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Voltage-dependent inhibition of the Na^+,K^+ pump by tetraethylammonium

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Abstract

Tetraethylammonium (TEA^+) is an effective inhibitor of a variety of K^+ channels, and has been widely used to reduce K^+-sensitive background conductances in electrophysiological investigations of the Na^+,K^+-ATPase. Here we demonstrate by combination of two-electrode voltage clamp (TEVC) and giant patch clamp of *Xenopus* oocytes, and measurements of the activity of purified ATPase of pig kidney that TEA^+ directly inhibits the Na^+,K^+-ATPase from the outside. The K_I value in TEVC experiments at 0 mV is about 10 mM increasing with more negative potentials. A similar voltage-dependent inhibition by TEA^+ was observed in the excised membrane patches except that the apparent K_I value at 0 mV is about 100 mM, a value nearly identical to that found for inhibition of purified kidney ATPase. The voltage-dependent inhibition can be described by an effective valency of 0.39 and is attributed to an interference with the voltage-dependent binding of K^+ at an external access channel. The apparent dielectric length of the access channel for K^+ is not affected by TEA^+.

Keywords: TEA; Na^+,K^+-ATPase; Oocyte; Pump current; Ouabain; (*Xenopus*)

1. Introduction

The Na^+,K^+ pump or Na^+,K^+-ATPase maintains the electrochemical gradients for Na^+ and K^+ across the membrane of animal cells by transporting three Na^+ ions out of the cell and two K^+ ions into the cell per ATP molecule hydrolysed. Due to a fixed $3Na^+/2K^+$ stoichiometry, the pump generates an outward-directed current of positive charges that can be measured under voltage-clamp conditions and serves as a measure of pump activity. To extract the current generated by the pump from total membrane current, the pump current was either blocked by specific inhibitors of the Na^+,K^+-ATPase such as cardiac glycosides, or activated by application of extracellular K^+. The voltage-clamp technique has been proven to be a particularly powerful method for functional investigation of the Na^+,K^+ pump [1,2]. What makes detection and analysis of pump current possible is a high density of pump molecules in certain types of cell membranes, and thus extensive investigations have been performed for more than 10 years with cells such as squid giant axon

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[3,4], cardiac cells [5–8] or *Xenopus* oocytes [9–11]. More recently, expression systems have become available and are now also used for electrophysiological studies on overexpressed Na^+,K^+-ATPases [12–14]. Even with a high density of pump molecules in the cell membrane, it is, nevertheless, essential to eliminate the background currents, which otherwise may complicate data interpretation. For instance, changes in the pump activity can lead to local changes in K^+ concentrations that in turn modify K^+ conductance and may lead to misinterpretation [15]. Therefore, to determine pump-mediated current based on the difference between total membrane current with active and with inhibited pumps, all other K^+-sensitive currents should be blocked. This can be achieved by addition of tetraethylammonium (TEA^+) and/or Ba^{2+}. On the other hand, it has been reported that TEA^+ directly [16–18] or TEA^+-sensitive K^+ currents [19] interfere with pump activity. We now present evidence showing that application of TEA^+ reduces the pump-mediated current in a voltage-dependent manner in *Xenopus* oocytes, and that the inhibition is a direct effect of TEA^+ on the pump.

2. Methods

The methods of oocyte preparation, measuring ouabain binding and voltage clamp are similar to those described previously [9,11,20].

2.1. Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetised with 1 g/l *m*-aminobenzoic acid ethylester methane sulfonate (MS222, Sandoz, Basel (Switzerland)). Parts of the ovary were removed and treated with collagenase. Full-grown prophase-arrested oocytes were selected for experiments. The cells were stored at 19°C in the oocyte Ringer's solution ORi (see Section 2) supplemented with antibiotics (in mg/l: 70 gentamicin or 25 streptomycin plus 20 penicillin). Experiments were performed after 1 to 5 days of incubation. Before two-electrode voltage-clamp and ouabain-binding experiments, oocytes were loaded with Na^+ by incubation in a '*Na-loading*' solution (see Section 2) for about 40 min. This procedure leads to an increase in cytoplasmic [Na^+] to more than 80 mM [21], a concentration that stimulates the pump maximally. After the Na^+ loading, oocytes were kept for at least 30 min in K^+-free Ringer's solution before being used in experiment.

Most experiments were done on the endogenous Na^+,K^+ pump of *Xenopus* oocytes, some also on the pump of electroplax of *Torpedo californica* expressed in the oocytes after injection of cRNA for the α and β subunit [12].

2.2. Measurements of ouabain binding

The number of ouabain binding sites represents the number of pump molecules per oocyte, and was determined by incubating Na^+-loaded oocytes for 20 min in K^+-free solution containing 2.5 μM [^{3}H]ouabain and 2.5 μM cold ouabain (see [22]).

2.3. Voltage-clamp experiments

Electrophysiological experiments were performed either on intact oocytes with the conventional two-electrode voltage clamp (TEVC) (see e.g. [9]) or on excised membrane patches with the giant patch technique using pipettes with tip diameter of 25–35 μm (see [23,24]). The protocol for characterisation of the Na^+ pump was the same as described previously [11]. In brief, steady-state membrane currents were recorded at the end of 200-ms rectangular voltage pulses (from −150 up to +50 mV in 10-mV increments) that were applied from a holding potential of −60 mV, and voltage dependencies were determined from the membrane currents at steady state. The current generated by the Na^+,K^+-ATPase was determined from the current either activated by extracellular K^+ or inhibited by 10 μM ouabain. Over the range of potentials applied in our investigations, the currents determined by these two assays are identical (see also [11]).

For analysis of the voltage-dependent stimulation of the pump by external K^+, current-voltage dependencies were determined at different external K^+ concentrations of 0.05, 0.1, 0.25, 0.5, 1 and 5 mM in Na^+-free solution. The concentration dependence of

pump current at different membrane potentials E was fitted by the equation:

$$I(E) = I_{max}\frac{[K^+]}{K^*_m(E) + [K^+]} \quad (1)$$

and the voltage dependence of apparent K^*_m value was determined by fitting the sum of two exponentials to the data:

$$K^*_m(E) = K_{m1}e^{z_1 EF/RT} + K_{m2}e^{z_2 EF/RT}. \quad (2)$$

An interpretation of this voltage dependency is that the external K^+ ions transported into the cell have to pass through an access channel within the pump molecule sensing a fraction z_i of the electric field [11,25]. For details see Appendix.

