Vasopressin V1 receptors on the principal cells of the rabbit cortical collecting tubule. Stimulation of cytosolic free calcium and inositol phosphate production via coupling to a pertussis toxin substrate.

M A Burnatowska-Hledin, W S Spielman

Research Article

The effects of arginine vasopressin (AVP) on the cytosolic free calcium concentration ([Ca2+]f) were examined in freshly immunodissected rabbit cortical collecting tubule cells using fluorescent Ca2+ indicators fura-2 and indo-1. The addition of AVP to a cell suspension resulted in a rapid and transient increase in the [Ca2+]f. The 1-deamino-8-D-AVP (dDVP), a V2 receptor agonist of AVP that stimulated adenosine 3',5' cAMP production in these cells, had no effect on [Ca2+]f and did not affect AVP-induced increase in [Ca2+]f. The AVP-induced increase in [Ca2+]f but not cAMP production was blocked by the V1 receptor antagonist, [1-(beta-mercapto-beta-beta-cyclopentamethylene propionic acid), 2-(O-methyl)tyrosine] Arg8-vasopressin. The AVP-stimulated increase in [Ca2+]f appeared to be largely due to Ca2+ release from intracellular stores as reduction of extracellular Ca2+ with EGTA had little if any effect on the AVP-induced increase in [Ca2+]f. This AVP-induced increase in [Ca2+]f was associated with an increase in inositol-1,4,5-trisphosphate production and appeared to involve a guanine nucleotide-binding protein (G), since the pretreatment of cells with pertussis toxin for 4-6 h inhibited this effect. Finally, measurements of [Ca2+]f in single cells suggest that only the principal cells of the collecting tubules respond to AVP with an increase in [Ca2+]f. In summary, these results demonstrate that the principal cells of the cortical collecting tubule possess two distinct receptor systems for vasopressin, the well-known V2 receptor […]

Vasopressin V₁ Receptors on the Principal Cells of the Rabbit Cortical Collecting Tubule

Stimulation of Cytosolic Free Calcium and Inositol Phosphate Production via Coupling to a Pertussis Toxin Substrate

Maria A. Burnatowska-Hledin and William S. Spielman
Department of Physiology, Michigan State University, East Lansing, Michigan 48824

Abstract

The effects of arginine vasopressin (AVP) on the cytosolic free calcium concentration ($[Ca^{+2}]_f$) were examined in freshly immunodissected rabbit cortical collecting tubule cells using fluorescent Ca^{+2} indicators fura-2 and indo-1. The addition of AVP to a cell suspension resulted in a rapid and transient increase in the $[Ca^{+2}]_f$. The 1-deamino-8-D-AVP (dDVP), a V_2 receptor agonist of AVP that stimulated adenosine 3',5' cAMP production in these cells, had no effect on $[Ca^{+2}]_f$ and did not affect AVP-induced increase in $[Ca^{+2}]_f$. The AVP-induced increase in $[Ca^{+2}]_f$ but not cAMP production was blocked by the V_1 receptor antagonist, [1-(beta-mercapto-beta-beta-cyclopentamethylene propionic acid), 2-(O-methyl)tyrosine] Arg⁸-vasopressin. The AVP-stimulated increase in $[Ca^{+2}]_f$ appeared to be largely due to Ca^{+2} release from intracellular stores as reduction of extracellular Ca^{+2} with EGTA had little if any effect on the AVP-induced increase in $[Ca^{+2}]_f$. This AVP-induced increase in $[Ca^{+2}]_f$ was associated with an increase in inositol-1,4,5-trisphosphate production and appeared to involve a guanine nucleotide-binding protein (G), since the pretreatment of cells with pertussis toxin for 4–6 h inhibited this effect. Finally, measurements of $[Ca^{+2}]_f$ in single cells suggest that only the principal cells of the collecting tubules respond to AVP with an increase in $[Ca^{+2}]_f$. In summary, these results demonstrate that the principal cells of the cortical collecting tubule possess two distinct receptor systems for vasopressin, the well-known V_2 receptor coupled to adenylate cyclase, and a V_1 receptor system that leads to the mobilization of cytosolic calcium, coupled through a pertussis toxin substrate (G protein) to a production of inositol phosphates.

Introduction

The action of vasopressin (VP)[1] to regulate water and sodium transport in the kidney has been largely linked with the cAMP messenger system through the activation of the V_2 receptor and adenylate cyclase activity (1–3). In the liver, vascular smooth muscle, and glomerular mesangial cells the action of VP has been linked to the activation of the V_1 receptor and phospholipase C activity responsible for the breakdown of phosphatidylinositol-4,5-biphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol with the resultant mobilization of calcium and activation of protein kinase C, respectively (4–8).

In the renal tubules from the medulla indirect evidence indicates the presence of both VP receptors (9). The presence of many cell types in the kidney, however, has complicated the direct investigation of the action of VP in specific cell types. Recently, both V_1 and V_2 receptor–dependent action of VP has been demonstrated in LLC-PK₁ cells, an established cell line of renal origin, raising the possibility of two receptors coexisting on the same cell (10, 11). Although LLC-PK₁ cells provide a useful model for transport and signal transduction studies (6, 12), ambiguity in the nephron site of origin and the constant concern that transformed cell lines undergo phenotypic changes make the physiological significance of the observation unclear.

