Chlorpropamide Action on Renal Concentrating Mechanism in Rats with Hypothalamic Diabetes Insipidus

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ABSTRACT To determine vasopressin (VP)-potentiating effect of chlorpropamide (CPMD), we studied the effect of CPMD in vivo and in vitro in kidneys and in specific tubule segments of rats with hypothalamic diabetes insipidus, homozygotes of the Brattleboro strain (DI rats). Rats on ad lib. water intake were treated with CPMD (20 mg/100 g body wt s.c. daily) for 7 d. While on ad lib. water intake, the urine flow, urine osmolality, urinary excretion of Na^+, K^+, creatinine, or total solute excretion did not change. However, corticopapillary gradient of solutes was significantly increased in CPMD-treated rats. Higher tissue osmolality was due to significantly increased concentration of Na^+, and to a lesser degree urea, in the medulla and papilla of CPMD-treated rats. Consequently, the osmotic gradient between urine and papillary tissue of CPMD-treated rats (Δ = 385±47 mosM) was significantly ($P < 0.001$) higher compared with controls (Δ = 150±26 mosM). Minimum urine osmolality after water loading was higher in CPMD-treated DI rats than in controls. Oxidation of [^{14}C]lactate to $^{14}CO_2$ coupled to NaCl cotransport was measured in thick medullary ascending limb of Henle's loop (MAL) microdissected from control and CPMD-treated rats. The rate of $^{14}CO_2$ production was higher (Δ + 113%±20; $P < 0.01$) in CPMD-treated MAL compared with controls, but $^{14}CO_2$ production in the presence of 10^{-3} M furosemide did not differ between MAL from control and from CPMD-treated rats. These observations suggest that CPMD treatment enhances NaCl transport in MAL. Cyclic AMP metabolism was analyzed in microdissected MAL and in medullary collecting tubule (MCT). MCT from control and from CPMD-treated rats did not differ in the basal or VP-stimulated accumulated of cAMP. The increase in cAMP content elicited by 10^{-6} M VP in MAL from CPMD-treated rats (Δ + 12.0±1.8 fmol cAMP/mm) was significantly ($P < 0.02$) higher compared with MAL from control rats (Δ + 5.1±1.0 fmol cAMP/mm). Preincubation of MAL dissected from Sprague-Dawley rats with 10^{-4} M CPMD in vitro increased cAMP accumulation in the presence of VP, but no such enhancement was found in preincubated MCT. Adenylate cyclase activity, basal or stimulated by VP, 5'guanylimidodiphosphate, or by NaF, assayed in isotonic medium did not differ between MAL or MCT from control rats and MAL or MCT from CPMD-treated rats. When assayed in hypertonic medium (800 mosM), the adenylate cyclase activity in the presence of 10^{-6} M VP was significantly higher in MAL of CPMD-treated rats. MAL and MCT from control and CPMD-treated rats did not differ in the activities of cAMP phosphodiesterase. The rate of [^{14}C]arachidonic acid conversion to [^{14}C]prostaglandin E_2 by medullary and papillary microsomes was not different between the control and CPMD-treated rats; likewise, there was no difference in accumulation of immunoreactive prostaglandin E_2 in the medium of in vitro incubated medullary or papillary slices prepared from control and CPMD-treated rats.

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Based on the findings recounted above, we propose a hypothesis that CPMD administration enhances the antidiuretic effect of VP, primarily by increasing medullary and papillary tonicity due to increased NaCl reabsorption in MAL. There is no evidence that CPMD sensitizes collecting tubules to the action of VP, at least at the cAMP-generation step. Therefore, increased antidiuretic response to VP in the kidneys of CPMD-treated DI rats is due to enhanced osmotic driving force for water reabsorption (lumen-to-interstitium osmotic gradient) in collecting tubules, rather than due to increased VP-dependent water permeability of tubular epithelium.

INTRODUCTION

The administration of chlorpropamide (CPMD) is well known to produce antidiuresis or to potentiate antidiuretic effect of vasopressin (VP) hypothalamic-neurohypophyseal diabetes insipidus (DI) (1–9). Earlier observations suggested that CPMD-induced antidiuresis is due in part to stimulation of VP release (1), but some other studies (2, 10, 11) did not find evidence for such an effect. Unquestionably, the major action of CPMD is enhancement of antidiuretic response of the kidney to endogenous or to exogenous VP (1–9). Based on studies of amphibian urinary bladder and on various preparations of renal medulla, several hypotheses were proposed attempting to explain VP-potentiating effect of CPMD. The central tenet of these hypotheses is the proposition that CPMD increases the sensitivity of target epithelium to VP. Since cyclic (c)AMP mediates transport effects of VP (12, 13), it was proposed (5, 14–16), as reviewed recently (8, 13), that CPMD increases cAMP accumulation in epithelial cells in response to VP.

First, it was proposed that CPMD-elicited enhancement of cAMP accumulation is due to inhibition of renal cAMP phosphodiesterase (cAMP-PDIE) (13, 15–17), or due to increased stimulation of renal medullary adenylate cyclase (AdC) by VP (5, 14, 18). Also, studies of CPMD action on amphibian urinary bladder could be interpreted as potentiation of VP effect at cAMP generation step (19–22). However, Omachi et al. (23) found that CPMD lowers the cAMP content of toad urinary bladder in response to VP.

Prostaglandins (PG), modulators of VP action (12, 13), were implicated in VP-potentiating effects of CPMD. Ozer and Sharp (19) proposed that CPMD decreases the inhibitory effect of PGE on VP-dependent generation of cAMP. A more recent study (24) suggested that inhibition of intraepithelial synthesis of PGE_2 at the PG cyclo-oxygenase step by CPMD and other sulfonylureas relieves a negative modulatory effect of PGE on hydroosmotic response to VP (24).

Several considerations motivated us to investigate further the CPMD action on renal tubules. First, it has been difficult to explain why not only CPMD but also some other sulfonylurea antidiabetic drugs, namely glyburide, which do not have VP-enhancing properties in mammalian kidney in vivo (8, 25–27), also inhibited the PGE_2 synthesis and enhanced VP-induced water flow in the amphibian bladder system in vitro in a way similar to CPMD (8, 24). Also, it appears puzzling that treatment of rats with CPMD either does not diminish (28), but even enhances (18) urinary excretion of PG in response to large doses of VP, or to its analogue [1-deamino,8-D-arginine]-vasopressin (DDAVP). All studies published to date investigated CPMD effect on renal cAMP metabolism in whole medullary and/or papillary preparations (5, 13–16, 18). It is only recent knowledge that these gross anatomical zones of the kidney contain at least two types of epithelia with VP-sensitive cAMP metabolism, namely ascending limb of Henle's loop, and epithelium of collecting tubules (12, 29–31). It is, therefore, uncertain in which one of these two tubule segments (or on both) CPMD may influence cAMP metabolism in response to VP. Finally, unexplored remained the question whether CPMD may act not only by changing VP-regulated water permeability of collecting tubules, but whether it also may have an effect on another fundamentally important factor for mammalian antidiuretic response—a corticopapillary gradient of solutes (32).

The results of our present study indicate that administration of CPMD to DI rats has no effect on VP-dependent cAMP accumulation in medullary collecting tubules (MCT), but that it does increase VP-dependent cAMP accumulation in medullary thick ascending limb of Henle's loop (MAL). Even more importantly, treatment with CPMD increases corticopapillary gradient of solutes, namely of Na^+ and urea, and stimulates NaCl transport-coupled oxidation of substrate in MAL. Based on these findings, we propose as a new hypothesis (Fig. 3) that CPMD administration enhances the antidiuretic effect of VP in renal medulla of DI rats primarily by increasing the osmotic driving force for water reabsorption in collecting tubules (Fig. 3).

