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Effects of Thyroid Hormones and Aldosterone on Mineralocorticoid Binding Sites in the Toad Bladder

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Summary. In the urinary bladder of the toad *Bufo marinus* triiodothyronine selectively inhibits the late effect of aldosterone on Na^+ transport. We have investigated whether T_3 might mediate its antimineralocorticoid action by controlling: i) the level of aldosterone binding sites in the soluble (cytosolic) pool isolated from tissues treated with T_3 (60 nM) for up to 20 hr of incubation; ii) the kinetics of uptake of ^{3}H-aldosterone into cytoplasmic and nuclear fractions after 2 or 20 hr of exposure to T_3. The number and the affinity of Type I (high affinity, low capacity) and Type II (low affinity, high capacity) cytosolic binding sites (measured at 0°C) did not vary significantly after 18 hr of exposure to T_3, while aldosterone-dependent Na^+ transport was significantly inhibited. In addition, T_3 did not modify the kinetics of uptake (90 min) of ^{3}H-aldosterone into cytoplasmic and nuclear fractions of toad bladder incubated *in vitro* at 25°C. By contrast, aldosterone itself was able to down-regulate its cytosolic and nuclear binding sites after an 18-hr exposure to the steroid hormone (10 or 80 nM). T_3 slightly (20%) but significantly potentiated the down regulation of nuclear binding sites. In conclusion, T_3 does not appear to have major effects on the regulation of the aldosterone receptor, which could explain in a simple manner its antimineralocorticoid action.

Key Words aldosterone · triiodothyronine · sodium transport · mineralocorticoid receptors · *bufo marinus* · down regulation

Introduction

In previous studies (Rossier, Rossier & Lo, 1979; Rossier et al., 1979), we have shown that thyroid hormones inhibited the aldosterone-dependent Na^+ transport *in vivo* and *in vitro*. We have demonstrated that this antagonism is probably mediated by specific nuclear receptors for triiodothyronine (T_3) (Geering & Rossier, 1981). The binding of T_3 to a nuclear receptor could lead to the induction of proteins (Ivarie, Morris & Eberhardt, 1980) which in turn could interfere at different steps of the action of aldosterone (Ludens & Fanestil, 1976; Crabbé, 1977; Marver, 1980): i) a *pretranscriptional step*, that is the binding of aldosterone to its cytoplasmic sites and/or the binding of the receptor-steroid complex to chromatin acceptor sites; ii) a *transcriptional step*, namely by inhibition of the synthesis of specific mRNAs coding for the aldosterone-induced proteins (AIPs); iii) a *translational step*, by prevention of the synthesis of AIPs; iv) a *post-translational step*, by interaction with the physiological expression of AIPs at specific cellular sites (i.e., plasma membranes, mitochondrion, etc.). In the present paper we have examined whether T_3 could antagonize the effects of aldosterone at a pretranscriptional level, by modulating the level and/or the affinity of mineralocorticoid binding sites which can be assessed in the urinary bladder of the toad and correlated with the sodium transport experiments (Rossier et al., 1983).

Materials and Methods

Reagents

1,2-^{3}H-aldosterone (specific activity 44 to 53 Ci/mM) was from Amersham. Aldosterone was a generous gift from Ciba-Geigy. All reagents were identical to those described in a previous paper (Geering, Gaeggler & Rossier, 1983).

Animals

Adult male and female *Bufo marinus* toads were obtained from C.P. Chase, Miami, Florida or from C. Sullivan, Nashville, Tennessee. They were handled before experiments as described (Geering et al., 1983).

Sodium Transport Measurement

Perfusion of the animal, incubation of the hemibladders, and measurement of electrophysiological parameters (short-circuit current, SCC; potential difference, PD; total tissue resistance, R) were carried out according to our previous publications (Rossier

et al., 1980). After one hour of preincubation, 3 different experimental protocols were used:

Protocol A: T_3 vs. Control

T_3 (60 nM) was added at $t_{-2\,hr}$ in both mucosal and serosal media incubating one set of hemibladders (test) and the diluent added (NaOH) to the paired controls.

Protocol B: (T_3 + Aldosterone) vs. Aldosterone

Protocol B was identical to protocol A but at t_0 aldosterone (80 nM) was added in both mucosal and serosal media incubating both sets of hemibladders.