The aim of the present study was to determine (a) if rabbit cortical collecting tubule (RCCT) cells possess a V_1 receptor coupled to the mobilization of cytosolic calcium ($[Ca^{+2}]_f$), in addition to the well established V_2 receptor coupled to adenylate cyclase; (b) the source for the changes in the $[Ca^{+2}]_f$; (c) which receptors are involved in the $[Ca^{+2}]_f$ mobilizing response; (d) if the increase in $[Ca^{+2}]_f$ is linked to IP₃ production; (e) the involvement of guanine nucleotide-binding protein in the VP-induced $[Ca^{+2}]_f$ response; and finally, (f) which cells of the rabbit collecting tubules (CT) are involved in the V_1-linked response.

Methods

Materials

DME, collagenase, fetal bovine serum, and trypsin were purchased from Gibco Laboratories, Grand Island, NY. Fura-2/AM and Indo-1/AM were purchased from Molecular Probes, Inc., Junction City, OR. The phosphodiesterase inhibitor, RO 20-1724, was a gift from Hoffman-La Roche, Nutley, NJ. PT was from List Biological Laboratories, Inc., Campell, CA. Arginine vasopressin (AVP) and 1-deamino-8-D-arginine vasopressin (dDVP) were obtained from Sigma Chemical Co. (St. Louis, MO), and [1-(beta-mercapto-beta-beta-cyclopentameth-

Address correspondence to Dr. Maria A. Burnatowska-Hledin, Department of Physiology, Giltner Hall, Michigan State University, East Lansing, MI 48824.

1. *Abbreviations used in this paper:* AVP, arginine vasopressin; $[Ca^{+2}]_f$, cytosolic free calcium; CT, collecting tubules; $d(CH_2)_5Tyr(Me)AVP$, [1-(beta-mercapto-beta-beta-cyclopentamethylene propionic acid), 2-(O-methyl)tyrosine] Arg⁸-vasopressin; dDVP, 1-deamino-8-D-arginine vasopressin; FITC-PNA, peanut lectin isothiocyanate; G, guanine nucleotide-binding protein; GTP, guanine nucleotide; IP₁, inositol-1-phosphate; IP₂, inositol-1,4-biphosphate; IP₃, inositol 1,4,5-triphosphate; PIP₂, phosphatidylinositol-4,5-biphosphate; PT, pertussis toxin; R, fluorescence ratio; RCCT, rabbit cortical collecting tubule; SSS, simplified saline solution; VP, vasopressin.

ylene propionic acid),2-(O-methyl)tyrosine] Arg⁸-vasopressin (d(CH₂)₅Tyr(Me)AVP) from Peninsula Laboratories, Inc., Belmont, CA. Myo-[³H]inositol was from New England Nuclear (Boston, MA). All other chemicals were from Sigma Chemical Co.

Isolation of the RCCT cells

To isolate relatively large numbers of virtually pure populations of RCCT cells, solid-phase immunoadsorption technology using monoclonal antibodies directed against cell surface antigens was used (13–15). Briefly, kidneys from 4–6-wk-old New Zealand white rabbits were removed, cortical tissue was dissected out, diced finely, and transferred to a 50-ml tube containing 0.1% collagenase in simplified saline solution (SSS; composition in millimolars: 145 NaCl, 5 KCl, 1 Na₂HPO₄, 0.5 MgCl₂, 5 glucose, 10 Hepes, 1 CaCl₂, pH 7.4), and incubated 30–45 min at 37°C in a shaker water bath. To remove collagenase the cell suspension was centrifuged at 1,000 g for 10 min, red blood cells were lysed with hypotonic saline (0.2%, 30 s), the suspension was filtered through a 250-μm Gelman wire mesh, and resuspended in 10% BSA in PBS (composition in millimolar: 137 NaCl, 8.1 Na₂HPO₄, 1.5 KCl, pH 7.3). The cell suspension was washed in PBS and overlayed on 100-mm plastic culture dishes coated with an IgG₃ (rct-30) antibody-specific against CT cells. After a few minutes plates were washed extensively with PBS and cells were detached from the plate and used for the particular study.

VP-induced cAMP formation

Freshly immunodissected RCCT cells suspended in SSS were transferred in 200-μl aliquots to 24-well plates and assayed for cAMP. Cells were incubated with the phosphodiesterase inhibitor RO 20-1724 (1 mM) for 15–20 min, and then with AVP or its analogues (100 μl, concentrated three times) for an additional 10–15 min at 37°C. The treatment was terminated with 0.2% HCl (75 μl). Cells were quick-frozen (−80°C), thawed, pH adjusted with 0.5 M Na₂HPO₄ (200 μl), and assayed in triplicate for cAMP by RIA (16). To determine protein concentration pelleted cells were dissolved with a solution containing 2% Na₂CO₃, 0.4% NaOH, 0.16% sodium tartrate, and 1% SDS, and assayed for protein using a modification of the Lowry method (15). The results are expressed as pmol cAMP/mg protein per 15 min.

Measurement of the $[Ca^{+2}]_f$

Cell suspension. $[Ca^{+2}]_f$ in cell suspension was measured using fura-2, basically as described previously (10). Freshly immunodissected cells were washed in SSS, counted, resuspended in 1 ml SSS containing 4 μM fura-2/AM, and incubated at 37°C in a water bath with continuous shaking for 30–40 min. Fluorescence was measured with a dual excitation wavelength spectrofluorometer with a computerized recording system in a temperature-controlled cuvette, continuously stirred with a magnetic stirrer (Spex Industries, Inc., Metuchen, NJ). Samples were excited at 340 and 380 nm and emission was monitored at 505 nm. The fluorescence ratios (R) were obtained by dividing the fluorescence excited at 340 nm by the fluorescence excited at 380 nm. $[Ca^{+2}]_f$ was calculated as described by Grynkiewicz et al. (16) and our previous study (10), using the formula:

