

Expression of Epithelial Na Channels in *Xenopus* Oocytes

LAWRENCE G. PALMER, IRENE CORTHESEY-THEULAZ,
HANS-PETER GAEGGELER, JEAN-PIERRE KRAEHENBUHL,
and BERNARD ROSSIER

From the Institute of Pharmacology, University of Lausanne, CH1005 Lausanne, Switzerland; and the Institute of Biochemistry, University of Lausanne, CH1066 Epalinges, Switzerland

ABSTRACT Epithelial Na channel activity was expressed in oocytes from *Xenopus laevis* after injection of mRNA from A6 cells, derived from *Xenopus* kidney. Poly A(+) RNA was extracted from confluent cell monolayers grown on either plastic or permeable supports. 1–50 ng RNA was injected into stage 5–6 oocytes. Na channel activity was assayed as amiloride-sensitive current (I_{Na}) under voltage-clamp conditions 1–3 d after injection. I_{Na} was not detectable in noninjected or water-injected oocytes. This amiloride-sensitive pathway induced by the mRNA had a number of characteristics in common with that in epithelial cells, including (a) high selectivity for Na over K, (b) high sensitivity to amiloride with an apparent K_i of ~100 nM, (c) saturation with respect to external Na with an apparent K_m of ~10 mM, and (d) a time-dependent activation of current with hyperpolarization of the oocyte membrane. Expression of channel activity was temperature dependent, being slow at 19°C but much more rapid at 25°C. Fractionation of mRNA on a sucrose density gradient revealed that the species of RNA inducing channel activity had a sedimentation coefficient of ~17 S. Treatment of filter-grown cells with 300 nM aldosterone for 24 h increased Na transport in the A6 cells by up to fivefold but did not increase the ability of mRNA isolated from those cells to induce channel activity in oocytes. The apparent abundance of mRNA coding for channel activity was 10-fold less in cells grown on plastic than in those grown on filters, but was increased two- to threefold by aldosterone.

INTRODUCTION

Renal and other epithelial cells express Na-selective, amiloride-sensitive channels in their apical membranes. The activity of these channels is under the control of the mineralocorticoid aldosterone (Garty, 1986; Eaton and Hamilton, 1988; Garty and Benos, 1988; Palmer, 1988). Since aldosterone exerts its major physiological effects by the induction of specific messenger RNA and protein species (Edelman, 1978; Garty 1986) the simplest mechanism through which the hormone could increase channel activity would be to induce the synthesis of mRNA coding for the channel.

Address reprint requests to Dr. Lawrence G. Palmer, Department of Physiology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

There is, however, indirect evidence suggesting that aldosterone might act by activating preexisting channels rather than by synthesizing new ones (see Garty, 1986). Furthermore, Asher and Garty (1988) have shown that the hormone increases apical Na permeability in the toad urinary bladder by at least two different mechanisms. The short-term (<3 h) effect of the steroid was not retained in vesicles subsequently isolated from the epithelial cells. Long-term stimulation, on the other hand, was retained in the vesicles, suggesting the possibility that this effect might involve *de novo* synthesis of the channels.

The *Xenopus* oocyte has been extensively used as a system for translating exogenous mRNA. In particular, these cells can synthesize transport proteins and insert them into their plasma membrane in functional form. They therefore provide a convenient and sensitive system for the assay of mRNA coding for ion channels and other membrane transporters. Recently, George et al. (1989) showed that epithelial Na channel function could be expressed in oocytes after injection of mRNA from A6 cells, a cell line from amphibian kidney that forms high-resistance, Na-reabsorbing epithelia in culture and which expresses amiloride-sensitive Na channels in its apical membranes. These authors used amiloride-sensitive uptake of radiolabeled Na as an assay for channel activity. Hinton and Eaton (1989) used electrophysiological methods to demonstrate the expression of amiloride-sensitive conductance from A6 cell mRNA.

Our objectives in this study were (a) to further characterize the channels induced in the oocyte with mRNA from A6 cells, using electrophysiological techniques, (b) to optimize conditions for the expression of channels, and (c) to use the oocyte as a quantitative assay system for the mRNA that produces channel activity, to examine whether aldosterone affects Na channels at the mRNA level.

METHODS

Cells

A6 cells from a subclone A62F3 were grown on either plastic culture dishes or on collagen-coated millipore filters as previously described (Paccolat et al., 1987). RNA was extracted from plastic-grown cells after they had reached confluence and begun to form domes, and from filter-grown cells after a high transepithelial resistance (>5 k Ω cm²) had been achieved.

RNA

RNA was extracted from A6 cells with a modification of the method described by Geering et al. (1985). Cells were rinsed free of culture medium and then scraped from their supports in the presence of 5% citric acid at 4°C. The cells were broken with a teflon-glass homogenizer using 20 up-and-down strokes with the pestle driven at 2,000 rpm. The homogenate was centrifuged for 5 min at 2,000 rpm in an HB-4 rotor. The pellet was rehomogenized with 5–10 strokes and recentrifuged. The two supernatants were then centrifuged for 30 min at 15,000 rpm in an SS-34 rotor. The supernatants from the high-speed spin were discarded and the pellets were resuspended in buffer containing 200 mM Tris HCl (pH 7.5), 5 mM EDTA, 2% sodium dodecyl sulfate, and 200 mg/ml heparin. This suspension was extracted with chloroform-phenol at room temperature. The aqueous phase was reextracted until no more material appeared at the interface between the two phases. The final aqueous phase was

precipitated with 0.1 vol 3 M Na acetate and 2.5 vol ethanol and stored at -20°C . Typically, ~ 100 mg RNA was recovered from one 60-mm petri dish, as judged from the optical density at 260 nm.

Poly A(+) RNA was obtained by passing the total pool of RNA through an oligo-dT sepharose affinity column in the presence of 300 mM NaCl (Geering et al., 1985). The column was eluted with NaCl-free buffer. 2–5% of the total RNA was recovered in the eluate as poly A(+) RNA.

Some preparations of poly A(+) RNA were fractionated on a sucrose density gradient as described previously (Geering et al., 1985). 100–150 mg RNA was layered on a continuous 5–20% sucrose gradient of volume 5 ml. The gradients were centrifuged at 16°C for 4 h at 45,000 rpm in an ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) with an SW 50.1 rotor. 14–16 fractions of equal volume were collected, mixed with 0.1 vol 3 M NaCl and precipitated with 2.5 vol ethanol. Fractions were resuspended in water and reprecipitated in ethanol as described above before use.

Oocyte Injections

Oocytes (Dumont stages V–VI) were obtained by partial ovariectomy of female *Xenopus laevis*. The oocytes were incubated in a modified Barth's solution (MBS) containing (in millimolar): 85 NaCl, 2.4 NaHCO_3 , 1 KCl, 0.8 MgSO_4 , 0.3 $\text{Ca}(\text{NO}_3)_2$, 0.4 CaCl_2 , and 12 HEPES, buffered to pH 7.2 with NaOH, plus penicillin (10 mg/ml) and streptomycin (5 mg/ml). After removal the oocytes were treated with 0.1% collagenase (type Ia, Sigma Chemical Co., St. Louis, MO) in Ca-free MBS for 1.5–3 h, until they could be separated with gentle mechanical agitation. They were incubated overnight in MBS to eliminate damaged oocytes. Surviving oocytes were injected with 50 nl H_2O containing up to 1 ng/nl mRNA using an automatic-pressure injection system (Inject+matic, J. Gabay, Geneva). The injected oocytes were incubated at either 19 or 25°C .

Electrophysiology

For measurement of Na channel activity, the oocytes were placed in a small lucite chamber and superfused continuously with Ringer's solution containing (in millimolar): 110 NaCl, 2 CaCl_2 , 5 HEPES, and 2.5 KOH, pH 7.5. Changes in the bathing solution were as noted in the Results.

The oocytes were impaled with two microelectrodes filled with 3 M KCl having resistances of 3–6 $\text{M}\Omega$. Control oocytes had resting potentials of -20 to -40 mV and total membrane resistances of 0.5–2 $\text{M}\Omega$. Oocytes injected with mRNA had similar resting potentials but often had much lower resistances. The membrane potentials were clamped to desired values, usually -60 to -100 mV, using a two-electrode voltage-clamp apparatus (Dagan Corp., Minneapolis, MN). Currents were monitored using a strip-chart recorder.

In most experiments the oocyte membrane was clamped either at -100 mV continuously or at -100 and -60 mV alternately with a frequency of 0.5 Hz. For measurement of current-voltage relationships two protocols were followed. In the first, a voltage ramp was applied to the command port of the clamp. In the other, the clamping voltage was changed in steps from a holding potential of -60 mV and held at the test voltages for 1–2 s.

Chemicals

Amiloride was a gift of Merck, Sharp and Dohme. Trypsin (Type I) and collagenase (Type Ia) were obtained from Sigma Chemical Co.