Protocol C: Aldosterone vs. Control

Aldosterone (80 nM) was added to one set of hemibladders (test) at t_0 and the diluent (ethanol) to the paired controls.

SCC and PD were measured at $t_{-2\,hr}$, t_0 and $t_{18\,hr}$. Results are expressed as SCC/hemibladder and $R \cdot$ hemibladder as described (Rossier et al., 1980).

At $t_{18\,hr}$, the tissues were cut off the glass canulas with a razor blade and washed 3 times in 50 ml of Ringer's (aldosterone-free) solution over a period of 120 min at 25°C. All subsequent steps were carried out at 0 to 2°C. The epithelial cells were scraped off the underlying tissue and their content in mineralocorticoid binding sites was measured.

Measurement of Aldosterone Binding Parameters in Cytosolic Fractions (Cell-Free, at 0°C)

Scraped epithelial cells were washed once in 5 ml of Ringer's solution and once in 5 ml of homogenization solution (solution A) containing: N-tris-(hydroximethyl)-methyl-2-amino-ethane (TES) 10 mM (pH 7.0 at 25°C), EDTA 1 mM, thioglycerol 12 mM, KCl 25 mM, sucrose 185 mM. EDTA was previously found to be critical for maximal recovery of mineralocorticoid binding sites (Rossier et al., 1983). Cells were ground in liquid nitrogen in a precooled mortar. Powdered cells were then transferred to a centrifuge tube and 500 μl of solution A (at 25°C) were added. Dithiothreitol (1 mM) was immediately added to the thawed sample which was then vigorously vortexed. The cytosolic fraction was obtained after 7 min centrifugation at 48,000 × g. The cytosolic fraction was quickly adjusted (by addition of solution A) to a final protein concentration between 3 and 5 mg/ml: protein content was estimated by UV spectrophotometry at 260/280 nm (Warburg & Christian, 1942). 120-μl aliquots were then incubated for 4 hr at 0°C in presence of increasing concentrations of ^{3}H-aldosterone (from 0.2 to 500 nM). Bound and free hormone were separated by the dextran-charcoal technique (Rossier et al., 1980). Binding parameters at equilibrium (4 hr) (Kd in nM = affinity, N_{max} in fM/mg cytosolic protein = maximum number of binding sites) were estimated as described (Claire et al., 1978), using a 5-parameter model:

$$B = \frac{Kd\,\mathrm{I} \cdot U}{N_{max}\,\mathrm{I} + U} + \frac{Kd\,\mathrm{II} \cdot U}{N_{max}\,\mathrm{II} + U} + \beta U$$

where B = bound aldosterone, U = unbound hormone = Total bound − Bound, Kd I = affinity for the first set of binding sites, N_{max} I = number of the first set of binding sites, Kd II and N_{max} II = affinity and number of the second set of binding sites, β slope of the nonspecific binding sites. In 3 control experiments, Kd I was 0.23, 0.46 and 0.26 nM, N_{max} I 40, 27 and 47 fmol/mg protein; Kd II 90, 50 and 60 nM, N_{max} II 354, 176 and 685 fmol/mg protein.

Data are displayed for convenience as Scatchard's plot. Preliminary experiments showed that T_3 could not compete directly with the binding of aldosterone to an isolated fraction at 0°C.

Uptake, Washout and Exchange of ^{3}H-Aldosterone in Bladders Incubated in Vitro at 25°C

The incubation and the preparation of cytoplasmic and nuclear fractions were prepared as described (Rossier et al., 1980):

Protocol D: Uptake of ^{3}H-Aldosterone after 2 or 18 Hours of Incubation in Presence of T_3

Five to 6 homologous pools of tissues were incubated with or without T_3 (60 nM) for 2 or 18 hr. The incubation was then continued in the presence of ^{3}H-aldosterone (10 nM) for various periods of time (5 to 90 min, at 25°C). Nonspecific binding was estimated at 90 min by measuring the ^{3}H-aldosterone bound in the presence of a 1000-fold excess of unlabeled hormone.

Protocol E: Washout of ^{3}H-Aldosterone from Tissue Labeled in Vitro at 25°C

The time-course of washout of labeled hormone was measured over a 240-min period in pools of tissues prelabeled with aldosterone during 120 min.

