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Extracellular pH modulates kinetics of the Na^+,K^+-ATPase

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Abstract

To investigate effects of pH on the Na^+,K^+-ATPase, we used the *Xenopus* oocytes to measure transient charge movements in the absence of extracellular K^+, and steady-state currents mediated by the pump as well as ATPase activity. The activity of purified Na^+,K^+-ATPase strongly depends on pH, which has been attributed to protonation of intracellular sites. The steady-state current reflects pump activity, the transient charge movement voltage-dependent interaction of external Na^+ ions with the pump molecule and/or conformational changes during Na^+/Na^+ exchange. The steady-state current exhibits a characteristic voltage dependence with maximum at about 0 mV at low external K^+ (≤ 2 mM) and with 50 Na^+. This dependency is not significantly affected by changes in external pH in the range from pH 9 to pH 6. Only below pH 6, the voltage dependence of pump current becomes less steep, and may be attributed to a pH-dependent inhibition of the forward pump cycle by external Na^+. External stimulation of the pump by K^+ in the absence of Na^+ can be described by a voltage-dependent K_m value with an apparent valency z_K. At higher external pH the z_K value is reduced. The transient current signal in the absence of external K^+ can be described by the sum of three exponentials with voltage-dependent time constants of about 50 ms, 700 μs and less than 100 μs during pulses to 0 mV. The charge distribution was calculated by integration of the transient current signals. The slowest component and the associated charge distributions do not significantly depend on external pH changes. The intermediate component of the transients is represented by a voltage-dependent rate constant which shows a minimum at about −120 mV and increases with decreasing pH. Nevertheless, the contribution to the charge movement is not altered by pH changes due to a simultaneous increase of the amplitude of this component. We conclude that reduction of external pH counteracts external K^+ and Na^+ binding.

Keywords: Sodium pump; ATPase activity; Transport; Electrophysiology; Proton ; *Xenopus* oocyte

1. Introduction

The Na^+,K^+-ATPase or Na^+,K^+ pump maintains the electrochemical gradients for Na^+ and K^+ across the cell membrane of animal cells by transporting three Na^+ ions out of the cell and two K^+ ions into the cell per ATP molecule hydrolysed. During transport the enzyme cycles between E1 conformation, with inwardly oriented cation binding sites, and E2 conformation, with outwardly oriented cation binding sites, driven by phosphorylation and dephosphorylation steps. As a result of the $3Na^+$–$2K^+$ stoichiometry the pump is electrogenic, and the generated current can serve as a measure for transport

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activity [1]. In the absence of extracellular K^+, the transport cycle is disrupted, but the ATPase can perform a half cycle mediating $3Na^+/3Na^+$ exchange. In this exchange mode partial reactions with associated charge movements can be studied by electrophysiological techniques [2].

To investigate structure–function relationships of the Na^+,K^+-ATPase, the function of the pump can be analysed after modifying the protein. It had been shown that the ATPase activity strongly depends on pH with a maximum in activity at about pH 7.5, suggesting the existence of two protonation sites [3]. The dependency on pH had been attributed to an effect on the equilibrium between the two conformations of the enzyme. As one of the possible candidates for protonation the glutamic acid residue Glu_{329} has been suggested since mutation of the Glu to Gln removes part of the pH dependence [4]. In flux measurements with dialysed squid axon [5] it could be demonstrated that sites of protonation are accessible form the cytoplasm, changes in the external pH were without any significant effect on transport activity. With our electrical measurements we obtained evidence that the external pH can effect partial reactions of the Na^+,K^+-pump cycle under non-saturating conditions. Part of the results has been presented recently [6,7].

2. Materials and methods

The methods of oocyte handling and voltage clamp are similar to those described previously [8]. All experiments were performed at room temperature (about 22°C).

2.1. Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetised with tricaine (MS222, Sandoz, Basel (Switzerland) 1 g/l). 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 oocyte Ringer's solution (ORi, see Section 2.4) containing antibiotics (in mg/l: 70 gentamicin or 25 streptomycin plus 20 penicillin). Experiments were performed after 1–5 days of incubation. Before two-electrode voltage clamp (TEVC) experiments oocytes were loaded with Na^+ by incubation in 'Na-loading' solution (see Section 2.4) for about 40 min, and thereafter stored for at least 30 min in 'post-loading' solution. This procedure leads to an increase in cytoplasmic $[Na^+]$ to more than 80 mM [9], and hence, the pump is maximally activated from the cytoplasmic side.

