Comparison of Na^+/K^+-ATPase Pump Currents Activated by ATP Concentration or Voltage Jumps

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ABSTRACT Using the giant patch technique, we combined two fast relaxation methods on excised patches from guinea pig cardiomyocytes to compare the rate constants of the involved reaction steps. Experiments were done in the absence of intra- or extracellular K^+. Fast ATP concentration jumps were generated by photolysis of caged ATP at pH 6.3 with laser flash irradiation at a wavelength of 308 nm and 10 ns duration, as described previously. Transient outward currents with a fast rising phase, followed by a slower decay and a small stationary current, were obtained. Voltage pulses were applied to the same patch in the presence or absence of intracellular ATP. Subtraction of the voltage jump-induced currents in the absence of ATP from those taken in the presence of ATP yielded monoexponential transient current signals, which were dependent on external Na^+ but did not differ between intracellular pH (pH_i) values 6.3 or 7.4. Rate constants showed a characteristic voltage dependence, i.e., saturating at positive potentials (~200 s^{-1}, 24°C) and exponentially rising with increasing negative potentials. Rate constants of the fast component from transient currents obtained after an ATP concentration jump agree well with rate constants from currents obtained after a voltage jump to zero or positive potentials (pH_i 6.3), and the two exhibit the same activation energy of ~80 kJ · mol^{-1}. For a given membrane patch, the amount of charge that is moved across the plasma membrane is roughly the same for each of the two relaxation techniques.

INTRODUCTION

As Na^+/K^+-ATPase (also referred to as the Na^+ pump) actively maintains the electrochemical gradients for Na^+ and K^+ across the plasma membrane, the enzyme is of essential importance for the survival of nearly all animal cells. For each molecule of ATP hydrolyzed, the enzyme expels three Na^+ ions in exchange for two K^+ ions. Thus the overall activity of Na^+/K^+-ATPase is electrogenic and leads to the generation of current—a property that has attracted the interest of electrophysiologists for decades. Although seemingly a rather simple entity, it has taken considerable effort to demonstrate the electrogenic behavior of the Na^+ pump and even more to identify the reaction step(s) in the enzymatic cycle, in which electrogenicity occurs; the latter is still a field of questions and controversy.

A variety of techniques have been used to study the electrogenic properties of Na^+/K^+-ATPase with high time resolution, including two-electrode voltage-clamp (Lafaire and Schwarz, 1986; Rakowski et al., 1989; Rakowski, 1993) and patch-clamp (Gadsby and Nakao, 1989) methods, potential-sensitive dyes (Clarke et al., 1989; Klodos and Forbush, 1988; Stürmer et al., 1989), and the bilayer technique (Fendler et al., 1985, 1987, 1993; Nagel et al., 1987; Borlinghaus et al., 1987; Wuddel and Apell, 1995). With the giant-patch technique (Hilgemann, 1989, 1995b) it has become possible to study the activity of Na^+/K^+-ATPase under the well-defined conditions of an excised patch (Hilgemann et al., 1991; Hilgemann, 1994; Friedrich et al., 1996).

In recent years two different relaxation techniques were developed to faciliate the measurement of electric currents generated by Na^+/K^+-ATPase in the millisecond range. The results, however, led to different conclusions about the rate constant(s) of the involved electrogenic step(s). One technique uses an ATP concentration jump whereby ATP is released from a nonhydrolyzable ATP analog ("caged ATP") by short irradiation with intense ultraviolet light (as pioneered by Kaplan et al., 1978; Forbush, 1984; and for electrical measurements by Fendler et al., 1985).

Another technique uses fast voltage steps and a specific protocol whereby the sodium pump current is obtained by subtracting currents with inhibited Na^+/K^+-ATPase from currents, which originate from the enzyme performing ATP-dependent Na^+/Na^+ exchange. Difference currents obtained in this manner can be attributed to electrogenic events in the Na^+/K^+-ATPase pump cycle (Nakao and Gadsby, 1986; Rakowski, 1993; Hilgemann, 1994). Fendler et al. (1987, 1993) reported a rate of at least 200 s^{-1} at 24°C and pH 6.2 for the electrogenic conformational change using purified Na^+/K^+-ATPase-containing membrane fragments adsorbed to a lipid bilayer membrane and the caged ATP technique, whereas Apell et al. (1987; see also Wuddel and Apell, 1995) concluded that the rate constant is slower, with ~20 s^{-1} in similar experiments (pH 7.0, 22°C). Nakao and Gadsby (1986) arrived at 200 s^{-1} at 35°C, and a value of ~40 s^{-1} at 20°C (pH_i 7.4) was reported by Gadsby et al. (1992) in voltage jump experiments on whole-cell patch-clamped heart cells. Hilgemann (1994), however, measured

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$\sim 600\ s^{-1}$ at 37°C (pH_i 7.4) in voltage jump experiments on giant excised patches from heart cells. Different rate constants were reported from these experiments, but it also has to be noted that the source of enzyme as well as the experimental conditions differed greatly.

Recently we developed an experimental procedure that allows the generation of fast ATP concentration jumps in giant-patch measurements on ventricular myocytes (Friedrich et al., 1996). Here we used this technique to combine ATP concentration jump and voltage jump experiments with the same enzyme and under identical conditions (if possible on the same membrane patch), to directly compare the rate constants obtained with these two different methods. The following data were all obtained in the absence of intra- or extracellular potassium, to slow down the (K^+-dependent) dephosphorylation of Na^+/K^+-ATPase. Internal sodium was always 40 mM, and external sodium was between 0 and 150 mM, as described in the text.

