REGULATION OF CYCLIC AMP METABOLISM BY PROSTAGLANDINS IN RABBIT CORTICAL COLLECTING TUBULE CELLS

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ABSTRACT

REGULATION OF CYCLIC AMP METABOLISM BY PROSTAGLANDINS IN RABBIT CORTICAL COLLECTING TUBULE CELLS

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In the rabbit cortical collecting tubule (RCCT), prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) at 1 n$\underline{M}$ inhibit arginine-vasopressin (AVP)-induced water reabsorption, while 100 n$\underline{M}$ PGE_1 and PGE_2 alone stimulate water reabsorption. Reported here are studies designed to investigate the molecular basis for the biphasic physiological action of PGE_1 and PGE_2 in the collecting duct. RCCT cells were isolated by immunoadsorption using a monoclonal antibody reactive to an ectoantigen unique to the collecting tubule.

In freshly isolated RCCT cells, PGE_1, PGE_2, and 16,16-dimethyl-PGE_2 (DM-PGE_2) stimulated cAMP synthesis at concentrations ranging from 0.1 to 10 $\mu\underline{M}$. Other prostaglandins including the synthetic PGE_2 analogue, sulprostone, failed to stimulate cAMP synthesis. Moreover, sulprostone did not antagonize PGE_2-stimulated cAMP formation. In contrast, PGE_2 and sulprostone at concentrations ranging from 1 to 100

nM, inhibited AVP-induced cAMP accumulation in freshly isolated RCCT cells. PGE_2, PGE_1, DM-PGE_2 and sulprostone at 100 nM were equally effective in inhibiting AVP-induced cAMP formation. Pertussis toxin pretreatment of RCCT cells blocked the inhibitory action of both PGE_2 and sulprostone, suggesting that this response is mediated by the inhibitory guanine nucleotide-binding regulatory protein, G_i. Moreover, sulprostone inhibited AVP-stimulated adenylate cyclase activity. These results suggest that PGE derivatives mediate either inhibition or activation of adenylate cyclase by stimulating different PGE receptors.

To further test this concept, PGE_2 binding to freshly isolated RCCT cell membranes was characterized. PGE_2 binding was saturable, specific, and reversible. Two different classes of PGE_2 binding were detected. [^{3}H]PGE_2 binding to the high affinity class of sites (Kd=10 nM) was increased by the GTP-analogue, GTPYS, while pertussis toxin pretreatment blocked the stimulatory action. In contrast, [^{3}H]PGE_2 binding to the low affinity class of sites (Kd=100 nM) was decreased by GTPYS; this inhibitory effect was not blocked by pertussis toxin pretreatment.

These results support our concept that RCCT cells express two different PGE receptors: (1) a high-affinity PGE/sulprostone receptor linked to the direct inhibition of adenylate cyclase through G_i, and (2) a low affinity PGE receptor coupled to the activation of adenylate cyclase mediated by the stimulatory guanine nucleotide-binding protein, G_s.

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ABBREVIATIONS

Abbreviations are: PG, prostaglandin; AVP, arginine-vasopressin; CA, calcitonin; PTH, parathyroid hormone; BK, bradykinin; IBMX, 3-isobutyl-1-methylxanthine; RO 20-1724, (±)-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; carbacyclin, 6-carbaprostaglandin I_2; sulprostone, 16-phenoxy-17,18,19,20-tetranor-PGE_2 methylsulfonylamide; PGE_2, PGE_1, $PGF_{2\alpha}$, and PGI_2, prostaglandins E_2, E_1, $F_{2\alpha}$ and I_2; [^{3}H]PGE_2, [5,6,8,11,12,14,15(N)-tritium]PGE_2; cAMP, adenosine 3',5'-cyclic monophosphate; DM-PGE_2, 16,16-dimethyl-prostaglandin E_2.

INTRODUCTION

Nearly twenty years ago, Grantham and Orloff demonstrated that PGE_1 at around 1 nM inhibits arginine-vasopressin (AVP)-induced water reabsorption in the rabbit cortical collecting tubule (93). They also found that 100 nM PGE_1 by itself stimulates water reabsorption. Subsequent studies have shown that animals pretreated with cyclooxygenase inhibitors excrete a hypertonic urine (155,156). In addition, stimulation of endogenous PGE_2 synthesis in isolated cortical collecting tubule segments with peptide hormones (90,96) or arachidonate (95) inhibited AVP-induced water reabsorption. These results support the general view that prostaglandins function *in vivo* to attenuate the hydroosmotic response stimulated by AVP in the cortical collecting duct.

The molecular mechanisms explaining the physiological actions of PGE derivatives in the collecting tubule are not precisely known. Since water reabsorption is mediated by cAMP (94), it seems plausible that PGE stimulates water reabsorption by increasing cellular cAMP levels by activating adenylate cyclase. In fact, PGE_2 does stimulate cAMP formation in collecting tubule cells from the rabbit, rat and dog (87,89,132,144).

A mechanistic explanation for the inhibitory action of PGE_2 on water reabsorption has proven to be difficult to establish, and the results have often seemed contradictory (87,119,120,138,144). PGE_2

inhibits AVP-stimulated cAMP accumulation in the rat cortical collecting tubule only in the absence of the cAMP phosphodiesterase inhibitor, IBMX (119,120), suggesting that PGE_2 may serve to activate a cAMP phosphodiesterase. However, there is preliminary evidence suggesting that PGE_2 inhibits adenylate cyclase activity in cultured rabbit cortical collecting tubule cells (138). Garcia-Perez and Smith observed that AVP-stimulated cAMP synthesis is inhibited in canine cortical collecting tubule cells, but only after a short pretreatment (10-20 minutes) with PGE_2 (87), suggesting that a heterologous desensitization mechanism may be involved (129-131). Finally, PGE_2 does not inhibit cAMP synthesis stimulated by AVP in cultured papillary collecting tubule cells (144). These discrepant results suggest that the PGE_2-inhibitory action on cAMP formation may be mediated by a number of different pathways.

Reported here are studies designed to explore the molecular mechanisms by which PGE_2 regulates cAMP metabolism in rabbit cortical collecting tubule cells. The lack of progress in determining the mechanism of action of PGE_2 in the collecting tubule is due, in part, to inadequate procedures for rapidly isolating large, homogeneous numbers of collecting tubule cells from the rabbit kidney cortex. We have developed an immunoadsorption technique for isolating collecting tubule cells from a heterogeneous mixture of collagenase-dispersed rabbit kidney cortical cells. Our results indicate that PGE_2: (a) inhibits AVP-stimulated cAMP accumulation at low concentrations by inhibiting adenylate cyclase, mediated by the inhibitory guanine nucleotide-binding regulatory protein of adenylate cyclase (G_i), (b) at high concentrations stimulates cAMP synthesis, probably by

activating adenylate cyclase mediated by the stimulatory guanine nucleotide-binding regulatory protein of adenylate cyclase (G_s), and finally (c), the inhibitory and stimulatory effects on cAMP synthesis appear to be mediated by two different receptors specific for E-series prostaglandins that are functionally coupled to G_i and G_s, respectively.

LITERATURE REVIEW

The intent of this review is first, to provide a summary of prostaglandin biochemistry, and second, to focus on aspects of prostaglandin biosynthesis and function pertaining to the regulation of water reabsorption and NaCl transport in the collecting duct and thick ascending limb of the mammalian kidney. The concepts that prostaglandins (a) are synthesized at or near their sites of action and (b) elicit these physiological responses by binding to specific prostaglandin receptors will also be developed. Since relatively little is known about transmembrane signaling pathways involving prostaglandin receptors, I will draw from the larger body of information on signal transduction processes coupled to other receptors, particularly those pathways involved in regulating cAMP metabolism.

Prostaglandin Biochemistry. The prostaglandins and thromboxanes, collectively known as prostanoids, are a family of oxygenated derivatives of 20-carbon polyunsaturated fatty acids. All prostaglandins contain a cyclopentane ring; the major differences among these derivatives arise from the nature and position of the oxygen-containing substituents in the ring, and are denoted by the letters A-I (1). In contrast to the prostaglandins, thromboxane A derivatives contain an oxane-oxetane moiety in place of the cyclopentane ring (1,2). In aqueous solutions, under physiological

conditions, the oxetane ring is readily hydrolyzed to form the hemiacetal derivative thromboxane B (2). In general, prostanoids contain either one, two or three carbon-carbon double bonds and are denoted by a numeric subscript (1).

In mammalian tissues, the most common substrate is 5,8,11,14 eicosatetraenoic acid, commonly named arachidonic acid, which is found primarily esterified at the sn-2 position of membrane phosphoglycerides (3). Prostaglandins synthesized from this precursor contain one cis and one trans carbon-carbon double bond at carbon atoms 5 and 13, respectively. Under certain dietary conditions (4), or in some cells and organs (5,6), 8,11,14 eicosatrienoic acid, 5,8,11,14,17 eicosapentaenoic acid or 7,10,13,16 docosetetraenoic acid replaces arachidonic acid as the eicosanoid precursor for prostaglandin biosynthesis.

Prostaglandin biosynthesis can be described in three steps (Fig. 1). In the first step, hormonal stimulation of cell surface receptors triggers the release of arachidonate from membrane phosphoglycerides by the action of phospholipases (7). This is considered an important regulatory step since the concentration of free arachidonate in the cell (<1 μM) is considerably lower than that required for prostaglandin formation.

The mechanism for arachidonate release is not clearly known. Arachidonate release and subsequent TxA_2 synthesis has been studied most extensively in platelets. In early work on the release mechanism, it was believed that arachidonate was hydrolyzed from phosphatidylinositol (PI) via the dual actions of PI-specific phospholipase C and a diacylglycerol lipase (7-9,10,11). However it

Figure 1. Prostaglandin biosynthetic pathway.

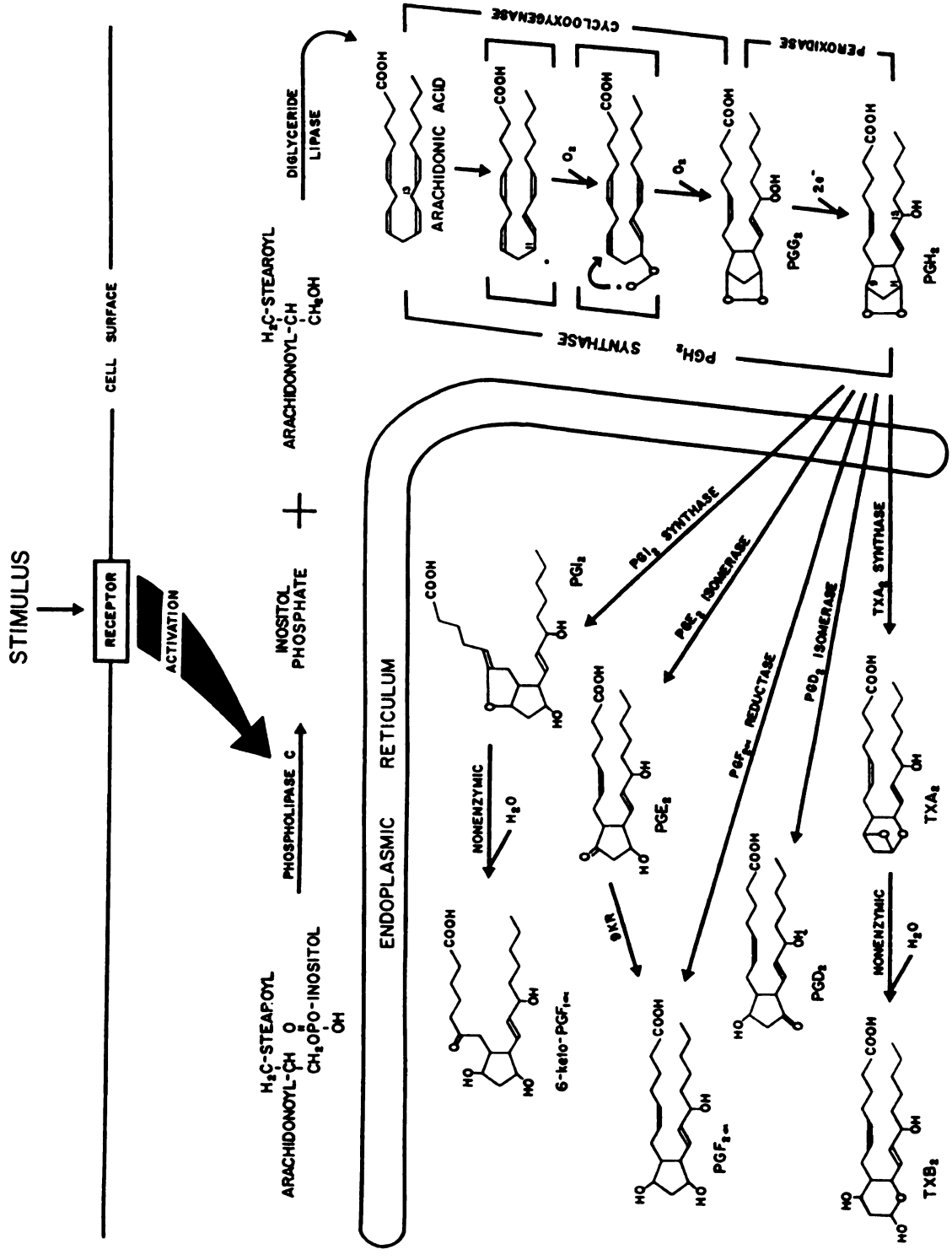
STIMULUS
RECEPTOR
CELL SURFACE
ACTIVATION
INOSITOL PHOSPHATE
PHOSPHOLIPASE C
DIGLYCERIDE LIPASE
ARACHIDONOYL-CH O CH2OPO-INOSITOL OH
H2C-STEAROYL ARACHIDONOYL-CH CH2OH
ENDOPLASMIC RETICULUM
CYCLOOXYGENASE
PEROXIDASE
PGH2 SYNTHASE
ARACHIDONIC ACID
PGG2
PGH2
PGI2
PGI2 SYNTHASE
PGE2 ISOMERASE
PGF2α REDUCTASE
PGD2 ISOMERASE
TXA2 SYNTHASE
NONENZYMIC
6-keto-PGF1α
PGE2
PGF2α
PGD2
TXA2
TXB2

Figure 1.

was later determined that arachidonate was also released from phosphatidylcholine (PC), suggesting that phospholipase A_2 is activated (10-12).

