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Amiloride-sensitive Na^+ conductance in native *Xenopus* oocytes

Wolf-Michael Weber *, Katja M. Liebold, Wolfgang Clauss

Institute for Animal Physiology, Justus-Liebig-Universität, Wartweg 95, D-35392 Giessen, Germany

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Abstract

Endogenous Na^+ conductances in the plasma membrane of oocytes of the South African clawed toad *Xenopus laevis* were investigated by microelectrode techniques and influx measurements. Removal of Na^+ from the bath solution under voltage clamp conditions led to a decrease in the clamp current indicating the existence of native Na^+ conductances. The observed current was voltage dependent but showed no marked rectification. Amiloride (10 μM) blocked this Na^+ current reversibly. However, amiloride analogues such as benzamil and phenamil had no effect on this Na^+ conductance. The Na^+/H^+-exchanger blocker EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride), another amiloride analogue, also had no effect thereby excluding a possible involvement of the Na^+/H^+ exchanger. The Na^+ mediated current had a reversal potential of about 50 mV suggesting high selectivity of these Na^+ conductances for Na^+ over other monovalent cations. When Na^+ was replaced by K^+ in the bath solution, amiloride had no effect on the clamp current over the whole potential range demonstrating that only Na^+ but not K^+ can enter the cell via the investigated conductances. In radio tracer experiments $^{22}Na^+$ influx into oocytes was nearly halved in presence of amiloride (10 μM), whereas benzamil and phenamil again failed to influence $^{22}Na^+$ influx. These results suggest that the endogenous amiloride-sensitive Na^+ conductance belongs to a new class of channels which is quite different from amiloride-sensitive epithelial Na^+ channels.

Keywords: Sodium ion conductance; Amiloride; Benzamil; Phenamil; EIPA; (*Xenopus* oocyte)

1. Introduction

The oocytes of the South-African clawed toad *Xenopus laevis* have proven to be an excellent model system for exploring many physiological processes at the single cell level. Furthermore, since several years the oocyte has been widely used for the expression and characterization of foreign transport systems including neurotransmitter receptors, ion channels and transport systems for various substrates (for review see: [1]). Their size and their capability to translate injected mRNA into proteins and to incorporate them in a functional form into its plasma membrane has made the oocytes to the favoured expression system. Because these cells were believed not to have endogenous Na^+ conductances worth to mention, they seemed especially suitable for the expression of the epithelial Na^+ channel. Amiloride, which inhibits epithelial Na^+ channels, was used as a probe to identify the channels in the oocyte membrane after functional expression following microinjection of mRNA derived from heterologous tissues. Some of the recently developed amiloride analogues, like benzamil or phenamil, show even higher affinity for the epithelial Na^+ channel than amiloride itself [2].

In the past years there have been several reports about the successful expression of epithelial Na^+ channels from different tissues in *Xenopus* oocytes (for review see: [3]). All these expressed sodium channels were inhibitable by amiloride and its analogues in micromolar doses.

Here we report, that oocytes themselves contain a native amiloride-sensitive Na^+ conductance. However, in contrast to the majority of expressed epithelial Na^+ channels reported previously, the currents produced by this endogeneous system in general are small and should not call in question the advantages of *Xenopus* oocytes as an excellent expression system for epithelial Na^+ channels. The insensitivity for benzamil and phenamil of the endogenous system clearly distinguishes this Na^+ conductance of the *Xenopus* oocyte from all known epithelial Na^+ channels.

* Corresponding author. E-mail: michael.weber@physzool.bio.uni-giessen.de. Fax: +49 641 7025878.

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2. Materials and methods

2.1. Oocytes

The following methods to obtain defolliculated oocytes were identical to those described in more detail previously [4]. Females of the clawed toad *Xenopus laevis* (purchased from Xenopus Limited, South Africa) were hypothermally anaesthetized, small pieces of ovary were removed and subsequently incubated in oocyte Ringer solution (ORi, see below) containing collagenase (1.5 U/ml, Serva, Germany), penicillin (80 μM) and streptomycin (30 μM) while shaking gently. After 7 h oocytes were washed 10 min in Ca^{2+} free ORi to remove the surrounding follicular cell layer. Only healthy looking full grown oocytes (Type V or VI) were used for further experiments which were performed at room temperature (22–24°C).

