J. Membrane Biol. 77, 15–23 (1984)

The Journal of
Membrane Biology

Effects of Thyromimetric Drugs on Aldosterone-Dependent Sodium Transport in the Toad Bladder

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Summary. Aldosterone increases transepithelial Na^+ transport in the urinary bladder of *Bufo marinus*. The response is characterized by 3 distinct phases: 1) a lag period of about 60 min, ii) an initial phase (early response) of about 2 hr during which Na^+ transport increases rapidly and transepithelial electrical resistance falls, and iii) a late phase (late response) of about 4 to 6 hr during which Na^+ transport still increases significantly but with very little change in resistance. Triiodothyronine (T_3, 6 nM) added either 2 or 18 hr before aldosterone selectively antagonizes the late response. T_3 *per se* (up to 6 nM) has no effect on base-line Na^+ transport. The antagonist activity of T_3 is only apparent after a latent period of about 6 to 8 hr. It is not rapidly reversible after a 4-hr washout of the hormone. The effects appear to be selective for thyromimetic drugs since reverse T_3 (rT_3) is inactive and isopropyldiiodothyronine ($isoT_2$) is more active than T_3. The relative activity of these analogs corresponds to their relative affinity for T_3 nuclear binding sites which we have previously described. Our data suggest that T_3 might control the expression of aldosterone by regulating gene expression, e.g. by the induction of specific proteins, which in turn will inhibit the late mineralocorticoid response, without interaction with the early response.

Key Words aldosterone · triidothyronine · thyromimetic drugs · sodium transport · toad bladder · *Bufo marinus*

Introduction

A major effect of aldosterone in the toad urinary bladder is to increase transepithelial sodium transport (Ludens & Fanestil, 1976; Crabbé, 1977; Marver, 1980). Recently, we reported that thyroid hormones had no effect on base-line sodium transport but antagonized the mineralocorticoid response in a dose-dependent manner (Rossier et al., 1979*a*; Rossier, Rossier & Lo, 1979*b*; Geering & Rossier, 1981). The antagonism was observed at triiodothyronine (T_3) concentrations (0.06 to 6 nM) which were compatible with a physiological rather than a pharmacological effect. This observation prompted us to look for the existence of T_3 nuclear binding sites. We did indeed observe high affinity (K_d = 57 pmol), low capacity (56 fmol/mg protein) nuclear binding sites for T_3 (Geering & Rossier, 1981) which could well be functionally related to the antimineralocorticoid action of T_3. Tata (1974), Greenberg, Najjar and Blizzard (1974) and Oppenheimer and Surks (1975) all present experimental evidence suggesting that T_3 mediates most of its effects through regulation of the gene expression of various specific proteins. Depending on the system, the latent period of the action of thyroid hormone varies from 6 to 12 hr. In the present paper we have examined in detail i) the time course of T_3 effect on sodium transport and tissue electrical resistance, ii) its specificity (by examining the relative biological activity of two related analogs (rT_3 and $isoT_2$) and iii) its reversibility.

Abbreviations. The abbreviations and trivial names used are: triiodothyronine or T_3, 3,5,3′-triiodo-L-thyronine; T_4 or thyroxine, 3,5,3′,5′-tetraiodo-L-thyronine; reverse T_3 or reverse triiodothyronine, 3,3′,5′-triiodo-L-thyronine; $isoT_2$ or isopropyldiiodothyronine, 3′,5-diiodo-3′-isopropyl-L-thyronine.

Materials and Methods

Adult male and female toads (*Bufo marinus*) were from C.P. Chase Co., Miami, Florida. They were kept in a terrarium with free access to a water tank kept at 25°C (± 1°C) and fed once a week with newborn mice. For 4 to 5 days preceding experiments, animals were kept in 0.045 M NaCl at 25°C (± 1°C). They were killed by double-pithing and perfused through the heart with a Ringer's solution (Ringer A) containing (in mM): NaCa 90, KCl 3, $NaHCO_3$ 25, $MgSO_4$ 0.9, KH_2PO_4 0.5, $CaCl_2$ 1, glucose 6 (osmolarity = 230 mOsm), and gentamicin 5 mg/liter. pH was 7.4 when the solution was gassed with 5% CO_2–95% O_2 at 25°C. For incubations, an amino acid-enriched Ringer's (Ringer B) was used. It contained in addition (mM): L-alanine 3.2, L-arginine 1.6, L-asparagine 0.7, L-cysteine 0.6, L-glutamine 2, L-glutamic acid 0.14,

glycine 0.9, L-histidine 0.6, L-isoleucine 0.3, L-leucine 0.4, L-lysine 0.4, L-methionine 0.19, L-phenylalanine 0.27, L-serine 0.7, L-threonine 0.9, L-tryptophan 0.04, L-tyrosine 0.5, L-valine 0.4, L-proline 0.4, L-aspartic acid 0.14, penicillin (25 mg/liter) and cefalotin (25 mg/liter). Solutions were filtered through Millipore filters (0.22 μ). All glassware was heat-sterilized, and gas was filtered through Millipore Cathivex filters.

Measurement of Na^+ Transport

Na^+ was measured by the short-circuit current (SCC) method in paired hemibladders mounted on glass canulas (Rossier et al., 1980). The everted sacs were filled with 5 to 10 ml of Ringer B and immersed in 80 ml of the same medium. After 60 min of preincubation ($t_{-2\,hr}$ or $t_{-18\,hr}$), thyromimetic drugs (T_3, $isoT_2$, rT_3) were added to the serosal side of the test hemibladders and the diluent (NaOH) to the paired controls. The incubation was either continued without change or in the presence of aldosterone (t_o) added 2 or 18 hr after the thyroid hormones. The following experimental groups were studied: 1) Aldosterone (80 nM) *vs.* control; 2) T_3 (6 nM) *vs.* control; 3) $isoT_2$ (6 nM) *vs.* control; 4) rT_3 (6 nM) *vs.* control; 5) T_3 (6 nM) + aldosterone (80 nM) *vs.* aldosterone (80 nM); 6) $isoT_2$ (6 nM) + aldosterone (80 nM) *vs.* aldosterone (80 nM); 7) rT_3 (6 nM) + aldosterone (80 nM) *vs.* aldosterone (80 nM); 8) T_3 (6 nM) *vs.* control for 18 hr followed by a 4-hr washout. Then both test and control hemibladders were challenged with aldosterone.

