J. Membrane Biol. 138, 65–76 (1994)

The Journal of
Membrane
Biology

Antidiuretic Hormone Action in A6 Cells: Effect on Apical Cl and Na Conductances and Synergism with Aldosterone for NaCl Reabsorption

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Received: 15 July 1993/Revised: 19 October 1993

Abstract. The effect of antidiuretic hormone on transepithelial Na^+ and Cl^- transport and its modulation by aldosterone (10^{-6} M) was studied in the *Xenopus laevis* distal nephron cell line A6-C1 by measuring transepithelial electrophysiological parameters and bidirectional anion fluxes. Vasotocin (or vasopressin) induced a biphasic increase in transepithelial short-circuit current (I_{sc}). Early and late effects were potentiated by aldosterone and could be mimicked by forskolin and BrcAMP, implicating cAMP as a mediator. The early increase in I_{sc} (maximum 1–2 min after hormone addition) was resistant to 50 μM amiloride. Electrophysiological experiments with apical ion substitutions or basolateral bumetanide (0.5 mM), as well as flux studies with $^{125}I^-$ or $^{36}Cl^-$, indicated that this current represented Cl^- secretion. The late increase in I_{sc} appeared with a lag of 2–5 min and was maximal after 15–25 min. It corresponded to an increase in Na^+ reabsorption, since it was amiloride sensitive. Bidirectional $^{36}Cl^-$ flux measurements in aldosterone-treated monolayers maintained under open-circuit conditions showed that the large vasotocin-induced increase in Cl^- permeability led, in these conditions, to a threefold increase of a baseline Cl^- reabsorption. This study shows that vasotocin induces in A6-C1 cells both a rapid increase in Cl^- permeability and a slower increase in Na^+ transport. The Cl^- permeability, which leads to Cl^- secretion under short-circuit conditions, contributes, under the more physiological open-circuit conditions, to the transport of Na^+ by allowing its co-reabsorption with Cl^-.

Key words: Cl^- channel — Na^+ channel — Ion fluxes — Hormonal regulation — Epithelial polarity — cAMP

Introduction

The final adjustment of urinary Na^+ concentration is controlled by mineralocorticoid hormones and antidiuretic hormone which act by regulating the Na^+ reabsorption across tight epithelia of distal segments of the nephron. Na^+ transport across these epithelia is mediated by the serial arrangement of an apical, amiloride-sensitive Na^+ channel through which Na^+ enters the cells and the basolateral Na^+,K^+-ATPase, which extrudes Na^+ and provides the driving force. Much of our knowledge about the mechanism by which hormones exert their action on this transport derives from studies on toad bladder, and more recently, on an amphibian kidney cell line (A6) [23, 25, 28, 33].

In these systems, aldosterone induces an increase in Na^+ reabsorption which is initiated after a lag period of approximately 45 min. This response is divided into an early and a late phase (later than 3 hr), based on electrophysiological and biochemical characteristics [2, 25]. Moreover, like other steroid hormones, aldosterone has in the long term a morphogenetic effect in vivo and a differentiation effect in in vitro cell culture systems [13, 18, 34].

Using the A6 cell model, it has been shown that, when used at concentrations at which both high and low affinity receptors for adrenal steroid hormones are occupied, aldosterone acts in a coordinated manner via transcriptionally mediated mechanisms at the level of the apical amiloride-sensitive Na^+ channel, as well as at that of the basolateral Na^+ pump and K^+ channel [12, 33]. However, the rate-limiting step for the regulation of Na^+ transport is the apical influx, provided that the Na^+ pump has a sufficient functional reserve. Using the patch-clamp technique, Kemendy, Kleyman and Eaton [14] have shown that aldosterone acts on the Na^+ conductance across the apical membrane of A6 cells by increasing the open probability and not the number of the active apical Na^+ channels.

The effect of antidiuretic hormone (arginine vasopressin in most mammalian species or arginine vasotocin in many nonmammalian vertebrates [1]) on Na^+ reabsorption appears to be mediated by a cAMP-dependent pathway. It is generally characterized by a transient maximum of Na^+ transport stimulation which

is observed after approximately 10–30 min of hormonal treatment [4, 10, 34]. Using the patch-clamp technique, Marunaka and Eaton [19] have demonstrated that, unlike in the case of aldosterone action, the increase in apical Na^+ conductance was mediated by an increase in the number of active channels, without significant effect on the open probability of single channels.

Chloride transport across distal nephron cells has not been studied to the same extent as Na^+ transport, although cAMP-stimulated Cl^- transport in collecting duct cells and A6 cells has been described several years ago [30, 39]. In addition, aldosterone-stimulated Cl^- transport has been observed in toad skin [5]. It is generally accepted that the regulated Cl^- transport of the mammalian collecting duct and the toad skin take place across intercalated and mitochondria rich cells, respectively, and not across the Na^+ transporting (principal) cells [5, 16]. In contrast, Cl^- transport across A6 cell monolayers (which was also observed with cells of clonal origin) was attributed to the same cells which also transport Na^+ [39]. More recently, Marunaka and Eaton [18] have studied apical Cl^- conductances in A6 cells at the single channel level and described two channels which could be involved in macroscopic Cl^- transport.

In the present study, the temporal relation between the vasotocin-induced regulation of Na^+ transport and Cl^- conductance and the effect of aldosterone on these parameters has been studied in highly differentiated A6 cell monolayers (A6-C1) maintained in the absence of serum. It is shown that vasotocin induces a rapid increase in transepithelial Cl^- conductance which is followed by a slower increase in that of Na^+. Both vasotocin effects are potentiated by a long-term aldosterone (10^{-6} M) treatment. While the increase in Cl^- permeability leads to Cl^- secretion under short-circuit conditions, Na^+ Cl^- co-reabsorption is stimulated in aldosterone-treated cells, when the monolayers are maintained in the more physiological open-circuit situation.

Materials and Methods

Cell Culture

Experiments were performed with A6 cells from the A6-C1 subclone (passage 109–124). This subclone was obtained by ring-cloning of A6-2F3 cells at passage 99 and was selected for its high transepithelial resistance and for its responsiveness to aldosterone and antidiuretic hormone [34, 35]. Cells were cultured on plastic dishes in 0.8 × concentrated, bicarbonate buffered Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (Flow) and 1% of a penicillin-streptomycin solution (GIBCO) at 28°C in 5% CO_2 atmosphere. Cells were split once a week 1/10 to 1/20 and the medium changed after three days. For culture on porous substrate, polycarbonate filters (Transwell, 0.4 μm pore size, 4.7 cm^2, Costar) were coated with a thin layer of bovine dermal collagen (Vitrogen 100, Collagen) which was polymerized with NH_3 vapor and crosslinked with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Cells were seeded on filters at two times the density of cells subcultured to confluency on plastic dishes. Inside and outside the filters were 2 and 2.5 ml, respectively, of the same medium as above. The upper medium was replaced the day after seeding and fresh medium was given on both sides after seven days. After 10 days, the medium was replaced by serum and bicarbonate-free 0.8 × DMEM, buffered with 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) to pH 7.4, and supplemented with 10^{-6} M aldosterone (Sigma) in the case of long-term treatment. Cells were placed in an incubator without CO_2 supplementation and the medium was routinely changed four days later. Experiments were generally performed 15 to 17 days after seeding. Fresh medium was always given the day before and 1–3 hr before the start of the experiment (preincubation). (Arginine)-vasotocin, (arginine)-vasopressin, amiloride, 8-bromoadenosine 3′-5′-cyclic monophosphate (BrcAMP) and aldosterone were purchased from Sigma, bumetanide was from Leo Pharm., and forskolin and 1,9-dideoxy forskolin from Calbiochem.

