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Papillary Collecting Tubule
Cells From Rabbit Kidney

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Frank Charles Grenier

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Doctor of Philosophy

William L. Smith

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PROSTAGLANDIN METABOLISM IN PAPILLARY COLLECTING TUBULE CELLS FROM RABBIT KIDNEY

By

Frank Charles Grenier

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ABSTRACT

PROSTAGLANDIN METABOLISM IN PAPILLARY COLLECTING TUBULE CELLS FROM RABBIT KIDNEY

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Homogeneous (>97%) populations of renal papillary collecting tubule (RPCT) cells were isolated from the rabbit kidney. RPCT cells were characterized as being derived from the collecting tubule on the basis of anatomical source, size, enzyme histochemistry, and cyclooxygenase antigenicity; in addition RPCT cells synthesized 3',5'-cyclic AMP (cAMP) in response to arginine vasopressin, formed hemicysts when grown to confluency and adhered with morphological asymmetry to Millipore filters.

Homogenates of RPCT cells when incubated with [^{3}H]-arachidonic acid formed 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGD_2. At

arachidonic acid concentrations below 2 μM the major product formed was 6-keto-$PGF_{1\alpha}$; at higher concentrations PGE_2 was the major radioactive prostaglandin formed.

A series of hormones were tested for their influence on the release of immunoreactive prostaglandins (iPG) by intact RPCT cells grown in monolayer culture; the major product (ca. 75%) under both bassal and stimulated conditions was $iPGE_2$. At very low concentrations ($\geq$ 10^{-10} M) bradykinin, lysyl-bradykinin and methionyl-lysylbradykinin all caused 3-5 fold increases in $iPGE_2$ formation. Significantly, neither arginine vasopressin (AVP) (10^{-7} M) nor desamino-AVP (10^{-7} M) caused prostaglandin release by RPCT cells. These results indicate that kinins can act directly on the collecting tubule to elicit PGE_2 formation; furthermore, the effect of kinins may be natriuretic since PGE_2 has been shown by others to inhibit Na^+ resorption by the medullary collecting tubule.

RPCT cells synthesized cAMP in response to AVP, parathyroid hormone and glucagon but not to adrenergic agents. In addition, exogenous PGE_2 and PGI_2, increased intracellular cAMP concentrations in RPCT cells.

The effects of PGE_2 and PGI_2 on AVP-induced cAMP production by RPCT cells were investigated. At 10^{-7} M AVP and 10^{-5} M PGE_2 or PGI_2, the increase in both total and intracellular cAMP levels was less than that expected for an additive response of AVP plus prostaglandin. This suggests that prostaglandins can partially block AVP-induced cAMP accumulation in the collecting tubule.

TO KEARSTIE

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INTRODUCTION

Various experimental approaches have been used to investigate how prostaglandins are involved with kidney processes including 1) whole animal clearance methods 2) perfusion of isolated kidneys, 3) micropuncture and stopped-flow techniques and 4) techniques using renal slices and renal homogenates (38,57). A drawback of all these methods, however, is that in each case many different kidney cells may influence any measurements made using these systems. It is virtually impossible to interpret such data on the individual cell level. Nevertheless, these methods have been useful in defining both the prostaglandin biosynthetic capacity of the kidney and the hormones which cause prostaglandin biosynthesis by the kidney (57).

Frustrated by the relative indirectness of these methods, Burg et al. developed a technique for microperfusing individual segments of kidney tubules (42). The tremendous advantage of this technique was its lack of complexity. Only one tubular cell type was being studied and therefore the interpretation of the data was greatly simplified. Using perfused collecting tubules, for example, Grantham et al. were able to demonstrate that PGE_1 inhibits arginine vasopressin (AVP)-stimulated water resorption in the collecting tubule (61). This information was unattainable prior to Burg's advancement.

The major limitation of Burg's method, however, is the difficulty in making biochemical measurements with isolated tubule segments.

Dissection of tubule segments is laborious and yields segments only 1-2 mm in length (42). It would require several hundred tubule segments (10^6 cells) to make many biochemical measurements (e.g. PGE_2 and 3',5'-cyclic AMP radioimmunoassays) that could be made easily with one partially confluent 24-well culture dish. Renal medullary interstitial cells, for instance, have been used in culture to make a series of biochemical measurements pertaining to prostaglandin metabolism (1). As with tubule segments though, the data obtained with cultured cells can also be cell specific.

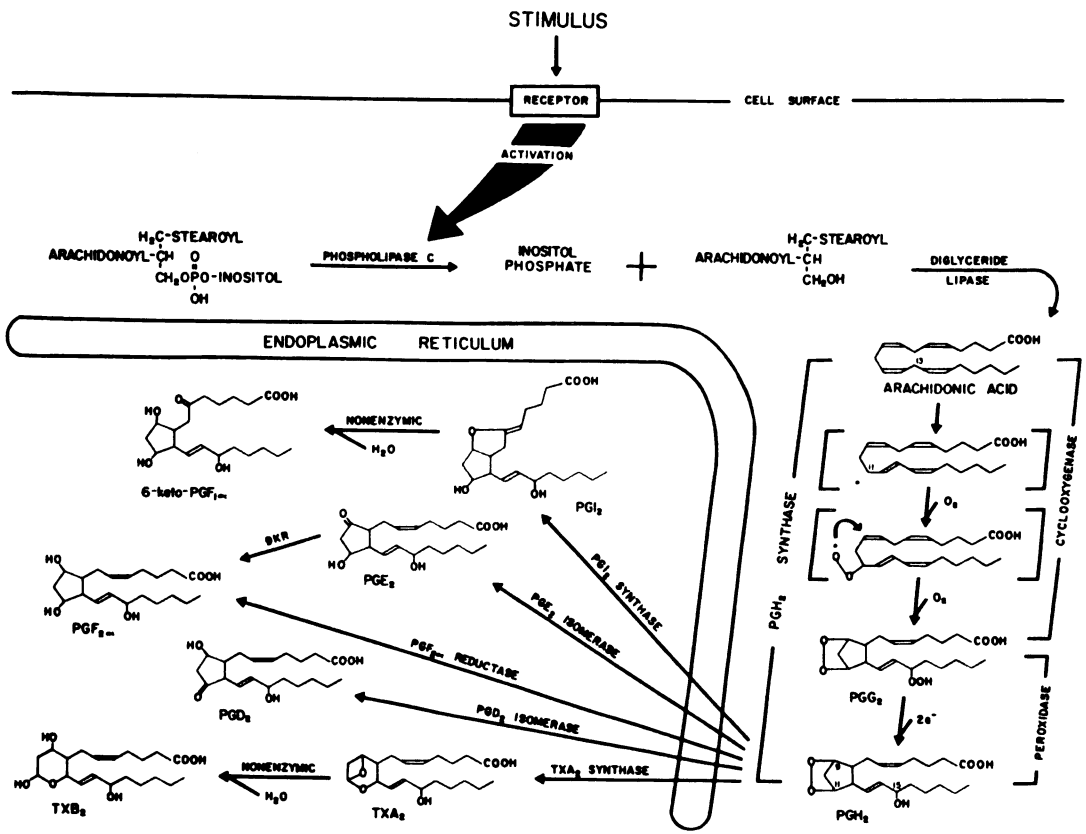
Our interest in investigating prostaglandin metabolism in isolated collecting tubule cells was based on two observations. The first was that immunohistochemical localization of the prostaglandin-forming enyzme, cyclooxygenase, demonstrated that the collecting tubule cell was the only tubular cell type that stained for the enzyme (52). The second was Grantham's observation that PGE_1 inhibits water resorption in kidney (61). Taken together, these data suggested that prostaglandins synthesized by the collecting tubule may be closely involved with kidney function. In order to test our hypothesis biochemically, we chose to isolate a population of collecting tubule cells from the rabbit kidney and to study prostaglandin metabolism in these cells.

LITERATURE REVIEW

Prostaglandin Metabolism. The pathways for the biosynthesis of various prostaglandins are illustrated in Fig. 1. The first step in this "arachidonate cascade" is the liberation of arachidonic acid from an esterified precursor in response to an exogenous stimulus. The precursor is most commonly a phospholipid. Triglycerides, even in triglyceride-rich cells such as renal medullary interstitial cells, are apparently not a primary source of arachidonic acid (1).

The release of arachidonic acid from phospholipids may occur via two different mechanisms. The first pathway involves the straightforward action of a phospholipase A_2 on a 2-arachidonyl-phosphoglyceride to yield free arachidonic acid. Bills et al. have shown that platelets contain a phospholipase A_2 activity which will preferentially catalyze the release of fatty acids from phosphatidylcholine containing arachidonic acid at the 2-position (2,3). A second pathway for arachidonate release also exists in platelets. A cytosolic phospholipase C specific for phosphatidylinositol catalyzes the cleavage of phosphatidylinositol, yielding diglyceride and inositol phosphate (4,5,6) and a diglyceride lipase then catalyzes the release of arachidonic acid from the diglyceride. This phospholipase C-diglyceride lipase pathway provides for the specific release of arachidonic acid because approximately 90% of the phosphatidylinositol in platelets contains arachidonic acid esterified at the 2-position

Figure 1. Overview of prostaglandin biosynthesis.


STIMULUS
RECEPTOR
CELL SURFACE
ACTIVATION
PHOSPHOLIPASE C
INOSITOL PHOSPHATE
DIGLYCERIDE LIPASE
ENDOPLASMIC RETICULUM
ARACHIDONIC ACID
NONENZYMIC
6-keto-PGF1α
PGI2
PGE2
PGF2α
PGD2
TXB2
TXA2
PGH2
PGG2
SYNTHASE
CYCLOOXYGENASE
PEROXIDASE

(7,8). It is not known whether one or both of these pathways are usually operational in cells other than platelets. Hong et al. have recently shown that BALB/3T3 cells contain a phospholipase A_2 activity which is activated by bradykinin, thrombin and the calcium ionophore, A23187; these effectors caused arachidonic acid release via a phospholipase A_2 activity and not by activation of a phospholipase C (9).

Free arachidonic acid released from phospholipids is converted to the endoperoxide, PGG_2, by a cyclooxygenase which is part of the PGH synthase. The reaction mechanism involves the abstraction of the L-hydrogen atom from carbon 13 of arachidonic acid, the addition of one molecule of oxygen at carbon 11 and, after a series of intramolecular rearrangements, the addition of a second molecule of oxygen at carbon 15 (10,11,12,13,14). PGG_2 is converted to another endoperoxide, PGH_2, by the peroxidase function of the PGH synthase which converts the 15-hydroperoxy prostaglandin to a 15-hydroxy prostaglandin. The half-lives of PGH_2 and PGG_2 in aqueous solutions at pH 7.5 are approximately 5 min (15).

PGH synthase (also called cyclooxygenase) was the first of the prostaglandin biosynthetic enzymes to be extensively characterized (16,17). It has been purified from sheep and bovine vesicular glands and is a dimer with a subunit molecular weight of 70,000 daltons (17). The cyclooxygenase activity of the enzyme requires an Fe^{2+} or Mn^{2+} containing porphyrin whereas the peroxidase activity is functional only with an Fe^{2+} containing porphyrin (18,19). The enzyme is located on the cytoplasmic side of the endoplasmic reticulum (20).

PGH_2 is normally converted to the biologically active prostaglandins, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane A_2). All these prostaglandins with the possible exception of PGD_2 are synthesized by the kidney. Enzymatic conversions to PGD_2 and PGE_2 are catalyzed by PGH-PGD and PGH-PGE isomerases (21,22,23,24) and conversions to PGI_2 and TxA_2 by PGI_2 and TxA_2 synthases (25,26). Presently, it is unknown if $PGF_{2\alpha}$ can be formed enzymatically directly from PGH_2; a 9-keto-PGE_2-reductase can convert PGE_2 to $PGF_{2\alpha}$ in some cells and this enzyme is present in the kidney (27). PGD_2, PGE_2 and $PGF_{2\alpha}$ can also be formed at significant rates non-enzymatically from PGH_2. Of the above enzymes only PGD_2 isomerase has been purified to homogeneity. The spleen enzyme is cytosolic, has a molecular weight of 30,000 daltons and requires glutathione (23). The brain enzyme is also cytosolic, but does not require glutathione and has a molecular weight of 85,000 (24).

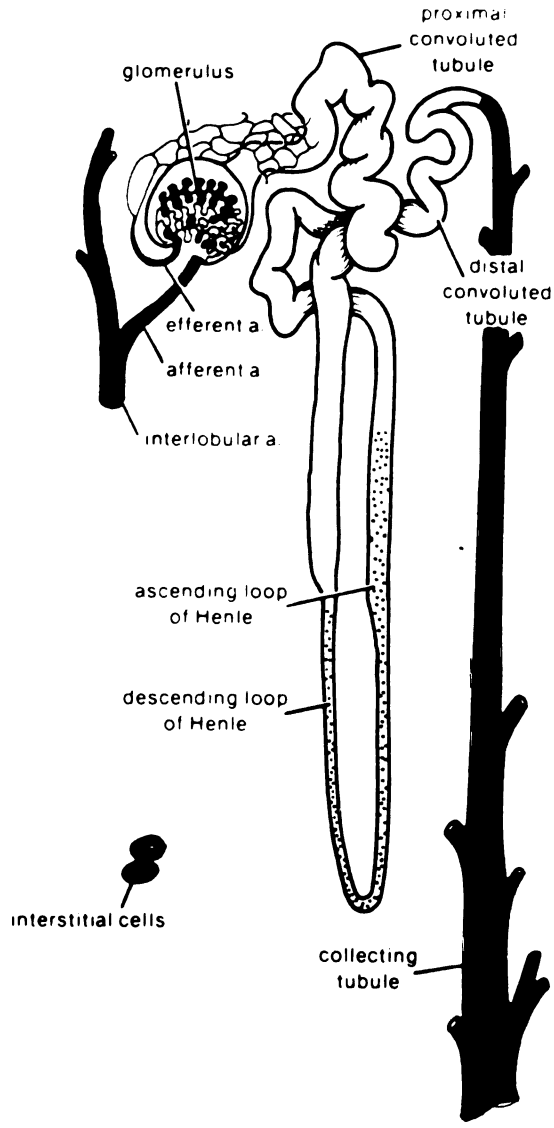
The catabolism of biologically active prostaglandins to inactive prostaglandins can occur either enzymatically or non-enzymatically. TxA_2 and PGI_2 are rapidly converted non-enzymatically to TxB_2 and 6-keto-$PGF_{1\alpha}$ (28,29). The half-lives of TxA_2 and PGI_2 in aqueous solution at pH 7.5 are approximately 30 sec and 5 min, respectively (30,31). PGI_2, PGD_2, PGE_2 and $PGF_{2\alpha}$ can be enzymatically inactivated by 15-hydroxy-prostaglandin dehydrogenase, which convert active 15-hydroxy-prostaglandins to inactive 15-keto-prostaglandins (32). 15-keto-prostaglandins can be further modified by 15-keto-prostaglandin Δ^{13} reductase (which reduces the 13,14 double bond) and 9-hydroxy-prostaglandin reductase (33,34). All of these catabolic enzymes are found in the mammalian kidney, but only

the NAD^+-dependent 15-hydroxy prostaglandin dehydrogenase has been studied in much detail. This enzyme is a soluble protein and is found principally in the kidney cortex in both the nephron and the vasculature (32,33,36). Outside the kidney this enzyme is found in large amounts in the lung and active prostaglandins escaping the kidney via the circulation can be catabolized there. The 9-keto-PGE_2 reductase may also be considered catabolic in the kidney because it converts active PGE_2 to $PGF_{2\alpha}$ which is essentially inactive in the kidney.

In the kidney, another type of functional inactivation may occur by sequestering biologically active prostaglandins in the lumen of the nephron. PGE_2, for example, is a potent natriuretic substance at the serosal surface of the collecting tubule, but is inactive at the lumenal surface (37). Prostaglandins are secreted and reabsorbed along much of the nephron, but have been shown to enter the nephron primarily at the level of the loop of Henle.

Kidney Function. The mammalian kidney has two primary functions. They are: a) to remove waste products, particularly those from protein metabolism, from the blood and b) to help regulate fluid and electrolyte balance in the body (39). The nephron is the excretory tubule which is responsible for the exchange of water and solutes from the glomerular filtrate and the passage of this fluid onto the collecting tubule at the distal end of the nephron (Fig. 2). From the collecting tubules, the filtrate is passed to the ureter and is evacuated as urine. Epithelial cells of the renal tubule change the composition of the urine through a combination of secretion and resorption.

Figure 2. Schematic representation of the functional unit of the kidney and the sites of prostaglandin synthesis in the kidney (shaded segments).


proximal convoluted tubule
glomerulus
distal convoluted tubule
efferent a
afferent a
interlobular a.
ascending loop of Henle
descending loop of Henle
interstitial cells
collecting tubule

The principal function of the collecting tubule is to accept hypo- or iso-osmotic filtrate from the distal convoluted tubule and to concentrate it under stimulation by the anti-diuretic hormone, arginine-vasopressin (AVP) (40). AVP is synthesized in the hypothalamus and is stored in granules in the posterior pituitary (41). Release of AVP into the vasculature is brought about by changes in the osmolality of the blood. Highly sensitive osmoreceptors in the hypothalamic nuclei can respond to as little as a 2% change in the osmotic pressure of the blood. The manipulation of the concentration of urine occurs by changing the Na^+ and water permeability of the collecting tubule lumenal membrane. Urine can be concentrated by passive removal of water down a concentration gradient between the relatively hypoosmotic urine and the hyperosmotic interstitium (40).

The binding of vasopressin to kidney plasma membranes has been examined in detail by Jard (43). The plasma membrane from pig kidney contains a lysine-vasopressin sensitive adenylate cyclase activity which is activated by vasopressin (half-maximal activation at 5×10^{-9} M) (43,44). Collecting tubule segments also have an adenylate cyclase activity which stimulated by arginine-vasopressin (45). The link between cAMP and increased permeability of the collecting tubule lumenal membrane is not presently understood, but probably involves a phosphorylation-dephosphorylation cycle(s) which alter membrane permeability (46).

Localization of Prostaglandin Synthesis in the Kidney. Lee et al. were the first to report that the renal medulla contained

prostaglandin-like material (47). Crowshaw later showed that prostaglandin biosynthesis was greater in the renal medulla than the renal cortex (48). Using sucrose density gradient centrifugation to fractionate kidney homogenates, Anggard et al. showed biosynthesis to be located primarily in the microsomal fractional of renal medullary cells (49). Because prostaglandins generally act near their sites of synthesis, defining the specific cellular sites of synthesis within the kidney can provide insights into prostaglandin function (50). Nutgeren et al. showed that medullary interstitial cells and medullary collecting tubule cells could synthesize prostaglandins (51). The histochemical assay used, however, involved staining for arachidonic acid-dependent peroxidase activity with 3,3'-diaminobenzidine and was both indirect and nonspecific.

Using a rabbit antibody raised against the rate-limiting enzyme of prostaglandin synthesis, cyclooxygenase, Smith et al. localized prostaglandin biosynthesis by immunohistochemistry (Fig. 2) (52). The enzyme was found in medullary and cortical collecting tubule cells, medullary interstitial cells and endothelial cells lining all arteries and arterioles in the rabbit, rat, sheep, cow and guinea pig kidneys. The enzyme was also detected in the parietal layer cells of Bowman's capsule in the rabbit and the mesangial cells in ovine and bovine glomeruli. In the hydronephrotic kidney, the enzyme was found in the thin limb of Henle cells (53). While this immunohistochemical technique does not eliminate the possibility that cyclooxygenase is present in cells other than those listed above, it does suggest that if the enzyme is present in other cells it probably exists at low concentrations.

Cyclooxygenase, for instance, is known to be present in vascular smooth muscle cells (54) which were not detected by this technique.

The particular prostaglandins synthesized by a given cell type can not be determined from the cyclooxygenase localization. One can use either of two approaches to determine which cells form which prostaglandins. The first is to isolate individual cell types from the kidney and to characterize the prostaglandins synthesized by these cells. The second is to raise antibodies against the enzymes that catalyze the formation of the active prostaglandins and to localize these enzymes immunohistochemically. Historically, the obstacle to this second approach has been that only the PGD_2 isomerase has been purified to homogeneity and thus only this enzyme would be suitable for raising antibodies using traditional immunological techniques. Recently, however, DeWitt et al. have been able to raise monoclonal antibodies against both PGI_2 synthase and TxA_2 synthase using hybridoma technology even though only impure enzyme preparations of these synthases were available (55). In the kidney, PGI_2 synthase was found in both vascular endothelial cells and smooth muscle cells and in a specialized type of interstitial cell found in the medullary interstitium (55). As with the localization of cyclooxygenase, this technique may not be sensitive enough to localize all the PGI_2 synthase containing cells in the kidney. TxA_2 synthase localization has just been begun.

Prostaglandin Function in the Kidney. Arterial blood entering the kidney contains prostaglandins at concentrations substantially below those known to cause most physiological effects in the kidney (e.g. < 50

pM for PGE_2 (56)). It is likely, therefore, that the effects of prostaglandins in the kidney are caused entirely by prostaglandins synthesized intrarenally. Prostaglandins synthesized in the kidney are known to be involved in controlling renal blood flow, the renin-angiotensin system, natriuresis, hypertension and water resorption (47, 58). Thus, prostaglandins synthesized in glomerular arterioles can affect renin release in juxtaglomerular cells (59,60), prostaglandins formed in the renal vasculature can attenuate decreases in renal blood flow caused by vasoconstrictors (57) and prostaglandins formed in the collecting tubule and possibly the renal medullary interstitial cells can regulate Na^+ and water transport in the collecting tubule (37,52,61). The particular prostaglandin which causes each of these effects has not been determined conclusively.

