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Stimulation of sodium transport by aldosterone and arginine vasotocin in A6 cells

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The effects of aldosterone and arginine vasotocin (AVT) on transepithelial Na^+ transport of cultured A6 cells were investigated. All experiments were performed with cells grown on Millicell™ culture-plate inserts for a period of 2–4 weeks in defined, serum-free medium. Omitting fetal bovine serum 2 days after seeding the cells on filters did not influence potential difference (PD) development or the hormonal responses tested. The cell layers were placed in an Ussing chamber for short-circuit current (I_{SC}) and transepithelial conductance (G) measurements. Base-line values were ($n = 93$): PD, 51.0 ± 0.2 mV (apical side negative); I_{SC}, 14.55 ± 0.06 $\mu A/cm^2$; G, 0.306 ± 0.001 mS/cm^2. I_{SC} and G were higher in cells pretreated with 10^{-7} M aldosterone for 24 h in the incubator, when compared to controls (I_{SC}, 28 ± 2 vs. 16 ± 2 $\mu A/cm^2$, G, 0.41 ± 0.04 vs. 0.26 ± 0.01 mS/cm^2, $n = 5$) and both remained stable for at least 6 h. In cells not treated with aldosterone, 10^{-7} M AVT increased I_{SC} within 1 min after addition, producing a maximum I_{SC} within 15 min which then declined to baseline levels over the next 5 h. Addition of AVT to aldosterone-pretreated cells resulted in a significantly greater peak increase in I_{SC} than in non-pretreated cells (change in I_{SC} compared to controls: 8.1 ± 0.4 vs. 4.9 ± 0.4 $\mu A/cm^2$, $n = 5$, $P < 0.001$), indicating a synergistic effect. A dose-response curve for amiloride obtained in the presence of AVT showed that amiloride completely inhibits I_{SC}. Pretreatment of the A6 cells with aldosterone for 24 h shifted the amiloride dose-response curve to the right, as expressed in a doubling of the apparent K_i value (from 0.17 ± 0.02 to 0.33 ± 0.04 μM). In conclusion, A6 cells grown in defined, serum-free medium express a greater than additive synergism between aldosterone and AVT in stimulating transepithelial Na^+ transport.

Introduction

A6 cells, originally derived from a whole mince of kidneys of the aquatic toad *Xenopus laevis*, have been extensively studied as a model system for characterizing transepithelial Na^+ transport in tight epithelia [1–10]. When grown on a permeable support, A6 cells form a monolayer exhibiting a high transepithelial resistance ranging from 2.7 to 11.4 $k\Omega cm^2$ [2,3,7,8,11,12] and active Na^+ transport from the apical to the basolateral side, which is reflected by the short-circuit current [13]. Na^+ transport can be reversibly blocked by amiloride and stimulated by hormones like aldosterone, arginine vasotocin (AVT) and insulin [2,6,13].

A6 cells have, until now, only been cultured in the continuous presence of serum [1–10], which generally contains an abundant number of

Abbreviations: AVT, arginine vasotocin; AVP, arginine vasopressin; ADH, anti-diuretic hormone; FBS, fetal bovine serum; PBS, phosphate-buffered saline

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hormones including aldosterone, deoxycorticosterone, insulin and triiodothyronine. Many of these hormones can directly or indirectly modulate the differentiation of the A6 cell culture and can potentially influence the expression of Na^+ transport systems in these cells [14]. In addition, instability in baseline I_{SC} from experiment to experiment has been attributed to differences in batches of FBS. Thus, some investigators select certain lots of serum on the basis of the high electrical resistance which they promote in the cultured epithelia in comparison to other lots of serum [10]. It seemed highly desirable to us to develop methods to maintain A6 cell cultures in a truly defined serum-free medium, in order to obtain a cell culture with a reproducible non-stimulated baseline of Na^+ transport.

There is ample evidence that arginine vasopressin (AVP) can stimulate Na^+ transport in variety of epithelia, including toad bladder [15], isolated perfused cortical collecting tubules of both rabbit [16] and rat [17] and in A6 monolayers [18]. In all these tissues, the response to AVP is almost instantaneous but transient, except in the rat cortical collecting tubules where the anti-diuretic hormone (ADH) induced increase in Na^+ transport persists for at least 3 h [17]. Aldosterone, like ADH, is also able to enhance Na^+ transport in a variety of tissues [15,17], including A6 cells [6], but by a different mechanism [19]. The time course of the effects of the two hormones differ markedly. Aldosterone promotes Na^+ transport across the toad bladder over a period of several hours [19], but only after a lag period of about 60 min [20]. Unlike ADH, aldosterone has additional long-term effects on Na^+ transport mediated via the induction of proteins [21], including metabolic effects [22] and possibly increased synthesis of new Na^+ channels [23].

Of particular interest is the interaction between aldosterone and AVP in their effect on Na^+ transport. From studies with isolated perfused rat cortical collecting tubules, it is known that pretreatment of the rats with desoxycorticosterone acetate, a synthetic mineralocorticoid, augments the ADH effect on Na^+ transport [17]. In the toad bladder, it was only after the bladders were depleted of endogenous aldosterone that subsequent aldosterone treatment resulted in a larger absolute enhancement in the increment in Na^+ transport upon adding ADH [24].