Electrical Measurements

Transepithelial electrical measurements were performed on Transwell^R rings in a modified Ussing chamber [21] using an automatic voltage-clamp apparatus [26] which was connected to a dual-channel recorder (Pharmacia LKB). The calomel voltage electrode pair and the Ag/AgCl current electrode pair were connected to the apical and the basolateral media by thin polyethylene tubings containing 3 M KCl-3% agarose. By convention, positive current corresponds to an apical to basolateral movement of positive charges across the epithelium. Transepithelial electrical resistance (R_{TE}) ($\Omega \times cm^2$) was calculated according to Ohm's law from the V_{TE} (mV) and the I_{sc} (μA cm^{-2}). The transepithelial conductance (G_{TE}) (μS cm^{-2}) is $1/R_{TE}$. The measurements were performed, unless otherwise stated, at room temperature (22–25°C) in serum-free and HEPES-buffered culture medium (0.8 × DMEM) containing (in mM): Na 100, Cl 96, K 4.3, Ca 1.4, Mg 0.7, SO_4 0.7, PO_4 0.7, glucose 20 plus vitamins and amino acids (~240 mOsm/kg H_2O). For some experiments (Fig. 1, Table 1), the cell monolayers were kept in open-circuit configuration and the transepithelial potential difference (V_{TE}) was monitored continuously. The short-circuit current (I_{sc}) was measured every 20 sec by clamping V_{TE} to 0 mV for 1 sec. For other experiments (Figs. 3, 4*A* and *B;* Tables 4 and 5), the monolayers were maintained under short-circuit conditions.

Apical Ion-Substitution Experiments

The apical medium was replaced with a solution containing (in mM) NaCl 100, Ca gluconate 1, Mg gluconate 0.5, K_2HPO_4 2, HEPES 10, glucose 5, and buffered to pH 7.4 with tris-(hydroxymethyl)aminomethane. The transepithelial potential difference and the short-circuit current was measured as described above. After stabilization of these parameters (approximately 15 min), the apical medium was replaced by a solution containing 10^{-5} M amiloride in control buffer or in buffer in which a fraction of the Cl^- was replaced by gluconate, or a fraction of Na^+ by tetramethylammonium (TMA), or a fraction of Na^+ by K^+. After an equilibration period of 15 min, current-voltage curves were obtained before, and after (1.5 to 3 min) the addition of vasotocin, by clamping the monolayer every 10 sec for 2 sec, from +50 to −50 mV at 10 mV intervals.

$^{125}I^-$-Flux Measurements

Culture conditions were the same as for electrophysiological measurements. A modified measurement chamber with a small opening was used which allowed adding the hormone and taking samples from both sides of the monolayer, without interrupting the voltage clamp. Experiments were performed at 26°C. Filters were preincubated for 1 hr in fresh 0.8 × DMEM buffered with HEPES. The monolayers were then maintained clamped at 0 mV and I_{sc} was measured continuously. After a few minutes, when the trace was stabilized, tracer amounts (15 μCi/ml) of Na^+ $^{125}I^-$ (17.4 Ci/mg, NEN) were added to one side of the filter culture. Amiloride (10^{-5} M) was added to the apical medium and after 15 min the medium, contralateral to the added radioactivity, was replaced by fresh medium (containing amiloride in the case of apical medium). The sampling of contralateral medium was started 10 min later. Aliquots of 1% of the chamber volume were taken every 3 min, after the medium had been mixed by shaking. Because of the lag period of approximately 30 sec, vasotocin (2.5×10^{-8} M) was given basolaterally 20 sec before the last aliquot of control medium was taken. The aliquots were counted in a gamma-counter (Pharmacia LKB) and the increases in cpm/3 min collection periods were converted into thousandths of the radioactivity given to the other side of the monolayer (arbitrary flux units).

$^{36}Cl^-$-Flux Measurements

Culture conditions and experimental setup were as for $^{125}I^-$-flux measurements. For experiments under open-circuit conditions, V_{TE} was monitored continuously. Both sides contained 2 ml of 0.8 × DMEM buffered with HEPES (96 mM Cl^-). On one side, 1.5 μCi/ml of $^{36}Cl^-$ tracer was added (13.9 mCi/g $H^{36}Cl$, neutralized with NaOH, NEN) and the voltage clamp was started in the experiments performed under short-circuit conditions. After a 20 to 30 min equilibration time, 1 ml of the contralateral medium was replaced quickly three times before the start of the sample collection. Samples were taken every 5 min by carefully aspirating 1 ml of medium which was immediately replaced by 1 ml of fresh medium (±vasotocin), without interrupting the voltage clamp or the V_{TE} measurement. The vasotocin treatment was initiated by adding 20 μl of a 100-fold concentrated stock solution in medium. The 1 ml samples and aliquots of bath medium diluted in 1 ml were mixed with 10 ml of scintillation fluid (Emulsifier-Safe, Packard) and counted in a scintillation counter (Kontron). The counts accumulated during the 5 min interval were calculated for each sample and converted in nEq cm^{-2} min^{-1} Cl^-.

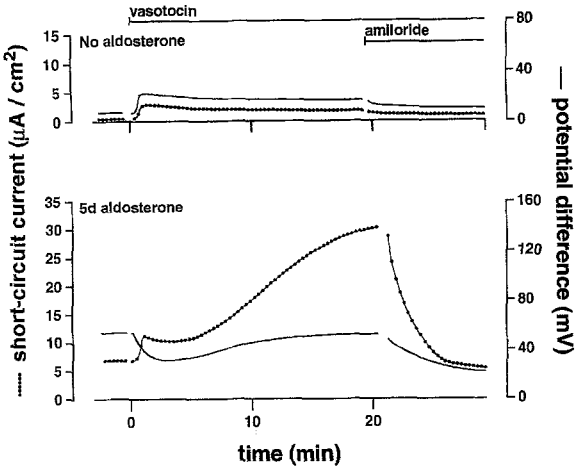

Fig. 1. Vasotocin action on transepithelial short-circuit current and potential difference and its potentiation by aldosterone. Monolayers of A6 cells were cultured on filters for a total of 15 to 18 days and maintained in the absence of serum and bicarbonate and in the absence or presence of aldosterone (10^{-6} M) for 5 to 8 days prior to the experiment. The preparations were maintained in open-circuit configuration during the recordings and the transepithelial short-circuit current (I_{sc}) was measured every 20 sec for 1 sec. Transepithelial short-circuit current and potential difference are redrawn from typical experiments. Vasotocin was added to the basolateral medium (2.5×10^{-8} M) and amiloride to the apical medium (5×10^{-5} M).

