J. Membrane Biol. 133, 213–226 (1993)

The Journal of
Membrane
Biology

Polarized Membrane Movements in A6 Kidney Cells Are Regulated by Aldosterone and Vasopressin/Vasotocin

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Summary. The polarity of cell-surface membrane movements and their regulation by adrenal steroid hormones (10^{-6} M aldosterone) and vasopressin or vasotocin were studied in A6 cells. This cell line is derived from the *Xenopus laevis* distal nephron and displays regulated Na^+ reabsorption but is devoid of regulated water transport. Apical and basolateral membrane movements and their hormonal regulation were characterized by measuring the uptake of the fluid phase marker horseradish peroxidase (HRP) and the secretion of proteins on both sides of cell monolayers cultured on filters. The intracellular accumulation of HRP was visualized by electron microscopy and quantified by the measure of cell-associated peroxidase activity. The rate of intracellular HRP accumulation corresponded to 0.01 nl/minute/filter (4.7 cm^2) from the apical side and was 20–32 times faster from the basolateral side. In contrast, the level of protein secretion was 3.5 times higher apically than basolaterally. Among the secreted proteins some were found to be secreted essentially apically, and others basolaterally. Vasotocin increased apical endocytosis (1.88-fold) and apical protein secretion (1.49-fold) in cells pretreated with aldosterone. Basolaterally, only the endocytosis was increased, and to a smaller extent (1.36-fold). These effects of vasotocin depended on aldosterone pretreatment and could be mimicked with forskolin and 8-bromoadenosine 3′:5′-cyclic monophosphate (BrcAMP). Measurements of intracellular cAMP levels showed that there was a rankorder correlation between the induced level of intracellular cAMP and that of apical endocytosis. This study shows that vasotocin has a polarized stimulatory action on apical endocytosis and protein secretion in A6 cells, and that the mediation of this action by cAMP is aldosterone dependent.

Key Words A6 cells · epithelial polarity · endocytosis · secretion · aldosterone · vasopressin

Introduction

Kidney epithelial cells form an active barrier between the extracellular compartment and the tubular lumen. They modulate the composition of the urine by transporting ions, other solutes, and water vectorially across this barrier. This directionality requires that epithelial cells have an asymmetric structure with specialized surface domains facing the different compartments. The separation of the domains is achieved by the tight junctions which also seal the paracellular space. Cell-surface membrane proteins involved in the vectorial transports, such as channels, exchangers, co-transporters or pumps, as well as enzymes, hormonal receptors and structural proteins, have been shown functionally and/or biochemically to have a polarized distribution between the lumen-facing apical membrane domain and the inside-facing basolateral one (Rodriguez-Boulan & Nelson, 1989; Simons & Wandinger-Ness, 1990; Mostov et al., 1992).

Using MDCK cells cultured on permeable filter supports, domain-specificity has also been demonstrated at the level of membrane movements. Indeed, the exocytosis of membrane proteins and soluble proteins from the secretory pathway is mediated by domain-specific vesicles (Wandinger-Ness et al., 1990) and the endocytosis and recycling of vesicles from and to the plasma membrane involves domain-specific endosomal compartments (Bomsel et al., 1989).

To maintain the body homeostasis, the cellular transport functions have to be continuously adapted to the changing conditions. In many cases transport is modulated by the change of the functional state of transport proteins already present in the plasma membrane. A second mechanism of transport regulation is based on the regulation of transport protein synthesis. A third possibility is the modulation of the number of transport proteins expressed at the cell surface by a translocation mechanism to and from an intracellular pool. This mechanism involves membrane movements to and from a specific plasma membrane domain. Two transport systems have been shown to be subjected to this third type of regulation in distal nephron cells: the proton pumps

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at the apical surface of intercalated cells and the water channel at the apical surface of principal cells. In these cases changes in endocytic activity accompany the changes in transport (Handler, 1988; Brown, 1989; Verkman, 1989). Whether the regulation of other transports across distal nephron cells, such as the Na^+ reabsorption, involves regulated membrane movements remains to be elucidated. However, exo and endocytosis take place also in epithelial cells in the absence of regulatory stimuli and play an important role for many other constitutive and regulated functions such as the apical secretion of mucous proteins, the basolateral secretion of basal membrane components, the replacement of cell surface proteins, receptor-mediated endocytosis, etc.

In the present study, surface membrane movements have been analyzed in A6 cells, a distal nephron cell line derived from the kidney of the clawed frog *Xenopus laevis*. These cells display Na^+ reabsorption which is stimulated by mineralocorticoid hormones and cAMP or hormones which elevate cell cAMP such as vasopressin or vasotocin. An increase in cell cAMP also induces in these cells a Cl^- permeability but no water transport (Perkins & Handler, 1981; Yanase & Handler, 1986). The absence of regulated water transport represents a simplification for the study of membrane movements which accompany hormonally induced changes in ion transport.

Here we show by measuring the fluid phase endocytosis and the protein secretion at both plasma membrane domains that A6 cells display polarized membrane movements. These are differentially regulated at the two sides, i.e., the stimulatory action of vasopressin or vasotocin on membrane movements is more pronounced apically than basolaterally. Furthermore, it is shown that the mediation of this effect by the cAMP second messenger pathway is dependent on the pretreatment of the cells with adrenal steroid hormone (10^{-6} M aldosterone).

Materials and Methods

Cell Culture and Electrical Measurements

Experiments were performed with A6 cells from the A6C1 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 responsiveness to aldosterone and antidiuretic hormone (Verrey et al., 1987). Cells were cultured on plastic dishes in 0.8 × concentrated, bicarbonate buffered Dulbecco's modified Eagle medium (DMEM) (GIBCO, Basel, Switzerland) supplemented with 10% fetal bovine serum (Flow Laboratories, Allschwil, Switzerland) 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 3 days. For culture on porous substrate, polycarbonate filters (Transwell, 0.4 μm pore size, 4.7 cm^2, Costar, Cambridge, MA) were coated with a thin layer of bovine dermal collagen (Vitrogen 100, Collagen, Palo Alto, CA) which was polymerized with NH_4OH and crosslinked with 2.5% glutaraldehyde in phosphate buffered saline (PBS). Filters were then extensively washed with H_2O and culture medium, and cells seeded on them 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 7 days. After 10 days the medium was replaced by serum-free, bicarbonate-free 0.8 × DMEM, buffered with 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) to pH 7.4 (~240 mOsm/kg H_2O), and supplemented with 10^{-6} M aldosterone (Sigma, Buchs, Switzerland) in the case of long-term treatment. Cells were placed in an incubator without CO_2 supplementation and the medium was routinely changed 4 days later. Experiments were generally performed 15 to 17 days after seeding and fresh medium was always given the day before.

Measurements of transepithelial potential difference (PD) and short-circuit current (SCC) were performed in a modified Ussing chamber according to published methods (Paccolat et al., 1987). Transepithelial electrical resistance (R) ($\Omega \times cm^2$) was calculated according to Ohm's law from the PD (mV) and the SCC ($\mu A/cm^2$). These electrical parameters were measured in serum-free and HEPES-buffered medium at room temperature. The SCC was approximately 2 to 5 μA/filter and the PD 4 to 10 mV for control cells, and 20 to 60 μA/filter and 30 to 70 mV, respectively, for aldosterone-treated cells. These measurements were performed on all filters. Those with an electrical resistance lower than 5,000 and 3,000 $\Omega \times cm^2$ for control and aldosterone-treated cells, respectively, were not used in subsequent experiments (1–2% of the filters).

