Expression of the Amiloride-blockable Na\textsuperscript{+} Channel by RNA from Control Versus Aldosterone-stimulated Tissue* 

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The amiloride-blockable Na\textsuperscript{+} channel was expressed in Xenopus oocytes injected with total RNA isolated from the toad urinary bladder. This system was used to investigate mechanisms that mediate the natriferic action of aldosterone. Incubation of the epithelium with aldosterone for 3 h doubled its channel activity but did not increase the ability of isolated RNA to express functional channels in oocytes. A 20-h incubation with the hormone produced an additional increase of Na\textsuperscript{+} transport across the intact epithelium and also augmented the channel activity expressed in oocytes by nearly 10-fold. The data are in agreement with our model that aldosterone enhances the apical Na\textsuperscript{+} permeability of tight epithelia by a short term activation of pre-existing channels, followed by chronic induction of new channel protein. Blocking methyl transfer reactions, previously shown to inhibit the natriferic action of aldosterone in tight epithelia, did not alter the basal or aldosterone-induced response in oocytes.

The corticosteroid aldosterone is a major regulator of electrolyte metabolism in all vertebrates (1). One of the best studied effects of this hormone is its ability to increase the rate of Na\textsuperscript{+} reabsorption across high resistance (tight) epithelia such as kidney distal tubules, descending colon, and urinary bladder (1–3). It is well established that this effect is primarily due to an increase in the luminal passive Na\textsuperscript{+} permeability by the activation or induction of amiloride-blockable Na\textsuperscript{+} channels (2, 4, 5).

Although the ability of aldosterone to augment the channel-mediated Na\textsuperscript{+} flux is well documented, the cellular processes involved are not understood. Since the hormonal effects are fully inhibited by blockers of either transcription or translation, it is obvious that de novo protein synthesis is required (6, 7). It is not clear, however, whether the induced proteins are new Na\textsuperscript{+} channels or regulatory proteins that function to modulate pre-existing channels. A number of groups have reported that an irreversible inhibition of basal channel activity by either proteolysis (8) or protein modifying reagents (9, 10) also blocks the response to aldosterone, added after the inhibitory reagent was removed. It was therefore concluded that the channels affected by the hormone are already present in the luminal surface (and therefore accessible to luminal modifiers) before the hormone was applied. Additional support for this idea was provided by demonstrating that stimulation of A6 cells with aldosterone does not produce an increase in the number of membranal amiloride binding sites (11) or in the level of antigen to an anti-Na\textsuperscript{+} channel antibody (12). A mechanism proposed to mediate the action of aldosterone on pre-existing apical channels is the methylation of either membrane proteins or lipids (13–16).

Recently, evidence was provided that the aldosterone-induced increase in luminal Na\textsuperscript{+} permeability may take place by two different mechanisms (17). The first mechanism dominates the natriferic response during the first 3–4 h of hormonal stimulation and is not affected by butyric acid or thyroid hormone. The other, seen only after a longer incubation, is fully blocked by both butyric acid and thyroid hormone. The data also suggest that these acute and chronic responses are mediated by different receptors to the hormone, i.e. the mineralocorticoid and glucocorticoid receptors, respectively (17). Since the above mentioned studies with irreversible channel inhibitors were all confined to relatively short hormone incubation periods, the possibility remains that the long term effect of aldosterone is de novo channel synthesis. This study addresses this hypothesis using an oocyte expression system as a tool to determine effects of aldosterone on the abundance of the mRNA that codes for the channel. This system is also used to determine whether a post-transcriptional methyl transfer reaction is involved in the hormonal action.

EXPERIMENTAL PROCEDURES

Toads (Bufo marinus of Mexican origin) were obtained from William A. Lemberger Co., Inc., Oshkosh, WI and kept at room temperature on wet grass beds. Animals were double pithed and the urinary bladder excised and briefly rinsed in NaCl Ringer's solution. They were either immediately lysed in guanidinium thiocyanate for RNA isolation (see below) or incubated in vitro ±aldosterone and then lysed. The in vitro incubation was done under conditions used for organ culture and therefore mimized cell necrosis and RNA degradation (18). Accordingly, bladder quarters were stretched over gelatin sponge rafts soaked with sterile NaCl Ringer's solution composed of 110 mM NaCl, 3.5 mM KCl, 0.5 mM MgCl\textsubscript{2}, 1.0 mM CaCl\textsubscript{2}, and 10 mM Tris-HCl (pH 7.3). The medium was also supplemented with 5 mM glucose, 5 mM pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, and either aldosterone (0.5 µM) or water diluent. The bladder quarters were incubated for 20 h at 22 °C and then immersed in a solution containing 4 mM guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. The lysed cells were scraped off the connective tissue with a sterile glass slide, and the guanidinium lysates were homogenized for 1 min using a Polytron tissue grinder (Kinematica GmbH, Lucerne, Switzerland) at maximal speed. Total RNA was isolated by the protocol of Chomczynski and Sacchi (19), and its quality was evaluated by electrophoresis through a 1% agarose-formaldehyde gel. Effects of aldosterone on Na\textsuperscript{+} transport across the intact epithelium were determined by monitoring the transepithelial short circuit current in hemihandcut mounts in Ussing chambers (20).