Protocol F: Exchange of Aldosterone at 25°C

To check whether bound aldosterone in the intact bladder was rapidly and freely exchangeable, we preincubated pools of tissue for 120 min in the presence of 10 nM ^{3}H-aldosterone or 10 nM unlabeled aldosterone. Then the media were exchanged (labeled *vs.* unlabeled and vice versa) and binding of ^{3}H-aldosterone in the cytoplasmic and nuclear fractions followed for the next 240 min.

Thin-Layer Chromatography (TLC)

The purity of ^{3}H-aldosterone was tested before use by TLC as described (Claire et al., 1979). ^{3}H-radioactivity recovered in the

incubation medium or in the cytoplasmic fraction were analyzed by the same method.

Results

Effects of T_3 on Base-Line and Aldosterone-Dependent Sodium Transport and Binding Parameters (Protocols A-C)

In this series of experiments, we measured the *Kd* and N_{max} of cytosolic binding sites isolated from tissues preincubated in 3 conditions (T_3 *vs.* Control; Aldosterone + T_3 *vs.* Aldosterone; Aldosterone *vs.* Control), thus verifying the effect of thyroid hormone on SCC and *R*. In a first attempt to detect an effect, we used a supramaximal concentration of T_3 (60 nM), a dose 10-fold higher than that used in a previous paper (Geering et al., 1983).

T_3 vs. Control (Protocol A)

After a 20-hr exposure to T_3, base-line SCC was slightly but not significantly inhibited (Table 1). However, as T_3 had relatively less effect on PD, there was a significant increase in *R*, which was not observed at concentrations of T_3 of 6 nM or less (Geering et al., 1983).

Figure 1 (panel *A*) displays the binding data measured in the same tissue at the end of the incubation as described in Materials and Methods. The Scatchard plot is curvilinear, suggesting heterogeneity of binding sites, but T_3 did not modify the plot. The best model describing the binding data involves 2 types of binding sites and a nonspecific component: Type I with a high affinity for aldosterone (*Kd* I = 0.3 nM), low capacity (N_{max} = 27 fmol/mg protein) and Type II with a low affinity (*Kd* II = 51 nM), high capacity (176 fmol/mg protein). Quite clearly, T_3, even at this rather high concentration, had no significant effect on the binding parameters. A second experiment (n = 12 paired hemibladders) without Na^+ transport measurements gave identical results (*data not shown*).

(Aldosterone + T_3) vs. Aldosterone (Protocol B)

The fact that T_3 alone had no effect on mineralocorticoid binding sites does not rule out the possibility that T_3 could regulate them, but only in presence of aldosterone. T_3 (60 nM) was able to significantly antagonize the aldosterone-dependent SCC and PD. As before, T_3 significantly increased *R* compared to the controls (aldosterone alone) (Table 1). The Scatchard plot (Fig. 1, panel *B*) and the binding parameters of this experiment indicate that again T_3 had no major effect on these conditions. An interesting observation was, however, an apparent decrease in the number of Type I binding sites, suggesting that aldosterone itself, in the course of an 18-hr incubation, could down regulate its own receptor. This was further investigated in Protocol C.

Aldosterone vs. Control (Protocol C)

As expected, aldosterone significantly increased aldosterone-dependent SCC and PD. At the end of the incubation, and as for Protocols A and B, tis-

Table 1. Effect of T_3 on transepithelial sodium transport (SCC), potential difference (PD) and tissue resistance (*R*)[a]