Uptake of Horseradish Peroxidase (HRP)

Monolayers of cells cultured on filters were preincubated 1 hr in uptake medium (UM: HEPES-buffered 0.8 × DMEM, 0.5% bovine serum albumin (BSA, Fluka, Buchs, Switzerland; Fraction V, #05488) adjusted to pH 7.4) at 28°C or on ice (for measurement of background activity). Hormones and mediators were added from stock solutions to the UM.

Apical uptake experiments were generally performed in 6-well cluster dishes. The uptake was started by replacing the apical UM with 400 μl HRP-UM and clusters were immediately transferred to the 28°C incubator. When the peroxidase activity in the basolateral medium was also measured, uptakes were performed on a drop of 200 μl UM placed on a piece of Parafilm. For basolateral uptakes, filters were placed on a 200 μl drop of HRP-UM and 400 μl of UM was put into the apical compartment. The uptake was stopped by transferring the filters to cluster-wells containing 2 ml ice-cold wash buffer (WB: 0.8 × PBS, 0.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.5% BSA, adjusted to pH 4.5 and filtered through a paper filter before use), and 1 ml WB was immediately added to the apical side. Filters were put onto a shaker at 4°C and the WB replaced twice. Filters were then cut out of the rings and washed another 5 times for 10 min with the same buffer.

Cell-associated HRP was extracted by placing the filters for 30 min at 4°C in tubes containing 0.5 ml of 1% Triton X-

100. The tubes were then vortexed and centrifuged for 2 min at 4°C before the solution was transferred to a new tube. The contralateral UM harvested for HRP determination were centrifuged 5 min at 1,000 × *g*. The supernatants were transferred to new tubes and adjusted to 1% Triton X-100.

Peroxidase activity was measured according to Steinman and Cohn (1972) using *o*-dianisidine (Sigma) as substrate. The absorbance at 495 nm was measured on a Shimadsu spectrophotometer. Using the specific activity of the HRP-UM, results were converted to volumes of medium. This allows the comparison of results obtained with media containing different levels of enzyme activity. Control experiments showed that the uptake level was similar with HRP-UM containing from 1 to 10 mg/ml HRP (*data not shown*).

In every experiment and for each set of filters (i.e., apical or basolateral uptake, different periods of uptake and filters with or without aldosterone pretreatment) duplicate filters were treated at 4°C to determine the background activity due to cellular activity and adsorption of HRP to the substrate and the cells. This background activity represented a limiting factor for the measurement of short uptake periods, since it amounted to approximately 25–50% of the total cell-associated activity measured after 10 min periods of apical uptake. This proportion was generally less then 20% for 30 min periods of apical uptake. The average of the background activity measured in duplicates was subtracted from each experimental value.

Secretion of Metabolically Labeled Proteins

Cells cultured on filters were rinsed twice on both sides with 0.8 × PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM) at room temperature. Labeling was 16 to 20 hr at 28°C in 0.8 × concentrated minimal essential medium (MEM) (GIBCO, MEM Select-Amin Kit) buffered with 20 mM HEPES to pH 7.4, lacking methionine and cysteine, and supplemented with 1/10 of 0.8 × concentrated, HEPES-buffered DMEM. The apical compartment contained 1 ml of that mixture and the basolateral compartment 1.5 ml supplemented with 50 μCi/ml of a mixture of [^{35}S]methionine and [^{35}S]cysteine (Translabel, ICN Flow, Allschwil, Switzerland). Cells were chased for 30 min in 0.8 × concentrated HEPES-buffered DMEM supplemented with 5 mM of each methionine and cysteine. This medium was replaced twice during that period to wash off labeled secreted proteins. For the collection of secreted proteins, the filter cups were transferred to a new multiwell plate and incubated for 30 min at 28°C with 600 μl of the same medium in both compartments and in the presence or absence of hormones or mediators. At the end of the incubation the apical and the basolateral media were transferred to tubes containing 5 μl of a 1 mg/ml BSA solution. After centrifugation at 3,000 rpm in an Eppendorf microfuge for 5 min at 4°C, 450 μl medium were transferred to a new tube and proteins precipitated with 10% (w/v) trichloroacetic acid (TCA). The pellets were washed twice with ethanol : ether (1 : 1) and resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 4.5% SDS, and 3% β-mercaptoethanol, and heated to 75°C for 5 min. Aliquots of 5 μl were then taken to count TCA precipitable secreted radioactivity in a Kontron Betamatic scintillation counter, using Emulsifier-Safe (Packard, Groningen, Netherlands) as scintillation fluid. The secreted proteins were resolved on 7.5% SDS-PAGE gels using a Bio Rad gel apparatus according to the manufacturers instructions. For fluorography, gels were soaked 30 min in Amplify (Amersham, UK) and exposed at −70°C on a Kodak X-OMAT AR film.

Measurement of Cellular cAMP

Filter-grown cells were preincubated in 0.8 × concentrated HEPES buffered DMEM (2 ml in the apical, and 2.5 ml in the basolateral compartment) for 30 min at 28°C in the presence or absence of 10^{-6} M aldosterone and 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX). The incubation was at 28°C and started by adding vasotocin, forskolin, phorbol 12-myristate 13-acetate (TPA), or diluent from stock solutions. The incubation was stopped by rinsing the filter-cups rapidly in ice-cold assay buffer (50 mM TrisHCl pH 7.4, 8 mM theophylline, 6 mM β-mercaptoethanol, supplemented with 70 mM NaCl) and freezing them in liquid nitrogen. Frozen filters were then cut out of the rings and immediately dipped into 50 μl of 1 N HCl in an Eppendorf tube. After the addition of 500 μl of NaCl-free assay buffer, cells were disrupted by sonicating for 3 × 1 sec at maximal power with a tip sonicator. The mixture was then neutralized with 50 μl of 1 N NaOH, centrifuged and aliquots of 50 or 10 μl were taken for the determination of the cellular cAMP content. A competition binding assay to bovine adrenal cAMP binding protein was performed as described by Brown et al. (1972).

Electron Microscopy

The monolayers of A6 cells used for electron microscopy were cultured in the same conditions and submitted to the same treatment as those used for uptake experiments. In some cases transparent Transwell-COL (Costar) filters were used. The filters were not cut out of the rings for the washing procedure, which was terminated by a wash in 0.8 × PBS-CM. The cells were then fixed in 2.5% glutaraldehyde, 0.8% paraformaldehyde in 50 mM cacodylate pH 7.4 at 4°C for 45 min and washed 3 × 5 min in 0.8 × PBS-CM. HRP was visualized cytochemically by using 3,3′-diaminobenzidine (DAB) as described by Herzog and Farquhar (1983). Briefly, filters were incubated for 90 min at room temperature in a TrisCl buffered solution (pH 7.4) containing 1 mg/ml DAB and 0.01% H_2O_2 and rinsed 3 × 5 min as above. After postfixation in 1% OsO_4 and 1% KFeCN in 50 mM cacodylate buffer for 90 min at 4°C, filters were rinsed in 0.8 × PBS-CM and pieces of filter cut out and dehydrated in a series of graded ethanol solutions and embedded in Epon 412 (Electron Microscopy Sciences, Fort Washington, PA). Ultra-thin sections of 60–100 nm were cut with a Reichert Ultratome, placed on a Formvar film (Sigma) and stained for 20 min with 2.5% magnesium uranyl acetate and for 45 min with 7.5% lead citrate. The sections were examined with a Philips EM 300 transmission electron microscope at 80 kV.

