Calcyclin Is an Early Vasopressin-induced Gene in the Renal Collecting Duct

ROLE IN THE LONG TERM REGULATION OF ION TRANSPORT*

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Long-term effects of arginine vasopressin (AVP) in the kidney involve the transcription of unidentified genes. By subtractive hybridization experiments performed on the RCCD1 cortical collecting duct cell line, we identified calcyclin as an early AVP-induced gene (1 h). Calcyclin is a calcium-binding protein involved in the transduction of intracellular signals. In the kidney, calcyclin was localized at the mRNA level in the glomerulus, all along the collecting duct, and in the epithelium lining the papilla. In RCCD1 cells and in m-IMCD3 inner medullary collecting duct cells, calcyclin was evidenced in the cytoplasm. Calcyclin mRNA levels were progressively increased by AVP treatment in RCCD1 (1.7-fold at 4 h) and m-IMCD3 (2-fold at 7.5 h) cells. In RCCD1 cells, calcyclin protein levels were increased by 4 h of AVP treatment. In vivo, treatment of genetically vasopressin-deficient Brattleboro rats with AVP for 4 days induced an increase in both calcyclin and aquaporin-2 mRNA expression. Finally, introduction of anti-calcyclin antibodies into RCCD1 cells by permeabilizing the plasma membrane prevented the long-term (but not short-term) increase in short-circuit current induced by AVP. Taken together, these results suggest that calcyclin is an early vasopressin-induced gene that participates in the late phase of the hormone response in transepithelial ion transport.

Arginine vasopressin (AVP) is a polypeptide hormone involved in the regulation of renal water and ion transport. In the collecting duct, AVP coordinately increases water and NaCl reabsorption by a two-step mechanism. The first mechanism is responsible for the short-term effects of AVP. These effects consist of the translocation of aquaporin-2 (AQP2) water channels and amiloride-sensitive sodium channels from intracellular pools to the apical membrane, promoting an increase in both sodium and water entry (1–4). This increase induces, in turn, a coordinate rise in transepithelial water and sodium reabsorption (5–10). These effects are rapid and transient because the effect is down-regulated after ~1 h in the rat collecting duct (11). In addition to this short-term effect, AVP induces a long-term effect. This effect consists of a late increase in transepithelial water, sodium, and chloride transport after several hours. This late effect is mediated through a transcriptional/translational mechanism and involves an increase in the mRNA and de novo synthesis of different proteins such as AQP2; the β and γ (but not α) subunits of ENaC (epithelial Na⁺ channel); the α1 (but not β1) subunit of Na⁺/K⁺-ATPase; and CFTR (cystic fibrosis transmembrane conductance regulator) (12–15). This long-term effect may involve the genomic pathway by activation of cAMP-responsive elements in the promoter region of these genes and may ensure a sustained increase in sodium, chloride, and water transport in this segment of the nephron. A recent study has focused on the effects of 4 h of treatment with vasopressin on the transcriptome of a mouse kidney cortical collecting duct cell line (16). Statistical comparison of the SAGE (serial analysis of gene expression) libraries revealed 48 vasopressin-induced transcripts and 11 vasopressin-repressed transcripts. A selection of the differentially expressed vasopressin-specific transcripts has been validated by Northern blot hybridization and by reverse transcription. Hepatocyte nuclear transcription factor-3α (VIT39 (vasopressin-induced transcript)) and receptor activity-modifying protein-3 (VIT48) have been suggested to be candidate proteins playing a role in physiological responses to vasopressin.

