J. Membrane Biol. 89, 173–183 (1986)

The Journal of
Membrane Biology

Thyroid Hormone Antagonizes an Aldosterone-Induced Protein: A Candidate Mediator for the Late Mineralocorticoid Response

A. Truscello, H.P. Gäggeler, and B.C. Rossier
Institut de Pharmacologie de l'Université de Lausanne, CH-1011 Lausanne, Switzerland

Summary. In the urinary bladder of the toad *Bufo marinus*, the basal rate of synthesis of a number of proteins was modulated in a bidirectional way (i.e., induced or repressed) by aldosterone and by triiodothyronine (T_3). Each hormone was therefore characterized by a distinct domain of response. When both hormones were added simultaneously, the two domains consistently overlapped at least for one protein, termed AIP-1, or aldosterone-induced protein 1 ($M_r \sim 65$ kilodaltons, $p_i = 6.7$, as analyzed by two-dimension gel electrophoresis). The physiological role of AIP-1 is unknown, but could be related to the late mineralocorticoid response. In five experiments, T_3 (60 nM, 18-hr incubation) consistently repressed AIP-1, while aldosterone-dependent sodium transport (late response) was significantly inhibited, as previously described. The repression of AIP-1 was also observed as early as 6 hr after aldosterone addition. In addition, sodium butyrate (3 mM), which was previously shown to also selectively inhibit the late mineralocorticoid response, was also able to repress AIP-1. Our results suggest that AIP-1 is one of the proteins involved in the mediation of the late mineralocorticoid response.

Key Words *Bufo marinus* · triiodothyronine · hormonal domain · aldosterone · sodium butyrate · transepithelial sodium transport

Introduction

In the urinary bladder of the toad *Bufo marinus*, we have shown that thyroid hormones inhibited the aldosterone-dependent Na^+ transport *in vivo* and *in vitro* (Rossier et al., 1979*a*, *b*; Geering et al., 1984). This antagonism appears to be mediated by specific nuclear receptors for triiodothyronine (T_3) (Geering & Rossier, 1981). The binding of T_3 to nuclear receptors is assumed to lead to the induction and/or repression of specific proteins (Ivarie et al., 1980) which could, in turn, interfere at different steps of the action of aldosterone (Rossier et al., 1984): 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 mRNA's coding for the aldosterone-induced proteins (AIP's); iii) *a translational step*, by prevention of the synthesis of AIP's; iv) *a post-translational step*, by interaction with the physiological expression of AIP's at specific cellular sites (i.e. plasma membrane, mitochondrion, etc.). In a previous paper (Rossier et al., 1984), we did not obtain any firm evidence for the first possibility, i.e. T_3 did not appear to display major effects on the regulation of the aldosterone receptor, which could explain in a simple manner its antimineralocorticoid action.

In the present study, we examined whether T_3 could antagonize in a selective manner the induction of proteins specifically controlled by aldosterone (AIP's).

Aldosterone is able to induce a number of cytosolic, membrane or mitochondrial proteins (Benjamin & Singer, 1974; Scott & Sapirstein, 1975; Edelman, 1978; Law & Edelman, 1978*a*,*b*; Geheb et al., 1981; Geering et al., 1982). This action is best detected by two-dimensional polyacrylamide gel electrophoresis (2D gel). As shown recently (Geheb et al., 1981), aldosterone consistently induced proteins (p_i 5.8 to 6.4, M_r 70 to 80 kD) which can be recovered either from a membrane-rich fraction or (at least for one of them) from the cytosolic fraction.

In the present study, we decided to use the same basic approach in order to determine the hormonal domain of aldosterone and T_3 in the toad bladder. Aldosterone-induced (and/or repressed) proteins could be selectively controlled by T_3. Thus, any overlapping in the action of both hormones could be at the site of interaction between the two hormones leading to the antagonism observed at the physiological level, i.e. transepithelial sodium transport.

Materials and Methods

Reagents

^{35}S methionine (1000 Ci/mmol) was from Amersham Corp. Aldosterone was a generous gift from Ciba-Geigy.

Animals

Adult male and female *Bufo marinus* were obtained from C. Sullivan, Nashville, Tennessee. They were handled before experiments as described (Geering et al., 1984).

Sodium-Transport Measurement

Perfusion of the animal, incubation of the hemibladder and measurement of electrophysiological parameters (short-circuit current (SCC) method) were carried out according to our previous publications (Geering et al., 1984). After 1-hr preincubation, six different protocols were used:

Protocol A: Aldosterone vs. Control

After 4-hr preincubation, aldosterone (80 nM) was added at t_0 to one set of hemibladder (test) and the diluent (ethanol) to paired controls. After 18-hr incubation, tissue labeling was started.

Protocol B: T_3 vs. Control

After 1-hr preincubation, T_3 (60 nM) was added at $t_{-3\,hr}$ in both mucosal and serosal media incubating one set of hemibladders (test) and the diluent (NaOH) was added to the paired controls. After 21-hr incubation, tissue labeling was started.

Protocol C: (T_3 + Aldosterone) vs. Aldosterone

Protocol C was identical to Protocol B, but at $t_{0\,hr}$ aldosterone (80 nM) was added in both mucosal and serosal media incubating both sets of hemibladders. PD and SCC were measured at $t_{-3\,hr}$, $t_{0\,hr}$ and $t_{18\,hr}$. Results are expressed as PD, SCC/hemibladders and R. hemibladders as described (Geering et al., 1984).

Protocol D: (T_3 + Aldosterone) vs. Aldosterone (6 hr)

T_3 (60 nM) was added at $t_{-3\,hr}$ in both mucosal and serosal media incubating one set of hemibladders and the diluent (NaOH) was added to the paired controls. At $t_{0\,hr}$, aldosterone (80 nM) was added to both mucosal and serosal media incubating both sets of hemibladders. PD, SCC and *R* were measured at $t_{-2\,hr}$ t_0 and $t_{6\,hr}$, time at which tissue labeling was started.

Protocol E: Butyrate vs. Control (10 hr)

Sodium butyrate (3 mM) was added at $t_{-4\,hr}$ in both mucosal and serosal media incubating one set of hemibladders (test) and the diluent (NaCl) was added to the paired controls. PD and SCC were measured at $t_{-4\,hr}$, t_0 and $t_{6\,hr}$, time at which tissue labeling was started.

Protocol F: (Butyrate + Aldosterone) vs. Aldosterone (6 hr)

Protocol F was identical to Protocol E, except that at t_0, aldosterone (80 nM) was added in both mucosal and serosal media incubating both sets of hemibladders. PD and SCC were measured at $t_{-4\,hr}$, t_0 and $t_{6\,hr}$, time at which tissue labeling was started.