The ATPase-activity measurements and some TEVC experiments were done with the endogenous Na^+,K^+ pump of *Xenopus* oocytes, the other TEVC experiments and all giant-patch-clamp experiments with the pump of electroplax of *Torpedo californica* expressed in the oocytes after injection of cRNA for the α and β subunit (20 ng per oocyte each) [10]. Higher accuracy in the analysis of transient currents was achieved if an ouabain-insensitive mutant was expressed, with Gln118 → Arg and Asn129 → Asp. In these experiments, the endogenous *Xenopus* pump could be blocked by 5 μM ouabain, which does not affect the mutant. In control experiments we found that the mutation was without significant qualitative effect on the pH dependencies investigated in this work.

2.2. Measurement of ATPase activity

Maximum hydrolytic activity of the Na^+,K^+-ATPase (ATPase activity) in yolk-free oocyte homogenates was determined spectroscopically as described in [11] using the pyruvate kinase/lactate dehydrogenase method in which the generation of ADP is coupled to oxidation of NADH. Oocytes were disrupted by passing through 200 μl Eppendorf pipette tips in ice-cold homogenisation buffer (HBO) containing (in mM): 20 Tris–HCl (pH 7.4), 5 $MgCl_2$, 5 NaH_2PO_4, 1 EDTA, 100 NaCl, 10 KCl, 1 DTT, PMSF 1, 5 μg/ml of each of leupeptin, pepstatin and antipain and with or without 1 mM ouabain. After homogenisation (10 oocytes/100 μl) yolk granules were removed by centrifugation of the samples twice at $150 \times g$ at 4°C for 5 min. Thirty μl of yolk-free oocyte homogenates were added to 0.8 ml of a test buffer A (in mM): 100 NaCl, 10 KCl, 5 $MgCl_2$, 25 imidazole (pH 7.2), 3 Na_2-ATP, 2 phosphoenol pyruvate, 0.31 or 0.06 NADH, and 6 μl pyruvate kinase (660 U/ml) (Sigma). ATPase activity of the sample was calculated from the difference between the slopes in the time course of adsorption change at 340 nm in the absence and in the presence of 1 mM ouabain.

2.3. Voltage-clamp experiments

The electrophysiological experiments were performed either on intact oocytes with the conventional TEVC (see, e.g., [1,8]) or on excised outside-out membrane patches with the giant-patch technique [12,13].

For TEVC microelectrodes were filled with 3 M KCl and had resistances of less than 0.5 MΩ. To avoid leakage of KCl into the cytoplasm, the pipette tips were filled with agar, which was achieved by briefly dipping the tip into heated agar and applying negative pressure to the pipette lumen. For giant-patch-clamp pipettes with tip diameters of 25–35 μm were prepared using the technique of filling the pipette tip with melted soldering glass as described previously [13]. Giga-seals with the fire-polished pipettes were established without additional coating.

The protocol for the characterisation of steady-state pump current by TEVC was the same as described previously [1,14]. In brief, steady-state membrane currents were recorded during the last 20% of 40 or 200 ms, rectangular voltage-clamp pulses (from −150 up to +30 mV in 10 mV increments) that were applied from a holding potential of −60 mV. The current signal was low-pass filtered at 300 Hz and sampled at 1 kHz.

The current generated by the Na^+,K^+-ATPase was determined as current activated by extracellular K^+, or inhibited by 5 μM ouabain for the endogenous or wild-type *Torpedo* pump or by 10 mM ouabain for the ouabain-insensitive mutant.