2.2. Electrophysiological measurements

For the electrophysiological measurements single oocytes were placed in a small plexiglas chamber (0.5 ml volume) and were superfused constantly. Voltage-clamp was performed by conventional two-microelectrode techniques [5] perfusing the oocytes constantly with ORi (see below) which contained 20 mM tetraethylammonium (TEA) as well as 5 mM $BaCl_2$, and 20 μM ouabain to block leak K^+ currents and the Na^+/K^+-ATPase, respectively. For determination of current–voltage relationships (*I–V* curves) steady state current was measured during the last 100 ms of 500 ms rectangular voltage pulses to different potentials. These pulses were applied from the holding potential (−60 mV) at a frequency of 0.25 Hz using a voltage-clamp amplifier (OC 725, Warner Instruments, Dixwell, USA) controlled by a personal computer connected via a CED 1401 interface (CED, Cambridge, UK). The software used for the performance of current–voltage curves and data acquisition was kindly given to us by Dr. W. Schwarz (Max-Planck-Institute for Biophysics, Frankfurt, Germany).

2.3. Influx measurements

Oocytes were placed in Eppendorf vials and washed two times with ORi containing ouabain (20 μM). The presence of this blocker was expected to inhibit the Na^+/K^+-ATPase and to allow maximal accumulation of $^{22}Na^+$ in the oocytes. At the beginning of the experiment each vial received $^{22}NaCl$ (10 μCi, Amersham, Braunschweig, Germany). After three hours at room temperature the oocytes were removed and washed in isotope-free ORi. Each oocyte was immediately placed individually in a scintillation vial, dissolved with 1% (w/v) SDS (sodium dodecyl sulfate) and radioactivity was counted in a liquid scintillation counter after addition of 2 ml scintillation fluid. Results are given as counts per min (cpm) per oocyte per 1 h.

2.4. Solutions

The composition of the oocyte Ringer solution (ORi) was (in mM): 90 NaCl, 3 KCl, 2 $CaCl_2$ and 5 *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Hepes, adjusted to pH 7.4). If lower Na^+ concentrations were used, Na^+ was replaced by *N*-methyl-D-glucamine (NMDG). Amiloride, benzamil, phenamil and EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride) were obtained from RBI (Köln, Germany); all other reagents were purchased from Sigma (Deisenhofen, Germany).

2.5. Statistics

Results, when not stated otherwise, are expressed as means ± standard error of the mean (S.E.), *n* is the number of oocytes and *N* the number of female donors.

3. Results

Defolliculated oocytes were clamped to −60 mV and the resulting holding current was recorded. Although

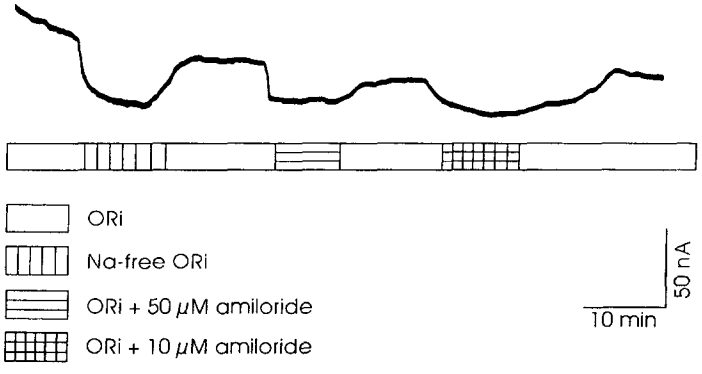

Fig. 1. Voltage clamp currents as responses to different concentrations of Na^+ and amiloride. The oocyte membrane was clamped to −60 mV. The measurement was performed in ORi with additional 20 μM ouabain, 5 mM Ba^{2+} and 20 mM TEA. Downward deflections represent decreases in inward currents. An individual current trace of a single oocyte representative of five typical experiments is shown ($n = 5$, $N = 5$).

