Vasopressin stimulates long-term net chloride secretion in cortical collecting duct cells

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Abstract The classical short-term effect (within minutes) of arginine vasopressin (AVP) consists in increasing sodium, chloride and water transport in kidney cells. More recently, long-term actions (several hours) of the hormone have been evidenced on water and sodium fluxes, due to transcriptional enhancement in the expression of their transporters. The present study demonstrates that AVP is also responsible for a long-term increase in net chloride secretion. In the $RCCD_1$ rat cortical collecting duct cell line, 10^{-8} M AVP induced, after several hours, an increase in net $^{36}Cl^-$ secretion. This delayed effect of AVP was inhibited by basal addition of 10^{-4} M bumetanide and apical addition of 10^{-4} M glibenclamide, suggesting chloride entry at the basal membrane through a $Na^+/K^+/2Cl^-$ and apical secretion through a chloride conductance. An original acute cell permeabilization method was developed to allow for entry of antibodies directed against the regulatory region (R) of the cystic fibrosis transmembrane regulator (CFTR) into the cells. This procedure led to a complete and specific blocking of the long-term net chloride secretion induced by AVP. Finally, it was observed that CFTR transcripts steady-state level was significantly increased by AVP treatment. Besides the well-documented short-term effect of AVP on chloride transport, these results provide evidence that in $RCCD_1$ cells, AVP induces a delayed increase in transepithelial net chloride secretion that is mediated by a $Na^+/K^+/2Cl^-$ co-transporter and CFTR.

Key words: Cystic fibrosis transmembrane regulator; $Na^+/K^+/2Cl^-$; Vasopressin; Kidney; Cortical collecting duct

1. Introduction

In the kidney, short-term effects of arginine vasopressin (AVP) are well documented: AVP is mainly involved in the regulation of water reabsorption, but also modulates sodium and chloride transport [1,2]. These effects are directly linked to activation of adenylate cyclase and rapid cAMP production. Recently, we have provided evidence for another mechanism which may be related to transcriptional effects of AVP. In this study [3], we showed that, in the rat cortical collecting duct (CCD) cell line $RCCD_1$, AVP leads to a delayed stimulation of Na^+ transport by increasing the de novo synthesis of certain subunits of the epithelial sodium channel and of the Na^+/K^+-ATPase. We hypothesized that this effect is in turn due to an effect of the hormone on gene expression through interaction of transcription factors on cAMP response elements. However, the stimulation of Na^+ reabsorption did not fully account for the long-term AVP-induced increase in ionic transport. Indeed, the delayed increase in short-circuit current observed after AVP treatment was only partially inhibited by the sodium channel blocker amiloride [3]. Therefore, we decided to search for another transport system upregulated by AVP. Since it has been shown that cAMP induces a short-term increase in net chloride secretion in several cellular models of the CCD [4–7], we decided to examine whether AVP could also exert long-term effects on chloride transport.

In this study, evidence is provided that AVP induces a delayed stimulation of net chloride secretion in $RCCD_1$ cells, which is blocked by inhibitors of $Na^+/K^+/2Cl^-$ and chloride channels, presumably cystic fibrosis transmembrane regulator (CFTR). Moreover, acute permeabilization of cells in the presence of an antibody (Ab) against CFTR prevented the AVP-induced net chloride secretion. Altogether, these results favor the view that AVP promotes a delayed increase in net chloride transepithelial secretion mediated by basal $Na^+/K^+/2Cl^-$ transporters and apical CFTR channels. Moreover, this effect is likely to involve an increase in the steady-state levels of CFTR mRNA.

2. Materials and methods

2.1. Cell culture

Experiments were performed on the $RCCD_1$ rat CCD cell line [8], between passages 24 and 42. Cells were grown on permeable supports coated with collagen (type 1 from rat tail, Institut Jacques Boy, France). The culture medium (2% fetal bovine serum (FBS)-DM) was as follows: HAM's F12:DMEM 1:1, $NaHCO_3$ 14 mM, glutamine 2 mM, dexamethasone 5×10^{-8} M, sodium selenite 3×10^{-8} M, transferrin 5 μg/ml, insulin 5 μg/ml, EGF 10 μg/ml, T_3 5×10^{-8} M, penicillin-streptomycin 10 U/ml, HEPES 20 mM, pH 7.4, FBS 2% (Gibco, Les Ulis, France). When cell confluence was reached (3–4 days after seeding), cells were incubated overnight in a minimum medium (MM: HAM's F12:DMEM 1:1, $NaHCO_3$ 14 mM, glutamine 2 mM, penicillin-streptomycin 10 U/ml, HEPES 20 mM, pH 7.4) before treatment with 10^{-8} M AVP on the basal side.