Results

Aldosterone has a stimulatory action on Na^+ reabsorption across A6 monolayers cultured on permeable supports whose amplitude depends on the culture conditions

Table 1. Vasotocin[a] effect and its aldosterone [c] dependence on transepithelial electrical parameters of A6-C1 monolayers maintained in open-circuit conditions[a]

Experimental condition	Short-circuit current (I_{sc})				Transepithelial conductance (G_{TE})		
	Baseline I_{sc} ($\mu A\ cm^{-2}$)	Early increase in I_{sc}[d] ($\mu A\ cm^{-2}$)	AMI[c]-sensitive I_{sc}[e] ($\mu A\ cm^{-2}$)	Relative increase in AMI-sensitive I_{sc}[f] (fractional change)	Baseline G_{TE} ($\mu S\ cm^{-2}$)	Early AVT effect[g] ($\mu S\ cm^{-2}$)	Late AVT effect[h] ($\mu S\ cm^{-2}$)
No aldosterone	0.5 ± 0.0[b]	2.3 ± 0.2	0.9 ± 0.2	1.7 ± 0.3	88 ± 19	160 ± 21	132 ± 18
6 hr aldosterone	3.0 ± 0.2	2.7 ± 0.2	5.2 ± 0.3	1.7 ± 0.0	112 ± 35	229 ± 47	229 ± 37
20 hr aldosterone	5.5 ± 0.1	3.9 ± 0.2	21.7 ± 3.3	3.9 ± 0.6	97 ± 12	248 ± 11	486 ± 48
5 day aldosterone	6.6 ± 1.4	3.7 ± 0.2	22.6 ± 2.6	3.6 ± 0.4	135 ± 5	317 ± 14	544 ± 46

[a] The recordings were performed as for Fig. 1 and the short-circuit current (I_{sc}) was measured every 20 sec. [b]The means of three experiments ± SE are indicated. [c]Aldosterone was 10^{-6} M bilaterally, vasotocin 2.5×10^{-8} M basolaterally, and amiloride (AMI) 5×10^{-5} M apically. [d]I_{sc} 1.5 min after AVT − baseline I_{sc}. [e]I_{sc} 20 min after AVT − I_{sc} 8 min after AMI. [f] AMI-sensitive I_{sc} 20 min after AVT/baseline I_{sc}. [g]1.5 min after AVT. [h]20 min after AVT.

Table 2. Comparison of vasotocin[c], forskolin and BrcAMP action on the short-circuit current (I_{sc})[a] in aldosterone-free and long term aldosterone-treated A6-C1 monolayers

Experimental condition	No aldosterone		5 day aldosterone	
	Early increase in I_{sc}[d] (μA cm^{-2})	Relative increase in amiloride-sensitive I_{sc}[e] (fractional change)	Early increase in I_{sc}[d] (μA cm^{-2})	Relative increase in amiloride-sensitive I_{sc}[e] (fractional change)
Vasotocin	2.4 ± 0.5[b]	1.7 ± 0.1	5.5 ± 0.7	3.3 ± 0.2
Forskolin	2.9 ± 0.2	3.3 ± 0.8	5.5 ± 1.0	3.5 ± 0.6
BrcAMP	3.1 ± 1.0	2.9 ± 0.9	4.4 ± 1.5	3.1 ± 0.4

[a] The monolayers were maintained in open-circuit conditions and the short-circuit current (I_{sc}) was measured every 20 sec as for Fig. 1. [b]The means of three experiments ± SE are indicated. [c]Aldosterone was 10^{-6} M bilaterally, vasotocin (AVT) was 2.5×10^{-8} M basolaterally, forskolin was 2×10^{-5} M basolaterally, 8-bromoadenosin 3′:5′-cyclic monophosphate (BrcAMP) was 3×10^{-3} M bilaterally, and amiloride 5×10^{-5} M apically. [d]I_{sc} 1.5 min after AVT − baseline I_{sc}. In the case of BrcAMP, the early increase in I_{sc} was measured 3 min after its addition. [e]I_{sc} 20 min after AVT/baseline I_{sc}.

[18, 21, 23, 38]. Using filter cultures of cells from the A6-C1 subclone, culture conditions were devised such that the baseline Na^+ transport, in the absence of hormone, was usually very low, allowing for changes of great magnitude upon addition of hormones. During a period of 10 days of culture in the presence of bicarbonate, CO_2 and fetal calf serum, high transepithelial resistance (R_{TE}) was established. The monolayers were then transferred for at least five days to serum-free and bicarbonate-free HEPES-buffered medium. In the series of filters used for Fig. 1 and Table 1, the baseline transepithelial short-circuit current (I_{sc}) was 0.51 ± 0.02 μA/cm^2, and the resistance 12,300 ± 2,200 Ω × cm^2. The I_{sc} was increased by a factor of 13 after long-term aldosterone (10^{-6} M) treatment (6.6 ± 1.3 μA/cm^2). It should be mentioned that the concentration of aldosterone used throughout this study (10^{-6} M) has been chosen to obtain maximal effects on Na^+ transport. At this concentration, aldosterone binds to both the high and the low affinity receptors for adrenal steroid hormones [29, 33, 37].

Biphasic Action of Vasotocin on the Transepithelial Short-Circuit Current

The addition of vasotocin (2.5×10^{-8} M) to the basolateral medium produced a biphasic increase in apical to basolateral short-circuit current (I_{sc}), whose second phase was only clearly visible when the filter was pretreated with aldosterone (10^{-6} M) and displayed, consequently, a high baseline I_{sc} (Figs. 1 and 4*A* and *B*). A similar response with the same maximal values was observed using arginine vasopressin, the antidiuretic hormone of most mammals. However, vasotocin was 100 times more potent than vasopressin ($K_{0.5} = 5 \times 10^{-10}$ M and $K_{0.5} = 5 \times 10^{-8}$ M, respectively). Therefore, vasotocin (the actual vasopressor hormone of the amphibian neurohypophysis [1]) was used throughout this study.

The experiments presented in Fig. 1 and Table 1 were performed on epithelia maintained under open-circuit conditions, and the short-circuit current was measured every 20 sec by clamping the transepithelial potential (V_{TE}) for 1 sec to 0 mV. In these conditions, vasotocin induced, after a lag period of approximately 30 sec, a rapid increase in I_{sc} and a large, concomitant increase in transepithelial conductance (G_{TE}). After an initial I_{sc} peak (2 to 7 μA cm^{-2}, 1–2 min after hormone addition) a slower decrease in I_{sc} followed, while the G_{TE} did not decrease to the same extent. This I_{sc} peak was followed by the onset of a late increase in I_{sc} which took place 2 to 5 min after hormone addition. This late increase was dependent on the pre-existing baseline I_{sc} (*see below*) and reached a maximum after 10 to 20 min. This late I_{sc} increase was accompanied in long-term aldosterone-treated cells by a further increase in G_{TE}.

