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Characteristics of the Na^+/K^+-ATPase from *Torpedo californica* expressed in *Xenopus* oocytes: A combination of tracer flux measurements with electrophysiological measurements

Wolfgang Schwarz and Quanbao Gu *

Max-Planck-Institut für Biophysik, Frankfurt am Main (F.R.G.)

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The Na^+/K^+-ATPase from electroplax of *Torpedo californica* was incorporated into the plasma membrane of *Xenopus* oocytes by microinjection of mRNA coding for the α- and β-subunit of the enzyme; the mRNAs were obtained by in vitro translation of cloned cDNAs (Noguchi et al. (1988) FEBS Lett. 225, 27–32). (1) Measurements of ouabain-sensitive membrane current revealed that the Na^+/K^+-ATPase of *Torpedo* is less sensitive to ouabain than the endogenous enzyme. (2) The ouabain-sensitive membrane currents in mRNA-injected oocytes exhibit similar voltage dependence as the currents generated by the endogenous ATPase of *Xenopus* oocytes; in particular, the current–voltage relation exhibits a maximum and a negative slope at potentials more positive than +20 mV. (3) A maximum can also be detected if the rate of $^{22}Na^+$ efflux is determined under different voltage-clamp conditions. If membrane current and rate of Na^+_2 efflux are determined simultaneously, a voltage-independent ratio between current and flux is obtained suggesting voltage-independent Na^+-K^+ stoichiometry. The data are compatible with a $3Na^+$-$2K^+$ stoichiometry.

Introduction

The oocytes of *Xenopus laevis* are particularly suited for the characterization of transport proteins that are not mediated by channels. This is because the large size of the oocytes (more than 1 mm in diameter) allows to perform flux measurements on the single cell, and this can be done under voltage-clamp conditions. In this respect a variety of primary and secundary active transport systems of the oocytes have been studied in detail in the past; these include Na cotransport systems [1–3] and the Na^+/K^+-ATPase [4,5]. The latter enzyme, often called sodium pump, is responsible for maintenance of the electrochemical gradients of Na^+ and K^+ across a cell membrane. It is generally accepted that, under physiological conditions, 3 Na^+ ions are transported out of the cell, and 2 K^+ ions into the cell per ATP molecule that is split. The imbalance of charges transported across the cell membrane leads to an outwardly directed net movement of positive charges. The activity of the Na^+/K^+-ATPase can be monitored, therefore, by measuring the influx of K^+, the efflux of Na^+, or the membrane current. The respective components generated by the ATPase are usually obtained from measurements in the

* Permanent address: Shanghai Institute of Cell Biology, Academia Sinica, Shanghai, China.

Abbreviations: ORI, oocyte Ringer solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DHO, dihydroouabain.

Correspondence: W. Schwarz, Max-Planck-Institut für Biophysik, Heinrich-Hoffmannstr. 7, D-6000 Frankfurt/M, F.R.G.

absence and presence of specific inhibitors of the ATPase, such as the cardiac glycosides.

The advantages of characterizing non-channel transport in the oocytes of *Xenopus* can be utilized also for transport systems not endogenously present in the oocytes, like the anion exchanger from red blood cells [6] or the Na^+/glucose cotransporter of epithelial cells [7,8]. This is due to the fact that the oocytes serve as a convenient expression system for foreign mRNA [9]. Recently, mRNA for the Na^+/K^+-ATPase cloned from cDNA of *Torpedo californica* electroplax has been injected into the oocytes [10]; a functional enzyme is incorporated into the cell membrane if mRNA for the α- and β-subunit are injected simultaneously.

In this contribution we present a functional characterization of the *Torpedo* Na^+/K^+-ATPase in the oocytes of *Xenopus* utilizing the above-mentioned advantages by combining measurements of isotope fluxes with electrophysiological techniques. This combination of techniques particularly demonstrates voltage-independent Na^+-K^+ stoichiometry and a negative slope in the voltage dependence of the Na^+–K^+ exchange.

Methods

The mRNAs for the α- and β-subunits of Na^+/K^+-ATPase obtained by in vitro translation of cloned cDNAs of *T. californica* electroplax [10] were kindly provided to us by Drs. M. Kawamura, M. Mishina, S. Noguchi and S. Numa. The methods of injecting mRNA, and measuring fluxes and current–voltage dependencies were identical to those described previously [11,12]. A brief description follows.

