiments were performed in Na^+-free solution and in the presence of high concentrations of Ba^{2+} and tetraethylammonium, to block all nonpump related K^+-sensitive currents. Pump currents and pump-mediated fluxes were determined as differences of currents or fluxes in solutions with and without extracellular K^+.

The voltage dependence of the K_m value for stimulation of the Na^+/K^+ pump by external $[K^+]$ shows significant species differences. Over the entire voltage range from -140 to $+20$ mV, the K_m value for the Na^+/K^+ pump of *Torpedo* electroplax is substantially higher than for the endogenous pump and exhibits more pronounced voltage dependence. For the *Xenopus* pump, the voltage dependence can be described by voltage-dependent stimulation by external $[K^+]$ and can be interpreted by voltage-dependent K^+ binding, assuming that an effective charge between 0.37 and 0.56 of an elementary charge is moved in the electrical field. An analogous evaluation of the voltage dependence of the *Torpedo* pump requires the assumption of movement of two effective charges of 0.16 and 1.0 of an elementary charge.

Application of 1,2-dioctanoyl-*sn*-glycerol (diC_8, 10–50 μM), which is known to stimulate protein kinase C, reduces the maximum activity of the *Xenopus* pumps in the oocyte membrane by 40% and modulates the voltage dependence of K^+ stimulation. For the endogenous *Xenopus* pump, the apparent effective charge increased from 0.37 to 0.51 of elementary charge and the apparent K_m at 0 mV increased from 0.46 to 0.83 mM. For the *Torpedo* pump, one of the apparent effective charges increased from 1.0 to 2.5 of elementary charge.

Injection of cAMP (final concentration 50 μM), which stimulates protein kinase A, has an effect opposite to stimulation of protein kinase C. The activity of the *Xenopus* Na^+/K^+ pump is elevated by 80%, and the voltage dependence of K^+ stimulation becomes less pronounced. For the endogenous pump the apparent effective charge decreased from 0.56 to 0.38 of elementary charge and the apparent K_m at 0 mV decreased from 0.78 to 0.65 mM. Also for the *Torpedo* pump, the effective charges and apparent affinities became reduced.

The data suggest that species differences in voltage-dependent stimulation of the Na^+/K^+ pump by external K^+ can account for differences in the steepness of the negative slope in the *I-V* relationships observed in different preparations. In addition, they suggest that the voltage dependence and the maximum activity of the Na^+/K^+ pump can be modulated by activation of protein kinases.

Key Words *Xenopus* oocyte · Na^+/K^+ ATPase · *Torpedo* electroplax · expression · current-voltage relationship · protein kinase

Introduction

Because the Na^+/K^+ pump operates at a $3Na^+ : 2K^+$ stoichiometry, an outward-directed current of positive charges is generated that can be measured under voltage-clamp conditions (for a review *see* DeWeer, Gadsby & Rakowski, 1988). It has been demonstrated that the stoichiometry remains constant under various experimental conditions (Gadsby, 1984; Schwarz & Gu, 1988; Rakowski, Gadsby & DeWeer, 1989); therefore, pump current is a measure of pump activity. The current-voltage (*I-V*) relationship of the Na^+/K^+ pump in oocytes of *Xenopus laevis* exhibits under physiological conditions a positive slope at negative potentials (Lafaire & Schwarz, 1985; Eisner, Valdeomillos & Wray, 1987; Rakowski & Paxson, 1988) and a negative slope at positive potentials (Lafaire & Schwarz, 1985; Lafaire & Schwarz, 1986; Schweigert, Lafaire & Schwarz, 1988). This voltage dependence suggests two voltage-dependent steps in the reaction

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cycle and has been demonstrated not only for the endogenous pump but also for the Na^+/K^+ pump of *Torpedo* electroplax expressed in the oocytes (Schwarz & Gu, 1988). Stimulation of pump current by membrane depolarization has been attributed to facilitated outward movement of positive charges during Na^+ translocation (Nakao & Gadsby, 1986). For the endogenous Na^+/K^+ pump, the negative slope has been attributed to voltage-dependent binding of extracellular K^+ prior to the translocation steps (Rakowski et al., 1991). Also for the Na^+/K^+ pump in cardiac Purkinje cells, a negative slope in the *I-V* relationship could be demonstrated that depends on concentration and species of the externally activating cation (Biehlen, Glitsch & Verdonck, 1991).

Second messengers released during intracellular signalling play a key role in regulation of membrane permeabilities. For the Na^+/K^+ pump, regulation of transport activity has been demonstrated for the effects of a variety of hormones. It has been suggested that stimulation of 3′ : 5′ cyclic AMP-dependent protein kinase (protein kinase A) by cAMP or of protein kinase C by elevation of diacylglycerol may directly lead to such hormonal pump regulation (Clausen & Flatman, 1977; Lynch et al., 1986)). Since passive membrane conductances are always modulated in parallel, indirect effects through modulation of intracellular ion composition cannot be excluded.

In this paper, we address the question whether species differences can account for the observation that negative slopes were reported only for the Na^+/K^+ pump in *Xenopus* oocytes and Purkinje cells but not for other preparations thus far. This we investigated by comparative analysis of the negative slope in *I-V* relationships for *Xenopus* and *Torpedo* pump current, and we analyzed the effects of stimulation of protein kinases A and C on pump currents of both species. A part of the results has been reported previously (Schwarz & Vasilets, 1991).

Materials and Methods

The methods of oocyte preparation, voltage clamp and efflux measurements were identical to those described previously (*see* Grygorczyk et al., 1989; Vasilets et al., 1990). For the convenience of the reader, they are briefly summarized below.

Oocytes

Females of the clawed toad *Xenopus laevis* were anesthetized with *m*-aminobenzoic acid ethylester 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 (stages V and VI, after Dumont (1972)) at room temperature (21°C). For expression of Na^+/K^+ ATPase from the electric organ of *Torpedo californica*, about 10 ng cRNA for both the α- and β-subunit (αT, βT) were injected per oocyte, and cells were incubated at 18°C for at least two days before an experiment. The cDNAs for the two subunits were kindly provided to us by Dr. M. Kawamura; the mRNA's were synthesized by H. Omay and Dr. H. Appelhans by in vitro transcription using Sal I-cleaved pSP62 as templates and SP6 polymerase.

Electrophysiological Measurements

I-V dependencies were determined by conventional two-microelectrode techniques (Turbo TEC Clamp, NPI, Tamm (FRG)) under control of a personal computer with a CED 1401 (CED, Cambridge (England)). From a constant holding potential of −60 mV, rectangular voltage pulses of 500 msec duration and varying amplitude from negative to positive potentials were applied every 4 sec, and steady-state currents were averaged during the last 100 msec. To reduce nonpump related K^+ currents, all solutions contained 5 mM $BaCl_2$ and 20 mM tetraethylammoniumchloride (TEA-Cl). Under these conditions, the current generated by the electrogenic Na^+/K^+ pump can be determined as a difference of total membrane current in solutions containing a distinct concentration of K^+ and in K^+-free solution (*see* Rakowski et al., 1991). For averaging currents from different oocytes, pump currents were normalized to the pump currents obtained at −120, −140 or −160 mV in 5 mM K^+-containing solution. The potential used for normalization was without effect on the results obtained by further analysis. Absolute currents are given in nA. For conversion into current densities, the surface area was estimated from measurements of membrane capacitance, assuming a specific capacitance of 1 $\mu F/cm^2$. An average of 0.18 cm^2 is obtained that is about 3.5 times larger than calculated from a spherical oocyte (for details *see* Vasilets et al., 1990).

Stimulation of Protein Kinases

To stimulate protein kinases in the oocytes, the cells were preincubated with specific activators of protein kinases or were microinjected with the activator. For this latter purpose, individual oocytes were impaled with a micropipette, and 50 nl of stock solution (*see below*) were injected by means of a nanoliter pump (*see* Grygorczyk et al., 1989). Final concentrations in cells, as given in Results, were calculated by assuming a total volume of an oocyte of 1 μl, which includes aqueous phase and intracellular compartments.

Flux Measurements

To estimate the rate of Na^+ efflux generated by the Na^+/K^+ pump, oocytes were microinjected with 20–50 nl $^{22}NaCl$ (about 70 MBq/ml). An oocyte is then placed in a perfusion chamber that is mounted on a Geiger-Müller tube, and loss of radioactivity by the efflux of $^{22}Na^+$ is monitored continuously under voltage clamp (Schwarz & Gu, 1988; Grygorczyk et al., 1989). The relative rate of efflux was determined from the exponential decline of radioactivity. From the relative rate of efflux (k), the number of Na^+ ions translocated per second across the oocyte membrane (I_{Na} (in C/sec)) was calculated according to

$$I_{Na} = F \cdot k \cdot [Na_i] \cdot \nu \quad (1)$$

with intracellular Na^+ concentration $[Na_i]$. For the volume of the oocyte ν the volume of the aqueous phase of about 0.5 μl was used rather than the total volume which, in particular, includes yolk particles.

As for the pump current, the pump-generated flux component was determined from the difference of efflux rates obtained in solutions with and without extracellular K^+.