$$[Ca^{+2}]_f = (R - R_0/R_s - R) \times (F_0/F_s)_{380} \times K_d.$$

The R_0 is the ratio at zero Ca^{+2} and R_s is the ratio at saturating Ca^{+2} concentrations. F_0 is the fluorescence at 380 nm at zero Ca^{+2} and F_s is the fluorescence at 380 nm at saturating Ca^{+2} concentration. The effective dissociation constant for the fura-2-Ca^{+2} complex, K_d, determined for fura-2 produced from fura-2/AM by RCCT cells as described previously (10), was 210 nM and was comparable to the 224-nM K_d value reported by Grynkiewicz et al. (16). Consequently, the 224-nM value was used to calculate $[Ca^{+2}]_f$. Calculated $[Ca^{+2}]_f$ values were corrected for autofluorescence and fura-2 leakage. Fura-2 leakage was determined by the addition of 0.1 mM MnCl₂ to the cell suspension. Whereas little change in fluorescence was observed immediately after cells were washed, it tended to increase with time. Consequently, cells were washed and resuspended in fresh medium several times

throughout the experiment. The addition of MnCl₂ consistently reduced basal $[Ca^{+2}]_f$ to similar levels.

Single cell. Single cell calcium concentrations were measured with an interactive laser cytometer equipped with a 5W argon laser (ACAS 470; Meridian Instruments, Inc., Okemos, MI). The optical system of ACAS 470 is designed to excite a sample with a laser beam at one wavelength and monitor emission at two wavelengths simultaneously using two photomultiplier tubes. Consequently, fluorescent calcium chelating indicators like Indo-1, with a single excitation at 356 nm and dual emission (Indo-1 free at 485 nm and Indo-1 bound to calcium at 405 nm), can be used to monitor changes in $[Ca^{+2}]_f$.

Cells were loaded with 2 μM Indo-1/AM as described for fura-2/AM and aliquots overlaid on 35-mm culture plates that had been modified to have a 10-mm diameter hole drilled in the bottom. A glass cover slip was cemented over the hole for use in the ultraviolet range. An area was chosen at random and several cells were scanned for the fluorescence at two wavelengths. After basal calcium concentration was obtained (2–3 scans) AVP was added and the scanning continued. The ratio of F_{405}/F_{485} was calculated for each cell and calcium concentration was derived from a standard curve that was generated using free acid Indo-1, 1 mM EGTA, 115 mM KCl, 20 mM NaCl, 1 mM MgSO₄, 10 mM 3-[N-morpholino] propane sulfonic acid, and varying concentrations of the Orion calcium standard. Total added calcium was converted to free calcium by a double quadratic formula where both Indo-1 and EGTA are interacting (Wade, M. H., and J. F. Holland, manuscript in preparation).

To determine whether the principal or the intercalated (mitochondria-rich) cells were responsible for the observed increase in $[Ca^{+2}]_f$ after AVP, peanut lectin isothiocyanate (FITC-PNA) which binds specifically to the intercalated cells (17) was added to the cell suspension during the last 20 min of incubation with Indo-1/AM. In those experiments the area scanned for Indo-1 fluorescence was also scanned using ACAS 470 interactive laser cytometer for FITC-PNA fluorescence at 488 nm excitation and emission at 515 nm and above.

Inositol phosphates production

Freshly immunodissected RCCT cells were suspended in 5 ml of medium 199 containing 200 mM glutamine, 0.5 mg/ml insulin, 0.34 mg/ml thyroid hormone, and 4 μM dexamethasone, and incubated with 15 μCi of myo[³H]inositol for 5 h at 37°C. Cells were centrifuged, resuspended in 10 ml of Hepes/Li buffer (concentration in millimolar: 4.7 KCl, 0.5 EDTA, 13 glucose, 20 Hepes/acid, 1.2 KH₂PO₄, 1.2 MgSO₄, 58 NaCl, 60 LiCl, 1.5 CaCl₂), and incubated for 15 min in a 37°C water bath. Cells were centrifuged again, resuspended in fresh Hepes/Li medium, and aliquoted (0.4 ml) into assay tubes. AVP (10⁻⁶ M) was added to the experimental tubes and controls received equal volume (0.1 ml) of Hepes/Li buffer. Cells were incubated in a 37°C shaking water bath for a specified time period and reaction was stopped with 0.5 ml of ice-cold 12% TCA. Vials were then frozen at −80°C.

Inositol phosphates were determined using the method described by Berridge et al. (18). Briefly, cells were defrosted, centrifuged for 2 min, and the supernatant separated for measurements of inositol phosphates using AG-1X8 resin columns (formate form, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA). Inositol-1-phosphate (IP₁) was eluted with 200 mM ammonium formate/100 mM formic acid, inositol-1,4-biphosphate (IP₂) with 400 mM ammonium formate/100 mM formic acid, and IP₃ with 1 M ammonium formate/100 mM formic acid. The pellet was resuspended in 0.5 ml solution containing 1 mM KCl, 10 mM myo-inositol, and 0.5 ml chloroform for phosphoinositide (lipid) measurement. The results were calculated as counts per minute of [³H]inositol phosphates/counts per minute ³H-lipids, and were expressed as percent change from appropriate control.

PT treatment

RCCT cells were isolated as described above and suspended in 2 ml of SSS. Half of the cell suspension was incubated in SSS with 10 μg/ml of PT and 0.1% BSA, while the other half was incubated with a vehicle and served as time control. Cells were incubated for 2, 4, or 6 h at 37°C

with continuous shaking. 40 min before the end of incubation 4 μM fura-2/AM was added and the incubation continued. At the end of the incubation period cells were washed in fresh SSS several times and $[Ca^{+2}]_f$ and cAMP production were determined as described above.