The oocytes

Females of the clawed toad *X. laevis* were anaesthetized on ice and parts of the ovary were removed. Full-grown oocytes arrested in the prophase of the first meiotic division (stage VI after Dumont [13]) were selected after collagenase treatment. For the expression of the α- and β-subunit of the Na pump of *Torpedo* in the oocytes, 10 or 20 ng of mRNA (0.5 μg/μl for each subunit) were injected into an oocyte, and the cells were incubated thereafter for 2–5 days at 16–18°C in Barth's solution. Before an experiment, the cells were usually preincubated for at least 6 h in K^+-free oocyte Ringer's solution (see below). This treatment reversibly blocks the normal Na^+–K^+ exchange mode of the ATPase and leads to an increase in intracellular Na^+ which stimulates pump activity. All flux and current measurements were performed at room temperature (20–22°C).

Recording and analysing data

The experiments were performed under control of an LSI 11/23 computer (see ref. 14). The oocytes were voltage-clamped by conventional two-microelectrodes technique. For determination of current–voltage dependencies, rectangular voltage pulses of varying amplitude and 500 ms duration were applied every 4 s, and steady-state current was averaged during the last 100 ms. Between the pulses, the holding potential was usually set to −70 mV.

For measurements of $^{22}Na^+$ efflux oocytes were injected with 20–50 nl $^{22}NaCl$ (about 70 MBq/ml) and placed in a perfusion chamber which was directly mounted on the window of a Geiger-Müller tube. The radioactivity remaining in the oocyte was continuously recorded with a rate meter at a time constant of 10 s; data points were averaged for 10 s. The rate of efflux was determined by fitting the exponential

$$R = R(t=0)\cdot\exp(-kt) \tag{1}$$

to data recorded during a period of fixed conditions. These conditions were maintained for at least 15 min.

The dependence of efflux rate and of current on the ouabain concentration can be described by Michealis-Menten kinetics

$$B = \frac{[\text{ouabain}]}{K_I + [\text{ouabain}]} \tag{2}$$

where B represents the degree of inhibition of the ouabain-sensitive component. After application of ouabain, data were analysed for the determination of efflux rates and currents after inhibition had come to a steady state; this was reached within 5–15 min after changing the bath solution and

could be judged by monitoring the changes in membrane potential.

For the mRNA-injected oocytes, where two populations of ATPase molecules with different K_I values are present, the number of expressed transport molecules was high enough to describe the concentration dependence also by Eqn. 2.

Solutions

The composition of the Barth's medium was: 90 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 5 mM Hepes (pH 7.6), 0.08 mM penicillin and 0.03 mM streptomycin. During the flux and voltage-clamp measurements an oocyte Ringer's solution (ORI) was usually used, with the composition: 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 5 mM Hepes (pH 7.4).

Results

Efflux measurements without voltage clamp

After expression of microinjected mRNA during the incubation period and after injection of ^{22}Na, Na^+ efflux is measured in the perfusion chamber by following the time-course of ^{22}Na remaining in the oocyte. In comparison to control oocytes which had not been injected with mRNA, a more rapid $^{22}Na^+$ efflux can be observed (Fig. 1A). The rate of efflux can be further increased (in control and injected oocytes) if intracellular Na^+ is elevated by K^+-free preincubation (Fig. 1B). If the oocytes are injected with mRNA, the rate of efflux is about 50–200% larger, depending on the amount of mRNA injected (see legend to Fig. 1B). Even if the length of experiment is extended to 4 h, the reduction of ^{22}Na in the cell can reasonably be described by a single exponential, indicating constant transport activity for the extrusion of Na^+.

The $^{22}Na^+$ efflux in the control as well as in the injected oocytes is mediated primarily by Na^+/K^+-ATPase. The Na^+/K^+-ATPase can specifically be inhibited by cardiac glycosides like ouabain or dihydroouabain (DHO). Fig. 2A demonstrates for the endogenous transport in control oocytes that 10 μM ouabain gives nearly complete inhibition of the ATPase; higher concentrations of ouabain (50 μM) give no further reduction of the rate of ^{22}Na efflux (see k values in legend to Fig. 2A). This is in agreement with observations on inhibition of $^{86}Rb^+$ influx [4] and of ATPase-generated current [12]. If oocytes are injected with mRNA, on the other hand, Na^+ efflux is much less affected by 10 μM ouabain, at least 100 μM are necessary for complete inhibition of the

