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THE ROLE AND REGULATION OF INTRARENAL PROSTAGLANDIN E_2 IN HYPERCALCEMIA

BY

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I dedicate this thesis to the memory of the person who inspired me to do science,

my first Science teacher,

my father,

Parminder Mangat

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ABSTRACT

In chronic hypercalcemia, inhibition of medullary thick ascending limb (mTAL) NaCl reabsorption is mediated by elevated intrarenal prostaglandin E_2 (PGE_2). However, the specific mechanisms by which PGE_2 exerts its effects on the mTAL are not known. Reabsorption of NaCl by the mTAL is driven by the active transport of Na^+ across the basolateral membrane via the Na^+-K^+-ATPase pump, coupled to uptake of Na^+ and Cl^- by the Na^+-K^+-$2Cl^-$ cotransporter. Inhibition of the Na^+-K^+-ATPase pump and/or the Na^+-K^+-$2Cl^-$ cotransporter could result in inhibition of NaCl transport. The study presented here demonstrates that basolateral Na^+-K^+-ATPase activity is not affected by hypercalcemia. We also show that increased extracellular Ca^{2+} and PGE_2 directly inhibit activity of the Na^+-K^+-$2Cl^-$ cotransporter.

The mechanisms and source of elevated PGE_2 in hypercalcemia are not understood. In rats fed dihydrotachysterol (DHT) to induce hypercalcemia, we measured the intrarenal expression of enzymes important in prostaglandin production, namely, cytosolic phospholipase A_2 ($cPLA_2$), prostaglandin H synthase-1 (PGHS-1) and prostaglandin H synthase-2 (PGHS-2).

The rate limiting step in prostaglandin synthesis is the PLA_2-mediated release of arachidonic acid from membrane phospholipids. Using Western blot analysis, we have shown expression of $cPLA_2$ in normal rat kidney tissue, with significant up-regulation after 3 days of hypercalcemia, suggesting increased endogenous availability of

arachidonic acid in the kidneys of these animals. We used immunohistochemistry to localize immunoreactive $cPLA_2$ mainly to interstitial cells of the inner and outer medulla, which are well known to synthesize and release prostaglandins, and to the macula densa and cortical thick ascending limb of the cortex.

PGHS-1 is expressed constitutively in almost all tissues including kidney and we found that PGHS-1 protein levels remained unchanged with hypercalcemia. Previous studies reveal that PGHS-1 functions primarily as a "house-keeping" protein. Thus, it appears that in hypercalcemia, PGHS-1 preserves its role as a constitutive protein, responsible for the basal production of prostaglandins.

Induction of PGHS-2, the other critical enzyme involved in prostaglandin synthesis, is generally associated with inflammation, mitogenesis and ovulation. PGHS-2 has also been shown to exist under unstimulated conditions in the kidney, and we have provided further support for this. In addition, we have demonstrated that PGHS-2 protein expression is significantly and maximally increased in hypercalcemic animals after 3 days of ingestion of DHT-containing diet, and displays a similar cellular pattern of distribution to $cPLA_2$. However, unlike $cPLA_2$, after the initial stimulation on day 3, PGHS-2 protein levels in the inner and outer medulla return to baseline levels.

Urinary PGE_2 excretion was significantly elevated in hypercalcemic rats after 5 days of DHT-containing diet. Previous studies have established that urinary levels of PGE_2 reflect intrarenal synthesis, derived mainly from the inner medulla. Expression

of $cPLA_2$ in the inner medulla is still stimulated at 5 days, suggesting it is responsible for increasing PGE_2 excretion.

Angiotensin II has been reported to stimulate PGE_2 production in kidney tubular epithelial cells, mesangial cells and interstitial cells. We investigated the possibility that Ang II AT_1 receptor activation occurs in hypercalcemia, and mediates the increase in intrarenal $cPLA_2$ and PGHS-2 protein expression. Our data strongly support this hypothesis. First, our studies demonstrated that the polydipsia associated with hypercalcemia was abolished by administration of losartan, a specific AT_1 Ang II receptor antagonist. Second, losartan prevented the increase in $cPLA_2$ expression in hypercalcemic animals in all kidney regions, and inhibited increased PGHS-2 expression in the inner medulla without affecting expression in the cortex and outer medulla. Finally, we demonstrated that losartan completely inhibited the increase in urinary PGE_2 excretion observed in rats with hypercalcemia for 5 days.

Taken together, these data indicate that in chronic hypercalcemia, inhibition of NaCl reabsorption is not due to inhibition of the Na^+-K^+-ATPase pump. Both increased Ca^{2+}_o and PGE_2 inhibit the Na^+-K^+-$2Cl^-$ cotransporter, and we propose that these factors may be involved in the inhibition of NaCl transport in hypercalcemia. Furthermore, we demonstrate that hypercalcemia stimulates intrarenal $cPLA_2$ and PGHS-2 protein expression. Our data suggest that stimulation of $cPLA_2$ expression, through Ang II activation of the AT_1 receptor in the inner medulla, largely mediates increased PGE_2 excretion in hypercalcemic animals.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
AVP	argenine vasopressin
BoPCaR	bovine parathyroid Ca^{2+}-sensing receptor
bNOS	brain (neuronal) nitric oxide synthase
Ca^{2+}_o	extracellular calcium
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CT	calcitonin
cTAL	cortical thick ascending limb
DAG	diacylglycerol
DHT	dihydrotachysterol
ENCC	electroneutral sodium chloride cotransporters
FITC	fluorescein-5-isothiocyanate
GFR	glomerular filtration rate
HC	hypercalcemia
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IMCD	inner medullary collecting duct
IP_3	inositol trisphosphate

mTAL	medullary thick ascending limb
PA	phosphatidic acid
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PGHS	prostaglandin H synthase
PGHS-1	prostaglandin H synthase-1
PGHS-2	prostaglandin H synthase-2
PKA	protein kinase A
PKC	protein kinase C
PTH	parathyroid hormone
RIA	radioimmunoassay
$sPLA_2$	secretory phospholipase A_2
TAL	thick ascending limb
TBS-T	tris buffered saline-Tween-20

INTRODUCTION

1. *INTRODUCTION*

The kidney performs a number of essential functions. It is involved in the maintenance of a constant extracellular environment required for adequate functioning of cells. This is achieved by excretion of waste products of metabolism such as urea, uric acid and creatinine, and by adjusting the urinary excretion of water and electrolytes to match net intake and endogenous production. The kidney can regulate the excretion of water and solutes such as Na^+, K^+ and H^+, by changes in tubular secretion or reabsorption. It also secretes hormones that are involved in (1) the regulation of systemic and renal hemodynamics (angiotensin II, renin, prostaglandins, bradykinin), (2) calcium, phosphorus and bone metabolism (1,25-dihydroxyvitamin D_3, or calcitriol), and (3) red blood cell production (erythropoietin).

An important function of the kidney, relevant to this thesis, is its ability to excrete a concentrated urine when conservation of water is necessary. A key step in this process is the generation of a hyperosmotic medullary interstitium by the reabsorption of NaCl without water in the medullary thick ascending limb of the loop of Henle (mTAL). Hypercalcemia has been associated with disturbances in renal concentrating ability and loop of Henle function, specifically inhibition of mTAL NaCl reabsorption, shown to be mediated by PGE_2. This results in excess excretion of free water, associated with ECF volume contraction and thirst.

In this Introduction, I will first review the morphology of the mammalian kidney, with special attention to the mTAL and its transport properties. I will then review the literature on hypercalcemia and prostaglandins. Finally, since angiotensin II has been reported to stimulate prostaglandin production and is a potent dipsogenic agent, we studied the role of angiotensin II in hypercalcemia, so I will present the relevant literature on this hormone.

1.1 RENAL MORPHOLOGY

A coronal section of the kidney (Figure 1) illustrates a cup-shaped renal cortex surrounding the renal medulla. The pyramid-like medulla has its upper portion, the papilla, projecting into the renal pelvis. The cortex comprises the cortical labyrinth and the medullary rays. The medulla is divided into an outer medulla (inner and an outer stripe) and an inner medulla. The innermost portion of the inner medulla forms the papilla. The cortex is densely penetrated by arteries (and the corresponding veins) whereas no arteries enter the medulla. The efferent arterioles of the juxtamedullary glomeruli descend into the medulla where they divide into the descending vasa recta, which, with the ascending vasa recta, make up the vascular bundles of the renal medulla. At intervals, descending vasa recta join the capillary plexus at the adjacent medullary level, most leaving the bundle within the inner stripe.

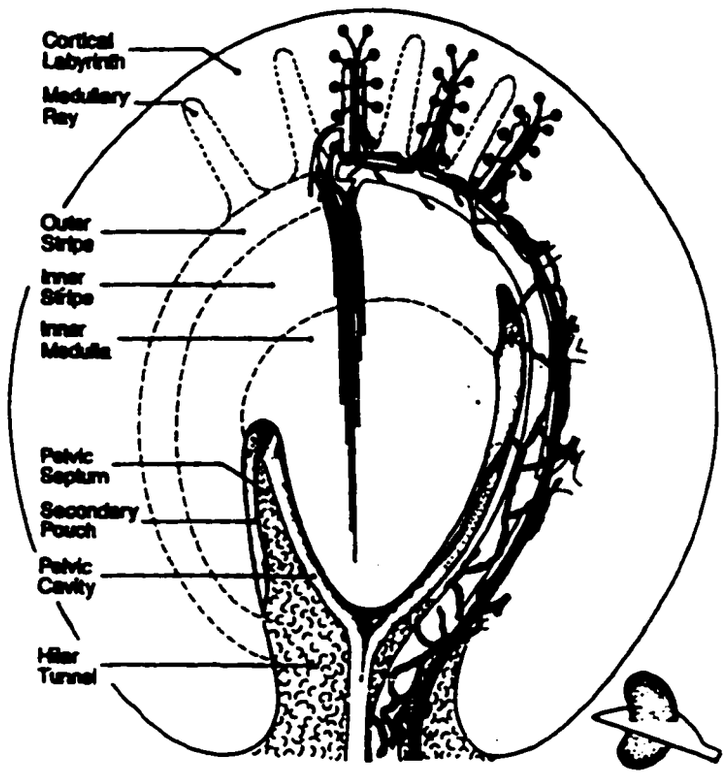


Figure 1. Schematic of a coronal section through the rabbit kidney.

From: Kriz and Kaissling, 1992.

The kidney is made up of specific structural units called nephrons (Figure 2). Each human kidney contains approximately 1 million nephrons, and each rat kidney, between 30,000 and 35,000. Three types of nephrons have been identified, based on the renal corpuscle location: superficial, mid-cortical and juxtamedullary. In addition, nephrons are also distinguished based on the lengths of the loops of Henle: short and long. In the rat, which has a greater number of short loops, the short loops all end at approximately the same level of the inner stripe, (that is, near the junction to the inner medulla) and only about 1500 out of about 10,000 long loops (15%) reach the lower half of the inner medulla.

1.2 THE THICK ASCENDING LIMB (TAL)

In mammalian kidneys, the TAL begins in the outer medulla at the border to the inner medulla, and ends just beyond the macula densa in the cortex. It is subdivided into a medullary (mTAL) and a cortical (cTAL) part. The TAL is a highly specialized segment, which exhibits transport properties ultimately responsible for the production of a concentrated urine during anti-diuresis and a dilute urine during water diuresis. The TAL reabsorbs 25% of the filtered NaCl at a rate similar to the proximal tubule, however, without the accompanying water absorption, to generate a hypertonic interstitium and a reduced luminal fluid osmolality. These processes are essential for the modulation of the urine concentrating mechanisms (Winters *et al.* 1991; Reeves & Andreoli, 1992).

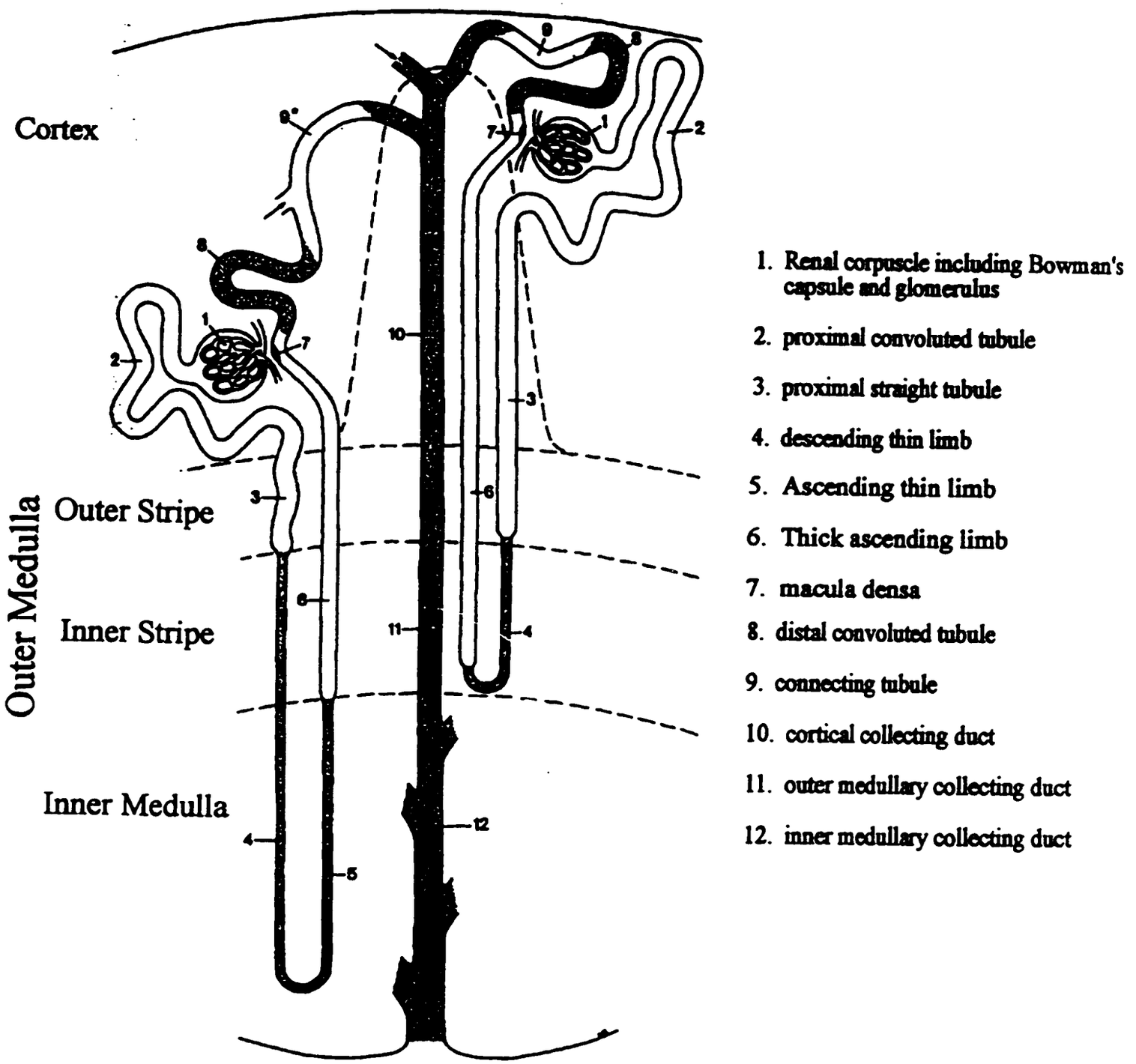


Figure 2. Schematic of nephrons and collecting duct, depicting a short-looped and a long-looped nephron. *Modified From: Kriz and Kaissling, 1992.*

Although it is situated in the medullary interstitium that has a minimal oxygen supply, metabolism in the mTAL is oxidative. The mTAL is susceptible to ischemic injury, and due to its high rate of O_2 consumption, ongoing transport along with decreased blood supply results in damage to the mTAL. This is considered a precipitating factor in the development of acute renal failure (Brezis et al., 1989).

The development of the *in vitro* perfusion technique allowed direct analysis of TAL transepithelial transport to be performed. Using this approach, Rocha and Kokko (1973) and Burg and Green (1973) were the first to describe four important features of salt absorption in rabbit mTAL and cTAL segments. First, it was demonstrated that net salt reabsorption resulted in a lumen-positive transepithelial voltage. This is in contrast to the proximal convoluted tubule and cortical collecting duct which have negative transtubular potential differences, and the descending limb of the loop of Henle where the potential is zero. Secondly, Cl^- transport was shown to occur against both electrical and chemical gradients, suggesting the presence of an active transport process. Na^+ was postulated to diffuse passively down the electrical gradient generated by the Cl^- movement (Rocha & Kokko, 1973). Thirdly, the net Cl^- and Na^+ absorption and the positive transepithelial voltage was shown to be dependent on the Na^+-K^+-ATPase pump (Rocha & Kokko, 1973) which has been located along the basolateral membranes of TAL cells (Katz et al., 1979). Finally, the TAL was demonstrated to possess a very low permeability to water, yet a high ionic conductance, in that it is three times more permeable to Na^+ than to Cl^-, findings that

were subsequently confirmed by Hebert and co-workers (Hebert *et al.* 1981c; Hebert *et al.* 1981a; Hebert *et al.* 1981b). Thus, the ability of the TAL to transport salt in the absence of water transport allows it to function as a diluting segment, and to provide energy for the development of an osmotic gradient within the renal interstitium (Hebert *et al.* 1987; Molony *et al.* 1989).

1.2.1 Transport in the TAL

Characterization of the transport properties of the TAL has been achieved through biochemical and electrophysiological studies on perfused microdissected TAL segments, isolated TAL cells and apical and basolateral membranes of the TAL. A proposed model for NaCl transport by this segment is illustrated in Figure 3 (Winters *et al.* 1991). According to the model, net Cl^- absorption is a secondary active transport process. All Cl^- reabsorption is mediated by an electroneutral Na^+:K^+:$2Cl^-$ co-transporter located along the TAL apical membrane, and is driven by the favourable electrochemical gradient for Na^+ entry. The Na^+ gradient is, in turn, maintained by the Na^+-K^+-ATPase pump located along the basolateral membrane. The reabsorbed K^+ is recycled across the apical membrane into the lumen by way of specific K^+ conductance channels. This apical K^+ recycling ensures the continuous supply of luminal K^+ for the operation of the Na^+:K^+:$2Cl^-$ co-transporter. In addition, it creates a lumen positive potential that allows 50% of luminal Na^+ to be reabsorbed in a passive

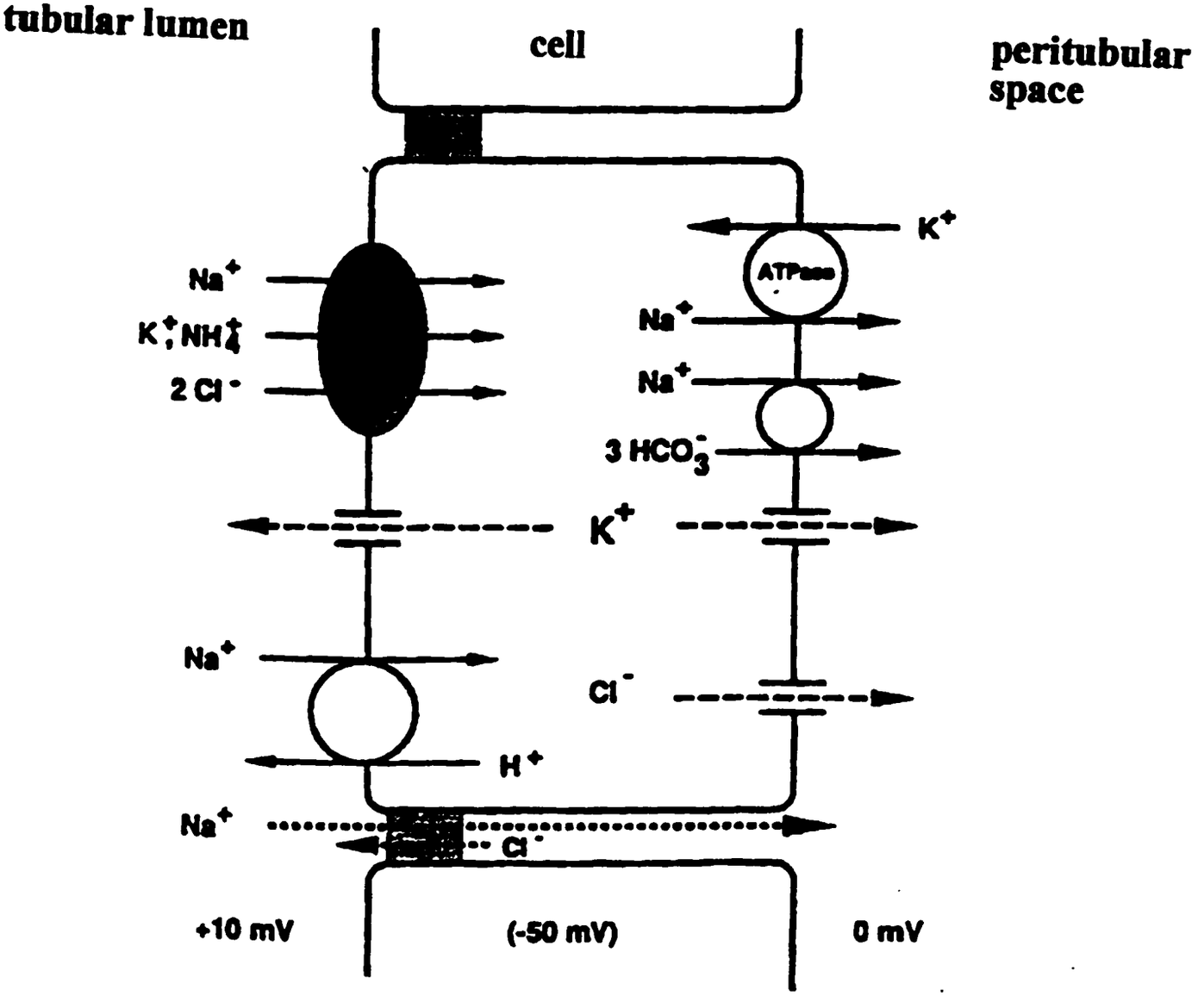


Figure 3. A model depicting the major elements of the mechanism of NaCl absorption by the TAL. Dashed lines indicate passive ion movements down electrochemical gradients. *Modified from: Winters et al., 1991.*

fashion via a paracellular pathway. Calcium and magnesium are also reabsorbed between cells in the TAL driven by the voltage. The paracellular pathway is cation selective with the majority of the current being carried by Na^+ movement from lumen to interstitium. In this manner, the efficiency of Na^+ transport is increased (Hebert et al., 1987), since for each Na^+ ion transported across the cell, which requires oxygen consumption and the hydrolysis of ATP, one Na^+ ion is transported via the paracellular pathway without any additional expenditure of energy. Reabsorbed Na^+ exits the cell via the Na^+-K^+-ATPase pump and the Cl^- via a basolateral conductive pathway (Cl^- channels). A basolateral K^+ conductance channel has been characterized, which most likely accounts for all KCl exit across the basolateral membrane (Hurst et al., 1992), with K^+ recycling occurring via the Na^+-K^+-ATPase pump.

The TAL also plays a role in acid-base balance by reabsorbing HCO_3 and transferring NH_4 synthesized by the proximal tubule to the interstitium for re-secretion by medullary collecting ducts (Good, 1990). Absorption by the $NaHCO_3$ route constitutes only a small component of Na transport (approximately 5-10% in the rat) and is mediated by an amiloride-sensitive Na^+/H^+ exchanger located along the apical membrane and a basolateral membrane $Na^+(HCO_3^-)_3$ co-transporter (Good, 1993). The rate of NaCl reabsorption by the macula densa cells in the cTAL provides the signal to the afferent arteriole that is responsible for autoregulation of glomerular filtration rate, renal blood flow and renin release, a phenomenon referred to as tubuloglomerular feedback. This NaCl uptake was also found to occur via the Na^+-

K^+-2Cl^- co-transporter (Reeves & Andreoli, 1992).

NaCl reabsorption in the mTAL is regulated by a variety of physical factors and hormones (Reeves & Andreoli, 1992). Stimulatory factors include vasopressin, glucagon, b-adrenergic agonists, high protein diet and mineralocorticoids. Inhibitory factors include increased extracellular calcium (hypercalcemia), PGE_2, peritubular hypertonicity and acidosis.

1.3 THE ELECTRONEUTRAL NA^+-K^+-CL^- COTRANSPORTERS

A specific pathway that allows the transport of Na^+, K^+ and Cl^- ions across cell membranes in a coupled, electrically neutral fashion, exists in a variety of animal tissues and serves a number of physiological functions. This Na^+-K^+-Cl^- co-transport was reported in epithelia of the TAL of the mammalian kidney (Gregor, 1985), the distal tubule of the amphibian kidney (Oberleithner *et al.* 1983), shark rectal gland (Hannafin *et al.* 1983), rabbit parotid (Turner *et al.* 1986) and flounder intestine (O'Grady *et al.* 1986), where it plays a role in the transport of salt and water. In other cells such as Ehrlich ascites tumor cells (Geck & Pfeiffer, 1985) and human red blood cells (Duhm & Gobel, 1984) it is involved in the regulation and maintenance of cell volume. In avian red blood cells, Na^+-K^+-Cl^- co-transport has also been shown to be

stimulated by b-adrenergic catecholamines (Haas *et al.* 1982) which may contribute to extrarenal K^+ regulation (Haas & McManus, 1985). In addition, reduced levels of this co-transporter in vascular smooth muscle cells may play a role in the pathogenesis of hypertension (O'Donnell & Owen, 1988).

Three groups of electroneutral Na^+-(K^+)-Cl^- transport systems have been identified. These have been classified according to whether K^+ accompanies Na^+ and Cl^- in the transport, and by their sensitivities to specific classes of inhibitors (Figure 4): (1) the benzothiadiazine (or thiazide)-sensitive Na^+-Cl^- cotransporter (TSC) (Ellison *et al.* 1987; Kunau *et al.* 1987); (2) the sulfamoylbenzoic acid (or bumetanide)-sensitive Na^+-K^+-$2Cl^-$ and Na^+-Cl^- symporters (BSC, NKCC) (Geck *et al.* 1980; Haas & Forbush III, 1987; Sun *et al.* 1991); and (3) the synchronous operation of the amiloride-sensitive Na^+-H^+ antiporter and the stilbene-sensitive Cl^--HCO_3^- exchanger (Kleyman and Cragoe, 1988; Turnberg *et al.* 1970). This latter pathway requires the synchronous function of two distinct membrane proteins, the Na^+-H^+ antiporter and the Cl^--HCO_3^- exchanger, so it is not a true coupled Na^+-Cl^- cotransporter. This double exchange system is inhibited by thiazides via their actions on carbonic anhydrase (see Figure 4) (Fried and Kunau, 1986).

The electroneutral Na^+-K^+-$2Cl^-$ cotransporters share a high degree of similarity in amino acid sequence (45-50%) and topology, despite their differences in types and number of ions transported and sensitivities to inhibitors. These proteins have a

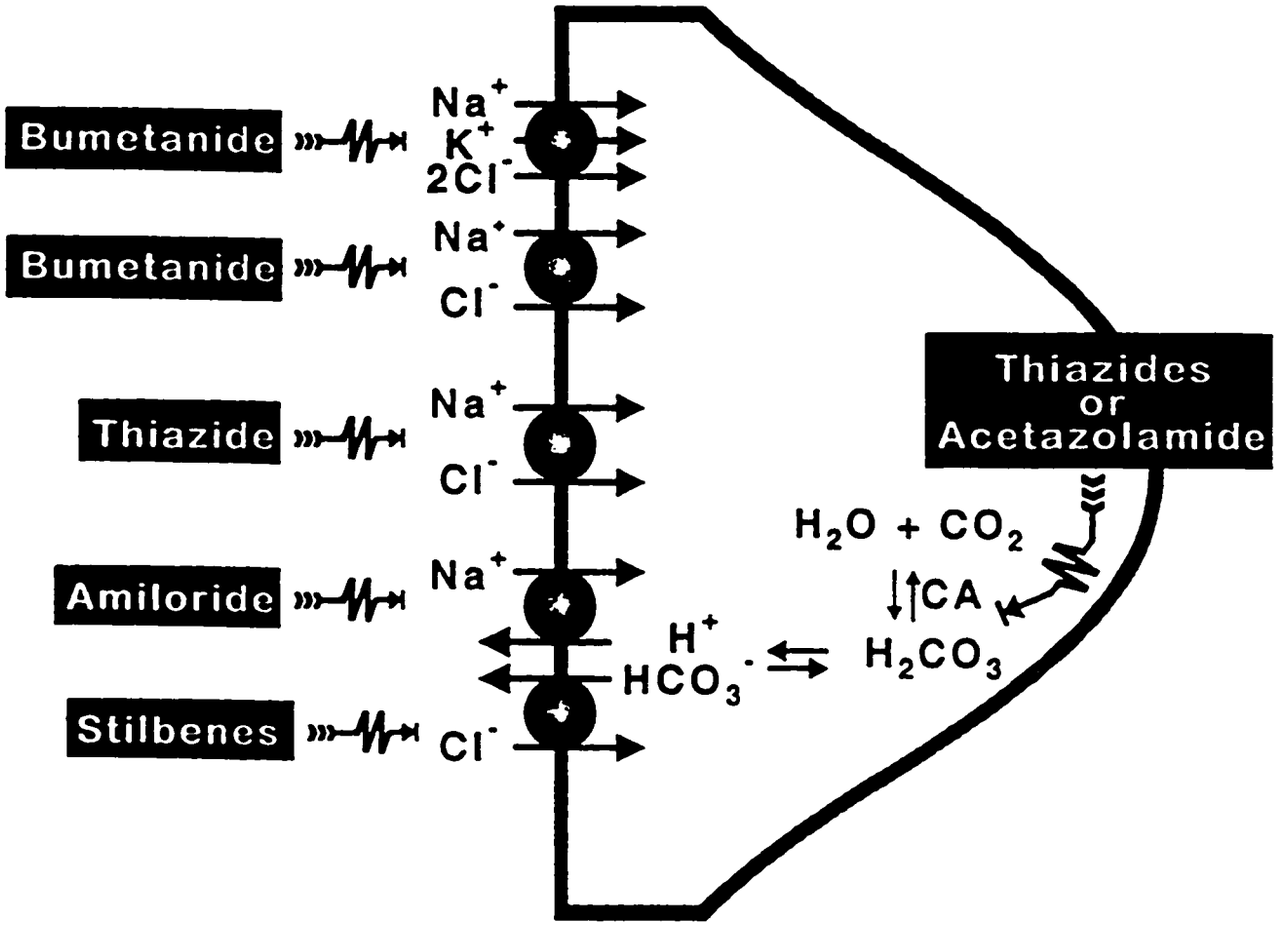


Figure 4. Classification of Na^+-K^+-$2Cl^-$ cotransport. The three groups of Na^+-K^+-$2Cl^-$ cotransporters are distinguished by their K^+-dependence and diuretic sensitivity. *From: Kaplan et al., 1996.*

predicted molecular weight for the nonglycosylated core protein of between 110-130 kDa, and consist of a central hydrophobic core region containing possibly 12 membrane-spanning domains, and long hydrophilic, intracellular amino and carboxyl termini (Kaplan *et al.* 1996). Furthermore, each protein contains from one to nine potential N-linked glycosylation sites on a large extracellular loop between the seventh and eighth membrane-spanning segments (Kaplan *et al.* 1996).

The Na^+-K^+-Cl^- cotransporter proteins can be divided further into three subgroups, ENCC1, ENCC2 and ENCC3, the Electroneutral Sodium-Chloride Cotransporters (Kaplan *et al.* 1996) (see Table 1 and Figure 5). The ENCC1 subgroup is formed by the mammalian renal and teleost thiazide-sensitive Na^+-Cl^- cotransporters and is found in the flounder bladder and apical membranes of the distal convoluted tubule of the mammalian nephron (Gamba *et al.*, 1994; Gamba *et al.*, 1993); the ENCC2 subgroup consists of the absorptive bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporters from rat, mouse and rabbit kidney and is found on the apical membranes of cortical and medullary thick ascending limbs of the loop of Henle (Gamba *et al.*, 1993; Payne and Forbush, 1994). Finally, the ENCC3 subgroup that includes the ubiquitous, basolateral, secretory bumetanide-sensitive Na^+-K^+-$2Cl^-$, was originally cloned from a mouse IMCD cell line, but has subsequently been found in many other tissues (Delpire *et al.*, 1994; Xu *et al.*, 1994).

In the kidney, bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport has also been detected in macula densa cells, though the molecular identity of the transporter in these cells is unknown (Lapointe *et al.* 1990; 1995). Proximal tubule cells in primary culture have also been shown to exhibit osmotically sensitive Na^+-K^+-$2Cl^-$ cotransport (Raat *et al.* 1994). Also, rat mesangial cells demonstrate bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport that can be activated by vasopressin and angiotensin II (Homma and Harris, 1991).

Studies addressing the affinity and stoichiometry of the Na^+-K^+-$2Cl^-$ co-transporter (Koenig *et al.* 1983) have reported a stoichiometry of 1 for Na^+, with Na^+ being accepted with the highest affinity, followed by Li^+. A stoichiometry of 1 was also established for K^+, however, this binding site is not specific. K^+ and Rb^+ have approximately equal affinities for this site. NH_4^+ and Cs^+ have also been found to bind to the K^+ site, but at lower affinities. The co-transporter also translocates two Cl^- ions, possessing two sites that differ in specificity and affinity. Binding studies using ^{3}H-bumetanide have identified a high- and low-affinity binding site in medullary plasma membrane vesicles (Forbush and Palfrey, 1983). The high-affinity site is specific for Cl^- and Br^-. Saturation of this site results in the low-affinity site exhibiting non-specific characteristics, with anion affinity for $Br^- > Cl^- > I^- > NO_3^-$ > thiocyanate.

Table 1. **The electroneutral Na^+-K^+-$2Cl^-$ cotransporter (ENCC) gene family.**

From: Kaplan et al., 1996.