The addition of the apical Na^+ channel blocker amiloride (5×10^{-5} M), 20 min after vasotocin addition, revealed that a substantial part of the induced I_{sc} was amiloride resistant and thus did not correspond to Na^+ transport. Since preliminary experiments had shown that, in the absence of vasotocin, generally more than 90% of the I_{sc} was inhibited by 5×10^{-5} M amiloride, the relative increase in amiloride-sensitive I_{sc} induced by vasotocin (20 min) could be approximated (Table 1). This calculation showed that the relative increase in Na^+ current was 1.7 to 3.9-fold.

That the early vasotocin-induced increase in I_{sc} corresponded to an amiloride-resistant current and that the late increase corresponded to the known cAMP-mediated effect on the amiloride-sensitive apical Na^+ channel could be seen when amiloride was added prior to vasotocin. In this case, the transient early increase in I_{sc} was not followed by a second increase. An example can be seen in Fig. 3*A* and *C* where the epithelia were, how-

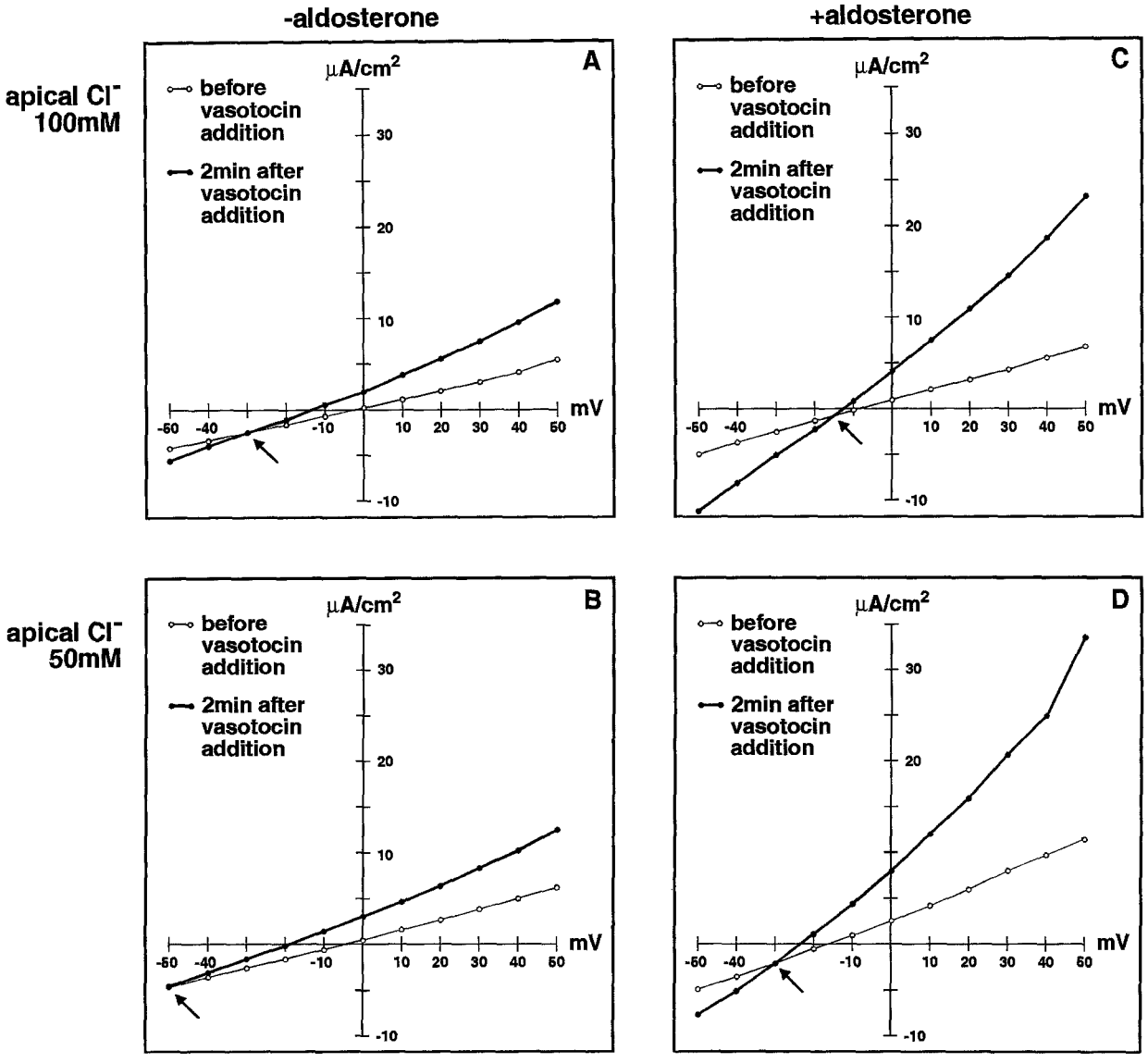

Fig. 2. Effect of apical ion substitutions on the reversal potential of the early vasotocin-induced current. Culture conditions were as for Fig. 1. The apical medium was replaced by a buffer containing 100 mM Cl^- (*A* and *C*) or 50 mM Cl^- and 50 mM gluconate (*B* and *D*), and 10^{-5} M amiloride. The monolayers were maintained under open-circuit condition and current-voltage curves were obtained before and 1.5 min after the addition of vasotocin (2.5×10^{-8} M) by clamping the monolayer every 10 sec for 2 sec at 10 mV intervals. Long-term pretreatment with 10^{-6} M aldosterone is indicated by +aldosterone.

Table 3. Effect of apical ion substitutions on the reversal potential of the early vasotocin-induced current

Experimental condition	−Aldosterone					+Aldosterone		
Apical Na^+ (mM)	100	100	100	96	20	100	100	100
Apical Cl^- (mM)	100	50	20	100	100	100	50	20
Apical K^+ (mM)	4	4	4	8	4	4	4	4
Reversal potential of the early vasotocin-induced current (mV)	−30.4 ± 2.8 $n = 5$	−47.3 ± 2.9 $n = 4$	−67.0 ± 4.5 $n = 3$	−32.3 ± 0.3 $n = 3$	−32.3 ± 0.9 $n = 3$	−17.7 ± 1.2 $n = 3$	−32.3 ± 1.5 $n = 3$	−52.7 ± 1.5 $n = 3$
Shift of the reversal potential (mV)		−16.9	−36.6	−1.9	−1.9		−14.6	−35.0

Given are the means (±SE, *n* experiments) of the transepithelial potential at which the transepithelial current 1–2 min after vasotocin addition is identical to that before hormone addition. Gluconate and tetramethylammonium were used to replace Cl^- and Na^+, respectively.

ever, maintained under continuous short-circuit conditions (V_{TE} clamped at 0 mV).

