Vasopressin Resistance in Chronic Renal Failure

Evidence for the Role of Decreased V_2 Receptor mRNA

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Abstract

Studies were performed to determine the mechanism underlying deficient arginine vasopressin (AVP)-stimulated adenylyl cyclase activity in chronic renal failure (CRF). As compared to control, principal cells cultured from the inner medullary collecting tubule of rats previously made uremic by 5/6 nephrectomy fail to accumulate cAMP when stimulated with AVP. CRF cells do respond normally to forskolin or cholera toxin and the defect in AVP responsiveness is not prevented by treatment with pertussis toxin, by cyclooxygenase inhibition, or by inhibition or down-regulation of protein kinase C. In contrast to their lack of responsiveness to AVP, CRF cells respond normally to other agonists of adenylyl cyclase such as isoproterenol or prostaglandin E_2. Plasma membranes prepared from the inner medullae of CRF rats exhibit a marked decrease in apparent AVP receptor number but no change in the apparent number of beta adrenergic receptors. Reverse transcriptase PCR of total RNA from the inner medullae of CRF animals reveals virtual absence of V_2 receptor mRNA; mRNA for α_s is present in normal abundance. These studies indicate that AVP resistance in CRF is due, at least in part, to selective down-regulation of the V_2 receptor as a consequence of decreased V_2 receptor mRNA. (*J. Clin. Invest.* 1995. 96:378–385.) Key words: down-regulation (physiology) • adenyl cyclase • urine concentrating ability • kidney tubules, collecting • vasopressin receptor

Introduction

One of the earliest clinical manifestations of chronic renal failure (CRF)[1] is a defect in urinary concentrating ability (1, 2). The pathogenesis of this defect is not well understood. The urinary concentrating defect in uremia is not due to deficient arginine vasopressin (AVP) secretion from the pituitary gland as both the neurophysin for AVP (3) and AVP itself (4) are found in increased quantity in the sera of patients with CRF. Furthermore, Tannen et al. have demonstrated persistent excretion of hypotonic urine by patients with CRF despite the administration of exogenous AVP (5) suggesting that the concentrating defect is at least in part due to impaired AVP responsiveness of the collecting duct. This has in fact been corroborated in vitro. Fine et al. examined the ability of AVP to increase the hydraulic conductivity of isolated perfused cortical collecting tubules (CCT) dissected from the remnant kidney of uremic rabbits. As compared to CCTs from normal rabbits, CCTs obtained from uremic animals exhibited markedly decreased responsiveness to either AVP or exogenous 8-bromo-cAMP (6). Similarly, CCTs from uremic rabbits exhibited impaired AVP-stimulated adenylyl cyclase (AC) activity. It is clear, therefore, that uremia causes AVP resistance at both pre and post cAMP sites. The studies described herein were aimed at elucidating the nature of the biochemical defect responsible for the impairment in AVP-stimulated cAMP generation in CRF.

Methods

Model of chronic renal failure. The model of CRF employed was that of the 5/6 nephrectomized rat. As previously reported (7, 8) male Sprague-Dawley rats were anesthetized with pentobarbital and subjected to right nephrectomy and ligation of the arterial supply to $\sim$ $^2/_3$ of the left kidney. Rats were allowed ad lib. access to standard rat chow and water until their death $\sim$ 3–6 wk later. Only animals with serum creatinine $\geq$ 1.0 mg/dl (normal $\leq$ 0.4) were studied. Of these, randomly selected animals were subjected to 24 h of dehydration. The mean maximal urinary concentration in these CRF animals (n = 10) was 904±52 mOsm/kg H_2O. Unoperated male Sprague-Dawley rats of comparable age to the CRF rats (at the time of death) were used as controls; maximal urinary concentration in these animals routinely measured $\geq$ 2,500 mOsm/kg H_2O.

Inner medullary collecting tubule cell culture. Inner medullary collecting tubule (IMCT) cells from control and CRF animals were cultured as previously described (9, 10). Male Sprague-Dawley rats are exsanguinated, their kidneys immediately removed using sterile technique and placed in ice-cold KRB (composition [mM]: NaCl 128, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1.2, $NaCH_3COO$ 10, NaH_2PO_4 2, glucose 10, Tris 10), pH 7.4, containing 100 U/ml penicillin and 100 μg/ml streptomycin base. The kidneys are then bisected and the inner medullae removed; infarcted tissue from CRF rats was discarded. Inner medullae from 4 to 12 rats are pooled, finely minced with a sterile razor blade, and incubated in 5 ml of 0.05–0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ) in KRB for 60–90 min at 37°C under a 5% CO_2 atmosphere. The tissue is gently agitated at 15–20-min intervals. After incubation, 2 vol sterile distilled water is added to lyse cells other than collecting tubule cells. The tissue is pelleted by centrifugation for 3 min in a tabletop clinical centrifuge, resuspended in 10% BSA (fraction V; Miles Laboratories Inc., Elkhart, IN), and centrifuged again. The remaining cell pellet is resuspended in 90% Ham F-12 10% Liebovitz L-15 medium

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1. *Abbreviations used in this paper:* AC, adenylyl cyclase; AVP, arginine vasopressin; CCT, cortical medullary collecting tubule; CRF, chronic renal failure; CT, cholera toxin; IMCD, inner medullary collecting duct; IMCT, inner medullary collecting tubule; PKC, protein kinase C; PM, plasma membrane; PT, pertussis toxin; RT, reverse transcription; V_2R, V_2 receptor.