Results

Apical and Basolateral Endocytosis

As a first approach to assessing the membrane movements at the apical and basolateral surfaces of A6 cells cultured on filters, we measured the cellular uptake of the fluid phase marker horseradish peroxi-

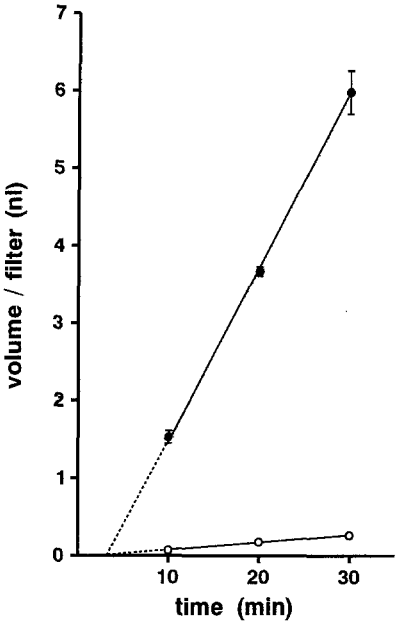

Fig. 1. Continuous uptake of horseradish peroxidase (HRP) from the apical and from the basolateral side of A6 cells cultured on filters. Monolayers of A6 cells cultivated on filters for 16 or 17 days and pretreated for 5 days with 1 μM aldosterone were incubated for the indicated times at 28°C with 10 mg/ml HRP given from the apical (○) or from the basolateral (●) side. After washing the cells at 4°C, cell-associated peroxidase activity was determined. Volumes of accumulated fluid are indicated. These volumes were obtained by converting peroxidase activities into corresponding fluid volumes using the specific activities of the HRP-media, and background values obtained for filters treated at 4°C were subtracted. Each point is the mean of 3 to 12 filters from 2 to 7 independent experiments. The bars represent SE. Straight lines have been drawn through the experimental points.

dase (HRP) (Steinman & Cohn, 1972; von Bonsdorff, Fuller & Simons, 1985; Bomsel et al., 1989). This approach gives information about the quantity of membrane retrieval from the cell surface and is an indirect indicator of exocytic activity, provided the membrane surface remains constant.

HRP was administered for different times of continuous uptake to the apical or basolateral side of cell monolayers pretreated with aldosterone. The amount of HRP accumulated in the cells during the incubation at 28°C was then determined by measuring the peroxidase activity which remained cell-associated after extensive cold washes. Background activity, which was measured in parallel filters incubated at 4°C, was subtracted.

Uptakes with different incubation times showed that much more HRP was accumulated in the cells during basolateral administration than during apical administration (Fig. 1). While the rate of intracellular accumulation of apically applied HRP was in the order of 0.01 nl/filter/min, that of basolaterally applied was higher by a factor of approximately 20. The rates of HRP accumulation from both sides were constant between 10 and 30 min of incubation, while they were somewhat lower during the first 10 min, suggesting the presence of a short lag period. A more precise definition of the early initial uptake in A6 cells, by measuring shorter uptake periods, was precluded by the relatively high background values (*see* Materials and Methods).

To address directly the question of whether the apically internalized marker is recycled to the same plasma membrane domain and/or transcytosed to the contralateral side, we measured the efflux of HRP after a 20 min loading period followed by washing of the cell surface at 4°C. Since filters had to be cut out of the rings for efficient washing, separate measurement of apically and basolaterally released HRP was not possible. However, the fact that in these conditions the total release of HRP during a 20 min incubation period at 28°C was very close to background confirmed that recycling to the plasma membrane was not a major route for endocytosed marker in A6 cells. Furthermore, this finding suggested that there is no major transcytotic pathway across A6 cells for the apically internalized marker.

Measurements of the appearance of transcytosed marker during the uptake of HRP were very difficult to interpret because of the leakage of HRP across most monolayers. However, the lowest values of contralateral HRP activity measured after long incubations with HRP, indicated that apical to basolateral transport amounted to 10% or less of the cell-associated HRP after 1 hr of apical uptake. An alternative explanation for the low level of measured transcytosis could be that basolaterally released HRP remained entrapped in the collagenized filter at the basal side.

As a confirmation that the cell-associated HRP measured with the enzyme assay corresponded to intracellular accumulation, we performed a qualitative analysis by electron microscopy. A6 cells cultured in the presence of aldosterone were incubated for 30 min with HRP administered either apically or basolaterally (in the presence of 25 nM basolateral vasotocin). After extensive washes with the same buffer as for the enzyme assay, cells were fixed with glutaraldehyde and peroxidase activity was revealed using 3,3'-diaminobenzidine (DAB) (Herzog & Farquhar, 1983). The electron micrographs in Fig. 2 show that HRP was internalized from both sides. However, the intracellular vesicular structures containing a dark precipitate were much more numerous when the marker was administered from the basolateral side, confirming that there was much more HRP uptake from the basolateral than from the apical