The electrophysiological data were expressed as SCC per hemibladder (Rossier et al., 1980) where SCC (A) = $SCC_t - SCC_o$. SCC_o was the value of SCC at the time of addition of aldosterone and SCC_t its value at any given time before or after SCC_o. Total tissue resistance (R in Ohm × hemibladder total surface) was calculated as the ratio of PD (mV) and SCC (mA). R was computed as described for SCC: $R = R_t - R_o$. R_o averaged 350 to 390 $\Omega \cdot$ hemibladder. It is a convenient way of expressing our results since the exact surface area is never measurable and since we are mainly interested in relative changes induced by various hormonal treatments. The errors on R measurement are obviously larger than on PD and on SCC, which were previously estimated to be within 5% (Rossier et al., 1980). However, the error on R is still small compared to the rather large changes in R induced by aldosterone (up to 40% of control values). The significance of the difference in the mean values ($SCC_{test} - SCC_{control}$ and $R_{test} - R_{control}$) was estimated by Student's paired t-test according to the method of Snedecor and Cochran (1971).

Plasma T_4

Plasma T_4 was measured by radioimmunoassay as described (Rossier et al., 1979*a*).

Material

Aldosterone was a generous gift of Ciba-Geigy SA. T_3 and rT_3 were from Henning, Berlin. $IsoT_2$ was kindly provided by Prof. E.C. Jörgensen, School of Pharmacology, University of California, San Francisco. All reagents were of analytical grade.

Results

Short- and Long-Term Effects of Aldosterone on Na^+ Transport

In order to insure stable experimental conditions for up to 30 hr of tissue incubation *in vitro*, the Ringer's solution (B) was supplemented with amino acids and additional antibiotics (*see* Materials and Methods). Figure 1 shows the effect of 80 nM aldosterone on transepithelial Na^+ transport (SCC) and total tissue resistance (R) after a short preincubation period *in vitro*. The mineralocorticoid tissue response was characterized by 3 phases: 1) a latent period of about 60 min during which neither SCC nor R were significantly changed; 2) an early response lasting 2 to 3 hr during which SCC increased rapidly and R fell concomitantly; 3) a late response where SCC still increased for the next 4 to 5 hr while R remained unchanged. A steady state of Na^+ transport was reached 6 to 8 hr after the addition of aldosterone and from then on the response was maintained as long as the hormone was present in the medium. Occasionally, SCC decreased slowly over the next 12 hr, but it always remained significantly higher than base-line values even after 20 hr of tissue incubation. The statistical evaluations of these effects are given in the legends of the various Figures.

It has been suggested that the tissue response to aldosterone might be more important and more reproducible after overnight tissue incubation in a steroid-free medium (Porter, Bogoroch & Edelman, 1964).

Figure 2 shows, however, that with the new experimental conditions used in this study, the magnitude of the tissue response to aldosterone was very similar after long-term preincubation. The latent period might be somewhat prolonged and the base-line more stable but the significance of these differences is difficult to assess in unpaired experiments.

Effect of T_3 on Base-Line and Aldosterone-Dependent Na^+ Transport

In a first experiment we tested whether T_3 could modify the base-line Na^+ transport over an extended tissue incubation period (20 hr). A dose of 6 nM T_3 was chosen since it has been shown previously that this concentration saturates the nuclear binding sites for T_3 in this tissue and produces a nearly maximal antimineralocorticoid response (Geering & Rossier, 1981).

As shown in Fig. 3, T_3 *per se* had no significant effect either on Na^+ transport or on tissue resistance. However, when T_3 (6 nM) was added to one

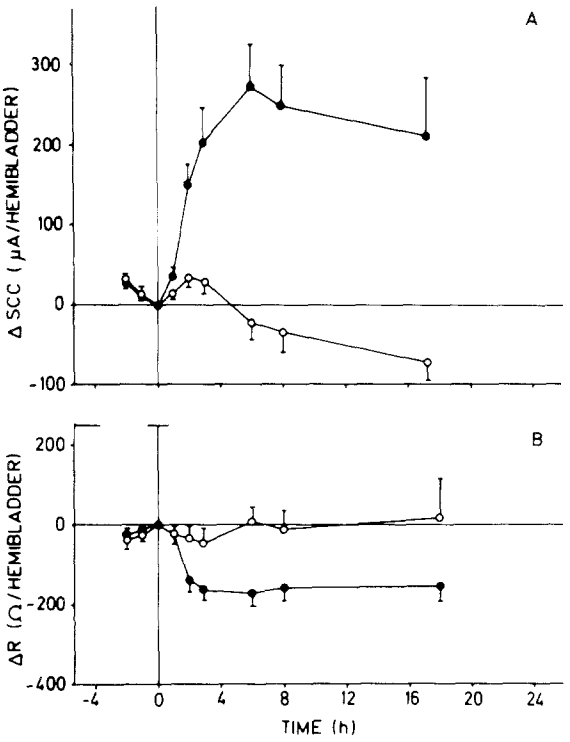

Fig. 1. Effects of aldosterone on transepithelial Na^+ transport (*A*) and transepithelial electrical resistance (*B*) in the toad bladder. Aldosterone (80 nM) was added at time 0 hr to test hemibladders (●) and diluent to paired controls (○). Na^+ transport is expressed as short-circuit current (SCC), as described in Materials and Methods. SCC_o (●) = 155 ± 33 μA; SCC_o (○) = 160 ± 27 μA ($P < 0.8$); R_o (●) = 385 ± 49 Ω; R_o (○) = 384 × 38 Ω ($P < 0.9$). *From t_o to t_3: SCC (early)* = $SCC_3 - SCC_o$. SCC (early) aldo (●) increased significantly (+220 ± 35 μA: $P < 0.001$) while SCC (early) control (○) did not (+22 ± 16 μA: $P < 0.3$). The net increase (aldo-control) is 198 ± 34 μA; $P < 0.001$). *R* (early) aldo (●) decreased significantly (−160 ± 27 Ω; $P < 0.001$) while *R* (early) control (○) did not (−38 ± 32 Ω; $P < 0.3$). The net decrease was thus: −122 ± 24 Ω; $P < 0.001$. *From t_3 to $t_{7\,hr}$: SCC (late)* = $SCC_7 - SCC_3$. SCC (late) aldo (●) increased significantly (+78 ± 26 μA; $P < 0.02$) while SCC (late) control (○) decreased significantly (−58 ± 18 μA; $P < 0.02$). The net increase (aldo-control) was 135 ± 38 μA ($P < 0.01$) during the late phase. *The total mineralocorticoid response on SCC.* SCC (total) = ($SCC_7 - SCC_o$) control = 344 ± 64 μA ($P < 0.001$). The early response (198 μA) represents 59% of the total response and the late response (136 μA) 41% of the total SCC response. By contrast, *R* (late) aldo (●) and *R* (late) (○) did not change significantly (−19 ± 9 Ω; and +34 ± 17 Ω; $P < 0.1$) and R_7 reached almost exactly the same level observed at R_o. The *total* response of *R R* (total) = ($R_7 - R_o$) aldo − ($R_7 - R_o$) control was −175 ± 25 Ω; $P < 0.001$. The early and the late response on *R* represent 70 and 30% of the total response, respectively