The molecular basis by which prostaglandins exert their effects have been carefully evaluated in only a few systems, none of which are in the kidney. Inhibition of platelet aggregation by PGI_2, PGE_1 and PGD_2 involves the interaction of these prostaglandins with specific receptors which ultimately affect adenylate cyclase activity in the platelet (62,63,64). PGE_2 may also function independently of a cAMP-mediated mechanism in WI-38 fibroblasts and S49 lymphoma cells in which amino acid and Mg^{2+} transport are affected, respectively (65,66). $PGF_{2\alpha}$ exerts its effect on the corpus luteum independently of a cAMP-mediated mechanism as well (67).

Prostaglandins and Water Resorption. Evidence for an antagonism between AVP-stimulated water resorption and prostaglandins has come from several sources. In hypophysectomized dogs pretreated with the

cyclooxygenase inhibitor, indomethacin, AVP caused a greater increase in urine osmolality than in untreated animals (68,69,70). Indomethacin pretreatment also potentiated AVP-stimulated cAMP synthesis suggesting that prostaglandins may block water resorption by inhibiting cAMP synthesis (70). Using perfused segments of rabbit cortical collecting tubules, Grantham et al. showed that AVP-stimulated water resorption is inhibited by 50% using 10^{-9}M PGE_1 (61). The phosphodiesterase inhibitor, theophylline, also caused water resorption, but PGE_1 potentiated this effect. PGE_1 alone caused a small increase in water resorption. These results suggested that PGE_1, like AVP, can cause cAMP synthesis in cortical collecting tubules which then leads to water resorption. Grantham postulated, however, that PGE_1 and AVP also compete for a common receptor which is linked to adenylate cyclase and that PGE_1 inhibits AVP-stimulated water resorption when both are present simultaneously.

Direct evidence for an inhibition of cAMP synthesis by prostaglandins, however, has not been clear cut. Edelman found that 10^{-7}M PGE_1 inhibited AVP-stimulated adenylate cyclase activity in crude homogenates of hamster papillae (71). Both Dousa and Torikai found, however, that 10^{-5}M PGE_2 had no effect on AVP-stimulated adenylate cyclase in isolated rat medullary collecting tubules (72,73). Using rat papillary collecting tubules, Dousa also found no inhibition of adenylate cyclase by PGE_2, but did observe an inhibition of total AVP-stimulated cAMP formation by 10^{-5}M PGE_2 when intact tubules were assayed (74). No inhibition was observed at concentrations below 10^{-5}M PGE_2. Neither PGE_1 nor PGE_2 stimulate cAMP synthesis at concentrations below 10^{-7}M in any renal test system.

The data on the interaction between AVP and PGE are conflicting. In the study by Grantham on isolated cortical collecting tubules, PGE_1 inhibits water resorption at 10^{-9} M which is one order of magnitude below the lowest concentration of PGE_1 or PGE_2 shown to inhibit cAMP formation in any other system. The potentiation of theophylline-stimulated water resorption by 10^{-9} M PGE_1 also suggests that PGE_1 can independently stimulate cAMP synthesis in the isolated tubule at a concentration two orders of magnitude below that reported in other systems. While there is little doubt that PGE_2 and PGE_1 can inhibit AVP-stimulated water resorption in the collecting tubule, there is still some question as to the concentrations at which PGE_2 is effective physiologically and whether a cAMP-independent mechanism of PGE_2 inhibition may also be important. Moreover, the importance of PGE_1 is doubtful because PGE_1 is probably not formed by the kidney in vivo.

The simplest model reported to explain the relationship between AVP and PGE_2 suggests that AVP simultaneously stimulates cAMP and PGE_2 synthesis in the collecting tubule. PGE_2 then serves as a feedback inhibitor of water resorption. The possible role of PGE_2 alone on water resorption is not known. The importance of other prostaglandins synthesized by the kidney, $PGF_{2\alpha}$, TxA_2, PGD_2 and PGI_2 on water resorption also remains to be investigated.

The Kallikrein-Kinin System and Prostaglandins. Kallikrein is a proteolytic enzyme which is widely distributed in body tissues and fluids, including the blood (41). Lysyl-bradykinin (kallidin) is formed intrarenally from kininogen by the action of kallikrein (75). The

source of kininogen for renal kinin synthesis, however, is presently unknown (75,76). Lysyl-bradykinin can be converted to bradykinin in the kidney by an aminopeptidase (76). Kininases are the enzymes which render these kinins biologically inactive. The most abundant renal kininase is probably the peptidyl dipeptidase, kininase II (77). Inhibition of kininase II with specific inhibitors results in substantially higher intrarenal levels of kinins suggesting that kininases are of major importance in regulating renal kinin levels (78).

Renal kallikrein is significantly different from plasma kallikrein and urinary kallikrein is generally believed to be a reflection of intrarenal kallikrein synthesis and not due to filtration of plasma kallikrein (78). Carretero et al. have shown that the inner cortex of the kidney contains the highest levels of kallikrein and Ward et al. have shown that kallikrein is associated with the plasma membrane fraction (79,80). Stopped-flow techniques have been used to localize kallikrein secretion to the level of the distal tubule (81). Release of kallikrein into the tubular fluid is well documented (82), but it is not known if release into the vasculature and interstitium also occurs. Due to the distal location of kallikrein secretion it has been proposed that kallikrein exerts its tubular effects primarily at the distal and collecting tubules (83).

Kallikrein and thus kinins are known to be related to sodium and water excretion by the kidney (84). For example, Mills et al. have reported that sodium excretion and kallikrein excretion are closely correlated in rabbits and rats (85). Decreased kallikrein excretion occurs in essential hypertension suggesting that retention of sodium

during hypertension may be partially regulated by kallikrein (86). Aldosterone, an adrenal cortical hormone which stimulates sodium resorption by the kidney, also causes kallikrein excretion (87). Kallikrein may thus serve to oppose the anti-natriuretic effect of aldosterone *in vivo*.

Consistent with the effect of kallikrein on sodium excretion, bradykinin infusion caused natriuresis in both the perfused dog kidney and the anesthesized dog (88,89,105). McGiff *et al*. were the first to show that intrarenal arterial infusion of bradykinin also caused the release of a PGE-like compound in the dog kidney (90). It has since been demonstrated that $PGF_{2\alpha}$ and PGI_2 are also released from the bradykinin-stimulated dog kidney (91,92). Sites within the kidney which are known to synthesize prostaglandins in response to bradykinin are the renal vasculature, the medullary interstitial cells and the papillary collecting tubule (1,93,94,95).

The importance of prostaglandins in mediating bradykinin-induced natriuresis has not been clear cut. In the perfused dog kidney, indomethacin did not inhibit bradykinin-induced natriuresis (96) while in the anesthesized dog, the prostaglandin synthesis inhibitor, meclofenamate, inhibited bradykinin-induced natriuresis (88). This discrepancy is typical of much of the data collected on the effect of prostaglandins on sodium transport in the kidney.

Large intravenous doses of PGE_2 have been shown to be natriuretic in both man and animals (97,98,99). In other work, cyclooxygenase inhibitors were found to increase, decrease or have no effect on sodium excretion in the conscious dog (100,101,102,103). In studies with *nephron* suspensions, renal slices and the perfused rabbit nephron, PGE

and PGF did not effect sodium transport (56,104). It was suggested therefore that prostaglandins caused natriuresis by causing changes in renal hemodynamics and not by direct tubular effects. Stokes and Kokko have found, however, that peritubular PGE_2 caused a natriuresis in isolated perfused cortical and medullary collecting tubules (37). Overall, while kinins can cause natriuresis and prostaglandin synthesis in the kidney, the significance of renal prostaglandin synthesis in relation to sodium excretion by the kidney remains unresolved.

Another function of bradykinin in the kidney that is apparently mediated, at least in part, by prostaglandins is bradykinin-induced diuresis. On intrarenal arterial infusion bradykinin caused diuresis in the dog and indomethacin pretreatment inhibited this diuresis (96). Moreover, bradykinin-induced diuresis could not be overcome by the simultaneous infusion of AVP (105,106). These data have been interpreted as meaning that bradykinin-induced prostaglandin synthesis may enhance diuresis by causing an antagonism of AVP-stimulated water resorption.

CHAPTER II

ISOLATION OF RENAL PAPILLARY COLLECTING TUBULE (RPCT) CELLS: CHARACTERIZATION OF THE PROSTAGLANDIN PRODUCTS FORMED BY CELL HOMOGENATES

Prostaglandins are formed by 4-5 different cells types in the kidney: medullary interstitial cells, endothelial cells of arteries and arterioles, glomerular epithelial and mesangial cells and both cortical and medullary collecting tubule cells. Homogenous populations of both renal medullary interstitial cells and vascular endothelial cells have been prepared and studies with these isolated cells have provided important insights into prostaglandin metabolism by these cells. Parallel studies with collecting tubules could lead to a further understanding of the function of prostaglandins in the kidney. In chapter I, a method is described for isolating collecting tubule cells and the characterization of the prostaglandin products formed by homogenates of these cells.

MATERIALS AND METHODS

Materials. Samples of PGD_2, PGE_2, $PGF_{2\alpha}$, TxB_2, 6-keto-$PGF_{1\alpha}$ and Flurbiprofen (dl-2-(fluoro-4-biphenylyl) propionic acid) were generous gifts of Drs. John Pike and Udo Axen of the Upjohn Company, Kalamazoo, Michigan. Unlabeled arachidonic acid was obtained from Nu-Chek Prep, Inc., Elysian, Minnesota and [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (60-100 Ci/mmol), L-[4,5-^{3}H(N)]-leucine (40-60 Ci/mmol) and D-[^{14}C(U)]-glucose (150-250 mCi/mmol) were purchased from New England Nuclear Corporation Boston, Massachusetts. Trypsin (1/250), Dulbecco's modified Eagle media, fetal calf serum, antibiotic-antimycotic (100x) and glutamine were purchased from Grand Island Biological Company. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was obtained from Miles Laboratories, Inc. Rabbit anti-cyclooxygenase serum and preimmune serum were prepared as described previously (52). Silica gel G was purchased from Merck. Nitroblue tetrazolium, NADH, bovine hemoglobin and EDTA were purchased from Sigma Chemical Company. Type CLS collagenase was from Worthington Biochemical Corporation. All other chemicals were reagent grade and were obtained from common commercial sources.

Isolation of renal papillary collecting tubule (RPCT) cells. New Zealand white rabbits (2-4 kg) were sacrificed by intravenous injections of lethal doses of Nembutal (0.5%) and the kidneys removed and placed in

phosphate-buffered saline (PBS). The papillae (0.7 g) were dissected from each kidney, transferred to Krebs-Ringer buffer (less Ca^{2+} and Mg^{2+} salts), pH 7.5, and minced finely into sections of 0.3-0.5 cm^3 with a razor blade at room temperature. The minced tissue was placed in 3 ml of Krebs-Ringer buffer, pH 7.5, containing 0.5 mg of trypsin per ml. The tissue was drawn up and down a disposable pipet (3 mm bore) 15-20 times per min for 5 min and filtered through a stainless steel mesh (0.25 mm^2 pore size). The initial filtrate normally contained 5-8 x 10^6 cells of which 95-99% were small non- collecting tubule cells. The remaining residue was removed from the filter and resuspended in 3 ml Krebs-Ringer buffer and again drawn up and down a disposable pipet 15-20 times per min (1 mm bore) until the tissue was completely dispersed (5 min). The trypsinized tissue was again filtered and the filtrate (containing 1-2 x 10^6 cells of which up to 40-80% were non-collecting tubule cells) diluted 1:2 with H_2O and allowed to stand for 4-5 min. Reducing the osmolity caused the lysis of all small cells. Krebs-Ringer buffer (3-4 ml) was then added to the suspension and the collecting tubule cells were collected by centrifugation at 500 x g for 2 min. The cell pellet was washed twice by resuspension and centrifugation in Krebs-Ringer buffer, pH 7.5. The final cell pellet was dispersed in Krebs-Ringer or another buffer and counted with a hemacytometer following staining with either trypan blue or erythrosine red at a final concentration of 0.04% (w/v) (107). The entire procedure normally required 90 min to complete. The overall purity of the cells isolated was judged to be greater than 97%.

[^{3}H]-leucine metabolism. Suspensions of RPCT cells were divided into 5 vials so that each contained 1-4 x 10^5 cells in 1 ml of Krebs-Ringer buffer, pH 7.5, containing a 1:50 dilution of antibiotic-antimycotic. Controls included 1 ml of buffer without cells and 1 ml of the cell suspension to which cycloheximide (1 µg/ml) was added. [^{3}H]-Leucine (2-4 µCi) was added to each vial, and the cells were incubated at 37°C under a 10% CO_2 atmosphere for different time intervals. To stop further metabolism of [^{3}H]-leucine, 100 µmoles of unlabeled leucine and 1 mg of bovine serum albumin were added to each vial followed immediately by 5 ml of ice cold 10% trichloroacetic acid. Each vial was left at 4°C for 40 minutes and centrifuged to pellet the protein. The supernatant was removed and the pellet washed with 30 ml of ice-cold 10% trichloroacetic acid on a Whatman GF/C glass fiber filter. The filter was dried and counted in 6 ml of Bray's scintillation fluid (108).

[^{14}C]-glucose metabolism. RPCT cells were isolated using a Ca^{2+} Mg^{2+}, HCO_3^- and glucose-free Krebs-Ringer buffer, pH 7.5. The final cell pellet was resuspended in Krebs-Ringer buffer lacking only HCO_3 and glucose. The cells were divided into 5 vials each containing 1-4 x 10^5 cells in 1 ml of buffer containing a 1:50 dilution of antibiotic-antimycotic. Two controls were performed, one without cells; the other, in which cells were incubated in the presence of NaN_3 (0.02%). [^{14}C]-Glucose (5 µCi) was added to each vial and the vials were sealed and incubated at 37°C. Glucose oxidation was measured as $^{14}CO_2$ trapped as $H^{14}CO_3^-$ on KOH-soaked filter paper which was placed in a plastic ladle above the media. After

different incubation times, 0.5 ml of 10% trichloroacetic acid was added to each vial and one hr later the filter paper was removed and counted in 6 ml of Bray's scintillation fluid.

NADH-diaphorase and anti-cyclooxygenase immunohistochemical staining. Staining was performed on cells adhered to coverslips. A few drops of a RPCT cell suspension (5 x 10^5 cells per ml of Dulbecco's modified Eagle media containing 10% fetal calf serum, a 1:50 dilution of antibiotic-antimycotic and 2 mM glutamine) were placed on each coverslip, and the samples incubated at 37°C under a 10% CO_2 atmosphere for 6-8 hr at which time approximately 40% of the cells had adhered. Coverslips with cells attached were washed with PBS, frozen in isopentane (-60°C) and then dried in a desiccator under water aspiration for 1 hr. The adhered RPCT cells were stained for NADH-diaphorase activity according to the procedure of Farber et al. (109). Immunohistochemical staining for the prostaglandin-forming cycooxygenase was also performed on cells quick frozen, as above, except that coverslips to which cells were adhered were dipped into chloroform/methanol (2/1, v/v at -10°C for 4-5 seconds and immediately lyophilized. After drying for 1 hr the adhered RPCT cells were stained for cyclooxygenase antigenicity as described previously (52,110). Fluorescence and bright-field microscopy was performed using a Leitz Orthoplan microscope. Photomicrographs were obtained with an Orthomat Camera using Kodak TriX Pan film (ASA 400).

Prostaglandin synthesis and characterization. RPCT cells or the small cells obtained in the first filtrate were suspended in 2 ml of 0.1

M tris-chloride, pH 7.5 containing 1 mM phenol. Ovine hemoglobin (0.15mg) was added to each sample of cells. The cells were homogenized in a glass pestle homogenizer at 4°C for 1-1 1/2 minutes and then added to 1ml of buffer containing [^{3}H]-arachidonate diluted to varying specific activities with unlabeled acid. A sample to which Flurbiprofen (10^{-4}M) was added served as a control. The vials were incubated with shaking for 15 min at 37°C and the reactions terminated by adding 21 ml of chloroform/methanol (1/1, v/v). The protein was removed by centrifugation and 9 ml of chloroform and 4.5 ml of 0.05 M HCl were added to the supernatant. The chloroform phase was removed and dried under a stream of N_2. The residues were dissolved in 100 µl of chloroform/methanol (1/1, v/v) and applied to thin layers of silica gel G (0.3 mm). Chromatography was performed in 2-butanone/isopropyl ether/methylene chloride/benzene/HOAc (40/40/5/5/1)-solvent system A-for 1 1/2 hr and standards visualized with I_2 vapor. Regions containing the $PGF_{2\alpha}$/6-keto-$PGF_{1\alpha}$, PGE_2, PGD_2, and TxB_2 standards were scraped into scintillation vials and counted, or radioscanning was performed on a Berthold Varian Aerograph Radioscanner. Individual prostaglandin derivatives were further characterized as follows.

PGE_2 and PGD_2. Radioactivity chromatographing with PGE_2 and PGD_2 in solvent system A was eluted from the silica gel with chloroform/methanol (1/1, v/v) and rechromatographed both in hexane/ethyl acetate/HOAc (40/40/1)-solvent system B-and chloroform/methanol/HOAc/H_2O (90/9/1/0.65)-solvent system C on thin layers of silica gel G. In addition, aliquots of radioactive material from both the PGE_2 and PGD_2 regions of chromatograms developed in

solvent system A were reduced by treatment with 5 mg of $NaBH_4$ in 0.5 ml of methanol containing 10 mg each of carrier PGE_2 and PGD_2. After 40 min at room temperature, 6.5 ml of chloroform/methanol (1/1, v/v), 3 ml of chloroform and 1.5 ml of 0.05 N HCl were added to each reaction vial. The lipid layer was removed, dried under N_2, redissolved in 100 µl of chloroform/methanol (1/1) and chromatographed in solvent system B.

PGF_2 and 6-keto-$PGF_{1\alpha}$. Material chromatographing in the combined $PGF_{2\alpha}$/6-keto-$PGF_{1\alpha}$ region of thin layer plates developed in solvent system A was eluted and rechromatographed in solvent systems B and C to resolve these two derivatives. The $PGF_{2\alpha}$/6-keto-$PGF_{1\alpha}$ region from chromatography in solvent system A was also treated with $NaBH_4$, as described above, except that 10 µg of PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were used as carriers in each reaction vial. Another aqliquot of the $PGF_{2\alpha}$/6-keto-$PGF_{1\alpha}$ region from chromatography in solvent system A was methylated by treating with ethereal diazomethane for 15 minutes at room temperature. The ether was removed under a stream of N_2 and the residue dissolved in 100 µl of chloroform/methanol (1/1) and chromatographed in solvent system C.

TxB_2. Radioactivity migrating with TxB_2 during chromatography in solvent system A was rechromatographed in solvent systems B and C.

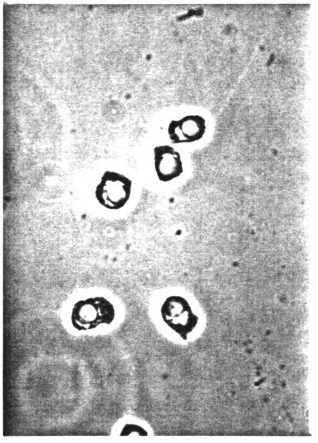
RESULTS AND DISCUSSION

Isolation and identification of collecting tubule cells. Rabbit renal papillae contain four major cell types. Three of these types, the medullary interstitial cells, the vascular endothelial cells and the epithelial cells comprising the thin segment of Henle's loop are small (diameter < 6.5μ) and thus can be distinguished by microscopic examination from the fourth cell type, the collecting tubule epithelial cell (RPCT), which has a diameter of > 10 μ. Dissociation of papillae by mincing and subsequent treatment with trypsin yielded mixtures of both large RPCT cells and contaminating small cell types. All small cells disappeared when the isotonic preparative media was diluted 1:2 with water and incubated for 3 min. Apparently, the small cells were preferentially lysed by this treatment while the number and morphology of the large cells was unaffected.

The size and shape of the large cells (Fig. 3) were similar to collecting tubule cells observed in histological sections of renal papillae (111). Two or three RPCT cells were often seen adhered along their long axis forming a slight arc. Strings of RPCT cells 10-15 cells in length were also observed.

Both the RPCT cells in histological sections and the isolated large cells stained positively for NADH-diaphorase activity. None of the small cells isolated were prominently stained for this enzyme. These observations are consistent with the distribution of NADH-diaphorase in

Figure 3. Phase contrast photomicrograph of trypsin-isolated RPCT cells in suspension. Magnification, x250.



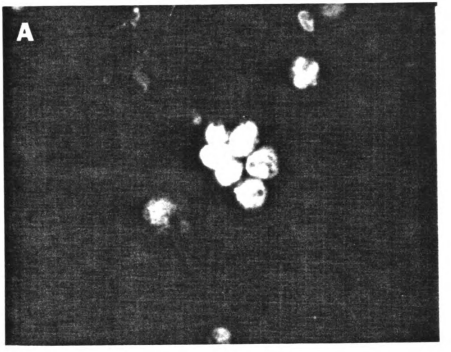
the renal medulla of the rabbit and serve to further confirm the identity of the isolated large cells as RPCT cells (109).