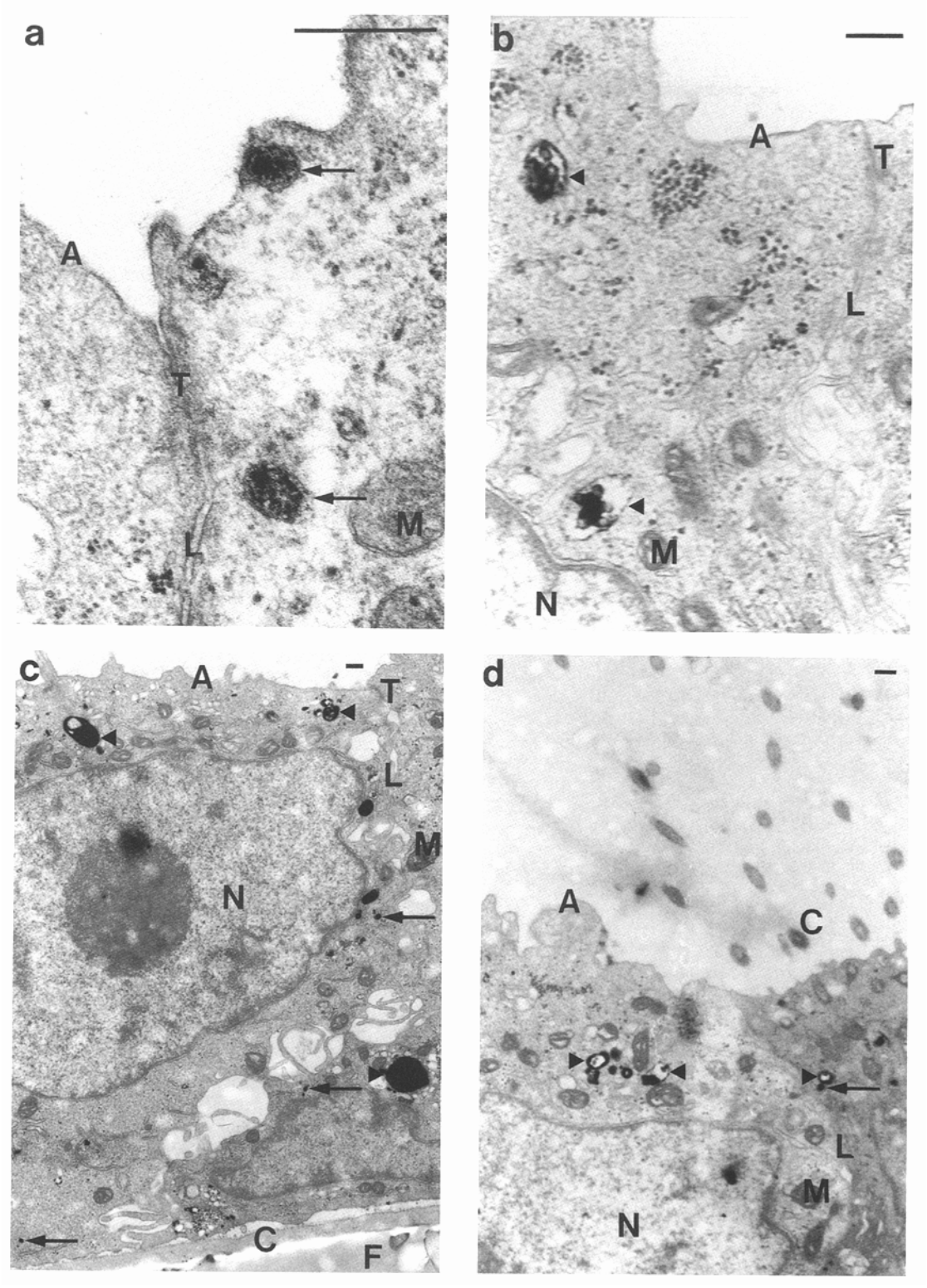

Fig. 2. Localization of horseradish peroxidase after apical (*a* and *b*) and basolateral (*c* and *d*) uptakes. Culture conditions were as for Fig. 1. Uptakes of HRP (10 mg/ml) at 28°C were for 30 min and in the presence of 25 nM basolateral vasotocin. After the washes, cells were fixed, peroxidase activity was revealed with 3,3′-diaminobenzidine (DAB), and cells processed for electron microscopy. Vesicular structures containing dark osmiophile precipitates of polymerized DAB are less numerous after apical (*a* and *b*) than after basolateral uptakes (*c* and *d*). Vesicles containing HRP are often localized near the apical membrane (apical uptake) or near the lateral membrane (basolateral uptake) (arrows). A large fraction of the label is found in multivesicular structures localized in the supranuclear region after uptakes from either side (arrowheads). *A*, apical membrane; *L*, lateral membranes; *F*, filter; *C*, collagen layer; *N*, nucleus; *M*, mitochondrion; *C*, cilium; *T*, tight junction. Bar = 0.25 μm.

side. Apically given HRP was found in small vesicles and multivesicular structures, presumably prelysosomes, which were confined to the apical, supranuclear region of the cells. Interestingly, basolaterally internalized material was, besides vesicles found in the vicinity of the lateral membrane, also found to a large extent in multivesicular structures localized to the supranuclear region. Despite the clonal origin of the cell line the morphology of the cells was not uniform. Certain cells showed apical cilia, whereas the majority had only small microvilli-like apical projections. Morphological heterogeneity in a recloned A6 cell line was reported previously by Moberly and Fanestil (1987). The uptake of HRP, however, was not restricted to cells with a specific morphology.

Apical and Basolateral Protein Secretion

As a second approach to assessing membrane movements, the secretion of metabolically labeled proteins into the apical and the basolateral medium was measured. This gives an indication on the level of exocytic activity. However, it is indirect and the amount of secreted proteins depends not only on the number of vesicles fusing with the plasma membrane but also on their content in labeled proteins which in turn depends on three factors. First, labeled proteins are expected to be more abundant in exocytic vesicles coming from the secretory pathway than in vesicles recycling to the surface from an endosomal compartment. Second, epithelial cells display a polarized secretion of proteins, and potentially a regulated secretory pathway. Hence, these cells contain different populations of secretory vesicles which can have different protein contents (Burgess & Kelly, 1987). Third, the degree of labeling of secreted proteins will depend on their methionine and cysteine content and on the timing of the labeling procedure.

A long labeling time was chosen (16–20 hr with [^{35}S]methionine and [^{35}S]cysteine, *see* Materials and Methods) to label the content of vesicles belonging to both a potential regulated secretory pathway, as well as to the constitutive pathway. After washing the surfaces of the cell monolayers during a short chase period (30 min), medium containing freshly secreted proteins was collected after a 30 min incubation. This timing was chosen to have a measure of the secretion rate of proteins synthesized during the labeling period (Low et al., 1991). The analysis of the secreted proteins by SDS-PAGE followed by fluorography revealed clear qualitative and quantitative differences in the pattern of the apically and basolaterally secreted proteins (Fig. 3). The presence of proteins with the same migration properties in apical and in basolateral medium suggested that several proteins were secreted on both sides, while others were found exclusively on one side. In this regard, the apically secreted proteins which appear as smears in the region between 45 and 66 kD are particularly striking. To quantify the amount of secreted proteins, aliquots of medium precipitated with trichloroacetic acid (TCA) were counted in a scintillation counter. For control monolayers, 3.5 times less counts were found in the basolateral than

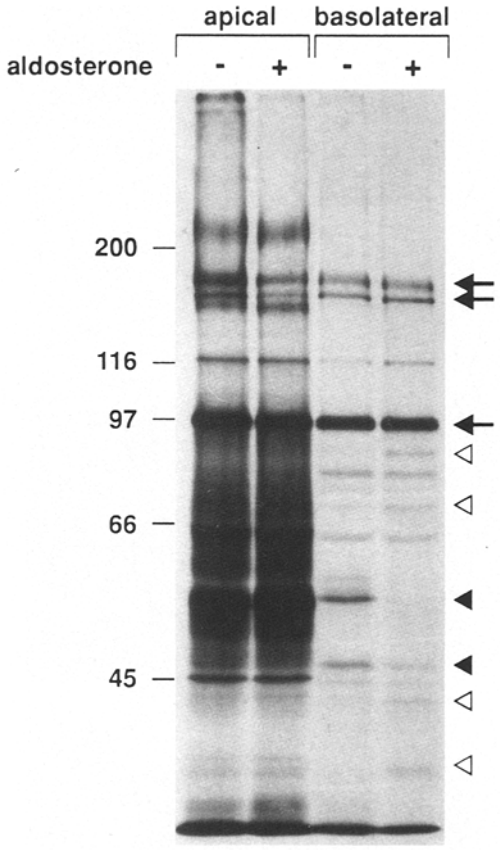

Fig. 3. Apical and basolateral protein secretion by A6 cells cultured on filters with or without aldosterone pretreatment. Culture conditions and aldosterone treatment (+aldosterone) were as for Fig. 1. Filters were labeled 20 hr with a mixture of [^{35}S]methionine and [^{35}S]cysteine. Cells were then washed and chased for a period of 30 min. Apical and basolateral media were collected after a 30 min incubation with equal quantities of apical and basolateral medium. To visualize the labeled secreted proteins, equal amounts of apical and basolateral medium were precipitated with trichloroacetic acid and run on a 7.5% SDS-polyacrylamide gel. A fluorograph is shown with the position and the molecular mass (in kD) of marker proteins. Arrows indicate bands which have similar intensities in apically and basolaterally secreted media. Arrowheads show basolateral bands which are modified by aldosterone treatment. Unfilled arrowheads indicate an increase, filled arrowheads a decrease of intensity.

