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Functional expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes

(A6 cells/epithelia/RNA/aldosterone)

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ABSTRACT **Expression of the amiloride-sensitive sodium channel was examined in *Xenopus* oocytes that were microinjected with A6 cell mRNA. Amiloride-inhibitable ^{22}Na flux could be measured in intact oocytes 2–3 days after injection with 25 ng of poly(A)$^+$ RNA isolated from aldosterone-treated A6 cells. The rate of ^{22}Na uptake was ≈15-fold greater in oocytes microinjected with 25 ng of poly(A)$^+$ RNA than in water-injected control oocytes. An increase in ^{22}Na uptake by mRNA-injected oocytes occurred whether the mRNA was isolated from A6 cells grown on a porous or nonporous support. In the presence of 4 mM NaCl, amiloride caused dose-dependent inhibition of ^{22}Na uptake in mRNA-injected oocytes, which was half-maximal at 6×10^{-8} M. Both 1 μM amiloride and 0.1 μM benzamil inhibited ^{22}Na uptake in mRNA-injected oocytes by >95%, whereas <50% inhibition occurred with 1 μM 5-(*N*-ethyl-*N*-isopropyl)amiloride. When A6 cell mRNA was size fractionated by sucrose density-gradient centrifugation, amiloride-sensitive ^{22}Na uptake was expressed predominantly by oocytes injected with mRNA from two contiguous fractions.**

High-resistance, sodium-absorptive epithelia have specific cells that express a nonvoltage-gated, amiloride-sensitive sodium channel on their apical plasma membrane. According to the model proposed by Koefoed-Johnson and Ussing (1), this channel facilitates transepithelial sodium transport by allowing sodium ions to enter the cell from the solution bathing the apical surface down a favorable electrochemical gradient. In model high-resistance epithelia, including frog skin, toad urinary bladder, and established epithelial cell lines of urinary epithelia (A6, TBM), both the mineralocorticoid aldosterone and the neuropeptide vasopressin increase the sodium permeability of the apical membrane by affecting the amiloride-sensitive sodium channel (2–5). Biochemical evidence suggests the amiloride-sensitive sodium channel is an integral membrane protein—possibly composed of multiple polypeptide subunits (5–8). However, at present it is not clear which subunits are required for channel function.

Recently, a method for isolating complementary DNA by using the *Xenopus* oocyte expression system (9) has been applied to the cloning of the intestinal Na^+/glucose cotransporter (10) and other integral membrane proteins (11–13). The first step in this approach is to establish assay conditions for specific detection of the transport protein of interest and to demonstrate that mRNA from a tissue known to express this transport protein directs its functional expression when microinjected into *Xenopus* oocytes.

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We report here the functional expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes microinjected with mRNA prepared from aldosterone-treated A6 cells. We believe this method will be valuable in isolating cDNA encoding the amiloride-sensitive sodium channel by expression cloning or in verifying the functional attributes of cDNA clones isolated by conventional antibody-directed screening techniques.

MATERIALS AND METHODS

Cell Culture. A6 cells were purchased from the American Type Culture Collection and recloned by limiting dilution at passage 77. The cells were initially grown in 150-cm^2 polystyrene tissue culture flasks and at confluence were passed to collagen-coated Nuclepore polycarbonate membranes (seeding density: 10^6 cells/cm^2). Cells were fed thrice weekly with amphibian medium containing 5% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml), according to Perkins and Handler (14). Before use in RNA extractions, the cells were exposed for 16 hr to medium containing 10^{-7} M aldosterone. After aldosterone treatment short-circuit current increased 3- to 4-fold above baseline, and transepithelial resistance fell 20–30%.

RNA Preparation. Total cytosolic RNA was prepared according to the method described by Geering *et al.* (15). Briefly, A6 cells were first homogenized in 5% citric acid, and then a microsomal fraction (isolated by differential centrifugation) was extracted 3 or 4 times with chloroform/phenol. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (16), reprecipitated twice with ethanol, and then finally dissolved in sterile water at 0.5 μg/μl.

Size fractionation of poly(A)$^+$ RNA prepared from aldosterone-treated A6 cells (grown in polystyrene tissue culture flasks) by sucrose density-gradient centrifugation was performed under nondenaturing conditions as described (15). Individual fractions were reprecipitated twice with ethanol and dissolved in sterile water at a final concentration of 0.2 μg/μl (concentration estimated by A_{260} units).