2.4. Measurements of ATPase activity

In addition to the determinations of transport activity, ATPase activity of purified Na^+,K^+-ATPase was measured in the presence and absence of different TEA^+ concentrations. The Na^+,K^+-ATPase was purified from the outer medulla of pig kidneys using the Procedure C of Jørgensen [26] with addition of 3 mM dithiothreitiol (DTT) during all purification steps. The ATPase activity was determined spectroscopically by the pyruvate kinase/lactate dehydrogenase assay [27]. The reaction mixture contained (in mM): 50 NaCl, 2 KCl, 5 $MgCl_2$, 25 imidazole (pH 7.2), 3 ATP (Tris salt), 2 phosphoenol pyruvate, different (TEA)Cl concentrations ranging from 5 to 200 mM, and also pyruvate kinase (660 U/ml) and NADH (0.32 mM) (both from Sigma) in a final volume of 0.8 ml. The reaction was started by addition of 2 μg of purified Na^+,K^+-ATPase.

2.5. Solutions

The solutions had the following compositions unless stated otherwise (in mM):

ORi: 90 NaCl, 2 KCl, 2 $CaCl_2$, 5 MOPS/Tris (adjusted to pH 7.4).

Na^+-loading solution: 110 NaCl, 2.5 sodium citrate, 5 MOPS/Tris (adjusted to pH 7.6).

Na^+-free test solutions: 90 tetramethylammonium (TMA^+) chloride, 0 to 5 KCl, 10 or 20 $BaCl_2$, 2 $NiCl_2$, 5 MOPS/Tris (adjusted to pH 7.8).

In the Na^+-*containing test solution* 50 mM (TMA)Cl was replaced by 50 mM NaCl, and either 0 or 2 mM KCl were present.

For the giant patch-clamp experiments the composition of the pipette and bath solutions were designed to mimic the ionic conditions for intact cells at the internal and external membrane surfaces.

Pipette solutions:

(Inside-out patch): 50 NaCl, 40 (TMA)Cl, 2 KCl, 10 $BaCl_2$, 2 $NiCl_2$, 5 MOPS/Tris (adjusted to pH 7.6).

(Outside-out patch): 90 NaCl, 10 KCl, 5 $MgCl_2$, 2 Na_2ATP, 2 EGTA, 5 MOPS/Tris (adjusted to pH 7.4).

Bath solutions:

(Inside-out patch): 90 NaCl, 10 KCl, 5 $MgCl_2$, 0 or 2 Na_2ATP, 2 EGTA, 5 MOPS/Tris (adjusted to pH 7.4).

(Outside-out patch): 50 NaCl, 40 (TMA)Cl, 0 or 2 KCl, 10 $BaCl_2$, 2 $NiCl_2$, 5 MOPS/Tris (adjusted to pH 7.6).

If solutions contained (TEA)Cl, it replaced corresponding concentrations of (TMA)Cl. All external test solutions contained $BaCl_2$ to reduce currents through K^+-selective channels (see [18]). The Ni^{2+} was added to block Na^+/Ca^{2+} exchange. To reduce background currents mediated by Ca^{2+}-activated channels (Cl^- channels), Ca^{2+} was omitted from the test solutions (see [28,29]). In the nominally Na^+- and K^+-free solutions, the actual concentrations were below 5 μM as determined by flame photometry.

3. Results

3.1. Two-electrode voltage-clamp measurements

To judge whether TEA^+ possibly interacts with the extracellular binding of Na^+ or K^+ to the pump molecule, we chose for our standard conditions 50 mM Na^+ and 2 mM K^+ in the external solutions. Under these conditions inhibition of the pump by external Na^+ and stimulation by external K^+ are strongly dependent on membrane potential [11,21]. These dependencies, therefore, may serve as sensitive parameters to assess effects of TEA^+.

A typical set of current-voltage dependencies in the

presence and absence of ouabain, and in the presence and absence of TEA^+ is illustrated in Fig. 1A. To confirm the reversibility of inhibition and to correct for possible drifts in membrane current, measurements were always performed first in the absence of