Statistical methods

Results are expressed as mean±SE. The n indicates number of independent isolations. Where indicated, the significance of differences between control and effector-treated cells was determined by the use of the analysis of variance.

Results

Effects of AVP and its analogues on $[Ca^{+2}]_f$. In freshly immunodissected RCCT cells basal $[Ca^{+2}]_f$ when corrected for fura-2 leakage was 116±0.2 nM ($n = 5$). The addition of AVP caused a dose-dependent increase in $[Ca^{+2}]_f$ (Fig. 1). In a representative tracing (Fig. 1) 10^{-11} M AVP had no effect on $[Ca^{+2}]_f$, while 10^{-10} M AVP increased $[Ca^{+2}]_f$ from 112 to 145 nM, 10^{-7} M from 126 to 182 nM, and 10^{-5} M from 91 to 183 nM. This effect was rapid (within 10 s) and transient, returning to basal levels within a few minutes (Fig. 2, *top*). The removal of extracellular calcium (3 mM EGTA) reduced the basal $[Ca^{+2}]_f$ from 118 to 52 nM but did not affect AVP-induced increase in $[Ca^{+2}]_f$ from 52 to 87 nM (Fig. 2, *bottom*). The calculated ED_{50} for AVP-induced increase in $[Ca^{+2}]_f$ was ~ 50

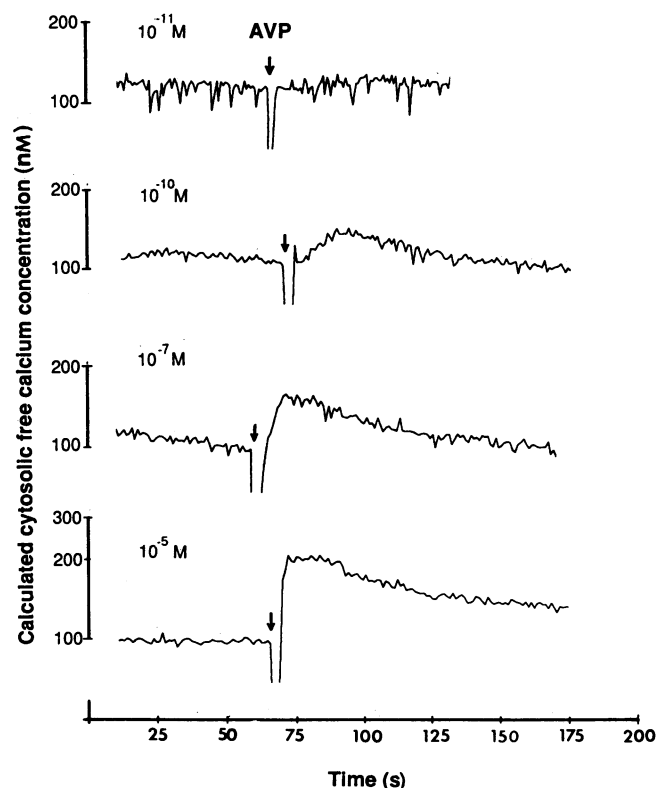*Figure 1.* Representative spectrofluorometric tracings of a dose-dependent increase in $[Ca^{+2}]_f$ in freshly immunodissected RCCT cells induced by AVP (10^{-11}–10^{-5} M) as determined with fura-2. Arrows indicate points of AVP addition. Calculated $[Ca^{+2}]_f$ (log scale) was corrected for fura-2 leakage. Long downward spikes indicate opening of the spectrofluorometer's shutter. Each dose was tested on at least three independent cell isolations. For further details see Methods and Results.
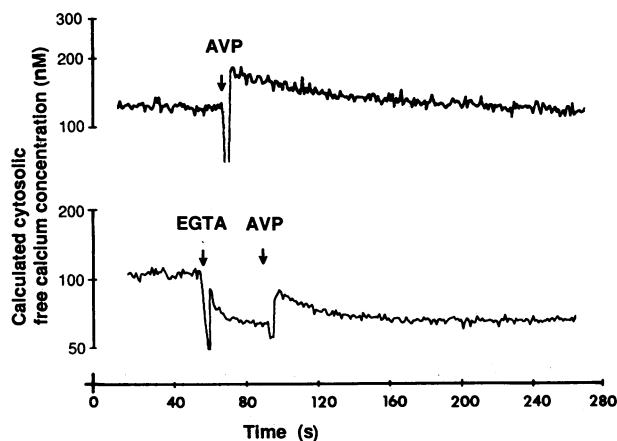
Figure 2. Effects of 10^{-6} M AVP on $[Ca^{+2}]_f$ in freshly immunodissected RCCT cells suspended in a medium containing 1 mM Ca^{+2} (*top*) and after addition of 3 mM EGTA (*bottom*) to remove extracellular Ca^{+2} ($n = 5$).

nM and was considerably higher than the ED_{50} for AVP-induced cAMP production for these cells (0.1 nM) (Fig. 3).

To determine whether the AVP-induced increase in $[Ca^{+2}]_f$ is linked to either the V_1 or V_2 receptor, the effects of a selective V_1 antagonist, $d(CH_2)_5Tyr(Me)AVP$, and a V_2 agonist, dDVP, on cAMP production and $[Ca^{+2}]_f$ were examined. As before, the addition of AVP to cell suspensions increased both cAMP production and the $[Ca^{+2}]_f$ (Fig. 4 and Fig. 5, *top*, respectively). dDVP (10^{-6} M), which increased cAMP production (Fig. 4), did not have any effect on basal $[Ca^{+2}]_f$ or AVP-stimulated $[Ca^{+2}]_f$ (Fig. 5, *middle*), while $d(CH_2)_5Tyr(Me)AVP$ (10^{-5} and 10^{-6} M), which had no effect on cAMP production (Fig. 4) and did not affect basal $[Ca^{+2}]_f$, completely inhibited AVP-induced increase in $[Ca^{+2}]_f$ (Fig. 5, *bottom*). SSS, which served as a vehicle, had no effect on $[Ca^{+2}]_f$ (Fig. 5, *top*).