Until now, there have been no reports on the interaction of aldosterone and AVP on Na^+ transport in A6 monolayers. Therefore, we studied in detail the time course of the effects of aldosterone and AVT, and their interaction in regulating Na^+ transport in A6 cells. In these experiments, A6 cells were cultured in a defined medium, i.e., in the absence of serum, for an extended period of time. We found that A6 cells grown in defined, serum-free medium exhibit hormone-dependent transepithelial Na^+ transport. There was a greater than additive synergism between aldosterone and AVT on transepithelial Na^+ transport when these cells were pretreated for 24 h with aldosterone prior to the experiment, quite comparable to that observed in the toad bladder [24] and rat cortical collecting tubule [17].

Parts of this study have been presented at the 20th annual meeting of the American Society of Nephrology, 1987, Washington DC, U.S.A.

Materials and Methods

Cell culture. A6 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 68. All experiments were carried out with passages 70–80. The cells were cultured on 15 cm tissue culture dishes (Falcon, Cockeysville, MD) at 28°C in a humidified incubator (Queue, Parkersburg, WV) in the presence of 1% CO_2 in air. The basic defined culture medium (referred to as A6 culture medium) was Dulbecco's modified Eagle's medium (formula No. 84-5022, Gibco, Long Island, NY) with a total Na^+ concentration of 86 mM, a HCO_3^- concentration of 8 mM and an osmolarity of 190 mOsm. To this medium were added 200 U/ml penicillin and 200 mg/ml streptomycin (Gibco, Long Island, NY). For maintaining the cells, 10% fetal bovine serum (FBS) (Gibco, Long Island, NY) was also added. A6 cells were fed twice a week and were split weekly. To split the cells, culture dishes were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4), followed by the addition of 3 ml of 0.1% trypsin and 1 mM EDTA in PBS to each 15 cm tissue culture dish. The cells detached from

the dish within 20 min at 28°C in the incubator and the trypsin action was subsequently inhibited by adding 22 ml of A6 culture medium including 10% FBS. The cells were then seeded on three tissue culture dishes (1 : 6 by surface area), and on six Millicell™-HA 30-mm culture-plate inserts (3 : 1 by surface area) (Millipore, Bedford, MA) placed in a 6-well tissue culture-plate (Costar, Cambridge, MA). 2 days after seeding on Millicell™ culture-plate inserts, the cells were fed with A6 culture medium alone (without 10% FBS) on both sides. From that time on, FBS remained excluded from the culture medium, unless otherwise indicated.

Electrical measurements. Transepithelial potential difference (PD) across the Millicell™ culture-plate inserts was determined in a sterile manner by placing two agar bridges in the solutions on either side of the filter. The agar bridges were connected via Ag-AgCl electrodes (IVM, Healdsburg, CA) to a voltage amplifier (Elenco Electronics Inc., Nothbrook, IL). Only monolayers with a PD of 35 mV or higher were used for experiments. The percentage of filters discared never exceeded 20%.

Measurement of transepithelial Na^+ transport, monitored by short-circuit current (I_{SC}), and by transepithelial conductance (G) was performed in an Ussing-type chamber, which has been described in detail by Rick et al. [25]. Briefly, the filter bottom of a Millicell™ culture-plate insert was removed with a scalpel. The filter was mounted between two plastic rings and placed between two Lucite half-chambers (exposed surface area 1.6 cm^2). Rubber O-rings were imposed between the half-chambers and the ring to produce a watertight seal. To minimize edge damage, silicon grease was applied to the ring facing the apical side of the monolayer. The chambers were connected to a flow-through system (total volume of each chamber was 35 ml) for continuous recirculation of the solution on each side (2.0 ml/min) with a roller pump (Isametic, Zürich, Switzerland). A potential-sensing electrode consisting of an agar bridge connected to an Ag-AgCl electrode, was mounted close to the monolayer in each chamber, and both chambers also contained a large Ag-AgCl electrode to pass current. The chamber electrodes were connected to an automatic voltage clamping device (Model VCC600, Physiological Instruments, Houston, TX), and the I_{SC} and G values were recorded on a strip-chart recorder (Cole Palmer, Chicago, IL). I_{SC} was measured by clamping the transepithelial potential to 0 mV. G was calculated from the current deflections in response to 10 mV pulses of 1 s duration every 20 s. The A6 monolayers were incubated for an initial control period in A6 Ringer (Dulbecco's modified Eagle's medium without penicillin or streptomycin, saturated with 1% CO_2/99% O_2). Naproxen ($5 \cdot 10^{-6}$ M), an inhibitor of prostaglandin synthesis, was always present in the A6 Ringer to avoid possible interference of prostaglandins on ion transport and therefore I_{SC} [5,10]. Once both I_{SC} and G were stable, usually after 60–90 min, the experiment was continued as described in the Results.