MATERIALS AND METHODS

Single ventricular cardiomyocytes from guinea pig were isolated according to well-established procedures (Isenberg and Klöckner, 1982; Collins et al., 1992). Cells were obtained by retrograde perfusion on a Langendorf-type apparatus with a low-Ca^{2+} Tyrode's solution containing collagenase (Sigma Type I or Worthington, 0.5 mg/ml), followed by dispersion with scissors and filtering through nylon mesh. Cells were stored in a K^+-rich solution (DS, see below) at +4°C for up to 3 days, where they developed plasma membrane protrusions or blebs. Gigaohm seals were obtained on these membrane blebs, the composition of which does not seem to differ from that of pure plasma membrane (Collins et al., 1992), as also described by Hilgemann (1989). Patch pipettes were pulled from borosilicate glass (Type N-51 A, 1.6 mm inner diameter, 2.2 mm outer diameter; Drummond Scientific Co., Broomall, PA) on a conventional two-stage puller (model PP-83; Narishige, Tokyo, Japan) to obtain tip opening diameters from 20 to 26 μm. The pipette tip was immersed in α-D,L-tocopherol acetate shortly before the pipette was filled with solution.

After obtaining a seal on the bleb from a ventricular myocyte, the patch was excised and continuously superfused with bath solution, and the patch current was recorded. As temperature control is essential for the determination of rate constants, a thermostatted perfusion system was used as described in a previous paper (Friedrich et al., 1996), in which the experimental setup is described in more detail. A XeCl excimer laser provided flashes of 10 ns duration at a wavelength of 308 nm. Via a quartz lightguide of 400 or 700 μm diameter, ultraviolet light was directed into the perfusion chamber to liberate ATP from caged ATP by photolysis in the close vicinity of the patch membrane (~100 μm). The energy input varied between 14 and 180 $mJ \cdot cm^{-2}$ (attenuated by neutral density filters), resulting in ATP release fractions between 3% and 30%. It should be noted that the ATP concentration jump experiments were carried out at pH 6.3, because the photolytic reaction involves a proton-dependent step, favoring ATP release at low pH. At pH 7.4, free $[Mg^{2+}] = 2$ mM, and 24°C, the photolytic release reaction has a time constant of ~25 ms, which limits time resolution and thus does not allow the measurement of intrinsic rate constants of enzymatic reactions (see our previous publication: Friedrich et al., 1996). At pH 6.3 photolytic release takes place within ~2 ms, which is sufficient to initiate fast electrogenic reaction steps. Voltage jump-induced transient currents of Na^+/K^+-ATPase, involved in ATP-dependent Na^+/Na^+ exchange (with extracellular and intracellular Na^+ present, but without extracellular K^+) were obtained by applying specific protocols of rectangular voltage pulses of alternating sign and 30 ms duration first in the absence and then in the presence of 500 μM ATP and again after the washing out of ATP. From a zero holding potential the potential range covered by the voltage pulse protocols was −200 mV to +140 mV, and from a −50 mV holding potential it was between −200 mV and +100 mV. If current responses to identical voltage pulses before and after activation by ATP did not reveal any changes with time, they were subtracted from currents responding to a voltage pulse in the presence of ATP to isolate voltage jump-induced transient currents. These difference currents could be fitted well by a monoexponential decay function to calculate the time constants of the signals. To exclude possible artifacts immediately after a voltage jump due to charging current spikes, usually the time range between 1 ms and 30 ms after the step was used for fitting and the initial current amplitude was calculated by extrapolation of the fitted curve to $t = 0$ ms. The amount of transiently moved charge was calculated as the product of the extrapolated initial amplitude and the time constant from the fit.

Experimental solutions

Tyrode for myocyte preparation

130 mM NaCl, 5.4 mM KCl, 0.3 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2$, 20 mM taurine, 5 mM creatine, 10 mM HEPES, 11 mM glucose, 36 μM $CaCl_2$, pH 7.35 with NaOH.

Storage solutions

DS: 134 mM KCl, 10 mM EGTA, 10 mM HEPES, 10 mM glucose, 2 mM $MgCl_2$, pH 7.4 with KOH, used for storage of guinea pig cardiac myocytes.

Bath solutions

B-7.4: Bath solution for conventional continuous-flow solution exchange experiments contained (in mM): 40 NaCl, 100 *N*-methyl-D-glucamine (NMG), 10 EGTA, 10 HEPES, 20 tetraethylammonium (TEA)-Cl, 2 $MgCl_2$, pH 7.4 adjusted with HCl.

B-6.3: Bath solutions for conventional continuous-flow solution exchange experiments or caged ATP photolysis at pH 6.3 contained (in mM): 40 NaCl, 100 NMG, 10 MES, 10 EGTA, 20 TEA Cl, and 2 $MgCl_2$, pH adjusted with HCl. For photolysis experiments, 1 mM glutathione or L-ascorbic acid was added together with caged ATP as protective agents against harmful effects of the by-products of the photolysis reaction on the activity of Na^+/K^+-ATPase. Ten units/ml hexokinase (Sigma, München, Germany), together with 1 mM glucose, was also added to ensure initially ATP-free conditions (usually caged ATP stock solutions contain 0.5% free ATP). Caged ATP (P^3-1-(2-nitro)phenylethyladenosine-5′-triphosphate, triethylammonium salt) was used in concentrations from 10 to 500 μM. It was synthesized as described previously (Fendler et al., 1985).

Pipette solutions

Two different pipette solutions (K^+-free) were used, containing (in mM):

P-Na: 145 NaCl, 10 HEPES, 2 $BaCl_2$, 2 $MgCl_2$, 0.5 $CdCl_2$, adjusted to pH 7.4 with NaOH.

P-NMG: 145 NMG, 10 HEPES, 5 KCl, 2 $BaCl_2$, 2 $MgCl_2$, 0.5 $CdCl_2$, adjusted to pH 7.4 with HCl.

All chemicals were of analytical or higher grade (Merck, Darmstadt, Germany).