Proposed gene name	Primary functions	Diuretic sensitivity	Original name	Source of cDNA	Amino acid number, predicted M_r
ENCC1	NaCl cotransport in absorptive epithelia	Thiazides	TSCfl	Flounder urinary bladder	1023 112-kDa
			rTSC	Rat kidney	1002 110-kDa
ENCC2	NaKCl cotransport in absorptive epithelia	Bumetanide	BSC1	Rat kidney (mouse kidney)	1095 120-kDa
			NKCC2	Rabbit kidney	1099 121 kDa
ENCC3	NaKCl cotransport in secretory epithelia or volume regulation during cell shrinkage	Bumetanide	BSC2	Mouse IMCD3 cells	1205 130-kDa
			NKCC1	Shark rectal gland	1192 130-kDa

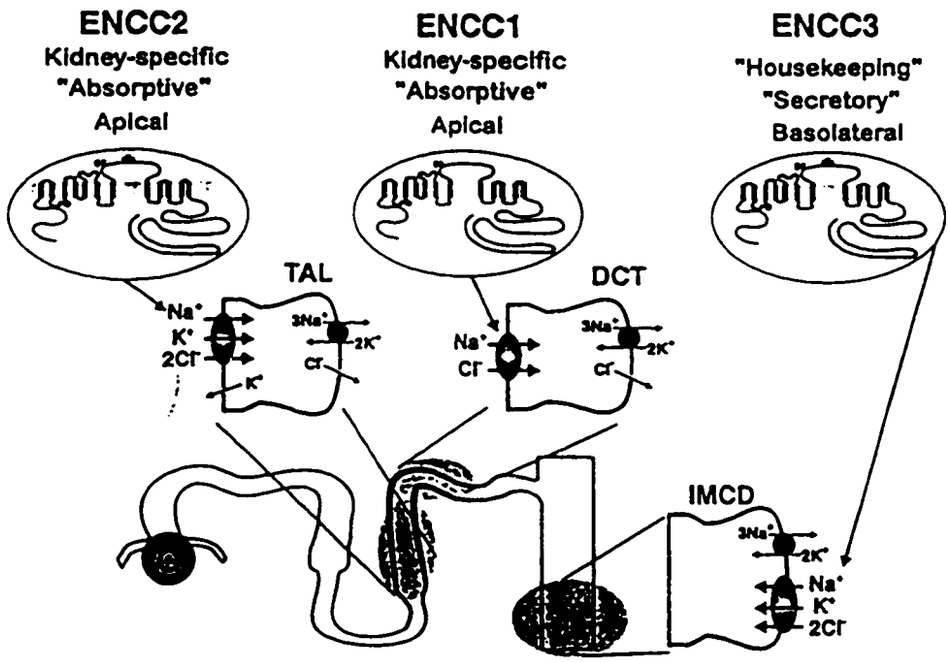


Figure 5. **Electroneutral Na^+-K^+-$2Cl^-$ cotransporters in the mammalian kidney.** *From Kaplan et al., 1996.*

The binding of bumetanide, a loop diuretic, to the Na^+:K^+:2Cl^- co-transporter has been shown to be stimulated by low Cl^- concentrations and inhibited by high Cl^- concentrations. From this, it has been postulated that the low-affinity Cl^- binding site is the target for the inhibitor (Haas & McManus, 1983).

1.3.1 Regulation of Na^+-K^+-2Cl^- cotransport

Na^+-K^+-2Cl^- cotransport is a passive process that does not consume energy directly, although ATP is consumed by the Na^+-K^+-ATPase pump and in phosphorylation processes required for transport (Kondo *et al.* 1987; Haas, 1989). Indeed, acute regulation of this transporter occurs mainly through phosphorylation/dephosphorylation mechanisms. Several potential phosphorylation sites for serine-threonine kinases (protein kinase C and cAMP-dependent protein kinase A) have been identified on ENCC2- and ENCC3-type Na^+-K^+-2Cl^- cotransporters, mainly in the putative carboxyl terminus (Lytle & Forbush III, 1992). The precise molecular mechanisms involved in phosphorylation remain to be elucidated.

Diverse stimuli have been shown to affect the Na^+:K^+:2Cl^- cotransporter in a variety of cell types. Some of these are included in Table 2. It appears that the activity

Table 2. **Regulation of Na^+:K^+:2Cl^- cotransport**

Modified from Kaplan et al., 1996.

Stimulus	Messenger	Effect	Cell type	Reference
Volume	PKC	Activation	Ehrlich	Vigne et al., 1994
	?	Activation	Rat brain endothelial cells	Vigne et al., 1994
PGE_2	cAMP-PKA	Activation	Osteoblast	Whisenant et al 1991
?	cAMP-PKA	Activation	Shark rectal gland	Lytle and Forbush, 1992
Norepinephrine	cAMP-PKA	Activation	Rat parotid acinar cells	Paulais and Turner, 1992
?	cAMP-PKA	Activation	Rat mesangial cells	Homma and Harris, 1991
Calcitonin	cAMP-PKA	Activation	Rabbit TAL cell line	Vuillemin *et al.*, 1992
?	PKC	Activation	Rat mesangial cells	Homma and Harris, 1991
?	PKC	Inhibition	Bovine endothelial cells	O'Donnell, 1991
?	cGMP	Activation	Rat mesangial cells	Homma and Harris, 1991
Endothelin	?	Activation	Rat brain endothelial cells	Vigne *et al.*, 1994
Angiotensin	Ca^{2+}	Activation	Rat mesangial cells	Homma and Harris, 1991
Vasopressin	Ca^{2+}	Activation	Bovine endothelial cells	O'Donnell, 1991

of the Na^+:K^+:2Cl^- cotransporter is modulated through receptor-mediated changes in intracellular messengers. Second messengers alter the activity of a variety of kinases that affect the phosphorylation state of the cotransport protein.

In addition to metabolic pathways indicating regulation via phosphorylation/dephosphorylation mechanisms, transport can also be regulated indirectly by other transport mechanisms located within either the same or opposite membrane. Indeed, inhibition of the basolateral Na^+-K^+-ATPase pump inhibits activity of the cotransporter by reducing the electrochemical gradient for Na^+ entry (Rocha & Kokko, 1973; Hebert *et al.* 1987).

In the mouse mTAL, bumetanide-sensitive transport has been reported to exist in two modes: a high efficiency K^+-dependent mode (1Na^+-1K^+-2Cl^-) and a low efficiency 1Na^+-1Cl^- mode (Sun *et al.* 1991). The mode of NaCl transport has been reported to be modulated by osmolality and vasopressin (AVP) (Sun *et al.* 1991). Under conditions of hypotonicity or in the absence of AVP, apical NaCl transport was reported to occur via the 1Na^+-1Cl^- mode (Sun *et al.* 1991). Upon exposure of rabbit mTAL cells to hypertonicity, or stimulation of perfused mouse mTALs with AVP, NaCl transport was shown to proceed via the K^+-dependent 1Na^+-1K^+-2Cl^- co-transporter mode (Sun *et al.* 1991) (Figure 6). In this mode, although the rate of Na^+ entry into the cell is similar to the 1Na^+-1Cl^- mode, the total rate of NaCl reabsorption is doubled (due to recycling of K^+ that results in the additional transport of Na^+ by the

paracellular pathway) (Sun *et al.* 1991).

Sun and co-workers demonstrated that the AVP-induced modulation of NaCl transport to the high efficiency state was mimicked by both direct activation of the catalytic unit of the adenylate cyclase system with forskolin, and the addition of the second messenger, cyclic AMP (Figure 6). These results led to speculation that phosphorylation of the $1Na^+:1Cl^-$ co-transporter or a regulator of the transporter, by a cAMP-dependent protein kinase may be involved.

Thus, K^+ dependence of the co-transporter appears to be subject to physiological regulation. Important physiological consequences emerge from this model. *In vivo*, the mTAL exists in an environment with a reduced oxygen tension (Hebert *et al.* 1987). Thus, it would greatly benefit the cells in this environment to exhibit a more efficient form of NaCl transport (Hebert *et al.* 1987). However, to date, there has been no molecular evidence to support this concept of mode switching in this segment.

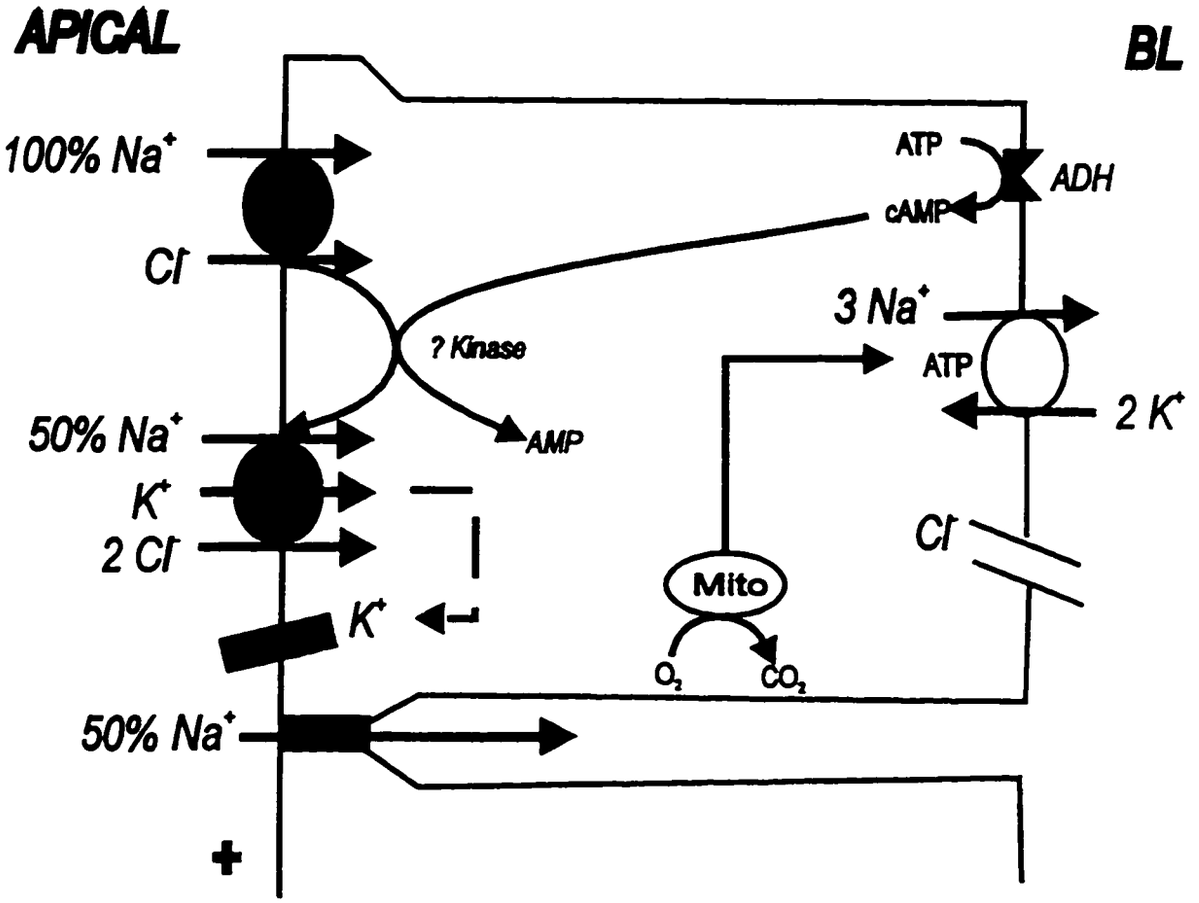


Figure 6. General model for the enhancement of the efficiency of NaCl transport by coupling K^+ to a loop diuretic-sensitive Na^+:Cl^- cotransport system. *Modified from: Sun et al., 1991.*

1.4 CALCIUM HOMEOSTASIS AND THE CA^{2+}-SENSING RECEPTOR

Intracellular and extracellular calcium (Ca^{2+}) ions exhibit multiple important roles in all organisms. Ca^{2+} is an important second messenger, involved in the regulation of processes such as cell division, hormone secretion and cell motility (Pietrobon *et al.* 1990). Since all intracellular Ca^{2+} ultimately derives from extracellular sources, the maintenance of a constant extracellular Ca^{2+} concentration is critical.

1.4.1 Calcium transport along the nephron

The normal plasma Ca^{2+} concentration is approximately 2.5 mM. Of this, 35-40% is bound to macromolecular proteins, mainly albumin, so that only 60-65% of plasma Ca^{2+} is filtered at the glomerulus. Of the Ca^{2+} that is ultrafiltered, approximately 10% is complexed as the Ca^{2+} salts of bicarbonate, phosphate, sulphate and citrate.

Most (65%) of the filtered Ca^{2+} is reabsorbed from the lumen of proximal tubules and returned to the circulation. The proximal convoluted tubule absorbs about 55% of the filtered Ca^{2+} and an additional 10% is absorbed in proximal straight tubules. The majority (90%) of Ca^{2+} absorption in this segment is passive and proceeds

via the paracellular pathway, as a consequence of Na^+ and Cl^- movement. The remainder (10%) is believed to traverse a cellular pathway. Ca^{2+} permeable channels have been described in proximal tubule cells, however, they are activated by membrane stretch and are thought to participate in cell volume regulation . Basolateral efflux may be mediated by Na^+/Ca^{2+} exchange .

Approximately 20% of the filtered Ca^{2+} is absorbed in mTAL and cTAL , by both passive and active transport pathways. Under resting conditions (in the absence of parathyroid hormone, PTH) passive paracellular Ca^{2+} transport prevails. However, after stimulation with PTH or calcitonin, both passive paracellular and active cellular absorption contribute to net Ca^{2+} absorption (Friedman & Gesek, 1993). Changes in the electrochemical driving force for Ca^{2+} determine the magnitude of paracellular absorption. The transepithelial voltage (electropositive in the lumen in this segment), which is the primary determinant of the driving force, is set by the rate of Na^+ absorption. Therefore, an increase in Na^+ absorption will cause the transepithelial voltage to also increase, and thus stimulate Ca^{2+} absorption. Conversely, inhibition of Na^+ transport (such as with furosemide or bumetanide) results in decreased passive Ca^{2+} absorption (Friedman & Gesek, 1993).

An additional 10% of filtered Ca^{2+} is absorbed in the distal convoluted tubules and connecting tubules. Passive Ca^{2+} absorption in these segments is negligible, so that absorption is believed to occur exclusively by an active, transcellular pathway. The

distal convoluted tubule is a major regulatory site for Ca^{2+} excretion in the urine.

Finally, the inner medullary collecting tubules are responsible for the absorption of approximately 1.5% of the filtered Ca^{2+}.

1.4.2 Calcium homeostasis

The homeostatic system that maintains stable levels of extracellular Ca^{2+} has two key elements (Brown, 1991). The first involves extracellular Ca^{2+}-sensing cells that recognize and respond to minute (1% to 2%) fluctuations in the extracellular ionized Ca^{2+} (Ca^{2+}_o) concentration. These cells include parathyroid cells and the thyroidal C cells, which respond to conditions of hypocalcemia by secreting more parathyroid hormone (PTH) and less calcitonin (CT), respectively. The second element is the effector tissue that modifies translocation of Ca^{2+} into or out of the extracellular fluid in response to changes in the circulating levels of calciotropic factors (Brown, 1991; Kurokawa, 1994).

PTH restores normocalcemia through direct and indirect actions on the bone, kidney and intestine (Figure 7) (Brown *et al.* 1995). In hypocalcemia, stimulation of PTH secretion increases release of bone and skeletal Ca^{2+}, enhances Ca^{2+} reabsorption by the distal nephron in the kidney, and stimulates the proximal tubular synthesis of

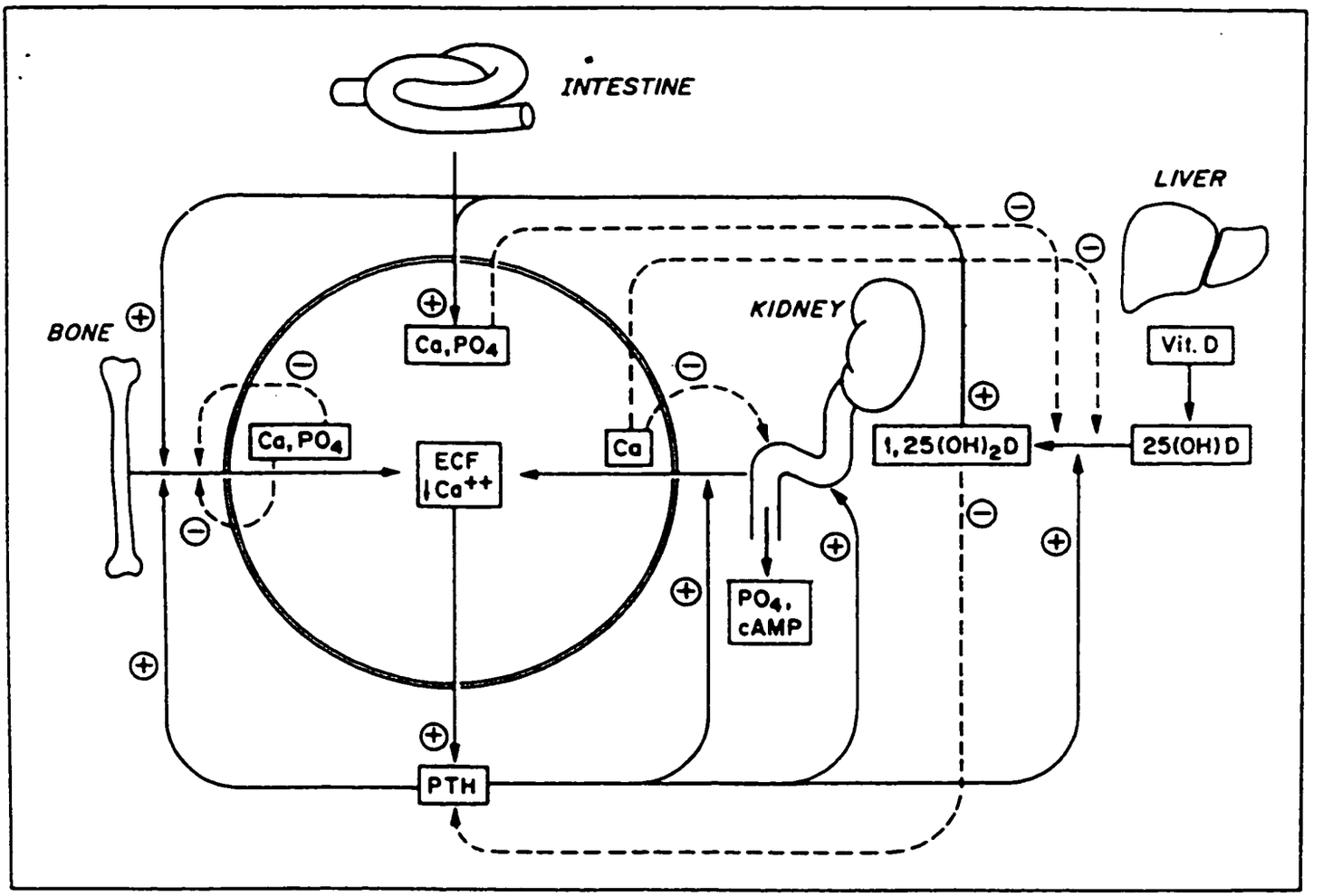


Figure 7. Actions of Ca^{2+}_o on its target tissues. *From Brown et al., 1995.*

calcitriol, the active form of vitamin D, ($1,25(OH)_2D$), which increases intestinal absorption of Ca^{2+} (Brown, 1991; Kurokawa, 1994). In this manner, Ca^{2+}_o is restored to normal levels, and PTH secretion is again normalized.

The mechanisms underlying the direct regulation of parathyroid cells by Ca^{2+}_o are still not defined. Recent evidence suggests that Ca^{2+}_o sensing involves a cell surface receptor. Brown et al. used expression cloning in *Xenopus laevis* oocytes to isolate and characterize a cDNA clone encoding a polyphosphoinositide (PI)-coupled form of bovine parathyroid Ca^{2+}_o-sensing receptor (BoPCaR1) (Brown *et al.* 1993).

This receptor is a member of the superfamily of G-protein-coupled receptors, and recognizes not only divalent cations, but also trivalent cations such as gadolinium (Gd^{3+}), and polycations such as neomycin (Brown *et al.* 1995). Transcripts for the receptor have been found in the parathyroid, and in cell types and tissues that appear to sense Ca^{2+}_o, such as the kidney and the thyroid (C cells) (Brown *et al.* 1993). This transcript in the C cells likely mediates the stimulatory effects of hypercalcemia on calcitonin secretion. Transcripts for the receptor have also been found in the brain (Brown *et al.* 1993). A Ca^{2+}_o-sensing receptor that shares a high degree of homology (greater than 90% identity at the amino acid level) with BoPCaR1 has been cloned from the rat kidney (Riccardi *et al.* 1995).

1.5 HYPERCALCEMIA

As described in the previous section, Ca^{2+} levels in the serum are normally kept within narrow limits by Ca^{2+} homeostatic mechanisms that involve an interplay of various hormonal and physiological influences. When this homeostatic control is impaired or lost, Ca^{2+} levels may increase above the normal range (hypercalcemia). Primary hyperparathyroidism and malignancy-associated hypercalcemia represent the most frequent causes of hypercalcemia in hospitalised patients. Approximately 20% to 40% of all patients with cancer are affected by hypercalcemia at some time during the course of their disease (Barri & Knochel, 1996). The risk of developing hypercalcemia varies among the different types of malignancy. Lung, renal, ovarian and breast cancer, and squamous cell carcinoma of the head and neck are most often complicated by hypercalcemia (Walls *et al.* 1995; Barri & Knochel, 1996).

The association of hypercalcemia with disturbances in renal concentrating ability, water intake and loop of Henle function has been widely recognized in both humans (Cohen *et al.* 1957; Gill & Bartter, 1961) and experimental animals (Epstein *et al.* 1958; Epstein *et al.* 1959). Chronic hypercalcemia is associated with a significant reduction in medullary solute content (Levi *et al.* 1983; Eigler *et al.* 1962; Work & Jamison, 1987), and more specifically, the NaCl component of the inner and the outer

medulla (Berl, 1987; Peterson *et al.* 1987; Levi *et al.* 1983). A minor component of the reduction in medullary solute content may be attributed to increased medullary blood flow (Levi *et al.* 1983). Reduced medullary solute content could be due to decreased solute delivery and/or decreased NaCl reabsorption by the mTAL.

One feature of the human kidney that differs from that of rat or dog is in the percentage nephrons that have long loops of Henle penetrating into the renal medulla and which, therefore, contribute to the elaboration of a concentrated urine: 100% in dogs, 30% in rats and only 10-14% in man. Those few nephrons that do contribute to the renal pyramid and, thereby, to the formation of a concentrated urine originate primarily in glomeruli in the juxtamedullary portion of the cortex. Accordingly, when injury to the kidney is confined to the medulla in man, concentrating ability may be severely impaired, while glomerular filtration rate may be only slightly lowered (Epstein *et al.* 1958).

Ca^{2+} is concentrated in the medulla of kidneys in the same way as is Na^{+}. Therefore, if high concentrations of Ca^{2+} were injurious, the cells of the medullary tubules most likely will be the first affected. In dogs with experimental hyperparathyroidism or rats with vitamin D intoxication, the earliest deposition of Ca^{2+} is indeed in the ascending loops of Henle, the distal convoluted tubules and the medullary collecting ducts (Carone *et al.*, 1960). In hypercalcemic humans, the medulla is predominantly affected, although with severe and long-standing hypercalcemic

disorders, proximal tubules and even cortical blood vessels and glomeruli also become calcified (Epstein, 1966). The degree of the concentrating defect appears to depend more on the duration of hypercalcemia and the extent of tissue deposition than on the exact level of serum Ca^{2+} (Epstein, 1966).

Alterations in arginine vasopressin (AVP) release or response, renal hemodynamics and renal tubular function have been examined as possible mediators of the concentrating defect. AVP has been shown to increase the transepithelial voltage and stimulate NaCl cotransport by the mTAL of the rat and mouse (Besseghir *et al.* 1986; Hall & Varney, 1980; Hebert *et al.* 1981b; Saski & Imai, 1980). Despite elevated plasma levels of AVP after dehydration, a renal concentrating defect was still found to persist in pair-fed, pair-watered hypercalcemic animals (Levi *et al.* 1983). In addition, the medullary collecting duct response to AVP is not impaired in hypercalcemic animals.

In vitro studies showed that an acute increase in Ca^{2+}_{o} inhibits AVP-stimulated cAMP production in the mouse mTAL but not in the medullary collecting duct (Takaichi *et al.* 1986). Furthermore, in the presence of normal extracellular $[Ca^{2+}]$, it was shown that AVP-stimulated increases in cAMP production were reduced in the mTAL segments but not in the inner or outer medullary collecting ducts isolated from hypercalcemic rats (Berl, 1987). Therefore, it was proposed that if variations in cAMP in the mTAL regulate NaCl transport, then TAL NaCl reabsorption would be

impaired in hypercalcemic animals (Peterson, 1990).

Studies conducted on isolated perfused loops of Henle subsequently showed that chronic hypercalcemia in rats was associated with inhibition of TAL NaCl reabsorption (Peterson, 1990). The mechanism underlying this impaired TAL NaCl reabsorption has not yet been fully elucidated. Renal PGE_2 excretion is strikingly elevated in hypercalcemic rats (Levi *et al.* 1983; Serros & Kirschenbaum, 1981), suggesting that PGE_2 may be involved in mediating this defective NaCl reabsorption.

Peterson and co-workers (Peterson *et al.* 1993) used rats with vitamin D-induced chronic hypercalcemia to test the hypothesis that PGE_2 does indeed mediate the defective TAL NaCl reabsorption. These investigators measured Cl^- reabsorption in isolated loop segments microperfused *in vivo* under various conditions. They hypothesized that if elevated PGE_2 impairs TAL NaCl reabsorption in chronic hypercalcemia, then an acute reduction in PGE_2 synthesis (with indomethacin, an inhibitor of cyclooxygenase activity) should restore normal transport to this segment. They found that indomethacin treatment did not affect Cl^- reabsorption in control rats. However, in hypercalcemic animals that demonstrated significantly lower rates of Cl^- reabsorption, indomethacin completely blocked this inhibition, so that Cl^- reabsorption rates were comparable to those observed in control animals. In addition, they demonstrated that forskolin and a cAMP analogue (8-p-CPT-cAMP), but not staurosporine (a PKC inhibitor) also reversed the inhibition within 20 min. Finally,

they reported that acute indomethacin treatment also normalized the elevated PGE_2 levels observed in the outer medulla of hypercalcemic animals. These results suggest that elevated endogenous PGE_2 mediates the hypercalcemia-induced inhibition of NaCl reabsorption at a step proximal to the generation of cAMP.

1.6 PROSTAGLANDINS

Although prostaglandin synthesis occurs in a variety of tissues, the kidney is a particularly rich source of prostanoids. In the mammalian kidney, prostaglandins are important physiologic modulators of salt and water homeostasis and vascular tone. In particular, when renal perfusion pressure is reduced and vasoconstrictors such as Ang II and norepinephrine perfuse the kidney, prostaglandins are formed to modulate the vasoconstriction and to maintain GFR (Wilson, 1992).

Prostaglandins are biologically active fatty acids belonging to a family of lipids called "eicosanoids." Eicosanoids are oxygenated derivatives of eicosatetraenoic acid, commonly known as "arachidonic acid." The availability of arachidonic acid is most often the rate-limiting step controlling the synthesis of eicosanoids. Figure 8 (Smith, 1992) illustrates an overview of the pathway for the deacylation of arachidonic acid from membrane phospholipids and oxygenation to various eicosanoids. The three

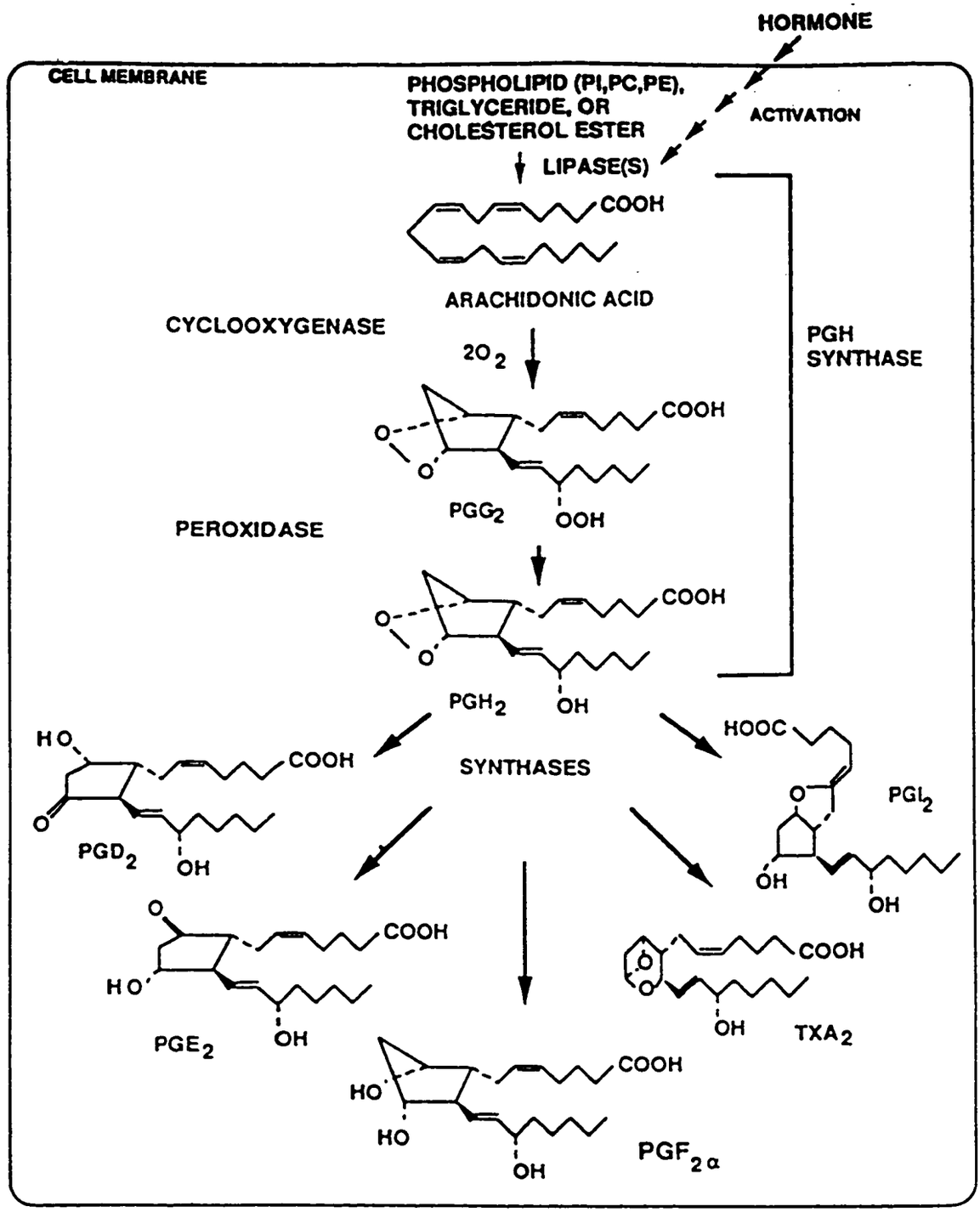


Figure 8. Pathway for the deacylation of arachidonic acid from membrane phospholipids and oxygenation to eicosanoids. *From: Smith, 1992.*

phases of prostaglandin production are as follows: (1) mobilization of arachidonic acid from cellular phospholipids via the action of phospholipase A_2 on phosphatidylethanolamine and phosphatidylcholine; (2) conversion of arachidonic acid to the prostaglandin endoperoxides, prostaglandin G_2 and then prostaglandin H_2 (precursors of the stable prostaglandins), by Prostaglandin H synthases (PGHS, "cyclooxygenase" or "COX") and (3) either isomerization or reduction of prostaglandin H_2 by specific synthases (isomerases) or reductases to the major biologically active prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), or thromboxane A_2 (TxA_2).

Most portions of the nephron are able to synthesize eicosanoids. Although differences exist between animal species, PGE_2 is the dominant (80-90% of the measured prostaglandins) renal prostaglandin in all species, and urinary concentrations are typically in the nanomolar range, which is well above the circulating (picomolar) concentrations. The proximal and distal convoluted tubules synthesize very small amounts of PGE_2. The thin descending limb of Henle, the medullary thick ascending limb of Henle and the medullary and cortical collecting tubules have been reported to possess a greater synthetic capacity (Farman *et al.* 1986; Farman *et al.* 1987). Medullary interstitial cells are also able to produce abundant amounts of PGE_2 (Pitcock, 1988).

PGE_2 exerts potent vasodilatory effects on the kidney. When infused into the arterial circulation, PGE_2 reduces blood pressure, and increases renal blood flow with

little effect on GFR (Memon *et al.* 1993). PGE_2 has also been shown to inhibit NaCl reabsorption in the mTAL and reduce water reabsorption along the length of the collecting tubule (Bonvalet *et al.*, 1987; Reeves and Andreoli, 1992).

1.6.1 Phospholipase A_2 (PLA_2)

Phospholipase A_2 may be grouped into distinct families of proteins that catalyze the hydrolysis of the ester linkage at the *sn*-2 position of membrane phospholipids to produce free fatty acids and lysophospholipids. To date, the best characterized PLA_2s have been the secreted enzymes from pancreas (Verheij *et al.* 1981), venoms (Dennis, 1983) and inflammatory cells and exudates (Kramer *et al.* 1988; Seilhamer *et al.* 1989), the $sPLA_2$s. $sPLA_2$s are small proteins (about 14 kDa) which nonselectively cleave any fatty acid from the *sn*-2 position of phospholipids and require millimolar Ca^{2+} levels for activity.

A high molecular mass (85 kDa) cytosolic phospholipase A_2 ($cPLA_2$) has been found in human, bovine and rabbit platelets (Kim *et al.* 1991a; Kim *et al.* 1991b; Takayama *et al.* 1991), rodent macrophage cell lines (Leslie *et al.* 1988), human monocytic cell lines (U937 and THP1) and rat kidney. In contrast to the $sPLA_2$s, the $cPLA_2$s are activated at submicromolar levels of Ca^{2+} and preferentially hydrolyze

phospholipids that have arachidonic acid at the *sn*-2 position, suggesting that their primary function is to initiate the biosynthesis of eicosanoids such as PGE_2. Isolation of the cDNA encoding $cPLA_2$ demonstrates that the enzyme has no homologous regions to the $sPLA_2$s. The amino acid sequence revealed a 45 amino acid stretch in the amino terminal region that showed homology to Ca^{2+}-dependent forms of protein kinase C and other Ca^{2+}-dependent membrane binding proteins .

A major process regulating $cPLA_2$ is the post-translational regulatory mechanism responsible for the rapid activation of enzyme activity. $cPLA_2$ contains a Ca^{2+}-dependent translocation domain such that it is found in the cytosol of cells in the absence of Ca^{2+}, and it translocates to the membrane in response to micromolar Ca^{2+} concentrations (Krause et al., 1991; Yoshihara and Watanabe, 1990). Activity of $cPLA_2$ is also increased by some agents that cause phosphorylation of the serine, threonine or tyrosine residues.

The mechanisms controlling expression of the $cPLA_2$ gene at the transcriptional level are less well understood. The $cPLA_2$ gene is expressed in a variety of human tissues and has been localized to chromosome 1 (Miyashita *et al.* 1995). The 5'-flanking region has a feature typical of a housekeeping gene in that it has no TATA box to direct transcription. In the absence of a TATA box, genes are dependent on other sequences to assemble the transcription machinery. Usually, a GC-rich region contains multiple SP1 sites, which can recruit transcription factors to the promoter region

(Miyashita *et al.* 1995). This GC-rich, TATA-boxless promoter forms the typical "housekeeping promoter" of genes. The human $cPLA_2$ gene promoter is not GC-rich and does not contain SP1 sites (Miyashita *et al.* 1995). However, it has been observed that TATA boxless genes can be transcriptionally regulated. Indeed, $cPLA_2$ mRNA is subject to regulation in some cell lines by mediators such as interleukin 1 (IL-1), tumour necrosis factor (TNF), glucocorticoids, epidermal growth factor (EGF), phorbol myristate acetate (PMA) and interferon-g (Gronich *et al.* 1994; Wu *et al.* 1994; Maxwell *et al.* 1993; Hoeck *et al.* 1993; Miyashita *et al.* 1995). There is evidence supporting regulation of the *rate* of transcription of the $cPLA_2$ gene (Wu *et al.* 1994) in addition to post-transcriptional mechanisms affecting mRNA stability (Tay *et al.* 1994). Therefore, this enzyme has the potential to be regulated at multiple levels.