2.2. $^{36}Cl^-$ transport studies

Transepithelial $^{36}Cl^-$ transport was determined across $RCCD_1$ cells cultured on 12 mm transwell filters (Costar). Both the apical to basal and the basal to apical fluxes were determined by adding 100 nCi/ml $^{36}Cl^-$ (Amersham, 89 μCi/ml) as a tracer at the apical side or at the basal side of the cells, allowing for determination of unidirectional chloride fluxes and subsequent calculation of net fluxes. After 10 min of incubation at 37°C, the medium was collected at the opposite side. Radioactivity was counted using a β-scintillation counter (Wallak, Pharmacia) and results were expressed in nmol/10 min/cm^2. In some experiments, cells were pre-incubated with bumetanide 10^{-4} M (Sigma) dissolved in 0.1% ethanol or glibenclamide 10^{-4} M (Calbiochem) dissolved in 0.1% DMSO in the apical or basal medium before

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determination of $^{36}Cl^-$ fluxes. Finally, some experiments were performed using a MM without bicarbonate (HAM's F12:DMEM HCO_3^--free, glutamine 2 mM, penicillin-streptomycin 10 U/ml, HEPES 20 mM, pH 7.4) to examine the influence of Cl^-/HCO_3^- exchanger in the phenomenon.

2.3. Permeabilization procedure

In series of experiments, cells were permeabilized in order to give access of the HCF703 Ab directed against the regulatory region (R) of CFTR [9,10] to its intracellular epitope before $^{36}Cl^-$ transport experiments. In these experiments, cells grown on 12 mm diameter transwell filters were incubated overnight in MM before treatment or not with 10^{-8} M AVP. After 4.5 h of treatment with or without AVP, the basolateral medium (1.5 ml) was maintained but the apical medium was replaced with the same volume (0.5 ml) of MM to which anti-CFTR Abs (1/4000) were added, in the absence or presence of the immunizing peptide (1.2 μg/ml stock in distilled water). The sequence of this peptide is NPINSIRKFS [9]. Multiwell dishes containing the filters were then deposed on a bath of ethanol previously cooled at −20°C by addition of dry ice. Under these conditions, cell freezing occurred within 10–15 min. Just after freezing, dishes were put back at 37°C in the incubator to allow for thawing. This procedure was designed to entry of the Ab and subsequent sealing of the apical membrane, thus permitting for further evaluation of its effect on transport properties of the epithelium. $^{36}Cl^-$ transport experiments were performed at 7.5 h as previously described. In preliminary experiments performed with trypan blue (data not shown), it was observed that >95% of the cells were permeabilized with this method. As a control, permeabilization was performed in the presence of a monoclonal anti-β-actin Ab (1/4000) (Sigma) which belongs to the same IgG isotype (mouse IgG1 isotype) as the anti-CFTR Ab used in this study.

2.4. Electron microscopy

Three hours after the permeabilization procedure (i.e. 7.5 h after AVP treatment), cells were immediately fixed in 2.5% glutaraldehyde in PBS at room temperature, then post-fixed in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were performed on transversally oriented monolayers and examined with a Philips EM 410 electron microscope. Morphological features of permeabilized cells were compared to those of non-permeabilized ones.

2.5. RNAse protection assay

A RNAse protection assay was performed on $RCCD_1$ cells grown on 24 mm transwell filters (Costar) and lysed with a guanidium thiocyanate solution (guanidium thiocyanate 4 M, Na citrate 25 mM, pH 7, sarcosyl 0.5%, β-mercaptoethanol 0.1 M). The protocol was as previously described [11]. Part of the rat CFTR cDNA (exon 10–13) subcloned into Bluescript was used to synthesize CFTR cRNA probe [12,13] (cRNA probe: 689 bp, protected fragment: 500 bp). β-Actin probe was synthesized using a cDNA inserted in a Bluescript plasmid [11] (cRNA probe: 158 bp). The β-actin mRNA signal was used as an internal standard to take into account the differences in gel loading. Antisense cRNA probes were synthesized with [^{32}P]UTP (15 TBq/mmol, Amersham, UK) using the Promega riboprobe kit. Gels were exposed to Kodak XOMAT AR 5 films. Signal quantification was performed using an image analyzer (Optilab, Graftek, Grenoble, France) and for each condition, results were normalized to the signal obtained with β-actin. To illustrate the effects of AVP, normalized levels of CFTR mRNA are provided as relative to the control levels without AVP.