Solutions

The compositions of the standard oocyte Ringer's solution (ORi) was (in mM): 110 NaCl, 3 KCl, 2 $CaCl_2$, 5 morpholinopropane sulfonic acid (MOPS, adjusted to pH 7.2). The test solutions contained (in mM): 90 tetramethylammonium chloride (TMA-Cl), 2 $CaCl_2$, 5 $BaCl_2$, 20 tetraethylammonium chloride (TEA-Cl), 5 MOPS (adjusted to pH 7.2), and varying (0–5) KCl. In solutions with K^+ concentrations below 1 mM the actual concentrations were confirmed by flame photometry; in nominally K^+-free solutions the contaminating level was below 5 μM. To increase pump activity, oocytes were usually preloaded with Na^+ by incubating the cells for at least 1 hr in solution that had the following composition (in mM): 110 NaCl, 2.5 sodium citrate, 5 MOPS (adjusted to pH 7.6) (*see* Rakowski et al., 1991). In the loaded oocytes, intracellular activity of Na^+ was about 80 mM after 2 hr incubation as measured by Na^+-selective microelectrodes. The stock solution of activators of protein kinases contained 1 mM cAMP dissolved in H_2O or 0.2 mM 1,2-dioctanoyl-*sn*-glycerol (diC_8) dissolved in dimethyl sulfoxide (DMSO). Before injection, stock solution of diC_8 was sonicated for 1 min. Injection of the DMSO into oocytes by itself was without effect.

Results

The negative slope of the endogenous Na^+/K^+ pump in *Xenopus* oocytes can be described in terms of a voltage-dependent K_m value for pump stimulation by external $[K^+]$ (Rakowski et al., 1991). This can be investigated most effectively over a wide potential range if release of Na^+ to the external medium is facilitated by using Na^+-free solutions. Therefore, all experiments described in this investigation were performed with Na^+-free media containing different concentrations of K^+. To block all nonpump mediated K^+-sensitive currents, the bath solutions contained 5 mM $BaCl_2$ and 20 mM TEA-Cl; under these conditions the pump current can be determined as the difference current measured in solutions with and without extracellular $[K^+]$ (Rakowski et al., 1991).

Voltage and K^+ Dependence of *Torpedo* Pump Currents and Fluxes

The protocol of a typical voltage-clamp experiment for the determination of *I-V* dependencies of the pump is shown in Fig. 1*A*; while the holding potential was set to −60 mV, holding current was monitored on a chart recorder. The experiment was started in K^+-free solution. After the preparation had stabilized, an *I-V* curve (*a*) was measured. The cell was then exposed to solutions with different K^+ concentrations, and for each concentration an *I-V* curve was measured (*b,d,f,h,i,l*). After each change to a new K^+ concentration, the bath was perfused again with K^+-free solution to obtain reference *I-V* curves (*c,e,g,k,m*) for linear drift corrections. A selection of *I-V* curves from this experiment is shown in Fig. 1*B*. In Fig. 1*C* the *I-V* curves for pump-

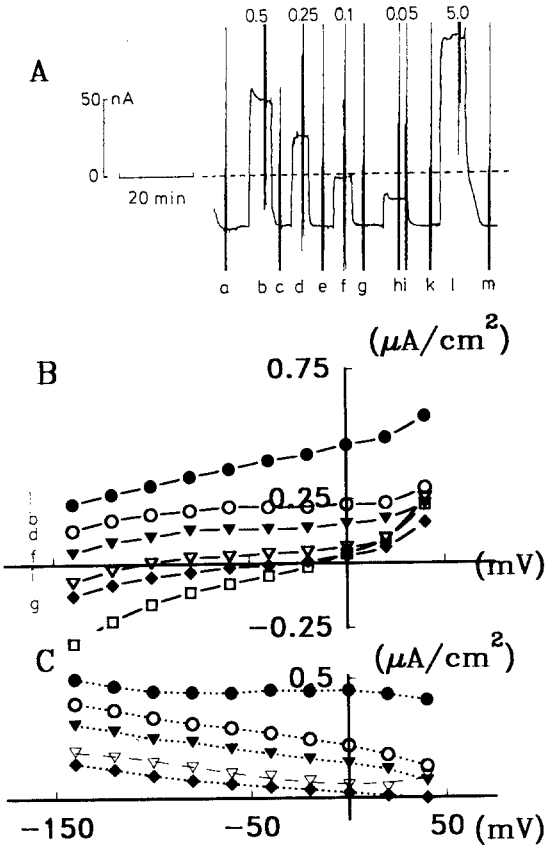

Fig. 1. (*A*) Chart record of holding current of a typical voltage-clamp experiment in Na^+-free solution. The holding potential was set to −60 mV. The letters *a* to *m* indicate where *I-V* measurements were performed. The experiment was performed on an oocyte injected with cRNAs for the α- and β-subunits of the Na^+/K^+ pump of *Torpedo* electroplax and was started in K^+-free solution. During the upward deflections of holding current (representing pump-generated current) the chamber was perfused with solution containing K^+ at concentrations given by the numbers in mM. (*B*) *I-V* curves of total membrane current at different K^+ concentrations. The letters refer to the measurements as indicated in *A*. (*C*) Difference *I-V* curves between measurements in solution with a distinct K^+ concentration and K^+-free solution; filled circles, 5 mM; open circles, 0.5 mM; filled triangles, 0.25 mM; open triangles, 0.1 mM; filled diamonds, 0.05 mM.

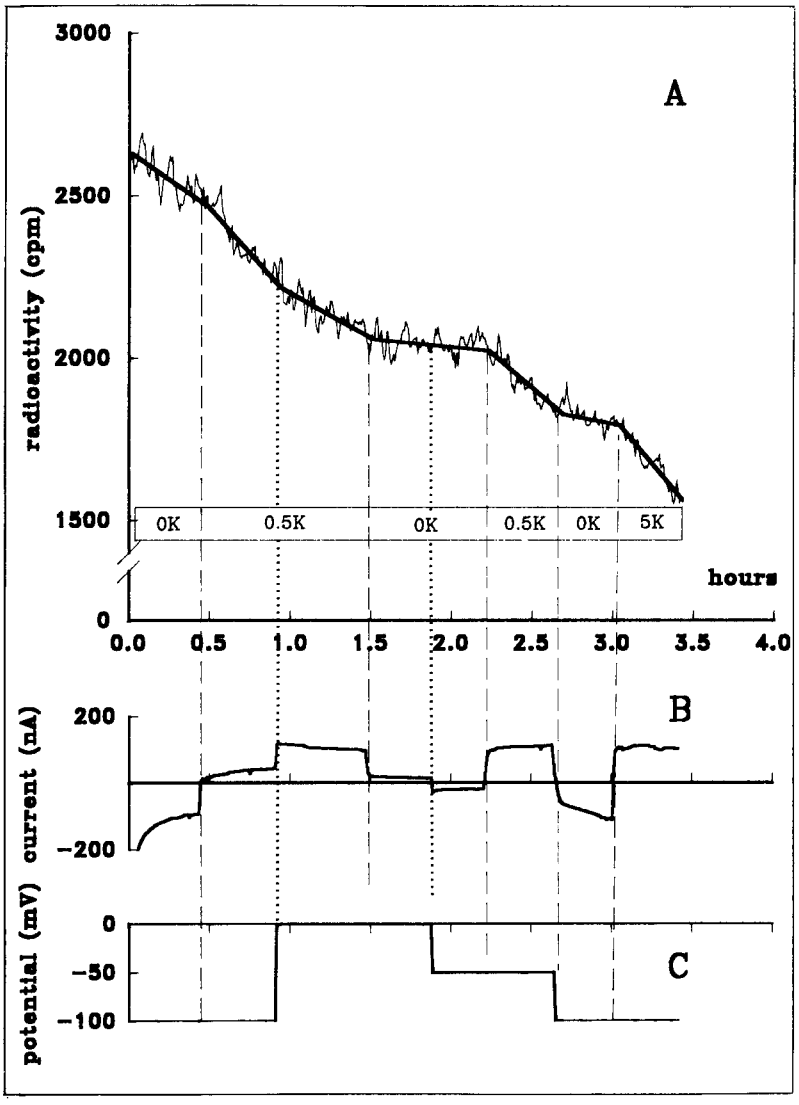

Fig. 2. (*A*) Loss of radioactivity from an oocyte after microinjection of ^{22}Na with 0 or 0.5 mM KCl in the wash solution. The efflux measurements were performed under voltage clamp as indicated in *C*. The rates of efflux (k) determined from the exponential decline of radioactivity were (in 10^{-6} sec^{-1})

$[K^+]$ (mM)	Clamp potential (mV)		
	0	−50	−100
0	0.01	0.01	19.5
0.5	33.2	66.8	70.8
5.0	—	—	95.1

(*B*) Holding current necessary to clamp the oocyte membrane to the potentials as indicated in *C*.

generated current are shown; it is demonstrated that in oocytes with Na^+/K^+ pump from *Torpedo* electroplax with high external $[K^+]$ (5 mM) in Na^+-free solution the pump current is nearly voltage-independent; in the absence of extracellular $[Na^+]$, also in squid axon (Rakowski et al., 1989) and ventricular myocytes (Nakao & Gadsby, 1989) nearly voltage-independent pump current is obtained. At lower concentrations of extracellular K^+ (3 mM or less) depolarization leads to pump inhibition; a similar observation has been made previously for the endogenous Na^+/K^+ pump in the oocytes (Rakowski et al., 1991) and in cardiac Purkinje cells (Biehlen et al., 1991).

