J. Membrane Biol. 62, 163–167 (1981)

The Journal of
Membrane Biology

Metabolism of Poly(A) (+)RNA in Toad Bladder Epithelial Cells

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Summary. Previous studies have characterized the induction of poly(A)(+)RNA synthesis by aldosterone during the latent period, preceding the increased active transepithelial sodium transport (measured as short-circuit current, SCC). To assess the role of aldosterone in the maintenance of the response in general and the metabolism of this RNA in particular, the decay of the increased SCC and of the newly synthesized poly(A)(+)RNA was monitored. On removal of the hormone, the SCC decayed with a half-life of 6.5 hr after a lag period of 2–3 hr. Studies on the disappearance from the cytoplasm of poly(A)(+)RNA synthesized in the first two hours after addition of aldosterone revealed a number of RNA species with diverse size decaying at a relatively slow rate after removal of aldosterone, and RNA sedimenting in the 10–14 S region decaying at a faster rate closely related to the decay in SCC. Maintenance of aldosterone in the media resulted in a much slower rate of decay of this 10–14 S. It is concluded that the decay of the 10–14 S poly(A)(+)RNA is closely related to the decay in SCC and the stability of this RNA is influenced by the retention of aldosterone in the medium.

Key words: Toad bladder, Na^+ transport, aldosterone, poly(A)(+)RNA, RNA metabolism

In a series of studies [8, 9, 14] the increased synthesis of poly(A)(+)RNA in the epithelial cells of the urinary bladder of the toad *Bufo marinus* in response to treatment with aldosterone has been described. There is increased incorporation of [^{3}H]-Uridine into poly(A)(+)RNA particularly in the 10–14 S region during that latent period. This reflects an accumulation of 10–14 S poly(A)(+)RNA in the cytoplasm (as indicated by hybridization with [H^3]-poly U). The synthesis of this putative mRNA can be related to the increased SCC and appears to be specific to the action of aldosterone. The implication of these and previous studies with metabolic inhibitors [reviewed in ref. 2] is that aldosterone, like other steroids, acts by affecting the transcription process. It has been suggested that steroid hormones not only stimulate the synthesis of RNA but may also influence the degradation of the mRNA in the cytoplasm [13], thereby having precise control over hormone-regulated events. In the experiments reported here, the metabolism of poly(A)(+)RNA induced by aldosterone has been studied and the decay of this RNA related to the decay of the physiological response and to the decay of other mRNA in the cell.

Materials and Methods

The incubation medium (frog Ringer's solution) contained (in mM): 90 NaCl, 3 KCl, 25 $NaHCO_3$, 0.5 $MgSO_4$, 0.5 KH_2PO_4, 1 $CaCl_2$ and 6 glucose; pH 7.5 (gassed with 95% O_2 5% CO_2), osmolarity 230 mosmol. Gentamicin was added to a final concentration of 5 μg/ml and the medium sterilized by filtration. Glassware was heat sterilized. All reagents were either reagent grade or spectroquality.

Male and female toads were collected locally in Brisbane and stored on damp sawdust. The bladders isolated from these animals showed a response in an increased SCC and an increased incorporation of precursors into RNA that was very similar both qualitatively and quantitatively to the response of animals obtained from North American suppliers. The pretreatment of animals, isolation of the bladder, measurement of the potential difference and SCC have been described previously [8]. Two to three hours after isolation of the bladder, aldosterone (final concentration $=7\times10^{-8}$ M) was added to both the serosal and mucosal media. Aldosterone was removed by aspirating the media and replacing with fresh media.