2.6. Statistical analysis

Results are expressed as mean values ± S.E.M. Student's *t* test for unpaired data and variance analyses was performed (when allowed by the *F* value, results were compared by the modified least significant difference).

3. Results

3.1. Effect of AVP on transepithelial Cl^- transport: short-term and long-term responses

The effect of AVP on net chloride secretion was examined by $^{36}Cl^-$ fluxes experiments after 10 min, 4 and 7.5 h of treatment with the hormone (Fig. 1). Fig. 1A is an example of an experiment realized under control conditions and after 7.5 h of AVP treatment. Both apical to basal (absorptive) and basal to apical (secretory) fluxes were evidenced, allowing for determination of the net flux. Under control conditions, a weak, non-significant, net chloride secretion was observed. Treatment of $RCCD_1$ cells for 7.5 h with 10^{-8} M AVP did not modify the absorptive $^{36}Cl^-$ flux but, in contrast, it significantly increased the secretory one, leading to a significant increase in net chloride secretion. Fig. 1B is the time-course of the AVP effect on chloride transport. Ten minutes of treatment with AVP resulted in a large increase in net chloride secretion, corresponding to the known short-term effect of the hormone. Four hours after AVP addition, no difference could be observed between the control and the AVP-treated cells, suggesting down-regulation of the short-term effect. After 7.5 h of AVP, net chloride secretion was clearly increased.

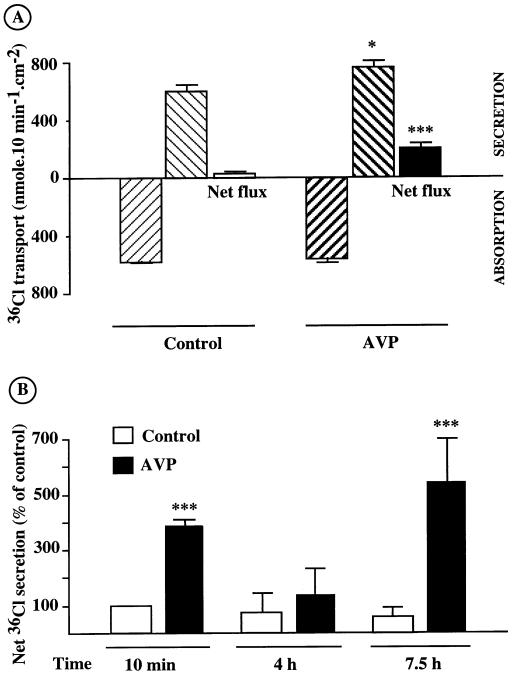

Fig. 1. Long-term effect of AVP on net transepithelial chloride transport in $RCCD_1$ cells. A: The effect of AVP 10^{-8} M was tested on the apical to basal (ABSORPTION) and the basal to apical (SECRETION) $^{36}Cl^-$ fluxes. Cells were incubated for 7.5 h in the presence of 10^{-8} M AVP (AVP) or under control conditions (Control) before determination of the $^{36}Cl^-$ fluxes. Each bar is the mean value of three filters. *, ***: $P<0.05$, $P<0.001$, AVP vs. Control. B: The time-course of the AVP effect was determined on the net transepithelial $^{36}Cl^-$ secretion by incubating cells for different times (10 min, 4 and 7.5 h) with (AVP) or without (Control) the hormone. Each bar is the mean value of 12–15 filters from five experiments. ***: $P<0.001$, AVP vs. Control.

3.2. Long-term AVP-induced net chloride secretion involves a basal $Na^+/K^+/2Cl^-$ and an apical chloride conductance

To determine which ionic transporters were potentially involved in the long-term net chloride secretion, we tested the effect of the $Na^+/K^+/2Cl^-$ blocker bumetanide [14] and of the CFTR-like Cl^- conductance inhibitor glibenclamide [15]. In addition, experiments were performed in a medium devoid of bicarbonate to block the activity of the Cl^-/HCO_3^- exchanger.