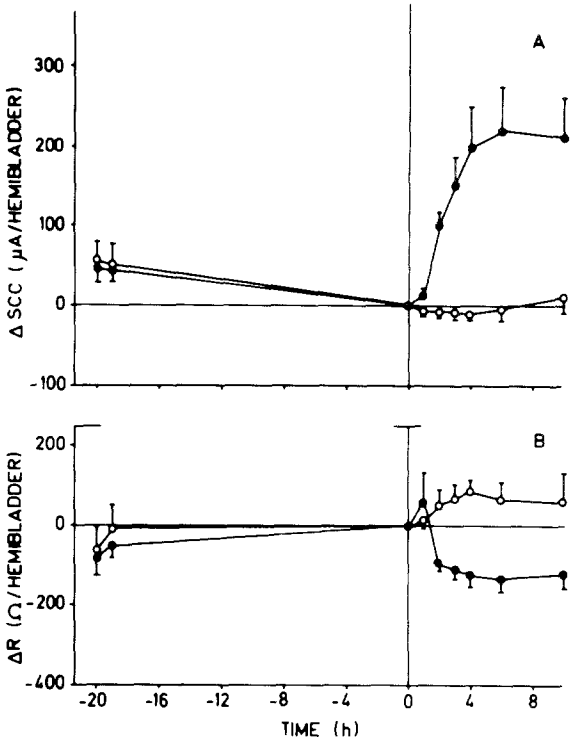

Fig. 2. Effects of aldosterone on transepithelial Na^+ transport (*A*) and tissue resistance (*B*) after preincubation of bladders for 20 hr in steroid-free medium. SCC_o control (○) = 69 ± 9 μA; SCC_o aldo (●) = 80 ± 13 μA (n = 10 pairs, $P < 0.3$); R_o control (○) = 365 ± 36 Ω; R_o aldo (●) = 373 ± 49 Ω ($P < 0.8$). *From t_o to $t_{2\,1/2}$ (early response)*: SCC (early) aldo (●) increased significantly (+125 ± 27 μA; $P < 0.005$) while SCC (early) control (○) did not change significantly (−9 ± 6 μA; $P < 0.3$). The net difference (aldo-control) was +134 ± 30 ± 30 μA ($P < 0.005$). *R* (early) aldo (●) fell significantly (−104 ± 20 Ω; $P < 0.005$) while *R* early control (○) increased (+70 ± 35 Ω; $P < 0.1$). The net difference (aldo-control) was −174 ± 41 Ω ($P < 0.005$). *From $t_{2\,1/2}$ to t_6 (late response)*: SCC (late) aldo (●) increased significantly (+97 ± 31 μA, $P < 0.02$) while SCC control (○) did not change (+6 ± 8 μA; $P < 0.5$). The net difference (aldo-control) was 91 ± 30 μA ($P < 0.02$). *R* (late) aldo (●) and *R* (late) control did not change significantly (−31 ± 15 Ω and −8 ± 45 Ω, respectively). The net difference (aldo—control) −23 ± 20 Ω was not significant. The *total* response of SCC was SCC (total) = ($SCC_6 - SCC_o$) aldo − ($SCC_6 - SCC_o$) control = 255 ± 59 μA ($P < 0.005$). The *early* (125 μA) accounted for 56% and the *late* response for 44%, respectively, of the total SCC response. The *total* response of *R R* (total) = ($R_6 - R_o$) aldo − ($R_6 - R_o$) control was −197 ± 60 Ω ($P < 0.001$. The early responses (−174 Ω) accounted for 78% and the late response (−23 Ω) for 22% respectively, of the total *R* response

set of hemibladders 2 hr before the addition of aldosterone, the Na^+ event was clearly impeded, but only after 6 to 8 hr of incubation (Fig. 4). Characteristically the drop in tissue resistance observed with aldosterone in the first 3 hr was not affected by T_3, thus indicating that the hormone operated rather in the late phase of the Na^+ transport response. We tested this hypothesis further by preincubating the tissue in the presence of T_3 for 16 hr before addition of aldosterone.

As shown in Fig. 5, the Na^+ transport response was inhibited after 3 to 4 hr but again the drop in

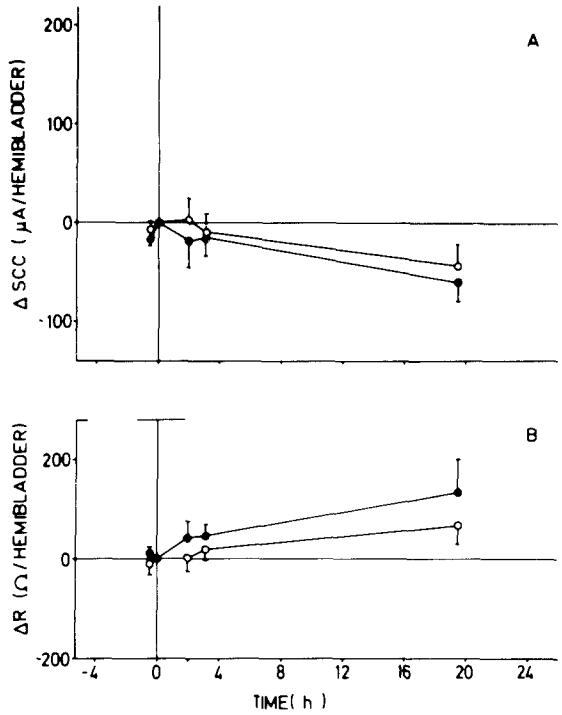

Fig. 3. Effects of triiodothyronine (T_3) on base-line Na^+ (*A*) and tissue resistance (*B*). T_3 (6 nM) was added at time 0 hr to test hemibladders (●) and diluent to paired controls (○). SCC and *R* were computed as described in Materials and Methods. SCC_o control (○) 156 ± 16 μA; SCC_o T_3 (●) = 166 ± 18 μA (n = 10 pairs, $P < 0.3$); $SCC_{20\,hr} = -16 \pm 16$ μA ($P < 0.3$); R_o control (○) = 317 ± 33 Ω; R_o T_3 (●) = 290 ± 29 Ω ($P < 0.3$); $R_{20\,hr} = 66 \pm 40$ Ω ($P < 0.2$)

