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Conditions for a backward-running Na^+/K^+ pump in *Xenopus* oocytes

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Current generated by the electrogenic Na^+/K^+ pump protein was determined in oocytes of *Xenopus laevis* as strophantidine-sensitive current measured under voltage clamp. Under conditions of reduced intracellular [Na^+] and [ATP], both to values below 1 mM, and in extracellularly K^+-free medium, the Na^+/K^+ pump seems to operate in a reversed mode pumping Na^+ into the cell and K^+ out of the cell. This is demonstrated by strophantidine-induced hyperpolarization of the membrane and inward-directed current mediated by the pump protein. In addition, strophantidine-sensitive uptake of $^{22}Na^+$ can be demonstrated under these conditions. The pump current decreases with membrane depolarization as expected for a pump cycle that involves inward movement of positive charges during Na^+ translocation.

Introduction

Under physiological conditions, the Na^+/K^+-ATPase transports three Na^+ ions out of the cell and two K^+ ions into the cell by splitting one ATP molecule per pump cycle. As has been shown previously in cardiac myocytes [1] and squid giant axon [2], increasing the inward-directed gradient for [Na^+] and outward-directed gradient for [K^+], and the [ADP]·[P_i]/[ATP] ratio seems to force the pump to operate in a backward-running mode. In contrast to these cells, the oocytes of *Xenopus laevis* do not allow replacement of the cytoplasmic medium. Nevertheless, the oocytes have turned out to be an excellent model system for investigation of the Na^+/K^+ pump. The cells exhibit a very high density of pump molecules in their plasma membrane [3,4] while passive conductances are low compared to most other cells (see Ref. 5). Therefore, transport mediated by the Na^+/K^+ pump can easily be studied by measurements of the electrogenic current [6], and $^{22}Na^+$ [7,8] or $^{86}Rb^+$ [3] transport can be measured in single cells. Also biochemical and morphological studies can be performed on single oocytes [3,4,9]. In this report we describe the conditions that allow one to investigate a reversely operating pump in the *Xenopus* oocytes. Some of the results have been reported in an abstract [10].

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Materials and Methods

Oocytes

To obtain the oocytes, females of the clawed toad *Xenopus laevis* were anesthetized with *m*-aminobenzoic acid ethyl ester methanesulfonate (MS222, Sandoz, Basel (Switzerland)). Parts of the ovary were removed and treated with collagenase to remove enveloping tissue. Experiments were performed with the full-grown prophase-I arrested oocytes (stage V and VI, after Dumont, [11]) at room temperature (21°C).

Electrophysiological measurements

Current-voltage dependencies were determined by conventional two-microelectrode techniques [6]. From a holding potential of −60 mV, rectangular voltage pulses of 500 ms duration and varying amplitude were applied from negative to positive potentials every 4 s, and steady-state currents were averaged during the last 100 ms. The intracellular Na^+ activity was determined by Na^+-selective microelectrodes. The electrodes were filled with liquid ion exchanger (cocktail ETH 227, Fluka AG, Buchs, Switzerland) and calibrated in solution containing 1 to 40 mM NaCl plus sufficient KCl to maintain [Cl^-] at 120 mM.

Tracer flux measurements

For measuring uptake of $^{22}Na^+$, individual oocytes were incubated in 0.7 ml K^+-free ORi solution (see below) containing $^{22}Na^+$-labeled NaCl (7.4 MBq/ml) for 20 min while kept under voltage-clamp conditions at −100 mV. To determine the amount of radioactivity taken up by single oocytes, the cells were washed six times in K^+-free ORi solution to remove contaminating $^{22}Na^+$. The individual oocytes were then dissolved in 0.1 ml 5% sodium dodecyl sulfate solution and radioactivity was determined in 2 ml of Qickszint 2000 (Zinsser, Frankfurt/M (F.R.G.)) by liquid scintillation counting.

Determination of nucleotide concentrations

The concentrations of adenine nucleotides were determined on an isocratic HPLC system using reverse-phase chromatography. After an oocyte was suspended in 200 μl 0.4 M perchloric acid and homogenized, the suspension was then centrifuged and the perchlorate anions were precipitated by addition of 80 μl 1.0 M K_2PO_4 medium (pH 6) to 160 μl supernatant. After centrifugation, the precipitate was removed, and the supernatant was used for nucleotide analysis [12]. The nucleotides were quantified from the peak height in the chromatogram.

Solutions

The composition of the oocyte Ringer's solution (ORi) was (in mM): 110 NaCl, 3 KCl, 2 $CaCl_2$, 5 morpholinopropanesulfonic acid (Mops, adjusted to pH 7.2). For K^+-free solution the KCl was omitted; the contaminating level of K^+ was below 5 μM as determined by flame photometry. For Na^+- and K^+-free medium the NaCl was replaced by 90 mM tetramethylammonium chloride (TMA-Cl). In some experiments, the solutions contained in addition 5 mM $BaCl_2$ and 20 mM tetraethylammonium chloride (TEA-Cl) for reduction of non-pump related currents [5]; in these solutions, extracellular [NaCl] was reduced to 90 mM.