Condition	*n* (pairs)	SCC (μA ± SE)			*P*[b]	PD_0	PD (MV ± SE)		*P*[b]	R_0	*R* (Ω ± SE)		*P*[b]
		SCC_0	SCC_{18}-SCC_0	ΔSCC_{18} test-cont			PD_{18}-PD_0	ΔPD_{18} test-cont			R_{18}-R_0	ΔR_{18} test-cont	
Protocol A:													
Control		181 ± 29	−95 ± 21			48.2 ± 5.9	−15.2 ± 4.6			290 ± 23	+144 ± 26		
	10			−55 ± 30	$P > 0.1$			−3.1 ± 3.1	$P > 0.4$			+189 ± 54	$P < 0.01$
T_3		212 ± 48	−150 ± 45			47.5 ± 6.5	−17.8 ± 5.5			252 ± 18	+333 ± 71		
Protocol B:													
Aldo		303 ± 49	+138 ± 82			60.1 ± 6.7	+19.6 ± 8.1			215 ± 19	+21 ± 24		
	10			−245 ± 84	$P < 0.02$			−29.9 ± 8.8	$P < 0.01$			+99 ± 36	$P < 0.025$
Aldo + T_3		254 ± 29	−107 ± 27			53.1 ± 5.3	−10.2 ± 4.0			225 ± 20	+120 ± 29		
Protocol C:													
Control		124 ± 15	−22 ± 9			36.9 ± 4.4	+2.4 ± 3.5			305 ± 19	+113 ± 28		
	9			+192 ± 28	$P < 0.001$			+31.6 ± 3.4	$P < 0.001$			−157 ± 36	$P < 0.01$
Aldo		141 ± 28	+170 ± 22			37.3 ± 5.0	+34 ± 4.3			290 ± 20	−43 ± 16		

[a] Sodium transport (SCC), potential differences (PD) and tissue resistance (*R*) were determined as described in Materials and Methods.
[b] Student's paired *t*-test.