Aldosterone (chromatographic grade) was purchased from Calbiochem (San Diego, CA), and a stock solution was prepared in 99% ethanol (final concentration of ethanol was always below 0.1%). Arginine vasotocin was from United States Biochemical Corp. (Cleveland, OH), 8-bromoadenosine 3′ : 5′-cyclic monophosphate (8-Br-cAMP) and naproxen were from Sigma (St. Louis, MO) and amiloride-HCl was a gift from Merck Sharp & Dohme Research Laboratories (West Point, PE).

Results are expressed as mean ± SE. N is the number of Millicell™ culture-plate inserts used. Statistical differences between the mean values were determined by analysis of variance. In case of a significant overall effect, the statistical analysis was continued by single degree of freedom contrasts. Unpaired Student's t-test was used to determine the difference between two independent groups.

Results

Fig. 1 shows the results of transepithelial PD measurements performed on A6 cells grown in the presence or absence of 10% FBS (except for the first 2 days after seeding during which 10% FBS was always present). Within 3 days, the cells developed a PD significantly different from zero ($P < 0.02$). Maximal PD was achieved after a period of 10–12 days and remained stable at a

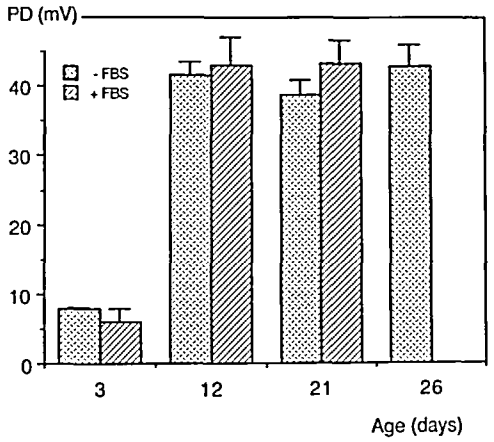

Fig. 1. Time course of development of transepithelial potential difference (PD) (apical side negative) across filter cups in the absence or presence of 10% defined fetal bovine serum (FBS). A6 cells were seeded (day zero) on filter cups at a density of 3:1 by area. Results are mean ± SE of seven Millicell™ culture-plate inserts.

value around 40 mV for at least 2 weeks. There was no significant difference in the development of PD in the presence or absence of FBS ($P > 0.48$).

In separate experiments with A6 cells, we observed no major differences in the transport parameters or hormonal responses when FBS was present during the culture of the A6 cells and the subsequent experiments, as shown in Table I. Basal I_{SC} and G values of both cell groups were determined. We refer to this point as zero time. The continuous presence of FBS during the culture of A6 cells increased the basal I_{SC} slightly ($P < 0.05$), compared to cells grown in the absence of serum. However, the basal G remained unaltered. At zero time AVT (10^{-7} M) was added to the basal side. There were no significant differences in the effect of AVT on I_{SC} or G between the A6 cells cultured in the presence or absence of FBS. At 60 min, amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side of the cells and 20 min later both I_{SC} and G were determined. The amiloride-insensitive I_{SC} and G were not significantly different between both cell groups.

All experiments described subsequently were performed with cells grown on filters for a period of 2–4 weeks with defined A6 culture medium in the absence of serum. The average electrical parameters, determined in Ussing chambers, were as follows: PD, 51.0 ± 0.2 mV (apical side negative); I_{SC}, 14.55 ± 0.06 $\mu A/cm^2$, G, 0.306 ± 0.001 mS/cm^2; $n = 93$.

Effect of 24-h aldosterone pretreatment

One group of cells not pretreated with aldosterone (−aldo) was fed with A6 culture medium containing aldosterone (10^{-7} M), while the other group, which was pretreated with aldosterone (+aldo) was fed with A6 culture medium only, on both sides 24 h prior to the experiment. In the aldosterone-prefed cells, but not in the non-pretreated cells, the hormone was

TABLE I

EFFECT OF AVT ON ELECTRICAL PARAMETERS OF A6 CELLS CULTURED WITH AND WITHOUT FBS

Values are means ± S.E. of four experiments. A6 cells were grown in the presence (+FBS) or absence (−FBS) of 10% FBS (except for the first 2 days after seeding during which 10% FBS was always present). The subsequent experiments were performed in the presence or absence of 10% FBS. Zero time is the time point at which both I_{SC} and G were stable, normally within 90 min after mounting the monolayer in the Ussing chamber. At zero time arginine vasotocin (AVT, 10^{-7} M) was applied to the basal side. Peak refers to peak value of I_{SC} and G after drug addition. Δ, peak value of I_{SC}, $G - I_{SC}$ or G at zero time. At 60 min amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side and I_{SC} or G were measured 20 min later. Significant differences between −FBS and +FBS groups were determined by an unpaired Student's t-test.