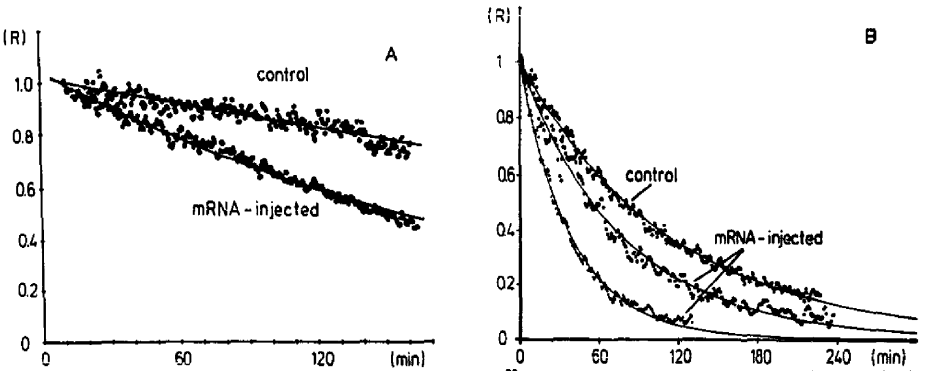

Fig. 1. Loss of radioactivity from different oocytes after injection of ^{22}Na. The symbols represent recorded radioactivity in the oocytes; for comparison of the different experiments the data are normalized. The solid lines represent least-squares fits of Eqn. 1 to the data. (A) Oocytes not preincubated in K^+-free solution; Control oocyte which had not been injected with mRNA ($k = (31 \pm 1) \cdot 10^{-6}$ s^{-1}, Expt. No. 020388/1), oocyte injected 2 days before the experiment with 10 ng ($k = (81 \pm 1) \cdot 10^{-6}$ s^{-1}, Expt. No. 100388/3). (B) Oocytes preincubated in K^+-free solution to raise intracellular Na^+; control oocyte which had not been injected with mRNA ($k = (144 \pm 1) \cdot 10^{-6}$ s^{-1}, Expt. No. 070188/1). Oocyte injected 2 days before the experiments with 10 ng ($k = (209 \pm 2) \cdot 10^{-6}$ s^{-1}, Expt. No. 070188/3) and 20 ng ($k = (417 \pm 3) \cdot 10^{-6}$ s^{-1}, Expt. No. 070188/2) of mRNA, respectively.

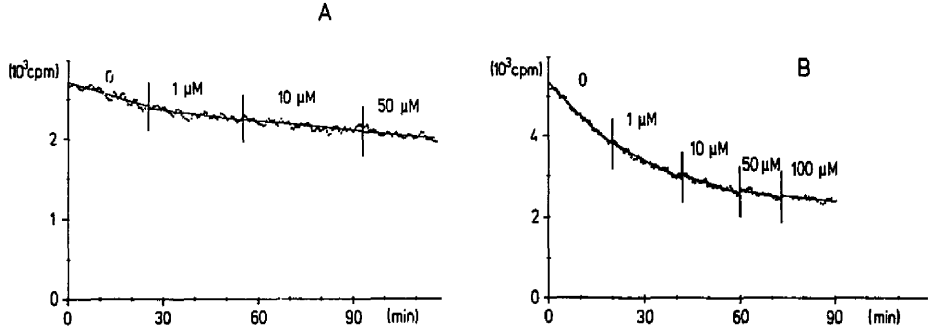

Fig. 2. Loss of radioactivity from two oocytes after injection of ^{22}Na with different concentrations of ouabain in the wash solution as indicated in the figure. The dots represent recorded data. Least-squares fits of Eqn. 1 were applied to the data to obtain k values (in 10^{-6} s^{-1}, see table below for the respective ranges). (A) control oocyte which had not been injected with mRNA (Expt. No. 161287/1). (B) Oocyte injected 2 days before the experiment with 10 ng of mRNA (Expt. No. 021287/3).

[Ouabain] (μM)	Control oocyte	Injected oocyte
0	81±4	289±5
1	42±4	201±6
10	29±3	126±8
50	31±6	86±4
100	-	55±1

ouabain-sensitive component (see k values in legend to Fig. 2B). In oocytes with expressed ATPase, the endogenous and the expressed enzyme are active, and both transport systems obviously have different K_I values. The k values determined from experiments as described in Fig. 2 cannot be used for an estimation of the K_I values, since partial inhibition of the Na^+ pump depolarizes the membrane, and this in turn stimulates pump activity [5]. Therefore, determination of concentration-dependent inhibition should be done under voltage clamp (see below).