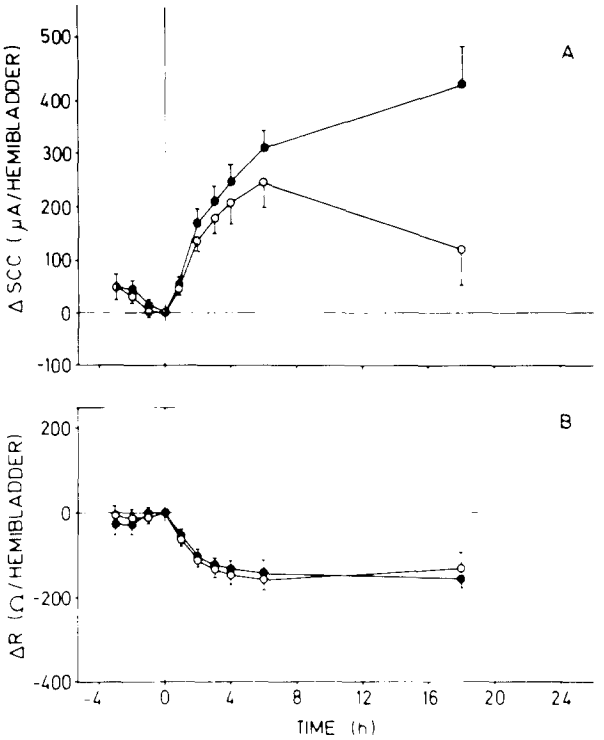

Fig. 4. Effects of T_3 on the action of aldosterone on transepithelial Na^+ transport and tissue resistance. T_3 was added at time −2 hr to test hemibladders (○) and diluent to paired controls (●). At time 0 hr aldosterone (80 nM) was added to both sets of hemibladders. *R* and SCC were computed as described in Materials and Methods. SCC_o aldo (●): 165 ± 24 μA; SCC_o aldo + T_3 (○): 162 ± 29 μA (n = 10 pairs; $P < 0.9$); R_o aldo (●): 334 ± 28 Ω; R_o + T_3 (○): 380 ± 40 Ω ($P < 0.3$); $SCC_{4\,hr}$ (test-control) = −40 ± 29 μA ($P < 0.2$); $R_{4\,hr}$ (test-control) = −20 ± 21 Ω ($P < 0.4$); $SCC_{18\,hr}$ (test-control) = −314 ± 44 μA ($P < 0.001$); $R_{18\,hr}$ (test-control) = +45 ± 49 Ω ($P < 0.4$)

tissue resistance was not at all antagonized by T_3. This experiment was repeated with a 10-fold higher concentration of T_3 which produced similar results. However, such high concentrations of T_3 acting during long incubation periods tended to significantly increase the tissue resistance above control values, as was shown in a companion paper (Rossier et al., 1983*a*).

Effects of T_3 Analogs on Base-Line and Aldosterone-Dependent Na^+ Transport

The specificity of the observed antagonism was assessed by testing 2 analogs of T_3 for their biological activity. i) rT_3, which has been shown to be biologically inactive in a number of experimental systems (Koerner et al., 1975) and which exhibits no affinity for the nuclear T_3 binding sites, and ii) $isoT_2$, which is a most potent thyromimetic drug with respect to both biological activity and ability to displace T_3 from its nuclear binding sites.

As shown in Fig. 6, rT_3 was biologically inactive in our experimental system at a concentration (6 nM) at which T_3 nearly displayed its full activity. On the other hand, $isoT_2$ at the same concentration (6 nM) effectively antagonized the late aldosterone response qualitatively similar to T_3 (Fig. 7).

Since base-line SCC values at t_o were comparable in all experimental hemibladders (Figs. 1, 3–7) and since T_3, rT_3, and $isoT_2$ groups were never significantly different from control groups, it was possible to construct a composite graph in which data are expressed in absolute values as μA per hemibladder (Fig. 8). Five experimental groups are represented: aldosterone, aldosterone + rT_3, aldosterone + T_3, aldosterone + $isoT_2$ and "Controls" (control, T_3, rT_3, $isoT_2$ groups combined). The following order of antimineralocorticoid activity can tentatively be established: $isoT_2 \geq T_3 > rT_3$. It

should be noted that even with the most active antagonist ($isoT_2$), SCC values were always significantly higher than in control conditions. Thus, it seems that thyromimetic drugs are not able to completely abolish the mineralocorticoid response.

Reversibility of the Antagonistic Activity of T_3

In this experiment one of two sets of hemibladders was pretreated for 20 hr with 6 nM T_3. The hemibladders were then extensively washed during 2 hr before challenging both sets with aldosterone. As shown in Fig. 9, the antimineralocorticoid activity persisted even in the absence of T_3. Typically, the effect of aldosterone on R was not at all modified by T_3 pretreatment while SCC was clearly inhibited.

Relationship Between Plasma T_4 and the Mineralocorticoid Response Measured *in Vitro*

In order to assess the physiological role of thyroid hormone as a potential antagonist *in vivo* of aldosterone, we measured plasma T_4 in 3 groups of animals at different times of the year and then checked the mineralocorticoid response in the same animals. As shown in the Table, mean plasma T_4 varied significantly from time to time over a fivefold range (0.6 to 3.1 nM). In all 3 groups, aldosterone elicited a significant response ($P < 0.001$), but the magnitude of the response varied significantly over a twofold range. The mineralocorticoid response was inversely related to the plasma T_4 concentration, suggesting that the aldosterone-T_3 interaction might also occur *in vivo*. The aldosterone response measured in the absence of thyroid hormone *in vitro* suggests that the effect of thyroid hormone has a long half-life and is not rapidly reversed by the incubation in a T_3-free medium.