GLOSSARY

AdC	adenylate cyclase
AVP	[8-arginine]-vasopressin
cAMP	adenosine 3',5'-cyclic monophosphate
cAMP-PDIE	cyclic AMP phosphodiesterase
CPMD	chlorpropamide
Cr	creatinine
DI	diabetes insipidus
DI rat	homozygote rat of Brattleboro strain with hypothalamic diabetes insipidus
Gpp(NH)p	5'guanylimidodiphosphate

KRB	Krebs Ringer buffer
MAL	medullary thick ascending limb of Henle's loop
MCT	medullary collecting tubule
MIX	1-methyl-3-isobutyl-xanthine
PG	prostaglandin(s), with the corresponding letter (E, F) for each series
U_{osm}	urine osmolality
U_xV	urinary excretion of solute X
VP	vasopressin

METHODS

Animals. Most experiments were conducted on the adult male homozygotes of Brattleboro strain of rats (weighing 200–300 g) with hereditary DI (further referred to as "DI rats") purchased from Blue Spruce Farms, Altamont, NY. Some studies were conducted on male adult Sprague-Dawley albino rats purchased from Bio-Lab, Inc., Minneapolis, MN. All animals were maintained in an air-conditioned room (21°–25°C) and allowed free access to tap water and standard diet (Purina laboratory rat chow, Ralston Purina Co., St. Louis, MO).

Experimental design. DI rats were housed in individual metabolic cages with free access to food and water ad lib. Urine was collected daily under oil. Experimental DI rats were treated for 7 d with CPMD in one daily subcutaneous injection in a dose 20 mg/100 g body wt; control DI rats received the same volume of vehicle (3, 5, 14, 18). CPMD for injection was dissolved in 0.9% NaCl slightly alkalinized with concentrated NaOH and then adjusted to pH 7.4 with HCl (3, 5, 18) to a final concentration 50 mg CPMD/ml. To ascertain that CPMD was effectively reabsorbed, a sample of blood for the determination of glucose and other analyses was obtained from the jugular vein, under light ether anesthesia, before and after 7-d treatment with CPMD. As expected, CPMD treatment resulted in −58% drop in ambient plasma glucose levels, but no significant change in plasma glucose was observed in vehicle-treated controls. Rats treated with CPMD did not show any signs of toxicity. They did not loose weight and their blood urea nitrogen and plasma creatinine (Cr) levels were in normal range and not different from controls (data not shown). Also, urine excretion parameters did not differ from controls (Table II). 2 h after the last injection of CPMD (or vehicle) the DI rats were lightly anesthetized with pentobarbital (6 mg/100 g body wt) and the kidneys were taken for further analyses, as described below, and the animals were killed.

The water-loading test was performed in control DI rats and CPMD-treated DI rats as described by Stoff et al. (33). After overnight fasting, the rats were given oral distilled water load of 5% of body weight, and urine flow and urine osmolality (U_{osm}) was determined in 30-min intervals.

As in our previous studies (29, 34, 35), the experiments were designed in such a way that CPMD-treated and control animals were handled simultaneously throughout the experiment on a paired basis, i.e., the control and CPMD-treated rat entered the experimental protocol simultaneously, the kidneys were removed and fluid and tissue samples as well as tubules were microdissected at the same time. Likewise, incubations, storage, and all analyses of samples from control and CPMD-treated animals were conducted simultaneously with the use of the same chemicals, radiochemicals, and standards in order to minimize variability.

Analysis of solutes in tissues and fluids. We used previously reported procedures (33, 36, 37). The kidneys were rapidly removed under anesthesia. The cortex, outer (red) medulla, and papilla were separated by razor blade and rapidly frozen by clamping with stainless steel tongs precooled by immersion in liquid N_2. Deep-frozen tissue was transferred to polyethylene (12 × 75 mm) test tubes filled with liquid N_2 and surrounded by dry ice. The tubes with tissues in the frozen state were weighed again with a Sartorius microbalance to determine wet weight. After overnight lyophylization, tubes were reweighed for the determination of dry weight. Lyophylized tissue was extracted for determination of solutes using in principle the method of Appleboom (36).

Approximately 1 ml of triple-distilled water heated at 100°C was added to each tube and tubes were then heated in a boiling water bath for 60 min. The test tubes with extracted tissue samples were cooled and reweighed to determine the final volume of added water. Samples were then centrifuged at 2,000 *g* for 10 min and aliquots of supernatant were used for the determination of Na^+, K^+, and urea. Water content in samples was expressed as percentage of wet weight. Total tissue osmolality was calculated as follows (33): Total osmolality [mosM/kg H_2O] = [urea] + 2 [Na^+] + 2 [K^+]. Urinary excretion of solutes (U_xV) was measured as in our previous studies (37–39). Total urinary excretion of sodium ($U_{Na}V$), potassium (U_KV), total solutes ($U_{osm}V$), and Cr ($U_{Cr}V$) was expressed per 100 g body wt per 24 h. Na^+ and K^+ was determined by atomic absorption photometry (model 951, Instrument Laboratories Corp. Palatine, IL), urea by the colorimetric method using Harleco kits (39). U_{osm} was measured with a Fiske osmometer (Fiske Associates, Inc., Burlington, MA); Cr was determined colorimetrically (40). Plasma glucose was determined with the hexokinase/glucose-6-phosphate dehydrogenase method (41), as in our previous report (38).

Microdissection of tubules. Tubular segments were dissected from vehicle-treated controls and from CPMD-treated DI rats, control, and drug-treated rats always in the same way, using the procedures described in detail in our previous studies (29, 34, 35, 42) with several recent minor modifications. Briefly, under light anesthesia with pentobarbital (6 mg/100 g body wt) the aorta was cannulated retrogradely with polyethylene PE-100 tubing (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ), the tip being placed just distal to the branching of the left renal artery. Composition of all solutions are described below. The left kidney was perfused to complete blanching with 5–10 ml of perfusion solution, subsequently followed by 20 ml of heparinized (heparin concentration 20 USP U/ml) collagenase medium for ~5 min and then sliced with a razor blade along the corticomedullary axis. The slices were then incubated in aerated collagenase medium (at 35°C for 45 min) then thoroughly rinsed in ice-cold microdissection medium and transferred to petri dishes for microdissection. Microdissection and all subsequent procedures were performed at 0°–4°C. Segments of MAL and MCT were carefully teased out from the inner stripe of the outer medulla with sharpened steel needles using a stereomicroscope (magnification × 30).

MAL and MCT were identified using the established criteria (31, 42, 43), as in our previous studies (29, 34, 35, 42). Some differences were observed between MAL and MCT dissected from DI rats (Brattleboro homozygotes) and the same segments dissected from Spraque-Dawley rats (42). MCT can be dissected with greater ease, and vice versa, MAL with greater difficulty from DI rats, compared with normal Sprague-Dawley rats (42). For example, using the same technique, dissected MAL were obtained in shorter fragments and MCT In longer fragments in Brattleboro DI rats than in Sprague-Dawley rats. This feature may be re-

lated to different morphology of medulla and medullary interstitium of DI rats reported earlier (44, 45). Dissected segments were aspirated and transferred onto small round fragments (~3 mm^2) of glass coverslip. The total length of tubules in the sample was then determined. Samples were placed on the stage of microscope (model 1053, American Optical Scientific Instruments, Warner-Lambert Co., Buffalo, NY) with a drawing attachment (camera lucida) and inspected under × 100 magnification. Tubules in the sample and 1-mm calibration grid were quickly drawn on white paper with a marker. Tubule length was then measured using a Dietzger planimeter from drawn pictures of samples. The measured samples were kept at 0°–4°C before incubations, when the assay was performed immediately, or before freezing for storage at −80°C.

Enzyme assays. Assays for AdC and cAMP-PDIE were performed on disrupted tubular segments. Tubular disruption (42, 43, 46) was performed as described in our previous studies (29, 34, 35, 42, 46): The microdissection medium was aspirated off each sample and replaced with 0.25 μl of hypoosmotic medium. All samples were then frozen rapidly on dry ice and stored at −80°C overnight. Samples were allowed to thaw at 4°C before being assayed for enzyme activities.

