Endogenous Prostaglandin E_2 Mediates Inhibition of Rat Thick Ascending Limb Cl Reabsorption in Chronic Hypercalcemia

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Abstract

The hypothesis that endogenous PGE_2 mediates defective thick ascending limb (TAL) Cl reabsorption (percent delivered load: FR_{Cl}%) in rats with vitamin D–induced chronic hypercalcemia (HC) was tested by measuring FR_{Cl}% in loop segments microperfused in vivo in HC and control rats treated acutely with indomethacin (Indo) or its vehicle, and obtaining the corresponding outer medullary [PGE_2]. Microperfusion conditions were developed in which FR_{Cl}% was exclusively furosemide sensitive. To determine the cellular mechanism, tubules were perfused acutely with forskolin (FSK), cAMP, or the protein kinase C inhibitor staurosporine (SSP). Outer medullary [PGE_2] in HC rats was 9 to 10 times greater than control and could be normalized by Indo. FR_{Cl}% was 20% lower in HC rats infused with vehicle, and Indo, FSK, and cAMP returned FR_{Cl}% to normal despite sustained HC. Indo or FSK had no effect on FR_{Cl}% in control rats and Indo did not prevent inhibition of FR_{Cl}% by luminal PGE_2 (1 μM). Luminal SSP (10^{-7}, 10^{-8} M) in HC did not return FR_{Cl}% to control values. We conclude that impaired TAL FR_{Cl}% in HC occurs at a pre-cAMP site and is due to endogenous PGE_2 and not to HC. (*J. Clin. Invest.* 1993. 91:2399–2407.) Key words: loop of Henle • indomethacin • prostaglandins • transport • kidney

Introduction

There is ample evidence from in vitro studies that PGE_2 can antagonize arginine vasopressin (AVP)[1] stimulation of thick ascending limb (TAL) NaCl reabsorption (1–4). More recently, PGE_2 has been shown to specifically inhibit transport-related ouabain-sensitive oxygen consumption in rabbit TAL cells (5), and decrease the NaK-ATPase activity of isolated medullary TAL (mTAL) in the rat (6). The mTAL should be exposed to PGE_2 synthesized by outer medullary interstitial cells and collecting ducts (3). In addition, TAL cells have been shown to have a limited capacity to synthesize PGE_2 (3, 5). One earlier free-flow micropuncture study indicated an effect of endogenous prostaglandins on loop of Henle Cl reabsorption in volume-expanded (10% body weight) animals (7). Interpretation of the results of these experiments, which rely exclusively on the use of cyclooxygenase inhibition, is complicated since arachidonic acid can be metabolized by the outer medulla via the cytochrome P-450 pathway (8, 9) and perhaps the lipoxygenase pathway (10). Metabolites of the P-450 pathway have been shown to inhibit ion transport in the TAL in vitro (11). Indomethacin (Indo) at high concentrations (10^{-4} M) has been shown to inhibit all three metabolic pathways (12). Due to the presence of the countercurrent system in the renal medulla, estimation of drug concentrations in vivo can be problematic. Therefore, despite significant research efforts in the area of PGE_2–AVP interactions, there is no evidence that chronic activation of renal PGE_2 production can cause inhibition of TAL transport, and there are no pathophysiological conditions in which inhibition of TAL NaCl reabsorption can be attributed to altered renal PGE_2 metabolism (3, 4, 13).

Chronic hypercalcemia (HC) in man and animals is associated with disturbances in water intake, renal concentrating ability, and loop of Henle function (14–16). Recently, in functionally isolated perfused loops of Henle using a microstop-flow technique, chronic hypercalcemia in rats was shown to be associated with inhibition of TAL NaCl reabsorption (14). What is the mechanism of impaired TAL NaCl reabsorption in chronic hypercalcemia? There is evidence from in vitro studies that acute increases in extracellular [Ca^{2+}] can directly inhibit AVP stimulated production of cAMP and thus could mediate the transport defect (17). In acute hypercalcemia, loop Cl reabsorption is decreased by an indomethacin-resistant mechanism (18). However, two previous studies have shown that renal PGE_2 excretion is strikingly elevated in rats with chronic hypercalcemia (15, 19). Evidence was provided from one of these studies that elevated PGs may underlie the AVP-resistant concentrating defect (19), however, the results of the other study did not support this hypothesis (15). It is possible that elevated PGE_2 and not the hypercalcemic state impairs TAL transport. Although renal excretion of PGE_2 is increased in rats with chronic hypercalcemia, the source of the PGE_2 is unknown. For example, given the capacity of inner medulla to synthesize PGE_2 (3), it is possible that PGE_2 is not elevated in the outer medulla and may not have a major impact on TAL function.

Therefore, the present study tested the hypothesis that endogenous PGE_2 mediates impaired TAL NaCl reabsorption in functionally isolated perfused nephrons in vivo in chronic hypercalcemia. Microperfusion conditions were developed in which Cl reabsorption was exclusively furosemide sensitive. TAL Cl reabsorption was measured in loop segments micro-

Parts of the results of this paper have appeared in abstract form: 1990. *Clin. Invest. Med.* 13(4):B75; 1992. *Clin. Invest. Med.* 15(4):A93; 1991. *J. Am. Soc. Nephrol.* 2(3):748; and 1992. *J. Am. Soc. Nephrol.* 3(3):457.

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Received for publication 24 October 1992 and in revised form 12 January 1993.

1. *Abbreviations used in this paper:* AVP, arginine vasopressin; dDAVP, 1-[desamino]-8-D-arginine vasopressin; DHT, dihydrotachysterol; ECF, extracellular fluid; FR_{Cl}%, fractional chloride reabsorption percent; FSK, forskolin; HC, hypercalcemia; Indo, indomethacin; mTAL, medullary thick ascending limb; PKC, protein kinase C; SSP, staurosporine; TAL, thick ascending limb.

J. Clin. Invest.

0021-9738/93/06/2399/09 $2.00
Volume 91, June 1993, 2399–2407

perfused in rats with chronic HC and pair-fed control rats treated with Indo or its vehicle. In hypercalcemic and control rats prepared under similar conditions, PGE_2 was extracted from the outer medulla and measured by RIA. Hypotheses regarding the underlying cellular mechanism by which TAL Cl reabsorption is inhibited in chronic HC were tested using luminal addition of forskolin (FSK), cAMP, and the protein kinase C inhibitor staurosporine (SSP). Further microperfusion experiments were performed to determine if Indo impairs the ability of PGE_2 to inhibit TAL transport in normal rats.

Methods

Studies were carried out using 109 male Sprague-Dawley rats weighing 280–350 g. Chronic HC was induced by feeding a normal electrolyte diet containing 4.25 mg/kg diet of dihydrotachysterol (DHT), a vitamin D analogue, for 4–6 d (14). Control measurements were made in rats pair fed (7 g/100 g body wt per d) the identical normal diet without DHT (14). All rats were given tap water to drink. In the microperfusion experiments to examine the effects of luminal furosemide or PGE_2, animals were fed standard rat chow.