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containing 10% FBS, 1% antibiotic, and hormonal supplements. Tissue is seeded into 24-well (16 mm diameter) replicate culture dishes (Falcon Labware; Becton Dickinson & Co., Lincoln Park, NJ) and then kept under a 5% CO_2 atmosphere at 37°C. After 24 h the cells are fed with the same medium that contains, however, only 1% FBS.

Studies on cAMP accumulation. Studies on cAMP accumulation were performed 72–96 h after initial culture while the cells were subconfluent. As previously reported from this laboratory (9, 10) these studies were performed in KRB at 300 mOsm/kg H_2O, pH 7.4, containing 0.5 mM isobutyl methylxanthine (IBMX) to inhibit phosphodiesterase. Media is aspirated from cells and replaced with 0.5 ml buffer, containing the desired effector substance(s). Incubations are done in an Isotemp dry bath at 37°C (Fisher Scientific Co., Pittsburgh, PA). As intracellular cAMP has been demonstrated to peak after a 5–10-min exposure to AVP, all incubations for cAMP measurements are performed for 7.2 min. At the end of this time the incubation is terminated by aspiration of the effector and the immediate addition of 0.3 ml of 0.01 N HCl to lyse the cells and liberate cellular cAMP. After 20 min, a 100-μl aliquot of acid is taken and immediately frozen for subsequent determination of cAMP content. cAMP is determined by radioimmunoassay (New England Nuclear, Boston, MA) with standards similarly dissolved in 0.01 N HCl. As treatment of the cells with HCl causes membrane fragmentation and loss of protein into the acid, a second 100-μl aliquot of the acid from each sample is taken for protein determination. The residual acid is aspirated off and the remaining tissue solubilized in 1% SDS. A 100-μl aliquot of the SDS is added to the second 100-μl aliquot of HCl and the combined protein content is determined by the method of Lowry (11). Studies on CRF and control tissues were performed in parallel after the same duration of culture using identical reagents and assay conditions. Values obtained in triplicate wells were meaned for an "*n*" of one. Results are expressed as femtomoles cAMP per microgram protein.

Scanning electron microscopy. Monolayers of control and CRF cells grown on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 M Na phosphate, pH 7.2 for 30 min at room temperature. They were then rinsed with 0.1 M Na phosphate, pH 7.2 and postfixed with 1% osmium tetroxide in 0.1 M Na phosphate, pH 7.2. The cells were then rinsed in water and dehydrated in ethanol (25–95%). They were then subjected to critical point drying in a critical point drier (Samdri: PVPs; Tousimis Research Corp., Rockville, MD), sputtercoated with chromium using an evaporator (502-A; Denton Vacuum Inc., Cherry Hill, NJ), and scanned with a scanning electron microscope (250 MK3; Cambridge Research & Instruments, Cambridge, MA).

Immunoblotting. Monolayers of control and CRF cells were lysed on ice in a buffer composed of 50 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 mM EDTA, 0.2 mM Na_3VO_4, 1 mM PMSF, 1.5 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton. The lysates were clarified by centrifugation and frozen at −70°C overnight. Each lysate (87 μg) was subjected to SDS-PAGE according to the method of Laemmli (12) and then transferred to nitrocellulose. The samples were then immunoblotted with a polyclonal rabbit antibody directed against mouse erythrocyte carbonic anhydrase II (CA-2; a generous gift from Dr. Paul Linser, Univerity of Florida, Gainesville, FL) (13) which has previously been shown to recognize CA-2 in intercalated cells of the rat inner medullary collecting duct (IMCD) (14). The signal was then detected with an alkaline phosphatase–conjugated goat anti–rabbit antibody.

Preparation of inner medullary plasma membranes. Inner medullary plasma membranes (PMs) from control and CRF rats were prepared from freshly isolated inner medullary collecting tubules according to the method of Stokes et al. (15). Inner medullae of 6–12 rats are minced finely and incubated at 37°C in 10 ml of a buffer consisting of (mM) 130 NaCl, 25 Hepes, 14 glucose, 3.2 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 1.8 KH_2PO_4, pH 7.4, containing 0.2% collagenase and 0.2% hyaluronidase. After 45 min, 0.001% DNase is added and incubation continues for another 30–45 min; during this time the cells are aspirated through a Pasteur pipette 10–12 times every 15 min. The resulting suspension is spun at 28 *g* for 2 min. The supernate is discarded, the pellet resuspended in the same buffer, and the procedure repeated twice. The pellet is then resuspended in a tissue buffer consisting of 20 mM Hepes, 25 mM sucrose, 0.1 mM EGTA, 0.05% BSA, pH 7.4, containing protease inhibitors (1 mM PMSF, 5 μg/ml leupeptin and 2 μg/ml pepstatin A). The cells are gently homogenized with 20–30 strokes of a loosely-fitting Dounce, spun at 800 *g* for 20 min, resuspended and spun twice more, then aliquoted in the same buffer and stored at −70°C until use.

Receptor binding assays. Maximum specific AVP binding was estimated in a buffer consisting of 50 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA, and 0.2% BSA. Assays contained 10 μl [^{3}H]AVP (sp act = 74.3 Ci/mmol; New England Nuclear) and 10 concentrations of cold AVP ranging from 1–40 nM. Binding in the presence of 10 μM cold AVP was determined to be nonspecific. In preliminary studies we determined that binding was time-dependent, saturable, and dissociable. Each assay tube contained 50 μg inner medullary PMs in a final volume of 100 μl; assays were performed in triplicate. The assays were incubated at 30°C for 30 min. Incubations were terminated by adding 0.5 ml ice-cold 2.5% BSA in 100 mM Tris followed by rapid vacuum filtration through glass fiber filters (GF/F; Whatman Laboratory Products Inc., Clifton, NJ). The filters were washed with 10% sucrose in 100 mM Tris at room temperature. Radioactivity trapped on the filters was quantitated using liquid scintillation counting.