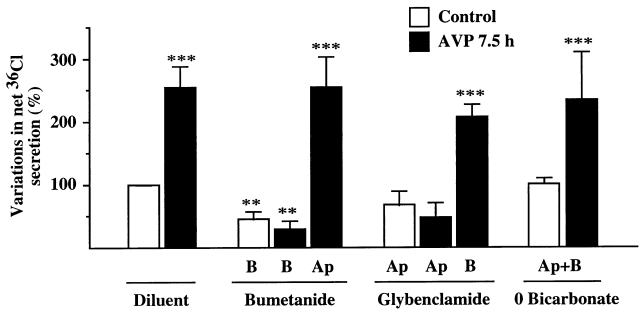

Fig. 2. Effect of bumetanide, glibenclamide and absence of bicarbonate on the long-term AVP-induced net chloride secretion. The effect of 10^{-4} M bumetanide and 10^{-4} M glibenclamide added either apically or basally was tested on the AVP-induced net chloride secretion at 7.5 h. Basal (B) addition of bumetanide blocked the AVP effect whereas apical addition (Ap) of the drug did not modify it. Conversely, apical but not basal addition of glibenclamide blocked the long-term effect of AVP. Finally, replacing the medium with a HCO_3^--free medium did not prevent the long-term AVP-induced net chloride secretion. Each bar represents the mean value of 9–12 filters from five experiments. **, ***: $P<0.01$, $P<0.001$, experimental vs. control without AVP.

Results are given in Fig. 2. As compared to control experiments (performed in the presence of the diluent only), addition of 10^{-4} M bumetanide on the basal side of the cells significantly reduced both the basal level of net chloride secretion and blocked completely the long-term response to AVP. In contrast, apical addition of 10^{-4} M bumetanide did not prevent the AVP-induced net chloride secretion. Apical addition of 10^{-4} M glibenclamide also totally blocked the long-term AVP-induced net chloride secretion, without a significant effect on the basal secretion. Finally, when experiments were performed in a medium devoid of bicarbonate, the effect of 7.5 h of treatment with AVP was not significantly different from that observed in a medium containing bicarbonate. Altogether, these experiments suggest that the long-term AVP-induced net chloride secretion may involve an entry of chloride at the basal membrane through a $Na^+/K^+/Cl^-$ cotransporter and chloride exit at the apical membrane through a glibenclamide-sensitive chloride conductance. The Cl^-/HCO_3^- exchanger does not appear to be involved in the phenomenon.

3.3. Putative role of CFTR in the delayed AVP-induced increase in net chloride secretion

Since glibenclamide is not a highly specific inhibitor of CFTR but inhibits also K^+ channels [16], experiments were designed to test whether the HCF703 anti-CFTR Ab [9,10] was able to block the long-term AVP effect on chloride transport. Indeed, such blocking properties have been described for anti-CFTR Abs directed against the regulatory region (R) [17]. As the epitope recognized by anti-CFTR Ab is intracellular, it was necessary to design an experiment that allows for cell entry of the Ab and subsequent resealing of the apical membrane before carrying out the transport experiments. Free access of the Abs to the R region of CFTR was yielded by cell permeabilization as described in Section 2 (When cells were not permeabilized, anti-CFTR Ab did not block net chloride secretion.) Examination of the ultrastructural characteristics of permeabilized cells by electronic microscopy revealed no major modification of the cellular aspect as compared to intact cells (Fig. 3A,B). Results of $^{36}Cl^-$ transport are shown in Fig. 3C. As in non-permeabilized cells, treatment of permeabilized $RCCD_1$ cells for 7.5 h with AVP resulted in a large increase in net $^{36}Cl^-$ secretion. Under these conditions, addition of anti-CFTR Ab totally blocked the AVP effect on net chloride secretion. Conversely, the effect of AVP was observed when the permeabilization was performed in the presence of the anti-CFTR Ab and an excess of immunizing peptide. Finally, when experiments were performed using another unrelated Ab of the same isotype (anti-β-actin Ab), the AVP effect on net chloride secretion was not inhibited. These data suggest that CFTR is involved in chloride transport in $RCCD_1$ cells and that CFTR plays a major role in the long-term effect of AVP on net chloride secretion.