Tissue Labeling and Cell Lysis

At $t_{18\,hr}$, the tissues were cut off the glass canulas with a razor blade and rinsed in fresh medium containing their respective hormones. They were then rinsed three times (3 × 5 min) in 10 ml of medium lacking methionine and finally incubated in 25-ml Erlenmeyer flasks containing 4 ml of media (lacking unlabeled methionine) supplemented with ^{35}S methionine (250 μCi/ml; ~1000 Ci/mmol) and the hormones. One to three hemibladders per flask were incubated at 25°C (pH 7.4, gassed with 5% CO_2/95% O_2) under constant shaking, for 60 min. Preliminary experiments showed that this incubation time was sufficient for proteins to be labeled at a specific activity high enough for analysis within 2 to 12 weeks, but still within the linear uptake of ^{35}S methionine into the total protein pools. This labeling protocol should therefore favor the detection of newly synthetized proteins induced by hormones, independently of any hormonal effect on their rate of degradation. Incorporation was stopped by rapidly cooling the sample on an ice bath, and all subsequent steps were carried out at 0 to 4°C. Epithelial cells were scraped off the underlying tissue and washed twice in 5 ml of bicarbonate-Ringer's solution. In order to determine the domain of each hormone and avoid any problem of recovery, no cell fractionation was used in this study.

Total cell extracts were prepared according to the method described by O'Farrell (1975), either immediately or after storage of the cells for up to 4 weeks in liquid nitrogen. Epithelial cells (from one to three hemibladders) were put into 120 μl sonication buffer. Sonication was performed 4 × 1 sec on a B-12 Branson sonifier (macrotip, position 4) on an ice bath with 10-sec cooling intervals. Then 15 μl DNAse (Sigma, Type I, 1 mg/ml) were added on the ice bath with intermittent vortexing. After 5 min, urea (162 mg, ultrapure) and 150 μl lysis buffer were added and mixed at 0°C until samples were solubilized. Sometimes, small pieces of connective tissue or cell debris were not completely solubilized by this procedure. Therefore, all samples were routinely spun in an Eppendorf centrifuge (at 0°C) for 2 × 2 min.

Supernatants were removed and aliquots (3 × 5 μl) taken and precipitated in 10% TCA for Lowry determination and measurement of incorporation of ^{35}S methionine into TCA-insoluble material. Same amounts of radioactivity (2.5×10^5 to 5×10^5 cpm, 10 to 40 μl aliquot, with no more than 200 μg per sample) were used for 2D gel electrophoresis.

Two-Dimensional Gel Electrophoresis

2D-gels were carried out according to O'Farrell (1975). Equilibrium isoelectric focusing was conducted at 6800 V hr. The second dimension was run on linear (8 to 15%) SDS-polyacrylamide

Table 1. Effects of T_3 on baseline and aldosterone-dependent sodium transport

Condition	n (pairs)	SCC (μA ± SE)			P^d	PD_0	PD (mV ± SE)		P^d
		SCC_0	SCC_{18h}-SCC_0	ΔSCC_{18h} test-cont.			$PD_{18h} - PD_0$	ΔPD_{18h} test-cont.	
Protocol A									
Control	9[a]	124 ± 15	− 22 ± 9	+192 ± 28	$P < 0.001$	36.9 ± 4.4	+ 2.4 ± 3.5	+31.6 ± 3.4	$P < 0.001$
Aldo		141 ± 28	+170 ± 22			37.3 ± 5.0	+34 ± 4.3		
Control	6[b]	193 ± 69	− 78 ± 9	+100 ± 37	$P < 0.05$	45.2 ± 7.8	−12.8 ± 6.8	+25.4 ± 6.2	$P < 0.001$
Aldo		191 ± 60	+ 29 ± 60			44.5 ± 8.0	+12.6 ± 5.1		
Control	15[c]	152 ± 29	− 45 ± 17	+158 ± 22	$P < 0.001$	40.2 ± 4.1	− 3.7 ± 3.8	+29.1 ± 3.2	$P < 0.001$
Aldo		161 ± 29	+113 ± 32			40.2 ± 4.3	+25.5 ± 4.2		
Protocol B									
Control	9[a]	181 ± 29	− 95 ± 21	− 55 ± 30	$P < 0.1$	48.2 ± 5.9	−15.2 ± 4.6	− 3.1 ± 3.1	$P > 0.4$
T_3		212 ± 48	−150 ± 45			47.5 ± 6.5	−17.8 ± 5.5		
Control	6[b]	164 ± 47	− 35 ± 18	− 73 ± 23	$P < 0.02$	45.3 ± 7.8	− 9.1 ± 7.2	−10.5 ± 3.9	$P < 0.05$
T_3		175 ± 52	−108 ± 37			44.5 ± 6.2	−19.6 ± 5.8		
Control	16[c]	174 ± 24	− 70 ± 16	− 60 ± 21	$P < 0.05$	47.1 ± 4.6	−11.9 ± 3.8	− 5.4 ± 2.8	$P > 0.1$
T_3		198 ± 35	−130 ± 31			46.4 ± 4.6	−17.2 ± 4.1		
Protocol C									
Aldo	10[a]	303 ± 49	+138 ± 82	−258 ± 84	$P < 0.02$	60.1 ± 6.7	+19.6 ± 8.1	−29.9 ± 8.8	$P < 0.01$
Aldo + T_3		254 ± 29	−107 ± 26			53.1 ± 5.3	−10.2 ± 4.0		
Aldo	6[b]	156 ± 28	+ 32 ± 30	−132 ± 21	$P < 0.005$	44.6 ± 3.4	+10.4 ± 3.3	−25.8 ± 4.2	$P < 0.005$
Aldo + T_3		178 ± 25	−100 ± 28			44.9 ± 4.2	−15.4 ± 5.6		
Aldo	16[c]	248 ± 37	+ 92 ± 52	−207 ± 55	$P < 0.005$	54.2 ± 4.7	+14.9 ± 5.3	−28.7 ± 5.7	$P < 0.001$
Aldo + T_3		226 ± 25	−115 ± 19			50.0 ± 3.7	−13.8 ± 3.1		

[a] From Rossier et al. 1984; [b] present study; [c] combined data from a and b, sodium transport was determined as described in Materials and Methods; [d] Student's paired t test.

slab gels. Isoelectric points were determined by slicing the cylindral gel (first dimension) into 5-mm sections and measuring the pH after a 10-min extraction into 2 ml of bidistilled water for 1 hr. For the determination of the apparent molecular weight in the second dimension, the following ^{14}C-labeled standards (Amersham) were used: phosphorylase *b* (92.5 and 94 Kd), bovine serum albumine (BSA, 69 Kd), ovalbumin (46 Kd), carbonic anhydrase, 30 Kd), and lysozyme (14.3 Kd).

Definition

Induction, repression and their derivation refer strictly to increases or decreases in the rate of synthesis of individual proteins (Ivarie et al., 1981). Their usage is not intended to imply a transcriptional mechanism of action by the hormones used in the present study.