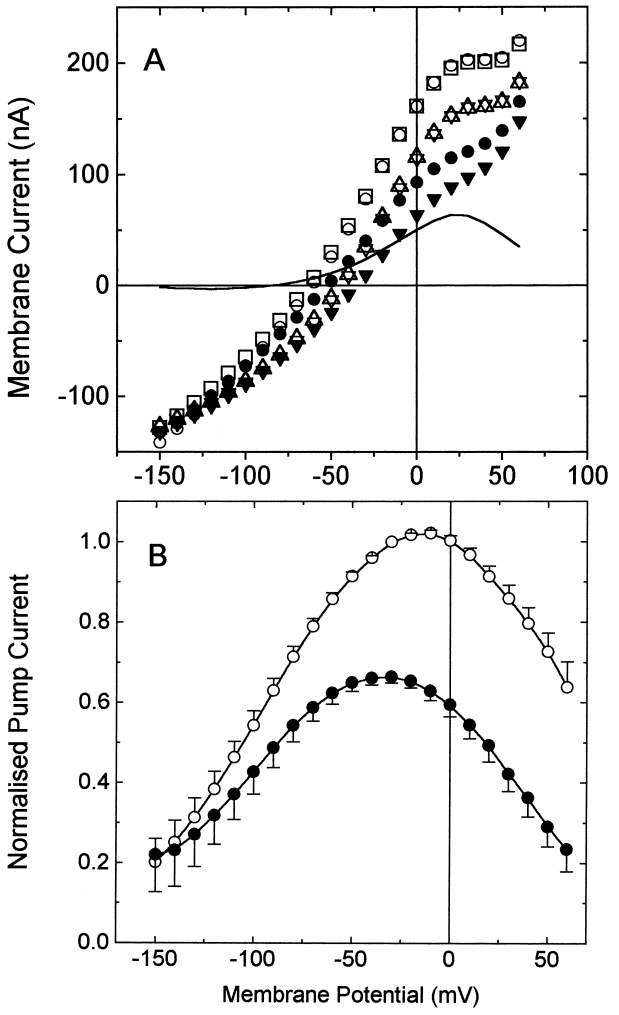

Fig. 1. Voltage dependence of membrane and pump currents, and inhibition by 20 mM TEA^+. A: Total membrane currents in the absence (circles and squares) and the presence (triangles) of 10 μM ouabain measured in a single oocyte. The open symbols represent data in the absence, and the filled symbols in the presence of 20 mM TEA^+. In the presence of TEA^+, 20 mM TMA^+ was replaced by TEA^+. 50 mM NaCl and 2 mM KCl was present in all solutions. The solid line represents the current blocked by TEA^+ in the presence of ouabain. B: Averaged current-voltage relations for pump current in the absence (open symbols) and presence (filled symbols) of 20 mM TEA^+. The current was determined as the difference of membrane current in the presence and absence of 10 μM ouabain as illustrated in A. Data represent averages of eight experiments ±S.E.M. All experiments were performed in the presence of 10 mM $BaCl_2$.

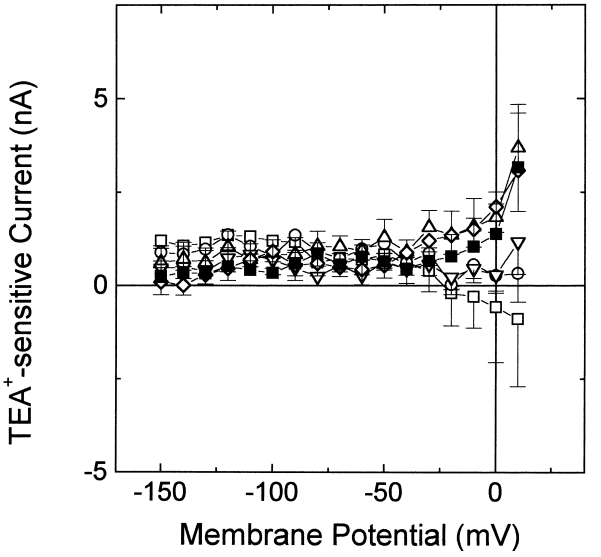

Fig. 2. Effect of TEA^+ on membrane current in the presence of Ba^{2+} and ouabain. Current-voltage dependencies determined as the difference of current in the absence and the presence of different concentrations of TEA^+: squares 1 mM, circles 2 mM, triangles up 5 mM, triangles down 10 mM, diamonds 20 mM, filled squares 40 mM. Solutions were Na^+-free and contained 5 mM KCl, 20 mM $BaCl_2$ and 10 μM ouabain. Data represent averages from 11 experiments ±S.E.M.

TEA^+, then in the presence, and again in the absence. For the absence of TEA^+ (open symbols), the figure shows the two sets of data that were obtained before and after the application of 20 mM TEA^+. These two sets of currents are nearly identical and demonstrate the reversibility of the inhibition of membrane current by TEA^+. Current related to the Na^+ pump was determined as the current component inhibited by 10 μM ouabain. The figure illustrates that the pump current in the presence of TEA^+ (difference between filled symbols) is smaller than the pump current in the absence of TEA^+ (difference between open symbols). Fig. 1B summarises the voltage dependencies of pump currents averaged from eight experiments. In the presence of 20 mM TEA^+, the ouabain-sensitive current is reduced. This inhibition increases with more positive potentials exceeding 60% at +50 mV. The data are normalised to the control pump current at −30 mV; at this potential, 20 mM TEA^+ reduces the current from 47 nA to 31 nA. In addition to its effect on pump current, TEA^+ blocks another current component as illustrated by the data obtained in the presence of ouabain (see Fig. 1A). The reversal potential

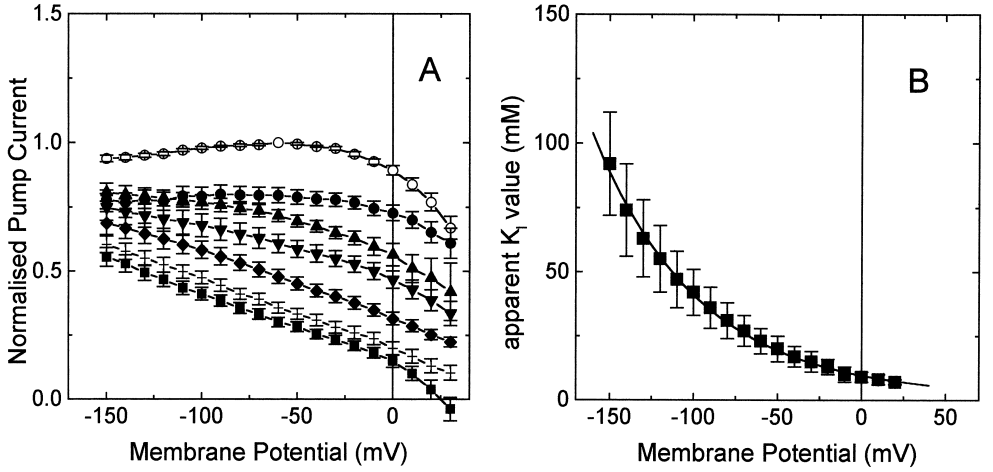

Fig. 3. Voltage dependence of pump current and its potential-dependent inhibition by TEA^+. A: Voltage dependence of pump current that was determined as current activated by 0.5 mM K^+ in Na^+-free solution with 100 mM (TMA)Cl and 20 mM $BaCl_2$, and in the absence (○) or presence of TEA^+: ●, 1 mM; ▲, 5 mM; ▼, 10 mM; ♦, 20 mM; +, 50 mM; ■, 100 mM. Data represent averages from ten experiments ±S.E.M. B: Voltage dependence of apparent K_I values (50% inhibition) for inhibition of pump current by TEA^+. The K_I values were determined from the [TEA^+] dependency at the respective membrane potential from the data presented in A. The solid line represents a fit of $K_I = K_{1/2} \times \exp(-z_{TEA}EF/RT) + K_\infty$ (fit parameters $K_{1/2} = 8.1 \pm 0.8$ mM, $z_{TEA} = 0.39 \pm 0.01$, $K_\infty = 1.6 \pm 1.2$ mM).

