ELSEVIER

Biochimica et Biophysica Acta 1509 (2000) 155–166

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

The current produced by the E779A mutant rat Na^+/K^+ pump α1-subunit expressed in HEK 293 cells

Stefan Zillikens [a], Günter Gisselmann [b], Helfried Günther Glitsch [a,*]

[a] *Arbeitsgruppe Muskelphysiologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany*
[b] *Lehrstuhl für Zellphysiologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany*

Received 18 April 2000; accepted 12 July 2000

Abstract

The current (I_p) generated by the wild-type or the glutamate (E) 779 alanine (A) mutant of the rat Na^+/K^+ pump α1-subunit expressed in HEK 293 cells was studied at 35°C by means of whole-cell recording in Na^+-free and Na^+-containing solution. Glutamate 779 is located in the fifth transmembrane domain of the α-subunit of the Na^+/K^+-ATPase. Compared with the wild-type, the E779A mutant exhibited an apparent K_o^+-affinity decreased by a factor of 3–4 both in Na^+-free and in Na^+-containing media. The competition of Na_o^+ and K_o^+ for cation binding sites of the pump remained unchanged. Similarly, in Na^+-free solution the shape of the I_p–V curves for various external K^+-concentrations ($[K^+]_o$) was essentially the same. However, in Na^+-containing solutions the shape of I_p–V curves from cells expressing the mutant of the rat α1-subunit clearly differed from the shape observed in cells expressing the wild-type, but voltage dependence of the pump current persisted. A prominent Na_o^+-activated, electrogenic Na^+-transport mediated by the pump, displaying little voltage dependence in the potential range tested (−80 to +60 mV), was present in the cells expressing the E779A mutant pump. The data suggest that exchanging E779 for A in the rat Na^+/K^+ pump α1-subunit causes a modest decrease in the apparent K_o^+ affinity and a profound, Na_o^+-dependent alteration in the electrogenicity of the mutant pump expressed in HEK 293 cells.

Keywords: Sodium pump; Pump current; Whole-cell recording; α1-Subunit; E779A mutant; HEK 293 cell; (Rat)

1. Introduction

The activation of the Na^+/K^+ pump of animal cells by extracellular K^+ (K_o^+) and its congeners has been well established for decades [1,2]. The location of the K^+-binding sites on the α1-subunit of the Na^+/K^+ pump remains, however, uncertain. The Na^+/K^+-ATPase is the molecular basis of the pump. According to Lingrel and co-workers [3,4], various amino acids within the transmembrane domains of the subunit affect the K_o^+-affinity of the enzyme. Among them, serine 775 of the fifth domain [5] along with aspartate 804 and 808 in the sixth domain [3] play a key role in the binding of K_o^+ to the enzyme. Interestingly, glutamate 779 which, like other amino acids, exerts a modest effect on the enzyme affinity for K_o^+, also affects the voltage dependence of the Na^+/K^+ pump activity. Argüello et al. [6] expressed a ouabain-resistant E779A mutant of the sheep Na^+/K^+-ATPase α1-subunit in HeLa cells and studied the exogenous pump using biochemical and electrophysiological methods. The electrophysio-

* Corresponding author. Fax: +49-234-321-4129;
E-mail: helfried.glitsch@ruhr-uni-bochum.de

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00289-3

logical studies were performed in Na^+-containing solutions. The authors reported a decrease of the apparent K_o^+-affinity of the Na^+/K^+ pump by a factor of 4 compared with the wild-type-ATPase. Furthermore, the current produced by the mutant pump displayed little voltage dependence between −100 and +60 mV. In addition the authors described for the first time an electrogenic Na_o^+/Na_i^+-exchange carried out by the pump, i.e., a pump current activated by external Na^+ (Na_o^+) in K^+-free solution.

Here, we report on the electrogenicity of the E779A mutant rat Na^+/K^+-ATPase α1-subunit expressed in HEK 293 cells. The experiments were conducted in Na^+-free and Na^+-containing media. The results differ in one important point from those reported by Argüello et al. [6]: the current generated by the rat mutant pump clearly exhibits voltage dependence both in Na^+-containing and Na^+-free media.

2. Materials and methods

2.1. Mutagenesis

Site-directed mutagenesis was performed by the unique site elimination technique [7] (U.S.E.-Kit, Pharmacia Biotech, Uppsala, Sweden). For site-directed mutagenesis the α1-subunit of the Na^+/K^+ ATPase from rat male brain [8] was cloned in the pRcCMV plasmid. The target mutagenic primer was complementary to the gene of interest and carried the intended mutation. Furthermore, an additional mismatch eliminated a MspI-restriction site in the vicinity of the target mutation (see Fig. 1). This mismatch served as an additional control for the successfully performed mutation as a result of the designed mutation-primer. This mutation did not alter the amino acid sequence of the enzyme. The mutation of the α1-subunit of the Na^+/K^+ pump was checked by MspI-restriction and ultimately by sequencing of a ±300 bp fraction of the target region of the mutated cDNA on both strands.

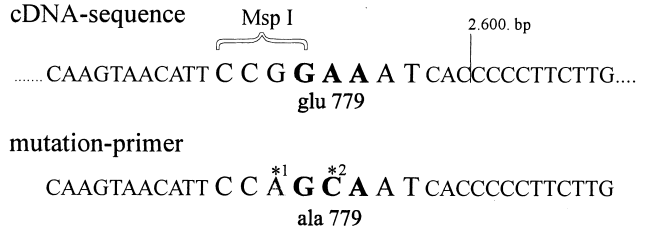

Fig. 1. The upper sequence represents a section of the cDNA of the α1-subunit of the rat Na^+/K^+ pump. The triplet printed in bold letters is coding for the glutamate in position 779. The lower nucleotide sequence depicted represents the mutation-primer. The substituted nucleotides are indicated by an asterisk.

2.2. Cell culture

HEK 293 cells were cultured in Minimal Essential Medium (MEM; Life Technologies, Eggenstein, Germany) supplemented with 10% (v/v) fetal bovine serum (Bio Whittaker, Verviers, Belgium), 20 mM L-glutamine, 1% (v/v) MEM non-essential amino acids, 100 000 IU/l penicillin, and 100 mg/l streptomycin (all from Life Technologies) in 25-cm^2 culture flasks at 37°C and 5% CO_2. The culture medium was changed every 4–5 days. Within approximately 7 days cells had grown to near confluency. Cells were split by trypsinization and then seeded in new culture flasks and culture dishes (diameter 35 mm, no. 1008, Falcon, Becton Dickinson, Plymouth, UK), at a density of 4000 cm^{-2}.