Isolated RPCT cells were subjected to immunohistofluorescence staining with rabbit anti-cyclooxygenase serum and rabbit pre-immune serum, respectively (Fig. 4). The RPCT cells stained much more intensely with the immune serum in agreement with staining seen in tissue sections (110,112).

Metabolic characterization of RPCT cells. The isolated RPCT cells excluded both trypan blue and erythrosine red dyes. A maximum of 10^6 RPCT cells were obtained per g of papillae by our method although the average yield was approximately half of that value. Figs. 5 and 6 illustrate the metabolism of [^{14}C]-glucose and [^{3}H]-leucine, respectively, by isolated cells. RPCT cells both oxidized [^{14}C]-glucose to $^{14}CO_2$ and incorporated [^{3}H]-leucine into trichloroacetic acid-precipitable material in a time-dependent fashion for about 2 h. Cells preincubated for 30 min with NaN_3 (0.02%) showed a 75-90% reduction in [^{14}C]-glucose oxidation. Incorporation of [^{3}H]-leucine into tricholoracetic acid-precipitable radioactivity was inhibited at least 80% when cells were preincubated with cycloheximide (1 µg/ml) for 30 min. In contrast, streptomycin, a prokaryotic protein synthesis inhibitor, at a concentration of 100 µg/ml did not block [^{3}H]-leucine incorporation.

Isolated RPCT cells adhered to both glass and plastic petri dishes. Approximately 40% of the isolated cells became attached within a few hours when incubated in Dulbecco's modified Eagle media supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotic-antimycotic.

Figure 4. Fluorescence photomicrographs of isolated RPCT cells treated with (A) anti-cyclooxygenase or (B) rabbit perimmune serumthen FITC-labeled goat anti-rabbit IgG. The rounding of the cells as compared to those in Fig. 3 was caused by chloroform/methanol fixation prior to staining. Magnification, x 250.


A

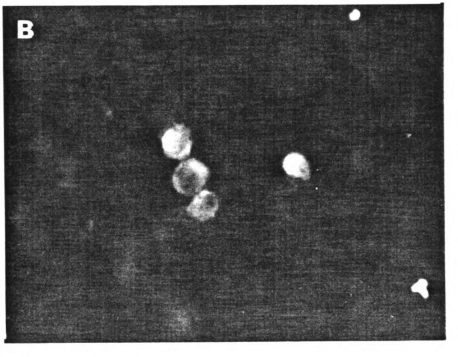

B

Figure 5. [^{14}C]-glucose oxidation by isolated RPCT cells. RPCT cells were incubated for the indicated times with [^{14}C]-glucose] and the formation of [^{14}C]-CO_2 measured as described in the text.

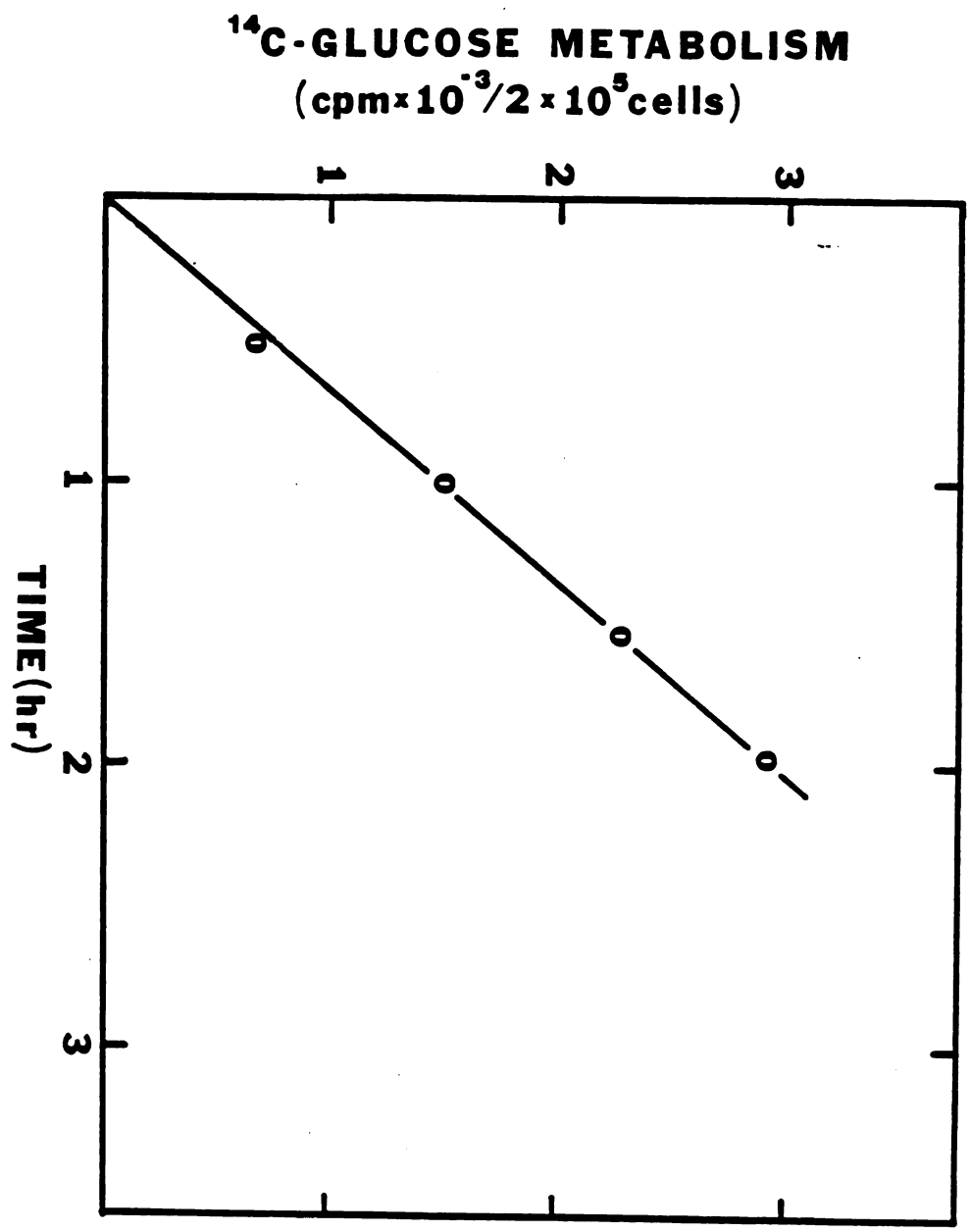
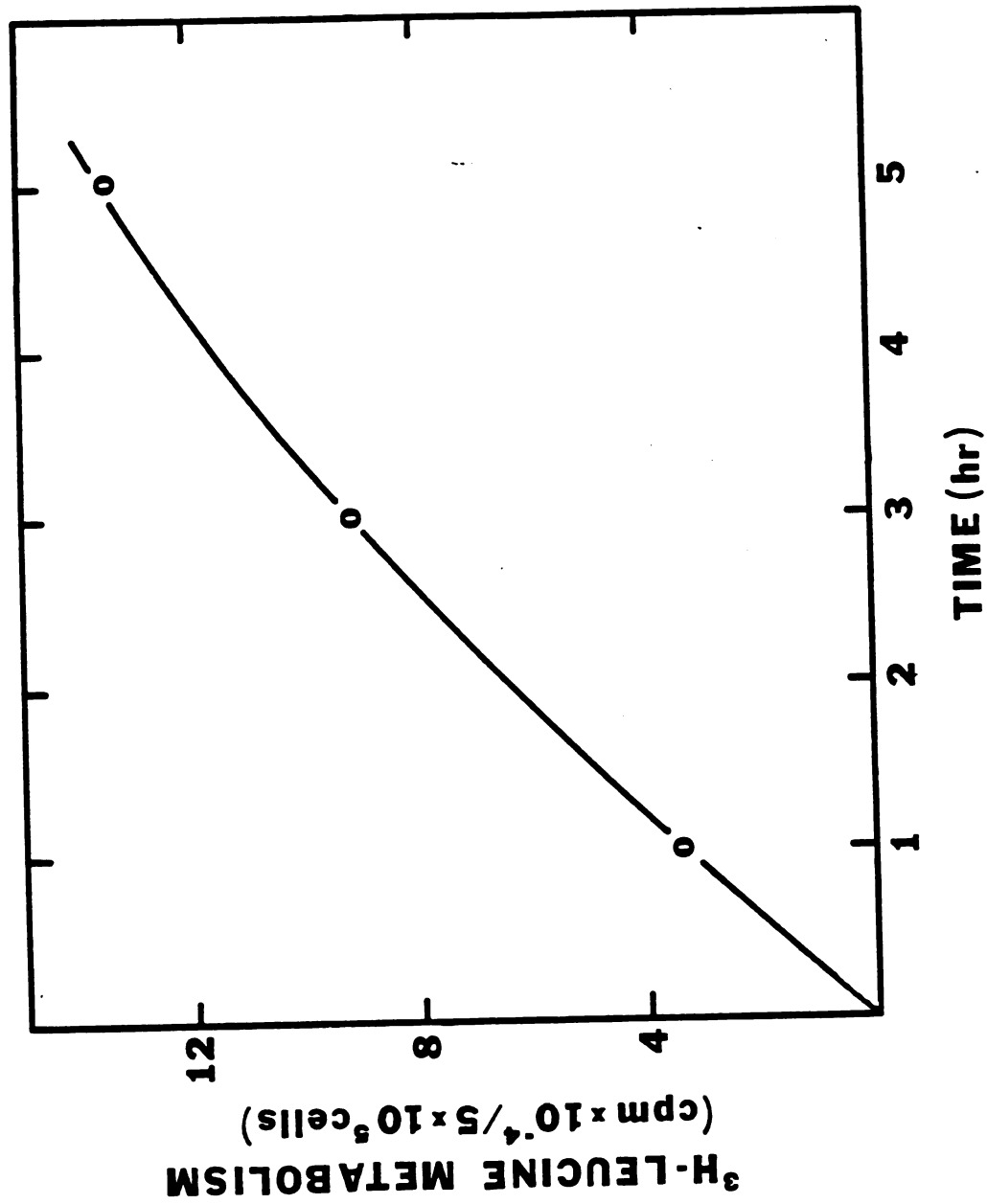

^{14}C-GLUCOSE METABOLISM
(cpm×10^{-3}/2×10^{5}cells)
1
2
3
TIME(hr)
1
2
3

Figure 6. [^{3}H]-leucine incorporation into trichloroacetic acid precipitable-products by isolated RPCT cells. RPCT cells were incubated for the indicated times with [^{3}H]-leucine and the formation of trichloroacetic acid precipitable-radioactivity measured as described in the text.


^{3}H-LEUCINE METABOLISM
(cpm x 10^{-4}/5 x 10^5cells)
12
8
4
TIME (hr)
1
2
3
4
5

The attached cells could be removed from these surfaces by treatment with trypsin (0.05% in PBS for 5 min) and subcultured on other petri dishes where they retained distinctive RPCT cell morphology and continued to exclude vital dyes. Subcultured RPCT cells when grown in a 10% CO_2 atmosphere still excluded vital dyes after 10-14 days during which time only the culture media was exchanged. No increase in cell number occurred during this time.

Attempts were also made to isolate RPCT cells following tissue dissociation in which EDTA (0.001-0.01%) was substituted for trypsin. EDTA treatment of minced papillae did provide relatively high yields of RPCT cells (6-10 x 10^6 cells per g papillae). The EDTA-isolated cells also had the same capacity as trypsin-isolated cells both to oxidize glucose and to convert exogenous leucine into protein. However, the EDTA-isolated cells were permeable to vital dyes, were lysed rapidly by treatment with trypsin and were unable to repair their permeability defect during a 48 hr incubation in culture media.

"Small" cell isolation. Relatively large numbers of small cells were obtained (5-8 x 10^6 cells per g of papillae) and were contaminated with only 1-5% CT cells. The small cells were spherical and approximately 6μ in diameter. None of these cells stained with vital dyes or for NADH-diaphorase activity. I made no attempt to distinguish between vascular endothelial cells, medullary interstitial cells and the epithelial cells of the thin loop of Henle.

Characterization of arachidonic acid metabolites formed by RPCT cells. Fig. 7 shows a radiochromatogram of the products synthesized

from [^{3}H]-arachidonic acid by CT cell homogenates. The major products synthesized by homogenates of both CT cells and small cells chromatographed with authentic PGD_2, PGE_2 and $PGF_{2\alpha}$ standards as expected (49,113). The identity of radioactivity chromatographing with PGD_2 and PGE_2 in solvent system A was verified by chromatography in solvent systems B and C and by reduction with $NaBH_4$ and chromatography in solvent system B. The two peaks of radioactivity seen in the $PGF_{2\alpha}$ region of the thin-layer plate (Fig. 7) were seldom resolved clearly. However, as described below, further examination indicated that 50% of the radioactivity migrating with $PGF_{2\alpha}$ was actually [^{3}H]-6-keto-$PGF_{1\alpha}$ while only 30% was found to be due to [^{3}H]-$PGF_{2\alpha}$. TxB_2 was not synthesized in significant quantities (<3%). When cell homogenates were incubated with [^{3}H]-arachidonic acid in the presence of Fluriprofen (10^{-4}M) only unreacted arachidonate was recovered indicating that all products were derived from the activity of the prostaglandin-forming cyclooxygenase and not a lipoxygenase. Thus, the small amount of radioactivity chromatographing in the monohydroxy acid region of the thin-layer plate (Fig. 7) is likely HHT and not a 20 carbon product (114). Data derived from incubations with five different RPCT homogenates are summarized in Table 1.

Material with R_f = 0.17-0.19 in solvent system A was eluted from the silica gel G with chloroform/methanol (1/1, v/v). The presence of both radioactive 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ was indicated by comparing the chromatographic properties of the eluted material with those of various other prostaglandin derivatives in solvent systems B and C (Table 2). Treatment of the radioactive material with ethereal diazomethane converted 40% of the radioactivity into a product which

Figure 7. A radiochromatogram of the products formed upon incubation of [^{3}H]-arachidonic acid (100 μM) with a RPCT cell homogenate. Products were separated by chromatography in solvent system A. Radioscanning was performed as described in the text. AA-arachidonic acid; RC-ricinoleic acid.

RADIOACTIVITY (^{3}H, cpm x 10^{-4})
0
2
4
6
0
5
10
15
DISTANCE FROM THE ORIGIN (cm)
$F_{2\alpha}$
6KF
E_2
TxB_2
D_2
RIA
AA

Table 1. Prostaglandin Products Formed From Arachidonic Acid by Cell Populations Isolated From Rabbit Renal Papillae[a]

	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	PGE_2	PGD_2	TxB_2
CT cell homogenates	9-20%	30-47%	13-28%	5-9%	1-2%
"Small" cell homogenates	12-13%	41-54%	17-26%	10-15%	2-3%

[a]Reactions were performed using [^{3}H]-arachidonic acid (0.02 μM). The results of 5 separate experiments are summarized.

Table 2. Characterization of 6-keto-$PGF_{1\alpha}$ (R_f Values)

	Metabolite	6-k-$PGF_{1\alpha}$[a]	PGE_2	PGD_2	$PGF_{2\alpha}$[a]
Solvent System A	0.17-0.19	0.29(100%)	0.28	0.41	0.17(100%)
Solvent System B	0.65-0.69	0.68(57%)	0.68	0.80	0.52(25%)
Solvent System C	0.23-0.26	0.25(62%)	0.24	0.35	0.15(28%)
Solvent System B ($NaBH_4$ treated)	0.65-0.69	0.68(51%)	0.52	0.51	0.52(35%)
Solvent System C (methyl esters)	0.40-0.43	0.41(40%)	0.41	0.51	0.24(36%)

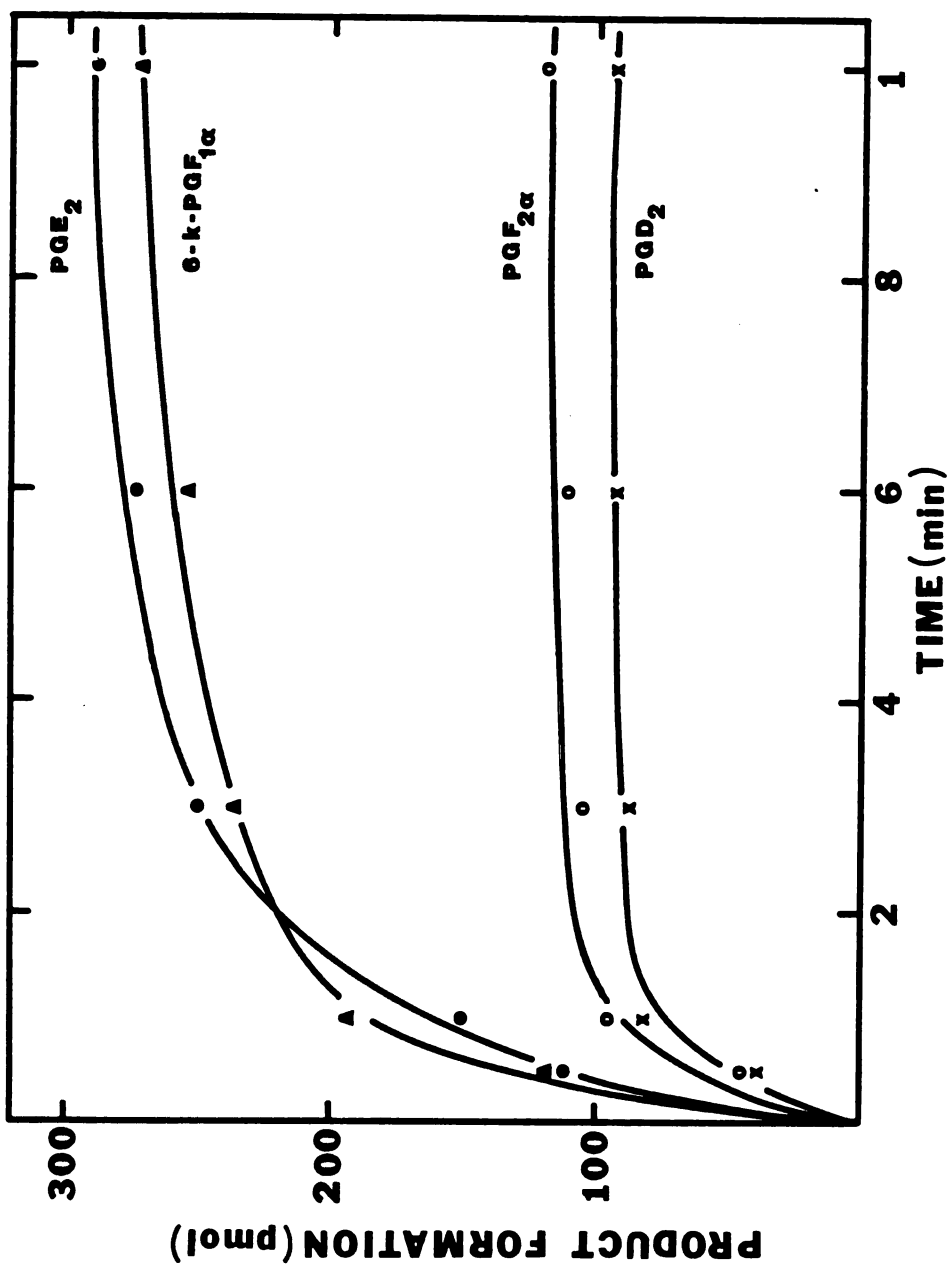
[a]Percentages in parentheses indicate the percentage of radioactivity with R_f = 0.17-0.19 in solvent system A which chromatographs with 6-keto-$PGF_{1\alpha}$ or $PGF_{2\alpha}$ in the subsequent solvent systems.

also chromatographed with the methyl ester prepared from 6-keto-$PGF_{1\alpha}$. $NaBH_4$ treatment of the radioactive metabolite and authentic 6-keto-$PGF_{1\alpha}$ failed to alter the chromatographic properties of either compound in agreement with the finding of Pace-Asciak (115), although PGD_2 and PGE_2 were reduced to PGF isomers by $NaBH_4$ (>90%) under these conditions.

We performed two control experiments which verified that 6-keto-$PGF_{1\alpha}$ formation was actually being catalyzed by the biosynthetic machinery originating from the intact RPCT cells and not by enzymes derived from cellular debris which might possibly have remained after hypotonic lysis of small papillary cells. In the first experiment, we examined the supernatant obtained from the final wash of RPCT cells, but found no prostaglandin biosynthetic activity. In the second experiment, we compared 6-keto-$PGF_{1\alpha}$ synthesis by homogenates of two different preparations of RPCT cells: one in which trypsin (0.5 mg/ml) was included and the other in which trypsin was excluded during the hypotonic lysis step. We reasoned that trypsin would inactivate any prostaglandin biosynthetic enzymes not sequestered within the intact plasma membrane. We found no differences in the amounts of the different radioactive products formed per cell by homogenates of RPCT cells which had been isolated by each of these two procedures.