AdC activity was measured according to the method (43) described in detail previously (29, 30, 34, 35, 42). Tubular samples (1–3-mm total tubular length) were incubated for 30 min at 30°C in a final volume of 2.5 μl consisting of the following (final concentrations): 0.25 mM [α-^{32}P]ATP (4 × 10^6 cpm/sample), 1 mM cAMP, 3.8 mM $MgCl_2$, 0.25 mM EDTA, 100 mM Tris HCl, 20 mM creatinine phosphate, and 1 mg/ml creatine kinase (pH 7.4). The osmolality of this incubation mixture was ~300 mosM (30, 35). Additionally, in some experiments (Results), NaCl and urea were added to this incubation mixture in a 1:2 molar ratio to attain a final osmolality of 800 mosM (30, 35). The reaction was stopped by freezing the slides on dry ice (29, 34, 42). Each coverslip plus frozen sample was transferred to individual precooled 12 × 75-mm glass test tubes followed by 150 μl of stop solution consisting of 3.3 mM ATP, 5 mM cAMP, 50 mM Tris HCl (pH 7.6), and [^{3}H]cAMP (1 × 10^4 cpm/sample) to monitor recovery and cAMP was separated from other products using Dowex-50 and aluminum oxide columns according to the method of Salomon et al. (43, 47). AdC activity was expressed as femtomoles cAMP per 30 min per millimeter of tubule (30, 43).

cAMP-PDIE activity was assayed by a previously described method (46). Tubular samples (0.5–1.5 mm) were incubated for 10 min at 37°C in 2.5 μl consisting of (final concentrations) 10 mM $MgSO_4$, 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.0 (Mg-EDTA-Tris buffer), and 10^{-6} M [^{3}H]cAMP. The medium osmolality was 300 mosM (30, 35). The reaction was stopped by placing the slides on dry ice, followed by transfer of each sample plus coverslip to individual precooled 12 × 75-mm glass test tubes; 100 μl of Mg-EDTA-Tris buffer were added to each tube while still frozen. All tubes were then immersed in a boiling water bath for 3 min, cooled on ice, and incubated for a further 15 min at 37°C in the presence of 50 μl of 5′-nucleotidase (snake venom from *Crotalus atrox;* 1 mg/ml). Nucleotides were separated from nucleosides on QAE-Sephadex columns as described in our previous studies (29, 30, 35, 46).

Measurement of cAMP accumulation in MAL and MCT. This was described in detail previously (29, 30, 34, 42). Approximately 6–8 mm of total tubule length of MAL constituted one sample for basal cAMP accumulation and 2–7 mm for [8-arginine]-vasopressin (AVP) stimulated cAMP accumulation. For MCT, 3.5–8.5 mm of tubule length for basal cAMP accumulation and 1.5–5 mm for AVP-stimulated cAMP accumulation were required. After measurement of tubule length, samples were placed in 2.5 μl of modified Krebs Ringer buffer (KRB) with or without test agents and hormone. In some experiments, medium 199 (Gibco Laboratories, Grand Island, NY) was used instead of KRB as an incubation medium. Results of experiments with the use of KRB or medium 199 were indistinguishible and therefore are presented together in the text. In some experiments (specified in the Results) incubations were conducted in hyperosmolar (800 mosM) incubation mixture. In such experiments either KRB or medium 199 was supplemented with NaCl and urea (1:2 molar ratio) to a final osmolality of 800 mosM (30, 35). All samples are incubated for 10 min at 30°C and the reaction was stopped by placing the slides on dry ice. Samples and coverslips were transferred while still frozen into precooled 12 × 75-mm glass test tubes and 100 μl of 50 mM Na^+ acetate added to each, and placed for 3 min in a boiling water bath (29, 34). Tubule samples and standards were acetylated and cAMP content determined by radioimmunoassay (RIA) (29, 30, 34). CPMD or medium 199 at concentrations higher than those used in the present experiments did not interfere with RIA for cAMP. The content of cAMP in the sample (tubules and incubation medium together) is expressed in femtomoles per millimeter of tubular length.

NaCl transport-coupled $^{14}CO_2$ production from [^{14}C]lactate in isolated MAL. $^{14}CO_2$ production from [U-^{14}C]lactate was measured according to a modification of the method of Le Bouffant et al. (48). The principle of the method consists in the continuous trapping, by simple diffusion into KOH, of the metabolic product $^{14}CO_2$ resulting from oxidative metabolism of a uniformly radiolabeled (U-^{14}C) substrate by isolated tubules, incubated in a bicarbonate-free solution (48). Freshly dissected MAL were transferred onto a 1-mm diam round coverslip placed on the concave bacteriological slide. Concave bacteriological slides contained one small round coverslip for the incubation mixture with tubules and another coverslip for the droplet of 0.3 M KOH, into which the $^{14}CO_2$ formed is continuously trapped in the course of incubation. After length of tubule segments in the sample was measured, samples were kept at 0°–4°C until the incubation. At the beginning of the assay the microdissection medium was aspirated from samples with a micropipette and replaced with 1.0 μl of metabolic medium that contained [^{14}C]lactate (4 × 10^5 cpm/sample) with or without added drugs, as specified in the Results. The 2-μl droplet of 0.3 M KOH was placed on another coverslip situated in the bottom of a well of a concave bacteriologic slide. Another concave bacteriologic slide was placed on the top creating a small chamber. Samples were allowed to equilibrate for 20–30 min at 0°–4°C and incubation was started as follows. The KOH droplet was removed by soaking into a piece of filter paper, then fresh 2 μl of KOH was added, chamber made from two concave bacteriologic slides was sealed by vaseline coating and by clamping with two bulldog clamps, and the sealed chamber was immersed into 30°C water incubation bath. The incubation time was usually 90–150 min; the same incubation time was used within one experiment. In preliminary experiments we established that $^{14}CO_2$ production from [U-^{14}C]lactate by MAL was proportional to incubation time (r = 0.98) at least up to 240 min. The reaction was stopped by placing the slides with samples on a block of dry ice. The coverslips with 0.3 M KOH, still frozen, were then quickly transferred with forceps into the scintillation vial for ^{14}C-radioactivity measurement by liquid scintillation

counting. Samples prepared in exactly the same way but without tubules were used as blanks and blank values were subtracted to calculate $^{14}CO_2$ produced by the MAL segments. In preliminary experiments, it was established that $^{14}CO_2$ production from [^{14}C]lactate is proportional ($r = 0.99$) to length of tubule in the sample up to 3 mm. The rate of $^{14}CO_2$ production is expressed in femtomoles of $^{14}CO_2$ generated from [U-^{14}C]lactate per 60 min millimeter of tubule length.

Several criteria have been used by others (48–51) to document that oxidation of substrates in thick ascending loop of Henle is closely coupled to lumen-to-interstitium NaCl cotransport (48–52). In preliminary experiments we confirmed, using similar criteria, that metabolism of [^{14}C]lactate to $^{14}CO_2$ in our system is coupled to continuing NaCl cotransport in MAL. Inclusion of loop diuretic furosemide or replacement of NaCl in the metabolic medium with equimolar LiCl resulted in marked suppression of [^{14}C]lactate oxidation (Table I). These results closely agree with observations from other laboratories (48–52).

Measurement of PG formation. The method used (53) was described in detail in our previous study (54). All preparative procedures were conducted at 0°–4°C. The kidney was removed under anesthesia, divided with a razor blade into outer (red) medulla and papilla (inner medulla), minced, and homogenized with a Polytron homogenizer at speed 5 in a 100 mM Tris-HCl buffer, pH 7.4 (1:3, wt/vol). The homogenate was centrifuged for 10 min at 10,000 *g*. The pellet was resuspended in the same volume of Tris-HCl buffer and was centrifuged again at 10,000 *g* for 10 min. Pooled supernatants were suspended in teflon-glass Potter homogenizer and were diluted into 1:1 with Tris-HCl buffer and then ultracentrifuged for 90 min at 100,000 *g*. The pellet, microsomal fraction, was resuspended in 500 μl of 100 mM Tris-HCl buffer, the aliquots were quickly frozen on dry ice, and were stored at −80°C. Protein content in the microsomal fraction was determined by the method of Lowry et al. (55) as in previous studies (37, 38, 54). In the preliminary experiments, it was ascertained that conversion of [^{14}C]arachidonic acid to [^{14}C]PGE_2 was linearly proportional to microsomal protein up to 30 μg/tube. Enzymatic reactions were carried out in Tris-HCl buffer (pH 7.5) for 10 min at 30°C. The incubation mixture (200 μl) consisted of the following (final concentrations): 10 μM [1-^{14}C]-arachidonic acid (8×10^5 cpm); 1 mM GSH, 1 mM L-epinephrine, and 100 mM Tris-HCl (pH 7.5) (53, 54). Incubations were terminated by adding 15 μl of 4 N formic acid. The mixture was extracted twice successively with 600 μl of chloroform/methanol (2:1, vol/vol; acidified with HCl to pH 3.5), and the extracted samples were dried under a stream of N_2 gas (53, 54). The dried residue was dissolved in 100 μl of chloroform/methanol (2:1, vol/vol; pH 3.5). Extracts were chromatographed on silica gel G thin-layer plates, as previously described (53, 54). Areas corresponding to $PGF_{2\alpha}$, PGE_2, and arachidonic acid (54) were scraped off the plates and the radioactivity was determined by liquid scintillation counting (54). Blank values were determined from complete incubation mixtures without added microsomes.