Microperfusion studies. Rats were anesthetized with Inactin 100 mg/kg body wt, i.p. and prepared for microperfusion studies as previously described (14); a brief summary follows. Right and left jugular veins were cannulated to allow infusion of solutions, anesthetic maintenance, and lissamine green. The left carotid artery was cannulated to monitor blood pressure and to allow for blood collections. A tracheostomy was performed. The left kidney was exposed, placed in a lucite cup, and warmed agar was placed around the kidney to form a well. The kidney was bathed in water-equilibrated paraffin oil. The ureter was cannulated for urine collections. Rats were infused intravenously at 1% body wt/h with 0.9% NaCl containing either Indo (5 mg/kg per h) or 0.14% Na_2CO_3 as a vehicle, after receiving a loading dose of either 5 mg/kg Indo or the corresponding amount of vehicle. Rats were infused with 0.9% NaCl without Indo or vehicle in microperfusion studies with furosemide, FSK, cAMP and SSP. Each rat was infused intravenously with Plasmanate (5% human plasma protein) in buffered saline at the rate of 1 ml/h except in the microperfusion studies with luminal furosemide. To ensure similar circulating levels of AVP, the animals were given a loading dose of 45 pg of the V_2 agonist, 1-[desamino]-8-D-arginine vasopressin (dDAVP), and the analogue was added to the Plasmanate infusion to deliver 45 pg/min (20). An arterial blood sample for the measurement of ionized calcium [Ca^{2+}] was taken at completion of the surgery, which usually required 25 min. Exclusion criteria for hypercalcemic rats was a blood [Ca^{2+}] $\geq$ 1.5 mM/liter and for normocalcemic rats was a blood [Ca^{2+}] $\leq$ 1.32 mM/liter. Reported blood [Ca^{2+}] was corrected to pH 7.40. In separate studies, to ensure that blood [Ca^{2+}] did not change in HC rats during the microperfusion experiments with Indo, [Ca^{2+}] was measured in HC rats treated with Indo or its vehicle (5 rats per group) at the time when microperfusion studies were initiated (t = 120 min) and then again 3 h later. For all studies, data were derived only from animals with mean arterial pressure $\geq$ 95 mmHg and a normal transit time of lissamine green dye (14).

After a 120-min equilibration period measured from the time of initiation of the systemic infusions, microperfusion of the loop of Henle was performed. A micropipette filled with perfusate stained with 0.05% FD&C Green No. 3 was inserted into surface proximal tubules at random and perfusion started and stopped to determine the latest surface proximal and earliest surface distal sites. Loops of Henle that had early distal nephron segments with the characteristic appearance of the distal convoluted tubule were selected for study. The same perfusion pipette was placed into the latest accessible surface proximal and perfused at 22 nl/min with a calibrated perfusion pump (Wolfgang Hampel, Neu-Isenburg, Germany). The loop perfusate was designed to minimize the contribution of the proximal tubule to Cl reabsorption by approximating equilibrium [NaCl] for this nephron segment (21). Mannitol was added to bring the osmolality to 300 mosmol/kg H_2O to impair water reabsorption in loop segments upstream from the TAL. A pipette was inserted proximal to the perfusion pipette and a sudan black–stained paraffin oil block was injected. The proximal segment upstream from the oil block was vented. The loop was perfused for 20 min before collections were made at the early distal site in studies in which PGE_2, FSK (22), and the protein kinase C inhibitor, SSP (23) were used. When 8-*p*-CPT-cAMP (cAMP) was used, the loop was perfused for 30 min. In all other studies, including those in which furosemide was added to the luminal perfusate, loops were perfused for 1–2 min to ensure satisfactory flow before a collection was obtained. At this time a sudan black–stained oil-filled collection pipette was inserted into the earliest distal tubule site, an oil block was injected, and a complete timed collection was obtained. In all experiments, samples were collected for a minimum of 1.5 min and a maximum of 3 min.

The perfusate consisted of the following (in mM): 93 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 1 NaH_2PO_4, 1 $CaCl_2$, 4 urea, 6 NaGluconate, and 61 mannitol. Sufficient carboxy [^{14}C]inulin that had been exhaustively dialyzed was added to yield 80–100 cpm/nl. In selected experiments the following additions to the perfusate and the resulting concentrations were: 1 mM furosemide, 0.1 mM FSK, 1 mM cAMP, (1) 10^{-7} and 10^{-8} M SSP, and 1 μM PGE_2 (1). PGE_2 was dissolved in 95% ethanol and underwent serial dilutions to obtain the desired concentration. The corresponding amount of ethanol vehicle was added to the perfusate for the control studies. SSP was dissolved in dimethylsulfoxide and FSK was dissolved in 95% ethanol; both were serially diluted in 0.9% saline to obtain the desired concentration in the perfusate.

Determination of outer medullary [PGE_2]. In these studies, rats were rendered hypercalcemic or pair fed the control diet for 5 d (5 rats per group). They were injected intraperitoneally with 2.7 ng dDAVP and Indo (5 mg/kg) or the corresponding amount of vehicle 2 h, 1 h, and 10 min before they were anesthetized. An arterial line was inserted and a sample taken for blood [Ca^{2+}]. The left kidney was rapidly removed, bisected, and placed in ice-cold Hanks' buffer containing 0.1 mM Indo (24). The time required to complete the process was < 10 s. Preliminary work indicated that unless both kidneys were removed simultaneously, PGE_2 increases dramatically in the kidney that is removed last. The inner medulla was rapidly excised, the cortex removed, and lastly the outer medulla carefully dissected on frozen buffer. The tissue was predominantly from the inner stripe of the outer medulla. It was transferred into preweighed plastic tubes containing 1 ml of Hanks' buffer with 0.1 mM Indo and weighed. Approximately 100 mg of outer medullary tissue was obtained from each kidney. The sample was homogenized in a small glass silanized tube using a teflon plunger for ~ 1 min. The homogenizer was cleaned, dried, and resilanized between samples. The homogenate was acidified with 1 N HCl (100 μl) and centrifuged at 4°C, 1,180 relative centrifugal force (g) for 30 min. The supernatant was extracted using Bond Elut C18 columns according to the procedure outlined in the RIA kit. Greater than 95% of [^{3}H]PGE_2 added to the homogenate could be recovered from the C18 column. The methanol extract was stored at −70°C for up to 2 wk. Approximately 100 μl was removed for the RIA, dried down at room temperature under a stream of nitrogen, and reconstituted in 1 ml of assay buffer. Each RIA measurement was performed in duplicate.