Xenopus Oocyte Microinjection. Ovarian lobes were dissected from anesthetized female *Xenopus laevis*, and oocytes were defolliculated by 2- to 3-hr incubation with 0.25% collagenase (type IIa, Sigma) in calcium-free modified Barth's saline (MBS; ref. 9). After overnight incubation at 18°C in MBS containing penicillin (10 units/ml) and streptomycin (10 μg/ml), individual oocytes (Dumont stage V and VI; ref. 23) were pressure injected with either 50 nl of sterile water or poly(A)$^+$ RNA (0.2–0.5 μg/μl). Microinjected

Abbreviation: EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride.

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oocytes were maintained at 18°C in antibiotic-containing MBS for 2–3 days.

Measurement of ^{22}Na Uptake. Groups of four to eight oocytes were placed into wells on a 96-well microtiter plate (Dynatech) and resuspended in 0.1 ml of assay buffer containing 100 mM choline chloride, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 5 mM Hepes·NaOH, pH 7.4 (final concentration, 4 mM Na^+). After 30-min preincubation on ice with or without amiloride (or analog), the assay was initiated by adding 2 μCi (20 μCi/ml; 1 Ci = 37 GBq) carrier-free $^{22}NaCl$ (Amersham; specific activity, 1052 mCi/mg of Na). Unless otherwise stated, after 90 min the radioactive assay solution was aspirated, and the oocytes were washed 4 times in 2.5 ml of 100 mM NaCl/5 mM Hepes·NaOH, pH 7.4. After the wash, individual oocytes were placed into plastic vials for γ counting. Results are expressed as the mean value ± SEM.

Chemicals. Amiloride, benzamil, and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) were provided by Merck Sharp & Dohme.

RESULTS

Initial experiments were done to verify the absence of an intrinsic amiloride-sensitive sodium-transporting system in native *Xenopus* oocytes. In eight experiments Na^+ uptake exhibited by noninjected or water-injected oocytes was 21.7 ± 1.5 pmol per oocyte·hr^{-1} (absolute ^{22}Na uptake was 119 cpm for 60-min incubation; n = 51 oocytes), and there was no endogenous amiloride-sensitive (10 μM) uptake. In contrast, sodium uptake by oocytes microinjected with 25 ng of poly(A)$^+$ RNA prepared from aldosterone-treated filter-grown A6 cells was ≈15 times greater than controls. Three days after microinjection, oocytes injected with A6 cell poly(A)$^+$ RNA exhibited Na^+ uptake of 304.6 ± 14.1 pmol per oocyte·hr^{-1} (n = 32 oocytes, three experiments) as compared with 21.0 ± 1.2 pmol per oocyte·hr^{-1} (n = 26 oocytes) for water-injected controls in the same experiments. In approximately one of every six experiments, Na^+ uptake by mRNA-injected oocytes was >1000 pmol per oocyte·hr^{-1} (>30 times control; see Fig. 1) without explanation other than intrinsic differences between batches of oocytes. An increase in Na^+ uptake could also be detected 2 days after microinjection of poly(A)$^+$ RNA (204.2 ± 34.2 pmol per oocyte·hr^{-1} in one experiment, n = 12), although there was a trend for greater oocyte-to-oocyte variability in ^{22}Na uptake. Sodium uptake by microinjected oocytes could be increased by injecting more poly(A)$^+$ RNA in the range between 0.3–25 ng per oocyte (Fig. 1).