RESULTS

Induction of Channel Activity

Voltage-clamp records from three oocytes are shown in Fig. 1. To assess Na channel activity, 5 μM amiloride was added to the superfusate. In noninjected oocytes, or in oocytes injected with 50 nl H_2O but no RNA, there was no measurable change in the

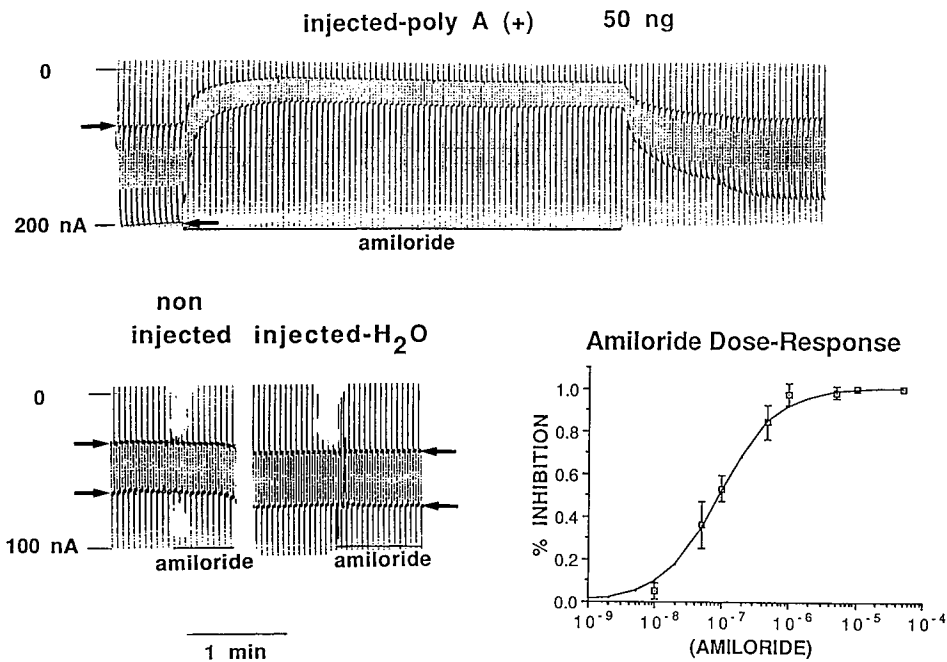


FIGURE 1. Voltage-clamp recordings from control and mRNA-injected oocytes. The upper panel shows currents from an oocyte injected with 50 ng poly A(+) RNA from A6 cells grown on plastic. The oocyte was clamped alternately at -100 and -60 mV. The steady-state inward currents at -100 and -60 mV are indicated by arrows. After application of amiloride ($5 \mu\text{M}$) the current at -100 mV fell from 200 to below 50 nA. The current at -60 mV fell from 70 to 10 nA. The effects of amiloride were reversible when the drug was removed from the superfusate. The panels on the lower left show similar recordings from a noninjected oocyte and from an oocyte injected with 50 nl H_2O . In neither case was an effect of amiloride observed. The lower right panel shows a dose-response curve for amiloride. The response is plotted as fractional inhibition of amiloride-sensitive current, assuming full inhibition at a concentration of $50 \mu\text{M}$. Data are given as means \pm SD for five to six determinations. The solid line is drawn according to Eq. 1 with $K_i = 88$ nM.

electrical properties of the oocyte membrane upon the addition of amiloride. The sensitivity of the method would have permitted us to detect an amiloride-sensitive current of 1–2 nA. The upper trace of Fig. 1 shows the response to amiloride of an oocyte injected with 50 ng mRNA. The initial currents were larger than those of the controls. In addition, the changes in current in response to changes in voltage followed a more complex time course (see Fig. 5 A below); hyperpolarization

induced a slow increase and depolarization a slow decrease in inward current. Steady-state values of current are indicated by arrows. Addition of 5 μM amiloride reduced currents at both -100 and -60 mV as well as the slow transients. Membrane conductance, measured as the difference in current between -100 and -60 mV, was greater in the mRNA-injected oocytes, and was reduced by amiloride.

The dose-response curve to amiloride is also shown in Fig. 1. When the currents were measured at -100 mV, 5 μM amiloride produced a nearly maximal response, whereas 0.1 μM produced a half-maximal response. The dose-response relationship could be fairly well described by the equation:

$$I_{\text{Na}}(A) = I_{\text{Na}}(0)/(1 + A/K_I) \quad (1)$$

where I_{Na} is the amiloride-sensitive current, A the concentration of amiloride, and K_I the apparent inhibition constant for the drug. The value of K_I that best fit these data was 82 nM.

The sensitivity of the mRNA-induced currents to amiloride depended on the transmembrane voltage as well as the extracellular Na concentration. Table I shows

TABLE I

[Na]	V_m	K_I
mM	mV	nM
110	-100	110 ± 40
110	-60	210 ± 90
110	-100	125 ± 30
11	-100	49 ± 8

Effect of extracellular Na concentration ([Na]) and membrane voltage (V_m) on the apparent K_I for amiloride. Data are given as means \pm SD for 11 oocytes (upper two lines) and 7 oocytes (lower two lines).

the values of K_I obtained by transforming Eq. 1 to:

$$[I_{\text{Na}}(0)/I_{\text{Na}}(A)] - 1 = A/K_I \quad (2)$$

The left side of Eq. 2 was plotted as a function of A , and the slope of the relationship was estimated using linear regression. K_I was measured in the same oocyte either at two different voltages (-100 and -60 mV) and 110 mM extracellular Na or at two different extracellular Na concentrations (110 and 11 mM) and -100 mV holding potential. Depolarization by 40 mV increased K_I from 110 to 210 nM. Reducing extracellular Na decreased K_I from 125 to 49 nM (Table I).

Selectivity

The ion selectivity of the amiloride-sensitive pathway was assessed in two ways. First, the ability of Na and K to carry inward, amiloride-sensitive currents was compared. Second, the reversal potential for amiloride-sensitive currents was investigated.

Fig. 2 shows the result of replacing all extracellular Na with K. In the presence of Na, 5 μM amiloride reduced currents and conductance reversibly, as in Fig. 1. In the presence of high extracellular K, membrane currents and conductance decreased and there was no response to amiloride. In the presence of amiloride, the membrane

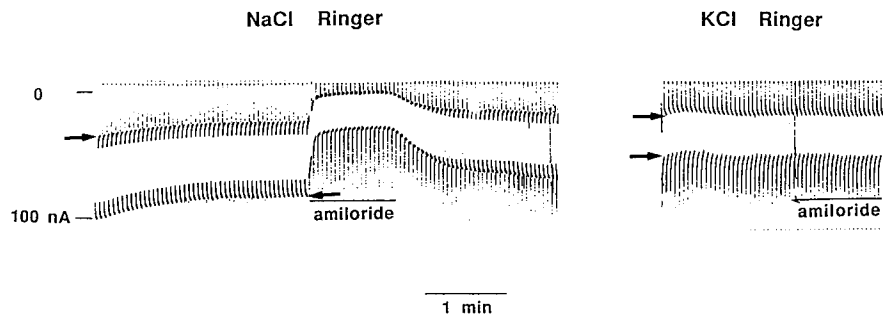


FIGURE 2. Selectivity of amiloride-sensitive current. The figure shows a voltage-clamp recording from a single oocyte injected with 50 ng poly A(+) RNA from cells grown on plastic. The oocyte membrane was clamped alternately to -100 and -60 mV. Steady-state currents are indicated by arrows. In the presence of standard NaCl Ringer's solution there was a large, reversible response to amiloride. When the Na in the solution was replaced by K, amiloride had no effect on the electrical properties of the membrane.

conductance was higher in KCl than in NaCl. This was also true of control oocytes, and presumably reflects the higher conductance of the native oocyte membrane to K. After returning to the normal high Na solution, the response to amiloride was again observed (not shown). Similar results were obtained with seven different oocytes, none of which had any detectable amiloride-sensitive current in high K, Na-free solution. Thus Na, but not K, can enter the cells through the amiloride-sensitive pathway.

Fig. 3 shows the current-voltage relationship of an RNA-injected oocyte obtained by applying voltage ramps to the membrane. The ramps were applied continuously while $5 \mu\text{M}$ amiloride was added to the superfusate. Two ramps were chosen, immediately before application of amiloride and immediately after a new steady state

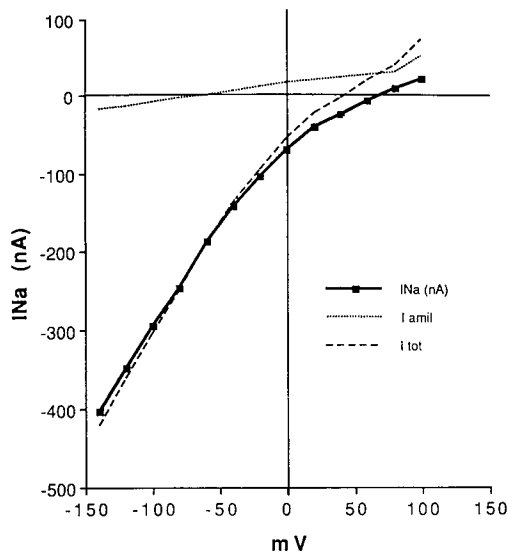


FIGURE 3. Current-voltage relationship of amiloride-sensitive current. Data are from a single oocyte injected with 50 ng poly A(+) RNA from cells grown on plastic. Ramps were applied to the voltage clamp to change the voltage between -140 to $+100$ mV over a period of 1 s. Currents from both ascending and descending ramps from three successive applications of amiloride were averaged. Currents measured in the absence (dashed line) and presence (dotted line) of amiloride are plotted as a function of voltage. The solid line through the square symbols represents the difference or amiloride-sensitive current. The line has no theoretical significance.

had been reached. The two ramps were obtained ~5 s apart. This method was chosen to minimize time-dependent changes in conductances of the oocyte membrane, and to optimize the measurement of the reversal potential for the amiloride-sensitive pathway. Currents from the second ramp were subtracted from those of the first at the same voltage, and the difference current was plotted as a function of voltage. Net inward current was negative.