Fig. 8 shows a time course for the production of various prostaglandin derivatives when isolated RPCT cell homogenates were incubated with 2.5 μM arachidonic acid. PGE_2 and 6-keto-$PGF_{1\alpha}$ were synthesized at initial rates of 150 pmol/min/10^6 cells. The reactions were complete within 10 min and prior to the complete

Figure 8. Time course for the biosynthesis of various prostaglandin products by a RPCT cell homogenate incubated with [^{3}H]-arachidonic acid (2.5 μM). Reactions were performed for the indicated times and the products analyzed as described in the text.

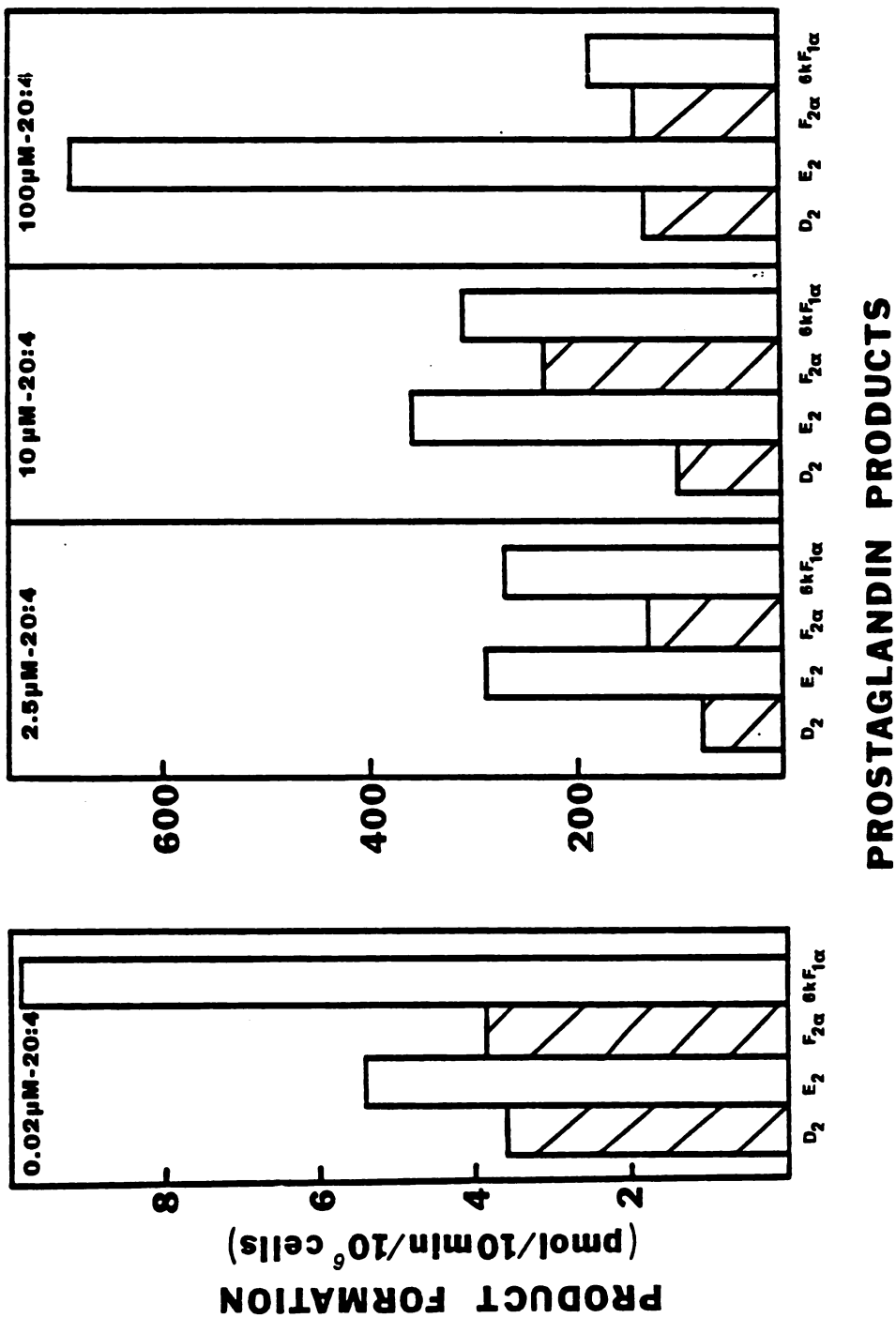

PGE2
6-k-PGF1α
PGF2α
PGD2
PRODUCT FORMATION (pmol)
300
200
100
2
4
6
8
1
TIME (min)

utilization of substrate, apparently reflecting the self-catalyzed destruction of the cyclooxygenase (116).

Fig. 9 compares the amounts of different prostaglandins synthesized at different initial concentrations of arachidonate. At concentrations of arachidonic acid of less than 2 μM, 6-keto-$PGF_{1\alpha}$ was the major product formed while at higher arachidonate concentrations, PGE_2 was the major product. Vane and coworkers (117) and Sun et al. (118) have made similar observations of the effect of substrate concentrations on the prostaglandin product distribution in other tissues. Apparently, the K_m of the PGI_2 synthase is much lower than that of the isomerase catalyzing PGE_2 formation. We suspect that the fall in overall 6-keto-$PGF_{1\alpha}$ production noted at high concentrations of arachidonic acid (e.g. 100 μM) may have resulted from the presence of small but inhibitory levels of hydroperoxide contaminants (119) in the substrate solution.

Although PGI_2 is known to be formed by the renal cortex (120,121), apparently by renal vascular endothelial cells (122), the renal medulla has previously been reported to form only the classical prostaglandin derivatives (1,49,113,123,124). Thus, it was somewhat surprising to find that isolated collecting tubule cells as well as populations enriched in medullary interstitial cells have the capacity to produce significant quantities of PGI_2. Since this work was reported, Dunn (124) and Silberbauer (125) have also reported that PGI_2 can be produced by the renal medulla.

Figure 9. Prostaglandin biosynthesis by RPCT cell homogenates at different initial concentrations of [^{3}H]-arachidonic acid. Incubations were performed for 15 min and the radioactive products measured as described in the text.


0.02μM-20:4
2.5μM-20:4
10μM-20:4
100μM-20:4
D2 E2 F2α 6kF1α
2
4
6
8
200
400
600
PRODUCT FORMATION
(pmol/10min/10^6 cells)
PROSTAGLANDIN PRODUCTS

CHAPTER III

KININ-INDUCED PROSTAGLANDIN SYNTHESIS BY RENAL PAPILLARY COLLECTING TUBULE CELLS IN CULTURE

In Chapter II, I presented a method for isolating RPCT cells following dissociation with trypsin and defined the prostaglandin biosynthetic capacity of these cells. In this chapter, I describe an improved isolation procedure for RPCT cells and the further characterization of these cells in terms of their morphology, enzyme histochemistry, responsiveness to hormones and growth in tissue culture. I also present the results of experiments designed to determine which prostaglandins are released by RPCT cells in culture and what hormonal factors stimulate the release of these prostaglandins.

MATERIALS AND METHODS

Materials. Trypsin (1/250), Dulbecco's modified Eagle media (DMEM), antibiotic-antimycotic (100x) and fetal bovine serum were all purchased from the Grand Island Biological Company. Collagenase (CLS II) was obtained from Worthington Biochemicals, Inc. Bradykinin triacetate, lysyl-bradykinin, bovine serum albumin, histamine, thrombin, glucagon, norepinephrine, isoproterenol, epinephrine, calcitonin, cholera toxin, arginine vasopressin (AVP), angiotensin II, NADPH, NADH, NADP, NAD and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Company. Flufenamic acid was from Aldrich Chemical Company. The Ca^{++} ionophore A23187 was obtained from Cal-Biochem. [^{3}H]PGD_2 (120 Ci/mmole), [^{3}H]arachidonic acid (130 Ci/mmole), [^{3}H]PGE_2 (130 Ci/mmole), [^{3}H]$PGE_{2\alpha}$ (120 Ci/mmole), [^{3}H]TxB_2 (150 Ci/mmole), [^{3}H]6-keto-$PGF_{1\alpha}$ (100 Ci/mmole), [^{3}H]L-leucine (60 Ci/mmole), [^{3}H]3',5'-cyclic AMP (31 Ci/mmole) and radioimmunoassay supplies for the 3',5'-cyclic AMP assay were all purchased from New England Nuclear Corp. Antisera against PGE_2 and $PGF_{2\alpha}$ were obtained from Miles Laboratories, Inc. Antisera against TxB_2 and 6-keto-$PGF_{1\alpha}$ were purchased from Immunalysis, Inc., Milton, MA. Prostaglandin standards were generously supplied by Dr. John Pike of the Upjohn Company. [^{3}H]PGH_2 was prepared essentially as described by Hamberg and Samuelsson (125). Dr. Roderick Walker of the University of Illinois, Chicago, graciously donated samples of desamino-arginine vasopressin

(dD-AVP) (126). Methionyl-lysyl-bradykinin was purchased from Chemical Dynamic Corp., South Plainfield, N.J. Parathormone (1-34 bovine parathyroid hormone (PTH), 6000 I.U./mg) was from Beckman Instruments. Glutaraldehyde, Araldite 502, Epon 812, OsO_4, 2,4,5-tri(dimethylamino-ethyl)-phenol and propylene oxide were from Electron Microscopy Sciences. Uranyl acetate was from Polysciences, Inc., Warrington, PA. MF cement, HAMK-024-12 filters and calf-skin collagen were obtained from Millipore Corp. All other chemicals were reagent grade or better and were purchased from common chemical sources.

Cell isolation. Collecting tubule cells were isolated under sterile conditions from renal papillae using a modification of the procedure described in Chapter II. The papillae were considered to be those portions of the medulla containing no thick ascending limbs. Papillae (0.5-0.8 g) were carefully dissected from rabbit kidneys, minced in a petri dish with a sterile razor blade and transferred to a plastic culture tube containing 4 ml of 0.1% collagenase in Krebs buffer (composition, in mM: 118 NaCl, 25 $NaHCO_3$, 14 glucose, 4.7 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$ and 1.8 KH_2PO_4), pH 7.3. The minced tissue was incubated for 1.5-2.5 hours at 37° under a 5% CO_2 atmosphere. The tissue was then gently agitated by drawing it up and down in a large bore 10 ml pipet 4-5 times. The cell suspension was filtered through a Gelman stainless steel mesh (Cat. No. 4320-1) and the filtrate diluted with 2 volumes of distilled H_2O to burst cells other than collecting tubule cells. The collecting tubule cells were pelleted by centrifugation on a tabletop clinical centrifuge. The cell pellet was resuspended in 10% bovine serum albumin in phosphate buffered saline

(PBS; composition in mM: 151 NaCl, 45 KH_2PO_4 and 2.5 NaOH), pH 7.2 and centrifuged again to pellet the cells. This latter step removed considerable amounts of debris from broken cells. The final cell pellet was resuspended in DMEM containing 10% fetal bovine serum and antibiotic-antimycotic (1/100) and an aliquot taken for counting. When using 24-well replicate culture dishes (16 mm, well diameter), cells were plated to a density of 6-9 x 10^4 cells per well and grown at 37° under a 10% CO_2 atmosphere.

Incubation of cells with effectors. Treatment of RPCT cells with effectors was done in triplicate using 24-well culture dishes. Cells were first rinsed free of media with Krebs buffer, pH 7.3, and then 0.6 ml of buffer containing an effector was added to the cells. The cells were incubated for the desired lengths of time at 37°C under a 5% CO_2 atmosphere. Typically, 0.02- 0.10 ml of buffer was removed from each well for radioimmunoassays. The remaining buffer was removed and discarded and 0.20 ml of 0.1% sodium dodecyl sulfate (SDS) added to each well. The sample was incubated at 37°C for 15 min and the solubilized protein assayed by the Lowry procedure (127) using bovine serum albumin as a standard.

When measuring the doubling time of cells, day 1 represented 18 hour old cells. On subsequent days, cells from 3 different wells were removed by treatment with PBS containing 0.03% EDTA, pH 7.3. The cells in one aliquot were counted with a hemacytometer, and the other aliquot was assayed for protein after the addition of 0.5 ml of 0.5% SDS. Media in all wells was changed daily. The effects of AVP and bradykinin on 3',5'-cyclic AMP and prostaglandin formation, respectively, were

determined prior to the treatment with PBS containing 0.03% EDTA, pH 7.3.

Prostaglandin radioimmunoassays. Immunoreactive prostaglandins, $iPGE_2$, $iPGF_{2\alpha}$, $iTxB_2$ and 6-keto-$PGF_{1\alpha}$ were measured with anti-PGE_2, anti- $PGF_{2\alpha}$, anti-TxB_2 and anti-6-keto-$PGF_{1\alpha}$ sera, respectively; $iPGD_2$ was measured using anti-TxB_2 sera with a cross-reactivity against PGD_2 of 2.5%. All the prostaglandin radioimmunoassays employed a single antibody to bind the prostaglandins and dextran T-70-coated charcoal (25 mg dextran, 250 mg charcoal, in 25 ml of PBS, pH 7.2 to precipitate any unbound prostaglandins). The sensitivities of the various assays were 10-500 pg for PGE_2 and $PGF_{2\alpha}$, 20-1,000 pg for 6-keto-$PGF_{1\alpha}$ and TxB_2 and 500-10,000 pg for PGD_2 when using the anti-TxB_2 sera. The cross-reactivities of the anti- sera with different prostaglandins are shown in Table 3.

The large amounts of $iPGE_2$ relative to other prostaglandins synthesized by RPCT cells allowed us to measure $iPGE_2$ directly using aliquots of incubation media without chromatographic separation from other products. The very low cross-reactivity of anti-$PGF_{2\alpha}$ sera with other prostaglandins also permitted measurements of $iPGF_{2\alpha}$ directly without isolating PGF_{α}. Because of the small amounts of 6-keto-$PGF_{1\alpha}$ formed and the cross reaction of PGE_2 with anti-6-keto-$PGF_{1\alpha}$ serum, it was necessary to purify 6-keto-$PGF_{1\alpha}$ prior to assay. To measure $iPGD_2$, PGD_2 was first purified by thin-layer chromatography in two solvent systems and then assayed using anti- TxB_2 serum. All the effectors used were checked to determine if they had an independent effect on the radioimmunoassays.

TABLE 3. Cross-reactivities of prostaglandin anti-sera with various prostaglandins.

Sera	[a]% Cross-reactivity				
	PGE_2	TxB_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	PGD_2
anti-PGE_2	100	<0.1	1.6	<0.1	1.5
anti-TxB_2	0.26	100	0.07	0.05	2.5
anti-$PGF_{2\alpha}$	<0.01	<0.01	100	<0.01	<0.01
anti-6-keto-$PGF_{1\alpha}$	2.1	<0.1	4.5	100	<0.3

[a]% Cross-reactivity was calculated after determining the amount of a prostaglandin derivative which would cause 50% displacement of the bound, radiolabeled parent hapten from the antibody (e.g. [^{3}H]PGE_2 from anti-PGE_2) relative to the amount of unlabeled parent antigen (e.g. PGE_2) needed to cause the same displacement.

When necessary, extraction of the incubation media and chromatographic separation of prostaglandins was performed as follows. Prostaglandins were extracted from 1 ml of Krebs buffer, pH 7.3, with 8 ml of chloroform/methanol (2/1; v/v) and 1.5 ml of 0.05 M HCl. The lipid layer was removed and the solvent evaporated under a stream of N_2 gas. The residue was dissolved in 0.10 ml of chloroform/methanol (1/1; v/v) and spotted on a silica gel G thin layer plate (Analtech). The prostaglandins were routinely resolved by chromatography in a solvent system containing isopropyl ether/2-butanone/benzene/methylene chloride/acetic acid (40/40/5/5/1; v/v/v/v/v). Regions of the sample with the same chromatographic mobilities as PGD_2 and 6-keto- $PGF_{1\alpha}$ standards were scraped from the plate and eluted from the silica gel with 5 ml of chloroform/methanol (1/1; v/v) and the solvent evaporated as above. Material extracted from the PGD_2 region of the first plate was spotted on a second silica gel G thin layer plate and subjected to chromatography in hexane/ethyl acetate/acetic acid (40/40/1; v/v/v) and the PGD_2 region scraped and eluted as above. Both 6-keto-$PGF_{1\alpha}$ and PGD_2 were redissolved in ethanol/PBS, pH 7.2 (1/4, v/v) and assayed. The recoveries of PGD_2 and 6-keto-$PGF_{1\alpha}$ throughout these procedures were monitored using [^{3}H]PGD_2 and [^{3}H]-6-keto-$PGF_{1\alpha}$, respectively, and averaged 65-70%.

3',5'-cyclic AMP radioimmunoassay. To quantitate extracellular 3',5'-cyclic AMP, samples were routinely assayed without purification of 3',5'-cyclic AMP by removing aliquots from incubation mixtures containing various effectors with RPCT cells. All effectors used were tested for potential interference with the radioimmunoassay.

To verify that it was 3',5'-cyclic AMP which was actually being measured, some samples were treated with phosphodiesterase: 0.05 ml of bovine phosphodiesterase (3 mg/ml) in 0.05 M tris-chloride, pH 7.7, was added to 0.1 ml aliquots removed from incubation mixtures in which 3',5'-cyclic AMP was generated and the sample incubated for 45 minutes at 37°. After destruction of phosphodiesterase, 3',5'-cyclic AMP levels were determined by radioimmunoassay and found to be reduced by more than 90%. Furthermore, in samples spiked with 5×10^5 dpm of [^{3}H] 3',5'-cyclic AMP and then treated with phosphodiesterase and extracted and chromatographed according to the procedures of Goldberg et al. (128), greater than 85% of the original tritium label was converted to products (ca. 85% 5'-AMP) with chromatographic mobilities different than 3',5'- cyclic AMP.

Measurements of intracellular 3',5'-cyclic AMP were performed as follows. At the appropriate times after addition of the effectors, the incubation buffer was aspirated. The cells were washed quickly with 1 ml of ice-cold PBS, pH 7.2, and then 350 µl of ice-cold 1% perchloric acid was added to each well. [^{3}H]3',5'-Cyclic AMP (3000 dpm) was added to each well and the samples incubated at 4° for 2.5 hr. The perchloric acid solution was then neutralized with 3 M KOH and centrifuged at 2000 x g for 10 min to remove particulate material. Supernatants derived from each sample were applied to columns of Dowex AG-1X8 (0.5 x 4.0 cm) equilibrated with 0.1 N formic acid; 3',5'-cyclic AMP was eluted with 5 ml of 2 N formic acid. The eluant was lyophilized and the residue redissolved in 250 µl of H_2O. An aliquot of this sample was used to determine the recovery of [^{3}H]3',5'-cyclic AMP (typically 60-70%), and the remainder used for radioimmunoassays.

Statistical Analyses. Experiments involved using 24-well culture dishes in which a minimum of 3 or 4 replicates and thus 6 or 8 treatments were analyzed for any effects. A completely random analysis of variance was used to test for differences between samples means at $p<0.05$ (129). Dunnett's test was used for comparing differences between effector means with the control mean (129). Error bars on the figures are $\pm$ SEM.

Electron microscopy. Samples examined by electron microscopy included renal papillary tissue and RPCT cells grown in monolayers attached to either culture dishes or Millipore filters (130). Primary fixation of all samples was performed for 24 hr at 4° in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. In the case of papillary tissue, cubes (ca. 1 mm^3) were cut from fresh tissue and immersed in fixative. Cultured RPCT cells were overlayed with the fixative and the fixation performed in culture dishes. To grow RPCT cells on millipore filters, one edge of a HAMK-024-12 Millipore filter was glued to the bottom of a culture dish using MF cement, the entire dish seeded with RPCT cells and the cells incubated on the filter for 3-4 days in culture media. Following fixation in glutaraldehyde all samples were washed 4-6 hr with frequent changes of 0.1 M sodium cacodylate, pH 7.4. The samples were then postfixed in 1% OsO_4 overnight at 4°. The samples were washed and then dehydrated by sequential exposure to 50%, 70%, 80%, 90% and 100% (3x) solutions of ethanol. After the third treatment with 100% ethanol, RPCT cells were removed from culture dishes by scraping with a rubber policeman, transferred to 4 dram screw top vials and collected by centrifugation at

500 x g for 2 min. To these cell pellets or tissue blocks or the entire millipore filter was added propylene oxide, which was, after a brief incubation period, then mixed with an equivalent volume of Epon-araldite resin (Epon 812:Araldite 502:dodecenyl succinic anhydride; 25/20/60; v/v/v), and the samples agitated for 4 hr at 24°. Extra resin was then added to provide a ratio of resin to propylyene oxide of 2, and the mixture agitated overnight. The samples were then collected by centrifugation and resuspended in Epon-Araldite resin to which had been added 0.024 volumes of 2,4,6-tri-(dimethylaminomethyl)phenol. The samples were placed in Beem capsules and incubated for 72 hr at 60°. The capsules were sectioned and the sections examined and photographed using a Phillips Model 201 transmission electron microscope. The sections were counterstained with 2% uranyl acetate in H_2O. Kodak EM4463 film was used for photography.

Enzyme Histochemistry. Histochemical staining for succinate dehydrogenase, NADH diaphorase and α-glycerophosphate dehydrogenase (109,131) was performed on quick-frozen RPCT cells cultured on glass cover slips and compared to staining of sections (10 μm) of rabbit kidney cut on Ames Lab-Tek cryotome. Light microscopy was performed on a Leitz Orthoplan microscope equipped with an Orthomat camera. Photomicroscopy was performed using Kodak TriX Pan Film (ASA 28).