In another series of experiments, we measured immunoreactive PGE_2 accumulated in the incubation medium of papillary and medullary tissue slices. Slices prepared by Stadie-Riggs slicer were quickly weighed and then incubated (100 mg wet wt/6 ml) in a medium containing 140 mM NaCl, 5 mM KCl, 10 mM Na acetate, 10 mg glucose, 2 mM NaH_2PO_4, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 0.01 mM arachidonic acid, and 20 mM Tris-HCl (pH 7.4). After 60-min incubation at 30°C, the incubation was stopped by acidification with 1 N HCl to pH 3.5. The incubation medium was extracted (1:3, vol/vol) with a mixture of chloroform/methanol (2:1, vol/vol; pH 3.5). The extract was dried under a stream of N_2 then suspended in 1 ml of 10 μM phosphate buffer (pH 7.4) and frozen and stored at −80°C until RIA for PGE_2. Blanks were run through the same procedure without the tissue. PGE_2 was determined in samples by specific RIA described in detail elsewhere (56, 57).

Statistical evaluation of the results was made with use of Student's *t* test, as specified in Results. $P > 0.05$ were considered nonsignificant.

Solutions and materials. The microdissection medium was a modified Hanks' balanced salt solution (HBSS) (43) that was used in our previous studies (29, 34, 35, 42), consisting of (in final concentrations): 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 1.0 mM $MgCl_2$, 10 mM Tris-HCl, 0.25 mM $CaCl_2$, pH 7.4. The composition of the collagenase medium was identical to that of the microdissection medium except that the $CaCl_2$ concentration was 1 mM and it included collagenase (0.1%, wt/vol), bovine serum albumin (0.1%, wt/vol), and hyaluronidase (0.1%, wt/vol). In some experiments, instead of modified HBSS, we used medium 199 as a microdissection medium or, after the addition of collagenase, instead of the above described collagenase medium. Results with the use of medium 199 in place of modified HBSS were indistinguishable. Medium 199 was, as mentioned above, used in some experiments instead of modified KRB in incubations for the determination of cAMP metabolism, with identical results. The hypoosmotic medium contained (in final concentrations): 1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% bovine serum albumin, and 1 mM Tris HCl (pH 7.4). Modified KRB contained 140 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$, 10 mM sodium acetate, 10 mM glucose, 20 mM Tris, 2.0 mM NaH_2PO_4, pH 7.4. Metabolic incubation mixture (metabolic medium) for $^{14}CO_2$ production (48) contained 140 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM sodium acetate, 5 mM glucose, 20 mM Tris, 2.0 mM NaH_2PO_4, and 5 mM of [U-^{14}C]lactate (4×10^5 cpm/sample); pH 7.4.

Bovine serum albumin, rattlesnake venom (*Crotalus atrox*), collagenase (type I, 150 U/mg), hyaluronidase (type 1–5, 500 NF U/mg), cAMP, and furosemide as well as other biochemicals were purchased from Sigma Chemical Co., St.

TABLE I

Effect of Furosemide and LiCl on $^{14}CO_2$ Generation from [^{14}C]Lactate by MAL

	$^{14}CO_2$		*P* value*
	fmol/mm/60 min		
Basal	5,160±1,170	(4)‡	
Furosemide (10^{-4} M)	2,710±480	(6)	0.05 < – < 0.1
Furosemide (10^{-3} M)	1,440±380	(4)	<0.02
LiCl§	1,540±200	(4)	<0.025

Experiments were conducted on MAL microdissected from albino Sprague-Dawley rats (for further details, see Methods).

* For significance of difference from basal value (*t* test).

‡ Mean±SEM; in brackets number of samples.

§ 140 mM NaCl in the incubation medium (Methods) was replaced by equimolar quantity of LiCl.

Louis, MO. Sodium heparin (Panheparin, 100 USP U/ml) was purchased from Abbott Laboratories, Chemical Division, North Chicago, IL; [α-^{32}P]ATP (25 Ci/mM) was purchased from ICN Nutritional Biochemicals, Cleveland, OH; [1-^{14}C]arachidonic acid (40 Ci/mM), [^{3}H]cAMP (30–50 Ci/mM), and [U-^{14}C]lactate (40 Ci/mM) also from ICN. Synthetic AVP (385 U/mg) and 1-methyl-3-isobutyl-xanthine (MIX) were purchased from Calbiochem Behring Corp., American Hoechst Corp., San Diego, CA. RIA kits for the measurement of cAMP content, and 5'-guanylimidodiphosphate (Gpp[NH]p) were purchased from Schwarz/Mann Div., Becton, Dickison & Co., Orangeburg, NY. CPMD was a generous gift from the Pfizer Laboratories, Div. of Pfizer Inc., New York. These and all other compounds and reagents were of the highest quality grade available and were purchased from standard suppliers.

RESULTS

The effects of CPMD on tissue and urinary solutes in vivo. Water and solute excretion in DI rats was studied in a group treated with CPMD or with placebo (Methods). In concordance with previous reports (3–5, 8, 18), while on ad lib. water intake, CPMD treatment did not alter urine flow, U_{osm}, $U_{osm}V$, $U_{Na^+}V$, and $U_{K^+}V$ (Table II). Likewise, at the end of the experiment CPMD-treated rats did not differ from controls in $U_{Cr}V$ (controls: 3.8±0.3 mg Cr/100 g body wt/24 h, n = 7; CPMD-treated: 4.2±0.2 Cr/100 g body wt/24 h, n = 7; mean±SEM). Analysis of solute content showed that concentrations of Na^+, and to a much lesser degree urea, were significantly enhanced in the medulla and in the papilla, but not in the cortex of CPMD-treated rats; tissue contents of K^+ and of H_2O did not change (Table III). Consequently, the total medullary and total tissue osmolality was significantly increased as a result of CPMD administration. The extent of differences between U_{osm} and papillary tissue osmolality was markedly, more than two times greater in CPMD-treated rats than in controls (Fig. 1).

Maximum diluting ability was determined by water-loading test in another group of animals (Methods). Before the water loading test, U_{osm} of control DI rats (300±33 mosM; mean±SEM; n = 5) did not differ from CPMD-treated DI rats (294±30 mosM; mean±SEM; n = 5). However, after the water load, minimal U_{osm} achieved by CPMD-treated DI rats (204±4 mosM; mean±SEM; n = 4) was significantly higher ($P < 0.005$, t test) than minimal U_{osm} in control DI rats (131±13 mosM; mean±SEM; n = 5).

Metabolism of cAMP in MCT and MAL. We found no difference in accumulation of cAMP, basal or stimulated with 1 μM AVP, between MCT from control DI rats and DI rats treated with CPMD (Table IV). Also, when assayed in medium of higher osmolality (800 mosM), the cAMP response to submaximal (0.2 nM) or maximal (1 μM) doses of AVP was not increased in MCT dissected from CPMD-treated rats (data not shown). On the other hand, in the presence of 1 μM AVP, the cAMP accumulation was markedly enhanced in MAL from CPMD-treated DI rats, compared with DI controls (Table IV). Similarly, cAMP accumulation in the presence of VP was enhanced in the MAL of normal rats preincubated in vitro with added 0.1 mM CPMD, however, the same preincubation with CPMD did not increase cAMP accumulation in MCT (Table V).

AdC activity in MCT or in MAL, basal or stimulated with submaximal and maximal doses of AVP, was not different between control DI rats and CPMD-treated DI rats (Table VI). Also no differences were observed between AdC activities in MCT and in MAL from control DI rats and CPMD-treated DI rats when as-

TABLE II

Excretion of Fluid and Solutes and U_{osm} in Control DI Rats, and DI Rats Treated for 7 d by Subcutaneous Injections of CPMD (20 mg/100 g body wt)

	U_{osm}		Urine flow		$U_{osm}V$		$U_{Na}V$		$U_{K}V$	
	A	B	A	B	A	B	A	B	A	B
	mosmol/kg H_2O		*ml/24 h/100 g body wt*		*mosmol/24 h/100 g body wt*		*meq/24 h/100 g body wt*		*meq/24 h/100 g body wt*	
Controls (n = 7)	257±38*	194±17	41.7±6.9	46.8±11.5	10.28±2.0	8.15±1.56	0.62±0.16	0.60±0.18	1.37±0.23	1.20±0.43
CPMD-treated (n = 7)	190±12	168±16	43.8±7.5	45.4±7.1	7.95±1.1	7.08±0.8	0.58±0.26	0.45±0.13	1.21±0.27	1.01±0.2

Values are from urine collections before initiation (A) and the last day (B) of CPMD treatment. For details, see Methods.
Values in any of the measured parameters were not significantly different between period before treatment (A) and period after treatment (B) or between controls and chlorpropamide-treated, group (t test).
Mean±SEM.