Analytical methods and calculations. Blood [Ca^{2+}] and pH were measured by ion-specific electrodes (model 634, Stat Analyzer; Ciba Corning, Medfield, MA). PGE_2 was measured by RIA. Volumes of collected tubular fluid samples during the microperfusion studies were measured using a calibrated constant-bore quartz glass capillary. A constant volume of perfusate and collectate for [^{14}C]inulin determination was counted in a liquid-scintillation spectrometer (model LS 3801; Beckman Instruments, Inc., Fullerton, CA) in scintillation fluor and appropriate quench corrections were applied. The [Cl] in the collected samples and the perfusate was measured by coulometric titration using a microtiter (model ET-1; World Precision Instruments, New Haven, CT). Perfusate samples were analyzed in the same manner as

the collected samples. The perfusion rate in vivo (PR) was calculated by the expression:

$$PR\ (nl/min) = CR\ (nl/min) \times C/P\ [Inulin],$$

where CR is the collection rate at the distal site and C/P [Inulin] is the ratio of [^{14}C]inulin in the collected sample dpm to [^{14}C]inulin in the perfusate. To eliminate samples contaminated by the addition of surface fluid and those samples obtained from loops in which the perfused segment included more distal portions of the nephron, data were derived only from experiments in which the C/P [Inulin] ratios were ≥ 0.95 and ≤ 1.15. In preliminary studies, a high correlation was found between elevation of C/P [Inulin] to values over 1.5 with high rates of Cl reabsorption when fluid was intentionally collected from sites in the cortical collecting duct. Fractional Cl reabsorption (FR_{Cl}%) was calculated as:

$$FR_{Cl}\% = [([Cl]_P \times PR) - ([Cl]_C \times CR)]/([Cl]_P \times PR),$$

where $[Cl]_P$ and $[Cl]_C$ are the [Cl] in the perfusate and collected sample, respectively. Chloride delivery was calculated as $PR \times [Cl]_P$. The absolute rate of Cl reabsorption, J_{Cl} was calculated as:

$$J_{Cl}\ (pmol/min) = ([Cl]_P \times PR) - ([Cl]_C \times CR),$$

where $[Cl]_P$ and $[Cl]_C$ are defined as above.

Student's *t* test for paired data was used to compare blood [Ca^{2+}] measured in the same animal. When only two treatment groups were involved in the experiment, Student's *t* test for unpaired data was used. The results presented in Table II and Figs. 2 and 4 were obtained from experiments designed to test specific hypotheses. To determine the statistical significance of differences between planned contrasts between the experimental groups and the untreated control group (vehicle treated), Dunn's procedure using Student's *t* distribution and the *t* statistic (also referred to as the Bonferroni *t* procedure) was used (25). For a single planned contrast in Table II and Fig. 2 to achieve significance at $P < 0.05$, the calculated value of *P* from the unpaired *t* test had to be < 0.00625 (i.e., 0.05/8 [the number of contrasts]. Although not required, one-way ANOVA was used to confirm that the between group variance exceeded the within group variance. The results of the one-way ANOVA are reported in the table and figure legends. To assess the statistical significance of the separate effects of dietary pretreatment and Indo on FR_{Cl}%, and outer medullary [PGE_2], multivariate analysis of variance was performed using the data from these four treatment groups. To determine if perfusion rate, collection rate, C/P [Inulin], $[Cl]_P$, Cl delivery, and $[Cl]_C$ were significant determinants of Cl reabsorption, linear-regression analysis was performed relating each of these factors to the corresponding Cl reabsorption, expressed as FR_{Cl}%, measured in the same loop segment. Data reported in Tables I–III were analyzed separately. $P < 0.05$ was considered statistically significant.

Reagents. Reagents were purchased or obtained from the following sources: normal electrolyte diet with and without DHT, ICN Nutritional Biochemicals (Cleveland, Ohio); DHT, Roxane Laboratories (Columbus, Ohio); standard rat chow, Purina, Woodstock, Ontario, Canada; Inactin anesthetic, BYK Gulden, Konstanz, FRG; FSK, 8-CPT-cAMP, PGE_2, and Indo Sigma Chemical Co. (St. Louis, MO); SSP, Kyowa Hakko USA (New York, NY); Furosemide, Abbott Laboratories (Montreal, Quebec, Canada); [^{14}C]inulin and Formula 963 scintillation fluor, New England Nuclear (Montreal, Quebec, Canada); Plasmanate, Cutter Laboratories (Berkeley, CA); vasopressin analogue, dDAVP, Bachem, California (Torrance, CA); FD & C Green No. 3 dye, Keystone Aniline and Chemical Co. (Chicago, IL); PGE_2 RIA assay kit (NEN KitNEK-020; New England Nuclear) and Bond Elut C18 columns, Varian Associates Inc. (Palo Alto, CA).

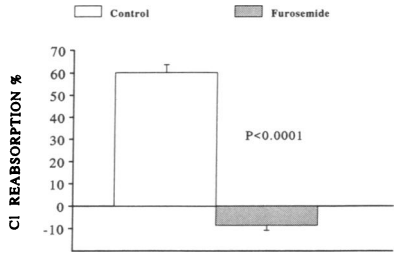

Figure 1. Effect of 1 mM furosemide addition to the luminal perfusate to abolish net Cl reabsorption in the functionally isolated perfused loop segment in control rats in vivo. Cl reabsorption is expressed as a percent of delivered Cl. Number of tubules per group is given in Table I. *P* value calculated using Student's *t* test for unpaired data.

Results

Assessment of furosemide-sensitive Cl reabsorption

Data collected during these microperfusion experiments obtained in normal rats are summarized in Table I and Fig. 1. In all the microperfusion studies, the microperfusion pump was set to deliver 22 nl/min. The actual perfusion rate calculated from the [^{14}C]inulin delivery is quite close to this value (Table I). It is also clear that the standard loop perfusate used in our studies virtually abolished fluid reabsorption by the loop segment. This fact is evident by comparing the volume perfused and the volume collected per min as well as the C/P [Inulin] ratio. To assess the contribution of the TAL to net Cl reabsorption under these experimental conditions, the fraction of loop Cl reabsorption that could be inhibited by the addition of furosemide (1 mM) to the luminal perfusate was determined. As documented in Table I, in control rats the loop segment reabsorbed 1,325±104.8 pmol/min Cl compared with a secretory flux of −179±53.6 when furosemide was added to the loop perfusate. The striking inhibition of transport was also reflected in the [Cl] of the collected fluid, which was 41.1±3.56 mM in the control condition and 104±4.44 mM in the loops exposed to furosemide. The $[Cl]_C$ of the collected fluid in the presence of furosemide was not significantly different than the

Table I. Effect of Luminal Furosemide on Cl Reabsorption in the Functionally Isolated Microperfused Loop Segment in Normal Rats

	Tubules	Perfusion rate	Collection rate	C/P* [Inulin]	Perfusate [Cl]	Chloride delivery	Collected [Cl]	J_{Cl}
		nl/min	*nl/min*		*mM*	*pmol/min*	*mM*	*pmol/min*
Control	7	22.2±0.52	21.1±0.48	1.07±0.013	96.8±0.83	2,185±55.1	41.1±3.56	1,325±104.8
		$P > 0.37$	$P > 0.60$	$P < 0.005$	$P > 0.96$	$P > 0.33$	$P < 0.001$	$P < 0.0001$
Control + furosemide (1 mM)	4	21.3±0.84	21.5±0.64	0.98±0.015	96.8±2.12	2,068±126.6	104.1±4.44	−179±53.6

P, probability value calculated using Student's t test for unpaired data. * Ratio of [^{14}C]inulin in the collected sample to the perfusate.