In this study, we have searched for mRNA induced by AVP treatment using the subtractive hybridization technique in the vasopressin-responsive RCCD1 cell line. Results show that the calcium-binding protein calcyclin is an AVP-induced protein. Interestingly, in the kidney, calcyclin is mainly expressed in the collecting duct and appears to be regulated by AVP in coordination with AQP2. Finally, calcyclin appears to be indispensable in allowing the long-term (but not short-term) response of the hormone in RCCD1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The RCCD1 rat cortical collecting duct cell line (17) and the m-IMCD3 mouse inner medullary collecting duct cells (a generous gift from Dr. P. Gascard, Berkeley University, Berkeley, CA) were used between passages 10 and 25. Cells were grown either on Transwell or Snapwell (Costar Corp.) filters coated with collagen (type 1 from rat tail; Institut Jacques Boy, Reims, France). The defined culture medium for RCCD1 cells was Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1), 14 mM NaHCO₃, 2 mM glutamine, 5 × 10⁻⁶ M dexamethasone, 3 × 10⁻⁸ M sodium selenite, 5 μg/ml transferrin, 5 μg/ml insulin, 10 μg/ml epidermal growth factor, 5 × 10⁻⁸ M triiodothyronine, 10 units/ml penicillin/streptomycin, and 2% fetal bovine serum (Invitrogen, Les Ulis, France). The culture medium for m-IMCD3 cells was Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1), 10 units/ml penicillin/streptomycin, 20 mM HEPES, pH 7.4, and 2% fetal bovine serum (Invitrogen, Les Ulis, France). The culture medium for m-IMCD3 cells was Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1), 10 units/ml penicillin/streptomycin, and 10% fetal bovine serum. The medium was changed every other day.

Animals—Male Sprague-Dawley rats (250–300 g) were used for in situ hybridization experiments. In addition, 16 male Brattleboro rats (250–300 g) were used for Northern blot experiments aimed at deter-
mining the effects of AVP on calcyclin mRNA expression. In these experiments, animals were treated with AVP (Sigma) using different protocols: either with a single intramuscular injection of AVP (2 µg in 0.9% NaCl) 3 h before killing or with an osmotic minipump (500 ng/day in 0.9% NaCl) for 4 days. Control rats were treated similarly, except that saline was used. Animals had free access to tap water and were anesthetized with pentobarbital before killing.

**PCR-based Suppression/Subtractive Hybridization**—To determine the mRNAs differentially expressed after vasopressin treatment in the cortical collecting duct, subtractive hybridization was performed on RCDs cells grown on Transwell filters in defined culture medium for 4 days, incubated overnight in minimum medium (Ham’s F-12/Dulbecco’s Modified Eagle’s medium (1:1), 14 mM NaHCO3, 2 mM glutamine, 10 units/ml penicillin/streptomycin, and 20 mM HEPES, pH 7.4), and then treated or not for 1 h basolaterally with 10-8 M AVP. Poly(A) mRNAs were extracted from RCDs cells treated or not with AVP using oligo(dT)15 covalently bound to magnetic beads (Dynal, Oslo, Norway). Double-stranded cDNA was then synthesized and digested with RsaI. To identify up-regulated sequences, these two pools of cDNA fragments derived from control and AVP-treated cells were subjected to subtractive hybridization and suppression PCR (PCR-Select™ cDNA Subtraction kit, CLONTECH) according to the manufacturer’s protocol. Adaptors were linked to the AVP-treated cDNA pool, and two successive hybridizations followed by extension to fill in ends were performed in the presence of an excess of cDNA without linkers from the untreated cells. A first PCR amplification using suppression PCR amplifies predominantly only differentially expressed sequences. A second PCR amplification reduced the background and further enriched differentially expressed cDNA fragments. “Forward” subtraction was performed when linkers were added to cDNA fragments obtained from hormone-treated cells, whereas “reverse” subtraction was performed when linkers were added to cDNA fragments obtained from control cells. PCR products were cloned into the pT-Adv vector using the AdvAmp™ PCR cloning kit (CLONTECH). Differential screening of the subtracted library was performed to eliminate false positives by hybridization with 32P-labeled probes prepared from forward and reverse subtracted cDNAs (PCR-Select Differential Screening kit, CLONTECH). Clones showing signal ratios of >5:1 (forward versus reverse subtracted probe) were further analyzed by DNA sequencing.