Results

In order to determine the domain of action of thyroid hormone and aldosterone, it was important to check the sodium transport responses in the same tissues submitted to further biochemical analysis. For each protocol, six pairs of hemibladders were used to measure the sodium transport (measured by the short-circuit current (SCC) method) and their potential difference (PD) at the time of addition of aldosterone ($t_{0\,hr}$) and 18 hr later ($t_{18\,hr}$). Supramaximal doses of T_3 (60 nM) and aldosterone (80 nM) were used throughout the study. Since this protocol is similar to that used in a previous study (Rossier et al., 1984), the SCC and PD of the present study are shown in Table 1 and compared to that previously obtained. The two series gave very similar results. In Protocol A (Aldo *vs.* Control), aldosterone elicited a significant increase of PD and SCC ($\Delta PD_{18\,hr} = +25.4 \pm 6.2$ mV, $P < 0.001$; $\Delta SCC_{18\,hr} = +100 \pm 37$ μA, $P < 0.05$; $n = 6$ pairs). In Protocol B (T_3 *vs.* control), T_3 significantly inhibited baseline SCC and baseline PD ($\Delta PD = 10.5 \pm 3.9$ mV, $P < 0.05$; $\Delta SCC = -73 \pm 23$ μA, $P < 0.02$; $n = 6$), an effect which was observed before but did not reach the level of significance. When combined together, it appears that the effect on PD is not significant ($\Delta PD = -5.4 \pm 2.8$ mV, $P < 0.1$), while there remained a slight significant decrease of SCC ($\Delta SCC = -60 \pm 21$ μA; $P < 0.05$; $n = 16$). As previously shown (Rossier et al., 1979*a*, *b*; Geering et al., 1984), this inhibition of the baseline sodium transport does not appear to be of cytotoxic nature, since T_3 significantly increased the electrical resistance during this period ($\Delta R_{18} = +135 \pm 35\Omega$; $P < 0.001$; $n = 16$). In Proto-

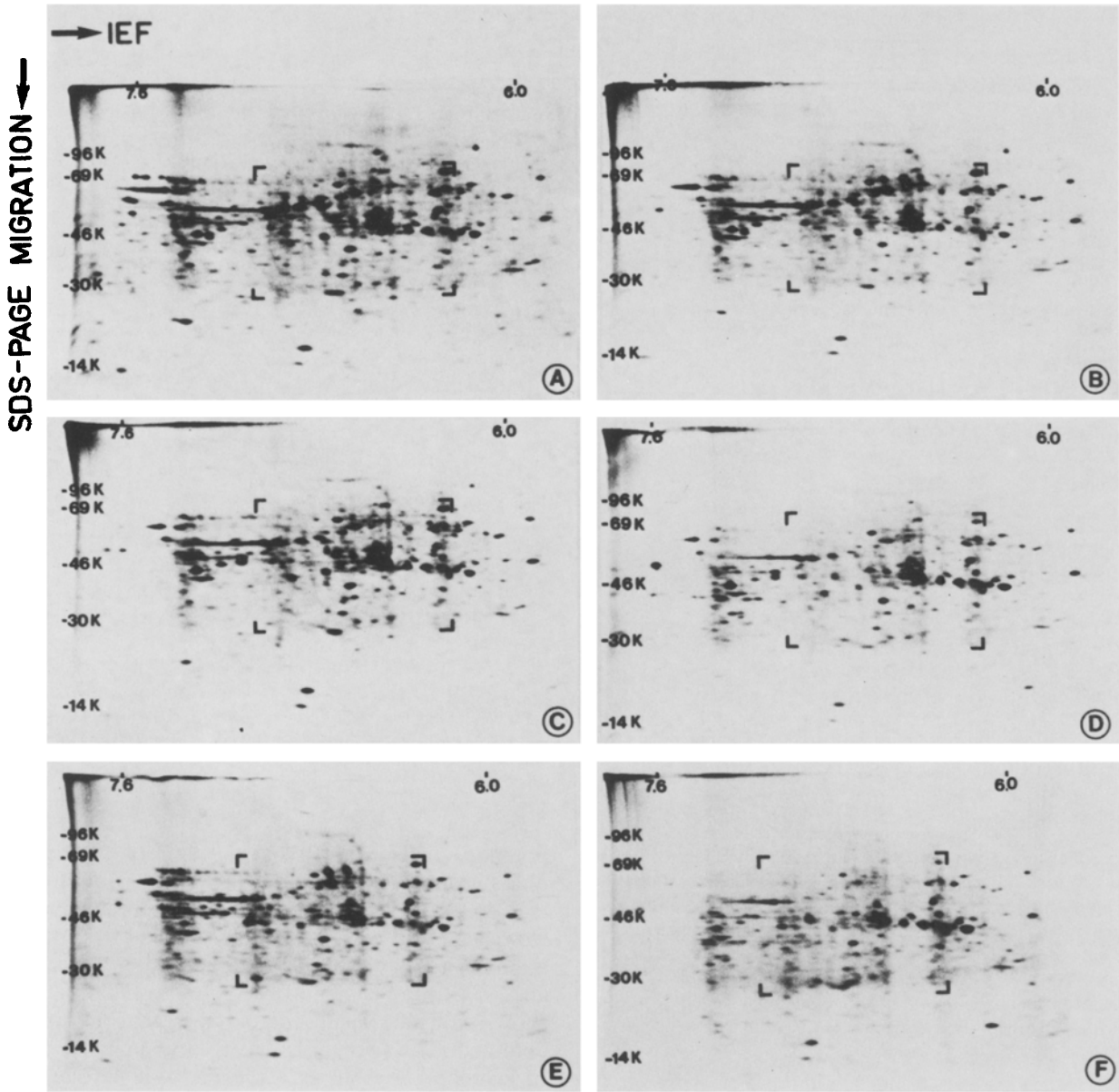

Fig. 1. Two-dimensional gel autoradiograms from toad urinary bladder cell proteins pretreated with aldosterone (18 hr, 80 nM) (Panel *B*) or with diluent (Panel *A*); with T_3 (20 hr, 60 nM) (Panel *D*) or with diluent (Panel *C*); with aldosterone (18 hr) + T_3 (20 hr) (Panel *F*) or with aldosterone alone (18 hr) (Panel *E*). Paired pools of epithelial cells (*A vs. B*; *C vs. D*; *E vs. F*) were labeled with ^{35}S methionine (250 μCi/ml) for 60 min at 25°C and lysed according to O'Farrell (*see* Materials and Methods). Regions of the gel (M_r 30 to 70 kD; p_i 6.2 to 7.0) which are blown up in Fig. 2 are indicated in brackets. Approximate M_r are indicated on the ordinate (SDS-PAGE migration). Approximate isoelectric points are indicated on the abscissa (IEF)

col C (aldosterone *vs.* (aldostèrone + T_3)), T_3 elicited its usual antagonism, significantly inhibiting the aldosterone-dependent PD and SCC ($\Delta PD = -25.8 \pm 4.2$ mV, $P < 0.005$; $\Delta SCC = -132\ \mu A \pm 21\ \mu A$, $P < 0.001$, $n = 6$ pairs). The physiological responsiveness of these tissues to aldosterone and T_3 having been checked, it was possible to determine their domain of action at the translational level (relative rate of synthesis) by labeling the overall proteins with ^{35}S methionine and analyzing the labeled products by 2 D gels, as described in Materials and Methods. In each protocol (A to C), three pairs of hemibladders were analyzed separately, while the three last pairs were pooled before labeling, leading to the analysis of four independent data. The results of a representative experiment are shown in Fig. 1