Many hormones that induce prostaglandin biosynthesis (13-19) also stimulate polyphosphoinositide turnover and inositol trisphosphate release (20-25) via activation of a phospholipase C with substrate specificity for phosphatidylinositol-4,5-bisphosphate (27-30). Release of inositol trisphosphate subsequently results in transient increases in cytosolic free calcium (27,31-33). Therefore it is possible that calcium mobilization may play an important role in stimulating arachidonate release, since both phospholipase C and phospholipase A_2 are activated by calcium (34). However, there is also evidence suggesting that agonist-induced arachidonate release may be mediated by receptors linked to the direct activation of phospholipase A_2 in some cells (35,36).

In the second step, the 15-hydroxy-prostaglandin endoperoxide, PGH_2, is formed from one equivalent of arachidonate and two equivalents of molecular oxygen by the action of the enzyme PGH synthase. PGH synthase is a membrane-bound protein (39,43), and is associated with the endoplasmic reticulum in most cells (44-46). There are two separate catalytic activities associated with the PGH synthase enzyme (37-39): (a) a cyclooxygenase activity which catalyzes the bis-dioxygenation reaction, forming the 15-hydroperoxy-prostaglandin endoperoxide, PGG, and (b) a hydroperoxidase activity which catalyzes the two-electron reduction of the 15-hydroperoxy-moiety of PGG to form PGH. Both the cyclooxygenase and hydroperoxidase activities copurify (38,39) and can be precipitated using

monoclonal antibodies (40). Moreover, both activities require heme (38,48,49). The PGH synthase enzyme is made up of a single polypeptide chain (41,42), indicating that the two different catalytic activities reside in the same molecule.

A group of drugs known as nonsteroidal anti-inflammatory agents are all competitive inhibitors of the cyclooxygenase, but not the peroxidase component (42,50,51). Some drugs, such as aspirin, flurbiprofen and indomethacin irreversibly inhibit cyclooxygenase (52,53). Of the three agents, only aspirin covalently reacts with cyclooxygenase, acetylating a serine residue at or near the catalytic site (42). The observation that acetylation does not correlate well with aspirin induced inactivation of the enzyme (54), and that both flurbiprofen and indomethacin do not covalently modify the protein (55) suggests that a change in the active-site of cyclooxygenase occurs prior to acetylation. Drugs such as ibuprofen are simple reversible inhibitors that compete with fatty acids for the binding site on the enzyme (51). Other agents with anti-oxidant properties, such as acetaminophen, at high concentrations inhibit cyclooxygenase activity by trapping free radical intermediates generated during catalysis (56).

In the third step, the formation of biologically active prostaglandins is catalyzed by the action of a number of different enzymes, for which PGH_2 serves as the substrate. Several of these enzymes have been purified and characterized.

The formation of PGD_2 from PGH_2 is catalyzed by PGH-PGD isomerases (69). PGD_2 is also formed nonenzymatically in aqueous solutions from PGH_2. Different forms of this enzyme have been

purified from rat spleen and rat brain (57,58). The enzyme isolated from rat spleen requires reduced glutathione for PGD_2 formation (58), whereas the brain enzyme requires no cofactor (57). Unlike all other prostaglandin forming enzymes, PGH-PGD isomerases are soluble proteins. It is worth noting that other proteins, such as serum albumin (57), also catalyze the formation of PGD_2 from PGH_2. Moreover, it is not clear if these enzymes are present in cells that contain PGH synthase, which catalyzes the synthesis of PGH_2 precursor. Therefore it is not certain if these proteins catalyze the formation of PGD_2 *in vivo*.

PGH-PGE isomerases catalyze the formation of PGE_2 from PGH_2. A number of distinct forms of this enzyme have been identified in microsomes prepared from sheep vesicular glands (43) and rabbit renal collecting tubule cells (59). The enzymatic formation of PGE is stimulated by reduced glutathione (86). Like PGD_2, PGE_2 is also formed nonenzymatically from PGH_2 in aqueous solution at pH 7.4.

PGI_2 is a potent platelet antiaggregatory and smooth muscle relaxing substance formed in vascular endothelial cells, and in both vascular and nonvascular smooth muscle by the action of PGI_2 synthase (47,60). This enzyme has been purified to apparent electrophoretic homogeneity from both bovine and porcine aorta (61,62). PGI_2 synthase is a monomeric protein with a molecular weight of 50,000 daltons. The protein requires heme for activity and is susceptible to inactivation by lipid hydroperoxides. PGI_2 is unstable in physiological solutions; the enol ether bond is readily hydrolyzed forming the biologically inactive metabolite, 6-keto-$PGF_{1\alpha}$.

In contrast to PGI_2, TxA_2 is a potent platelet proaggregatory and smooth muscle constricting substance (63,64). TxA_2 formation is

catalyzed by TxA synthase which is found in high concentrations in platelets, macrophages and lung (69). Porcine TxA synthase has been purified to apparent electrophoretic homogeneity, and like PGI_2 synthase, is a heme-containing enzyme (65,66). As mentioned previously, TxA_2 is rapidly hydrolyzed in aqueous solution ($t_{1/2}$ = 30 seconds at 37°) to form the less biologically active product, TxB_2.

Unlike other prostaglandins formed from PGH_2, $PGF_{2\alpha}$ biosynthesis requires a two-electron reduction of PGH_2. No heat labile activity catalyzing $PGF_{2\alpha}$ formation has been detected (69). In addition, the cofactor for this reaction is unknown. $PGF_{2\alpha}$ may be synthesized from PGE_2 *in vivo* by reduction of the 9-keto group catalyzed by a 9-keto prostaglandin reductase (67). Considerable quantities of $PGF_{2\alpha}$ are present in renal venous blood and urine (68).

Prostaglandins are rapidly and efficiently catabolized to inactive forms (69). The first step in the degradation of prostaglandins is the oxidation of the hydroxyl group at C-15 to form the 15-keto derivative, catalyzed by an NAD-dependent 15-hydroxy prostaglandin dehydrogenase localized largely in the lung and kidney (69). These prostaglaninds are further catabolized by the action of a reductase that catalyzes the reduction of the carbon-carbon double bond at C-13. NADPH is the electron donor for this reaction. Other prostaglandin catabolizing enzymes include 9-hydroxyprostaglandin dehydrogenase and prostaglandin E-9-keto-reductase (69). The resulting products catalyzed by these enzymes undergo both β-oxidation and ω-oxidation in the liver, and are excreted by the kidney (69).

Prostaglandin Biosynthesis and Compartmentation in the Kidney. Measurements of PGE_2 and $PGF_{2\alpha}$ entering the renal artery indicate that the concentrations of these prostaglandins are too low to account for their physiological actions in the kidney (68). Studies reported by Ferreira and Vane indicate that PGE_2 and $PGF_{2\alpha}$ do not accumulate in the arterial blood, but are rapidly degraded to biologically inactive metabolites (70). These observations support the broader view that prostaglandins are not circulating hormones, but are autacoids, or hormone-like substances targetted at or near their sites of synthesis (70,71). In light of this concept, determination of the specific cellular locations of prostaglandin synthesis might also predict the sites of action and possible physiological roles of these metabolites in various segments of the nephron.

The cellular sites of prostaglandin formation have been investigated using either histochemical (72) or immunocytochemical (45,73-75) techniques designed to localize the PGH synthase enzyme, or by measuring prostaglandin synthesis in microdissected nephron and vascular segments (18,47,76,77) or isolated cells (78-81). Based on these studies, prostaglandins are formed by seven different cell types in the kidney (Fig. 2). These include vascular endothelial and smooth muscle cells, glomerular mesangial and epithelial cells, medullary interstitial cells, thin descending limb cells, and collecting tubule cells. There is also some evidence indicating that the medullary thick ascending limb forms PGE_2 (79,81). However, it is clear that the cells with the greatest capacity to synthesize prostaglandins are collecting tubule cells, particularly those localized in the medullary portion (73,75).

Figure 2. Cellular sites of prostaglandin biosyntheis in the mammalian nephron (G, glomerulus; EA, efferent arteriole; AA, afferent arteriole; IA, intralobular artery; PCT, proximal convoluted tubule; PR, pars recta; DTL, descending thin limb; ATL, ascending thin limb; MTAL, medullary thick ascending limb; CTAL, cortical thick ascending limb; DCT, distal convoluted tubule; CCT, cortical collecting tubule; MCT, medullary collecting tubule; PCT, papillary collecting tubule; IC, interstitial cells). The darkened areas represent sites of prostaglandin biosynthesis which include: vascular smooth muscle and endothelial cells, glomerular mesangial and epithelial cells, interstitial cells, collecting tubule cells and cells of the ascending and descending thin limbs.

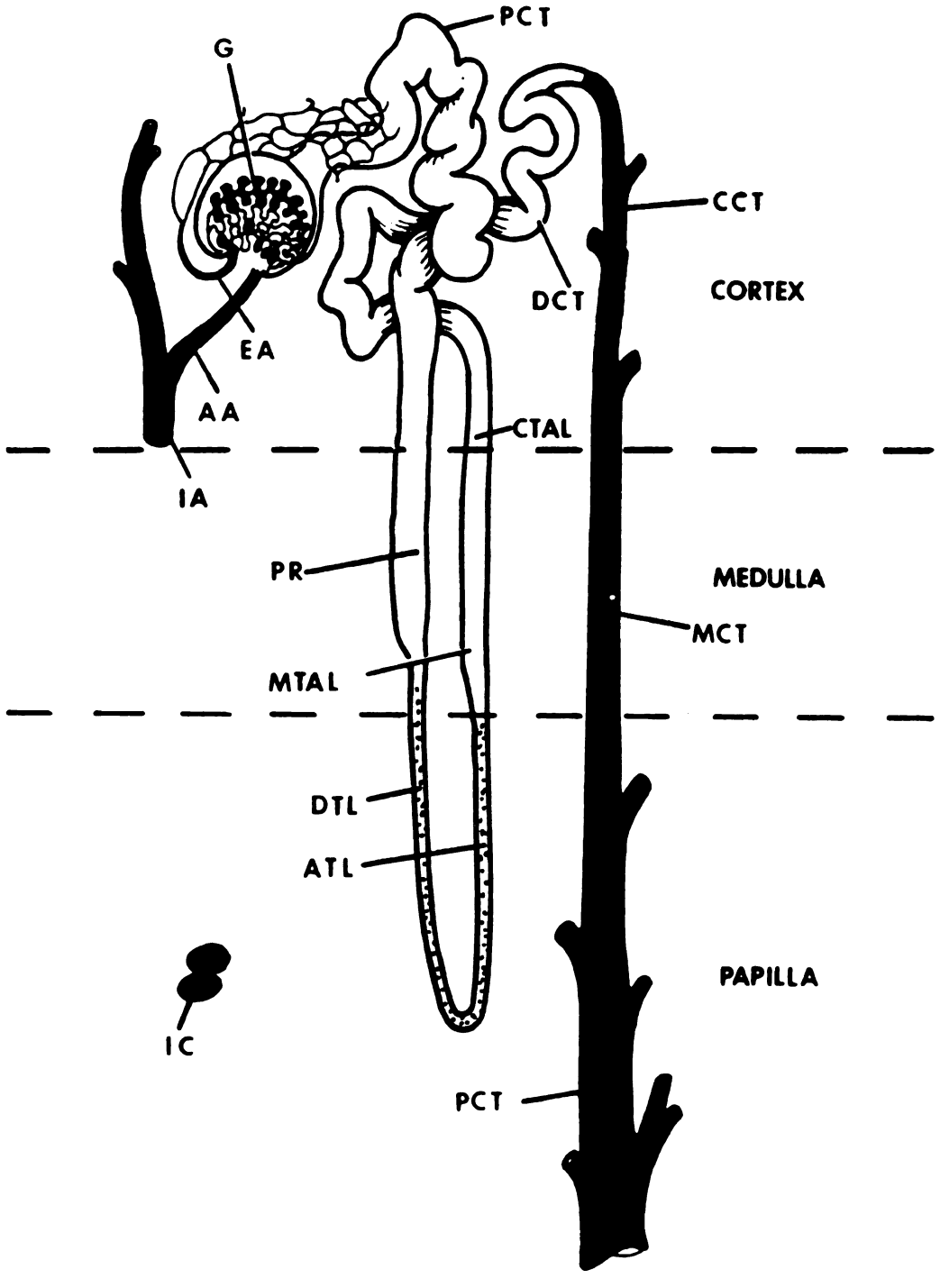
PCT
G
CCT
CORTEX
DCT
EA
AA
CTAL
IA
PR
MEDULLA
MCT
MTAL
DTL
ATL
PAPILLA
IC
PCT

Figure 2.

The major prostaglandin derivative produced by renal cyclooxygenase-containing cells is PGE_2 (76,78,80,82). However, substantial amounts of other prostaglandins are also formed. Glomerular mesangial and epithelial cells produce PGE_2, and small amounts of 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$ and TxB_2 (80,82). Kirschenbaum et al. observed that microdissected rabbit cortical collecting tubule segments synthesize small amounts of 6-keto $PGF_{1\alpha}$, TxB_2 and $PGF_{2\alpha}$ in addition to PGE_2 (18). Using homogenates of freshly isolated rabbit papillary collecting tubule cells, Grenier and Smith also observed that 6-keto $PGF_{1\alpha}$ was synthesized in addition to PGE_2, using arachidonate as the substrate (78). In fact using low concentrations of arachidonate (<2 μM), 6-keto $PGF_{1\alpha}$ was the major product. It was later determined that PGE_2 synthesis was enzymatic and required reduced glutathione (19). However, monoclonal antibodies that react with putative PGH-PGE isomerases from ovine seminal vesicles did not cause immunocytochemical staining in collecting tubule epithelia (83). The reasons for this result are not clear; however, it is possible that the concentration of the enzyme in this tissue is too low to detect, or perhaps that there are tissue-specific PGH-PGE isomerases.

Even though 6-keto-$PGF_{1\alpha}$ is formed in rabbit collecting tubule cells, monoclonal antibodies that specifically react with PGI_2 synthase failed to stain collecting tubule epithelia in tissue sections where staining was clearly visible in capillary endothelial and smooth muscle cells (75). We speculate that the collecting duct synthesizes both PGE_2 and small amounts of PGI_2 concurrently. PGI_2 in turn may "signal" the endothelial cells in the adjacent vasculature that prostaglandins are being formed by the collecting tubule. The

concept that prostaglandins may mediate intercellular communication among medullary tubule, endothelial and interstitial cells in order to coordinate different renal transport processes was first proposed by Stokes (84).