During some flux experiments presented in Fig. 4*A* and *B*, the epithelia were maintained under short-circuit conditions in the absence of amiloride. In these conditions, though a biphasic response of the I_{sc} to vasotocin was observed, there was a major difference in the amplitude of the late increase compared to that observed in epithelia maintained under open-circuit conditions. In epithelia pretreated with long-term aldosterone there was, just after the transient I_{sc} peak, a decrease in total I_{sc} below the baseline level (Fig. 4*B*). This decrease was possibly due to a transient change in Na^+ transport secondary to the acute early amiloride-resistant current. The late increase in I_{sc} was also less pronounced, compared to that under open-circuit conditions. This could have been due, in these high transport-rate conditions, to a rate-limiting effect of the basolateral Na^+,K^+-ATPase and to the consequent feedback inhibition at the level of the apical Na^+ channel [8, 22].

Both the early and the late vasotocin-induced increases in I_{sc} were potentiated by long-term aldosterone treatments. After such a treatment, which *per se* produced a 13-fold increase in I_{sc}, the early induced peak of I_{sc} was increased by a factor of 1.5 to 2 (Figs. 1, 3*A* and *C*, 4*A* and *B* and Tables 1 and 2). In contrast, the late, amiloride-sensitive increase in I_{sc} was roughly proportional to the pre-existing I_{sc}, indicating that there was not only an additive but a synergistic effect on the Na^+ transport. Furthermore, the relative increases were larger in those filters which had received aldosterone for 20 hr and 5 days (3.9 and 3.4-fold increase, respectively) as compared to the control filters and those with 6 hr of aldosterone treatment (1.8 and 1.7-fold increase, respectively).

In summary, the two effects which compose this biphasic action of vasotocin could be dissociated by their timing, their sensitivity to amiloride, and by their dependence on pre-existing, aldosterone-induced amiloride-sensitive I_{sc}, and both components were potentiated by a long-term aldosterone treatment.

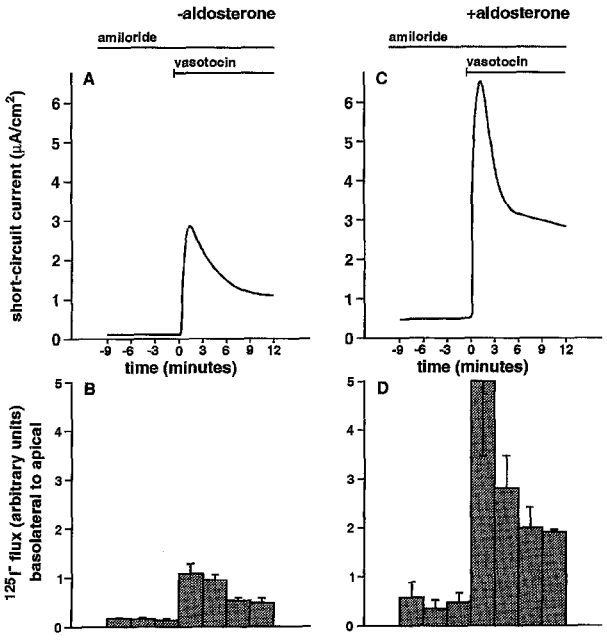

Fig. 3. $^{125}I^-$-flux induced by vasotocin and its potentiation by aldosterone. Culture conditions were as for Fig. 1. Amiloride (10^{-5} M) was given to the apical medium and the monolayers were maintained under short-circuit condition during the experiment. Recordings of the I_{sc} from typical experiments on a control monolayer (−aldosterone) and a monolayer pretreated for 5 days with 10^{-6} M aldosterone (+aldosterone) are shown in *A* and *C*, respectively. *B* and *D* show the mean (±SE, $n = 3$) of the basolateral to apical $^{125}I^-$-fluxes given in arbitrary units.

Mimicking of the Vasotocin Effects with Forskolin and BrcAMP

To evaluate whether the observed induction of ion transport by vasotocin could be mediated by cAMP, I sub-

Table 4. Effect of basolateral bumetanide[a] on the I_{sc} induced by vasotocin in amiloride-treated, aldosterone-free A6-C1 monolayers maintained in short-circuit conditions

Experimental condition	No bumetanide ($\mu A\ cm^{-2}$)	0.5 mM bumetanide ($\mu A\ cm^{-2}$)	Change induced by bumetanide (fractional change)
Baseline amiloride-resistant I_{sc}	0.16 ± 0.05	0.18 ± 0.08	1.1 ± 0.1
Amiloride-resistant I_{sc} 1.5 min after AVT (I_{sc} ~1.5 min after AVT — baseline I_{sc})	3.8 ± 0.4	2.0 ± 0.1	0.53 ± 0.05
Amiloride-resistant I_{sc} 15 min after AVT (I_{sc} 15 min after AVT — baseline I_{sc})	1.6 ± 0.2	0.35 ± 0.05	0.23 ± 0.03

[a] Monolayers were maintained in short-circuit conditions; basolateral bumetanide (5×10^{-4} M) or diluent and apical amiloride (5×10^{-5} M) or diluent were given 20 and 10 min before the addition of vasotocin (AVT, 2.5×10^{-8} M), respectively.

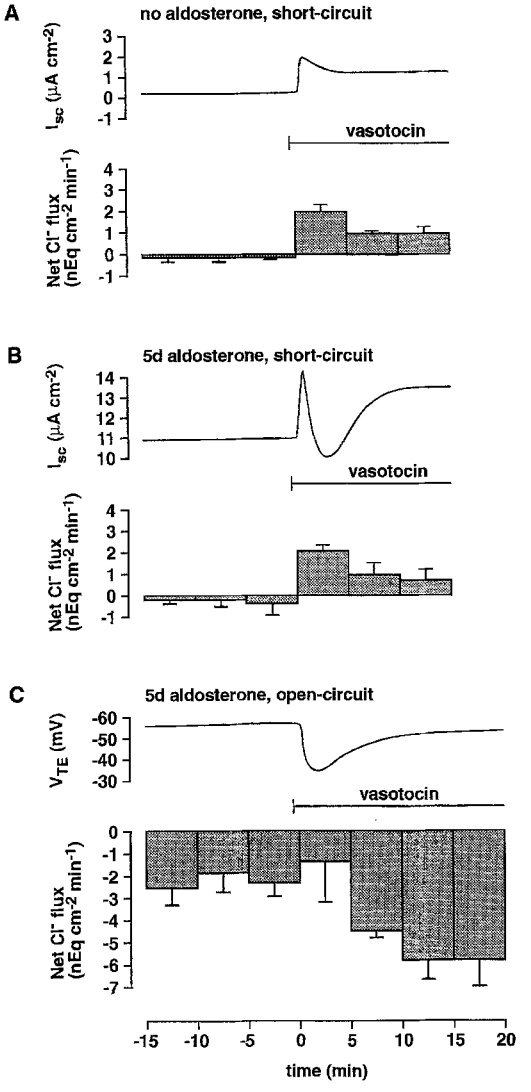

Fig. 4. Net $^{36}Cl^-$ fluxes induced by vasotocin under short-circuit and open-circuit conditions. Culture conditions were as for Fig. 1. For each experimental condition, the trace of the monitored electrical parameter from a typical experiment is shown: Continuous short-circuit condition, I_{sc} (*A* and *B*); continuous open-circuit condition, V_{TE} (*C*). The net basolateral to apical $^{36}Cl^-$ fluxes represent the average ± SE of the net fluxes calculated in three individual bidirectional flux measurements. Experiments were performed on control monolayers (no aldosterone, *A*) and monolayers pretreated for 5 to 8 days with 10^{-6} M aldosterone (5 day aldosterone, *B* and *C*).