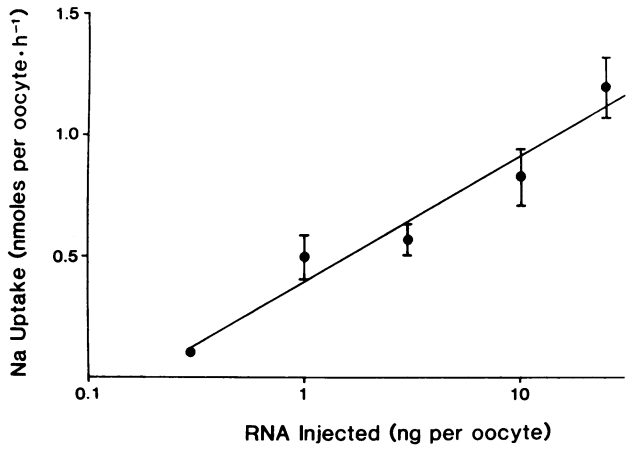

FIG. 1. Relationship between expression of Na^+ uptake and the amount of RNA microinjected into *Xenopus* oocytes. Oocytes were microinjected with 50 nl of A6 poly(A)$^+$ RNA (0.006–0.5 μg/μl) and then assayed 3 days later for ^{22}Na uptake as described. The amount of RNA injected per oocyte is shown on a logarithmic scale. Each point represents the mean ± SEM for 8 to 10 oocytes (error bars were omitted when smaller than the data symbol). In this experiment Na^+ uptake by water-injected oocytes was 32.7 ± 5.9 pmol per oocyte·hr^{-1}.

Oocytes microinjected with 25 ng of poly(A)$^+$ RNA prepared from aldosterone-treated A6 cells that were grown on a nonpermeable support (polystyrene flask) also exhibited a marked increase in amiloride-sensitive Na^+ uptake. In one experiment, oocytes injected with poly(A)$^+$ RNA from plastic-grown A6 cells exhibited Na^+ uptake of 280.8 ± 34.2 pmol per oocyte·hr^{-1} (n = 15 oocytes) compared with 317.9 ± 15.8 pmol per oocyte·hr^{-1} (n = 16 oocytes) for oocytes injected with poly(A)$^+$ RNA from filter-grown A6 cells.

Fig. 2 shows the time course of Na^+ uptake in oocytes microinjected with either 25 ng of poly(A)$^+$ RNA from filter-grown cells or 50 nl of sterile water. Sodium uptake in RNA-injected oocytes is linear with time up to 90 min and is inhibited ≈80% by 0.5 μM amiloride. In other experiments, the level of inhibition of Na^+ uptake by 0.5 μM amiloride ranged from 88 to 94% (n = 12 oocytes) and for 1 μM amiloride the level of inhibition ranged from 97 to 99% (n = 19 oocytes). Inhibition of Na^+ uptake by amiloride was dose dependent with half-maximal inhibition (K_i) at 6×10^{-8} M when measured in the presence of 4 mM NaCl (Fig. 3). This finding is in excellent agreement with the published value of K_i for amiloride inhibition of ^{22}Na flux in vesicles prepared from aldosterone-treated, filter-grown A6 cells (17) and for inhibition of short-circuit current in intact A6 cell monolayers (18).

Additional proof that ^{22}Na uptake as measured by our assay occurs exclusively by means of expression of the epithelial sodium channel and not by expression of another amiloride-sensitive Na^+ transport protein comes from examining the effects of amiloride analogs with different specificities for inhibiting the sodium channel, Na^+/H^+ exchanger, and the Na^+/Ca^{2+} exchanger (Fig. 4). Benzamil (0.1 μM), which has ≈10-fold greater potency for inhibiting the epithelial Na^+ channel than amiloride as well as reduced affinity for inhibition of other Na^+-coupled ion transporters (19), inhibits Na^+ uptake 96% in oocytes injected with poly(A)$^+$ RNA