A number of different hormones stimulate prostaglandin synthesis in the kidney. For example, angiotensin II stimulates prostaglandin synthesis in the glomerulus (85), medullary interstitial cells (15,16) and papillary collecting tubule cells (19).

Kinins (e.g. bradykinin, lysyl-bradykinin) also stimulate prostaglandin release in endothelial cells (86), medullary interstitial cells (15,16) and collecting tubule cells (17,19,87-90). Interestingly, bradykinin appears to stimulte PGE_2 release in the rabbit cortical collecting tubule only when added to the basolateral side (90). In canine cortical collecting tubule cells, bradykinin stimulates PGE_2 synthesis only when added to the apical side (87).

The antidiuretic hormone, arginine-vasopressin (AVP) also stimulates prostaglandin release in medullary interstitial cells (15,16) and cortical collecting tubule cells (17,87). There is some evidence suggesting that AVP-induced water reabsorption and AVP-stimulated PGE_2 release in the collecting tubule are mediated by different receptors. For example, AVP induces PGE_2 release when added to either the apical or basolateral side of canine cortical collecting tubule cell monolayers (87). However, AVP will only stimulate cAMP synthesis when added to the basolateral side (87). Moreover, AVP-stimulated PGE_2 release was inhibited by the specific pressor antagonist d-cyclo-o-methyl-tyrosine-arginine vasopressin in rat

medullary slices (91,92), suggesting that a "pressor-like" V_1-vasopressin receptor mediates prostaglandin release.

Physiological Action of Prostaglandins in the Kidney. The physiological actions of PGE_2 on tubular transport functions in the renal nephron are largely localized to the thick ascending limb of Henle's loop and the collecting duct. Direct effects of PGE_2 on tubular function have been studied using microdissected perfused nephron segments. Grantham and Orloff demonstrated that low concentrations of PGE_1 inhibit water reabsorption stimulated by AVP in the rabbit cortical collecting tubule (93). Since cAMP is the second messenger that mediates water reabsorption (93,94), it was postulated that PGE_1 blocked the hydroosmotic response by inhibiting cAMP accumulation stimulated by AVP (93,94). Curiously, high concentrations of PGE_1 alone also stimulated water reabsorption, and addition of the cAMP phosphodiesterase inhibitor, theophylline, augmented this response (93). These results suggested that PGE_1 may also stimulate cAMP synthesis.

It was later demonstrated that endogenous PGE_2 synthesis stimulated by addition of arachidonate (95) or bradykinin (90,96) to the bathing solution inhibited AVP-induced water reabsorption in isolated rabbit cortical collecting tubule segments. PGE_2 may also inhibit NaCl reabsorption in the cortical collecting tubule (97,98).

In the rabbit thick ascending limb, PGE_2 inhibits NaCl absorption (99). PGE_2 also inhibits NaCl reabsorption stimulated by AVP in the mouse medullary thick ascending limb (100-102). AVP-induced NaCl reabsorption is also mediated by cAMP (100-102).

G-Proteins Mediating Hormonal Regulation of cAMP. There is growing evidence that guanine nucleotide-binding (G) proteins mediate a variety of different signal transduction pathways that are activated by many hormone receptors (103). Currently, five different G-proteins have been identified: (1) the stimulatory G-protein of adenylate cyclase (G_s), (2) the inhibitory G-protein of adenylate cyclase (G_i), (3) the G-protein mediating light activation of cGMP phosphodiesterase from rod outer segments (transducin), (4) the G-protein mediating hormonal activation of ion channels, and (5) G-proteins mediating hormonal activation of phospholipase(s) that specifically catalyze the phosphodiesteratic cleavage of phosphatidylinositol-4,5-bisphosphate to inositol triphosphate and diacylglycerol (103). In addition, other G-proteins that either directly or indirectly mediate the activation of cAMP phosphodiesterases have also been detected (104-108). The earliest advances in understanding G-protein function were born of studies investigating the stimulation and inhibition of adenylate cyclase.

Agonist-induced activation and inhibition of adenylate cyclase is mediated by receptors that are functionally coupled to guanine nucleotide-binding regulatory proteins. Both stimulatory (G_s) and inhibitory (G_i) G-proteins have been identified and extensively characterized (103).

Both of these proteins are alpha-beta-gamma heterotrimers and are peripherally bound to the cytoplasmic surface of the plasma membrane. The most obvious differences between these proteins are realized by comparing the alpha subunits: G_s-alpha is slightly larger in size (M_r = approximately 45,000 daltons) and is susceptible to

ADP-ribosylation catalyzed by cholera toxin: G_i-alpha is smaller than G_s (M_r = approximately 41,000 daltons) and is susceptible to ADP-ribosylation catalyzed by pertussis toxin (103). The beta/gamma subunits of both of these proteins appear to be identical (M_r, beta = 35,000 daltons; M_r, gamma = 10,000 daltons) and are functionally interchangeable (103). In addition, both G_s and G_i bind and hydrolyze GTP, and require Mg^{++} and GTP for activity (103).

As mentioned above, G-proteins are differentially susceptible to ADP-ribosylation catalyzed by bacterial toxins. Pertussis toxin catalyzes the NAD-dependent ADP-ribosylation of G_i which inactivates the protein (103,111). In some cases, pertussis toxin also blocks phosphoinositide turnover and subsequent calcium mobilization (112), suggesting that pertussis toxin-reactive G-proteins sometimes mediate these signal transduction pathways as well. Cholera toxin catalyzes the ADP-ribosylation of the stimulatory guanine nucleotide-binding regulatory protein (G_s) of adenylate cyclase, which unlike pertussis toxin, irreversibly activates the protein (103,109,110).

G-proteins undergo a regulatory cycle (103). In brief, GTP binds to the alpha subunit promoting the dissociation of alpha- from beta/gamma-subunits, activating the protein. The bound GTP is hydrolyzed, GDP dissociates, and the subunits reassociate to form the inactive heterotrimeric protein (103). Agonist-receptor binding apparently increases the steady-state levels of the activated form of these proteins (103). This regulatory cycle may also serve as a mechanism to prevent persistent hormonal inhibition or stimulation of adenylate cyclase (103).

In the case of G_s, the molecular entity that activates adenylate cyclase is the Mg^{++} and GTP-bound form of the alpha subunit (103). However, the manner in which G_i inhibits adenylate cyclase is not clearly known. Gilman and co-workers have speculated that the dissociated beta/gamma subunit of G_i may indirectly inhibit adenylate cyclase by inactivating the alpha subunit of G_s; an abundance of free beta/gamma subunits would drive the reassociation of free G_s-alpha to form the oligomeric, inactive protein (113). This hypothesis is based on the observations that (a) reconstitution of purified oligomeric G_i mediates hormone-induced inhibition of adenylate cyclase, (b) the non-hydrolyzable GTP analogue, GTPγS induces subunit dissociation and activation of the protein, and (c) purified beta/gamma subunit from G_i inhibits both basal and forskolin-stimulated adenylate cyclase in platelet membranes (114,115). In addition, G_i is present in considerable excess over G_s in many different cell types examined. Therefore, one can envision G_i as a reservoir of beta/gamma subunits that are released when stimulated by the appropriate agonists.

It is important to note, however, that this model is not intended to rule out other possible inhibitory mechanisms. As discussed above, indirect inhibition of adenylate cyclase by hormone-induced "release" of beta/gamma subunits, as proposed by Gilman and co-workers (113-115), requires cellular membranes to contain functional G_s. However, Jakobs and Schultz have shown that somatostatin causes inhibition of forskolin-activated adenylate cyclase in mutants of S49 lymphoma cells (116); these cells do not express functional G_s (116). Moreover, purified beta/gamma subunits which inhibit platelet adenylate cyclase do not inhibit adenylate cyclase in cyc^- membranes

(117). Therefore, inhibition of adenylate cyclase by somatostatin in S49 cell membranes occurs via another mechanism that probably does not involve "release" of free beta/gamma subunits from G_i. However, modest inhibition of forskolin-activated adenylate cyclase is observed when cyc^- membranes are reconstituted with purified, activated alpha subunits of G_i (117). These results suggest that G_i-alpha may directly inhibit adenylate cyclase.

G-proteins also mediate either directly or indirectly, the activation of cAMP phosphodiesterases. For example, muscarinic agonists stimulate cAMP degradation in human astrocytoma cells (104,105) via a pathway that induces calcium mobilization and subsequent activation of a calcium/calmodulin-dependent cAMP phosphodiesterase. A similar pathway activated by a_1-adrenergic agonists has been identified in rat heart ventricular cells (106). All of these pathways appear to be mediated by a G-protein(s) that is insensitive to pertussis toxin (104-106). Stimulation of insulin receptors (107,108) and A_1 adenosine receptors (108) directly activates a particulate cAMP phosphodiesterase in 3T3-L1 adipocytes. These signal transduction pathways appear to be mediated by a pertussis toxin-sensitive G-protein.

Effects of PGE on cAMP Synthesis in the Collecting Tubule and Thick Ascending Limb. AVP induces water reabsorption in the collecting tubule or NaCl reabsorption in the thick ascending limb by stimulating cAMP synthesis (93,94,100-102). Therefore, it is reasonable to postulate that PGE_2 inhibits these responses by blocking intracellular cAMP accumulation. During the last two decades,

appreciable efforts directed at testing this hypothesis have been made (94). The available data indicate that PGE_2 inhibits AVP-stimulated cAMP accumulation; however, the characteristics of these inhibitory actions vary, suggesting that there may be several mechanisms whereby PGE_2 may (a) stimulate cAMP degradation, (b) directly inhibit adenylate cyclase or (c) induce a heterologous desensitization of hormone-activated adenylate cylcase.

Edwards et al. (118) and Torikai and Kurakawa (119) reported that PGE_2 inhibits cAMP accumulation stimulated by AVP in microdissected collecting tubule segments. However, this inhibitory effect was observed only in the absence of the cAMP phosphodiesterase inhibitor, IBMX. These data suggest that PGE_2 may stimulate cAMP degradation in these cells. In fact, PGE_2 stimulates cAMP phosphodiesterase activity in P-815 mastocytoma cells (120).

PGE_2 may induce cAMP degradation by stimulating a subclass of specific PGE_2 receptors linked to signal transduction pathways that are analogous to those coupled to α_1-adrenergic receptors or muscarinic receptors. α_1-Adrenergic agents stimulate cAMP degradation in rat cardiomyocytes by increasing the concentration of cytosolic free calcium, subsequently activating a calcium-calmodulin dependent cAMP phosphodiesterase (106). A similar pathway coupled to muscarinic receptors has also been identified in human astrocytoma cell lines (104,105). Both of these pathways are mediated by guanine nucleotide-binding regulatory proteins that are insensitive to pertussis toxin (104-106).

In addition, PGE_2 mobilizes calcium in glomerular mesangial cells (121) and presumably stimulates calcium mobilization in certain smooth

muscle beds acting via a specific subclass of PGE receptors (122,123). Thus, it is conceivable that PGE_2 may stimulate specific PGE receptors that are functionally linked to an as yet unidentified G-protein that may mediate cAMP phosphodiesterase activation, possibly by promoting calcium mobilization. It should be emphasized that the mechanism presented here is largely speculative. In fact, there are other signal transduction pathways that may activate cAMP phosphodiesterases (107,108). More work is needed to adequately address this possibility.

A second type of inhibition by PGE_2 has been observed mainly in isolated thick ascending limb segments. Torikai and Kurakawa have demonstrated that, in contrast to the rat cortical collecting tubule, PGE_2 inhibits AVP-induced cAMP accumulation in the rat thick ascending limb in the presence of IBMX (119). This result suggests that PGE_2 may directly inhibit adenylate cyclase. Recently, Watanabe et al. have solubilized a PGE_2 receptor from the outer medulla of dog kidney that is physically coupled to a pertussis toxin-reactive G-protein (124), supporting the concept that PGE_2 inhibits adenylate cyclase by stimulating a receptor that is coupled to G_i. In addition, this receptor shares certain unique characteristics -- notably that GTP stimulates PGE_2 binding -- with a PGE_2 receptor identified in hamster adipocytes (125). In hamster and human adipocytes, PGE_2 inhibits catecholamine-stimulated adenylate cyclase (126,127). Consistent with this mechanism are the observations of Culpepper (102). He observed that pertussis toxin blocks the inhibitory action of PGE_2 on NaCl reabsorption stimulated by AVP in mouse medullary thick ascending limb segments. Further studies are required to determine precisely, the

molecular mechanism of the PGE_2-inhibitory action in the thick ascending.

There is some evidence suggesting that PGE_2 may also inhibit adenylate cyclase in the rabbit cortical collecting tubule. Bradykinin inhibits AVP-induced water reabsorption in the rabbit cortical collecting tubule by a prostaglandin-dependent mechanism, and pertussis toxin blocks this inhibitory effect (96). One possible interpretation of this result is that pertussis toxin blocks the inhibition of AVP-stimulated adenylate cyclase by PGE_2. However, pertussis toxin has also been shown to inhibit arachidonate release in platelets (35). Therefore, pertussis toxin may have inhibited the release of PGE_2.

Thus, PGE_2 may block cAMP accumulation in some renal epithelia by stimulating specific receptors functionally coupled to the direct inhibition of adenylate cyclase mediated by G_i.

Another type of inhibition by PGE_2 that is different from the effects seen in microdissected rat tubule segments (119) has been observed in canine cortical collecting tubule (CCCT) cells in culture (87). Garcia-Perez and Smith observed that PGE_2 and $PGF_{2\alpha}$ inhibit AVP-induced cAMP formation in CCCT cells pretreated with the cAMP phosphodiesterase inhibitor, IBMX (87). However, this inhibitory effect was not observed until after a 10-20 minute preincubation. The time-dependent nature of the inhibitory action of PGE_2 is typical of heterologous desensitization of hormone-sensitive adenylate cyclase by PGE_2 in other cell types (128-130). Therefore the results of Garcia-Perez and Smith suggest that the adenylate cyclase system in CCCT cells may undergo analogous regulatory control by PGE_2. In

general, the net effect of heterologous desensitization is to dampen the hormonal activation of adenylate cyclase (128-130). The precise molecular mechanism of this process is not known (130).