mitted control and long-term (5 days) aldosterone-treated monolayers to the same protocol as for Fig. 1, and replaced vasotocin with the adenylate cyclase activator forskolin or by a membrane-permeant analogue of cAMP (BrcAMP) (Table 2). In aldosterone-treated cells, the effect of forskolin (2×10^{-5} M) was very similar to the effect of vasotocin, both qualitatively and quantitatively (Table 2), while its inactive analogue, 1,9-dideoxy forskolin had no effect on the electrical parameters (*data not shown*). BrcAMP (3 mM) had a similar effect. However, the early increase in I_{sc} was slower, possibly due to the slow diffusion of this analogue into the cells. Interestingly, in aldosterone-free cells the effect on the amiloride-sensitive short-circuit current was clearly larger with forskolin and BrcAMP than with vasotocin. This difference could be due to the potentiation by long-term aldosterone (10^{-6} M) of the signal transduction upstream of the adenylate cyclase [34].

Effect of Apical Ion Substitutions on the Early Vasotocin-Induced Current

The large decrease of the R_{TE} induced by vasotocin occurred simultaneously with a parallel increase in I_{sc} measured with symmetrical apical and basolateral solutions. Only a change in the transcellular conductance (G_{TE}) can explain both phenomena, since an increase in the paracellular conductance alone would not induce any change of I_{sc} under these conditions. Furthermore, considering the apical to basolateral resistance ratio in A6 cells of 0.8 or larger [38], a major increase in apical membrane conductance is needed to explain the change in transcellular conductance, although a simultaneous change in basolateral membrane conductance cannot be excluded.

To determine the ionic selectivity of the amiloride-resistant conductance induced by vasotocin in the apical membrane, I performed ionic replacement experiments. Transepithelial current/voltage (*I/V*) curves were obtained in the presence of 10^{-5} M apical amiloride immediately before and 1.5 to 3 min after addition of vasotocin (Fig. 2). The difference between the current before and after vasotocin addition is the vasotocin-induced transepithelial current. The reversal potential of the vasotocin-induced current is given by the intersection of the two *I/V* curves. Mean values of this vasotocin-induced current reversal potential with different apical solutions are shown in Table 3. Reducing the Na^+ concentration from 100 to 20 mM did not produce any significant change of the reversal potential. This was not surprising considering that the experiments were performed in the presence of the Na^+ channel blocker amiloride. It shows, however, that vasotocin did not induce an amiloride-resistant Na^+ conductance. Increasing the apical K^+ concentration from 4 to 8 mM did not induce either any change in the reversal potential of the vasotocin-induced current. In contrast, a 50 or 80% reduction of the apical Cl^- concentration (gluconate replacement) induced a −16.9 and −36.6 mV change, respectively, in vasotocin-induced current reversal potential. These values are close to the values calculated for a Cl^--selective conductive pathway (−17.7 and −41.2 mV, respectively). Although the apical mem-

Table 5. Effect of vasotocin on bidirectional Cl^- fluxes across A6-C1 monolayers maintained in short-circuit or open-circuit conditions

Experimental condition	No aldosterone	5 day aldosterone (10^{-6} M)	
	Short-circuit conditions	Short-circuit conditions	Open-circuit conditions
AP → BL Cl^- flux (nEq cm^{-2} min^{-1})[b]			
10–5 min before vasotocin	0.65 ± 0.13[a]	1.3 ± 0.3	2.6 ± 0.8
0–5 min after vasotocin	1.8 ± 0.1	6.5 ± 0.5	10.4 ± 1.5
10–15 min after vasotocin	2.3 ± 0.0	8.2 ± 0.5	11.2 ± 1.5
BL → AP Cl^- flux (nEq cm^{-2} min^{-1})			
10–5 min before vasotocin	0.58 ± 0.13	1.1 ± 0.3	0.59 ± 0.09
0–5 min after vasotocin	3.6 ± 0.4	8.6 ± 0.8	9.0 ± 0.8
10–15 min after vasotocin	3.2 ± 0.3	8.9 ± 0.4	5.3 ± 0.7
Net Cl^- flux (nEq cm^{-2} min^{-1}, BL → AP)			
10–5 min before vasotocin	−0.07 ± 0.21	−0.16 ± 0.20	−2.0 ± 0.7
0–5 min after vasotocin	1.8 ± 0.4	2.0 ± 0.3	−1.4 ± 1.8
10–15 min after vasotocin	0.87 ± 0.33	0.68 ± 0.58	−5.9 ± 0.8
Cl^- current (calculated, μA cm^{-2})			
10–5 min before vasotocin	−0.11 ± 0.34	−0.26 ± 0.33	−3.2 ± 1.1
0–5 min after vasotocin	2.9 ± 0.6	3.3 ± 0.5	−2.1 ± 2.8
10–15 min after vasotocin	1.4 ± 0.5	1.1 ± 0.9	−9.4 ± 1.3
I_{sc} over baseline (measured, μA cm^{-2})			
7.5 min before vasotocin			
~1.5 min after vasotocin[c]	3.1 ± 0.2	6.7 ± 0.5	
12.5 min after vasotocin	2.0 ± 0.1	6.4 ± 0.4	

[a] Three bidirectional flux experiments were performed for each condition. Mean values ± SE are indicated. [b] AP → BL stands for apical to basolateral. [c] Peak value of I_{sc} ~1.5 min after vasotocin addition.

brane potential and the intracellular ion concentrations were not controlled under the present experimental conditions, these results strongly suggest that the amiloride-resistant ionic pathway opened by vasotocin was chloride selective.

Inhibition of the Early Vasotocin-induced I_{sc} by Basolateral Bumetanide

A Na^+,K^+,2Cl^--cotransport system blockade to 96–98% by 5×10^{-4} M bumetanide has been described in A6 cells [7, 39]. Since this transport system could be involved in the basolateral influx of Cl^-, I tested the effect of basolateral bumetanide in conditions in which I postulated that the vasotocin-induced increase in I_{sc} was due to Cl^- secretion (basolateral to apical transport). To that effect, experiments were conducted in the presence of apical amiloride and under short-circuit conditions (Table 4). Bumetanide (5×10^{-4} M) inhibited, after a preincubation of 20 min, 47% of the transient I_{sc} peak, and more importantly, 77% of the amiloride-resistant current measured 15 min after hormone addition. This observation further supported the idea that the amiloride-resistant vasotocin-induced I_{sc} was carried by Cl^- ions. That the early I_{sc} peak was inhibited to a lesser extent than the amiloride-resistant current 15 min later could have been due to the initial secretion of the intracellular Cl^-.