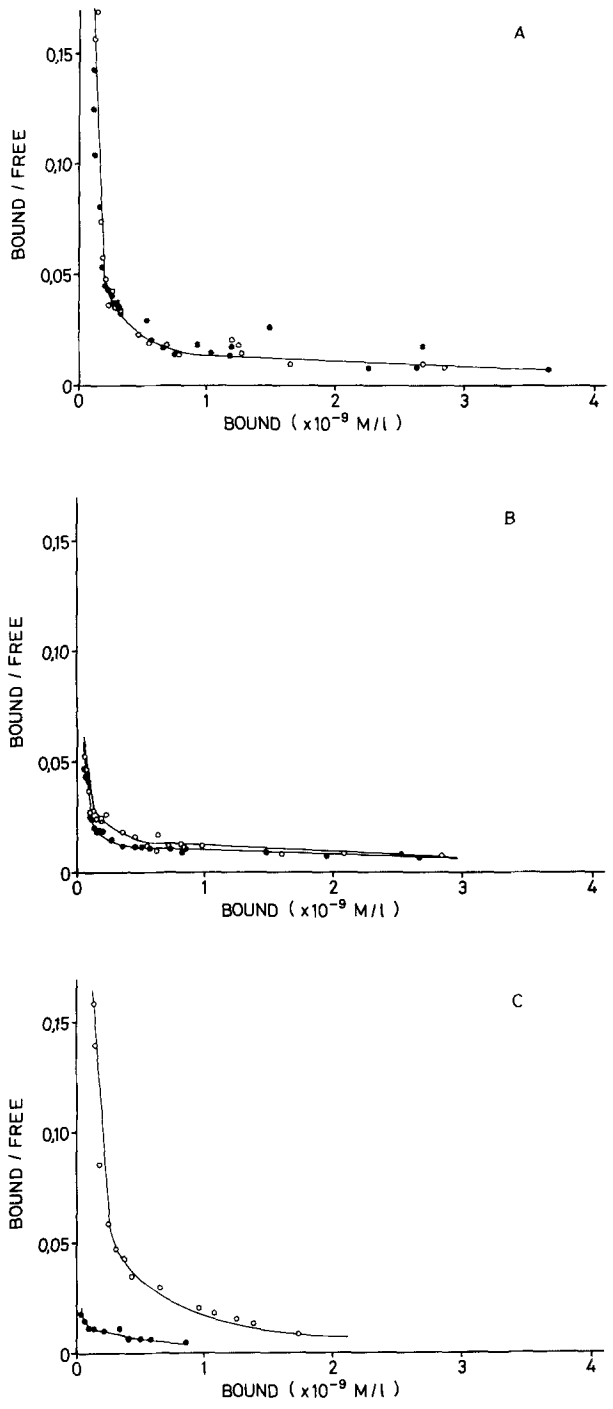


Fig. 1. Scatchard plots of binding assays performed in cytosol of toad bladder epithelial cells from Protocols A to C and prepared as described in Materials and Methods. Binding parameters (Kd_I, Kd_{II}, N_I, N_{II}) and curve fitting were computed as described in Materials and Methods. Kd_I and Kd_{II} varied nonsignificantly from 0.2 to 0.8 nM and from 60 to 150 nM, respectively, in the various experimental conditions. *Panel A* T_3 (●) *vs.* Control (○): $N\max_I$ (○): 40 ± 3 fmol/mg prot *vs.* $N\max_I$ (●): 33 ± 3 fmol/mg prot (n.s.); $N\max_{II}$ (○): 354 ± 46 fmol/mg prot *vs.* $N\max_{II}$ (●): 425 ± 47 fmol/mg prot (n.s.); β (nonspecific) was 0.0049 (± 0.0007) in (○) *vs.* 0.0040 (± 0.0007) in (●). *Panel B* Aldo + T_3 (●) *vs.* Aldo (○): $N\max_I$ (○): 9 ± 1 fmol/mg prot *vs.* $N\max_I$ (●): 12 ± 1 fmol/mg prot (n.s.); $N\max_{II}$ (○): 271 ± 21 fmol/mg prot *vs.* $N\max_{II}$ (●): 195 ± 26 fmol/mg prot (n.s.); β (○) was 0.0043 ± 0.0005 *vs.* (●) 0.0047 ± 0.0003 (n.s.). *Panel C* Aldo (●) *vs.* control (○): $N\max_I$ (○): 47 ± 1 fmol/mg prot *vs.* $N_{max\,I}$ (●): 2 ± 1 fmol/mg prot; $N\max_{II}$ (○): 685 ± 15 fmol/mg prot *vs.* $N\max_{II}$ (●): 185 ± 20 fmol/mg prot. The more than 20-fold and 30-fold decrease of $N\max_I$, respectively, in test tissues were both significantly different from control values ($P < 0.01$). β (●) was 0.0005 ± 0.0002 *vs.* (○) 0.0013 ± 0.0005 (n.s.)

sues were washed extensively in aldosterone-free medium for 120 min.

In this experiment (Fig. 1, panel *C*) one can observe a marked decrease in the content of Type I binding sites which amounted to only 5% of the control values and to a lesser extent in that of Type II binding sites (40% of control). In order to ensure that most of the endogenously bound aldosterone was removed by our procedure, we first checked whether aldosterone can be easily and completely exchanged (Protocol D) and compared the time course of washout of labeled hormone to its uptake (Protocol E).

As shown in Fig. 2 (panels *A* and *B*), aldosterone (10 nM) was readily exchangeable in the intact tissue at 25°C in the two types of experiment (labeled hormone exchanged against unlabeled hormone, panel *A*, or the reverse order, panel *B*). Clearly the cytoplasmic pool was exchanged at a faster rate than the nuclear pool, but equilibrium was surely reached within 120 min for both pools. As shown in Fig. 3 (panel *A*), the cytoplasmic uptake of ^{3}H-aldosterone was so rapid that maximum binding was already observed at the earliest time point (1 min). By contrast the nuclear uptake could be easily resolved kinetically. Half-maximum saturation (10 nM aldosterone) occurred at 15 min and an apparent steady state was reached within 120 min. If the tissue was washed out at this time (Fig. 3, panel *B*), the disappearance of ^{3}H-aldosterone was extremely rapid and complete within 40 min. The disappearance of ^{3}H-aldosterone from the nuclear pool proceeded at a slower rate which reached an apparent steady state after 120 min, which was, however, just above the nonspecific background. From these data, a 120-min washout would appear to be quite sufficient to remove aldosterone specifically bound to the cytoplasmic sites.