Effects of AVP on inositol phosphate production. In an attempt to determine if VP stimulated a phospholipase C–mediated hydrolysis of phosphoinositides we examined the effects of AVP on formation of inositol phosphates in RCCT cells that were prelabeled with [³H]inositol. VP stimulated the formation of labeled [³H]IP₃, [³H]IP₂, and [³H]IP₁. As shown in Fig. 6, 10^{-6} M AVP increased IP₃ production within the first 10 s. This increase of 32% from the control level was followed
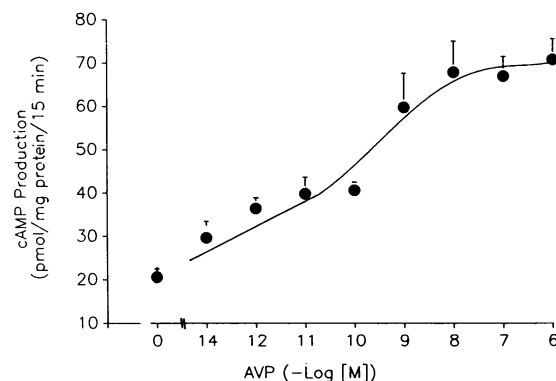
Figure 3. Dose-response effect of AVP on cAMP production in RCCT cells. Each point represents a mean±SE of three experiments.
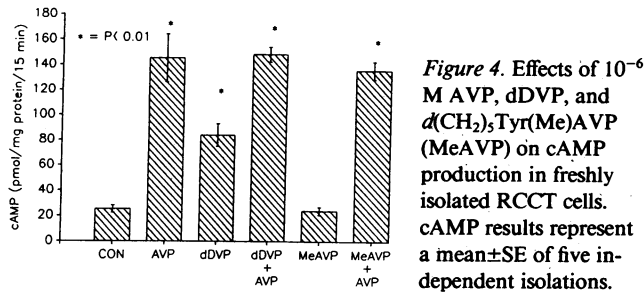
Figure 4. Effects of 10^{-6} M AVP, dDVP, and d(CH$_2$)$_5$Tyr(Me)AVP (MeAVP) on cAMP production in freshly isolated RCCT cells. cAMP results represent a mean±SE of five independent isolations.

by a small decline at 30 s (17% over the control) followed by a continuous increase over the next 5 min (56% increase). A subsequent peak was observed at 15 min (53% increase). The increases in IP$_2$ were similar to those in IP$_3$ production but lower in magnitude (Fig. 6, *middle*). Unlike the rapid increases in IP$_3$ and IP$_2$ observed in the first 10 s after AVP, a small decline in IP$_1$ production was observed in the first 60 s, followed by a gradual increase over the next 20 min (Fig. 6, *bottom*). A similar oscillatory pattern in inositol phosphate production was observed in three additional experiments in which the three metabolites were not separated (data not shown). The increased formation of [^{3}H]IP$_3$, detected as early as 10 s after addition of AVP, suggests that VP did stimulate a phospholipase C–mediated hydrolysis of [^{3}H]PIP$_2$.

Effects of PT on the AVP-stimulated $[Ca^{+2}]_f$. To examine the role of the regulatory G protein in AVP-induced increase in [Ca^{+2}]$_f$, freshly isolated cells were pretreated with PT. Whereas 2-h preincubation had no effect on AVP-induced increase in [Ca^{+2}]$_f$ (results not shown), the effect of AVP was virtually abolished after 4-h (Fig. 7) and 6-h (results not shown) incubation. The pretreatment with PT had no effect on AVP-induced increase in cAMP production. (In time controls 10^{-6} M AVP increased cAMP from 34.5 to 68.7 pmol/mg
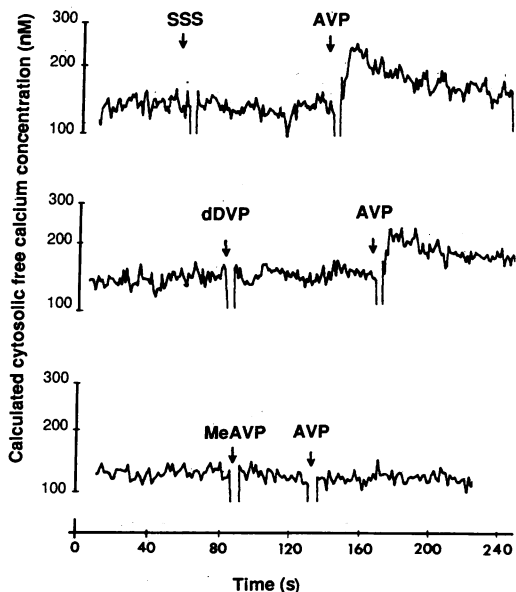

Figure 5. Representative spectrofluorometer tracings demonstrating the effects of 10^{-6} M AVP on [Ca^{+2}]$_f$ after addition of SSS (vehicle, *top*), 10^{-6} M dDVP (*middle*), and 10^{-6} M d(CH$_2$)$_5$Tyr(Me)AVP (MeAVP), *bottom*), in freshly immunodissected RCCT cells. Both analogues were tested on four independent cell isolations.