Measurements of Potential Differences. Lexan polycarbonate tubing (5/8" x 3/4") was cut into 1/2" cylinders and the surfaces of the cylinders sanded smooth. The cylinders were boiled in Alconox for 1 hour, rinsed with distilled H_2O and allowed to dry. A thin film of

silicone cement was put on one surface of the cylinder. A polycarbonate Nucleopore filter (0.8 μm; Cat. No. 111109) was then attached to this edge and the cement allowed to dry overnight. Three dabs of silicone cement were put on the filter edges of the cylinder in order to form a tripod so that a space was present below the filter when the unit rested on a flat surface. Approximately 2 mm of clearance was satisfactory.

The filter unit was washed with 75% ethanol and then 0.08 ml of a collagen solution (1/1 mixture of 8 mg/ml calf-skin collagen and 0.1% acetic acid) was spread uniformly across the inner surface of the filter. The collagen solution was allowed to dry overnight. The collagen was fixed by exposing the entire unit to vapors of concentrated NH_4OH (30%) for 1 hr. The collagen-coated filter units were stored in petri dishes at 37°C under a humid 5% CO_2 atmosphere. Prior to seeding the filters with cells, the filter unit was rinsed with DMEM containing 10% fetal bovine serum. Cells were seeded at a density of 1-2 x 10^6 cells/cm^2 of filter, in order to insure confluency. The unit was then placed in a 100 mm^2 petri dish containing 12 ml of media and incubated at 37° under a 5% CO_2 atmosphere.

Measurements of potential differences were made in either DMEM containing 10% fetal bovine serum or in 20 mM tris-chloride, pH 7.2 containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 140 mM NaCl. Buffer was added to the inside and outside of the filter unit such that the fluid levels were equal and thus no hydrostatic pressure was present. The inside and outside solutions were connected to two solutions of 3 M KCl by 12 inch salt bridges. The salt bridges were made by drawing a hot mixture of 3 M KCl and 3% bacterial grade agar into polyethylene tubing (PE 240) and allowing the agar to solidify. The bridges were stored immersed in 3 M

KCl. The two 3 M KCl solutions were connected to a Corning model 10 pH meter with two Fischer calomel electrodes (Cat. No. 13-639-52). A potential of zero was determined by placing both agar bridges in either the inside or outside solutions. A potential difference was measured by moving one bridge back to the other solution.

Madin-Darby canine kidney (MDCK) cells used as positive controls for potential difference measurements were obtained from the American Type Culture Collection and were grown in DMEM containing 10% fetal bovine serum at 37° under a 5% CO_2 atmosphere.

RESULTS

Histological Characterization of Isolated Papillary Collecting Tubule (RPCT) Cells. We previously isolated RPCT cells from rabbit kidney by a procedure which involved mincing of the papillae, agitation of the tissue with 0.05% trypsin and then hypotonic lysis of the dispersed cells; an average of 10^6 RPCT cells were obtained per g of papillae. We have since found that we can increase the cell yield 10-fold by substituting a milder 1.5-2.5 hr incubation with collagenase (0.1%) at 37° for the trypsin-agitation step of the earlier procedure. Approximately 60% of the isolated cells adhered to plastic petri dishes. When grown to confluency (8-10 days) and examined by light microscopy, RPCT cells were polygonal (Fig. 10), and resembled cells in confluent cultures of Madin-Darby canine kidney (MDCK) cells, a transformed epithelial cell line apparently derived from the canine distal tubule (130,132). Cells having the highly elongated appearance of smooth muscle cells were seen occasionally in some RPCT cell cultures but never represented more than 0.5% of the total cells in one day cultures.

Fig. 11A-C are electron micrographs comparing the morphological properties of collecting tubules in situ (Fig. 11A) with RPCT cells grown in monolayers on Millipore filters (Fig. 11B and 11C). The isolated RPCT cells had intact plasma membranes and the mitochondrial and nuclear membranes were well-preserved (Fig. 11B). There was little vacuolization induced by the isolation procedure. Lipid droplets were

apparent in approximately 20% of both the isolated cells and the intact tubules. RPCT cells which were grown on Millipore filters resembled the parent cells in having relatively few microvilli but those present studded the apical surface (Fig. 11B) suggesting polarity in adhesion and growth. This same pattern of adherence has also been observed with MDCK cells (130). Cell junctions were consistently present between the apical and basolateral surfaces of RPCT cells grown on Millipore filters (Fig. 11C).

Hemicysts commonly observed in cultures of MDCK cells (132) were seen in cultures of RPCT cells which had been grown to confluence without media changes (Fig. 12); addition of ouabain (10^{-5} M) to the media prevented formation of the hemicysts. We observed electrical potential differences of 0.5-1.0 mV (apical surface negative) in each of five separate experiments with cultures of MDCK cells seeded on collagen-coated Nucleopore filters. However, we detected a potential difference (0.4 mV, apical surface negative) with only one of five different cultures of RPCT cells. There are two possible explanations for our inability to achieve consistent results in measuring potential differences with RPCT cells. First, the quantities of RPCT cells required to cover the relatively large Nucleopore discs used for potential difference measurements could only be obtained after 2-3 cell passages during which time increasing contamination (> 10%) of nonpolygonal fibroblast-like cells had occurred; and second, unlike MDCK cells, RPCT cells do not form a stable confluent monolayer, but instead begin to detach and die upon reaching confluency (see below, Figure 15).

Figure 10. Phase photomigraph of RPCT cells grown eight days in monolayer culture. Magnification, x 250. Nucleus (N).

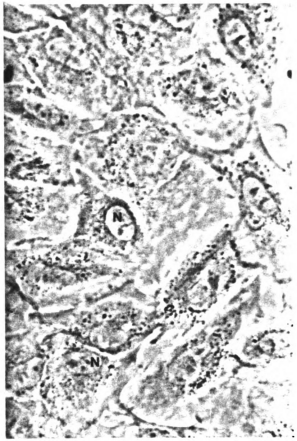
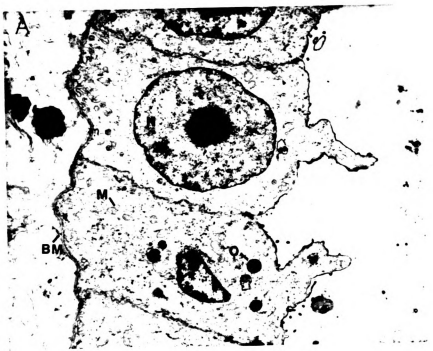
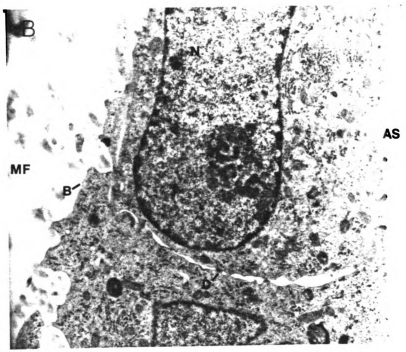

N
N

Figure 11. Electron micrographs of (A) intact collecting tubule from rabbit renal papillae (magnification, X4,500), (B) isolated RPCT cells grown in Millipore filters (magnification, X12,250) and (C) junctional complex of RPCT cells grown on Millipore filter (magnification X20,000). Nucleus (N), mitochondria (M), basement membrane (BM), oil droplets (O), rough endoplasmic reticulum (RER), basolateral surface (B), apical surface (AS), Millipore filter (MF), desmosome (D), tight junction (TJ), and gap junction (GJ).


A
M
BM


B
AS
MF
B

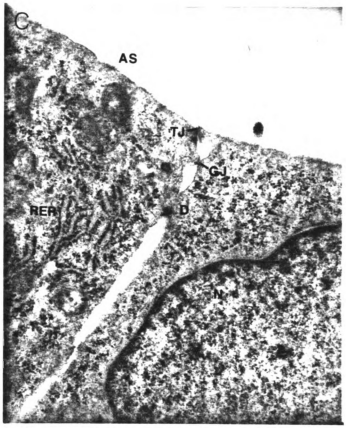
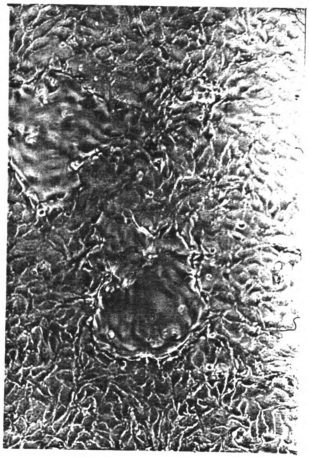

C
AS
TJ
C-J
RER
D

Figure 12. Hemicysts present in culture of RPCT cells grown to confluence without changing the growth medium. Magnification, X80.



The RPCT cells gave intense and uniformly positive staining for both NADH diaphorase and α-glycerophosphate dehydrogenase activities but did not stain for succinate dehydrogenase activity. This pattern of staining distinguishes collecting tubules from other epithelial cells of the papillae and inner medulla (133).

Biochemical Characteristics of RPCT Cells. We had previously identified isolated RPCT cells in suspension as being derived from the collecting tubules on the basis of tissue source, cell diameter and immunohistochemical properties. To determine if monolayer cultures of RPCT cells retained differentiated biochemical characteristics expected for collecting tubules, we measured the ability of one day old RPCT cells to synthesize 3',5'-cyclic AMP in response to a variety of effectors (Table 4). Agents capable of increasing 3',5'-cAMP levels at relatively high concentrations ($\geq 10^{-7}$ M) were AVP, dD-AVP, lysine vasopressin, cholera toxin, oxytocin, glucagon and, unexpectedly, parathyroid hormone; in contrast, adrenergic agents, calcitonin and histamine were ineffective. Fig. 13 compares the concentration dependence of the AVP-, parathyroid hormone- and oxytocin-induced increases in extracellular 3',5'-cyclic AMP. As anticipated, the most active agent on a molar basis was AVP. The concentration dependence observed with AVP is similar to that seen with intact tubules dissected from the outer medulla (134).

Fig. 14 shows the time course of the AVP-induced appearance of both intracellular and extracellular 3',5'-cyclic AMP. There was an approximately linear increase in extracellular 3',5'-cyclic AMP during the 60 min incubation period whereas intracellular 3',5'-cyclic AMP

TABLE 4. Formation of 3',5'-cyclic AMP by RPCT cells in response to hormonal effectors

Effector	Concentration (M)	[a]Extracellular 3'5'-cyclic AMP (fmoles/µg cell protein)	[a]Intracellular 3',5'-cyclic AMP (fmoles/µg cell protein)
None	---	28±4.3	3±0.4
IBMX (control)	10^{-4}	48±6.1	5±0.5
bradykinin	10^{-7}	55±9.1[b]	12±1.3
AVP	10^{-7}	200±16.2*	41±3.9*
dD-AVP	10^{-7}	142±16.4*	51±6.1*
cholera toxin	10^{-7}	105±12.1*	14±2.3
norepinephrine	10^{-5}	63±12.5	3±0.4
epinephrine	10^{-5}	58±11.2	–
isoproterenol	10^{-5}	68±3.9	–
calcitonin	10^{-7}	57±6.1	–
histamine	10^{-5}	58±6.7	–
angiotensin II	10^{-7}	47±5.0	–
parathyroid hormone	10^{-7}	138±11.4*	–
oxytocin	10^{-7}	123±14.9*	17±2.0
lysine vasopressin	10^{-7}	187±19.3*	–
glucagon	10^{-7}	122±15.2*	–

[a] Values represent means ± SEM; those marked with an asterisk (*) indicate a significant change from control values ($p<0.05$). Incubations with effectors were performed for 60 min at 37° in the presence of IBMX (10^{-4} M) and 3'5'-cyclic AMP assayed as described in the text.

[b] The response to bradykinin was determined following pretreatment of RPCT cells for 1 hr with 0.5 mM aspirin; in the absence of this pretreatment, bradykinin (or PGE_2, 10^{-7} M) caused a 2 fold increase in 3'5'-cyclic AMP formation (extracellular); the response to other effectors was the same before and after pretreatment.

Figure 13. Release of cAMP into media by RPCT cells incubated with different concentrations of AVP (O———O), oxytocin (Δ———Δ) and PTH (●———●). Incubations were performed at 37° for 60 min with IBMX (10^{-4} M) and the indicated concentrations of effectors. Radioimmunoassays for extracellular cAMP were performed as described in the text.

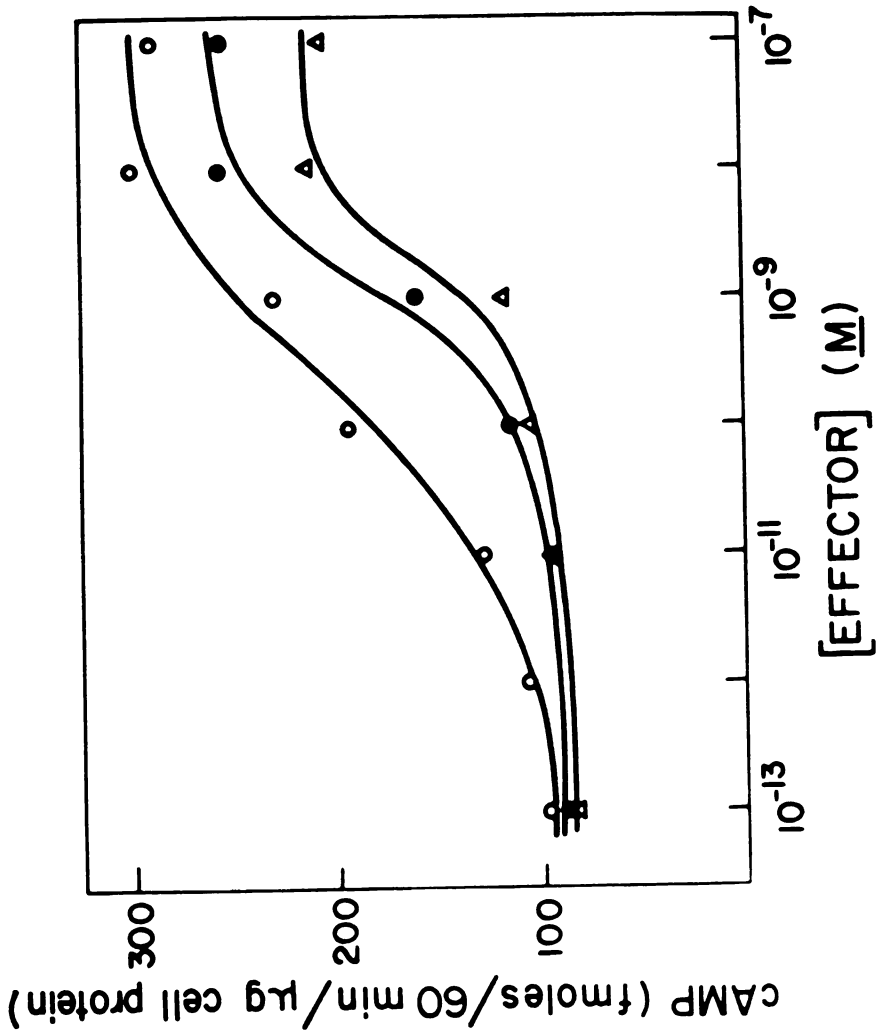
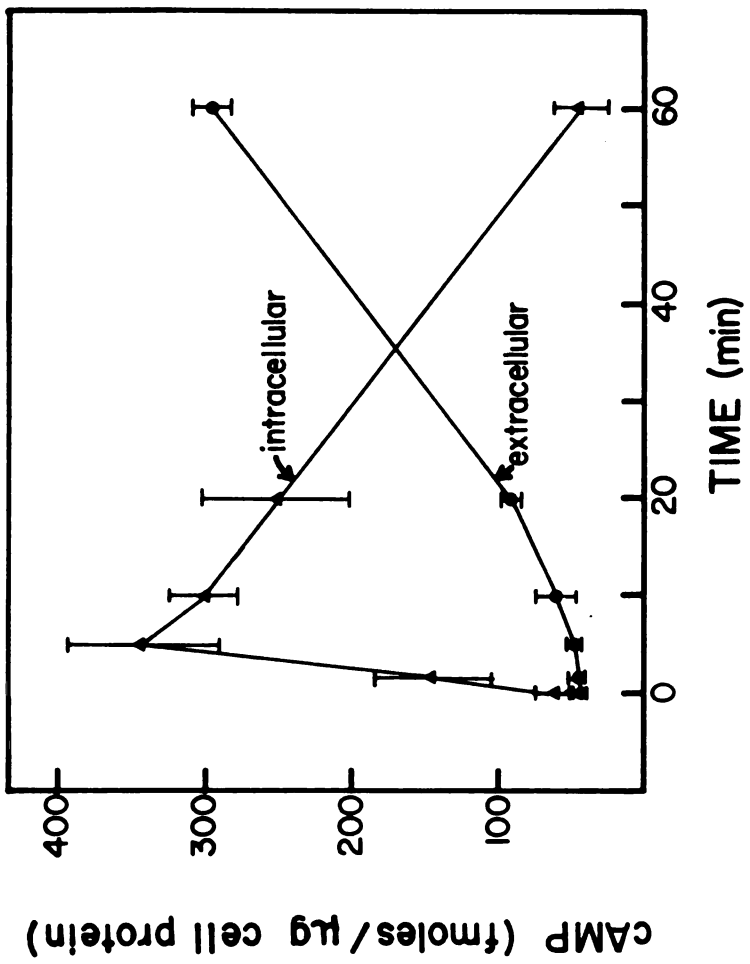

cAMP (fmoles/60 min/μg cell protein)
300
200
100
10⁻¹³
10⁻¹¹
10⁻⁹
10⁻⁷
[EFFECTOR] (M)

Figure 14. Time course of the formation of intracellular (▲——▲) and extracellular (●——●) cAMP by RPCT cells in response to 10^{-7} M AVP. Incubations were performed for the indicated times in the presence of IBMX (10^{-4} M) and radioimmunoassays for cAMP were performed as described in the text.


cAMP (fmoles/µg cell protein)
400
300
200
100
0
20
40
60
TIME (min)
intracellular
extracellular

levels peaked during the first 5 min with AVP and then gradually decreased.

Growth of RPCT Cells in Monolayer Culture. After a lag period of 3-4 days there was a four-fold increase in both cell number and cellular protein in culture dishes seeded with freshly isolated RPCT cells (Fig. 15). After 10 days the cells reached confluence and both cell protein and cell number began decreasing. The AVP-responsiveness characteristic of differentiated collecting tubules and noted with one day old RPCT cells was retained by RPCT cells maintained in tissue culture for up to 10 days; 3-4 fold increases in 3',5'-cyclic AMP were elicited with 10^{-7} M AVP. Furthermore, ten-day-old RPCT cells were similar in size and appearance to the original cells and stained positively for NADH diaphorase. In addition, the rate of $iPGE_2$ synthesis was increased to the same extent (3-4 fold) by treatment with bradykinin (10^{-9} M) in cultures of both one- and ten-day-old RPCT cells. Thus, several differentiated characteristics of collecting tubule cells were retained when RPCT cells were grown almost to confluency in primary monolayer culture.

Prostaglandin Biosynthesis by RPCT cells in Response to Various Effectors. Table 5 summarizes the results of a series of experiments in which the influence of a number of effectors on the synthesis of $iPGE_2$ by RPCT cells was determined. Agents which consistently increased $iPGE_2$ synthesis by RPCT cells after brief (10 min) incubation times included the kinin derivatives, the Ca^{++} ionophore A23187, cholera toxin, and thrombin. No significant response was elicited by

Figure 15. RPCT cell growth in monolayer culture. 24-well cluster dishes were seeded with approximately 30,000 CT cells per well. Protein content and cell number were determined at the indicated times as described in the text.