of this current at −80 to −90 mV (see solid line in Fig. 1A) suggests that K^+ is the charge carrier although the solutions contained 10 mM $BaCl_2$. Increasing the Ba^{2+} concentration to 20 mM leads to inhibition of this current to less than 1 nA. Since the goal was to investigate possible effects of TEA^+ on the Na^+,K^+ pump and since it was shown that TEA^+ blocks other K^+-sensitive currents in the oocytes [18,19], all further experiments were done in the presence of 20 mM $BaCl_2$ to remove all contributions from K^+-selective channels. TEA^+ by itself had only negligible effects on other membrane currents in the presence of 20 mM Ba^{2+}. If the pump is blocked by ouabain, the difference between membrane current in the absence and presence of the TEA^+ is less than 5 nA over the entire potential range investigated as shown in more detail in Fig. 2.

Since the inhibition becomes more pronounced at more positive potentials and since at these potentials the activity of the Na^+ pump is dominated by potential-dependent stimulation by external K^+ [11], we investigated the inhibition by TEA^+ in more detail under conditions where the stimulation by K^+ becomes particularly visible, i.e. in Na^+-free solution with 0.5 mM K^+. Voltage dependencies of the pump current were determined under these conditions in the presence of different TEA^+ concentrations ranging from 0 to 100 mM (Fig. 3A). The apparent K_I value for inhibition by TEA^+ was determined for each membrane potential according to

$$I_{pump}([TEA]) = I_{max}\frac{K_I^*}{K_I^* + [TEA]}. \quad (3)$$

The voltage dependence of the K_I^* values (Fig. 3B) can be described by a potential-independent component of $K_\infty = 1.6$ mM plus an exponentially dependent component with a $K_{1/2}(0\ mV)$ of about 8.1 mM and an effective valence z_{TEA} of about 0.39 as if a positive charge has to be moved in an outward direction through part of the electric field during the voltage pulse. This means that a movement of TEA^+ into an access channel cannot account for the voltage dependence.

A very sensitive parameter for detecting changes in pump activity has turned out to be the apparent K_m value for pump stimulation by external K^+ [30,31]. To calculate the K_m values, pump current was determined in Na^+-free solution and in the presence of different concentrations of external K^+ in the absence (Fig. 4A) or presence (Fig. 4B) of 20 mM TEA^+. Pump current at 5 mM K^+ was determined at the beginning and at the end of each experiment; the nearly identical values illustrate the reliability of

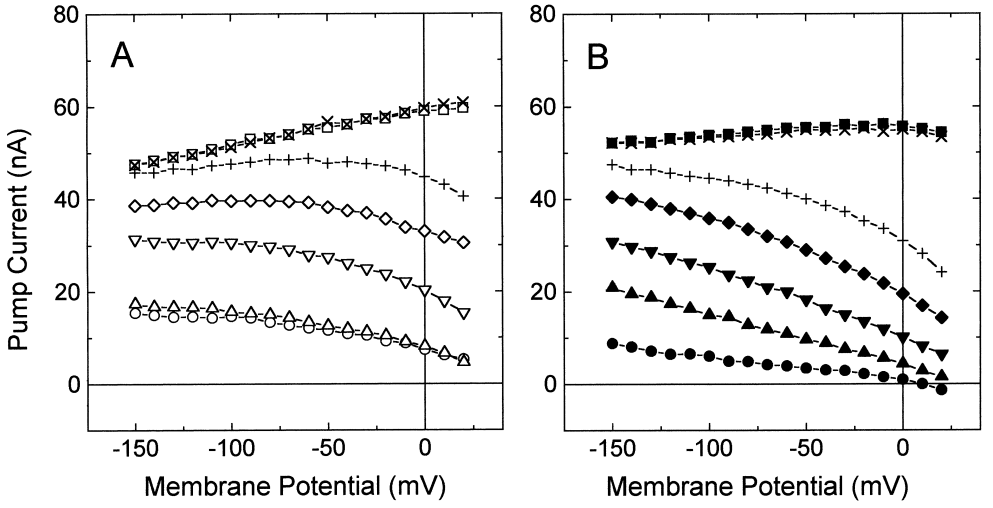

Fig. 4. Voltage dependence of pump current at different external K^+ concentrations in the absence (A) and presence (B) of 20 mM TEA^+. Data were obtained from the same oocyte first in the absence, then in the presence of TEA^+. All measurements were made in Na^+-free solutions. Pump currents were determined as current activated by different K^+ concentrations (squares, crosses: 5 mM, plus signs: 1 mM, diamonds: 0.5 mM, triangles down: 0.25 mM, triangles up: 0.1 mM, circles: 0.05 mM). The double determinations at 5 mM were obtained from measurements at the beginning and at the end of the respective experiment.

the experiments lasting for about one hour. The apparent K_m values were determined according to Eq. 1 for each membrane potential. In the presence of TEA^+, the K_m values are elevated over the entire potential range (Fig. 5) as if the TEA^+ counteracts the interaction of K^+ with the pump molecule thus decreasing its apparent affinity. As in our previous investigations on pumps expressed in the oocytes, the voltage dependencies of K_m were fitted by a double exponential (Eq. 2). TEA^+ affects only the component with the more pronounced voltage dependence by increasing the K_{m1} value at 0 mM from 0.31 mM to 0.63 mM; the voltage dependence, represented by the effective valence z_1, is hardly affected (see legend to Fig. 5). The voltage dependence of inhibition by TEA^+ and the change of the voltage-dependent K_m