In summary, PGE_2 may block cAMP accumulation in collecting tubule and thick limb epithelia by stimulating specific receptors linked to different transmembrane signaling pathways: (1) a PGE receptor coupled to a G-protein mediating activation of cAMP phosphodiesterases and (2) a PGE receptor linked to direct inhibition of adenylate cyclase mediated by G_i. In addition to these receptor-effector coupling pathways, PGE may induce heterologous desensitization of adenylate cyclase via a mechanism that is, as yet, unknown. It is not clear if each of these different pathways are specific for certain species, or if these mechanisms are present only in specific types of renal cells. For example, a PGE receptor linked to activation of cAMP phosphodiesterases may predominate in the rat collecting tubule (118,119), another PGE receptor coupled to G_i mediating the inhibition of adenylate cyclase may predominate in the thick ascending limb of rat (119), mouse (100-102) and possibly dog (124), and heterologous desensitization may be most important in the canine cortical collecting tubule (87). However there is precedent for multiple adenosine receptors coupled to different signal transduction pathways that inhibit hormone-induced cAMP accumulation in cloned 3T3-L1 adipocytes (108).

In addition to inhibitory effects on cAMP accumulation, PGE_2 also stimulates cAMP synthesis in different renal cell types. For example, PGE_2 stimulates cAMP formation in the collecting tubule and thin limb of Henle's loop (131). PGE_2 also stimulates cAMP synthesis in the

cortical and medullary thick ascending limb of rabbit and mouse (81), but not rat (131). Usually the concentration of PGE_2 required to stimulate cAMP synthesis is higher than that needed to inhibit cAMP accumulation induced by other hormones (132).

The stimulatory effect of PGE_2 is probably mediated by specific receptors coupled to G_s. Precedents for this view come from work on a frog erythrocyte PGE receptor (133), where PGE_2 binding is inhibited by GTP, and from work on the human platelet PGI_2/PGE_1 receptor, where PGE_1 stimulates GTPase activity that is sensitive to cholera toxin (134).

Smith and Garcia-Perez (135) have speculated that cAMP synthesized in response to high concentrations of PGE_2 may inhibit hormone-induced prostaglandin synthesis, and there is some recent evidence to support this concept in rabbit papillary collecting tubule cells (136).

Summary. PGE_2 synthesized in the collecting tubule is a physiologically important factor in regulating water and sodium chloride reabsorption. The model presented in Fig. 3 illustrates our current concepts of the molecular mechanism of action of PGE_2 in the renal collecting duct and thick ascending limb. PGE_2 apparently acting via specific receptors linked to distinct transmembrane signaling pathways inhibits NaCl secretion in the thick ascending limb, and water reabsorption in the collecting duct by blunting cAMP synthesis stimulated by AVP. In turn, high concentrations of PGE_2 may inhibit prostaglandin synthesis by stimulating cAMP formation via specific receptors coupled to the activation of adenylate cyclase.

Figure 3. Proposed mechanism of action of PGE_2 in the collecing duct and thick ascending limb. Prostaglandin E biosynthesis is stimulated by hormones, such as bradykinin or AVP, binding to specific receptors linked to arachidonate release in the collecting duct. PGE_2 synthesized by the collecting duct inhibits water reabsorption at this site, or NaCl reabsorption in the medullary thick ascending limb, stimulated by AVP. The proposed mechanisms for these inhibitory actions are to blunt cAMP accumulation by either inhibiting adenylate cyclase activation, or by stimulating cAMP degradation by activating phosphodiesterases. These pathways may be mediated by PGE receptors linked to guanine nucleotide-binding regulatory proteins. Stimulation of cAMP formation by PGE_2 may inhibit further prostaglandin release, presumably by inhibiting arachidonate release. The abbreviations used are: BK, bradykinin; AVP, arginine-vasopressin; V_1, V_1-type vasopressin receptor; V_2, V_2-type vasopressin receptor; G_s, stimulatory guanine nucleotide-binding regulatory protein of adenylate cyclase; G_i, inhibitory guanine nucleotide-binding regulatory protein of adenylate cyclase; G_x, putative G-proteins regulating cAMP degradation.

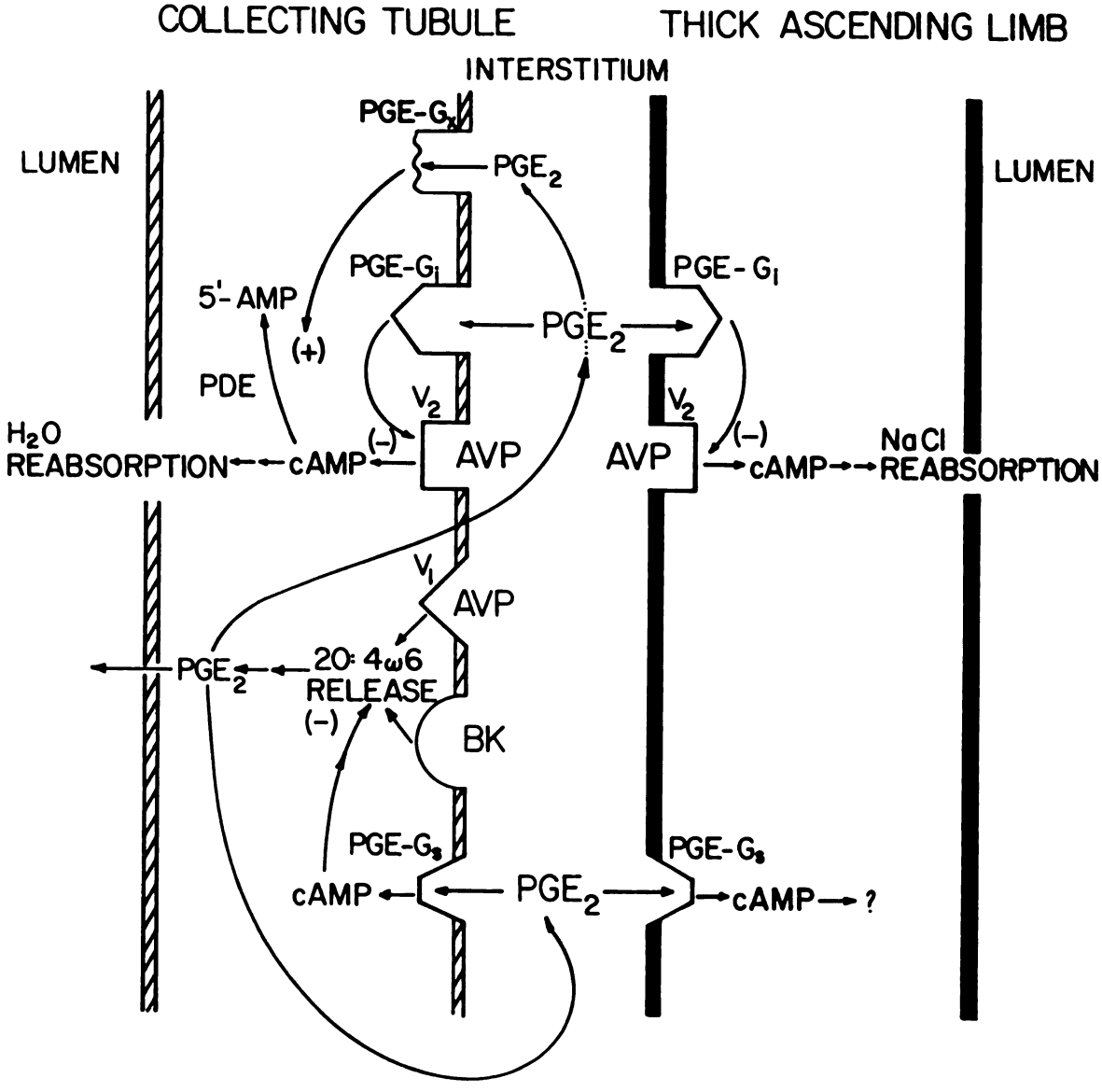
COLLECTING TUBULE
THICK ASCENDING LIMB
INTERSTITIUM
LUMEN
LUMEN
PGE-G$_x$
PGE$_2$
PGE-G$_1$
PGE-G$_1$
5'-AMP
PGE$_2$
(+)
PDE
V$_2$
V$_2$
H$_2$O
REABSORPTION
cAMP
(-)
AVP
AVP
(-)
cAMP
NaCl
REABSORPTION
V$_1$
AVP
PGE$_2$
20:4ω6
RELEASE
(-)
BK
PGE-G$_s$
PGE-G$_s$
cAMP
PGE$_2$
cAMP
?

Figure 3.

These PGE receptors may be linked to guanine nucleotide-binding proteins that mediate these responses, forming a family of PGE receptors analogous to other hormone-receptor families, such as adenosine and catecholamines.

CHAPTER II

IMMUNODISSECTION AND CULTURE OF RABBIT CORTICAL COLLECTING TUBULE CELLS

This chapter describes the preparation of monoclonal antibodies that react specifically with a cell surface antigen in the rabbit renal collecting tubule including the arcades and the use of these antibodies to isolate rapidly large quantities of cortical collecting tubule cells. These cells grow rapidly in culture and retain many of the hormonal, morphological and histochemical properties expected of collecting tubule epithelia. This study demonstrates that immunodissected rabbit cortical collecting tubule (RCCT) cells may serve as a useful model for investigating the molecular mechanisms of actions of prostaglandins in the collecting duct.

METHODS

Materials. Hypoxanthine, penicillin G, streptomycin sulfate, aminopterin, thymidine, fluorescein isothiocyanate (FITC)-labeled peanut lectin, bradykinin triacetate, isoproterenol, calcitonin, bovine serum ablumin, arginine-vasopressin (AVP), parathyroid hormone (PTH), collagenase, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. Trypsin, Dulbecco's modified Eagle's medium (DMEM), collagenase, and fetal bovine serum were purchased from Grand Island Biological Company. PGE_2 and $PGF_{2\alpha}$ were purchased from Cayman Chemical. Prostaglandin D_2 (PGD_2) was from Upjohn Diagnostics. Prostaglandin I_1 (PGI_1) was a gift from Dr. John Pike of Upjohn. NCTC 109 medium was from MA Bioproducts. Normal horse serum was from Flow Laboratories. FITC-labeled rabbit anti-mouse immunoglobulin G (IgG), anti-cAMP, allotype specific rabbit anti-mouse IgG and immunoglobulin M (IgM) sera, and rabbit anti-PGE_2 serum were purchased from Miles Laboratories. Anti-human Tamm-Horsfall serum was from Cappel Laboratories. [^{3}H]PGE_2 (130 Ci/mmol) and ^{125}I-cAMP were from New England Nuclear. Protein A-Sepharose was from Pharmacia. Other chemicals were reagent grade or better purchased from common commercial sources.

Preparation of anti-rabbit collecting tubule monoclonal antibodies. Four-week-old female Swiss mice (Charles Rivers) were immunized intraperitoneally at 2-week intervals with 10^7 rabbit renal

cortical cells (prepared as described below) suspended in 0.2 ml of 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). Three days after the third innoculation, the mice were killed by cervical dislocation and their spleens were removed under sterile conditions. Mouse lymphocyte-myeloma cell fusions were performed as described earlier (139,142) using the SP2/0-Ag14 plasmacytoma developed by Galfre et al. (142) and the resulting hybridomas grown in hypoxanthine, aminopterin, and thymidine (HAT) medium (139).

Media, from wells found by visual inspection to have growing hybridomas, were screened 12-20 days after the cell fusions. Media were tested for the presence of an antibody, which when used in indirect immunocytofluorescence, would cause the staining of collecting tubule cells (17). Cryotome sections from rabbit kidney were prepared as previously described (17,152). Each section was overlayed with 0.3 ml of a 1:2 dilution of a hybridoma medium in phosphate-buffered saline (PBS)(composition in mM: 151 NaCl, 45 KH_2PO_4, and 2.5 NaOH), pH 7.3, and incubated for 30 minutes. the sections were washed to remove excess first antibody, overlayed with a 1:20 dilution of FITC-labeled rabbit anti-mouse IgG in PBS, pH 7.3 and incubated for an additional 30 minutes. The sections were finally washed with PBS, pH 7.3, and examined by fluorescence microscopy using a Leitz Orthoplan microscope. Photomicroscopy was performed with an Orthomat camera using Kodak Tri-X pan film (ASA 400).

Cells from wells containing medium that showed positive collecting tubule cell staining were cloned by limiting dilution (146,151). Ouchterlony double-diffusion analyses were performed to

determine antibody subclass. Hybridoma media (40 µl) were tested against rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM sera (40 µl) in 1.5% agar at 24°C for 16-24 hours (17,139).

Purification of mouse IgG_3 from rct-30 culture medium. As discussed below, the rct-30 hybridoma line secretes a mouse IgG_3 designated [IgG_3(rct-30)]. IgG3(rct-30) was isolated as follows. The rct-30 line was grown in IgG-free hypoxanthine and thymidine (HT) medium (17,139). The medium was adjusted to pH 8.0, and then applied to a protein A-Sepharose column (1x5 cm). The column was eluted stepwise with 0.1 M buffers of pH 8.0 (sodium phosphate) and pH 4.5 and 3.5 (sodium citrate) (139). IgG_3(rct-30) was eluted at pH 4.5. Fractions containing IgG_3(rct-30) were pooled, immediately titrated to pH 8.0, sterilized by filtration, and stored at -20°C. The protein concentration was determined by measuring the absorbance at 280 nm (an absorbance at 280 nm of 1.45 is equivalent to 1 mg/ml (141)).

Preparation of culture dishes coated with IgG_3(rct-30). All procedures were performed under sterile conditions. Each culture dish was incubated for 4 hours at 24°C with 5 ml of 50 mM Tris-HCl, pH 9.5 containing 200 µg of purified IgG_3(rct-30). Immediately before use, the antibody solution was aspirated from the dishes, which were then washed three times with 5 ml of 1% bovine serum albumin in PBS, pH 7.3.

Isolation and culture of RCCT cells. RCCT cells were isolated from kidneys of 3.5 to 4-lb. New Zealand White rabbits killed by intravenous injection of a lethal dose of 7.5% (w/v) sodium pentobarbital solution. Kidneys were removed under sterile conditions and washed free of excess blood. Cortical tissue was carefully dissected

from each kidney and pooled (4-5 g). The tissue was minced with a sterile razor blade, transferred to a 50-ml conical tube containing 25 ml of 0.1% (w/v) collagenase in Krebs buffer, pH 7.3, and incubated at 37°C with constant shaking for 45-60 minutes. After the incubation period, the cell suspension was centrifuged at 200 g for 5 minutes. The pellet was resuspended in 35 ml of PBS. The resulting cell suspension was pipetted through Gelman wire-mesh filter holders and centrifuged again as described above. Finally, the pellet was resuspended in 10 ml of 10% bovine serum albumin in PBS, pH 7.3, and the cells were collected by centrifugation.