**In Situ Hybridization**—For in situ hybridization studies, kidneys from Sprague-Dawley rats were fixed for 15 min in 4% paraformaldehyde with 5 mM MgCl2 and then kept at 4°C in 0.9% NaCl. Animals were anesthetized with pentobarbital before killing. From Sprague-Dawley rats were fixed for 15 min in 4% paraformaldehyde and 0.9% NaCl. Cortical collecting duct, subtractive hybridization was performed on the mRNAs differentially expressed after vasopressin treatment in the medulla, and the only structure that was labeled was the collecting duct (C, arrows). Finally, in the papilla (D), the collecting duct cells and the epithelium lining the papilla (arrows) strongly expressed calcyclin mRNA.

In **Western Blot Experiments**—Cells were plated on 24-mm Transwell filters and cultured for 3 days in defined culture medium before incubation overnight in minimum medium. Cells were then treated or not basolaterally for 4 h at 37°C with 10-8 M AVP. Cells from each filter were rinsed and scraped at 4°C in phosphate-buffered saline before addition of lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Nonidet P-40, 50 mM Tris, pH 7.5, 10 µg/ml protease inhibitor mixture (Sigma), and 0.5 mM phenylmethylsulfonyl fluoride) and incubation for 30 min at 4°C. Supernatants (after centrifugation at 12,000 × g) corresponding to each filter were submitted to SDS-PAGE (15%) using the Laemmli buffer system before transfer onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was then pretreated for 1 h at room temperature with 5% milk in Tris-buffered saline plus 0.1% Tween 20 and incubated with the anti-calcyclin antibody (1:250) overnight at 4°C, followed by incubation with a secondary antibody conjugated to peroxidase (1:20,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Proteins were visualized using the ECL or ECL Plus detection kit (Amersham Biosciences). Results were normalized to the signal obtained by Western blotting of β-actin in the same cell samples. To this end, the membrane was stripped and incubated overnight at 4°C with 5% milk in Tris-buffered saline plus 0.1% Tween 20 and then for 1 h at room temperature with an anti-β-actin antibody (1:10,000; Santa Cruz Biotechnology, Inc.) before incubation with the peroxidase-conjugated secondary antibody (1:20,000) for 1 h at room temperature and ECL visualization.

**Immunocytochemistry**—Immunocytochemical experiments were performed with a rabbit polyclonal anti-calcyclin antibody (Swiss Swant Laboratories, Bellinzona, Switzerland) diluted 1:200. RCDs cells and mIMCD3 cells were grown to confluence on collagen-coated Transwell filters (12-mm diameter) and then fixed in paraformaldehyde for 30 min at room temperature, washed, and incubated at room temperature for 1 h with the anti-calcyclin antibody: A secondary antibody (goat anti-rabbit Fab fraction (1:200); Jackson ImmunonovaResearch Laboratories, Inc.) coupled to the fluorochrome Cy5 (red fluorescence) was used to visualize the signal. In these experiments, the nucleus was stained with Sytox (green fluorescence; Molecular Probes, Inc.). The sections of cells were then photographed using confocal laser scanning microscopy (Leica TCS4D apparatus).

**Northern Blot Experiments**—Total RNA (10–20 µg) was extracted from RCDs, or mIMCD3 cells cultured on 24-mm diameter Transwell filters or from Brattleboro rat kidneys, run on a 0.8% denaturing gleyzal-agarose gel, and blotted onto nylon membranes (Hybond-N, Amer sham Biosciences) as previously described (19). The membranes were then hybridized with random-primer [α-32P]dCTP-labeled probes for the clone encoding calcyclin (276 bp, nucleotides 51–327), AQP2 (330 bp, nucleotides 638–968), or GAPDH (851 bp, nucleotides 20–871) as an internal control. The membranes were exposed to Biomax MR films (Kodak) and analyzed with an Instant Image (Packard Instrument Co.).
Calcyclin Is a Vasopressin-induced Protein

**FIG. 2.** Confocal localization of calcyclin in RCCD$_1$ cells. Calcyclin was localized in RCCD$_1$ cells grown on porous filters and cultured overnight in minimum medium (see “Experimental Procedures”) by immunofluorescence using a specific anti-calcyclin antibody (in red). The nuclei were stained in green. Whereas almost no staining was observed when the experiment was performed without the primary antibody (anti-calcyclin antibody) (A–C), a clear staining was observed in its presence (D–F). The $xz$ reconstruction of the cells (F) shows that calcyclin was localized in the cytoplasmic compartment of the cells. Bars = 30 (A), 10 (B), and 5 (C) $\mu$m.