Isolation and Analysis of [^{3}H]-RNA

RNA was labeled with [5-^{3}H]-Uridine (5 μCi/ml) obtained from Radiochemical Center, Amersham U.K. added to the serosal side of the bladder. In the studies of the time course of [^{3}H]-Uridine

0022-2631/81/0062-0163 $01.00

incorporation, bladders were divided into four equal pieces and incubated in 25-ml Erlenmeyer flask containing 15 ml of Ringer's and [^{3}H]-Uridine (5 μCi/ml) continually oxygenated with 95% O_2 5% CO_2. The experiment was arranged so that all portions of the bladders were incubated for the same period of time, i.e. in the case of a 24-hr experiment the control portions were incubated with [^{3}H]-Uridine for the final 2 hr of the experiment. Incubations for other time points were staggered to finish at this time. After incubation, the bladders were washed with ice-cold frog Ringer's solution. All subsequent steps were carried out at 0–4 °C. The removal and homogenization of the bladder epithelial cells, and extraction of the RNA has been described previously [8]. After precipitation with ethanol at −20 °C overnight, the poly(A)(+) RNA was isolated by (oligo-dt)-cellulose chromatography and analyzed by sucrose gradient centrifugation [14]. The 5–20% sucrose gradients were fractionated using a Gilson precision peristaltic pump. Twenty-nine fractions were collected from each gradient. Sedimentation coefficients were calculated from cytoplasmic RNA markers run on parallel gradients. The [^{3}H] content was determined with a Beckman LS250 liquid scintillation spectrometer using a Toluene/Triton-X100 scintillation mixture (4 g PPO, 15 mg POPOP per liter, 69% Toluene/31% Triton). [^{3}H] was counted at 40% efficiency.

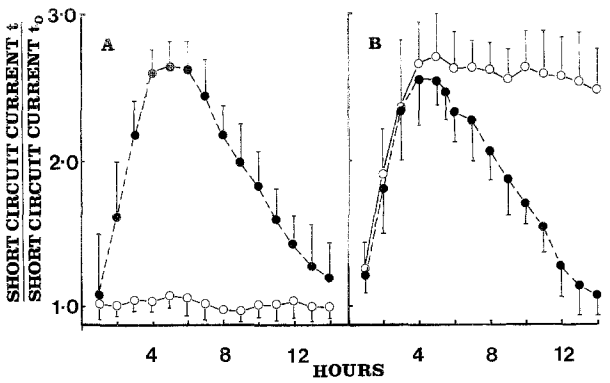

Fig. 1. The decay of short-circuit current after removal of aldosterone. Paired hemibladders ($n=10$) were preincubated for 3 hr in frog Ringer's before addition of aldosterone (7×10^{-8} M) at t_0. 3 hr after addition of the hormone, the media were changed. *Panel A:* --●-- aldosterone removed at t_3; -○- control. *Panel B:* ---●--- aldosterone removed at t_3; -○- aldosterone retained throughout. Points indicate mean ± SEM

Results

Decay of the Aldosterone-Induced Changes in Short-Circuit Current

To estimate the half-life of the aldosterone-induced response, the hormone was removed after 3 hr, when the increase in SCC was well established. The decline in SCC after removal of the hormone will reflect a combination of phenomena; dissociation of the hormone-receptor complex from the nuclear material, degradation of induced RNA and inactivation of any protein or phospholipid mediators of the hormone response.

At least ten individual animals were used in each study. Only those bladders showing a distinct response (an increase in SCC of 50% or more 3 hr after addition of aldosterone) were used. Three hours after adding the hormone the incubation media were aspirated and replaced with frog Ringer's containing no aldosterone. This procedure removed 90% of the hormone from the incubation media and bladder tissue (as indicated by recovery of [^{3}H]-aldosterone). A small perturbance of the SCC occurred immediately after this manipulation but the SCC recovered to an expected value within 30 min.

The data from this study (Fig. 1) show a steady rise in SCC after the latent period, the full response developing 4–5 hr after addition of the hormone. Removal of the hormone resulted in decay of the SCC that was approximately exponential but became apparent 2 hr after removal of the hormone. The half-life of the response after removal of the hormone was calculated as 6.5 hr.