Studies conducted in a variety of systems have demonstrated that a number of agonists do result in arachidonic acid release with subsequent eicosanoid production. These agonists include bradykinin, vasopressin, serotonin, acetylcholine and norepinephrine (Mukherjee et al., 1994). There is no evidence to date that $cPLA_2$ is up-regulated in hypercalcemia.

1.6.2 Prostaglandin H synthase-1 and -2 (PGHS-1, PGHS-2)

Prostaglandin H synthase (PGHS) is another key enzyme involved in prostaglandin synthesis. PGHS exhibits both cyclooxygenase (*bis*-oxygenase) activity catalyzing prostaglandin G_2 formation from arachidonic acid, and peroxidase activity that reduces the 15-hydroperoxyl group of prostaglandin G_2 to the 15-OH of prostaglandin H_2. PGH_2 is then further metabolized by specific isomerases or synthetases to various derivatives including PGE_2.

Many cell types *in vitro*, when stimulated by mitogens, have been shown to synthesize increased amounts of PGHS, or cyclooxygenase ("COX") (Mitchell *et al.*, 1995). Stimulation of cell division by mitogens activates a number of intracellular events, including the protein synthesis-independent transcription of primary response, or immediate-early genes. In 1990, "COX" was identified as an immediate-early gene induced by phorbol esters, which led to the realization that "COX" exists in at least two discrete isoforms, PGHS-1 (cox-1) and PGHS-2 (cox-2).

The two isozymes are important pharmacologically as targets of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (Smith and DeWitt, 1995). Aspirin inhibits PGHS-1 and PGHS-2 by acetlyating Ser-529 and Ser-516 respectively, thus preventing arachidonic acid from reaching the catalytic site (Lecomte *et al.*, 1994).

1.6.2.1 PGHS gene structure

PGHS-1 and PGHS-2 are encoded by separate genes located on different chromosomes: chromosomes 2 and 9 for the murine and human PGHS-1, respectively, and chromosome 1 for both murine and human PGHS-2. The human PGHS-2 gene shares approximately 60% nucleotide sequence homology with PGHS-1, with differences in the first two and last exons (Jones et al., 1993).

The first PGHS to be genetically cloned was the "classical" cyclooxygenase, PGHS-1, from sheep seminal vesicles and the cDNA was used to clone the murine and human genes. The genomic region that encodes the transcription unit covers approximately 22 kilobases of DNA. Northern blot analyses revealed that these clones hybridize to 2.8 Kb mRNA species which code for proteins with a molecular weight of approximately 72 kDa, containing 600 to 602 amino acids . Sequence homology between species is about 90% at the nucleotide and amino acid level. As is often observed in developmentally regulated "housekeeping" genes, the PGHS-1 gene lacks a TATA box. To date, almost nothing is known about the regulation of PGHS-1 gene expression.

The PGHS-2 gene has been cloned from chicken, rat, mouse and human (Herschman, 1994). The PGHS-2 gene is smaller than that of PGHS-1 (8.3 kb). The PGHS-2 mRNA is approximately 4.0-4.5 kb in length and codes for a 72 kDa protein

(Tazawa *et al.* 1994). The 5'-untranslated region of this gene reveals the features of a primary response gene. It contains a TATA box 30 bp upstream and putative binding motifs for NF-kB, NF-IL6, C/EBP, AP2, SP1, and CRE within the 290 bp upstream from the transcription start site (Tazawa *et al.* 1994). Transient expression of these constructs linking this 290 bp region to the luciferase gene has demonstrated that the promoter activity resides in this region (Tazawa *et al.* 1994). Corresponding regions in the rat, mouse and chicken have also shown similar promoter activity (Xie *et al.* 1993; Sirois *et al.* 1993). The 3'-untranslated region of PGHS-2 mRNA contains multiple AUUUA motifs, sequence motifs identified as being responsible for shortening the half-life of mRNA (DeWitt & Meade, 1993a).

1.6.2.2 PGHS protein structure

PGHS-1 and PGHS-2 are homodimeric, heme-containing, glycosylated proteins, each with two catalytic sites. The PGHS-1 molecule comprises three distinct domains: an epidermal growth factor-like domain, a membrane binding unit and a catalytic domain. Within the catalytic domain, the sites for peroxidase and cyclooxygenase are adjacent but spatially distinct. Analysis of the crystal structure reveals that the cyclooxygenase active site is at the apex of a long, hydrophobic channel which widens at this site to form a cavity. Tyr-385, the putative radical acceptor, is

located in the centre of the cyclooxygenase cavity. The channel and cavity are lined by nonpolar residues with only two charged residues (Arg-120 and Glu-524). Ser-530 lies at the point where the channel widens. Four potential N-glycosylation sites have been identified in the ovine PGHS-1, with glycosylation of Asn-410 being required for the expression of the peroxidase and cyclooxygenase activities (Ruegg & Burgess, 1989; Yanagihara *et al.* 1991).

Within a species, there is approximately 60% amino acid identity between the deduced amino acid sequences of PGHS-1 and PGHS-2. The most notable difference between the two PGHS proteins is that PGHS-2 contains a cassette of 18 amino acids near the C-terminus of the enzyme that is absent in PGHS-1 (Sirois and Richards, 1992).

Even though PGHS-1 and PGHS-2 are only 60% homologous, the residues lining the cyclooxygenase channel and cavity are almost all conserved, so that their crystal structures are essentially superimposable. Both enzymes also exhibit similar K_m and V_{max} values for the metabolism of arachidonic acid (Ruegg & Burgess, 1989). Both PGHS-1 and PGHS-2 are bound to the lumenal surface of the endoplasmic reticulum and contiguous outer membrane of the nuclear envelope.

1.6.2.3 Expression of PGHS-1 and PGHS-2

PGHS-1 has been termed the "constitutive" isozyme and PGHS-2, the "inducible" isoform, yet recent evidence suggests that this may be an oversimplification. Although PGHS-1 is detected in most tissues at levels which remain constant, levels have been shown to change during development (Brannon *et al.* 1994). Furthermore, expression of PGHS-1 can be down-regulated in endothelial cells in response to acidic fibroblast growth factor and up-regulated in mast cells treated with stem cell factor and dexamethasone (Smith *et al.* 1996; Samet *et al.* 1995). PGHS-1 mediates basal production of PGE_2 (Langenbach *et al.* 1995), and within the mouse kidney, is constitutively expressed in arteries and arterioles, glomeruli, collecting ducts and medullary interstitial cells, with no immunoreactivity detected to date in proximal or distal convoluted tubules, loop of Henle or macula densa (Harris *et al.* 1994; Smith & Bell, 1978).

PGHS-2 mRNA and protein is undetectable in most tissues, however, its expression is stimulated in a variety of cell types. Evidence exists for PGHS-2 induction by serum, growth factors, cytokines and phorbol esters in fibroblasts (Kujubu *et al.* 1991; Kujubu *et al.* 1993; O'Banion *et al.* 1992), vascular smooth muscle (Ruegg & Burgess, 1989) and endothelial cells (Habib *et al.* 1993); by lipopolysaccharide (LPS) in monocytes and macrophages (Lee *et al.* 1992) and by chorionic gonadotrophin in ovarian follicles (Sirois and Richards, 1992). In each cell type, the induction of

PGHS-2 requires protein synthesis and, where tested, expression of PGHS-2 mRNA and protein is inhibited by the anti-inflammatory steroid, dexamethasone. Many of the agents that stimulate PGHS-2 expression act on receptors that have an intracellular tyrosine kinase domain, activation of which results in the phosphorylation of proteins(Corbett *et al.* 1996). PGHS-2 is also constitutively expressed in brain, testes, tracheal epithelia and the kidney (Yamagata *et al.* 1993; Smith *et al.* 1996; Harris *et al.* 1994). PGHS-2 immunoreactivity in the adult rat kidney has been localized to the macula densa, cortical thick ascending limb and interstitial cells of the inner medulla, with no immunoreactivity in arterioles, glomeruli or collecting ducts (Harris *et al.* 1994).

Gene knock-out studies (Morham *et al.* 1995; Dinchuk *et al.* 1995) demonstrate that PGHS-2-null mice have a shortened survival time. These investigators found that kidneys from mutant mice were small, pale and had a granular appearance on the capsular surface. The observed nephropathy was characterized by areas of abnormal subcapsular parenchyma comprised of small immature glomeruli and tubules, consistent with nephron hypoplasia. Analysis of tissue from 3-day old mice to 6-week and older mice revealed these postnatal renal developmental abnormalities progressively deteriorated with increasing age.

Therefore, PGHS-2 appears to play an important role in renal development, and displays a multitude of functions, depending on the tissue and stage of animal development. The role of PGHS-2 within the kidney remains unclear.

1.6.3 Renal prostaglandin receptors

Following intracellular synthesis, prostanoids exit the cell, probably via facilitated diffusion, or perhaps via the recently cloned human prostaglandin transporter (Lu *et al.*, 1996). Although this transporter is expressed in the kidney, specific renal localization has not been reported to date. Upon exiting the cells, prostanoids act on the parent cell and/or the neighbouring cells in an autocrine and/or paracrine fashion through specific G protein-linked prostanoid receptors (Smith, 1992). Local production of prostaglandins may be stimulated by several hormones including angiotensin II, vasopressin and bradykinin, and the prostanoids may thereby mediate or modulate the actions of these hormones (Dunn & Hood, 1977; Bonvalet *et al.* 1987).

Prostaglandins can regulate both renal hemodynamics and epithelial water and solute transport. Each prostaglandin has been shown to exert distinct effects on these targets. In addition, a single prostaglandin, such as PGE_2, can have multiple and at times opposing functional effects on a target tissue. An example of this is PGE_2's action as a vasodilator in some vascular beds and a vasoconstrictor in others (Breyer *et al.* 1996). These diverse effects are accounted for by multiple receptor subtypes for individual prostaglandins. The nomenclature has been standardized so that all PGE_2 receptors are referred to as "E-prostanoid" receptors, or EP receptors. Subtypes are designated by subscript so that EP_1, EP_2, EP_3, and EP_4 refer to different isoforms of PGE_2 receptors.

The family of EP receptors couple to their intracellular effectors via G proteins, and possess seven transmembrane hydrophobic domains (Henderson *et al.* 1990). These receptors bind their ligands with the seven transmembrane a-helices, and couple to G-proteins via their intracellular sequences, in particular the third intracellular loop and the proximal portion of the C-terminal tail (O'Dowd *et al.* 1989).

The EP_1 receptor has been localized mainly to the cortical collecting duct (CCD), where PGE_2 has been shown to inhibit Na^+ transport by a mechanism coupled to the release of Ca^{2+} from intracellular stores (Hebert *et al.* 1991a; Hebert *et al.* 1993). This receptor most likely exerts its effects by coupling to diacylglycerol/ inositol phosphate (IP_3). The G_i-protein coupled EP_2 receptor has been detected in lung and placental tissue, with only very low levels being expressed in the kidney (Regan *et al.* 1994). The EP_4 receptor subtype, also a G_s-coupled receptor, has been shown, by in situ hybridization, to be expressed in low levels in the glomerulus, suggesting it may participate in the glomerular vasodilatory effects of PGE_2 (Breyer *et al.* 1996; Edwards, 1985).

The EP_3 receptor is unique among the EP receptors in that multiple alternatively spliced variants have been reported to exist (Breyer *et al.* 1996). These receptor isoforms are identical throughout the seven transmembrane domain, differing in the C-terminal amino acid sequence and/or in the 3' untranslated region of the

cDNA (Breyer *et al.* 1996). Although the splice variants may cause changes in signal transduction responses, most EP_3 receptors couple to a G_i-protein to decrease cAMP via inhibition of adenylate cyclase (Hebert *et al.* 1993; Sugimoto *et al.* 1992; Goureau *et al.* 1992).

In situ hybridization and reverse transcription polymerase chain reaction (RT-PCR) in microdissected rat nephron segments have been used to determine the intrarenal distribution of EP_3 receptor mRNA (Breyer *et al.* 1993; Takeuchi *et al.* 1993). High levels of EP_3 mRNA were detected in mTAL, with lower levels of expression evident in the MCD and CCD. While EP_3 mRNA was detected in rat cortical TAL, no significant expression was apparent in rabbit cortical TAL. The differential expression of EP_3 mRNA in the cortical versus medullary TAL in the rabbit corresponds to the effect of PGE_2 on ion transport in these regions, since PGE_2 inhibits Cl^- absorption in the rabbit medullary TAL, but has no effect on cortical TAL Cl^- transport (Stokes, 1979). This suggests that activation of the EP_3 receptor may cause inhibition of NaCl reabsorption in the TAL (Breyer *et al.* 1996).

1.7 ANGIOTENSIN II

Angiotensin II (Ang II) is a multifunctional hormone, regulating blood pressure, plasma volume, neuronal function and dipsogenic responses . Ang II stimulates a variety of physiologic responses that support arterial blood pressure and renal function, and contributes to the pathogenesis of hypertension, arterial disease, cardiac hypertrophy, heart failure and diabetic renal disease (Goodfriend *et al.*, 1996).

Ang II plays a major role in regulating renal blood flow (RBF) and glomerular filtration rate (GFR). The three potential regulatory sites of the glomerular circulation, afferent and efferent blood vessels and glomerular capillary surface area, are targets for the actions of Ang II (Schramek *et al.* 1995). Ang II has been reported to increase renal release of vasodilatory prostaglandins such as PGE_2, which attenuate the vasoconstrictor actions of Ang II.

Ang II is derived from the protein precursor angiotensinogen by the sequential action of proteolytic enzymes, including a reaction catalyzed by angiotensin-converting enzyme (ACE), which occurs in plasma as well as in tissues such as the kidneys, brain, adrenal gland and ovaries (Johnston, 1992). Synthetic capability for all components of the renin-angiotensin system has been identified within the kidney, in the glomerulus and proximal tubule (Hollenberg and Fisher, 1995). In situ hybridization has been

used to localize angiotensinogen, renin and ACE mRNA within the kidney. Renin and Ang II have been co-localized to juxtaglomerular cells. Measurement of cortical tissue Ang II levels demonstrates that tissue concentrations are much higher than plasma. Therefore, the renal effects mediated by Ang II may be due to local production of this hormone.

1.7.1 Angiotensin II receptors

The effects of Ang II on target organs are mediated by plasma membrane receptors, of the G-protein-coupled variety, with seven transmembrane domains (Griendling *et al.* 1996). Thus, the receptor is a pivotal control point for the regulation of tissue-specific functions. Several subtypes of Ang II receptors have been cloned and antagonists to these receptors have been developed. Two major receptor subtypes have been characterized and classified as AT_1 and AT_2 receptors. These receptors are functionally distinct, with an amino acid sequence homology of only 30%. The gene for the AT_1 receptor is located on human chromosome 3, and for the AT_2 receptor on chromosome X (Szpirer *et al.* 1993; Koike *et al.* 1994).

AT_1 receptors mediate most of the known functions of Ang II. These receptors demonstrate selective inhibition by biphenylimidazoles, typified by losartan, and are insensitive to tetrahydroimidazopyridines, such as PD123177 (Bumpus *et al.* 1991).

AT_1 receptors show higher affinities for Ang I and Ang II than for Ang III . In the rat and mouse, two AT_1 receptor variants, AT_{1A} and AT_{1B}, which differ in 18 amino acids have been identified. These variants exhibit 94% overall sequence identity. These two cloned receptors display an open reading frame of 1077 bp, encoding a 359-amino acid, 41-kDa protein. cDNAs encoding AT_1 receptors in the human, rabbit, dog and pig have also been cloned (Griendling *et al.* 1996) and show more than 90% homology to the rat and bovine AT_1 receptor at the amino acid level. Pharmacological studies of the expressed cDNAs demonstrate that AT_{1A} and AT_{1B} mRNAs code for proteins with almost identical ligand-binding affinities and receptor-effector coupling.

1.7.1.1 Tissue distribution

Tissue distribution of AT_{1A} and AT_{1B} receptors has been examined only at the mRNA level since these receptors are pharmacologically indistinguishable. Both subtypes have been identified in the spleen, liver and kidney (Kakar *et al.* 1992b; Sandberg *et al.* 1992). AT_{1A} receptors predominate in vascular smooth muscle, heart, brain, lung and kidney, whereas AT_{1B} is the major receptor subtype in the pituitary and adrenal glands (Kakar *et al.* 1992b; Kakar *et al.* 1992a; Sandberg *et al.* 1992; Kitami *et al.* 1992).

AT_2 receptors are most highly expressed in the developing fetus, with a decrease in expression after birth (Feuillan *et al.* 1993). The exact role of AT_2 receptors in development still remains to be determined. AT_2 receptors have also been detected in skeletal muscle, ovary, adrenal medulla (Feuillan *et al.* 1993) and brain (Tsutsumi & Saavedra, 1991). RT-PCR has been used to demonstrate low levels of expression in liver, lung, heart and kidney (Ichiki & Inagami, 1995).

1.7.1.2 Signal transduction

Depending on the cell type, AT_1 receptors have been shown to be coupled to activation of phospholipases A_2, C and D, to voltage-dependent Ca^{2+} channels, and to inhibition of adenylate cyclase (Griendling *et al.* 1996). These effectors couple to the receptor via G-proteins, which also differ depending on the cell type. AT_1 receptors may also be coupled directly to kinases initiating tyrosine phosphorylation cascades (Marrero *et al.* 1994).

The major signalling pathway in kidney proximal tubular cells is the coupling of Ang II to phospholipase A_2 (Morduchowicz *et al.* 1991). This coupling has also been observed in vascular smooth muscle and mesangial cells as measured by the production of prostaglandins (Rao *et al.* 1994; Schlondorff *et al.* 1987). Ang II can also activate a phosphatidylinositol-specific phospholipase C, releasing inositol trisphosphate (IP_3)

and diacylglycerol (DAG), and a phosphatidylcholine-specific phospholipase D, generating phosphatidic acid (PA) and choline. IP_3 then releases Ca^{2+} from intracellular stores, resulting in the activation of calmodulin-dependent enzymes. In the presence of these increased intracellular Ca^{2+} levels, DAG, produced either by phospholipase C or by the sequential activation of phospholipase D and phosphatidic acid phosphohydrolase, activates the protein kinase C enzymatic cascade (Tsuda *et al.* 1993). Activation of phospholipase C by Ang II is transient, whereas activation of phospholipase D is sustained. In this manner, the activation of protein kinase C which mediates specific cellular functions, is prolonged (Tsuda *et al.* 1993).

Adenylate cyclase is coupled to the AT_1 receptor via G_i, a pertussis toxin-sensitive G protein (Pobiner *et al.* 1985; Anand-Srivastava, 1989). The coupling of AT_1 receptors to adenylate cyclase is mainly inhibitory, resulting in the attenuation of cAMP generation and the responses it regulates (Pobiner *et al.* 1985). Cyclic AMP, through activation of protein kinase A, phosphorylates cellular proteins and regulates a wide variety of cellular functions, including Na^+ transport in proximal tubular cells (Douglas *et al.* 1990).

The signal transduction mechanisms used by AT_2 receptors are not completely understood. Three types of second messenger activation have been proposed, depending on the tissue type studied: inhibition of cGMP production (Bottari *et al.*

1992), activation of K^+ channels (Kang *et al.* 1992), and inhibition of phosphotyrosine phosphatase (Bottari *et al.* 1992).

1.7.1.3 Regulation of receptor expression

One of the most potent regulators of AT_1 receptors is Ang II itself (Gunther *et al.* 1980). However, regulation by other factors such as ACTH, insulin, estrogen and growth factors has also been reported (Takayanagi *et al.* 1992; Kakar *et al.* 1992a; Nickenig & Murphy, 1994). Regulation has been demonstrated to occur at the transcriptional and posttranscriptional levels (Sandberg, 1994; Nickenig & Murphy, 1994). Although transcriptional regulation has been shown, the specific promoter sites are not yet known. The mechanisms underlying posttranscriptional regulation are also still unclear, though induction of a destabilizing factor has been postulated (Nickenig & Murphy, 1994). This is consistent with the presence of AUUUA signals in the 3' untranslated regions of all Ang II AT_1 receptor mRNAs (Sandberg, 1994).

1.7.1.4 AT_1 Receptor desensitization

In many systems, the response to Ang II is tachyphylactic, such that the action of Ang II is transient, with the target tissue becoming refractile to further stimulation by the agonist. Two mechanisms put forward to explain this desensitization

phenomenon are receptor internalization and receptor phosphorylation.

Angiotensin receptors have been shown to be subject to phosphorylation, a reversible effect, which does not affect the binding of Ang II, but instead uncouples the receptors from the signal transduction apparatus (Oppermann *et al.* 1996). Although the exact site of phosphorylation is unknown, several sequences in the tail of the receptor could serve as susbstrates for G protein receptor kinases.

Sequestration and internalization of the AT_1 receptor requires binding of an active hormone agonist, and is not stimulated when an antagonist is bound (Hunyady *et al.*, 1994). The AT_1 receptor contains a region rich in serine and threonine in the carboxy-terminal cytoplasmic tail which is essential for internalization (Hunyady *et al.* 1994). In cultured vascular smooth muscle cells, radioactive Ang II was shown to be internalized with a half life of 1.5 min, with 90% of the receptor being internalized by 10 min (Griendling *et al.* 1996; Anderson *et al.* 1993). Once the agonist binds to the receptor, the ligand-receptor complex appears to migrate within the plane of the membrane to concentrate in coated pits (Anderson *et al.* 1993), becomes internalized in small vesicles, and subsequently becomes associated with lysosomes. In this manner, internalization of the agonist-receptor complex contributes to the rapid desensitization of the cells to further agonist exposure.

1.8 RATIONALE AND OBJECTIVES OF STUDY

The association of hypercalcemia with disturbances in renal concentrating ability, water intake and loop of Henle function has been widely recognized (Cohen *et al.* 1957; Gill & Bartter, 1961; Epstein *et al.* 1959; Epstein *et al.* 1958). Studies in rats show that chronic hypercalcemia is associated with inhibition of thick ascending limb NaCl reabsorption (Peterson, 1990). NaCl reabsorption by the medullary thick ascending limb (mTAL) is considered to be the most important component of the renal concentrating and diluting mechanisms. It is established that reabsorption of NaCl by mTAL is driven by active transport of Na^+ across the basolateral membrane via the Na^+-K^+-ATPase pump coupled to uptake of Na^+ and Cl^- by the bumetanide-sensitive Na^+-K^+-$2Cl^-$ co-transporter, and that Cl^- exit is via specific basolateral Cl^- (conductance) channels. Therefore, net reabsorption of NaCl by the mTAL can be decreased by either inhibiting the apical uptake step or by inhibiting the basolateral exit step.

Renal prostaglandin E_2 (PGE_2) excretion is elevated in hypercalcemic rats (Levi *et al.* 1983; Serros & Kirschenbaum, 1981), suggesting that PGE_2 may be involved in mediating this defective NaCl reabsorption. In rats with vitamin D-induced chronic hypercalcemia, outer medullary PGE_2 levels are increased, and PGE_2 appears to mediate the inhibition of thick ascending limb NaCl transport (Peterson *et al.* 1993).

The availability of arachidonic acid is a rate-limiting step controlling the synthesis of prostaglandins. Release of arachidonic acid from membrane phospholipids is regulated primarily by phospholipase A_2. PGHS is another key enzyme involved in prostaglandin synthesis. Mammalian cells contain two isoforms of PGHS (PGHS-1 and PGHS-2). PGHS-1 mediates basal production of PGE_2 (Langenbach *et al.* 1995) and PGHS-2 has been termed the "inducible" form of the enzyme, since its expression is often undetectable unless induced by mitogenic or proinflammatory agents (Kujubu *et al.* 1991; Kujubu *et al.* 1993; O'Banion *et al.* 1992; Habib *et al.* 1993; Lee *et al.* 1992). In the kidney, however, PGHS-2 immunoreactivity has been detected in the absence of inflammation (Harris *et al.* 1994). Angiotensin II has been reported to increase PGE_2 production in a number of systems (Schrameck *et al.*, 1995).

The specific mechanisms by which PGE_2 exerts its effects on NaCl transport in the mTAL in hypercalcemia are not known. Furthermore, the source, mechanisms and stimulus responsible for elevated renal PGE_2 levels in hypercalcemia are unknown.

AIM OF STUDY

The overall purpose of my thesis was to investigate the mechanisms involved in regulating NaCl transport and PGE_2 production in hypercalcemia. Specifically, I had 3 major aims:

1. To determine if changes in apical and/or basolateral transport steps are responsible for inhibiting NaCl transport in the mTAL.
2. To determine the effect of hypercalcemia on $cPLA_2$, PGHS-1 and PGHS-2 protein expression.
3. To investigate the role of Ang II AT_1 receptors in hypercalcemia.

This thesis is divided into 3 sections to address these aims. My specific objectives and hypotheses to be tested were as follows:

SECTION 1. To determine if changes in apical and/or basolateral transport steps are responsible for inhibiting NaCl transport in the mTAL

Objective 1. To test the hypothesis that hypercalcemia-induced inhibition of mTAL Cl^- reabsorption is due to inhibition of the basolateral Na^+-K^+-ATPase pump.

Objective 2. To test the hypothesis that inhibition of mTAL Cl^- reabsorption in hypercalcemia is due to inhibition of the apical Na^+-K^+-$2Cl^-$ cotransporter.

Objective 3. To test the hypothesis that high Ca^{2+}_o and/or PGE_2 inhibit the mTAL apical Na^+-K^+-$2Cl^-$ cotransporter.

SECTION 2. To determine the effect of hypercalcemia on $cPLA_2$, PGHS-1 and PGHS-2 protein expression

Objectives 4. To test the hypothesis that expression of intrarenal $cPLA_2$, PGHS-1 and/or PGHS-2 proteins is increased in hypercalcemia.

Objective 5. To determine, by immunohistochemistry, the location of expression of intrarenal $cPLA_2$ and PGHS-2 in hypercalcemia.

SECTION 3. To investigate the role of Ang II AT_1 receptors in hypercalcemia

Objective 6. To test the hypotheses that intrarenal Ang II levels are increased in hypercalcemia.

Objective 7. To determine the effect of blockade of AT_1 receptors on intrarenal $cPLA_2$ and PGHS-2 protein expression, urinary PGE_2 excretion, and water intake in hypercalcemia.

METHODS

2. *METHODS*

2.1 TREATMENT OF ANIMALS

All animal studies were conducted on male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing 250-320 g. The animals were individually housed in temperature and humidity-controlled rooms with 12 h light and 12 h dark cycles. Upon arrival , the rats were given a 5 day acclimatization period with free access to tap water and Purina Rat Chow. Following this acclimatization period, the animals were divided into two groups, control and hypercalcemic, and their diets replaced as described below. Daily water intake and body weight were recorded for all animals.

At a predetermined time during the study period, rats were anesthetized with Somnitol (60 mg/kg i.p.) as per a protocol approved by the Animal Care Committee (University of Ottawa). Immediately prior to sacrifice, cardiac puncture was performed to obtain blood for measurement of ionized Ca^{2+} and pH by ion-specific electrodes (Stat Analyzer, model 634; Ciba Corning, Medfield, MA). The analyzer corrected the reported blood Ca^{2+} to pH 7.40.

2.1.1 Control group

The animals in this group were fed a commercially obtained control diet (ICN Nutritional Biochemicals, Cleveland, OH) [in g/kg: $MgCl_2.6H_2O$, 37.1; KCl, 8.1; KH_2PO_4, 17.8; corn starch, 199.0; casein purified high nitrogen, 300.0; butter, 35.0; $CaCO_3$, 13.0; NaCl, 10.0; sucrose, 380.0] for up to 5 days. Since food intake of hypercalcemic animals has been shown to be lower than that of rats on the control diet (Levi *et al.* 1983), to ensure similar caloric, protein and mineral intake between the two groups, food intake of control rats was matched to hypercalcemic rats. To achieve this, the rats in the control group were allowed 7g of diet per 100 g body weight per day, an amount equal to that consumed by hypercalcemic rats. Rats were allowed free access to tap water.

2.1.2 Hypercalcemic group

Hypercalcemia was induced in rats by feeding them an identical standard diet (described above) to which the vitamin D analogue dihydrotachysterol (Philips Roxan, Columbus, Ohio), was added at a concentration of 4.25 mg/kg of diet (DHT-containing diet). Such supplementation with vitamin D has been demonstrated to increase serum Ca^{2+} significantly (Levi *et al.* 1983). Rats were allowed free access to both the diet and water for 1 to 5 days.

2.2 TISSUE DISSECTION AND MEDULLARY THICK ASCENDING LIMB TUBULE ISOLATION

The method used for the isolation of medullary thick ascending limb (mTAL) tubule suspensions from rat kidneys was modified from one previously described by Trinh-Trang-Tan et al (Trinh-Trang-Tan *et al.* 1986).

Male Sprague-Dawley rats (300-350 g body weight) were anaesthetized with somnitol, 60 mg/Kg (i.p.). The abdominal cavity was opened, the kidneys removed by excision as rapidly as possible, and placed in Hanks balanced salt solution (HBSS) [137.9 mM NaCl, 1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose] on ice. The kidneys were sliced evenly into ~ 1 mm slices and placed into ice-cold HBSS. Each slice was then dissected under a dissecting microscope at 12X magnification. The papilla was first removed and discarded and the inner stripe of the outer medulla dissected from the outer stripe.

Tissue pieces derived from the inner stripe of the outer medulla were then passed through a polypropylene filter (opening 1 x 1 mm) to obtain small cubes of tissue of fairly uniform size. Tissue was then incubated in a shaking water bath at 37°C, in 10 ml HBSS containing collagenase (Type A, Boehringer Mannheim, 0.07% wt/vol), for four periods of 15 min each. At the end of each 15 min digestion period, the tissue was triturated three times through 190 P.E. tubing (attached to a 15g luer

adapter and a 10-cc syringe) to release any digested tubules and 1 min was allowed for sedimentation of incompletely digested tissue pieces. The supernatant containing isolated cells and dissociated tubules was then transferred to an HBSS solution containing 1.5% bovine serum albumin (BSA) on ice. Ten millilitres of fresh HBSS-collagenase solution was added to the remaining tissue pieces for the next 15 min incubation period at 37°C. In this manner, the outer medullary tissue was serially digested from the surface inward.

At the end of all digestion periods, the supernatants were pooled and passed through a 100 mm pore-diameter nylon sieve. This allowed isolated cells, thin limbs and collecting ducts to pass through the sieve with the collagenase solution, leaving the thick ascending limbs trapped on the sieve. The tubules were then collected by rinsing the sieve with HBSS-BSA solution. The tubule suspension was centrifuged for 5 min at low speed (80 x g), the supernatant discarded and the pellet resuspended in fresh HBSS to yield an mTAL tubule suspension. All glassware used in this preparation was siliconized with Sigmacote (Sigma). This treatment prevents the mTAL tubules from sticking to the glass surface (Sigma).

The viability of the cells in the mTAL tubule suspension was assessed using the trypan blue exclusion test. Using pipette tips with the ends cut to a 1 mm wide opening, 10 ml of the mTAL suspension was placed onto a microscope slide together with 10 ml trypan blue. The proportion of viable mTAL in the preparation was

assessed by differentiating between those nuclei which did not take up the trypan blue dye (viable) and those which did (non-viable).

2.3 PROTEIN DETERMINATION

The Bio-Rad protein assay, a modification of the Bradford method (Bradford, 1976), was used for the measurement of protein. This dye-binding assay is based on the principle that a differential colour change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to basic and aromatic amino acid residues (especially arginine) occurs. Thus, a colour change occurs (from red to blue) which is proportional to the amount of protein that is bound.

An aliquot of the sample is digested to soluble protein with 1N NaOH . The protein sample is then centrifuged at 1500 x *g* for 5 min and an aliquot (- volume depends on the type of sample being assayed) of the supernatant removed for protein determination. The sample is brought up to a volume of 800 ml with distilled water. A water blank (800 ml dH_2O), an NaOH blank (volume of NaOH present in sample aliquot assayed plus dH_2O) and a set of standards made from BSA (1.25, 2.5, 5.0, 10.0

and 20.0 mg/ml) are also prepared. Biorad protein assay dye reagent (200 ml) is then added to all tubes and the absorbance of each sample measured at 595 nm using a Beckman spectrophotometer. A standard curve is generated from the optical density of the five standard concentrations at 595 nm plotted against the protein values in mg. The standard curve is used to determine the concentration of the protein in the unknown samples.

2.4 ANTI-TAMM-HORSFALL ANTIBODY LABELLING OF mTAL TUBULES

The mTAL tubules isolated as described above were labelled with anti-Tamm-Horsfall antibody and evaluated by indirect immunofluorescence. The tubules were suspended in HBSS and 150 mg of the tubule suspension was transferred to glass slides. The slides were allowed to air dry (approximately 1 hour) and the tubules subsequently fixed and permeabilized with 50% acetone and 50% methanol for 5 min. Care was taken not to allow the sections to dry at any time during the remainder of the procedure. The slides were then washed in PBS for 3 x 5 min.

All sections were first stained for DNA using 50 ml of the DNA-binding fluorochrome Hoechst 33258 at a concentration of 0.05 mg/ml for 30 min (in the dark, at room temperature). Following a 3 x 5 min wash in PBS, 50 ml of goat

antiserum to human uromucoid (Tamm-Horsfall glycoprotein, Organon Teknika Corporation, 1:50 dilution) was placed on the sections and left to incubate for 30 min at room temperature. At the end of this time the slides were washed in PBS for 3 x 5 min and then incubated with 50 ml of fluorescein-5-isothiocyanate (FITC)-conjugated rabbit affinity purified antibody to goat IgG (Organon Teknika Corporation, 1:20 dilution) for 30 min in the dark. Finally, the slides were again washed with PBS for 3 x 5 min and mounted with 90% glycerol. Control slides consisted of (1) omission of primary antibody (goat anti-human uromucoid antiserum, replaced with PBS) and (2) omission of conjugated second antibody (FITC-conjugated rabbit anti-goat IgG).

Specimens were viewed with a Zeiss Axioplan Universal microscope equipped with a fluorescence illuminator with an HBO 50 mercury lamp and filters for FITC (excitation: 450/490 nm; emission: 515 nm) and Hoechst 33258 (excitation: 355/425 nm; emission: 460 nm) and a Zeiss MC100 camera. The photographs were taken using Kodak Tri-X pan 400 ASA film and subsequently developed and printed. This procedure was conducted three times on three different mTAL preparations.

2.5 DETERMINATION OF Na^+-K^+-ATPASE ACTIVITY IN mTAL TUBULES FROM CONTROL AND HYPERCALCEMIC RATS

Rats were fed control or DHT-containing diet for 5 days for this study. Prior to anaesthesia, the rats were injected with 2 mg/kg BW furosemide (i.p.) to inhibit activity of the Na^+-K^+-$2Cl^-$ cotransporter. Once removed, both kidneys were placed into HBSS containing 1 mM furosemide and the mTAL tubules isolated as described above. Treatment of animals with furosemide at this concentration has been shown to improve viability of the isolated segments since there is a decrease in ATP utilization (Sun *et al.* 1991). An aliquot was removed for protein determination.