oocytes were believed not to contain detectable Na^+ conductances, addition of the diuretic amiloride (50 μM) to the bath solution caused a significant decrease of the holding current. Even smaller concentrations of amiloride (10 μM) could block these conductances completely in a reversible way (Fig. 1). The same decrease in holding current could be observed when Na^+ was removed from the bath solution. Subsequent readdition of Na^+ led to the former current value again. The Na^+ mediated, amiloride-blockable current amounted up to 29.5 ± 1.6% or 42.8 ± 4.1 nA ($n = 5$, $N = 5$) of the total holding current at a clamp potential of −60 mV.

Difference curves obtained from current–voltage relationships (I–V curves) in presence and absence of amiloride (50 μM) yielded the current mediated by the amiloride-sensitive Na^+ conductances (Fig. 2A). This current was voltage-dependent and showed a reversal potential of about 50 mV as expected for Na^+ selective conductances and also predicted by the constant field equation [6]. Difference curves acquired in presence and absence of Na^+ yielded the same results (not shown).

Some analogues of amiloride, such as benzamil and phenamil, are reported to inhibit Na^+ conductances with even higher affinity [7]. I–V curves obtained in absence and presence of the amiloride analogue benzamil (10 μM) showed no difference indicating that benzamil has no inhibitory potency on the amiloride-sensitive current over the whole voltage range (Fig. 2B). The same results were obtained with another amiloride analogue, phenamil (10 μM, Fig. 2C). Again, I–V curves recorded in presence and in absence of this blocker were identical demonstrating that this blocker too, has no effect on endogenous Na^+ conductances in the plasma membrane of oocytes.

The ion selectivity of the amiloride-sensitive conductance was further elucidated by replacing sodium by potassium in the bath solution and testing the ability of amiloride to inhibit currents under both experimental conditions. When Na^+ was replaced by K^+, the amiloride induced change on the clamp current disappeared over the whole voltage range. Current–voltage relationships with high K^+ concentrations in presence and absence of amiloride (50 μM) showed no difference, indicating high selectivity of the amiloride-sensitive conductance for Na^+ over K^+ (Fig. 3) Thus, sodium, but not potassium, can enter the oocyte through the amiloride-sensitive pathway.

Xenopus oocytes in situ are surrounded by a tight layer of follicular cells. In presence of these follicle cells the