2.3. Transfection and establishment of a stable transfected cell line

HEK 293 cells were transfected with the eucaryotic expression plasmid pR α1 derived from pRc/CMV (Invitrogen, San Diego, USA). This vector contained the wild-type and E779A-mutant of the α1-subunit of the rat Na^+/K^+-ATPase. Two days prior to transfection HEK 293 cells were seeded to a density of $\sim 1\times10^5$ in a culture flask (25 cm^2). The transfection was performed by means of Ca^{2+} phosphate coprecipitation [9]. The 'transfection mix' consisting of 250 μl 2×Hepes-buffered saline (in mM: 280 NaCl, 2.8 Na_2HPO_4, 50 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.2 (NaOH)), and 250 μl of 250 mM $CaCl_2$ solution containing either 14 μg of the wild-type- or E779A-plasmid was added drop by drop to the cells in the culture flasks containing 2.5 ml medium. Following a 3-h incubation period at 37°C and 5% CO_2, the cells were washed twice with phosphate-buffered saline (in mM: 0.9

$CaCl_2$, 2.7 KCl, 1.5 KH_2PO_4, 0.5 $MgCl_2$, 106 NaCl, 6.5 Na_2HPO_4, pH 7.2 (NaOH)). The cells were cultured for 2 days. Due to the fact that the transfected cells expressed the cardiac steroid-insensitive isoform of the rat Na^+/K^+-ATPase, stable transfected HEK 293 cells were obtained by the addition of 5 μM ouabain to the culture medium. This concentration of ouabain inhibits ~95% of the pump current (I_p) generated by the endogenous expressed Na^+/K^+ pumps. Non-transfected cells in medium containing 5 μM ouabain died within 2 days. This unique system is independent of the existence of an antibiotic resistance gene. After approximately five passages a polyclonal stably transfected cell line was established.

2.4. Whole-cell recording

A culture dish (diameter 35 mm, Becton Dickson) containing HEK 293 cells was placed on the stage of an inverted microscope (IM 35, Zeiss, Oberkochen, Germany) and perfused with bathing solution prewarmed to 37°C by means of a Peltier element. Solution flow, driven by gravity, was 2–3 ml/min. An outlet, which was positioned opposite to the inlet, kept the solution level constant in the dish. The cell under study was additionally superfused at 0.4 ml/min via a multibarreled pipette (inner diameter: 0.5 mm) with one of the solutions from reservoirs fixed 20 cm above the stage and connected to the pipette via plastic and teflon tubes. Solution changes controlled by electromagnetic valves (The Lee Company, Westbrook, USA) were completed within 1 s. The temperature of the solution in the vicinity of the cell studied was 35°C. The ventricular myocytes were whole-cell voltage-clamped [10] with pipettes made from borosilicate glass capillaries (GC150 TF-10, Clark Electromedical Instruments, Reading, UK) and back-filled with pipette solution. The filled pipettes had an initial resistance between 2.5 and 6 MΩ. Potential differences between the pipette and the superfusion medium were set to zero just before patch formation. In order to establish a gigaohm seal the pipette was positioned at the cell surface by means of a micromanipulator (FT 103, Narishige London, UK). Thereafter, gentle suction was applied to the pipette. Following establishment of the seal a second suction pulse ruptured the cell membrane beneath the pipette's tip and established the whole-cell configuration of the patch-clamp technique. The myocytes were voltage clamped by means of an EPC-7 patch-clamp amplifier (List Medical, Darmstadt, Germany). A PC was connected to the amplifier via 12-bit AD- and DA-converters, respectively. Voltage protocols were generated and the resulting membrane currents were recorded by the program ISO2 (MFK, Niedernhausen, Germany). The currents were low-pass filtered at 200 Hz and digitized at 1 kHz. The cell capacitance (C_m) was estimated by means of a depolarizing pulse of 60 mV starting from −70 mV. The capacitance varied widely between 18 and 65 pF. A mean value of 30.6 ± 7.6 (S.E.M.) pF was obtained from 25 cells in which the activation of the pump current I_p by external K^+ at 0 mV was studied. I_p was estimated as current activated by K_o^+ or inhibited by ouabain (10^{-2} M).

2.5. Solutions

The composition of the sodium-containing bathing solution was (in mM): 144 NaCl, 0–10.8 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes, pH 7.4 (NaOH). The sodium-free medium contained (mM): 144 choline chloride, 0–10 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes, 5×10^{-3} atropin sulfate, pH 7.4 (TEAOH). The bathing solution also contained 2–4 mM $BaCl_2$ and 5 mM $NiCl_2$ in order to block K^+- and Ca^{2+}-conductances [11,12], respectively. The pipette solution was composed of (in mM): 110 CsCl, 40 NaCl, 10 NaOH, 3 $MgCl_2$, 6 EGTA, 16 Hepes, 10 Mg-ATP, pH 7.4 (CsOH).

2.6. Drugs

Digitoxigenin (Fluka Chemie, Buchs, Switzerland) was diluted from a 10^{-3} M stock solution containing 10% ethanol to a final concentration of 5×10^{-6} M in the bathing solution. At this concentration the drug blocked the endogenous Na^+/K^+ pumps of the HEK 293 cells almost completely ($K_{0.5}$: ~10^{-6} M). The ethanol concentration of the bathing solution never exceeded 0.5‰ and had itself no effect on the membrane current of the cells. Ouabain (Sigma, Deisenhofen, Germany) was dissolved in the external media to obtain a final concentration of 10^{-2} M. This drug is a specific Na^+/K^+ pump inhibitor.

2.7. Statistics and curve fitting

Whenever possible, data are presented as ± S.E.M. *n* indicates the number of myocytes studied. Error bars are only shown in the figures if they exceed the size of the symbols. Differences between the data points were analyzed by Student's two-tailed, unpaired *t*-test and considered significant if $P \leq 0.05$.

The curves fitted to the data in Fig. 4 obey the Hill equation:

$$I_p = I_{p(max)}/[1 + (K_{0.5}/[K_o^+]^{n_H})$$

where $I_{p(max)}$ denotes the maximal pump current I_p, $K_{0.5}$ is $[K^+]_o$ for half-maximal I_p activation, and n_H represents the Hill coefficient.

3. Results

3.1. Endogenous and exogenous pump current I_p

Preliminary experiments on three HEK 293 cells in a medium containing 144 mM Na^+ and 5.4 mM K^+ resulted in an apparent K_d value (K_d') of 9×10^{-7} M for I_p inhibition by digitoxigenin. Therefore, 5×10^{-6} M digitoxigenin is expected to inhibit almost completely the pump current (~95%). Fig. 2 shows the membrane current of a HEK 293 cell transfected with the wild-type of the cardiac glycoside-insensitive α1-subunit of the rat Na^+/K^+ pump. The membrane potential is clamped to 0 mV (V_c). The cell is superfused with Na^+-free, choline$^+$-containing solution. The horizontal lines above the current trace indicate changes of the external K^+ concentration. After estimation of I_p by switching from K^+-containing to K^+-free medium 5×10^{-6} M digitoxigenin is added to the solution (arrow above the lines) in order to block I_p generated by endogenous Na^+/K^+ pumps. However, a K_o^+-activated pump current is still present under these conditions, and amounts to about 75% of I_p in digitoxigenin-free medium. The current is further inhibited by 10^{-4} M digitoxigenin. Judging from the K_o^+-activated current amplitudes, the exogenous pump molecules produce about three