Data analysis

The concentration of ATP released after photolysis of caged ATP was calculated according to the following equation:

$$\eta = 1 - e^{-\kappa E} \quad (1)$$

In this formula η is the fraction of ATP released from a given initial concentration of caged ATP, E is the energy density of the applied ultraviolet light flash (expressed in $mJ \cdot cm^{-2}$), and κ is an empirical

constant, which was determined to be 0.002 $cm^2 \cdot mJ^{-1}$ by means of a luciferin/luciferase assay (ATP-Bioluminescenz CLS; Boehringer, Mannheim, Germany).

Photolytically induced transient current signals were fitted with a model function including two exponentially decaying components and a constant:

$$I(t) = A_1 \cdot e^{-(t-t_0)/\tau_1} + A_2 \cdot e^{-(t-t_0)/\tau_2} + I_\infty \qquad (2)$$

with the constraint

$$A_1 + A_2 + I_\infty = 0 \quad \text{for} \quad t = t_0 > 0$$

In this equation, I_∞ is the stationary current for $t \to \infty$, and t_0 is a time offset that was introduced to account for a possible lag phase due to the time-dependent release of ATP from caged ATP (a typical value for t_0 was 1 ms at pH 6.3). As data analysis on the basis of Eq. 2 yields time constants τ_1 and τ_2, the inverse of these values will be referred to as rate constants k_1 and k_2 throughout the text.

To test quantitative agreement with BLM measurements, the rate constant k_2 ($= \tau_2^{-1}$), which describes the binding/dissociation of ATP/caged ATP to/from the enzyme, was calculated using Eq. 3 (cf. Fendler et al., 1993):

$$\tau_2^{-1} = k_a^+ \cdot \eta \cdot K_{0.5} \cdot \frac{c_c^0}{(1-\eta) \cdot c_c^0 + K_{0.5}} \qquad (3)$$

$$\text{with} \quad K_{0.5} = \frac{K_{0.5}^c}{(1-\eta)}$$

In this equation c_c^0 is the initial concentration of caged ATP before photolysis, η is the released fraction of ATP, k_a^+ the rate constant of ATP binding to the enzyme, $K_{0.5}$ the ATP binding constant, and $K_{0.5}^c$ the caged ATP binding constant.

Voltage dependence of the amount of transported charge during the on or off phase of a voltage jump-induced current signal was fitted according to an equation that represents the analytic expression of a Boltzmann distribution (cf. Sagar and Rakowski, 1994):

$$Q_{(V)} = Q_{min} + \Delta Q_{max} \cdot \{1 + e^{[z_q(V_q - V) \cdot F/(R \cdot T)]}\}^{-1}; \qquad (4)$$

$$\Delta Q_{max} = Q_{max} - Q_{min}$$

In this equation F is the Faraday constant, R is the molar gas constant, T is the absolute temperature, V_q is the potential at the midpoint of the curve, and z_q is the apparent valence, i.e., the part of the membrane potential that is sensed by a unit charge during transport across the membrane.

Voltage dependence of the observed rate constant of voltage jump-induced signals was fitted by application of the following formula (cf. Sagar and Rakowski, 1994):

$$k_{(V)} = k_1 + k_{-1(V)} = k_1 \cdot \{1 + e^{[z_k(V_k - V) \cdot F/(R \cdot T)]}\} \qquad (5)$$

In this equation V_k is the potential, at which $k_{(V)} = 2 \cdot k_1$, and z_k is the apparent valence, as defined above.

For the calculation of time constants from the ATP concentration jump experiments, the program ORIGIN (Microcal Software, Northampton, MA) was used for fitting the data traces. Voltage-induced transient currents were fitted using PClamp 6 analysis software. Fits according to Eqs. 4 and 5 and all other calculations were performed using the features provided by the ORIGIN program.

RESULTS

Transient currents obtained with ATP concentration jumps

Recently (Friedrich et al., 1996) we described a method of obtaining fast changes in ATP concentration on an excised giant patch and showed time-resolved sodium pump currents in the presence of saturating extracellular K^+ (5 mM). Now we extend this study to measurements in the absence of extracellular (and intracellular) K^+. Photolytic release of ATP at pH 6.3 resulted in fast transient outward currents with a fast rising phase followed by a slower decay and a relatively small stationary outward current (see Fig. 1). The signals could be fitted by a function with two exponential components and a constant (Eq. 2). The stationary outward current with an amplitude of ~1 pA in the absence of external K^+ appears to be relatively large (~10%) as compared to pump currents typically measured with saturating external K^+ in giant-patch experiments with comparable pipette-tip openings (~20 μm). It was absolutely dependent on internal Na^+ and ATP but not on external Na^+, exhibited an ATP dependence with an apparent K_m of ~3 μM, and showed a temperature dependence (in the temperature range 10–30°C) with an activation energy of ~60 $kJ \cdot mol^{-1}$ (data not shown).

Fig. 1 shows the current responses after the release of five different ATP concentrations (induced by irradiation of five different caged ATP concentrations with the same fraction η released at 21°C) in the same patch, demonstrating the fast rise of current and a slower relaxation to a stationary current. To test quantitative agreement with predictions from a kinetic model presented by Fendler et al. (1993), we fitted the data according to a model function (Eq. 2) with a k_1 ($= \tau_1^{-1}$) fixed at 150 s^{-1} (21°C), k_2 ($= \tau_2^{-1}$) was obtained according to Eq. 3 (from which the reciprocal time constant of the binding/dissociation reaction of ATP/caged ATP to/from the enzyme can be calculated), and the amplitudes A_1, A_2, and I_∞ were allowed to vary freely. The resulting fit curves are included in Fig. 1 and show that the data can be well described quantitatively by kinetic model parameters that originated from measurements with a different enzyme (*Electrophorus electroplax*) and a different experimental technique (BLM-measurements; Fendler et al., 1993). Thus, following the argumentation of Fendler et al. (1987, 1993) for the assignment of the observed rate constants to partial reactions of the Na^+/K^+-ATPase pump cycle, we conclude that the slower rate constant k_2, which shows substrate dependence, is determined by caged ATP dissociation and ATP binding, whereas k_1, which at a given release fraction η is not dependent on ATP concentration, reflects the electrogenic transport step (or an electroneutral step preceding and thus rate-limiting an even faster electrogenic step).