Maximum specific beta adrenergic receptor binding was estimated in a buffer consisting of 20 mM Hepes, 1 mM $MgCl_2$, and 154 mM NaCl (buffer A) as described by Zahniser et al (16). Assays contained 50 μl ^{125}I-cyanopindolol (sp act = 2,200 Ci/mmol; New England Nuclear) and 11 concentrations of cold isoproterenol ranging from 0.5–100 nM. Binding in the presence of 1 μM cold 1-propranolol was determined to be nonspecific. Each assay tube contained 50 μg inner medullary PMs in a final volume of 250 μl; assays were performed in triplicate. The assays were incubated at 37°C for 36 min. Incubations were terminated by adding 3 ml ice-cold buffer A followed by rapid vacuum filtration through glass fiber filters (Whatman Laboratory Products Inc.). The filters were washed with 10 ml buffer A at room temperature. Radioactivity trapped on the filters was quantitated using gamma counting. For both AVP and cyanopindolol binding was saturable, dissociable, and only one class of receptor was identified by Scatchard transformation (17) of the binding data. Maximum uptake of the ligand is expressed as pmol/mg protein.

Relative quantitation of mRNA levels. Inner medullae of individual control and CRF animals were homogenized in liquid nitrogen and total RNA extraction was carried out by the method of Chomczynski and Sacchi (18). Total RNA was measured by spectrophotometry (OD_{260}). 1.0 μg total RNA was dissolved in 4 μl pyrocarbonicaciddiethyl ester (DEPC)-treated water and 16.0 μl of a reverse transcriptase (RT) master mix was added to each sample. The master mix contained 0.01 M dithiothreitol (Sigma Chemical Co., St. Louis, MO), 1 × PCR buffer (Perkin-Elmer Cetus Corp., Norwalk, CT), 1.0 mM deoxynucleotides (Perkin-Elmer Cetus Corp.), 5.0 mM $MgCl_2$, 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus Corp.) and 0.5 μM antisense oligonucleotide for either V_2 or α_s (Macromolecular Resources, Fort Collins, CO). The template in these reactions was always from the same RNA preparation. Reactions were incubated at 42°C in a thermal cycler (Perkin-Elmer Cetus Corp.) for 90 min.

After reverse transcription and heat inactivation of the reverse transcriptase, 8.0 μl of the RT product was combined with 82.0 μl of a PCR master mix. The master mix contained 1 × PCR buffer, 0.2 mM deoxynucleotides, 1.5 mM $MgCl_2$ and sense and antisense primers for either V_2 or α_s in a total volume of 100 μl. The mixture was overlaid with 100 μl of mineral oil to prevent evaporation during the high temperature incubation. In an effort to minimize formation of primer dimers we performed a "hot start" at 94°C for 10 min before the addition of 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer Cetus Corp.) in 10 μl 1 × PCR buffer. The PCR cycle consisted of 2 min denaturing at 94°C, 2 min primer annealing at 58°C, and 2 min primer extension at 72°C.

40 cycles were performed followed by a 7-min final extension at 72°C. The primers used were: V_2 antisense, 5′-TGAGGCATCTGT-

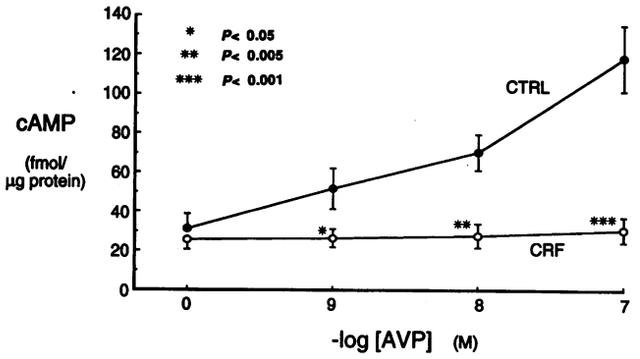

Figure 1. Dose-response to AVP in control (*CTRL*) and CRF IMCT cells ($n = 6$).

CCCAGTTGCTTCC-3′; V_2 sense, 5′-ATCCGGAAGCTCCTCTGG-AAAGACC-3′ (19); G protein α_s subunit antisense, 5′-CCAGCA-AGCTTIGTRTCIRYIGCRCAIGT-3′; and α_s sense, 5′-CCAGCG-GTACCGAYGTIGGIGSICARBG-3′ (20). In these primers, R = A or G, Y = C or T, S = G or C, and B = A or C. 10-μl aliquots were removed after 25, 30, 35, and 40 cycles. Samples were stored at 4°C until the time of analysis.

Equal volumes (8 μl) of PCR product were electrophoresed in 1.0% agarose (Seakem LE; FMC Bioproducts, Rockland, ME) at 72 V for 2–3 h using a horizontal gel apparatus (Owl Scientific Plastics, Inc., Mechanicsburg, PA). Gels were stained for 20 min in 0.5 μg ethidium bromide/ml and then destained in deionized water. The bands were visualized using a UV Transilluminator (UVP, Inc., San Gabriel, CA) and the gel was then photographed. Negative controls containing 8 μl of DEPC-treated water, 82 μl of master mix, and 10 μl of 0.1 × Taq polymerase yielded no observable product after 40 cycles.