Currents through the amiloride-sensitive pathway (I_{Na}) were inward at all voltages more negative than +50 mV. At more positive voltages, currents were small and difficult to measure accurately, but appeared to reverse at ~+60 mV. Since these values are close to that expected for the Nernst potential for Na in amphibian oocytes (Palmer et al., 1978), the results also suggest a high selectivity of the amiloride-sensitive pathway for Na over K.

Data from this oocyte were particularly satisfactory because I_{Na} was large, leak currents were rather small even at positive voltages, outward amiloride-sensitive currents could be detected, and amiloride was applied three times with essentially similar results. In three other preparations in which data could be obtained at positive potentials, currents became zero between +40 and +90 mV. With most oocytes leak currents became much larger than amiloride-sensitive currents at positive membrane voltages, making the determination of reversal potentials difficult.

Na Dependence of I_{Na}

Oocytes were clamped at -100 mV while the extracellular Na concentration was reduced in stages by substitution of *N*-methyl-D-glucamine (NMDG) for Na. This procedure was then repeated in the presence of amiloride and I_{Na} was measured as the difference in current at each Na concentration. As can be seen in Fig. 4, I_{Na} was a saturable function of extracellular Na. Half-maximal values of I_{Na} were obtained at a Na concentration of ~10 mM. The amiloride-insensitive current also decreased (not shown) as Na was decreased, indicating the presence of a conductance to Na not blocked by the drug. This conductance was also observed in control oocytes.

Voltage Dependence of I_{Na}

The I_{Na} -*V* curve of Fig. 3 was obtained using rather slow, continuous changes in membrane voltage. Thus this method should yield information on the steady-state *I*-*V* relationship of the channels. By making step changes in the membrane voltage, the time dependence of the response to the channels to voltage could be studied. We found that this method was less reliable than that of the voltage ramp in the region of positive membrane potentials, where I_{Na} is very small. Therefore only data at negative potentials are reported.

Fig. 5 A illustrates the currents observed after a step in voltage from -60 to -120 mV. The current trace shows a capacitive transient which decays to a minimal current level within 100 ms. Then, over a period of 1-2 s, the current increases (becomes more negative) to a new steady-state level. This late increase in current is completely abolished by amiloride, demonstrating that it represents an increase in current flowing through Na channels.

These results were analyzed further by measuring I_{Na} at two times. First, the "instantaneous" current was obtained by linear extrapolation of the rising current

phase to the beginning of the voltage change. Second, steady-state current values were obtained at the end of the pulse. I_{Na} was calculated at both times from the change in I with amiloride.

In Fig. 5 *B*, values of instantaneous and steady-state currents are plotted against membrane voltage for a holding potential of -60 mV. At -60 mV the two currents are equal by definition. At more negative voltages the steady-state I_{Na} exceeds the instantaneous I_{Na} . At less negative values the steady-state I_{Na} is smaller.

I_{Na} depends on the number of Na channels in the membrane (N), the current through individual channels (i), and the probability of the channel being open (P_o):

$$I_{\text{Na}} = iNP_o \quad (3)$$

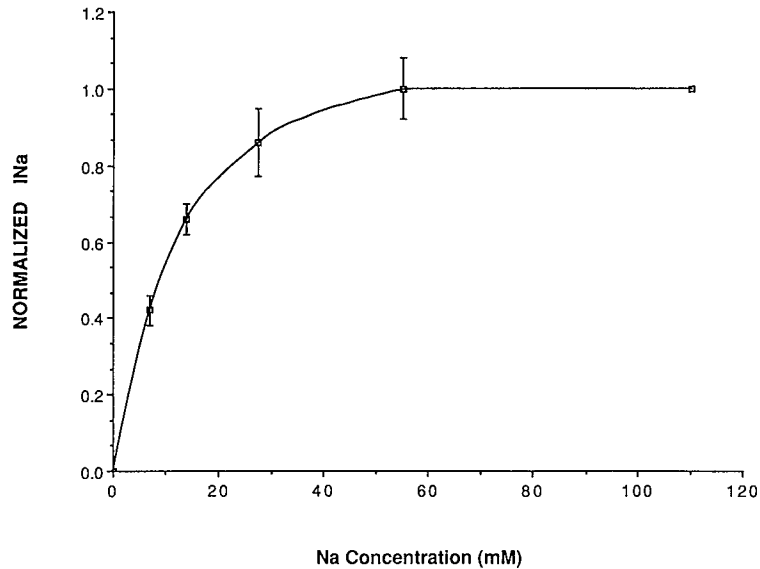


FIGURE 4. Dependence of I_{Na} on Na concentration. Currents were measured at a voltage of -100 mV in the presence and absence of amiloride at six different Na concentrations from 0 to 110 mM. Na was replaced with NMDG keeping Cl concentration, ionic strength, and osmolarity constant. The difference currents were normalized to the value obtained with 110 mM Na. Data are plotted as means \pm SD for six determinations. The solid line has no theoretical significance.

If i and N remain constant throughout the voltage pulse, the time-dependent change in I_{Na} will reflect changes in P_o . Assuming that the measurement of instantaneous I_{Na} is a good estimate of the current before P_o changes in response to the voltage change, then:

$$\text{“instantaneous” } I_{\text{Na}}(V) = i(V)NP_o(-60)$$

and

$$\text{“steady-state” } I_{\text{Na}}(V) = i(V)NP_o(V).$$

Thus the ratio of the currents gives an estimate of the relative probability of the channel being open:

$$\frac{\text{steady-state } I_{\text{Na}}(V)}{\text{instantaneous } I_{\text{Na}}(V)} = \frac{P_o(V)}{P_o(-60)}$$

This ratio is plotted as a function of V in Fig. 5 *D*. P_o increases with hyperpolarization, roughly doubling between -60 and -120 mV. P_o decreased with depolarization. Because the absolute values of I_{Na} became much smaller as the voltage approached zero, these data are less reliable.

Finally, as shown in Fig. 5 *C* and *D*, the voltage dependence of P_o was considerably reduced when the extracellular Na concentration was 11 mM instead of the normal 110 mM. In four oocytes for which similar data were obtained, P_o increased between -60 and -140 mV by 2.06 ± 0.05 -fold with 110 mM Na and by 1.27 ± 0.06 -fold with 11 mM Na.

Expression of Channels

Temperature. The rate of appearance of Na channel activity was temperature dependent (Fig. 6). In this experiment, oocytes were injected with 50 ng mRNA and incubated at either 19 or 25°C. All measurements of I_{Na} were made at room temperature (24–26°C) with 110 mM extracellular Na and a membrane voltage of -100 mV.

When the oocytes were kept at 19°C up until the electrical measurements were made, I_{Na} was measurable after 48 h but values were <5 nA. In contrast, oocytes kept at 25°C had much larger values of I_{Na} that reached a maximum of over 40 nA 24 h after injection. Also shown in the figure are data from oocytes incubated for 6 h at 25°C after preincubation for 0, 48, or 72 h at 19°C. Preincubation substantially increased the currents that were measured after 6 h at the elevated temperature. In a second experiment no I_{Na} could be measured after 6 h at 25°C immediately after injection, whereas the same incubation 72 h after injection increased I_{Na} from 27 to 85 nA.

The rate of expression of channels was estimated at the two temperatures in five experiments using three batches of oocytes (Table II). The rate at 19°C was calculated as the absolute value of I_{Na} divided by the time of incubation (24–72 h). The rate at 25°C was determined as the change in I_{Na} divided by the time of incubation (~ 6 h). While there were large variations in the rates of expression at both temperatures, in each case there was a large increase in the rate, averaging ~ 60 -fold, after warming to 25°C.

This temperature-dependent activation I_{Na} could reflect either activation of channels that had been previously inserted into the membrane in inactive form, or a temperature-sensitive step in the synthesis, processing, or translocation of channels to the plasma membrane. To distinguish these two possibilities, we used an approach devised by Garty and Edelman (1983). This protocol makes use of the ability of trypsin to inactivate apical Na channels in the intact toad urinary bladder. When added to the medium bathing the intact cells, the enzyme will attack channels already present in the membrane. An effect of preexposure to trypsin on the subsequent activation of channels is considered as evidence that inactive channels in the plasma membrane are involved in the response.