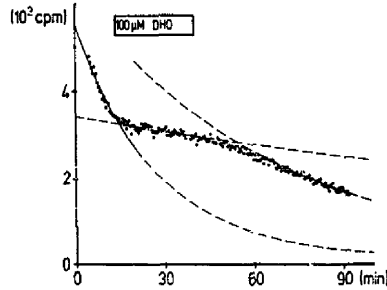

Fig. 3. Loss of radioactivity from an oocyte after injection of ^{22}Na. The oocyte had been preincubated for 4 days after injection of 10 ng mRNA. The symbols represent recorded data, the lines least-squares fits of Eqn. 1 to the data in the respective range. The calculated k values (in 10^{-6} s^{-1}) are before DHO application: 603±13, during application of 100 μM DHO: 68±4, and after 30 min of washout: 243±3 (Expt. No. 171287/8).

For the endogenous Na^+/K^+-ATPase in the *Xenopus* oocytes, dihydroouabain (DHO) had been demonstrated to be a reversible inhibitor [5]. Also the expressed ATPase from *Torpedo* is reversibly inhibited by DHO. This is demonstrated in Fig. 3: 100 μM DHO inhibits the efflux of $^{22}Na^+$, and after washout the efflux gradually recovers. But, even after 30 min of washing with DHO-free solution, reversibility is not complete. This slow recovery from inhibition by DHO does not provide advantages over inhibition by ouabain in flux experiments, and we used ouabain in all other experiments described in this paper.

Measurements of membrane current

To determine K_I values for the inhibition of the Na^+ pump by ouabain the ouabain-sensitive membrane current has been determined at a con-

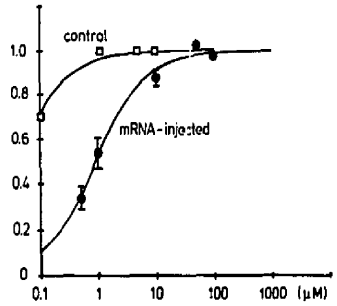

Fig. 4. Inhibition of ouabain-sensitive membrane current as a function of ouabain concentration. Open squares represent values determined from 2–5 experiments on control oocytes (S.E. is within the size of the symbols; data were taken from Schweigert et al. [12]). Filled circles represent values (±S.E.) from 3–5 experiments on oocytes injected with 10 ng 2 days before the experiment. Solid lines represent least-squares fits of Eqn. 2 to the data: $K_I = (0.04 \pm 0.01)$ μM (for control oocytes); $K_I = (0.90 \pm 0.12)$ μM (for injected oocytes).

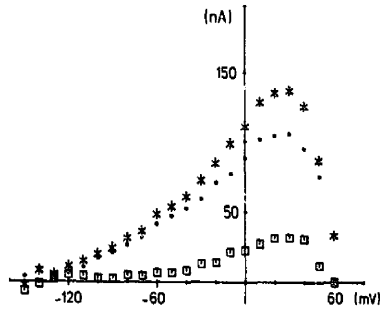

Fig. 5. Voltage dependence of ouabain-sensitive current determined as the difference between current in solution without and with either 1 (squares) or 100 μM (asterisks) ouabain. The dots represent the difference current between 1 and 100 μM ouabain. The oocyte was injected 3 days before the experiment with 10 ng mRNA (Expt. No. 300688/-).

stant membrane potential. The dependence of the degree of pump inhibition on ouabain concentration is shown in Fig. 4 for control and mRNA-injected oocytes. The data demonstrate that the K_I value for the injected oocytes (0.9 μM) is more than one order of magnitude higher than for the control oocytes (0.04 μM).

The current-voltage dependence of the endogenous Na^+/K^+-ATPase in the oocytes has been investigated in the past in detail (see, for example, Ref. 12). This enzyme is characterized by increasing transport activity with depolarization, and often a maximum at about +20 mV can be detected. Fig. 5 shows the voltage dependence of the current component of an injected oocyte that is inhibited by 1 μM (squares) and 100 μM (asterisks) ouabain. The difference between the two curves (filled dots) represents the voltage dependence of pump current mediated only by the *Torpedo* ATPase. A maximum at about +20 mV can be detected for this transport system, also.