2.5.1 Permeabilization of tubules

It was necessary to permeabilize the tubules before activity of the Na^+-K^+-ATPase pump could be measured, in order for the ^{32}P-ATP to access the cell interior. Two methods were employed: (1) a combination of freeze/thaw and osmotic shock treatment. An aliquot of tubule suspension was centrifuged and resuspended in distilled water. The sample tube was then submersed alternately in liquid nitrogen and a 37°C water bath three times; (2) treatment with the antibiotic alamethacin. Both methods have been reported to permeabilize membranes (Doucet *et al.* 1979; Grossman & Hebert, 1988). An aliquot of the tubule suspension was incubated with 5 ml/ml

alamethacin at 37°C for 15 min.

Both methods increased the amount of $^{32}P_i$ measured in the activity assay (see Results section) as compared to non-permeabilized tubules. Treatment with alamethacin was found to be more reproducible and easier to perform than the freeze/thaw method, and so, was the method used for permeabilizing tubules in subsequent assays.

2.5.2 Na^+-K^+-ATPase activity assay

Na^+-K^+-ATPase activity was assayed using a phosphate release assay, using g-^{32}P labelled ATP as a tracer (method obtained from Dr. Yves Berthiume, University of Montreal, Que). The assay uses the property of activated charcoal to specifically adsorb any noncleaved substrate (ATP) and one of the products (ADP) but not the liberated $^{32}P_i$.

An aliquot of the permeabilized tubule suspension (150 mg protein) was added to 800 ml assay buffer [100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM sodium azide (NaN_3), 50 mM Tris (pH7.4)], with or without 5 mM ouabain. NaN_3 is used to inhibit mitochondrial ATPase, thus reducing background ATPase activity. The tubes were incubated for 20 min on ice to allow for ouabain binding, and

then transferred to a 37°C circulating water bath for 15 min to allow for temperature equilibration.

The assay was initiated by adding 100 ml of 40 mM ATP in assay buffer containing 5 mCi / ml ^{32}P-ATP (Amersham, 5,000 Ci/mmol) to each tube. An aliquot was removed from each tube at 2 minute intervals and pipetted into a filtration column (Quik-Sep Columns, Isolab) containing cold stop solution [1 M perchloric acid, 0.35 M NaH_2PO_4]. Perchloric acid inactivates the enzyme and the NaH_2PO_4 blocks nonspecific phosphate binding sites on the plastic column. Activated charcoal solution (2 % w/vol) was then added, to selectively bind ATP but not P_i. The funnel was vortexed, the cap removed, and allowed to drip into an empty test tube. An aliquot of the eluate was added to liquid scintillation cocktail and counted for ^{32}P in a beta liquid scintillation counter.

2.5.3 Calculations

An ATP standard (40 mM ATP) was used to calculate the average specific activity of the isotope for each assay. To calculate nmol P_i released, the following equation was used:

nmol P_i released = [cpm of sample (cpm) / specific activity (cpm/nmol)].

The nmol P_i values thus obtained were normalized to protein concentration and

plotted against time. Linear regression analysis was conducted, and the value of the slope represents nmol P_i released per minute, or activity.

2.6 MEASUREMENT OF $Na^+:K^+:2Cl^-$ COTRANSPORTER ACTIVITY BY ^{86}Rb UPTAKE

Since the $Na^+:K^+:2Cl^-$ cotransporter transports ^{86}Rb in place of K^+, bumetanide-sensitive ^{86}Rb uptake was used to measure cotransporter activity. The experiments were performed in the presence and absence of ouabain to determine the contribution of the Na^+-K^+-ATPase pump to ^{86}Rb influx. These experiments were conducted in rat mTAL tubule suspensions and on a mouse mTAL cell line, as described below.

2.6.1 mTAL tubule suspensions

Medullary thick ascending limb tubules from control and hypercalcemic rats (maintained on diet for 5 days) were isolated as described above. In some experiments, rats were infused for 5 min with 10% mannitol, 0.45% NaCl through the jugular vein, since this procedure has been reported to improve the patency of the isolated tubules and thereby improve access to the apical membrane (Chamberlin *et al.* 1984). ^{86}Rb uptake was performed on the tubule suspensions as described by Sun and co-workers (1991), with some modifications.

An aliquot (300 mg) of tubule suspension resuspended in uptake solution [composition: 100 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 20 mM mannitol, 1 mM $CaCl_2$, 20 mM $BaCl_2$, 5 mM RbCl, 10 mM Hepes, pH 7.4] was incubated at 37°C for 20 min, with or without 5 mM ouabain and 100 mM bumetanide. ^{86}Rb uptake was initiated with the addition of 100 ml ^{86}Rb solution containing 20 mCi/ml ^{86}Rb (Amersham, 880 mg Rb / ml) in 5 mM RbCl. Uptake was allowed to proceed for up to 180 sec.

At specific time points, aliquots were removed from the reaction tube and added to ice cold stop solution [140 mM KCl, 10 mM Hepes, pH 7.4] on filters (Millipore, 0.45 mM). The tubules were washed with the stop solution for a total of 4 times. The filter containing the tubules was then transferred to a scintillation vial containing 2 ml dH_2O. The tubules were lysed with a combination of hypotonic shock and freeze-thawing by placing the vial alternately in liquid nitrogen and a 37°C water bath. A sample was removed for protein concentration determination, and another for ^{86}Rb determination by liquid scintillation counting.

2.6.2 ^{86}Rb Uptake in a mouse medullary thick ascending limb cell line

A mouse medullary thick ascending limb cell line was obtained from Dr. Glen Nagami's laboratory (University of California, Los Angeles, California). These cells require maintenance in an atmosphere containing 35% O_2 and 5% CO_2 since growth in the presence of elevated oxygen tension was determined to be essential for the expression of differentiated properties (Igarashi *et al.* 1996).

We received mouse TAL cells at passage # 388. Cells were split, grown and maintained in DMEM / F-12 medium supplemented with 7% fetal bovine serum as described above. The cells were kept in a modular incubator chamber (Billups-Rothenberg Inc. CA) which was gassed daily with 35% O_2 and 5% CO_2. Since the effect of high Ca^{2+} on ^{86}Rb uptake was to be studied in these cells, some cells were grown in media containing 1.65 - 1.7 mmol/L ionized Ca^{2+} for 3 days (ionized Ca^{2+} of normal medium was 1.0 - 1.12 mmol/L). The ionized Ca^{2+} concentration of the uptake solutions for the cells exposed to high Ca^{2+}_o was also adjusted to be between 1.65 and 1.7 mmol/L.

^{86}Rb uptake experiments were conducted on these TAL cells using the method described by Homma et al. (Homma *et al.* 1990). Cells were grown to 90-100 % confluence on plastic culture dishes. The cells were incubated in uptake solution [140

mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.1 mmol/L (normal Ca^{2+}) or 1.7 mmol/L (high Ca^{2+}) ionized Ca^{2+}, 5 mM glucose, 25 mM Hepes/Tris (pH 7.4)] with or without 100 mM bumetanide (Calbiochem) or 1 mM PGE_2 for 30 min at 37°C.

Uptake was initiated by adding 5 mCi/ml ^{86}Rb and uptake allowed to proceed for up to 30 min at 37°C for the time course experiments. For all other experiments, ^{86}Rb uptake was conducted for 10 min. Uptake was terminated by aspirating off the uptake solution and adding 2 ml ice cold 100 mM $MgCl_2$ buffered with 10 mM Hepes/Tris, pH7.4. Cells were rapidly rinsed 4 times with this buffer and allowed to air dry. The cells were then solubilized in 2 ml 1 N NaOH. An aliquot was removed for protein determination, and the remainder added to liquid scintillation cocktail, and ^{86}Rb counted.

2.7 Investigation of PGHS-1, PGHS-2 and $cPLA_2$ Protein Expression using Western Blot Analysis

Rats were maintained on control or DHT-containing diets for up to 5 days. Animals were anesthetized and one kidney was rapidly removed and immediately placed in 4 °C homogenization buffer [50 mM Hepes, (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 10% v/v glycerol]. The kidneys were decapsulated and

cut into 1 mm slices. The inner medulla, outer medulla and cortex were dissected from each slice, separated and placed into fresh buffer and homogenized using a polytron for 30 seconds. Tissue homogenates were centrifuged at 10,000 x g for 5 min and protein concentrations of the supernatants were measured. The supernatants were stored in aliquots at -70°C until used.

Total protein equivalents for each lysate (5 mg inner medulla; 10 mg outer medulla and 20 mg cortex) were heated at 100 °C for 5 min in sample buffer containing 63 mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 2% mercaptoethanol and 1 mg/ml of bromophenol blue. The samples were separated on 7.5% SDS/polyacrylamide minigels using the Mini PROTEAN II electrophoretic apparatus and transferred to nitrocellulose membranes using the Mini Trans blot electrophoretic transfer system (Bio-Rad Laboratories Ltd., Mississauga, Ont.).

Membranes were blocked overnight at room temperature with a solution of 5% fat-free dried milk in tris-buffered saline-0.1% Tween-20 (TBS-T). Separate membranes were incubated at room temperature with either a polyclonal PGHS-1 rabbit IgG (1:1000 dilution) (Oxford Biomedical Research Inc., Oxford, MI) for 18 h, a rabbit anti-murine polyclonal antibody to PHGS-2 (1:1000 dilution) (Cayman Chemical Co., Ann Arbor, MI) for 18 h or with polyclonal $cPLA_2$ antiserum for 1 h (1:2000 dilution) (kindly donated by The Genetics Institute, Cambridge, MA). The membranes were washed with TBS-T and incubated with goat anti-rabbit IgG conjugated to horseradish

peroxidase (1:2000 dilution) (Amersham, Oakville, Ont) for 30 min at room temperature.

Immunoreactive proteins were visualized on autoradiography film, using the enhanced chemiluminescence (ECL) method (Amersham, Oakville, Ont). This method is based on the ability of horseradish peroxidase (HRP) / hydrogen peroxide to catalyze oxidation of a cyclic diacylhydrazide, luminol, in alkaline conditions. Immediately following oxidation, the luminol is in an excited state which decays to ground state via a light emitting pathway. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by the HRP in the presence of chemical enhancers such as phenols. The light produced by this reaction peaks after 5 - 20 min after which it decays with a half-life of approximately 60 min. The maximum light emission is at a wavelength of 428 nm which can be detected by exposure to blue-light sensitive autoradiography film (Hyperfilm ECL).

2.8 INTRARENAL LOCALIZATION OF cPLA$_2$ AND PGHS-2 PROTEIN IN HYPERCALCEMIA

Kidneys from control and hypercalcemic rats were removed, immediately fixed in 10% formalin, embedded in paraffin and sections (4 mm) were mounted on glass slides.

For immunostaining, the sections were permeabilized with 1% SDS followed by incubation with 1% BSA to minimize non-specific antibody binding. The sections were then incubated for 24 h at 4 °C with either the polyclonal cPLA$_2$ antiserum (1:100 dilution), or the rabbit anti-murine antiserum (1:50 dilution) for PGHS-2 immunolocalization. Sections were also incubated with a polyclonal rabbit anti-mouse antibody to neuronal nitric oxide synthase (bNOS) (1:100 dilution), known to localize to the macula densa (Bachmann *et al.* 1995). Some sections were thereafter incubated with goat antiserum to human uromucoid (1:100 dilution, Tamm-Horsfall glycoprotein) (Organon Teknika Corporation, Scarborough, Ont.), a marker for thick ascending limb (Kikeri *et al.* 1990), for 30 min at room temperature.

Primary antibody binding for PGHS-2 or cPLA$_2$ was visualized with FITC-conjugated goat anti-rabbit IgG (1:50 dilution) (Amersham, Oakville, Ont). Goat anti-mouse IgG conjugated to texas red (1:100 dilution) was used for the detection of bNOS protein. Donkey anti-sheep IgG conjugated to cyanine 3.18 (1:50 dilution, Cy3.18)

(Bio/Can, Mississauga, Ont) was used for the detection of Tamm-Horsfall protein.

The specificity of immunostaining was determined by substitution of the primary antibody with nonimmune serum, omission of the primary antibody, and omission of the secondary antibody. All slides were mounted in glycerol containing p-phenylenediamine to inhibit fading. Sections were viewed and photographed using a Zeiss Axioplan Universal microscope equipped with a fluorescence illuminator and an HBO 50 mercury lamp.

2.9 INVESTIGATION OF THE ROLE OF ANGIOTENSIN II IN HYPERCALCEMIA

2.9.1 Measurement of intrarenal angiotensin II levels

Rats were fed the control or DHT-containing diet for 3 days and intrarenal levels of Ang II were measured by radioimmunoassay, after separation by high performance liquid chromatography (HPLC) using the method of Ruzicka et al. (Ruzicka *et al.* 1995).

2.9.1.1 Tissue Extraction

Briefly, kidneys were perfused *in situ* with phosphate-buffered saline (pH 7.4) to remove blood, and then removed and snap-frozen in liquid nitrogen. Snap-frozen kidneys were weighed, minced, and then boiled for 20 min in 1 M acetic acid. Tissue was then homogenized for 1 min using a hand-held homogenizer (Tissue Tearor, Biospec Products Inc., Racine, WI), and centrifuged at 10,000 x *g* for 10 min at 4°C.

The aqueous phase was removed and passed through a C18 Sep-Pak cartridge that had been preconditioned with 10 ml dH_2O, 10 ml 100% methanol, followed by 10 ml dH_2O, at a rate of 5 ml/min. The sample was applied to the cartridge at a rate of 1 ml/min, and the cartridge rinsed with 5 ml 0.1% acetic acid. The Ang II was eluted with 6 ml 4% acetic acid in ethanol, at a rate of 1 ml/min. Eluants were dried in a Speed Vac concentrator for further analysis.

2.9.1.2 High Performance Liquid Chromatography (HPLC)

HPLC separation of Ang II was kindly performed with the assistance of the laboratory of Dr. F. Leenen (Univ. of Ottawa), according to their published protocol (Ruzicka *et al.* 1995). Briefly, the dried eluant was dissolved in mobile phase, centrifuged and the supernatant injected. Ang II was separated on a CSC-Spherisorb-ODS2 C-18 column, 15 x 0.46 cm, particle size 5 mm (CSC, Montreal, Quebec) in a

gradient system consisting of Waters 510 HPLC pumps controlled by Waters workstation (Millipore, Millford, MA), a Rheodyne 7125 injector (Rheodyne, Cotati,CA) and a Spectra/Chrom CF-1 fraction collector (Spectrum, Houston, TX). The mobile phase was 0.05 M sodium acetate, pH 5.6, and methanol with a linear gradient from 40 to 80% methanol in 38 min from injection, at a flow rate of 1.0 ml/min. Elution time for Ang II was 12 min. Fractions were collected every 2 min into polypropylene tubes containing 50 ml 10% glycerol and 100 ml 0.05 M Tris buffer, pH 7.4. Fractions were subsequently dried overnight in a Speed Vac concentrator.

2.9.1.3 Radioimmunoassay for Ang II

Following HPLC, Ang II levels were measured in the eluants by radioimmunoassay (RIA). RIA buffer was composed of 50 mM Tris/HCl, pH 7.5 containing 0.3% BSA. Standard curves were prepared using Asn^{1}-Val^{5}-Ang II as substrate (Sigma, St. Louis, MO). To control for the presence of mobile phase, all standards were added to 2 ml of mobile phase (with 100 ml buffer and 50 ml 10% glycerol) and dried in a manner identical to the samples being measured. For the RIA, 300 ml of antibody solution in RIA buffer and 50 ml of tracer [^{125}I-angiotensin II, adjusted to give 4000-5000 cpm / tube] (Amersham, Oakville, Ont.), in RIA buffer were added to the dried samples. The antiserum concentration was adjusted to yield

40-45% of specific binding after 18 h incubation at 4°C. Following this incubation, bound and free tracer were separated using dextran-coated charcoal (non-specific binding was less than 1%). The radioactivity of the bound tracer was measured with a gamma counter. Results are expressed as fmol Ang II / g kidney.

2.9.2 Effect of blockade of AT_1 receptors in hypercalcemia

For these studies, rats were placed on the control or DHT-containing diet for 3 days. In addition, some rats were given losartan (kindly donated by Merck & Co. Inc., Rahway, NJ) in the drinking water. Water intake was measured daily and the amount of losartan administered adjusted to ensure the rats ingested 15 mg/kg/day (Kline & Liu, 1994). Blood ionized Ca^{2+} was also measured in these animals.

The animals were divided into four groups: (1) control group, in which rats received the control diet; (2) hypercalcemic group, in which rats were fed the DHT-containing diet; (3) control + losartan group, in which rats received the control diet and losartan in the drinking water; (4) hypercalcemic + losartan group, in which rats were given the DHT-containing diet and losartan in the drinking water.

Kidneys from these rats were removed and analyzed by western blot as described in Section 2.7. Furthermore, urinary PGE_2 in these animals was measured

by radioimmunoassay as described in the following section.

2.10 URINARY [PGE_2] DETERMINATION

Rats were placed in metabolic cages and their urines collected. Since urinary PGE_2 levels show a circadian rhythm, urines were collected over a 24 h period. To each urine collection bottle, 2 ml oil was added to prevent urine evaporation, and thymol to inhibit the growth of micro-organisms.

2.10.1 Urine extraction

Urines were acidified to pH 4.0 with HCl and centrifuged at 1500 x *g* for 10 min. The supernatant was removed and the pellet discarded. A C-18 reverse phase cartridge was activated by rinsing with 5 ml methanol and then with 5 ml dH_2O, at a rate of 5 ml/min. The sample (supernatant) was then passed through the column at a rate of 1 ml/min. The cartridge was then rinsed with 5 ml dH_2O followed by 5 ml of HPLC grade hexane. Finally, PGE_2 was eluted from the sample with 5 ml ethyl acetate containing 1% methanol. The ethyl acetate was then evaporated to dryness under a gentle stream of dry nitrogen.

2.10.2 PGE_2 radioimmunoassay

PGE_2 was measured using a commercially available radioimmunoassay (RIA) kit (DuPont NEN). The basic principle of the radioimmunoassay is competitive binding, whereby a radioactive antigen competes with a non-radioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from the samples or standards and a fixed amount of tracer (labelled antigen) are allowed to react with a constant and limiting amount of antibody, decreasing amounts of tracer are bound to the antibody as the amount of unlabelled antigen is increased.

The procedure used was as follows. The dried urine extract was reconstituted in RIA assay buffer [0.9% NaCl, 10 mM EDTA, 0.3% bovine g-globulin, 0.05% Triton-X-100, 0.05% sodium azide, 25.5 mM $NaH_2PO_4.H_2O$, 24.5 mM $Na_2HPO_4.7H_2O$, pH 6.8]. One hundred ml of the reconstituted sample or standard was added to 100 ml each of antiserum (rabbit anti-PGE_2, 1:18 dilution) and tracer solution (^{125}I-PGE_2, 1:25 dilution) and incubated overnight at 4°C.

At the end of the overnight incubation, 1 ml of cold precipitating reagent [16% polyethylene glycol and 0.05% sodium azide in 50 mM phosphate buffer, pH 6.8] was added and the samples were incubated in an ice bath for 30 min. In this manner, separation of the antibody-antigen complexes from free antigen was achieved by

precipitation of the antibody-bound tracer with polyethylene glycol in the presence of carrier immunoglobulin.

The samples were then centrifuged at 4°C at 2000 x *g* for 30 min. The supernatants containing the unbound antigen were decanted, and the pellet containing the antibody-antigen complex counted in a gamma counter. A standard curve was constructed using known amounts of PGE_2, and sample PGE_2 concentrations were determined by interpolation.

2.11 DENSITOMETRIC AND STATISTICAL ANALYSES

The intensity of bands obtained from Western blot analysis was quantified by scanning densitometry using a software image analysis program (Image 1.47). Protein bands from hypercalcemic rats were compared to the intensity of proteins from pair-fed control rats. An arbitrary density of 1 was assigned to the band from the control rat kidney. Measurements were not made at saturation levels of chemiluminescence. Preliminary experiments revealed that band intensity on autoradiographs for $cPLA_2$, PGHS-1 and PGHS-2 increased linearly with quantity of protein loaded.

Data are expressed as means ± SE. Significance was assigned at $P < 0.05$. Statistical analysis of the Western blots was performed using the nonparametric Kruskal-Wallis test in combination with the Dunn's multiple comparison post-hoc test to examine the differences between individual group means. All other data were analyzed using a one way analysis of variance (ANOVA) followed by the Tukey post-hoc test.

RESULTS

3. RESULTS

3.1 THE HYPERCALCEMIC RAT MODEL

Hypercalcemia was induced by feeding rats a DHT-containing diet for up to 5 days. To ensure that rats were ingesting the diet, blood ionized Ca^{2+} and water intake were measured in both control and hypercalcemic animals.

3.1.1 Blood ionized calcium

Blood ionized Ca^{2+} concentration, presented in Figure 9A, was significantly elevated after 2 days of diet (control 1.33 ± 0.03 mmol/l vs hypercalcemic 1.52 ± 0.01 mmol/l; $P < 0.05$; n = 4) and increased to a maximum on day 3 (1.65 ± 0.05 mmol/l; $P < 0.001$ vs day 0; n = 4). Levels remained significantly elevated in DHT-fed rats for the remainder of the study period (1.69 ± 0.02 mmol/l, day 5; $P < 0.001$ vs day 0; n = 5).

3.1.2 Water intake

Water intake was measured over 24 h periods in control and hypercalcemic rats. As shown in Figure 9B, compared to pair-fed control rats, water intake was significantly elevated in hypercalcemic rats after 3 days on the DHT diet ($P < 0.01$). After 5 days of DHT diet, water intake in hypercalcemic rats was significantly higher ($P < 0.001$) than both pair-fed controls and hypercalcemic rats fed the DHT diet for 3 days.

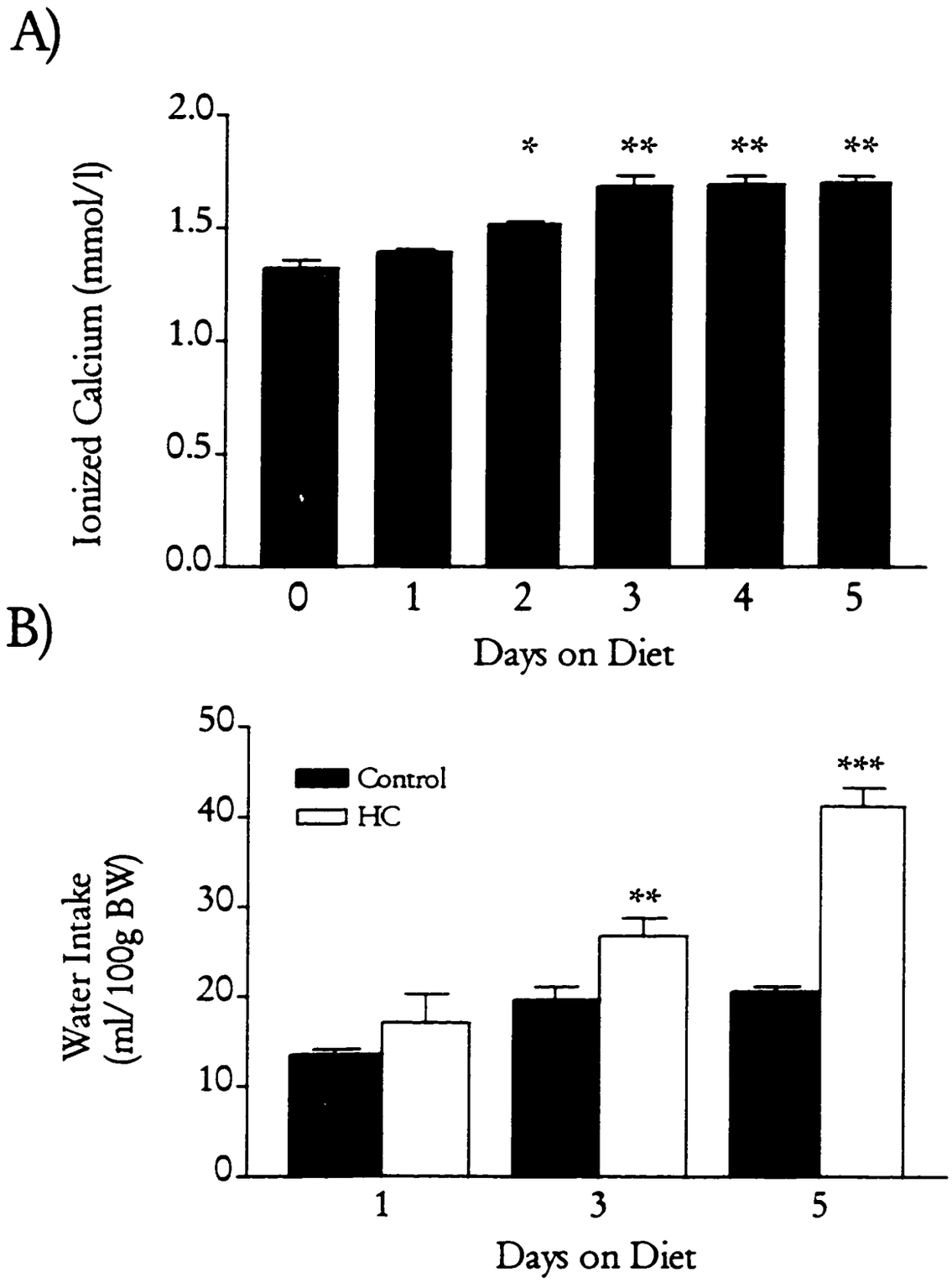


Figure 9. Effect of hypercalcemia on ionized Ca^{2+} and water intake. A. Ionized Ca2+ levels in rats fed DHT-containing diet for 0 to 5 days. B. Water intake of rats fed control (hatched bars) or DHT-containing diet (open bars) for 1, 3 and 5 days. **P <0.01 vs. control, ***P < 0.001 vs. control.

SECTION 1. Aim: To determine if changes in apical and/or basolateral transport steps are responsible for inhibiting mTAL NaCl transport in hypercalcemia

3.2 mTAL TUBULE SUSPENSIONS

The aim of these experiments was to isolate suspensions of mTAL segments from the inner stripe of the outer medulla of the rat.

The method utilized was rapid (2h), and the amount of fresh mTAL tissue obtained from 4 rat kidneys was approximately 3 mg. When viewed with standard light microscopy, the suspensions consisted of mTAL tubules of uniform diameter that were 50 - 150 mm in length. The suspensions were free of red blood cells and proximal straight tubules, but were found to contain a few fragments of thin limbs (approximately 5% of total).

Phase-contrast micrographs of a single tubule under varying magnifications are illustrated in Figure 10. These tubules are morphologically similar to images of isolated perfused mTAL segments reported by Hebert et al (1986) and those isolated from the mouse (Kikeri et al., 1990).

Figure 11 illustrates segments of the tubule suspension that were labelled with a

Figure 10. Phase contrast micrographs of a single isolated mTAL tubule under different magnifications. A. Magnification, x400; Objective, x20; Scale bar, 10 mm. B. Magnification, x800; Objective, x40; Scale bar, 10 mm. C. Magnification, x2000; Objective, x100 (oil); Scale bar, 10 mm. Note the infoldings in the basolateral membrane, especially evident in C.

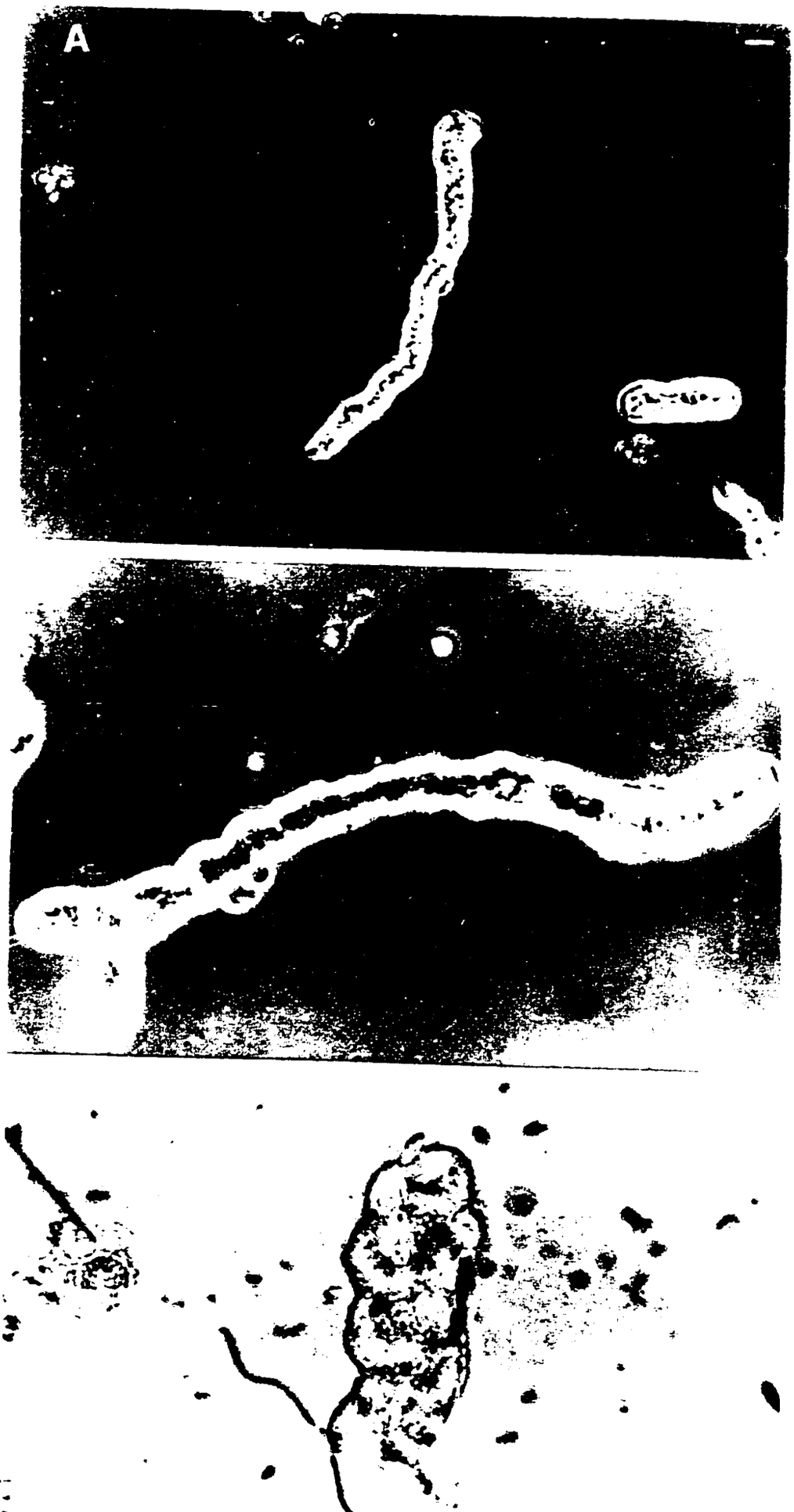

A

Figure 11. Segments of mTAL tubule suspension fluorescently labelled to reveal purity of the preparation. The same tubules in these photomicrographs were stained for DNA and Tamm-Horsfall. **A.** Phase-contrast micrograph of isolated tubules. **B.** Photomicrograph illustrating staining of tubules with the DNA-binding fluorochrome, Hoechst 33258, to identify all tubules present. **C.** Immunofluorescence micrograph of tubules stained with anti-Tamm-Horsfall antibodies to specifically locate mTAL tubules. mTALs show intense staining (thick arrow, C) as compared to thin limbs (thin arrow, C). Magnification, x400; Objective, x20; Scale bar, 10 mm. Immunostaining shown here is representative of 4 experiments.

DNA-binding fluorochrome (Hoechst 33258) and an anti-Tamm-Horsfall antibody. As shown in Figure 11B, the tubules observed in the phase-contrast micrograph (Figure 11A) stained intensely for DNA, clearly showing the labelling of many nuclei of epithelial cells found within the tubules. In addition, more than 90% of the tubules in suspension stained with anti-Tamm-Horsfall antibodies by indirect immunofluorescence (Figure 11C). The thick ascending limbs fluoresced intensely with this technique (large arrow, Figure 11C), whereas thin limbs exhibit only background fluorescence (small arrow, Figure 11C).

Prior to performing experiments with the tubule suspension, an aliquot was removed and the viability of the preparation examined by trypan blue exclusion. Using this technique, the tubule suspension was only used when there was no uptake of trypan blue into cells of the segments, indicating that a viable preparation had been achieved.

3.3 ASSESSMENT OF BASOLATERAL TRANSPORT BY MEASUREMENT OF Na^+-K^+-ATPASE ACTIVITY IN mTAL TUBULES

The purpose of these studies was to test the hypothesis that hypercalcemia-induced inhibition of TAL Cl^- reabsorption is due to inhibition of activity of the Na^+-K^+-ATPase pump.

mTALs were isolated from control animals and used to first establish a technique for permeabilization of tubules. Two methods were employed: a combination of freeze/thaw and osmotic shock (FT) and treatment with alamethacin (Ala). The effect of these manipulations on P_i released from mTAL tubules is shown in Figure 12. Compared to untreated tubules (non-permeabilized), use of either method for permeabilization resulted in a significant ($P < 0.05$, n=4) increase in P_i released. Since treatment with alamethacin was easier to perform and as reproducible as the freeze/thaw method, it was the method of choice for subsequent experiments.

To determine if activity of the Na^+-K^+-ATPase pump was altered in hypercalcemia, mTAL tubules were isolated from rats fed the control or DHT-containing diet for 5 days, and assayed for Na^+-K^+-ATPase-mediated P_i production.

Tubules isolated from both control and hypercalcemic rats demonstrated a time-dependent increase in P_i release over a period of 10 min (Figure 13A,B). This release is due to total ATPase activity in the tubules. Preliminary experiments using concentrations of ouabain from 1 mM to 7 mM revealed that maximal inhibition of the Na^+-K^+-ATPase pump in this system was attained with 5 mM ouabain. Incubation of tubules with 5 mM ouabain decreased the amount of P_i released by 63% (2 min) to 71% (10 min) (Figure 13, n=5).

