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Biochimica et Biophysica Acta 1325 (1997) 197–208

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Comparison of ouabain-sensitive and -insensitive Na/K pumps in HEK293 cells

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Received 19 August 1996; revised 11 December 1996; accepted 17 December 1996

Abstract

The Na/K pump current I_p of single HEK293 cells either untransfected (endogenous I_p) or transfected with the $\alpha 1$ subunit of the rat Na/K pump (exogenous I_p) was investigated in Na-containing solution by means of whole-cell recording at 30°C. The endogenous I_p was irreversibly blocked by 10^{-4} M ouabain or $2 \cdot 10^{-4}$ M dihydro-ouabain (DHO). Its density amounted to 0.33 pA pF^{-1} at 0 mV and 5.4 mM K_o. It was half maximally activated at 1.5 mM K_o and increased linearly with depolarization over the entire voltage range studied (−80 to +60 mV). In contrast, HEK293 cells stably transfected with cDNA for the cardiac glycoside-resistant $\alpha 1$ subunit of the rat Na/K pump showed an I_p in the presence of 10^{-4} M ouabain and $2 \cdot 10^{-4}$ M DHO, respectively. This exogenous I_p was reversibly blocked by 10^{-2} M ouabain. Half maximal activation of the exogenous I_p occurred at 1.7 mM K_o. Its amplitude increased linearly with depolarization at negative voltages but remained almost constant at positive membrane potentials. Comparison with the I_p of isolated rat cardiac ventricular myocytes strongly suggests that the exogenous I_p in HEK293 cells is generated by the $\alpha 1$ subunit of the rat Na/K pump since it displays identical properties. Therefore, HEK293 cells represent an expression system well suited for the electrophysiological analysis of recombinant, cardiac glycoside-resistant Na/K pumps by means of whole-cell recording.

Keywords: Sodium pump; Whole-cell recording; HEK293 cell; Transfection; Expression system; Rat $\alpha 1$ subunit

1. Introduction

In recent years HEK293 cells (human embryonic kidney cells), a mammalian cell line, have been used as a system for the heterologous expression of transporters and ion channels [1,2]. Due to their small volume, the cells are well suited for studies on endogenous and exogenous ion channels and transporters by means of whole-cell recording. In contrast to conventional voltage-clamping on larger cells (e.g., *Xenopus* oocytes) the patch-clamp technique [3] allows a good control of the composition of the cell interior and renders possible a change of the intracellular solution during the experiment [4]. Apart from some fundamental data (e.g. Ref. [1]), information about the electrophysiological characteristics of HEK293 cells is scanty. It is, however, clear that endogenous, voltage-activated Ca or Na channels are absent in these cells [2]. The aim of the experiments described below was two-fold. First, we studied the current produced by the endogenous Na/K pump molecules in order to contribute to the electrophysio-

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PII S0005-2736(96)00259-3

logical characterization of the cells. Second, we tested whether HEK293 cells could express exogenous Na/K pumps and thus could provide a system for the analysis of the Na/K pump current generated by exogenous pump molecules by means of whole-cell recording. Such a system would markedly facilitate the electrophysiological analysis of the Na/K pump structure-function relationship. Most recently, Yamamoto and co-workers [5] reported on a successful electrophysiological analysis of recombinant, mutated Na/K pumps expressed in HeLa cells. Their study concentrated on the kinetics of K_o- and cardiac glycoside-binding to wild-type and mutated pump molecules. The experiments described below, however, characterize different aspects of endogenous and recombinant Na/K pumps in HEK293 cells. In addition the properties of the rat $\alpha 1$ subunit in recombinant and wild-type pumps are compared. Some of the results have been published in abstract form [6].

2. Materials and methods

2.1. Cell culture

HEK293 cells were maintained in culture flasks (25 cm^2) with Minimal Essential Medium (MEM; Life Technologies GmbH, 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 I.U./l penicillin, and 100 mg/l streptomycin (all from Life Technologies, Eggenstein, Germany) at 37°C and 5% CO_2. The culture medium was replaced every 2–3 days. When the cells were grown to near confluency, they were trypsinized and seeded at a density of 4000 cm^{-2} in a new culture flask.

2.2. Transfection and selection of stably transfected cells

The eucaryotic expression plasmid pRα1 was used for the transfection of the HEK293 cells [7]. It is derived from the plasmid pRc/CMV (Invitrogen Corporation, San Diego, USA) and contains the cDNA of the $\alpha 1$ subunit of the rat Na/K pump under the control of the enhancer-promotor regions of the immediate early gene from the human cytomegalovirus. HEK293 cells were transfected with pRα1 by means of the Ca phosphate coprecipitation method [8]. Shortly, two days before transfection $\approx 1 \cdot 10^5$ HEK293 cells were seeded in a culture flask. On the day of transfection, 250 μl 2 × Hepes-buffered saline (in mM: 280 NaCl, 2.8 Na_2HPO_4, 50 N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (Hepes), pH 7.2 (NaOH)) were gently mixed with 250 μl of a 250 mM $CaCl_2$ solution containing 14 μg pRα1. Five min later, 240 μl of the resulting solution were added dropwise to the cells in the culture flask (2.5 ml culture medium). The cells were kept in the incubator (37°C, 5% CO_2) for 3 h. Afterwards they were washed twice with phosphate-buffered saline (in mM: 0.9 $CaCl_2 \cdot 2\ H_2O$, 2.7 KCl, 1.5 KH_2PO_4, 0.5 $MgCl_2 \cdot 6\ H_2O$, 106 NaCl, 6.5 Na_2HPO_4, pH 7.2 (NaOH)) and kept in culture medium for two days. To obtain stably transfected cells, 10^{-4} M dihydro-ouabain (DHO) was then permanently added to the medium.

Mock transfection (plasmid pRc/CMV only) revealed that all cells died within two days in the DHO-containing culture medium. This observation excludes the possibility that HEK293 cells became ouabain-resistant by mutations of their endogenous $\alpha 1$ subunit.