Flux measurements under voltage clamp

If fluxes are to be compared with currents it is very useful if both types of data can be obtained from the same oocyte. For this purpose the holding potential can be clamped to different values during an efflux experiment, while radioactivity in the oocytes, clamp potential and clamp current are recorded in the absence and presence of 100 μM ouabain. In our control oocytes $^{22}Na^+$ efflux was usually too slow to detect significant potential-dependent changes of the rate of efflux.

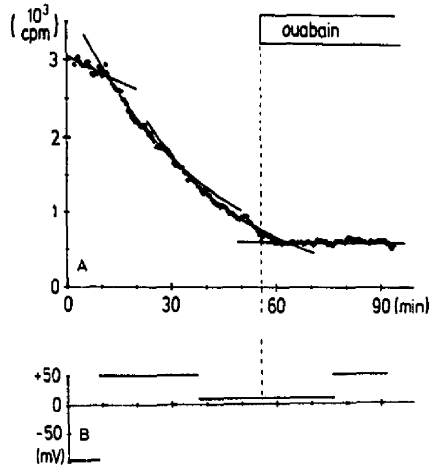

Fig. 6. Efflux experiment under varying voltage-clamp conditions. The oocyte has been injected with 10 ng mRNA 3 days before the experiment. $^{22}Na^+$ efflux, membrane current and holding potential were recorded simultaneously. (A) Loss of radioactivity from an oocyte after injection of ^{22}Na. The dots represent recorded data. Least-squares fits of Eqn. 1 were applied to the data to obtain k values (in 10^{-6} s^{-1}) for the respective voltage ranges: −100 mV: 127±30, +50 mV: 443 ±6, +10 mV: 552±15; in the presence of 100 μM ouabain nearly no efflux was detectable. (B) Holding potential during the flux measurements. (Expt. No. 030388/4.)

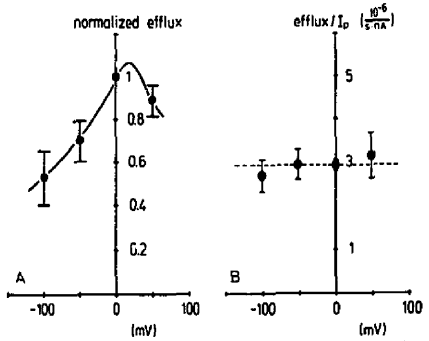

Fig. 7. (A) Voltage dependence of the rate of ouabain-sensitive Na^+ efflux. Data are average values (±S.E.) from 2–6 different experiments and normalized to the value at 0 mV; the line is drawn by eye. (B) Ratio between ouabain-sensitive Na^+ efflux and current for different membrane potentials from three experiments.

Oocytes injected with mRNA have 2- to 3-times larger efflux rates (see above), and fig. 6 demonstrates that potential-dependent changes can be detected in these cells. Depolarization from −100 mV increases the rate of efflux; but at more positive potentials (+50 mV) the rate is smaller than at +10 mV (see k values in the figure legend). This characteristic is maintained if the ouabain-sensitive component is extracted. Fig. 7A summarizes the results of six experiments for the ouabain-sensitive efflux, where voltages of −100, − 50, 0 and +50 mV were investigated. Like the current–voltage curve, the flux-voltage curve also exhibits a maximum. If currents and fluxes are recorded from the same oocytes at different membrane potentials, ratios between the rate of Na^+ efflux and pump current can be obtained. Fig. 7B demonstrates that there is no significant potential dependence of this ratio, which is a measure of the Na^+-K^+ stoichiometry.

Discussion

If oocytes of *X. laevis* are injected with mRNA specific for the α- and β-subunit of the Na^+/K^+-ATPase of *T. californica*, a functional enzyme is incorporated into the oocyte membrane. This has been demonstrated previously by measurements of ouabain-sensitive ATPase activity in microsomes and $^{86}Rb^+$ uptake, as well as by ouabain binding [10]. In this paper we prove in addition the functional formation of *Torpedo* ATPase by measurements of ouabain-sensitive $^{22}Na^+$ efflux and membrane current. In most experiments 10 ng of mRNA were injected, which led to an increase in pump-mediated flux of at least 50%. If injected with 20 ng, the ouabain-sensitive fluxes are about 3-times larger than in control oocytes.