Table 1. Effect of aldosterone on apical and basolateral horseradish peroxidase (HRP) uptake and protein secretion, and on transepithelial electrical parameters

	HRP Uptake		Protein Secretion		Transepithelial Electrical Parameters		
	Apical	Basolateral	Apical	Basolateral	Potential difference	Short-circuit current	Resistance
	nl/filter/10 min		Fraction of apical secretion of control cells		mV	$\mu A/cm^2$	$\Omega \cdot cm^2$
−Aldosterone	0.102 ± 0.006	3.22 ± 0.33	1.00	0.283 ± 0.051	15.1 ± 2.8	1.2 ± 0.2	11,600 ± 450
+Aldosterone	0.116 ± 0.008 $n = 16$	2.42 ± 0.30** $n = 4$	1.09 ± 0.11 $n = 7$	0.242 ± 0.044* $n = 7$	66.6 ± 4.1** $n = 31$	10.3 ± 0.6** $n = 31$	6,300 ± 200** $n = 31$

The presence or absence of 10^{-6} M aldosterone in the culture medium for five days prior to the experiment is indicated by + and − aldosterone. The cell-associated peroxidase activities were converted into corresponding fluid volumes using the specific activities of the HRP media, and the values were corrected for background activity. The means ± SE and the number of filters (n) are indicated. Paired two-tailed t-tests of +aldosterone *vs.* −aldosterone: $^*P \leq 0.02$, $^{**}P \leq 0.001$; other means are not different at the 0.1 level.

in the apical medium on average, indicating that more labeled secretory proteins are selectively targeted to the apical than to the basolateral surface (Fig. 3 and Table 1).

Effect of Aldosterone on Apical and Basolateral Membrane Movements

It is known that aldosterone increases the transepithelial Na^+ reabsorption across A6 cells and other tight epithelia. This effect starts after a lag period of ~1 hr and can be divided in an early (~3 hr) and a late effect (~12–24 hr) (Rossier et al., 1985; Paccolat et al., 1987; Verrey, 1990). Furthermore, aldosterone can also have morphogenetic effects, as do other steroid hormones (Wade et al., 1990; Kaissling & Le Hir, 1991). To determine whether aldosterone also affects membrane movement, experiments were performed on cell monolayers which had been maintained for 5 days in serum-free HEPES-buffered medium, in the presence or absence of 10^{-6} M aldosterone, a concentration at which both high and low affinity receptors for adrenal steroid hormones are occupied (Watlington et al., 1982; Verrey, 1990) (*see* Materials and Methods for detailed culture conditions). This treatment produced an eightfold increase in transepithelial short-circuit current, which in these conditions represents essentially the transepithelial reabsorption of Na^+ ions (Perkins & Handler, 1981 and *unpublished observations*). The parameters for membrane movements were modified much less than the Na^+ transport (Table 1). However, the basolateral HRP uptake and the basolateral secretion of proteins, were always slightly lower in aldosterone-treated cells than in control cells. This effect of aldosterone corresponded to an increase of the ratio of apical to basolateral endocytosis and protein secretion by a factor of 1.51 and 1.27, respecively. Aldosterone also had an effect on the pattern of secreted proteins observed on SDS-PAGE (Fig. 3). Prominent changes, such as the appearance or disappearance of major bands, were seen only in the basolaterally secreted proteins. However, similar changes in apically secreted proteins could have been masked by the dense labeling of other proteins. It is not clear whether these changes are due to altered synthesis and secretion or to a difference in proteolytic processing of secreted proteins.

Aldosterone-Dependent Effect of Vasopressin and Vasotocin on Membrane Movements

A6 cells have been used as a model to study the stimulatory action of antidiuretic hormone on Na^+ reabsorption in distal kidney cells (Bindels, Schafer & Reif, 1988; Preston, Muller & Handler, 1988; Sariban-Sohraby et al., 1988; Marunaka & Eaton, 1991). It is known that antidiuretic hormone (arginine vasopressin, AVP) and arginine vasotocin (AVT), which is the actual vasopressor peptide hormone secreted by the neurohypophysis in amphibian (Acher, 1974), stimulate the synthesis of cAMP in A6 cells. It appears that the effect of these neuropeptides on Na^+ reabsorption is mediated by this cellular messenger (Bindels et al., 1988; Marunaka & Eaton, 1991).

The uptake of HRP at the apical side of cell monolayers pretreated with aldosterone was determined in the presence or absence of basolateral AVP

Table 2. Regulation of cAMP accumulation and membrane movements in A6 cells cultured on filters with or without aldosterone

Experimental condition	−Aldosterone					+Aldosterone				
	Cellular cAMP (pmol/filter)	Apical		Basolateral		Cellular cAMP (pmol/filter)	Apical		Basolateral	
		HRP uptake (fract. change)	Protein sec. (fract. change)	HRP uptake (fract. change)	Protein sec. (fract. change)		HRP uptake (fract. change)	Protein sec. (fract. change)	HRP uptake (fract. change)	Protein sec. (fract. change)
Control	1.2 ± 0.5 $n = 5$	1.0	1.0	1.0	1.0	1.7 ± 1.1 $n = 5$	1.0	1.0	1.0	1.0
2.5^{-8} M vasotocin	33 ± 5 *** $n = 5$	1.11 ± 0.04 * $n = 20$	1.17 ± 0.08 $n = 6$	1.03 ± 0.02 $n = 4$	1.12 ± 0.09 $n = 6$	466 ± 22 *** $n = 5$	1.88 ± 0.09 *** $n = 33$	1.49 ± 0.16 * $n = 6$	1.36 ± 0.07 ** $n = 10$	0.98 ± 0.07 $n = 6$
$2 \cdot 10^{-5}$ M forskolin	80 ± 6 *** $n = 5$	1.11 ± 0.07 $n = 8$	1.49 + 0.09 ** $n = 6$	ND	1.25 ± 0.06 * $n = 6$	193 ± 34 ** $n = 5$	1.63 ± 0.09 *** $n = 10$	1.54 ± 0.07 *** $n = 7$	ND	1.07 ± 0.09 $n = 7$
$2 \cdot 10^{-5}$ M ddforskolin	ND	0.94 ± 0.09 $n = 6$	1.11 ± 0.07 $n = 4$	ND	1.30 ± 0.05 * $n = 4$	ND	1.00 ± 0.05 $n = 4$	1.03 ± 0.06 $n = 4$	ND	1.25 ± 0.08 $n = 4$
$3 \cdot 10^{-3}$ M BrcAMP		ND	1.54 ± 0.01 *** $n = 3$	ND	1.24 ± 0.09 $n = 3$		1.29 ± 0.12 $n = 6$	1.38 ± 0.11 * $n = 4$	ND	1.36 ± 0.10 * $n = 4$
$3 \cdot 10^{-7}$ M TPA	0.9 ± 0.4 $n = 4$	ND	1.77 ± 0.18 * $n = 4$	ND	1.18 ± 0.05 * $n = 4$	5.0 ± 1.8 $n = 4$	4.91 ± 1.15 * $n = 6$	2.25 ± 0.68 $n = 5$	ND	1.14 ± 0.05 * $n = 5$

The means ± SE are given for n filters. HRP uptake and protein secretion are expressed as the means of the fractional changes (test/control) calculated for n test preparations *vs.* their own control preparations. Absolute control values of HRP uptakes are given in Table 1. Aldosterone treatment was as in Table 1, other hormones and factors were given for 10 or 30 min for cAMP measurements and protein secretion, respectively, and 5 min prior to the start of HRP uptakes (10 min uptakes). BrcAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; ddforskolin, 1,9-dideoxy forskolin; TPA, phorbol 12-myristate 13-acetate. Paired two-tailed t-tests of test *vs.* control: $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.0005$, other means are not different from the control at the 0.05 level.

or AVT (given 5 min before the start of the uptake), as a first step in determining whether these hormones have an effect on membrane movements in A6 cells. It was shown that both hormones increased the HRP uptake by a factor of 1.5 to 2.5 (Table 2, and *data not shown*). Next, the dose-response for the apical uptake by both hormones was compared. The maximal effect for AVP and AVT was the same, while AVT ($K_{0.5} = 0.9 \times 10^{-9}$) was nearly 100 times more potent than AVP. This result is analogous to our observation at the level of the transepithelial short-circuit current where the difference of potency of both hormones is similar (*data not shown*). AVT was generally used in subsequent experiments because of the similarity of the effect of both hormones and because it is the physiological and more potent hormone in *Xenopus laevis* (Acher, 1974). A concentration of 25 nM AVT was used which elicited a submaximal response for apical HRP uptake.