TABLE III
Tissue Content of Na, K, Urea, and Water in control DI Rats, and in DI Rats Treated with CPMD

	Controls	CPMD treated	P value*
Na (*meq/kg H_2O*)			
Cortex	61.2±0.9 (9)‡	65.8±6.0 (9)	NS
Medulla	68.8±3.6 (9)	105.9±6.2 (9)	<0.001
Papilla	117.1±14.5 (10)	171.9±18.4 (10)	<0.05
K (*meq/kg H_2O*)			
Cortex	66.1±6.7 (9)	64.7±7.2 (9)	NS
Medulla	68.0±3.8 (9)	76.8±5.7 (9)	NS
Papilla	47.7±4.0 (10)	65.5±8.7 (10)	NS
Urea (*mmol/kg H_2O*)			
Cortex	6.4±0.6 (9)	7.7±0.7 (9)	NS
Medulla	10.9±1.9 (9)	15.7±1.3 (9)	<0.05
Papilla	26.4±3.6 (10)	39.1±4.3 (10)	<0.05
Water content (*% of tissue wet wt*)			
Cortex	76.4±0.8 (9)	77.1±0.6 (9)	NS
Medulla	83.0±0.9 (9)	82.3±0.7 (9)	NS
Papilla	87.0±0.7 (10)	87.1±0.9 (10)	NS
Total solutes (*mosmol/kg H_2O*)			
Cortex	273.8±20.2 (9)	268.9±16.3 (9)	NS
Medulla	284.5±11.2 (9)	381.4±20.1 (9)	<0.001
Papilla	354.0±32.0 (10)	513.9±41.9 (10)	<0.01

* For significance of differences from controls (*t* test).
‡ Mean±SEM; number of animals is indicated in brackets. For further details see Methods.

sayed in the presence of nonhormonal stimuli, i.e., in the presence of 0.1 mM Gpp(NH)p or 10 mM NaF (data not shown). Activities of AdC were also assayed in medium with higher osmolality (800 mosm). The AdC activity assayed in the presence of 1 μM AVP in MAL from CPMD-treated DI rats (383±38 fmol cAMP/30 min per mm tubule length; mean±SEM, n = 15 samples from four experiments) was significantly ($P < 0.005$; t test) higher than AdC activity in MAL from control DI rats (211±32 fmol/30 min per mm tubule length; mean±SEM, n = 22 samples from four experiments); the basal activities of AdC were not different. AdC activity, basal or stimulated by 1 μM AVP, measured in hyperosmolar medium (800 mosM), showed no difference between MCT of control DI rats and MCT from CPMD-treated rats.

Activity of cAMP-PDIE (expressed in femtomoles cAMP per millimeter per min; mean±SEM of n samples from five experiments) in control MCT (68.8±3.1; n = 24) was not different from MCT in CPMD-treated DI rats (68.0±3.1; n = 25). Likewise, cAMP-PDIE activity in MAL from control DI rats (62.1±3.1; n = 28) did not differ from MAL from CPMD-treated DI rats (63.1±4.4; n = 24).

^{14}C-Substrate oxidation in MAL. The rate of $^{14}CO_2$ production from [^{14}C]lactate in MAL microdissected from CPMD-treated DI rats was markedly elevated (Δ + 113%±33; $P < 0.05$; paired t test; four experiments) compared with MAL from DI controls (Table VII). When $^{14}CO_2$ production from [^{14}C]lactate in MAL was measured with or without the presence of maximum inhibitory dose (1 mM) of furosemide, only the $^{14}CO_2$ production from [^{14}C]lactate that was suppressible only furosemide was higher in CPMD-treated MAL (Fig. 2).

PG synthesis. PG synthesis in medulla and papilla of CPMD-treated rats was first assessed by conversion of [^{14}C]arachidonic acid to [^{14}C]PGE_2 (Methods) catalyzed by PG cyclo-oxgenase contained in microsomal fractions of medulla and papilla (53). The conversion rate of [^{14}C]arachidonic acid to [^{14}C]PGE_2 (expressed in nanomoles [^{14}C]PGE_2 per 20 min per milligram protein; mean±SEM) by microsomal fraction from papilla of CPMD-treated DI rats (119±51; n = 6) did not differ from microsomal fraction of control DI rats (97±26; n = 6). Also, the conversion rate of microsomes from medulla of CPMD-treated DI rats (42±11; n = 6) was not lower than in control DI rats (39±15; n = 6).

Accumulation of immunoreactive PGE_2 in incuba-

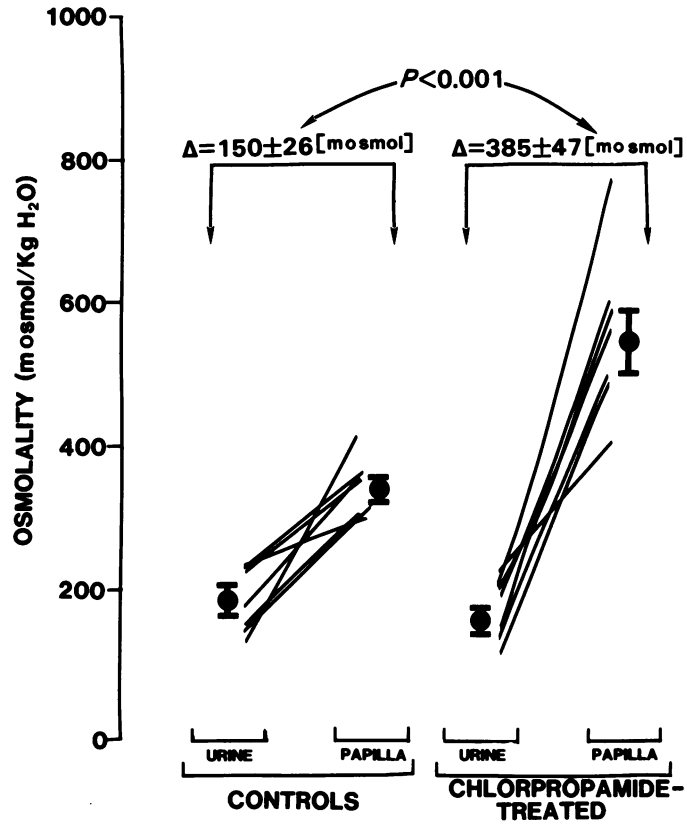

FIGURE 1 Difference between U_{osm} before killing and corresponding papillary osmolality in individual control DI rats (*left* side), and DI rats treated with chlorpropamide (*right* side); for each group $n = 7$. U_{osm} was not different between the two groups, urine-to-papilla osmotic gradient (Δ [mosmoles]) is significantly ($P < 0.001$, t test) greater in chlorpropamide-treated DI rats.

tion medium (Methods) of papillary slices from kidneys of CPMD-treated rats (3.03±1.52 ng PGE_2/mg wet wt; mean±SEM, $n = 3$) was not different from papillary slices from control DI rats (2.92±1.0 ng PGE_2/mg wet wt; mean±SEM, $n = 3$). Likewise, no difference was found in accumulation of PGE_2 in medium from incubated medullary slices from CPMD-treated DI rats and control DI rats (data not shown).

DISCUSSION

As recounted in the Introduction a number of hypotheses were proposed to explain the mechanism by which CPMD enhances antidiuretic effect of VP. In the present study cellular actions of CPMD were, to our knowledge, for the first time directly analyzed specifically in MCT and in MAL of mammalian kidney. We also examined the hitherto unexplored effect of CPMD on renal corticopapillary gradient of solutes. All our studies with in vivo CPMD administration were conducted in DI rats devoid of endogenous VP (58), to exclude the possibility of CPMD interaction with even small amounts of endogenous VP that might be released even in well water-hydrated normal animals.