2.3. Preparation of single rat ventricular myocytes

Isolation of single myocytes was carried out according to Bechem and co-workers [9]. Briefly, adult female Wistar rats were anaesthesized with diethylether and killed by decapitation. The aorta was cannulated before the heart was excised, mounted on a Langendorff apparatus, and perfused at 37°C for several minutes with the following solutions (in mM): (1) Ca-free solution: 204 sucrose, 35 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 2.0 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 Hepes; (2) enzyme solution: 204 sucrose, 35 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 0.1 EGTA, 10 Hepes; 0.5 mg/ml bovine serum albumine (BSA; Sigma, Germany), 0.4 mg/ml collagenase B (Boehringer Mannheim, Germany), 10 μl/ml elastase (Serva, Heidelberg, Germany), 0.3 mg/ml protease (Sigma, Germany), 0.15 mg/ml

DNase (Sigma, Germany). The pH of the oxygenated solutions was 7.4. The digested ventricles were cut into pieces and gently stirred at 20–22°C in tissue wells containing a nominally Ca-free solution with the following composition (in mM): 204 sucrose, 35 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 10 Hepes; 1.0 mg/ml BSA, 0.3 mg/ml DNase, pH 7.4. [Ca] was increased to 1.3 mM by stepwise replacement with Hanks' medium 199 (PAA, Linz, Austria) supplemented with 100.000 I.U./l penicillin, 100 mg/l streptomycin, and 250 μg/l amphotericin B (all from Sigma, Germany). Isolated myocytes were transferred to culture dishes (∅ 35 mm) and used for the electrophysiological experiments either immediately after isolation or after one day in culture (37°C, 3% CO_2).

2.4. Whole-cell recording

A culture dish (∅ 35 mm) with either HEK293 cells or rat ventricular myocytes was fixed to the stage of an inverted microscope (Diaphot TMD, Nikon, Tokyo, Japan). The dish was perfused with extracellular solution from a reservoir connected to the dish by plastic tubes. It was placed about 100 cm above the stage. Solution flow, driven by gravity, was 1 ml/min. The solution level in the dish was held constant by means of an outlet opposite to the inlet from the reservoir. A multibarreled pipette (inner diameter: 500 μm) was placed 100–200 μm from the cell under investigation. The cell was continuously superfused at a rate of 0.4 ml/min with one of the solutions from reservoirs connected with the pipette via plastic tubes. The reservoirs were about 30 cm above the stage. Solution changes, controlled by electromagnetic valves (The Lee Company, Westbrook, USA), had a time constant of 200–600 ms [10]. The temperature in the vicinity of the cell was 30°C when superfused with one of the test solutions. Patch pipettes were made from borosilicate glass capillaries (GC150TF-10, Clark Electromedical Instruments, Reading, UK) and back-filled with the pipette solution. They had an initial resistance between 2.5 and 7 MΩ for experiments with HEK293 cells and between 1 and 2.5 MΩ for experiments with rat ventricular myocytes. The tip potential between the patch pipette and the extracellular solution was compensated before establishing a GΩ seal ($\geq$ 10 GΩ). Gentle suction was applied to a patch pipette positioned at the cell membrane to obtain the whole-cell configuration of the patch-clamp technique [3]. The cells were voltage-clamped by means of an EPC-7 patch clamp amplifier (List Medical, Darmstadt, Germany). The amplifier was connected to a personal computer via 12 bit AD- and DA-converters, respectively. Currents were low-pass filtered at 200 Hz and digitized with 1 kHz (200 Hz in early experiments). The programme ISO2 (MFK, Niedernhausen, Germany) was used to generate the voltage protocols and to record the resulting currents. The cell capacitance was determined by a programme routine that applied depolarizing and hyperpolarizing voltage ramps 10 mV in amplitude starting from a holding potential of 0 mV. HEK293 cells displayed membrane capacitances between 20 and 85 pF, whereas rat ventricular myocytes showed values between 60 and 160 pF.

2.5. Solutions for whole-cell recording

The extracellular solution for whole-cell recording contained (in mM): 144 NaCl, 0–10.8 KCl, 5 $NiCl_2$, 2 $BaCl_2$, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes, pH 7.4 (NaOH). Barium and nickel served to suppress potassium and calcium conductances, respectively, as well as the sarcolemmal Na/Ca exchanger. DHO (Roth, Karlsruhe, Germany) and ouabain (Fluka Chemie AG, Buchs, Switzerland) were added from 10^{-2} M stock solutions. The DHO stock solution was aqueous, whereas the ouabain stock solution contained 10% (v/v) ethanol. In experiments, in which [ouabain] was 10^{-2} M, the drug was solved directly in the extracellular solution. In this case, the superfusate contained 2% (v/v) ethanol. Control experiments revealed that this [ethanol] had no influence on the membrane current under the present experimental conditions.

The pipette solution was composed of (in mM): 110 CsCl, 40 NaCl, 10 NaOH, 3 $MgCl_2$, 6 EGTA, 16 Hepes, 10 MgATP, pH 7.4 (CsOH). Assuming a contamination of $\approx 1 \cdot 10^{-6}$ M Ca, free [Ca] and [Mg] were calculated to be $1.3 \cdot 10^{-11}$ M and $2.4 \cdot 10^{-3}$ M, respectively [11]. The solution contained cesium to block potassium conductances and a high [Na] to strongly activate the Na/K pump at internal sites.

2.6. Statistics

Whenever possible data are presented as mean ± S.E.M. The S.E.M. is only shown in the figures when it exceeds the size of the symbol. *n* indicates the number of cells studied. Differences between data points were checked by Student's two-tailed, unpaired *t*-test and considered significant if $P < 0.05$.

3. Results

3.1. The endogenous I_p

The Na/K pump is an ATP-consuming enzyme, that transports 3 Na ions out of and 2 K ions into the cell per each ATP molecule split. The net movement of one positive charge out of the cell during each Na/K pump cycle thus produces an outward current. The Na/K pump current I_p depends on $[K]_o$ and can be blocked by cardiac glycosides, specific inhibitors of the Na/K pump. Fig. 1 shows the identification of the Na/K pump current of HEK293 cells (endogenous I_p). The holding potential V_C is 0 mV. Horizontal lines above the current trace indicate changes of the superfusion solution. In Fig. 1A, a HEK293 cell is initially superfused with extracellular solution, that contains 144 mM Na and 5.4 mM K. Switching to K-free solution (0 K) immediately shifts the holding current by about 20 pA in the inward direction. The following application of 10^{-4} M ouabain results in an inward current shift of the same amplitude but with a slower time course. Fig. 1B displays a similar experiment with another HEK293 cell. At the beginning the cell is superfused with a solution containing 5.4 mM K. K-free medium instantaneously blocks an outward current of 10 pA. $2 \cdot 10^{-4}$ M DHO more slowly blocks an outward current component of the same amplitude. As can be seen in Fig. 1A,B, under the chosen experimental conditions the cardiac glycoside-sensitive outward current is virtually identical to the current activated by K_o. This outward current is the current generated by the Na/K pump. However, in contrast to K-free medium the block of I_p by cardiac glycosides was nearly irreversible. Even after 5–10 min in cardiac glycoside free solution, there was no recovery of I_p. The I_p density at 5.4 mM K_o and 0 mV amounted to 0.33 ± 0.02 pA · pF^{-1} ($n = 28$).