Statistical analysis. Comparisons between groups were by the unpaired t test. In all circumstances $P < 0.05$ was considered significant. Data are presented as mean±SEM.

Results

Response to AVP. Basal cAMP levels were the same in cells cultured from control and CRF animals in this (31.0±7.4 vs 25.4±4.9 fmol/μg protein; $n = 6$, NS) and all other experiments in this study. The response to AVP, however, was markedly different. As seen in Fig. 1, control cells exhibit a dose-depen-

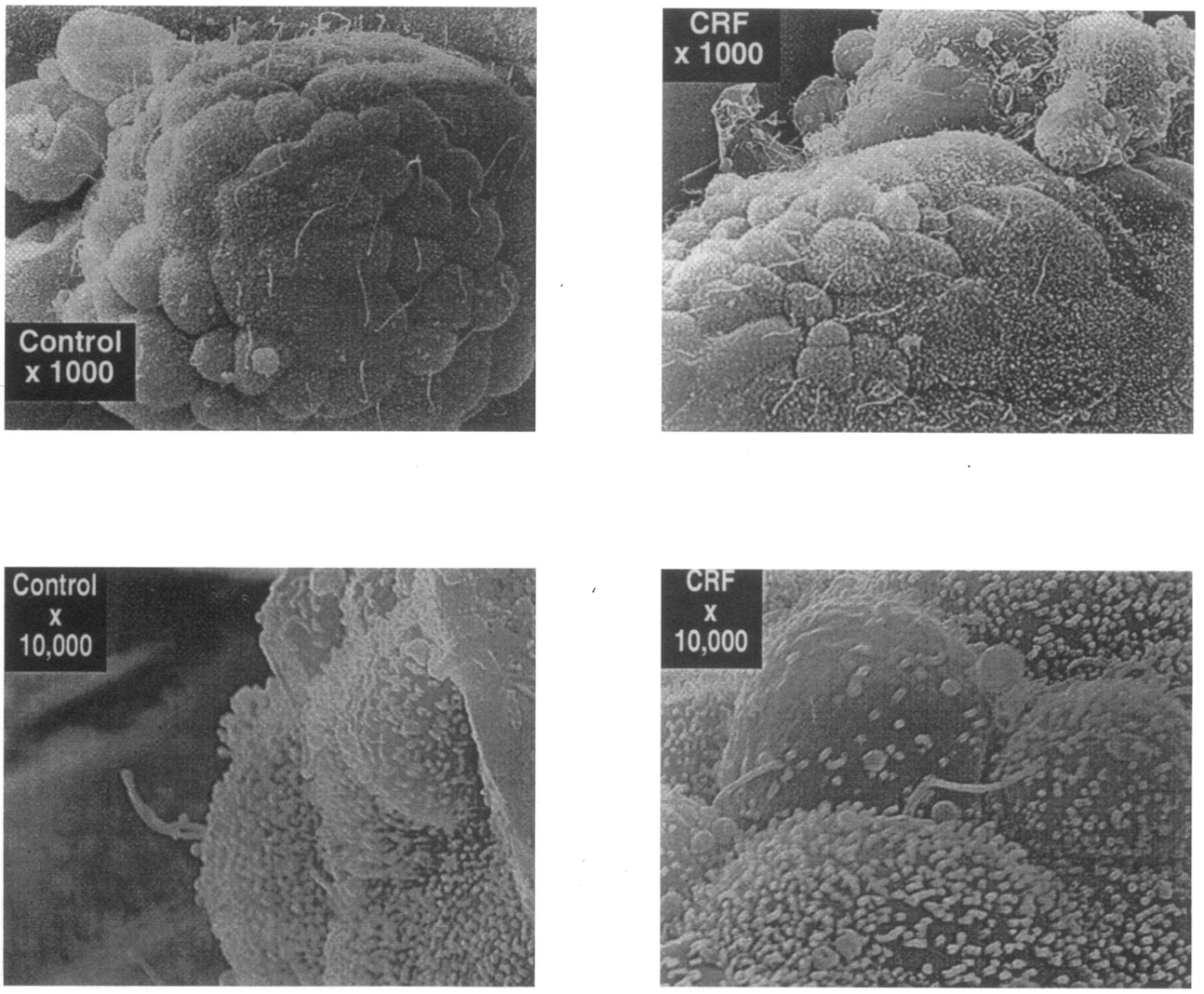

Figure 2. Scanning electron micrographs of control and CRF IMCT cells.

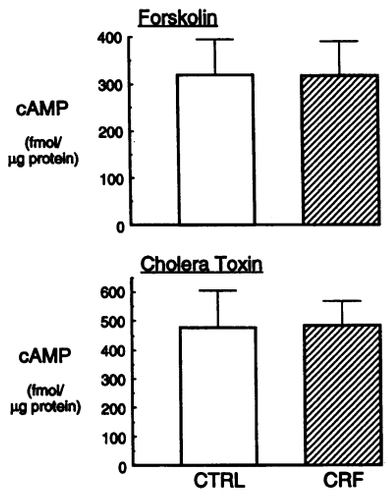

Figure 3. Responses to forskolin (*top;* 10 μM, n = 4) and cholera toxin (*bottom;* 100 ng/ml × 90 min, n = 3) in control and CRF IMCT cells.