Table II. Microperfusion Data Collected from Functionally Isolated Loop Segments In Vivo During Experiments using Systemic Indo Administration with the Standard Loop Perfusate or Luminal Forskolin in HC and Control Rats and 1-8-CPT cAMP or staurosporine in HC rats

	Tubules	Perfusion rate	Collection rate	C/P* [Inulin]	Perfusate [Cl]	Chloride delivery	Collected [Cl]	J_{Cl}
		nl/min	*nl/min*		*mM*	*pmol/min*	*mM*	*pmol/min*
Control + vehicle	11	22.8±0.30	22.5±0.34	1.01±0.015	98.8±0.87	2,251±33.4	45.0±1.75	1,237±42.5
Control + Indo	9	22.4±0.51	21.3±0.81	1.06±0.018	98.9±1.24	2,199±64.9	43.8±3.39	1,273±67.3
Control + FSK (0.1 mM)	6	21.8±0.38	20.5±0.48	1.07±0.19	105.7±1.57	2,303±68.4	44.2±2.66	1,410±93.9
HC + vehicle	12	22.8±0.27	21.5±0.49	1.07±0.018	97.4±0.20	2,212±29.2	57.8±2.22§	972±52.5‖
HC + Indo	11	21.3±0.34	20.3±0.50‡	1.09±0.011§	99.6±1.62	2,159±63.6	46.8±2.15	1,225±62.5
HC + FSK (0.1 mM)	6	22.5±0.78	20.4±0.88	1.11±0.019§	103.1±0.67	2,315±89.4	53.9±3.06	1,210±67.1
HC + cAMP (1 mM)	7	22.5±0.24	21.6±0.18	1.05±0.015	101.6±1.44	2,292±23.5	50.6±4.73	1,201±107.9
HC + SSP (10^{-7} M)	7	22.5±0.36	22.3±0.61	1.01±0.018	103.3±0.08§	2,327±37.2	59.4±2.32‖	998±70.1¶
HC + SSP (10^{-8} M)	5	21.8±0.57	19.4±0.73§	1.09±0.024	99.9±1.41	2,142±55.7	60.8±4.35‖	954±58.9‡
ANOVA**		NS	<0.010	<0.005	<0.001	NS	<0.0001	<0.0005

* Defined in Table I. ‡ $P < 0.05$, § $P < 0.01$, ‖ $P < 0.001$, ¶ $0.05 < P < 0.1$, vs. Control + vehicle, calculated using Dunn's Procedure with the t statistic for planned contrasts. ** Significance level of the ANOVA with nine groups.

perfusate $[Cl]_P$ ($P > 0.10$, Table I). The perfusion rates, collection rates, [Cl] of the perfusate, and Cl delivery to the loop segment were not significantly different between the control and furosemide groups, however, a statistically significant difference in the concentration ratio of [^{14}C]Inulin in the collected and perfused fluid was observed. If the extent of fluid reabsorption by the loop was the major determinant of Cl reabsorption and not the presence of furosemide, then one would predict the existence of a significant correlation between J_{Cl} or FR_{Cl}% and C/P [Inulin]. In this, and in all the subsequent microperfusion studies, there was no correlation between the C/P [Inulin] with J_{Cl} or FR_{Cl}%. In every case, the slopes of the lines were not statistically significant and the R^2 value for these regression analyses was ≤ 4.7%. This was also the case for the perfusion rate, collection rate, Cl delivery, and perfusate [Cl]. The small statistically significant differences in these variables can not account for the observed differences in Cl reabsorption. In contradistinction, as one might predict, there was a highly significant correlation between the [Cl] of the collected fluid ($[Cl]_C$) and the amount of Cl reabsorbed expressed either as J_{Cl} or FR_{Cl}%. The slope of the line relating FR_{Cl}% to $[Cl]_C$ calculated from the data presented in Table II was significant at the level $P < 0.00001$, the correlation coefficient was −0.894 and the R^2 value was 79.9%.

To reduce the variation due to differences in Cl delivery and in the length of the perfused loop segments, loop Cl reabsorption is expressed as a fraction of the amount delivered to the segment, i.e., FR_{Cl}%, in Fig. 1. Normal loops of Henle reabsorb 60±3.6% of the delivered Cl, which is reversed to a small degree of secretion −8.6±2.2 by the addition of 1 mM furosemide to the luminal perfusate.

Mechanism underlying impaired TAL Cl reabsorption in HC: effects of Indo, forskolin, cAMP, staurosporine, and PGE_2

After 5 d of consuming the DHT-containing diet, blood [Ca^{2+}] was on average 1.57±0.06 mM (SD) versus 1.29±0.06 mM (SD) in pair-fed control rats. The exclusion criteria used in the study prevented the values from overlapping. Table II and Fig. 2 summarize microperfusion data collected during experiments in which Indo or its vehicle was infused systemically, FSK was added to the luminal perfusate in control and HC rats, or cAMP or SSP was perfused through individual loops of Henle in HC rats. Table III and Fig. 3 summarize the experiments with PGE_2 in normal control rats. All of these animals were infused with dDAVP (see Methods) to ensure constant circulating levels of AVP.

Effect of indomethacin in control and HC rats. If elevated PGE_2 impairs TAL Cl reabsorption in chronic HC, then an acute reduction in PG synthesis should lead to a restoration of normal transport in this segment. In these experiments, HC and pair-fed control rats had been given a loading dose and

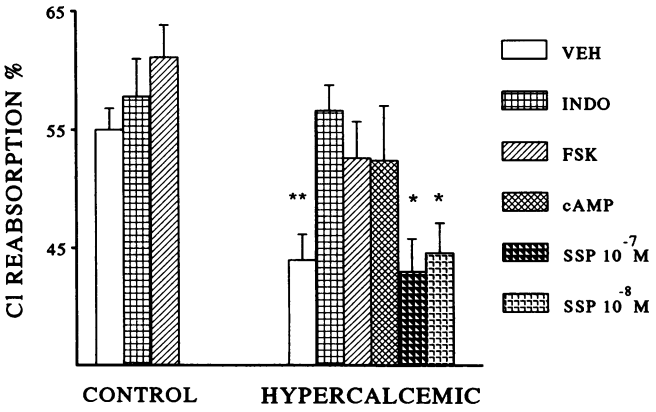

Figure 2. Effect of systemic Indomethacin (*Indo*) with the standard luminal perfusate or luminal forskolin (*FSK*, 0.1 mM) in HC and control rats and luminal cAMP (1 mM) or staurosporine (*SSP*, 10^{-7} and 10^{-8} M) in HC rats on Cl reabsorption in functionally isolated microperfused loop segments in vivo. Cl reabsorption is expressed as a percent of delivered Cl. Number of tubules per group is given in Table II. $P < 0.0001$ for ANOVA with nine groups. *$P < 0.05$, **$P < 0.01$ vs. control + vehicle using Dunn's procedure with the t statistic for planned contrasts.