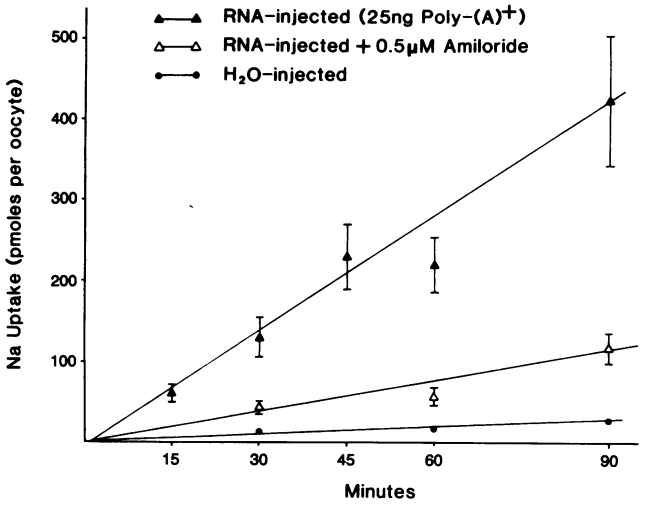

FIG. 2. Time course of Na^+ uptake in microinjected oocytes. Oocytes were microinjected with either water or A6 poly(A)$^+$ RNA. Three days after injections, Na^+ uptake by either water-injected (●) or RNA-injected oocytes (▲, △) was measured at various times. Oocytes microinjected with RNA were assayed either with (△) or without (▲) 0.5 μM amiloride. Each point represents the mean ± SEM for four to eight oocytes (error bars were omitted when smaller than the data symbol).

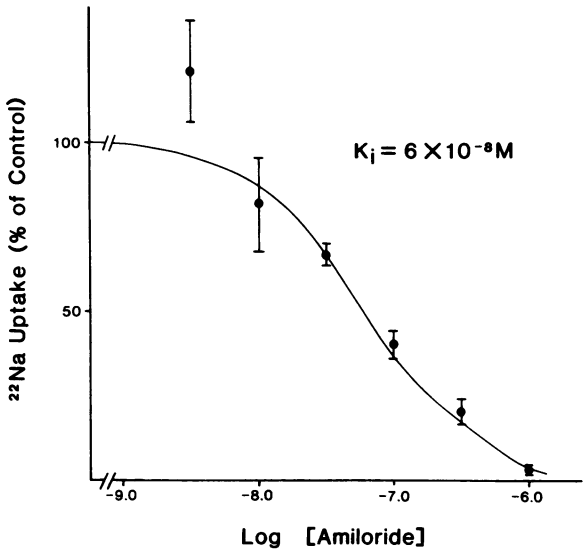

FIG. 3. Concentration-dependent inhibition of Na^+ uptake in RNA-injected oocytes by amiloride. Oocytes were microinjected with 25 ng of A6 poly(A)$^+$ RNA 3 days before ^{22}Na uptake measurements were done. Groups of oocytes were assayed in the presence of various concentrations of amiloride, and Na^+ uptake data are expressed as a percent of the mean value obtained without the drug. Half-maximal inhibition (K_i) occurred at 6×10^{-8} M amiloride. Each point represents the mean ± SEM of three experiments and a total of 18–22 oocytes.

prepared from aldosterone-treated, filter-grown A6 cells. In contrast, 1 μM EIPA, an analog with reduced activity against the epithelial sodium channel but greater potency for inhibiting Na^+/H^+ and Na^+/Ca^{2+} exchange (19), inhibits Na^+ uptake in oocytes injected with poly(A)$^+$ RNA from filter-grown A6 cells only 16%. The profile of inhibition of Na^+ uptake by amiloride, benzamil, and EIPA is similar for oocytes injected with poly(A)$^+$ RNA isolated from aldosterone-treated A6 cells grown either on collagen-coated porous membranes or in plastic flasks, and there was no statistically significant difference between these two groups ($P > 0.1$, Student's t test).

To exclude the possibility that expression of amiloride-sensitive Na^+ uptake in microinjected oocytes is a nonspecific effect of RNA injection, we performed experiments using size-fractionated poly(A)$^+$ RNA from aldosterone-treated, plastic-grown A6 cells. In Fig. 5 we demonstrate that amiloride-sensitive Na^+ uptake is expressed predominantly by oocytes microinjected with 10 ng of fractions 8 or 9. Sodium uptake exhibited by oocytes injected with 10 ng of other fractions (1–6 or 11–13) was not significantly different from water-injected oocytes. An ethidium bromide-stained formaldehyde/agarose minigel (data not shown) revealed the molecular size range of the two fractions expressing amiloride-sensitive Na^+ uptake to lie between 1.4 and 4.4 kilobases (kb). Because size separation was performed under nondenaturing conditions, we were unable to determine from this experiment a more narrow molecular mass range of mRNA encoding the amiloride-sensitive sodium channel or whether multiple mRNAs are required for functional expression.