In full agreement with previous reports by other investigators (3–5, 18, 28), we observed that administration of CPMD alone to DI rats maintained on ad lib. water intake caused no change (Table II) in urine flow, U_{osm}, U_KV, $U_{Na}V$, $U_{osm}V$, and $U_{Cr}V$. However, medullary and papillary tissue osmolality, mainly Na^+ content, was increased and consequently corticopapillary gradient of solutes was significantly enhanced in CPMD-treated rats (Table III). Even in total absence of VP, residual basal water permeability of collecting tubules permits some degree of osmotic equilibration (32, 59). Increased tissue osmolality of medulla and papilla due to CPMD treatment (Table III) did not result in a detectable difference in U_{osm} in DI rats on ad lib. water intake (Table II). However, under conditions of water loading (Results), CPMD-treated DI

TABLE IV

Accumulation of cAMP in MCT and in MAL from Control DI Rats, and in DI Rats Treated with CPMD

	MCT			
	Controls		CPMD-treated	
Experiment	Basal	1 μM AVP	Basal	1 μM AVP
1	6.8±2.5 (3)*	49.3±17.4 (3)	—	61.8±17.5 (4)
2	11.9±1.3 (3)	46.8±1.7 (2)	12.05±3.9 (4)	54.8±27.9 (3)
3	6.4±1.2 (6)	59.8±14.0 (6)	7.3±0.6 (6)	58.2±9.0 (6)
4	11.4±2.6 (4)	60.8±3.1 (6)	7.1±0.8 (3)	64.8±4.0 (6)
5	11.2±1.6 (4)	69.8±5.1 (6)	16.6±3.0 (6)	54.4±4.2 (6)
6	9.6±2.1 (3)	199.1±6.4 (3)	3.4±0.1 (2)	71.8±8.5 (3)
7	—	149.0±42.7 (4)	8.0±1.7 (4)	132.2±7.1 (4)
Mean±SEM	8.9±0.9	86.1±10.9‡	10.1±1.3	69.4±5.7‡
n‖	23	30	25	32

	MAL			
	Controls		CPMD-treated	
Experiment	Basal	1 μM AVP	Basal	1 μM AVP
1	3.6±0.1 (5)*	4.2±0.6 (6)	5.6±0.7 (3)	18.8±5.0 (6)
2	1.6±0.3 (3)	6.4±1.7 (5)	4.2±1.4 (4)	16.3±5.1 (6)
3	7.1±0.8 (6)	15.5±1.5 (6)	4.6±2.0 (3)	20.8±4.1 (3)
4	2.8±0.7 (4)	9.0±1.5 (5)	3.7±1.0 (5)	9.3±1.1 (5)
5	1.3±0.3 (4)	6.4±1.6 (4)	3.6±1.7 (4)	11.3±1.5 (3)
6	2.4±0.7 (4)	8.3±1.8 (3)	2.1±0.2 (4)	19.2±2.3 (4)
Mean±SEM	3.5±0.5	8.4±1.0‡	3.9±0.5	14.5±1.8‡§
n‖	26	29	23	27

Tubules were incubated in a medium containing 0.5 mM MIX, with 1 μM AVP added or without hormone (basal). Osmolality of the incubation medium was 300 mosM. All values are expressed as femtomoles cAMP per millimeter tubule length (for details see Methods).

* Denotes mean±SEM; number of samples is indicated in parentheses.

‡ Denotes values significantly ($P < 0.001$; t test) different from corresponding basal.

§ Significantly higher ($P < 0.005$; t test) than values of controls in the presence of AVP.

‖ n denotes total number of samples.

rats had higher minimal U_{osm}, suggesting that the effect of increased papillary osmolality and tubule-to-interstitium gradient is manifested under these conditions. These findings appear to be analogous to observations of Stoff et al. (33) in DI rats, and Pokracki et al. (10) on water-hydrated CPMD-treated normal rats.

The mechanism by which CPMD increases corticopapillary gradient of solutes should be briefly considered. Since CPMD increased corticopapillary gradient (Table III) of solutes in DI rats devoid of VP, the VP as a factor promoting solute gradient buildup (45, 58, 60) is obviously not involved. This feature suggests that CPMD treatment, directly or indirectly, influenced some other factor(s) in the renal countercurrent concentrating system that is essential to build up and maintain medullary and papillary hypertonicity (32, 59). The medullary and papillary hypertonicity depends on several processes, but primarily on the lumen-to-interstitium transport of NaCl in thick ascending limb of Henle's loop (32, 35); blockade of this NaCl transport in rat MAL by "loop diuretics" (61) is expected to abolish corticopapillary solute gradient (32, 59). Therefore, we considered a possibility that CPMD treatment intensified NaCl reabsorption in MAL and caused increased deposition of Na^+ and other solutes into medullary and papillary interstitium.

We addressed this question at least in an indirect way. Several recent studies (49–52) documented that oxidative metabolism of thick ascending limb of

TABLE V

Accumulation of cAMP in MCT and in MAL Microdissected from Normal (Sprague-Dawley) Rat Preincubated In Vitro for 90 min without (Controls) or with 0.1 mM CPMD

	MCT	
Experiment	Controls	CPMD-preincubated
1	28.0±3.1 (6)*	30.30±1.7 (6)
2	34.3±0.9 (5)	36.4±7.3 (6)
3	20.5±3.0 (6)	20.4±2.1 (6)
Mean±SEM	27.2±2.0	29.0±2.9
n‡	17	18
	MAL	
Experiment	Controls	CPMD-preincubated
1	2.71±0.34 (4)*	5.68±1.24 (3)
2	2.43±0.42 (6)	3.92±0.6 (6)
3	2.37±0.34 (5)	2.87±0.04 (6)
Mean±SEM	2.49±0.21	3.92±0.45§
n‡	15	15

Tubules were first preincubated with or without CPMD in medium 199 adjusted to osmolality 800 mosM with NaCl and urea for 90 min and then further incubated, with 1 μM AVP, added for an additional 10 min. All values are expressed as femtomoles cAMP for millimeter tubule length (for further details see Methods).

* Denotes mean±SEM; number of samples in parentheses.

‡ Denotes total number of samples.

§ Denotes a value significantly ($P < 0.01$; t test) different from controls.

Henle's loop (48–52) is closely coupled with NaCl cotransport (48–52), a finding supported by several lines of evidence. Blocking of luminal NaCl entry into cells by loop diuretics (48–52) and/or removal of transported ions Na^+, Cl^-, or both (48–52), causes a marked decrease in oxidative metabolism of these epithelial cells, which is manifested and detected either as decreased oxygen consumption (49–52) or decreased $^{14}CO_2$ generation from ^{14}C-labeled mitochondrial substrates (48). Our preliminary experiments (Table I) indeed indicated that the rate of oxidative metabolism coupled to NaCl cotransport could be assessed also in MAL microdissected from rat kidney by measuring $^{14}CO_2$ formation for [^{14}C]lactate (48). Another variant of the measurement of $^{14}CO_2$ production from radiolabeled substrates was successfully applied in studies of oxidative metabolism in various microdissected tubular segments, including MAL (62), showing the feasibility of this method. We found that in MAL dissected from CPMD-treated rats, the rate of [^{14}C]lactate oxidation to $^{14}CO_2$ is about doubled when compared with controls (Table VII, Fig. 2). The observation that increased $^{14}CO_2$ production from [^{14}C]lactate in MAL from CPMD-treated rats is not found when 1 mM furosemide was added indicates that the increased oxidative metabolism in MAL is indeed coupled to NaCl cotransport (Fig. 2). We therefore suggest, with all due caution concerning indirectness of this evidence, that our findings are compatible with the possibility that CPMD treatment enhances NaCl reabsorption in MAL (Fig. 3 *C*). Consequently, the increased NaCl reab-

TABLE VI

Activity of AdC in MCT and in MAL from Control DI Rats, and from DI Rats Treated with CPMD

	MCT					
	Controls			CPMD-treated		
	Basal	0.2 nM AVP	1 μM AVP	Basal	0.2 nM AVP	1 μM AVP
Mean±SEM	134.8±12.9	196.5±16.3	1762.0±164.5	102.5±7.9	226.5±20.1	1734.7±134.9
*n**	17	18	18	18	15	18
	MAL‡					
	Controls			CPMD-treated		
	Basal	0.2nM AVP	1 μM AVP	Basal	0.2 nM AVP	1 μM AVP
Mean±SEM	42.7±4.8	49.1±8.7	604.0±72.6	30.8±5.1	41.5±3.7	606.0±62.1
*n**	17	16	18	17	14	15

Enzyme activity is expressed in femtomoles cAMP/30 min per mm tubule length. Incubation mixture had osmolality 300 mosM; for further details, see Methods.