		Zero time	AVT peak	Δ	Amiloride
I_{SC} ($\mu A/cm^2$)	−FBS	15 ±2	18 ±3	4 ±2	0.2 ±0.1
	+FBS	20 ±1 *	24 ±1	4 ±1	0.6 ±0.1
G (mS/cm^2)	−FBS	0.33±0.04	0.44±0.05	0.11±0.04	0.20±0.06
	+FBS	0.37±0.04	0.55±0.05	0.18±0.02	0.18±0.06

* $0.01 < P < 0.05$.

also present on the basal side during the subsequent experiment in the Ussing chamber. Basal I_{SC} and G of both cell groups were determined when the electrical parameters were stable, usually within 90 min after mounting of the monolayer in the Ussing chamber, as shown in Figs. 2A and 3A. We refer to this point as $t = 0$ min. Pretreatment with aldosterone for 24 h increased the basal I_{SC} by 75% (from 16 ± 2 to 28 ± 2 $\mu A/cm^2$, $P < 0.05$, $n = 5$) and increased G by 87% (from 0.26 ± 0.01 to 0.41 ± 0.04 mS/cm^2, $P < 0.01$, $n = 5$). Both I_{SC} and G remained stable for at least 6 h in both +aldo and −aldo cells. Hence, the aldosterone-induced increase in I_{SC} and G persisted during the whole experiment.

The time course of the effect of AVT (10^{-7} M) on I_{SC} and G was determined for the −aldo and +aldo groups, as shown in Figs. 2B and 3B. Upon addition of AVT to the basal side of cells prefed with medium only (−aldo), both I_{SC} and G increased rapidly, followed by a slow decline towards baseline levels over the next 5 h. The effect of AVT started approx. 1 min after addition of the drug and reached a maximum in 16 ± 2 min. The cells prefed with aldosterone (+aldo) reacted in a similar fashion to the addition of AVT, except that I_{SC} remained slightly elevated above the value at zero time, even after 5 h of incubation with the drug.

8-Br-cAMP ($5 \cdot 10^{-4}$ M) mimicked several of the above described actions of AVT, as shown in Figs. 2C and 3C, except that the response to 8-Br-cAMP on I_{SC} peaked at 35 ± 6 min, which is a significantly slower response compared to the reaction to AVT ($P < 0.01$). In addition, the effect of 8-Br-cAMP on G, in both +aldo and −aldo cells, appeared to be sustained in contrast to the effect of AVT.

The data described above are summarized in Tables II and III. Control experiments, i.e., without the addition of AVT or 8-Br-cAMP, were performed for both −aldo and +aldo cells and there were no spontaneous increases (Δpeak) in either I_{SC} or G ($P > 0.50$). AVT and 8-Br-cAMP caused a greater peak increase (Δpeak) in both I_{SC} ($P < 0.001$) and G ($P < 0.01$) when the cells were pretreated with aldosterone (+aldo) compared to non-pretreated cells (−aldo). However, this synergism between aldosterone and

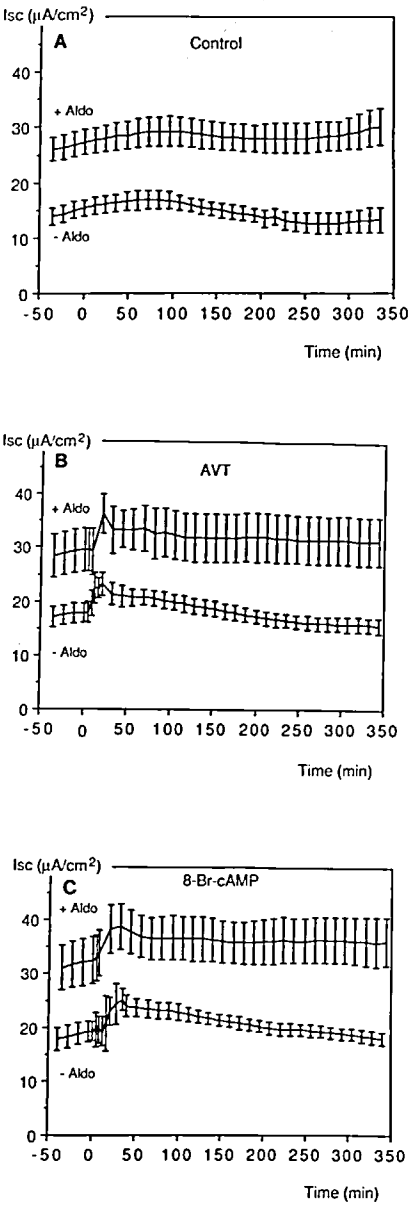

Fig. 2. Time course of control (A), arginine vasotocin (AVT) (B) and 8-bromoadenosine 3′:5′-cyclic monophoshate (8-Br-cAMP) (C) stimulation of I_{SC} in A6 cells. Cells were fed 24 h prior to the experiment with A6 Ringer containing either 10^{-7} M aldosterone (+aldo) or vehicle (−aldo). In the aldosterone-pretreated group, 10^{-7} M aldosterone was also present at the basal side during the experiment. Zero time is the time point at which both I_{SC} and G were stable, normally within 90 min after mounting the monolayer in the Ussing chamber. At zero time AVT (10^{-7} M), or 8-Br-cAMP ($5 \cdot 10^{-4}$ M) were added to the basal side, or no additions were made (control). Results are mean ± S.E. of five (control, 8-Br-cAMP) and six (AVT) experiments.