Table III. Effect of 1 μM PGE_2 Addition to the Luminal Perfusate on TAL Cl Reabsorption in Indomethacin-treated Normocalcemic Rats

	Tubules	Perfusion rate	Collection rate	C/P* [Inulin]	Perfusate [Cl]	Chloride delivery	Collected [Cl]	J_{Cl}
		nl/min	*nl/min*		*mM*	*pmol/min*	*mM*	*pmol/min*
Ethanol vehicle	11	22.2±0.52	20.5±0.52	1.07±0.020	100.5±1.18	2,240±43.8	46.7±3.53	1,266±88.2
		$P > 0.63$	$P > 0.34$	$P > 0.92$	$P > 0.29$	$P > 0.28$	$P < 0.025$	$0.05 < P < 0.088$
PGE_2 (1 μM)	8	22.6±0.41	21.1±0.33	1.07±0.021	103.0±2.59	2,314±51.5	59.9±3.76	1,043±88.2

P, probability value calculated using Student's *t* test for unpaired data. * Defined in Table I.

then infused for 2 h with Indo or the Na_2CO_3 vehicle. Loops of Henle were perfused with the standard perfusate. In other HC rats pretreated in a similar manner with Indo or vehicle, blood $[Ca^{2+}]$ measured at a time equivalent to the start of the microperfusion experiments (initial) was elevated and remained unchanged for the subsequent 3-h period (final): (HC + V [n = 5], initial = 1.65±0.034 vs. final = 1.64±0.047, $P = 0.92$ and HC + Indo [n = 5], initial = 1.63±0.064 vs. final = 1.63±0.059, $P = 0.95$).

In control animals, the [Cl] of fluid emerging from the TAL ($[Cl]_C$) was 45.0±1.75 mM, and the segment reabsorbed Cl (J_{Cl}) at the rate of 1,237±42.5 pmol/min. Treatment of these pair-fed control rats with Indo had no effect on Cl reabsorption. In contrast, as shown on line 4 of Table II, HC rats infused with the vehicle had significantly lower rates of Cl reabsorption (972±52.5 pmol/min, $P < 0.001$) and the $[Cl]_C$ of fluid emerging from the TAL was significantly higher (57.8±2.22 mM, $P < 0.01$) compared with the control group. After treatment with Indo in HC rats, despite sustained hypercalcemia, J_{Cl} increased 26% and reached values similar to those measured in control rats (1,225±62.5 pmol/min). FR_{Cl}% is illustrated in Fig. 2. The results show the inhibition of TAL Cl reabsorption in HC animals ($P < 0.01$) and the achievement of control rates of transport after acute treatment with the PG synthesis inhibitor, Indo. Multivariate analysis of variance of the effects of the dietary pretreatment and Indo on TAL Cl reabsorption using these four groups showed statistically significant ($P < 0.05$) interaction between these two factors. The analysis indicates that Cl reabsorption is significantly decreased by HC and Indo increases TAL Cl reabsorption only in HC animals.

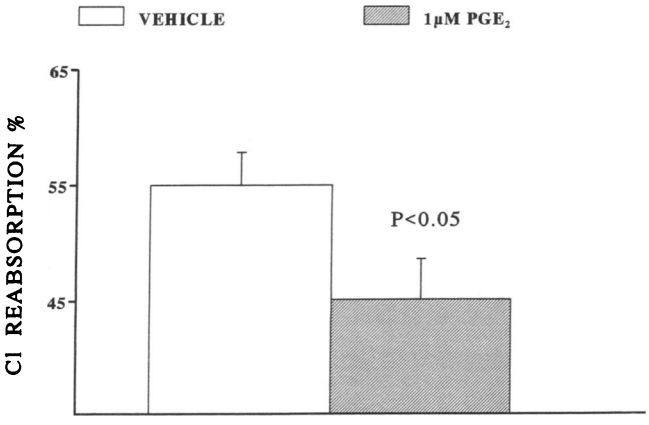

Figure 3. Effect of 1 μM PGE_2 in the luminal perfusate to inhibit net Cl reabsorption in functionally isolated microperfused loop segments in normal rats in vivo. Cl reabsorption is expressed as a percent of delivered Cl. Number of tubules per group is given in Table III. *P 0.035 calculated using Student's *t* test for unpaired data.

Effects of forskolin and cAMP on Cl reabsorption. If the mechanism by which PGE_2 impairs TAL Cl reabsorption in HC involves a reduction in cAMP production, then raising intracellular cAMP via stimulation of the adenylate cyclase catalytic unit should reverse the transport defect. To test this hypothesis, loops of Henle in control and HC rats were perfused for 20 min with the standard perfusate containing 0.1 mM FSK. As shown in Table II, Cl reabsorption in HC rats after exposure to FSK increased to values similar to those measured in control rats. Fig. 2 illustrates the increase in FR_{Cl}% in HC rats treated with FSK in the luminal perfusate and lack of a significant effect of FSK to stimulate Cl reabsorption in control rats.

The above results indicate that sustained HC does not impair the mechanism by which cAMP produces its effect on TAL transport. Logically, provision of cAMP should have the same effect as treatment with FSK, however, by itself would not indicate at which step PGE_2 impairs TAL function. The hypothesis that PGE_2 impairs TAL transport at a pre-cAMP step was tested in HC rats in which cAMP was provided by perfusing the loop segment with 8-*p*-CPT-cAMP for 30 min. Preliminary results showed variable stimulation when loops were perfused for only 20 min. In these experiments, the perfusion and collection rates, C/P [Inulin], $[Cl]_P$, and Cl delivery were all similar to control values. Stimulation of Cl reabsorption by cAMP is evidenced by measurement of J_{Cl} and $[Cl]_C$ similar to those measured in control rats (Table II). The effect of cAMP to increase FR_{Cl}% is illustrated in Fig. 2.