The activation energy (E_A) for the two reciprocal time constants of the photolytically induced transient current signals was determined in a set of experiments in which caged ATP was photolysed with a constant release fraction η at different temperatures (in the range from 20° to 35°C) on the same membrane patch (see Arrhenius plots in Fig. 2). The fast rate constant k_1 showed an E_A of ~84 $kJ \cdot mol^{-1}$; for k_2 we obtained an E_A of ~66 $kJ \cdot mol^{-1}$. These values compare well with results from Fendler et al. (1993), who reported an E_A of 101 $kJ \cdot mol^{-1}$ for τ_1^{-1} and 68 $kJ \cdot mol^{-1}$ for τ_2^{-1} (measured in the temperature range

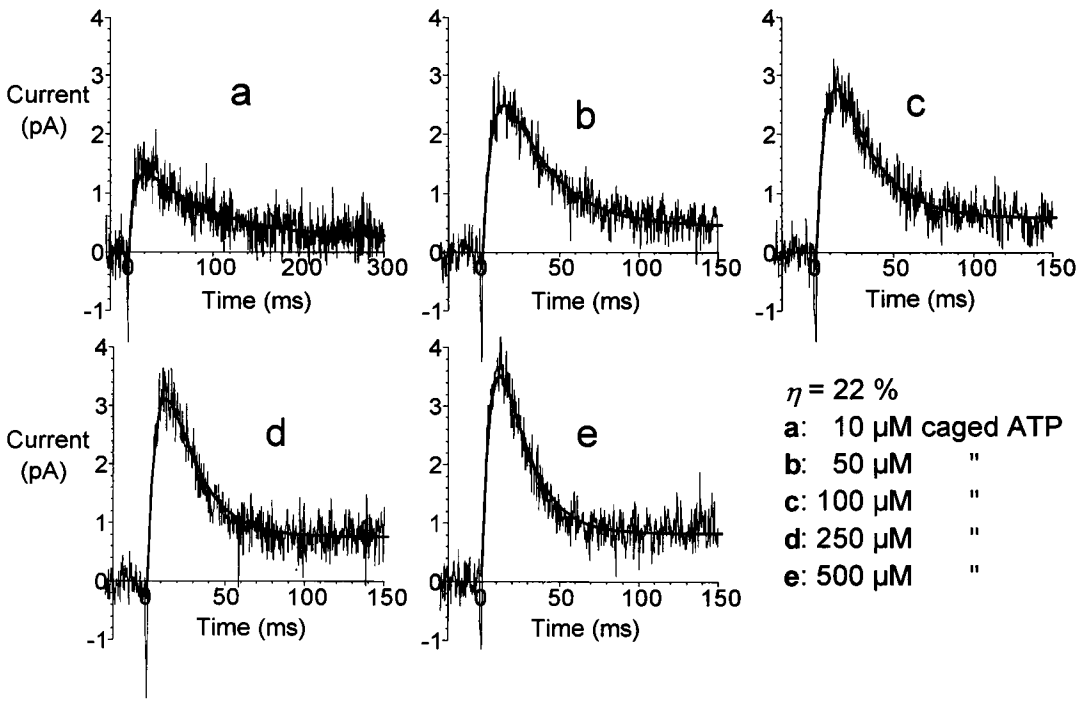

FIGURE 1 Pump current signals after photolytic release of ATP from caged ATP at pH 6.3, 21°C, $[Na^+]_i$ = 40 mM, $[Na^+]_o$ = 0, $[K^+]_o$ = 0 mM, a release fraction η = 0.22, and variable initial caged ATP concentrations (*a*: 10 μM, *b*: 50 μM, *c*: 100 μM, *d*: 250 μM, *e*: 500 μM). All traces were recorded from the same patch. Included are fits to the data according to Eq. 2, with rate constants k_1 (= τ_1^{-1}) = 150 s^{-1} and k_2 (= τ_2^{-1}) determined according to Eq. 3 (determined by Fendler et al., 1993). Bath solution: B-6.3 + caged ATP as stated. Pipette solution: P-NMG.

between 3°C and 23°C) in experiments using purified enzyme attached to an artificial lipid bilayer.

Transient currents obtained with voltage jumps

To compare the obtained rate constants from ATP concentration jump experiments to the reported slower rate constants derived from whole-cell measurements of Na^+/K^+-ATPase under Na^+/Na^+ exchange conditions (i.e., with high, 150 mM extracellular $[Na^+]$ and no extracellular K^+), we undertook similar experiments on excised giant patches. The procedure is shown in Fig. 3, *a* and *b*: variable voltage pulses were applied to the membrane in the absence of ATP, in the presence of ATP, and again after the washing out of ATP. If the current responses to test pulses in the absence of ATP did not reveal changes with time, they were subtracted from voltage pulse-induced currents in the presence of ATP, to isolate voltage-induced transient currents of Na^+/K^+-ATPase, as exemplified in Fig. 3 *c*. These voltage-induced transient currents can be fitted by a monoexponential decay function, and the obtained rate constants show a characteristic dependence on extracellular $[Na^+]$ and on the applied membrane potential (Fig. 5 *a*), as has been shown before (Nakao and Gadsby, 1986; Hilgemann, 1994). As ATP concentration jump experiments had to be carried out at pH 6.3, we checked the dependence of the voltage jump-induced rate constant on intracellular pH for the direct comparison of the rate constants obtained with the two different