Mature female Xenopus laevis frogs were purchased from Xenopus I. Frogs were maintained and oocytes were removed and defolliculated as described (21, 22). Oocytes were injected with 50-nl aliquots of total RNA, incubated for 3 days at 22 °C in ND96 medium (96 mM...
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NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 5 mM HEPES¹ pH 7.6, and then assayed for ³²Na⁺ uptake as follows. Groups of 12–15 oocytes were placed in Eppendorf vials and washed twice with a 1-ml medium containing 100 mM choline chloride, 1 mM MgCl₂, 1 mM CaCl₂, 40 mM HEPES, and 5 mM Tris-HCl (pH 7.4). The lack of Na⁺ in the bathing solution is expected to inhibit the Na⁺/K⁺-ATPase and allow maximal accumulation of ³²Na⁺ in the oocytes. At time 0, the solution was replaced with 50 μl of fresh medium containing ²²NaCl (carrier-free, 10 μCi/ml), ³³H]inulin (20 μM, 5 μCi/ml), and either amiloride (or ethyl-isopropyl amiloride) or water diluent. Oocytes were incubated for 90 min at 22°C. During this period the internal radioactivity increased linearly with time. At the end of the incubation oocytes were washed with four 1-ml volumes of nonradioactive ice-cold medium containing 100 μM amiloride. Each oocyte was placed individually in a scintillation vial, dispersed in 5 ml of xylene-based scintillation fluid, and counted for ³²Na⁺ and ³³H radioactivity. Leaky or incompletely washed oocytes (less than 5% of the oocytes assayed) were identified by high levels of ³³H radioactivity and not included in the data averaged. Data were expressed as means ± S.E. of the uptake per oocyte. Each experiment was repeated several times using at least two different RNA preparations. Data from different experiments were averaged by expressing each flux as a percentage of the uptake under control conditions in the same experiment.

Protein carboxymethylation in oocytes was determined as described (14). Groups of 50 oocytes injected with RNA ± the methylating agent S-adenosyl-L-homocysteine (AdoHcy) were incubated with 0.5 mCi/ml [methyl-³²H]methionine for 48 h. They were washed in ND96 medium and homogenized using 15 strokes in a Dounce-type tissue grinder (Whetond Inds., Millville, NJ). Yolk granules were removed by a 10-min centrifugation at 12,000 × g through a layer of 1.5 M sucrose in ND96 medium (23). The cytosol (upper layer) and plasma membrane (medium-sucrose interface) were collected. Proteins were precipitated in trichloroacetic acid and washed three times. Amiloride was a gift from Merck Sharp and Dohme. All conventional chemicals were analytical grade.

RESULTS

Amiloride-blockable Na⁺ channels were previously expressed in Xenopus oocytes injected with RNA from A6 cells (25–27) and chicken lower intestine (22). However, since we were interested in comparing the early and late actions of aldosterone, characterized before only in the toad urinary bladder, we expressed channels from this model epithelium as well. Injecting oocytes with toad bladder RNA evoked a large Na⁺ permeability not seen in water-injected oocytes (Fig. 1). This pathway was effectively blocked by 1.0 μM amiloride but was insensitive to a similar concentration of ethyl-isopropyl amiloride. Na⁺ fluxes measured in the presence of increasing concentrations of amiloride indicated a half-maximal inhibitory concentration of 90.1 nM (Fig. 2). Both the high affinity to amiloride and the relative insensitivity to ethyl-isopropyl amiloride strongly suggest that the pathway expressed in oocytes is the epithelial Na⁺ channel (28). Blocking transcription with 50 μg/ml actinomycin D had no effect on the development of the amiloride-blockable Na⁺ permeability (Fig. 1). Thus, the injected RNA does not have any effect on the development of the amiloride-blockable Na⁺ flux could readily be detected in the RNA-injected oocytes. RNA isolated from toads whose plasma aldosterone was raised for 3 h only expressed Na⁺ channel activity that was not significantly different from the one

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AdoHcy, S-adenosyl-L-homocysteine; Isc, transepithelial short circuit current.
expressed by RNA from control, saline-injected animals (Fig. 3). On the other hand, a 20-h incubation with the hormone produced more than a 2-fold increase in the amiloride-sensitive flux expressed by the isolated RNA. The data in Fig. 3 represent measurements in a single RNA preparation using four animals for each experimental condition. The same results were obtained in another independent RNA preparation using the same protocol.