Table 2. Effects of T_3 (60 nM), aldosterone (80 nM) and sodium butyrate (3 nM) on the incorporation of ^{35}S methionine into the total pool of proteins of toad bladder epithelial cells

Experiment	Tissue	Time of incubation (hr)	^{35}S methionine incorporated[a] into protein Specific activity (cpm · 10^6/mg protein)					
			Control	Aldo	T_3	(Aldo + T_3)	Butyrate	But + T_3
1 Pilot	4 quarters of bladder	18	3.64	3.56	5.21	4.44	—	—
2 Prot A[b]	3 paired hemibladders	18	3.63	4.42	—	—	—	—
			3.31	2.99	—	—	—	—
			6.75	4.74	—	—	—	—
Prot B[c]	3 paired hemibladders	18	2.43	—	3.32	—	—	—
			5.89	—	9.14	—	—	—
			2.97	—	5.55	—	—	—
Prot C[d]	3 paired hemibladders	18	—	1.15	—	1.64	—	—
			—	3.56	—	7.47	—	—
			—	2.79	—	5.54	—	—
3 Prot A[b]	1 pool of 3 paired hemibladders	18	3.97	2.69	—	—	—	—
Prot B[c]	idem	18	3.41	—	2.84	—	—	—
Prot C[d]	idem	18	—	3.54	—	2.31	—	—
Prot D	1 paired hemibladder	6	—	3.44	—	3.88	—	—
Prot E	1 paired hemibladder	6	3.00	—	—	—	3.61	—
Prot F	1 paired hemibladder	6	—	—	—	—	3.71	3.30

[a] Incorporation of ^{35}S methionine (200–250 μCi/ml, specific activity > 1000 Ci/mM) was carried out for 60 min at 25°C as described in Materials and Methods. Epithelial cells were scraped and lysed according to O'Farrell. Five μl aliquots (triplicate) were taken from each homogenate and precipitated overnight with 100 μl of TCA 10% (vol/vol). Precipitates were washed twice in cold 5% TCA and then dissolved in 150 μl NaOH 1N. Forty μl of the dissolved precipitate were neutralized with HCl 1N and ^{35}S radioactivity was counted after addition of 10 ml of Biofluor (Packard®). One hundred μl were used for protein determination according to Lowry. Specific activities were compared in protocols A–C with a Student's paired *t* test in experiments 1 to 4 (18 h).

[b] Protocol A: Control = 4.26 ± 0.63 (×10^6 cpm/mg prot) *vs.* Aldo = 3.68 ± 0.40 (×10^6 cpm/mg prot) (mean ± SE; $n = 5$ pairs; $P > 0.3$).

[c] Protocol B: Control = 3.67 ± 0.60 (×10^6 cpm/mg prot) *vs.* T_3 = 5.21 ± 1.11 (×10^6 cpm/mg prot) (mean ± SE; $n = 5$ pairs; $P > 0.1$).

[d] Protocol C: Aldo = 2.92 ± 0.47 (×10^6 cpm/mg prot) *vs.* (Aldo + T_3) = 4.28 ± 1.06 (×10^6 cpm/mg prot) (mean ± SE; $n = 5$ pairs; $P > 0.3$).

and the summary of data in Tables 2 and 3. In addition, the results of a pilot (18-hr incubation) experiment (without sodium transport measurement) and a 6-hr incubation (with sodium transport measurement) (Protocol D) are also included in these tables.

As shown in Table 2, aldosterone and T_3 had minimal and no significant effects on the specific activity of the total protein pool, suggesting that these hormones have no major effect on the overall rate of protein synthesis and/or on the uptake of ^{35}S-methionine into the precursor cytosolic pool. Therefore, the selective induction or repression of protein was studied by loading the same amount of radioactivity on the first dimension (isoelectric focusing).

As shown in Fig. 1 (panels *A* and *B*), the overall pattern of protein synthesis was at first sight not markedly influenced by aldosterone (Protocol A: aldo *vs.* control). However, upon closer examination, a number of changes in spot intensities can be observed throughout the gel. Depending on the time of exposure of the X-ray film (from 10 days to 3 months), it is possible to resolve more than a thousand different proteins in this type of gel. In this experiment, ten proteins can be easily identified as AIP's, but interestingly, at least five were repressed (ARP's). In the pilot experiment, one major spot (AIP-1) was already observed and appeared to be repressed by T_3. This region of the gel is enlarged in Fig. 2 (Panels *A* and *B*). Three AIP's (AIP-1, AIP-2, AIP-3) are clearly observed. They cluster in the same region of the gel but with distinct molecular weights and isoelectric points (Table 3). AIP-1 has an apparent M_r of about 65,000 daltons and a p_i of about 6.7. AIP-2 has a slightly higher molecular weight (68,000 daltons) and the same p_i (6.7). AIP-3 has a still higher molecular weight (~69,000 daltons), but a more acidic p_i (~6.6). The basal rate (in control tissues) of synthesis of AIP-2 and AIP-3 appeared to be very low, while that of AIP-1 was readily detectable. On the same area of the gel, it is also evident that a least two proteins are repressed by aldosterone. ARP-1 has an apparent M_r of 58,000 ($p_i = 7.0$), while ARP-2 is of lower molecular weight (33,000 daltons, $p_i = 6.8$). It is also apparent that a large number of spots (for instance the major spot at