Decay of Poly(A)(+)RNA Synthesized after Aldosterone Treatment

These experiments were designed to monitor the metabolism of the poly(A)(+)RNA after removal of the hormone. Bladders from 10 animals were divided into 4 equal pieces. The pooled quarter-bladders were pulsed with [^{3}H]-Uridine for the first 2 hr after addition of aldosterone followed by removal of the hormone and a chase with excess unlabeled Uridine for various times [8]. This experimental design restricts any experiment to 3 time points and a control (no aldosterone). However, the specific activities of the control incubations were consistent (control total RNA specific radioactivity, 565 cpm/μg and 605 cpm/μg; control poly(A)(+)RNA, 8,700 cpm/μg and 9,600 cpm/μg), allowing a composite picture to be built up from the two experimental protocols (Fig. 2). In experiment 1 total cytoplasmic RNA and poly(A)(+)RNA was extracted from the control tissue (2 hr [^{5}H]-Uridine, no aldosterone), bladders pulsed for 2 hr with [^{3}H]-Uridine in the presence of aldosterone, and bladders pulsed with [^{3}H]-Uridine for 2 hr followed by a chase for 3 hr and 8 hr. In experiment 2, RNA was isolated from control tissue and bladders pulsed and chased for a total of 6, 14 and 24 hr. An increase in the incorporation of [^{3}H]-Uridine into cytoplasmic RNA, particularly poly(A)(+)RNA after aldosterone treatment has been reported previously [1, 8, 14]. The experiments graphed in Fig. 2 confirm this observation. There is an increase of over 50% in the specific activity of both total cytoplasmic RNA and poly(A)(+)RNA isolated up to 12 hr after hormone treatment. Removal of the hormone with the addition of excess unlabeled Uridine resulted in

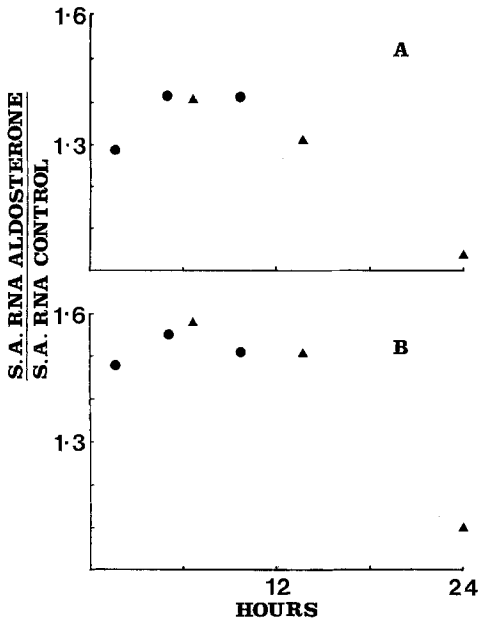

Fig. 2. Change in specific radioactivity of unfractionated cytoplasmic RNA and poly(A)(+)RNA isolated by (oligo-dt) cellulose chromatography. Bladders were divided into 4 pieces. The pieces pooled ($n=10$) and incubated with aldosterone (7×10^{-8} M) and [^{3}H]-Uridine for 2 hr. The aldosterone and [^{3}H]-Uridine were removed and incubation continued for the indicated time with 1,000-fold excess of Uridine. *Panel A:* Total cytoplasmic RNA from experiment 1 (●) (2, 5 & 10 hr after addition of hormone) and from experiment 2 (▲) (6, 14 & 24 hr after addition). *Panel B:* Poly(A)(+)RNA from experiment 1 and experiment 2