For analysis of the dependence of the endogenous pump current on external K^+, current–voltage dependencies were measured under TEVC 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 the different membrane potentials E was fitted by:

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

using a Hill coefficient of $n = 1.3$ (see [1,15]). The voltage dependence of the apparent K_m value was analysed by fitting an exponential to the data:

$$K_m(E) = K_m(0)e^{z_K EF/RT} \quad (2)$$

$K_m(0)$ is the apparent K_m value for pump stimulation by external K^+ at 0 mV, z_K represents an effective valence. An interpretation of this voltage dependency is that the two K^+ ions transported into the cell have to pass an access channel within the pump molecule and sensing a fraction z_K of the electrical field, which may be considered as a measure for the dielectric length of the access channel [14,16].

In the absence of external K^+, the pump cannot complete the cycle but mediates $3Na^+/3Na^+$ exchange. In this mode no steady-state current is generated, but a transient current can be detected in response to a voltage jump; the associated charge movements have been attributed to movements of the transported Na^+ ions in part of the electrical field in the membrane and/or to charge movements during conformational changes (see, e.g., [17]). To analyse kinetics of the charge movements we had to increased the number of pump molecules in order to obtain sufficiently large current signals. This we achieved by expressing the ouabain-insensitive *Torpedo* pumps in the oocytes. Signals in response to voltage-clamp pulses of 40 ms duration were recorded in giant outside-out membrane patches. Contributions from endogenous pumps were blocked by the presence of 5 μM ouabain. The current signal was low-pass filtered at 10 kHz and sampled at 20 kHz.

2.4. Solutions

The solutions for oocyte incubation had the following composition if not 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); Post-loading solution: 100 NaCl, 1 $CaCl_2$, 20 TEACl, 5 $BaCl_2$, 5 MOPS/NaOH (adjusted to pH 7.6).

For TEVC measurements the following solutions were used. Na^+-free test solutions: 90 tetramethylammonium (TMA^+) chloride, 0–5 KCl, 20 TEACl, 5 $BaCl_2$, 5 $NiCl_2$, 5 buffer adjusted to the respective pH value. In the Na^+-containing test solution (for determination of steady-state pump current), 40 mM

TMACl was replaced by 50 mM NaCl, and either 0 or 2 mM KCl were present (for measurement of transient currents): TMACl was replaced by 100 mM NaCl, and the pump-mediated signal was determined by subtraction of current measured in the presence of 100 μM ouabain.

The giant-patch-clamp experiments were performed in the outside-out configuration. The pipette solution was composed to mimic the ionic conditions for intact, Na^+-loaded cells at the internal membrane surface: 70 NaCl, 10 KCl, 5 $MgCl_2$, 5 Na_2ATP, 5 EGTA, 5 MOPS (adjusted by about 25 mM NaOH to pH 7.4). The bath solution contained 100 NaCl, 20 TEACl, 5 $BaCl_2$, 5 buffer adjusted to the respective pH value. All test solutions in the experiments with expressed ouabain-insensitive *Torpedo* pumps contained 5 μM ouabain to block the endogenous *Xenopus* pump. The pump-mediated signal was determined by subtraction of current measured in the presence of 10 mM ouabain.

For adjustment of pH to 8 and 9 tricine was used, and to pH 5 and 6 MES was used, instead of MOPS. All external test solutions contained the $BaCl_2$ and TEACl to reduce current through K^+-selective channels (see [18]); to reduce background currents mediated by Ca^{2+}-activated channels (Cl^- channels), no Ca^{2+} was added to the external test solutions (see [19,20]). The Ni^{2+} was added to block Na^+/Ca^{2+} exchange. In the nominally Na^+- and K^+-free solutions, the actual concentrations were below 5 μM as determined by flame photometry.

3. Results

The hydrolytic activity of Na^+,K^+-ATPase in oocyte homogenates strongly depends on pH. The endogenous *Xenopus* ATPase in oocyte homogenates shows a pronounced maximum in its activity at about pH 7 (Fig. 1) under conditions of optimised concentrations of Na^+, K^+, and ATP (cf. Section 2). Inhibition of ATPase activity at high pH can be attributed to deprotonation, inhibition at low pH to protonation of intracellular sites (compare [3,5,21]). In the intact cells, extracellular pH only slightly affects transport activity; maximum pump current determined in Na^+-free solution with 5 mM K^+ is much less affected by the changes in external pH (Fig. 1).