The pellet from the last centrifugation step was resuspended in PBS, pH 7.3, to yield a final volume of 6 ml. Aliquots (1 ml) of the cell mixture were transferred to six tissue culture dishes that were previously coated with IgG_3(rct-30) and incubated for 1-3 minutes at 24°C. The dishes were washed 3-5 times with PBS, pH 7.3, to remove nonadherent cells. Cells remaining bound to the culture dishes were grown in RCCT cell medium (DMEM) medium containing 10% fetal bovine serum; 165 U penicillin G/ml; 70 U streptomycin sulfate/ml; and 2 mM glutamine) at 37°C under a water-saturated 7% CO_2 atmosphere. When the cells had reached confluency, typically after 4-5 days in culture, they were detached from the plates by treatment for 30 minutes with 0.05% (w/v) ethylenediaminetetraacetic acid (EDTA) in Krebs buffer, pH 7.3 without calcium or magnesium. The cells were then gently washed from the plate, collected by centrifugation, and then resuspended in RCCT media at a concentration of 10^5 cells/ml. The cells were then seeded into 24-well culture dishes at a density of 10^5 cells/well ($4x10^4$ cells/mm^2). The cells were cultured for 3-5 days prior to

performing experiments, during which the medium was changed once. Experiments involving measurements of cAMP or PGE_2 formation were carried out using passaged cells grown in 24-well culture dishes.

Preparation of cloned hybridoma cell line (rct-30A). Confluent monolayers of RCCT cells grown in 100 mm plastic culture dishes were removed by treatment with 0.1% trypsin and 0.05% EDTA in PBS, pH 7.3, were injected intraperitoneally on days 1, 14, and 28 into a 4-week-old female Swiss mouse. Three days after the third immunization, splenocytes from the immunized mouse were fused with SP2/0-Ag14 myeloma cells as described above. Culture medium from each well containing hybridoma colonies was screened by indirect immunofluorescence for specific staining of rabbit kidney and cultured RCCT cells essentially as described above. Hybridomas from positive wells were cloned by limiting dilution.

Hormone-induced PGE_2 release. All treatments were done in triplicate using cells grown in 24-well culture dishes. The medium was aspirated from each well, and the cells were washed once with Krebs buffer, pH 7.3. Cells were treated with various hormones or effectors in Krebs buffer, pH 7.3, and incubated for 1 hour at 37°C under a water-saturated 7% CO_2 atmosphere. Following treatment, the medium was removed from each well and PGE_2 levels were determined by radioimmunoassay as previously described (59).

Hormone-induced cAMP formation. All treatments were performed in triplicate using cells grown in 24-well culture dishes. Culture medium was removed by aspiration, and the cells were washed once with Krebs buffer, pH 7.3 at 37°C under a water-saturated 7% CO_2 atmosphere. After pretreatment, the media were aspirated and the cells were

treated with the same buffer containing various hormones and effectors at 24°C for 10 minutes. Treatments were terminated by adding an equal volume of 5% (w/v) trichloroacetic acid. The cells were quick-frozen, then thawed and allowed to stand at 4°C for at least 60 minutes. The samples were extracted three times with 5 volumes of water-saturated ether, and the residual ether was evaporated under a stream of N_2 for 5 minutes at 24°C. Samples were assayed for cAMP by radioimmunoassay as described by Frandsen and Krishna (140).

Protein determination. Following removal of media for cAMP and PGE_2 determinations, any remaining liquid was aspirated from each well and the cell protein was solubilized from a small volume of a solution containing 2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, and 1% sodium dodecyl sulfate. After 24 hours at 24°C, aliquots were assayed for protein using a modification of the Lowry method (145).

Statistical analyses. In each experiment, a completely random analysis of variance was used to test for differences among treatment groups at $P<0.05$. The Student-Newman-Keuls test was used, when applicable, to compare treatment means. All values are expressed as the means ± standard error (SE).

Flow cytometric analysis of peanut lectin binding to RCCT cells. Confluent primary or confluent first-passage RCCT cells were detached from tissue culture dishes by treatment with a solution containing 0.1% trypsin and 0.05% EDTA, pH 7.3. The cells were collected by centrifugation and resuspended in a 1:20 dilution of FITC-labeled peanut lectin in 1.5 ml of PBS, pH 7.3, and incubated for 20 minutes at 24°C. Cells were again collected by centrifugation and resuspended in PBS, pH 7.3. Control cells were incubated in PBS, pH 7.3 in the

Table 1

Properties of Mouse Monoclonal Antibodies Reactive with Collecting Tubules[a]

Hybridoma	Mouse Antibody Type	Reactivity with Tubule Segments from Different Species					
		cow	dog	guinea pig	mouse	rabbit	rat
rct-10	IgM	N.D.[b]	N.D.	N.D.	N.D.	CCT	N.D.
rct-30	IgG_3	N.D.	N.D.	CCT MCT CNT MTAL	N.D.	CCT MCT	MCT
rct-40	IgG_1	N.D.	N.D.	CCT MCT MTAL TLH	N.D.	CCT MCT	CCT MCT

[a] Determined by indirect immunofluorescence as described in the text.

[b] Abbreviations: N.D., none detected; CCT, cortical collecting tubule; MCT, medullary collecting tubule; CNT, connecting tubule; MTAL, medullary thick ascending limb; TLH, thin limb of Henle.

absence of lectin. The resulting cell populations were analyzed by flow cytometry using an Ortho Cytofluorograph 50 H. Fluorescence measurements were obtained using an argon ion laser at 0.2 W at 488 nm.

Electron microscopy. RCCT cells were isolated and grown to confluency. The cells were fixed for 24 hours at 4°C with 2% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2. Cells were washed, postfixed with OsO_4, and embedded as previously described (17). The sections were counterstained with 2% uranyl acetate in water, examined, and photographed using a Phillips model 201 transmission electron microscope.

RESULTS

Preparation of monoclonal antibodies to rabbit collecting tubules. Spleens from mice immunized with rabbit kidney cortical cells were fused with SP2/0-Ag14 plasmacytoma cells. Media from each of 768 wells containing growing hybridomas were assayed for the presence of an antibody that reacts specifically with the collecting duct of rabbit kidney by indirect immunofluorescence microscopy. Cells from three wells gave a positive response to this test, and were cloned. The clones were designated rct-10, rct-30 and rct-40. Table 1 summarizes the immunochemical properties of the immunoglobulins secreted by each of these clones. The specificities of the antibodies secreted by each clone were different, suggesting that each antibody is directed against a different antigen. The immunoglobulin secreted by rct-30 was used to stain sections of rabbit kidney medulla (Fig. 4A). Fluorescence staining was most intense on the basolateral surfaces of the collecting tubule. It is not certain whether this antibody is directed against an intrinsic membrane protein or a component of the extracellular matrix. The antibody secreted by rct-30 stained all cells of the cortical and medullary collecting tubule including the arcades (Fig. 4B), and therefore, did not discriminate between principal and intercalated cells. The tubular sites that stained with rct-30 were identified based on cell morphology, staining distribution in the medullary rays, and

Figure 4. Fluorescence photomicrographs of rabbit kidney sections stained with culture medium from hybridoma cell line, rct-30 (X180). Cells were stained as described in the text. Fluorescent-stained cells are collecting tubule epithelia in (A) medulla and (B) cortex of rabbit kidney.

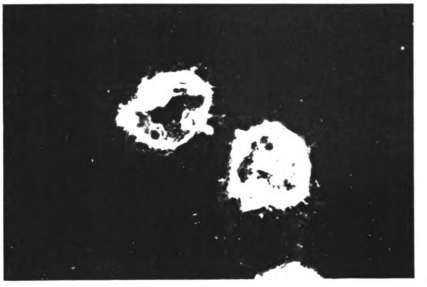

Figure 4A.

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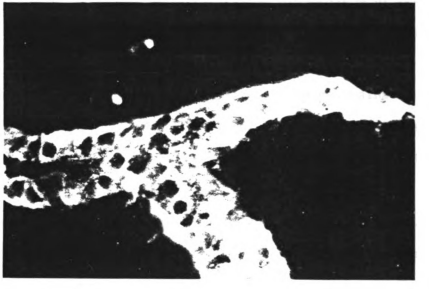

Figure 4B.

coincidence of immunofluorescent staining with histochemical staining for both NADH diaphorase (148) and PGH synthase antigenically (152).

The immunoglobulin secreted by rct-30 was determined to be a mouse IgG_3 using Ouchterlony double diffusion analyses of culture medium against subclass and allotype specific sera. The hybridoma rct-30 grew more rapidly and secreted more immunoglobulin than either rct-10 or rct-40. Purification of IgG_3(rct-30) was accomplished by selectively eluting the immunoglobulin from a Protein A-Sepharose column using 0.1 M sodium citrate, pH 4.5. The purified IgG_3(rct-30) was used to isolate rabbit cortical collecting tubule epithelia.

Isolation of rabbit cortical collecting tubule (RCCT) cells. The procedure for isolating RCCT cells was similar to the method previously developed for isolating canine cortical collecting tubule cells (17). Polystyrene tissue culture dishes (100 mm) were coated with 200 µg of purified IgG_3(rct-30). Optimal dispersions of rabbit renal cortical cells were achieved by incubating the minced tissue with 25 ml of 0.1% (w/v) collagenase in Krebs buffer, pH 7.3, at 37°C with constant shaking, yielding single cells and short chains of cells (approximately $1x10^8$ cells, total; $2x10^7$ cells were applied to each dish). Using the immunoadsorption technique described above, $1x10^6$ cells were routinely isolated ($2x10^5$ cells/dish). Less than 10^3 cells bound to dishes coated with control mouse IgG (IgG_{2b}(cyo-7), Ref. 139) or with bovine serum albumin alone. Moreover, no cell growth was observed in the control dishes after culturing for 4-5 days. The appearance of RCCT cells shortly after immunoadsorption is shown in Fig. 5A. RCCT cells grew rapidly in culture and reached confluence in 4-5 days (Fig. 5B). At this time, there were an average of $2x10^6$

Figure 5. Phase contrast photomicrographs of collecting tubule cells at different growth stages in primary culture. A: 1-2 hours after immunoadsorption (X85); B: 4-5 days in culture (X230); C: 50 days in culture (X115).

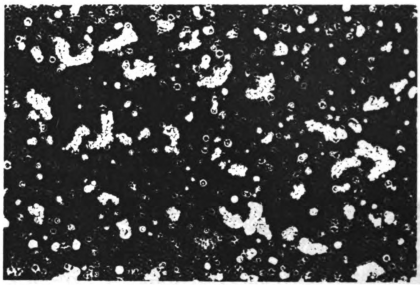

Figure 5A.

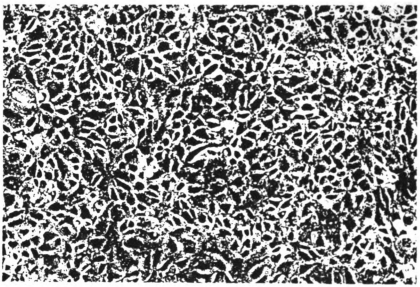

Figure 5B.

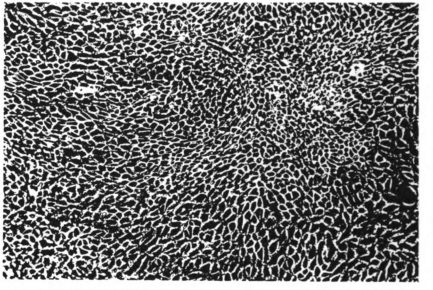

Figure 5C.

cells per dish. An additional 2-3 fold increase in cell number was observed after longer culture periods. No significant changes in cell morphology were detected after 50 days in primary culture (Fig. 5C). RCCT cells passaged after 4-5 days grew rapidly, reaching confluence after seeding at a density of $4x10^4/mm^2$. No changes in cell morphology were observed. The doubling time for nonconfluent cells was 32 hours.

Histochemical and antigenic properties of RCCT cells. Freshly isolated RCCT cells were removed from the culture dishes by trypsinization and stained with IgM(rct-10), IgG_1(rct-40) or IgG_3(rct-30) by indirect immunofluorescence. In each case >95% of the cells were found to be reactive with the anti-collecting tubule antibodies. However, by the time the RCCT cells reached confluence in primary culture, they were unreactive with any of the anti-collecting tubule antibodies, suggesting that: (a) the RCCT cells no longer expressed the intrinsic membrane antigens which with these antibodies react, (b) the antibodies are directed against an extracellular matrix protein that is not secreted by the cells or (c) that noncollecting tubule epithelia had overgrown the culture. This third possibility was eliminated by performing the following experiments. We first prepared and cloned another hybridoma cell line prepared from a fusion of SP2/0-Ag14 myeloma cells and splenic lymphocytes from mice immunized with cultured RCCT cells. The antibody secreted by this cell line was designated IgG_2(rct-30A) (Fig. 6). When cryostat sections of rabbit kidney cortex were stained with this antibody and examined using indirect immunofluorescence, only the collecting tubule and arcades were reactive. Thus, passaged RCCT cells and cortical collecting

Figure 6. Fluorescence photomicrograph of nonconfluent first-passage cultures of RCCT cells stained with medium from rct-30A hybridoma cultures.

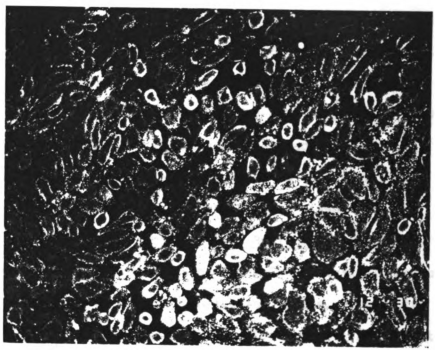

Figure 6.

tubules share an antigen which is undetectable in other renal cortical epithelia. In addition, both freshly isolated and primary cultures of RCCT cells were unreactive with an antibody that specifically reacted with rabbit proximal convoluted tubules (W.S. Spielman, unpublished observation). Cultured RCCT cells were also unreactive with antibodies against Tamm-Horsfall protein under conditions in which this antibody stained the medullary thick ascending limb cells in rabbit kidney sections. Both primary and passaged cultures of confluent RCCT cells stained uniformly for NADH diaphorase activity, but did not stain for succinate dehydrogenase activity. This histochemical staining pattern is identical to that seen with collecting tubules in sections of rabbit renal tissue (148).