TABLE II

EFFECT OF AVT AND 8-Br-cAMP ON THE SHORT-CIRCUIT CURRENT OF A6 CELLS WITH AN WITHOUT ALDOSTERONE PRETREATMENT FOR 24 HOURS

Values are given as $\mu A/cm^2$ and are means ± S.E. Cells were fed 24 h prior the experiment with A6 medium containing either 10^{-7} M aldosterone (+aldo) or vehicle (−aldo). Zero time is the time point at which both I_{SC} and G were stable, normally within 90 min after mounting the monolayer in the Ussing chamber. At zero time, arginine vasotocin (AVT, 10^{-7} M) or 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP, $5 \cdot 10^{-4}$ M) was applied to the basal side, or no additions were made (control). Subsequently, I_{SC} was determined at different times points (peak and 330 min). Peak refers to peak value of I_{SC} after drug addition (AVT at $t = 16 \pm 2$ min; 8-Br-cAMP at $t = 35 \pm 4$ min). Δ = peak value of $I_{SC} - I_{SC}$ at zero time or I_{SC} at 330 min $- I_{SC}$ at zero time. At 350 min amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side and I_{SC} was measured 10 min later. The number of experiments (n) is given in parentheses. Significant differences between −aldo and +aldo groups were determined by analysis of variance. In case of a significant overall effect the statistical analysis was continued by single degree of freedom contrasts.

		Zero time (I_{SC})	Peak		330 min		Amiloride (I_{SC})
			I_{SC}	Δ	I_{SC}	Δ	
Control ($n = 5$)	−aldo	16±2	17±2 [a]	0.5±0.5	13±2	−3.0±1.8	0.8±0.1
	+aldo	28±2 *	29±3 **	1.1±0.4	30±3 **	2.4±0.7 *	1.6±0.2 *
AVT ($n = 6$)	−aldo	18±2	23±2	4.9±0.4	15 ≡ 2	−2.5±0.9	0.5±0.1
	+aldo	29±4 *	38±4 **	8.1±0.4 ***	31±5 ***	2.0±1.9 *	1.4±0.2 **
8-Br-cAMP ($n = 5$)	−aldo	19±2	24±2	5.2±0.5	18±1	−1.5±2.4	1.3±0.4
	+aldo	32±4 **	40±4 **	7.7±0.3 ***	36±5 ***	4.0±0.9 *	3.0±0.4 ***

* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$.

[a] Because there was no obvious peak I_{SC} in control cells, we chose 25 min as a reference time for comparison to AVT and 8-Br-cAMP groups.

TABLE III

EFFECT OF AVT AND 8-Br-cAMP ON CONDUCTANCE OF A6 CELLS WITH AND WITHOUT ALDOSTERONE PRETREATMENT FOR 24 HOURS

Values are given as (mS/cm^2) and are means ± S.E. Cells were fed 24 h prior to the experiment with A6 medium containing either 10^{-7} M aldosterone (+aldo) or vehicle (−aldo). Zero time is the time point at which both I_{SC} and G were stable, normally within 90 min after mounting the monolayer in the Ussing chamber. At zero time, arginine vasotocin (AVT, 10^{-7} M) or 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP, $5 \cdot 10^{-4}$ M) was applied to the basal side, or no additions were made (control). Subsequently, G was determined at several time points (peak and 330 min). Peak refers to values of G after drug addition when I_{SC} peaked (AVT at $t = 16 \pm 2$ min; 8-Br-cAMP at $t = 35 \pm 4$ min). Δ = peak value of $I_{SC} - I_{SC}$ at zero time or I_{SC} at 330 min $- I_{SC}$ at zero time. At 350 min, amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side and G was measured 10 min later. The number of experiments (□) is given in parentheses. Significant differences between −aldo and +aldo groups were determined by analysis of variance. In case of a significant overall effect, the statistical analysis was continued by single degree of freedom contrasts.

		Zero time (G)	Peak		330 min		Amiloride (G)
			G	Δ	G	Δ	
Control ($n = 5$)	−aldo	0.26±0.01	0.26±0.01 [a]	−0.01±0.01	0.27±0.03	0.01±0.05	0.10±0.01
	+aldo	0.41±0.04 **	0.41±0.03 **	0.00±0.01	0.39±0.03	−0.02±0.02	0.08±0.02
AVT ($n = 6$)	−aldo	0.28±0.03	0.42±0.04	0.14±0.01	0.24±0.02	−0.04±0.02	0.09±0.01
	+aldo	0.48±0.02 ***	0.79±0.04 ***	0.31±0.05 **	0.44±0.02 **	−0.04±0.03	0.10±0.03
8-Br-cAMP ($n = 5$)	−aldo	0.28±0.02	0.51±0.04	0.23±0.03	0.38±0.08	0.10±0.04	0.08±0.02
	+aldo	0.41 ≡ 0.05 **	0.84±0.12 ***	0.43±0.09 **	0.62±0.10 *	0.21±0.07 *	0.10±0.02

* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$.

[a] Because there was no obvious peak I_{SC} in control cells, we chose 25 min as a reference time for comparison to AVT and 8-Br-cAMP groups.