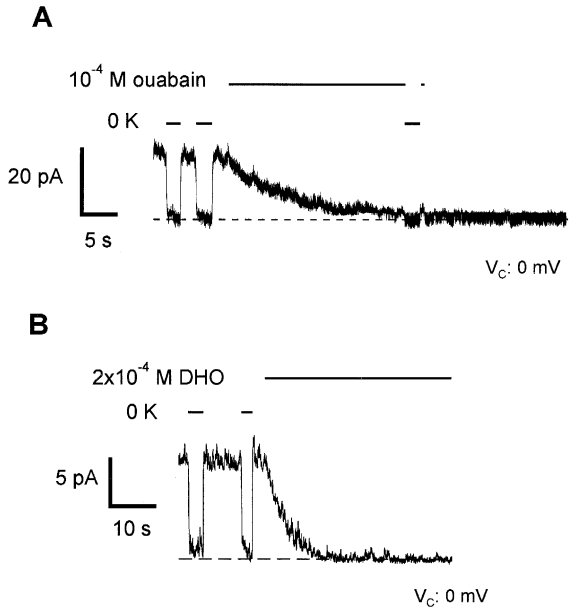

Fig. 1. Identification of the endogenous I_p in HEK293 cells. I_p is identified as current inhibited by cardiac glycosides or K-free solution. In this and the following figures solution changes are indicated by horizontal lines above the current traces and the horizontal calibration mark denotes zero current level. The holding potential V_C is 0 mV. (A) Initially, the superfusate contains 5.4 mM K_o. Application of K-free solution or of a medium containing 10^{-4} M ouabain (5.4 mM K_o) results in an identical inward shift of the membrane current. Thus, the amplitudes of I_p estimated by either procedure are identical. (B) Different cell. The I_p amplitudes estimated by application of a K-free medium or a solution containing $2 \cdot 10^{-4}$ M dihydro-ouabain (DHO; 5.4 mM K_o) are almost the same.

The following experiments were all performed at a holding potential of 0 mV, except for the measurements of the Na/K pump current-voltage (I_p-V) relationships.

The concentration-dependent inhibition of the endogenous I_p by DHO is illustrated in Fig. 2. Fig. 2A shows the effect of $5 \cdot 10^{-6}$ M DHO on the endogenous I_p of a HEK293 cell. At the beginning of the record the cell is superfused with a solution containing 5.4 mM K. Solution changes are indicated by the bars above the current trace. Switching to K-free solution reveals an I_p amplitude of 12 pA. A [DHO] of $5 \cdot 10^{-6}$ M slowly shifts the holding current by 2.5 pA in the inward direction, i.e., it inhibits 21% of the initial I_p. Changing the superfusate to K-free solution during the steady-state inhibition by DHO causes a further inward shift of the current by 9.5 pA, corre-

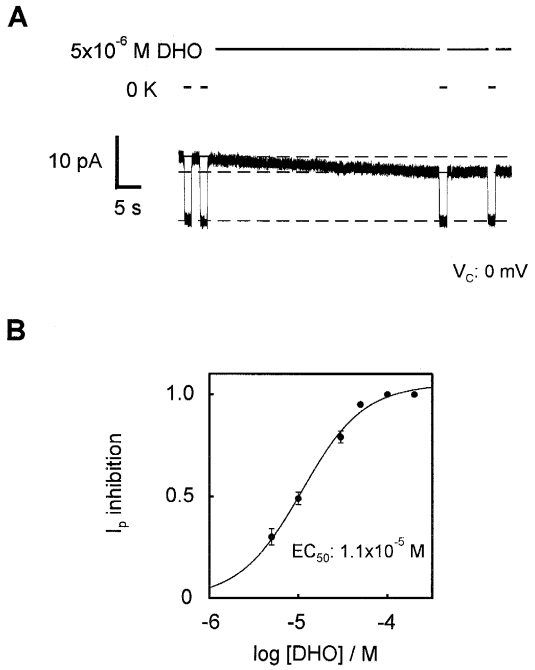

Fig. 2. Inhibition of I_p by DHO in native HEK293 cells. (A) Original record. At the beginning the cell is superfused with a solution containing 5.4 mM K_o. I_p amplitudes are estimated by short pulses of K-free solution. Application of $5 \cdot 10^{-6}$ M DHO (with 5.4 mM K_o) reduces the initial I_p amplitude (12 pA) by 2.5 pA or 21%. (B) Concentration–response curve of steady-state I_p inhibition by DHO (with 5.4 mM K_o). Complete inhibition of I_p in a K-free medium is arbitrarily set to 1. Data are shown as means ± S.E.M. ($n \geq 3$). Error bars are given only if they exceed the size of the symbols. The sigmoid curve fitted to the data represents a logarithmic version of the Hill equation. Half maximal inhibition of I_p (EC_{50}) occurs at $1.1 \cdot 10^{-5}$ M DHO with a Hill coefficient n_H of 1.2.

sponding to 79% of the initial I_p amplitude. The application of various [DHO] on 21 HEK293 cells resulted in the concentration–response curve presented in Fig. 2B. The relative steady-state inhibition of I_p by DHO is plotted versus the logarithm of the [DHO]. Each data point represents the mean of at least three measurements. The sigmoid curve fitted to the data obeys a logarithmic version of the Hill equation. According to this fit half maximal I_p inhibition (EC_{50}) occurs at $1.1 \cdot 10^{-5}$ M DHO with a Hill coefficient n_H of 1.2, in line with the notion that one DHO molecule binds to one Na/K pump molecule.