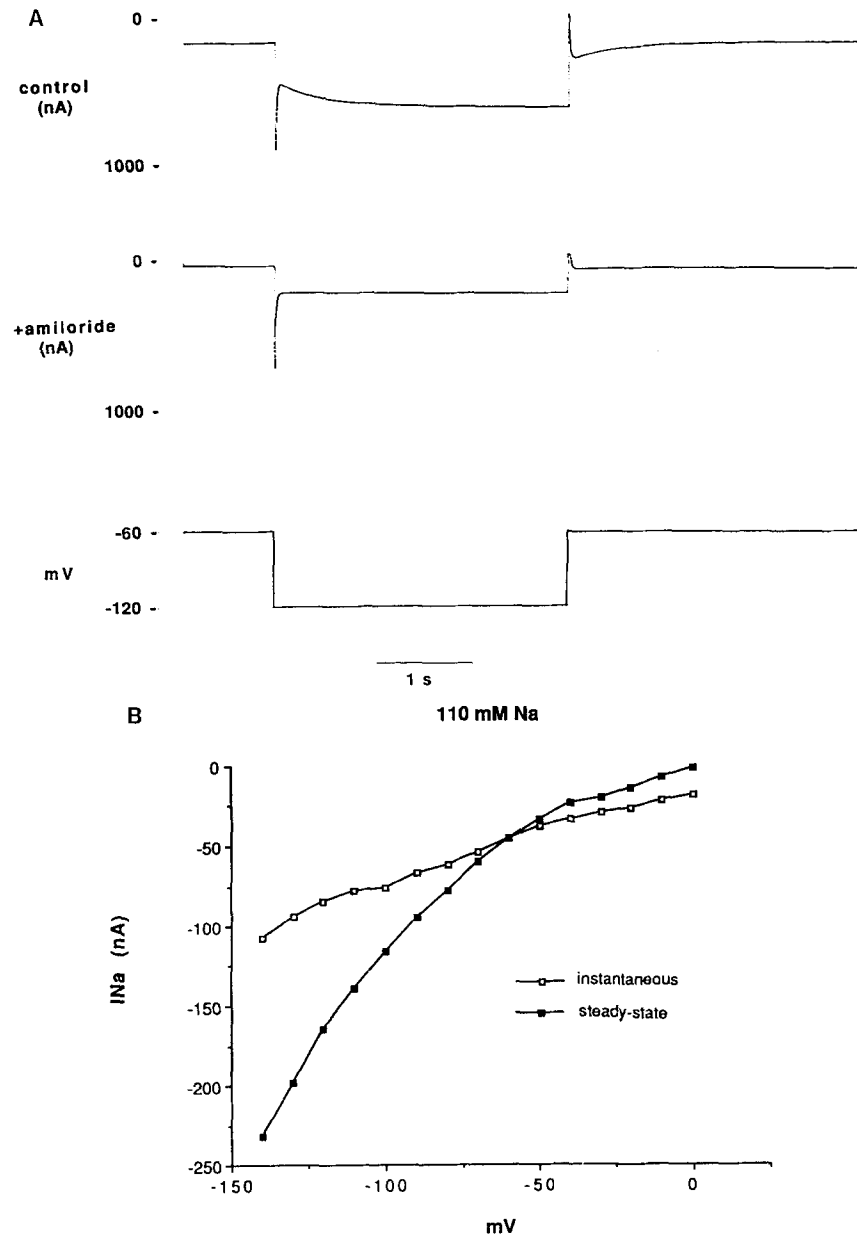
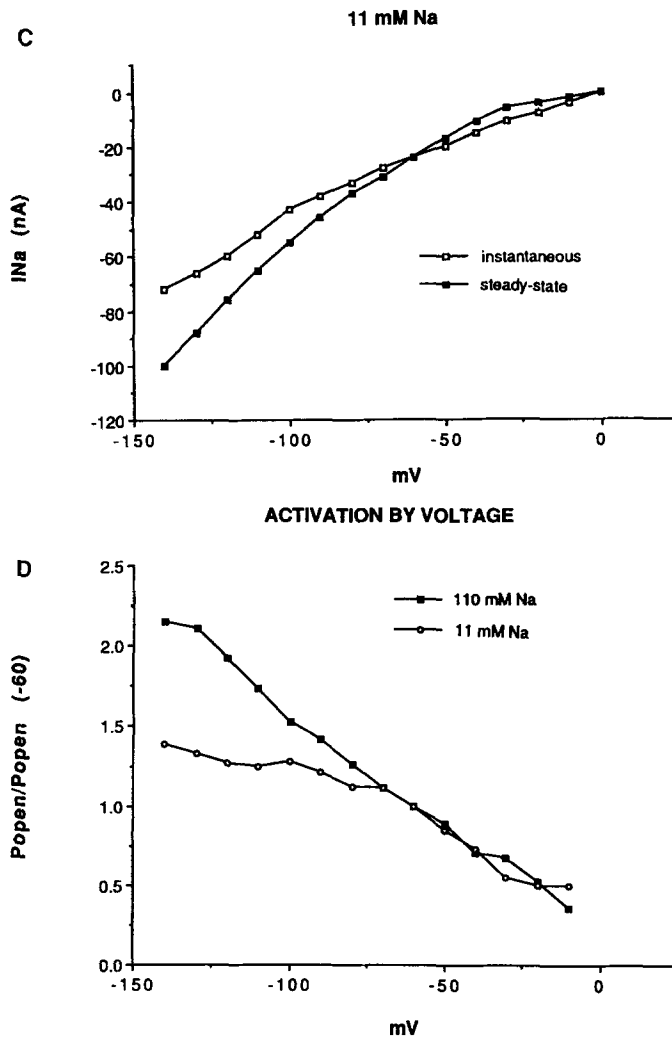


FIGURE 5. Voltage dependence of amiloride-sensitive currents. (A) A single oocyte injected with 50 ng poly A(+) RNA from cells grown on plastic was clamped at -60 mV in the presence of 110 mM external Na. The voltage was changed to -120 mV as indicated on the bottom trace. The top and middle traces show the current responses in the absence and presence of 5 μ M amiloride, respectively. (B) Instantaneous (*open symbols*) and steady-state (*filled symbols*) amiloride-sensitive currents were measured as a function of voltage as described in the text. The holding potential was -60 mV.



(C) Currents were determined as in *B* but in the presence of 11 mM Na + 99 mM NMDG. (D) The ratio of the steady-state to the instantaneous current is plotted as a function of voltage using the data in parts *B* and *C*. This ratio is assumed to be equal to that of the open probabilities at the holding potential (-60 mV) and the test potentials as explained in the text.

We first determined if Na channels in the oocyte membrane were susceptible to trypsinization. A 15-min exposure of the oocytes to 1 mg/ml trypsin at 25°C reduced I_{Na} by 80% (Table III). Oocytes incubated under the same conditions but in the absence of trypsin served as time controls and showed no decrease in I_{Na} . Oocytes that were exposed to trypsin in the presence of 5 μM amiloride retained 76% of their original I_{Na} measured after removal of both the trypsin and the amiloride. Thus amiloride was able to partially protect the channels against trypsin, as shown previously for the toad bladder channels (Garty and Edelman, 1983).

We then trypsinized oocytes that had been injected with mRNA and incubated at

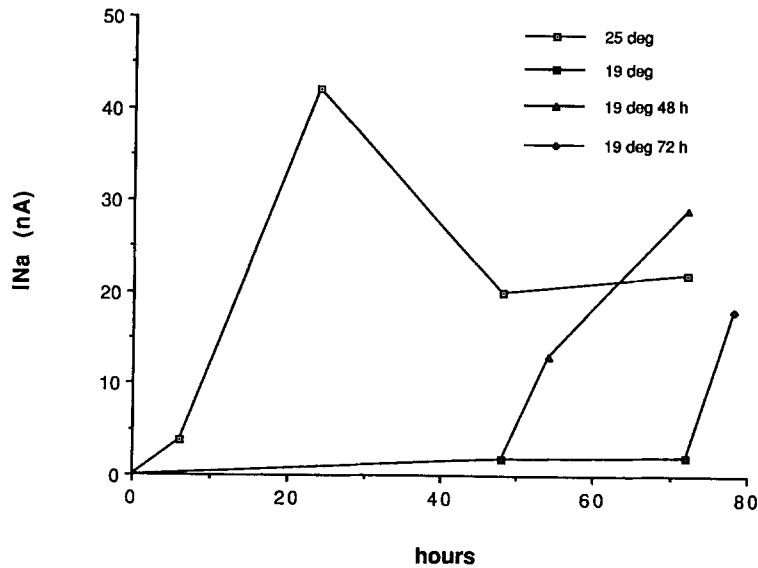


FIGURE 6. Effect of temperature on the induction of amiloride-sensitive current in oocytes. Oocytes were injected with 50 ng poly A(+) RNA and incubated at either 19 or 25°C as shown. In all cases I_{Na} was measured at 25°C. Data are given as means for five to eight oocytes at each point.

19°C for 48 h (Fig. 7). These oocytes were exposed to the enzyme at 1 mg/ml for 15 min at 25°C, in the presence or absence of amiloride. They were then incubated for 6 h at 25°C after which I_{Na} was measured. In this experiment the oocytes developed more channel activity at 19°C than did those in the experiment of Fig. 6. Nevertheless there was a large activation of I_{Na} during the 6-h warming period. The main point of Fig. 7 is that oocytes pretreated with trypsin developed I_{Na} levels that were as high as those not treated with the enzyme. Thus the channels activated by raising the temperature were resistant to trypsin before activation. This is consistent with the hypothesis that these channels were sequestered in an intracellular compartment at 19°C.

TABLE II

Experiment	Time 19°	Rate 19°	Rate 25°	Ratio
20	48	0.04	1.8	4.5
20	72	0.03	2.7	9.0
21	48	0.33	1.7	5.2
22	24	0.03	2.3	7.7
22	72	0.38	9.7	2.5
Mean ± SEM		0.16 ± 0.08	6.7 ± 3.0	58 ± 11

Temperature dependence of rate of appearance of I_{Na} . Oocytes were injected with 50 ng mRNA and incubated at 19°C for 24–72 h, as indicated. The rate of appearance of I_{Na} , defined as the total I_{Na} divided by the time of incubation, is given in units of nA/h. The oocytes were then warmed to 25°C for 6 h. The rate of appearance at this temperature is defined as the increase in I_{Na} divided by 6.