DISCUSSION

The purpose of this study was to develop a functional expression assay which would be useful in the isolation and characterization of cDNA encoding the amiloride-sensitive sodium channel. The experiments described here demonstrate the expression of amiloride-inhibitable Na^+ uptake in *Xenopus* oocytes microinjected with mRNA from aldosterone-treated A6 cells and the absence of significant Na^+ uptake in native or water-injected oocytes. In this study, Na^+ uptake by mRNA-injected oocytes as measured by our ^{22}Na flux assay was mediated exclusively by an amiloride-sensitive pathway (97–99% inhibition with 1 μM amiloride). The concentration of amiloride required for half-maximal inhibition of Na^+ uptake by mRNA-injected oocytes ($K_i = 6 \times 10^{-8}$ M) was of a magnitude considered characteristic for inhibition of the epithelial sodium channel and not typical for other amiloride-inhibitable Na^+-coupled ion transporters (i.e., Na^+/H^+ antiporter, Na^+/Ca^{2+} exchanger, and Na^+/K^+-ATPase) (19). Furthermore, the K_i for amiloride described in our study agrees with published observations of K_i for amiloride inhibition of ^{22}Na uptake in A6 cell vesicles measured with similar Na^+ concentration (17) and for the inhibition of short-circuit current in intact A6 monolayers (18). In addition, we demonstrated that the degree of inhibition of Na^+ uptake in mRNA-injected oocytes by both benzamil and EIPA conforms to previously published observations of these two amiloride analogs on inhibition of sodium transport mediated by the epithelial sodium channel (20, 21). These data are consistent with the functional expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes and its specific detection by our ^{22}Na flux assay.

In our RNA size-fractionation experiment, we demonstrated the expression of amiloride-sensitive ^{22}Na uptake in oocytes microinjected with mRNA in the 1.4- to 4.4-kb

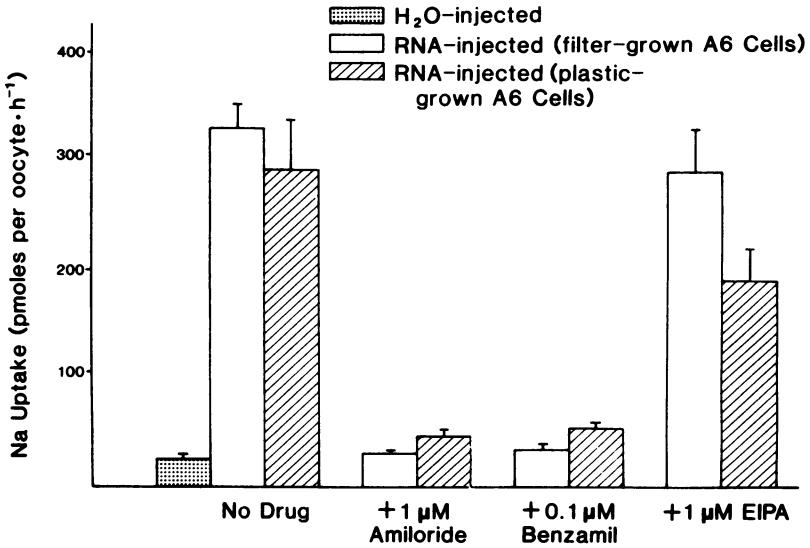

FIG. 4. Inhibition of Na^+ uptake in RNA-injected oocytes by amiloride, benzamil, and EIPA. Oocytes were microinjected with 25 ng of A6 poly(A)$^+$ RNA from aldosterone-treated A6 cells grown either on collagen-coated membranes (open bars) or in polystyrene tissue culture flasks (hatched bars). Sodium uptake was measured 3 days later with 1 μM amiloride, 0.1 μM benzamil, or 1 μM EIPA or without drug. The magnitude of Na^+ uptake by water-injected oocytes is shown by the dotted bar. Each bar represents the mean ± SEM for 8–16 oocytes.