3.4. AVP increases CFTR mRNAs accumulation

The effect of 10^{-8} M AVP was evaluated on the accumulation of mRNA encoding CFTR by a RNAse protection assay (Fig. 4). An example of a RNAse protection assay is shown in Fig. 4A. A protected band of an expected size (500 nucleotides) was detected in control $RCCD_1$ cells and its expression was increased 7.5 h after AVP addition. Smaller bands were also present but without apparent correlation with CFTR mRNA expression. Quantification of results (radioactivity of the 500 nucleotides band, normalized to the

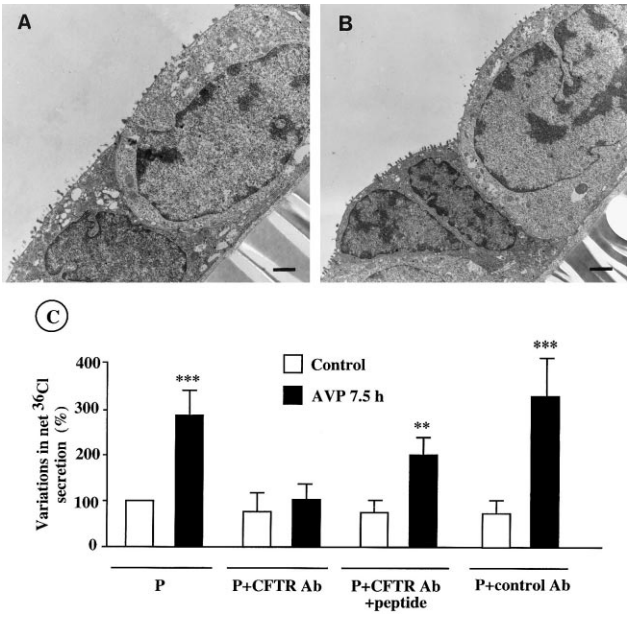

Fig. 3. Effect of anti-CFTR Ab on the AVP-induced increase in net $^{36}Cl^-$ secretion. The effect of anti-CFTR Abs directed against the regulatory region (R) was tested on $^{36}Cl^-$ secretion after the cell permeabilization procedure (see Section 2). A and B illustrate the ultrastructural aspect of $RCCD_1$ cells in the absence (A) or presence (B) of the permeabilization procedure. No major alteration of the cell structure was observed. The increase in $^{36}Cl^-$ secretion observed after 7.5 h of incubation with AVP was blocked by pre-incubating cells with the anti-R CFTR Ab (1/4000) after cell permeabilization (see Section 2). Anti-CFTR Ab was added after 4.5 h of treatment with AVP. The blocking effect of the Ab was significantly impaired in the presence of an excess of the immunizing peptide (peptide). It was not reproduced by another Ab from the same isotype (anti-β-actin Ab; 1/4000: control Ab). Each bar is the mean value of 9–12 filters from six experiments. **, ***: $P<0.01$, $P<0.001$, AVP 7.5 h vs. Control.