The Na^+/K^+-ATPase from *Torpedo* differs from the endogenous enzyme with respect to ouabain sensitivity. For oocytes injected with mRNA, the pump-mediated Na efflux is less sensitive to ouabain than in control oocytes. If K_I values for inhibition of pump activity are to be determined, measurements have to be performed under voltage-clamp conditions. It has been demonstrated for the endogenous ATPase that ouabain binding is voltage-independent [15], but inhibition of the pump by ouabain depolarizes the membrane. This in turn stimulates the still operating pump molecules, and without voltage clamp apparently higher K_I values were obtained. For the endogenous system K_I values have been determined from current measurements at a constant membrane potential [12] (see Fig. 4) of the order of 0.1 μM. If the same type of experiments are performed with mRNA-injected oocytes, a K_I value of 1 μM is obtained. Large differences in the sensitivity of the Na^+/K^+-ATPase for ouabain between different animal species have been reported (see, for example, Ref. 16), and even within the same tissue, different types of receptor for ouabain have been detected that are ascribed to an α^+ and an α isoform of the subunit of the Na^+/K^+-ATPase [17]. Hence, the difference in the K_I values we find between the endogenous and the *Torpedo* ATPase in the *Xenopus* oocytes is not surprising. In measurements of the activity of ATPases from *Xenopus* oocytes and *Torpedo* electric organ, Tekeda et al. [18], on the other hand, found similar K_I values. This may be due to different substrate conditions. Differences in the K_I values for the two ATPases could indeed be detected in activity measurements by G. Schmalzing (personal communication) at low K^+ (3–5 mM); at higher K^+ concentrations the sensitivity of the *Xenopus* ATPase to ouabain but not of the *Torpedo* ATPase is reduced, leading to similar K_I

values (about 0.1 μM), in agreement with the observations made by Takeda et al. [18]. Different dependencies of ouabain sensitivity on K^+ have also been reported for ATPases of red blood cells from different species [19].

The difference in ouabain sensitivity of pump-mediated currents and fluxes supports the view that the injection of the mRNA indeed leads to expression of *Torpedo* ATPase rather than to a stimulated expression of the endogenous enzyme. In addition, the difference provides the advantage that the two types of ATPase can be separated for their characterization in the oocytes. The endogenous system is completely inhibited with 1 μM ouabain, while under these conditions a large portion of the *Torpedo* ATPase is still active.

The current that can be inhibited by increasing the ouabain concentration from 1 to 100 μM in mRNA-injected oocytes is current purely generated by the *Torpedo* ATPase. Fig. 5 shows similar characteristics in the potential dependence as for the endogenous pump; in particular, the pump current increases with depolarization, and at potentials more positive than +20 mV the *I–V* curve has a negative slope. In the past it has been discussed whether the negative slope could be an artifact due to ouabain-induced changes in K^+ conductance (see Ref. 20). But the flux measurements with ^{22}Na demonstrate the same voltage dependence also for the Na^+ efflux, supporting the view that the negative slope represents a characteristic of the pump-mediated current.

For the endogenous pump current the maximum in the *I–V* cannot always be detected (see Ref. 12); under these conditions a negative slope can also not be detected in flux and current measurements with the expressed enzyme. This has been attributed to seasonal variations of an intracellular parameter.

Simultaneous measurements of Na^+ efflux and of net charge transfer (i.e., current) generated by the Na^+/K^+-ATPase allow estimation of the Na^+-K^+ coupling ratio (for a review see Ref. 20). For the endogenous Na^+/K^+-ATPase in the *Xenopus* oocytes Eisner et al. [21] estimated a $3Na^+$-$2K^+$ stoichiometry. Since we have no accurate value for the intracellular Na^+ activity (a_{Nai}), accurate estimation of the Na^+ efflux is not possible, and values for the stoichiometry become questionable. Rough estimates of a_{Nai} by ion-selective microelectrodes gave values of about 10 mM in the oocytes with *Torpedo* ATPase; this would be compatible with 2.8 Na^+ ions being transported outward for one net charge, and a 3 Na^+-$2K^+$ stoichiometry seems likely. In any case, comparison of the rates of efflux with the currents demonstrate a voltage-independent ratio (see Fig. 7B), indicating voltage-independent stoichiometry for the voltage range of −100 to +50 mV. This is in line with a report on squid axon by DeWeer et al. [22], who found voltage-independent stoichiometry between −60 and 0 mV.

Acknowledgements

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