Results and Discussion

An outward-directed gradient for $[K^+]$ and an inward-directed gradient for $[Na^+]$ will provide conditions for the Na^+/K^+ pump to operate in a reversed mode. To steepen the outward-directed gradient for $[K^+]$, K^+ is omitted from the external medium. To steepen the inward-directed gradient for $[Na^+]$, oocytes were first incubated in Na^+- and K^+-free medium. This treatment reduced the intracellular Na^+ activity from 8.0 ± 0.7 mM to 0.9 ± 0.2 mM (see Fig. 1A). 4 to 6 h of incubation are sufficient to obtain nearly maximum reduction in $[Na^+]$. With these oocytes, a steep gradient for $[Na^+]$ was obtained by having an external medium with 90 or 110 mM Na^+.

To increase the $[ADP] \cdot [P_i]/[ATP]$ ratio, Na^+-depleted oocytes were incubated in solution containing 1 mM 2,4-dinitrophenol (DNP). This pretreatment leads to a reduction of [ATP] and an increase of [ADP]. For quantification of intracellular [ATP] and [ADP], an isocratic HPLC system was used [12]. Separation of the nucleotides was achieved by reverse-phase chromatography. The time-course of [ATP] and [ADP] changes is shown for averaged data in Fig. 1B. After about 1.5 h major changes that occur have completed. For the experiments described below, oocytes were incubated, therefore, for at least 90 min in the DNP-containing solution.

Strophantidine is a specific and reversible inhibitor of the Na^+/K^+ pump in the *Xenopus* oocytes. In cells with reduced intracellular $[Na^+]$ and elevated $[ADP] \cdot$

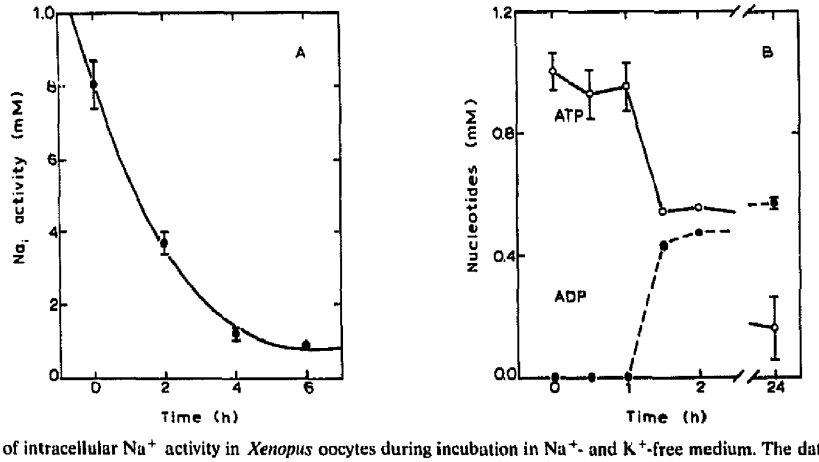

Fig. 1. (A) Decline of intracellular Na^+ activity in *Xenopus* oocytes during incubation in Na^+- and K^+-free medium. The data are averages ± S.E. from five determinations. (B) Time course of changes in intracellular [ATP] and [ADP] after subjecting the oocytes to 1 mM DNP. Data are averages ± S.E. from 2–8 determinations.

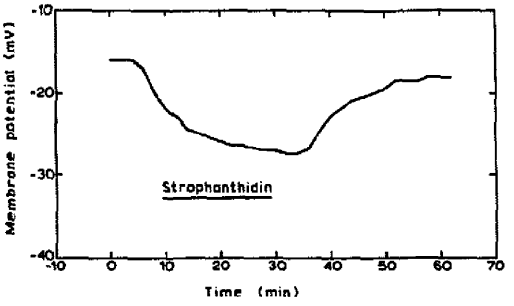

Fig. 2. Reversible hyperpolarization of membrane potential by application of 10 μM strophantidine to an external K^+-free bath solution.

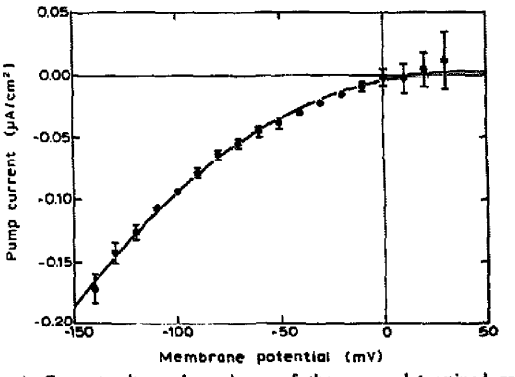

Fig. 4. Current-voltage dependence of the pump determined as described in Fig. 3. Data are averages ± S.E. of five experiments. For averaging currents from different oocytes, pump currents were normalized to the pump currents obtained at −100 mV. Pump activity was expressed as density of pump-generated current assuming a surface area of 0.18 cm^2 [13].