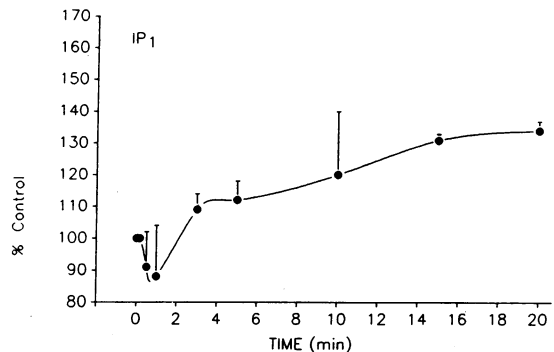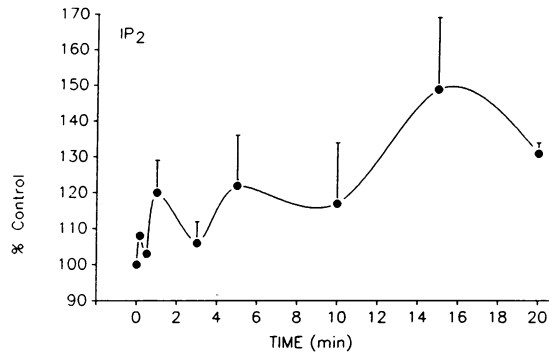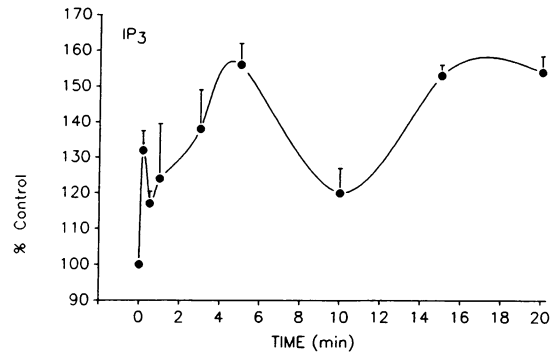Figure 6. Effects of 1 μM AVP on the production of IP$_3$ (*top*), IP$_2$, (*middle*), and IP$_1$ (*bottom*) in RCCT cells. Results are means of two independent experiments performed in duplicate.

protein per 15 min and in PT–treated cells from 40.6 to 77.2 pmol/mg protein per 15 min.)

Measurement of $[Ca^{+2}]_f$ *in response to AVP in individual RCCT cells.* Because of the nonhomogenous nature of the RCCT cell population, we examined changes in [Ca^{+2}]$_f$ in response to AVP at a single-cell level. As shown in several representative experiments in Fig. 8, only some of the cells responded to AVP. Of 57 cells examined, 72% demonstrated an increase in [Ca^{+2}]$_f$ in response to AVP. To determine which cells of the CT were involved in the AVP-induced increase in [Ca^{+2}]$_f$, intercalated cells were stained with FITC-PNA. Our results indicate that cells that responded to AVP did not stain for PNA, suggesting that the principal cells were responsible for the observed effects of AVP on [Ca^{+2}]$_f$. 85% of the PNA negative cells demonstrated an increase in [Ca^{+2}]$_f$, and none of the PNA positive cells responded to AVP.

Discussion

The present study demonstrates that addition of AVP to freshly immunodissected RCCT cells causes a rapid and tran-
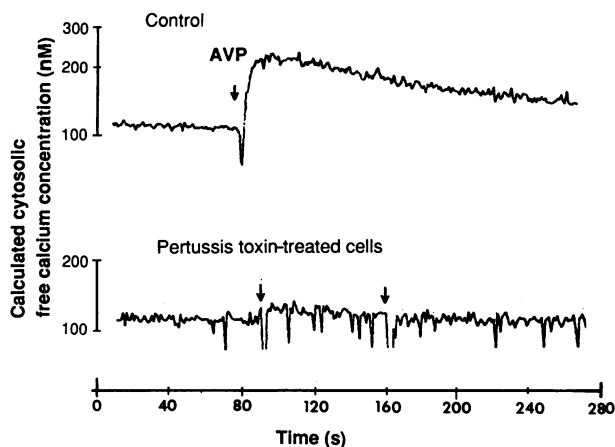
Figure 7. $[Ca^{+2}]_f$ response to AVP (10^{-6} M) in control (*top*) and after pretreatment of freshly isolated RCCT cells with PT (10 μg/ml) for 4 h (*bottom*). Arrows indicate points of AVP addition. Experiments were repeated on cells from at least four separate isolations.
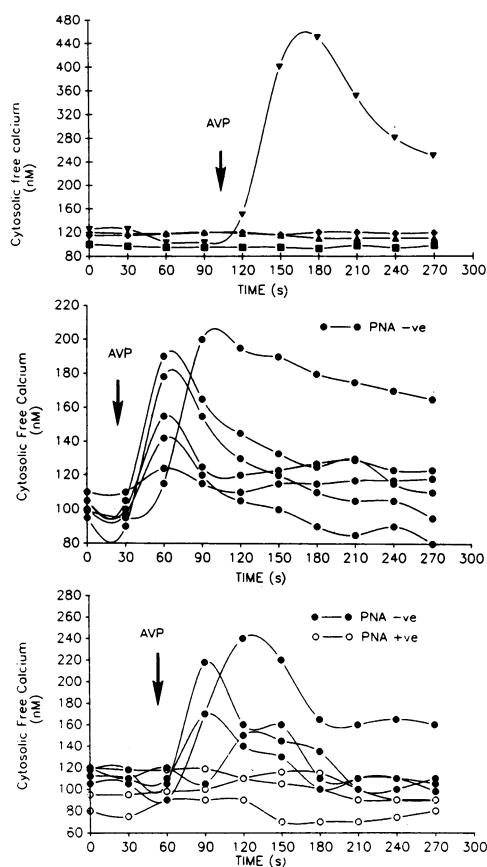