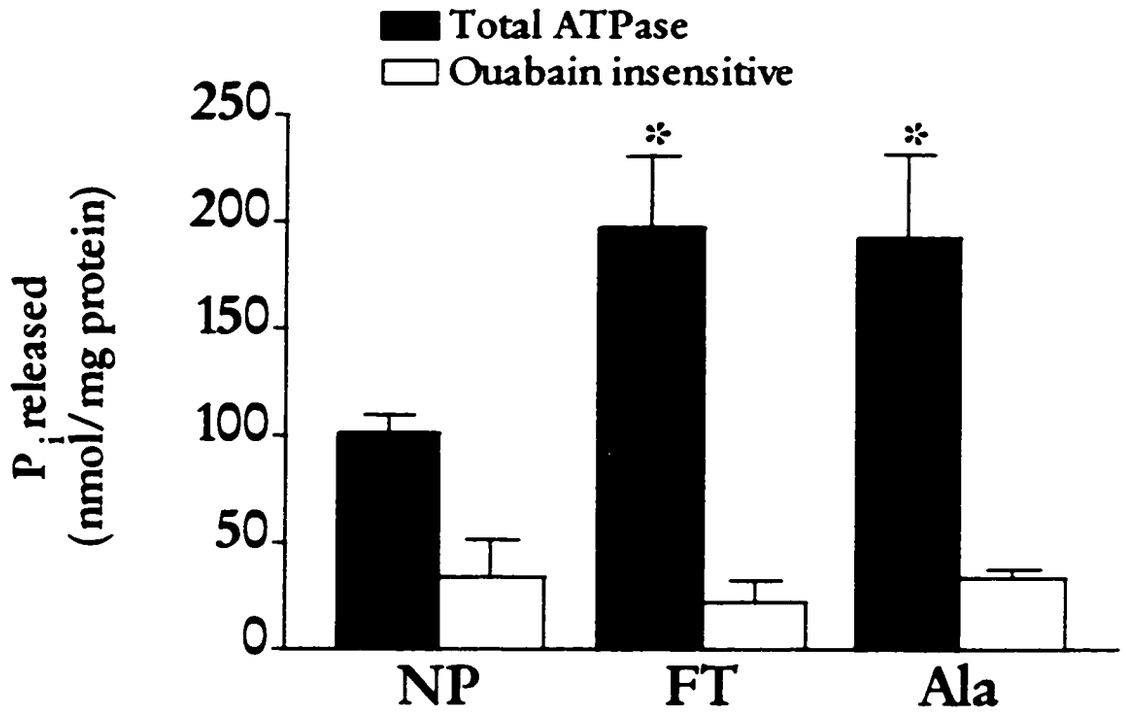
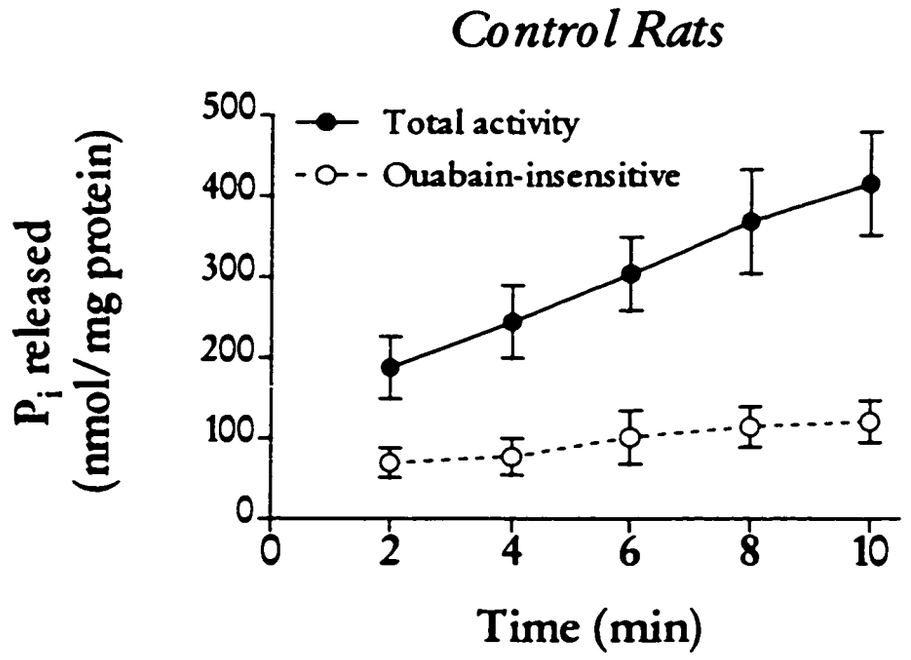


Figure 12. Permeabilization of mTAL tubules. Tubules were treated in the following manner: NP, not permeabilized; FT, freeze/thaw and osmotic shock treatment; Ala, incubated with alamethacin. Solid bars represent Pi release due to total ATPase activity, open bars reflect ouabain-insensitive Pi production (n=4). *P <0.05 vs. NP.

A)



B)

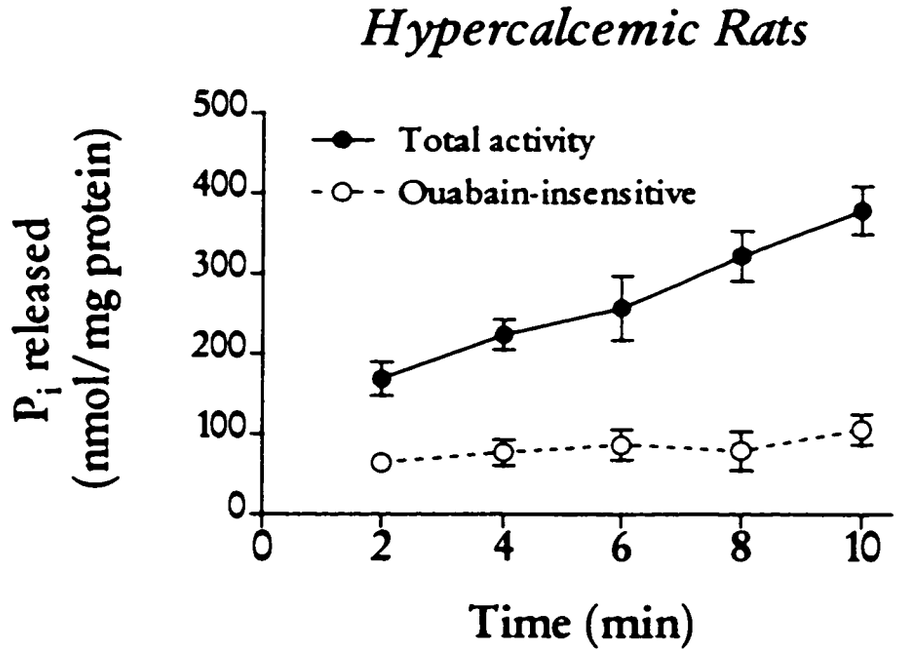


Figure 13. Total and ouabain-insensitive ATPase activity in mTALs isolated from control (A) and hypercalcemic (B) rats. Solid lines represent P_i release due to total ATPase activity. Dashed lines reflect ouabain-insensitive P_i release (n=5).

The difference between total ATPase activity and that not inhibited by ouabain reflects the contribution of the Na^+-K^+-ATPase pump to P_i release. Na^+-K^+-ATPase activity from control and hypercalcemic mTAL tubules is depicted in Figure 14. There was no difference in P_i released between the two groups. Linear regression analysis was conducted for each group, to determine the value of the slope, which represents nmol P_i released per minute, or Na^+-K^+-ATPase activity. The slopes were not significantly different between the two groups (control: 22.0 ± 4.9 vs. hypercalcemic: 21.7 ± 3.4 nmol P_i released/min; *P* = NS; n = 5).

Therefore, these data suggest that there is no difference in activity of the Na^+-K^+-ATPase pump in mTALs isolated from control and hypercalcemic rats after 5 days.

3.4 ^{86}Rb Uptake Studies to Investigate Apical Na^+-K^+-$2Cl^-$ Cotransporter Activity

The aim of these studies was to test the hypothesis that inhibition of TAL Cl^- reabsorption in hypercalcemia is due to inhibition of the apical Na^+-K^+-$2Cl^-$ cotransporter.

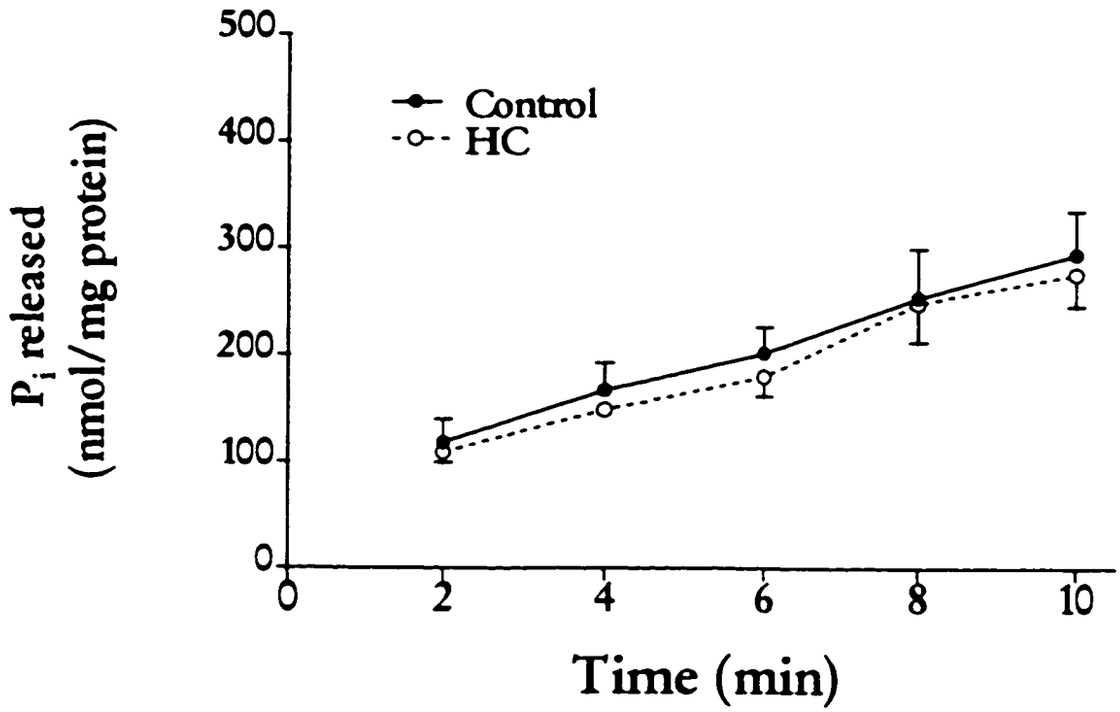


Figure 14. Na^+-K^+-ATPase acivity in control and hypercalcemic (HC) mTAL tubules. This graph illustrates ouabain-sensitive P_i release from mTAL tubules isolated from control (solid lines) and hypercalcemic (dashed lines) rats (n=5). These results are derived from Figure 13.

3.4.1 ^{86}Rb uptake in medullary thick ascending limb tubules

In preliminary experiments, mTAL tubules were isolated from control rat kidneys and ^{86}Rb uptakes performed as described in *Methods*. Uptakes were conducted for up to 120 sec. A previous study showed that infusion of rats with mannitol improves the patency of the lumen and access to the apical membrane (Chamberlin *et al.* 1984). Therefore, we infused rats with 10% mannitol prior to removal of the kidneys. Although ^{86}Rb uptake was observed in these tubules, there was no significant time-dependent increase in ^{86}Rb uptake over the 180 sec time period studied (Figure 15, n = 10). Furthermore, bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransport, failed to inhibit ^{86}Rb uptake in this preparation.

3.4.2 Assessment of Na^+-K^+-$2Cl^-$ cotransporter activity in a mouse mTAL cell line

Since we were unable to measure Na^+-K^+-$2Cl^-$ cotransporter activity in mTAL tubule suspensions, we used a mouse mTAL cell line to study the activity of this cotransporter. We used this model to determine if exposure of mTAL cells to high extracellular Ca^{2+} (Ca^{2+}_o) and/or PGE_2, two important factors in hypercalcemia, affect the activity of the Na^+-K^+-$2Cl^-$ cotransporter.

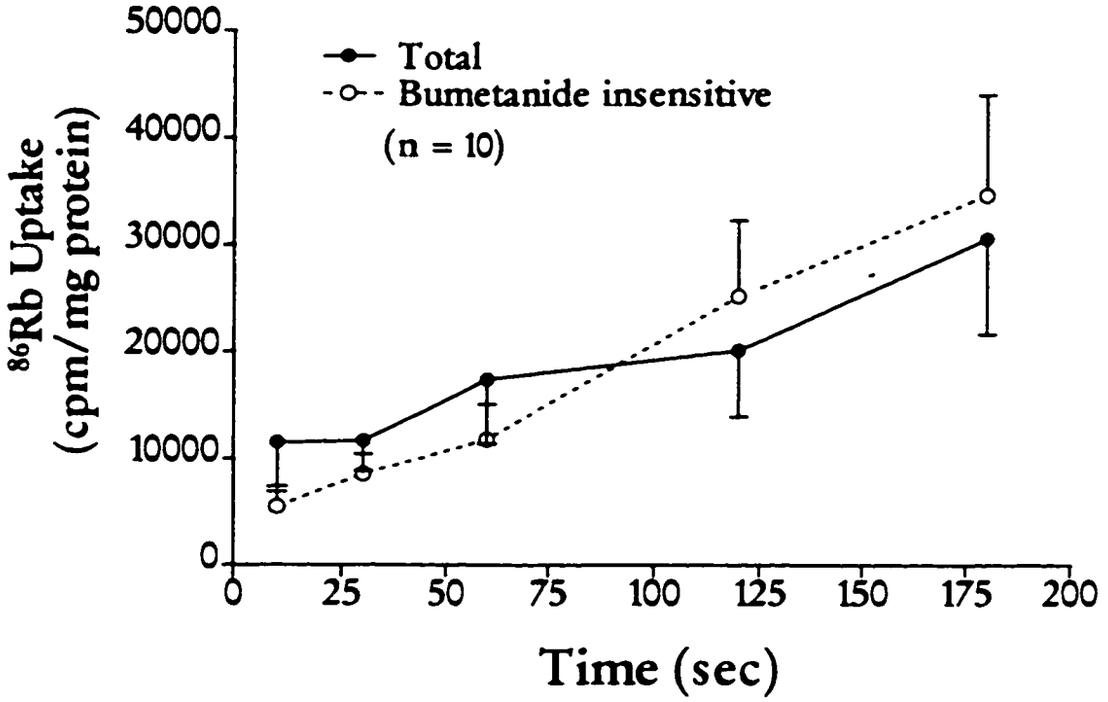


Figure 15. Time course of ^{86}Rb uptake in mTAL tubules. Solid lines represent total ^{86}Rb uptake, broken lines depict ^{86}Rb uptake in the presense of bumetanide.

3.4.2.1 Time course of ^{86}Rb uptake

To determine if the mouse medullary thick ascending limb cell line exhibited bumetanide-sensitive ^{86}Rb uptake, and to establish an appropriate uptake time for subsequent experiments, uptakes were performed over a 30 min time period, in the presence and absence of ouabain and bumetanide.

The results from these experiments are presented in Figure 16. In the absence of inhibitors, ^{86}Rb uptake was linear for approximately 15 min, and then began to plateau. Uptake in the presence of ouabain alone resulted in significant inhibition of ^{86}Rb influx by 73% over the time period studied ($P < 0.001$ vs. total influx at 10 min and 30 min). In cells treated with ouabain and bumetanide, ^{86}Rb uptake was significantly inhibited by a further 19% ($P < 0.001$ vs total and ouabain-insensitive influx).

Since there was significant ^{86}Rb uptake into the cells after 10 min of incubation, a time point which was also in the linear portion of the curve, it was chosen as the uptake time for subsequent experiments.

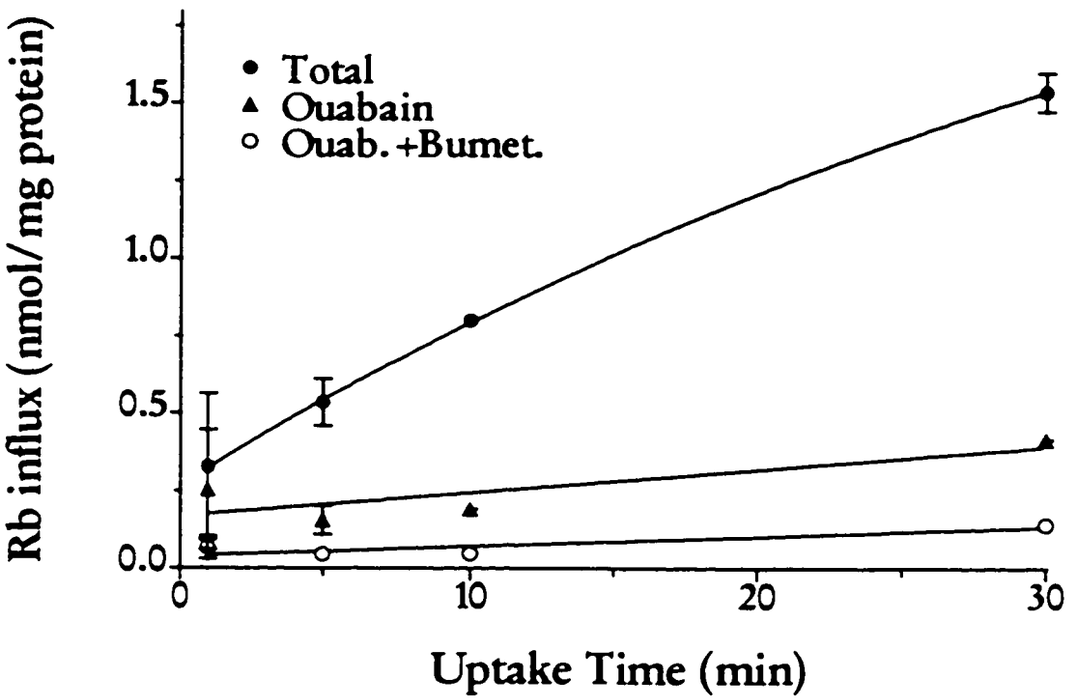


Figure 16. Time course of ^{86}Rb uptake in mouse mTAL cells. ^{86}Rb uptake was allowed to proceed for up to 30 min and total (closed circles), ouabain-insensitive (closed triangles) and bumetanide + ouabain-insensitive (open circles) Rb influx was measured (n=3).

3.4.2.2 Effect of high Ca^{2+}_o and PGE_2 on Na^+-K^+-2Cl^- cotransporter activity

The aim of these experiments was to determine if high levels of Ca^{2+}_o and/or PGE_2 affect the activity of the Na^+-K^+-2Cl^- cotransporter. As described in the *Methods* section, mouse medullary thick ascending limb cells were grown in medium containing normal (1.0-1.12 mmol/L, NC) or high levels of Ca^{2+}_o (1.65-1.7 mmol/L, HC) for 3 days and incubated with or without PGE_2 (1 mM, 30 min) prior to performing uptake experiments.

Uptake of ^{86}Rb in cells exposed to normal Ca^{2+}_o is illustrated in Figure 17. The figure demonstrates the proportion of total ^{86}Rb influx which is sensitive to ouabain and bumetanide in the absence (Figure 17A) and presence (Figure 17B) of PGE_2. Similarly, Figure 18 illustrates total, ouabain-sensitive and bumetanide-sensitive ^{86}Rb influx in mTAL cells exposed to high Ca^{2+}_o in the absence (Figure 18A) and presence (Figure 18B) of PGE_2. Total ^{86}Rb uptake was variable between groups. In order to compare the different conditions, data are expressed as a percentage of total ^{86}Rb uptake.

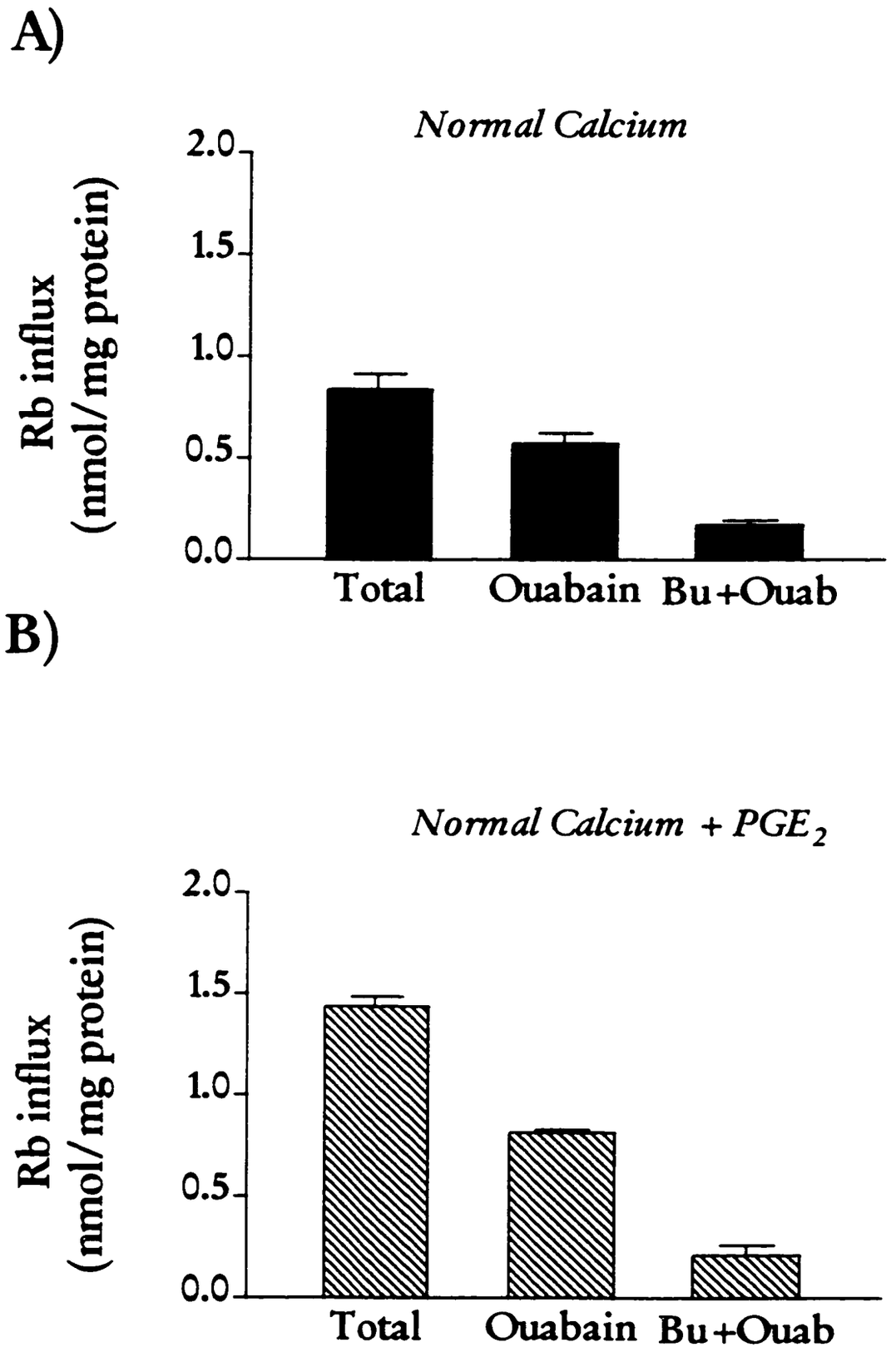


Figure 17. Effect of normal Ca^{2+} and PGE_2 on Rb influx in mouse mTAL cells. A. Cells were exposed to normal Ca^{2+}_o levels for 2 days and Rb influx measured for 10 min in the absence (total) or presence of ouabain (2 mM) and ouabain + bumetanide (100 uM) (Bu+Ouab). B. As in (A) except cells were exposed to PGE_2 (1 uM) for 30 min prior to, and during, uptake measurements (10 min).

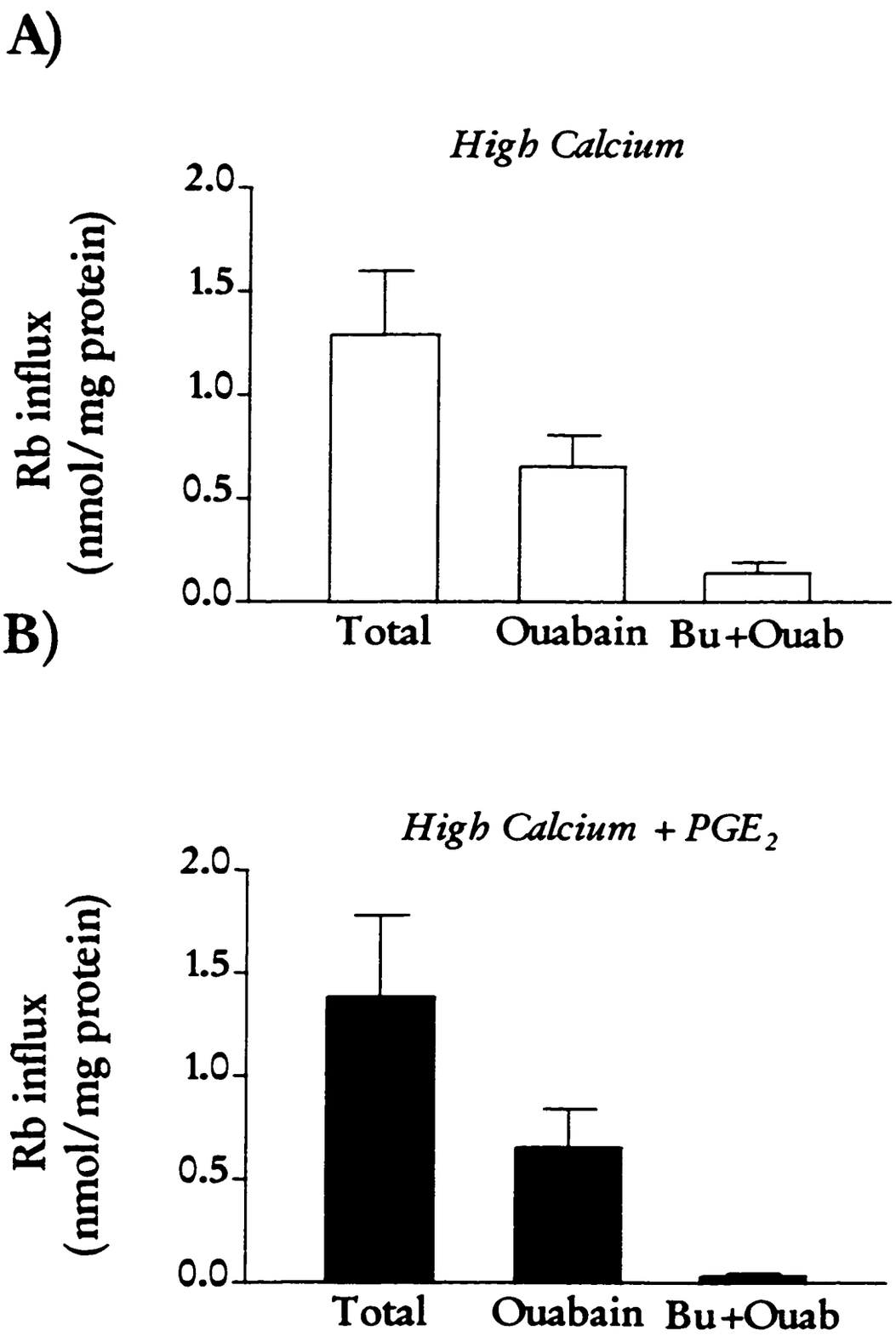


Figure 18. Effect of high Ca^{2+}_o and PGE_2 on Rb influx in mouse mTAL cells. A. Cells were exposed to high Ca^{2+}_o levels for 2 days and Rb influx measured for 10 min in the absence (total) or presence of ouabain (2 mM) and ouabain + bumetanide (100uM) (Bu+Ouab). B. As in (A) except cells were exposed to PGE_2 (1 uM) for 30 min prior to, and during, uptake measurements (10 min).

The effect of Ca^{2+}_o and PGE_2 on Na^+-K^+-ATPase activity (ouabain-sensitive ^{86}Rb influx) in mTAL cells is illustrated in Figure 19. The percentage of ouabain-sensitive ^{86}Rb influx in cells exposed to normal levels of Ca^{2+}_o (NC) was not significantly different from cells exposed to normal Ca^{2+}_o and PGE_2 (NC + PGE_2), suggesting that PGE_2 has no effect on Na^+-K^+-ATPase activity under these conditions.

In mTAL cells exposed to high Ca^{2+}_o, a significant ($P < 0.05$) increase in the percentage of ouabain-sensitive ^{86}Rb influx was observed, compared to normal Ca^{2+}_o (NC) conditions. This suggests that high Ca^{2+}_o stimulates Na^+-K^+-ATPase activity. PGE_2 had no further effect on ouabain-sensitive ^{86}Rb influx in mTAL cells exposed to high Ca^{2+}_o (HC + PGE_2).

In this mouse mTAL cell line, bumetanide-sensitive ^{86}Rb influx constituted 25.7 ± 1.0 % of the total ^{86}Rb influx (Figure 20). Under conditions of normal Ca^{2+}_o, PGE_2 significantly reduced this influx to 14.2 ± 2.7 % ($P < 0.001$). Exposure of cells to high Ca^{2+}_o significantly decreased bumetanide-sensitive ^{86}Rb influx to 13.2 ± 2.0 % ($P < 0.001$ vs. NC). Furthermore, exposure of cells to both high Ca^{2+}_o and PGE_2 resulted in a further significant decrease in bumetanide-sensitive ^{86}Rb influx to 1.9 ± 0.8 % of total influx ($P < 0.001$ vs. HC).

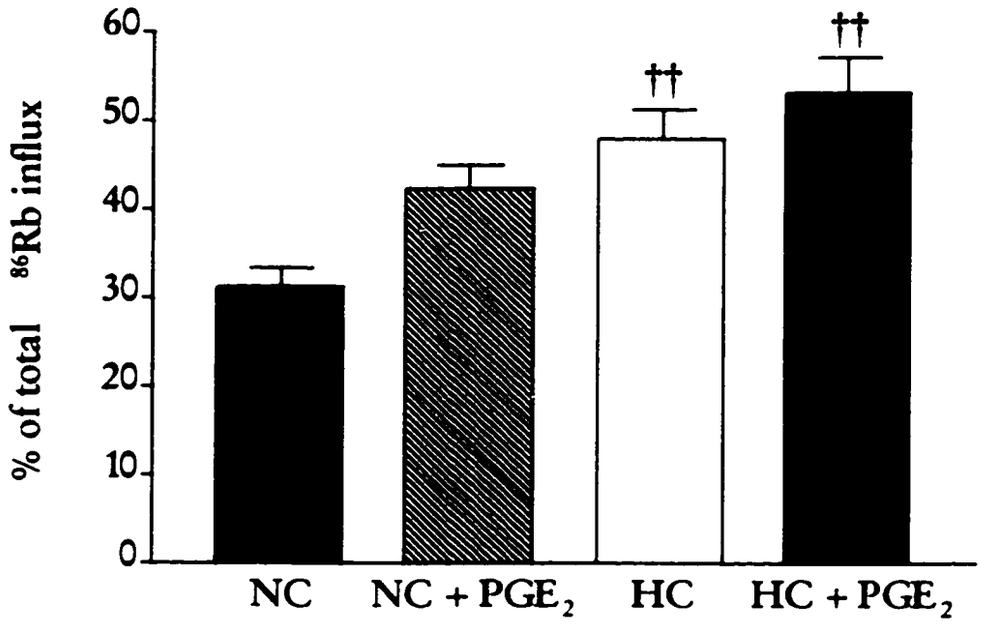


Figure 19. Effect of Ca^{2+}_o and PGE_2 on Na^+-K^+-ATPase activity in mouse mTAL cells. Ouabain-sensitive ^{86}Rb uptake is expressed as a percentage of total ^{86}Rb uptake in cells treated with normal Ca^{2+}_o (NC), normal Ca^{2+}_o + PGE_2 (NC+PGE_2), high Ca^{2+}_o (HC) and high Ca^{2+}_o + PGE_2 (HC+PGE_2). P <0.01 vs NC and NC + PGE_2.

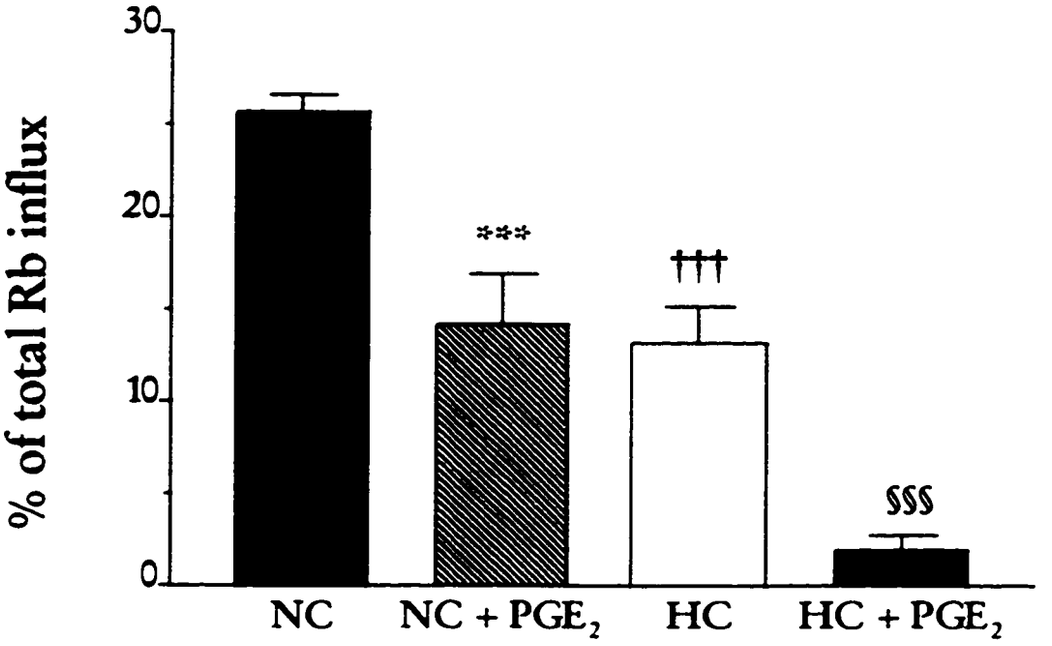


Figure 20. Effect of Ca^{2+}_o and PGE_2 on Na^+-K^+-$2Cl^-$ activity in mouse mTAL cells. Bumetanide-sensitive ^{86}Rb ubtake is expressed as a percentage of total ^{86}Rb uptake in cells treated with normal Ca^{2+}_o (NC), normal Ca^{2+}_o + PGE_2 (NC+PGE_2), high Ca^{2+}_o (HC) and high Ca^{2+}_o + PGE_2 (HC+PGE_2). ***P <0.001 vs NC; †††P <0.001 vs NC; §§§P <0.001 vs. NC, NC + PGE_2, HC.

In summary, these data show that exposure of mTAL cells to elevated Ca^{2+}_o increased activity of the Na^+-K^+-ATPase pump, and inhibited the Na^+-K^+-$2Cl^-$ cotransporter. We also demonstrate that PGE_2 had no effect on Na^+-K^+-ATPase activity, but inhibited the Na^+-K^+-$2Cl^-$ cotransporter. Finally, treatment of mTAL cells to both high Ca^{2+}_o and PGE_2 almost completely abolished activity of the Na^+-K^+-$2Cl^-$ cotransporter.