Effects of T_3 on the Kinetics of Uptake of ^{3}H-Aldosterone

So far, we have not excluded the possibility that T_3 may prevent the nuclear uptake of the steroid-re-

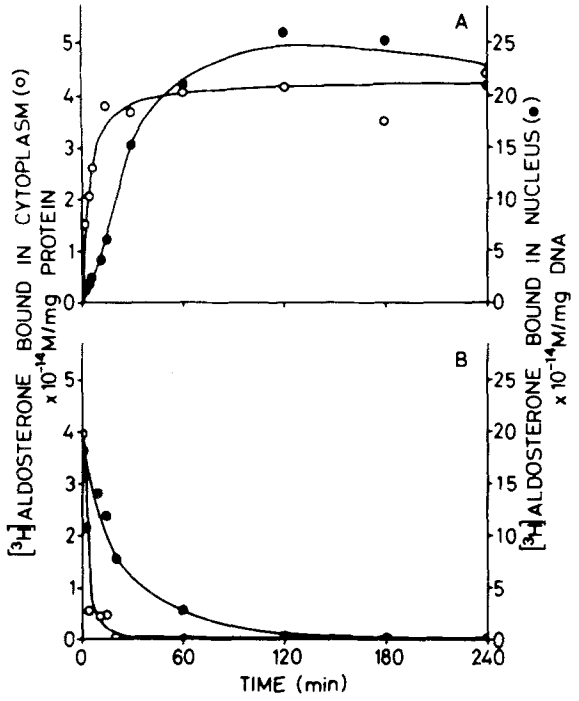


Fig. 2. Exchange of aldosterone in intact bladders incubated *in vitro* at 25°C. (protocol D). *Panel A.* Homologous pools of tissue were preincubated for 120 min at 25°C in the presence of 10 nM unlabeled aldosterone. At t_0, media with unlabeled hormone were exchanged against an identical medium containing 10 nM ^{3}H-aldosterone and the incubation continued for various periods of time (*abscissa*). On the *left ordinate*, the amount of ^{3}H-aldosterone specifically bound to the cytoplasm (○) is indicated. On the *right ordinate*, the amount of ^{3}H-aldosterone specifically bound in nucleus (●) is indicated. *Panel B.* The protocol is identical to that of Panel A but the tissues were first incubated in medium containing the labeled hormone and replaced at t_0 with unlabeled medium

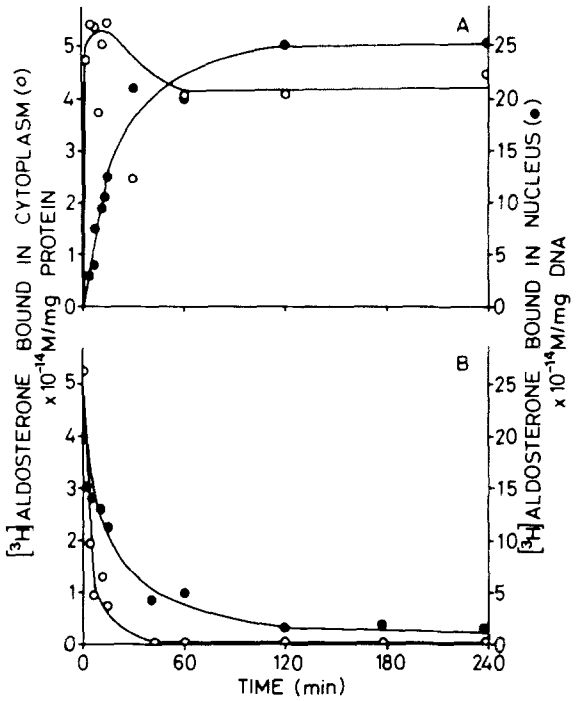


Fig. 3. Kinetics of uptake of ^{3}H-aldosterone and washout of labeled aldosterone in intact bladders incubated *in vitro* at 25°C. *Panel A.* After a 120-min preincubation in steroid-free medium, the uptake of ^{3}H-aldosterone into the cytoplasmic (○) and nuclear (●) pool was followed for up to 240 min. *Panel B.* After a 120-min preincubation in the presence of 10 nM ^{3}H-aldosterone, the washout of the labeled hormone from the cytoplasmic (○) and nuclear (●) pools was followed for up to 240 min

ceptor complex. We have therefore measured this uptake in tissues which have been exposed to T_3 for either 2 or 18 hr. As shown in Fig. 4, T_3 did not have any detectable effect.