In the mRNA-injected oocytes, the number of pump molecules is sufficiently high to allow detection and analysis of pump-generated Na^+ efflux by measuring the release of injected $^{22}Na^+$ from a single cell under voltage-clamp conditions (Schwarz & Gu, 1988). Figure 2*A* shows the release of radioactivity with time from an oocyte during a typical experiment. For at least 20 min, the loss of radioactivity is monitored under constant conditions; then the holding potential (*see* Fig. 2*C*) or the K^+ concentration (*see* horizontal bar in Fig. 2*A*) is changed, and loss of radioactivity is continued to be monitored. From the exponential time course, the relative rate of efflux is estimated. The flux component generated by the Na^+/K^+ pump is determined as the difference of efflux rates in solutions with and witout extracellular $[K^+]$. The voltage dependence of the efflux rate of the pump at 0.5 mM K^+ averaged from a series of experiments is shown in Fig. 3*A*. Similar to the pump current at 0.5 m*M* K^+, the rate of Na^+ efflux decreases with membrane depolarization.

Since the flux measurements were performed under voltage-clamp conditions, pump-generated

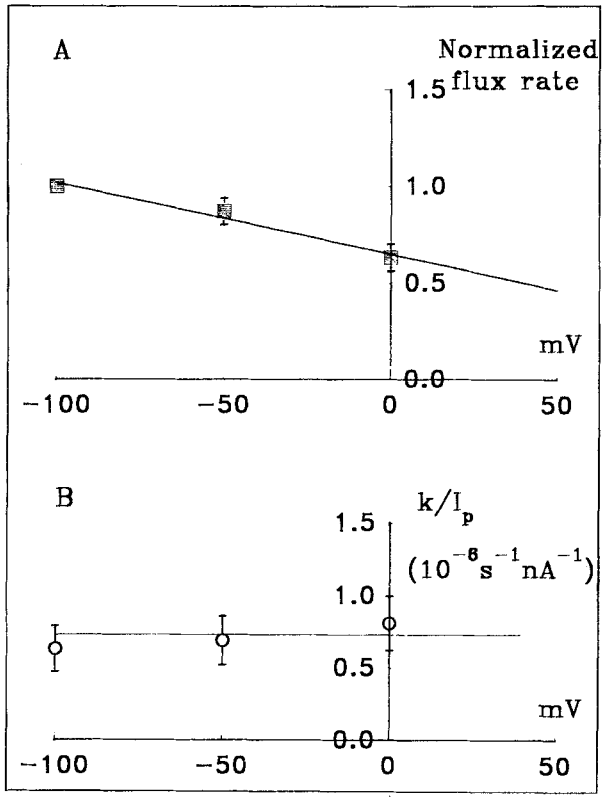

Fig. 3. (*A*) Voltage dependence of relative rates of pump-mediated ^{22}Na efflux in 0.5 mM K^+ obtained from five different experiments. The line represents a linear fit to the data points. (*B*) Ratio of efflux rate (k) to pump current (I_p) normalized to −100 mV. The data represent averages (±SEM) of four experiments.

currents could be determined in this type of experiments in parallel on the same oocyte. The holding current necessary to clamp the oocyte membrane to the respective holding potential (Fig. 2*C*) was monitored continuously during the experiment (Fig. 2*B*). Pump-generated current was determined as the K^+-sensitive current either from this type of record or from *I-V* measurements as described in Fig. 1 before or at the end of the flux experiment. Figure 3*B* presents a comparison of the currents with the fluxes and indicates similar voltage dependencies. In fact, a nearly constant value is obtained if the ratio between flux rate and current is plotted *versus* potential; this suggests voltage-independent $Na^+ : K^+$ stoichiometry. Assuming an intracellular Na^+ concentration of 80 mM, and an aqueous volume of the oocytes of 0.5 μl (*see* Materials and Methods), the rate of efflux can be converted into the number of Na^+ ions translocated per second across the oocyte membrane (I_{Na}, *see* Eq. (1)). In Fig. 4, I_{Na} is plotted *versus* the number of net charges translocated per second that is given by the pump current I_p. A linear fit to the data has a slope that is compatible with 3 Na^+ ions per 1 net charge, or in other words with a $3Na^+ : 2K^+$ stoichiometry. This demonstrates that omission of external Na^+ and reduction of external $[K^+]$ do not effect the normal $3Na^+/2K^+$ pump mode and that the negative slopes in the *I-V* relationships represent voltage dependence of the normal mode of $3Na^+/2K^+$ pumping. From the number of ouabain binding sites (*see below*) and the stoichiometry the turnover number of the pump cycle can be estimated to about 30 sec^{-1} for a pump current of 200 nA.

Separation of Currents Generated by the Xenopus and the Torpedo Pump

The pump current measured in mRNA-injected oocytes represents the sum of current generated by the endogenous *Xenopus* pump and by the expressed *Torpedo* pump. To separate these two components, the number of pump molecules was determined in control and mRNA-injected oocytes. This can be achieved by measurements of ouabain binding to the plasma membrane of single oocytes with radioactively labeled ouabain (Richter, Jung & Passow, 1984; Schmalzing, Kröner & Passow, 1989). Control oocytes of the same batches of oocytes as used in voltage-clamp experiments exhibit (12.8 ± 2.0) × 10^9 molecules/cell (about 700 per μm^2); oocytes injected with the cRNA for the α- and β-subunits of the *Torpedo* pump exhibit (38.9 ± 5.5) × 10^9 molecules/cell (about 2200 per μm^2); these data were averaged from six different batches of oocytes. It has been demonstrated recently that the β-subunit of *Torpedo* (βT) (Schmalzing et al., 1991) can assemble with excess of endogenous *Xenopus* α-subunits (αX) (Geering et al., 1989) to form about 50% additional functional pump molecules. Since coinjection of cRNA encoding αT with cRNA encoding βT leads to a threefold increase in pump molecules, it is unlikely that a significant fraction of ($\alpha X \beta T$) is formed. We assume, therefore, that in oocytes injected with cRNA encoding $T\alpha$ and $T\beta$ 30% of the pump molecules are endogenous pumps and 70% *Torpedo* pumps. To determine voltage and $[K^+]$ dependencies of the *Torpedo* pump current, we corrected the total pump current for the contribution of the endogenous pump assuming the same voltage and $[K^+]$ dependencies for the *Xenopus* pump molecules in the cRNA-injected oocytes as in the control oocytes. The procedure of current separation will allow to make qualitative and with certain restrictions also quantitative comparison between the *Xenopus* and *Torpedo* pump.

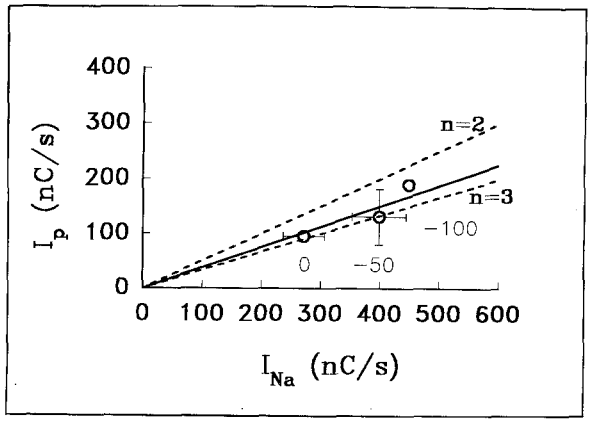

Fig. 4. Relationship between pump-mediated net charge transfer (obtained from the pump current I_p) and Na^+ transfer I_{Na} (obtained from efflux rates assuming an intracellular Na^+ activity of 80 mM (*see* Materials and Methods)). The data points represent averages (±SEM) from four experiments. The dotted lines have slopes of 1/2 and 1/3, respectively; the solid line is a linear fit to the data with a slope of 1/2.7.