Effect of SSP in HC rats. If the mechanism by which PGE_2 impairs TAL Cl reabsorption in HC depends on PKC activation, then treatment of loops of Henle with SSP should return transport to control values. As shown on lines 8 and 9 of Table II and Fig. 2, perfusion of the loop of Henle with standard perfusate containing either 10^{-7} or 10^{-8} M SSP had no significant effect on $[Cl]_C$ or FR_{Cl}% in HC rats. As mentioned previously, the calculated value of FR_{Cl}% minimizes variation of the data attributed to differences in Cl delivery and length of the perfused segment and is the most appropriate way to express and compare Cl reabsorption. In addition, $[Cl]_C$ is the most significant determinant of furosemide-sensitive Cl reabsorption in all of the experimental conditions. J_{Cl} measured in HC TAL exposed to SSP 10^{-8} M was reduced compared with control data, however, the difference at 10^{-7} M was of borderline statistical significance. The finding that $[Cl]_C$ and FR_{Cl}% are

still significantly different compared with values measured in control rats (Table 2, line *1*) argues against an effect of SSP 10^{-7} M on TAL Cl reabsorption in HC rats. Although this protein kinase C inhibitor was without effect in our studies, the same preparation of SSP was used in an adjacent laboratory and produced significant changes in inner medullary collecting duct function in vitro (26). Fig. 2 summarizes the effects of HC to inhibit TAL Cl reabsorption and Indo, FSK, and cAMP, but not SSP (10^{-7}, 10^{-8} M) to reverse the defect.

Studies with PGE_2 in normal rats

To ensure that the effect of Indo treatment in HC rats was due to decreased production of PGE_2 and not to an effect of Indo to antagonize the action of PGE_2 on TAL Cl reabsorption, loop perfusion experiments were performed in normal rats treated with Indo and PGE_2 was added to the loop perfusate. These animals were also infused with dDAVP to ensure constant circulating levels of AVP. The loops were perfused for 20 min with the modified loop perfusate containing 1 μM PGE_2 or the ethanol vehicle. The effect of PGE_2 on loop Cl reabsorption is presented in Table III and Fig. 3. Exposure of normal loops of Henle to PGE_2 for 20 min in vivo resulted in a 28% increase in $[Cl]_C$ in fluid emerging from the TAL ($P = 0.022$) and a decrease in FR_{Cl}% reabsorption from 55.7±3.03% (vehicle) to 45.1±3.51% (PGE_2, $P = 0.035$). J_{Cl} was lower in tubules perfused with PGE_2, but the difference was of borderline statistical significance ($P = 0.089$).

Outer medullary [PGE_2]

Fig. 4 shows that PGE_2 extracted from the outer medulla of HC rats is 9 to 10 times greater ($n = 5$ rats per group, $P < 0.015$) than concentrations measured in control rats. The concentration of PGE_2 in HC rats infused with the vehicle was 4.7±1.03 compared with 0.56±0.20 ng/g wet wt in the corresponding control group. In HC rats, but not in controls, acute exposure to Indo, similar to that used in the microperfusion experiments, decreased PGE_2. Outer medullary [PGE_2] in HC rats decreased to 1.6±0.45 ng/g wet wt, a value not statistically different from 0.52±0.20 ng/g wet wt obtained in control rats given the vehicle. Multivariate analysis of variance indicates statistically significant interaction between HC and Indo treatment ($P = 0.0163$) i.e., HC increases [PGE_2] and Indo reduces it only in HC animals.

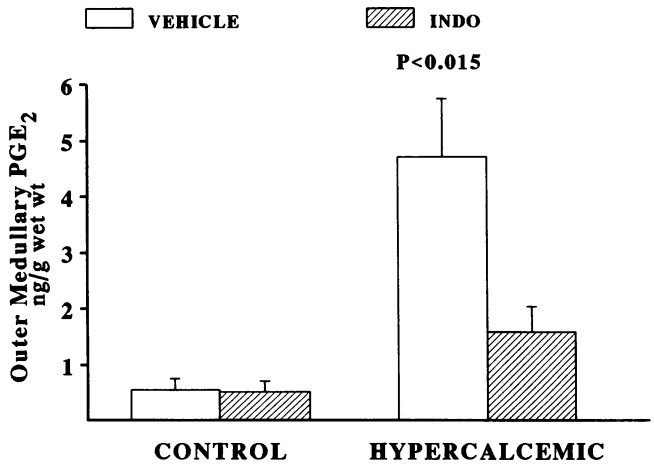

Figure 4. PGE_2 extracted from the outer medulla measured by RIA expressed as ng/g wet wt is elevated in HC rats (*VEHICLE*) compared with control rats. Acute treatment with the cyclooxygenase inhibitor indomethacin rapidly reduced PGE_2 to values measured in control rats. Number of animals per group = 5. $P < 0.0003$, for the ANOVA with four groups. Probability value over the data bar indicates statistical difference vs. control + vehicle calculated using Dunn's procedure with the *t* statistic for planned contrasts. Multivariate ANOVA indicates that HC stimulates outer medullary PGE_2, and Indo decreases PGE_2 only in HC animals.

Discussion

The results of the present microperfusion study of the integrated response of the TAL in vivo demonstrate that acute PG synthesis inhibition in HC animals, but not in controls, is associated with stimulation of TAL Cl reabsorption and a normalization of transport despite sustained HC. In animals treated in a similar manner we demonstrate that endogenous PGE_2 in the outer medulla of HC rats is elevated 9 to 10 times compared with control rats and can be reduced to normal after acute treatment with Indo. This is the first report of a pathophysiological condition in which inhibition of TAL Cl reabsorption can be attributed to increased renal PGE_2 production. The issues to be addressed include (*a*) the specificity of the method used to assess TAL Cl reabsorption, (*b*) endogenous PGE_2 impairment of TAL Cl reabsorption in HC in vivo, (*c*) the hypotheses regarding the signal transduction pathway by which PGE_2 impairs Cl reabsorption, and (*d*) the effect of elevated extracellular fluid (ECF) [Ca^{2+}] on TAL Cl reabsorption.

Assessment of TAL Cl reabsorption in vivo

Microperfusion conditions. We have used the technique of continuous microperfusion of the loop of Henle in functionally isolated cortical nephrons in vivo. Under these conditions, glomerular filtrate is diverted and control of luminal fluid composition and delivery is permitted. In addition, the segment of interest remains within the renal interstitium. The segment consists of a portion of the proximal convoluted tubule, the pars recta, thin descending limb, TAL (medullary and cortical portions, which may be as long as 3 mm [27]), and a portion of the distal convoluted tubule. Each of these regions has distinct fluid and electrolyte permeabilities and transport characteristics. In contrast to other microperfusion studies, we have manipulated the perfusate composition to minimize Cl reabsorption in nephron segments proximal to the TAL. This was achieved by limiting perfusate [Na] to 110 mM and [Cl] to 100 mM ([$NaHCO_3$] was 10 mM). In the rat, no net reabsorption or secretion of NaCl and water occur at a luminal [NaCl] of 95 mM (21). To further prevent fluid reabsorption, mannitol was added. In other respects, the perfusate composition resembles late proximal fluid and the perfusion rate used is only slightly higher than flow rates measured at late proximal sites.