AVT stimulated the HRP uptake on both sides of the cells pretreated with aldosterone. However, this effect was not symmetrical, since the increase was more pronounced apically (1.88-fold) than basolaterally (1.36-fold) (Table 2). This AVT-induced stimulation of endocytosis was to a large extent dependent on the pretreatment of the cells with aldosterone. To determine whether this potentiation of the AVT effect was an early or a late effect of aldosterone, the AVT effect on HRP uptake was measured after different times of aldosterone treatment.

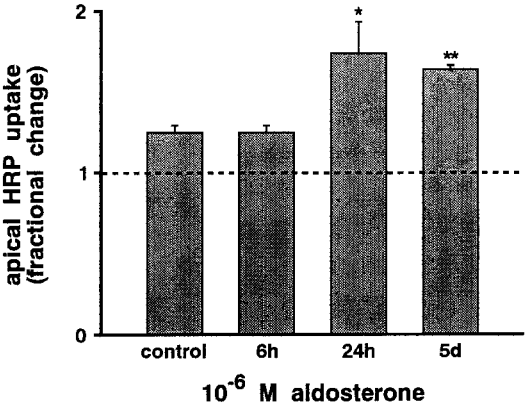

Fig. 4. Time course of aldosterone effect on the stimulation of apical HRP uptake by vasotocin. Culture conditions and measurements of endocytosed HRP were as for Fig. 1. Cells were pretreated for the indicated time with 1 μM aldosterone. Vasotocin (25 nM) was given 5 min before the start of 10 min uptake periods. The means of fractional changes (test/control) from 4–6 filters (2–3 independent experiments) are indicated. The error bars represent SE and the result of two-tailed t-tests performed *vs.* control values are indicated: $*P \leq 0.02$, $**P \leq 0.0001$.

While the Na^+ transport was already increased by a factor of 4 after 6 hr of aldosterone treatment, the potentiation of the AVT effect required a longer time and appeared to be maximal after an aldosterone treatment of 24 hr (*see* Fig. 4). This potentiation can

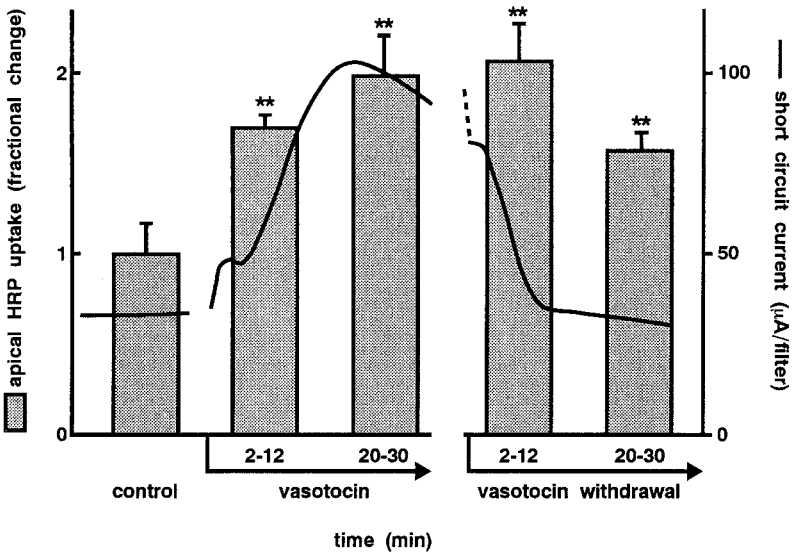

Fig. 5. Time course of the effect of vasotocin on apical HRP uptake. Culture conditions, aldosterone treatment and measurements of HRP uptakes were as for Fig. 1. Uptakes of 10 min were at the indicated times after the addition of 25 nM vasotocin or after the withdrawal of 25 nM vasotocin (18 min treatment). The means of fractional changes (test/control) from 5–6 filters (two independent experiments) are indicated. The error bars represent SE and the result of two-tailed *t*-tests performed *vs.* control values are given (**$P \leq 0.001$). Test values from the different time points are not significantly different from each other at the 0.05 level. The transepithelial short-circuit current, which was measured approximately every 20 sec for 1 sec, was redrawn from a typical experiment as a continuous curve.

therefore be considered as a late effect of aldosterone (Rossier et al., 1985).

To compare the time course of the AVT action on the ion transport activity and on membrane movements, the effect of different preincubation and withdrawal times of AVT on apical uptakes of HRP, administered for periods of 10 min, was measured (Fig. 5). The stimulation of the HRP uptake was a relatively slow process, since it was higher when the uptake was performed 20–30 min than 2–12 min after basolateral hormone addition. Furthermore, AVT withdrawal did not produce an acute change in endocytosis, since the level of HRP uptake was the same in the presence or absence of AVT, when the filters have been preincubated for 20 min with this hormone. A curve representing the effect of AVT on the short-circuit current has been superposed on the bars representing the HRP uptake to allow a comparison of the time course of both effects.

To test the AVT effect on the protein secretion, we administered the hormone to the cells at the beginning of the 30 min collection time. This treatment had a clear effect on the quantity of labeled proteins released into the apical medium (Table 2). Interestingly, this effect was restricted to the apical side and depended to a large extent on the pretreatment of the cells with aldosterone, as did the increase in HRP uptake described above. The intensity of all bands was not modified to the same extent. However, the total pattern on SDS-PAGE did not show major changes, unlike those expected if a separate population of vesicles with a specific protein content was induced to fuse with the plasma membrane (Fig. 6). Nevertheless, this observation is compatible with both the stimulation of secretion by the constitutive pathway as well as with the triggering of secretion by a regulated pathway. In this latter case the protein content of the vesicles of the regulated pathway would have to be similar to that of the vesicles of the constitutive one.

Role of cAMP in the Mediation of the Vasotocin Effect on Membrane Movements

The aim of the next experiments was to investigate the role of cAMP in mediating the action of AVT on the membrane movement and to determine whether the potentiation of this effect by aldosterone was due to an effect on the synthesis of cAMP or to an effect upstream or downstream of it.

The fact that AVT also stimulates the synthesis of cAMP under our experimental conditions was confirmed by the large accumulation of this cellular messenger which was observed after AVT addition to aldosterone pretreated cells and in the presence of the phosphodiesterase inhibitor 3-isobutyl-methyl-xanthine (IBMX) (Table 2 and Fig. 7). In the absence of IBMX, the AVT-induced cAMP level obtained in aldosterone-treated cells was approximately 25 times lower than that obtained in its presence. To allow the quantification of smaller effects, such as those observed with cells cultured in the absence of aldosterone, IBMX was routinely used to amplify the response.