Principal and intercalated cells in RCCT cell populations. Since indirect immunofluorescence staining using IgG_3(rct-30) is uniform for all cells of the rabbit cortical collecting tubule, we expected that RCCT cell populations would contain a mixture of both intercalated and principal cells. In the cortical collecting tubule of the normal rabbit kidney, the mixture of principal cells to intercalated cells is 3:1 (144,149). Peanut lectin has been reported to react only with intercalated cells in the collecting duct of the rabbit kidney cortex (144). Therefore, FITC-labeled peanut lectin was used to assess the relative numbers of principal and intercalated cells in RCCT cell cultures. In subconfluent primary cultures of RCCT cells, 27% of 352 cells were stained with FITC-labeled peanut lectin (Fig. 7). Fluorescent labeling of passaged RCCT cells with FITC-labeled peanut lectin was quantitated by flow cytometry (data not shown). Approximately 25% of 10^5 cells (two separate runs) exhibited fluorescence

Figure 7. Fluorescence (A) and phase contrast (B) photomicrographs of primary cultures of RCCT cells stained with FITC-labeled peanut lectin (X425).

Figure 7A.

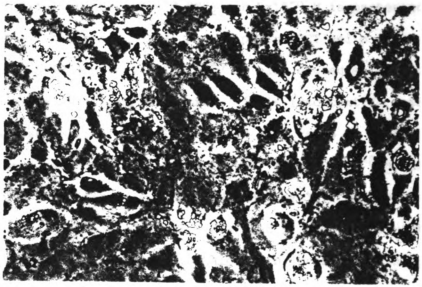

Figure 7B.

attributable to staining with the peanut lectin. Based on these studies it was apparent that there were discrete populations of RCCT cells that were either reactive or unreactive with FITC-labeled peanut lectin in a ratio of about one to three. Confluent primary cultures of RCCT cells were examined by transmission electron microscopy, and two different collecting tubule-like cells were indistinguishable based on the absence (principal cells) or presence (intercalated cells) of large numbers of mitochondria or lack of microvilli (data not shown) (150).

Hormonal responses of RCCT cells. Passaged RCCT cells were tested for their ability to form cAMP and PGE_2 in response to various hormones and effectors. As shown in Fig. 8, cAMP levels were increased three- to five-fold when RCCT cells were treated with PGE_2, isoproterenol or AVP. Half-maximal increases were detected at concentrations of $5x10^{-9}$ M AVP, 10^{-6} M PGE_2 and $5x10^{-8}$ M isoproterenol. In the case of AVP treatment, cAMP production in RCCT cells reached a maximum after 5-10 minutes. PTH (10^{-6} M) also caused a two- to three-fold increase in cAMP synthesis. It is important to note that the maximal responses of freshly isolated RCCT cells to hormone treatment were similar; isoproterenol (10^{-6} M), AVP (10^{-6} M) and PGE_2 (2.8×10^{-5} M) significantly increased cAMP levels from a control value of 10.2±0.3 to 34.4±0.9, 27.5±2.3, and 38.0±0.6 fmol cAMP/10 minutes/μg protein, respectively. Calcitonin, prostaglandin D_2, prostaglandin I_1 and prostaglandin $F_{2\alpha}$ all failed to stimulate cAMP synthesis in RCCT cells.

Figure 8. Effects of (A) arginine-vasopressin (AVP), (B) PGE_2 on cAMP formation in cultured RCCT cells, and (C) isoproterenol. *Significantly different from controls ($P<0.05$).

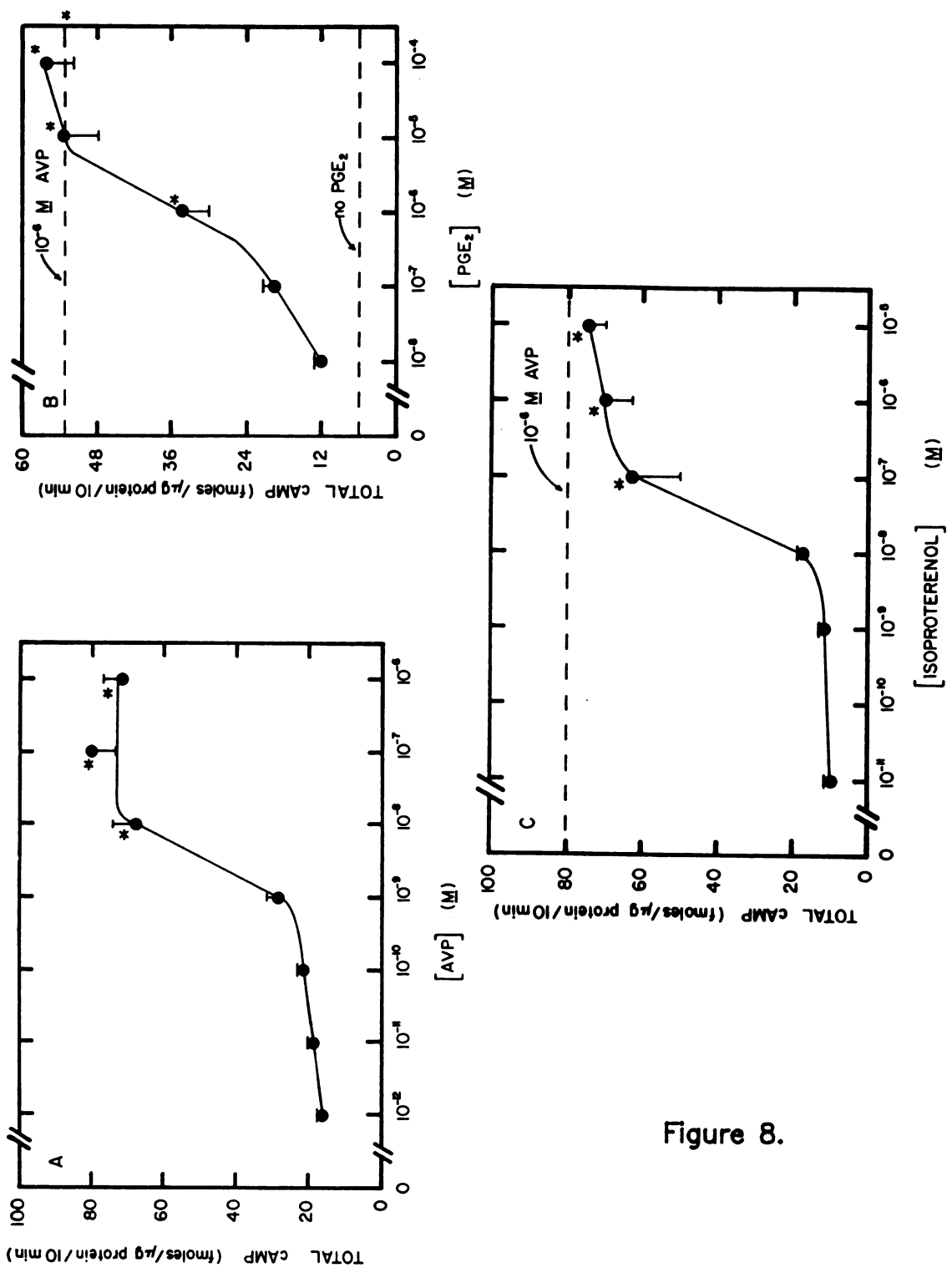
A
B
C
TOTAL cAMP (fmoles/μg protein/10 min)
[AVP] (M)
[PGE₂] (M)
[ISOPROTERENOL] (M)
10⁻⁸ M AVP
no PGE₂

Figure 8.

Bradykinin caused a five- to sevenfold increase in PGE_2 release from RCCT cells (Fig. 9). Half-maximal increases in PGE_2 release were obtained with 10^{-8} to 10^{-7} M bradykinin. Neither AVP nor isoproterenol increased PGE_2 synthesis above control levels.

Figure 9. Effect of bradykinin on PGE_2 formation in cultured RCCT cells. *Significantly different from control values ($P<0.05$).

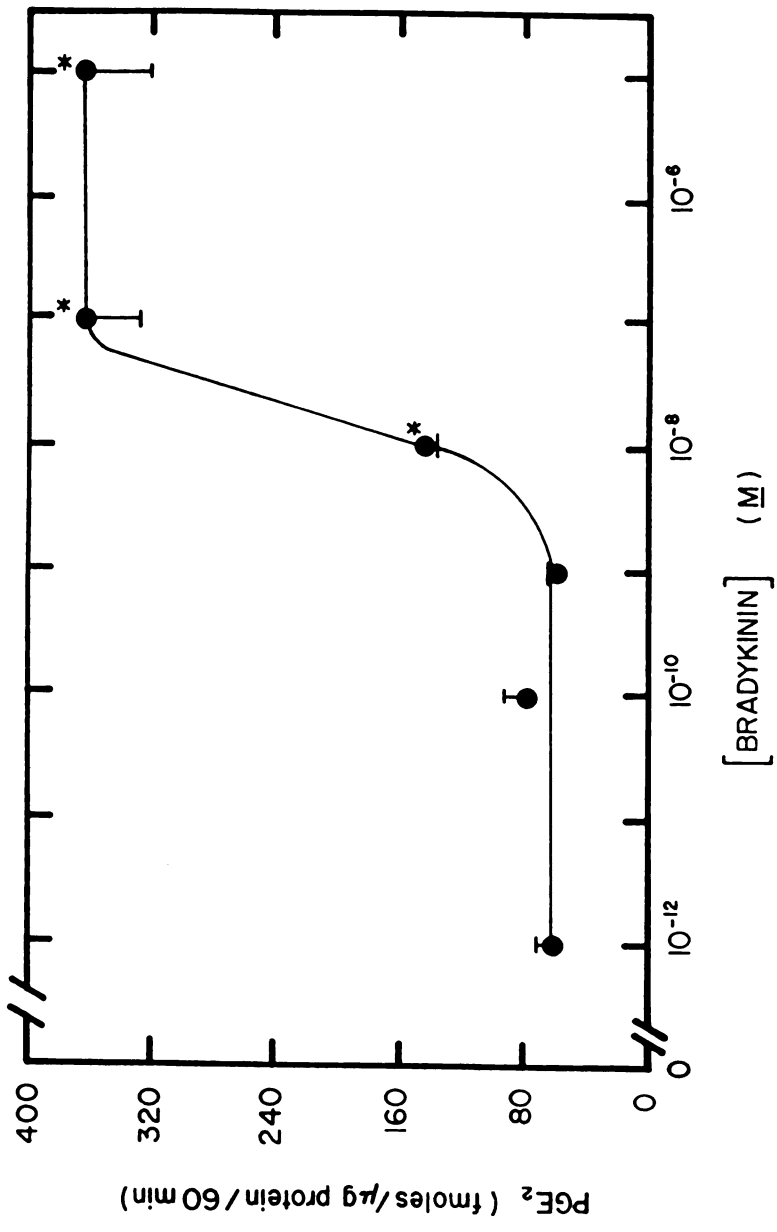
PGE2 (fmoles /µg protein / 60 min)
400
320
240
160
80
0
10-12
10-10
10-8
10-6
[BRADYKININ] (M)

Figure 9.

DISCUSSION

The purpose of this study was to develop a method for rapidly isolating large numbers of hormonally responsive collecting tubule cells from rabbit kidney cortex. Although collecting tubule segments can be isolated by microdissection (137,138,153), this procedure requires several months of training, and cells cultured from microdissected tubule segments dedifferentiate at least to the extent of losing the ability to form prostaglandins in response to hormones (138). The immunodissection method described here can be learned in a week, yields a minimum of 10^6 cells, and after culturing provides cells that are responsive to hormones in a manner similar to those reported for freshly microdissected rabbit cortical collecting tubules (119,147).

It should be noted, however, that RCCT cells form cAMP in response to PTH treatment. PTH has been reported not to stimulate adenylate cyclase activity in microdissected cortical collecting tubule segments, but does stimulate cAMP synthesis in the collecting tubule arcades (connecting tubule), which also exhibit hormonal responsiveness similar to epithelia of the collecting duct (147). Thus, RCCT cells may contain connecting tubule epithelia. However, rabbit papillary collecting tubule cells were also found to form cAMP in response to PTH treatment (59). Unlike rabbit papillary collecting

tubule cells, RCCT cells synthesize cAMP in response to isoproterenol, but not to D- or I-series prostaglandins (59,153).

RCCT cells synthesized PGE_2 in response to bradykinin (77), but not AVP treatment (data not shown). This finding is consistent with other reports which indicate that AVP-induced PGE_2 release in microdissected collecting tubule segments is dependent on the age and strain of rabbits from which the tubules are derived (18,77).

Although RCCT cell preparations exhibit hormonal responses expected for collecting tubule epithelia, some degree of dedifferentation may occur as the cells are cultured. Of note in this regard is that after 4-5 days in primary culture, RCCT cells no longer can be stained with any of the three original monoclonal antibodies which are reactive with freshly isolated cells (Table 1).

To determine the degree of homogeneity of passaged RCCT cells, we prepared another monoclonal antibody (IgG_2(rct-30A)) since the original anti-collecting monoclonal antibodies were unreactive with cultured cells. IgG_2(rct-30A) caused immunofluorescent staining of both passaged RCCT cells (98%) and collecting duct epithelia in the rabbit renal cortex. This result suggests that passaged RCCT cells are free of contamination from epithelia derived from other renal cortical cells. Consistent with this view are the observations that: (a) the passaged RCCT cells stained uniformly for NADH diaphorase activity, and failed to stain for succinate dehydrogenase activity (148); (b) RCCT cells examined by light microscopy exhibited morphological characteristics expected for collecting tubule epithelia and lacked fibroblast growth; (c) RCCT cells examined by electron microscopy were found to be composed of epithelia having the

appearance of principal and intercalated cells (150); (d) RCCT cells did not stain with monoclonal antibodies directed against rabbit proximal tubules or with anti-human Tamm-Horsfall serum which is reactive with the rabbit thick ascending limb; (e) both freshly isolated and cultured RCCT cells synthesized cAMP in response to AVP, PGE_2, and isoproterenol treatment (119,147); and (f) a relatively constant proportion of freshly isolated and passaged cells were reactive with FITC-labeled peanut lectin (144,149).