We next studied the dependence of the endogenous I_p on $[K]_o$. For this purpose solutions containing various [K] were applied to the cells. An original record of such an experiment is shown in Fig. 3A. A HEK293 cell is superfused with solutions containing 0, 1.35, 2.7, or 5.4 mM K, as indicated by the lines above the current trace. The membrane current is shifted in the outward direction with increasingly higher $[K]_o$. The difference in holding current between K-containing and K-free solution represents the I_p amplitude at the respective $[K]_o$. I_p amplitudes were normalized to the amplitude at 5.4 mM K_o and plotted versus $[K]_o$. The result is shown in Fig. 3B. Each data point is the mean of 2–12 measurements. The curve fitted to the data points represents the Hill equation:

$$I_p = I_{p(max)} \cdot [K]_o^{n_H} / \left(K_{0.5}^{n_H} + [K]_o^{n_H}\right). \quad (1)$$

The maximal Na/K pump current ($I_{p(max)}$) is 1.21, half-maximal activation of I_p occurs at 1.5 mM K_o ($K_{0.5}$), and the Hill coefficient n_H amounts to 1.2.

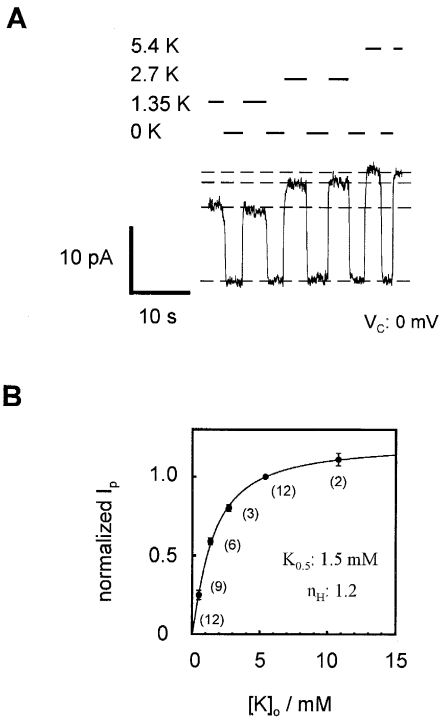

Fig. 3. Activation of the endogenous I_p by K_o. (A) Original record. Increasing external K concentrations (horizontal bars) evoke an increasing outward component of membrane current (bottom trace). Note that the K_o-activated outward current tends to saturate at high $[K]_o$. (B) Normalized concentration-response curve of I_p activation by K_o. I_p amplitudes were normalized to the amplitude at 5.4 mM K_o, which was arbitrarily set to 1. Numbers in parentheses indicate the number of cells studied. The curve fitted to the data obeys the Hill equation (Eq. (1)): $I_{p(max)} = 1.21$; $K_{0.5} = 1.5$ mM K_o; $n_H = 1.2$.

It is well known that I_p is voltage-dependent (review: Ref. [12]). Fig. 4 illustrates the voltage-dependence of the endogenous I_p, which was determined by means of the protocol depicted in Fig. 4A. The upper trace in Fig. 4A represents the voltage protocol, whereas the lower trace shows the resulting membrane current of a HEK293 cell. The cell was clamped to voltages between −80 and +60 mV in 20 mV steps. Changes in the membrane potential result in capacitative artifacts visible as rapid upward and downward deflections in the current trace, respectively. The cell is continuously superfused with a solution containing 5.4 mM K. During each clamp potential the superfusate is temporarily changed to K-free solution, as marked by the horizontal bars above the current trace. K-free solution shifts the membrane current in the inward direction at each potential tested. This is shown in further detail in Fig. 4B. Magnified current traces from Fig. 4A are presented. They were obtained during superfusion with K-free solution. For clarity bars indicating changes to K-free solution are left. Two traces at zero potential are shown, the first from the beginning and the second from the end of the record in Fig. 4A. Since the difference current at this potential remains constant, changes of the seal resistance during the record can be excluded. It is clear from Fig. 4B that the difference in membrane current between K-containing and K-free solution increases with increasing membrane potential. This difference current represents I_p because K-activated and cardiac glycoside-sensitive current are identical under the present experimental conditions (cf. Fig. 1). Control experiments (not shown) confirmed that this is not only true for 0 mV but for all potentials in the voltage range studied. I_p amplitudes were normalized to the amplitude at 0 mV and plotted versus membrane potential to yield the I_p-V relationship shown in the inset in Fig. 4C. Normalized I_p amplitudes range from 0.41 at −80 mV to 1.32 at +60 mV. The I_p-V curve is nearly linear over this voltage range. The main Fig. 4C illustrates the mean I_p-V relationship for the endogenous I_p. Mean values of 4–15 measurements for each potential are presented. Normalized I_p amplitudes are 0.55 ± 0.03 ($n = 10$) at −80 mV and 1.38 ± 0.05 ($n = 4$) at +60 mV. This mean I_p-V curve is almost linear over the entire voltage range studied.