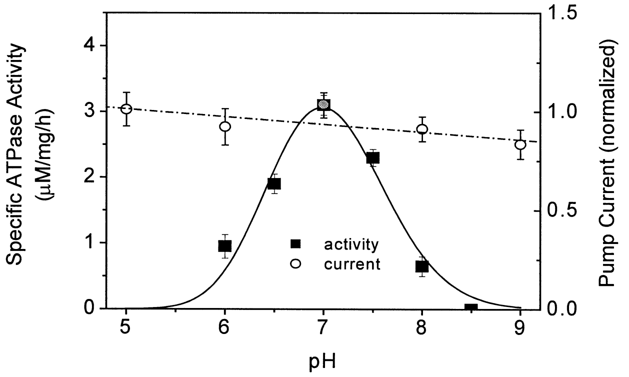

Fig. 1. Dependence of maximum ATPase activity in oocyte homogenates (filled symbols) on pH of solution and of maximum pump current (open circles) on external pH. Data represent averages of 7–11 measurements (mean ± S.E.M.). The lines are drawn to fit the data. Currents were normalised to pH 7, −60 mV ($I = 26 \pm 3$ nA).

3.1. Two-electrode voltage-clamp experiments

Effects of extracellular pH on the endogenous pump were investigated in more detail under non-saturating conditions. In externally 50 mM Na^+ and 2 mM K^+, the pump current shows a maximum at about 0 mV (see Fig. 2A). The decline of pump current with negative as well as with positive potentials has been attributed to voltage-dependent interaction of the external cations with the pump molecule; negative potentials counteracting the release of Na^+, and positive potentials counteracting the binding of K^+ [22]. For the range of pH 6–9 the pump current is hardly affected over a wide range of potentials. In Fig. 2B the data of Fig. 2A are redrawn for a number of selected potentials; in the range of pH 6–9 only at depolarised (0 mV) and positive (+30 mV) potentials does a slight pH dependency become apparent. This suggests that the potential-dependent interaction of external K^+ is affected since voltage dependence of pump activity is governed at these potentials by external K^+. In the absence of external Na^+, this can be described by a voltage-dependent apparent K_m value for pump stimulation by the external K^+ with an apparent valency z_K (see Eq. 2 in Section 2). The K_m values at pH 7 and pH 8 clearly differ (Fig. 3), showing less pronounced voltage dependence at pH 8 ($z_K = 0.42$) compared to pH 7 ($z_K = 0.76$).

Further acidification down to pH 5 leads to a pronounced stimulation of pump activity at negative potentials (Fig. 2). Since external Na^+ dominates the potential dependency of pump current at negative potentials, lowering pH seems to counteract the potential-dependent inhibition of the pump cycle by external Na^+. Na^+ dependency of the ATPase can be investigated most effectively by analysing in the absence of external K^+ the transient charge movements mediated by the pump in the $3Na^+/3Na^+$ exchange mode. For sufficient accuracy of the measurements, pump-mediated signals from the endogenous pumps are not large enough. A sufficiently high signal can be obtained by expression of additional pumps by injection of cRNA. Fig. 4A shows the transient 'on' and 'off' signals in response to a voltage pulse from a holding potential of −60 mV to +10 mV at pH 6 and pH 9 in TEVC experiments for oocytes with *Torpedo* pumps. The transient at pH 9 appears to be not as fast as that at pH 6, but kinetics can be analysed with sufficient accuracy in the oocytes only with the giant patch (see below).

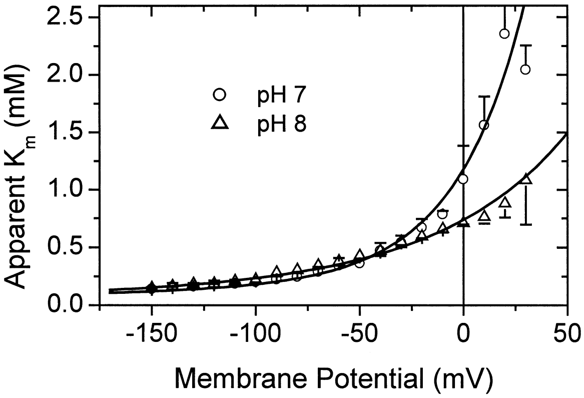

Fig. 3. Dependence of apparent K_m value for stimulation of the endogenous pump by external K^+ in the absence of Na^+ at pH 7 (○) and pH 8 (△). Data represent averages from four experiments (mean ± S.E.M.), and were fitted by Eq. 2. The fitted parameters are $K_m(0) = 0.98$ and 0.54 mM, $z_K = 0.76$ and 0.42 for pH 7 and pH 8, respectively.