$[P_i]/[ATP]$ ratio, application of 10 μM strophantidine to K^+-free external solution leads to a reversible hyperpolarization of the membrane (Fig. 2). This demonstrates inhibition of an electrogenic transport mode of the pump. The hyperpolarization suggests that strophantidine inhibits a net inward current of positive charges which one would expect from an electrogenic backward-running pump that transports more Na^+ ions into the cell than K^+ ions out of the cell.

The pump-generated current is a measure for transport activity and can be determined in voltage-clamp experiments. Fig. 3A shows the voltage dependence of total membrane currents in presence (filled symbols) and absence (open symbols) of 10 μM strophantidine. Pump current is assumed to be represented by the component that is inhibited by strophantidine (Fig. 3B). Fig. 4 summarizes the voltage dependence of pump current averaged from a series of experiments. The inward current density at −100 mV is about 0.1 $\mu A/cm^2$ assuming a surface area of the oocyte of 0.18 cm^2 [13]. For a backward-running 3 Na^+/2 K^+ pump, this would corresponds to an uptake of Na^+ of about 0.6 pmol per s and oocyte.

Strophantidine-sensitive uptake of $^{22}Na^+$ can indeed be demonstrated. Total and strophantidine-insensitive uptake of $^{22}Na^+$ was determined in the absence and presence of 50 μM strophantidine, respectively, and is assumed to represent Na^+ uptake mediated by the pump. The oocytes exhibit pump-mediated uptake of 1.55 ± 0.25 pmol Na^+ per s and oocyte. This value is larger but still compatible with the 0.6 pmol estimated from the current measurements and assuming 3 Na^+/2 K^+ stoichiometry. The existing difference could, at least in principle, result from a superimposition of pumps mode with other stoichiometries; an electrically silent Na^+/Na^+ exchange would contribute to $^{22}Na^+$

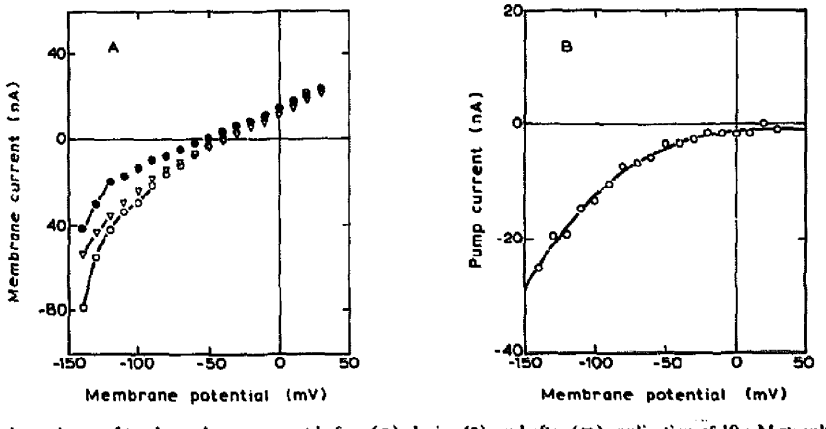

Fig. 3. (A) Voltage dependence of total membrane current before (○), during (●), and after (▽) application of 10 μM strophantidine to K^+-free bath solution. (B) Voltage dependence of pump-generated current determined as difference of current averaged from current measured before and after application of strophantidine and of current measured during application of strophantidine.

uptake but not to current; nevertheless, the drastically reduced intracellular [Na^+] makes this mode of pumping not very likely. It has been suggested recently that the Na^+/K^+ pump molecule can be converted into a cation-selective channel by treatment with palytoxin ([14], and Rettiger, J., Habermann, E. and Schwarz, W., unpublished data); a contribution of such a transport mode would, on the other hand, further reduce the uptake of Na^+ estimated from the measured current. We, therefore, prefer to attributed the difference between measured and calculated Na^+ uptake to the fact that flux and current measurements were obtained from different batches of oocytes.

For the forward-running pump in the *Xenopus* oocytes, stimulation of pump activity by membrane depolarization had been demonstrated [6]. It has been suggested that a voltage-dependent step involved in Na^+ translocation, though not rate-limiting, determines the probability of an enzyme intermediate that enters the rate-limiting step [1,15]. Consequently, the current generated by a backward-running pump could be inhibited by depolarization, which indeed is observed. Inward-directed pump current and its inhibition by depolarization has also been detected in whole-cell patch-clamp experiments on cardiac myocytes [1] and in internally dialysed squid giant axons [2] and has been taken as evidence for a backward-running pump.

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