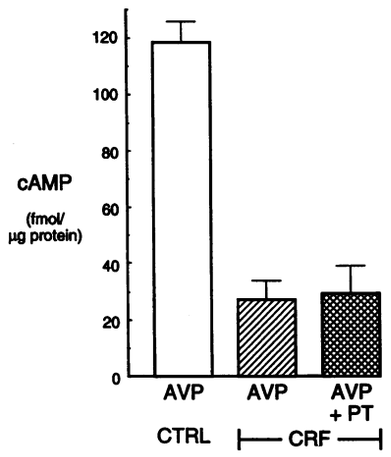

Figure 4. Effect of pertussis toxin (PT; 10 ng/ml × 16 h) on AVP responsiveness in CRF IMCT cells (n = 5).

dent response to AVP with cAMP accumulation increasing to 118.5±16.8 fmol/μg protein at 10^{-7} M AVP. In contrast, CRF cells exhibit no response to AVP as cAMP accumulation remains at 30.6±6.4 fmol/μg protein at 10^{-7} M AVP (n = 6; P < .005 vs control).

Histological and immunochemical characterization. The IMCD is composed of two cell types, principal cells and intercalated cells. Only the principal cells produce cAMP in response to AVP. Therefore, the failure of CRF cells to respond to AVP could be due to selection of intercalated rather than principal cells in the culture process. To eliminate this possibility we examined the control and CRF cells by scanning electron microscopy. As seen in Fig. 2 the two cultures are identical in appearance demonstrating ciliated cells with apical microvilli characteristic of principal cells from the early portion of the IMCD (21). It should be noted that this appearance is similar to that previously reported in cultured rat IMCT cells by Yagil et al. (22).

To further characterize these cells as principal rather than intercalated cells we immunoblotted for the presence of carbonic anhydrase type II (CA-2) which is expressed by intercalated cells but not principal cells (21). A 29.5-kD band corresponding to CA-2 was detected in the M1 cell line, a heterogeneous population of cortical collecting duct cells containing 10–20% intercalated cells, but was not seen in either control or CRF IMCT cells (data not shown). Several other bands were detected in IMCT cells, none of which was more prominent in CRF cells as compared to control. These findings indicate that the failure of CRF cultures to respond to AVP is not due to selective enrichment of CRF cultures with intercalated cells.

Role of the catalytic unit (C). To determine whether the decrease in AVP-stimulated cAMP accumulation in CRF cells is due to impaired function of C, we examined the response to forskolin a plant product that directly stimulates C (23). As seen in the top of Fig. 3, forskolin-stimulated cAMP accumulation is identical in control and CRF cells (319.5±76.2 vs 318.7±71.7 fmol/μg protein; n = 4, NS) indicating that catalytic unit function in CRF cells is intact.

Role of the stimulatory guanine nucleotide-binding protein, G_s. To determine whether the decrease in AVP-stimulated cAMP accumulation in CRF cells is due to deficient G_s activity we examined the response to cholera toxin (CT). CT catalyzes ADP ribosylation of an arginine residue in the region of the α chain of G_s responsible for its GTPase activity. ADP ribosylation of this residue markedly reduces GTPase activity, leaving the G protein in its GTP-bound or activated state which then stimulates C (24). Therefore, decreased responsiveness to CT would suggest either a quantitative deficiency of or a functional defect in G_s. However, as seen in the bottom of Fig. 3, CT-stimulated cAMP accumulation is identical in control and CRF cells (479.0±126.8 vs 486.7±81.6 fmol/μg protein; n = 3, NS) indicating that G_s function in CRF cells is intact.

Role of the inhibitory guanine nucleotide-binding protein, G_i. To determine whether the decrease in AVP-stimulated cAMP accumulation in CRF cells is due to enhanced activity of G_i we examined the response to pertussis toxin (PT). PT catalyzes ADP-ribosylation of a cystine residue in the α chain of G_i; ADP-ribosylation prevents inhibitory input to C (24). Therefore, if pretreatment of CRF-cells with PT were to result in restoration of the response to AVP a G_i-mediated mechanism would be invoked. However, as seen in Fig. 4, pretreatment with PT in a fashion that does reverse G_i-mediated effects in cultured rat IMCT cells (10) did not increase the response to AVP in CRF cells (29.4±9.7 vs 27.3±6.7 fmol/μg protein; n = 5, NS).

Role of prostaglandins. Studies by several investigators have suggested that PGs, particularly PGE_2, may inhibit the hydroosmotic response to AVP (25, 26). It is well recognized that subjects with renal failure often depend on increased levels of vasodilatory PGs (e.g., PGE_2) to maintain glomerular filtration rate (27). Though studies on the cultured normal rat IMCD do not support inhibition of AVP-stimulated cAMP generation by PGE_2 (28), we considered the possibility that excess PG production may be responsible for the lack of AVP-responsiveness in CRF. Therefore, we examined the response of CRF cells to AVP in the background of cyclooxygenase inhibition with 5 μM flurbiprofen which we have previously shown to decrease ionophore-stimulated PGE_2 production by > 97% (29). However, as seen in Fig. 5, cyclooxygenase inhibition did not increase the response to AVP in CRF cells (29.0±10.5 vs 34.0±9.9 fmol/μg protein; n = 3, NS).