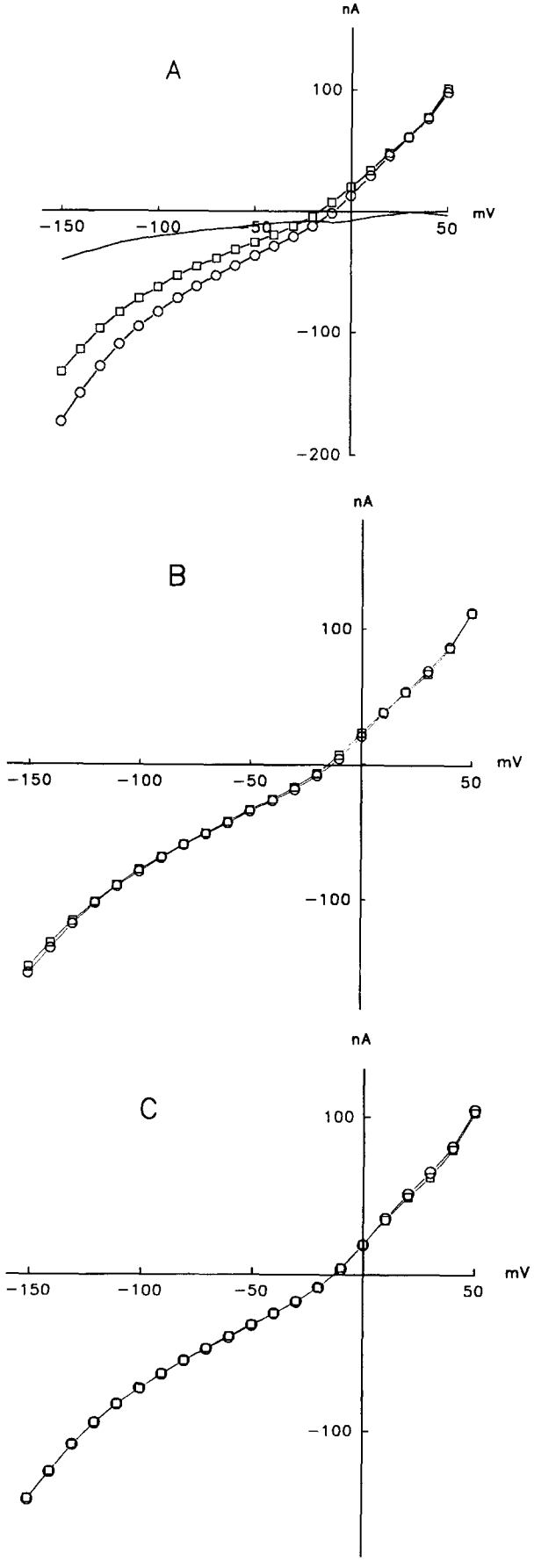

Fig. 2. Current–voltage relationships of different oocytes. (A) I–V curves in absence (circles) and presence (squares) of amiloride (50 μM). The solid line is the difference between the curves and represents the amiloride-sensitive portion of the Na^+ mediated current. Shown is a typical result out of a pool of three experiments ($n = 3$, $N = 3$). (B) I–V curves in absence (circles) and presence (squares) of benzamil (10 μM, $n = 3$, $N = 3$). (C) I–V curves in absence (circles) and presence (squares) of phenamil (10 μM, $n = 3$, $N = 3$).

oocytes possess an endogenous Na^+/H^+ exchanger, which is also blocked by amiloride [8]. Because this exchanger requires amiloride doses of about 1 mM to be completely blocked, it is improbable that the effects of low doses of amiloride were due to the sodium-proton exchanger. Nevertheless, we performed some experiments with EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride) to exclude this possibility. This amiloride analogue is a poor sodium channel blocker but is known to be the most effective blocker of the sodium proton exchanger [9,10]. Addition of EIPA in quite high doses (100 μM) to the bath solution showed no inhibitory effect on the clamp-current indicating that the Na^+/H^+ exchanger is not present in defolliculated oocytes which we used for all our experiments. However, in some oocytes the clamp-current was even slightly increased and returned to its former value when EIPA was removed from the bath solution.

In uptake experiments the accumulation of $^{22}Na^+$ in oocytes and the inhibition of the isotope influx by different blockers were determined. Extrusion of $^{22}Na^+$ by endogenous Na^+/K^+-ATPases was prevented by addition of ouabain (20 μM) to the uptake solution. After 3 h of incubation time the oocytes showed considerable accumulation of $^{22}Na^+$ when no additional amiloride was in the uptake solution (Fig. 4). In the presence of amiloride (10 μM) $^{22}Na^+$ uptake was nearly halved, another strong evidence for the presence of an amiloride-sensitive Na^+ conductance. Addition of benzamil (10 μM) or phenamil (10 μM) to the bath solution showed no inhibition of $^{22}Na^+$ influx and yielded nearly the same results as in the absence of these amiloride analogues indicating the existence of an Na^+ conductance which is sensitive to amiloride, but insensitive for the amiloride analogues benzamil and phenamil. These data obtained by influx measurements are in perfect accordance with the above mentioned electrophysiological results. The inability of benzamil or phenamil to block Na^+ influx into oocytes and the marked inhibition of Na^+ influx by amiloride gives clear evidence for the fact that oocytes possess a Na^+ conductance which is different from the well known epithelial Na^+ channel.