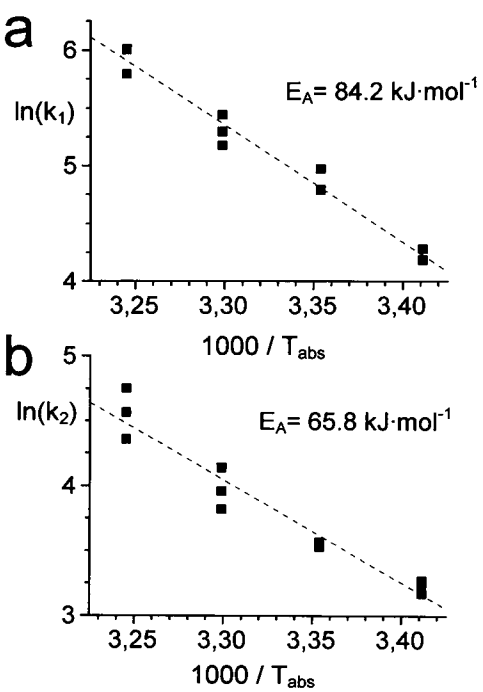

FIGURE 2 Arrhenius plot for the observed rate constants k_1 and k_2 of transient currents from ATP concentration jump experiments (η = 25%) carried out several times at four different temperatures between 20°C and 35°C on the same patch from a guinea pig cell. The activation energies were calculated from linear regression: $E_A(k_1)$ = 84.2 kJ · mol^{-1}, $E_A(k_2)$ = 65.8 kJ · mol^{-1}. Bath solution: B-6.3 + 40 μM caged ATP. Pipette solution: P-Na.

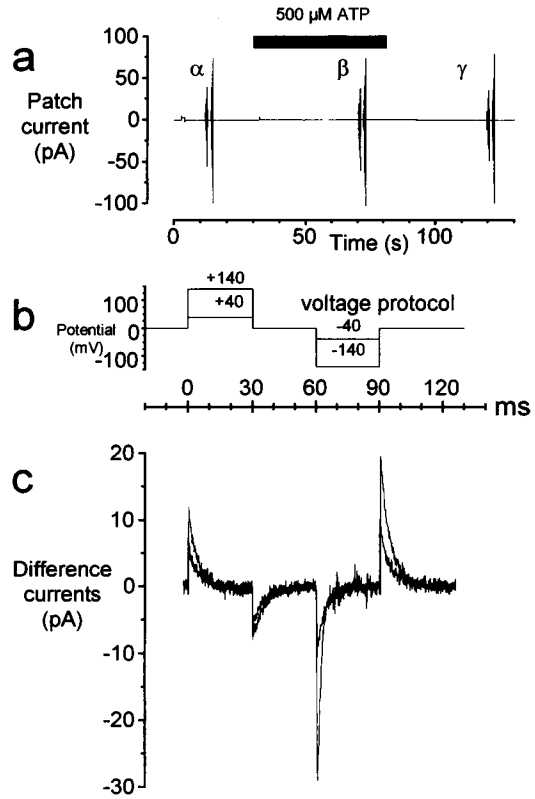

FIGURE 3 (*a*) Procedure for obtaining voltage jump-induced transient currents of Na^+/K^+-ATPase; the patch current at low time resolution is shown. Current signals are responses to voltage protocols as indicated in *b*, rectangular voltage pulses of alternating sign and successively increasing amplitude were applied first in the absence of ATP (α), then in the presence of 500 μM ATP (β) and as a control in the absence of ATP again (γ). (*c*) Difference currents obtained by subtraction of corresponding patch currents in α from those in β (or γ from β) in *a*. Four voltage jumps (to $\pm$40 mV and to $\pm$140 mV) are shown that yield eight transient current signals (on and off phases), which could be fitted well by a monoexponential decay function. Bath solutions: B-7.4 with or without 500 μM ATP. Pipette solution: P-Na. 24°C.

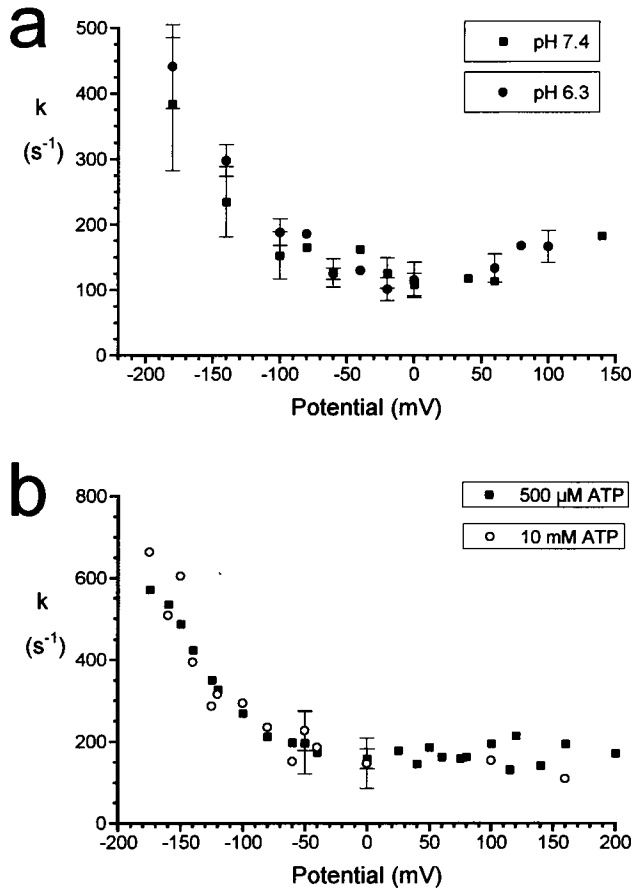

FIGURE 4 (*a*) Voltage dependence of fitted rate constants (values are means $\pm$ SD) from voltage jump experiments at pH_i 6.3 and 7.4 on the same patch. Bath solutions: B-6.3 and B-7.4. The pipette solution contained no K^+ and 40 mM Na^+, 100 mM NMG (21°C). (*b*) Voltage dependence of fitted rate constants from voltage jump experiments with 500 μM ATP or 10 mM ATP on the same patch. Data at 0 mV and -50 mV were obtained from the transient currents of the off phases of voltage jumps back to the holding potential (0 mV or -50 mV, respectively) and indicate the amount of experimental scatter. Bath solution: B-7.4 + ATP as stated. Pipette solution: P-Na. 24°C.