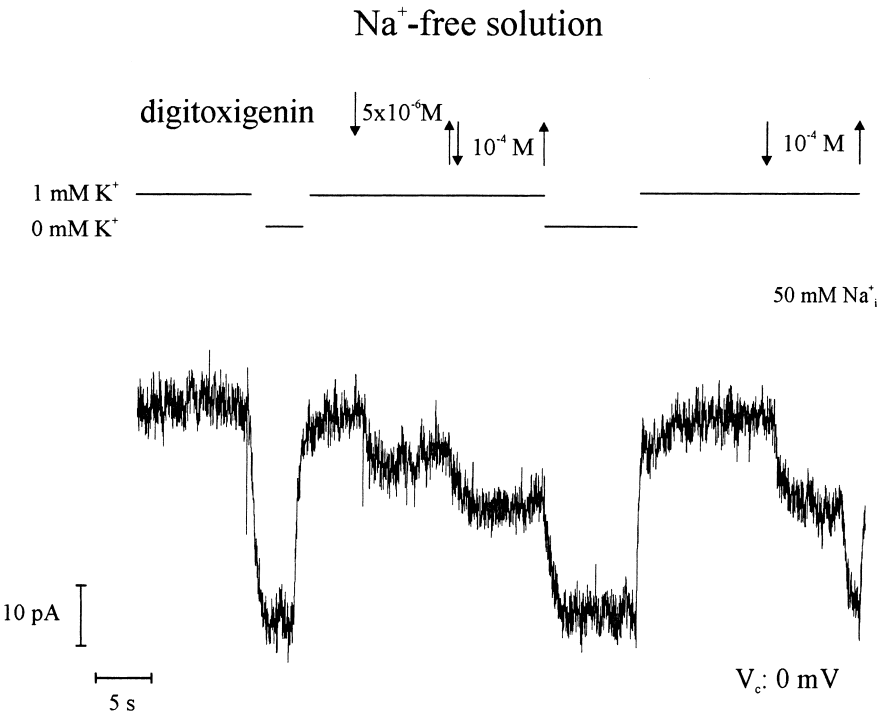

Fig. 2. Whole-cell recording from a HEK 293 cell transfected with the rat wild-type α1-subunit of the Na^+/K^+ pump in Na^+-free medium. I_p persisting in the presence of 5 μM digitoxigenin is almost completely generated by the expressed ouabain-insensitive exogenous Na^+/K^+ pump. Arrows indicate the presence of digitoxigenin in the external solution and horizontal lines above the current trace illustrate the respective $[K^+]_o$.

times more I_p than the endogenous HEK 293 cell Na^+/K^+-ATPase molecules.

3.2. Identification and K_o^+ dependence of I_p produced by exogenous rat Na^+/K^+ pumps

Fig. 3 demonstrates that the K_o^+-activated outward current of transfected HEK 293 cells superfused with a Na^+-free medium containing 5×10^{-6} M digitoxigenin is in fact I_p generated by exogenous pumps. Again, solution changes are indicated by horizontal lines above the current trace. A high concentration (10 mM) of the specific Na^+/K^+ pump inhibitor ouabain completely blocks the current activated by 1 mM K_o^+ and produced by the glycoside-insensitive rat Na^+/K^+ pump α1-subunit expressed in the cells. In addition, Fig. 3 depicts the procedure for measuring I_p as a function of $[K^+]_o$. As can be seen, I_p activated by 10 mM K^+ is slightly larger in the cell than the pump current evoked by 1 mM K_o^+. Finally, it is clear from Fig. 3 that estimations of I_p as K_o^+-activated or ouabain-sensitive current yield an identical amplitude.

Using the procedure depicted in the left-hand part of Fig. 3, we studied the I_p activation by external K^+ in HEK 293 cells expressing either the wild-type or the E779A mutant of the rat Na^+/K^+ pump α1-subunit. The experiments were carried out in Na^+-free or Na^+-containing solution including 5×10^{-6} M digitoxigenin. Fig. 4 presents the results. The upper panel (Fig. 4A) shows the activation of I_p by K_o^+ in Na^+-free medium. I_p activated by 10 mM K_o^+ was arbitrarily set to 100%. It amounted to 0.74 ± 0.31 pA/pF ($n=18$) in HEK cells expressing the wild-type and to 0.50 ± 0.17 pA/pF ($n=4$) in cells containing the mutant α1-subunit of the rat Na^+/K^+ pump. I_p at other $[K^+]_o$ is given correspondingly. The Hill equations fitted to the data suggest a $[K^+]_o$ for half-maximal I_p activation (apparent $K_{0.5}$ value) of 0.17 mM for HEK 293 cells expressing the wild-type and of 0.58 mM for cells expressing the E779A mutant. The Hill coefficient (n_H) equated to 1.03 and 1.55, respectively. Fig. 4A shows that the apparent affinity of the mutant for external K^+ is lower by a factor of ~3 than the affinity of the wild-type α1-subunit. A similar result was obtained from measurements on transfected cells in Na^+-containing solution. In Fig. 4B I_p-activation was plotted as a function of $[K^+]_o$ normalized to I_p at 10.8 mM K_o^+, which was thought to represent the maximal I_p in HEK-cells expressing the wild-type. At this $[K^+]_o$, I_p density amounted to 0.67 ± 0.16 pA/pF ($n=13$) in cells containing the wild-type of the rat α1-subunit and to 0.48 ± 0.21 pA/pF ($n=13$) in HEK 293 cells expressing the mutant. It has been known for a long time that the apparent affinity of the Na^+/K^+ pump for K_o^+ is lower in Na^+-containing than in Na^+-free solution. Accordingly, the $K_{0.5}$ value for the I_p acti-

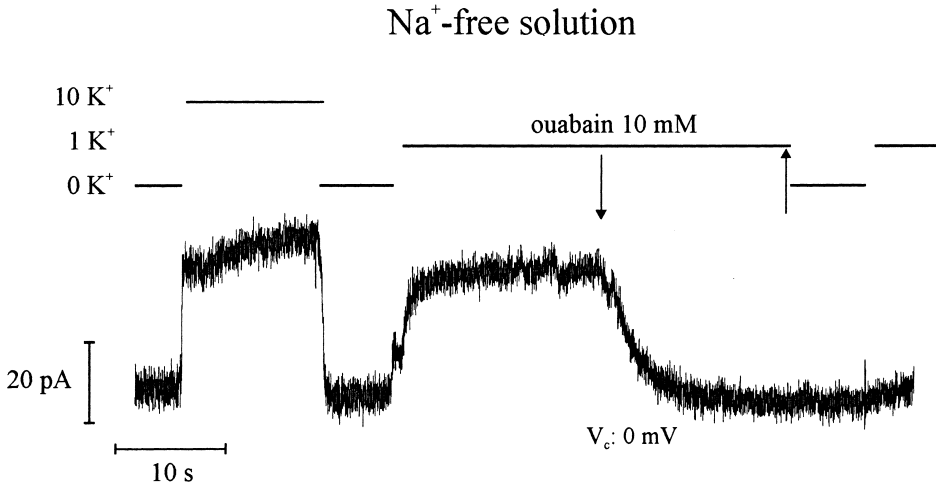

Fig. 3. Membrane current of a HEK 293 cell expressing the wild-type of the rat Na^+/K^+ pump in Na^+-free solution containing 5 μM digitoxigenin to suppress endogenous I_p. The ouabain (10 mM)-sensitive current is identical to the K_o^+-activated I_p. Arrows indicate the presence of ouabain in the external solution and horizontal lines above the current trace illustrate the respective $[K^+]_o$.