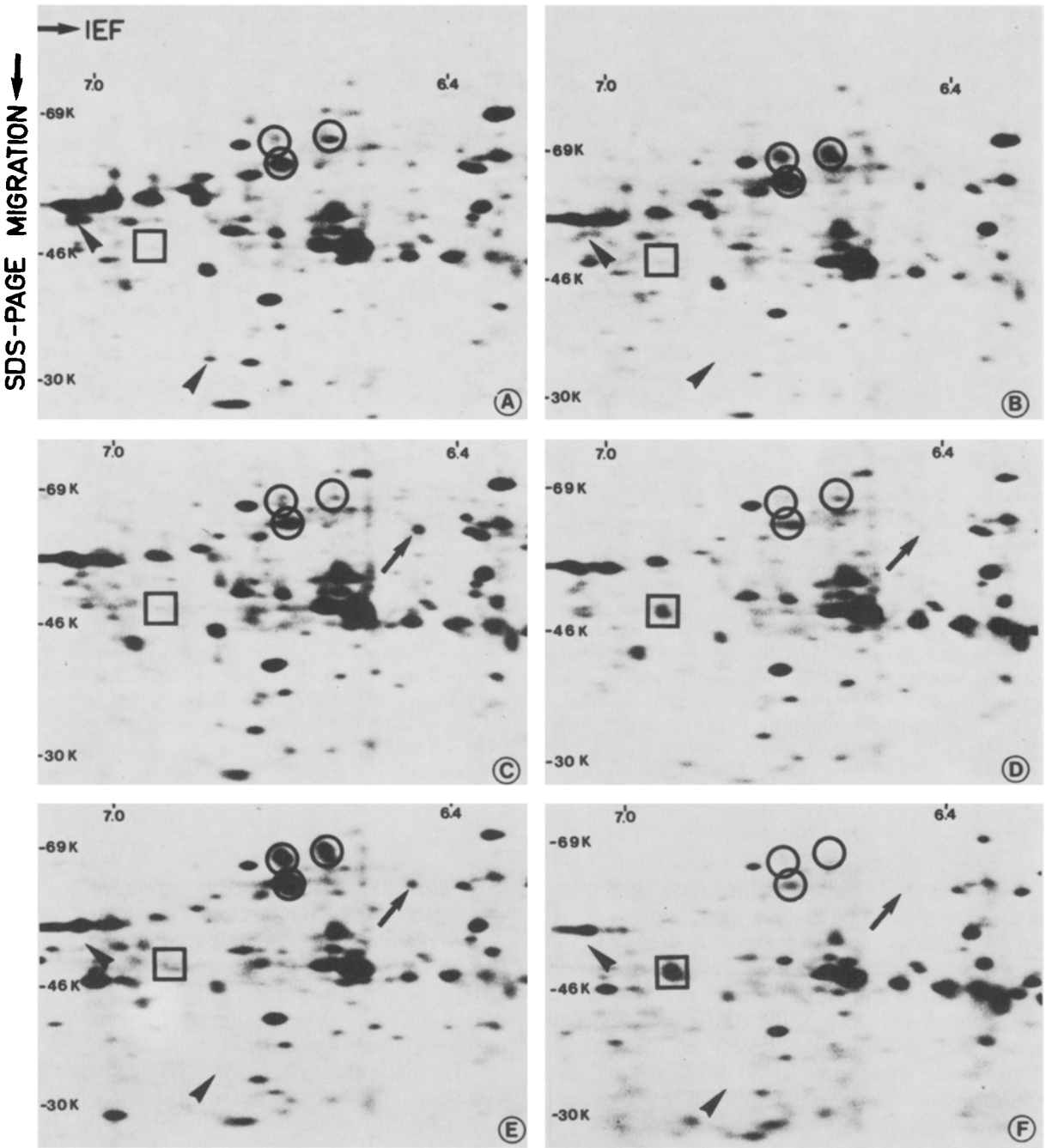

Fig. 2. Regions (Mr 30 to 70 Kd; p_i 6.2 to 7.0) of two-dimensional gel autoradiogram of Fig. 1. Panels *A* and *B*: control *vs.* aldo. Panels *C* and *D*: control *vs.* T_3. Panels *E* and *F*: aldo *vs.* (aldo + T_3). AIP-1, AIP-2, AIP-3 are circled. ARP-1 and ARP-2 are indicated by closed arrowheads, TIP-1 by an open square and TRP-1 by a closed arrow. *See* Table 3 for summary of data

47,000 daltons, p_i = 6.5) are not modulated by aldosterone and can serve as reference spots.

In Fig. 1 (Panels *C* and *D*), the same type of analysis was conducted (Protocol B, T_3 *vs.* Control). In the region of interest for further analysis (Fig. 2, *C* and *D*), one can observe a thyroid-induced protein (TIP-1) with an apparent M_r of ~52,000 daltons (p_i = 6.9) and one thyroid-repressed protein (TRP-1: M_r 63,000, p_i = 6.4). It is apparent that the AIP-1, AIP-2 and AIP-3 are also slightly repressed by T_3.

Finally, when given together (Protocol C: aldo *vs.* (aldo + T_3)), T_3 and aldosterone induced and repressed the expected proteins. Of special interest, however, was that AIP-1, AIP-2 and AIP-3 were markedly inhibited by T_3. This repression cannot be

Table 3. Effects of aldosterone and T_3 on the relative rate of synthesis of seven proteins analyzed by 2D gel electrophoresis

Figure symbols[a]	Incubation condition (18 and 6 hr)			Approximate M_r and p_i		
	Aldosterone *vs.* control	T_3 *vs.* control	(Aldosterone + T_3) *vs.* Aldo	M_r		p_i
Open circle	+ (6/6)	0 (6/6)	− (6/6)	AIP-1	65000	6.7
Open circle	+ (2/6)	0 (6/6)	− (2/6)	AIP-2	68000	6.7
Open circle	+ (2/6)	0 (6/6)	− (2/6)	AIP-3	69000	6.6
Closed arrow head	− (6/6)	0 (6/6)	0 (6/6)	ARP-1	58000	7.0
Closed arrow head	− (6/6)	0 (6/6)	0 (6/6)	ARP-2	33000	6.8
Open square	0 (6/6)	+ (5/6)	+ (5/6)	TIP-1	52000	6.9
Closed arrow head	0 (6/6)	− (6/6)	− (6/6)	TRP-1	63000	6.4

[a] Symbols as indicated in Figs. 1–3.
+ means induced, 0 means no change, and − repressed by reference to the paired control. In parentheses, number of observations showing the specific change out of 6 experiments (5 experiments: 18 hr incubation; 1 experiment: 6 hr incubation).

explained by some kind of gel artifact, since, on the same area, thyroid hormone had its expected *stimulatory* effect, on one protein (TIP-1).

In Table 3, a summary of the data obtained in the course of five independent experiments (for each protocol) is displayed. From this table, it is apparent that AIP-1 is consistently inhibited by T_3 in all conditions tested. It is also clear that T_3 had specific and consistent effect of its own, especially as far as TIP-1 and TRP-1 are concerned. ARP-1 and ARP-2 were also consistently detected throughout the present study, but were never affected by T_3. Within the limited number of spots analyzed here, only AIP-1 seems to be a real candidate as one of the proteins mediating the physiological antagonism between T_3 and aldosterone. AIP-1 was also inhibited in a 6-hr incubation (Protocol D) (*data not shown*).

Finally, since this protein could be important in determining the late mineralocorticoid response, it was of interest to test whether sodium butyrate, another antagonist of this response (Truscello et al., 1983), could selectively modulate the synthesis of AIP-1. As shown in Fig. 3 (Protocol F), sodium butyrate was able to selectively inhibit AIP-1 (and AIP-2 and -3 as well) at a time when the late mineralocorticoid response was already inhibited, as shown previously. Interestingly, butyrate displayed its own domain of action (butyrate induction or repression).