relaxation techniques. Fig. 4 *a* demonstrates that the rates do not differ between pH_i 6.3 and 7.4. Analysis of the transferred charge showed that its value also did not change within this pH range (data not shown).

It must be noted that different concentrations of ATP were used in our giant-patch measurements (500 μM), as compared to whole-cell experiments (10 mM) by Nakao and Gadsby (1986). If high nucleotide concentrations were able to slow down the electrogenic step, this might explain the differences in the observed rate constants. To address this question, we carried out voltage-jump protocols in the presence of 500 μM ATP or 10 mM ATP on the same membrane patch, but differences in the voltage dependence of the obtained rate constants could not be observed (as shown in Fig. 4 *b*).

The observed rate constant in voltage jump experiments is the sum of the forward and backward rate constants in a simple two-state model (cf. Nakao and Gadsby, 1986; Sagar and Rakowski, 1994). As this sum increases with increasing hyperpolarization but reaches a plateau at positive potentials, it can be concluded that the backward rate constant is strongly voltage dependent, and the plateau value reflects the forward rate constant (Nakao and Gadsby, 1986). This analysis yields a forward rate constant of $\sim$150 s^{-1} at 0 mV and pH 7.4 (21°C; see Fig. 5 *a*), very similar to the rate for the electrogenic event (or step preceding and rate-limiting the electrogenic step), seen with photolysis of caged ATP at 0 mV and pH_i 6.3 (21°C) in the same patch (Fig. 5 *c*).

In another set of experiments, voltage jump-induced transient currents were measured at different temperatures. Fig. 6 *a* shows data from an experiment in which voltage jump protocols were applied on the same patch at three different temperatures. The Arrhenius plot for the forward rate constants (means of values at zero or positive potentials) of the

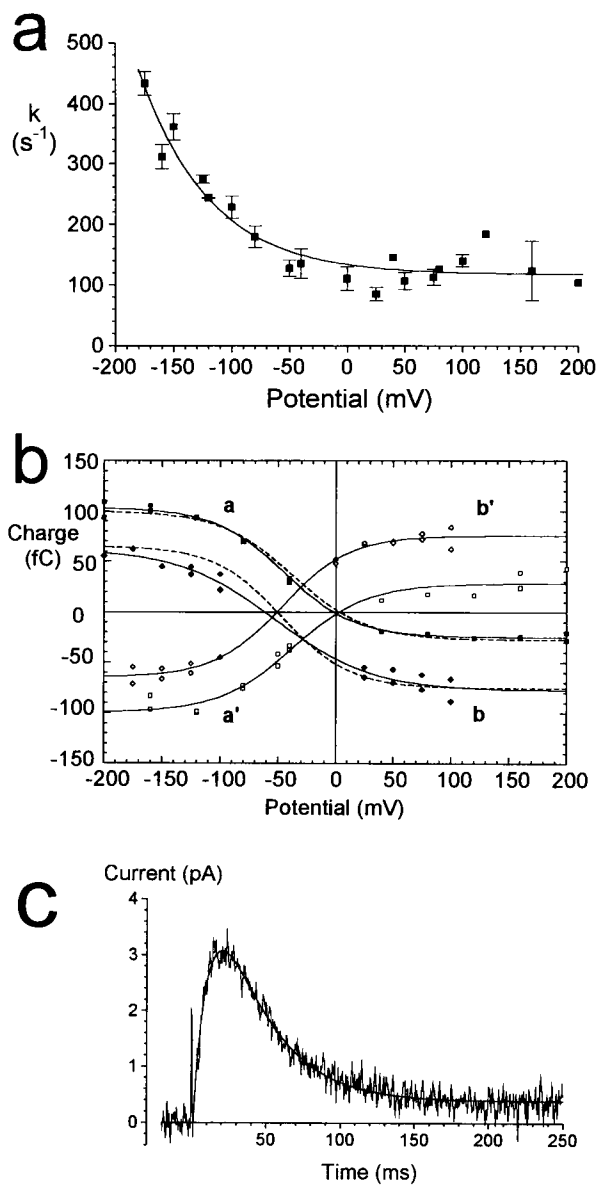

FIGURE 5 (*a*) Voltage dependence of fitted rate constants of the voltage jump experiment in the presence of 145 mM Na_o^+, no K_o^+ (pipette solution: P-Na) at 21°C, pH_i 7.4. Values are means ± SD from two independent runs of voltage protocols on the same patch. Included is a fit to the data according to Eq. 5 (with the following values: k = 116 ± 11 s^{-1}, z_k = 0.42 ± 0.07, V_k = −116 ± 15 mV). (*b*) Voltage dependence of charge, transferred during transient currents (calculated from fitted parameters: initial amplitude at time of voltage step multiplied by the time constant) from two independent runs of voltage protocols on the same patch. Included are fits to the data according to Eq. 4 (*solid line*, fit parameters as stated in Table 1) referring to four data sets: *a*: 0 mV holding potential between voltage pulses, off phases of voltage steps; *a'*: 0 mV, on phases; *b*: −50 mV, off phases; *b'*: −50 mV, on phases. The dashed lines were obtained by multiplication of the fitted curves corresponding to data sets *a'* and *b'* with (−1) for comparison of the amount of charge translocated during the on or the off phase of a voltage pulse. (*c*) Transient current signal from a photolysis experiment on the same patch (bath solution: B-6.3 + 50 μM caged ATP, η = 25%). Included is a fit to the data according to eq. 2 with the following parameters: A_1 = −8.5 ± 0.4 pA, τ_1 = 11.9 ± 0.4 ms, A_2 = 8.1 ± 0.4 pA, τ_2 = 31 ± 0.7 ms, I_∞ = 0.38 ± 0.01 pA, and with $Q = A_1 \cdot \tau_1 + A_2 \cdot \tau_2$ follows for the amount of transported charge: Q = 151 fC.