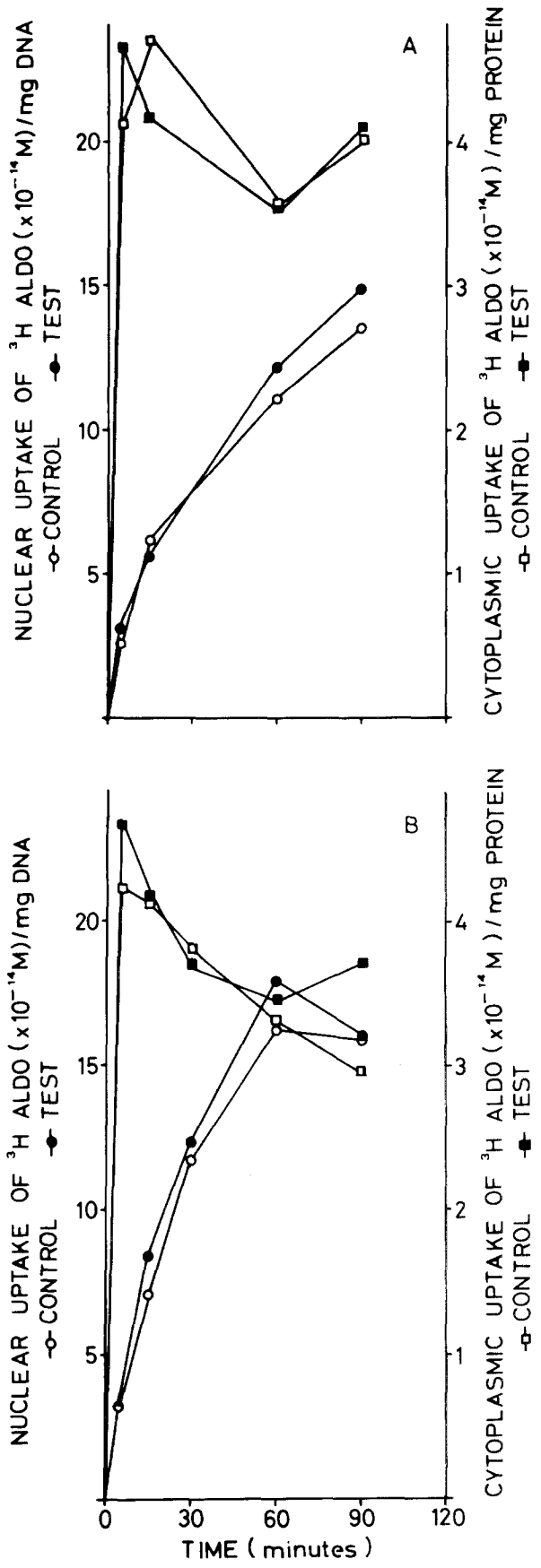
Effects of T_3 on the Down Regulation of Nuclear Binding Sites Induced by Aldosterone

We incubated homologous pools of tissue with ^{3}H-aldosterone (10 nM) for 18 hr with or without T_3 (60 nM). The data of seven paired experiments (Table 2) indicate that not only the cytoplasmic but also the nuclear binding sites were two- to threefold lower than the tissues in which the uptake was measured after 2 or 18 hr of preincubation (data from Figs. 2, 3 and 4 for comparison). In addition, T_3 potentiated the down regulation of mineralocorticoid binding sites induced by aldosterone. The difference between the two cytoplasmic pools was not significant but there was a small (−20%) but significant ($P < 0.005$) difference in the nuclear pool. This decrease was not merely due to ligand metabolism, since analysis of ^{3}H-radioactivity both in the incubation media and the cytoplasmic fractions showed that more than 90% of the radioactivity migrated at the same position (by TLC) as an unlabeled aldosterone standard. T_3 did not change the chromatography pattern.

Discussion

The T_3-Aldosterone Antagonism Cannot be Simply Explained by a Major Effect on the Regulation of Mineralocorticoid Binding Sites

With two different experimental approaches, we were unable to detect any significant effect of T_3 on aldosterone binding sites. Within the limits of our present technology, one would like to conclude that



T_3 interacts with the effects of aldosterone at a step beyond the receptor-steroid interaction with chromatin acceptor sites. Of course negative findings should always be taken with caution since the methods presently available for studying the aldosterone receptor are still limited, pending its purification and the availability of specific probes such as antibodies detecting the molecule independently of its binding properties. A positive finding was the potentiation of the down regulation of nuclear binding sites induced by aldosterone (Table 2). We feel that this effect is not likely to play an important role in the observed antagonism. First, its magnitude, in absolute and relative terms, is small compared to the effect of aldosterone alone. Second, the marked down regulation of Type I and to a lesser extent that of Type II binding sites induced by aldosterone alone was not accompanied by a proportional decrease in aldosterone-dependent Na^+ transport measured after 18 hr of incubation. In other words, the down regulation of the physiological response might be delayed and become apparent only at a later time, since the aldosterone-dependent sodium transport clearly depends not only directly on the occupancy and the number of receptors available, but also on the biological half-lives of the two major intermediates, namely mRNAs and AIPs.