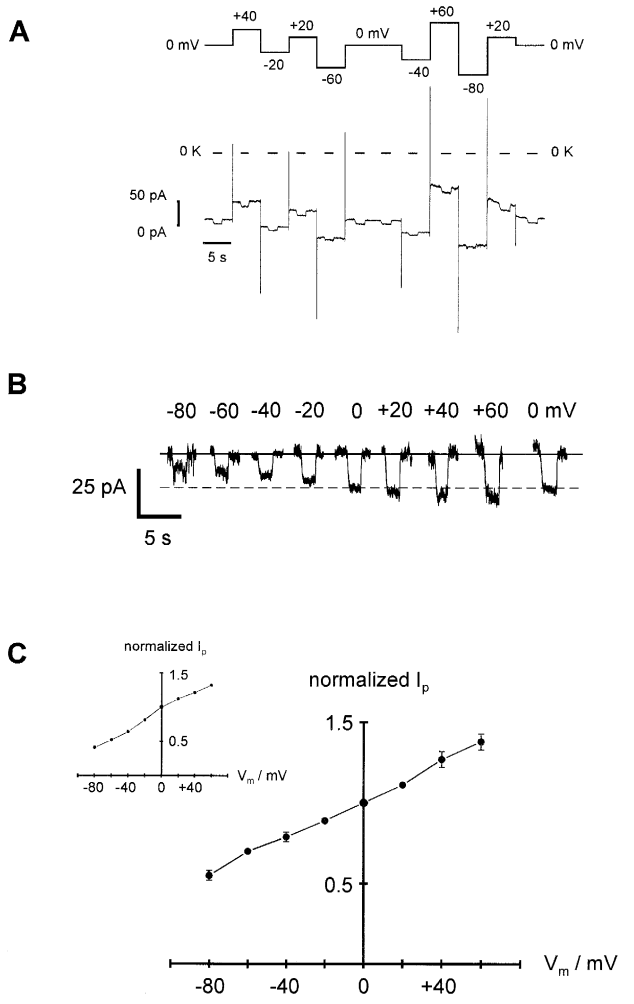

Fig. 4. I_p-V curve of native HEK293 cells. (A) Estimation of I_p at various membrane potentials (top line). The superfusate contains 5.4 mM K_o. I_p is measured as current reactivated by K_o after a short pulse of a K-free medium, indicated by the short lines above the current trace (bottom). During the application of K-free solution the membrane current is shifted in the inward direction at each potential tested. (B) Enlarged current traces from (A), ordered by membrane potential. Bars indicating changes to K-free medium are left. I_p is estimated as current reactivated by 5.4 mM K_o after superfusion with K-free medium. I_p increases with depolarization. (C) Normalized mean I_p-V relationship of native HEK293 cells. I_p amplitudes are normalized to the corresponding amplitude at zero potential. Data represent means ± S.E.M. ($n = 4$–15). The inset shows the normalized I_p-V curve obtained in the experiment illustrated in (A) and (B).

3.2. The exogenous I_p

After the characterization of the cardiac glycoside-sensitive endogenous I_p, we investigated

whether a cardiac glycoside-resistant I_p could be detected in HEK293 cells transfected with the $\alpha 1$ subunit of the rat Na/K pump. Fig. 5 displays the membrane current of a HEK293 cell transfected with the $\alpha 1$ subunit of the rat Na/K pump and cultured for 44 days in a medium containing 10^{-4} M DHO. This [DHO] blocks the endogenous I_p (cf. Fig. 2B). The horizontal lines above the current trace indicate changes of the superfusate. At the beginning of the record the cell is superfused with a solution containing 5.4 mM K_o *plus* $2 \cdot 10^{-4}$ M DHO. As can be seen from Fig. 5 a K-free solution or a solution containing 5.4 mM K plus 10^{-2} M ouabain block an almost identical outward component of the membrane current. Thus, a Na/K pump current is present under conditions which completely block the endogenous I_p. The density of this Na/K pump current amounted to 0.16 ± 0.02 pA · pF^{-1} ($n = 11$). In contrast to the irreversible blockade of the endogenous I_p by ouabain or DHO the I_p inhibition by ouabain in transfected cells is reversible. I_p recovers in the presence of a solution containing $2 \cdot 10^{-4}$ M DHO. Obviously, the transfected cells expressed Na/K pumps exhibiting a much lower ouabain sensitivity than the endogenous Na/K pumps. Assuming one-to-one binding of ouabain to the Na/K pump an estimation of the apparent K_d value (K'_d) for I_p inhibition by ouabain can be derived from the kinetics of ouabain-binding and -unbinding (for a detailed description of this procedure see Ref. [13]). Consequently, I_p inhibition and recovery were presumed to proceed monoexponentially. The time constant of I_p inhibition (τ) amounted to 1.3 s, whereas that for I_p recovery (τ_{off}) was 64.9 s. Hence, the K'_d value was calculated to be $2.0 \cdot 10^{-4}$ M. Similar results were obtained on two other cells (K'_d values: 2.5 and $2.7 \cdot 10^{-4}$ M, respectively). The mean K'_d for the inhibition of the exogenous I_p by ouabain was $(2.4 \pm 0.2) \cdot 10^{-4}$ M ($n = 3$). Since rat Na/K pumps are known to be ouabain-insensitive, these results strongly suggest that the transfected cells expressed a functional Na/K pump which contained the rat $\alpha 1$ subunit and displayed, therefore, the characteristic ouabain-resistance.

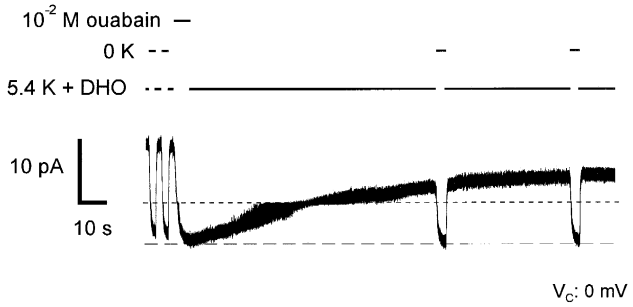

Fig. 5. Blockade of the exogenous I_p by 10^{-2} M ouabain is (partially) reversible. Original current record (bottom) from a HEK293 cell stably transfected with the rat $\alpha 1$ subunit. The cell is continuously superfused with a medium containing 5.4 mM K_o *plus* $2 \cdot 10^{-4}$ M DHO except for short periods of K-free or ouabain-containing (10^{-2} M) solution, as marked by the bars above the current trace. I_p is estimated either as current blocked by K-free solution or as current inhibited by 10^{-2} M ouabain (5.4 mM K_o). Both estimates are nearly identical. At the end of the record I_p amounts to $\approx 75\%$ of the initial amplitude. The apparent increase and decrease, respectively, of the current noise in passing zero current level (dashed line) is an artifact due to the data acquisition programme.

The K_o-dependence of the exogenous I_p was determined in media containing different $[K]_o$ *plus* either 10^{-4} M ouabain or $2 \cdot 10^{-4}$ M DHO to block the endogenous I_p. Raising $[K]_o$ resulted in an outward shift of the holding current (not shown). I_p was estimated as the difference in the holding current measured during application of a K-free solution. I_p amplitudes were normalized to the I_p amplitude at 5.4 mM K_o and plotted versus $[K]_o$. Fitting with the Hill equation (Eq. (1)) yielded a maximal I_p of 1.33, a $K_{0.5}$ of 1.7 mM K_o, and a Hill coefficient of 1. These values hardly differ from those obtained for the endogenous I_p (cf. Fig. 3B).

The voltage dependence of the exogenous I_p in transfected HEK293 cells was studied as shown in Fig. 4 for the endogenous I_p. The cells were continuously superfused with solutions containing 5.4 mM K_o plus $2 \cdot 10^{-4}$ M DHO or 10^{-4} M ouabain, respectively. I_p was estimated as K_o-activated current at membrane potentials between -80 and $+60$ mV in 20 mV steps. For this purpose a K-free solution was applied to the cell under study for a few seconds at each membrane potential. Measurements on 6 cells (summarized in Fig. 7, open circles) revealed that I_p increased with depolarization at negative voltages. However, it remained essentially constant at positive potentials in contrast to the endogenous I_p of untransfected cells which increased linearly over the entire range of membrane potentials (Fig. 4C). Normalized mean amplitudes of the exogenous I_p varied between

0.42 ± 0.04 at -80 mV ($n = 2$ only) and 1.09 ± 0.03 at $+60$ mV ($n = 4$ only).