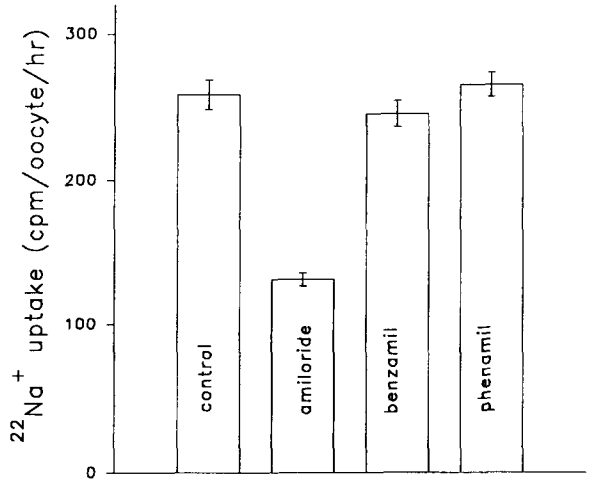

Fig. 4. Radio-isotope influx measurements with $^{22}Na^+$. Oocytes were incubated for 3 h in $^{22}Na^+$ containing ORi. $^{22}Na^+$ uptake was measured in absence (control) and in presence of amiloride (10 μM), benzamil (10 μM), and phenamil (10 μM), respectively. All solutions contained additionally 20 μM Ouabain. In each case, mean ± S.E. of $^{22}Na^+$ uptake into 30 oocytes from three different females is depicted.

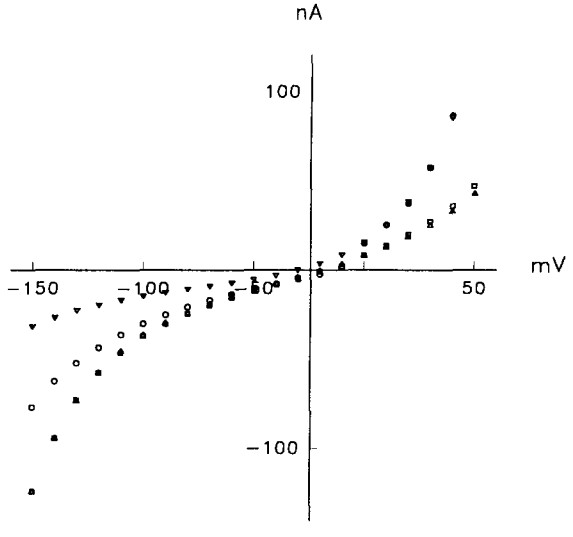

Fig. 3. Selectivity of the amiloride-sensitive Na^+ conductance. *I–V* curves in presence (triangles) and absence (circles, squares) of amiloride (10 μM). In presence of Na^+ (circles) amiloride inhibited a great part of the Na^+ conductance (downward triangles). When Na^+ was replaced by K^+ (squares), application of amiloride had no influence on the *I–V* curve (upward triangle). Shown is a typical result ($n = 4$, $N = 4$).

4. Discussion

The aim of this study was to characterize an endogenous Na^+ conductance that we found in control experiments during studies dedicated to investigate the expression of epithelial Na^+ channels in *Xenopus* oocytes. This Na^+ conductance in native oocytes was mentioned shortly in a previous paper [5], where we showed that removal of Na^+ from the bath solution led to a decrease in the clamp current. After Na^+ readdition the clamp current reached the former value. This current decrease was independent of the presence of Ni^{2+} (5 mM) or quinine (500 μM). Both blockers had no effect on the clamp current indicating that the endogenous Na^+/Ca^{2+}-exchanger could not be responsible for the observed effect. Because of the presence of ouabain (20 μM) in all solutions that we used, it can be excluded, that the Na^+/K^+-ATPase is involved in the observed effects.