Down Regulation of the Mineralocorticoid Binding Site by Aldosterone

Our data (Fig. 1C and Table 2) strongly suggest that aldosterone might regulate its own receptor in the toad bladder. The present results are in accordance with the findings of Claire and his colleagues (1981) obtained in the rat using *in situ* perfusion of the kidneys. As in the present study, Type I binding sites appeared to be down regulated more than Type II. As our experimental system allows to correlate biochemical and physiological events in the same tissue, it will be clearly interesting to study, in the future, the relationship between the down regulation of mineralocorticoid binding sites and its physiological counterpart, namely the possible down regulation of aldosterone-dependent Na^+ transport.

Fig. 4. Effect of T_3 on the cytoplasmic and nuclear uptake of ^{3}H-aldosterone in intact bladders preincubated either for 2 hr (Panel *A*) or for 18 hr (Panel *B*) in the presence of T_3 (60 nM). On the left ordinate, ^{3}H-aldosterone specifically bound to the cytoplasm is denoted by squares, □ (control) and ■ (T_3). On the right ordinate ^{3}H-aldosterone specifically bound to the nucleus is denoted by circles, ○ (control) and ● (T_3)

Table 2. Effect of T_3 (60 nM) on the binding of ^{3}H-aldosterone after an 18-hr incubation in the presence of the labeled hormone, compared with the 90-min uptake of ^{3}H-aldosterone with no prior aldosterone incubation

Condition	*n*	^{3}H-aldosterone specifically bound in:					
		Soluble pool (cytoplasm) ($\times 10^{-14}$ mol/mg prot)			Nucleus ($\times 10^{-14}$ mol/mg DNA)		
		18-hr uptake[a]	90-min uptake[b]		18-hr uptake[a]	90-min uptake[b]	
			A	*B*		*A*	*B*
Control	7 pairs[a]	1.96 ± 0.25	4.02	2.96	6.93 ± 1.50	13.8	15.8
T_3		1.67 ± 0.22	4.15	3.71	5.54 ± 1.35	15.1	16.0
ΔT-Control		−0.29 ± 0.29			−1.39 ± 0.31		
		$P > 0.4$			$P < 0.005$		

[a] 18-hr ^{3}H-aldo uptake: Cell fractionation was carried out as described in Materials and Methods. Specific binding is the amount of bound ^{3}H-aldosterone which can be displaced by a 1000-fold excess of unlabeled aldosterone. Nonspecific binding (20% of total binding in the soluble pool and 5% in the nucleus) was not significantly modified by T_3. Values are mean ± SE, *P* refers to comparison of Control and T_3 by paired Student's *t*-test.

[b] 90-min ^{3}H-aldo uptake: Values from Fig. 4 are given for comparison. A: 2-hr preincubation (± T_3) (panel *A*, Fig. 4); B: 18-hr preincubation (± T_3) (panel *B*, Fig. 4).

In conclusion, in order to understand the mechanism of the T_3-aldosterone antagonism, we should concentrate our efforts on the possibility that T_3-induced protein(s) act at the transcriptional and/or translational level. We have recently demonstrated that aldosterone is an inducer of (Na^+, K^+)-ATPase in the toad bladder (Geering et al., 1982). By contrast, T_3 was totally inactive and did not prevent the induction of the sodium pump by aldosterone. Recently a number of aldosterone-induced proteins have been identified by two-dimensional gel electrophoresis (Geheb, Huber, Hercker & Cox, 1981). Such a system might be useful in the future to approach this problem, and preliminary experiments indicate that T_3 might selectively regulate the rate of synthesis of several aldosterone-induced proteins (Truscello, Geering, Gaeggeler & Rossier, 1983).

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