Discussion

AIP-1: An Overlapping Domain for T_3 and Aldosterone

In the present paper, we examined whether thyroid hormone could selectively modulate the induction of protein by aldosterone as analyzed by two-dimensional gel electrophoresis. As was proposed by Ivarie et al. (1981) for glucocorticoid hormone, it is clear that aldosterone can selectively control the expression of a restricted number of genes, thereby determining what is termed a hormonal domain. A set of aldosterone-induced proteins (membrane-bound and cytosolic) have been previously described by Geheb et al. (1981). The proteins described cluster within the same range of molecular weight (70,000 to 80,000 daltons) and isoelectric points (5.8 to 6.4). The three major AIP's described in this paper correspond rather well to this category (AIP-1, -2 and -3 with M_r between 65,000 and 69,000 daltons and a p_i between 6.6 and 6.7), since it is obviously not possible to standardize such gels with a great accuracy. We did not attempt to identify the cell compartment in which these AIP's were localized, since we intended to determine a hormonal domain as proposed by Ivarie et al. (1981). In a manner analogous to that described by Ivarie et al. (1981) for glucocorticoid, we also observed that aldosterone is able to repress a number of proteins such as ARP-1 and ARP-2, findings which were consistently observed throughout our study.

Likewise, T_3 was able to induce and repress its own set of proteins, such as TIP-1 and TRP-1, thus demonstrating its specific hormonal domain. Since we knew from our previous studies (Geering et al., 1984; Rossier et al., 1984) that T_3 antagonized aldosterone-dependent sodium transport, it was of interest to determine whether an overlap between the two hormonal domains could be detected. Clearly, AIP-1 (and to a lesser extent AIP-2 and AIP-3) could represent such a common site of regulation for the two hormones. The selectivity of the interaction is ensured by the observation that TIP-1 was induced whether aldosterone was present or not. At the present time, we do not know what the physiological roles of AIP-1, -2 and -3 are. Our data, however, suggest that these proteins might be involved

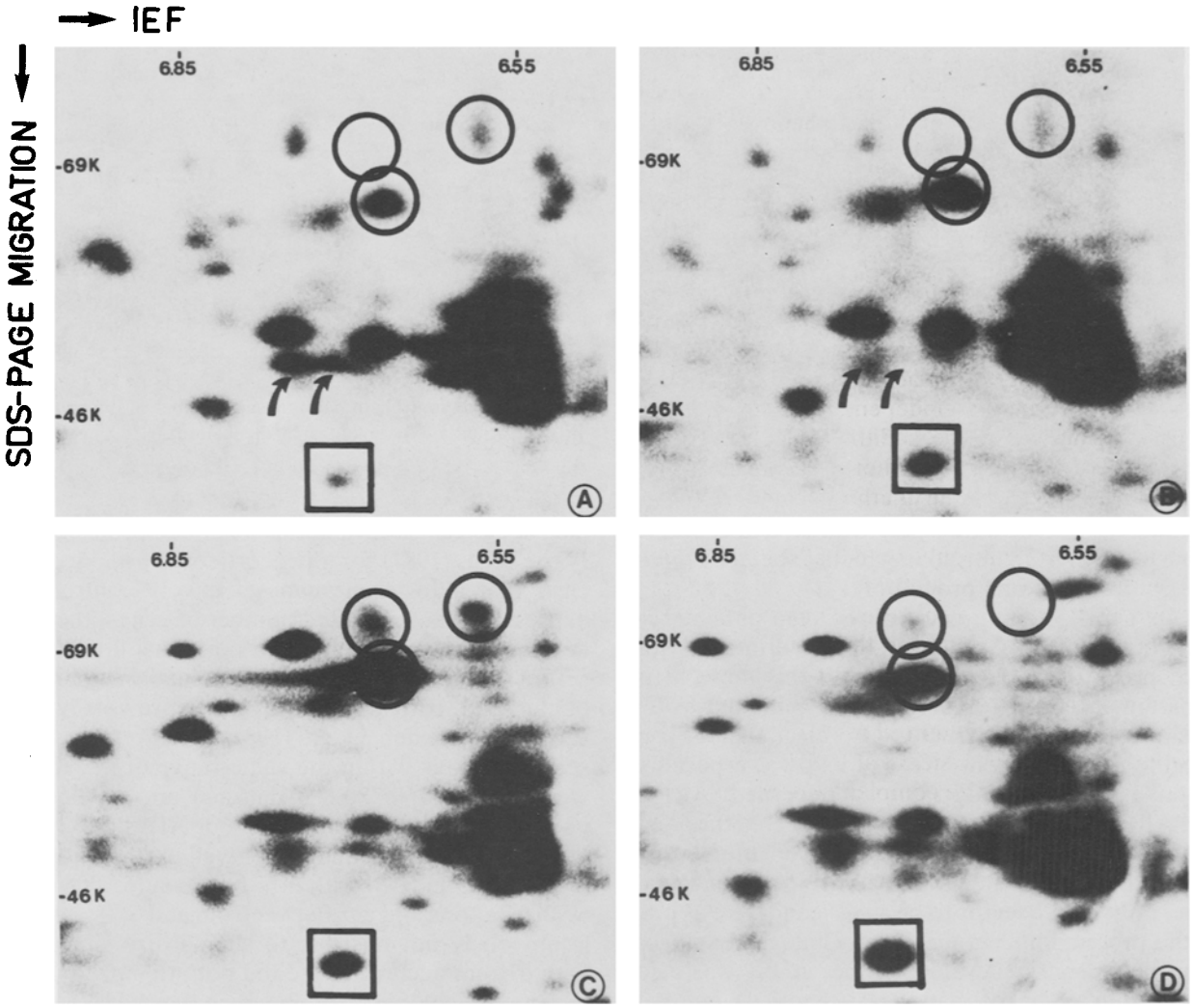

Fig. 3. Two-dimensional gel autoradiogram from toad urinary bladder proteins from one paired hemibladder pretreated with aldosterone (6 hr; 80 nM) (Panel *C*) or aldosterone (6 hr; 80 nM) + sodium butyrate (10 hr; 3 mM) (Panel *D*) (Protocol *F*) or with sodium butyrate (10 hr; 3 mM) (Panel *B*) or with diluent (10 hr; 3 mM NaCl) (Panel *A*) (Protocol *E*). AIP-1, AIP-2 and AIP-3 are circled. BRP-1 and BRP-2 (butyrate repressed proteins) are indicated by closed arrowheads. BIP-1 (butyrate induced protein) is squared. Sodium transport measurement: *Protocol E* butyrate (test) *vs.* diluent (control): $PD_{6\,hr}$ (test) = 49.5 mV *vs.* $PD_{6\,hr}$ (control) = 56.6 mV; $SCC_{6\,hr}$(test) = 93 μA *vs.* $SCC_{6\,hr}$(control) = 105 μA. *Protocol F* Butyrate + Aldo (test) *vs.* aldo (control): $PD_{6\,hr}$(test) = 46.4 mV *vs.* $PD_{6\,hr}$(control) = 67.8 mV; $SCC_{6\,hr}$(test) = 180 μA *vs.* $SCC_{6\,hr}$(control) = 240 μA