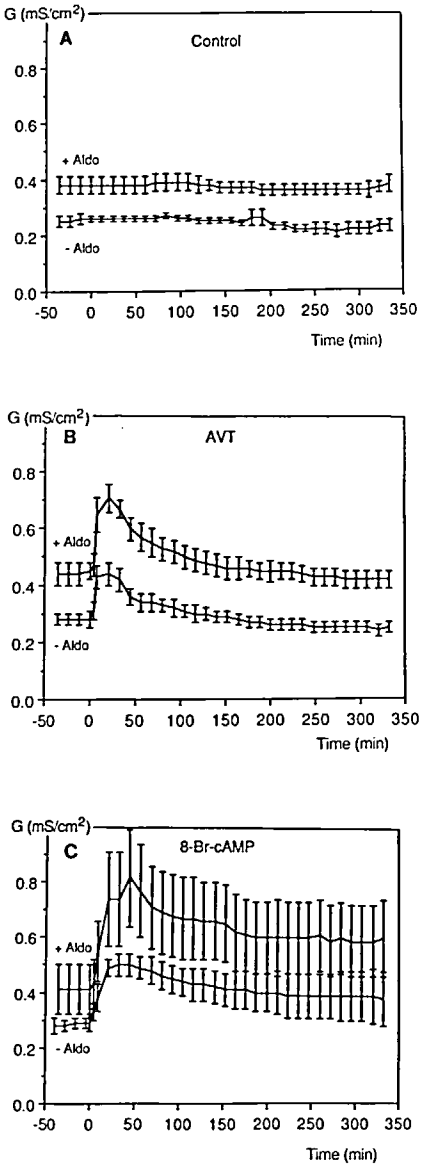

Fig. 3. Time course of control (A), arginine vasotocin (AVT) (B) and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP) (C) stimulation of G in A6 cells. Cells were fed 24 h prior to the experiment with A6 Ringer containing either 10^{-7} M aldosterone (+aldo) or vehicle (−aldo). In the aldosterone pretreated group, 10^{-7} M aldosterone was also present at the basal side during the experiment. Zero time is the time point at which both I_{SC} and G were stable, normally within 90 min after mounting the monolayer in the Ussing chamber. At zero time AVT (10^{-7} M), or 8-Br-cAMP ($5 \cdot 10^{-4}$ M) were added to the basal side, or no additions were made (control). Results are mean ± S.E. of five (control, 8-Br-cAMP) and six (AVT) experiments.

AVT and between aldosterone and 8-Br-cAMP on I_{SC} was only modest, since the percent increase in the I_{SC} upon addition of AVT or 8-Br-cAMP was not significantly different with or without aldosterone pretreatment (AVT, 29 ± 3 vs. $28 \pm 4\%$; 8-Br-cAMP, 25 ± 4 vs. $28 \pm 3\%$, $P > 0.88$). Finally, aldosterone pretreatment also caused I_{SC} to remain elevated at $t = 330$ min, in the control, AVT and 8-Br-cAMP group, in contrast to non-treated cells ($P < 0.005$). However, I_{SC} at $t = 330$ min was not significantly different between the control versus AVT or 8-Br-cAMP groups ($P > 0.55$) and this holds for both the aldosterone-pretreated and non-treated cells. Therefore, we could not show a sustained response to AVT or 8-Br-cAMP in the absence of prior aldosterone treatment.

At 330 min, amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side of the cells and 20 min later both I_{SC} and G were determined as shown in Tables II and III. The amiloride-insensitive I_{SC} was doubled ($P < 0.0001$) by pretreatment with aldosterone for 24 h in all three groups, i.e., control, AVT and 8-Br-cAMP. However, G in the presence of amiloride was not significantly different between −aldo and +aldo cells ($P > 0.89$). Interestingly, the amiloride-insensitive I_{SC} in the presence of 8-Br-cAMP was slightly but significantly elevated compared to control or AVT groups in both −aldo and +aldo cells ($P < 0.001$).

Effect of 4 h aldosterone pretreatment

Part of the study was repeated with cells pretreated with aldosterone for only 4 h prior to the addition of AVT. Although 4 h of incubation with aldosterone was long enough to raise I_{SC} significantly ($P < 0.005$), it was too short to cause a larger than additive synergism. The AVT-induced increase in I_{SC} in cells pretreated with aldosterone for 4 h was not different when compared to non-pretreated cells, as shown in Table IV. Of note, the amiloride-insensitive I_{SC} was significantly ($P < 0.02$) increased by aldosterone pretreatment for 4 h.

Dose-response curve for amiloride

Fig. 4 shows a typical experiment in which a submaximal dose of amiloride (10^{-7} M) was added to the apical side. Upon addition of amiloride,

TABLE IV

EFFECT OF AVT ON SHORT-CIRCUIT CURRENT OF A6 CELLS WITH AND WITHOUT ALDOSTERONE PRETREATMENT FOR 4 HOURS

Values (in $\mu A/cm^2$) are means ± S.E. obtained from 13 experiments. At zero, time aldosterone (10^{-7} M, +aldo) or vehicle (−aldo) was added to the basal side and 4 h later AVT (10^{-7} M) was applied in addition to the basal side. At 5 h amiloride ($5 \cdot 10^{-5}$ M) was added to the apical side and I_{SC} was measured 10 min later. Peak AVT, peak value of I_{SC} with AVT; Δ peak, peak value of I_{SC} with AVT − I_{SC} at 4 h. Significant differences between −aldo and +aldo groups were determined by an unpaired Student's *t*-test.