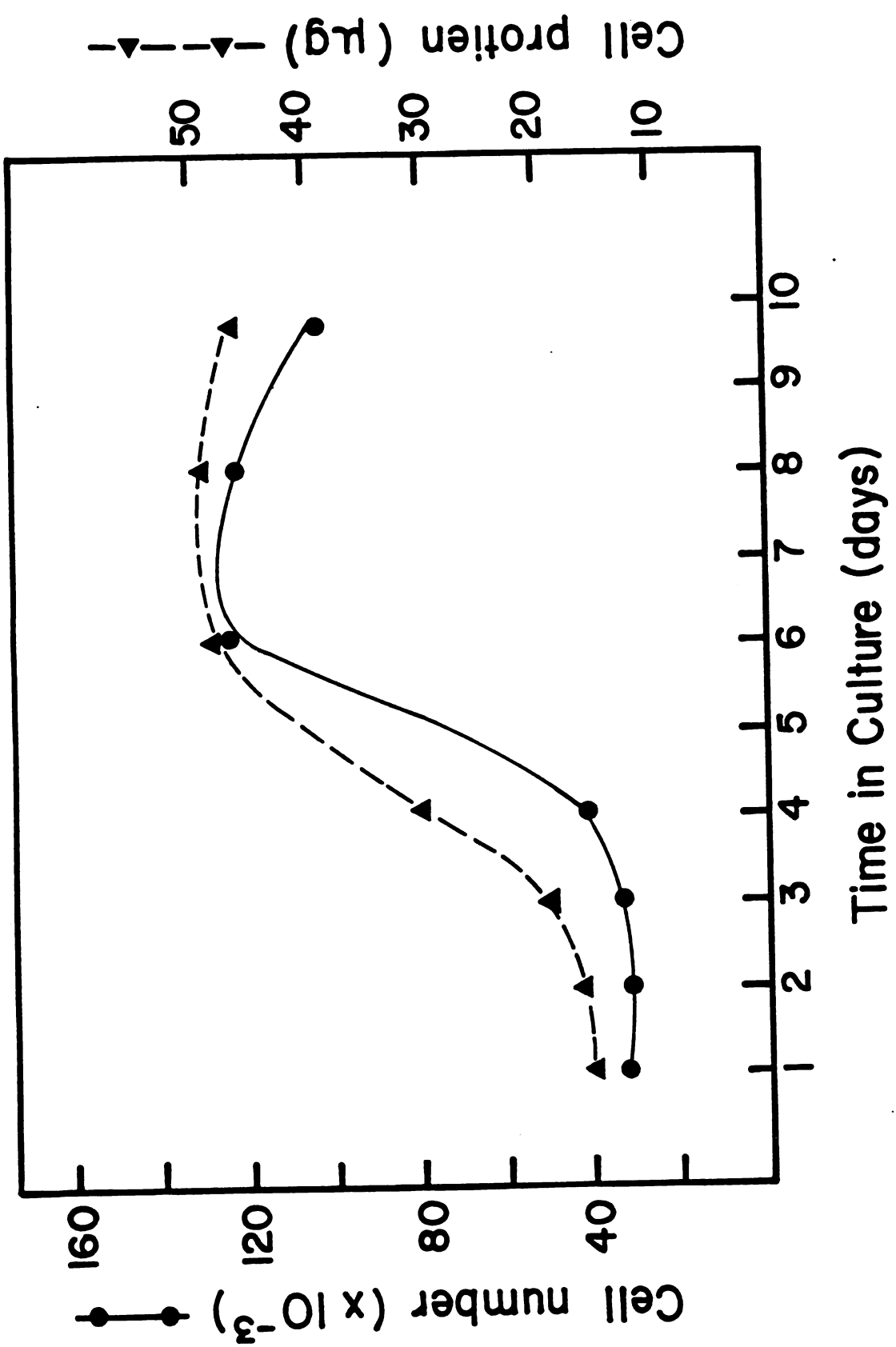

Cell protien (µg)
50
40
30
20
10
Cell number (x 10⁻³)
160
120
80
40
1
2
3
4
5
6
7
8
9
10
Time in Culture (days)

TABLE 5. Synthesis of $iPGE_2$ by RPCT cells in response to hormonal effectors.

Effector	Concentration (M)	[a] $iPGE_2$ (fmoles/μg cell protein) Incubation Time (min) 10	60
None		45±3.5	85±9.2
IBMX	10^{-4}	44±3.9	82±8.9
bradykinin	10^{-7}	184±15.1*	334±26.1*
lysyl-bradykinin	10^{-7}	196±12.8*	305±23.7*
methionyl-lysyl-bradykinin	10^{-7}	190±20.7*	320±27.6*
AVP	10^{-7}	59±7.6	102±10.2
dDAVP	10^{-7}	49±5.2	115±10.7
cholera toxin	10^{-7}	125±15.1*	237±23.2*
norepinephrine	10^{-5}	57±6.8	154±16.2*
epinephrine	10^{-5}	67±4.2	165±15.7*
isoproterenol	10^{-5}	63±3.2	90±6.9
thrombin	2.5 u/ml	108±10.9*	154±13.3*
A23187	10^{-7}	147±9.2*	430±36.7*
histamine	10^{-5}	55±3.4	ND
angiotensin II	10^{-7}	75±8.6	140±10.3*

[a]Values represent means ± SEM; those marked with an asterisk (*) indicate a significant change from control values ($p<0.01$). $iPGE_2$ was measured as described in the text.

parathyroid hormone (10^{-7} M), calcitonin (10^{-7} M), histamine, AVP, dD-AVP, lysine vasopressin (10^{-7} M), oxytocin (10^{-7} M) or isoproterenol even after 60 min incubation periods at relatively high concentrations of each of these agents. Angiotensin II, epinephrine and norepinephrine each increased iPGE formation up to 2-fold but only after a 60 min incubation.

Homogenates of RPCT cells synthesize various prostaglandins in different proportions from arachidonic acid depending on the initial fatty acid substrate concentration. Therefore, we characterized the prostaglandins formed by intact RPCT cells in response to several effectors to determine if any of these agents caused the preferential formation of a prostaglandin derivative other then PGE. We found that the types of prostaglandins formed by RPCT cells under basal conditions and in response to bradykinin, AVP, dD-AVP, A23187, angiotensin II, epinephrine and norepinephrine were very similar in all cases (Table 6). The major product was of the E series (70-75%). Smaller amounts of $iPGF_{2\alpha}$ (ca. 15%), $iPGD_2$ (5-10%) and i-6-keto-$PGF_{1\alpha}$ (5%) were also found. No $iTxB_2$ was detected under any conditions. These results indicate that there was no differential effect by any agent tested on the synthesis of any particular class of prostaglandin.

We performed two sets of experiments to determine if significant catabolism of PGE formed by RPCT cells could occur during the incubation of these cells with effectors. In the first experiment, [^{3}H]PGE_2 was added in Krebs buffer, pH 7.3, to intact RPCT cells at a concentration (10^{-7} M) designed to mimic conditions which occur following synthesis of $iPGE_2$ by cells in response to bradykinin (10^{-8} M). Following 3 hr incubations at 37° without effectors or

TABLE 6. Characterization of prostaglandins formed by RPCT cells in response to hormonal effectors.

Effector	Concentration (M)	[a]Immunoreactive Prostaglandin (fmoles/μg cell protein/60 min)				
		PGE_2	$PGF_{2\alpha}$	PGD_2	6-keto-$PGF_{1\alpha}$	Total
None	---	55 (75)	11 (15)	4 (5)	3 (4)	73
bradykinin	10^{-7}	182*(73)	37*(15)	17*(7)	12*(5)	248*
AVP	10^{-7}	56 (73)	11 (14)	6 (8)	4 (5)	77
dD-AVP	10^{-7}	65 (76)	13 (15)	4 (5)	3 (4)	85
epinephrine	10^{-5}	61 (70)	12 (14)	10*(11)	4 (5)	87
norepinephrine	10^{-5}	80 (75)	15 (14)	6 (6)	5 (5)	106
A23187	10^{-7}	187*(75)	36*(14)	17*(7)	10*(4)	250*
angiotensin II	10^{-7}	69 (70)	14 (14)	10*(10)	5 (5)	98

[a]RPCT cells were incubated with the indicated concentrations of effectors for 60 min and prostaglandins quantitated by radioimmunoassay as described in the text. Values obtained were rounded to the nearest whole number. Values denoted by an asterisk (*) were significantly different from the control; numbers in parenthesis are percentages of total prostaglandins formed. $iTxB_2$ was below the limits of detection.

with AVP (10^{-7} M), bradykinin (10^{-8} M), angiotensin II (10^{-7} M) or epinephrine (10^{-5} M), prostaglandins were extracted and separated by thin-layer chromatography and the mobility of the radioactivity with respect to prostaglandin standards determined. In all cases, greater than 90% of the original radioactivity was recovered and of this material more than 90% cochromatographed with PGE_2. In a second experiment, homogenates of RPCT cells (10^7) were prepared in 1 ml of 0.1 M tris-chloride, pH 7.4, containing 10^{-4} M NADH, 10^{-4} M NADPH, 10^{-4} M NAD, 10^{-4} M NADP, 10^{-4} M flufenamic acid and 10^{-7} M [^{3}H]PGE_2, and the sample was incubated for 3 hr at 37°. Following this incubation, less than 5% of the added radioactivity cochromatographed with products other than PGE_2. Under the same conditions using guinea pig lung homogenates (1 mg protein/ml) more than 60% of the added [^{3}H]PGE_2 was converted to less polar products. These results indicate that no significant catabolism of prostaglandins occurs in RPCT cells during our experiments and thus, the results obtained in assaying the parent prostaglandins accurately reflect prostaglandin formation by RPCT cell incubates.

PGE_2, PGD_2 and $PGF_{2\alpha}$ all can be formed from the prostaglandin endoperoxide, PGH_2, nonenzymically in varying ratios depending on the experimental conditions (125). Therefore, we tested RPCT cells for PGH-PGE isomerase activity, the enzyme which catalyzes the formation of PGE_2 from PGH_2. [^{3}H]PGH_2 (30 μM) was incubated with homogenates of both freshly isolated and nine day old RPCT cells (0.6 mg protein/ml) in 0.1 M tris-chloride, pH 7.4, containing 5 mM reduced glutathione. The rate of formation of [^{3}H]PGE_2 was 2 pmoles/min/μg of cell protein with both freshly isolated and nine day

old cells. No synthesis of [^{3}H]PGE_2 above that seen with no-enzyme controls was observed with cell homogenates which had been boiled (100° for 2 min). The specific activity of PGH-PGE isomerase in RPCT cells is 100 times the specific rate of PGE_2 release observed with bradykinin-treated cells (Fig. 15). Our results establish that PGH-PGE isomerase activity is present in RPCT cells and suggest that PGE_2 released by intact cells is actually synthesized through an enzyme-catalyzed isomerization of PGH_2.

iPGE Biosynthesis by RPCT Cells in Response to Bradykinin. The kinin derivatives were the only effectors of potential physiological importance that caused a rapid increase in the synthesis of prostaglandins by RPCT cells (Table 5). Therefore, we examined the effects of the kinins in more detail. Increased synthesis of $iPGE_2$ by RPCT cells could be detected within 1 min of exposing the cells to bradykinin (Fig. 16). By 3 min a maximal 4-6 fold increase relative to the control had occurred. Bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin exhibited similar dose-response curves with half-maximal responses occurring at concentrations of approximately 10^{-11} M (Fig. 17). Thus, RPCT cells are extremely sensitive to induction of prostaglandin formation by kinins. By comparison, half-maximal responses to bradykinin in Swiss mouse 3T3 cells occur at approximately 10^{-8} M bradykinin (135). At concentrations of 10^{-8} M, the responses of RPCT cells to bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin were not additive.

The release of arachidonic acid and PGE_2 from 3T3 cells induced by bradykinin is reportedly blocked by inhibitors of RNA and protein

Figure 16. Time course of the $iPGE_2$ synthesis by RPCT cells in monolayer culture. Incubations were as described in the text with (●———●) and without (▲———▲) bradykinin (10^{-8} M).

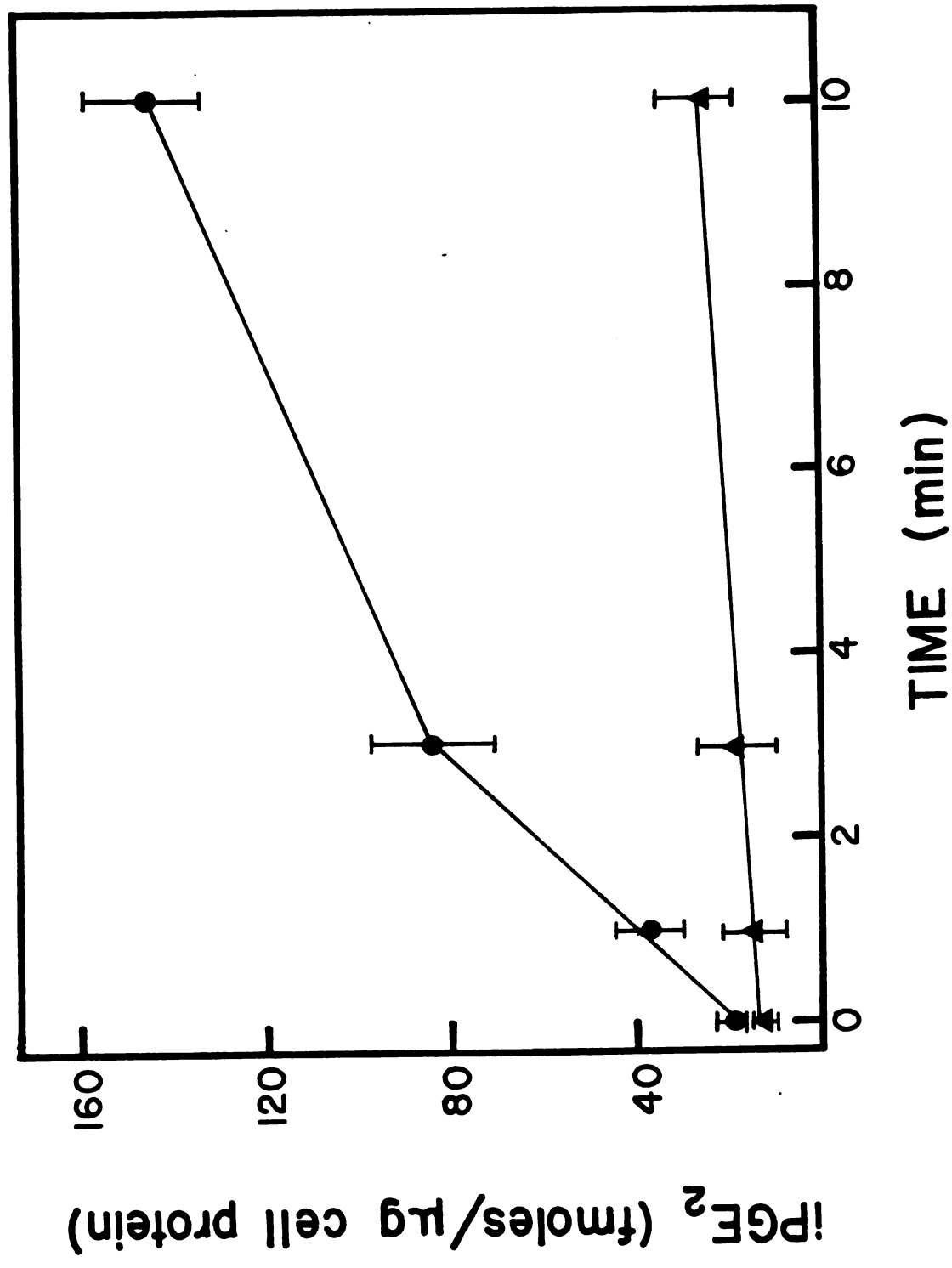
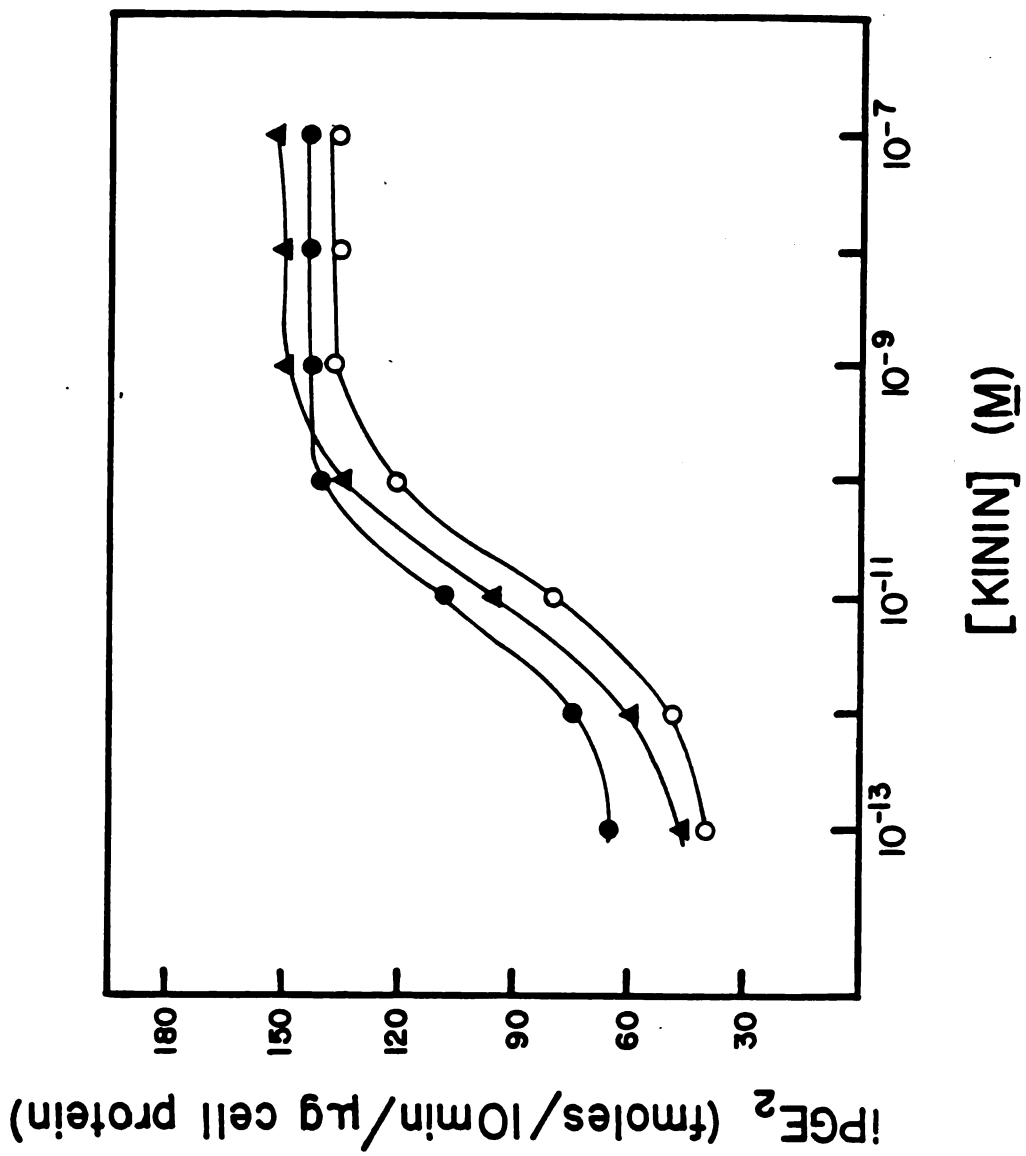

iPGE2 (fmoles/µg cell protein)
160
120
80
40
0
2
4
6
8
10
TIME (min)

Figure 17. Dependence of $iPGE_2$ synthesis on kinin concentration. Incubations were performed for 10 min at 37° with the indicated concentrations of bradykinin (O———O), lysyl-bradykinin (▲———▲) and methionyl-lysyl-bradykinin (●———●) and $iPGE_2$ analyzed as described in the text.


iPGE2 (fmoles/10min/μg cell protein)
180
150
120
90
60
30
10-13
10-11
10-9
10-7
[KININ] (M)

synthesis (136). However, we found that even when RPCT cells were preincubated for 60 min with cycloheximide (5 μg/ml), subsequent formation of $iPGE_2$ in response to bradykinin was unaffected for up to 90 min; under these conditions, incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material decreased from 10,600 cpm in the absence of inhibitor to 700 cpm in the presence of cycloheximide. Thus, unlike 3T3 cells, RPCT cells do not contain a protein, undergoing rapid turnover, which is involved in the release of fatty acid precursors of prostaglandins.

DISCUSSION

The collecting tubule appears to be the only region of the renal tubule where prostaglandins are synthesized in healthy kidneys (51,52,123). We have segregated renal papillary collecting tubule cells from other prostaglandin- forming cells of the papillae so that we could determine what factors influence prostaglandin metabolism in the collecting tubule.

Based on simple morphological and histochemical criteria, these primary RPCT cell cultures were comprised of at least 97% collecting tubule epithelia. The properties which identify these cells as being derived from the collecting tubule are as follows: (a) the cells are isolated from papillae carefully dissected from the outer medulla; (b) RPCT cells uniformly stain positively for NADH diaphorase and α-glycerophosphate dehydrogenase activities and cyclooxygenase antigenicity and negatively for succinate dehydrogenase activity; papillary collecting tubule cells exhibit this unique pattern of staining but cells of the thin limb and descending limb of Henle's loop, medullary interstitial cells, capillaries and venules do not (52,131); (c) the average diameter of the isolated cells measured on cell suspensions both prior to adherence and after trypsinization of adherent cells is 12-15 μm -- as expected for collecting tubules, which are approximately twice the diameter of any other papillary cell; (d) RPCT cells contain AVP-responsive adenylate cyclase activity and the

half-maximal concentration of AVP necessary for activation (10^{-10} M) is similar to that reported by others for isolated collecting tubule segments both with respect to AVP-dependent adenylate cyclase responsiveness (135) and to water permeability (61); RPCT cells also release 3',5'-cyclic AMP in response to glucagon but not adrenergic agents as expected for collecting tubules (137,138); and (e) morphological characteristics such as the size of the nuclei, numbers of lipid droplets, relative paucity of mitochondria, asymmetric growth on millipore filters and formation of hemicysts are properties expected of collecting tubule epithelial cells. The only characteristic of RPCT cells which was not anticipated from previous studies of collecting tubules was the increased production by the isolated cells of 3',5'-cyclic AMP in response to parathyroid hormone. Although parathyroid hormone does not activate adenylate cyclase activity in the cortical or inner medullary collecting tubule (139), our results raise the possibility that the papillary collecting tubule is responsive to this hormone.

A number of properties of MDCK cells, a transformed epithelial cell line derived from the canine distal tubule, have been described previously (130,132). RPCT cells are similar to MDCK cells morphologically, in the formation of hemicysts and in their responses to most effectors of adenylate cyclase activity (132). The most notable differences are that parathyroid hormone increases 3',5'-cyclic AMP synthesis by RPCT but not MDCK cells and that oxytocin and AVP are equipotent in their actions on MDCK but not on RPCT cell adenylate cyclases (132).