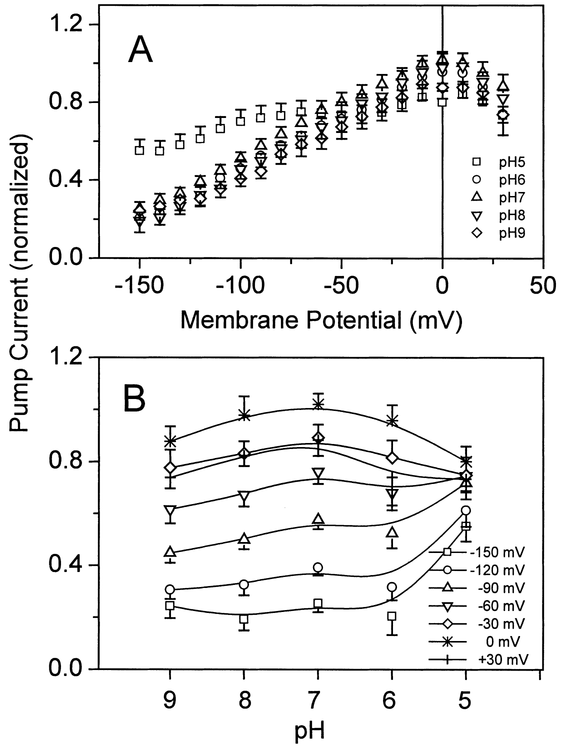

Fig. 2. Dependence of endogenous pump current on membrane potential at different external pH (A). Pump-mediated current was determined as the current activated by external application of 2 mM K^+ in the presence of 50 mM Na^+. Data represent averages of 7–11 experiments (mean ± S.E.M.). Currents were normalised to pH 7, 0 mV ($I = 31 \pm 4$ nA) (B) Same data as in A, but plotted for a series of selected potentials versus pH. The lines are drawn to fit the data.

Nevertheless, we measured first under TEVC for different external pH values the amount of charges Q that are moved in the electrical field in response to a voltage pulse by integrating the transient signal. The voltage dependence $Q(E)$ of the charges moved during on responses is shown for three pH values in Fig. 4B, and follows a Fermi equation:

$$Q(E) = Q_{-\infty} + \frac{Q_{+\infty} - Q_{-\infty}}{1 + e^{-z_f(E-E_{1/2})F/RT}} \quad (3)$$

with maximally moved charge $Q_{max} = Q_{+\infty} - Q_{-\infty}$. z_f is the effective valency and represents the dielectric length times the valence of the moved charge; $E_{1/2}$ is the midpoint potential. The voltage dependence can be fitted by the same set of parameters independent of the pH value (see legend to Fig. 4B).

3.2. Giant-patch-clamp experiments

Though the amount of charges moved and the voltage dependence of their distribution are not significantly affected by changes in pH, the measurements of the transient currents of whole oocytes (compare Fig. 4A) suggested differences. Therefore, we analysed the effect of pH on the time courses. Because of the large size of the oocytes, and hence its large capacitance, the time resolution of the transients does not allow accurate analysis [23]. We, therefore, performed experiments on giant membrane patches. We used the ouabain-resistant mutant of the pump of *Torpedo* electroplax which allows easy subtraction of contributions of the endogenous pumps by blocking with 5 μM ouabain, which does not effect the mutated *Torpedo* pump.