TABLE III

Treatment	Fraction of initial I_{Na}
None	1.06 ± 0.09
Trypsin	0.18 ± 0.05
Trypsin + amiloride	0.76 ± 0.08

Effect of trypsin on I_{Na} . I_{Na} was measured before and 15 min after incubation with no added enzyme, 1 mg/ml trypsin, and 1 mg/ml trypsin + 5 μ M amiloride. The ratios of the currents at the two times are indicated as means \pm SEM for 7 to 10 oocytes.

Size of mRNA. To enrich the mRNA preparation with respect to channel-inducing activity we separated mRNA on a continuous sucrose density gradient. The distribution of total mRNA along the gradient, measured as optical density at 260 nm, is plotted in Fig. 8 (*dotted line*). There is a peak of RNA at fraction 10, corresponding to 16 Svedberg units (S).

Equal amounts of RNA from each fraction were injected into oocytes to test for induction of Na channel activity. There was a distinct peak of activity about three-fold over unfractionated mRNA produced by injection of fraction 9 (17 S). Fraction 10 (16 S) also had activity that was significantly enriched compared with unfractionated mRNA.

Growth substrate. We compared the ability of mRNA from cells grown on plastic and permeable supports to induce Na channel activity in oocytes. The results of three different experiments with slightly different protocols are summarized in Table IV. In the first experiment, filters were seeded with cells at twice the density of that used to seed plastic petri dishes (1.5×10^6 cells/cm²), the cells were then grown to confluence, and preincubated with serum- and steroid-free medium for 24 h before extraction of RNA. The purpose of this protocol was to study the effect of aldosterone on these cells (see below). They were compared with cells from a previous passage grown to confluence on plastic petri dishes in the continuous presence of serum. In the second experiment, cells from the same batch were seeded onto plastic or filters but at different densities (5×10^4 cells/cm² for plastic, 1.5×10^6 cells/cm²

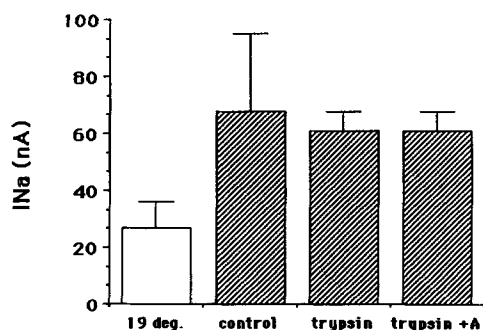


FIGURE 7. Effect of trypsin on activation of I_{Na} . Oocytes were injected with 50 ng poly A(+) RNA and incubated for 48 h at 19°C. In one set of oocytes I_{Na} was measured just after warming the cells to 25°C (*open bar*). Three other sets of oocytes were incubated for 15 min in the presence of no enzyme (*control*), 1 mg/ml trypsin (*trypsin*) and 1 mg/ml trypsin + 5 μ M amiloride (*trypsin + A*). These oocytes were then rinsed and incubated for an additional 4–6 h at 25°C before determination of I_{Na} . Data represent means \pm SEM for 8 to 11 oocytes.

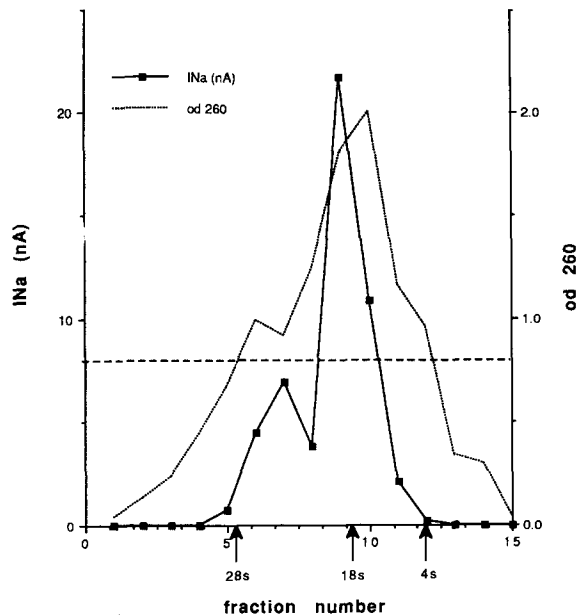


FIGURE 8. Separation of poly A(+) RNA on a sucrose density gradient. 170 μg RNA was layered onto a 5–20% continuous sucrose gradient. After centrifugation, the gradient was collected in 16 fractions. Fraction 1 is from the bottom (heavy end) of the gradient. The dotted line indicates the amount of RNA recovered in each fraction, measured as the optical density at 260 nm. Oocytes were injected with 50 ng of RNA from the various fractions, and I_{Na} was determined at -100 mV. Data points indicate mean values of I_{Na} for 8 to 12 oocytes. The mean value of I_{Na} for unfractionated poly A(+) RNA was 8 nA. For calibration, an identical gradient was loaded with 45 μg poly A(–) RNA. Three peaks of optical density were

observed and assumed to correspond to 4 S, 18 S, and 28 S. The positions of the peaks are indicated by arrows.

for filters). Serum was removed from both sets of cells 24 h before harvesting. In the third experiment, cells were seeded onto filters and plastic at the same density (5×10^4 cells/cm²) and treated as discussed in the “size of mRNA” section.

The results of all three experiments were similar. Recovery of total RNA and poly A(+) RNA was similar for plastic- and filter-grown cells. However, when equal amounts of mRNA from the two preparations were injected into oocytes, that from the filter-grown cells induced about 10 times as much channel activity measured as I_{Na} at -100 mV.

Aldosterone. To assess the possibility that aldosterone induces mRNA coding for the Na channel in A6 cells, we compared the abilities of mRNA from control and aldosterone-stimulated cells to elicit I_{Na} in oocytes. Cells were grown to confluence

TABLE IV

Experiment	RNA	I_{Na}	
		Plastic	Filter
	ng	nA	nA
1	25	11 \pm 3	147 \pm 45
2	10	9 \pm 3	106 \pm 24
3	2.5	1.4 \pm 0.5	19 \pm 4

Induction of I_{Na} in oocytes by mRNA from cells grown on plastic and filters. Equal amounts of RNA extracted from either plastic-grown or filter-grown cells were injected into oocytes, and I_{Na} was measured at -100 mV. The three experiments represent three different extractions of RNA, as explained in the text. Data represent means \pm SEM for 8 to 12 oocytes.

on filters or on plastic. The medium was replaced with one that was free of serum and contained either no added steroids (controls) or 300 nM aldosterone. After 24 h the cells were harvested and mRNA was extracted. The recoveries of total RNA and poly A(+) RNA from control and aldosterone-treated cells were the same. Equal amounts of mRNA were injected into oocytes and I_{Na} was assessed.

In filter-grown cells, equal amounts of mRNA from control and aldosterone-stimulated cells induced the same level of I_{Na} in oocytes. I_{Na} is plotted as a function of the amount of RNA injected in Fig. 9. The dose-response relationships were essentially identical for the two conditions.

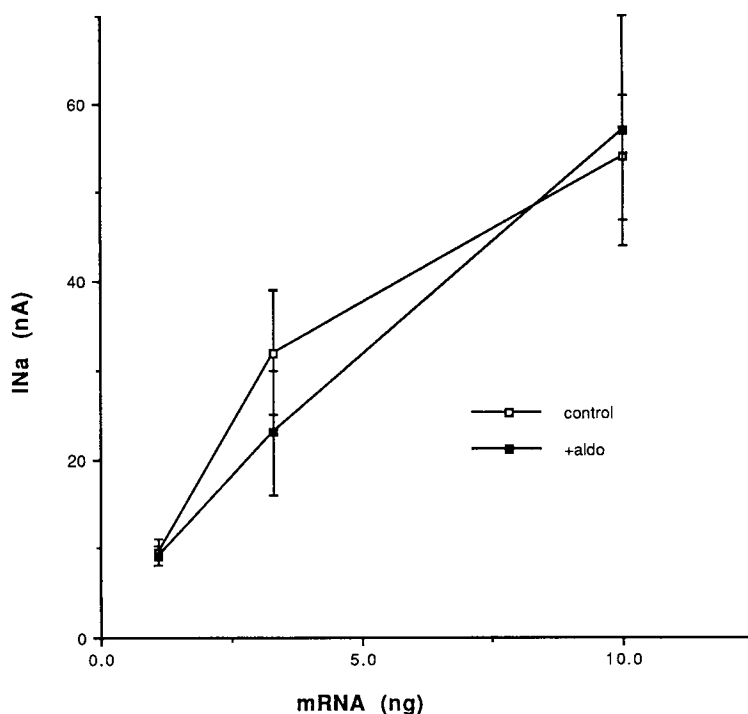


FIGURE 9. Effect of aldosterone. Cells were grown to confluence on filters. Transepithelial electrical parameters were measured, and the medium bathing the cells was replaced with one containing no serum and either no steroids or 300 nM aldosterone. After 24 h electrical parameters were remeasured and RNA was extracted. Oocytes were injected with various quantities of poly A(+) RNA from control and aldosterone-treated cells. I_{Na} was measured at -100 mV. Data show means \pm SEM for eight oocytes.