**FIG. 3.** Confocal localization of calcyclin in m-IMCD$_3$ cells. Calcyclin was localized in m-IMCD$_3$ cells (in red). The nuclei were stained in green. Experiments were performed under the same conditions as described for RCCD$_1$ cells (see the legend to Fig. 2). No staining was observed in the absence of the anti-calcyclin antibody (data not shown). In its presence, a clear signal was evidenced (A) in the cytoplasm of the cells (B). Bars = 10 $\mu$m.

Blue as a marker of cell permeabilization showed that $>$95% of the cells were permeabilized under these conditions. In control experiments, the same protocol was performed, except that rabbit IgG was used in place of the anti-calcyclin antibody.

To study the short-term effects of AVP (15 min), cells were permeabilized after incubation in minimum medium, incubated for 4.5 h at 37 °C to allow membrane resealing, and then mounted in the voltage-clamp system (Costar Corp. and World Precision Instruments, Inc.). To study the long-term effects of AVP (7.5 h), cells were first treated with AVP. After 3 h of incubation, cells were permeabilized and then further incubated for 4.5 h, always in the presence of AVP, at 37 °C before mounting in the voltage-clamp system. In this system, cells were bathed on each side with 8 ml of minimum medium that was thermostated at 37 °C and that was circulated by a gas lift (95% O$_2$ and 5% CO$_2$ mixture). $I_{sc}$ ($\mu$A/cm$^2$) was measured by clamping the transepithelial voltage to 0 mV for 1 s. To study the short-term effects of AVP, $I_{sc}$ was determined before and after 15 min of incubation with $10^{-8}$ M AVP. To study the long-term effects of AVP, $I_{sc}$ was determined in cells treated or not with $10^{-8}$ M AVP for 7.5 h.

**Statistical Analysis—**Results are expressed as means ± S.E. Statistical analysis was performed using Student’s $t$ test for unpaired data according to the experiments.

**RESULTS**

Calcyclin Is a Differentially Expressed Gene in RCCD$_1$ Cells Treated with Vasopressin—PCR-based subtractive hybridization was used to establish a library of cDNAs representing early (1 h) vasopressin-regulated mRNAs in RCCD$_1$ cells. To this end, mRNAs corresponding to 24-mm diameter filters treated or not for 1 h with $10^{-8}$ M AVP were prepared with the poly(A) isolation kit and used in a PCR-based suppression/subtractive hybridization experiment (see “Experimental Pro-
The selected clones were sequenced and analyzed by homology searches using the BLAST program. Among these sequences, we have identified a clone highly homologous to calcyclin (GenBank™/EBI accession Number AJ132717), which was further studied.

In the Nephron, Calcyclin Is Expressed in Collecting Duct Cells—To examine the expression of calcyclin in the kidney, in situ hybridization experiments were performed on kidneys from Sprague-Dawley rats. The results are presented in Fig. 1. Calcyclin mRNA was expressed all along the collecting duct and also in the glomerulus and in the epithelium lining the papilla. In the collecting duct, calcyclin mRNA was expressed at low levels in the cortical part, with a progressive increase along the medulla and the papilla.

Immunocytochemical Localization of Calcyclin in RCCD1 and m-IMCD3 Cells—The expression of calcyclin was examined in two different models of collecting duct cells: the RCCD1 cell line, corresponding to cortical collecting duct cells, and the m-IMCD3 cell line, corresponding to papillary collecting duct cells. Calcyclin was expressed in both models and appeared to be present essentially in the cytoplasmic compartment (Figs. 2 and 3, respectively).