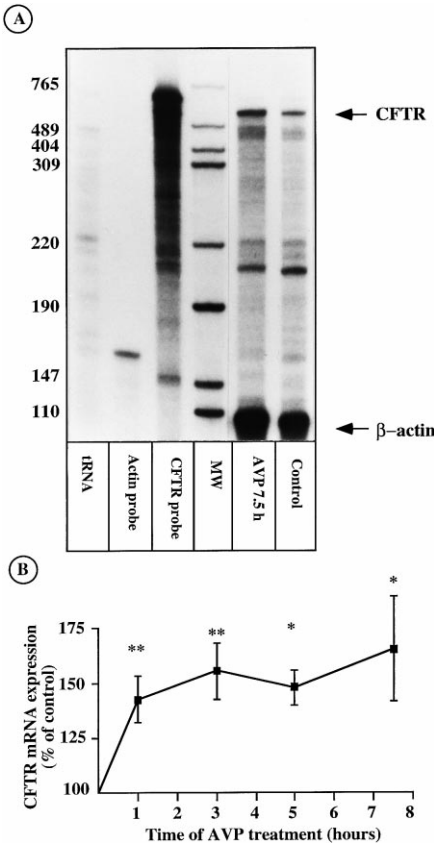

Fig. 4. Effect of AVP on the amount of mRNA encoding CFTR. The amount of mRNA encoding CFTR was determined by a RNAse protection assay. A: First lane: hybridization of CFTR and actin probes with yeast tRNA. Second and third lanes: probes encoding β-actin and CFTR. MW: molecular weight markers. Lanes 5 and 6: hybridization of CFTR and actin probes with lysates of cells, treated (AVP) or not (Control) with AVP for 7.5 h; protected fragments are indicated by arrows. B gives the kinetics of the increase in CFTR mRNA in response to AVP. Each point is the mean value of 5–7 determinations. *, **: $P<0.05$, $P<0.01$ AVP vs. Control.

actin signal) allows us to examine CFTR mRNA levels in cells treated for various delays with 10^{-8} M AVP, as compared to untreated cells. A significantly increased level of CFTR transcripts was observed 1 h after the addition of 10^{-8} M AVP. The mRNA levels then remained stable up to 7.5 h (Fig. 4B).

4. Discussion

The polypeptide hormone AVP is known to act in the renal collecting duct to promote water and sodium reabsorption [1,2]. This effect is rapid (few minutes) and involves binding to V_2 membrane receptors and cAMP cascade activation. More recently, it has been shown that AVP also modulates chloride transport, although the orientation of the net flux (absorptive versus secretory) appears to vary according to the experimental models. In some cases, it was reported that AVP addition results in net chloride reabsorption [18–20]. Other reports document a net chloride secretion in the terminal portions of the nephron [21–24], which is stimulated by cAMP [25–27]. In the amphibian A6 cell line (a model of the distal nephron), Chalfant et al. [4] showed that AVP promotes both net chloride secretion and sodium reabsorption. Yanase and Handler also showed that adenosine 3′,5′-cyclic monophosphate stimulates net chloride secretion in this epithelium [5]. Morris et al. [6] recently reported that AVP stimulates Cl^- secretion in A6 cells. Stimulation of net chloride secretion by AVP was also demonstrated in Madin-Darby canine renal cells (MDCK), another model of the distal nephron [7]. Actually, since the electrochemical driving force for Cl^- across the apical membrane is approximately zero under basal conditions [28], the reabsorption or, conversely, the secretion of chloride may largely depend on hormonal stimulation as well as on the cell's ionic context. The nature of the chloride transporters involved in the AVP effect remains under discussion. There are several arguments in favor of the involvement of the CFTR in chloride movements. Although the precise role of CFTR in the kidney is largely unknown, several studies have shown that CFTR is expressed in this organ and in particular in the collecting duct [29–31]. In primary cultures of rabbit cortical collecting duct (CCD), E. Nagy et al. speculated that AVP could rapidly (<10 min) increase Cl^- transport through CFTR Cl^- channels [18]. In the M_1 mouse CCD cell line, Letz and Korbmacher [32] demonstrated that forskolin was able to stimulate an apical Cl^- conductance and suggested that the stimulated channels were CFTR-like Cl^- channels. In the inner medullary collecting duct, both Husted et al. [25] and Vandorpe et al. [26] presented evidence for an involvement of CFTR in anion secretion in response to cAMP. Finally, Moyer et al. demonstrated that cAMP stimulates CFTR-mediated Cl^- secretion in MDCK cells by activating channels resident in the apical membrane [33]. Our results also report a short-term effect of AVP on net chloride secretion in the $RCCD_1$ cell line.

In addition to this short-term action, evidence has been provided for long-term effects of AVP. Indeed, it appears now that the cascade of events linked to cAMP generation involves a series of complex processes affecting cAMP-responsive transcription factors which lead to regulation of gene expression. cAMP-dependent proteins (CREB, CREM, ATF1) have been shown to bind to specific cAMP-responsive elements (CRE) located in the promoter region of several genes [34]. Such a transcriptional regulatory pathway may well be implicated in the physiological action of AVP, since expression of some transporters and channels has been shown to depend on AVP. This results in delayed and sustained modifications in transport events. Long-term increase in the expression of the water channel AQP_2 has been reported to occur after AVP treatment or dehydration [35,36]. Similarly, we have shown, in a previous study, that sodium reabsorption was increased several hours after AVP exposure in the $RCCD_1$ renal cell line [3]. This late effect of AVP was preceded by de novo synthesis of β- and γ-subunits of the amiloride-sensitive epithelial sodium channel ENaC and of the α_1 subunit of Na^+/K^+-ATPase.