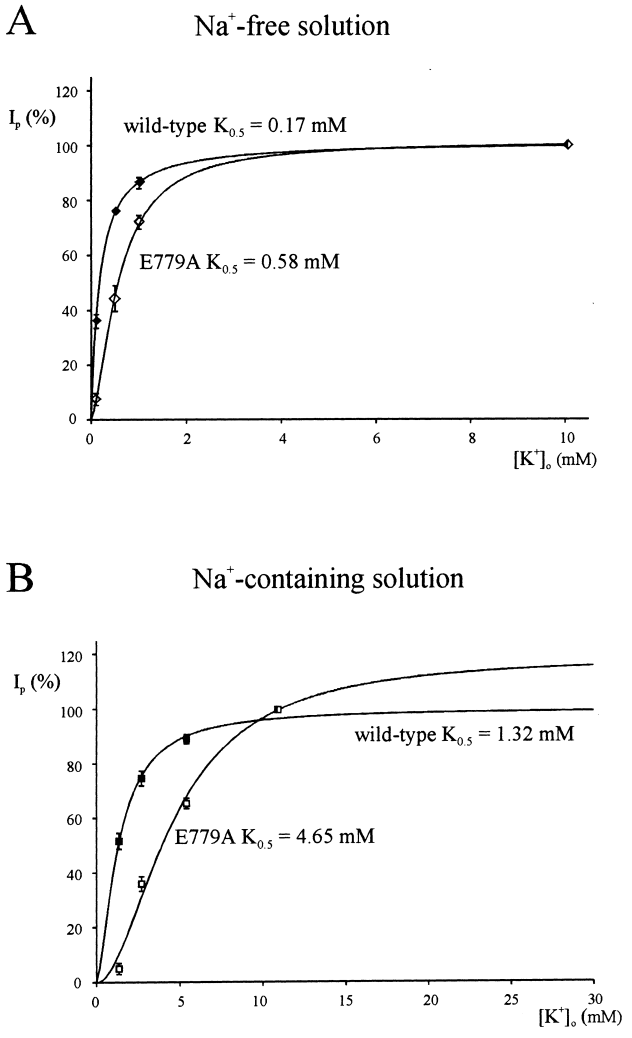

Fig. 4. Activation of I_p as a function of the extracellular K^+-concentration in Na^+-free (A) and Na^+-containing (B) solution ($V_c = 0$ mV). Pump currents generated by 10 mM K_o^+ in Na^+-free and 10.8 mM K_o^+ in Na^+-containing solutions were arbitrarily set to 100%. Mean values of data points were fitted by a Hill equation. Wild-type (filled symbols): $n = 3$–6; E779A mutant (open symbols): $n = 8$–9.

vation by K_o^+ was increased by almost the same factor in HEK 293 cells expressing one of the two rat α1-subunits. This increase in $K_{0.5}$ value in Na^+-containing solutions probably reflects a competition between Na_o^+ and K_o^+ for external cation binding sites of the Na^+/K^+ pump. Thus, it appears from the data shown in Fig. 4 that the competition remains unchanged in the mutant pump, though the apparent affinity for external K^+ is decreased. The reduced K_o^+ affinity for the E779A mutant pump is the reason why maximal I_p activation is reached only at a $[K^+]_o$ much higher than 10 mM (Fig. 4B). The Hill coefficient was calculated to be 1.21 for the wild-type and 1.9 for the mutant pump.

3.3. I_p generated by pumps containing the mutant rat α1-subunit is voltage-dependent

According to Argüello et al. [6] HeLa cells expressing the E779A mutant of a ouabain-resistant α1-subunit of the sheep Na^+/K^+ pump exhibit a voltage-independent I_p in Na^+-containing media at K_o^+ concentrations between 2 and 50 mM. By way of contrast, cells expressing the wild-type sheep α1-subunit display normal I_p–V curves under these conditions. I_p increases with depolarization (positive slope of the I_p–V relationship) at $[K^+]_o \geq 5$ mM and decreases (negative slope) at 0.5 or 1 mM K_o^+. We studied the voltage dependence of I_p generated by the α1-subunit of the rat Na^+/K^+ pump expressed in HEK 293 cells. The cells were superfused with Na^+-free or Na^+-containing solutions. The media contained 5×10^{-6} M digitoxigenin. Fig. 5 shows I_p–V curves measured in Na^+-free solutions containing 0.1–10 mM K_o^+. The upper part (Fig. 5A) represents I_p–V relationships of HEK 293 cells expressing the wild-type of the rat α1-subunit. Mean I_p densities were plotted versus membrane potential. The I_p–V curves were obtained as differences between membrane current-voltage relationships measured in K_o^+-containing or K_o^+-free media by voltage ramps (+60 to −80 mV in 1.5 s). The curves clearly indicate that I_p is voltage-dependent. As expected from I_p–V relationships of other cells in Na^+-free solution, I_p increases with depolarization at $[K^+]_o \geq K_{0.5}$ value (curves observed at 0.5 to 10 mM K_o^+), but decreases at $K_o^+ \leq K_{0.5}$ value (I_p–V relationship at 0.1 mM K_o^+) [13–15]. HEK 293 cells expressing the E779A mutant of the rat Na^+/K^+ pump α1-subunit display a quite similar dependence of I_p on $[K^+]_o$ and membrane potential (Fig. 5B). However, a closer inspection of the curves reveals that I_p at 0.5 mM K_o^+ is almost voltage-independent, in contrast to the pump current of cells expressing the wild-type of the rat α1-subunit (compare Fig. 5A).