This study demonstrates that immunodissection is an effective method for isolating large numbers of collecting tubule cells from rabbit renal cortical tissue. Moreover, both freshly isolated and cultured RCCT cells may be useful for investigating the molecular mechanism of action of prostaglandins.

CHAPTER III

DIFFERENTIAL REGULATION OF cAMP METABOLISM BY PROSTAGLANDINS IN RABBIT CORTICAL COLLECTING TUBULE CELLS

Grantham and Orloff were the first to demonstrate that low concentrations of PGE_1 inhibit arginine-vasopressin (AVP)-induced water reabsorption in the perfused rabbit cortical collecting tubule (93); they also found that at higher concentrations PGE_1 alone stimulated water reabsorption. Since water reabsorption in the collecting tubule is mediated by cAMP (93,94), it is likely that the inhibitory and stimulatory effects of PGE_1 and PGE_2 (93-95) on water reabsorption result from the ability of these prostaglandins to regulate differentially cellular cAMP levels.

Reported here are studies designed to determine the effects of PGE_2 on cAMP metabolism in highly purified populations of rabbit cortical collecting tubule (RCCT) cells. Our results indicate that at low concentrations, E-series prostaglandins, including the PGE analogue, sulprostone, act primarily via a G_i-dependent mechanism to inhibit directly adenylate cyclase activity. However at high concentrations, PGE derivatives excluding sulprostone, stimulate adenylate cyclase.

METHODS

Materials. Commercial reagents were purchased from the following sources: [γ-^{32}P]ATP (3000 Ci/mmol), [2,8-^{3}H]cAMP (30 Ci/mmol) and ^{125}I-cAMP (>100 Ci/mmol) from ICN Radiochemicals; pyruvate kinase, creatine phosphokinase, myokinase, potassium phosphoenol pyruvate, phosphocreatine, GTP, IBMX, Dowex 50W-hydrogen cation exchange resin (200-400 mesh), 8% cross linkage) and neutral alumina (type WN-3) from Sigma; collagenase, RPMI 1640 and fetal bovine serum from Grand Island Biological Company (Gibco); PGE_2, PGE_1, $PGF_{2\alpha}$ and DM-PGE_2 from Cayman Chemical Company; PGD_2 from Upjohn Diagnostics; flurbiprofen and carbacyclin were gifts from Dr. John Pike of Upjohn; AVP from Calbiochem; transferrin from Boehringer-Mannheim; goat anti-cAMP serum and Safety-Solve scintillation fluid from Research Products International; pertussis toxin from List Biological Laboratories; RO 20-1724 cAMP phosphodiesterase inhibitor was a gift from Hoffman-LaRoche; sulprostone was a gift from Berlex Laboratories.

Isolation of RCCT cells. RCCT cells were isolated by immunoadsorption as described in Chapter II using a monoclonal antibody specific for a cell surface antigen unique to the collecting tubule. In experiments using freshly isolated cells, all buffers contained the cyclooxygenase inhibitor, flurbiprofen (10 μM). Immunodissected RCCT cells were washed from the plates, collected by centrifugation, resuspended in HKRS, pH 7.3 (composition: 140 mM NaCl, 14 mM glucose,

10 mM HEPES, 4.7 mM KCl, 1.8 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.8 mM $MgCl_2$), and transferred to test tubes or 96-well tissue culture clusters (0.1 ml/well). Cells to be cultured were resuspended in RPMI 1640 medium containing transferrin (5 µg/ml) and 3% fetal bovine serum, transferred to 24-well tissue culture clusters, and incubated at 37°C for 3 to 5 days, or until confluent, in a water-saturated 7% CO_2/air atmosphere. The RCCT cells were then washed twice and cultured for 1 or 2 more days in the same medium without serum (156). Each isolation (4 rabbit kidneys) routinely yielded enough cells to seed 72 wells for culture or immediate use.

Pertussis toxin treatments. Freshly isolated RCCT cells were resuspended in RPMI 1640, distributed into 96-well plates and pretreated for 4 hours at 37°C with vehicle or pertussis toxin (final concentration = 1 µg/ml). For cultured RCCT cells, the medium was aspirated from each well and replaced with RPMI 1640 and incubated for 4 hours at 37°C with or without pertussis toxin (1 µg/ml).

Effector stimulated cAMP synthesis. RCCT cells were treated for 10 minutes at room temperature with various effectors in HKRS, pH 7.3, containing 10 µM RO 20-1724 unless noted otherwise in the figure legends. Treatments were stopped by acidification with 0.1 ml of 0.1 M HCl and temporarily stored at -80°C. Cultured RCCT cells were incubated for 4 hours with 10 µM flurbiprofen in RPMI 1640 medium prior to treating with effectors. Cyclic AMP was quantitated by radioimmunoassay as described by Frandsen and Krishna (140). The pH of the samples was adjusted to 6.2 with 0.5 M Na_2HPO_4 prior to performing the assay.

Effector-stimulated adenylate cyclase activity. Freshly isolated RCCT cells were suspended in a modified HKRS, pH 7.5 (composition: 145 mM NaCl, 10 mM HEPES, 5 mM glucose, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), and transferred to test tubes. The cells were pretreated with vehicle or sulprostone for 10 minutes at 24°C and the samples were frozen on dry ice. Adenylate cyclase activity was assayed immediately after thawing the samples using a modification of the method of Meeker and Harden (157). The reaction mixture in a final volume of 0.15 ml contained: 0.1 mM [γ-^{32}P]ATP (60 cpm/pmole; 0.5 µCi/assay), 1 mM [^{3}H]cAMP (30,000 cpm/assay), 100 µM GTP, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.1 mg/ml myokinase, 5 mM $MgCl_2$, 2 mM EGTA, 150 mM NaCl, 25 mM HEPES, pH 7.5, and varying concentrations of AVP. The reaction was started by adding the reaction mixture to each tube. Samples were incubated at 30°C for 30 minutes, and the reactions stopped by adding 0.85 ml of 5% (w/v) trichloroacetic acid. Cyclic AMP was separated from the ATP in two steps using Dowex 50-X8 cation exchange and neutral alumina chromatography (158). Each sample was transferred to Dowex columns (2 ml bed volume) pre-equilibrated with deionized water and washed with 3 ml of deionized water to remove the ATP. The cAMP was eluted directly into the alumina columns (1.5 g/column) with 10 mls of deionized water. The cAMP was washed from the alumina columns with 4 ml of 50 mM Tris-HCl (pH 8) buffer. The eluates were collected in scintillation vials, and quantitated by liquid scintillation counting. Recovery of cAMP was 50-60%.

Protein determinations. Total cellular protein was determined using a modification (145) of the Lowry procedure (159).

Statistical analyses. Values in all experiments are expressed as the mean ± the standard error. Differences among treatment groups were determined using a completely random analysis of variance ($P<0.05$). The student-Newman-Keul's test or lsd test were used to compare treatment means, when applicable.

RESULTS

Effect of prostaglandins on cAMP synthesis in freshly isolated RCCT cells. In freshly isolated RCCT cells, PGE_2, PGE_1, and DM-PGE_2 stimulated cAMP synthesis at concentrations ranging from 0.1 to 10 μM (Fig. 10). The potencies of PGE_2 and PGE_1 were roughly equal (EC_{50} = 0.1 μM), whereas DM-PGE_2 was slightly less potent (EC_{50} = 1 μM). The PGE_2 analogue, sulprostone, did not stimulate cAMP formation (Fig. 10); moreover, 100 μM sulprostone did not inhibit cAMP formation stimulated by 1 μM PGE_2. No stimulation of cAMP synthesis was detected when RCCT cells were treated with 10 μM PGD_2, $PGF_{2\alpha}$, or the stable PGI_2 analogue, carbacyclin indicating that prostaglandin-induced cAMP formation in RCCT cells is specific for the E-series prostaglandins tested, except sulprostone.

Effect of prostaglandins on AVP-stimulated cAMP formation in freshly isolated RCCT cells. PGE_2 inhibits AVP-stimulated cAMP formation (Fig. 11) over a range of concentrations less than those that substantially stimulate cAMP synthesis (>0.1-100 nM). Sulprostone, which does not stimulate cAMP accumulation, also inhibited AVP-induced cAMP synthesis and was as potent, or perhaps slightly more potent than PGE_2 (EC_{50}, sulprostone = 1 nM; EC_{50}, PGE_2 = 3 mM). Maximal inhibition of the AVP response typically occurred at concentrations between 10 and 100 nM.

Figure 10. Concentration dependence of cAMP synthesis stimulated by different PGE derivatives in freshly isolated RCCT cells. RCCT cells were treated with varying concentrations of PGE_2 (o), PGE_1 (o), DM-PGE_2 (□) or sulprostone (□) for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each point represents the mean of triplicate determinations.

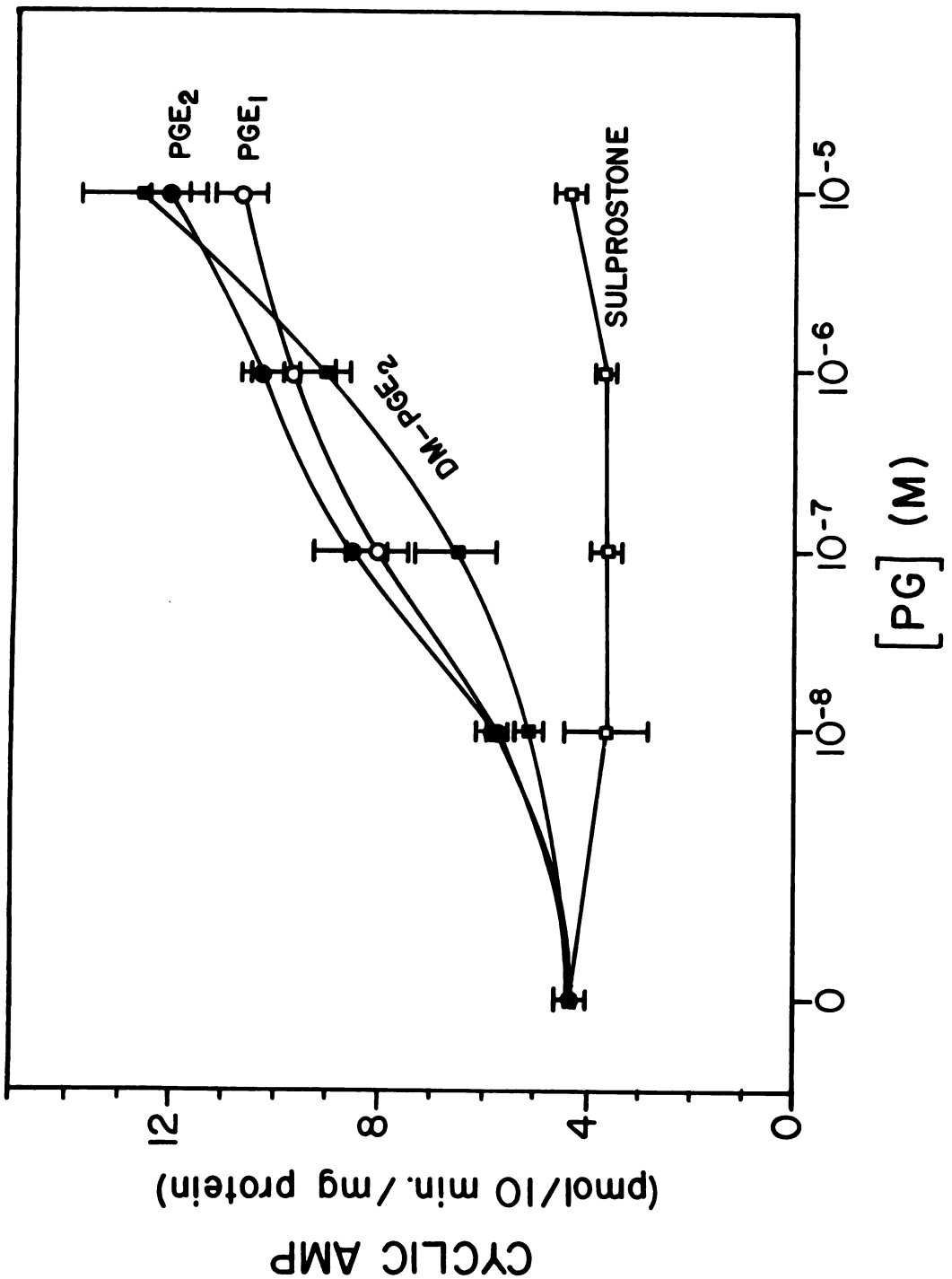
CYCLIC AMP
(pmol/10 min./mg protein)
0
4
8
12
PGE2
PGE1
DM-PGE2
SULPROSTONE
0
10-8
10-7
10-6
10-5
[PG] (M)

Figure 10.

Figure 11. Concentration dependence for inhibition by PGE_2 or sulprostone of AVP-induced cAMP accumulation in freshly isolated RCCT cells. RCCT cells were treated with varying concentrations of either PGE_2 or sulprostone alone (o) or in combination with 10 nM AVP (o) for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each point represents the mean of triplicate determinations.