The results of three such experiments are summarized in Table V. In all three experiments the potency of the mRNA needed to induce I_{Na} in oocytes was unaffected by aldosterone. In two out of the three preparations, we also measured short-circuit current (I_{sc}) across the epithelia both before and after aldosterone stimulation. This parameter is a measure of net transepithelial Na transport in A6 cells (Perkins and Handler, 1981) and therefore is a good indicator of the physiolog-

ical response of the cells to the steroid. The stimulation of I_{sc} by aldosterone was assessed as:

$$[I_{sc}(\text{aldo}, 24)/I_{sc}(\text{control}, 24)]/[I_{sc}(\text{aldo}, 0)/I_{sc}(\text{control}, 0)]$$

where 0 and 24 refer to the times at which I_{sc} was measured, before and 24 h after the addition of aldosterone. The hormone-treated cells had short-circuit currents that were 4.9 and 2.6 times those of controls in the two experiments.

In contrast to these results with filter-grown cells, we found that aldosterone increased the ability of mRNA from plastic-grown cells to induce I_{Na} in oocytes. In

TABLE V

Experiment	RNA	I_{Na}		I_{sc}	
		Control	+ Aldo	Control	+Aldo
		nA		$\mu A/cm^2$	
1	31	16 ± 3	19 ± 7		
	10	30 ± 11	23 ± 9		
	30	52 ± 9	54 ± 9		
2	3	37 ± 8	59 ± 14	8.1 ± 0.4	6.9 ± 0.5
				(t = 0)	
	10	107 ± 20	106 ± 20		
	30	73 ± 10	99 ± 14	4.8 ± 0.3	20.1 ± 2.8
				(t = 24 h)	
	1.1	9.5 ± 1.5	9.1 ± 1.1		
3	3.3	32 ± 7	23 ± 7		
	10	54 ± 7	57 ± 13		
	2.5	19 ± 4	22 ± 6	7.4 ± 0.1	7.5 ± 0.3
				(t = 0)	
			6.3 ± 0.3	16.6 ± 0.4	
			(t = 24 h)		

Induction of I_{Na} in oocytes by mRNA from control and aldosterone-treated cells. Cells were grown to confluence on filters. At $t = 0$ short-circuit current (I_{sc}) was measured across the epithelium (experiments 2 and 3 only), after which the growth medium was replaced with one that was serum free and contained no steroids (control) or 300 nM aldosterone. I_{sc} was remeasured after 24 h, after which RNA was extracted. I_{Na} was measured at -100 mV. Experiments 1–3 represent three different RNA extractions. Data are given as mean + SEM for 8 to 10 oocytes.

two experiments, the increase in I_{Na} was two- to threefold greater in hormone-treated cells compared with controls (Table VI).

DISCUSSION

Expression of Amiloride-sensitive Na Channels in Oocytes

In our most active oocyte preparations, we found mean levels of amiloride-sensitive current in mRNA-injected oocytes of ~ 100 nA at -100 mV. Since control oocytes had amiloride-sensitive currents of < 2 nA, this represents transport through channels whose activity was induced by the mRNA. Similar results have been obtained by

other investigators using mRNA from A6 cells (George et al., 1989; Hinton and Eaton, 1989) from respiratory epithelial cells (Kroll et al., 1989).

If we assume values for single-channel current (i) of ~ 0.5 pA and average open probability of ~ 0.5 as measured for Na channels in rat cortical collecting tubule under similar conditions (Palmer and Frindt, 1988), then the number of induced channels (N) can be calculated from Eq. 3 to be $\sim 4 \times 10^5$ /oocyte. Assuming that the oocytes are spheres with diameter 1.2 mm, I_{Na} can be expressed as a current density. Thus 100 nA/oocyte corresponds to $2 \mu\text{A}/\text{cm}^2$ of nominal oocyte surface area. This density is an order of magnitude lower than that measured in maximally stimulated A6 cells, which as shown in Table V can reach $20 \mu\text{A}/\text{cm}^2$.

Characteristics of Na Channels in Oocytes

We examined the properties of the amiloride-sensitive conductance induced in the oocytes that could be assessed using the two-electrode voltage-clamp system. In general, there was a good correlation between these properties and those observed

TABLE VI

Experiment	RNA	I_{Na} (nA)	
		Control	+Aldo
	ng	nA	nA
2	10	9 ± 3	31 ± 6
2*	10	18 ± 4	30 ± 6
3	2.5	1.4 ± 0.5	3.6 ± 0.8
3	10	4.0 ± 0.8	8.2 ± 1.4

Induction of I_{Na} in oocytes by mRNA from control and aldosterone-treated cells. Cells were grown to confluence on plastic petri dishes and treated as described in Table V. Poly A(+)RNA was extracted from the cells and either 2.5 or 10 ng was injected into the oocytes. I_{Na} was measured at -100 mV. Experiments 2 and 3 represent different extractions. Experiments 2 and 2* represent two different injections of the same mRNA extraction into different batches of oocytes. Data are given as means \pm SEM for 8 to 10 oocytes.

for the amiloride-blockable Na channels in the apical membranes of tight epithelia. Thus, as far as we can tell, the channels are faithfully expressed in the oocyte system. Some of these properties are discussed in more detail below.

Amiloride sensitivity. The apparent K_i for amiloride to block Na channels in the oocytes was $\sim 10^{-7}$ M (Table I). This value is similar to that found for Na channels in intact epithelia (Benos, 1982).

In many epithelia, the affinity of amiloride for the Na channel increases with decreasing Na concentration. In the toad bladder, the interaction between Na and amiloride appeared to be competitive, and the apparent K_i for Na inhibition of amiloride block was ~ 50 mM (Palmer, 1984). Assuming similar competitive interactions in the oocyte, an increase in the apparent K_i for amiloride from 49 nM at a Na concentration of 11 mM to 125 nM at 110 mM Na corresponds to an apparent K_i for Na of 58 mM, similar to that found in the toad bladder.

Hyperpolarization of the membrane voltage (V_m) also enhances the apparent amiloride affinity in the toad bladder (Palmer, 1984; Warncke and Lindemann,

1985). The relationship between apparent K_1 for amiloride and V_m could be described by the equation:

$$K_1(V) = K_1(0) \exp(\delta RTV_m/F)$$

Values of δ were found to be from 0.15 to 0.3 (Palmer, 1984; Warncke and Lindemann, 1985). Assuming that the same relationship holds in the oocyte, δ can be calculated from the decrease in apparent K_1 for a 40-mV hyperpolarization (Table I) to be ~ 0.41 . Thus the voltage dependence of amiloride block in the oocyte is in the same direction as, but somewhat steeper than, that in the toad bladder.

Saturation of I_{Na} . In A6 cells, as in a number of other epithelia, Na transport through apical Na channels is a saturable function of the Na concentration in the apical medium (Sariban-Sohraby et al., 1983). The apparent K_m value for amiloride-sensitive Na uptake into the cells was 18 mM. Similar apparent K_m values have been obtained in a variety of epithelia using electrical techniques (Garty and Benos, 1988; Palmer, 1988). In the oocytes, the apparent K_m value for I_{Na} at a membrane potential of -100 mV was ~ 10 mM (Fig. 4). This phenomenon could reflect either saturation of single-channel currents (Olans et al., 1984; Palmer and Frindt, 1988) or a down-regulation of the number of conducting channels (Van Dreissche and Lindemann, 1979). Our data in the oocytes do not permit distinction between these two possibilities. Whatever the mechanism of saturation, it appears to be an integral part of the channel since it is expressed along with the Na conductance itself in the oocytes.

Selectivity. In many tight epithelia the amiloride-sensitive Na channels are highly selective for Na over K (Palmer, 1978). In A6 cells the situation appears to be more complicated as Eaton and Hamilton (1988) have observed both poorly selective (3:1) and highly selective ($>20:1$) amiloride-sensitive channels using the patch-clamp technique. The lower selectivity channels were seen predominantly in cells grown on plastic. In oocytes, we found that amiloride-sensitive channels were highly selective for Na over K even when induced by mRNA from A6 cells grown on plastic (see Figs. 2 and 3).

One possible explanation for these findings is that the two channel types may represent different modifications of the same protein. Thus conditions in oocytes, and perhaps also in many native epithelia, may favor the highly selective form. Another possibility is that the two channels arise from two entirely different species of mRNA which are expressed at different rates in oocytes.

Voltage dependence of P_o . The Na channels in the oocytes were activated by hyperpolarization of the cell membrane. The simplest interpretation of this finding is that the inherent kinetics of the channel are voltage dependent, so that the probability of being open is increased. The finding of very slow intrinsic kinetics of Na channels in both rat cortical collecting tubule (Palmer and Frindt, 1988) and A6 cells (Eaton and Hamilton, 1988) is consistent with the observed time course of this effect, which occurred over a period of ~ 1 s. It is also conceivable that the distribution of ions across the cell membrane changes during this period. Redistribution of Na itself would be expected to produce the opposite result as increased Na influx during hyperpolarization would lead to depletion of the permeant ion from the extracellular space and accumulation in the cell, thus reducing the driving force

and the inward current. On the other hand, the distribution of regulatory ions could be affected by the voltage.