in the late action of aldosterone, that is during the phase in which sodium transport increases without changes in transepithelial electrical resistance (Geering et al., 1984). We have previously shown that aldosterone increases the biosynthesis rate of the alpha and beta subunits of Na,K-ATPase in the toad bladder (Geering et al., 1982) during this late mineralocorticoid response. In the same study it was shown, however, that T_3 was inactive. In other words, T_3 did not change the basal rate of Na,K-ATPase synthesis nor the aldosterone-dependent rate of synthesis. In addition, it can be easily shown that Na,K-ATPase is not resolved in the gel system used here (Geering et al., *unpublished observations*). While it is clear that Na,K-ATPase is one of the AIP's appearing during the late mineralocorticoid response (Geering et al., 1982), this study shows that other proteins might also play an important role in this response. Indeed, there are at least three reasons to believe that AIP-1, -2 and -3 belong to a set of proteins physiologically relevant to the late response. First, our experimental protocol (labeling after 6 or 18 hr of aldosterone incubation) would automatically favor the detection of such proteins. Second, the fact that these AIP's are precisely antagonized by T_3, which selectively inhibits

the late response, is compatible with this idea. Third, sodium butyrate, another selective inhibitor of the late mineralocorticoid response (Truscello et al., 1983) was also able to repress the induction of AIP-1, AIP-2 and AIP-3. It is noteworthy to recall that sodium butyrate, which acts by a different mechanism than T_3, also differs from T_3 in that it is itself able to antagonize the induction of Na,K-ATPase (Truscello et al., 1983). A clear and definitive understanding of the physiological role of AIP's, ARP's, TIP's, and TRP's cannot be given before these gene products are defined in molecular terms. Until this has been achieved, it is, of course, impossible to exclude independent parallel pathways accounting for the sodium transport effects unrelated to the induced and/or repressed protein, as defined by our current methodology. From this point of view, one should bear in mind that the 2-D gel analysis may still only reveal 10% of the gene products controlled by aldosterone and/or thyroid hormones. It should also be emphasized that our observations are restricted to the amphibian model and that we do not know whether they will apply to the mammalian kidney, since it is established that thyroid hormone has acquired new functions in homeotherm animals (Rossier et al., 1979*a*). Our data, however, emphasize the complexity of the action of aldosterone which is presented as a model in the next section.

Mechanism of Action of Aldosterone: A Complex Response

The relationship between induced (or repressed) proteins and the physiological response is by no means easy to establish. It is, however, tempting to speculate that various AIP's and ARP's act at different intracellular sites, mediating transepithelial sodium transport. As illustrated in Fig. 4, AIP's and ARP's could be divided into two categories: those involved in a constitutive pathway for sodium transport (sites 1, 3, 5) and those involved in a regulatory pathway. The regulatory pathway (sites 2, 4, 6) is directly implicated in the transport of sodium across the epithelial cell at three critical sites.

The first site is the *apical membrane* locus of the *amiloride-sensitive sodium channel*, which a number of authors have found to be involved in the early response to aldosterone (Cuthbert & Shum, 1975; Lewis & Wills, 1981; Palmer & Edelman, 1981; Palmer et al., 1982; Garty & Edelman, 1983; Kipnowski et al., 1983; Sariban-Sohraby et al., 1983).

The second site is the *basolateral membrane*, locus of the *ouabain-sensitive sodium pump*. An

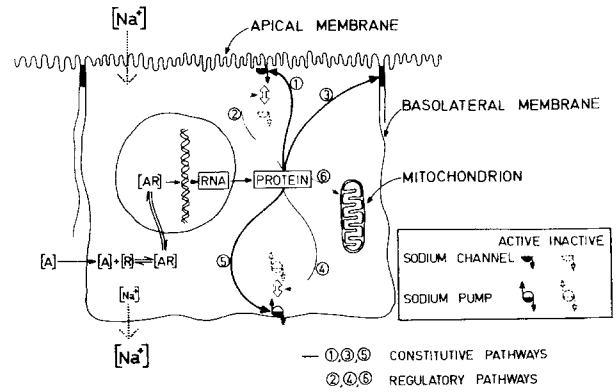

Fig. 4. Model of the mechanisms of action of aldosterone. An epithelial cell is schematically represented. Aldosterone (A) crosses the plasma membrane and links to its "cytosolic" (soluble) receptor (R). The complex (AR) is "activated" and links to chromatin acceptor sites in the nucleus. Upon this interaction, transcription is induced or repressed. Induced or repressed proteins (AIP's or ARP's) mediate an increased transepithelial ion transport at different sites. Constitutive pathway: sites 1, 3, 5. Regulatory pathway: sites 2, 4, 6. *See* explanation in Discussion

equally large number of data support the idea that Na,K-ATPase is involved in either the early mineralocorticoid response (Schmidt et al., 1975; Doucet & Katz, 1981; Petty et al., 1981) or the late one (Garg et al., 1981; Handler et al., 1981; Natke & Stoner, 1982; Geering et al., 1985; O'Neil & Hayhurst, 1985).

The third site is the *tight junction apparatus* which controls the overall leakiness of the tissue. Only scarce data so far are available concerning this last site (Uhlich et al., 1969; Paccolat et al., 1984).

The regulatory pathway involves a set of molecular events (e.g. phosphorylation-dephosphorylation, acylation, transmethylation) which modulate the constitutive pathway. Three sites of regulation could be envisaged (*see* 2, 4, 6 in Fig. 4).

First, the insertion (and/or the expression) of the sodium channel at the apical membrane. Along these lines, it has recently been suggested that the action of aldosterone might be mediated by a transmethylation reaction occurring in vesicles that carry an amiloride-sensitive sodium transport (Sariban-Sohraby et al., 1984).

Second, the insertion (and/or the expression) of the sodium pump at the basolateral membrane could be regulated by an AIP which could be induced during the early or late phase of aldosterone action.

Third, sodium transport could also be regulated either by controlling the energy supply (ATP) (Cortas et al., 1984) to the sodium pump or the ATP/ADP ratio, which in turn could modify the apical sodium permeability. A number of data support this concept (Kinne & Kirsten, 1968; Kirsten et al.,

1968; Law & Edelman, 1978*b*; Marver & Schwartz, 1980; Beauwens & Crabbé, 1983).

Finally, one should consider the possibility that the time course of induction and repression of various proteins may well vary for each protein. Thus, the early or the late mineralocorticoid response could be sequentially controlled by different AIP's and ARP's. The complexity of such a pleiotropic response will be understood once a combined molecular and genetic approach is available for tackling this problem.

We acknowledge the excellent assistance of J. Bonnard in preparing and typing the manuscript. The authors also thank D. Fanestil and J.P. Kraehenbuhl for their helpful suggestions and criticism of the manuscript. This work was supported by Swiss National Science Foundation No. 3.419-0.83.