3.3. The contribution of the endogenous and exogenous I_p to the total I_p of transfected HEK293 cells

The contribution of endogenous and exogenous Na/K pumps to the total I_p of transfected HEK293 cells was estimated in the following way. First, the transfected cells were cultured for 2–3 days in a DHO-free medium in order to abolish the inhibition of the endogenous Na/K pumps which was induced by the DHO-containing culture medium. Afterwards the total I_p of the cells was estimated as K_o-activated current. The total I_p density amounted to 0.20 ± 0.03 $pA \cdot pF^{-1}$ ($n = 7$). The total I_p was then inhibited in two steps. Application of a superfusate containing 5.4 mM K plus 10^{-4} M ouabain completely blocked the endogenous I_p of the HEK293 cells. Subsequent superfusion with a solution containing 5.4 mM K plus 10^{-2} M ouabain blocked the residual I_p which was most probably generated by pump molecules containing the exogenous $\alpha 1$ subunit of the rat Na/K pump. Fig. 6 illustrates this procedure. Fig. 6A displays an original record from a HEK293 cell. Changes of the superfusate are indicated by the horizontal lines above the current trace. At the beginning of the experiment the total I_p is estimated as the current inhibited by K-free solution. It amounts to 15 pA. The application of 10^{-4} M ouabain shifts the membrane current in the inward direction by 5.2 pA or 35% of the total I_p. The residual 65% (9.8 pA) of the total I_p are then blocked by 10^{-2} M ouabain. As a mean from 7 cells 37 ± 3% of the total I_p were inhibited by 10^{-4} M ouabain. Therefore, at least ≈ 63% of the total pump current were generated by Na/K pumps containing the exogenous, ouabain-resistant rat $\alpha 1$ subunit. If 10^{-4} M ouabain blocked some of these pump molecules, the contribution of the exogenous pumps to the total I_p would be even larger. For comparison, similar measurements were carried out on rat ventricular myocytes. It is known from electrophysiological and biochemical studies [14,15] that about 70–80% of the Na/K pumps in these cells contain the ouabain-resistant $\alpha 1$ subunit. Fig. 6B shows the membrane current of a rat ventricular cell. Initially, the total I_p is estimated as current inhibited by K-free solution. I_p amounts to 60 pA.

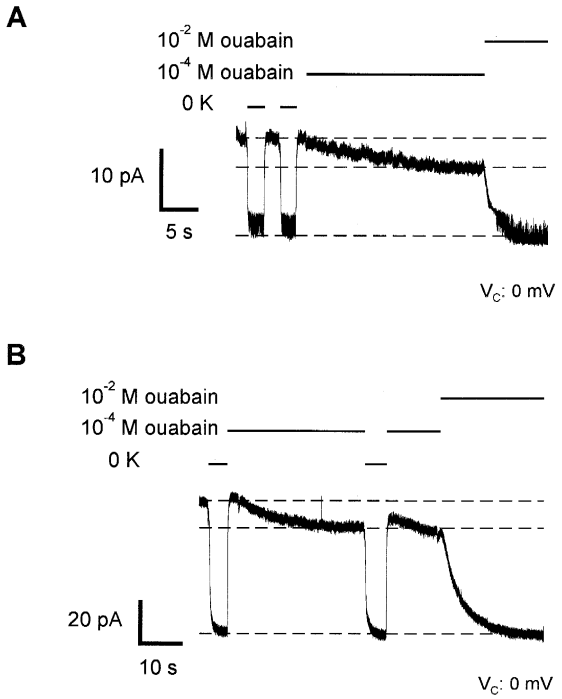

Fig. 6. Contribution of ouabain-insensitive I_p to the total I_p of transfected HEK293 cells and native rat ventricular myocytes. (A) Contribution of the exogenous, ouabain-resistant I_p to the total I_p in a stably transfected HEK293 cell. The cell was cultured for two days in DHO-free medium in order to abolish inhibition of the endogenous Na/K pumps. Original record of membrane current (bottom trace). Solution changes are indicated by the horizontal lines above the current trace. At the beginning of the record the cell is superfused with a medium containing 5.4 mM K_o. Total I_p is estimated twice as current blocked by K-free solution. Application of 10^{-4} M ouabain (with 5.4 mM K_o) diminishes the total I_p by 35%. The residual I_p is blocked by 10^{-2} M ouabain (with 5.4 mM K_o). Thus, the exogenous, ouabain-resistant I_p contributes at least 65% to the total I_p of the cell. (B) Contribution of ouabain-insensitive Na/K pump molecules to the total I_p of a rat ventricular myocyte. Application of different media is visualized by the bars above the current trace. Initially, the superfusate contains 5.4 mM K_o. Total I_p is measured as current blocked by K-free solution. A medium containing 10^{-4} M ouabain (with 5.4 mM K_o) inhibits 17% of the total I_p. Complete block of I_p occurs following application of 10^{-2} M ouabain (with 5.4 mM K_o). The ouabain-insensitive Na/K pumps contribute at least 83% to the total I_p of the myocyte. Dashed lines in (A) and (B) are drawn to facilitate the identification of the I_p components.

10^{-4} M ouabain inhibits 10 pA or 17% of the total I_p. The remaining pump current is completely blocked by 10^{-2} M ouabain. This component is most probably due to the ouabain-resistant pump molecules containing the $\alpha 1$ subunit. Corresponding experiments

on 5 myocytes revealed that $23 \pm 4\%$ of the total I_p were blocked by 10^{-4} M ouabain. Thus, 77% (at least) of the total I_p were generated by pump molecules containing the ouabain-resistant $\alpha 1$ subunit. These results are in quantitative agreement with the reports mentioned above. It is, however, difficult to exclude that a small portion of the pumps containing the $\alpha 1$ subunit were already blocked by 10^{-4} M ouabain.