Quantitatively, 70 mV change in voltage was required to double the apparent P_o . A similar voltage dependence was observed at the single-channel level for Na channels in the apical membrane of the rat cortical collecting tubule (Palmer and Frindt, 1988). In that study, P_o increased by 2.3%/mV hyperpolarization between 0 and -100 mV membrane potential in cell-attached patches. A similar activation of highly selective Na channels has been observed in A6 cells (Marunaka, Y., and D. C. Eaton, personal communication). In contrast, the low-selectivity channels from A6 cells were activated by depolarization of the apical membrane (Hamilton and Eaton, 1985). Thus this property of the channels expressed in the oocytes also resembles that of the highly selective variety of Na channels in the original epithelium.

Factors Affecting Channel Expression

We have explored optimization of the expression of amiloride-sensitive conductance with respect to the temperature of incubation of the oocytes and the size of the injected mRNA.

Temperature. The strong dependence of the rate of expression of Na conductance on temperature was surprising. In early studies of the translation of exogenous mRNA by oocytes the cells were incubated at 19°C (Gurdon et al., 1971), presumably to extend the life of the oocyte in vitro. Subsequently, most studies using oocytes to express foreign proteins have similarly used incubation temperatures below 20°C. Indeed, we also found that oocytes tended to degenerate more rapidly at 25°C, but in most preparations this effect was more than offset by the increased rate of expression.

In principle the strongly temperature-dependent step could involve the synthesis of protein, the translocation of the protein to the plasma membrane, or the activation of channels that have already reached the membrane. The results of the trypsinization experiments argue against the latter possibility. The finding that pretreatment with trypsin does not prevent or blunt the subsequent activation by raising the temperature implies that at 19°C, before activation, trypsin does not have access to its cleavage site. The simplest interpretation is that the channels have not yet arrived at the plasma membrane under these conditions. However, an alternative interpretation, which we cannot rule out, is that the channels are present in the membrane in a conformation that is both nonconducting and trypsin resistant.

It is unlikely that protein synthesis *per se* could account for all of the temperature dependence. First of all, the rate of appearance of Na conductance at the plasma membrane at 25°C was more rapid after the oocytes had been preincubated at lower temperatures for 2–3 d (Fig. 6). This implies that at least one rate-limiting step, presumably including that of the synthesis of protein, can be accomplished at the lower temperature. Second, studies of the rate of synthesis of cytoplasmic proteins indicated an activation energy of 25–30 kcal/mol (Hunt et al., 1969). This would imply a two- to threefold increase in the rate for a 6°C increase in temperature. This will certainly contribute to the activation of the overall process, but is probably not the only factor involved since the overall rate increases by more than 50-fold (Table II).

The movement of membrane components between cytoplasmic and plasma membrane compartments can be highly temperature sensitive. The fusion of endosomes with lysosomes is arrested below 20°C (Dunn et al., 1980; Weigel and Oka, 1982). Perhaps more directly related to the present situation, Fambrough (1983) reported that the transport of newly synthesized acetylcholine receptors to the cell surface of cultured chick muscle did not occur below 25°. However, we do not know if membrane trafficking in poikilotherms has a similar temperature dependence.

Garty and Asher (1985) found that amiloride-sensitive Na fluxes into apical membrane vesicles from toad bladder cells depended strongly on the temperature at which the cells were incubated before homogenization. Warming the cells from 18 to 25°C greatly enhanced the fluxes subsequently measured in vesicles, provided that the Ca-ion activity in the medium was low. Furthermore, this temperature-dependent effect also appeared to involve the movement of channels between intracellular and plasma membrane compartments, since irreversible inhibition of membrane-associated channels with the arginyl-specific reagent phenylglyoxal did not affect the subsequent activation process (Garty et al., 1988). It is therefore conceivable that regulation of the movement of Na channels into and out of the plasma membrane is similar in toad bladder and in the oocyte.

Size of the mRNA. The results of the fractionation of mRNA on a sucrose density gradient indicate that the mRNA molecules responsible for eliciting channel activity are of moderate size. The most active fractions corresponded to a sedimentation coefficient of 16–17 S. These fractions were equal to or slightly lighter than those which contained the highest absolute amount of mRNA. Similar results have been obtained by George et al. (1989).

Although it is not possible to unambiguously determine the size of the message from its sedimentation coefficient alone, a relationship between *S* and molecular weight (*M*) valid for single-stranded polynucleotides is (Mahler and Cordes, 1966):

$$S = 0.01 M^{0.55}$$

Thus a sedimentation coefficient of 16–17 S would correspond to a molecular weight of 68–75 kD or a message length of 2.0–2.2 kb. Making the unlikely assumption that the message contains no noncoding regions, such a message would code for a protein with a molecular weight of at most 70–80 kD. This overestimate should be viewed with caution, since the gradients were run in nondenaturing conditions, in which the shape of the RNA molecule is not known, and were not designed to specifically address this question. In addition, the distribution of channel-inducing activity on the gradient was quite broad.

Benos et al. (1987) have purified Na channel proteins from A6 cells based on their ability to bind amiloride analogues. The protein consists of an oligomer of molecular weight 730,000 D which separates under reducing conditions into five or six components of 315, 150, 95, 71, 55, and 35 kD.

If the mRNA that induces channel activity in oocytes codes for one or more of these peptides, it is most likely to be those of low molecular weight. Thus it is possible that only one or more of the smaller components of the protein complex is required for the transport of Na. Another possibility is that higher molecular weight components of the channel are already present in the oocytes and only the smaller

components are necessary to make a functional channel. A third possibility is that the channels are present in their entirety in an inactive or sequestered form, and that the injected mRNA codes for a regulatory protein involved in the activation or translocation of the molecule.

Regulation of mRNA Levels in A6 Cells

Two conditions that are thought to regulate the activity of Na channels in the apical membrane of the A6 cell include the substrate on which the cells are grown (Sariban-Sohraby et al., 1983) and the mineralocorticoid hormone aldosterone (Perkins and Handler, 1981; Paccolat et al., 1987). We have used the oocyte system to address the question of whether the amount of mRNA coding for channel activity might play a role in regulating the channels under these conditions.

Growth substrate. Sariban-Sohraby et al. (1983) found that A6 cells grown on plastic express no measurable amiloride-sensitive Na uptake across their apical surfaces. Hamilton and Eaton (1985) observed amiloride-sensitive channels in cells grown on plastic using the patch-clamp technique, but these channels were rather rare and were poorly selective for Na over K. Thus to express normal levels of highly selective channels the cells must be grown on permeable supports. Our results suggest that this phenomenon could arise in large part from changes in the levels of mRNA coding for channel activity. mRNA from filter-grown cells induced up to 10 times higher levels of I_{Na} as that from plastic-grown cells (Table IV).

These results differ somewhat from those of George et al. (1989) who found no significant difference in the channel-inducing activity of mRNA from plastic-grown and filter-grown cells. The differences can be partially explained by their use of aldosterone to stimulate the cultures. The difference between filter-grown and plastic-grown mRNA was diminished by the hormone in our study since it appeared to induce channel mRNA in the latter but not in the former condition.

Aldosterone. Aldosterone is an adrenal steroid hormone which acts directly on tight epithelial cells to increase the rate of Na transport. It is widely accepted that one of the important sites of aldosterone's action is on the apical Na channels (Garty, 1986; Palmer, 1988). The hormone depends on RNA and protein synthesis to exert its effects, and a number of proteins that are "induced" by aldosterone have been identified (see Garty, 1986). Thus one possible mechanism by which aldosterone could increase Na transport would be to increase the rate of biosynthesis of the channels. Under these assumptions the amount of mRNA coding for the Na channel protein(s) should increase in proportion to the total amount of RNA in the cells. Although there is some indirect evidence that the major effect of aldosterone is to activate preexisting channels rather than to synthesize new ones (Garty, 1986), the possibility that mRNA coding for the Na channels is induced has not been tested directly.

The action of aldosterone has a complex time course which can be divided into at least two phases, based on experiments with toad bladder and A6 cells (Geering et al., 1985). The early phase lasts for about 3 h and is correlated with binding of the steroid to high affinity "type I" receptors. The later phase, defined between 3 and 24 h requires occupancy of both type I and lower affinity type II receptors. This phase

can be selectively blunted by Na butyrate or thyroid hormone (Truscello et al., 1983; Geering et al., 1984).

Although it was previously thought that stimulation of apical Na channels was accomplished mostly during the early phase (Spooner and Edelman, 1975), more recent data suggest that apical Na permeability continues to rise during the late response (Palmer and Speez, 1986). Furthermore, Asher and Garty (1988) showed that vesicles isolated from toad bladder retained the hormone-dependent increase in Na permeability only during the late phase. They argued that Na channels were affected by the hormone during both phases but by different mechanisms. The late response could involve either a permanent covalent modification of the channels or else *de novo* synthesis of the channel proteins.

We therefore focussed our studies of the effect of aldosterone on mRNA on conditions appropriate for the late phase, 24 h after challenge of the cells with a high concentration (300 nM) of the hormone. Our data indicate that mRNA coding for channel activity is not induced by aldosterone even during the late response. This finding is in agreement with a recent study by Kleymann et al. (1989) in which it was found that the number of binding sites in A6 cells for a covalent amiloride analogue was unchanged after stimulation with aldosterone for 24 h. These results are consistent with the idea that the major effect of the hormone is to synthesize a protein involved in the activation of preexisting channels. During the late response this could involve covalent modifications of the channel protein (Asher and Garty, 1988). One candidate for such a modification is methylation of the protein or associated lipids (Sariban-Sohrabay et al., 1984).