	I_{SC} ($\mu A/cm^2$)	
	−aldo	+aldo
$t = 4$ h	12 ±1	19 ±2 **
Peak AVT	16 ±2	23 ±2 *
Δ peak	4.0±0.6	4.0±0.4
AVT+amiloride	0.9±0.1	1.9±0.3 *

* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$.

I_{SC} decreased very rapidly, reached a minimum and then increased slightly before it stabilized. The action of amiloride was reversible, although not completely. Upon removal of amiloride, I_{SC} increased and stabilized at a slightly lower value. We also studied the effect of amiloride (from 10^{-9} to $5\ 10^{-4}$ M) in the continuous presence of 10^{-7} M AVT on I_{SC} in A6 cells pretreated for 24 h with aldosterone and in non-pretreated cells.

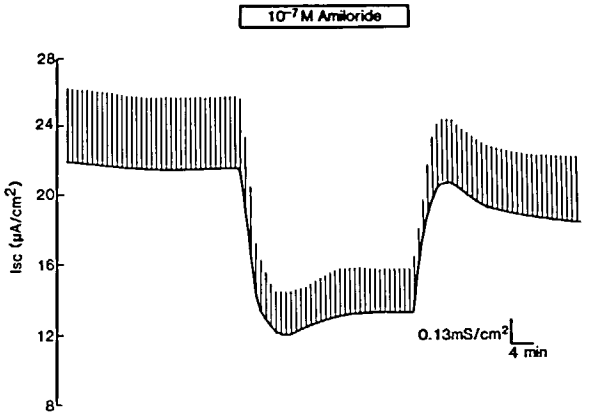

Fig. 4. Original recording illustrating the reversible effect of a submaximal doses of amiloride (10^{-7} M, apical side) on I_{SC} and G in A6 cells. The continuous trace is the I_{SC} and the pulses are current deflections in response to 10 mV pulses from which G was calculated.

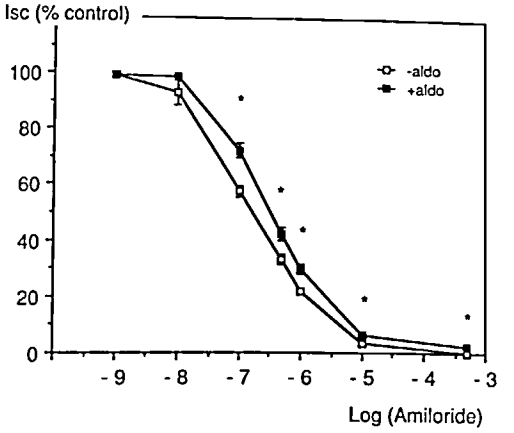

Fig. 5. Dose-response curve for amiloride. Amiloride was added to the apical side in the continuous presence of AVT (10^{-7} M, basal side). Control, non-pretreated A6 cells; Aldo, 24 h aldosterone-pretreated cells. Results are mean ± S.E. of six experiments.

Amiloride was added to the apical side 60 min after the addition of AVT and the minimum I_{SC} was subsequently determined. These results are shown in Fig. 5. In non-pretreated cells, I_{SC} can be inhibited by amiloride in a dose-dependent way, with complete inhibition occurring at $5 \cdot 10^{-4}$ M. Pretreatment with aldosterone shifted the amiloride dose-response curve to the right, as expressed in a doubling of the apparent K_i value from 0.17 ± 0.02 to 0.33 ± 0.04 μM ($P < 0.05$).

Discussion

Our results are the first description of the time course of the effects of AVT and 8-Br-cAMP on I_{SC} and G in A6 cells grown in a defined, serum-free medium. In addition, it is also the first demonstration of a synergistic effect between aldosterone and AVT in this preparation.

A6 cells seeded on Millipore filter cups developed a transepithelial PD rapidly, and a maximal and stable PD was established within 12 days. Removal of FBS from the culture medium 2 days after seeding did not affect the electrical properties of the cells for at least 3 weeks nor did it affect the hormonal responses. In contrast, Verrey et al. [8] were unable to maintain A6 cells in serum-free media for an extended period of time. We are not certain whether A6 cells can truly

grow in the defined, serum-free medium, because they were seeded at greater than confluent density. On the other hand, A6 cells certainly retain the ability to form tight epithelial sheets with a high transepithelial resistance and they could be used for at least 4 weeks with no indication of deterioration. Our preparation offers the ability to study Na^+ transport under truly hormone-free conditions. Previously reported values of non-stimulated I_{SC} [2,27] were, in general, lower than ours, which averaged 14.55 ± 0.06 $\mu A/cm^2$, and the transepithelial resistance was higher in these reports. Therefore, these differences appear to be due to higher rates of transepithelial Na^+ transport in our preparation, which might be due to an increased Na^+ permeability of the apical membrane, or augmented operation of the basolateral Na/K-ATPase pump, or both [21].