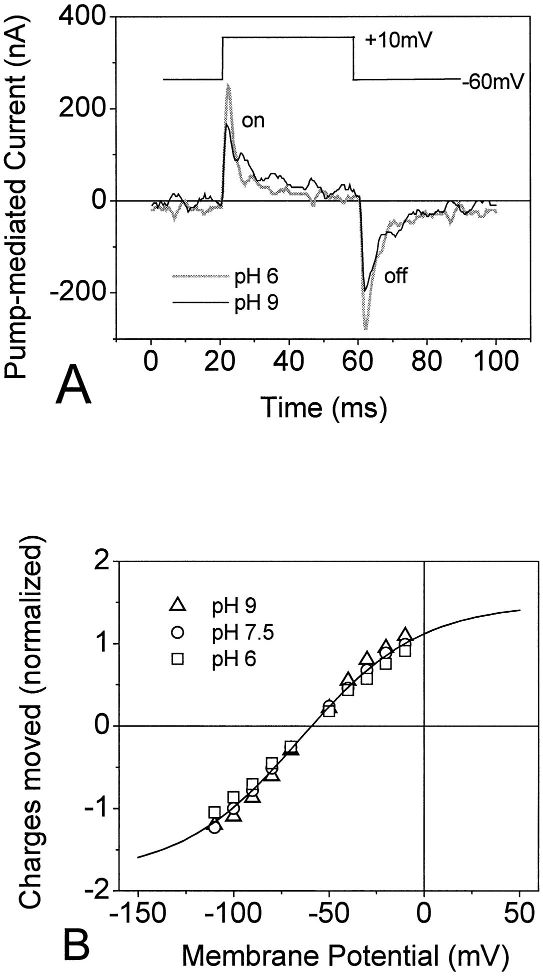

Fig. 4. (A) Transient current mediated by the wild-type *Torpedo* pump in the absence of extracellular K^+ and induced by a voltage pulse (inset) at pH 6 and pH 9, respectively. The pump-mediated current was determined as the difference of current in the presence and absence of 100 mM Na^+, which is identical to the difference obtained by measurements without and with ouabain. (B) Voltage-dependent distribution of charges moved transiently in response to voltage pulses applied under TEVC from the holding potential ($V_H = -60$ mV) to oocytes with *Torpedo* pumps at different pH values. The pump-mediated transients were determined as the difference of current in the presence and absence of 100 mM Na^+. The charges were determined by integration of the transients. Data represent averages of 3–9 experiments (mean ± S.E.M.), and were fitted simultaneously by Eq. 3 (continuous line) with $Q_{max} = 3.9$ (corresponding to 3.2 ± 0.2 nC), $E_{1/2} = -54$ mV, and $z_f = 0.74$.