Two caveats to this conclusion should be mentioned. First, Hinton and Eaton (1989) found that long-term (72 h) withdrawal of steroids from the culture medium leads to a fall in mRNA coding for channel activity. Second, we found that aldosterone did increase the channel-inducing ability of mRNA from cells grown on plastic. We do not yet know the time course or concentration dependence of these effects. Thus it seems likely that steroid action on these cells could be even more complicated than that discussed above.

Despite these caveats, our major conclusion is that while using a protocol in which the induction of Na transport by the hormone can be clearly demonstrated, an increase in short-circuit current is observed without a concomitant increase in the abundance of mRNA coding for Na channel activity in oocytes. The simplest interpretation is that the channels themselves are not aldosterone-induced proteins.

This project was carried out while L. G. Palmer was on sabbatical leave from Cornell University Medical College. It was supported by grants from the Zyma Foundation, Nyon, Switzerland, by the Swiss National Science Foundation, and by the National Institutes of Health (DK-27847). The work was done during the tenure of an Established Investigatorship of the American Heart Association (L. G. Palmer).

Original version received 25 October 1989 and accepted version received 26 January 1990.

REFERENCES

- Asher, C., and H. Garty. 1988. Aldosterone increases the apical Na⁺ permeability of toad bladder by two different mechanisms. *Proceedings of the National Academy of Sciences*. 85:7413-7417.

- Benos, D. J. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. *American Journal of Physiology*. 242:C131–C321.
- Benos, D. J., G. Saccomani, and S. Sariban-Sohrab. 1987. The epithelial sodium channel: subunit number and location of the amiloride binding site. *Journal of Biological Chemistry*. 262:10613–10618.
- Dunn, W. A., A. L. Hubbard, and N. N. Aronson, Jr. 1980. Low temperature selectively inhibits fusion between pinocytotic vesicles and lysosomes during heterophagy of ¹²⁵I-asialofetuin by the perfused rat liver. *Journal of Biological Chemistry*. 255:5971–5978.
- Eaton, D. C., and K. L. Hamilton. 1988. The amiloride-blockade sodium channel of epithelial tissues. In *Ion Channels*. T. Narahashi, editor. Plenum Publishing Corp., New York, NY. 1:251–282.
- Edelman, I. S. 1978. Candidate mediators in the action of aldosterone on Na⁺ transport. In *Membrane Transport Processes*. Vol. 1. J. F. Hoffman, editor. Raven Press, New York, NY. 125–140.
- Fambrough, D. M. 1983. Biosynthesis and intracellular transport of acetylcholine receptors. *Methods in Enzymology*. 96:331–352.
- Garty, H. 1986. Mechanism of aldosterone action in tight epithelia. *Journal of Membrane Biology*. 90:193–205.
- Garty, H., and C. Asher. 1985. Ca²⁺-dependent, temperature-sensitive regulation of Na⁺ channels in tight epithelia. A study using membrane vesicles. *Journal of Biological Chemistry*. 260:8330–8335.
- Garty, H., and D. H. Benos. 1988. Characteristic and regulatory mechanism of the amiloride-blockade Na⁺ channel. *Physiological Reviews*. 68:309–373.
- Garty, H., and I. S. Edelman. 1983. Amiloride-sensitive trypsinization of apical sodium channels. Analysis of hormonal regulation of sodium transport in toad bladder. *Journal of General Physiology*. 81:785–803.
- Garty, H., O. Yeger, and C. Asher. 1988. Sodium-dependent inhibition of the epithelial sodium channel by an arginyl-specific reagent. *Journal of Biological Chemistry*. 263:5550–5554.
- Geering, K., M. Claire, H.-P. Gaeggeler, and B. C. Rossier. 1985. Receptor occupancy vs. induction of Na⁺-K⁺-ATPase and Na⁺ transport by aldosterone. *American Journal of Physiology*. 248:C102–C108.
- Geering, K., H.-P. Gaeggeler, and B. C. Rossier. 1984. Effects of thyrometric drugs on aldosterone-dependent sodium transport in the toad bladder. *Journal of Membrane Biology*. 77:15–23.
- Geering, K., D. I. Meyer, M.-P. Paccolat, J.-P. Kraehenbuhl, and B.C. Rossier. (1985). Membrane insertion of a and b-subunits of Na⁺, K⁺-ATPase. *Journal of Biological Chemistry*. 260:5154–5160.
- George, A. L., O Staub, K. Geering, B. C. Rossier, J.-P. Kraehenbuhl, and T. R. Kleyman. 1989. Functional expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes. *Proceedings of the National Academy of Sciences*. 86:7295–7298.
- Gurdon, J. B., C. D. Lane, H. R. Woodland, and G. Marbaix. 1971. Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature*. 233:177–182.
- Hamilton, K. L., and D. C. Eaton. 1985. Single channel recordings from the amiloride-sensitive Na⁺ channel. *American Journal of Physiology*. 249:C200–C207.
- Hinton, C. F., and D. C. Eaton. 1989. Expression of amiloride-blockade sodium channels in *Xenopus* oocytes. *American Journal of Physiology*. 257:C825–C829.
- Hunt, T., T. Hunter, and A. Munro. 1969. Control of haemoglobin synthesis: rate of translation of the message RNA for the a and b chains. *Journal of Molecular Biology*. 43:123–133.

- Kleyman, T. R., E. J. Cragoe, Jr., and J.-P. Kraehenbuhl. 1989. The cellular pool of Na⁺ channels in the amphibian cell line A6 is not altered by mineralocorticoids. *Journal of Biological Chemistry*. 264:11995–12000.
- Kroll, B., W. Bautsch, S. Bremer, M. Wilke, B. Tümmler, and E. Frömter, 1989. Selective expression of an amiloride-inhibitable Na⁺ conductance from mRNA of respiratory epithelium in *Xenopus laevis* oocytes. *American Journal of Physiology*. 257:L284-L288.
- Mahler, H. R., and E. H. Cordes. 1966. Biological Chemistry. Harper and Row Publishers, New York, NY. p. 152.
- Olans, L., S. Sariban-Sohraby, and D. J. Benos. 1984. Saturation behavior of single, amiloride-sensitive Na⁺ channels in planar lipid bilayers. *Biophysical Journal*. 46:831–835.
- Paccolat, M.-P., K. Geering, H.-P. Gaeggeler, and B.C. Rossier. 1987. Aldosterone regulation of Na⁺ transport and Na,K-ATPase in A6 cells: role of growth conditions. *American Journal of Physiology*. 252:C468–C476.
- Palmer, L. G. 1984. Voltage-dependent block by amiloride and other monovalent cations of apical Na channels in the toad urinary bladder. *Journal of Membrane Biology*. 80:153–165.
- Palmer, L. G. 1987. Ion selectivity of epithelial Na channels. *Journal of Membrane Biology*. 96:97–106.
- Palmer, L. G. 1988. Regulation of NaCl transport in tight epithelia. *Advances in Comparative and Environmental Physiology*. 1:292–309.
- Palmer, L. G., T. J. Century, and M. M. Civan. 1978. Activity coefficients of intracellular Na⁺ and K during development of frog oocytes. *Journal of Membrane Biology*. 40:25–38.
- Palmer, L. G., and G. Frindt. 1988. Conductance and gating of epithelial Na channels from rat cortical collecting tubule: effects of luminal Na and Li. *Journal of General Physiology*. 92:121–138.
- Palmer, L. G., and N. Speez. 1986. Stimulation of apical Na permeability and basolateral Na pump of toad urinary bladder by aldosterone. *American Journal of Physiology*. 250:F273–F281.
- Perkins, F. M., and J. S. Handler. 1981. Transport properties of toad kidney epithelia in culture. *American Journal of Physiology*. 241:C154–C159.
- Sariban-Sohraby, S., M. B. Burg, and R. J. Turner. 1983. Apical sodium uptake in toad kidney epithelial cell line A6. *American Journal of Physiology*. 245:C167–C171.
- Sariban-Sohraby, S., M. Burg, W. P. Wiesmann, P. K. Chiang, and J. P. Johnson. 1984. Methylation increases sodium transport into A6 apical membrane vesicles: possible mode of aldosterone action. *Science*. 225:745–746.
- Spooner, P. M., and I. S. Edelman. 1975. Further studies on the effect of aldosterone on electrical resistance of toad bladder. *Biochimica et Biophysica Acta*. 406:304–314.
- Truscello, A., K. Geering, H.-P. Gaeggeler, and B. C. Rossier. 1983. Effects of butyrate and histone deacylation on aldosterone-dependent Na⁺ transport in the toad bladder. *Journal of Biological Chemistry*. 258:3388–3395.
- Van Driessche, W., and B. Lindemann. 1979. Concentration dependence of currents through single sodium-selective pores in frog skin. *Nature*. 282:519–520.
- Warncke, J., and B. Lindemann. 1985. Voltage dependence of Na channel blockage by amiloride: relaxation effects in admittance spectra. *Journal of Membrane Biology*. 86:255–265.
- Weigel, P. H., and J. A. Oka. 1982. Endocytosis and degradation mediated by the asialoglycoprotein receptor in isolated rat hepatocytes. *Journal of Biological Chemistry*. 257:1201–1207.