Of particular interest is the observation that the effect of AVT on Na^+ transport in A6 cells could be augmented by pretreatment with aldosterone for 24 h. The enhancement in Na^+ transport produced by AVT is greater in aldosterone-treated A6 monolayers when compared to non-pretreated cells. This synergistic effect between both hormones indicates different mechanisms of action. The mechanisms by which either of these hormones increase Na^+ transport is presently the subject of considerable speculation. Both ADH and aldosterone increase the number of apical membrane Na^+ channels [22,23], the rate-limiting factor in transepithelial Na^+ transport. Aldosterone, however, has additional ways of increasing transepithelial Na^+ transport [28] including a direct effect on Na/K-ATPase protein synthesis. The effect of aldosterone on the apical membrane Na^+ channels [8,22] is presumably part of the so-called early response, whereas the induction of pump-protein synthesis takes place primarily during the late response [8,21]. The fact that in the present study the absolute, and not the percent, increase of the I_{SC} with AVT or 8-Br-cAMP was significantly greater in aldosterone-pretreated versus non-pretreated cells, may indicate that the mechanism of action of these drugs is the same with or without aldosterone, but that the baseline is different. Of interest in this respect is our finding that treatment with aldosterone for 4 h failed to show a synergism between AVT and aldosterone, possibly because the base-line I_{SC} was not yet sufficiently increased by a 4 h aldosterone stimulation. One could argue that AVT creates a greater effect in the aldosterone-conditioned cells because of the presence of a greater number of AVT-sensitive but inactive Na^+ channels. Alternatively, the synergistic effect might be due to increased membrane expression of newly formed pump units. In addition, it has been shown in the toad bladder [21] that the effects of ADH and aldosterone on the synthesis of basolateral Na/K-ATPase are not additive, indicating that the synergism could occur at the level of the activation of apical membrane Na^+ conductance.

The potentiation of Na^+ transport by AVT and aldosterone was also observed when exogenous 8-Br-cAMP was used in place of AVT, suggesting that the augmentation of Na^+ transport is mediated by a rise in cellular cAMP. Our results are qualitatively similar to those reported recently in another amphibian epithelium, the toad bladder. Girardet et al. [21] demonstrated that simultaneous addition of aldosterone and an ADH analog, oxytocin, led to a potentiation of the action on Na^+ transport. This effect of oxytocin could be mimicked by cAMP. In addition, they demonstrated a sustained response to oxytocin lasting up to 20 h, while cAMP failed to reproduce this sustained effect on Na^+ transport. The authors suggested a dual effect of oxytocin at the receptor level; one mediated by the adenylate cyclase, which could be mimicked by exogenous cAMP, and the other by a different second messenger. In our present study, however, no difference in the effects of AVT or 8-Br-cAMP was found. The observations by Girardet et al. [21] may, therefore, not be applicable to the A6 cell line or, alternatively, AVT and oxytocin are different hormones in terms of their effects on Na^+ transport.

Our finding that the amiloride-insensitive I_{SC} after cAMP is larger than in control cells is consistent with an earlier study by Perkins and Handler [13]. They confirmed their observation in a second study [10] by stimulating both amiloride-insensitive I_{SC} and chloride secretion with forskolin, leading them to postulate the presence of a cAMP-induced chloride secretion.

The action of a submaximal concentration of amiloride on Na^+ transport can be divided into

two phases. An early phase consisting of a reversible inhibition of I_{SC}, which reached a minimum within 8 min. This minimum was never maintained, however, and in a second phase I_{SC} increased slightly to reach a new steady-state value. One possible explanation for this observation is a so-called Na^+ self-inhibition [29]. According to this hypothesis, the Na^+ channel in the apical membrane can either exist in a conducting state or can be blocked by Na^+. Addition of a submaximal concentration of amiloride will block the conducting Na^+ channels, but in the meantime will release channels from the Na^+ self-inhibition by shifting the equilibrium between conducting and non-conducting channels towards a larger number of conducting channels. The non-conducting channels that are converted to conducting channels would also be blocked by amiloride but the total conductance would increase slightly because of the large number of blocked channels that could 'flicker' open. This phenomenon might explain the biphasic response to amiloride addition and removal. In non-pretreated A6 cells, I_{SC} can be completely inhibited by amiloride, with a half-maximal inhibition constant of 0.17 μM, indicating that I_{SC} is solely caused by Na^+ transport, presumably through Na^+ channels in the apical membrane. Surprisingly, pretreatment with aldosterone doubled the apparent inhibition constant for amiloride. Presently, there are several possible explanations of this observation. Aldosterone could induce a second form of the channel with a lower amiloride affinity, or if aldosterone were to increase the ratio of blocked versus conducting channels, the apparent affinity for amiloride should also decrease [29]. Alternatively, it is also possible that aldosterone gives rise to a small I_{SC} not caused by Na^+ transport through the amiloride-sensitive Na^+ channel. Since Na^+ uptake was not measured directly but estimated by I_{SC}, the participation of other ions involved in active transport can not be totally ruled out. At present, we are unable to distinguish between these possibilities.

In summary, A6 cells grown in a defined, serum-free medium exhibit high rates of hormone-dependent transepithelial Na^+ transport. There is a greater than additive effect between aldosterone and AVT on Na^+ transport if the cells are pretreated for 24 h with aldosterone prior to the experiment.

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