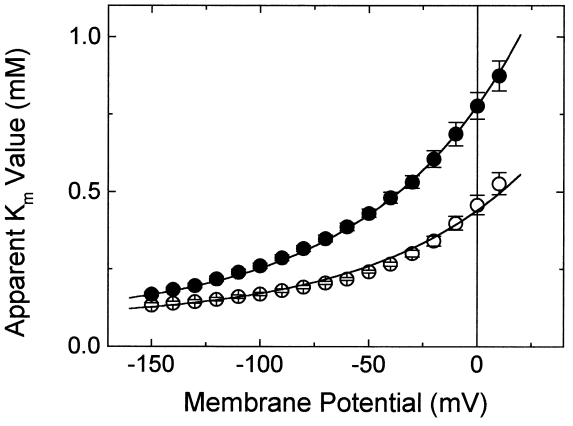

Fig. 5. Effect of TEA^+ on voltage dependence of apparent K_m value for pump stimulation by external K^+. The values were obtained from current-voltage dependencies measured in solutions without external Na^+ and with different K^+ concentrations as illustrated in Fig. 4. Open symbols refer to data obtained without, filled symbol with 20 mM TEA^+. Data represent averages of seven experiments ± S.E.M. The solid lines are fits of Eq. 2 to the data with the following parameters: For control: $K_{m1}(0) = 0.31+0.01$, $K_{m2}(0) = 0.14+0.01$, $z_1 = 0.38+0.02$, $z_2 = 0.09+0.01$; for 20 mM TEA: $K_{m1}(0) = 0.63+0.01$, $K_{m2} = 0.14+0.01$, $z_1 = 0.34+0.01$, $z_2 = 0.09+0.01$.

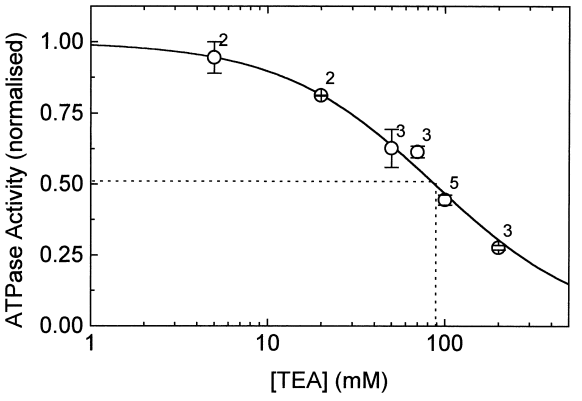

Fig. 6. Inhibition of ATPase activity by TEA^+. Data represent normalised average values ± S.E.M. with the number of experiments indicated by the numbers near the symbols. The solid line represents a least-squares fit of Eq. 3 to the data with a K_I value of 88 ± 5 mM.

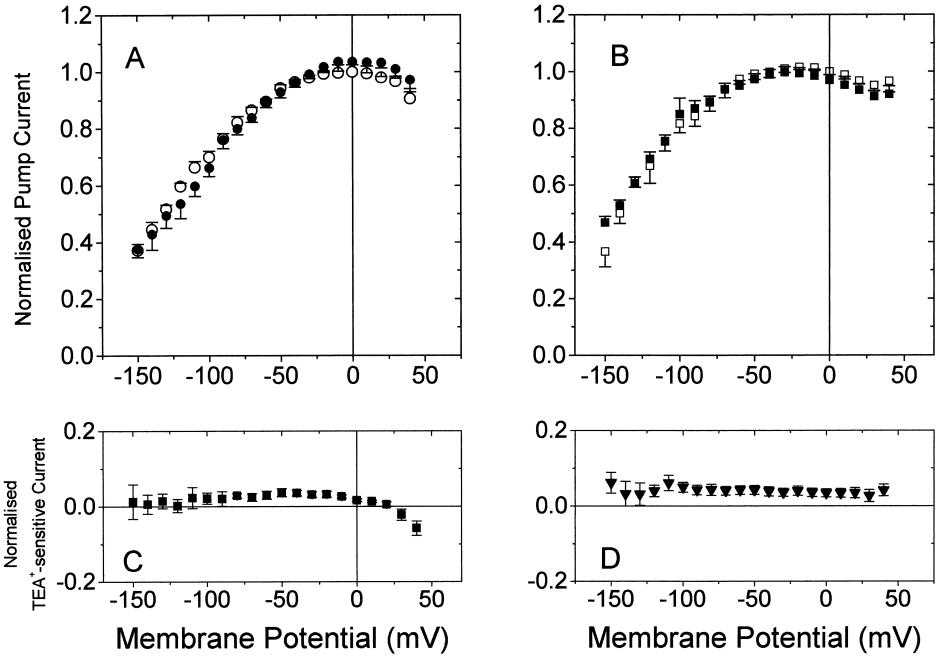

Fig. 7. Current-voltage dependencies in outside-out (A, C) and inside-out (B, D) giant membrane patches. In outside-out configuration, pump current (A) was determined as current activated by 2 mM external K^+ in solution with 50 mM Na^+. In inside-out configuration, pump current (B) was determined as current activated by 2 mM Na_2ATP. C and D represent the current difference in the absence and presence of 20 mM TEA^+ in the external or internal medium, respectively, with the pump blocked by 10 μM ouabain. Data represent averages of seven and eight experiments, respectively, and are normalised to pump current in the absence of TEA^+ at 0 mV.

value for pump stimulation by external K^+ are indications for an external site of action for the TEA^+ affecting K^+ binding.

3.2. ATPase activity measurements

Inhibition of ATPase activity by TEA^+ has been reported for microsomal preparations of rat brain [17]. To exclude the possibility of inhibition of a K^+-selective channel associated with the pump [19], we performed measurements of ATPase activity of purified Na^+,K^+-ATPase from pig kidney. Fig. 6 shows that ATPase activity decreases with increasing concentration of TEA^+. The K_I value of about 90 mM is clearly higher than that obtained for inhibition of pump current, but is compatible with the value reported for inhibition of ATPase activity in the microsomal preparations [17].