Fig. 6A shows I_p–V curves of HEK 293 cells expressing the wild-type of the rat α1-subunit. The curves were obtained in Na^+-containing solution in-

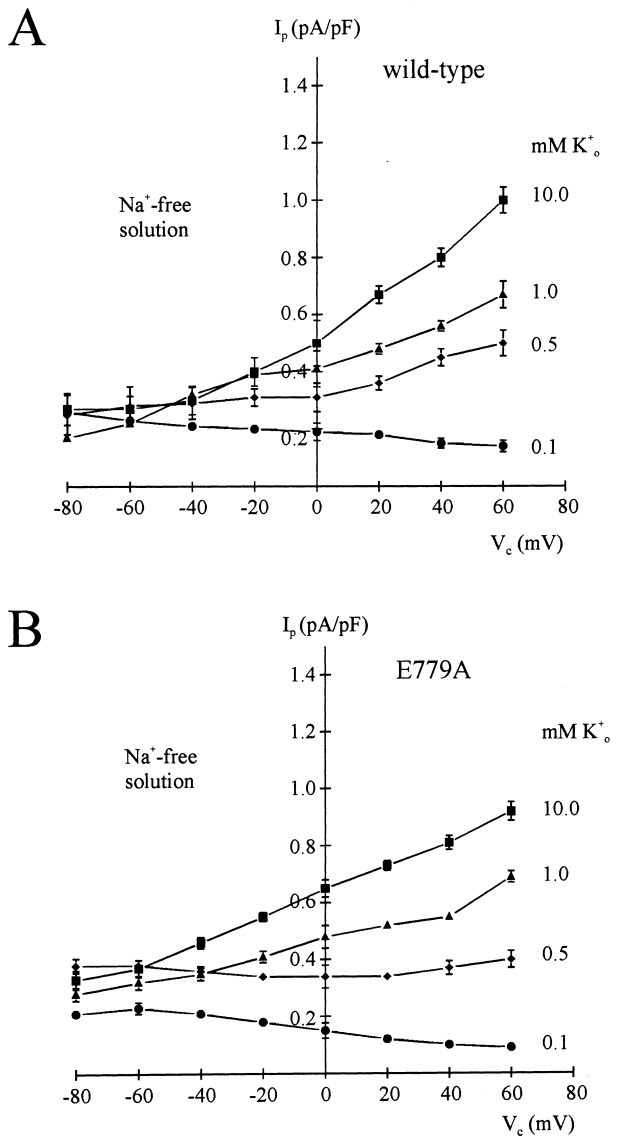

Fig. 5. I_p–V relationships of HEK 293 cells expressing the α1-subunit of the rat Na^+/K^+ pump. Measurements in Na^+-free media containing 0.1 mM to 10 mM K^+_o. Pump current densities are plotted versus clamped membrane potential (V_c). (A) I_p–V curves of cells expressing the wild-type of the rat α1-subunit ($n=3$–6). (B) I_p–V curves of HEK 293 cells expressing the E779A mutant ($n=7$–11). Note the shallow positive slope of the curves at $[K^+]_o=0.5$ mM in A (wild-type) and the voltage-independent I_p at $[K^+]_o=0.5$ mM in B (mutant).

cluding 5×10^{-6} M digitoxigenin at various $[K^+]_o$. I_p densities were plotted versus membrane potential. Not only the I_p amplitude at 0 mV, but also the shape of the I_p–V relationship varies with the external K^+-concentration. At low $[K^+]_o$ the curves display a negative slope over the entire voltage range tested (circles, 1.35 mM K^+_o; diamonds, 2.7 mM K^+_o). A region of negative slope has been described earlier for other cells in Na^+-containing media, especially at low $[K^+]_o$ [16]. The I_p–V curves of the HEK 293 cells exhibit a positive slope at higher $[K^+]_o$ (triangles, 5.4 mM K^+_o; squares, 10.8 mM K^+_o) where the external K^+-binding sites of the pump molecules are almost saturated (compare Fig. 4). This is in accordance with observations in other cell species [13]. The variation of the I_p–V relationships with $[K^+]_o$ is more clearly seen in Fig. 6C where the I_p amplitudes at various membrane potentials were normalized to their respective amplitudes at 0 mV and plotted versus voltage. The negative slope of the I_p–V curves at low $[K^+]_o$ and the positive slope at higher $[K^+]_o$ are easily recognized. Interestingly, HEK 293 cells expressing the E779A mutant of the rat Na^+/K^+ pump α1-subunit exhibit I_p–V relationships, where the general shape remains qualitatively unchanged at the various K^+_o concentrations between 2.7 and 10.8 mM K^+_o (Fig. 6B). Again, I_p densities at various $[K^+]_o$ were plotted versus membrane potential. However, irrespective of the $[K^+]_o$ at which the I_p–V curves were obtained, the curves display little voltage dependence between −80 and −20 mV. A distinct positive slope is observed at 5.4 and 10.8 mM K^+_o at positive potentials. Of course, the I_p-density varies with the $[K^+]_o$ at 0 mV. If compared with Fig. 6C, the normalized I_p–V relationships shown in Fig. 6D emphasizes the smaller K^+_o effect and the reduced voltage dependence of I_p in cells expressing the E779A mutant of the α1-subunit. Nevertheless, a voltage dependence of the pump current amplitude does exist for 5.4 mM ($P=0.0001$) and 10.8 mM K^+_o ($P=0.03$) in contrast to the observations of Argüello and co-workers [6] on HeLa cells expressing the E779A mutant of a ouabain-insensitive sheep Na^+/K^+ pump α1-subunit. The data presented in Fig. 6 reveal remarkable, quantitative differences in the K^+_o dependence of the I_p–V curves observed in HEK 293 cells expressing either the wild-type or the mutant rat Na^+/K^+ pump α1-subunit, if measured in Na^+-containing media, whereas the differences are small for cells in Na^+-free solutions (Fig. 5). Thus, extracellular Na^+-dependent processes affect the I_p–V relationship of the HEK 293 cells expressing the E779A mutant in an unexpected way.