TABLE 1 Fit parameters of transferred charge

Data set	ΔQ (in fC)	V_q (in mV)	z_q
a	129 ± 4	−45 ± 3	0.83 ± 0.09
a'	128 ± 11	−37 ± 6	0.80 ± 0.22
b	140 ± 14	−52 ± 11	0.61 ± 0.11
b'	140 ± 7	−46 ± 7	0.87 ± 0.11

observed transient currents from these experiments exhibits an activation energy of ~80 kJ mol^{-1} (see Fig. 6 *b*).

The amount of charge transferred during a voltage step is equal for the on and the off phase of a voltage pulse and is dependent on voltage, reaching saturating values (see Fig. 5 *b*) at both extremely positive or negative potentials. The corresponding data points were fitted by a Boltzmann distribution function (see Eq. 4). From the fit parameters a value for the amount of transported charge can then be computed, 129 fC in the case of Fig. 5 *b*. This compares well with the charge transported through a transient current in a photolysis experiment on the same membrane patch (see Fig. 5 *c*).

A fit of the current trace from Fig. 5 *c* with a model function according to Eq. 2 yields a set of parameters, from which the transported charge Q can be approximated by the integral of the two exponential components: $Q = A_1 \cdot \tau_1 + A_2 \cdot \tau_2$. Because its amplitude is so small, the influence of the stationary current has been neglected to yield this approximation. This calculation yields a charge of 151 fC, which is about the same as the result derived from the voltage jump experiment (Fig. 5 *b*). Together with the good agreement between the values of the rate constants and the activation energies, these observations suggest that the same charge translocating process is being studied in the two different approaches.

DISCUSSION

Partial reactions in the Na^+ limb of the consecutive Na^+ and K^+ transport by Na^+/K^+-ATPase were investigated by measuring the transport-related current after sudden perturbations of an equilibrium. The giant patch technique allows observation of ion pumps under the well-defined conditions of an excised patch and the use of laser flash photolysis for very fast ATP concentration jumps (~500 s^{-1} at 24°C, 2 mM Mg^{2+}, pH 6.3; cf. Walker et al., 1988; Barabás and Keszthelyi, 1984). This technique (Friedrich et al., 1996) allowed us to extract kinetic parameters of the pump cycle under voltage clamp conditions.

Voltage jumps and ATP concentration jumps, under nearly identical conditions, on the same membrane patch (containing Na^+/K^+-ATPase) revealed the same rate constant for the electrogenic Na^+ translocation (or a step preceding, thus rate-limiting the electrogenic event) of ~200 s^{-1} at 24°C and 0 mV, confirming our earlier conclusion from experiments with Na^+/K^+-ATPase at saturating extracellular potassium (Friedrich et al., 1996). The amounts