Success in preventing fluid reabsorption (J_v) is evident by comparing collected rates with perfused rates, which differed by < 0.2–3 nl/min at the perfusion rate of 22 nl/min. This is in clear contrast to other loop segment studies where J_v was ≥ 50% of the perfused rate (18, 28). In addition, the [Cl] of fluid emerging from the loop segment ($[Cl]_C$) and the absolute rate of Cl reabsorption in our control animals were substantially lower compared with values reported in a previous study (18). The $[Cl]_C$ of collected fluid measured in control rats dur-

ing continuous microperfusion of the loop (45 mM) is similar to the limiting [Na] estimated in previous in vivo microstopflow conductivity studies (14, 20).

Specificity of furosemide. Using our modified tubule perfusate and exclusion criteria, the total amount of Cl reabsorbed between the perfusion and collection pipettes could be abolished by 1 mM furosemide in the perfusate. Can effects of furosemide on other transport systems in the TAL or other nephron segments in the perfused region account for this finding? Because in the present study, Cl was measured and not Na, a reduction in $NaHCO_3$ reabsorption due to inhibition of carbonic anhydrase activity would not have caused an overestimate of the contribution of the TAL. Other studies showing that furosemide can inhibit fluid reabsorption in proximal nephron segments (29) were performed using a luminal [NaCl] of 155 mM, nearly 60 mM higher than the limiting gradient that can be established by this segment (21). The perfusate [Cl] in the present study was chosen to approximate the limiting gradient and, in so doing, prevent net NaCl and water reabsorption by the proximal tubule. Although it is possible that furosemide may affect thin descending limb function (30), this segment is short (extends the length of the inner stripe of the outer medulla) and its contribution to net Cl reabsorption is quite small in comparison to that of the TAL. Therefore, Cl reabsorption reported in the present study largely reflects net uptake in medullary and cortical TAL in vivo.

TAL inhibition is mediated by endogenous PGE_2 in chronic HC

Measurement of outer medullary [PGE_2]. The results of the present study show for the first time, rapid reversal of impaired TAL Cl reabsorption using a PG synthesis inhibitor. Acute treatment with Indo in the rat has been shown to decrease renal PGE_2 excretion by 95% (7). However, given the capacity of the inner medulla to synthesize PGE_2 (3, 24), it is possible that urinary PGE_2 excretion largely reflects inner medullary production and the medullary TAL may not be exposed to inhibitory concentrations. Therefore, it was essential to establish the presence of elevated PGE_2 in the outer medulla of HC rats. In the present study, we show that [PGE_2] in the outer medulla of HC rats is 9 to 10 times greater than that measured in controls. Furthermore, acute treatment with Indo, similar to that used in the microperfusion studies, reduced PGE_2 in the HC rats to control concentrations. As noted previously, the statistical analysis indicates that HC stimulates outer medullary PGE_2 and Indo decreases it only in HC animals.

Estimation of [PGE_2] in vivo. Using the [PGE_2] expressed in ng/g wet wt of medullary tissue from which the PG was extracted, one can estimate the concentration in outer medullary tissue assuming 80% of tissue wet wt is water (kidney water content = 80% [31] and inner medullary water content = 87% [32] in normal rats). The estimated concentration in the untreated HC animals is $16.9\pm3.65 \times 10^{-9}$ M and in the control rats is ~ 12% of this value, i.e., $1.97\pm0.709 \times 10^{-9}$ M. If one assumes that PGE_2 is distributed primarily in ECF due to its polarity, the concentration would increase by ~ 66%. How closely do these values reflect actual tissue concentrations in vivo? Since control rats were pretreated with a high dose of Indo before removal of the tissue, the contribution of biosynthesis that occurred from the time of tissue excision, cooling, and penetration of Indo into the tissue from the isolation medium can be estimated. The close correspondence between the values measured in control animals with and without Indo pretreatment ($1.97\pm0.709 \times 10^{-9}$ M, control vehicle vs. $1.84\pm0.709 \times 10^{-9}$ M, control + Indo) argues against a significant component of biosynthesis to contaminate the estimate of outer medullary [PGE_2] in vivo. As mentioned previously, acute pretreatment of HC rats with Indo had a profound impact on the amount of PGE_2 extracted from the outer medulla. Indo pretreatment in HC rats reduced outer medullary PGE_2 to values similar to those measured in control tissue ($5.6\pm1.60 \times 10^{-9}$ M). It is well known that medullary collecting ducts and interstitial cells are responsible for the bulk of medullary PGE_2 synthesis (3, 24). However, since the TAL (3, 5, 8) and thin descending limb (33) have a limited capacity to synthesize PGE_2, the source of PGE_2 produced in HC that inhibits TAL Cl reabsorption can not be deduced.

The concentrations of PGE_2 in the outer medulla measured in the present study are in marked contrast to an earlier study by Stahl et al. (24) in rabbits in which PGE_2 extracted from the outer medulla was more than 1,000 times greater. It should be appreciated that in addition to performing the measurements in tissue derived from rabbits, a different method was used to extract and measure PGE_2. Since Indo pretreatment was not used, it is not possible to estimate the contribution of biosynthesis after excision of the tissue and exposure to Indo in the isolation medium.

Lack of interaction between Indo and PGE_2 on TAL Cl reabsorption in vivo. As a further test of the hypothesis that PGE_2 mediates the defect in TAL Cl reabsorption, loops of Henle in normal animals were perfused with 1 μM PGE_2. There was an 18% reduction in FR_{Cl}% and an increase in the $[Cl]_C$ in tubules perfused with PGE_2. The ability of exogenous PGE_2 to impair TAL Cl reabsorption in rats treated with Indo provides evidence that Indo does not impair the mechanism by which PGE_2 exerts its effect on the TAL. This supports the claim that correction of TAL Cl reabsorption in response to Indo in HC is due to the reduction in [PGE_2] and not to the presence of Indo.

Acute exposure of the TAL in normal rats to 1 μM PGE_2 added to the luminal perfusate was associated with inhibition of Cl reabsorption similar in magnitude to that observed in untreated HC rats (HC vehicle). This finding suggests that elevated PGE_2 in vivo is sufficient to impair TAL Cl transport by itself, i.e., an elevation in ECF and/or intracellular fluid [Ca^{2+}] is not required.