Specificity of AVP defect. In addition to responding to AVP, rat IMCT cells also produce cAMP in response to PGE_2 (28) or isoproterenol (10). To determine whether deficient cAMP accumulation in CRF is specific to the AVP receptor or is common to all cyclase-linked receptors we examined the response of CRF IMCT cells to these agonists as well. As seen

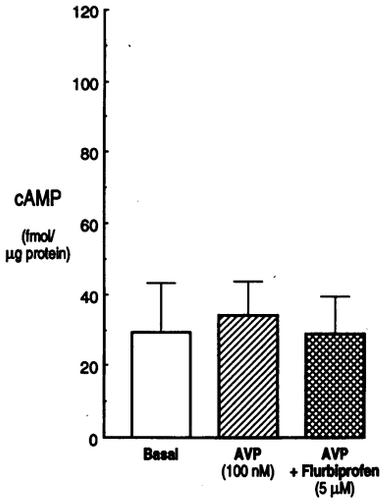

Figure 5. Effect of cyclooxygenase inhibition (5 μM flurbiprofen × 15 min) on AVP responsiveness in CRF IMCT cells (n = 3).

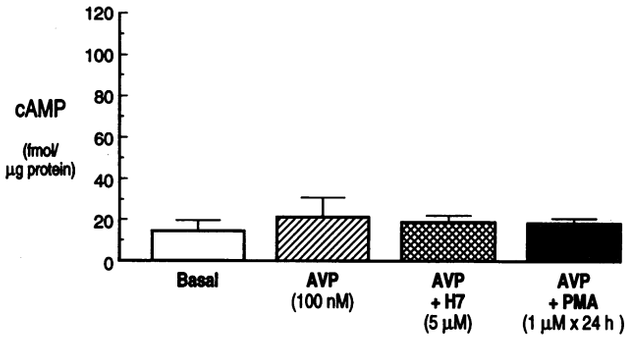

Figure 7. Effects of acute inhibition (50 μM H7 × 15 min) or chronic down-regulation (1 μM PMA × 24 h) of PKC on AVP responsiveness in CRF IMCT cells (n = 3).

in Fig. 6, CRF cells respond normally to both PGE_2 (181.0±58.6 vs 167.4±45.6 fmol/μg protein; n = 4, NS) and isoproterenol (97.8±22.7 vs 101.7±19.1 fmol/μg protein; n = 4, NS). Therefore, the defect in CRF IMCT cells is unique to AVP.

Role of protein kinase C. The remnant kidney of animals subjected to 5/6 nephrectomy undergoes compensatory renal hypertrophy which has been shown to be associated with an increase in protein kinase C (PKC) activity (30). We have previously demonstrated that activation of PKC inhibits AVP-stimulated cAMP accumulation in rat IMCT cells without affecting the responses to PGE_2 or isoproterenol (31). We, therefore, sought to determine whether impaired AVP-stimulated cAMP accumulation in CRF is due to enhanced PKC activity. We examined the response of CRF cells to AVP when PKC was inhibited acutely by a 5-min preincubation with H7 [1-(5-isoquinolinysulfony)-2-methylpiparazine; Calbiochem-Behring Corp., Sun Valley, CA] (29, 32) and when it was chronically down-regulated by overnight treatment with PMA (33, 34). As seen in Fig. 7, neither acute inhibition of PKC with H7 (18.5±3.5 fmol/μg protein; n = 3, NS) nor its chronic down-regulation with PMA (18.3±2.4 fmol/μg protein; n = 3, NS) enhanced the response to AVP in CRF cells (21.2±9.4 fmol/μg protein) indicating that AVP resistance in these cells is not due to enhanced activation of PKC. Furthermore, since the ability of PKC to inhibit AVP-stimulated cAMP accumulation in rat IMCT cells is itself mediated by G_i (31) the failure of PT to enhance AVP responsiveness in CRF cells (see above; Fig. 4) also argues against a role for PKC in the resistance to AVP.

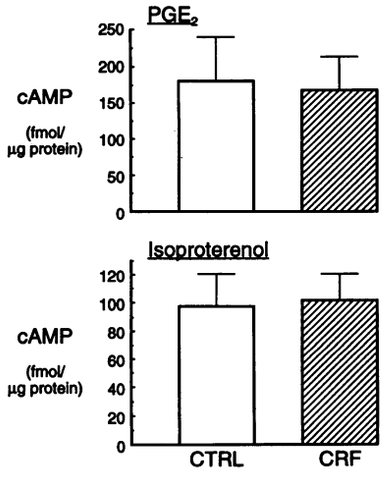

Figure 6. Responses to PGE_2 (*top;* 10 μM, n = 4) and isoproterenol (*bottom;* 10 μM, n = 4) in CTRL and CRF IMCT cells.

Receptor binding studies. The observation that CRF cells display an impaired response to AVP with normal responsiveness to isoproterenol or PGE_2 (see above; Fig. 6) suggests the possibility of selective down-regulation of the AVP receptor in CRF cells. Therefore, we estimated binding characteristics of the AVP and beta adrenergic receptors in CRF tissues. We have been unable to prepare plasma membranes from cultured rat IMCT cells without destroying the AVP receptor in the process; to our knowledge no other investigators have had success in this endeavor either. Therefore, these studies were performed in plasma membranes prepared from freshly isolated IMCT as described previously (31). We performed preliminary studies aimed at demonstrating that the PMs from CRF animals exhibit the same pattern of AC activation as do the cultured CRF cells. As seen in Table I, inner medullary plasma membranes from CRF rats exhibit a modest and statistically nonsignificant decrease in basal AC activity. Forskolin elicits a 12-fold response in control tissues and 14-fold in CRF tissues; in contrast, the response to AVP is 11-fold in control membranes but only 4-fold in CRF tissues. Thus, inner medullary plasma membranes from CRF rats exhibit an impaired response to AVP but respond normally to forskolin. [^{3}H]AVP binding was examined in these same inner medullary plasma membrane preparations.