3.3. Patch-clamp experiments

It has been suggested previously that TEA^+ can permeate the oocyte membrane [19]. Measurements

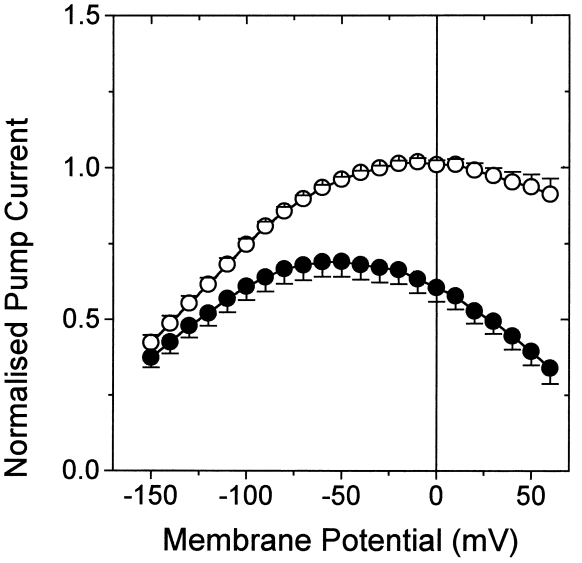

Fig. 8. Effect of TEA^+ on current mediated by expressed pumps of *Torpedo* electroplax. Pump current was determined as the current activated by 2 mM external K^+ in a solution containing 50 mM Na^+ in the absence (○) and presence (●) of 20 mM TEA^+. Data represent averages from nine experiments ± S.E.M.

with TEA^+-sensitive microelectrodes support this view (data not shown). Therefore, the question arose whether the effect of TEA^+ on pump current is mediated from the external medium or from the cytoplasm. Though the above results, and the fast onset and recovery of the effects in the time range of solution exchange support an external access site, we performed measurements of K^+- and TEA^+-sensitive currents in excised inside-out (Fig. 7A, C) and outside-out (Fig. 7B, D) giant membrane patches. Surprisingly, in contrast to the measurements on intact oocytes, 20 mM TEA^+ has no significant effect on the pump current in either configuration (Fig. 7A, B). In line with the results obtained with the two-electrode voltage clamp, TEA^+ has also no effect on other membrane currents (Fig. 7C, D). The lack of effect cannot be due to the ionic composition of the solutions used because in these giant patch-clamp experiments, we applied extracellularly the same solutions as in the two-electrode clamp experiments, and we tried to mimic the cytoplasmic conditions as close as possible.

A possible difference between the two types of experiments is that the two-electrode measurements were done with the endogenous Na^+,K^+ pump of the *Xenopus* oocytes while the patch-clamp experiments were done with the pumps of *Torpedo* electroplax expressed in the oocytes. But the *Torpedo* pump current was also blocked by TEA^+ when measured in intact oocytes (Fig. 8). Similarly to the endogenous

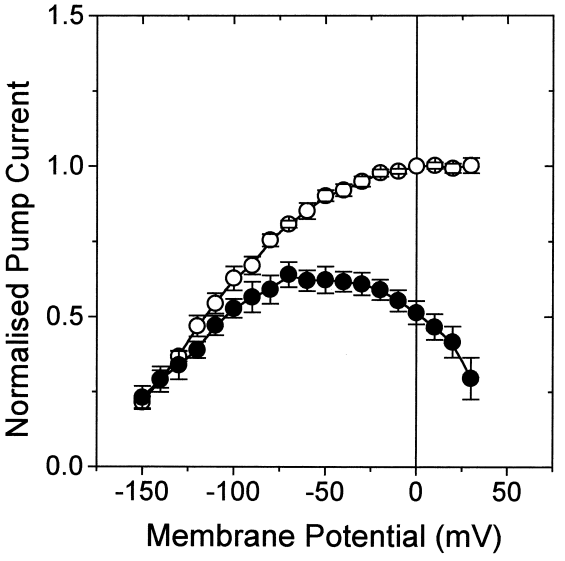

Fig. 9. Effect of 100 mM extracellular TEA^+ on pump current in outside-out membrane patches. Pump current was determined as the current activated by 2 mM external K^+ in a solution containing 50 mM Na^+ without (○) or with 100 mM TEA^+ (●). Data represent averages of seven and nine experiments, and are normalised to pump current in the absence of TEA^+ at 0 mV.