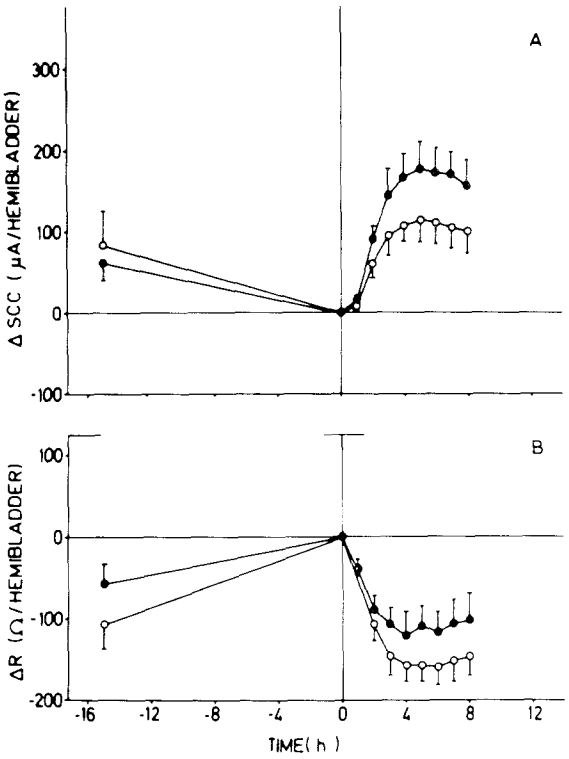

Fig. 5. Effects of T_3 on the action of aldosterone on transepithelial Na^+ transport (A) and tissue resistance (B). T_3 (6 nM) was added at time −16 hr to test hemibladders (○) and diluent to paired controls (●). At time 0 hr aldosterone (80 nM) was added to both sets of hemibladders. SCC and R were computed as described in Materials and Methods. SCC_o test (○) = 121 ± 17 μA; SCC_o control (●) = 97 ± 18 μA ($n = 10$; $P < 0.2$); R_o test (○) = 380 ± 34 Ω; R_o control (●) = 302 ± 36 Ω ($P < 0.05$); $SCC_{2\,hr}$ (test-control) = −31 ± 12 μA ($P < 0.05$); $SCC_{7\,hr}$ (test-control) = −67 ± 18 μA ($P < 0.01$); $R_{2\,hr}$ (test-control) −21 ± 21 Ω ($P < 0.4$); $R_{7\,hr}$ (test-control) = −47 ± 30 Ω ($P < 0.1$)

Discussion

Time Course of Aldosterone Effect on Sodium Transport and Electrical Resistance

In the present experimental conditions, the electrophysiological parameters (SCC and R) could be followed up for long periods of time. The 3 distinct phases in the action of aldosterone—that is a latent period, an early response with an increase of SCC and a fall of R, and finally a late response with further increase of SCC and little change of R—are easily recognized (Figs. 1 and 2). Our data confirm the study of Spooner and Edelman (1975) obtained in a different experimental setup (SCC and R were measured in classic Ussing's chambers over a 6-hr period). The concept of a dual action of aldosterone has been questioned by others (Civan & Hoffmann, 1971). We feel, however, that our incubation conditions are closer to the physiological ones and permit a better conservation of tissue properties without bacterial contamination. In addition, bladders mounted as sacks have a higher base-line resistance (Walser, Butler & Hammond, 1969) and are less subject to edge damage—an important point when studying the effects of aldosterone on tissue resistance.

It should be pointed out that the early and the late responses are clearly continuous and overlapping processes. The beginning of the early response is easily defined by the end of the latent period and

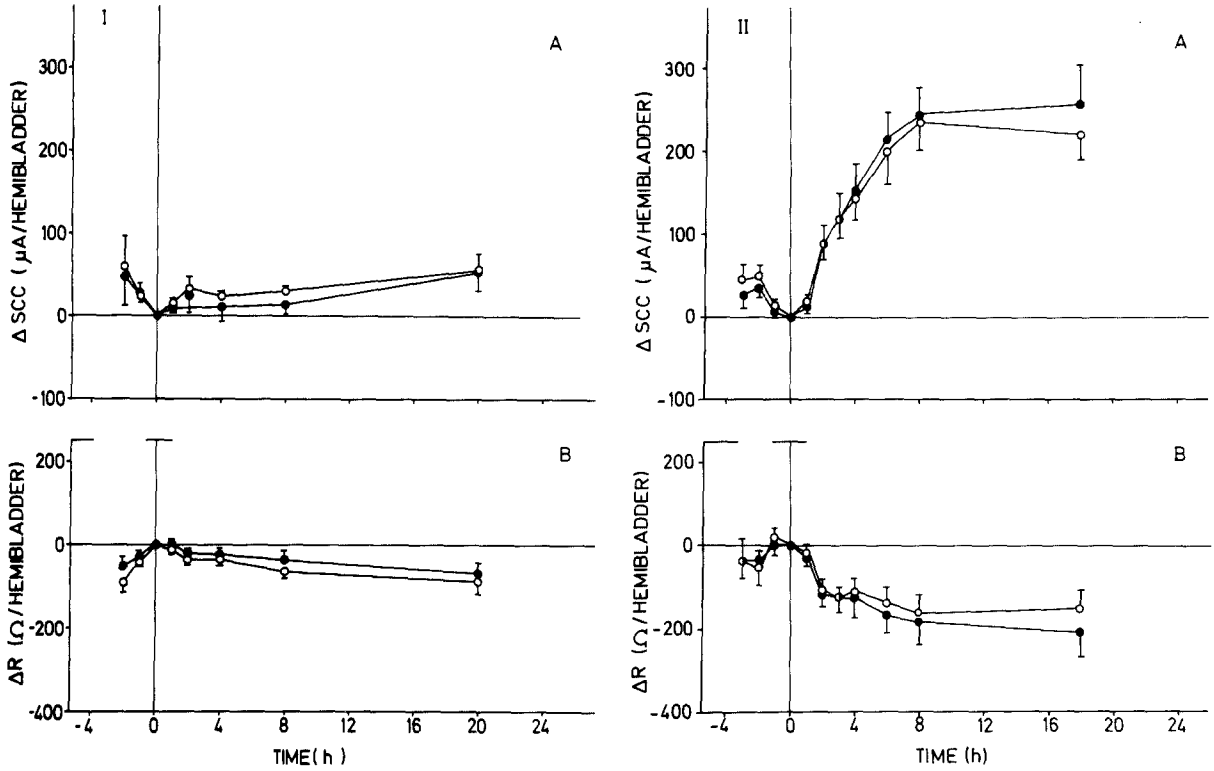

Fig. 6. Effects of reverse T_3 (rT_3) on base-line Na^+ transport (*IA*) and tissue resistance (*IB*) and effects of rT_3 on the action of aldosterone on transepithelial Na^+ transport (*IIA*) and tissue resistance (*IIB*). rT_3 (6 nM) was added at time −2 hr to test hemibladders (●) and diluent to paired controls (○). At time 0 hr aldosterone (80 nM) was added to both sets of hemibladders (*II*). SCC and *R* were computed as described in Materials and Methods. *Panel IA:* SCC_o control (○) = 158 ± 40 μA (diluent); SCC_o test (●) = 146 ± 26 μA (n = 10, $P < 0.6$) (rT_3); $SCC_{20\,hr}$ (test-control) = −16 ± 32 μA ($P < 0.6$). *Panel IB*: R_o control (○) = 370 ± 24 Ω (diluent); R_o test (●) = 368 ± 37 Ω ($P < 0.9$) (rT_3); $R_{20\,hr}$ (test-control) = −10 ± 25 Ω ($P < 0.8$). *Panel IIA*: SCC_o control (●) = 142 ± 50 μA (aldo alone); SCC_o test (○) = 144 ± 61 μA (n = 10, $P < 0.9$) (aldo + rT_3); $SCC_{18\,hr}$ (test-control) = −37 ± 35 μA ($P < 0.3$). *Panel IIB*: R_o control (●) = 414 ± 55 Ω (aldo alone); R_o test (○) = 418 ± 50 Ω (aldo + rT_3); $R_{18\,hr}$ (test-control) = +61 ± 62 Ω ($P < 0.3$)