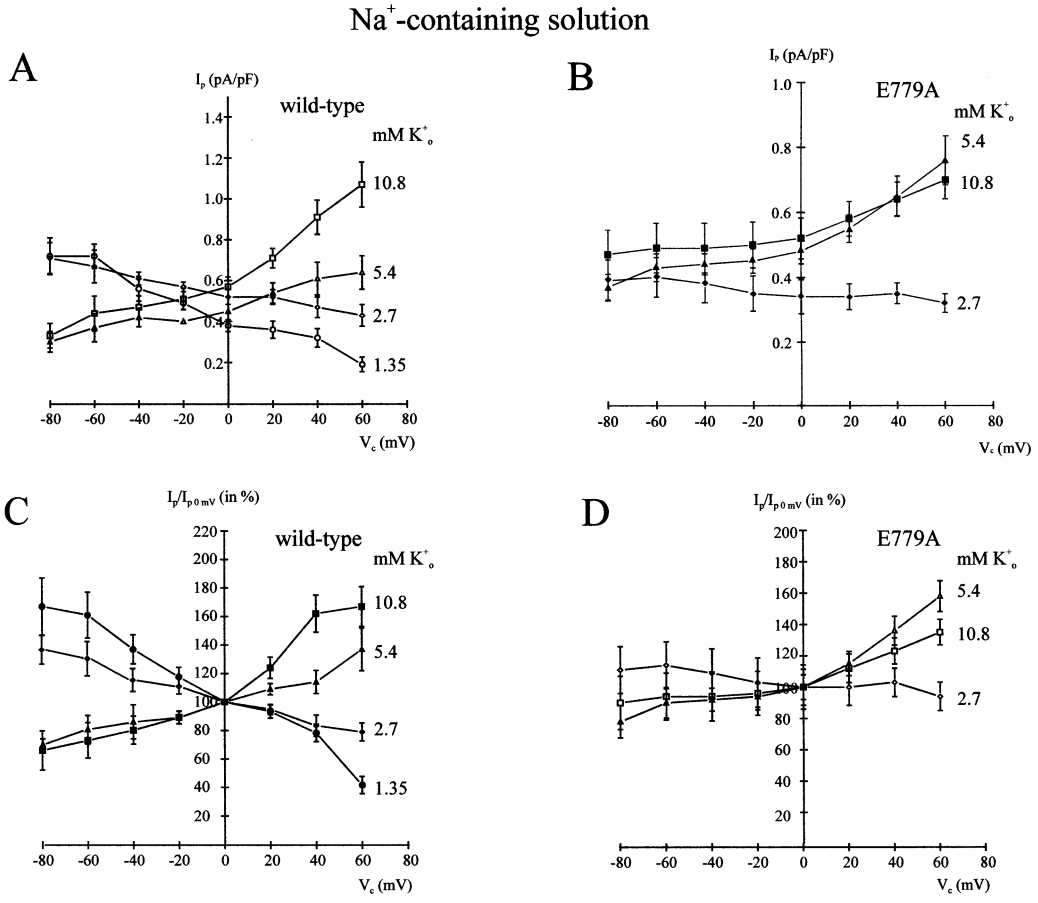

Fig. 6. I_p–V relationships of HEK 293 cells expressing the α1-subunit of the rat Na^+/K^+ pump. Measurements in Na^+-containing solutions at $[K^+]_o$ between 1.35 and 10.8 mM. Pump current densities are plotted versus membrane potential. (A) I_p–V curves of cells expressing the wild-type of the rat α1-subunit ($n=5$–6). (B) I_p–V curves of HEK 293 cells expressing the E779A mutant ($n=7$–9). I_p is nearly voltage-independent between −80 and −20 mV, but increases at more positive potentials for $[K^+]_o > 2.7$ mM. Note different scaling of the ordinates in A and B. (C) Normalized I_p–V relationships. Data from A. I_p amplitudes normalized to the respective I_p amplitudes at 0 mV. (D) Normalized I_p–V relationships. Data from B. I_p amplitudes normalized to the respective I_p amplitudes at 0 mV.

3.4. A Na_o^+-activated I_p in HEK 293 cells expressing the E779A mutant of the rat α1-subunit

A unique Na_o^+/Na_i^+-exchange has been discovered by Argüello et al. [6] in HeLa cells expressing the E779A mutant of the sheep $Na^+/^+$ pump α1-subunit. We do confirm the existence of this unusual mode of electrogenic cation transport via the Na^+/K^+ pump. HEK 293 cells expressing the E779A mutant of the rat pump α1-subunit also exhibit a ouabain-sensitive Na_o^+/Na_i^+-exchange. As can be seen from Fig. 7A the amplitude of I_p generated by these cells in Na^+-free solution is roughly the same regardless of whether I_p is estimated as K_o^+-activated or ouabain-sensitive current. This is also true for HEK 293 cells expressing the wild-type of the rat α1-subunit (see Fig. 3). However, a contrasting result is obtained from I_p measurements in Na^+-containing media. The pump current of cells expressing the mutant rat pump is much smaller when estimated as K_o^+-activated rather than as ouabain-sensitive current (Fig. 7B). The latter estimation yielded an I_p which is ∼3 times larger than K_o^+-activated current. By way of contrast, the amplitude of I_p derived by both procedures in Na^+-containing solution are nearly identical for HEK 293 cells expressing the wild-type (Fig. 7C). Figs. 7A,B

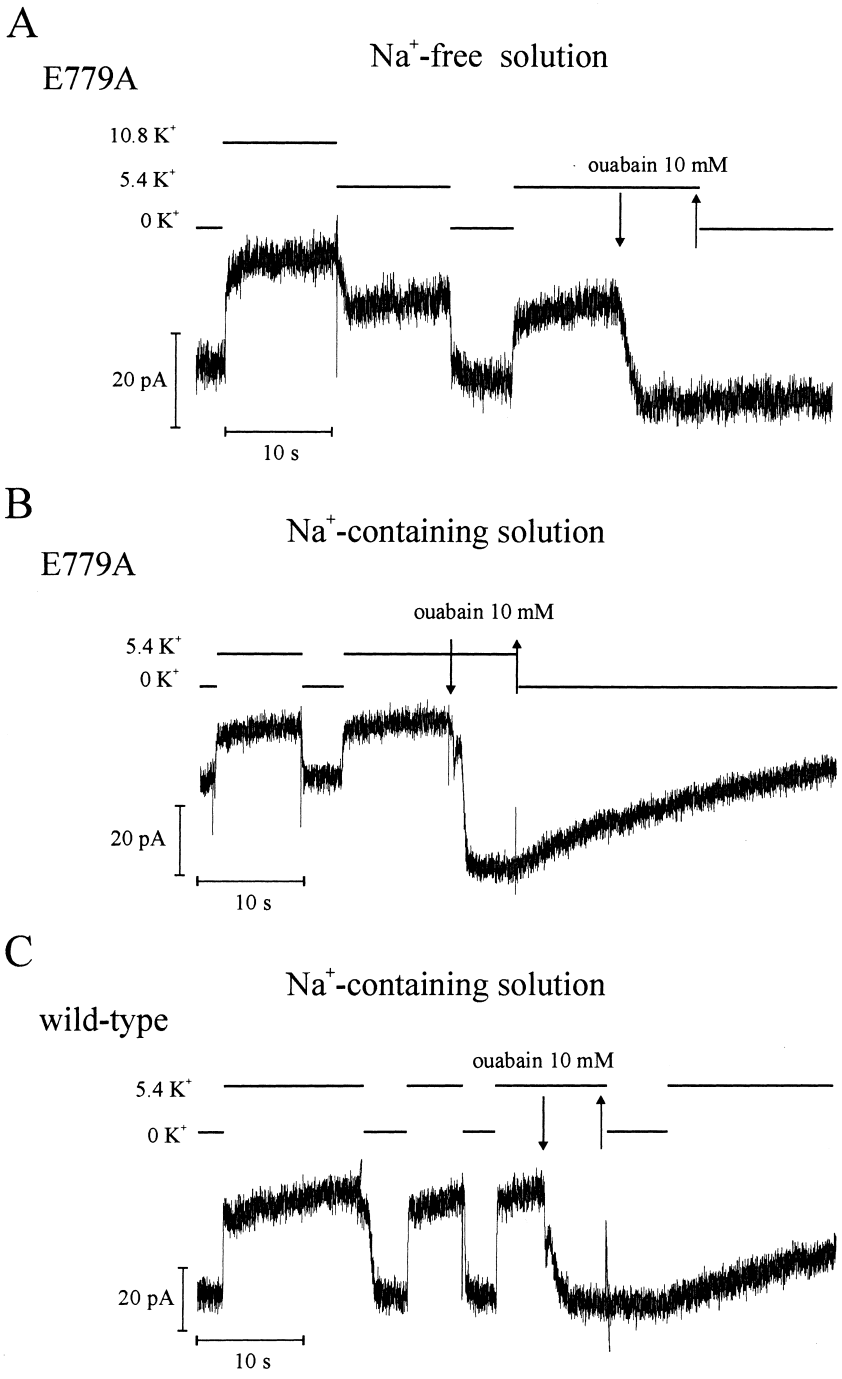

Fig. 7. Na_o^+-activated pump current in HEK 293 cells expressing the E779A mutant of the rat α1-subunit of the Na^+/K^+ pump. $V_c = 0$ mV. (A) In Na^+-free solution I_p estimated as K_o^+-activated or ouabain (10 mM)-sensitive current is about the same. Note the drift in baseline current. (B) In Na^+-containing medium the ouabain-inhibited current is approximately three times larger than the K_o^+-activated I_p. (C) In Na^+-containing solution K_o^+-activated and ouabain-sensitive current of a cell expressing the wild-type are nearly identical.