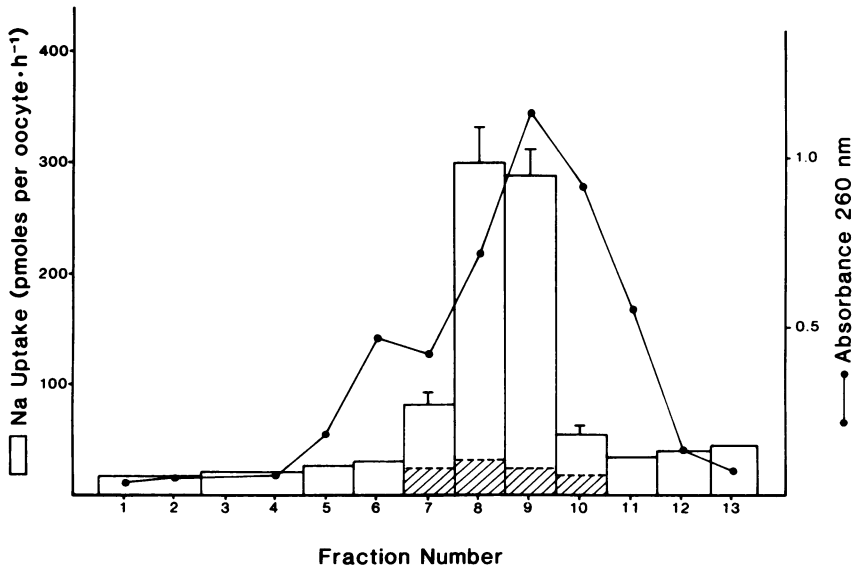

FIG. 5. Sodium uptake by oocytes microinjected with size-fractionated A6 cell mRNA. After heating (68°C, 3 min), 150 μg of A6 poly(A)$^+$ RNA was loaded on a 5–20% sucrose density gradient and centrifuged 4 hr at 45,000 rpm (SW-55Ti rotor). Fractions were collected by puncturing the bottoms of the tube, and each fraction was reprecipitated twice with ethanol. The RNA concentration of each fraction was adjusted to 0.2 μg/μl, and separate groups of oocytes were microinjected with 10 ng of each fraction. Sodium uptake was assayed 3 days after microinjection. The amount of RNA recovered from each fraction is indicated by the solid line (A_{260}), and mean Na^+ uptake of six to eight oocytes is represented by the vertical bars. Fraction number 1 is the bottom of the gradient (highest molecular mass mRNA species). In fractions exhibiting Na^+ uptake greater than water-injected oocytes, error bars (SEM) are shown, and the magnitude of amiloride-insensitive (1 μM) Na^+ uptake is shown by the hatched regions.

molecular size range. The predicted molecular mass range of single nascent polypeptides encoded by mRNA in this size distribution is ≈50–160 kDa and could include many putative subunits that have been identified in either purified or affinity-labeled sodium channels (5–8). Based on these observations we feel it is unlikely that functional expression of the amiloride-sensitive sodium channel depends upon translation of the large α subunit (≈300 kDa) described by Benos *et al.* (8). It is conceivable that expression of amiloride-sensitive ^{22}Na uptake in our experiments resulted from the translation of a regulatory subunit acting on endogenous sodium channels and not from synthesis of channel-forming polypeptides *per se*, although we were unable to demonstrate any endogenous amiloride-sensitive sodium conductive pathway in native or water-injected oocytes.

We have also demonstrated that expression of the amiloride-sensitive sodium channel in *Xenopus* oocytes is possible with mRNA isolated from aldosterone-treated A6 cells grown on solid supports. In our initial experiments, we cultured A6 cell monolayers on collagen-coated membranes. As with many other epithelial cell lines, the degree of cellular differentiation is greater when the cells are grown on a porous support (22). Previous studies of ^{22}Na flux in intact A6 cell monolayers found that little or no amiloride-sensitive Na^+ uptake is expressed when these cells are cultured on a nonporous support (18). Despite these previously reported observations of A6 cells grown on solid supports, our experiments clearly show the expression of amiloride-sensitive Na^+ uptake by oocytes microinjected with mRNA isolated from aldosterone-treated A6 cells grown on either a porous or nonporous surface. We believe these results suggest that mRNA encoding one or more essential components of the amiloride-sensitive sodium channel is present in aldosterone-treated A6 cells regardless of the type of support. The absence of amiloride-sensitive Na^+ transport in A6 cells grown on a solid support might be due to translational or posttranslational events rather than the absence of mRNA encoding the sodium channel.

We conclude that the amiloride-sensitive sodium channel can be functionally expressed and specifically detected in *Xenopus* oocytes microinjected with mRNA from aldosterone-treated A6 cells. These observations should be helpful in isolating and characterizing cDNA encoding this important epithelial ion channel.

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