We have demonstrated that RPCT cells can be grown and maintained in monolayer culture for at least 10 days with no major changes in

differentiated cell characteristics such as morphology, histochemical properties, AVP-dependent 3',5'-cyclic AMP production and bradykinin-dependent prostaglandin formation. Thus, RPCT cells, when grown in primary monolayer culture, provide a rational model for studying the biochemical events associated with water and inorganic ion transport which appear to be the principal functions of the collecting tubule (57,140). However, as with any primary cell culture system, one cannot eliminate the possibility that certain properties of the parent cells are modified or lost during isolation and culture.

The results of our studies on prostaglandin formation by RPCT cells suggest that kinins can act directly on the medullary collecting tubule to cause PGE_2 biosynthesis. The concentration at which each of the major renal kinins -- bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin (141) -- cause half-maximal stimulation of prostaglandin synthesis in RPCT cells (10^{-11} M) is four orders of magnitude lower than that required for prostaglandin synthesis in other cell culture systems (135) and is less than one tenth of the concentration needed for half-maximal contraction of uterine smooth muscle (142). Thus, RPCT cells are extraordinarily responsive to kinin derivatives. Since the concentrations of kinins in urine (141) are greater than necessary to cause maximal iPGE by RPCT cells, we suspect that kinins may act at the blood surface of the collecting tubule (75) to induce prostaglandin formation. Kinin- induced prostaglandin synthesis by renal collecting tubules may cause a natriuresis (133) since PGE_2 does inhibit both active Na^+ resorption by medullary collecting tubule segments (37) and passive Na^+ resorption by the anatomically adjacent medullary thick ascending limb (143).

Epinephrine, norepinephrine and angiotensin II increased prostaglandin formation by RPCT cells, but only by 2-fold, at most, and only after extended treatment times at relatively high concentrations. We speculate, therefore, that these agents are not major physiological effectors of prostaglandin synthesis by collecting tubules. Both sympathetic nerve stimulation and angiotensin II do increase prostaglandin synthesis in intact kidneys, and we suspect that these agents act principally on the renal vasculature (144) and/or glomerular epithelial and mesangial cells (145).

Although AVP does induce prostaglandin formation in the renal medulla (45,146) and the anuran bladder (147), neither AVP nor the nonpressor analog dD-AVP (126) had any effect on the rate of synthesis of prostaglandins by RPCT cells during a 60 min incubation period; yet, under these same experimental conditions both AVP and dD-AVP do cause a 3-4 fold increase in the production of 3',5'-cyclic AMP. These results suggest that AVP when acting as an antidiuretic agent has no short term, acute influence on prostaglandin synthesis by medullary collecting tubules.

The results of our product characterizations indicate that PGE_2 is the major prostaglandin released by medullary collecting tubules and that PGE_2 is synthesized enzymatically. The lack of PGE_2 catabolism by RPCT cells suggests that this prostaglandin must be inactivated at other cellular sites either in the kidney or, after entering the venous drainage, by the lung. PGI_2 is formed at significant rates by RPCT cell homogenates, especially at low concentrations of arachidonic acid. However, the lack of appreciable 6-keto-$PGF_{1\alpha}$ release in these current studies suggests that either (a) PGI_2 synthase activity

is lost during acclimation of RPCT cells to monolayer culture conditions or (b) that PGI_2 synthase activity is simply not expressed by intact cells.

CHAPTER IV

INTERRELATIONSHIPS AMONG BRADYKININ, PGE_2, VASOPRESSIN AND cAMP IN RENAL PAPILLARY COLLECTING TUBULE CELLS

In the previous chapter I described studies on prostaglandin metabolism by RPCT cells in culture. PGE_2 was found to be the major prostaglandin product and PGE_2 synthesis was shown to be induced by low concentrations of renal kinins, but not by AVP. In this chapter, I describe the results of experiments directed toward understanding the effects of prostaglandins alone and in combination with AVP on cAMP metabolism by RPCT cells.

MATERIALS AND METHODS

Materials. Trypsin (1/250), Dulbecco's modified Eagle media (DMEM), antibiotic-antimycotic (100X) and fetal bovine serum were all purchased from Grand Island Biological Company. Collagenase (CLS II) was obtained from Worthington Biochemicals, Inc. Bradykinin triacetate, bovine serum albumin, cholera toxin, arginine vasopressin (AVP) and 3-isobutyl-1 methylxanthine (IBMX) were purchased from Sigma Chemical Company. [^{3}H]arachidonic acid (130 Ci/mmole), [^{3}H]PGE_2 (130 Ci/mmole), [^{3}H]3',5'-cyclic AMP (31 Ci/mmole) and radioimmunoassay supplies for the 3',5'-cyclic AMP assay were all purchased from New England Nuclear Corp. Antiserum against PGE_2 was obtained from Miles Laboratories, Inc. Prostaglandin standards, PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2, were either donated by or purchased from the Upjohn Company. All other chemicals were reagent grade or better and were purchased from common chemical sources.

Cell isolation. Renal papillary collecting tubule (RPCT) cells were isolated from rabbit papillae as described in Chapter III. The final cell pellet obtained was resuspended in DMEM containing 10% fetal bovine serum and antibiotic-antimycotic (1/60) and an aliquot taken for counting. Typically the RPCT cells were seeded in 24-well cluster culture dishes (16 mm, well diameter) at a density of 6-9 x 10^4 cells per well and grown at 37°C under a 10% CO_2 atmosphere.

Incubation of cells with effectors. Treatment of RPCT cells with effectors was done in triplicate or quadruplicate using 24-well culture dishes. Cells were first rinsed free of media with Krebs buffer (composition, in mM: 118 NaCl, 25 $NaHCO_3$, 14 glucose, 4.7 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$ and 1.8 KH_2PO_4), pH 7.3 with or without IBMX depending on the experiment being done. Effectors were then added to the cells in Krebs buffer. In experiments in which intracellular cyclic AMP was measured, 0.3 ml of Krebs buffer (± effectors) was added to each well for the desired time intervals. For $iPGE_2$ assays, 0.3-1.0 ml Krebs buffer ± effectors was added to each well and 0.03-0.10 ml was removed for radioimmunoassay at the desired time intervals. Cell protein was assayed by the Lowry procedure following the solubilization of cell protein by adding 0.20 ml of 0.1% sodium dodecyl sulfate to the wells and incubation at 37°C for 10 min. Bovine serum albumin was used as the protein standard.

$iPGE_2$ radioimmunoassay. Immunoreactive PGE_2, $iPGE_2$, was measured using anti-PGE_2 antibody with an assay yielding a sensitivity range of 10-500 pg for PGE_2. The large amount of $iPGE_2$ synthesized by RPCT cells relative to other prostaglandins allowed us to measure $iPGE_2$ directly without chromatographic separation from the other prostaglandins present. The $iPGE_2$ radioimmunoassay employed a single antibody to bind $iPGE_2$ and dextran T-70 coated charcoal (25 mg dextran, 250 mg charcoal in 25 ml phosphate buffered saline, pH 7.2) to precipitate any unbound $iPGE_2$. All the effectors used were checked to determine if they had an independent effect on the radioimmunoassay.

3',5'-cyclic AMP radioimmunoassay. Measurements of intracellular cAMP were performed as follows. At the appropriate times after addition of the effectors, the incubation media was aspirated. 0.35 ml of ice-cold 6% trichloracetic acid (TCA) was added to each well and the entire culture dish frozen in a -70°C freezer. After 15 min the dish was thawed, [^{3}H]3',5'-cyclic AMP (5000 dpm) added to each well and the samples incubated at 4°C for 2 h. The TCA solution was then removed from the wells and extracted 4 times with 5 ml portions of water-saturated diethyl ether. The residual ether was removed by placing the samples in a 65°C water bath for 15 min and then the samples were lyophilized. The lyophilized residue was redissolved in 0.15-0.50 ml 0.05 M sodium acetate, pH 6.2. An aliquot of this sample was used to determine [^{3}H]cAMP recovery (typically 80-90%) and another aliquot used for radioimmunoassay. ^{125}I cpm from the radioimmunoassay were counted on a Beckman gamma counter in order to exclude interfering [^{3}H]-cAMP cpm.

Stirring experiments. To make measurements on cells that had been stirred several modifications were necessary. 1 ml of RPCT cells in DMEM containing 10% fetal bovine serum were seeded at a density of 6-9 x 10^4 cells per 35 mm culture dish. The culture dishes were tilted at an angle such that the RPCT cells only adhered to approximately 35-40% of the surface area of the dish. Prior to treatment of the RPCT cells with effectors the cells were rinsed free of media as described above. Effectors were then added to the cells in 1 ml of Krebs buffer and the buffer mixed vigorously by one of two methods. In the first method the buffer was pipetted up and down continually for the entire time interval

of the experiment. In the second method a small stir bar (15 mm x 1.5 mm) was used to stir the buffer. Extreme care must be taken to regulate the speed of stirring to ensure the stir bar remains on the portion of the culture dish not containing cells. Any cells that contact the stir bar are immediately removed from the surface of the culture dish. After mixing by either method, the Krebs buffer was removed and 1 ml of 6% TCA was added to the dish. The measurement of cAMP was then done as described above except that one additional extraction with diethyl ether was done.

Statistical analyses. Experiments involved using 24-well culture dishes in which 3 or 4 replicates and thus 8 or 6 treatments were analyzed for any effects. A completely random analysis of variance was used to test for differences between sample means at $p < 0.05$ (129). Dunnett's test was used for comparing differences between the effector means and the control mean.

RESULTS

The Effect of Prostaglandins on Intracellular cAMP Concentrations in RPCT Cells. Fig. 18 illustrates the dose response curves for the effects of PGI_2, PGE_2, PGD_2 and $PGF_{2\alpha}$ on intracellular cAMP levels in RPCT cells incubated in the presence of 10^{-4} M IBMX. With all effectors tested, the maximal increase in intracellular cAMP concentrations occurred at 10 min. In the absence of phosphodiesterase inhibitors no changes in cAMP levels were observed. PGI_2 and PGE_2 caused 3.5- and 2-fold increases in cAMP, respectively. Half-maximal increases in cAMP concentrations occurred at approximately 5×10^{-6} M for both PGI_2 and PGE_2. Neither PGD_2 nor $PGF_{2\alpha}$ altered intracellular cAMP concentrations. The effects of PGI_2 and PGE_2 at 10^{-5} M were additive, suggesting that two distinct receptor sites exist in RPCT cells. Pretreatment of RPCT cells with aspirin (1 mM for 1.5 h) did not change the relative increase in intracellular cAMP synthesis observed with either PGE_2 or PGI_2; however, with aspirin the levels of cAMP were depressed approximately 30% in both control and treated cells presumeably due to suppression of endogenous prostaglandin synthesis.

The Effect of Bradykinin on Intracellular cAMP Levels in RPCT Cells. Fig. 19 illustrates the time courses for the increases in intracellular cAMP levels caused by the additions of PGE_2 (10^{-5} M

Figure 18. Dependence of intracellular cAMP formation on prostaglandin concentration. Incubations were performed for 10 min at 37°C and cAMP assayed as described in the text.

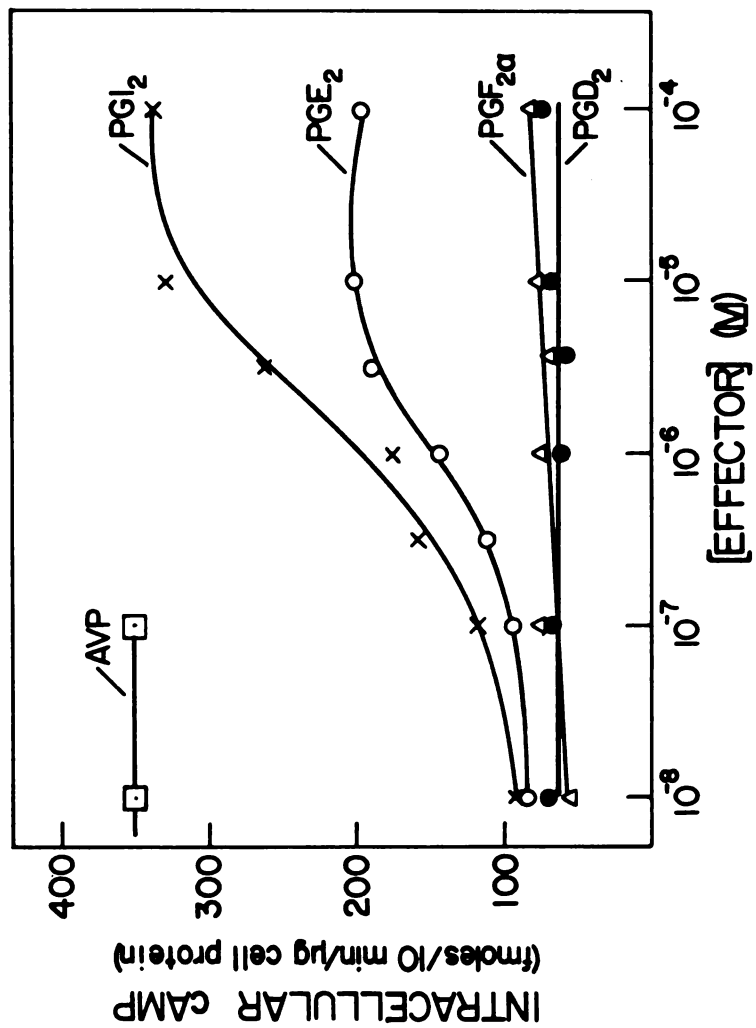
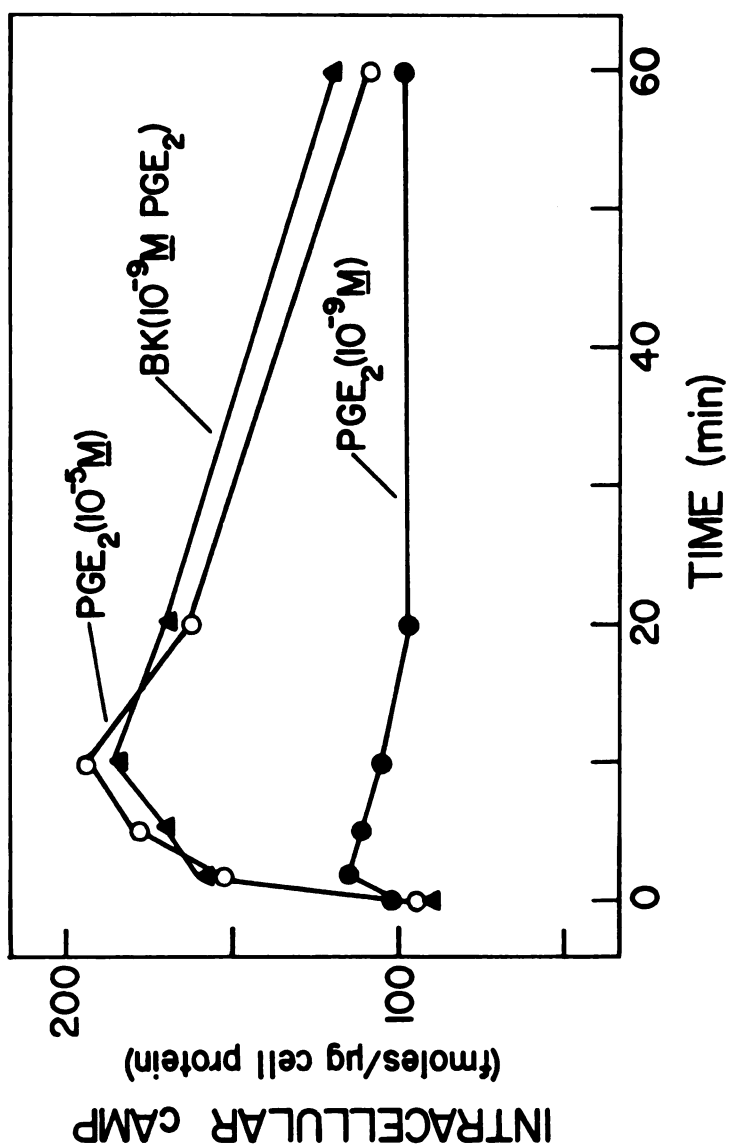

INTRACELLULAR cAMP
(fmoles/10 min/µg cell protein)
400
300
200
100
AVP
PGI2
PGE2
PGF2α
PGD2
10-8
10-7
10-6
10-5
10-4
[EFFECTOR] (M)

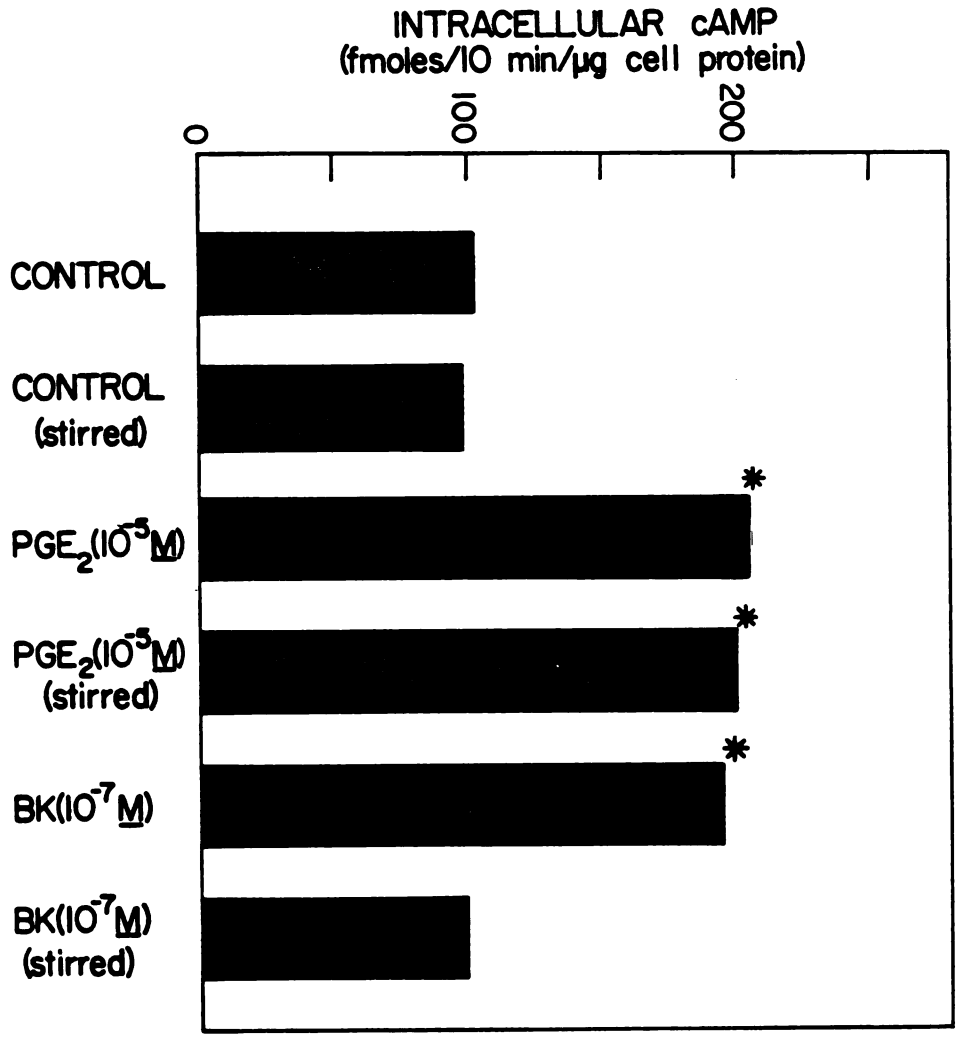
Figure 19. Time course for PGE_2 (10^{-5} M and 10^{-9} M) and bradykinin (10^{-7} M) stimulated intracellular cAMP formation by RPCT cells. cAMP was assayed as described in the text.


INTRACELLULAR cAMP
(fmoles/µg cell protein)
200
100
0
20
40
60
TIME (min)
PGE2(10-5M)
BK(10-9M PGE2)
PGE2(10-9M)

or 10^{-9} M) or bradykinin (10^{-7} M). In all cases the peak intracellular cAMP concentration occured 10 min after the addition of effectors. The effect of bradykinin on cAMP accumulation was completely inhibited by pretreatment of the RPCT cells with aspirin (1 mM per 1.5 h). In the absence of aspirin, the concentration of $iPGE_2$ present in the media following a 10 min incubation with bradykinin was typically 10^{-9} M or two orders of magnitude below the lowest concentration of exogenous PGE_2 or PGI_2 that would stimulate cAMP synthesis. Moreover, a ten-fold dilution of the media surrounding the cells (so the extracellular concentration of PGE_2 was 10^{-10} M in response to bradykinin) did not affect the ability of bradykinin to increase intracellular cAMP levels two fold.

To investigate the basis for the apparent concentration differential between the effects on cAMP of prostaglandins synthesized endogenously and prostaglandins added exogenously, we performed the following experiment. RPCT cells were first seeded on culture dishes lying at an angle so that the cells adhered to only one side of each dish. This allowed the media to be mixed with a stir bar situated on the opposite side of the dish without disrupting the cells mechanically. Different dishes containing RPCT cells were then incubated with the desired effector (PGE_2 (10^{-5} M) or bradykinin (10^{-7} M)) in the presence and absence of stirring. As shown in Fig. 20, stirring eliminated the increase in cAMP concentrations caused by bradykinin but not by exogenous PGE_2. These results imply that in the presence of bradykinin and in the absence of stirring, prostaglandins formed by RPCT cells can accumulate at locally high concentrations in extracellular spaces near the cell membranes and that by increasing the rate of

Figure 20. Effect of stirring on intracellular cAMP formation caused by PGE_2 (10^{-5} M) or bradykinin (10^{-7} M). cAMP was assayed after incubation for 10 min at 25°C as described in the text.