SECTION 2. Aim: To determine the effect of hypercalcemia on $cPLA_2$, PGHS-1 and PGHS-2 protein expression

3.5 INVESTIGATION OF $cPLA_2$, PGHS-1 AND PGHS-2 PROTEIN LEVELS IN HYPERCALCEMIA

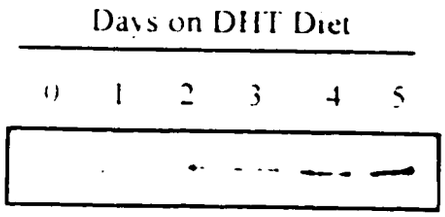
To determine whether intrarenal expression of PGHS-1, PGHS-2 and $cPLA_2$, is affected by hypercalcemia, Western blots were performed on homogenates of inner medulla, outer medulla and cortex from rats fed the DHT-containing diet for up to 5 days.

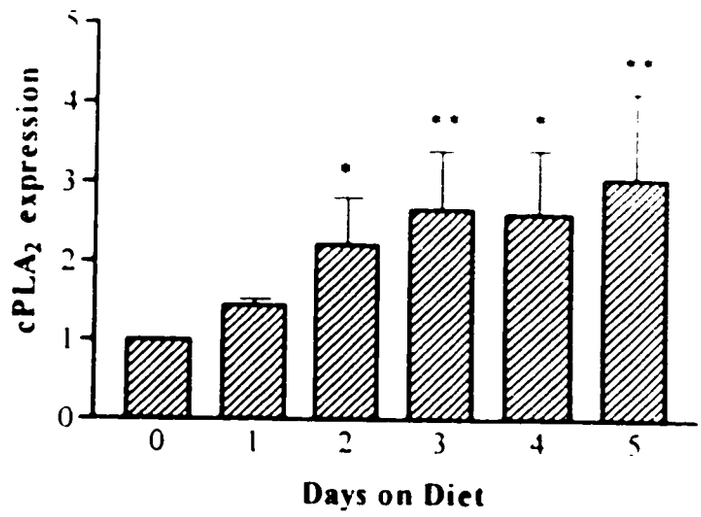
3.5.1 $cPLA_2$ expression in hypercalcemia.

Using a specific antiserum for $cPLA_2$, Western blot analysis revealed an immunoreactive protein with a molecular mass of approximately 110 kDa (Figure 21).

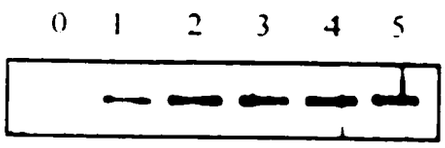
Figure 21. Expression of $cPLA_2$ in hypercalcemic rats. Representative Western blots (left) and densitometric analyses (right) demonstrating protein expression in the (**A**) inner medulla (5 mg protein/well), (**B**) outer medulla (10 mg protein/well) and (C) cortex (20 mg protein/well) of rats fed DHT-containing diet for 0 to 5 days. Densitometric values represent the means ± SE of 4-6 experiments. $^{*}P < 0.05$ vs. day 0, $^{**}P < 0.01$ vs. day 0.

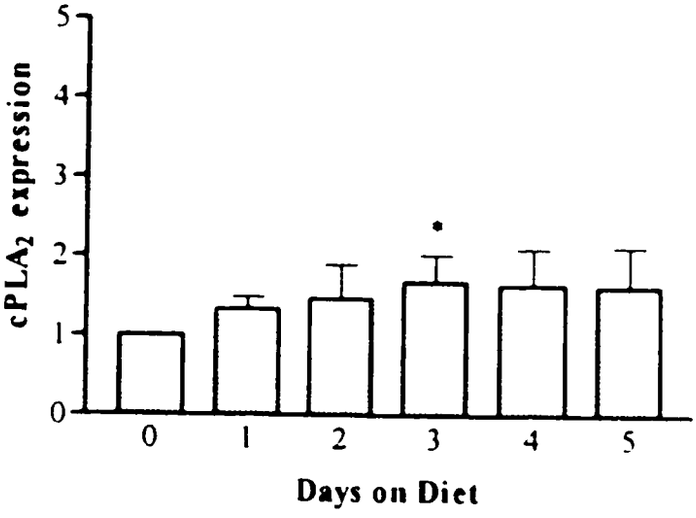
A Inner Medulla



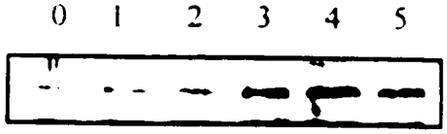


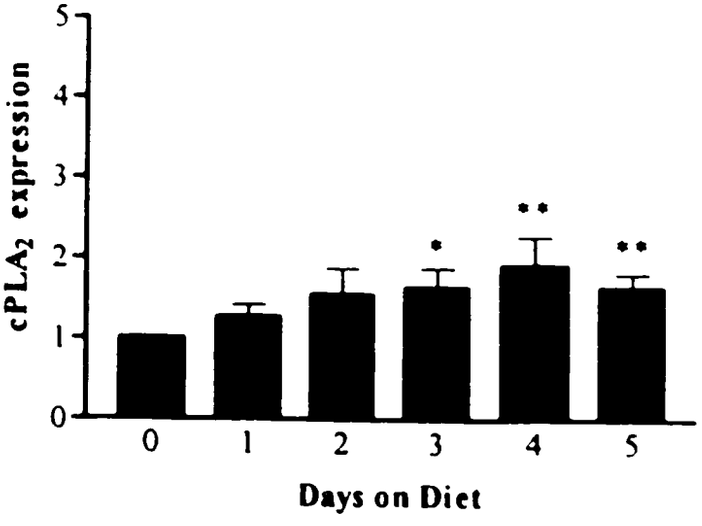
B Outer Medulla





C Cortex





In the inner medulla, outer medulla and cortex, there were significant increases in $cPLA_2$ protein expression at 3 days on the DHT diet (fold increase vs control: inner medulla 2.7 ± 0.7; $P < 0.01$; n = 4; outer medulla 1.5 ± 0.3; $P < 0.05$; n = 6; cortex 1.6 ± 0.2; $P < 0.05$; n = 6). $cPLA_2$ protein expression in the inner medulla and cortex remained significantly increased at day 4 and 5 of the study. However, levels of $cPLA_2$ protein in the outer medulla at days 4 and 5 of DHT diet were not statistically different from control values.

3.5.2 PGHS-1 expression in hypercalcemia

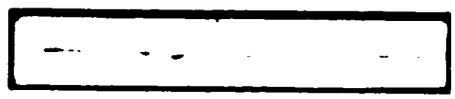
PGHS-1 protein was detected on Western blots as a single band with a molecular mass of approximately 75 kDa in kidneys from both control and hypercalcemic rats. There was no significant change in expression of this protein with hypercalcemia over the period studied in any of the kidney regions (Figure 22). Therefore, no further studies were performed with this protein, with respect to immunolocalization and effect of losartan on protein expression.

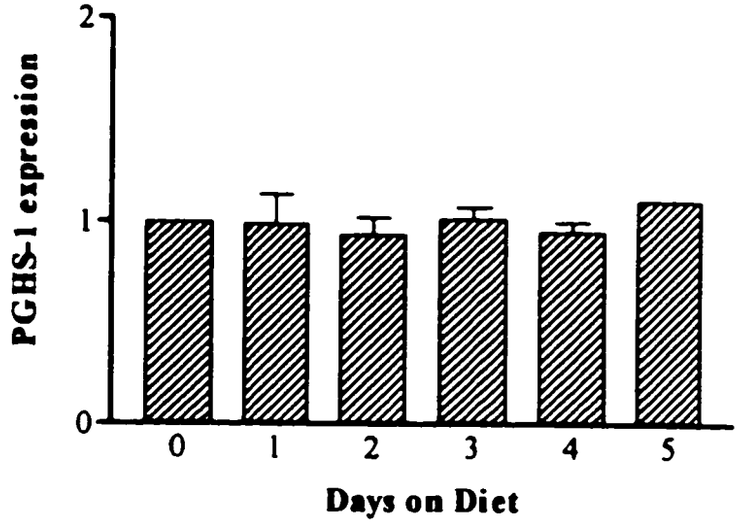
3.5.3 PGHS-2 expression in hypercalcemia

PGHS-2 was expressed in kidneys from both control and hypercalcemic rats, and appeared as a single band at approximately 72 kDa on most blots and some times as a

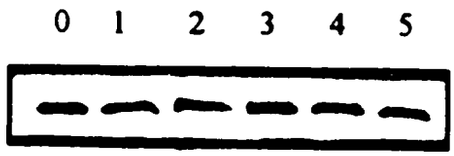
Figure 22. Intrarenal PGHS-1 expression in hypercalcemia. Representative Western blots (left) and densitometric analyses (right) demonstrating levels of PGHS-1 protein in the (A) inner medulla (5 mg protein/well), (B) outer medulla (10 mg protein/well) and (C) cortex (20 mg protein/well) of rats fed DHT-containing diet for 0 to 5 days. Densitometric values represent the means ± SE of 3 experiments.

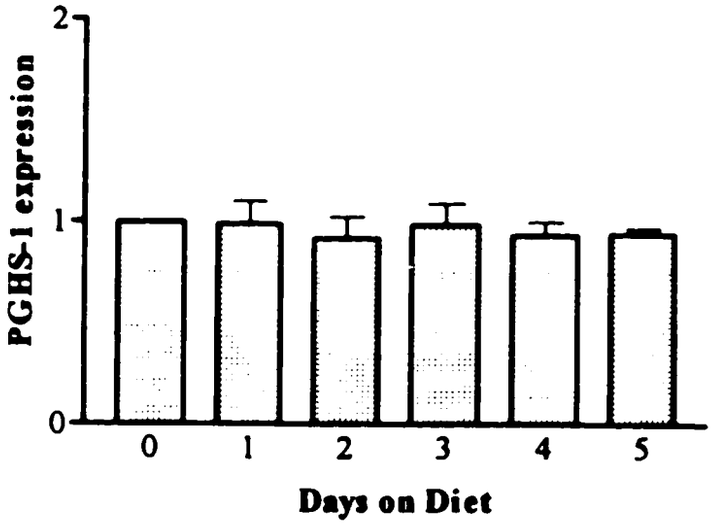
A. Inner Medulla



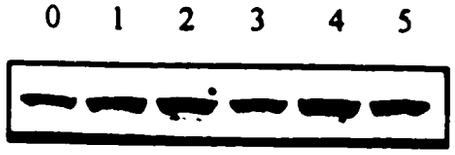


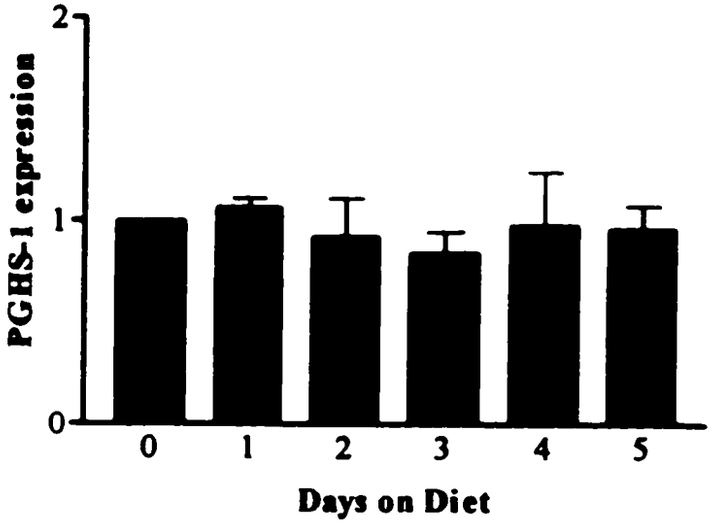
B. Outer Medulla





C. Cortex





doublet (due to glycosylation). PGHS-2 protein expression in both inner and outer medulla was significantly increased after 3 days on the DHT diet (fold increase vs control: inner medulla 1.8 ± 0.2; $P < 0.05$; n = 3; outer medulla 2.3 ± 0.2; $P < 0.05$; n = 3), and returned to control levels by 5 days (Figure 23 A,B). PGHS-2 expression in the cortex (Figure 23C) was also increased on day 3 (fold increase vs control: 15.7 ± 7.9; $P < 0.01$; n = 6) and remained significantly elevated up to day 5.

3.6 INTRARENAL LOCALIZATION OF cPLA$_2$ AND PGHS-2 PROTEIN IN HYPERCALCEMIA

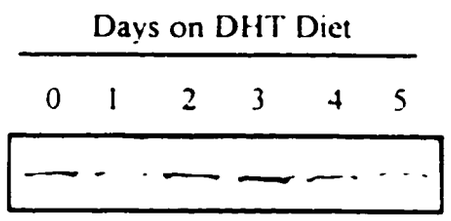
In order to determine which cells might mediate the changes in expression of PGHS-2 and cPLA$_2$ in hypercalcemia, immunofluorescence studies were performed.

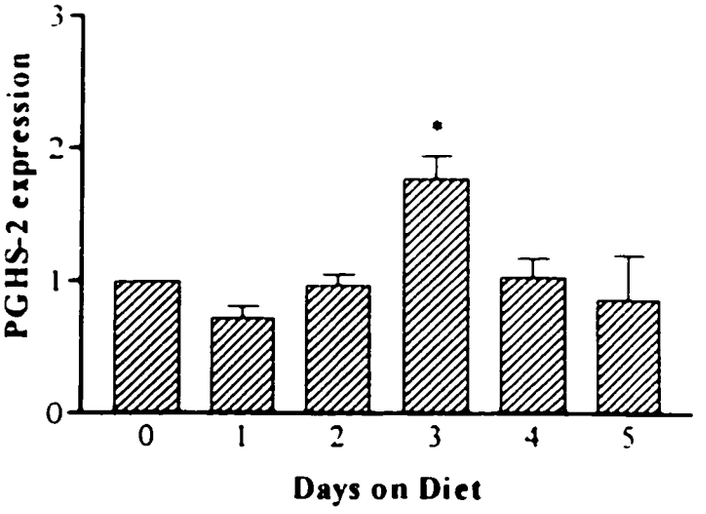
3.6.1 Inner medulla

In the inner medulla, cPLA$_2$ immunoreactivity was detected mainly in interstitial cells, with a faint signal in epithelial cells of the inner medullary collecting ducts (Figure 24A). PGHS-2 immunoreactivity was also localized to medullary interstitial cells (Figure 24B). In these kidney sections, interstitial cells displayed the characteristic morphological features previously described (Lemley & Kriz, 1991), in

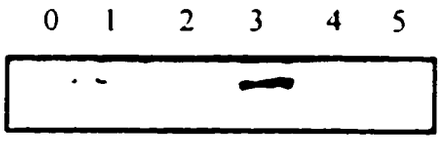
Figure 23. Time course for expression of PGHS-2 in hypercalcemia. PGHS-2 expression was assessed by Western blots (left) and densitometric analyses (right) in the **(A)** inner medulla (5 mg protein/well), **(B)** outer medulla (10 mg protein/well) and **(C)** cortex (20 mg protein/well) of rats fed DHT-containing diet for 0 to 5 days. Densitometric values represent the means ± SE of 3-6 experiments. $*P < 0.05$ vs. day 0, $**P < 0.01$ vs. day 0.

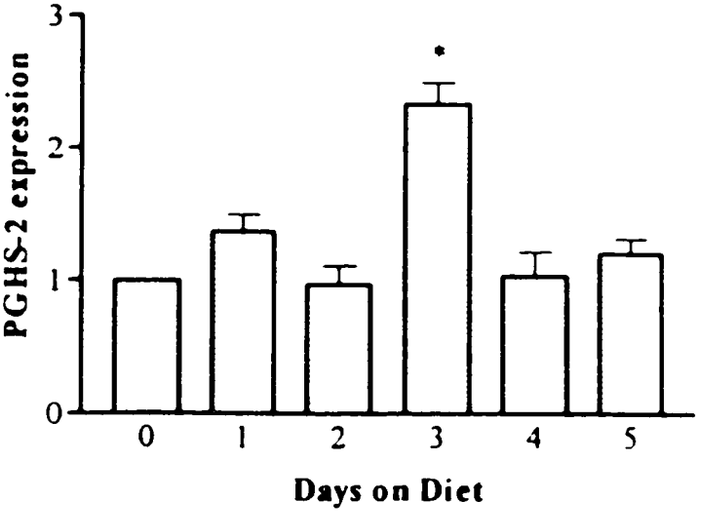
A. Inner Medulla



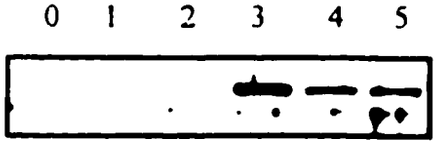


B. Outer Medulla





C. Cortex



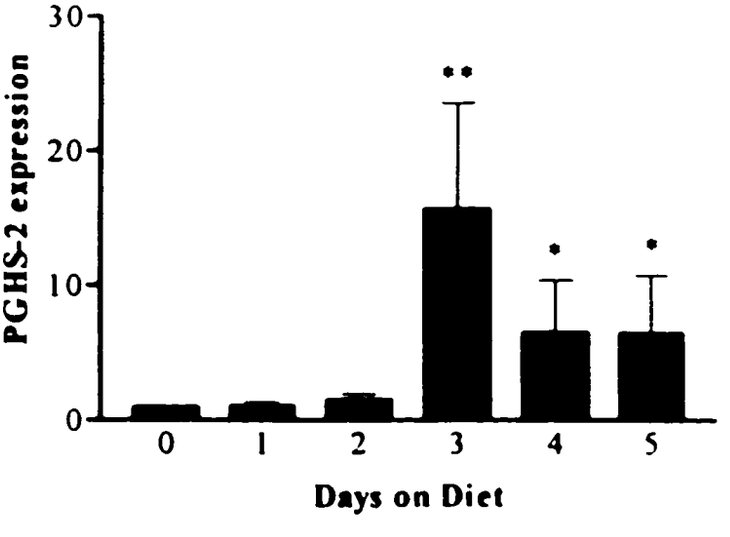


Figure 24. $cPLA_2$ and PGHS-2 immunostaining in the inner medulla. (A) $cPLA_2$ immunofluorescence is found mainly in interstitial cells with a faint signal in inner medullary collecting ducts (arrowheads). (B) PGHS-2 immunofluorescence showing positive staining in interstitial cells (arrowheads). (C) Immunofluorescence of the same field as in (A) and (B) demonstrating co-localization of $cPLA_2$ and PGHS-2 to interstitial cells in the inner medulla (arrowhead). Immunostaining shown here is representative of 5 experiments. Similar results were observed in control rats (not shown). The pictures shown are copies of the original. Magnification, x 360.

that they were arranged between tubules and vasa recta, in the wide interstitial spaces, like the rungs of a ladder.

Co-localization of immunoreactivity in these sections was achieved by using a dual filter which allowed double exposure of the rhodamine and fluorescein filters. As illustrated in Figure 24C, $cPLA_2$ and PGHS-2 immunoreactivity was clearly co-localized to interstitial cells in the inner medulla, with only $cPLA_2$ staining on the apical membranes of medullary collecting ducts.

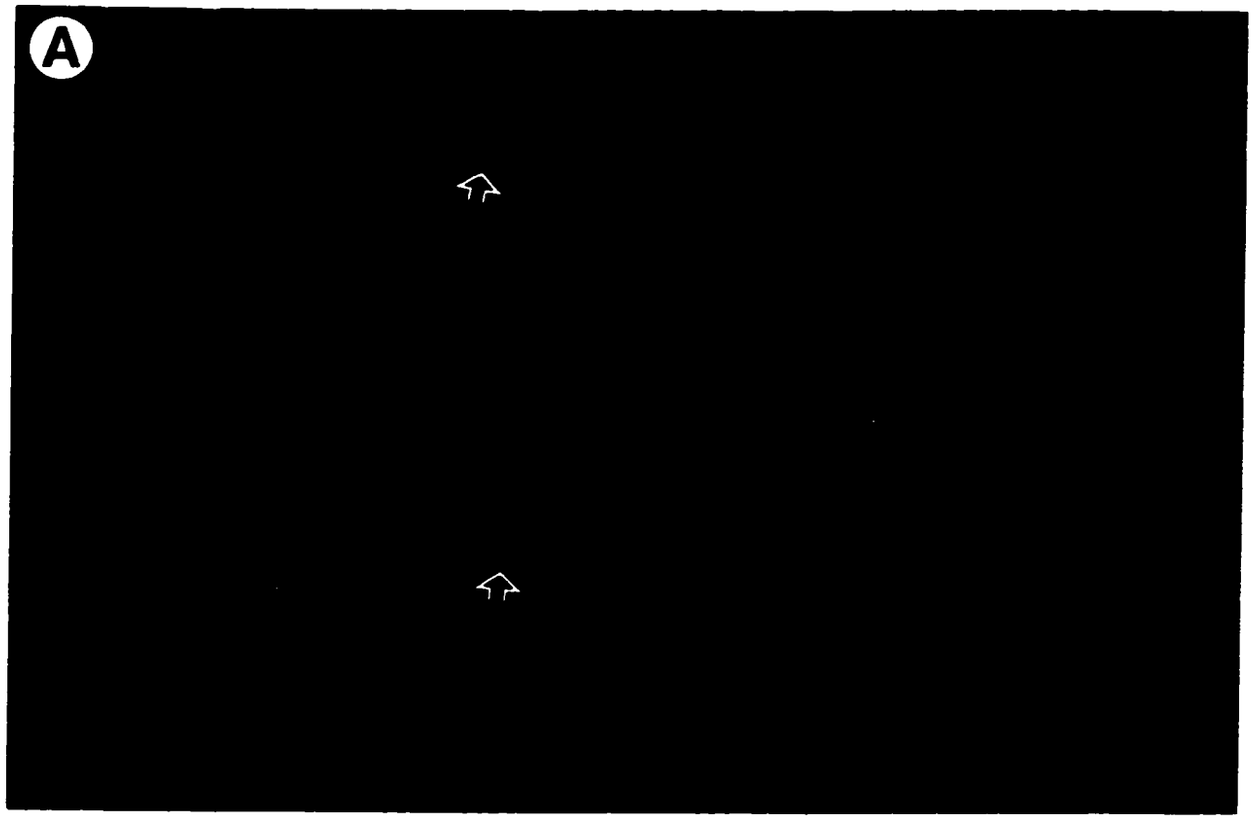
3.6.2 Outer medulla

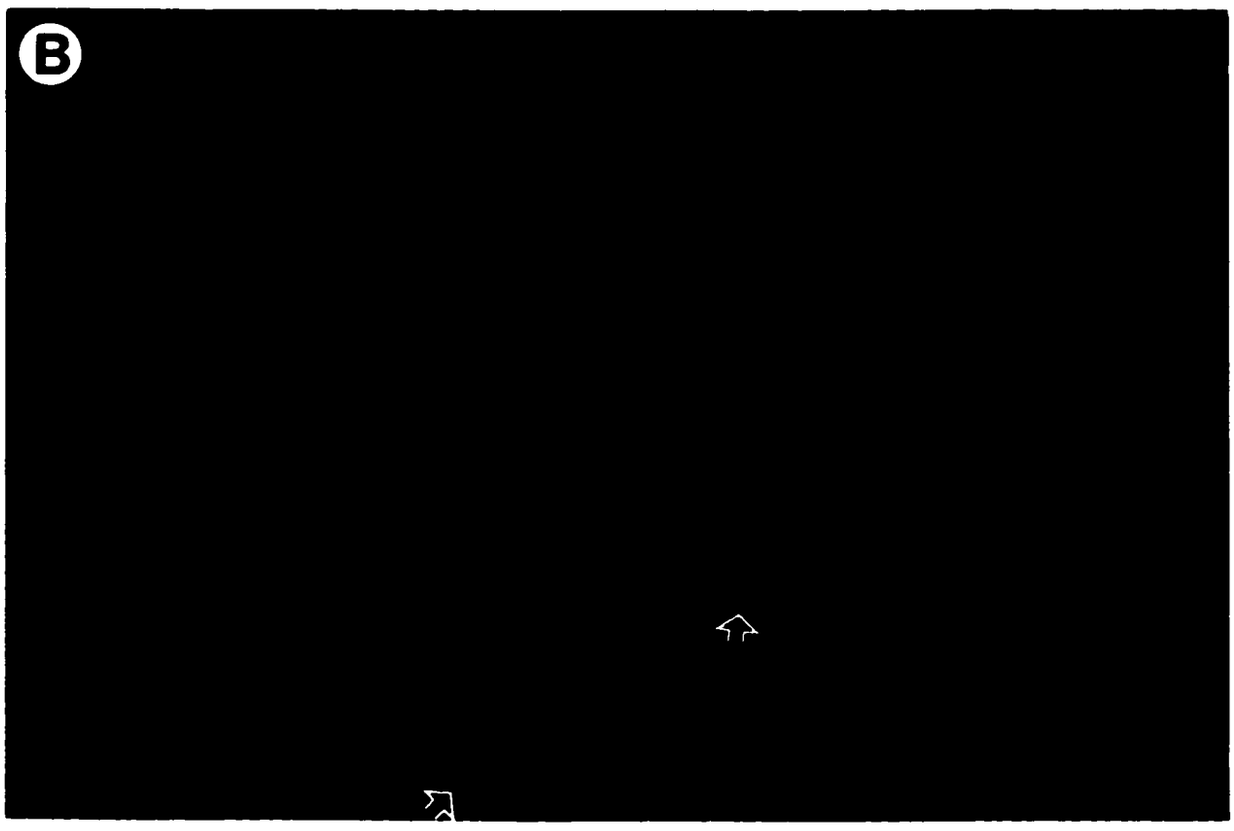
In the outer medulla, both $cPLA_2$ and PGHS-2 immunoreactivity was detected mainly in the interstitial cells (Figure 25A,B). Co-localization was not possible in this region, due to the difference in intensity of staining between the two antibodies.

3.6.3 $cPLA_2$ and PGHS-2 expression in the cortex

In the cortex, the cortical thick ascending limb was identified as the tubular structures showing positive staining for Tamm Horsfall protein. The macula densa is a densely nucleated area, where the epithelial cells are more narrow and their nuclei lie closer together. The macula densa has been reported to stain positively for neuronal

Figure 25. $cPLA_2$ and PGHS-2 immunostaining in the outer medulla. (A) $cPLA_2$ immunofluorescence demonstrating staining of interstitial cells (arrowheads). (B) Positive staining for PGHS-2 was localized mainly to interstitial cells (arrowheads). Immunostaining shown here is representative of 5 experiments. Similar results were observed in control rats (not shown). Magnification, x 360.


A


B

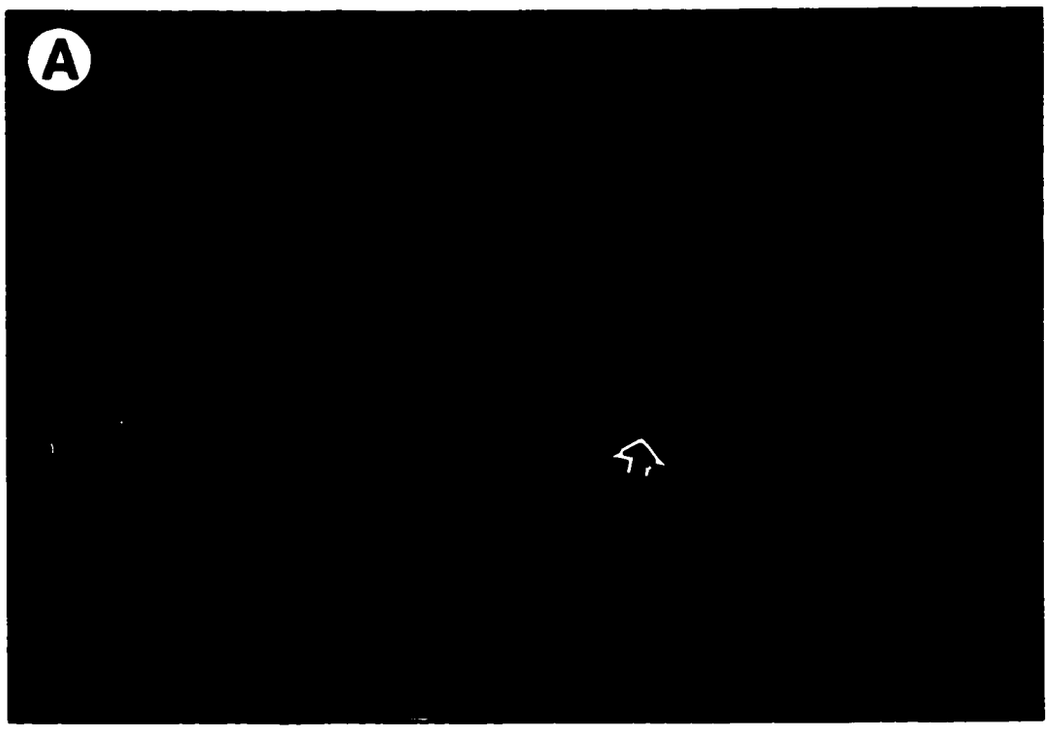
nitric oxide synthase (bNOS) (Bachmann *et al.* 1995), which we used as a marker for these cells.

The apical membranes of cortical thick ascending limbs showed distinct $cPLA_2$ immunoreactivity (Figure 26). In addition, positive $cPLA_2$ staining was detected in the macula densa region (Figure 27), as identified by co-localization with bNOS.

The cortex stained for PGHS-2 in the Tamm-Horsfall positive cortical thick ascending limb (Figure 28). Intense staining for PGHS-2 was also observed in the macula densa region (Figure 29) and in the adjoining cortical thick ascending limb. Interestingly, not all the cells of the cortical thick ascending limb demonstrated positive immunoreactivity for PGHS-2. This pattern of expression is in agreement with a previous report by Harris et al., in rat kidney (Harris *et al.* 1994).

No differences in immunolocalization between control and hypercalcemic rat kidneys were observed. Immunostaining was deemed specific since all control sections (substitution of the primary antibody with nonimmune serum, omission of the primary antibody and omission of the secondary antibody) were negative for immunofluorescence.

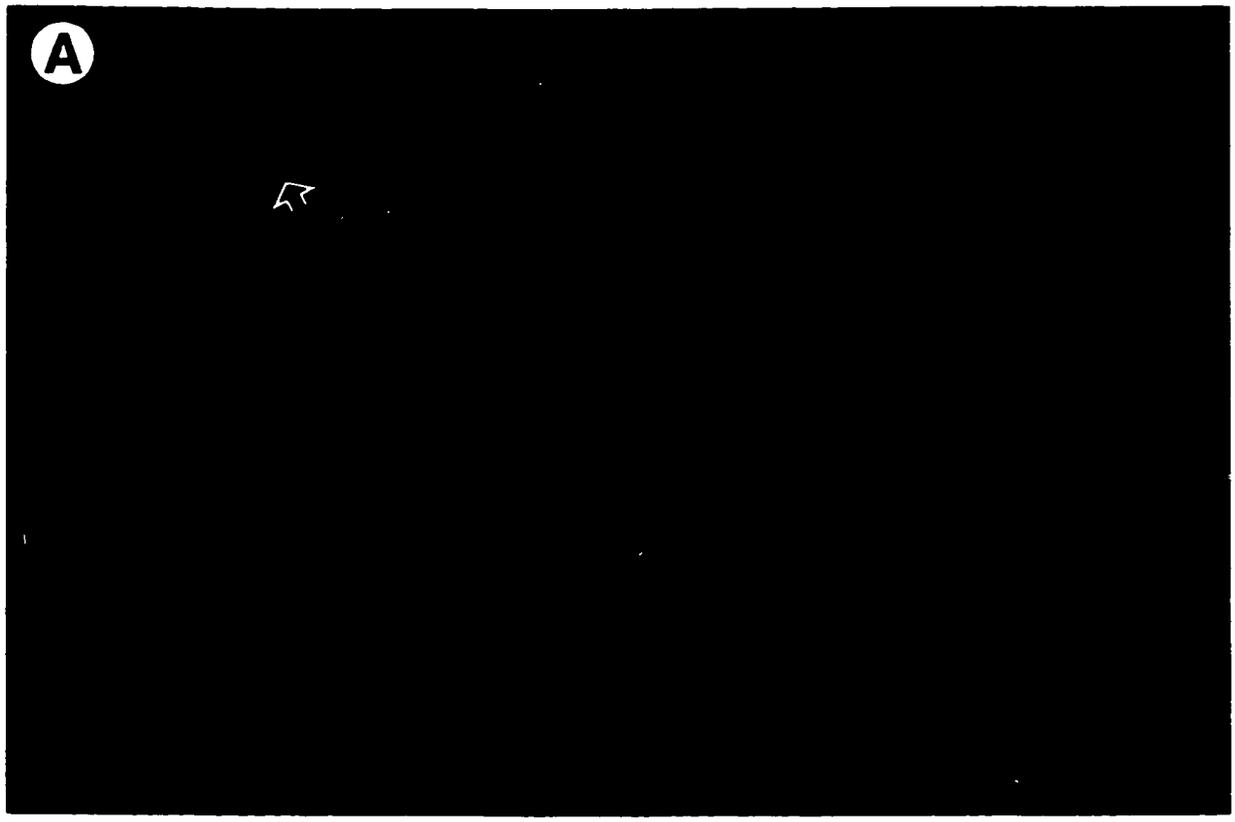
Figure 26. Immunostaining of $cPLA_2$ and Tamm-Horsfall protein in hypercalcemia (A) Tamm-Horsfall staining of cortical thick ascending limbs of the cortex (arrowheads). (B) $cPLA_2$ immunofluorescence of the same field as in (A), showing distinct apical staining of cortical thick ascending limbs (arrowheads). Immunostaining shown here is representative of 4 experiments. Similar results were observed in control rats (not shown). Magnification, x360.


A


B

Figure 27. Immunostaining of $cPLA_2$ and bNOS protein in hypercalcemia (A) bNOS staining of macula densa in the cortex (arrowhead). (B) $cPLA_2$ immunofluorescence in the cortex. (C) Immunofluorescence of the same field as in (A) and (B) demonstrating co-localization of $cPLA_2$ and bNOS to the macula densa in the cortex (arrowhead). Immunostaining shown here is representative of 4 experiments. Similar results were observed in control rats (not shown). Magnification, x 360 .

Figure 28. PGHS-2 and Tamm-Horsfall immunostaining in kidney tissue from hypercalcemic rats. (A) Tamm-Horsfall staining of cortical thick ascending limbs of the cortex (arrowheads). (B) PGHS-2 immunofluorescence of the same field as in (A), showing staining of cortical thick ascending limb (arrowhead). Immunostaining shown here is representative of 4 experiments. Identical localization was observed in all three regions of control rats (not shown). Magnification, x360.