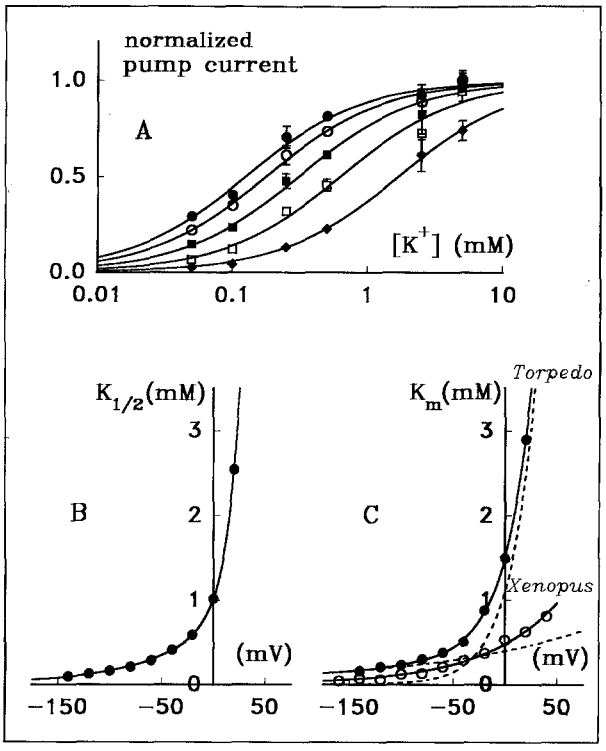

Fig. 5. (*A*) Dependence of pump current in oocytes injected with cRNAs of *Torpedo* electroplax on K^+ concentration at different membrane potentials. The currents were normalized to maximum current obtained by fitting Eq. (3) to the data (solid lines). Data points represent average values ±SEM from five experiments; for symbols without error bar, the deviations are less than the symbol size. Filled circles, −140 mV; open circles, −100 mV; filled squares, −60 mV; open squares, −20 mV; filled diamonds, +20 mV. (*B*) Voltage dependence of the apparent $K_{1/2}$ value determined from data as presented in *A*. (*C*) Voltage dependence of apparent K_m values for stimulation by $[K^+]$ of the *Torpedo* (filled symbols) and *Xenopus* (open symbols) pump. Data for *Torpedo* pump were obtained as described in Results. The solid lines represent least-squares fits of Eqs. (4) and (5) to the *Xenopus* and *Torpedo* data, respectively; the broken lines represent the two components necessary to describe the *Torpedo* data (fitted parameters; *see* Table 1 (*Control I*) and Table 2 (*Control*). The data for the *Xenopus* pump were taken from Rakowski et al., 1991.

Voltage Dependence of K_m for Stimulation of Torpedo Pump Current

To describe the dependence of pump current on membrane potential and K^+ concentration, apparent voltage-dependent K_m values for stimulation of pump current (I_p) by external $[K^+]$ were introduced. For noninjected oocytes, the $[K^+]$ dependence of I_p was fitted for different membrane potentials by

$$I_p = I_{max} \cdot [K^+]^n/([K^+]^n + K_m^n) \quad (2)$$

with a Hill coefficient of $n = 1.3$ and voltage-dependent K_m values (Rakowski et al., 1991). We assume that the dependence of *Torpedo* pump current on $[K^+]$ and membrane potential can be described by an analogous expression. Figure 5*A* shows $[K^+]$ dependencies of total pump current of mRNA-injected oocytes for a number of selected potentials. The voltage dependence of the K^+ concentration for half maximum stimulation ($K_{1/2}$ values) is summarized in Fig. 5*B*. To calculate the voltage dependence of an apparent K_m value for the *Torpedo* pump, we fitted the following equation to the normalized data as shown in Fig. 5*A* based on the 30 to 70% ratio between endogenous and *Torpedo* pumps (*see above*):

$$I_p = I_p^T + I_p^X = 0.7 \cdot [K^+]/([K^+] + K_m^T) + 0.3 \cdot [K^+]^{1.3}/([K^+]^{1.3} + K_m^{X1.3}). \quad (3)$$

The superscripts *T* and *X* refer to *Torpedo* and *Xenopus*, respectively. The K_m^X values were calculated from the parameters given in Table 1 (*Control I*), and the K_m^T values were then obtained from fits of Eq. (3) to data. For the contribution by the *Torpedo* pump, a Hill coefficient of $n = 1$ was used which gave best fits to the data at all potentials. The voltage dependence of these corrected K_m values is shown in Fig. 5*C*, and the parameters pertaining to the fitted curve are listed in Table 2 (*Control*).

The voltage dependence of K_m for the endoge-

Table 1. Fitted parameters of Eq. (2) for the voltage dependence of the apparent K_m-value of *Xenopus* pump stimulation by external $[K^+]$[a]

	Control I	diC_8	Control II	cAMP
$K_m(0)$ (mM)	0.48	0.83	0.78	0.65
z^*	0.37	0.51	0.56	0.38

[a] Control I parameters were taken from (Rakowski et al., 1991), control II parameters were obtained from experiments performed in parallel to those with cAMP injection.

Table 2. Fitted parameters of Eq. (3) for the voltage dependence of the apparent K_m-value of *Torpedo* pump stimulation by external $[K^+]$

	Control	diC_8	cAMP
A_1 (mM)	1.11	0.10	0.43
z_1^*	1.03	2.46	0.93
A_2 (mM)	0.40	0.78	0.18
z_2^*	0.16	0.16	0.10

nous Na^+/K^+ pump could be described by a single exponential (*see* open symbols in Fig. 5*C*)

$$K_m = K_m(V = 0\,\text{mV}) \cdot \exp(z^* \cdot VF/RT) \qquad (4)$$

with an apparent affinity at $V = 0$ mV of $K_m = 0.46$ mM and an effective charge for stimulation by $[K^+]$ of $z^* = 0.37$ of an elementary charge (*see* Table 1, *Control I*); F, R, and T have their usual meanings. These values for the *Xenopus* pump were obtained from the data presented by Rakowski et al. (1991). In contrast to the observations with the endogenous pump, the voltage dependence of the *Torpedo* pump cannot be described by a single exponential. An adequate fit is obtained by the sum of two exponentials (*see* broken lines in Fig. 5*C*)

$$K_m = A_1 \cdot \exp(z_1^* VF/RT) + A_2 \cdot \exp(z_2^* \cdot VF/RT) \qquad (5)$$

with an apparent K_m value at $V = 0$ mV of $A_1 + A_2 = 1.5$ mM, and with effective charges of about 1.0 and 0.16 of an elementary charge, respectively (*see* Table 2, *Control*).

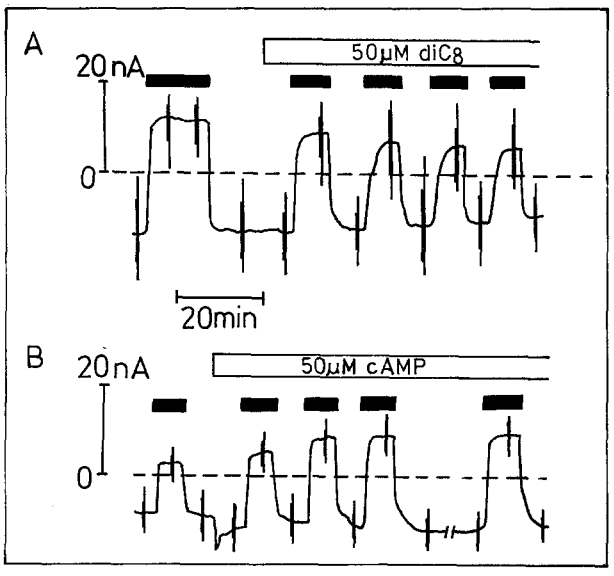

Fig. 6. Chart records of holding current from oocytes that were clamped to −60 mV and perfused with Na^+-free solution without or with 5 mM K^+ (upward deflections of holding current which represent pump current stimulated by the 5 mM K^+ (black bars)). (*A*) At the time indicated the 50 μM diC_8 was added to the superfusion medium. (*B*) At the time indicated the 50 μM cbAMP was added to the superfusion medium.

Modulation of Pump Activity by Protein Kinases

Extracellular application of activators of protein kinase C leads to downregulation of the Na^+/K^+ pump that has been attributed to an endocytotic removal of pump molecules from the plasma membrane (Vasilets et al., 1990, 1991). The resulting reduction in surface membrane is particularly pronounced with the phorbol ester phorbol 12-myristate 13-acetate (PMA). It has been suggested that the pump molecules remaining in the membrane are modified by the stimulation of protein kinase C (Vasilets et al., 1990). With the diacylglycerol analog diC_8, the induced endocytotic process followed by measurements of membrane capacitance shows large variability. Occasionally, changes in membrane surface may be negligible, in other instances the surface may be reduced by up to 50%. Figure 6*A* shows a chart record of holding current at −60 mV where pump activity is maximally stimulated during temporary applications of 5 mM K^+ to the Na^+-free solution. The maximum pump activity decreases after superfusion with 50 μM diC_8 and reaches steady state after about 1 h. In the experiments illustrated in Fig. 6*A*, no change in membrane capacitance was detected. To average data from different experiments, pump activity was expressed as pump current density, which will correct for possible changes in the number of pump molecules due to endocytotic changes in surface area. On the average (*see* Fig. 7), maximum pump activity decreased to 59% 1 hr after

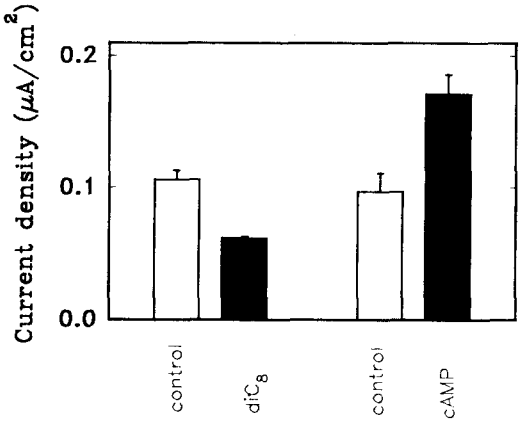

Fig. 7. Density of *Xenopus* pump current stimulated by 5 mM external $[K^+]$ at −60 mV. Current densities were calibrated by measurements of membrane capacities and assuming a specific capacitance of 1 $\mu F/cm^2$. diC_8 refers to oocytes that were pretreated with 50 μM diC_8 for 1 hr, and cAMP to oocytes that were microinjected with cAMP (final concentration 50 μM); controls refer to untreated oocyte of the respective batches. Data represent averages ±SEM from five experiments.