Figure 8. Representative tracings from the experiments examining the effects of 10^{-6} M AVP on $[Ca^{+2}]_f$ at the single-cell level. Cells were loaded with Indo-1/AM and scanned for its fluorescence using an interactive laser cytometer as described in Methods. Each panel represents results from one scanned field and each tracing represents changes in $[Ca^{+2}]_f$ in an individual cell. Despite comparable basal $[Ca^{+2}]_f$ levels, the heterogeneity in the magnitude of the response can be clearly seen among the cells. Cells shown in the middle and the bottom panels were also stained with FITC-PNA to identify the intercalated cells. All cells in the middle panel were PNA negative and all responded to AVP. In the bottom panel three cells stained positive for PNA, but only the PNA-negative cells responded to AVP. Cells shown in the top panel were not stained with FITC-PNA. $[Ca^{+2}]_f$ was examined in at least 57 cells from five independent isolations, and cells from three independent isolations were stained with PNA.

sient increase in the $[Ca^{+2}]_f$. The removal of extracellular Ca^{+2} (EGTA added) had no effect on AVP-induced increase in $[Ca^{+2}]_f$ suggesting that an intracellular source of Ca^{+2} was involved. This effect of AVP appears to be linked to a V_1 receptor as the V_1 antagonist, $d(CH_2)_5Tyr(Me)AVP$, which had no effect on basal $[Ca^{+2}]_f$ or cAMP production, inhibited the AVP-induced increase in $[Ca^{+2}]_f$ but not cAMP production. dDVP, a V_2 agonist that stimulated cAMP production, had no effect on $[Ca^{+2}]_f$. These results strongly suggest the presence of two receptor systems in RCCT cells similar to that we have recently reported for LLC-PK$_1$ cells (10).

Further, measurements of AVP-induced changes in $[Ca^{+2}]_f$ in single cells indicate that the V_1 receptor can be found on the principal cells of the collecting tubule as only cells that did not stain with PNA responded to AVP with an increase in $[Ca^{+2}]_f$. It cannot be ascertained from these studies, however, whether intercalated cells lack a V_1 receptor. Although cells positive for PNA did not respond to AVP, it is possible that PNA itself might have affected the response. Also, other mitochondria-rich cells of the CT that do not stain with PNA were not identified.

Our results also indicate that AVP stimulates rapid IP$_3$ production, suggesting that as in other cells (4, 5) this increase in IP$_3$ may be involved in increasing $[Ca^{+2}]_f$. The significance of the oscillatory nature of the IP$_3$ production is not clear, but is not unique to RCCT cells. A similar pattern has been observed in adrenal glomerulosa cells (19).

It has now been accepted that some calcium-utilizing pathways are mediated by the membrane GTP binding protein regulated phospholipase C activity. In this scheme, the agonist-receptor interaction results in GTP binding to the alpha subunit of a G protein, activating phospholipase C to cleave PIP$_2$ to IP$_3$ and diacylglycerol, and a subsequent increase in $[Ca^{+2}]_f$ and kinase C activity, respectively (20, 21). The support for the involvement of GTP-binding protein comes predominantly from studies using PT, a specific modifier of some G proteins, which, as a result of ADP-ribosylation of the alpha subunit of G protein, inhibits the ability of some Ca^{+2} mobilizing agonists to promote PIP$_2$ hydrolysis (20, 21). Whereas in the liver AVP induced PIP$_2$ breakdown, and IP$_3$

and diglycerol production and subsequent increase in $[Ca^{+2}]_f$ do not appear to involve G protein (4), our results indicate that pretreatment of RCCT cells with PT does abolish AVP-induced increase in $[Ca^{+2}]_f$. The effect of PT appears to be time dependent since at 2 h, a time at which PT had an inhibitory effect in some but not all cell systems (4, 21), it had no effect on RCCT cells. Further, studies by Dr. William K. Sonnenburg and Dr. William L. Smith (manuscript in preparation) showed that in membranes from freshly immunodissected RCCT cells, PT ribosylated a 41-kD protein. In those experiments RCCT cell membranes were incubated with [^{32}P]NAD in the presence and absence of PT (16 μg/100 μg membrane protein) for 2 h at 30°C and analyzed by SDS-PAGE followed by autoradiography. PT catalyzed the ADP-ribosylation of a 41-kD protein. When intact cells were preincubated for 4 h

with the toxin, there was no labeling of protein during the in vitro ADP-ribosylation, consistent with the toxin substrate having already been ADP-ribosylated in the intact cells.

The physiological significance of a dual receptor system using both adenylate cyclase and a PT-sensitive, calcium-mobilizing pathway is not clear. The VP-regulated water and sodium transport have been linked to the stimulation of adenylate-cyclase activity (2, 3, 6), while the AVP-mediated PG synthesis in the glomerular mesangial cells and in interstitial cells has been linked to increased PIP_2 turnover (22) and increased $[Ca^{+2}]_f$ (6–8, 22). Whether the effect of AVP on $[Ca^{+2}]_f$ in RCCT cells is linked to the PG synthesis or to water and sodium transport, or both, remains to be determined.

The significance of the difference in the ED_{50} of AVP at the V_2 and V_1 receptors in the principal cells remains to be determined. It is possible that the heterogeneity in the calcium response observed at the single cell level may be responsible, in part, for the higher ED_{50} at the V_1 receptor. It is also possible that like the action of glucagon in the liver, for example, classically believed to operate through the cAMP system and now shown to involve IP_3-Ca^{+2} system, the stimulation of the lower affinity receptor system regulates the high affinity receptor (23, 24). The presence of V_1 and V_2 receptors on the same cell type further underscores the possibility of such interaction.