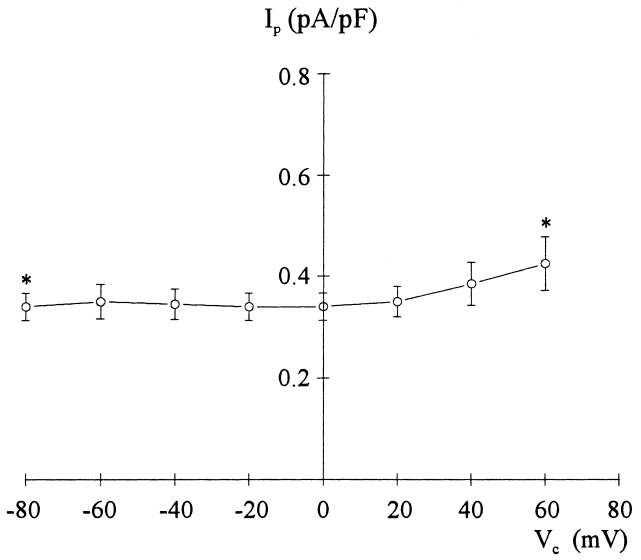

Fig. 8. Voltage dependence of the Na_o^+-activated pump current in HEK 293 cells expressing the E779A mutant of the rat α1-subunit. Mean I_p densities at −80 mV and +60 mV (asterisks) are not significantly different ($P = 0.16$; $n = 16$).

reveal a Na_o^+-activated, ouabain-sensitive current in HEK 293 cells expressing the E779A mutant of the rat α1-subunit. This current is most probably due to electrogenic Na_o^+/Na_i^+-exchange carried out by the mutant pump. We studied the voltage dependence of the Na_o^+-activated pump current by means of voltage ramps (from +60 to −80 mV within 1.5 s) in cells superfused with K^+-free, Na^+-containing medium with or without 10 mM ouabain. The difference current representing the Na_o^+-activated pump current is plotted versus membrane potential in Fig. 8. I_p amplitudes were normalized to the respective cell capacitance ($n = 16$). The Na^+-activated pump current exhibits little voltage dependence. There might be a very small positive slope of the I_p–V relationship at positive potentials. However, the I_p densities at −80 mV and +60 mV (asterisks) are not significantly different ($P = 0.16$). In addition, we found little dependence of the Na_o^+-activated I_p on voltage when the current was estimated as the difference between ouabain (10 mM)-inhibited current at 10.8 mM K_o^+ and the K_o^+-activated pump current. At zero potential the Na_o^+-activated I_p amounted to 0.34 ± 0.028 pA/pF ($n = 4$) and was about 65% of the total (ouabain-sensitive) I_p.

4. Discussion

Site-directed mutagenesis of amino acids located in the transmembranal domain V and VI of the Na^+/K^+ pump led to the conclusion that residues in these domains are of varying importance for the cation binding to the enzyme [3,4]. A combined biochemical and electrophysiological study on a ouabain-resistant E779A mutant of the sheep Na^+/K^+ pump α1-subunit expressed in HeLa cells revealed the absence of the normal voltage dependence of I_p and its variation by K_o^+ in Na^+-containing solution. Simultaneously, the apparent K_o^+-affinity of the mutant pump in Na^+-containing solution was decreased by a factor of 3–4 if compared with the wild-type [6]. Furthermore, the authors noted the presence of an electrogenic Na_o^+/Na_i^+-exchange via the sheep mutant pump expressed in HeLa cells.

4.1. The E779A mutant of the rat Na^+/K^+ pump α1-subunit displays reduced K_o^+ affinity but preserved Na_o^+/K_o^+-competition

As described above, we studied the ouabain-insensitive wild-type and E779A mutant of the rat Na^+/K^+ pump α1-subunit expressed in HEK 293 cells superfused with Na^+-free or Na^+-containing media. Compared with the wild-type the apparent K_o^+-affinity of the mutant pump was lower by a factor 3–4 in both Na^+-free and Na^+-containing solution. As mentioned before, a similar decrease of the K_o^+-affinity was observed in Na^+-containing solution by Argüello et al. [6] on a different E779A mutant pump expressed in a different cell species. Since Na^+-containing solution reduced the apparent K_o^+-affinity of both the wild-type and the E779A mutant of the rat α1-subunit expressed in HEK 293 cells to nearly the same extent (Fig. 4), the competition between Na_o^+ and K_o^+ for external cation binding sites of the pump seems to be unaffected in the mutant pump. The reduced K_o^+ affinity and the unchanged Na_o^+/K_o^+-competition might be interpreted as an indication of a hindered access for Na_o^+ and K_o^+ to the external cation binding sites of the E779A mutant pump. This hindrance might be due to the loss of a carboxyl group in the mutated α1-subunit. However, in view of the very similar voltage dependence of I_p at various $[K^+]_o$ in cells expressing either the wild-type or

the mutant of the rat α1-subunit and superfused with Na^+-free media (Fig. 5), a major structural alteration of an access channel to the external cation binding sites of the mutant pump seems unlikely.

4.2. The I_p–V relationship of the mutant pump is nearly unchanged in Na^+-free media

As can be seen from Fig. 5A and B, the I_p–V curves recorded in Na^+-free solutions are quite similar in HEK 293 cells expressing the wild-type or the E779A mutant of the rat pump α1-subunit. The I_p–V relationship displays an extended region of negative slope at 0.1 mM K_o^+ and a positive slope over the whole voltage range studied at high $[K^+]_o$ (>0.5 mM K_o^+). It is considered that at low extracellular K^+ concentration binding of K_o^+ to the pump is rate limiting for pump cycling and determines the shape of the I_p–V curve [15]. This is because K^+-binding probably occurs at the bottom of an access channel connecting the cation binding sites of the pump with the extracellular space. $[K^+]_o$ at the bottom probably varies with membrane potential (see [17] for the concept). Positive potentials lower the local K_o^+ concentration and thereby the activation of I_p, whereas negative membrane potentials increase $[K^+]_o$ at the bottom of the channel and thereby lead to enhanced I_p activation (Fig. 5A,B).