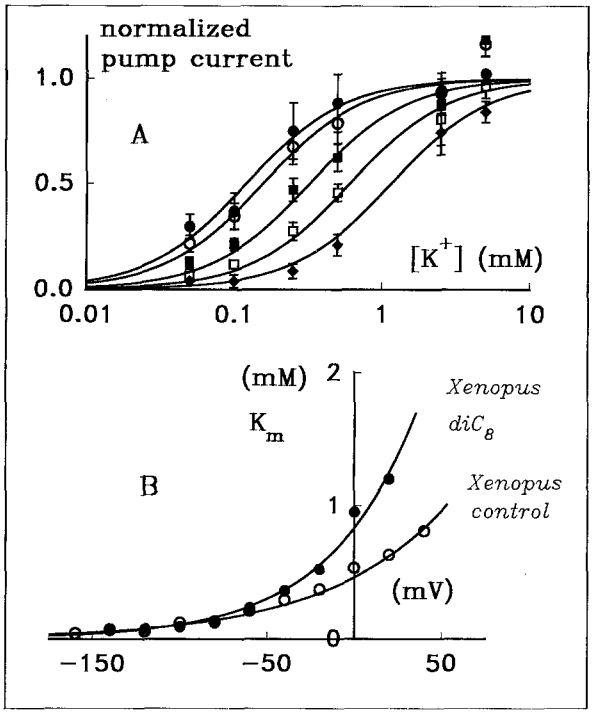

Fig. 8. (*A*) Dependence of *Xenopus* pump current on K^+ concentration at different membrane potentials. Oocytes were injected with 50 nl diC_8 solution (final concentration 10 μM) 1 hr before the first *I-V* measurement. The currents were normalized to maximum current obtained by fitting Eq. (2) with $n = 1.3$ to the data (solid lines). Data points represent average values ±SEM from five experiments; for symbols without error bar, the deviations are less than the symbol size. Filled circles, −140 mV; open circles, −100 mV; filled squares, −60 mV; open squares, −20 mV; filled diamonds, +20 mV. (*B*) Voltage dependence of the apparent K_m value determined as shown in *A*. The solid line represents a least-squares fit of Eq. (4) (fitted parameters, *see* Table 1). Control data of non-diC_8 injected oocytes were taken from Rakowski et al., 1991.

diC_8 application, and corresponds to a decrease of turnover rate from about 100 translocations per sec to about 60 per sec.

To activate protein kinase A, oocytes were microinjected with cAMP to give final intracellular concentration of 50 μM or were superfused with 50 μM dbcAMP. Figure 6*B* shows that in contrast to the stimulation of protein kinase C, stimulation of protein kinase A leads to an increase in maximum transport activity determined at 5 mM K^+ and −60 mV, steady state is already reached after about 30 min. This stimulation of pump current is accompanied by a slight increase in number of ouabain binding sites of maximally 14%. Membrane capacitance is slightly reduced by 15%. The difference in maximum pump current in Fig. 6*A* and *B* before treatment with the activators of protein kinases reflects variability among oocytes from different females. Figure 7 again shows averaged pump current densities; injection of cAMP or superfusion with dbcAMP leads to an increase of the maximum pump activity by about 78%.

Modulation of Voltage Dependence of K_m by Stimulation of Protein Kinase C

The results described above demonstrate that maximum transport activity is modified by stimulation of protein kinases. This raises the question whether or not this modification also involves changes of voltage dependence. Since superfusion with 50 μM diC_8 can lead to pronounced reduction of pump current as well as membrane capacitance, we investigated the effect of diC_8 by injection of small amounts of diC_8 (10 μM final concentration) which does not produce changes in membrane capacitance. Figure 8 summarizes the results of the dependence on $[K^+]$ and membrane potential. The fairly large error bars in Fig. 8*A* are the result of the much smaller pump currents in these cells ($I_p^{max} = 0.14\ \mu A/cm^2$, *compare* Fig. 1*C* for an mRNA-injected oocyte). The voltage dependence of the K_m values obtained from fits of Eq. (2) to averaged data can be described by a single exponential (Fig. 8*B*) as in control oocytes that have not been injected with diC_8 (*see* open symbols in Fig. 5*C*), but the effective charge has increased to 0.51 of an elementary charge and also the

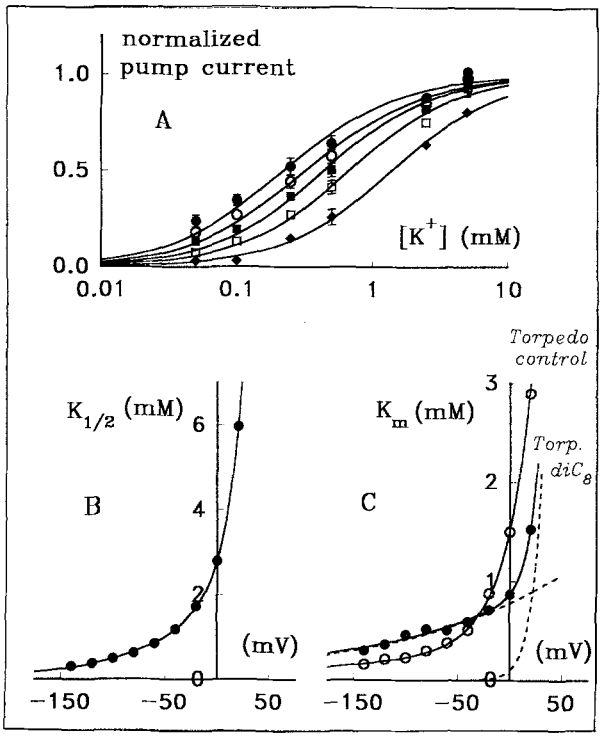

Fig. 9. (*A*) Dependence of pump current in oocytes injected with cRNAs of *Torpedo* electroplax on K^+ concentration at different membrane potentials. Oocytes were injected with 50 nl diC_8 solution (final concentration 10 μM) 1 hr before *I-V* measurements were started. The currents were normalized to maximum current obtained by fitting Eq. (3) to the data (solid lines). Data points represent average values ±SEM from five experiments; for symbols without error bar, the deviations are less than the symbol size. Filled circles, −140 mV; open circles, −100 mV; filled squares, −60 mV; open squares, −20 mV; filled diamonds, +20 mV. (*B*) Voltage dependence of the apparent $K_{1/2}$ values determined from data as shown in *A*. (*C*) Voltage dependence of apparent K_m values for stimulation by $[K^+]$ of the *Torpedo* pump in diC_8-injected (filled symbols) and noninjected (open symbols) oocytes. *Torpedo* pump currents were separated from total pump currents as described in Results. The solid lines represent least-squares fits of Eq. (5); the broken lines represent the two components necessary to describe the *Torpedo* data in diC_8-injected oocytes (fitted parameters, *see* Table 2 (*Control* and *diC*$_8$). The control data are the same as shown in Fig. 5.

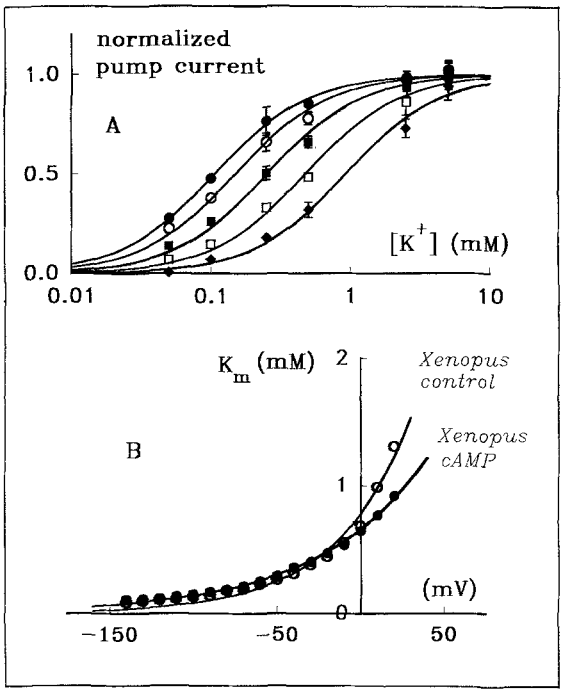

Fig. 10. (*A*) Dependence of *Xenopus* pump current on K^+ concentration at different membrane potentials. Oocytes were injected with 50 nl cAMP solution (final concentration 50 μM) 0.5–1 hr before the first *I-V* measurement. The currents were normalized to maximum current obtained by fitting Eq. (2) with $n = 1.3$ to the data (solid lines). Data points represent average values ±SEM from five experiments; for symbols without error bar, the deviations are less than the symbol size. Filled circles, −140 mV; open circles, −100 mV; filled squares, −60 mV; open squares, −20 mV; filled diamonds, +20 mV. (*B*) Voltage dependence of the apparent K_m value determined as shown in *A* (filled circles). The solid line represents a least-squares fit of Eq. (4) (fitted parameters, *see* Table 1). In addition, control data of non-cAMP injected oocytes from the same batches are shown.