is characterized by the classic fall in *R*. The end of the early response is variable and occurs 2 to 4 hr after aldosterone addition when the effect of aldosterone on *R* is near maximum. The beginning of the late response cannot be easily determined. It certainly overlaps the early phase since the overall response *appears* monotonous. Data from Figs. 5 and 9 would suggest that this phase can start as early as 120 min after aldosterone addition. Interestingly, in the toad bladder of *Bufo bufo*, the two phases are easily distinguished (Rossier et al., *unpublished observations*). The end of the late response can be defined when the maximum overall response is reached. Again the time at which this maximum is observed is quite variable: from as early as 6 hr in some experiments (Figs. 1, 2, 5, 9) to as late as 18 hr in others (Figs. 4 and 6). The reasons for the large variations observed in the 3 phases of aldosterone action (i.e., latent period, early and late response) remain obscure.

The existence of two effects of aldosterone which can be separated on an electrophysiological basis raises interesting questions concerning the mechanism of action of the hormone. First, is the late response specific to the steroidal action? In other words, is it related to a primary effect of aldosterone or is it only secondary to the early increase in sodium permeability, which would directly trigger an overall adaptive change in the capacity of the epithelium to transport sodium? Second, how should we revise our current hypothesis on the manner in which aldosterone acts at the subcellular level? We would like to discuss the effect of thyromimetic drugs in this context since these drugs might in the long run shed some light on this problem.

Thyroid Hormones and Related Analogs Antagonize Selectively the Late Mineralocorticoid Response

As shown previously, T_3 (6 nM) and related analogs had no significant effect on SCC or *R* over a 20-hr period of incubation (Fig. 3). However, they inhibited a significant fraction of the aldosterone-dependent SCC with no change of *R* (Figs. 4 and 5). Even after 18 hr of exposure to T_3, the early response to aldosterone was not inhibited. The effect of T_3 was specific to thyromimetic drugs since $isoT_2$ was active whereas rT_3 was not. It appears that the early

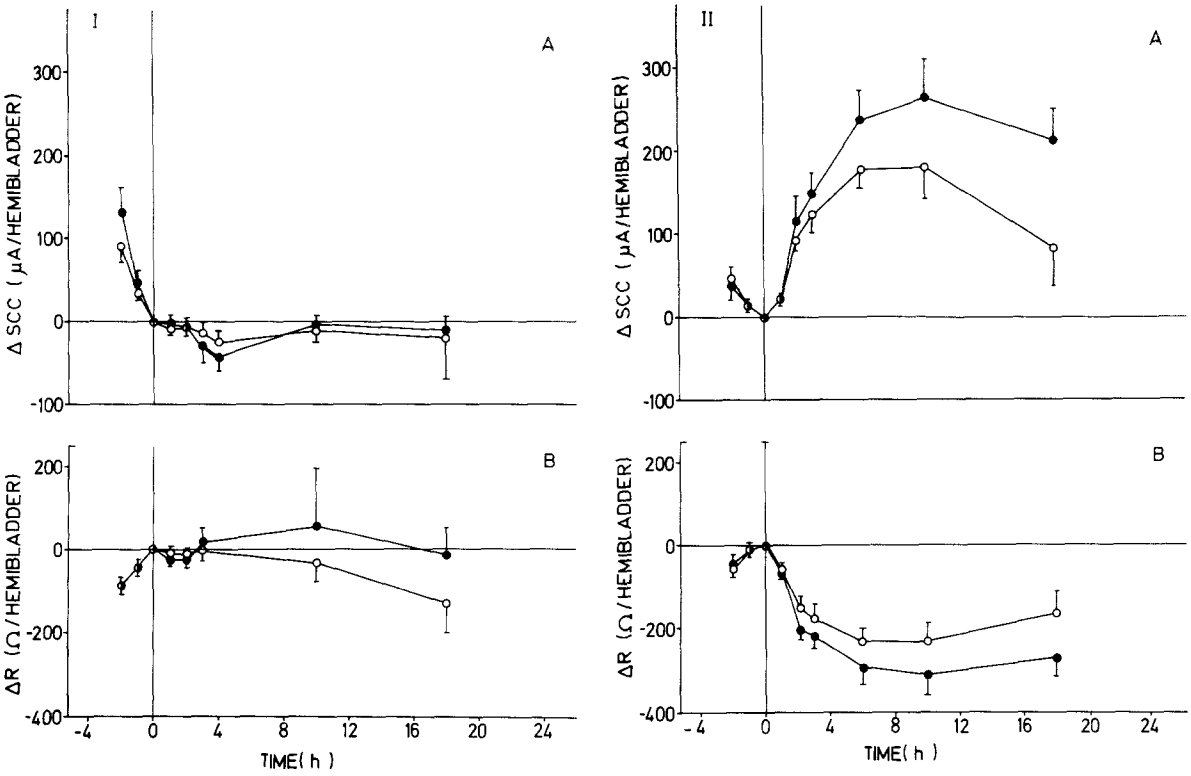

Fig. 7. Effects of isopropyldiiodo-thyronine ($isoT_2$) on base-line Na^+ transport (*IA*) and tissue resistance (*IB*) and effects of $isoT_2$ on the action of aldosterone on transepithelial Na^+ transport (*IIA*) and tissue resistance (*IIB*). $IsoT_2$ was added at time −2 hr to test hemibladders (●) and diluent to paired controls (○) (*I*). At time 0 hr aldosterone (80 nM) was added to both sets of hemibladders (*II*). SCC and *R* were computed as described in Materials and Methods. *Panel IA:* SCC_o control (○) = 123 ± 54 μA (diluent); SCC_o test (●) = 105 ± 22 μA ($n = 10$, $P < 0.7$) (iso − T_2); $SCC_{20\,hr}$ (test-control) = +11 ± 51 μA ($P < 0.9$). *Panel IB*: R_o control (○) = 469 ± 68 Ω (diluent); R_o test (●) = 391 ± 27 Ω ($P < 0.2$) (iso − T_2); $R_{20\,hr}$ (test-control) = +118 ± 85 Ω ($P < 0.2$). *Panel IIA*: SCC_o control (●) = 98 ± 15 μA (aldo alone); SCC_o test (○) = 97 ± 21 μA ($n = 10$, $P < 0.9$) (aldo + iso − T_2); $SCC_{3\,hr}$ = −25 ± 25 μA ($P < 0.4$): $SCC_{18\,hr}$ = −133 ± 39 μA ($P < 0.01$). *Panel IIB:* R_o control (●) = 535 ± 44 Ω (aldo alone); R_o test (○) = 512 ± 47 Ω (aldo + iso − T_2); $R_{3\,hr}$ = +38 ± 31 Ω ($P < 0.2$); $R_{18\,hr}$ = +106 ± 38 Ω ($P < 0.025$)