Calcyclin mRNA Expression Is Increased by AVP Treatment in RCCD1 Cells—Fig. 4A shows a representative Northern blot experiment aimed at determining the time course of calcyclin mRNA induction by 10^{-8} M AVP in RCCD1 cells. Whereas GAPDH mRNA expression was not modified, calcyclin mRNA expression increased progressively with the time of AVP exposure. Fig. 4B illustrates the mean values of five different experiments. Calcyclin mRNA induction significantly increased as soon as 1 h after AVP addition, with a maximal effect after 4 h (~70% increase). Thereafter, calcyclin mRNA expression returned to the control level (at 24 h).

Calcyclin Protein Level Is Increased by AVP Treatment in RCCD1 Cells—Fig. 6A shows a representative Western blot experiment aimed at determining the effect on calcyclin expression of 4-h 10^{-8} M AVP treatment in RCCD1 cells. Calcyclin protein expression was largely increased by AVP treatment. Fig. 6B illustrates the mean values of three different experiments, taking into account the expression of β-actin.
Calcyclin Is a Vasopressin-induced Protein

Calcyclin mRNA Expression Is Increased in Parallel with AQP2 mRNA Expression in Brattleboro Rat Whole Kidneys—To examine the in vivo effect of AVP on the expression of renal calcyclin mRNA, Northern blot experiments were performed with RNA obtained from Brattleboro rat whole kidneys treated or not for 4 days with AVP (see "Experimental Procedures"). The expression of calcyclin and AQP2 mRNAs was significantly increased by AVP treatment. Fig. 7A shows a representative experiment using an osmotic minipump. Four control rats were treated in a similar fashion, except that only diluent (0.9% NaCl) was used. A representative experiment of five different experiments is shown in A (each lane corresponds to one animal), and the quantified data are given in B. Both calcyclin and AQP2 mRNAs were significantly increased by AVP. *, p < 0.05; ***, p < 0.001 (AVP versus control).

The short-term effect of AVP on renal ion and water transport have been widely documented (5–10). By contrast, very limited information is available on the long-term effects of this hormone. In addition to the long-term effects of AVP on water transport associated with an increase in AQP2 synthesis (12, 26), we have recently shown that AVP exerts a delayed stimulation of sodium and chloride transport in collecting duct cells (13, 14). This effect depends on transcription of several transporters of sodium and chloride, in particular ENaC, Na-K-ATPase, and CFTR. As a matter of fact, the cAMP- or intracellular Ca2+-induced
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Calcyclin is a 10.5-kDa protein that belongs to the family of calcium-binding proteins (28–31). Calcium-binding proteins are divided into two groups: the first group is constituted by annexins, and the second group by EF-hand proteins such as calmodulin. Calcyclin belongs to this second group. It was first identified as a cell cycle-dependent protein highly induced by growth conditions (28), but its precise role remains unknown. Different studies have shown that calcyclin is expressed only in fibroblasts and epithelial cells and that it can associate with other proteins, in particular annexins (annexin-2, -6, and -11) (32, 33). Calcyclin, as annexins, may be involved in exocytosis phenomena. In this way, it has been shown in numerous studies that modulation of intracellular calcium plays an important role in the regulation of exocytosis and that calcium-binding proteins can act as transducing proteins in coupling stimulus to exocytosis. It has also been shown that, in vitro, calcyclin can bind actin-binding proteins such as caldesmon, tropomyosin, and calponin (34–36).

Our data obtained by in situ hybridization show that calcyclin was localized mainly in the collecting duct cells in the kidney, with additional staining in the glomerulus and in the epithelium lining the papilla. In a previous study, Lewington et al. (37) showed that calcyclin is present in glomeruli and distal tubules. Our results are compatible with those data. At the cellular level, it has been suggested that calcyclin could be localized in the cytoplasmatic compartment of the cells under basal conditions and that it could be targeted to two different membranous compartments, the plasma membrane and the nuclear membrane, in response to an increase in intracellular calcium (38). In our experiments performed in RCCD1 and m-IMCD3 cells incubated under basal conditions (Figs. 2 and 3), calcyclin was localized in the cytoplasmatic compartment. Further studies will be necessary to examine whether AVP treatment or modifications of the intracellular Ca2+ concentration modify this localization.