The above experiment provides the first indication that a chronic but not short term exposure to aldosterone increases the abundance of an mRNA species that evokes channel activity in oocytes. Yet, correlating this response with the long term action of the hormone requires an in vitro incubation with aldosterone under conditions comparable with those used to establish the time course of increase in channel activity (22). Therefore, a procedure that enables the isolation of functional RNA from toad bladder after a long in vitro incubation was established (see “Experimental Procedures”) and used to further analyze effects of aldosterone on expression of channel activity in oocytes. Results from three different RNA preparations, each pooling material from four animals, are depicted in Fig. 4. The data clearly demonstrate that also under in vitro conditions, a 3-h incubation with aldosterone does not significantly increase the ability of isolated RNA to express functional channels in oocytes. On the other hand, a 20-h exposure to the hormone evokes more than a 9-fold increase in the channel expression. An aldosterone-induced increase in Na⁺ transport can be detected in toad bladder already 60-90 min after the hormonal application, and a 3-h incubation usually doubles Isc (Fig. 3, insert, as well as many previous papers reviewed in Refs. 2 and 4). Thus, the hormonal response detected in oocytes cannot account for the initial natriferic effect.

The observed increase in channel activity in oocytes does not necessarily represent a direct effect of aldosterone on channel expression. An alternative explanation is that this response is secondary to the stimulation of Na⁺ transport seen already after a short term incubation, i.e. the hormone-induced increase in apical permeability modifies an intracellular parameter (e.g. the cytoplasmic Na⁺ activity, ATP/ADP ratio, etc.), which in turn alters gene expression. To test for such an indirect effect, we have examined the effects of aldosterone added to tissue that was bathed in a Na⁺-free Ringer's solution, and 60 min later 0.5 μM aldosterone was added to the serosal compartment of one hemibladder from each pair (t = 0). The epithelia were maintained open circuited, and Isc was measured approximately every 20 min. The figure depicts changes in Isc relative to its value at t = 0 (Isc/Isc(t = 0)) in four experiments (means ± S.E.).
TABLE I
Effects of aldosterone in NaCl versus Na+-free Ringer's solution

Tissue excised from a single animal was divided into four bladder quarters. Two pieces were maintained for 20 h in a normal NaCl Ringer's solution (110 mM NaCl) with and without 0.5 μM aldosterone and the other two in a modified Ringer's in which NaCl was replaced by choline chloride, with and without aldosterone. RNA was isolated and assayed in oocytes as above. The experiment was repeated for three RNA preparations, and data were averaged as in Fig. 4.

| Treatment                        | 32Na+ uptake | -Amiloride | +Amiloride |
|----------------------------------|--------------|------------|------------|
|                                  | % of control value |            |            |
| NaCl Ringer's solution           |              |            |            |
| Control                          | 100          | 30 ± 14    |            |
| Aldosterone treated              | 300 ± 105    | 32 ± 7     |            |
| Choline Ringer's solution        |              |            |            |
| Control                          | 134 ± 26     | 49 ± 19    |            |
| Aldosterone treated              | 309 ± 44     | 32 ± 5     |            |

TABLE II
Effects of AdoHcy on channel expression and protein carboxymethylation

Oocytes were injected with 50-nl aliquots of total RNA ± 6 mM AdoHcy. Since the oocyte diameter is <1.3 mm, the internal AdoHcy concentration should be >200 μM. AdoHcy-injected oocytes also received 300 μM methylation inhibitor in the bathing medium. Some of the oocytes were incubated in ND96 medium (±AdoHcy) and assayed for 32Na+ uptake 3 days later. The rest also received 0.5 mCi/ml [methyl-3H]methionine and were assayed 48 h later for protein carboxymethylation, as described under "Experimental Procedures." Ami, amiloride.

| 32Na+ uptake |                   |                   |
|--------------|-------------------|-------------------|
|              | -Ami +Ami -Ami +Ami |                  |
|              | cpm/oocyte        |                   |
| No additions | 505 ± 93 130 ± 13 | 2853 ± 326 300 ± 76 |
| + AdoHcy     | 540 ± 97 206 ± 46 | 2659 ± 376 212 ± 24 |
|              |                   | Volatile C3H8    |
| Membrane     |                   |                   |
| No additions | 62.7             | 31.9             |
| + AdoHcy     | 106.2            | 47.8             |
| Cytosol      |                   |                   |
| No additions |                   |                   |
| + AdoHcy     |                   |                   |

uptake. Some of the injected oocytes were also incubated with [methyl-3H]methionine to determine effects of AdoHcy on protein carboxymethylation. It was found that injecting oocytes with AdoHcy and including it in their incubating medium does not significantly inhibit expression of channel activity (Table II). Under the same conditions, however, AdoHcy lowered protein carboxymethylation by about 50%. Thus, a methyl transfer reaction is not essential for the expression of basal and aldosterone-induced channel activity in oocytes.