K_m value at 0 mV is elevated (*compare* data in Table 1, *Control I − diC*$_8$).

Figure 9 summarizes the effect of the diC_8 injection on the potential dependence of pump stimulation by external $[K^+]$ in mRNA-injected oocytes. The $[K^+]$ dependence of the normalized pump current is plotted for a number of selected potentials in Fig. 9*A*. The voltage dependence of the $K_{1/2}$ values for stimulation by $[K^+]$ is shown in Fig. 9*B*. The $[K^+]$ and voltage dependence of pure *Torpedo* pump current was obtained as before by fitting Eq. (3) to data as shown in Fig. 9*A*. The resulting voltage dependence of K_m^I can still be described by two exponentials (Fig. 9*C*) as in non-diC_8 injected oocytes (Fig. 5*C*), but one of the effective charges increased by a factor of more than 2; the K_m value at 0 mV ($A_1 + A_2$) becomes reduced (*compare* data in Table 2, *Control − diC*$_8$). Since the two exponential components are differently affected, the K_m^I value may be elevated or reduced by the diC_8 treatment depending on the membrane potential.

Modulation of Voltage Dependence of K_m by Stimulation of Protein Kinase A

In addition to the activator of protein kinase C, diC_8, we investigated whether the activator of protein kinase A, cAMP, also modulates the voltage dependence of the apparent K_m value for pump stimulation by external $[K^+]$. Figure 10*A* shows the $[K^+]$ depen-

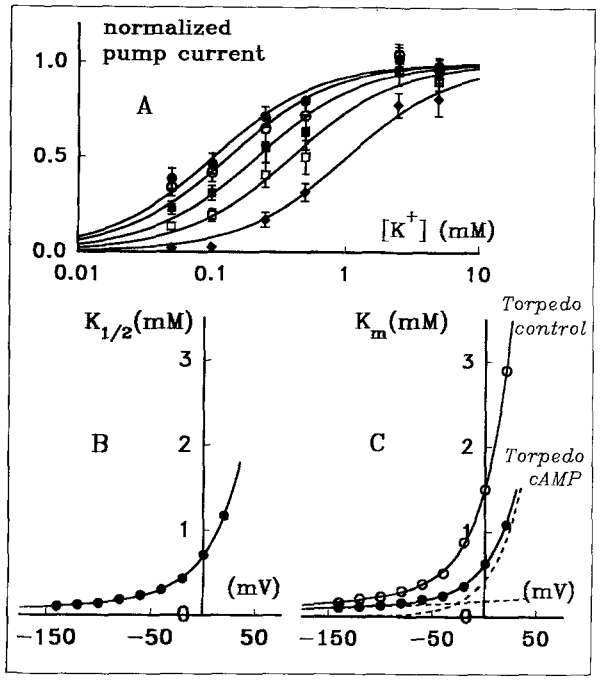

Fig. 11. (*A*) Dependence of pump current in oocytes injected with cRNAs of *Torpedo* electroplax on K^+ concentration at different membrane potentials. Oocytes were injected with 50 nl cAMP solution (final concentration 50 μM) 1 hr before *I-V* measurements were started. The currents were normalized to maximum current obtained by fitting Eq. (3) to the data (solid lines). Data points represent average values ±SEM from five experiments; for symbols without error bar, the deviations are less than the symbol size. Filled circles, −140 mV; open circles, −100 mV; filled squares, −60 mV; open squares, −20 mV; filled diamonds, +20 mV. (*B*) Voltage dependence of the apparent $K_{1/2}$ values determined from data as shown in *A*. (*C*) Voltage dependence of apparent K_m values for stimulation by $[K^+]$ of the *Torpedo* pump in cAMP-injected (filled symbols) and noninjected (open symbols) oocytes. *Torpedo* pump currents were separated from total pump currents as described in Results. The solid lines represent least-squares fits of Eq. (5); the broken lines represent the two components necessary to describe the *Torpedo* data in cAMP-injected oocytes (fitted parameters, *see* Table 2 (*Control* and *cAMP*). The control data are the same as shown in Fig. 5.

dence of pump currents in oocytes containing only endogenous *Xenopus* pumps for a number of selected membrane potentials. The voltage dependence of the apparent K_m values is plotted in Fig. 10*B* in comparison to control data for non-cAMP injected oocytes of the same batches. In contrast to injection of diC_8, cAMP injection leads to slight reduction of the K_m value at 0 mV from 0.78 to 0.65 but strongly reduces the effective charge from 0.56 to 0.38 of an elementary charge; resulting in less pronounced voltage dependence (*see* Table 1).

The effect of cAMP on the voltage dependence of the K_m value was also investigated for the *Torpedo* pump. Figure 11*A* shows the $[K^+]$ dependence of pump currents for a number of selected membrane potentials. The voltage dependence of the apparent $K_{1/2}$ values is plotted in Fig. 11*B*. The K_m values for the *Torpedo* pump were again determined by fits of Eq. (3) to data as shown in Fig. 11*A*. Figure 11*C* shows the voltage dependence of these K_m values in comparison to control data of non-cAMP modified *Torpedo* pumps. As for the *Xenopus* pump, the effective charges are reduced; in addition, the K_m value at 0 mV ($A_1 + A_2$, *see* Eq. (5)) is reduced by 60% (*compare* data in Table 2, *Control* − *cAMP*).

Discussion

Since the $3Na^+/K^+$ pump molecule transports charges across the cell membrane and undergoes conformational changes, voltage-dependent steps in the reaction cycle of the pump may modulate transport activity. The positive slope in the *I-V* relationship of the Na^+/K^+ pump that has been observed in most preparations has been attributed to positive charges moving outwardly during steps leading to external Na^+ liberation (Nakao & Gadsby, 1986). The existence of further voltage-dependent steps in the reaction cycle has been doubted for a long time (*see* e.g. (DeWeer et al., 1988)). On the other hand, the negative slope that was demonstrated in the oocytes of *Xenopus laevis* suggested the existence of a second voltage-dependent step that contributes to transport activity at least in these cells (Lafaire & Schwarz, 1986; Schweigert, Lafaire & Schwarz, 1988). Recently we could demonstrate that variations in the external $[K^+]$ modulate the steepness of the negative slope (Rakowski et al., 1991); this has been attributed to voltage-dependent stimulation of the pump by external $[K^+]$. Similar results were recently obtained from whole cell recordings on rabbit cardiac Purkinje cells (Biehlen et al., 1991). These authors demonstrate that the steepness of the negative slope not only depends on K^+ concentration but also on the cation species applied for pump stimulation. A negative slope can be detected if the cation concentration is below the respective K_m at −20 mV.

Detailed studies of dependence of pump current on ion composition have also been performed in experiments on Guinea pig ventricular myocytes (Gadsby, Bahinski & Nakao, 1989). This careful analysis of the data gave no indication for voltage-dependent stimulation by external $[K^+]$ in these cells. The question arises whether this discrepancy represents species differences in pump protein or rather differences in the environment in which the pump molecules operate.

Comparison of the *Torpedo* Pump with the Endogenous *Xenopus* Pump

Since the oocytes of *Xenopus* can be used as an expression system for foreign mRNA, these cells allow one to characterize pumps from different sources in the same environment. A separation of pump currents generated by the endogenous *Xenopus* pump and the *Torpedo* pump is based on determinations of the number of pump molecules in control and mRNA-injected oocytes. Comparison of the *I-V* curves for the endogenous *Xenopus* pump with the *Torpedo* pump shows that in both cases a negative slope is obtained at positive potentials (Schwarz & Gu, 1988). This negative slope is due to voltage-dependent stimulation of pump current by external $[K^+]$ (*see* Fig. 1*C*). However, the voltage dependencies of the activities of the two species of pump molecules are different (for comparison *see* Fig. 5*C*). It is obvious that description of the voltage dependence of K_m for the *Torpedo* pump requires two exponentials. The most characteristic observation is that the K_m values are higher for the *Torpedo* than for the *Xenopus* pump at all membrane potentials. This is due to a higher K_m value at 0 mV of 1.5 mM compared to 0.46 mM and to more pronounced voltage dependence (compare *Control* data in Tables 1 and 2). While the voltage dependence for the *Xenopus* pump can be described by an effective charge between 0.36 and 0.56 of an elementary charge moved during pump stimulation by $[K^+]$ (*see also* Rakowski et al., 1991), two effective charges have to be assumed for the description of voltage-dependent stimulation of the *Torpedo* pump with a dominating component of about 1 of an elementary charge. This demonstrates that Na^+/K^+ pumps from different species may show different degrees of voltage dependence, particularly as is demonstrated here for the negative-slope region of the *I-V* relationship.