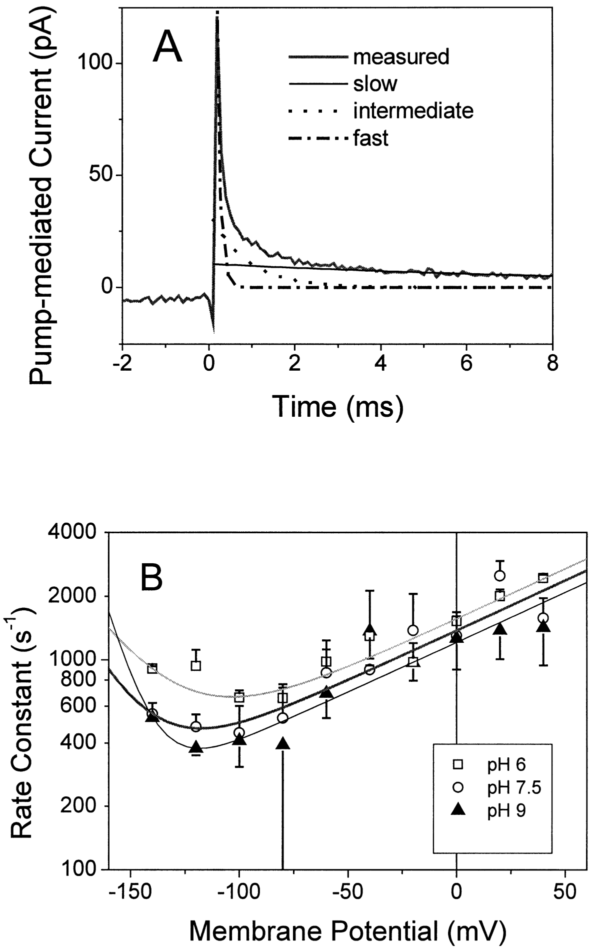

Fig. 5. (A) Transient signal in response to a potential jump from −30 mV to 0 mV (on response) at pH 7.5 in giant patch with ouabain-insensitive *Torpedo* pumps. The transient was fitted by the sum of three exponentials with time constants of 0.09 ms, 0.9 ms and 11 ms for the fast, intermediate and slow component, respectively. (B) Voltage dependence of the intermediate rate constant at different external pH values. The lines represent fits of:

$$k = 10^3 \cdot (e^{z_1(E-E_1^*)F/RT} + e^{-z_{-1}(E-E_{-1}^*)F/RT})$$

which corresponds to Eq. 4. Fit parameters are listed in Table 1.

Fig. 5A shows a selected transient ‘on response’ at pH 7.5 for a voltage pulse from −30 mV to 0 mV.

Table 1
Parameter for description of the voltage-dependent rate constants for transient charge movements in *Torpedo* pumps at different external pH according to Eq. 4 for the intermediate component and Eq. 5 if fitted by a single exponential (data from [23])

	Intermediate component			Single exponential
	pH 6	pH 7.5	pH 9	pH 7.4
z_1	0.27	0.27	0.27	
k_1^0 (s^{-1})	1562	1378	1209	140
E_1* (mV)	−41	−30	−18	
z_{-1}	0.88	1.20	2.1	0.45
k_{-1}^0 (s^{-1})	4.2	0.30	0.0018	48
E_{-1}* (mV)	−156	−169	−155	−60

The time course can be described by the sum of three exponentials: a fast component that can hardly be resolved with a time constant of less than 100 μs, an intermediate component with a time constant of about 1 ms, and a slow one with a time constant of more than 10 ms. At pH 7.5 the slow time constant appears to be voltage-insensitive (not illustrated). At low and high pH this component exhibits high degree of variability, and therefore, was not analysed in detail. The intermediate time constant τ, on the other hand, shows clear voltage dependence at all pH values. The voltage dependence of the rate constant $k = 1/\tau$ can be described by the sum of two voltage-dependent rate constants (Fig. 5B and Table 1) and shows a minimum at $E_{\min} \approx -120$ mV. This suggests a transition with voltage-dependent forward (k_1) and backward (k_{-1}) rates leading to the transient charge movement:

$$k = k_1^0 e^{z_1 EF/RT} + k_{-1}^0 e^{-z_{-1} EF/RT} \qquad (4)$$

The voltage dependence of the distribution of the charges for the intermediate component has been determined by integration of this component. Similarly to the results of TEVC, the voltage dependence is not significantly affected by pH, and this despite of the pH dependence of the rate constant of the intermediate component. But this can be attributed to a compensatory change in the amplitude of the intermediate component. A fit of the voltage dependence of the intermediate component by Fermi Eq. 3 yields for all pH values the same $E_{1/2}$ and z_f values. While z_f with 0.73 is similar to the value obtained under TEVC (Fig. 4B) for the total signal of wild-type *Torpedo* pump, the $E_{1/2}$ value is shifted by 50 mV to negative potentials.

4. Discussion

It has been shown long time ago that the activity of the Na^+,K^+-ATPase strongly depends on pH with optimum activity at about pH 7.5 [3–5]. Inhibition of ATPase activity at lower as well as at higher pH has been taken as evidence that two sites of protonation are involved [3] that are most likely accessible form the cytoplasm [5]. Breitwieser et al. [5] have demonstrated by measurements of Na^+ and K^+ isotope fluxes in squid giant axon that transport depends on internal pH but is nearly insensitive to changes in external pH. Also the ATPase activity in the oocyte homogenates shows the strong dependency on pH with a maximum at about pH 7, while pump current is much less effected by the external pH (Fig. 1). The decrease of ATPase activity at high pH is removed if the putative intramembranous residue Glu_{329} is mutated to Gln [4], and it has been suggested that this amino acid is important for protonation.