References

Beauwens, R., Crabbé, J. 1983. Evidence for concerted effects of aldosterone on a target sodium-transporting epithelium. *J. Clin. Invest.* **72:**928–934

Benjamin, W.B., Singer, I. 1974. Aldosterone-induced protein in toad urinary bladder. *Science* **186:**269–272

Cortas, N., Abras, E., Arnaout, M., Mooradian, A., Muakasah, S. 1984. Energetics of sodium transport in the urinary bladder of the toad. Effect of aldosterone and sodium cyanide. *J. Clin. Invest.* **73:**46–52

Cuthbert, A.W., Shum, W.K. 1975. Effects of vasopressin and aldosterone on amiloride binding in toad bladder epithelial cells. *Proc. R. Soc. London B* **189:**543–575

Doucet, A., Katz, A.I. 1981. Short-term effect of aldosterone on Na-K-ATPase in single nephron segments. *Am. J. Physiol.* **241:**F273–F278

Edelman, I.S. 1978. Candidate mediators in the action of aldosterone on Na^+ transport. *In:* Membrane Transport Processes. J.F. Hoffman, editor. Vol. 1, pp. 125–140. Raven, New York

Garg, L.C., Knepper, M.A., Burg, M.B. 1981. Mineralocorticoid effects on Na-K-ATPase in individual nephron segments. *Am. J. Physiol.* **240:**F536–F544

Garty, H., Edelman, I.S. 1983. Amiloride-sensitive trypsinization of apical sodium channels. Analysis of hormonal regulation of sodium transport in toad bladder. *J. Gen. Physiol.* **81:**785–803

Geering, K., Claire, M., Gaeggeler, H.P., Rossier, B.C. 1985. Receptor occupancy vs. induction of Na^+-K^+-ATPase and Na^+ transport by aldosterone. *Am. J. Physiol.* **248:**C102–C108

Geering, K., Gaeggeler, H.P., Rossier, B.C. 1984. Effects of thyromimetic drugs on aldosterone-dependent sodium transport in the toad bladder. *J. Membrane Biol.* **77:**15–23

Geering, K., Girardet, M., Bron, C., Kraehenbühl, J.P., Rossier, B.C. 1982. Hormonal regulation of (Na^+,K^+)-ATPase biosynthesis in the toad bladder. Effect of aldosterone and 3,5,3′-triiodo-L-thyronine. *J. Biol. Chem.* **257:**10338–10343

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

Geheb, M., Huber, G., Hercker, E., Cox, M. 1981. Aldosterone-induced proteins in toad urinary bladders. Identification and characterization using two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem.* **256:**11716–11723

Handler, J.S., Preston, A.S., Perkins, F.M., Matsumura, M., Johnson, J.P., Watlington, C.O. 1981. The effect of adrenal steroid hormones on epithelia formed in culture by A6 cells. *Ann. N.Y. Acad. Sci.* **372:**442–454

Ivarie, R.D., Baxter, J.D., Morris, J.A. 1981. Interaction of thyroid and glucocorticoid hormones in rat pituitary tumor cells. *J. Biol. Chem.* **256:**4520–4528

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, New York

Kinne, R., Kirsten, R. 1968. Der Einfluss von Aldosteron auf die Aktivität mitochondrialer und cytoplasmatischer Enzyme in der Rattenniere. *Pfluegers Arch.* **300:**244–254

Kipnowski, J., Park, C.S., Fanestil, D.D. 1983. Modification of carboxyl of Na^+ channel inhibits aldosterone action on Na^+ transport. *Am. J. Physiol.* **245:**F726–F734

Kirsten, E., Kirsten, R., Leaf, A., Sharp, G.W.G. 1968. Increased activity of enzymes of the tricarboxylic acid cycle in response to aldosterone in the toad bladder. *Pfluegers Arch.* **300:**213–225

Law, P.Y., Edelman, I.S. 1978*a*. Effect of aldosterone on incorporation of amino acids into renal medullary proteins. *J. Membrane Biol.* **41:**15–40

Law, P.Y., Edelman, I.S. 1978*b*. Induction of citrate synthase by aldosterone in the rat kidney. *J. Membrane Biol.* **41:**41–64

Lewis, S.A., Wills, N.K. 1981. Localization of the aldosterone response in rabbit urinary bladder by electrophysiological techniques. *Ann. N.Y. Acad. Sci.* **372:**56–63

Marver, D., Schwartz, M.J. 1980. Identification of mineralocorticoid target sites in the isolated rabbit cortical nephron. *Proc. Natl. Acad. Sci. USA* **77:**3672–3676

Natke, E., Jr., Stoner, L.C. 1982. Na^+ transport properties of the peritubular membrane of cortical collecting tubule. *Am. J. Physiol.* **242:**F664–F671

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:**4007–4021

O'Neil, R.G., Hayhurst, R.A. 1985. Sodium-dependent modulation of the renal Na-K-ATPase influence of mineralocorticoids on the cortical collecting duct. *J. Membrane Biol.* **85:**169–179

Paccolat, M.P., Geering, K., Gaeggeler, H.P., Rossier, B.C. 1984. A toad kidney epithelial cell line (A6); a suitable experimental model for studying the late mineralocorticoid response with regard to sodium transport and (Na^+,K^+)-ATPase biosynthesis. *Kidney Int.* **25:**987 (Abstr.)

Palmer, L.G., Edelman, I.S. 1981. Control of apical sodium permeability in the toad urinary bladder by aldosterone. *Ann. N.Y. Acad. Sci.* **372:**1–14

Palmer, L.G., Li, J.H.-Y., Lindemann, B., Edelman, I. 1982. Aldosterone control of the density of sodium channels in the toad urinary bladder. *J. Membrane Biol.* **64:**91–102

Petty, K.J., Kokko, J.P., Marver, D. 1981. Secondary effect of aldosterone on Na-K-ATPase activity in the rabbit cortical collecting tubule. *J. Clin. Invest.* **68:**1514–1521

Rossier, B.C., Claire, M., Rafestin-Oblin, M.E., Gäggeler, H.P., Geering, K. 1984. Effects of thyroid hormones and aldosterone on mineralocorticoid binding sites in the toad bladder. *J. Membrane Biol.* **77:**25–32

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

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

Sariban-Sohraby, S., Burg, M.B., Turner, R.J. 1983. Apical sodium uptake in toad kidney epithelial cell line A6. *Am. J. Physiol.* **245:**C167–C171

Sariban-Sohraby, S., Burg, M., Wiesmann, W.P., Chiang, P.K., Johnson, J.P. 1984. Methylation increases sodium transport into A6 apical membrane vesicles: Possible mode of aldosterone action. *Science* **225:**745–746

Schmidt, U., Schmid, H., Schmid, H., Dubach, U.C. 1975. Sodium- and potassium-activated ATPase. A possible target of aldosterone. *J. Clin. Invest.* **55:**655–660

Scott, W.N., Sapirstein, V.S. 1975. Identification of aldosterone-induced proteins in the toad's urinary bladder. *Proc. Natl. Acad. Sci. USA* **72:**4056–4060

Truscello, A., Geering, K., Gäggeler, H.P., Rossier, B.C. 1983. Effects of butyrate on histone deacetylation and aldosterone-dependent Na^+ transport in the toad bladder. *J. Biol. Chem.* **258:**3388–3395

Uhlich, E., Baldamus, C.A., Ullrich, K.J. 1969. Einfluss von Aldosteron auf den Natriumtransport in den Sammelrohren des Saugetierniere. *Pfluegers Arch.* **308:**111–126

Received 16 April 1985; revised 6 September 1985