Next, we tested whether the stimulation of HRP uptake and of protein secretion could be mediated by an increase in cellular cAMP. This was indeed the case, since both effects could be mimicked by the use of forskolin (20 μM, basolateral), an activator of the adenylate cyclase, while the inactive analogue

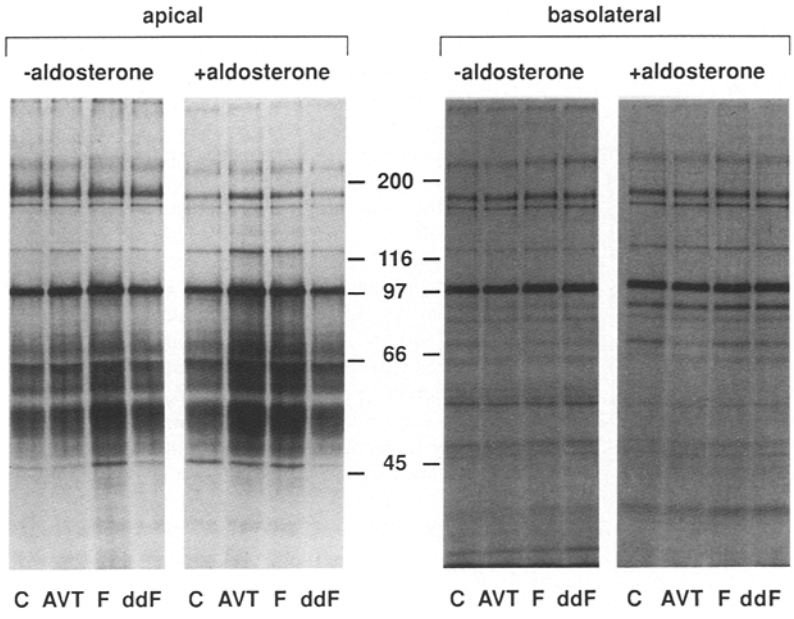

Fig. 6. Effect of vasotocin and forskolin on apical and basolateral protein secretion in A6 cells cultured on filters. Cell culture, aldosterone pretreatment (+aldosterone) and labeling with a mixture of [^{35}S]methionine and [^{35}S]cysteine were as for Fig. 3. After a 30 min period of washes and chase, the incubation was started by adding equal amounts of fresh medium to the apical and basolateral side. The basolateral medium was supplemented with diluent (*C*), 25 nM vasotocin (*AVT*), 20 μM forskolin (*F*) or 20 μM of 1,9-dideoxy forskolin (*ddF*). The media were collected after 30 min and processed as for Fig. 3. A fluorograph from a 7.5% SDS-polyacrylamide gel electrophoresis is shown and the position and molecular mass (in kD) of marker proteins are indicated.

1,9-dideoxy forskolin (ddforskolin) was, as expected, not effective (Table 2 and Fig. 7). One exception was a small stimulation of the basolateral protein secretion which was observed after the administration of both forskolin and its dideoxy analogue, and for which we have no explanation.

The role of cAMP in the stimulation of membrane movements in A6 cells was further confirmed by the use of 8-bromoadenosine 3′ : 5′-cyclic monophosphate (BrcAMP, 3 mM apical and basolateral), a membrane-permeable analogue of cAMP which also increased the membrane movements in aldosterone-pretreated cells (*see* Table 2). The somewhat lower induction, compared to forskolin and AVT, could be due to a slower initiation of the effect, as it was observed at the electrophysiological level (*data not shown*).

The demonstration that AVT produces an increase in cellular cAMP and that the tested physiological effects could be mimicked by using forskolin and a membrane-permeable analogue of cAMP, was a good indication that the cAMP pathway was involved in the mediation of the observed effects. However, it is not excluded that AVT (and/or AVP) can stimulate also other pathways which can influence the membrane movements. Indeed, it has been described that low doses of AVP can produce an activation of the inositol phospholipid pathway in A6 cells cultured for 2 days on filters (Casavola et al., 1992). In some experiments, we have made the observation that phorbol 12-myristate 13-acetate (TPA), a stimulator of protein kinase C, had a large effect on apical membrane movements, although it did not induce an accumulation of cellular cAMP (Table 2). This is an indication that these movements are not under the sole control of the cAMP/kinase A pathway. However, there was a rankorder correlation of the magnitudes of apical endocytosis stimulation with the levels of cAMP accumulation (Fig. 7), while there was no further increase in protein secretion above the level observed for forskolin-treated "minus aldosterone" cells. These observations suggested that the cAMP pathway played the dominant role for mediating the vasotocin-induced regulation of membrane movements.

The dependence of A6 cells on aldosterone for developing the capability of responding to an increase in cAMP by increasing apical protein secretion was tested by the administration BrcAMP in cell monolayers which had not been pretreated with aldosterone (Table 2). Interestingly, the response was similar to that in aldosterone-treated cell monolayers, indicating that the mediators downstream of cAMP, as well as the secretion machinery, were not dependent on the presence of aldosterone.

The use of forskolin with and without aldosterone pretreatment showed that the adenylate cyclase was functional in both situations (Table 2 and Fig. 7). However, the level of cAMP accumulation and, to some extent, that of the apically induced membrane movements were systematically lower in mi-

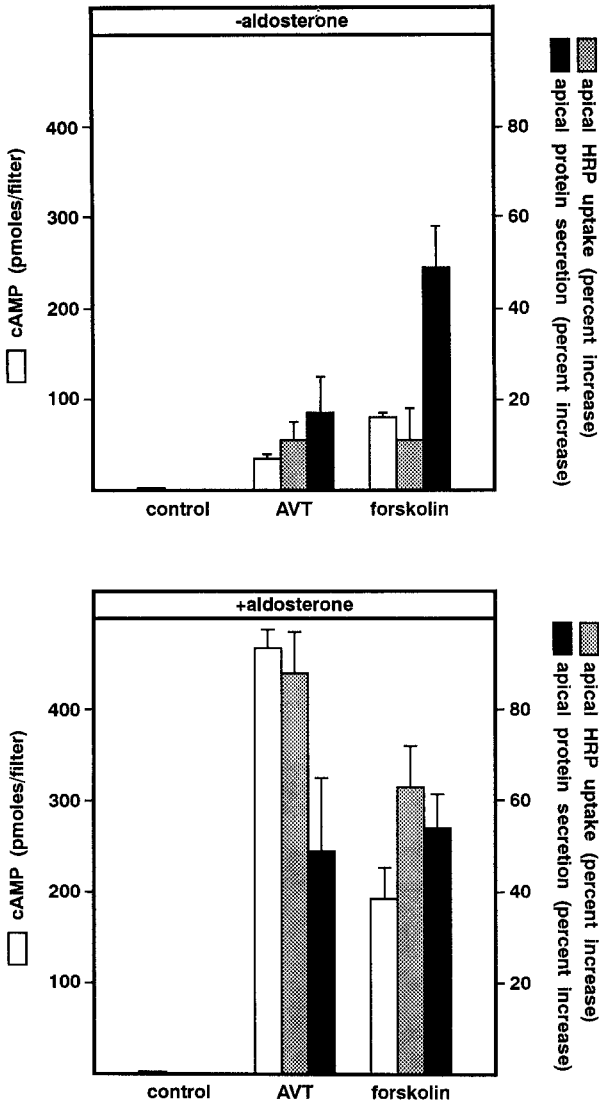

Fig. 7. Comparison of the level of cAMP accumulation with that of apical membrane movements induced by vasotocin and forskolin. The data are taken from Table 2 and changes in HRP uptake and protein secretion are shown as percent increase. The error bars represent SE.

nus-aldosterone cells, suggesting that aldosterone increases the level of the functional adenylate cyclase.