As seen in Table II, inner medullary PMs from CRF animals exhibit no significant change in binding affinity for [^{3}H]AVP (19.5±4.0 vs 10.0±1.0 nM; n = 3, NS); this modest decrease

Table I. Adenylyl Cyclase Activity in Inner Medullary Plasma Membranes from Control and CRF Rats

	Basal	AVP	Forskolin
Control	17.6±4.2	188.7±19.2	219.7±20.5
CRF	11.7±7.2	46.8±12.2	166.0±14.2
P	NS	< 0.005	NS

pmol/mg protein per 10 min; n = 3.

Table II. Binding Characteristics of Inner Medullary PMs from Control and CRF Animals

	[^{3}H]AVP		^{125}I-cyanopindolol	
	Control	*CRF*	*Control*	*CRF*
Kd (nM)	19.5±4.0	10.0±1.0*	10.4±3.5	10.0±1.6*
Maximum uptake (pmol/mg protein)	4.70±0.62	1.36±0.66‡	1.06±0.35	0.89±0.13*

* NS; ‡ $P < 0.025$; $n = 3$.

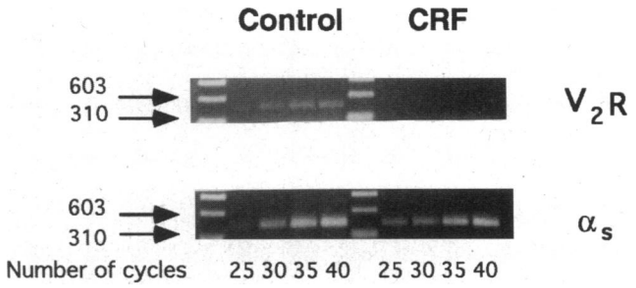

Figure 8. RT-PCR products (25–40 cycles) for V_2R (*top*) and α_s (*bottom*) from total RNA of control and CRF inner medullae. This result is representative of two experiments.

in Kd would, if anything, be expected to augment the response to AVP. Inner medullary PMs from CRF animals do, however, exhibit a marked decrease in maximum AVP uptake (4.70±0.62 vs 1.36±0.66 pmol/mg protein; $n = 3$, $P < 0.025$). In contrast to the impaired [^{3}H]AVP binding, CRF cells exhibit normal ^{125}I-cyanopindolol binding characteristics with no change in either affinity (10.4±3.5 vs 10.0±1.6 nM; $n = 3$, NS) or maximum uptake (1.06±0.35 vs 0.89±0.13 pmol/mg protein; $n = 3$, NS). Therefore, the decrease in maximum uptake for the AVP receptor is not simply a consequence of cellular hypertrophy in the presence of CRF. Thus, CRF is associated with selective down-regulation of the AVP receptor in the IMCD.

Relative quantitation of V_2 receptor (V_2R) mRNA. Having demonstrated a marked decrease in apparent AVP receptor number in CRF tissues we next examined the relative levels of V_2 receptor mRNA in inner medullae from control and CRF animals using RT-PCR. As an internal standard we measured the levels of α_s mRNA in the same samples. The use of an endogenous internal standard controls for possible degradation in the RNA preparation. We chose this particlar mRNA as our control for two reasons: (*a*) the previous finding that CT-stimulated cAMP accumulation is the same in control and CRF cells (Fig. 3, *bottom*) suggests that the α_s protein, and presumably its message, are present in equal amounts in the two tissues, and (*b*) the cDNA for α_s yields an amplification product similar in length to the V_2R amplification product so that differences in efficiency of the PCR are minimized. Fig. 8 shows the PCR products for V_2R and α_s from the inner medullae of control and CRF rats after 25, 30, 35, and 40 cycles. In control animals V_2R message of the expected size (449 bases; the identity of the PCR product has subsequently been verified by sequence analysis) is detectable after only 25 cycles of PCR. There is no detectable V_2R signal in the CRF samples even after 40 cycles. In contrast, using the same RNA preparations, mRNA for α_s was amplified to a similar degree from control and CRF tissues. This suggests that the down-regulation of V_2R observed in CRF takes place at the level of transcription.

Discussion

In cortical collecting ducts microdissected from uremic rabbits, Fine et al. observed impaired AC activity in response to AVP but normal stimulation by NaF (6). These data suggested a defect at the level of the AVP receptor. In contrast, Bonilla-Felix et al. (35) found no defect in the AVP responsiveness of cortical collecting tubules isolated from rabbits that had undergone 75% renal ablation. It should be noted, however, that in that study both the severity and duration of uremia were substantially less than in the study by Fine et al. The experimental conditions in the present study were comparable to those of Fine et al. and our findings of severely blunted AVP responsiveness in both inner medullary PMs and cultured IMCT cells from CRF animals are consistent with theirs. We extended these observations by undertaking a systematic examination of each component of the receptor–enzyme complex aimed at definitively identifying the site responsible for AVP unresponsiveness in CRF. We have demonstrated that the lack of AVP responsiveness in CRF cells is not due to an alteration in the function of the catalytic unit of AC or of either the stimulatory or inhibitory guanine nucleotide-binding regulatory proteins. AVP resistance is not mediated by a cyclooxygenase product as preincubation with flurbiprofen fails to restore the response to AVP.