* *n* denotes total number of samples from three experiments. None of the values was significantly different between controls and corresponding values of CPMD-treated rats.

‡ All values in MAL were significantly ($P < 0.001$; t test) lower than corresponding values in MCT.

TABLE VII

Oxidation of [^{14}C]Lactate to $^{14}CO_2$ in MAL of Control DI Rats, and DI Rats Treated with CPMD

Experiment	Controls	CPMD-treated
1	2,010±304 (6)*	3,432±723 (5)
2	1,857±804 (3)	2,278±387 (6)
3	1,375±246 (6)	1,722±267 (8)
4	1,359±202 (8)	4,220±609 (8)
Mean±SEM	1,598±168	2,903±312‡
n§	23	27

Results are expressed in femtomoles of $^{14}CO_2$ generated from [^{14}C]lactate per 60 min per millimeter of tubule length. For further details, see Methods.

* Mean±SEM femtomoles; number of samples is in parentheses.

‡ Significantly ($P < 0.005$; *t* test) different from controls.

§ Total number of samples.

sorption in MAL results in an enhanced deposition of Na^+ in medullary interstitium and, according to currently accepted models of countercurrent concentrating system (32, 59) will also result in increased corticopapillary gradient of solutes (Table III). Our interpretation is also compatible with our finding (Table III) that increased total papillary osmolality in CPMD-treated DI rats is mainly contributed to by enhanced content of NaCl (>60%) rather than urea (<10%).

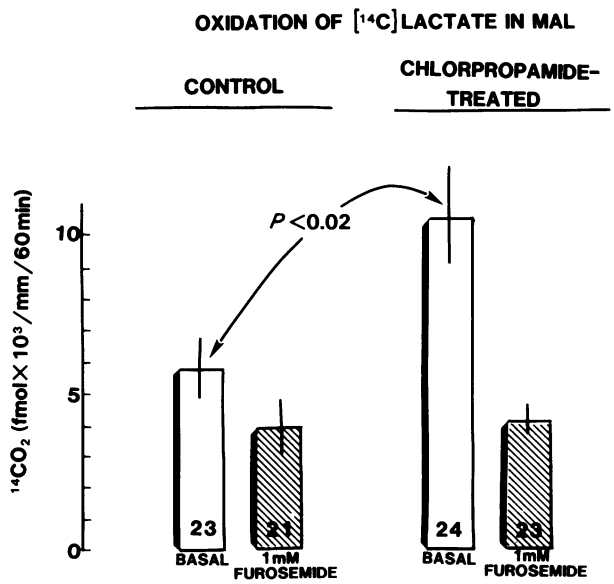

FIGURE 2 $^{14}CO_2$ formation from [^{14}C]lactate in MAL dissected from control DI rats and from chlorpropamide-treated DI rats. Each bar represents mean±SEM the number of samples (from three independent experiments) indicated at the bottom of the columns. (□): $^{14}CO_2$ production without the addition of an inhibitor. (▨): $^{14}CO_2$ production in the presence of maximum inhibitory concentration (1 mM) of furosemide. The rate of $^{14}CO_2$ production in MAL from chlorpropamide-treated DI rats was significantly (*t* test, $P < 0.02$) higher compared with control DI rats. The rate of $^{14}CO_2$ production in the presence of furosemide was not significantly different between the two groups.

Micropuncture (63) and microperfusion (64) studies indicated that PG, namely PGE_2, can inhibit NaCl reabsorption in MAL; moreover, the question of the role of PG was raised in studies of CPMD action conducted on amphibian urinary bladder (19, 24). We found no evidence that treatment with CPMD in vivo would inhibit PG synthesis in renal medulla and papilla. It is noteworthy that the effect of CPMD treatment—in terms of enhanced $^{14}CO_2$ formation from [^{14}C]lactate in MAL—persists in extensively rinsed microdissected tubules; in addition, epithelial cells of MAL are virtually devoid of PG-synthetizing capacity (54). Finally, two different sulfonylureas, CPMD and glyburide (24), both inhibited PG synthesis and potentiated the effect of VP in urinary bladder in vitro in an identical way (24). However, in contrast to CPMD, glyburide (8, 25–27) does not potentiate VP and rather causes diuresis in man and in experimental animals (8, 25–27). All these considerations suggest that, unlike in amphibians in vitro, the inhibition of PG synthesis is not a basis of CPMD actions on mammalian kidney observed in vivo.

Analysis of VP-sensitive cAMP metabolism in MCT brings no evidence for the proposition that CPMD treatment increases antidiuretic effect of VP by sensitizing collecting tubules to the VP action at the step of cAMP generation (Tables IV–VI). Assuming that the parts of the collecting tubule system adjacent proximally and distally to MCT behave in a similar way as MCT does, our observations suggest that CPMD does not potentiate the hydroosmotic effect of VP in mammalian collecting tubules, at least not at the step of cAMP generation. In contrast to the present findings, we have shown in our preceding studies that well-known PG cyclo-oxygenase inhibitors enhanced the stimulation of AdC in MCT by VP (54). Admittedly, our results do not rule out the theoretical possibility, for which there is currently no evidence, that CPMD potentiates action of VP in collecting tubules at the steps subsequent to cAMP generation.

Enhanced accumulation of cAMP in MAL observed in response to VP (Tables IV, V) is of interest from two points of view. First, numerical prevalence of MAL (compared with MCT) in the rat kidney medulla (12, 31) may explain why in some earlier studies the enhanced VP-dependent cAMP formation was observed in whole renal medullary tissue preparations from CPMD-treated animals (5, 14, 18) or in medullary slices preincubated with CPMD (16), even when VP-dependent cAMP generation in MCT is not in-

creased (Tables IV–VI). It is not clearly evident by which mechanism the VP-dependent cAMP accumulation in MAL is increased. Increased sensitivity of AdC is possible, but we found enhanced stimulation of AdC in MAL by AVP only under certain testing conditions (hypertonic medium). It is plausible that cellular factor(s) other than enzyme activities per se (12, 13), such as increased availability of substrate (ATP) or of cofactor(s) (guanosine triphosphate, Mg) may account for a higher rate of cAMP formation in situ.

Second, VP was shown recently to stimulate NaCl reabsorption in MAL (65–67). Therefore, CPMD treatment, by enhancing the VP-dependent cAMP accumulation in MAL, may possibly further stimulate NaCl reabsorption in this tubular segment. However, it should be stressed that CPMD treatment increased corticomedullary gradient of solutes (Table III, Figs. 1 and 3) and enhanced NaCl cotransport-coupled [^{14}C]lactate oxidation in MAL (Fig. 2, Table VII) even in the total absence of VP in DI rats. Therefore, the observed effects of CPMD treatment to increase papillary tonicity, a putative basis of the enhanced antidiuretic response to VP (Fig. 3), could and should occur even in the kidneys where the cAMP system of MAL is insensitive to VP, as hitherto appears to be the case of the human kidney (68).

The results of the present studies taken in aggregate provide a basis for the following working hypothesis concerning CPMD action in the mammalian kidney (Fig. 3). Most likely, the main effect of CPMD treatment is an increase in corticopapillary gradient of solutes, namely sodium; this effect is independent of VP. Our finding of CPMD-enhanced NaCl cotransport-coupled [^{14}C]lactate oxidation in MAL suggests that an increased rate of NaCl reabsorption in MAL is a primary mechanism for the buildup of greater medullary and papillary hypertonicity. This increased lumen-to-interstitium NaCl transport (Fig. 3 *A*, *C*) in MAL leads secondarily to enhanced deposition of urea (32, 59), according to current models of renal countercurrent concentrating mechanism (32, 59). Sensitivity of collecting tubules to hydroosmotic action of VP at the cAMP step is not enhanced by CPMD. As a result of CPMD treatment, the higher lumen-to-interstitium transepithelial osmotic gradient in collecting tubules provides greater osmotic driving force for water reabsorption (Fig. 3 *C*) but this can be manifested on ad lib. water intake only when exogenous or endogenous VP increases water permeability of the collecting tubule wall (Fig. 3 *D*). The proposed mechanism accounts for the fact that in the total absence of VP, as in DI rats or in DI patients, CPMD alone does not increase U_{osm} or decrease urine flow, since it neither mimics nor potentiates VP effect on water permeability of collecting tubules (Fig. 3 *C*). Increased interstitial tonicity thus causes that the same quantum of VP administered or released into the circulation, which increases water permeability of collecting tubules to the same degree, will result in greater antidiuresis, i.e., higher U_{osm} and lower urine flow, in the CPMD-treated kidney than in the nontreated kidney (Fig. 3 *B* and *D*).