A simple explanation for voltage-dependent stimulation, as has been pointed out by Rakowski et al. (1991), would be that the binding sites for the K^+ ions are located within an external vestibule of the pump molecule so that the K^+ ions must move through part of the electrical field to reach their binding sites. If, for the Na^+/K^+ pump of *Torpedo* electroplax, the binding sites were located deeper inside the pump molecule, the two K^+ ions would most likely have to move in single file to reach their binding sites through an access channel. The first K^+ ion would have to cross a larger fraction of the electric field than the second ion entering the access channel, and two different effective charges would be obtained (for illustration *see* Fig. 12). This picture would be in line with the sequential release of occluded K^+ or Rb^+ from two distinct transport sites

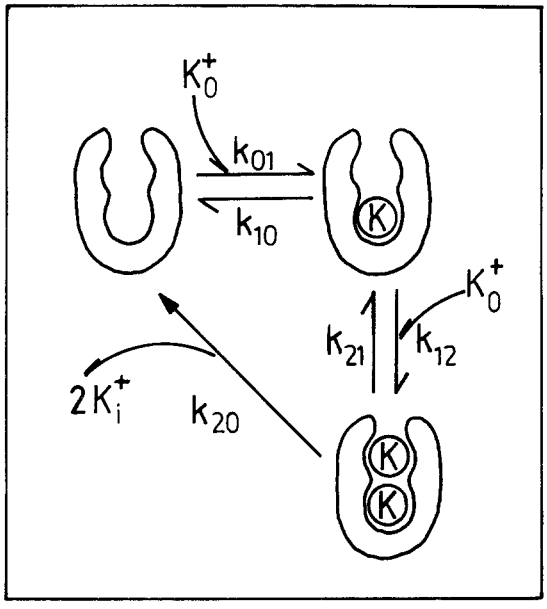

Fig. 12. Simplified reaction diagram for the pump cycle, assuming sequential binding of extracellular K^+. The k_{01} and k_{12} represent the rates of binding, the k_{10} and k_{21} of release of the first and second K^+ ion, respectively (for more details *see* Appendix). k_{20} lumps together all other transition rates involved in forward pumping.

(Forbush, 1987, 1988). Using this interpretation in the simplified reaction cycle of the foreward-running pump, K^+ binding may be described by the diagram shown in Fig. 12. Making some further simplifying assumptions (*see* Appendix), the dependence of pump current on $[K^+]$ and voltage can be described by

$$I_p = \frac{k_{20}[K^+]}{(k_{20}/k_{01}^*)\exp(z_1 VF/RT) + (k_{20}/k_{12}^*)\exp(z_2 VF/RT) + [K^+]}. \quad (6)$$

The asterisks indicate the voltage-independent factors of the respective rate constants. z_1 and z_2 represent the effective charges of the first and second K^+ ion moved during the binding step in the electrical field. Comparison with Eq. (5) yields:

$$K_m = (k_{20}/k_{01}^*)\exp(z_1 VF/RT) + (k_{20}/k_{12}^*)\exp(z_2 VF/RT) \quad (7)$$

$$A_1 = k_{20}/k_{01}^*$$

and

$$A_2 = k_{20}/k_{12}^*.$$

Therefore, the two components used to describe the voltage dependence of the K_m value of *Torpedo* pump stimulation by external $[K^+]$ may be inter-

preted by K_m values for the binding of the first and second K^+ ion. Further quantitative evaluation of the data is not performed because this would demand more accurate subtraction of endogenous pump current from total pump current than described in Results. Qualitatively, the pump molecule of *Torpedo* electroplax could represent one extreme with respect to structural species differences with a very pronounced influence of the access channel on K^+ binding. In pump molecules like those in ventricular myocytes (Nakao & Gadsby, 1989), K^+ binding sites could be closer to the membrane surface so that the K^+ ions have nearly voltage-independent access. This could explain why negative slopes in the *I-V* relationships of Na^+/K^+ pumps could not clearly be demonstrated in those preparations. The endogenous pump of *Xenopus* oocytes would then have an intermediate structure. Although the α-subunits of different species show a high degree of homology, pronounced sequence diversity is found in the N-terminal region, which has been suggested to be involved in intracellular cation binding (*see* e.g. Lingrel et al., 1990). Therefore, alternatively, species differences in pump stimulation by external K^+ could also result from differences in the cation affinity. In addition, different interactions of the *Xenopus* and of the *Torpedo* pump with the cytoskeleton, other membrane proteins or membrane lipids cannot be excluded.

Modulation of Voltage Dependence of *Xenopus* Pump Current by Protein Kinases

In addition to these suggested species differences, modulation of the voltage dependence can be achieved by stimulation of protein kinases. For the *Xenopus* pump, stimulation of protein kinase C not only leads to an endocytotic removal of pump molecules (Vasilets et al., 1990) but remaining pump molecules are reduced in their maximum pump activity (measured as pump current density in presence of 5 mM K^+; *see* Fig. 7). In addition, the voltage dependence of the K_m values of the remaining pumps becomes more pronounced (Fig. 8) and can be attributed to a reduced apparent affinity and an increase of the effective charge (Table 1) as if binding more strongly senses the electrical field. To account for regulation of pump activity by protein kinase C, direct phosphorylation of the pump molecule has been suggested (Bertorello & Aperia, 1989). Such phosphorylation could indeed be observed (Chibalin et al., 1991), and, in particular, phosphorylation of serine and threonine residues of Na^+/K^+ ATPase by protein kinase C had been demonstrated (Lowndes, Hokin-Neaverson & Bertics, 1990). As has been discussed for explaining the differences between the *Torpedo* and *Xenopus* pumps, differences in intermolecular interactions may result in modulated transport activity and K^+ binding characteristics. For example, an indirect effect involving possibly phosphorylation of cytoskeletal components (Kitamura et al., 1989; Gonda et al., 1990) could account for conformational changes in the pump molecule.

In contrast to the effect of stimulation of protein kinase C, stimulation of protein kinase A by cAMP results in stimulation of pump activity (measured as pump current density; *see* Fig. 7), and voltage-dependent stimulation by external $[K^+]$ becomes less pronounced. Opposite to the observations after stimulation of protein kinase C, the effective charge moved during K^+ binding becomes reduced as if the binding site is located less deep in the electrical field. The apparent affinity for K^+ binding at 0 mV is only slightly reduced.

Activation of the protein kinase C pathway or of the adenylate cyclase system has been considered to account in several instances for hormonal regulation of pump activity (Clausen & Flatman, 1977; Lynch et al., 1986). On the other hand, hormonal stimulation will usually also result in alterations of intracellular ion compositions and membrane potential that indirectly could modulate pump activity. In our experiments, intracellular concentration changes can be excluded due to the large size of the oocytes and changes of the membrane potential can be ruled out since voltage clamp was applied. The data suggest direct modification of the pump molecule or of cellular components that interact with the pump leading to modulation of pump activity and K^+ binding and that stimulation of protein kinase C and A exhibit opposite effects.

Modulation of Voltage Dependence of *Torpedo* Pump Current by Protein Kinases

To obtain information on the *Torpedo* pump, current generated by the endogenous *Xenopus* pump was subtracted from total pump current. The parameters for the voltage-dependent K^+ binding obtained for the *Torpedo* pump, of course, depend on subtraction of the correctly estimated current generated by the *Xenopus* pump. Variations in the ratio of the numbers of *Xenopus* and *Torpedo* pumps modify the absolute values of these parameters, but qualitative interpretations are still possible. An increase of effective charge, at least for the component that is more sensitive to the electrical field, is obtained after injection of diC_8, and after injection of cAMP a decrease of the K_m value at 0 mV for pump stimulation by external $[K^+]$ is obtained.