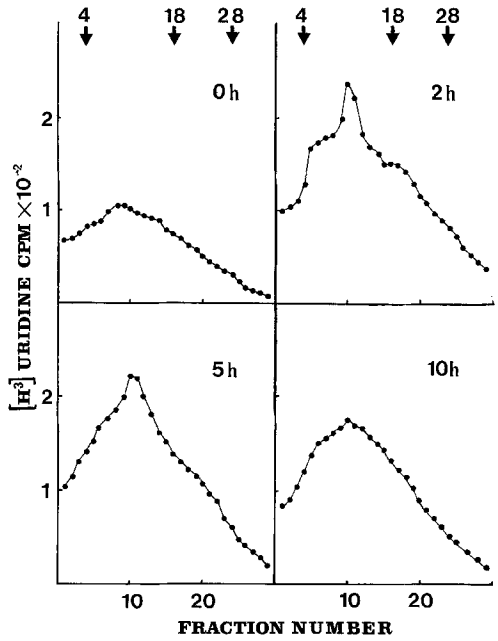

Fig. 3. Sucrose gradient analysis of poly(A)(+)RNA isolated at various times after removal of aldosterone and a chase with excess unlabeled Uridine. The panels represent [^{3}H] activity in poly(A)(+) RNA isolated in experiment 1 as described in legend to Fig. 2. Bladder pieces were pulsed with [^{3}H]-Uridine in the presence of 7×10^{-8} M aldosterone followed by a chase for various times with excess unlabeled uridine. *S* values were calculated from cytoplasmic RNA centrifuged in parallel

a disappearance of the labeled RNA from the cytoplasm, approaching control levels 24 hr after the start of the experiment. Although no accurate assessment of the half-life of the cytoplasmic RNA can be made from the data available, the decay of the labeled RNA in the cytoplasm after removal of the hormone has a much longer half-life than the decay in the SCC. One possible explanation for this anomoly is revealed in the sucrose gradient analysis of the poly(A)(+) RNA from these experiments (Figs. 3 and 4). The presence of aldosterone resulted in increased incorporation into most species of poly(A)(+)RNA but particularly into the fraction sedimenting between 10–14 S (Figs. 3 and 4). This 10–14 S RNA is present after a chase of 3 hr but is absent after an 8-hr chase. At this time (8-hr chase) other species of poly(A)(+) RNA sedimenting throughout the gradient were still heavily labeled (Fig. 3). A similar picture is seen in experiment 2 (Fig. 4), the distinct peak of labeled RNA sedimenting at around 12 S is seen after a 4-hr chase but is absent 14 hr after hormone addition (a 12-hr chase). Twenty-four hours after the start of the experiment the profile of RNA resembles the control.

The rapid disappearance of the 10–14 S poly(A)

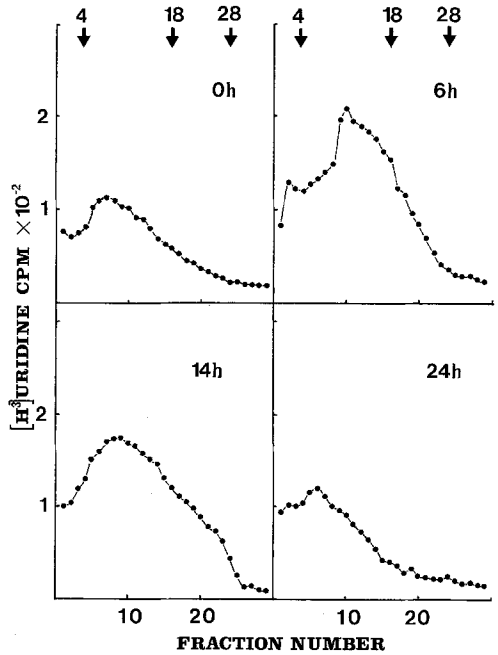

Fig. 4. Sucrose gradient analysis of poly(A)(+)RNA isolated at various times after removal of aldosterone and a chase with excess unlabeled uridine. The panels represent [^{3}H] activity in poly(A)(+) RNA isolated in experiment 2 described in legend to Fig. 2. *S* values were calculated from cytoplasmic RNA centrifuged in parallel