Table. Relationship between plasma T_4 of adult *Bufo Marinus* toads and the mineralocorticoid response measured *in vitro* in the urinary bladder of the same animal

Group	Time	N	Plasma T_4[a] (nM + SE)	Na^+ transport response[b]		ΔSCC test-control (ΔμA at t 6 hr) ± SE	*P* (paired *t* test)
				SCC_o control (μA ± SE)	SCC_o test (μA ± SE)		
A	April	15	1.6 ± 0.2	125 ± 25	154 ± 45	81 ± 17	$P < 0.001$
B	July	16	0.6 ± 0.2	114 ± 19	113 ± 18	159 ± 33	$P < 0.001$
C	October	15	3.1 ± 0.1	98 ± 11	108 ± 13	68 ± 9	$P < 0.001$

[a] Plasma T_4 was measured by radioimmunoassay as described in Methods. All groups differ significantly from each other (unpaired Student's *t* test): A *vs.* B, $P < 0.005$; B *vs.* C, $P < 0.001$; A *vs.* C, $P < 0.001$.

[b] The sodium transport response (aldosterone 10 nM at t_o) was measured in paired hemibladders from each group, incubated *in vitro* as described in Methods. $SCC_{test\text{-}control}$ is the mineralocorticoid response at t_{6h}. The significance of the mean differences *between* groups was estimated by unpaired Student's *t* test: A *vs.* B $P < 0.05$; A *vs.* C $P < 0.6$; B *vs.* C $P < 0.025$. At t_o no groups were significantly different from each other.

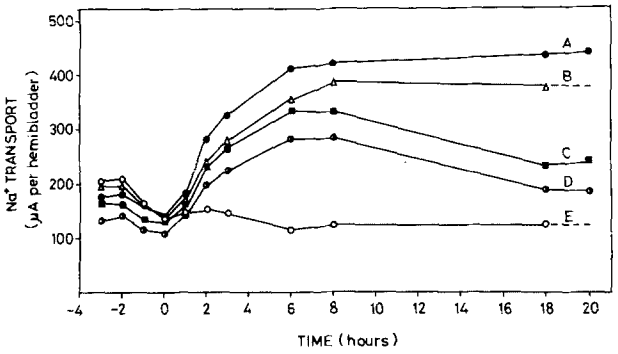

Fig. 8. Mineralocorticoid activity of aldosterone in the toad bladder and antagonism of T_3 and thyromimetic drugs. 6 nM rT_3 (*B*), T_3 (*C*) or $isoT_2$ (*D*) were added to hemibladders at time −2 hr and diluent to paired controls (*A*, *E*). At time 0 hr, 80 nM aldosterone was added to *A*, *B*, *C* and *D*. Na^+ transport is expressed as short-circuit current in absolute values. Data are from experiments shown in Figs. 1, 3, 4, 6 and 7 and pooled as defined in Materials and Methods. (*A*) Group Aldo; (*B*) Group rT_3; (*C*) Group T_3; (*D*) Group $isoT_2$; (*E*) Group "control." *Unpaired t-test: A vs. B*, not significant; *A vs. C*, *D*, or *E*, $P < 0.01$ or less; *C vs. E*, $P < 0.001$; *D vs. E*, $P < 0.01$

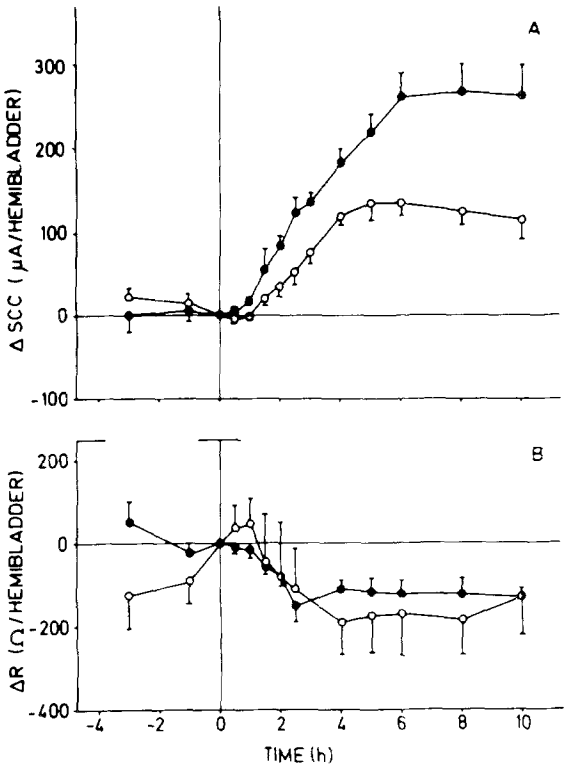

Fig. 9. Lack of reversibility of T_3 antagonism on aldosterone-induced Na^+ transport and tissue resistance. T_3 (6 nM) was added at time −20 hr to one set of hemibladders (○) and diluent to controls (●). At time −4 hr both sets of hemibladders were washed up to time 0 hr when aldosterone (80 nM) was added to both sets of hemibladders (○, ●). SCC and *R* are computed as described in Materials and Methods. SCC_o control (●) = 112 ± 17 μA; SCC_o test (○) = 84 ± 23 μA ($n = 10$; $P < 0.2$); $SCC_{2\,hr}$ = +47 ± 13 μA ($P < 0.01$); $SCC_{10\,hr}$ = +148 ± 36 μA ($P < 0.005$). R_o control (●) = 336 ± 22 Ω; R_o test (○) = 550 ± 121 Ω ($n = 10$; $P < 0.1$); $R_{2\,hr}$ = −4 ± 131 Ω ($P < 0.9$); $R_{10\,hr}$ = −1 ± 118 Ω ($P < 0.9$)

increase in sodium permeability and the fall in resistance cannot simply and directly trigger a secondary and adaptive change in transepithelial sodium transport. Interestingly, another drug, sodium butyrate, which can modify gene expression (probably at the transcriptional level) (Truscello, Geering, Gaeggeler & Rossier, 1983) is also able to selectively antagonize the late mineralocorticoid response without altering the early response (Rossier, Truscello & Geering, 1982). Antimineralocorticoid drugs previously described completely inhibit the aldosterone response: spirolactone by interaction at receptor level (Rossier et al., 1983*b*), actinomycin D by blocking transcription and cycloheximide by inhibiting protein synthesis (Edelman, Bogoroch & Porter, 1963).