INTRACELLULAR cAMP
(fmoles/10 min/µg cell protein)
0
100
200
CONTROL
CONTROL
(stirred)
PGE2(10-5M)
PGE2(10-5M)
(stirred)
BK(10-7M)
BK(10-7M)
(stirred)
*
*
*

movement of newly formed prostaglandins away from the cell surface by stirring, the effects of endogenous prostaglandins on cAMP accumulation are abolished.

Effect of PGE_2 on AVP Stimulated Intracellular cAMP Accumulation. Fig. 21 illustrates the time courses for the effects of PGE_2 (10^{-5} M), AVP (10^{-9} M) and AVP plus PGE on the accumulation of cAMP in RPCT cells. At 5, 10 and 20 min the amounts of intracellular cAMP observed in response to AVP or AVP plus PGE_2 are not significantly different. Since PGE_2 alone causes a 2-fold increase in intracellular cAMP concentrations, the results indicate that the effects of AVP and PGE_2 are not additive. Results similar to those shown in Fig. 4 were also obtained when 10^{-7} M AVP was substituted for 10^{-9} M AVP and when bradykinin (10^{-7} M) was substituted for PGE_2 (10^{-5} M).

Fig. 22 illustrates the effect of AVP, PGE_2 and bradykinin on intracellular cAMP levels in RPCT cells 10 min after the addition of the effectors. With either AVP plus PGE_2 or AVP plus bradykinin, the observed levels of intracellular cAMP are significantly lower (40-50%) than the levels expected based on the calculated additivity of the cAMP responses due to PGE_2 or bradykinin plus AVP. At concentrations of PGE_2 less than 10^{-7} M, no effect on AVP stimulated cAMP synthesis could be detected. The inhibition of AVP stimulated cAMP synthesis by bradykinin was eliminated by pretreatment of RPCT cells with aspirin (1 mM for 1.5 h). PGI_2 also inhibits AVP-stimulated cAMP synthesis by RPCT cells in a manner similar to that observed with PGE_2.

Figure 21. Time course of the effect of 10^{-5} M PGE_2 on intracellular cAMP synthesis stimulated by AVP (10^{-9} M). cAMP was assayed as described in the text.

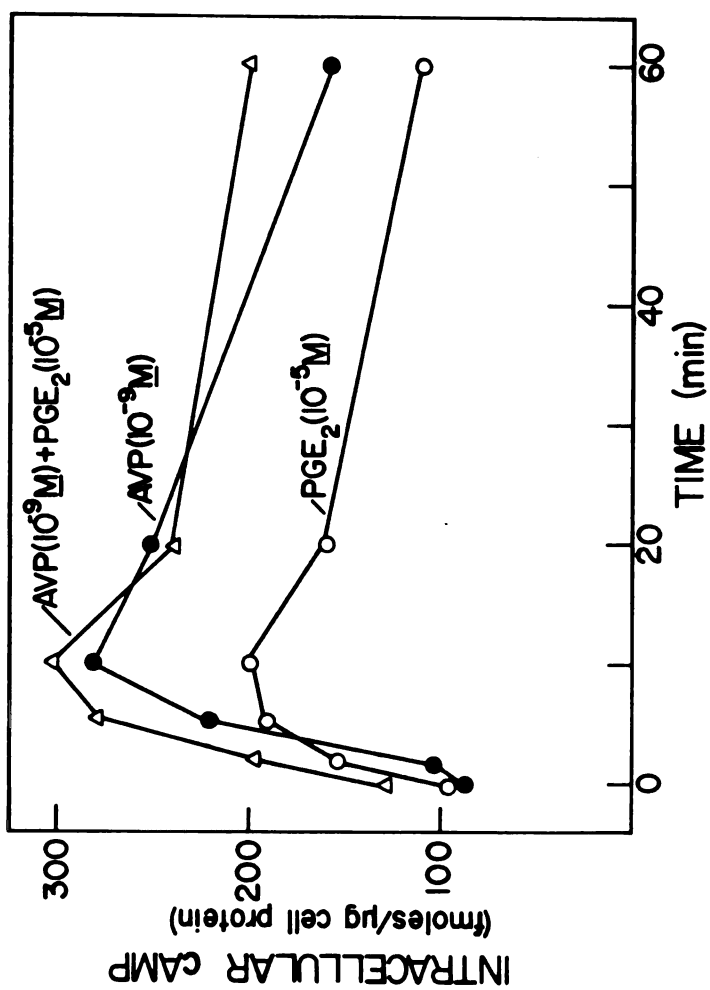
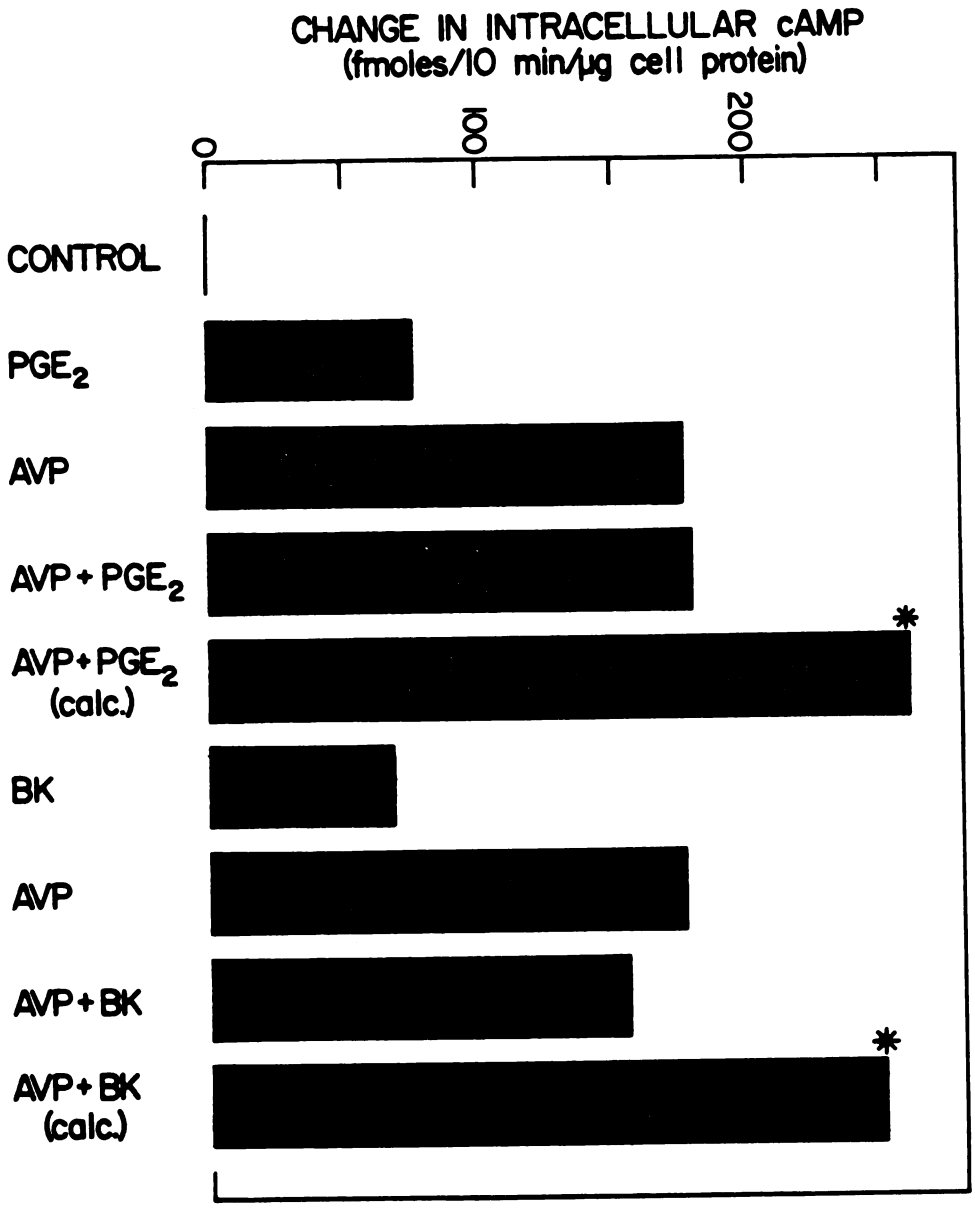

INTRACELLULAR cAMP
(fmoles/μg cell protein)
300
200
100
0
20
40
60
TIME (min)
AVP(10⁻⁹M)+PGE₂(10⁻⁵M)
AVP(10⁻⁹M)
PGE₂(10⁻⁵M)

Figure 22. Effect of AVP (10^{-7} M), PGE_2 (10^{-5} M), and bradykinin (10^{-7} M) on intracellular cAMP synthesis by RPCT cells. Incubations were performed for 10 min at 37°C and cAMP assayed as described in the text.

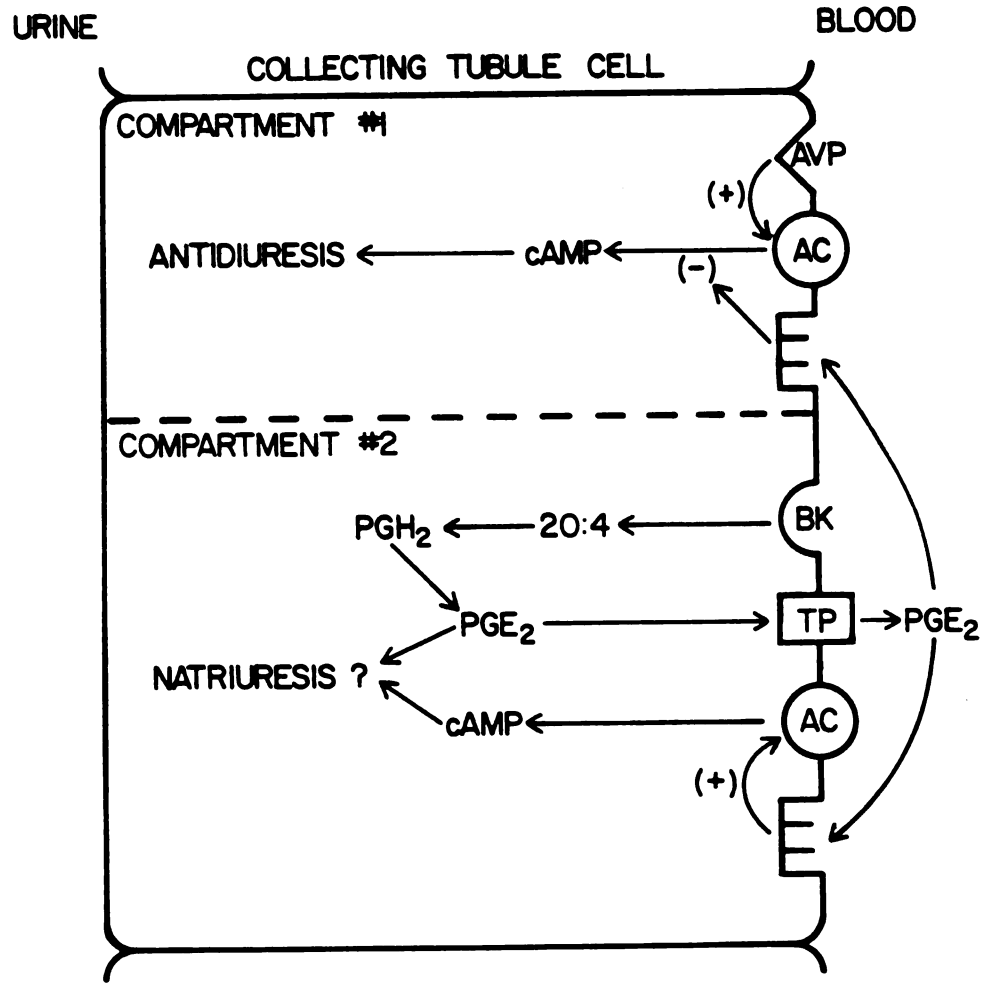

CHANGE IN INTRACELLULAR cAMP
(fmoles/10 min/µg cell protein)
0
100
200
CONTROL
PGE2
AVP
AVP + PGE2
AVP+PGE2
(calc.)
BK
AVP
AVP + BK
AVP+BK
(calc.)
*
*

DISCUSSION

A model illustrating the interrelationships of prostaglandins, bradykinin, cAMP and AVP in RPCT cells is illustrated in Fig. 23. Bradykinin, but not AVP, caused $iPGE_2$ biosynthesis by RPCT cells. Receptors for PGE_2 on RPCT cells are envisioned to be present both exogenously and on the serosal surface of RPCT cells. Stokes observed that only PGE_2 added to the serosal surface of cortical collecting tubules caused natriuresis; lumenal PGE_2 had no effect (37). In addition, our data suggests that PGE_2 synthesized due to bradykinin exits the cell and acts exogenously. While no known functions of prostaglandins synthesized due to bradykinin currently exist in RPCT cells, two potential functions are to mediate bradykinin-induced natriuresis and diuresis.

The reported natriuretic effect of bradykinin in the kidney is probably due to an effect on renal hemodynamics and an effect on tubular sodium transport (37,56,104). Stokes and Kokko have shown that exogenous PGE_2 inhibits sodium backflux, but not sodium efflux in the perfused rabbit cortical collecting tubule (37). Bradykinin-stimulated PGE_2 formation may cause a similar effect in the papillary collecting tubule. The means by which PGE_2 causes natriuresis in the cortical collecting tubule, however, is unknown. cAMP synthesized due to PGE_2 by RPCT cells may be involved in this process. PGE_2 caused

Figure 23. Model illustrating the effects of prostaglandins, bradykinin (BK), cAMP and AVP in RPCT cells.


URINE
BLOOD
COLLECTING TUBULE CELL
COMPARTMENT #1
AVP
(+)
ANTIDIURESIS
cAMP
AC
(-)
COMPARTMENT #2
PGH2
20:4
BK
PGE2
TP
PGE2
NATRIURESIS ?
cAMP
AC
(+)

natriuresis in the cortical collecting tubule at the same concentration at which PGE_2 caused cAMP synthesis by RPCT cells.

Bradykinin has also been shown to cause diuresis in the perfused dog kidney (89). Inhibition of prostaglandin synthesis with indomethacin abolishes bradykinin-induced diuresis (96) implying prostaglandins mediate bradykinin-induced diuresis somewhere in the kidney and as a possible corollary that prostaglandins synthesized due to bradykinin may inhibit anti-diuresis caused by AVP. In fact when bradykinin and AVP are simultaneously infused in the dog, bradykinin-induced diuresis is not inhibited (105,106). One role of PGE_2 synthesized due to bradykinin by RPCT cells thus may be to inhibit AVP-stimulated cAMP synthesis and thereby inhibit water resorption and enhance diuresis.

The interaction between AVP and prostaglandins in collecting tubules remains confused. Our data suggests that PGE_2 may cause a decrease in AVP-stimulated cAMP biosynthesis. However, our data also indicates that AVP and AVP plus PGE_2 cause an equivalent amount of total intracellular cAMP biosynthesis by RPCT cells. In order for water resorption to be inhibited by PGE_2, cAMP biosynthesis must ostensibly decrease.

One speculative explanation for how this could occur would be that two compartments of cAMP biosynthesis existed in RPCT cells. In a two compartment model total intracellular cAMP could be unchanged, but cAMP synthesis could decrease in compartment 1 (which controls water resorption) while increasing in compartment 2 in the presence of AVP plus PGE_2. The possibility that PGE_2 could inhibit AVP-stimulated water resorption independently of an inhibition of cAMP synthesis is

unlikely in lieu of the work of Grantham et al. Using perfused rabbit cortical collecting tubules, Grantham et al. showed that PGE_1 inhibited AVP-stimulated water resorption, but not cAMP-stimulated water resorption (61). This data indicated that PGE_1 inhibited water resorption by inhibiting cAMP biosynthesis. Neither Grantham nor Dorisa found any evidence suggesting PGE_1 or PGE_2, respectively, could stimulate phosphodiesterase in the collecting tubule (61,74).

There remains, however, several discrepancies between our data and Grantham's data. Grantham observed that PGE_1 inhibited AVP-stimulated water resorption at 10^{-9} M. We did not observe an inhibition of AVP induced cAMP biosynthesis below 10^{-6} M PGE_2. Additionally, Grantham used PGE_1 and not PGE_2 and he used cortical collecting tubules and not papillary collecting tubules. Either of these differences could be responsible for the difference in the sensitivities in these two systems. The isolation and culturing of RPCT cells may have caused a loss of sensitivity by these cells.

In order to resolve if the non-additive response of AVP plus PGE_2 is due to the inhibition of AVP-induced cAMP biosynthesis several experiments could be done. First, one could measure AVP-stimulated adenylate cyclase activity in plasma membrane fractions from RPCT cells and determine if PGE_2 or PGE_1 can inhibit this activity directly. Secondly, if one could find an effector which could cause an additive effect with AVP on cAMP biosynthesis in RPCT cells, the non-additive effect of AVP plus PGE_2 would appear to be specific. And lastly, one could try to find PGE_2 analogues which do not affect cAMP biosynthesis alone, but which do inhibit AVP-stimulated cAMP biosynthesis.

Additionally, PGE_1 should be tested to determine if it affects RPCT cells at a lower concentration than PGE_2.

SUMMARY DISCUSSION

A number of important points have been presented in the previous chapters. A brief synopsis of these observations is presented below.

Isolation of RPCT cells. A convenient procedure for isolating homogeneous populations (97%) of RPCT cells was developed. The cells obtained were shown to be derived from the collecting tubule by a series of morphological, histochemical and biochemical tests. RPCT cells isolated by this new procedure provide a reasonable model in which to study prostaglandin metabolism in collecting tubule cells. In addition, the cell yields obtained (10^7 cells/g papillae) allow one to make biochemical measurements with RPCT cells which cannot be made with isolated tubule segments.

RPCT cells biosynthesize cAMP in response to AVP and parathyroid hormone. The cyclic nucleotide theory of AVP-induced water resorption predicts that AVP-stimulated cAMP synthesis precedes water resorption. RPCT cells, as expected, synthesized cAMP in response to AVP. The half-maximal response to AVP occured at 10^{-10} M which was consistent with previously reported values for AVP-induced cAMP synthesis and water resorption in collecting tubules.

Parathyroid hormone was also shown to elevate extracellular cAMP levels which has not been previously reported in papillary collecting tubules.

RPCT cells can synthesize 6-keto-$PGF_{1\alpha}$. The capacity of RPCT cells to synthesize 6-keto-$PGF_{1\alpha}$ was the first indication that the kidney medulla can synthesize PGI_2. RPCT cells in culture, however, synthesized predominantly PGE_2 (70-75%) under conditions of basal and stimulated prostaglandin biosynthesis.

Bradykinin, but not AVP, caused $iPGE_2$ biosynthesis by RPCT cells. The current model of collecting tubule function predicts that AVP would cause prostaglandin biosynthesis in collecting tubule cells. AVP, however, did not induce prostaglandin biosynthesis by RPCT cells. The only important physiological stimulator of prostaglandin biosynthesis found was bradykinin. Bradykinin caused half-maximal increase in prostaglandin biosynthesis at 10^{-11} M which was three orders of magnitude below the values reported to cause prostaglandin synthesis by both 3T3 cells and vascular endothelial cells. Because PGE_2 causes natriuresis in the isolated cortical collecting tubule and bradykinin causes natriuresis in the kidney, one could speculate that bradykinin induced PGE_2 biosynthesis may cause natriuresis in the papillary collecting tubule.

PGE_2 and PGI_2 stimulated cAMP biosynthesis by RPCT cells. PGE_2 and PGI_2, but not PGD_2 and $PGF_{2\alpha}$, caused cAMP biosynthesis by RPCT cells. The responses to PGE_2 and PGI_2 were

additive suggesting that two separate receptors exist for PGE_2 and PGI_2. PGE_2 and PGI_2 may also have distinct functions in RPCT cells.

Prostaglandins cause cAMP biosynthesis through exogenous cell receptors. Bradykinin induced prostaglandin biosynthesis caused cAMP biosynthesis by RPCT cells. These prostaglandins can exit the cell and cause cAMP biosynthesis by interaction with exogenous cell receptors. The maintenance of the extracellular concentrations of PGE_2 (10^{-5}-10^{-6}) necessary to cause cAMP biosynthesis is probably achieved by sequestering endogenously synthesized prostaglandins is an unstirred layer around the RPCT cells.

PGE_2 inhibition of AVP-stimulated cAMP biosynthesis. The observed biosynthesis of cAMP by RPCT in response to AVP plus PGE_2 is approximately 40% less than the calculated amount of biosynthesis expected based on the assumption that the responses to AVP and PGE_2 along should be additive. This non-additive response, however, did not occur at a concentration below 10^{-6} M PGE_2. 10^{-6} M PGE_2 is three orders of magnitude greater than level of PGE_1 which inhibited AVP-stimulated water resporption in the cortical collecting tubule. This discrepancy in sensitivity in these systems remains to be determined.

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