Concern might be raised regarding the concentration of 1 μM used in the luminal perfusate, in view of the fact that the concentration of PGE_2 measured in the outer medulla in the untreated HC rats was found to be ~ 1,000 times lower. However, it is widely accepted that receptors for PGE_2 are located in the basolateral membrane, and all previous in vitro studies demonstrating clear effects of PGE_2 on salt or water transport have all added PGE_2 on the basolateral side (1, 2, 3, 26, 34, 35). However as demonstrated by Culpepper and Andreoli (1) 1 μM PGE_2 exerted a similar degree of inhibition on transepithelial voltage whether present in the bath or the luminal perfusate in the isolated mouse mTAL in vitro. There is also evidence from other studies that the loop of Henle is permeable to PGE_2 (36). Recognizing that it may be difficult for interstitial [PGE_2] to increase in vivo due to inability to control the basolateral side of the segment, we assumed that the PGE_2 would

diffuse out of the loop segment during the 20-min perfusion and reach concentrations on the basolateral side sufficient to impair TAL Cl reabsorption. It is not possible to predict the concentration of PGE_2 achieved in the vicinity of the basolateral membrane of a single TAL segment perfused during an experiment. However, it is unlikely that the concentration would be as high as 1 μM.

Signal transduction pathway

Adenylate cyclase-cAMP system. Previously, Culpepper and Andreoli (1) showed that PGE_2 antagonized AVP stimulation of Cl reabsorption in mouse mTAL at concentrations as low as 10^{-11} M. Half-maximal inhibition was achieved at 10^{-7} M. Subsequently they demonstrated that PGE_2 inhibition of Cl reabsorption occurred via a pre-cAMP mechanism since FSK or exogenous cAMP was able to reverse inhibition, and cholera toxin at high concentrations (10^{-10} M) prevented the action of PGE_2 in this nephron segment (2). More recently, PGE_2 membrane receptors coupled to pertussis toxin–sensitive G_i have been identified in mTAL and cortical TAL from rabbits (37). In these cells, concentrations of $PGE_2 \leq 10^{-8}$ M cause decreased production of cAMP in response to AVP, calcitonin, or glucagon. To test the hypothesis that the inhibitory action of PGE_2 in HC was due to decreased generation of cAMP, the lumen of the loop segment was perfused with FSK. This diterpine derivative increases cAMP primarily by direct activation of the adenylate cyclase catalytic unit (22). After acute exposure to FSK, TAL Cl reabsorption in HC animals returned to values similar to control. FSK had no significant effect in control rats. As predicted, provision of cAMP in HC animals also returned TAL Cl reabsorption to normal. The results of these experiments with FSK and cAMP support the claim that PGE_2 in HC acts at a pre-cAMP step. This finding also provides the functional relevance of an earlier observation by Berl et al. (38) that cAMP production in response to AVP by mTAL derived from HC rats is decreased.

Phosphotidylinositol system. PGE_2 has been shown to exert effects on the cortical collecting duct of the rabbit via three signal transduction pathways; two which are coupled to adenylate cyclase via G_s and G_i, and the third coupled to the phosphotidylinositol system through G_p, which leads to protein kinase C (PKC) activation (34, 35, 39). To test the hypothesis that PGE_2 inhibition of Cl reabsorption in the TAL may be due to PKC activation, SSP was used to inhibit PKC activity. Since SSP is not a selective PKC inhibitor (40, 41), we tested the effect of luminally applied 10^{-7} and 10^{-8} M SSP on Cl reabsorption in vivo. As shown in Fig. 2, there was no detectable improvement in TAL Cl reabsorption in HC animals. Bioactivity of SSP was confirmed using a different preparation in an adjacent laboratory (26). The possibility remains that failure of SSP to affect TAL transport may be due to the fact that the PKC inhibitor was added on the luminal side. However, if PGE_2 inhibits TAL transport by a PKC-dependent post-cAMP mechanism or a mechanism independent of cAMP, then increased production of cAMP by FSK or provision of cAMP would not restore Cl reabsorption to normal values. Therefore, it is unlikely that endogenous PGE_2 inhibits TAL Cl reabsorption via a PKC-dependent pathway.

Elevated ECF [Ca^{2+}] and TAL Cl reabsorption

Previous in vitro studies have shown that an elevation of [Ca^{2+}] to 2 mM in the incubation medium decreases AVP-stimulated cAMP production in mouse mTAL (17). Since glucagon- and forskolin-stimulated cAMP production were also diminished, the authors proposed that the catalytic subunit of adenylate cyclase was directly inhibited by the increase in external [Ca^{2+}]. The authors mention that preincubation of the mTAL segments with Indo did not prevent the inhibitory action of the acute increase in [Ca^{2+}]. In the present study, despite the presence of a sustained increase in ECF [Ca^{2+}] to > 1.5 but < 2.0 mM, Indo, FSK, and exogenous cAMP were able to reverse inhibition of TAL Cl reabsorption in chronic hypercalcemic rats. This indicates that the increments in ECF [Ca^{2+}] commonly observed in HC do not directly impair Cl reabsorption and do not appear to impair the catalytic subunit of adenylate cyclase in the TAL in vivo. It is not unreasonable to propose that the acute and chronic effects of increased ECF [Ca^{2+}] may differ. However, our results suggest that any direct action of increased ECF [Ca^{2+}] to impair TAL transport by decreasing cAMP production is not sustained in animals with chronic HC.

In summary, our study of the integrated response of the TAL in vivo establishes the link between altered renal PGE_2 production and inhibition of TAL Cl reabsorption. Data derived from these studies indicate that outer medullary [PGE_2] in normal rats is $\sim 2 \times 10^{-9}$ M and that inhibition of TAL Cl reabsorption is present when [PGE_2] increases to approximately 17×10^{-9} M. Acquisition of this information now permits reevaluation of in vitro data regarding physiological versus pharmacological effects of PGE_2 on medullary nephron segment function. Our results are consistent with previous suggestions that endogenous PGE_2 has little effect on the function of the TAL under normal conditions. We conclude that increased endogenous PGE_2, and not HC per se, mediates inhibition of TAL Cl reabsorption at a step proximal to the generation of cAMP and probably does not involve activation of protein kinase C. These findings are consistent with the results of in vitro studies regarding the mechanism of action of PGE_2 in the mTAL (1, 2). However, current thinking regarding the mechanism by which HC impairs TAL function will have to be reevaluated (4). In addition, it is possible that the sustained 20% decrease in TAL Cl reabsorption mediated by PGE_2 contributes to the urinary concentrating defect associated with HC (14). Lastly, the mechanism by which PGE_2 via decreased cAMP causes inhibition of TAL Cl reabsorption may involve decreased Na/K-ATPase activity (6) or events at the luminal membrane involving the Cl uptake step (42, 43).

Acknowledgments

We thank Dr. Richard L. Hébert for the SSP and Dr. Steven Nadler for the 8-*p*-CPT-cAMP. The authors appreciate the critical input offered by Dr. Nadler, Dr. Hébert, and Dr. Kevin Burns at various times during the performance of this work.

This work was supported by a grant from the Medical Research Council of Canada (MA 7745). Andrea J. McKay, a Ph.D. candidate, is currently the recipient of a Medical Research Council Studentship. DHT was generously supplied by Roxane Laboratories.

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