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References

Bertorello, A., Aperia, A. 1989. Na^+-K^+-ATPase is an effector protein for protein kinase C in renal proximal tubule cells. *Am. J. Physiol.* **256:**F370–F373

Biehlen, F.V., Glitsch, H.G., Verdonck, F. 1991. The dependence of sodium pump current on extracellular monovalent cations in isolated rabbit cardiac Purkinje cells. *J. Physiol.* **442:**169–180

Chibalin, A.V., Lopina, O.D., Petukhov, S.P., Vasilets, L.A. 1991. Phosphorylation of Na,K-ATPase by proteinkinase C and cAMP-dependent protein kinase. *Biol. Membr. (in press)*

Clausen, T., Flatman, J.A. 1977. The effect of catecholamines on Na-K transport and membrane potential in rat soleus muscle. *J. Physiol.* **270:**383–414

DeWeer, P., Gadsby, D.C., Rakowski, R.F. 1988. Voltage dependence of the Na-K pump. *Annu. Rev. Physiol.* **50:**225–241

Dumont, J.N. 1972. Oogenesis in *Xenopus laevis* (Daudin): I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* **136:**153–180

Eisner, D.A., Valdeomillos, M., Wray, S. 1987. The effects of membrane potential on active and passive sodium transport in *Xenopus* oocytes. *J. Physiol.* **385:**643–659

Forbush, B. 1987. Rapid release of 42K or 86Rb from two distinct transport sites on the Na,K-pump in the presence of P_i or vanadate. *J. Biol. Chem.* **262:**11116–11127

Forbush, B. 1988. The interaction of amines with the occluded state of the Na,K-pump. *J. Biol. Chem.* **263:**7979–7988

Gadsby, D.C. 1984. The Na/K pump of cardiac cells. *Annu. Rev. Biophys. Bioenerg.* **13:**373–398

Gadsby, D.C., Bahinski, A., Nakao, M. 1989. Voltage dependence of Na/K pump current. *Curr. Top. Membr. Transp.* **34:**269–288

Geering, K., Theulaz, I., Verry, F., Haeuptle, M.T., Rossier, B.C. 1989. A role for the β-subunit in the expression of functional Na^+-K^+-ATPase in *Xenopus* oocytes. *Am. J. Physiol.* **257:**C851–C858

Gonda, Y., Nishizawa, K., Ando, S., Kitamura, S., Minoura, Y., Nishi, Y., Inagaki, M. 1990. Involvement of protein kinase C in the regulation of assembly-disassembly of neurofilaments in vitro. *Biochem. Biophys. Res. Commun.* **167:**1316–1325

Grygorczyk, R., Hanke-Baier, P., Schwarz, W., Passow, H. 1989. Measurements of erythroid band 3 protein-mediated anion transport in mRNA-injected oocytes of *Xenopus laevis*. *Methods Enzymol.* **173:**453–466

Kitamura, S., Ando, S., Shibata, M., Tanabe, K., Sato, C., Inagaki, M. 1989. Protein kinase C phosphorylation of desmin at four serine residues within the non-Á-helical head domain. *J. Biol. Chem.* **264:**5674–5678

Lafaire, A.V., Schwarz, W. 1985. Voltage-dependent, ouabain-sensitive current in the membrane of oocytes of *Xenopus laevis*. *In:* The Sodium Pump: 4th International Conference on Na,K-ATPase. J.M. Glynn and J.C. Ellory, editors. pp. 523–525. The Company of Biologists, Cambridge (UK)

Lafaire, A.V., Schwarz, W. 1986. Voltage dependence of the rheogenic Na^+/K^+ ATPase in the membrane of oocytes of *Xenopus laevis*. *J. Membrane Biol.* **91:**43–51

Lingrel, J.B., Orlowski, J., Shull, M.M., Price, E.M. 1990. Molecular-genetics of Na,K-ATPase. *Prog. Nucl. Acid Res. M. B.* **38:**37–89

Lowndes, J.M., Hokin-Neaverson, M., Bertics, P.J. 1990. Kinetics of phosphorylation of Na^+/K^+-ATPase by protein kinase-C. *Biochim. Biophys. Acta* **1052:**143–151

Lynch, C.J., Wilson, P.B., Blackmore, P.F., Exton, J.H. 1986. The hormone-sensitive hepatic Na^+-pump: Evidence for regulation by diacylglycerol and tumor promotors. *J. Biol. Chem.* **261:**14551–14556

Nakao, M., Gadsby, D.C. 1986. Voltage dependence of Na translocation by the Na/K pump. *Nature* **323:**628–630

Nakao, M., Gadsby, D.C. 1989. [Na] and [K] dependence of the Na/K pump current-voltage relationship in Guinea pig ventricular myocytes. *J. Gen. Physiol.* **94:**539–565

Rakowski, R.F., Gadsby, D.C., DeWeer, P. 1989. Stoichiometry and voltage dependence of the sodium pump in voltage-clamped, internally dialyzed squid giant axon. *J. Gen. Physiol.* **93:**903–941

Rakowski, R.F., Paxson, C.L. 1988. Voltage dependence of Na/K pump current in *Xenopus* oocytes. *J. Membrane Biol.* **106:**173–182

Rakowski, R.F., Vasilets, L.A., LaTona, J., Schwarz, W. 1991. A negative slope in the current-voltage relationship of the Na^+/K^+ pump in *Xenopus* oocytes produced by reduction of external $[K^+]$. *J. Membrane Biol.* **121:**177–187

Richter, H.-P., Jung, D., Passow, H. 1984. Regulatory changes of membrane transport and ouabain binding during progesterone-induced maturation of *Xenopus* oocytes. *J. Membrane Biol.* **79:**203–210

Schmalzing, G., Kröner, S., Passow, H. 1989. Evidence for intracellular sodium pumps in permeabilized *Xenopus laevis* oocytes. *Biochem. J.* **260:**395–399

Schmalzing, G., Omay, H., Kröner, S., Appelhans, H., Schwarz, W. 1991. Expression of exogenous β1 subunits of Na,K pump in *Xenopus laevis* oocytes raises pump activity. *In:* The Sodium Pump: Recent Developments. P. DeWeer and J.H. Kaplan, editors. pp. 55–59. Rockefeller University Press, New York

Schwarz, W., Gu, Q. 1988. Characteristics of the Na^+/K^+-ATPase from *Torpedo californica* expressed in *Xenopus* oocytes: A combination of tracer flux measurements with electrophysiological measurements. *Biochim. Biophys. Acta* **945:**167–174

Schwarz, W., Vasilets, L.A. 1991. Variations in the negative slope of the current-voltage relationship of the Na^+/K^+ pump in *Xenopus* oocytes. *In:* The Sodium Pump: Structure, Mechanism, and Regulation. P. DeWeer and J.H. Kaplan, editors. pp. 327–338. Rockefeller University Press, New York

Schweigert, B., Lafaire, A.V., Schwarz, W. 1988. Voltage dependence of the Na-K ATPase: Measurements of ouabain-dependent membrane current and ouabain binding in oocytes of *Xenopus laevis*. *Pfluegers. Arch.* **412:**579–588

Stein, W.D. 1976. An algorithm for writing down flux equations for carrier kinetics, and its application to co-transport. *J. Theor. Biol.* **62:**467–478

Vasilets, L.A., Maedefessel, K., Schmalzing, G., Schwarz, W. 1991. Inhibition of the Na^+/K^+ pump in *Xenopus* oocytes by diacylglycerol analogs. *In:* The Sodium Pump: Recent Devel-

opments. P. DeWeer and J.H. Kaplan, editors. pp. 89–93. Rockefeller University Press, New York

Vasilets, L.A., Schmalzing, G., Mädefessel, K., Haase, W., Schwarz, W. 1990. Activation of protein kinase C by phorbol ester induces down regulation of the Na^+/K^+-ATPase in oocytes of *Xenopus laevis*. *J. Membrane Biol.* **118:**131–142

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Appendix

For the description of the potential dependence of the pump cycles that involves potential-dependent K^+ binding, the reaction scheme presented in Fig. 12 has been assumed. The assumption of sequential binding sites in an access channel is in line with the observation by Forbush (1987) of sequential cation release of occluded K^+ or Rb^+ to the external medium. The dependence on $[K^+]$ and membrane potential of the rate constants for the K^+ movements is described by

$$k_{01} = [K^+]\, k_{01}^* \exp(-z_1 VF/RT)$$

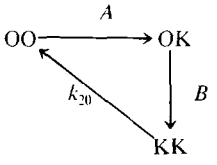
$$k_{10} = k_{10}^* \exp(y_1 VF/RT)$$

$$k_{12} = [K^+]\, k_{12}^* \exp(-z_2 VF/RT)$$

$$k_{21} = k_{21}^* \exp(y_2 VF/RT). \tag{A1}$$

The asterisks indicate the potential-independent rate constants, the z_i, y_i are effective charges and represent the fraction of potential drop acting on the transition of the K^+ ion from the energy minimum to the energy maximum. k_{20} lumps together all rate constants leading to final completion of the reaction cycle.

This scheme may be reduced to a simple cycle diagram (Stein, 1976):



Transport activity I is then represented by

$$I = \frac{k_{20}}{1 + k_{20}/B + k_{20}/A} \tag{A2}$$

with

$$B = \frac{k_{12}\, k_{20}}{k_{21} + k_{20}} \tag{A3}$$

$$A = \frac{k_{01}\, B}{k_{10} + B} = \frac{k_{01} \cdot k_{12} \cdot k_{20}}{k_{10}k_{21} + k_{10}k_{20} + k_{12}k_{20}}. \tag{A4}$$

Release of the cation bound first has been demonstrated to be a slow process; therefore, we assume that terms having k_{10}^* as parameter can be neglected. For further simplification, we make the assumptions that binding of the first K^+ ion strongly facilitates binding of the second, which allows us to neglect terms with k_{21}/k_{12}.

This leads to

$$I = \frac{k_{20}[K^+]}{[K^+] + (k_{20}/k_{01}^*)\exp(z_1 VF/RT) + (k_{20}/k_{12}^*)\exp(z_2 VF/RT)}. \tag{A5}$$

This equation represents the same expression as Eq. (5) which was used to describe the voltage dependence of the K_m value for stimulation of the *Torpedo* pump by external K^+ (*see* Eq. (7)).