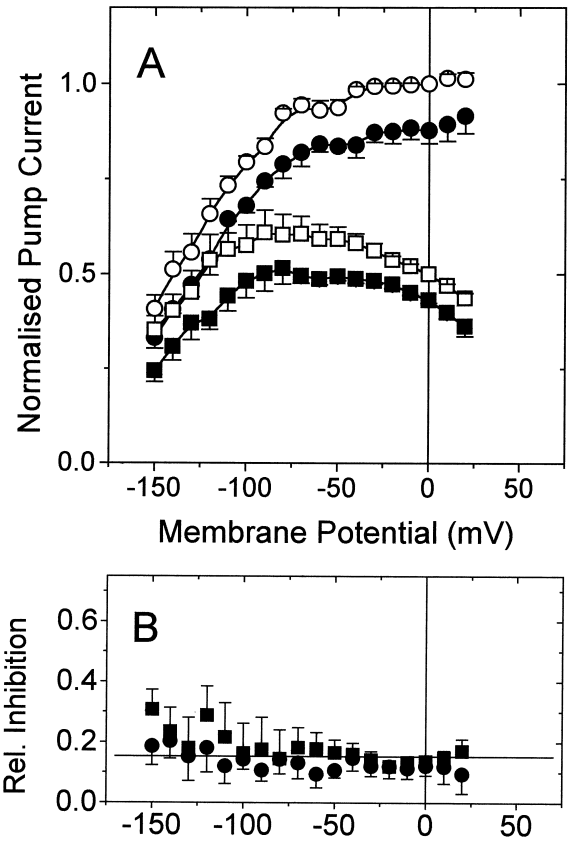

Fig. 10. Effect of 100 mM intracellular TEA^+ on pump current in inside-out membrane patches in the absence or presence of 100 mM extracellular TEA^+. A: Pump current was determined as the current activated by 2 mM internal ATP in solution containing 50 mM Na^+ without (open symbols) or with 100 mM TEA^+ (filled symbols). Experiments were performed in the presence (squares) or absence (circles) of 100 mM extracellular TEA^+. Currents are normalised to pump current in the absence of intracellular TEA^+ at 0 mV. For better comparison of the two data sets, currents in the presence of 100 mM extracellular TEA^+ (squares) were scaled down after normalisation to get a value of 0.5 at 0 mV in the absence of intracellular TEA^+. The pipette (extracellular) solution consisted of (in mM): 50 NaCl, 2 KCl, 100 (TMA)Cl or (TEA)Cl, 10 $BaCl_2$, 2$NiCl_2$, 5 MOPS/Tris, pH 7.6. Data represent averages of four experiments. B: Relative inhibition of pump current by 100 mM internal TEA^+ in the presence (squares) or absence (circles) of 100 mM external TEA^+.

pump, 20 mM TEA^+ increasingly inhibits the pump with more positive potentials in presence of 50 mM Na^+ and 2 mM K^+. At the potential for normalisation of pump current (−30 mV) TEA^+ reduced the current to 65% from 158 nA to 103 nA, and at +50 mV to less than 40%.

Since the measurements of ATPase activity led to a K_I value of about 90 mM, we tested whether such high concentrations have also to be used in the excised membrane patches to see an inhibition. Indeed, 100 mM TEA^+ clearly leads to a reduction of the pump current (Fig. 9) with 50% inhibition at 0 mV. The current-voltage curves in the absence and presence of the 100 mM TEA^+ in the giant patch are very similar to those in the absence and presence of 20 mM in the intact oocyte (Fig. 8). This strong and voltage-dependent effect of 100 mM TEA^+ was only observed if the TEA^+ was applied to the bath solution in the outside-out configuration. In the inside-out configuration, application of 100 mM TEA^+ to the bath solution produced a voltage-independent inhibition of less than 20% (compare open and filled symbols in Fig. 10A). The degree of inhibition is independent of the presence of external TEA^+ (Fig. 10B).

3.4. Ouabain binding

It has been reported [17] that TEA^+, like K^+, counteracts ouabain binding. This differs from what we found in *Xenopus* oocytes; the number of ouabain binding sites (B_{max}) does not change significantly if 20 mM TEA^+ is added to the incubation medium (Table 1). If B_{max} is taken as a measure for the number of pump molecules, this means a density of about 330 per μm^2 assuming a total surface area of 0.2 mm^2. The density of ouabain binding sites is not affected if higher ouabain concentrations are used. To exclude that a reduction in ouabain binding sites is counteracted by an exocytotic incorporation of new pump molecules into the surface membrane, we determined the membrane surface area from the membrane capacitance. The capacitance was calculated by integration of the transient current in response to the rectangular voltage pulses [22]. The data show (see Table 1) that the membrane surface area does not change due to TEA^+ treatment.

Table 1
Effect of TEA^+ on number of ouabain binding sites (B_{max}) and membrane capacitance

	Bound ouabain (normalised)	Capacitance (nF)
Control	1.00 ± 0.05 (n = 25)	211 ± 8 (n = 10)
20 mM TEA^+	1.07 ± 0.04 (n = 25)	206 ± 8 (n = 10)

4. Discussion

Controversial results for effects of TEA^+ on the Na^+,K^+-ATPase were reported previously. Zemková et al. [17] presented evidence that TEA^+ inhibits electrogenic and ATPase activity of the Na^+ pump in diaphragm muscles and that it counteracts ouabain binding. The authors concluded that TEA^+ competes with K^+ for binding to the Na^+,K^+ pump molecule and reduces the number of accessible ouabain binding sites. In contrast, Huang et al. [19], who also used the oocytes of *Xenopus*, proposed that TEA^+ acts on a K^+-selective channel that is somehow coupled to the Na^+,K^+-ATPase and that inhibition of the pump by ouabain also inhibits this K^+ channel. In their frame of interpretation, the elevated ouabain-sensitive current in the absence of TEA^+ should, therefore, be attributed to the indirect ouabain sensitivity of the pump-coupled K^+ channel.

4.1. Measurements with intact oocytes

The results of our investigation on intact *Xenopus* oocytes confirm those obtained by Huang et al. [19] showing an apparent voltage-dependent inhibition of ouabain-sensitive current. Taking into account that the effective valencies z_1 and z_2 for the pump stimulation by external K^+ differ considerably Eq. A2 becomes identical to that given in the legend to Fig. 3 with:

$$K_\infty = K_I,$$

$$K_{1/2} = [K^+]k^*_{01}/k_{20} + k^*_{01}/k^*_{12},$$

and

$$z_{TEA} = z_1.$$

The K^+ dependency and potential dependencies

suggest an apparent interaction between the external K^+ and TEA^+. This conclusion can be drawn from the finding that TEA^+ increases the apparent K_m values for pump stimulation by external K^+ (Fig. 5). The voltage dependence of inhibition by TEA^+ (Fig. 3B) with $z_{TEA} = z_1$ can then be attributed to the potential dependence of the interaction of K^+ with the pump molecule with the binding of TEA^+ being voltage-independent ($K_\infty = K_I$). Two additional facts support the view of a voltage-independent intrinsic TEA^+ binding: (1) the effective valencies of the K_m in the absence ($z = 0.38$) and presence ($z = 0.34$) of 20 mM TEA^+ are nearly identical, and (2) at positive potentials, that counteract K^+ binding, inhibition by TEA^+ becomes potential-independent. An interaction between K^+ and TEA^+ was also suggested by Zemková et al. in their experiments on diaphragm muscles [17], in line with our findings in the intact oocytes and the above interpretation.