Effect of Vasotocin on $^{125}I^-$ Fluxes

Fluxes of $^{125}I^-$ were measured as an alternative approach to confirm that the early increase in I_{sc} was due to the opening of an anion permeability. The filters were maintained short-circuited and with apical amiloride (10^{-5} M) during the experiment to favor the anion secretion and to avoid the possible effects of changes in Na^+ conductance. In these conditions (Fig. 3*A* and *C*), the baseline short-circuit current was low in both control and aldosterone-treated monolayers, and the transient nature of the early I_{sc} peaks appeared more clearly than under open-circuit conditions and in the absence of amiloride. The high specific activity of $^{125}I^-$ had the advantage of allowing a good time resolution of the fluxes. However, a limitation was that the quantification was not meaningful, because of the different properties of I^- compared to the physiologically dominant halide Cl^-. Vasotocin induced a large increase in

basolateral to apical $^{125}I^-$ flux which had a parallel time course to that of the I_{sc} (Fig. 3*B* and *D*). The apical to basolateral flux of $^{125}I^-$, which showed a larger variability between experiments, tended to decrease after the addition of vasotocin (*data not shown*). These experiments confirmed that an anion permeability was opened by vasotocin and strongly suggested that an anion flux was carrying the amiloride-resistant I_{sc}.

Net $^{36}Cl^-$ Fluxes and their Potential-dependent Reversal

To obtain more quantitative information about the anion fluxes induced by vasotocin and to test their possible role in the Na^+ transport regulation, bidirectional $^{36}Cl^-$-fluxes were measured in the absence of amiloride and under both short-circuit and open-circuit conditions (Fig. 4 and Table 5). Vasotocin produced a 4.5- and 7.5-fold increase in the average bidirectional Cl^- fluxes (average of apical to basolateral and basolateral to apical fluxes as a measure for the Cl^- permeability) in aldosterone-free and aldosterone-treated monolayers, respectively (Table 5). Similar to the electrical conductance (*see above*), the permeability did not decrease significantly after its initial rapid increase. However, under short-circuit conditions, the net basolateral to apical flux of $^{36}Cl^-$ decreased rapidly after a transient peak, as did the amiloride-resistant I_{sc} (*see* Fig. 3*A* and *C*). Calculation of the current carried by the net Cl^- flux (*see* Table 5) showed that it could fully account for the acute increase in amiloride-resistant I_{sc}. However, in these experiments (absence of amiloride), the changes in amiloride-resistant I_{sc} are superimposed on changes due to modifications of the Na^+ transport (*see* Fig. 4 and above), thereby limiting the possibility of comparison at later time points. Hence, it is also interesting to compare the calculated Cl^- currents (Table 5) with the measured amiloride-resistant I_{sc} (Fig. 3). Indeed, there is a close correspondence of these values, particularly in the aldosterone-free condition (where blocking the Na^+ channel with amiloride presumably does not induce a large increase in apical membrane potential; i.e., no major change in driving force for Cl^- secretion).

Aldosterone-treated cells maintained under open-circuit conditions have an apical membrane which is depolarized by the Na^+ conductance and show an apically negative V_{TE}. This provides a driving force for apical Cl^- influx and transepithelial reabsorption. Indeed, under these conditions I observed a baseline apical to basolateral Cl^- flux (Fig. 4*C* and Table 5). Although the addition of vasotocin increased the permeability to Cl^- to a similar extent as under short-circuit conditions, the net induced Cl^- flux was, in this case, in the opposite direction, that is apical to basolateral (Cl^- reabsorption). It corresponded to a significant threefold increase in the calculated Cl^- current from 3.2 (baseline) to 9.4 $\mu A\ cm^{-2}$ (*t*-test: $P \leq 0.01$) despite a slight reduction of the transepithelial driving force (V_{TE}). This net flux increase appeared to be slightly delayed and its time course correlated better with the increase in Na^+ conductance than with that of Cl^-. This delay could have been partly due to technical limitations of tracer studies, such as differences in intracellular tracer concentration before hormone addition in apical to basolateral *vs.* basolateral to apical measurements. However, it could also have corresponded to a "real"delay. Indeed, secondary permeability changes at the level of the basolateral membrane might have played a role, and, more importantly, the driving force for apical Cl^- influx was expected to increase in parallel with the late increase in Na^+ conductance, which does increase the driving force for Cl^- reabsorption because of its depolarizing action at the level of the apical membrane (*see* late increase in apically negative potential, Fig. 4*C*).

Discussion

Nature of the Conductive Pathway Involved in the Early Vasotocin Effect

In this paper, three lines of experimental evidence from different approaches are presented which support the conclusion that the early vasotocin-induced, amiloride-resistant current is carried by Cl^- and is due to the opening of an apical Cl^--selective conductive pathway.

First, the results from transepithelial electrophysiological and ionic substitution experiments taken together support the above conclusion: (i) The vasotocin-induced amiloride-resistant transepithelial current occurs in the presence of symmetrical apical and basolateral media and under short-circuit conditions (Figs. 1, 3, 4; Tables 1, 2, 4, 5). This observation cannot be explained by the opening of a paracellular pathway. (ii) This current appears simultaneously with a large drop in R_{TE} (Fig. 1, Table 1). Such a decrease in R_{TE} can only be explained by a large increase in apical or paracellular conductance, since the apical to basolateral resistance ratio is 80% or more in A6 cells [38]. However, a simultaneous change of basolateral membrane conductance could occur also. (iii) The reversal potential of this current is shifted only when Cl^- is substituted in the apical solution (Fig. 2, Table 3). This result is expected for a Cl^--selective conductive pathway.

Second, experiments with the Na^+,K^+,$2Cl^-$ co-transport system inhibitor bumetanide also support the conclusion that a transcellular Cl^- transport carries the early vasotocin-induced I_{sc}. Indeed, under experimental conditions which favor Cl^- secretion, blocking of the Na^+,K^+,$2Cl^-$ co-transport system, which is the putative

basolateral entry pathway for Cl^-, inhibits the amiloride-resistant short-circuit current to a large extent (Table 4).

Third, the time course of the vasotocin-induced anion fluxes parallels that of the induced I_{sc} (Figs. 3 and 4) and furthermore, the calculated net Cl^- fluxes can fully account for the acutely induced amiloride-resistant I_{sc}.

Native epithelia, such as the mammalian collecting duct or the amphibian skin also have, besides the Na^+ transporting (principal) cells, intercalated or mitochondria rich cells, respectively. In these epithelia, regulated Cl^- transport has generally been attributed to the second cell subpopulation [5, 16]. Interestingly, the A6-C1 cell line used in this study, as well as some cell lines tested in the study by Yanase and Handler [39], were of clonal origin. This supports the notion that in the case of A6 cells both apical conductances (for Na^+ and Cl^-) are present in the same cells [39]. However, the measurements have not been performed at the single cell level, and morphological criteria show that the differentiation of the A6-C1 cells on filters is not homogenous [34]. Therefore, it is not excluded that the two apical conductances are located in two cell types which have undergone a differential differentiation in culture.