Our present results indicate that the aldosterone response is complex and pleiotropic. Like glucocorticoids (Ivarie, Morris & Eberhardt, 1980), aldosterone might regulate the gene expression of at least 2 sets of proteins (Truscello et al., 1983). The induction of early gene products would not be sensitive to T_3 or sodium butyrate, while the induction of the late ones would.

How Does T_3 Control the Late Mineralocorticoid Response?

The present data cannot offer an answer to this question but, together with the identification of a specific nuclear binding site for thyroid hormone in the toad bladder, are consistent with the following induction hypothesis: binding of T_3 to specific nuclear acceptor sites is followed by induction of RNAs and proteins responsible for its antimineralocorticoid effect. The apparent lag time for the effect of T_3 (about 6 to 8 hr) is compatible with what is known about the protein induction controlled by thyroid hormone. The lack of reversibility after a 4-hr washout speaks for the induction of proteins with rather long half-lives.

With the techniques presently available (for example two-dimensional electrophoresis gel) it will be possible to examine the occurrence of thyroid-induced protein in this tissue. One obvious function of such a protein which can be easily studied in this tissue would be the control of mineralocorticoid receptors. The results of this first attempt to characterize the mechanism of the aldosterone-T_3 interaction will be reported in a companion paper.

This work was supported by grant no. 3-646-80 from the Swiss National Science Foundation.

References

Civan, M.M., Hoffman, R.E. 1971. Effect of aldosterone on electrical resistance of toad bladder. *Am. J. Physiol.* **220:**324–328

Crabbé, J. 1977. The mechanism of action of aldosterone. *In:* Receptors and Mechanism of Action of Steroid Hormones. J.R. Pasqualini, editor. Part 2, pp. 513–568. M. Dekker, New York, Basel

Edelman, I.S., Bogoroch, R., Porter, G.A. 1963. On the mechanism of action of aldosterone on sodium transport: The role of protein synthesis. *Proc. Natl. Acad. Sci. USA* **50:**1169–1177

Geering, K., Rossier, B.C. 1981. Thyroid hormone-aldosterone antagonism on Na^+ transport in toad bladder. Evidence for a triiodothyronine nuclear receptor. *J. Biol. Chem.* **256:**5504–5510

Greenberg, A.H., Najjar, S., Blizzard, R.M. 1974. Effects of thyroid hormone on growth, differentiation, and development. *In:* Handbook of Physiology, Section 7: Endocrinology, Vol. 3: Thyroid. M.A. Greer and D.H. Solomon, editors. pp. 377–389. American Physiological Society, Washington, D.C.

Ivarie, R.D., Morris, J.A., Eberhardt, N.L. 1980. Hormonal domains of response: Actions of glucocorticoid and thyroid hormones in regulating pleiotropic responses in cultured cells. *In:* Recent Progress in Hormone Research. R.O. Greep, editor. Vol. 36, pp. 195–239. Academic Press, New York

Koerner, D., Schwartz, H.L., Surks, M.I., Oppenheimer, J.H. 1975. Binding of selected iodothyronine analogues to receptor sites of isolated rat hepatic nuclei. High correlation between structural requirements for nuclear binding and biological activity. *J. Biol. Chem.* **250:**6417–6423

Ludens, J.H., Fanestil, D.D. 1976. The mechanism of aldosterone function. *Pharmacol. Ther. B* **2:**371–412

Marver, D. 1980. Aldosterone action in target epithelia. *Vitam. Horm. (N.Y.)* **38:**55–117

Oppenheimer, J.H., Surks, M.I. 1975. Biochemical basis of thyroid hormone action. *In:* Biochemical Actions of Hormones. G. Litwack, editor. Vol. 3, pp. 119–157. Academic, New York

Porter, G.A., Bogoroch, R., Edelman, I.S. 1964. On the mechanism of action of aldosterone on sodium transport: The role of RNA synthesis. *Proc. Natl. Acad. Sci. USA* **52:**1326–1333

Rossier, B.C., Claire, M., Oblin, M.E., Gaeggeler, H.P., Geering, K. 1983*a*. Effects of thyroid hormones and aldosterone on mineralocorticoid binding sites in the toad bladder. *J. Membrane Biol.* **77:**25–32

Rossier, B.C., Claire, M., Rafestin-Oblin, M.E., Geering, K., Gaeggeler, H.P., Corvol, P. 1983*b*. Binding and antimineralocorticoid activities of spirolactones in toad bladder. *Am. J. Physiol.* **244:**C24–C31

Rossier, B.C., Gaeggeler, H.P., Brunner, D.B., Keller, I., Rossier, M. 1979*a*. Thyroid hormone-aldosterone interaction on Na^+ transport in toad bladder. *Am. J. Physiol.* **236:**C125–C131

Rossier, B.C., Geering, K., Gaeggeler, H.P., Claire, M., Corvol, P. 1980. Testosterone: A specific competitive antagonist of aldosterone in the toad bladder. *Am. J. Physiol.* **239:**F433–F439

Rossier, B.C., Rossier, M., Lo, C.S. 1979*b*. Thyroxine and Na^+ transport in toad: Role in transition from poikilo- to homeothermy. *Am. J. Physiol.* **236:**C117–C124

Rossier, B.C., Truscello, A., Geering, K. 1982. Antimineralocorticoid effect of sodium butyrate and triiodothyronine: Evidence for two pathways in the action of aldosterone on Na^+ transport in the toad bladder. *In:* Biochemistry of Kidney Functions, INSERM Symposium No. 21. F. Morel, editor. pp. 225–232. Elsevier, Amsterdam

Snedecor, G.W., Cochran, W.G. 1971. Statistical Methods. (6th ed.) The Iowa State University Press, Ames

Spooner, P.M., Edelman, I.S. 1975. Further studies on the effect of aldosterone on electrical resistance of toad bladder. *Biochim. Biophys. Acta* **406:**304–314

Tata, J.R. 1974. Growth and developmental action of thyroid hormones at the cellular level. *In:* Handbook of Physiology, Section 7: Endocrinology, Vol. 3: Thyroid. M.A. Greer and D.H. Solomon, editors. pp. 469–478. American Physiological Society, Washington, D.C.

Truscello, A., Geering, K., Gaeggeler, H.P., Rossier, B.C. 1983. Effects of butyrate on histone deacetylation and aldosterone-dependent Na^+ transport in the toad bladder. *J. Biol. Chem.* **268:**3388–3395

Walser, M., Butler, S.E., Hammond, V. 1969. Reversible stimulation of sodium transport in the toad bladder by stretch. *J. Clin. Invest.* **48:**1714–1723

Received 5 January 1983; revised 12 May 1983