The large yield of cells obtained by the immunodissection technique may be further increased by growing cells in culture. This approach, although used successfully, may present some problems with identifying specific physiologic and biochemical functions at the cellular level. Our attempts to reproduce the results in cultured RCCT cells underscore this problem. Whereas the effect of AVP on the $[Ca^{+2}]_f$ was originally observed in cultured RCCT cells, it has not been consistent and at present cannot be demonstrated (results not shown). Whether the response has been lost because of culture conditions, or a preferential growth of intercalated cells, or both remains to be determined.

In summary, these results demonstrate that cells of the cortical CT possess two distinct receptor systems for VP, the well-known V_2 receptor coupled to adenylate cyclase, and a PT-sensitive V_1 receptor system that leads to the mobilization of cytosolic calcium through a G protein coupled to phospholipase C–dependent production of inositol phosphates. The two types of VP receptors of the CT appear to be colocalized on the principal cell.

Acknowledgments

References

1. Grantham, J. J., and M. B. Burg. 1966. Effect of vasopressin and cyclic AMP on permeability of isolated collecting tubule. Am. J. Physiol. 211:255–259.

2. Handler, J. S., and J. Orloff. 1981. Antidiuretic hormone. Annu. Rev. Physiol. 43:611–624.

3. Morel, F. 1981. Sites of hormone action in the kidney. Am. J. Physiol. 240:F159–F164.

4. Williamson, J. R., R. H. Cooper, S. K. Joseph, and A. P. Thomas. 1985. Inositol triphosphate and diacylglycerol as intracellular second messengers in liver. Am. J. Physiol. 248:C203–C216.

5. Nabika, T., P. A. Velletri, W. Lovenberg, and M. A. Beaven. 1985. Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and Arg vasopressin in vascular smooth muscle cells. J. Biol. Chem. 260:4661–4670.

6. Ausiello, D. A., K. L. Skorecki, A. S. Verkman, and J. V. Bonventre. 1987. Vasopressin signalling in kidney cells. Kidney Int. 31:521–528.

7. Bonventre, J. V., K. Skorecki, J. I. Kreisberg, and J. Y. Cheung. 1986. Vasopressin increases cytosolic free calcium concentration in glomerular mesangial cells. Am. J. Physiol. 251:F94–F102.

8. Scharschmidt, L. A., and M. J. Dunn. 1983. Prostaglandin synthesis by rat glomerular mesangial cells in culture. Effects of angiotensin II and arginine vasopressin. J. Clin. Invest. 71:1756–1764.

9. Wuthrich, R. P., and M. B. Vallotton. 1986. Prostaglandin E2 and cyclic AMP response to vasopressin in renal medullary tubular cells. Am. J. Physiol. 251:F499–F505.

10. Burnatowska-Hledin, M., and W. S. Spielman. 1987. Vasopressin increases cytosolic free calcium in LLC-PK_1 cells through a V_1 receptor. Am. J. Physiol. 253:F328–F332.

11. Tang, M. J., and J. M. Weinberg. 1986. Vasopressin induced increases of cytosolic calcium in cultured renal tubular cells (LLC-PK_1). Am. J. Physiol. 251:F1090–F1095.

12. Roy, C., and D. A. Ausiello. 1981. Characterization of (8-lysine) vasopressin binding sites on a pig kidney cell line (LLC-PK_1). J. Biol. Chem. 256:3415–3422.

13. Garcia-Perez, A., and W. L. Smith. 1983. Use of monoclonal antibodies to isolate cortical collecting tubule cells: AVP induces PGE release. Am. J. Physiol. 244:C211–C220.

14. Smith, W. L., and A. Garcia-Perez. 1985. Immunodissection: use of monoclonal antibodies to isolate specific types of renal cells. Am. J. Physiol. 248:F1-F7.

15. Spielman, W. S., W. K. Sonnenburg, M. Allen, K. Gerozissis, and W. L. Smith. 1986. Immunodissection and culture of rabbit cortical collecting tubule cells. Am. J. Physiol. 251:F348–F357.

16. Grynkiewicz, G., M. Poenie, and R. Tsien. 1985. A new generation of calcium indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440–3450.

17. Kriz, W., and B. Kaissling. 1985. Structural organization of the mammalian kidney. In The Kidney. D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 265–306.

18. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslpo, and R. F. Irvine. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. 212:473–482.

19. Ball, T., A. J. Baukal, G. Guillemette, and K. J. Catt. 1988. Multiple pathways of inositol polyphosphate metabolism in angiotensin-stimulated adrenal glomerulosa cells. J. Biol. Chem. 263:4083–4091.

20. Berridge, M. J. 1985. The molecular basis of communication within the cell. Sci. Am. 253:142–152.

21. Nakamura, T., and M. Ui. 1985. Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release and histamine secretion in mast cells by islet activating protein, pertussis toxin. J. Biol. Chem. 260:3584–3593.

22. Troyer, D. A., J. I. Kreisberg, D. W. Schwertz, and M. A. Venkatachalam. 1985. Effects of vasopressin on phosphoinositides and prostaglandin production in cultured mesangial cells. Am. J. Physiol. 249:F139–F147.

23. Petersen, O. H., and C. Bear. 1986. Two glucagon transducing systems. Nature (Lond.). 323:18.

24. Wakelam, M. J., G. J. Murphy, V. J. Hruby, and M. D. Houslay. 1986. Activation of two signal-transduction systems in hepatocytes by glucagon. Nature (Lond.). 323:68–71.