4.3. Na_o^+ profoundly alters the electrogenicity of the E779A mutant rat pump expressed in HEK 293 cells

Comparison between Fig. 6A,C and B,D reveals that the voltage dependence of I_p in HEK 293 cells expressing the E779A mutant is reduced but not abolished in Na^+-containing solution. I_p seems to be nearly voltage-independent at negative potentials between −20 and −80 mV. This finding might suggest that a voltage-independent partial reaction of the pump cycle is rate limiting for the pump cycling in the voltage range mentioned. Generally speaking, the K_o^+ effect on the voltage dependence of I_p generated by the mutant pump in cells superfused with Na^+-containing media is diminished. Thus, extracellular Na ions profoundly alter the characteristics of electrogenic Na^+-pumping in HEK 293 cells expressing the E779A mutant of the rat α1-subunit. This conclusion is supported by the observation that cells expressing the E779A mutant pump carry out a Na_o^+-activated, electrogenic Na^+-transport via the exogenous pump, most probably electrogenic Na_o^+/Na_i^+-exchange. A pump current produced by Na_o^+-activated Na^+ pumping has been described before by Argüelllo et al. [6] and Peluffo et al. [18] for HeLa cells expressing a ouabain-insensitive E779A mutant of the sheep Na^+/K^+ pump α1-subunit. Electrogenic Na_o^+/Na_i^+-exchange represents, in addition to Na^+/K^+-exchange, a new, second mode of Na^+-pumping which generates a steady-state pump current. At 10.8 mM K_o^+ and 0 mV the Na_o^+-activated exchange produces about 65% of the ouabain-inhibited pump current of HEK 293 cells expressing the mutant pump. The Na_o^+/Na_i^+-exchange is hardly, if at all, voltage-dependent between −80 and +60 mV (Fig. 8). According to our preliminary data the density of I_p generated by electrogenic Na_o^+/Na_i^+-exchange does not vary with $[K^+]_o$ between 2.7 and 10.8 mM K_o^+. However, the Na_o^+-activated I_p was always larger if measured as ouabain-sensitive current in K^+-free solution than if calculated as the difference between ouabain-sensitive current in K^+-containing medium and the respective K_o^+-activated current. This might suggest that in solutions containing 2.7 mM K_o^+ to 10.8 mM K_o^+ a nearly constant fraction of the mutant pump is unable to participate in electrogenic Na_o^+/Na_i^+-exchange. An electrogenic Na_o^+/Na_i^+-exchange is not mediated by the wild-type of the rat pump expressed in HEK 293 cells (Fig. 7C), where Na_o^+ inhibits the I_p activation by K_o^+ (Fig. 4). Thus, the Na^+-binding sites which are involved in I_p activation by Na_o^+ in the E779A mutant are probably different from the binding sites for external monovalent cations usually found in wild-type Na^+/K^+ pumps.

4.4. Concluding note

In conclusion, I_p measurements on HEK 293 cells expressing either the wild-type or the E779A mutant of the rat Na^+/K^+ pump α1-subunit revealed a decrease in the apparent K_o^+ affinity of the mutant pump in Na^+-free and Na^+-containing solutions. The competition between Na_o^+ and K_o^+ for extracellular cation binding sites of the pump, at which I_p is activated by K_o^+, remained unaffected by the muta-

tion. Similarly, the shape of the I_p–V relationship at various $[K^+]_o$ in Na^+-free medium was essentially unchanged ruling out a major structural alteration of the access channel to the K_o^+-binding sites of the mutant pump. In Na^+-containing solutions the voltage- and K_o^+-dependence of I_p was reduced in cells expressing the mutant pump. However, in contrast to the observations of Argüello and co-workers [6], voltage dependence of I_p clearly persisted in cells expressing the mutant. A Na_o^+-activated electrogenic Na^+ transport via the mutant rat pump exists in HEK 293 cells, similar to the electrogenic Na_o^+/Na_i^+-exchange first prescribed by the aforementioned authors for HeLa cells expressing a E779A mutant of the sheep pump. The Na_o^+-activated I_p displayed little voltage dependence between −80 and +60 mV. Thus, in comparison with the wild-type, the mutation E779A in the rat Na^+/K^+ pump α1-subunit expressed in HEK 293 cells caused a modest decrease of the apparent affinity to K_o^+ but a far-reaching Na_o^+-dependent change of the electrogenicity of the pump.

Acknowledgements

We are indebted to Professor J.B. Lingrel (Cincinnati) for a generous gift of the plasmid pRα1. The authors would like to thank Mrs Kirsty Sendhoff for critical reading of the manuscript.

References

[1] I.M. Glynn, J. Physiol. 462 (1993) 1–30.
[2] P. De Weer, in: D.W. Seldin, G. Giebisch (Eds.), The Kidney: Physiology and Pathophysiology, Raven Press, New York, 1992, pp. 93–112.
[3] T.A. Kuntzweiler, J.M. Argüello, J.B. Lingrel, J. Biol. Chem. 271 (1996) 29682–29687.
[4] J.B. Lingrel, J.M. Argüello, J. Van Huysse, T.A. Kuntzweiler, Ann. N. Y. Acad. Sci. 834 (1997) 194–206.
[5] J.M. Argüello, J.B. Lingrel, J. Biol. Chem. 270 (1995) 22764–22771.
[6] J.M. Argüello, R.D. Peluffo, J. Feng, J.B. Lingrel, J.R. Berlin, J. Biol. Chem. 271 (1996) 24610–24616.
[7] E.C. Conley, J.R. Saunders, Mol. Gen. Genet. 194 (1984) 211–218.
[8] G.E. Shull, J. Greeb, J.B. Lingrel, Biochemistry 25 (1986) 8125–8132.
[9] C.M. Gorman, Curr. Opin. Biotechnol. 1 (1990) 36–47.
[10] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Pflügers Arch. 391 (1981) 85–100.
[11] D. DiFrancesco, J. Physiol. 314 (1981) 359–376.
[12] S. Mechmann, L. Pott, Nature 319 (1986) 597–599.
[13] R.F. Rakowski, L.A. Vasilets, J. LaTona, W. Schwarz, J. Membr. Biol. 121 (1991) 177–187.
[14] F.V. Bielen, H.G. Glitsch, F. Verdonck, J. Physiol. 465 (1993) 699–714.
[15] R.F. Rakowski, D.C. Gadsby, P. De Weer, J. Membr. Biol. 155 (1997) 105–112.
[16] F.V. Bielen, H.G. Glitsch, F. Verdonck, J. Physiol. 442 (1991) 169–189.
[17] P. Läuger, Electrogenic Ion Pumps, Sinauer Associates, Sutherland, MA, 1991, pp. 1–313.
[18] R.D. Peluffo, J.B. Lingrel, J.M. Argüello, J.R. Berlin, Ann. N. Y. Acad. Sci. 834 (1997) 339–342.