The goal of the present study was to examine whether AVP could also exert a long-term effect on net chloride secretion. We have previously reported [3] that the delayed increase in ion transport induced by AVP was only partially sensitive to amiloride, suggesting that besides sodium transport, another pathway was concerned. Evidence is provided in the present report that AVP promotes a delayed stimulation of net chloride secretion in $RCCD_1$ cells. This increased net chloride secretion appears to occur through a basal $Na^+/K^+/2Cl^-$ co-

transporter and, likely, the apical CFTR. Indeed, it is blocked by basal addition of bumetanide and apical administration of glibenclamide. In addition, an Ab raised against the regulatory region (R) of CFTR can block net chloride secretion in these cells, suggesting that CFTR is indeed important in the described chloride secretory pathway. Moreover, an increase in the steady-state level of CFTR mRNA appears to precede the increase in chloride transport. The $Na^+/K^+/2Cl^-$ appears to be a major component of chloride entry into the cells in a variety of epithelia which secrete chloride [37]. In $RCCD_1$ cells, one might speculate that it corresponds to the secretory form of the $Na^+/K^+/2Cl^-$ (BSC2) which is expressed in the basolateral membrane of cells from the rat collecting duct [38,39]. Interestingly, it has been reported that in intestinal HT29 cells, cAMP was able to stimulate net chloride secretion at both short- (minutes) and long-term (8 h) [40]. Authors showed that the long-term secretion involved basal $Na^+/K^+/2Cl^-$ and apical CFTR and that it was mediated by an increase in the CFTR copy number at the mRNA level and de novo protein synthesis. In our study, we also observed an increase in the amount of mRNA encoding the CFTR. Thus, similar transport systems and regulatory pathways appear to be involved, although in two different epithelia. In particular, CFTR appears to play a major role in this chloride secretion mechanism. Interestingly, the CFTR mRNA level was already increased near maximal after 1 h of AVP treatment, whereas the physiological effect on chloride secretion was apparent only after several hours. It can be inferred that the increase in CFTR expression is not the limiting factor for the AVP-induced increase in Cl^- secretion. Indeed, one can speculate that the basolateral entry through the $Na^+/K^+/2Cl^-$ could be rate limiting. Alternatively, since AVP concomitantly increases sodium entry through the apical sodium channel (ENaC) [3], thus decreasing the driving force for apical Cl^- secretion, other transport pathways (as K^+ or H^+ secretion) may be involved in this mechanism to allow for chloride secretion. As a second hypothesis, one can propose that, even if the increase in the CFTR mRNA level is a rapid event, the following steps leading to an effective increase in chloride transport, i.e. mRNA maturation, protein translation, post-transcriptional events and CFTR expression in an active form in the membrane, require some delay. Along this line, such delay was observed between the AVP-induced increase in the steady-state level of mRNA encoding the β- and γ-subunits of ENaC and α_1 subunit of the Na^+/K^+-ATPase (1–3 h) and the observed effect on Na^+ reabsorption (6–7 h) [3]. In particular, the number of ENaC present in the apical membrane of the cells was not modified 4 h after AVP treatment, but was significantly increased after 7.5 h [3].

Interestingly, CRE sequences have been identified in the promoter region of CFTR [41,42]. But surprisingly, limited information is available in the literature on transcriptional regulation of CFTR. Based on promoter sequence analysis and on CFTR transfection in different cell types, evidence has been provided that both cAMP and the tumor-promoting agent phorbol myristate acetate (PMA) influence CFTR transcript levels [41,43–45]. While PMA appears to down-regulate these transcripts in T84 colonic cells [45], cAMP, acting through PKA, is a positive regulator of CFTR gene expression [43]. Our experiments extend these findings to the response to AVP, a peptide hormone with an important regulatory role in the kidney.

Together, the present findings suggest that AVP exerts a delayed control of chloride transport in CCD cells, in addition to its short-term effect. Here, we provide evidence that exposure of $RCCD_1$ cells to AVP leads after several hours to an increase in net chloride secretion and that this effect might involve CFTR. It will be of major interest to elucidate precisely the molecular events producing these effects.

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