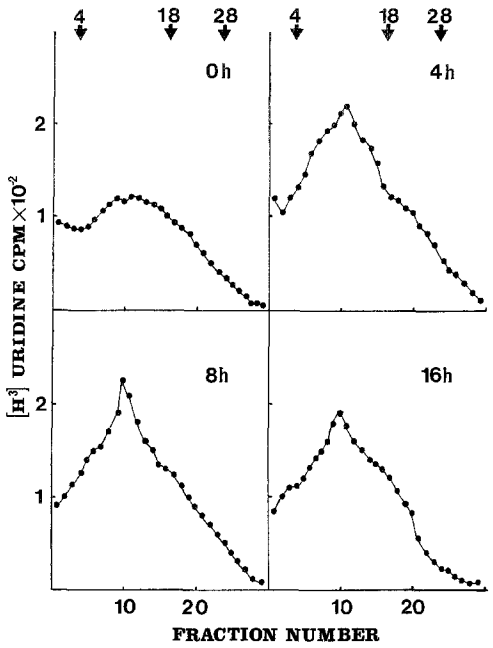

Fig. 5. Sucrose gradient analysis of poly(A)(+)RNA isolated after a 2-hr pulse of [^{3}H]-Uridine and a chase with excess unlabeled Uridine for various times. Aldosterone (7×10^{-8} M) was maintained throughout the incubation. *S* values were calculated from cytoplasmic RNA centrifuged in parallel

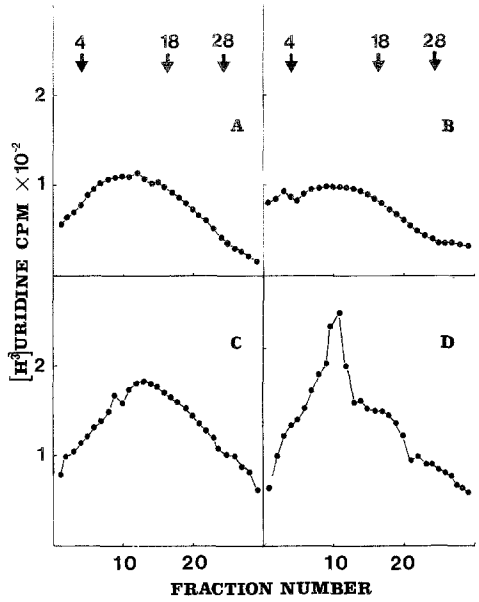

Fig. 6. Sucrose gradient analysis of poly(A)(+)RNA isolated from a single time point experiment. *Panel A:* Quarter-bladders pulsed with [^{3}H]-Uridine for 2 hr and the RNA extracted immediately. *Panels B, C & D:* Quarter-bladders pulsed with [^{3}H]-Uridine followed by 12-hr chase. *B.* No aldosterone. *C.* Aldosterone for 2 hr only. *D.* Aldosterone maintained throughout chase

(+)RNA from the cytoplasm did not occur when aldosterone was retained in the medium throughout the incubation although the [^{3}H]-Uridine was removed and excess Uridine added 2 hr after the start of the experiment (Fig. 5). The RNA sedimenting between 10–14 S is present, although reduced, 14 hr after the start of the experiment. This is in marked contrast to the situation when the hormone is removed (Fig. 4). The interpretation of the experiments shown in Figs. 3, 4 and 5 require comparison between groups of animals. To confirm these results in a single group of animals, a paired experiment was conducted (Fig. 6). Quarter bladders were treated in the following way; [^{3}H]-Uridine for 2 hr followed by phenol extraction (Fig. 6*A*); [^{3}H]-Uridine for 2 hr followed by a 12-hr chase (Fig. 6*B*); [^{3}H]-Uridine for 2 hr in the presence of aldosterone followed by a 12-hr chase in the absence of aldosterone (Fig. 6*C*); [^{3}H]-Uridine for 2 hr followed by a 12-hr chase in the presence of aldosterone (Fig. 6*D*). The two control incubations (Fig. 6*A* & *B*) served to confirm that the incubation for a total of 14 hr did not markedly alter the labeling characteristics. There was a small decrease in specific activity of the poly(A)(+)RNA during the 12-hr chase (12,014 cpm/μg in tissue extracted immediately and 9,614 cpm/μg after 12-hr chase). This may be due to a loss of viability of the epithelial cells or reflect a slow decay of the labeled RNA in the control tissue. The profiles of the poly(A)(+)RNA are similar showing little evidence of degradation of the poly(A)(+)RNA during the incubation. Fig. 6*C* & *D* confirm the experiments described above. The presence of aldosterone in the media has a stabilizing effect on the 12–14 S poly(A)(+)RNA (Fig. 6*D*). Withdrawal of aldosterone results in the rapid loss of this 12–14 S RNA from the cytoplasm.