3.4. I_p-V relationships of transfected and not-transfected HEK293 cells and of rat ventricular myocytes

Fig. 7 displays mean I_p-V relationships of normal (●; $n = 4$–15) and transfected (○; $n = 4$–6, $n = 2$ only at -80 mV) HEK293 cells. For comparison the I_p-V curve of rat ventricular myocytes is also presented (□; $n = 4$–10). For each series of experiments the I_p amplitudes measured at the various potentials were normalized to the respective amplitude at 0 mV (cf. Fig. 4). The pump current of the native (untransfected) HEK293 cells, i.e., the endogenous I_p, increases almost linearly with depolarization

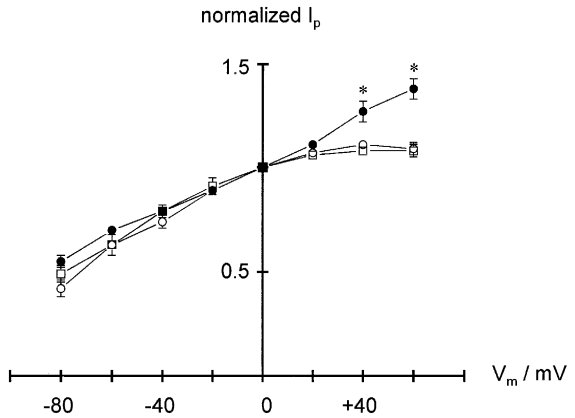

Fig. 7. Normalized mean I_p-V relationships of HEK293 cells and rat ventricular myocytes. In each series of experiments the I_p amplitudes measured at the various membrane potentials are normalized to the corresponding amplitude at zero potential, arbitrarily set to 1. The endogenous I_p (●) of native HEK293 cells ($n = 4$–15) exhibits a linear voltage dependence over the entire range of membrane potentials studied. In contrast, the I_p-V curves of transfected HEK293 cells (exogenous I_p: ○; $n = 4$–6; $n = 2$ only at -80 mV) and of rat ventricular myocytes (□; $n = 4$–10) show nearly constant I_p amplitudes at positive voltages. Both curves are almost identical. Asterisks mark significantly different I_p amplitudes ($P < 0.05$).

over the entire voltage range studied. This is in contrast to the I_p-V relationship of the transfected HEK293 cells and to the I_p-V curve of the ventricular myocytes. Both curves are nearly identical. They show a positive slope at negative membrane potentials but little variation at positive voltages. The similarity between both I_p-V curves suggests once more that the transfected HEK293 cells express Na/K pumps which contain the rat $\alpha 1$ subunit like the majority of the rat cardiac Na/K pump molecules [16].

4. Discussion

In recent years, molecular biological approaches enabled physiologists to examine structure-function relationships of the Na/K pump. Most studies, however, used biochemical or conventional voltage clamp methods to characterize recombinant Na/K pumps. A cell system suitable for the investigation of recombinant pump molecules by means of the patch clamp technique would be a powerful tool for the electrophysiological analysis of structure-function relationships of the Na/K pump. Therefore, the aim of the present study was to establish such a system. We chose HEK293 cells, a human cell line widely used for the expression of recombinant proteins. These cells are well suited for whole-cell recording, because of high seal resistances of up to 60 GΩ and because of their relatively small size. Membrane capacitance ranged between 20 and 85 pF, in good agreement with an earlier report [1]. Little is known about the Na/K pump of these cells, so we first characterized the Na/K pump current of HEK293 cells (endogenous I_p).

4.1. Characteristics of the endogenous I_p

The endogenous I_p was determined as current blocked by the cardiac glycosides ouabain or DHO. I_p density at 0 mV amounted to 0.33 pA · pF^{-1} in a solution containing 5.4 mM K_o. This value is rather small compared to excitable cells like ventricular myocytes, where the I_p density is around 1 pA · pF^{-1} or beyond [17]. Half maximal steady-state I_p inhibition occurred at $1.1 \cdot 10^{-5}$ M DHO (5.4 mM K_o) with a Hill coefficient of 1.2, suggesting one-to-one

binding to the Na/K pump as previously demonstrated by Hermans and co-workers [17]. Similar values were obtained in guinea pig ventricular myocytes ($1.4 \cdot 10^{-5}$ M DHO (5.4 mM K_o); [17]) or in cardiac Purkinje cells (canine: $0.4 \cdot 10^{-5}$ M DHO (8 mM K_o) [18]; rabbit: $3.5 \cdot 10^{-5}$ M DHO (10.8 mM K_o) [13]). In human endothelial cells half maximal steady-state inhibition of I_p occurred at $2.1 \cdot 10^{-5}$ M DHO (11 mM K_o) [19]. In the same study, a nearly irreversible I_p inhibition by DHO was observed. This was also the case for the endogenous I_p of the HEK293 cells. Similarly, the endogenous I_p of HeLa cells, a human cell line, was blocked nearly irreversibly by ouabain, digitoxin, and digoxin [5]. The reason for this nearly irreversible I_p blockade is not known. To our knowledge such an irreversible I_p inhibition has only been observed in the human cell types mentioned above raising the possibility that this is a property of human Na/K pumps.

Under our experimental conditions I_p (i.e., the outward current blocked by cardiac glycosides) was identical to the current activated by K_o. Half maximal activation of I_p occurred at 1.5 mM K_o with a Hill coefficient of 1.2. Comparable results were obtained for rat and guinea-pig ventricular myocytes [17,20,21], for rabbit sino-atrial node cells [22], for cardiac Purkinje cells [18], for human endothelial cells [19], and for various other cell types (cf. Ref. [23]). Obviously, K_o-activation of I_p does not differ substantially between different cell types and species.

The endogenous I_p was voltage dependent. I_p increased linearly over the whole voltage range studied, i.e., between -80 and $+60$ mV. This monotonic increase argues for only one voltage-dependent partial reaction in the pump cycle under the present experimental conditions (cf. Ref. [12]). The main voltage-dependent step is most probably located in the Na-translocating branch of the pump cycle [24,25] and is favoured by more positive membrane potentials. An I_p-V relationship similar to that in HEK293 cells was found in human endothelial cells [19]. I_p increased linearly between -150 and $+50$ mV in these cells. Such linear I_p-V curves are in contrast, however, to those obtained in the majority of cell types, including various cardiac cells, where I_p increases linearly with depolarization at negative membrane potentials but shows little voltage dependence at positive potentials [12,22,26–28]. The lack of voltage dependence at positive potentials can be explained by assuming that Na translocation is no longer rate-determining at these voltages. For the linear I_p-V curves of HEK293 and human endothelial cells it means that Na translocation must be rate-determining even at positive potentials up to $+60$ mV.