The rat IMCD has been reported to express V_{1a} receptor mRNA the level of which, in contrast to that of V_2 receptor mRNA, is not decreased when plasma AVP increases during dehydration (36). V_{1a} receptor occupancy might promote phosphoinositide hydrolysis with subsequent stimulation of PKC which has been shown by us and others to impair the hydroosmotic response to AVP (32, 37, 38). This does not appear to be the case, however, as neither acute inhibition nor chronic down-regulation of PKC restored the response to AVP in CRF cells.

Binding studies with [^{3}H]AVP reveal a > 70% decrease in maximum uptake for the AVP receptor in inner medullary plasma membranes from CRF rats with no decrease in maximum uptake for beta adrenergic receptors in the same PMs. The lack of change in beta adrenergic receptor uptake indicates that the decrease in maximum uptake for the AVP receptor is not simply a consequence of cellular hypertrophy in the presence of CRF but is, indeed, specific to AVP. In support of this observation, RT-PCR shows that, in contrast to mRNA for α_s which is present at normal abundance, message for the V_2 receptor is nearly absent in CRF PMs (in a second experiment not pictured in this manuscript a very faint band of V_2 receptor product was detected after 35 cycles of PCR from a CRF animal). The persistence of some [^{3}H]AVP binding despite near elimination of V_2 receptor mRNA may reflect the presence of V_{1a} receptors in these plasma membranes (see above). Taken together, the binding and RT-PCR data indicate that the site of the pre-cAMP defect in CRF is the V_2 receptor which is selectively down-regulated in CRF. It should be noted that the near total elimination of V_2R complicates the interpretation of the experiments aimed at examining downstream components of the signal transduction pathway; a defect in one of these components might be

obscured by the lack of stimulatory input from the receptor. The persistence of normal signaling in response to both PGE_2 and isoproterenol makes this unlikely but we cannot unequivocally rule out a role for other factors in addition to the decreased receptor number in vivo.

This study did not address the mechanism(s) responsible for the decreases in V_2 receptor mRNA and apparent number. The available data does not allow us to exclude the possibility of decreased stability of V_2 receptor mRNA. Decreased stability of message might explain the apparent further loss of V_2 receptor function in cultured CRF cells (Fig. 1) compared to inner medullary PMs from CRF animals (Table I). Alternatively, the observed changes in V_2 receptor message and apparent number may be due to decreased transcription. One may speculate that this is a process of homologous desensitization consequent to retained solutes and elevated levels of plasma AVP. This hypothesis is consistent with data in the study of Terada et al. in which rats subjected to 72 h of dehydration had increased plasma AVP levels and V_2 receptor mRNA in the IMCD was decreased by > 50% (36). Studies will be undertaken to define the molecular mechanisms underlying these phenomena; definitive studies on the regulation of V_2 receptor transcription must await identification of the promoter for the V_2 receptor gene.

It is interesting that IMCT cells cultured from CRF animals appear to exhibit "memory," i.e., they display the same selective defect in AVP responsiveness as do the original IMCT. This property facilitated the performance of experiments examining the roles of guanine nucleotide-binding proteins using bacterial toxins which could not readily be done in plasma membranes. Though memory is best recognized as a characteristic of immune cells (39) it has been found in other tissues as well. For example, it is known that adult mice bearing a myosin light chain-chloramphenicol acetyltransferase (CAT) transgene exhibit a rostrocaudal gradient in the expression of CAT with more caudal muscles expressing higher levels of the enzyme (40). It has recently been shown that the level of expression of CAT by muscle cells cultured from adult mice reflects the rostrocaudal position of the muscles from which the cells are derived (41). Cultured IMCT cells have also been previously shown to exhibit memory. Kohan et al. demonstrated differences in endothelin production by isolated IMCD of spontaneously hypertensive or Wistar-Kyoto rats. IMCT cells cultured from these rats produce endothelin in proportion to the level of production observed in the parental strain in vivo (42). The molecular mechanism(s) underlying this process of memory remains to be determined. One possibility is that of a methylation-dependent decrease in DNA transcription which has been linked to a number of processes associated with alterations in the efficiency of gene transcription. These include X-inactivation, chromosomal imprinting, and some tissue-specific patterns of gene expression (43). Our understanding of the mechanism responsible for the memory observed in the IMCT of CRF animals will have to await elucidation of the molecular mechanisms underlying decreased V_2 receptor transcription in this state.

Finally, it is appropriate that we consider the relevance of our findings to the urinary concentrating defect in uremic man. Patients with CRF exhibit a high filtered load of solute per remaining functioning nephron (44); this solute diuresis is a major cause of the inability to maximally concentrate urine. Disruption of the medullary architecture due to interstitial fibrosis and scarring may also contribute to the lack of maximal antidiuresis by preventing the generation of a hypertonic medullary interstitium. Neither of these defects, however, would result in the elaboration of urine that is hypotonic relative to plasma were the collecting duct appropriately responsive to vasopressin. It is the occurrence of hyposthenuria despite adequate circulating vasopressin that is explained by the present findings.

In summary, the studies reported herein demonstrate selective decreases in V_2 receptor message and protein product in the inner medullae of rats with CRF. The decrease in V_2 receptor number appears to be responsible for AVP resistance in this disease state. Further studies will be needed to determine whether CRF also affects post-cAMP components of the hydroosmotic response to AVP such as the water channel.

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