A

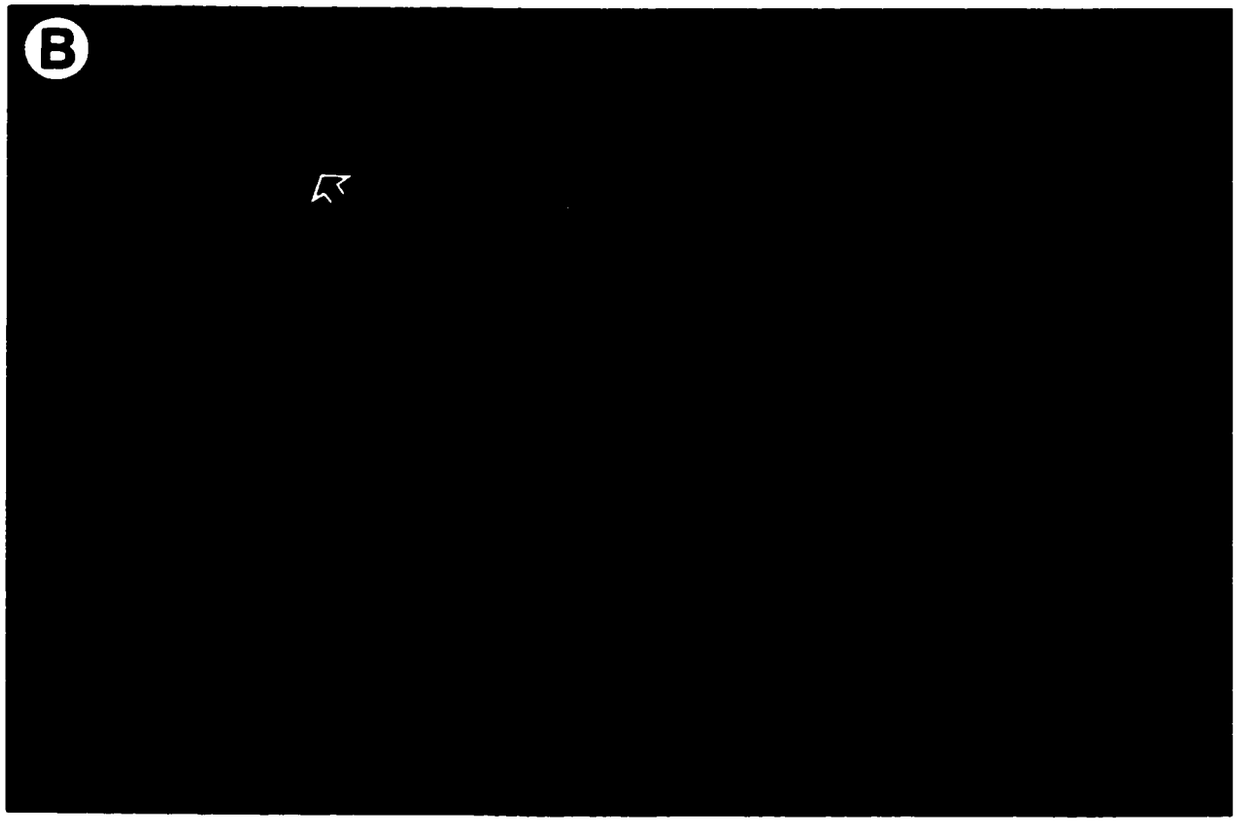

B

Figure 29. PGHS-2 and bNOS immunostaining in kidney tissue from hypercalcemic rats (A) bNOS staining of macula densa in the cortex (arrowhead). **(B)** PGHS-2 immunofluorescence of the same field as in (A), showing staining of macula densa, as identified by the clustering of nuclei, and cortical thick ascending limb (arrowhead). **(C)** Co-localization of bNOS and PGHS-2 demonstrating staining in macula densa (arrowhead). Immunostaining shown here is representative of 4 experiments. Identical localization was observed in all three regions of control rats (not shown). Magnification, x 360.

SECTION 3. Aim: To investigate the role of angiotensin II in hypercalcemia

Ang II has been reported to increase prostaglandin production in a number of cellular systems (Douglas & Hopfer, 1994; Zusman & Keiser, 1980; Siragy & Carey, 1996). In our previous studies, circulating plasma renin levels did not increase significantly in hypercalcemic rats with access to water, although pair-water feeding was associated with significantly higher levels (Peterson, 1990).

3.7 INTRARENAL ANGIOTENSIN II IN HYPERCALCEMIA

Studies were performed to investigate the effect of hypercalcemia on intrarenal levels of Ang II, determined by HPLC and radioimmunoassay. After 3 days of hypercalcemia, a time point at which we observed increased expression of cPLA$_2$ and PGHS-2, intrarenal Ang II levels did not significantly differ from control values (Figure 30). These values were comparable to those previously reported in control animals (Yosipiv & el-Dahr, 1996).

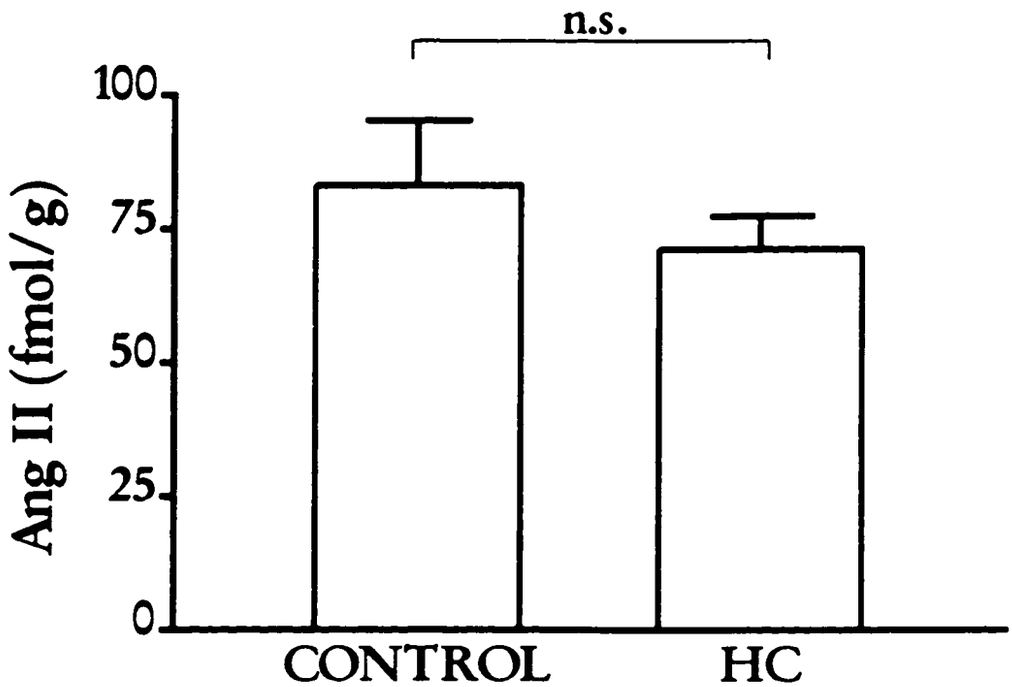


Figure 30. Intrarenal Ang II levels in control and hypercalcemic rats. Rats were fed control (C) or DHT-containing (HC) diet for 3 days. Ang II levels were measured by HPLC and radioimmunoassay. Data are expressed as means ± SE. (n = 4).

3.8 ROLE OF ANGIOTENSIN II AT_1 RECEPTORS IN HYPERCALCEMIA

To determine whether AT_1 Ang II receptor activation plays a role in the regulation of $cPLA_2$ and PGHS-2 in hypercalcemia, losartan, a specific AT_1 Ang II receptor antagonist, was administered in the drinking water (15 mg/kg/day) of rats on control and DHT diets for 3 days, a time at which these proteins were significantly elevated in hypercalcemia (Figures 21,23).

3.8.1 Effect of losartan on serum Ca^{2+} and water intake

Losartan had no effect on serum Ca^{2+} in hypercalcemic rats (hypercalcemic: 1.62 ± 0.05 mmol/l vs hypercalcemic + losartan: 1.61 ± 0.06 mmol/l; P = NS; n = 5) (Figure 31A). Water intake of all animals was measured daily and the dose of losartan adjusted to ensure that 15 mg/kg/day was being administered. Water intake was significantly increased in hypercalcemic animals (control: 19.6 ± 1.4 ml/100 g body weight/day vs. hypercalcemic: 27.4 ± 2.3 ml/100 g body weight/day; $P < 0.05$; n = 5) in agreement with previous studies (Peterson, 1990; Levi *et al.* 1983). Treatment of hypercalcemic animals with losartan significantly reduced water intake (hypercalcemic + losartan: 20.4 ± 1.1 ml/100 g body weight/day; P = NS vs control; n = 11) (Figure 31B).

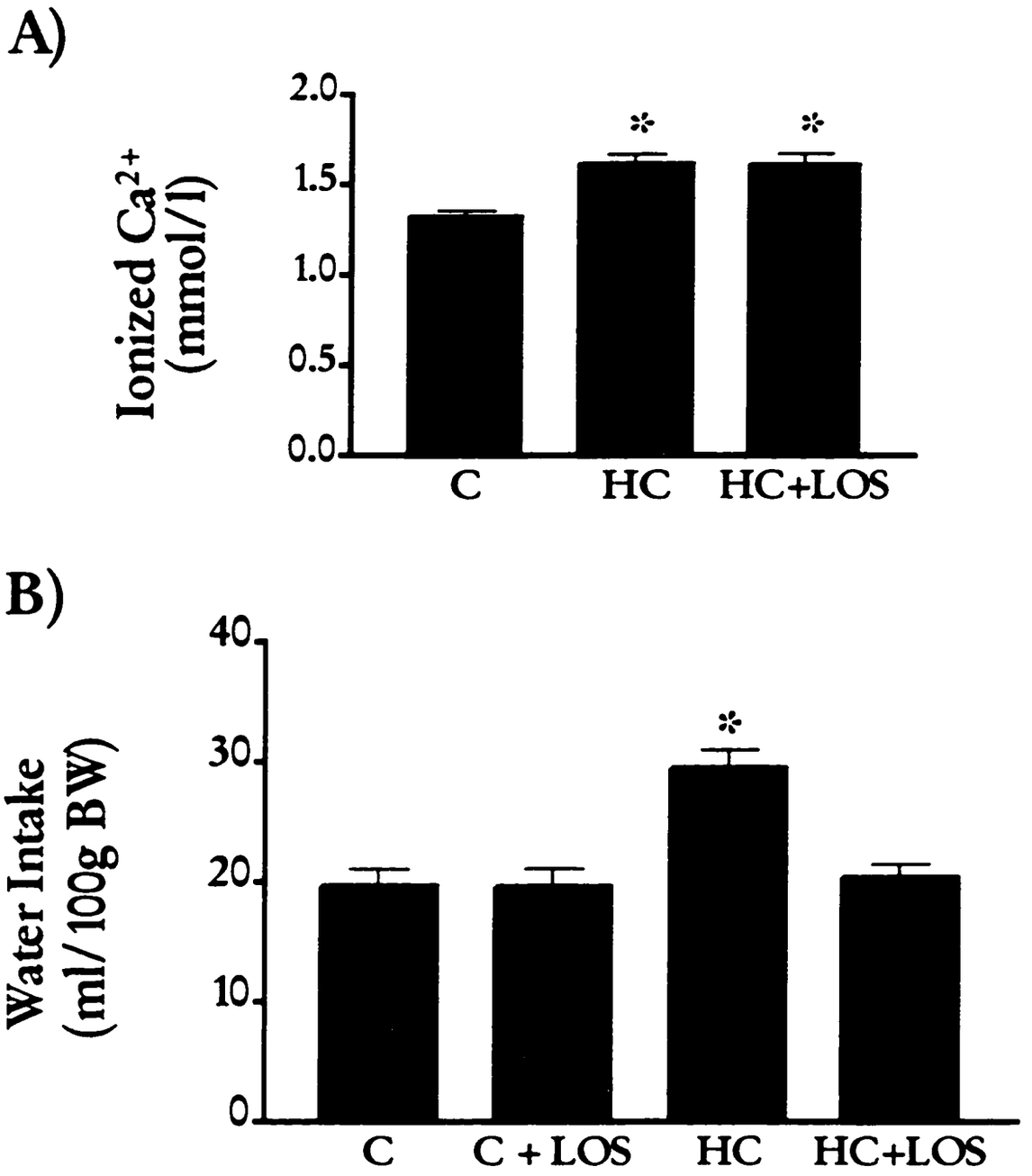


Figure 31. Effect of losartan on blood ionized Ca^{2+} levels and water intake. Ionized Ca^{2+} (A) and water intake (B) was measured in rats fed the control diet (C) alone, the control diet with losartan in the drinking water (C+LOS), the DHT-containing diet (HC) and the DHT-containing diet and losartan in the drinking water (HC+LOS). Data are expressed as means ± SE. *P <0.05 vs control.

3.8.2 Effect of losartan on $cPLA_2$ and PGHS-2 protein expression in hypercalcemia

As shown in Figure 32, in rats on control diet, $cPLA_2$ protein expression was not affected by losartan. In hypercalcemic rats, however, losartan treatment significantly inhibited the increase in $cPLA_2$ protein in the inner medulla, outer medulla and cortex.

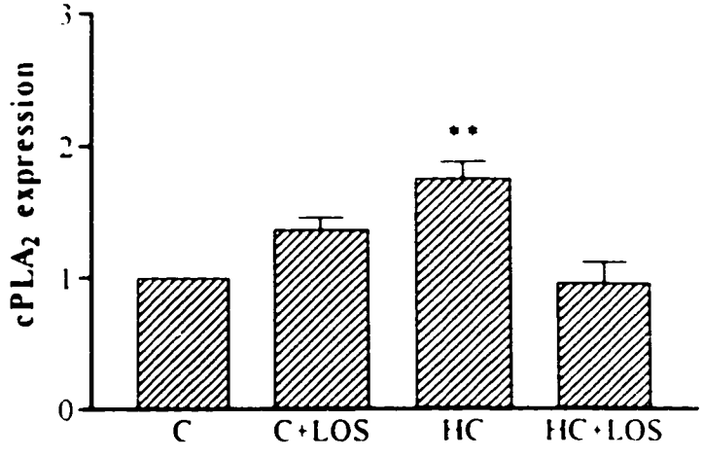
Losartan also abolished the increase in PGHS-2 expression in the inner medulla of hypercalcemic rats, but had no effect on increased expression of PGHS-2 in the cortex and outer medulla (Figure 33).

3.8.3 Effect of losartan on urinary PGE_2 levels

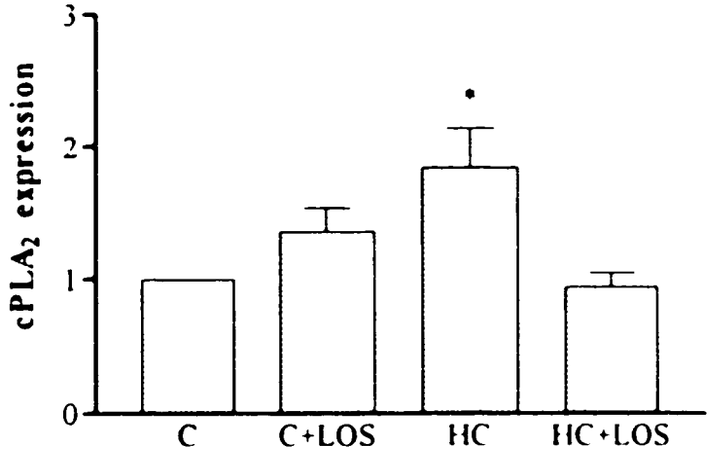
Urinary excretion of PGE_2 between day 2 and day 3 did not differ significantly between control and hypercalcemic rats (control: 417 ± 116 ng/day vs. hypercalcemic: 535 ± 193 ng/day; P = NS; n = 4). In contrast, and in agreement with previous reports (Levi *et al.* 1983; Serros & Kirschenbaum, 1981), urinary PGE_2 excretion measured at 5 days was significantly elevated in hypercalcemic rats as compared to controls (control: 347 ± 22 ng/day vs. hypercalcemic: 1706 ± 210 ng/day; $P < 0.001$; n = 3) (Figure 34). In hypercalcemic rats treated with losartan the increase in urinary PGE_2 was completely prevented (hypercalcemic + losartan: 705 ± 119 ng/day; P = NS vs control; n = 4).

Figure 32. Effect of losartan on $cPLA_2$ protein expression in hypercalcemia. Representative Western blots (left) and densitometric analyses (right) of protein expression obtained from rats fed the control diet alone (C), the control diet with losartan in the drinking water (C+LOS), the DHT-containing diet (HC) and the DHT-containing diet and losartan in the drinking water (HC+LOS) for 3 days. **(A)** inner medulla (5 mg protein/well), **(B)** outer medulla (10 mg protein/well) and **(C)** cortex (20 mg protein/well). $*P < 0.05$ vs. control, $**P < 0.01$ vs. control.

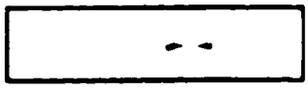
A. Inner Medulla



B. Outer Medulla



C. Cortex



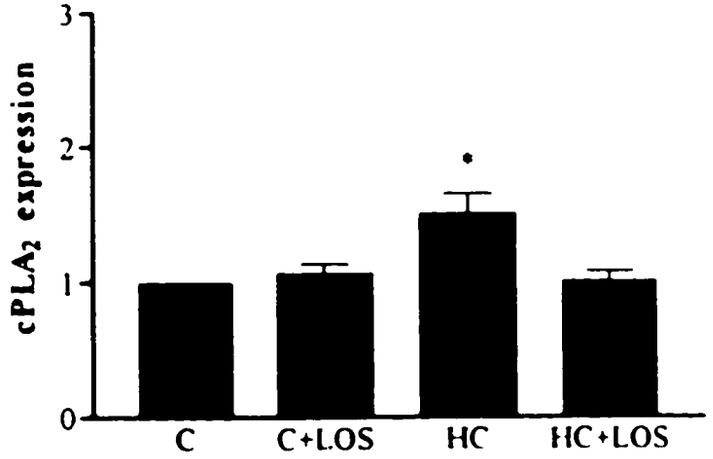
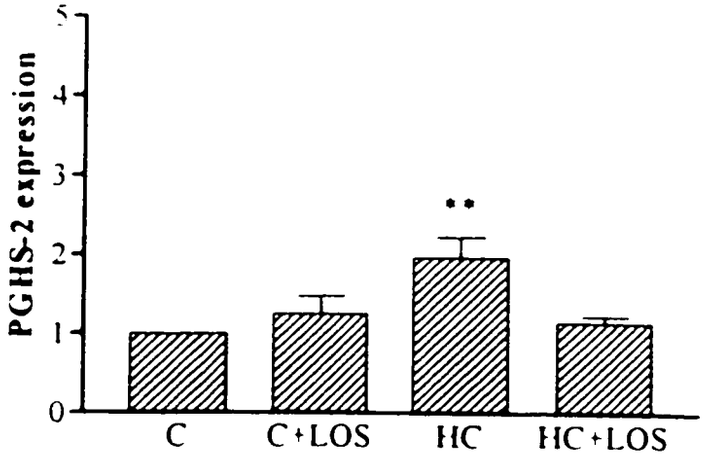
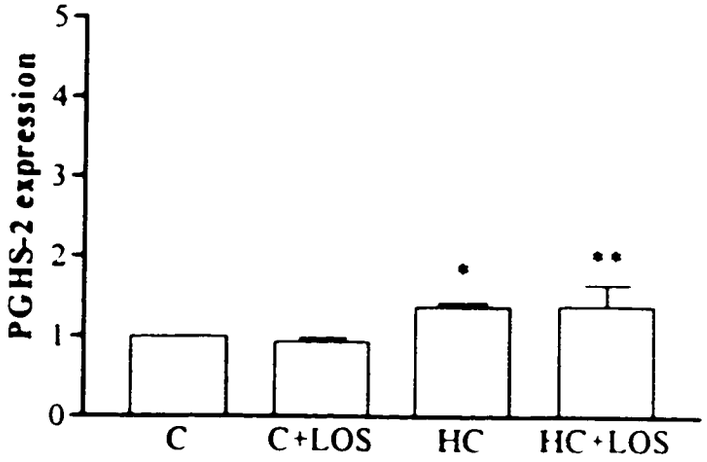


Figure 33. Effect of losartan on PGHS-2 protein expression in hypercalcemia. Representative Western blots (left) and densitometric analyses (right) demonstrating results obtained in rats fed the control diet alone (C), the control diet with losartan in the drinking water (C+LOS), the DHT-containing diet for 3 days (HC) and the DHT-containing diet and losartan in the drinking water for 3 days (HC+LOS). (A) inner medulla (5 mg protein/well), (B) outer medulla (10 mg protein/well) and (C) cortex (20 mg protein/well). $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control.

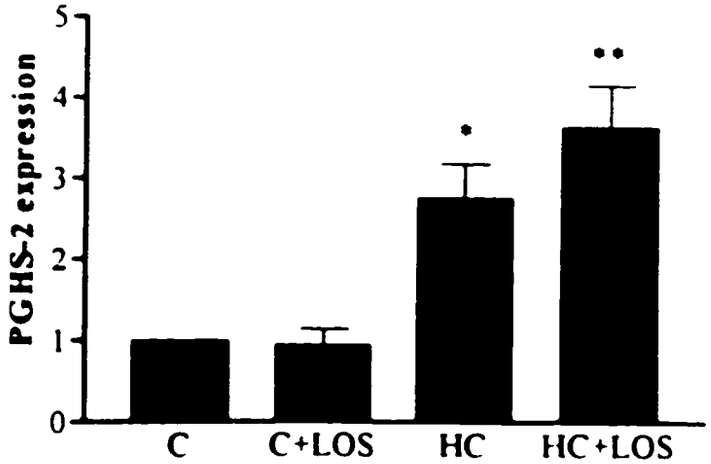
A. Inner Medulla



B. Outer Medulla



C. Cortex



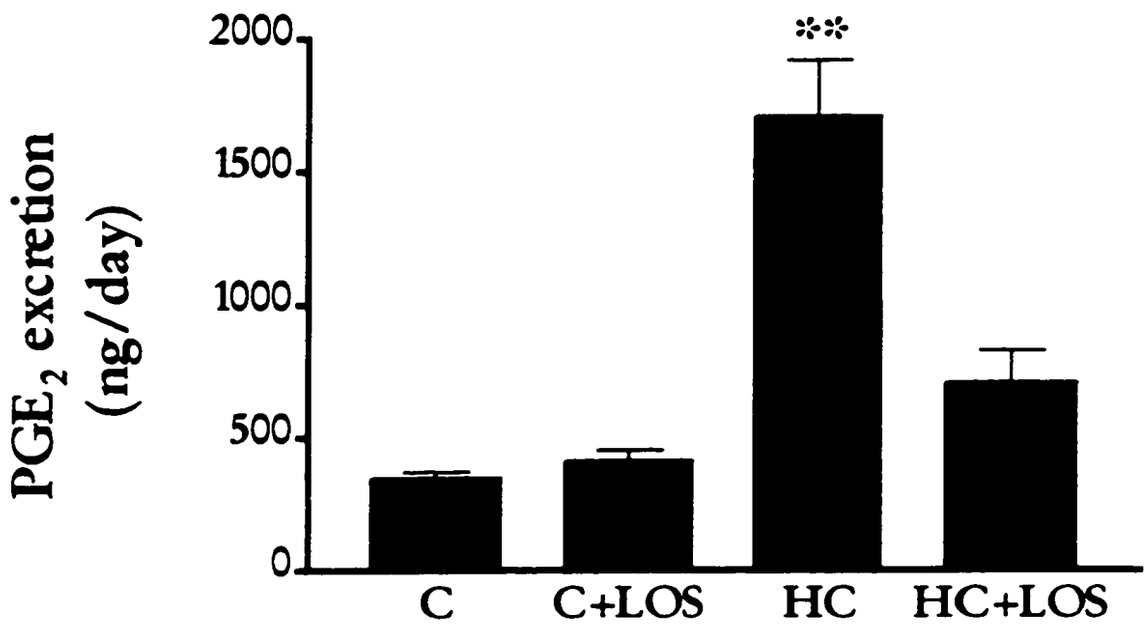


Figure 34. Effect of losartan on PGE_2 excretion. Urine was collected over a 24 h period (day 4 to day 5) from rats fed the control diet (C) alone, the control diet with losartan in the drinking water (C+LOS), the DHT-containing diet (HC) and the DHT-containing diet and losartan in the drinking water (HC+LOS). PGE_2 was measured by radioimmunoassay as described in Methods. Data are expressed as means ± SE. **P $<$0.001 vs C, C+LOS and HC+LOS.

DISCUSSION

4. DISCUSSION

4.1 OVERVIEW

Chronic hypercalcemia is associated with inhibition of mTAL NaCl reabsorption (Peterson, 1990). In rats with vitamin D-induced hypercalcemia, intrarenal PGE_2 levels are significantly elevated, and have been shown to mediate the inhibition of mTAL NaCl transport. However, the specific mechanisms by which PGE_2 exerts its effects on the mTAL in hypercalcemia are not known. Reabsorption of NaCl by the mTAL is driven by the active transport of Na^+ across the basolateral membrane via the Na^+-K^+-ATPase pump, coupled to uptake of Na^+ and Cl^- by the Na^+-K^+-$2Cl^-$ cotransporter. Inhibition of the Na^+-K^+-ATPase pump and/or the Na^+-K^+-$2Cl^-$ cotransporter could result in inhibition of NaCl transport. The study presented here demonstrates that basolateral Na^+-K^+-ATPase activity is not affected by hypercalcemia. We also show that increased extracellular Ca^{2+} and PGE_2 directly inhibit activity of the Na^+-K^+-$2Cl^-$ cotransporter.

There have been no studies addressing the regulation of enzymes involved in PGE_2 synthesis, or determination of the source of increased PGE_2 in hypercalcemia. The results contained within this thesis demonstrate that hypercalcemia causes an up-regulation of intrarenal $cPLA_2$ and PGHS-2, the two main enzymes involved in

prostaglandin synthesis, without altering intrarenal localization, as determined by immunofluorescence. Furthermore, we have demonstrated a role for Ang II, acting on AT_1 receptors, in mediating this up-regulation and in increasing urinary PGE_2 excretion in hypercalcemia.

My Discussion will be divided into the 3 aims of the study as outlined in the *Rationale and Objectives*, and presented in the *Results* Sections.

SECTION 1. Determine if changes in apical and/or basolateral transport steps are responsible for inhibiting mTAL NaCl transport in hypercalcemia

4.2 THE HYPERCALCEMIC RAT MODEL

Hypercalcemia was induced in rats by feeding a diet containing a vitamin D analogue, dihydrotachysterol (DHT). We and others (Levi *et al.* 1983) have shown that blood Ca^{2+} levels and water intake were significantly elevated by two days. These two events have previously been reported to be independent of, and not directly responsible for, the concentrating defect that is evident by day three, since the defect persists in pair-watered hypercalcemic animals, and can be induced by PGE_2 in rats with normal blood Ca^{2+} levels (Levi *et al.* 1983).

The effects observed in the present study are likely due to increased plasma calcium levels, and not due to direct effects of Vitamin D in the kidney. Receptors for Vitamin D have been localized predominantly to the distal convoluted tubule, connecting segment, initial cortical collecting duct and proximal tubule (Liu *et al.* 1994), regions in which we detected no appreciable immunoreactivity for $cPLA_2$ or PGHS-2.

4.3 mTAL TUBULE ISOLATION

In order to investigate the effects of hypercalcemia on mTAL Na^+-K^+-ATPase pump and Na^+-K^+-$2Cl^-$ cotransporter activity, it was necessary to isolate a purified preparation of mTAL segments. Although microdissection of individual segments is a frequently used and elegant technique, it is time consuming and requires the use of sophisticated biochemical techniques because of the very small amount of tissue obtained. We chose to use tubule suspensions as our tool for these studies, as the procedure for isolation is rapid, and a large number of tubules can be obtained.

Although methods have been published which describe the isolation of mTAL tubule suspensions (Kikeri et al., 1990; Chamberlin et al., 1984; Eveloff et al., 1980), our attempts at using a protocol described for rat mTAL isolation (Trinh-Trang-Tan *et*

al. 1986) were not successful. Tubules isolated using this method were not deemed viable (as assessed by trypan blue exclusion), and the yield obtained was lower than that published. Since the collagenase used in our laboratory was different from that used by others (Trinh-Trang-Tan *et al.* 1986), we modified collagenase concentration and digestion periods so that within two hours, we were able to isolate viable tubules with a yield greater than with methods published to date.

Precise dissection of the inner stripe of the outer medulla (a region containing primarily thick ascending limbs), before collagenase digestion, avoids contamination by other tubule segments. Digestion of the tissue is achieved with collagenase because mTAL tubules are resistant to this enzyme, so that mTAL tubules are dispersed intact, whereas any other nephron segments and vascular elements should be reduced to single cells or cell fragments.

The purity of the preparation was assessed by comparing fluorescence of all tubules in the preparation with that obtained with a specific marker for thick ascending limb. The DNA-binding fluorochrome, Hoechst 33258 was used to stain all types of tubules and cells in the suspension. This compound is *Bis*-benzimidazole, a fluorescence dye that specifically binds to double-stranded DNA in nuclei.

We used Tamm-Horsfall protein to specifically stain thick ascending limbs. Tamm-Horsfall protein is synthesized, stored and secreted primarily by thick

ascending limb cells. It has previously been reported that anti-Tamm-Horsfall antibody selectively recognizes mTAL cells among the dispersed cells obtained by collagenase treatment of the rabbit renal outer medulla (Allen et al., 1988). This suggests that Tamm-Horsfall protein does not bind nonspecifically to other cell types after collagenase digestion.

In the present study, when compared with the total number of tubules present as revealed by phase-contrast microscopy and DNA fluorescence, greater than 90% of the tubules in the suspensions stained with anti-Tamm-Horsfall antibodies, determined by indirect immunofluorescence. Collecting tubules were not observed, the major contaminants being segments of thin limb of the loop of Henle. Although rat nephrons are predominantly of the short loop variety, these contaminating thin limbs most likely originate from long looped nephrons. Long looped nephrons have been shown to have a more interdigitating epithelium, and, therefore, probably possess greater resistance to digestion than thin limbs of short loops (Kriz, 1981).

Thus, we have successfully isolated a high yield of a nearly pure suspension of viable segments of rat mTAL, for use in the study of this specialized nephron segment.

4.4 THE BASOLATERAL Na^+-K^+-ATPASE PUMP

Na^+-K^+-ATPase is an integral membrane protein that catalyzes the exchange of Na^+ and K^+ ions across the plasma membranes of most eukaryotic cells. In the kidney, this enzyme is the principal driving force for net Na^+ reabsorption and for the secondary active transport of other ions and nutrient solutes. Structurally, Na^+-K^+-ATPase is a heterodimer comprising at least two subunits (a, b). The a-subunit contains the ATP catalytic domain and the ion transporting function that allows the outward movement of 3 Na^+ ions in exchange for 2 K^+ ions.

To determine if hypercalcemia-induced inhibition of mTAL NaCl reabsorption is caused by inhibition of the basolateral Na^+-K^+-ATPase pump, we measured the activity of this pump in mTAL tubules isolated from control and hypercalcemic rats.

No difference was observed in Na^+-K^+-ATPase activity in tubules isolated from control and hypercalcemic rats, suggesting that inhibition of this pump was not responsible for the inhibition of NaCl reabsorption in hypercalcemia. However, we cannot rule out the possibility that the lack of inhibition is due to recovery of the transport defect in the tubules, which may have occurred during the isolation procedure and/or during the time between isolation and the onset of the experiment.

To further investigate factors controlling Na^+-K^+-ATPase activity in the mTAL, we used a mouse mTAL cell line. Using this model, we determined if exposure of mTAL cells to high extracellular Ca^{2+} (Ca^{2+}_o) and/or PGE_2, two important factors in hypercalcemia, affect the activity of the Na^+-K^+-ATPase pump. Ouabain-sensitive ^{86}Rb uptake was used as an index of pump activity.

Cells grown in a high Ca^{2+}_o environment demonstrated a stimulation of ouabain-sensitive ^{86}Rb uptake compared with those exposed to normal Ca^{2+}_o. Cell surface Ca^{2+}-sensing receptors have been localized to the mTAL (Riccardi *et al.* 1995), and a recent study by Paulais et al. (Paulais *et al.* 1996) demonstrated that in microdissected mTAL tubules, stimulation of these receptors by elevated Ca^{2+}_o induced a transient, though not consistent, increase in intracellular Ca^{2+} (Ca^{2+}_i), which they speculate is linked to reduced cAMP generation by these segments. Indeed, the Ca^{2+}-sensing receptor has been shown in parathyroid cells to couple to inhibition of adenylate cyclase through an inhibitory G-protein, G_i (Brown *et al.* 1995). Furthermore, an acute increase in Ca^{2+}_o has been reported to decrease cAMP production in the mouse mTAL by direct inhibition of the catalytic unit of adenylate cyclase (Takaichi *et al.* 1986). Therefore, exposure of mTAL cells to high Ca^{2+}_o may stimulate the Ca^{2+}-sensing receptor to inhibit adenylate cyclase and cAMP production.

Could this decreased cAMP production affect the Na^+-K^+-ATPase pump ? In

the mTAL, Na^+-K^+-ATPase activity has been reported to be inhibited by activation of adenylyl cyclase or by exogenous cAMP (Satoh *et al.* 1993a). This effect was abolished by inhibitors of either cAMP-dependent protein kinase A (PKA) or of PLA_2, suggesting that the increment in cAMP is not the final step in the regulation of Na^+-K^+-ATPase activity. It has been proposed that in the mTAL, cAMP-mediated activation of PKA stimulates PLA_2 to release arachidonic acid, and it is the metabolites of the arachidonic cascade which inhibit Na^+-K^+-ATPase pump activity (Satoh *et al.* 1993a; Satoh *et al.* 1993b). From this evidence, we can speculate that elevated Ca^{2+}_o may result in decreased intracellular cAMP-mediated PKA activation, which would cause stimulation of the Na^+-K^+-ATPase pump, as we observed.

Studies have shown that products of the cyclooxygenase or cytochrome P-450 pathways inhibit renal Na^+-K^+-ATPase. PGE_2 has been shown to inhibit the Na^+-K^+-ATPase pump in the rabbit and rat mTAL (Lear *et al.* 1990; Wald *et al.* 1990), cortical collecting duct and inner medullary collecting duct (Cohen-Luria *et al.* 1994). However, our work shows that exposure of mTAL cells to PGE_2 did not affect ouabain-sensitive ^{86}Rb uptake. The studies performed on mTAL cells used much higher concentrations of PGE_2 (100 mM, 30 mM) (Wald *et al.* 1990; Lear *et al.* 1990) than we used (1 mM), so the effects they report may be nonspecific, and comparing results may not be accurate.

In support of our findings, other investigators have also challenged the reported

direct effect of PGE_2 on pump inhibition (Sejersted *et al.* 1984; Cantley *et al.* 1991). Indeed, prostaglandins do not inhibit purified Na^+-K^+-ATPase preparations (Satoh *et al.* 1993a), suggesting that this inhibitory effect most likely occurs indirectly, perhaps by decreasing luminal Na^+ entry. A study by Kaji et al. also reports that the inhibition of Na^+-K^+-ATPase pump activity by PGE_2, measured by ouabain-sensitive ^{86}Rb uptake, was secondary to reduced Na^+ entry into the cells, and, therefore, not a direct effect (Kaji *et al.* 1996).

In summary, we have shown that the Na^+-K^+-ATPase pump is not affected by chronic hypercalcemia in our model, and is not likely to be responsible for inhibition of NaCl transport in the mTAL. Furthermore, we propose that in our *in vitro* cell culture model, high Ca^{2+}_o may activate the Ca^{2+}-sensing receptor to inhibit cAMP production, which may be responsible for the stimulation of Na^+-K^+-ATPase pump activity we observed. Finally, we demonstrate that PGE_2 does not affect mouse mTAL Na^+-K^+-ATPase pump activity.