Oocytes, voltage-clamped to negative holding potentials near the resting potential (−60 mV), respond to removal of external sodium with decrease of the clamp current. This Na^+ mediated current is sensitive to the potassium sparing diureticum amiloride. 10 μM amiloride was suffi-

cient to block entirely the endogenous Na^+ conductance in *Xenopus* oocytes. The low amiloride concentrations that we used argue against a participation of the Na^+/H^+ exchanger or the stretch-activated cation channel (SAC) inhabiting the oocyte membrane. Both of these transport systems need about 0.5 mM amiloride or more to be affected [11,12]. In order to further exclude a role of Na^+/H^+-exchangers, we used the amiloride analogue EIPA in high concentrations (100 μM). This pyrazinecarboxamide has no detectable effect on epithelial Na^+ channels but is the most effective inhibitor of Na^+/H^+ exchangers [7,13]. Because EIPA had no effect on the electrophysiological properties of the oocyte membrane, it is quite reasonable that oocytes don't have Na^+/H^+ exchangers in a detectable amount. This finding is in good accordance with the results of Towle et al. [8], who reported that defolliculated oocytes show no Na^+/H^+ exchange activity. Only non collagenase treated oocytes surrounded by their follicular cells, so-called follicles, exhibited detectable Na^+/H^+ exchange activities. The data of Towle et al. [8] and our own findings give strong evidence that Na^+/H^+ exchangers could not be responsible for the amiloride-induced changes in the clamp current.

The amiloride-blockable Na^+ conductances are characterized by a high selectivity for Na^+. The Na^+ mediated currents which are sensitive to amiloride exhibit a reversal potential of about 50 mV. This value correspond to the reversal potential predicted by the constant field equation for a conductance selectively permeable to sodium ions [6]. Moreover, this amiloride-sensitive Na^+ conductance shows a high selectivity for Na^+ over K^+. Potassium ions cannot enter the cell through the endogenous amiloride-blockable conductance as demonstrated when Na^+ is replaced by K^+. When K^+ is the main ion in oocyte bathing solution amiloride fails to induce any detectable effect on the holding current. Difference curves in the presence of Na^+ and when Na^+ is substituted by K^+ are close to zero indicating high selectivity for Na^+ over K^+.

Amiloride-sensitivity is one of the characteristics of Na^+ channels found in various tight epithelia in vertebrates [3,14] and invertebrates [15,16]. These channels are inhibited by submicromolar doses of amiloride. Some amiloride analogues, such as benzamil and phenamil, block the epithelial Na^+ channel with essentially higher affinities. Surprisingly, the endogenous amiloride-blockable Na^+ conductance in the oocytes shows absolutely no sensitivity for benzamil and phenamil. These findings are very striking, because up to now, all described amiloride-sensitive Na^+ channels were also blockable by benzamil and phenamil. The Na^+ conductance present in oocytes therefore seems to be quite different from the classical amiloride-sensitive Na^+ channel known from the most epithelia.

In conclusion, *Xenopus* oocytes do have an endogenous Na^+ conductance which is amiloride-sensitive, but different from the well known epithelial Na^+ channel recently cloned [17–19]. Its insensitivity for benzamil and phenamil clearly distinguishes this conductance from the epithelial Na^+ channel. The latter shows even higher affinity for those blockers than for amiloride [20]. From our present experimental data we cannot distinguish whether the endogenous Na^+ conductance is a different protein or is regulated in another way than the epithelial Na^+ channel.

Although the currents produced by this conductance are small, one should be aware of this transport system when considering to express foreign Na^+ channels in *Xenopus* oocytes. If amiloride is used as probe for the level of expression of these Na^+ channels in oocytes a low response to the blocker not necessarily argues for an expression at all. The answer to amiloride also could be produced by the endogenous Na^+ conductance. Therefore, to distinguish between endogenous Na^+ conductances and expressed Na^+ channels, we suggest to use benzamil as a probe especially when the expression results only in low signals.

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