The difference in cAMP accumulation and in the induction of membrane movements between aldosterone-treated and control cell monolayers was much larger after AVT administration than after that of forskolin. This indicated that a large part of the potentiation effect of aldosterone on the AVT effect was due to an increase of the efficiency of the mediation upstream of the adenylate cyclase.

Discussion

Differences in Membrane Movements at the Apical and the Basolateral Side of A6 Cells

This study shows that A6 cells cultivated on filters display a clear polarity of their membrane movements. The endocytosis of a fluid phase marker, the secretion of proteins, and the hormonal regulation of these membrane movements display membrane-domain specificity.

The quantitative difference in HRP accumulation at the apical and at the basolateral surface of A6 cells corresponds to the observation made in MDCK cells by von Bonsdorff et al. (1985) and by Bomsel et al. (1989). These authors observed an approximately 30-fold higher fluid phase marker accumulation after 1 hr application from the basolateral side compared to application from the apical side. However, in contrast to the present observation in A6 cells, there is a rapid initial uptake on both sides of MDCK cells which is followed by a plateau like period (Bomsel et al., 1989). This rapid initial uptake can be explained by the filling of an early endosomal compartment which is followed by recycling and transcytosis of marker to the cell surfaces. Hence, the total uptake rates in MDCK cells, calculated by adding the transcytosed and the recycled volumes of fluid to the volume accumulated in the cells, was only 3.6 times larger basolaterally than apically and corresponded to the differences in surface area (Bomsel et al., 1989). This means that the large difference in accumulation of marker resulted from very active recycling and transcytosis of apically applied marker (only about 10% remained intracellular) and from a comparatively higher transfer of basolaterally applied marker to the late endosome. The present results show that A6 cells have, unlike MDCK cells, not very active recycling and transcytotic pathways. Hence, the total uptake rate is not much different from the rate of marker accumulation. The quantity of marker accumulation is similar in MDCK and in A6 cells tested at their respective optimal culture temperature (37 and 28°C, respectively). Hence, in these conditions it appears that the total endocytic activity is lower in A6 cells, particularly at the level of the apical membrane. The localization of a large proportion of the marker endocytosed from opposite sides to supranuclear multivesicular structures, suggests that both endocytic pathways converge to common late endocytic compartments. This is in agreement with observations made in MDCK cells (Bomsel et al., 1989).

Implication of the Membrane Movements for the Regulation of Ion Transports

Vasopressin, the mammalian antidiuretic hormone and vasotocin, its equivalent in amphibian and other vertebrates, stimulate the synthesis of cAMP in fully differentiated monolayers of A6 cells cultured on filters (Lang et al., 1986; Preston et al., 1988). These hormones and cAMP analogues can elicit in A6 cells an increase in transepithelial short-circuit current which appears to depend on the culture conditions (Bindels et al., 1988). This increase in short-circuit current has been attributed to increases in Na^+ reabsorption and in Cl^- secretion (Perkins & Handler, 1981; Yanase & Handler, 1986; Bindels et al., 1988). Patch-clamp experiments have shown that vasopressin and cAMP increase in A6 cells the number of open apical, amiloride-blockable Na^+ channels and that cAMP increases the open probability of an apical Cl^- channel (Marunaka & Eaton, 1990, 1991). In our culture conditions AVT induces a rapid increase in Cl^- permeability (Cl^- secretion in short-circuit condition) and a slower, aldosterone-dependent ~3-fold increase in Na^+ reabsorption which starts after a lag of 2–3 min and reaches a maximum after approx. 20 min (Fig. 5 and *data not shown*).

It has been recently suggested that changes in apical endocytosis can mediate changes in Cl^- permeability in Cl^- secretory cells by modifying the channel density at the cell surface (Bradbury et al., 1992; Sorscher et al., 1992). However, in A6 cells the regulation of membrane movements by cAMP does not appear to mediate the early increase in short-circuit current, which corresponds to an increase in Cl^- permeability (Fig. 5 and *data not shown*). Indeed, the observed changes in membrane movements appear to be slower and require pretreatment with aldosterone (10^{-6} M), while a significant early increase in short-circuit current is observed in the absence of aldosterone (Table 2 and *data not shown*).

In many tight epithelia AVP and AVT induce an increase in H_2O permeability which is mediated by the fusion of vesicles which contain H_2O channels with the apical membrane (Handler, 1988; Brown, 1989; Verkman, 1989). By analogy to this process and based on circumstantial evidence, it has been suggested that the vasopressin- and vasotocin-induced increase in apical permeability to Na^+ was mediated by the translocation of intracellular channels to the plasma membrane (Garty & Benos, 1988; Marunaka & Eaton, 1990; Els & Helman, 1991; Palmer, 1992; Schafer & Hawk, 1992). The temporal correlation found in this study between the AVT action on Na^+ transport (amiloride blockable short-circuit current) and on apical membrane movements, neither supports nor excludes the role of a translocation event, since similar changes in membrane movements elicited by TPA did not induce the same changes in Na^+ transport. Only studies using tools specific to the structural elements of the channel can clarify this point. As yet, studies with antibodies directed against elements of the channel have brought conflicting results (Tousson et al., 1989; Kleyman, Coupaye-Gerard & Ernst, 1991 (*Abstr.*); Smith et al., 1991 (*Abstr.*)).

Role of Aldosterone in the Regulation of Membrane Movements by Vasotocin

Corticoid hormones have been shown to potentiate the action of AVP or AVT in tight epithelia by several mechanisms. One possible level of synergism is the effector machinery which mediates a physiological action, as observed in A6 cells and other tight epithelia, for the action of aldosterone on cAMP-mediated induction of Na^+ transport (Girardet et al., 1986; Preston et al., 1988; Schafer & Hawk, 1992). In this study it is shown that vasotocin increases membrane movements in A6 cells. In this case, however, aldosterone, which has no significant effect on the baseline activity, does not potentiate the cAMP action. This indicates that the machinery needed for membrane movements and their regulation by cAMP is functional in the absence of aldosterone. Using forskolin and BrcAMP, it was shown that in this case the potentiation of the vasotocin action is due to an increase in functional adenylate cyclase and to an increase in efficiency of the mediation upsteam of the adenylate cyclase. This latter point corresponds to the effect observed by Preston et al. (1988) after the addition of 1 μM dexamethasone to fully differentiated A6 monolayers. This indicates that this effect could be mediated by the low affinity receptor for mineralocorticoid hormones which resembles the mammalian glucocorticoid receptor (Watlington et al., 1982). Indeed, at the concentration of aldosterone given in this study, which was chosen because it produces a maximal response at the level of Na^+ transport and at that of Na^+,K^+-ATPase mRNA accumulation, both receptor types are occupied (Verrey, 1990). In conclusion, aldosterone potentiates the AVT action on membrane movements in A6 monolayers by enhancing the cAMP production induced by the hormone.

We wish to thank Heini Murer, Jürg Biber and Ian Forster for critically reading the manuscript, Christian Gasser for the graphic

and photograhic work, and Kurt Drickamer in whose laboratory F.V. performed preliminary experiments to this study. This work was supported by Grant 31.30132.90 from the Swiss National Science Foundation.

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Received 27 October 1992; revised 14 January 1993