DISCUSSION

The mineralocorticoid aldosterone is the principal regulator of Na+ reabsorption in tight epithelia. It acts primarily by increasing the apical Na+ permeability enabling a higher rate of Na+ entry from the lumen. In the amphibian urinary bladder the increase in Na+ channel activity, measured as an increase in the transepithelial short circuit current, is observed already 1 h after the hormonal application and peaks several hours later (2). More recently, evidence was provided that this response sums up two processes that take place in different time scales and increase the luminal channel activity by different mechanisms. The current study used functional expression of Na+ channels in Xenopus oocytes to further assess this possibility and determine whether either of the two processes postulated is associated with an increase in the abundance of mRNA coding for the channel. It was found that a 20-h application of aldosterone, either in vivo or in vitro, largely increases the ability of isolated RNA to express an amiloride-blockable conductance in oocytes. On the other hand, a relatively short incubation with the hormone, which is sufficient to double the channel activity in the intact epithelium, has no effect on the expression of functional channels in oocyte. This result supports the hypothesis that the acute and chronic natriferic processes involve different transcriptional events and suggests that only the latter one could be accounted for by induction of new channels.

Experiments done along similar lines using A6 cells produced somewhat complicated results. Hinton and Eaton (25) found that total RNA derived from cells that were depleted of aldosterone for 72 h evoked much lower amiloride-blockable currents than RNA obtained from hormone-repleted cells. Palmer et al. (27), on the other hand, reported that such an effect can be seen only in cells grown in plastic bottom dishes, conditions under which they have no amiloride-blockable permeability (30). If cells are cultivated on a porous support and form polarized Na+-transporting epithetium, no effect of aldosterone on channel expression in oocytes is detected, even though ISC is largely increased. The reason for the dependence of the above response on cell support and the apparent differences between the native and cultured epithelium is not clear. One possibility is that A6 cells preserve only part of the hormonal response, i.e. the short term effect not associated with increased expression in oocytes. Alternatively, it is possible that in A6 cells an aldosterone-induced increase in the channel message can be detected only if translation of channels is inhibited (i.e. by growing cells on a non-permeable support).

The most likely interpretation of the present data is that the aldosterone-induced mRNA species that increases expression of channels in oocytes for the channel protein itself. In this case, it could be concluded that aldosterone induces transcription of new Na+ channels in a time domain that corresponds to its long term natriferic action. Alternatively, it is also possible that expression of functional channels is limited by an aldosterone-induced regulator co-expressed with the channel protein from toad bladder RNA. In this case the data suggest that enhanced induction of this factor takes place after a prolonged incubation with the hormone and is responsible for its chronic natriferic action. We consider this possibility less likely although we cannot exclude it at the moment. Attempts to address this issue by determining the number of amiloride binding sites in oocytes injected with control versus aldosterone-stimulated RNA were so far unsuccessful. The reason appears to be the very low abundance of channels in the oocyte membrane (about 5% of their density in the native cells (27)).

One of the mechanisms proposed to mediate the natriferic action of aldosterone is methylation of either the channel protein or lipids around it. This model is based on the following observations made in cultured epithelia. (a) The methyl donor S-adenosyl-L-methionine increases the amiloride-sensitive Na+ permeability of membrane vesicles isolated from control, but not from aldosterone-stimulated cells (13). (b) Inhibiting cellular transmethylation with 3-deazaadenosine blocks the aldosterone-induced increase in channel activity (14, 15). (c) Aldosterone enhances incorporation of radioac-
This blocker also inhibits RNA capping; it will block every process that depends on new transcription. Distinction between transcriptional and post-translational methylation can be done in oocytes injected with already methylated RNA. Such experiments summarized in Table II have demonstrated that injecting oocytes with AdoHcy does not diminish the response to aldosterone even though the blocker inhibits protein carboxymethylation by more than 50%. We therefore suggest that a methyl transfer reaction does not mediate the chronic action of the hormone detected in this system. It is of course possible that methylation mediates only the short-term response not detected in oocytes. Also, since carboxymethylation was not fully inhibited by AdoHcy one may speculate that the methyl transfer reaction involved in the regulation of channels is relatively insensitive to AdoHcy.

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