In spite of its stimulation of NaCl reabsorption in MAL, CPMD treatment alone does not detectably decrease U_{osm} or increase urine flow (1, 3–5) in the final urine (Table II, Fig. 3 *A*, *C*). The most likely explanation is a secretion of NaCl into lumen of distal cortical convoluted tubules documented in a recent study (69), which causes that hypotonicity of tubular fluid achieved in a "diluting segment" (mainly MAL) cannot be maintained in distal cortical segments. Accordingly, although enhanced NaCl reabsorption in MAL contributes by buildup of medullary and papillary osmolality to greater antidiuretic response to VP, it does not enhance in the same time dilution of final urine.

Many points pertaining to the proposed CPMD action on urine concentration still remain to be clarified. In particular, it remains to be determined in future studies whether CPMD always acts on tubules directly, or through some secondary mechanism(s) after its administration in vivo. The question naturally arises whether renal effects of CPMD may be a consequence of its action on insulin secretion and hypoglycemia. Sulfonylureas other than CPMD, i.e., tolazamide, glyburide, and acetohexamide have hypoglycemic and other metabolic effects analogous to CPMD, but these compounds do not enhance VP-induced antidiuresis; on the contrary, they are diuretic in DI rats and DI men (8, 25–27). These comparisons argue strongly, albeit indirectly, against the notion that hypoglycemic action of CPMD is the basis of the observed renal effects. However, elucidation of molecular mechanism of CPMD effect will require future detailed studies. Also, the proposed effect of CPMD to stimulate NaCl reabsorption in MAL (Fig. 3) ought to be confirmed in direct studies on isolated MAL. Finally, a theoretical possibility that CPMD might increase renal tissue tonicity also through an effect on medullary blood flow (32, 59) or might act on other tubular sites (8) remains to be explored.

Thus, in conclusion, the present study suggests that CPMD increases antidiuretic effect of VP in mammalian kidney by increasing medullary and papillary osmolality, primarily by acting on thick ascending limb of Henle's loop. The proposed mechanism of CPMD action, if validated by future studies, could lead to search for more potent and more specific compounds that would stimulate selectively NaCl transport

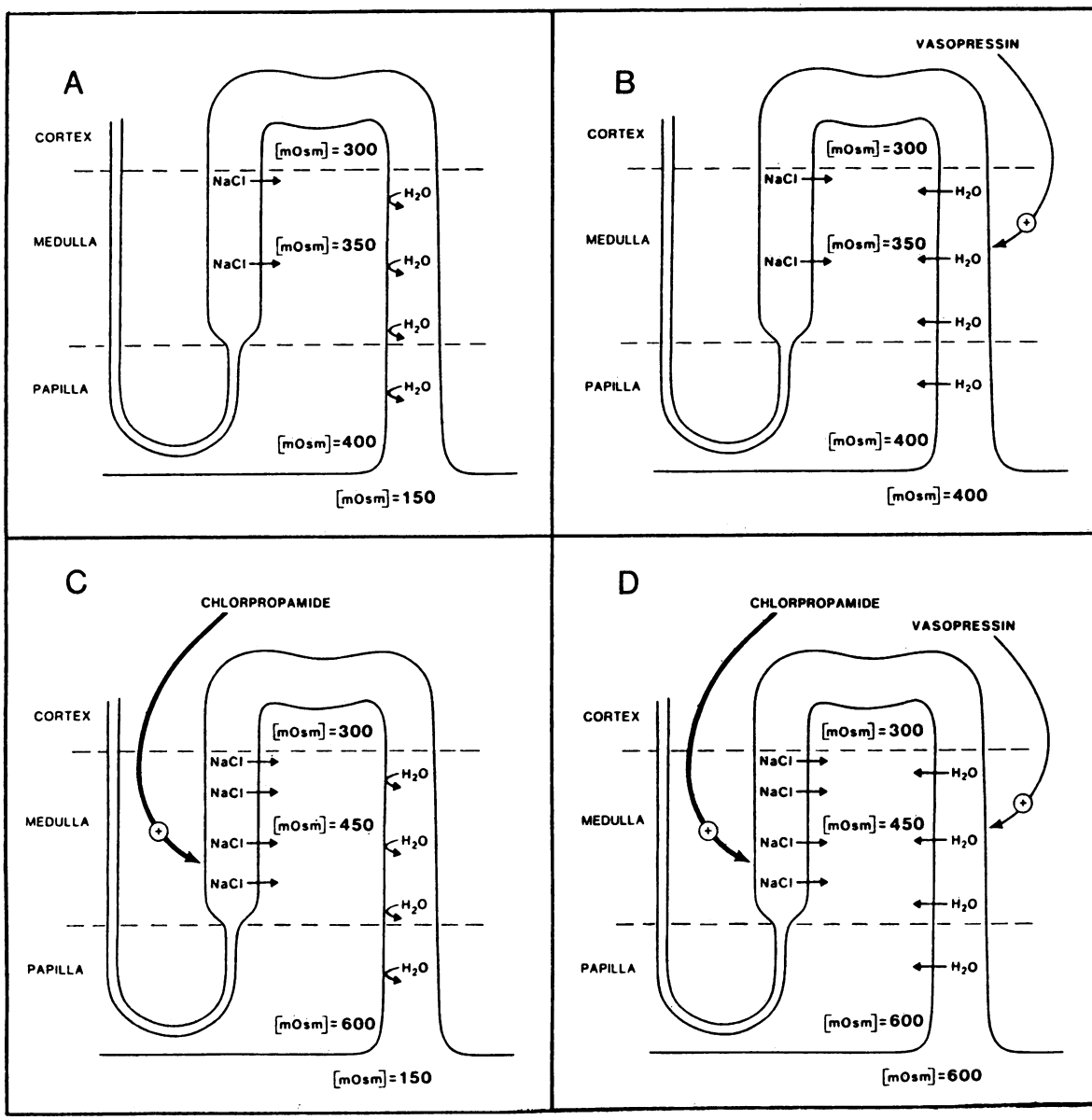

FIGURE 3 Schematic outline of the proposed mechanism by which CPMD enhances antidiuretic response to VP in hypothalamic diabetes insipidus. The small but distinct corticomedullary gradient of solutes is maintained in DI rats (Table III), even in the total absence of VP (45, 58) primarily by NaCl reabsorption in MAL. (*A*) In the absence of VP tubular fluid cannot equilibrate osmotically with interstitium since collecting tubules are virtually impermeable to H_2O; as a consequence the final urine is hypotonic (Table II). (*B*) The administration of VP increases permeability of collecting tubule to water permitting equilibration and resulting in hypertonic urine (1, 3–5); maximal U_{osm} is limited by osmolality of papillary interstitium. (*C*) Administration of CPMD causes an increase of NaCl reabsorption from MAL into interstitium and consequently an increase of medullary and papillary osmolality (Table III). However, in the absence of VP virtual impermeability of collecting tubules to water does not allow osmotic equilibration and, in spite of higher papillary tonicity, the urine remains hypotonic (compare with *A*). (*D*) VP is administered to CPMD-treated DI animals, in which corticopapillary gradient of solutes is increased (as in *C*). VP increases water permeability of collecting tubules, permits osmotic equilibration and results in generation of hypertonic urine. The higher osmotic driving force (collecting tubule lumen-to-interstitium osmotic gradient) results in higher U_{osm} and lower urine flow (1, 3–5, 8) compared with VP effect without CPMD treatment (*B*).

in MAL—"loop antidiuretics." CPMD and other putative compounds with analogous mode of action could be of potential use in the treatment of those urinary concentrating defects due to washout of medullary and papillary solutes (32, 59, 70).

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