4.2. The heterologous expression of Na/K pump molecules in various cells

The heterologous expression of cardiac glycoside-resistant Na/K pumps in cells expressing glycoside-sensitive endogenous pumps is an established method for analyzing the structure–function relationship of the Na/K-ATPase. For example, HeLa cells have been widely used for the expression of glycoside-resistant, mutated α subunits of the rat or sheep Na/K pump in order to study the functional differences between various α subunits and the significance of distinct amino acids for cation pumping (e.g., Refs. [29–31]). These studies were mainly carried out by means of biochemical methods. Similarly, *Xenopus laevis* oocytes represent a suitable expression system for recombinant Na/K pump molecules [32,33]. The properties of the exogenous pump molecules can be measured both by biochemical and conventional electrophysiological methods. In the present study HEK293 cells were chosen for the expression of Na/K pump molecules containing the cardiac glycoside-resistant rat $\alpha 1$ subunit of the pump.

4.3. The identification of exogenous Na/K pumps in HEK293 cells

We kept the transfected HEK293 cells for several weeks in media containing 10^{-4} M DHO, which completely blocked the endogenous glycoside-sensitive Na/K pumps. Under these conditions a Na/K pump current was identified (Fig. 5), suggesting the functional expression of pump molecules containing the transfected glycoside-resistant $\alpha 1$ subunit of the rat Na/K pump. Similar to the rat cardiac Na/K-ATPase (e.g., Ref. [14]) these pumps were blocked only by the extremely high ouabain concentration of 10^{-2} M (Fig. 5). The functional expression of Na/K pumps containing the rat $\alpha 1$ subunit implies the assembly of the exogenous α and the endogenous β subunits of the molecules. Such an assembly has been

reported before from *Xenopus* oocytes [34], COS-1 cells [35], and HeLa cells [30]. The assembly of the rat $\alpha 1$ subunit and the endogenous β subunit in HEK293 cells is not unexpected since human and rat α subunits are nearly homologous [33]. The total I_p density of the transfected HEK293 cells amounted to 0.20 $pA \cdot pF^{-1}$ or 61% of the I_p density of untransfected cells. Similarly, Putnam and co-workers [36] reported that the total ATPase activity of HeLa cells transfected with the rat $\alpha 1$ subunit was about 60% of the controls. Since exogenous and endogenous α subunits compete for assembly with the available endogenous β subunits, increased I_p density in the transfected cells would be surprising. Furthermore, the procedure of transfection per se tends to diminish the level of expressed pump molecules [35,37]. In contrast to these findings, however, is the observation of Yamamoto and co-workers [5] that the Na/K pump current densities in native and transfected HeLa cells were almost identical. Our results illustrated in Fig. 6A revealed that at least 63% of the total I_p density in transfected HEK293 cells are generated by pump molecules containing the rat $\alpha 1$ subunit. This is in line with the observation that 51% of the Na/K-ATPase activity of COS-1 cells transfected with the rat $\alpha 1$ subunit are caused by pumps containing the rat subunit [35]. According to Putnam and co-workers [36] less than 50% of the total Na/K-ATPase activity in HeLa cells transfected with various rat α subunits are due to molecules containing the exogenous subunit. However, in HeLa cells transfected with a mutated sheep $\alpha 1$ subunit up to 90% of the total Na/K-ATPase activity are produced by pumps containing the sheep subunit [29]. Finally, in HeLa cells transfected with a mutated human or sheep $\alpha 1$ subunit the whole pump current is apparently generated by recombinant pump molecules [5].

4.4. Characteristics of the exogenous I_p

In contrast to the inhibition of I_p by ouabain in native HEK293 cells the blockade of I_p by the cardiac glycoside in transfected cells was reversible. Estimation of the apparent K_d value (K'_d) from the kinetics of ouabain-binding and -unbinding yielded $2.4 \cdot 10^{-4}$ M, a concentration that completely blocks the endogenous I_p. Since both the time constant for solution exchange (200–600 ms) as well as that for I_p inhibition (1.1–1.4 s) are similar, the calculated K'_d value has to be considered a rough estimate that tends to overestimate the 'correct' K'_d. Despite this limitation, the value obtained by our procedure agrees fairly well with the K'_d value ($3 \cdot 10^{-4}$ M) reported by Berlin and co-workers [14] for the inhibition of I_p by ouabain in rat ventricular myocytes under slightly different conditions (15 mM Na_{pip}; 15 mM K_o). Both the reversibility and the K'_d value of the I_p inhibition by ouabain clearly demonstrate the expression of pump molecules containing the rat $\alpha 1$ subunit in the transfected HEK293 cells. Similarly, the shape of the I_p-V curve in transfected HEK293 cells strongly suggests the functional expression of pumps with the rat subunit. Like the I_p-V relationship of rat ventricular myocytes, the I_p-V curve of transfected HEK293 cells displays little variation of I_p at positive membrane potentials, whereas the I_p-V relationship of native HEK293 cells is almost linear over the entire range of potentials tested (Fig. 7). However, I_p activation by K_o is similar in native and transfected HEK293 cells. The $[K]_o$ required for half maximal I_p activation amounted to 1.5 mM in native cells and to 1.7 mM in transfected cells. Comparable values are reported for rat ventricular myocytes ([20]: 2.9 mM K_o; [17]: 1 mM K_o).

In conclusion, the shape of the I_p-V relationship and the effect of ouabain on I_p, but not the kinetics of I_p activation by K_o can be used to differentiate between endogenous Na/K pumps and exogenous pump molecules containing the rat $\alpha 1$ subunit in HEK293 cells. The different ouabain-sensitivity and voltage dependence of the rat Na/K pump as well as the activation of the rat I_p by K_o are well conserved in the recombinant Na/K pumps in HEK293 cells, demonstrating that this cell line is suited for the expression and whole-cell patch clamp analysis of the ouabain-resistant $\alpha 1$ subunit of the rat Na/K pump.

Acknowledgements

We are indebted to Professor Jerry Lingrel (Cincinnati) for a generous gift of the plasmid pR$\alpha 1$. The authors would like to thank Professors Barry W. Ache (Daytona) and Wolfgang Schwarz (Frankfurt) for helpful comments on an earlier draft of the manuscript.

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