This early amiloride-resistant vasotocin effect, as well as the late amiloride-sensitive effect are also produced by forskolin and by a membrane-permeant cAMP analogue (BrcAMP) (Table 2), and we have previously shown that vasotocin produces under our culture conditions in A6-C1 cells an increase in cellular cAMP [34]. This suggests that vasotocin produces both components of its effect via a cAMP-mediated pathway. The results presented here are consistent with the observation by Yanase and Handler [39] that agents which increase cellular cAMP in A6 cells induce a basal to apical Cl^- transport.

As potential mediators of this Cl^- transport across the apical membrane, two anion channels have been identified by patch-clamp analysis [18]. One candidate is a 8 pS, nonrectifying channel, with characteristics compatible with it being the cystic fibrosis transmembrane conductance regulator (CFTR) [3]. The other channel, having a 3 pS unit conductance, though activated by cAMP, shows an outward rectifying current/voltage relation which appears to be incompatible with an efficient Cl^- secretion. However, it could play a major role for the Cl^- reabsorption observed under open-circuit conditions [18].

The data presented in this paper do not allow me to exclude entirely the possibility that besides the Cl^- conductance described above, other ion pathways play a role, possibly transiently, in the mediation of the early vasotocin-induced increase in I_{sc} and drop in transepithelial resistance (R_{TE}). Yet, in agreement with a report of Matsumoto and Eaton [20], there is no indication from experiments presented here that a change in apical K^+ conductance could mediate a part of the observed drop in R_{TE}. This contrasts with the situation observed in rat cortical collecting duct where cAMP plays a role in maintaining secretory K^+ channels open [36]. The large increase in G_{TE} and in bidirectional Cl^- fluxes induced by vasotocin could also suggest that, besides the increase in apical conductance, a simultaneous increase in paracellular conductance takes place. No experiments which allow me to exclude that possibility have been performed. However, an important participation of the paracellular pathway appears unlikely since the induced conductive pathway is highly selective for Cl^- over Na^+ (*see* Table 3).

Delayed Induction of Na^+ Transport by Vasotocin

Besides the increase in H_2O permeability, the classical response to antidiuretic hormone in distal nephron principal cells and toad bladder granular cells is the increase in Na^+ reabsorption. A6 cells, which do not display H_2O transport, have been used as a model to study the mechanism by which vasopressin or vasotocin induces this regulation [4, 19, 23, 24, 27, 31]. In the present study, it is shown that the vasotocin-induced increase in amiloride-sensitive I_{sc} starts after a lag period of 2 to 5 min, as opposed to a lag of approximately 30 sec for the early induced amiloride-resistant component.

The mechanism by which vasopressin or vasotocin induce an increase in Na^+ transport has been extensively studied. Based on circumstantial evidence and on the fact that vasopressin induces an increase in the number of open Na^+ channels, it has been postulated that channels or channel elements are translocated from an intracellular vesicular pool to the cell surface by fusion of these vesicles with the apical plasma membrane [6, 8, 9, 17, 18, 22, 28]. The observation that the increase in Na^+ transport appears with a lag and is relatively slow (maximum after 15 to 25 min) would be compatible with such a mechanism [34]. However, it will only be possible to verify this hypothesis by using tools specific to the structural elements of the channel. As yet, studies using antibodies have brought conflicting results [15, 31, 32].

Potentiation of Vasotocin Action on Na^+ and Cl^- Transport by Aldosterone

A synergism of the aldosterone and the vasotocin action takes place at the level of the Na^+ transport and can be readily explained by the differential action of the two hormones on the open probability and on the number of active channels, respectively [14, 19]. In this study it is indeed observed that, when cells are maintained un-

der open-circuit conditions, the magnitude of the vasotocin-induced increase in amiloride-sensitive I_{sc} is much larger than expected for an additive effect on the preexisting amiloride-sensitive I_{sc}. However, the relative increase in I_{sc} is lower in control monolayers and in monolayers treated for 6 hr with aldosterone, compared to long-term aldosterone-treated monolayers. This difference indicates that there is a second level of potentiation of the vasotocin action, besides the synergistic mechanism at the level of the apical Na^+ channel. This finding is analogous to our previous observation that long-term aldosterone potentiates the vasotocin action on apical membrane movements by increasing the quantity of cAMP produced in response to vasotocin [34]. This aldosterone effect on the transduction machinery upstream of cAMP could also mediate the second level of Na^+ transport potentiation mentioned above. This hypothesis is supported by the fact that long-term aldosterone does not produce such a further potentiation when forskolin or BrcAMP are used instead of vasotocin (Table 2).

Long-term aldosterone (10^{-6} M) also potentiates the increase in both the early vasotocin-induced amiloride-resistant short-circuit current and Cl^- permeability (Tables 1, 2, and 5). A possible explanation is also the potentiation of cellular cAMP accumulation by aldosterone [34]. Long-term aldosterone could also induce an increase in the number of the apical Cl^- channels. Furthermore, the larger potentiation observed at the level of the vasotocin-induced I_{sc} could also be due in part to aldosterone-induced differences in the driving force for the apical Cl^- secretion and/or of the basolateral influx of Cl^- [11, 12].

Implications of the Vasotocin-induced Anion Transport for the Na^+ Reabsorption

The rate-limiting step for Na^+ reabsorption is the apical Na^+ influx, provided that the basolateral Na^+ transport capacity is sufficient. This flux depends on the permeability of the apical membrane for Na^+ and on a favorable electrochemical potential. Under open-circuit conditions, which resemble the situation in a kidney tubule, the stimulation of A6 cells by aldosterone leads to a depolarization of the apical membrane and to a large increase in the apically negative transepithelial potential. These are both due to the increase in apical Na^+ conductance. However, the depolarization of the apical membrane decreases the driving force for apical Na^+ influx. Hence, the induction of an apical permeability for Cl^- by vasotocin creates a much more favorable situation for the Na^+ transport by displacing the apical membrane potential towards the reversal potential of Cl^- ions, and thereby reinforcing the electrical part of the driving force for Na^+ influx [18]. It is shown in the present study that under open-circuit conditions the late increase in Na^+ conductance is accompanied by a threefold increase in Cl^- reabsorption which must correspond to an increase in net Na^+ Cl^- co-reabsorption to maintain the electroneutrality of the transport. In this situation of Na^+ Cl^- co-reabsorption, the apical influx of Cl^- could be mediated to a large extent by the 3 pS outwardly rectifying Cl^- channel described by Matsumoto and Eaton [18], and the basolateral efflux of Cl^- could be mediated by the inducible Cl^- conductance described by Granitzer, Nagel and Crabbé [11]. In conclusion, both the early and the late component of the vasotocin response contribute to an increase in transepithelial Na^+ reabsorption by producing an increase in the co-reabsorption of Na^+ and Cl^-.

I wish to thank Uschi Bolliger for her skillful technical assistance, J.D. Horisberger for helpful discussions and suggestions and for reading the manuscript, J. Beron, J. Biber, and I. Forster for reading the manuscript, and Christian Gasser for the art work. This work was supported by Grant 31.30132.90 from the Swiss National Science Foundation.

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