4.2. Measurements with membrane patches or purified ATPase preparations

The K_I values we obtained for the inhibition of pump current differ considerably from those obtained for inhibition of ATPase activity. The values for current inhibition are completely compatible with those reported by Huang et al. [19] for current inhibition in *Xenopus* oocytes, and the values for inhibition of ATPase activity of the purified enzyme agree with those reported by Zemková et al. [17] for activity inhibition of microsomal preparations. Since in the excised membrane patches high concentrations of TEA^+, similar to those for inhibition of ATPase activity, are necessary to block pump current, differences in characteristics between intact cells and excised patches or in the experimental conditions should account for the differences in the K_I values. For the ATPase measurements the ionic conditions are clearly different to those for the two-electrode measurements where the pump molecule is properly oriented in the membrane and faces different substrate compositions, but in the patch-clamp experiments we tried to mimic the conditions of the intact cell, and nevertheless, much higher TEA^+ concentrations have to be applied for inhibition compared to the intact cells. A still existing difference is that the intact oocyte has a pronounced microvillous structure and cytoplasmic domains of the pump molecule interact with the cytoskeleton and regulatory factors. These conditions could not be mimicked in both the patch-clamp and the ATPase measurements, and therefore, may account for the difference in K_I values.

4.3. Conclusion

Our results demonstrate that TEA^+ directly inhibits the Na^+,K^+-ATPase and interferes with K^+ binding. An indirect effect through inhibition of a K^+-selective channel can be excluded since TEA^+ inhibits also the activity of the purified enzyme. The voltage dependence of inhibition by TEA^+ can completely be attributed to the voltage-dependent pump stimulation by external K^+, which suggests also an external site for TEA^+. The site for the slight inhibition by intracellular TEA^+ at 100 mM is independent of the extracellular site. TEA^+ could interact independently of voltage with the external site to block the entry of K^+ into the access channel. The voltage-dependent interaction of K^+ with its binding site, as described by the effective valence z, is consequently not affected. An important and essential conclusion from our results is that TEA^+ does not significantly affect the apparent dielectric length of the access channel.

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Appendix

For the description of the potential-dependent stimulation of the pump by K^+ and inhibition by

TEA^+, we used an extended version of the reaction diagram we developed previously [31]. The transport cycle involves a sequential binding of two K^+ ions to an access channel (Fig. 11A). Inhibition of the pump by TEA^+ is assumed to occur only when the pump molecule is not occupied by K^+ and is described by a potential-independent binding of TEA^+. The dependence of the transition rates on $[K^+]$, membrane potential, and $[TEA^+]$ is described by:

$$k_{01} = k^*_{01}[K^+]e^{-z_1 EF/RT},$$

$$k_{12} = k^*_{12}[K^+]e^{-z_2 EF/RT},$$

$$k_{03} = k^*_{03}[TEA^+],$$

where the asterisks indicate potential-independent rate constants, and z_1 and z_2 are effective valencies and represent the fraction of potential drop acting on the first and second K^+ ion, respectively. A positive z value corresponds to an inward movement of a positive charge. In Fig. 11, k_{20} lumps together all rate constants leading to the final completion of the reaction cycle. The scheme for the transport cycle may be reduced to a simple cycle diagram (Fig. 11B) with

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

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

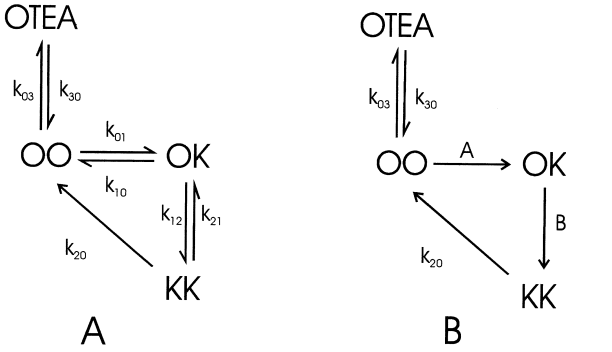

Fig. 11. Simplified reaction diagram for the pump cycle assuming sequential binding of extracellular K^+. A: k_{01} and k_{12} represent the rates of binding, k_{10} and k_{21} the release of the first and second K^+ ion, respectively. k_{20} lumps together all other transition rates involved in forward pumping. k_{03} and k_{30} represent rates of binding and release of TEA^+, respectively. B: Further reduction of the diagram in A to a simple cycle diagram (for details see text).

Transport activity I in the presence of TEA^+ is then represented by

$$I = \frac{k_{20}}{1+\frac{k_{20}}{A}+\frac{k_{20}}{B}+\frac{k_{20}k_{03}}{Ak_{30}}}.$$

As discussed previously [31], we made additional simplifications by neglecting terms with k_{10} and k_{21}/k_{12}, which leads to:

$$I = \frac{k_{20}[K^+]}{\left[[K^+]+\left(1+\frac{[TEA^+]}{K_I}\right)\frac{k_{20}}{k^*_{01}}e^{z_1 EF/RT}+\frac{k_{20}}{k^*_{12}}e^{z_2 EF/RT}\right]}$$

with $K_I = k_{30}/k^*_{03}$.

Stimulation by K^+ and inhibition by TEA^+ were described by Eq. 2 and the equation given in the legend to Fig. 3, respectively. In terms of the theoretical description, the apparent K^*_m and K^*_I values can be expressed by:

$$K^*_m = \left(1+\frac{[TEA^+]}{K_I}\right)\frac{k_{20}}{k^*_{01}}e^{z_1 EF/RT}+\frac{k_{20}}{k^*_{12}}e^{z_2 EF/RT}$$

$$\equiv K_{m1}e^{z_1 EF/RT}+K_{m1}e^{z_1 EF/RT}, \quad \text{(A1)}$$

$$K^*_I = K_I\left(1+[K^+]\frac{k^*_{01}}{k_{20}}e^{-z_1 EF/RT}+\frac{k^*_{01}}{k^*_{12}}e^{-(z_1-z_2)EF/RT}\right)$$

$$\overset{z_1>z_2}{\cong} K_\infty + K_{1/2}e^{Z_{TEA}EF/RT}. \quad \text{(A2)}$$

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