Only a few studies concerning the hormonal regulation of calcyclin have been reported. The promoter of calcyclin has been reported to contain a serum-responsive element (39), and calcyclin is generally considered to be a growth-regulated gene (28). In the rat kidney, it has been shown that calcyclin is induced after ischemic injury (37). In this study, we have shown that AVP increased the amount of mRNA encoding calcyclin in RCCD1 cells, in m-IMCD3 cells, and in Brattleboro rat kidney. In addition, we have shown that calcyclin protein expression was increased in RCCD1 cells after 4 h of treatment with 10-8 M AVP. Interestingly, the time course of the phenomenon appears to be different in the two cell lines and in the rat kidney. In RCCD1 cells derived from the cortical collecting duct, as in m-IMCD3 cells derived from the inner medullary collecting duct, calcyclin mRNA was rapidly and transiently increased. A significant effect was observed as soon as 0.5–1 h after AVP treatment (Figs. 4 and 5). This result is in accordance with the fact that calcyclin mRNA was evidenced as an AVP-induced gene in RCCD1 cells by subtractive hybridization after 1 h of AVP treatment. The time course shows that the maximal increase was observed after 4–7.5 h of treatment and then declined at 24 h. After 24 h of treatment with AVP, the amount of mRNA encoding calcyclin was not different between control and AVP-treated cells. In contrast, the results obtained with Brattleboro rat whole kidneys showed no increase in calcyclin mRNA 3 h after injection of AVP, but a clear effect after 4 days of AVP treatment (Fig. 7). In the same way, note that AQP2 mRNA was not significantly increased after 3 h of AVP treatment, but largely increased after 4 days. The differences observed in the time course and in the magnitude of the AVP effect between cell models and Brattleboro rat kidneys might be related to differences between in vitro and in vivo models. The complexity of animal models, the delay in the response to the hormone when administrated intramuscularly, and the presence of several complementary hormonal regulatory systems might explain a delayed effect on calcyclin and AQP2 mRNA expression in the rat. In this way, it should be noted that hormonal effects on the amount of mRNA encoding newly synthesized proteins are often delayed when studied in animal models rather than in cell lines.

The precise role of calcyclin in epithelial cells is unknown. In a previous work (20), calcyclin has been described to be involved in the Ca2+-dependent secretion of insulin in pancreatic cells. Interestingly, it was shown that introduction into pancreatic beta-cells of anti-calcyclin antibodies by permeabilizing the plasma membrane prevented insulin secretion, a result that clearly defined calcyclin as a key protein in the exocytosis process. In our study, introduction of anti-calcyclin antibodies into permeabilized RCCD1 cells supported a specific role of calcyclin in the long-term regulation of ion transport (Fig. 8). Indeed, anti-calcyclin antibodies prevented the AVP-induced increase in INa observed after 7.5 h of treatment, whereas it was without effect on the short-term effect of the hormone (15 min). The localization of calcyclin in the cytoplasmatic compartment of RCCD1 cells and the possibility that it could move to the nucleus upon certain stimuli as described in other studies (38) suggest different hypotheses concerning its role. In the first hypothesis, calcyclin could be implicated in the process leading to the transcription of other genes (transcriptional role). In this way, by binding calcium, calcyclin could play a role in the Ca2+-induced activation of Ca2+-responsive elements through nuclear kinases such as Ca2+/calmodulin-dependent protein kinase (40). Alternatively, or additively, the established interaction of calcyclin with the calcium signaling pathways suggests that calcyclin could be involved in the delivery of newly synthesized proteins (due to the transcriptional effect of AVP) to the apical and/or the basolateral membrane of the cells (exocytotic role) (41). Further experiments will be necessary to test these hypotheses.

In conclusion, we have identified calcyclin as a new AVP-induced gene in the collecting duct. In addition, our experiments suggest that calcyclin could play an important role in the long-term response of the hormone in transepithelial ion transport.

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