These findings would indicate that aldosterone not only induced the synthesis of the poly(A)(+)RNA but also affects the breakdown of the RNA in the cytoplasm.

Discussion

The time course of the total physiological response looks remarkably similar to the time course of the increase and decay of citrate synthase activity in rat kidney after a single injection of aldosterone into the adrenalectomized rat [4] and to the decay of SCC in toad bladder epithelium using a different experimental protocol reported by Lahav et al. [5]. The response is rapid and the decay of the response after removal of the hormone is relatively quick. This reflects the dynamic response of both the kidney and toad bladder epithelium to aldosterone.

An idea of the role of aldosterone in regulating the breakdown of the induced poly(A)(+)RNA is given by a direct analysis of the time course of the RNA breakdown (Figs. 2, 3 and 4). The results show an increase in poly(A)(+)RNA with a variety of sedimentation coefficients that decay with a long half-life when the hormone is removed, but also an increased incorporation into RNA sedimenting in the 10–14 S region decaying with a much shorter half-life more related to the decay in the SCC. A rough approximation of 5 hr can be made for the half-life of the 10–14 S poly(A)(+)RNA. Most other estimates of the half-lives of poly(A)(+)RNA have come from studies of cultured human or mouse cells in states of rapid proliferation. The decay in HeLa cells is complex but can be approximated by assuming a (stochastic) first-order decay and by assuming that 33% of the RNA has a half-life of 1 hr while the remainder has a half-life of 24 hr [11]. Half-lives of 24 hr or greater have been reported for globin mRNA in rabbit reticulocytes [10] and ovalbumin mRNA in hen oviduct [7]. It would seem in the stable, i.e. nonproliferating, epithelial cell of the toad bladder that the hormone-dependent mRNA is turned over at a slower rate than some mRNA in proliferating cells in culture but faster than in cells at various stages of their differentiation process.

The implication of this rapid decay of the 10–14 S poly(A)(+)RNA when compared to the decay of the bulk of the poly(A)(+)RNA is a selective destabilization of one particular RNA population (10–14 S). This concept of selective destabilization has been proposed as a mechanism by which globin mRNA accumulates during erythoid differentiation [6]. In the toad bladder epithelial cells, the presence of aldosterone may act to stabilize the 10–14 S poly(A)(+)RNA in the cytoplasm. The data presented here do not indicate if this stabilization is through some transcriptional or post-transcriptional event. Post-transcriptional control has been suggested to explain the effects of Actinomycin D on the glucocorticoid-induced synthesis of tyrosine aminotransferase in hepatoma cell cultures, where the antibiotic appears to stabilize either the enzyme or its mRNA [3, 12, 13].

These studies do not take into account the translational efficiency of the cytoplasmic poly(A)(+)RNA. There is no monitor of the translation product of the RNA; however the decay of the 10–14 S poly(A)(+)RNA is reasonably closely related to the decay of the SCC and strongly suggests that this mRNA is the prime mediator of the hormone response, the synthesis of which is an important regulator of sodium transport and thus the response of the epithelial cells to aldosterone.

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Received 1 July 1980; revised 18 November 1980