4.5 THE NA^+-K^+-2CL^- COTRANSPORTER

We performed studies to determine if inhibition of TAL NaCl reabsorption in chronic hypercalcemia is due to inhibition of the apical Na^+-K^+-$2Cl^-$ cotransporter. Activity of the cotransporter was assessed in mTAL tubules by measuring bumetanide-

sensitive ^{86}Rb uptake.

Although ^{86}Rb uptake in mTAL tubules was demonstrated, bumetanide-sensitive uptake was not detected. This cannot be due to poor viability and transport capability of this preparation, since Na^+-K^+-ATPase activity was successfully measured. Since the Na^+-K^+-ATPase pump is located on the basolateral membrane and the Na^+-K^+-$2Cl^-$ cotransporter is situated on the apical membrane, we propose that access to the apical membrane in this isolated tubule preparation was not possible, perhaps due to sealing of the ends of the segments. Therefore, a different model was required for these studies. We chose a cell culture system, which would allow us to manipulate conditions so that we could attempt to elucidate the factors involved in regulating Na^+-K^+-$2Cl^-$ cotransport.

We used a mouse mTAL cell line to assess Na^+-K^+-$2Cl^-$ cotransport (bumetanide-sensitive ^{86}Rb uptake) under conditions of normal or high Ca^{2+}_o, and in the absence or presence of PGE_2. Our data reveal that PGE_2 inhibited the Na^+-K^+-$2Cl^-$ cotransporter in cells exposed to normal Ca^{2+}_o. Exposure of cells to high Ca^{2+}_o also resulted in inhibition of the co-transporter. Interestingly, treatment of cells with both high Ca^{2+}_o and PGE_2 resulted in a further, almost complete, inhibition of Na^+-K^+-$2Cl^-$ co-transport. These observations do not permit us to deduce the signal transduction pathway involved in this mediating effect, although we can speculate on the mechanisms.

As discussed in the previous section, there is evidence from *in vitro* studies in mouse mTAL, that acute increases in Ca^{2+}_o directly suppress cAMP production (Takaichi *et al.* 1986). Furthermore, Ca^{2+}_o may exert its actions in the mTAL via stimulation of the Ca^{2+}-sensing receptor, which may be coupled to inhibition of adenylate cyclase through an inhibitory G-protein, G_i (Brown *et al.* 1995). In this manner, intracellular cAMP would be reduced, which could result in inhibition of the Na^+-K^+-$2Cl^-$ co-transporter. Sun et al (Sun *et al.* 1991) proposed a model that suggests that in the mouse mTAL, decreased cAMP may prevent phosphorylation of the Na^+-K^+-$2Cl^-$ cotransporter such that it remains in the Na^+-Cl^- "mode," resulting in reduced NaCl transport. We did not determine whether "mode switching" is occurring in our model.

Although increased Ca^{2+}_o alone may be able to reduce NaCl transport via direct inhibition of the Na^+-K^+-$2Cl^-$ cotransporter, under chronic conditions, such as in our model of hypercalcemia, elevated Ca^{2+}_o is not likely to be the sole factor responsible for inducing the NaCl transport defect. Indeed, Peterson et al. (Peterson *et al.* 1993) were able to reverse inhibition of mTAL Cl^- reabsorption with indomethacin, forskolin and exogenous cAMP in chronic hypercalcemic rats, demonstrating the importance of cAMP and PGE_2. Furthermore, NaCl inhibition in this model was induced with exogenous PGE_2 in rats with normal Ca^{2+}_o (Peterson *et al.* 1993).

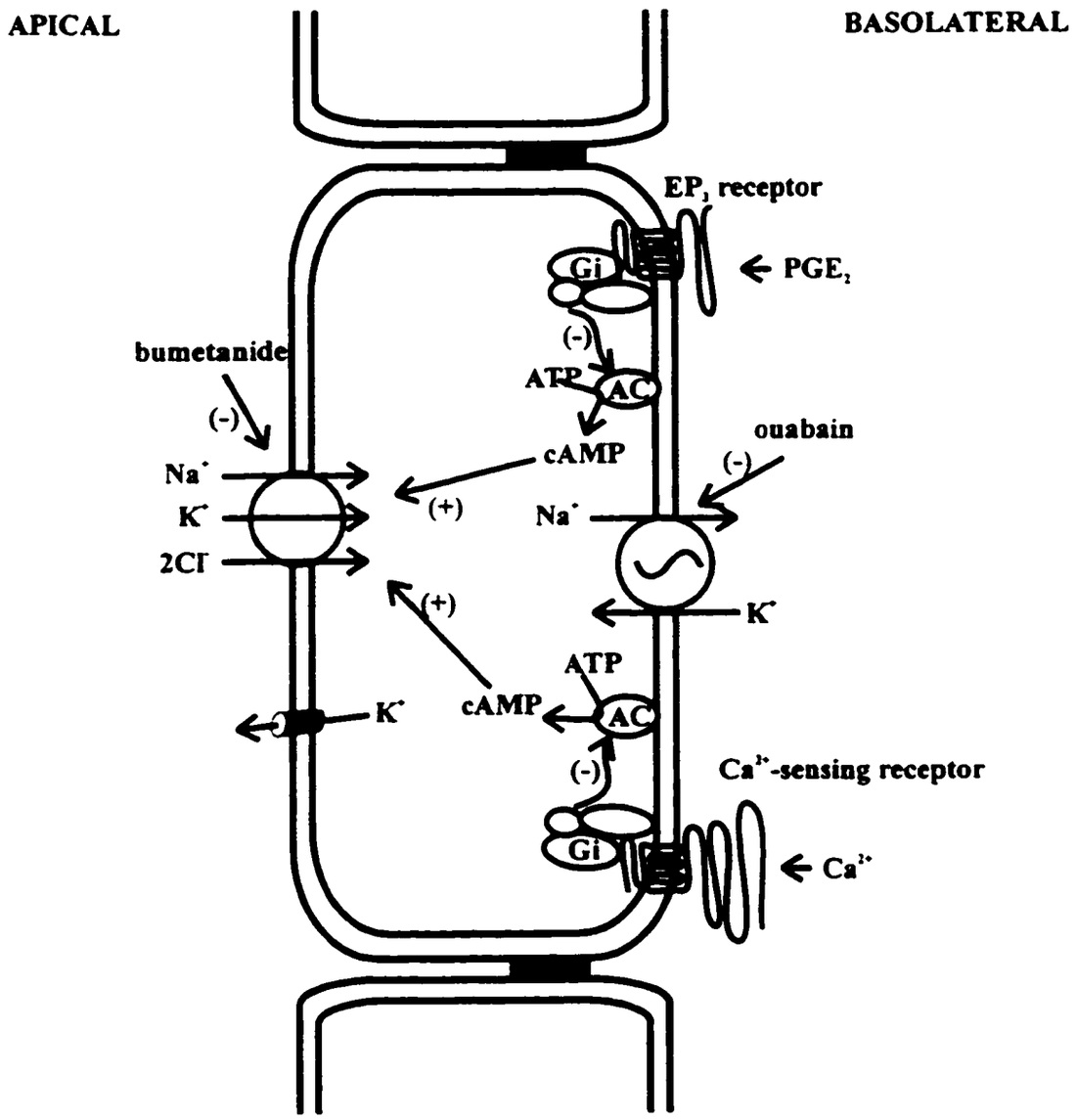
Although PGE_2 excretion has been shown to be significantly elevated in rats with chronic hypercalcemia (5 days or more on the DHTdiet), we demonstrated that PGE_2 excretion is not significantly elevated after 3 days of consuming the DHT diet - a time when the concentrating defect is apparent and blood ionized Ca^{2+} levels are significantly increased. From this, we suggest that increased Ca^{2+}_o may be involved in the inhibition of the co-transporter in the initial stages of hypercalcemia. Support for this concept is provided by Galla et al. (Galla *et al.* 1986), who showed that in rat, acute increases in Ca^{2+}_o reduce mTAL Cl^- reabsorption by an indomethacin-resistant mechanism. Therefore, PGE_2 most likely is involved in mediating long-term, chronic NaCl transport inhibition.

The inhibition of the Na^+-K^+-$2Cl^-$ cotransporter by PGE_2 demonstrated in this work has also been recently reported by Kaji et al (Kaji *et al.* 1996). These investigators showed that the inhibitory effect of PGE_2 was not mediated by an action on cytosolic Ca^{2+}, nor did it affect the affinity of the cotransporter for external Na^+, K^+ or Cl^-. However, PGE_2 did reduce the V_{max}, suggesting that the number of transporter sites were reduced. Since PGE_2 reduced [^{3}H] bumetanide binding, the authors propose that PGE_2 inhibits bumetanide-sensitive K^+ influx by down-regulating the number of Na^+-K^+-$2Cl^-$ cotransporters. The specific mechanisms by which PGE_2 induces this effect remain to be investigated.

Our observations demonstrating the inhibitory actions of high Ca^{2+}_o and PGE_2 on bumetanide-sensitive ^{86}Rb influx have not been previously reported. Our results show that high Ca^{2+}_o and PGE_2 exert a synergistic effect on Na^+-K^+-$2Cl^-$ cotransporter inhibition, suggesting that they are acting through different pathways, though both may cause a reduction in intracellular cAMP. We cannot, however, rule out the possibility that high Ca^{2+}_o may affect another factor which results in increased PGE_2 production, and so, still be responsible for the observed chronic effects.

A schematic summarizing our findings is illustrated below.

Figure 35. Proposed model for NaCl transport inhibition by high Ca^{2+}_o and PGE_2. Extracellular Ca^{2+} stimulates the Ca^{2+}-sensing receptor, which is coupled to an inhibitory G protein, G_i, to inhibit cAMP production. Reduced cAMP will decrease activity of the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter, thereby inhibiting NaCl transport. PGE_2 also inhibits the Na^+-K^+-$2Cl^-$ cotransporter, via activation of the EP_3 receptor, which is coupled to inhibition of adenylate cyclase (AC) and cAMP production.



SECTION 2. Aim: To determine the effect of hypercalcemia on $cPLA_2$, PGHS-1 and PGHS-2 protein expression

4.6 $cPLA_2$, PGHS-1 AND PGHS-2 PROTEIN EXPRESSION IN HYPERCALCEMIA

In chronic hypercalcemia, inhibition of mTAL Cl^- reabsorption has been linked to increased PGE_2 levels in the kidney (Peterson *et al.* 1993). Furthermore, we have shown that this may occur through inhibition of the Na^+-K^+-$2Cl^-$ cotransporter. The aim of the next series of studies presented in this thesis was to investigate the regulation of enzymes involved in PGE_2 synthesis, and to determine the source of increased PGE_2 in hypercalcemia.

The rate limiting step in prostaglandin synthesis is the PLA_2-mediated release of arachidonic acid from membrane phospholipids (Mukherjee *et al.* 1994). $cPLA_2$ has been isolated and purified from kidney tissue (Gronich *et al.* 1990), and its activity studied in isolated glomerular mesangial cells (Schramek *et al.* 1994). The *in vivo* regulation of renal $cPLA_2$, and its specific cellular localization in the kidney, however, remains unclear.

In the present studies, expression of $cPLA_2$ was evident in normal rat kidney tissue, and we demonstrated significant up-regulation after 3 days of hypercalcemia,

suggesting increased endogenous availability of arachidonic acid in the kidneys of these animals. In addition, we localized immunoreactive cPLA$_2$ mainly to interstitial cells of the inner and outer medulla, which are well known to synthesize and release prostaglandins (Brown *et al.* 1980; Kester *et al.* 1994), and to the macula densa.

Induction of PGHS-2, the other critical enzyme involved in prostaglandin synthesis, is associated with inflammation, mitogenesis and ovulation (Smith & DeWitt, 1995; Mitchell *et al.* 1995). The expression of PGHS-2 mRNA *in vitro* parallels that of *c-fos*, leading to the classification of PGHS-2 as an immediate early gene (Wu, 1996). Rapid, transient activation of PGHS-2, and subsequent return to baseline levels, has been observed in a variety of cultured cell systems (Kujubu *et al.* 1993; DeWitt & Meade, 1993b), including kidney mesangial cells (Kester *et al.* 1994). However, to date, the renal expression and regulation of this enzyme *in vivo* has not been fully elucidated.

PGHS-2 is expressed under unstimulated conditions in the kidney (Harris *et al.* 1994), and the present work provides further support for this. In addition, we demonstrate that the expression pattern of PGHS-2 in this *in vivo* model is consistent with observations in cultured cells. In this regard, we report that PGHS-2 protein expression was significantly and maximally increased in hypercalcemic animals after 3 days of ingestion of DHT-containing diet, and displayed a similar cellular pattern of distribution as cPLA$_2$.

However, unlike $cPLA_2$, PGHS-2 protein levels in the inner and outer medulla decrease after the initial stimulation on day 3, suggesting that $cPLA_2$, which is still stimulated at this time, is responsible for increasing PGE_2 production in these regions.

We can speculate as to the mechanisms responsible for the pattern of PGHS-2 expression. A "suicide inactivation" phenomenon has been described for prostaglandin H synthase (PGHS) (Egan *et al.* 1976). These studies do not discriminate between PGHS-1 and PGHS-2, since at the time that they were performed, the two isoforms had not been described. Addition of excess arachidonic acid to microsomal enzyme preparations exhibiting PGHS activity results in a rapid, transient burst in oxygen consumption as PGG_2 is formed (Egan *et al.* 1976). Little is known about the mechanisms resulting in the rapid fall in oxygenase activity, which causes inactivation of this enzyme before all the arachidonic acid is consumed. The rapid decline in PGHS-2 protein expression we observed in the present work could also be due to suicide inactivation.

Thus, even though levels of $cPLA_2$ are still significantly elevated in hypercalcemia after day 3, suggesting continued and increased availability of endogenous arachidonic acid, PGHS-2 may display this suicide inactivation in hypercalcemia, to decrease PGHS-2 protein levels after the initial stimulation on day 3.

In hypercalcemia there may be an increase in turnover of PGHS-2 at the later time points (after day 3), whereby inactive enzyme is replaced with active enzyme. In this manner, turnover of PGHS-2 may be increased while the absolute levels of the protein remain constant.

In the cortex of hypercalcemic animals, levels of PGHS-2 remained significantly elevated until day 5, so that this region appears to be more resistant to the autoinactivation phenomenon we suggest may be ocurring in the inner and outer medulla. This suggests the presence of additional regulatory signals or factors for PGHS-2 expression (in the macula densa and cortical thick ascending limb cells) which differ from those influencing interstitial cells of the inner and outer medulla.

PGHS-1 is expressed constitutively in almost all tissues including kidney (Harris *et al.* 1994; Smith & Bell, 1978). In the present work, we found that PGHS-1 protein levels remained unchanged with hypercalcemia in all kidney regions. Previous studies reveal that PGHS-1 functions primarily as a "house-keeping" protein (Langenbach *et al.* 1995). Thus, it appears that in hypercalcemia, PGHS-1 preserves its role as a constitutive protein, responsible for the basal production of prostaglandins, which are most likely involved in the rapid response to stimulation by agonists.

SECTION 3. Aim: To investigate the role of angiotensin II in hypercalcemia

4.7 ROLE OF ANGIOTENSIN II IN HYPERCALCEMIA

Ang II is well known to be a potent dipsogenic agent (Iyer *et al.* 1995; Kraly & Corneilson, 1990). Ang II has also been reported to stimulate PGE_2 production in kidney tubular epithelial cells, mesangial cells and interstitial cells (Douglas & Hopfer, 1994; Zusman & Keiser, 1980; Siragy & Carey, 1996). In vascular smooth muscle cells, Ang II induces phosphorylation of PLA_2 and thereby increases its activity (Rao *et al.* 1994). Since polydipsia and increased PGE_2 production are associated with chronic hypercalcemia, and we demonstrated stimulation of $cPLA_2$ protein in this condition, we investigated the role of Ang II in our model of hypercalcemia.

Local production of Ang II has been reported in the kidney (Hollenberg & Fisher, 1995). In order to determine if hypercalcemia affects Ang II production, we measured intrarenal levels of this hormone. In our previous studies, circulating plasma renin levels did not increase significantly in hypercalcemic rats with access to water, although pair-water feeding was associated with significantly higher levels (Peterson, 1990; Levi *et al.* 1983). Furthermore, our data indicate that intrarenal Ang II levels, measured at a time when expression of $cPLA_2$ and PGHS-2 was increased, are also unchanged with hypercalcemia. This suggests that increased production of intrarenal

Ang II is not responsible for the observed increased expression of $cPLA_2$ and PGHS-2 reported in this work.

Next, we investigated the possibility that Ang II AT_1 receptor activation occurs in hypercalcemia, and mediates the observed effects. Our data strongly support this hypothesis.

In these studies, losartan, a non-peptide, oral AT_1 receptor antagonist, was administered to control and hypercalcemic rats. Losartan interacts with amino acids in the transmembrane domains of AT_1 receptors, thus preventing the binding of Ang II (Goodfriend *et al.* 1996). Siragy and Carey recently demonstrated that losartan blocked the increase in renal interstitial fluid PGE_2 levels resulting from both sodium depletion and administration of Ang II (Siragy & Carey, 1996).

The present studies demonstrate that increased water intake, which is consistently observed in hypercalcemic rats (Peterson, 1990; Peterson *et al.* 1993), was abolished by administration of losartan. Therefore, our data indicate that the polydipsic behaviour in hypercalcemia is mediated by binding of Ang II to the AT_1 receptor. Administration of losartan in the rat has been shown to centrally inhibit Ang II binding in the subfornical organ, median eminence, area postrema, and other areas of the brain that are within the blood-brain barrier, such as the hypothalamic paraventricular nucleus, the median preoptic neurons, the bed nucleus of the stria

terminalis and the nucleus of the solitary tract (Li *et al.* 1993; Song *et al.* 1991). It has yet to be determined which central area(s) mediate drinking behaviour in hypercalcemic rats.

Losartan also prevented the increase in $cPLA_2$ protein of hypercalcemic animals in all regions of the kidney, and inhibited increased PGHS-2 production in the inner medulla without affecting expression in the cortex and outer medulla.

Finally, previous studies have established that urinary levels of PGE_2 reflect intrarenal synthesis, derived mainly from the inner medulla (Taverner *et al.* 1984). We demonstrate that losartan completely inhibited the increase in urinary PGE_2 excretion observed in rats with hypercalcemia for 5 days. We did not observe any change in urinary PGE_2 levels on day 3, a time when both $cPLA_2$ and PGHS-2 were stimulated. Thus, there appears to be a lag time between stimulation of expression of these enzymes and the appearance of increased urinary excretion of PGE_2.

Taken together, these data suggest that stimulation of $cPLA_2$ expression, through Ang II activation of the AT_1 receptor in the inner medulla, largely mediates the increased PGE_2 excretion in hypercalcemic animals.

Our data do not, however, permit precise intrarenal localization of sites of action of Ang II in hypercalcemia. Specific binding sites for Ang II (Mujais *et al.* 1986),

AT_1 receptor mRNA (Terada *et al.* 1993) and AT_1 receptor protein (Harrison-Bernard *et al.* 1997) have been detected in all tubular segments, suggesting that Ang II may bind to receptors on cortical and medullary thick ascending limb segments and thereby directly stimulate $cPLA_2$ protein expression. We cannot, however, rule out the possibility that Ang II mediates its effects in an indirect fashion by altering intrarenal hemodynamics.

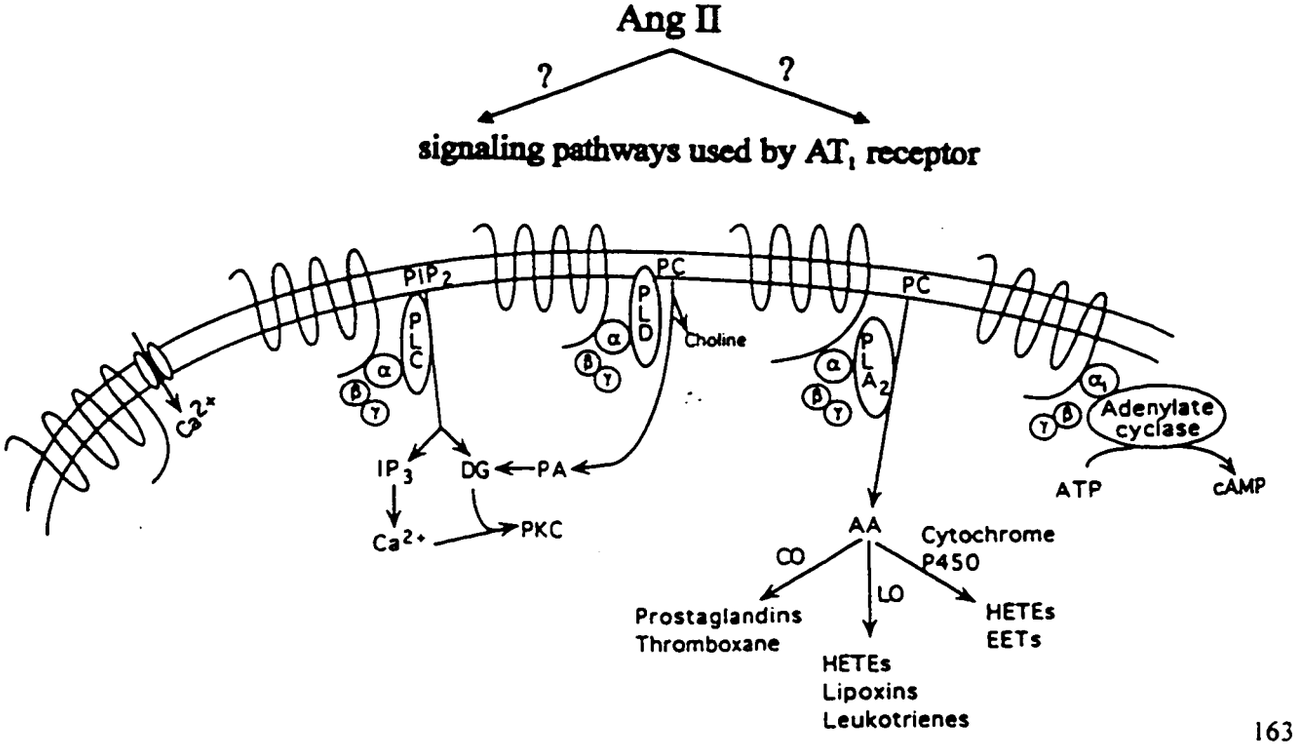
In the present study, $cPLA_2$ immunoreactivity was detected in interstitial cells of the inner and outer medulla. Zhou et al. have detected Ang II binding sites in type-1 interstitial cells of the inner stripe of the outer medulla, by electron microscopic emulsion autoradiography (Zhuo *et al.* 1994). By autoradiography, this group was unable to detect Ang II binding sites in the inner medulla, although AT_1 Ang II receptor mRNA has been localized to inner medullary collecting duct by reverse transcription-polymerase chain reaction (Terada *et al.* 1993). Furthermore, Harrison-Bernard et al. recently used a monoclonal antibody to demonstrate positive AT_1 receptor immunoreactivity in various regions of the kidney, including macula densa cells and medullary collecting ducts (Harrison-Bernard *et al.* 1997). Thus, since we also localized $cPLA_2$ to cells of the inner medullary collecting duct, it is possible that the intrarenal expression of this protein with hypercalcemia is partly due to AT_1 receptor stimulation on inner medullary collecting duct cells.

Similarly, although PGHS-2 protein expression was stimulated in all regions of

the kidney by hypercalcemia in the present study, losartan abrogated only the expression in the inner medulla. Since PGHS-2 was immunolocalized only to interstitial cells of the inner medulla, the results suggest the possibility that these cells do indeed contain AT_1 Ang II receptors, or that alteration in renal blood flow mediates the effects of Ang II.

We can speculate on the signaling pathways utilized by the AT_1 receptor (see Figure 36 below) to mediate the observed changes in $cPLA_2$ and PGHS-2 expression in hypercalcemia.

Figure 36. Signaling pathways used by the AT_1 receptor. *Modified from: Griendling et al., 1996.*



The AT_1 receptor has been shown to be coupled to PLA_2. Although this pathway may be activated initially by Ang II to increase $cPLA_2$ activity, it has been established that the AT_1 receptor becomes desensitized to stimulation by Ang II. Therefore, in order for up-regulation of protein expression to occur, activation of a pathway ultimately leading to actions on the nucleus may be required. The AT_1 receptor can also couple to phospholipase C and phospholipase D which results in the activation of protein kinase C. Since activation of phospholipase D has been shown to be sustained, this pathway may be responsible for ultimate activation of $cPLA_2$ and PGHS-2 protein expression.

Although the methods used in the present studies were appropriate for the parameters being measured, we must be mindful of their limitations. For example, some of the procedures used required processing of tissue samples prior to measurement - we cannot discount that such processing may have altered the results from those that would have been observed if these parameters were measured directly *in vivo*. Furthermore, certain protocols required whole kidney measurements to be made (e.g. Ang II levels), so that changes occurring in only a specific region of the kidney may not be detected, requiring the use of a more sensitive technique. Therefore, we can only speculate as to how the data we obtained *in vitro* corresponds to the *in vivo* condition.

CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

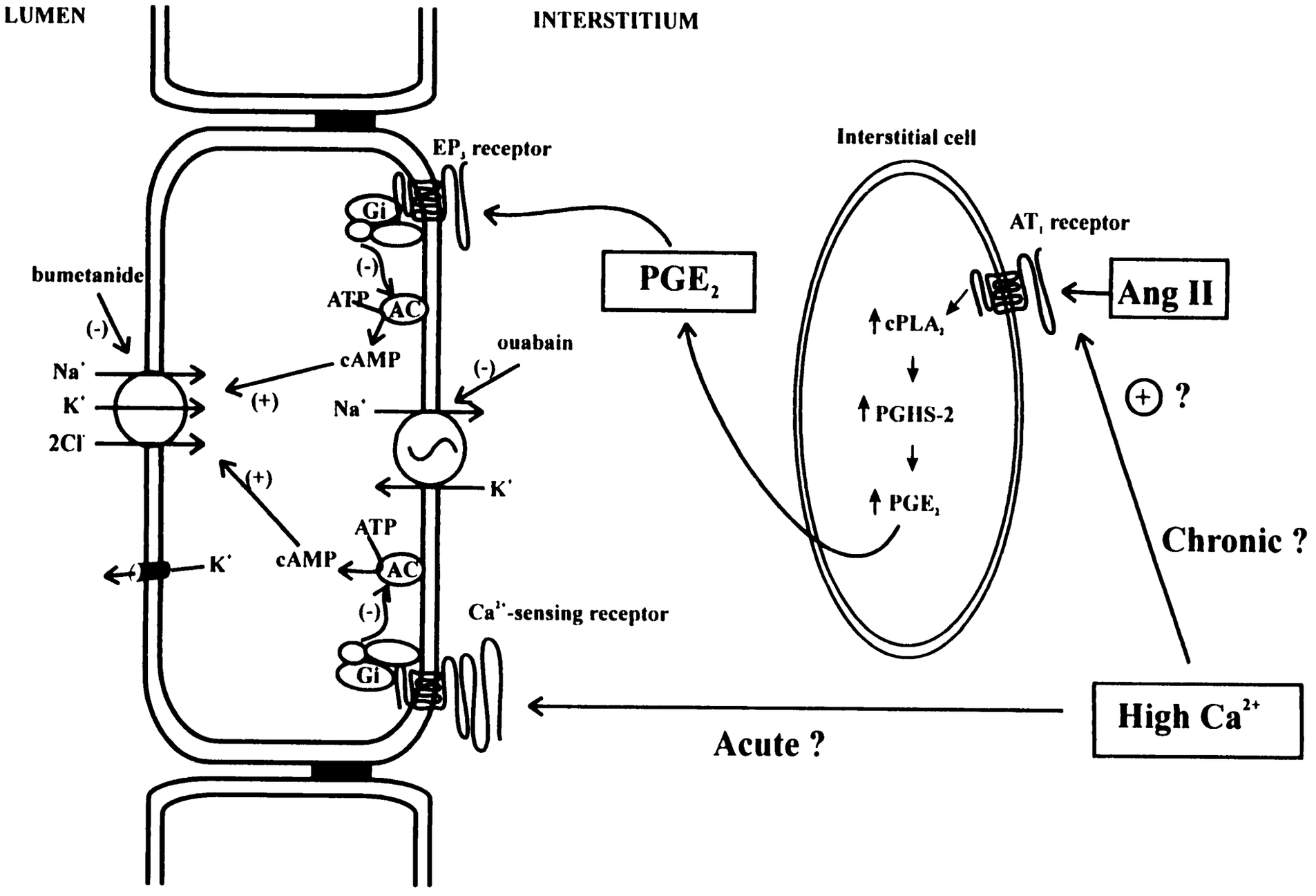
In summary, we have shown that in our model of chronic hypercalcemia, inhibition of NaCl reabsorption is not due to inhibition of the Na^+-K^+-ATPase pump. Both increased Ca^{2+}_o and PGE_2 inhibit the Na^+-K^+-$2Cl^-$ cotransporter, and we propose that these factors may be involved in the inhibition of NaCl transport in hypercalcemia.

We have shown that hypercalcemia up-regulates intrarenal $cPLA_2$ and PGHS-2 protein expression, two proteins critical in the synthesis of prostaglandins. Our data suggest that enhanced expression of $cPLA_2$ may account for the elevated renal PGE_2 levels in hypercalcemia. Furthermore, we have demonstrated an important role for Ang II, acting on AT_1 receptors, in mediating hypercalcemia-induced stimulation of intrarenal $cPLA_2$ and PGHS-2 expression, and in increasing renal PGE_2 production.

We use our *in vivo* and *in vitro* observations to propose the following hypothetical series of events which may ultimately lead to the inhibition of NaCl transport in the mTAL of hypercalcemic animals (Figure 37).

During the early stages of hypercalcemia (2 to 3 days on DHT-containing diet), elevated Ca^{2+}_o may stimulate Ca^{2+}-sensing receptors on mTAL cells to inhibit the Na^+-

Figure 37


LUMEN
INTERSTITIUM
EP1 receptor
Gi
(-)
ATP
AC
cAMP
bumetanide
(-)
Na+
K+
2Cl-
(+)
ouabain
(-)
Na+
K+
(+)
ATP
cAMP
AC
K+
(-)
Ca2+-sensing receptor
Gi
PGE2
Interstitial cell
AT1 receptor
Ang II
cPLA2
PGHS-2
PGE2
+ ?
Chronic ?
High Ca2+
Acute ?

K^+-$2Cl^-$ cotransporter via inhibition of cAMP production. After 3 days of DHT-containing diet, elevated Ca^{2+}_o may stimulate Ang II, acting on AT_1 receptors on interstitial cells, to increase $cPLA_2$ and PGHS-2 protein expression, resulting in increased PGE_2 production. PGE_2, in turn, via activation of the EP_3 receptor, may inhibit adenylate cyclase and cAMP production, which would cause inhibition of the Na^+-K^+-$2Cl^-$ cotransporter.

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6. BIBLIOGRAPHY

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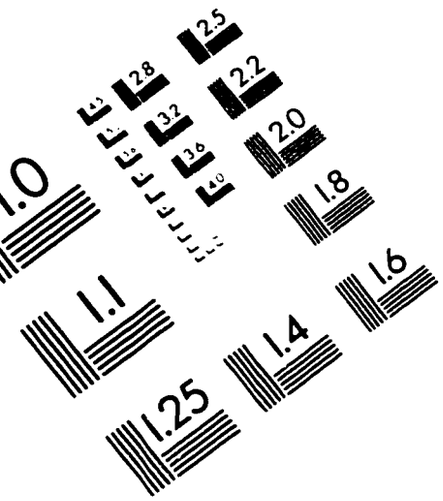
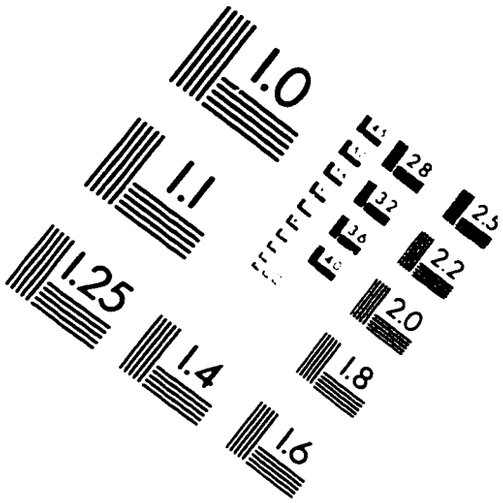
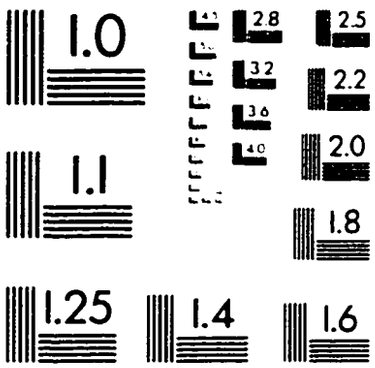
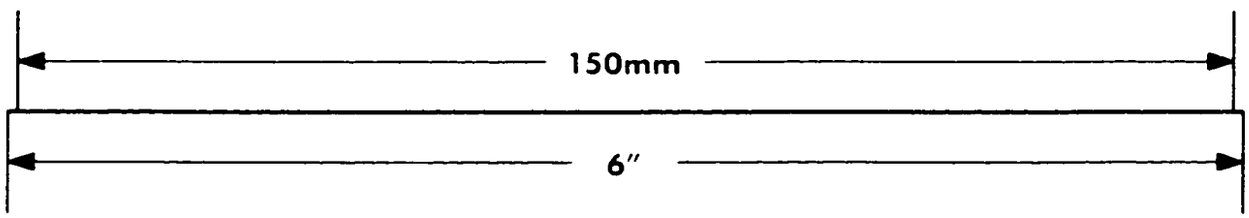
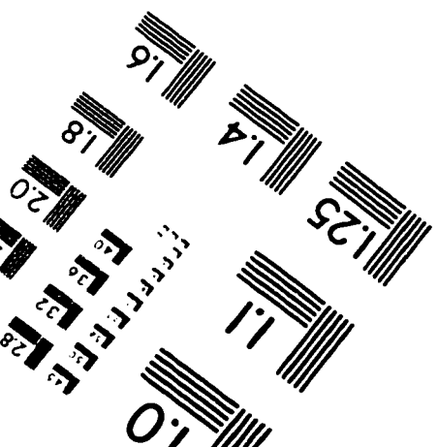
IMAGE EVALUATION TEST TARGET (QA-3)

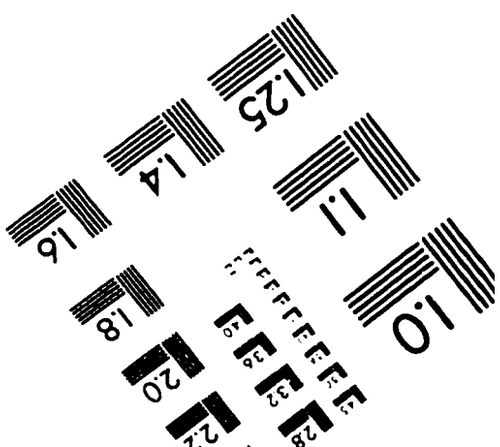












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