All these measurements were performed under conditions of optimised ATPase or transport activity, or close to physiological conditions. From the extracellular side, the ATPase is regulated by the voltage-dependent interaction of Na^+ and K^+. Our results give evidence that these interactions can be modified by changes in pH. Inspection of the pH dependency at different voltages (Fig. 2B) yields a slight dependence at positive potentials, also with a maximum around pH 7. One might speculate that these changes in transport result from slight alterations of intracellular pH following extracellular pH changes, but measurements of intracellular pH with pH-sensitive microelectrodes have shown that even long-lasting exposure to pH 6.1 or 8.3 has practically no effect on the intracellular pH [24] though changes in close vicinity to the internal membrane surface can of course not be excluded. At the positive potentials and at external $K^+ \leq 2$ mM, pump activity is governed by the potential-dependent interaction with K^+, suggesting that protons interact with K^+ binding. Effects of external pH on K^+ interaction is also supported by the finding that the apparent valency for K^+ binding in the absence of extracellular Na^+ becomes reduced with increasing pH (see Fig. 3).

Effects of pH on the Na^+-translocating limb of the pump cycle can most efficiently be studied in the absence of extracellular K^+. Under these conditions the pump performs Na^+/Na^+ exchange with transient charge movements. Since the transients are too fast to be analysed kinetically by TEVC, we applied the giant-patch-clamp technique. This on the other hand made it necessary to overexpress pumps by injection of cRNA for the α and β subunit of the *Torpedo* pump. For the α subunit we used the ouabain-insensitive mutant which exhibits qualitatively similar pH dependence as the wild-type pumps. This excludes the possibility that the Asp_{129} is a site of protonation/deprotonation that could account for the observed effects.

In giant patches of *Xenopus* oocytes, the transient current exhibits three exponential components that may be attributed to reaction steps that are associated with translocation of Na^+ by the transport protein [25–31]. With sufficient accuracy we could analyse only the intermediate component which, on the other hand, shows clear pH dependency. Rate constants in the same range of 100–2000 s^{-1} have been detected also in other preparations and have been attributed to a conformational change associated with release and rebinding of extracellular Na^+ [2,25,26,30–34]. In contrast to our findings for the intermediate component, the voltage dependence of the rate constants could be described by the sum of a voltage-independent forward rate (associated with extracellular Na^+ release) and a voltage- and Na_o^+-dependent backward rate [2,25,26,30,32]:

$$k = k_1^0 \bullet (1 + e^{-z_{-1}(E - E^*_{-1})}) \tag{5}$$

This kind of voltage dependence we also obtain if the transient were fitted by a single exponential (not shown, but see values in Table 1 and [23]). The intermediate component of the *Torpedo* pump is dominated by a voltage-dependent forward rate k_1, but at extremely negative potentials a voltage-dependent backward rate k_{-1} becomes also apparent.

Although the amount of movable charges of the intermediate component and their voltage-dependent distribution are not affected by pH changes, the transient becomes faster at lower external pH. At present we do not know the reason for this modulation. Since the three exponential components reflect three distinct kinetic steps in Na^+ translocation, modulation by pH may reflect different accessibility of the Na^+-binding sites by protonation–deprotonation steps from the extracellular side. Exchange of protons becomes more pronounced at negative potentials where the transients are governed by the forward rate k_1.

We conclude that reduction of pH down from pH 8 to pH 7 counteracts external K^+ binding. Since both external K^+ as well as Na^+ interact with the E2P conformation of the ATPase also external Na^+ interaction can be expected to be modulated by the external pH. Indeed, further lowering to pH 5 clearly counteracts Na^+ interaction. The next step in this investigation will be identification of the sites by site-directed mutagenesis. The experiments were particularly designed to gain new information on structure–function relationships. Since the steady-state transport activity of the Na^+,K^+ pump is hardly affected by changes in external pH, this characteristic may be of physiological importance in stabilisation of Na^+ and K^+ transport at variations of pH in blood serum during, e.g., respiratory acidosis or alkalosis.

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