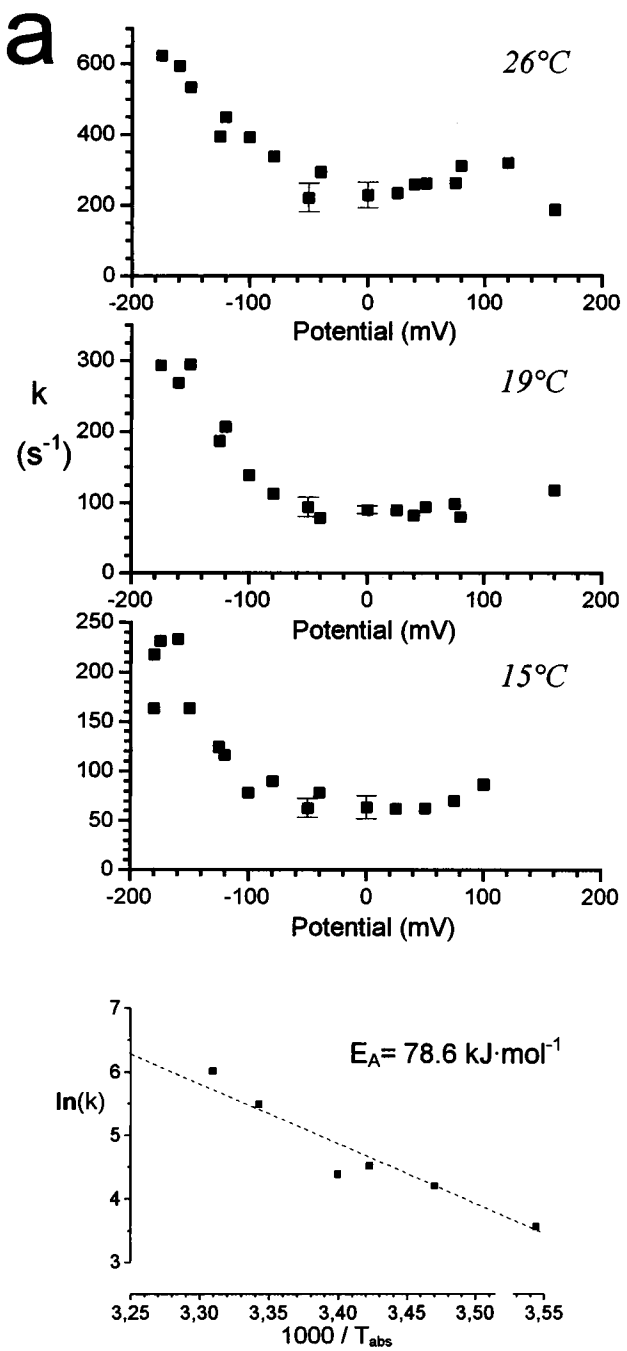

FIGURE 6 (*a*) Voltage dependence of fitted rate constants of a voltage jump experiment, obtained at three different temperatures (as indicated) on the same membrane patch (145 mM Na_o^+, pH_i 7.4, pipette solution: P-Na). Data at 0 mV and −50 mV were obtained from the transient currents of the off phases of a voltage jump back to the holding potential (0 mV or −50 mV, respectively) and indicate the amount of experimental scatter. (*b*) Arrhenius plot for the fitted rate constants (mean of values at zero or positive potentials) from several experiments as in *a*, covering different temperature ranges. Straight line fit to the data points leads to an activation energy of 78.6 kJ · mol^{-1}.

of charge that could be transported by the whole ensemble of Na^+/K^+-ATPase molecules within a membrane patch evoked by a sudden ATP concentration jump or a voltage jump are the same, and the activation energies for the rate constant of the step that is rate-limiting the electrogenic charge translocation process, obtained with these two different methods, also are identical. These observations indicate that the same process is observed with the two different relaxation techniques.

From this equivalence, together with the finding that the rate constants in voltage jump-induced transient currents did not differ between pH_i 6.3 and 7.4, it can be concluded that the reaction step that is rate-limiting the charge-translocating process proceeds with a rate constant of 200 s^{-1} at 24°C, at pH 6.3 and 7.4.

The data presented here corroborate the results of Fendler et al. (1987, 1993) for pig kidney and eel electric organ Na^+/K^+-ATPase and now also for guinea pig Na^+/K^+-ATPase in heart cells: 1) Rapid release of ATP from caged ATP results in transient current signals with a fast rising phase followed by a slower decay and a stationary current. Whereas the fast rate constant is not dependent on the ATP concentration, the slow component, which exhibits strong substrate dependence, reflects caged ATP dissociation from the enzyme and ATP binding to the enzyme. Transient currents from giant-patch experiments on heart cell Na^+/K^+-ATPase can be well described by a parameter set derived from the work of Fendler et al. (1993), obtained on a different enzyme preparation with a different experimental technique under otherwise comparable conditions. 2) The major electrogenic event (or the step that is rate-limiting the electrogenic event) proceeds with a rate constant of at least 200 s^{-1} at 24°C and 0 mV (pH_i 6.3 and 7.4). This was found in combined voltage jump and ATP concentration jump experiments. The small stationary current, which can be measured even in the absence of extracellular K^+, suggests a slow overall turnover rate, indicating that the rate-limiting step of the pump cycle must be in the K^+ transporting limb under these conditions.

The value for the rate constant of the charge translocating reaction step (or the step that is rate-limiting charge translocation) is in good agreement with previously published figures from caged ATP experiments at pH 6.2 (Fendler et al., 1987, 1993), voltage jump experiments on oocytes (Rakowski, 1993; Holmgren and Rakowski, 1994), and giant patches from heart cells (Hilgemann, 1994).

Why the electrogenic step seems to be slower in whole-cell patch-clamp measurements of heart cells (cf. Nakao and Gadsby, 1986) remains an interesting question. This discrepancy arises not only from the observed rate constants. It also becomes apparent from estimations of pump densities in whole-cell with respect to giant-patch measurements on heart cells. Nakao and Gadsby (1986) calculated a pump density of 1200 μm^{-2} from measurements of the moveable charge in voltage jump experiments on whole-cell patch-clamped myocytes, whereas Collins et al. (1992) reported the pump density to be in the range of 1200–3200 μm^{-2} from ATP-induced current transients measured on cardiac excised giant-patch membranes. This difference may be due to a specific cardiac cellular down-regulation, which is

functional under whole-cell conditions, but not in excised patches. This is also suggested by the observation of a slow activation process, which appears in pump-current measurements in giant-patch experiments upon first application of ATP and Na^+-containing solution (Hilgemann, 1995a; Friedrich et al., 1996).

Apell et al. (1987) and Wuddel and Apell (1995) found from caged ATP experiments, carried out with the bilayer technique, a much slower rate constant of 25 s^{-1} (20°C) for the electrogenic step. Again, it must be pointed out that the choice of conditions under which caged ATP photolysis experiments are carried out is crucial for the time resolution. If conditions are chosen (10 mM Mg^{2+}, pH 7.2, 20°C; Wuddel and Apell, 1995) such that ATP release proceeds with a rate constant as slow as 40 s^{-1}, the assignment of the observed rate constants to either ATP release or an intrinsic reaction step of the enzyme may not be unequivocal. Variation of the substrate concentration in caged ATP experiments, however, clearly reveals substrate dependence of the slow rate constant, which excludes the possibility that k_2 might represent an intrinsic reaction of Na^+/K^+-ATPase.

Recently we reported a bell-shaped dependence of the Na^+/K^+-ATPase pump current on intracellular pH under saturating substrate conditions (Friedrich et al., 1996). Our voltage jump experiments show, however, that lowering the pH to 6.3 does not slow down the rate-limiting sodium transport step. Therefore we suggest, in agreement with Forbush and Klodos (1991), that lowering cytoplasmic pH may slow down the potassium translocating step and/or the phosphorylation reaction—a choice that is beyond the reach of this study. Further voltage jump experiments over an extended pH range might elucidate the basis for the observed overall pH dependence.

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