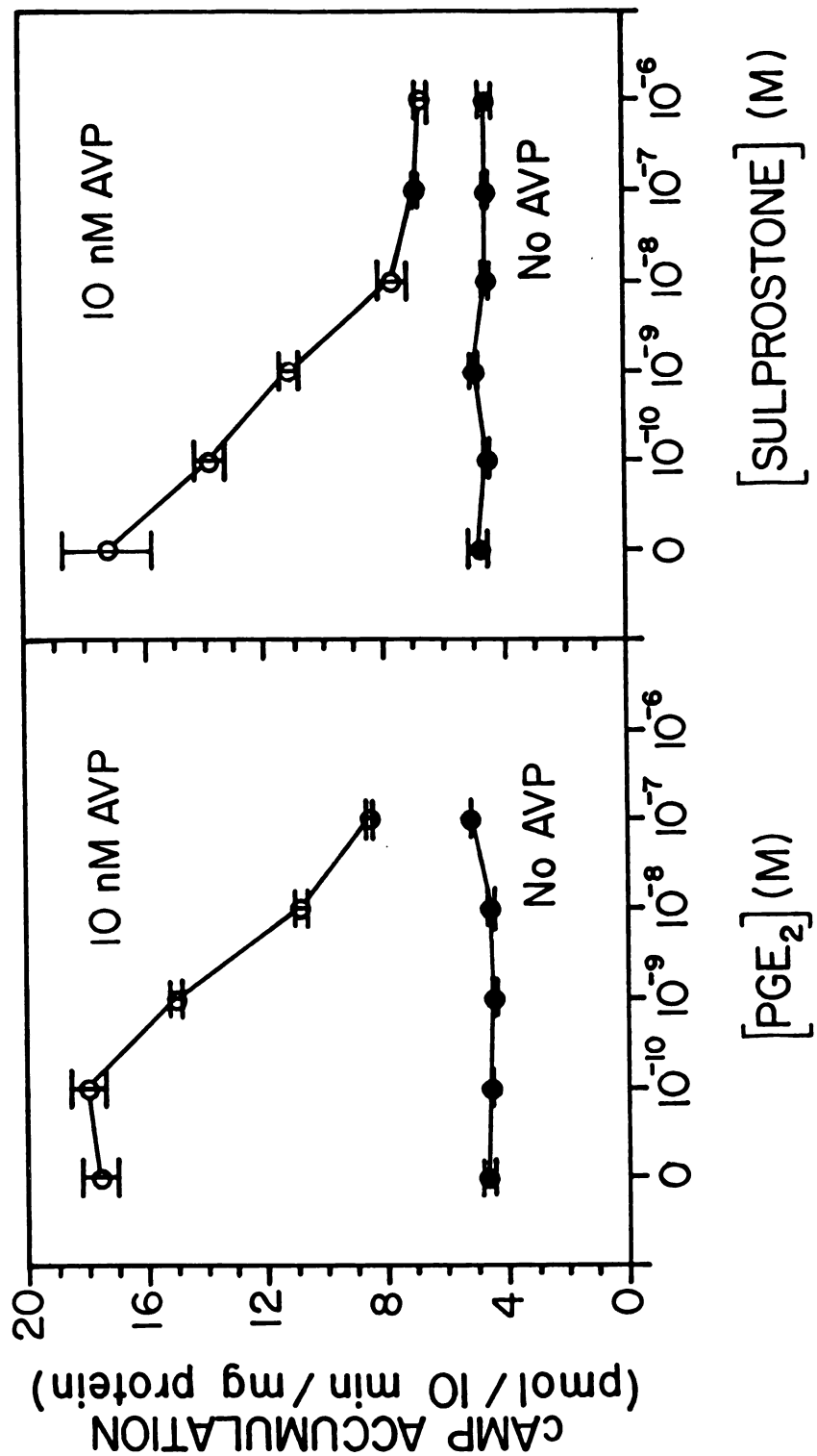
cAMP ACCUMULATION
(pmol / 10 min / mg protein)
20
16
12
8
4
0
10 nM AVP
No AVP
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
[PGE$_2$] (M)
[SULPROSTONE] (M)

Figure 11.

The specificity of the PGE_2/sulprostone-inhibitory action was determined by comparing the ability of different prostaglandins with PGE_2 and sulprostone to blunt AVP-stimulated cAMP synthesis. The effect of different E-series prostaglandins on basal and AVP-stimulated cAMP accumulation is shown in Fig. 12. Although PGE_2, PGE_1 and DM-PGE_2 at 30 nM stimulated cAMP synthesis in the absence of AVP, all were equally potent in inhibiting AVP-induced cAMP formation. Moreover, the E-series prostaglandins blocked AVP-stimulated cAMP synthesis to nearly the same extent as sulprostone, when the difference between the treatment groups receiving no AVP and 10 nM AVP are compared. The effects of $PGF_{2\alpha}$, carbacyclin and PGD_2 on AVP-induced cAMP formation are shown in Fig. 13. Carbacyclin and $PGF_{2\alpha}$ at 100 nM decreased AVP-stimulated cAMP synthesis, but not to the same extent as 100 nM sulprostone. PGD_2 (100 nM) had no significant effect on the AVP response.

Characterization of the PGE/sulprostone-inhibitory action in freshly isolated RCCT cells. Experiments were formulated to determine if treatments of freshly isolated RCCT cells with PGE_2 or sulprostone either inhibit adenylate cyclase and/or stimulate cAMP degradation. In four separate experiments, pertussis toxin pretreatment of freshly isolated RCCT cells blocked sulprostone-induced inhibition of AVP-stimulated cAMP synthesis by 50 to 90%. The most pronounced effect of pertussis toxin is shown in Fig. 14. Pertussis toxin pretreatment attenuated the inhibitory effect of PGE_2 and sulprostone to the same extent. These results indicate that PGE_2/sulprostone-induced inhibition of AVP-stimulated cAMP synthesis is mediated by a pertussis toxin-sensitive G-protein.

Figure 12. Inhibition by different PGE derivatives of AVP-induced cAMP accumulation in freshly isolated RCCT cells. RCCT cells were treated with 30 nM sulprostone, PGE_2, DM-PGE_2, PGE_1 or no prostaglandin, alone or in combination with 10 nM AVP for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each value represents the mean of triplicate determinations. *,$Significantly different from the control value (ie., 10 nM AVP alone). Values sharing a common symbol are not significantly different ($P<0.05$).

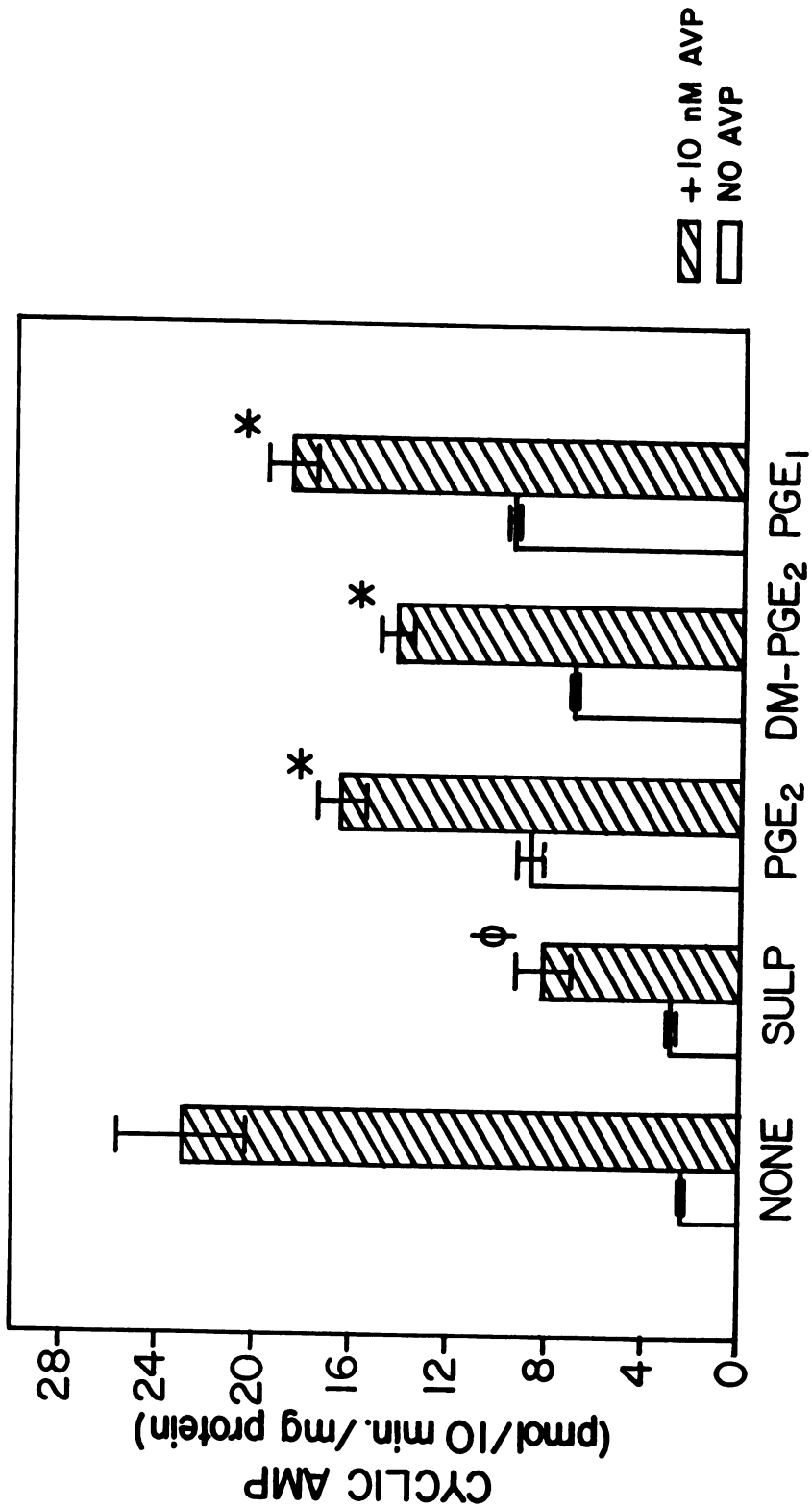
+10 nM AVP
NO AVP
CYCLIC AMP
(pmol/10 min./mg protein)
0
4
8
12
16
20
24
28
NONE
SULP
PGE2
DM-PGE2
PGE1
*
*
*
Φ

Figure 12.

Figure 13. Inhibition by different prostaglandins of AVP-induced cAMP accumulation in freshly isolated RCCT cells. RCCT cells were treated with 100 nM sulprostone, carbacyclin (carba), $PGF_{2\alpha}$, PGD_2 or no prostaglandin, alone or in combination with 10 nM AVP for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each value represents the mean of triplicate determinations. *,$Significantly different from the control value (i.e., 10 nM AVP alone). Values sharing a common symbol are not significantly different ($P<0.05$).

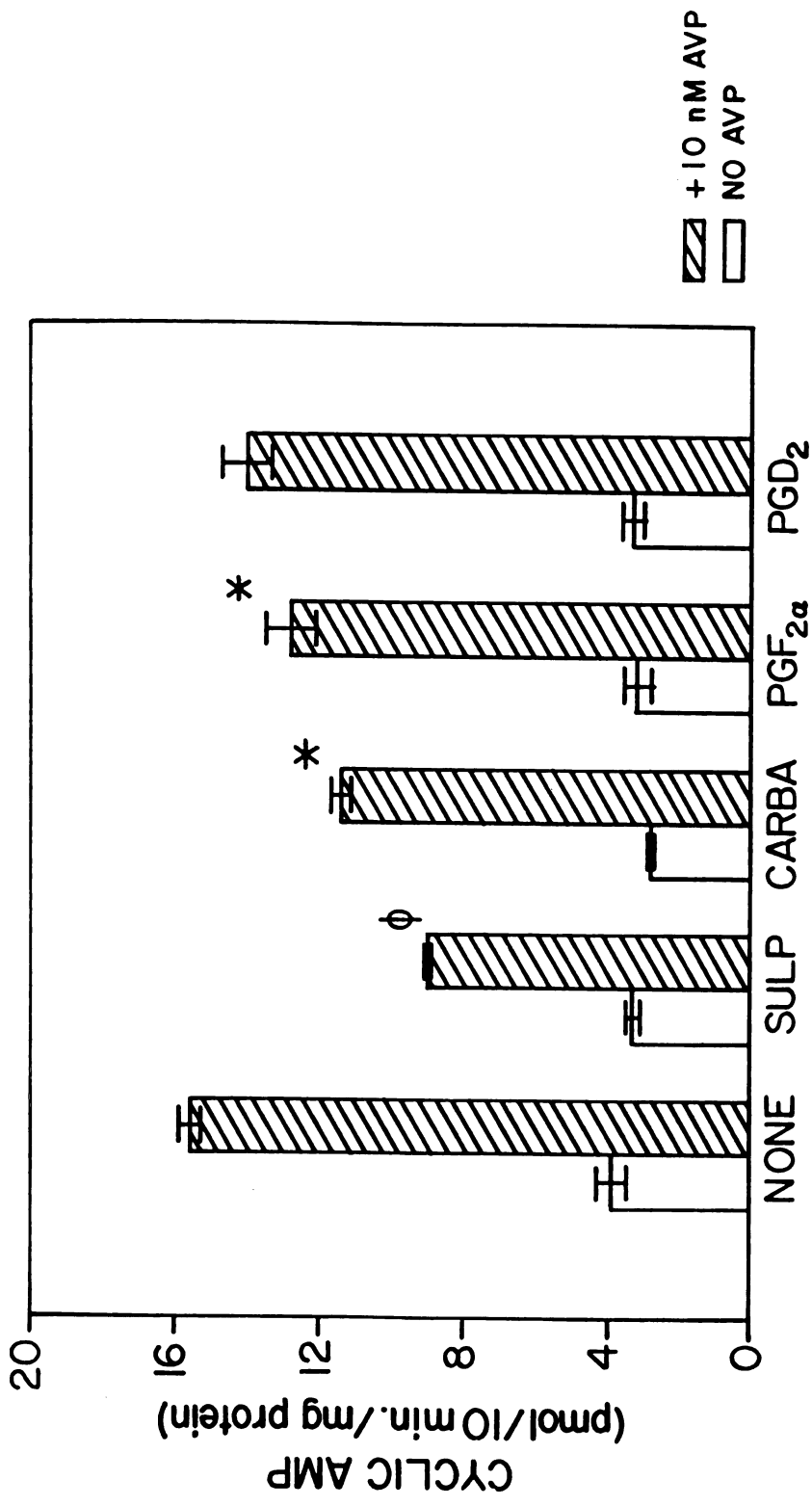
+10 nM AVP
NO AVP
CYCLIC AMP
(pmol/10 min./mg protein)
0
4
8
12
16
20
NONE
SULP
CARBA
PGF2α
PGD2
*
*
Φ

Figure 13.

Figure 14. Pertusis toxin treatment blocks inhibition by sulprostone of AVP-induced cAMP accumulation in freshly isolated RCCT cells. RCCT cells were pretreated for 4 hours with pertussis toxin (1 μg/ml) or vehicle at 37°C, followed by treatment with either 0 or 100 nM sulprostone, alone or in combination with 10 nM AVP for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each value represents the mean of triplicate determinations. *Significantly different from the control value (i.e., 10 nM AVP alone)($P<0.05$).

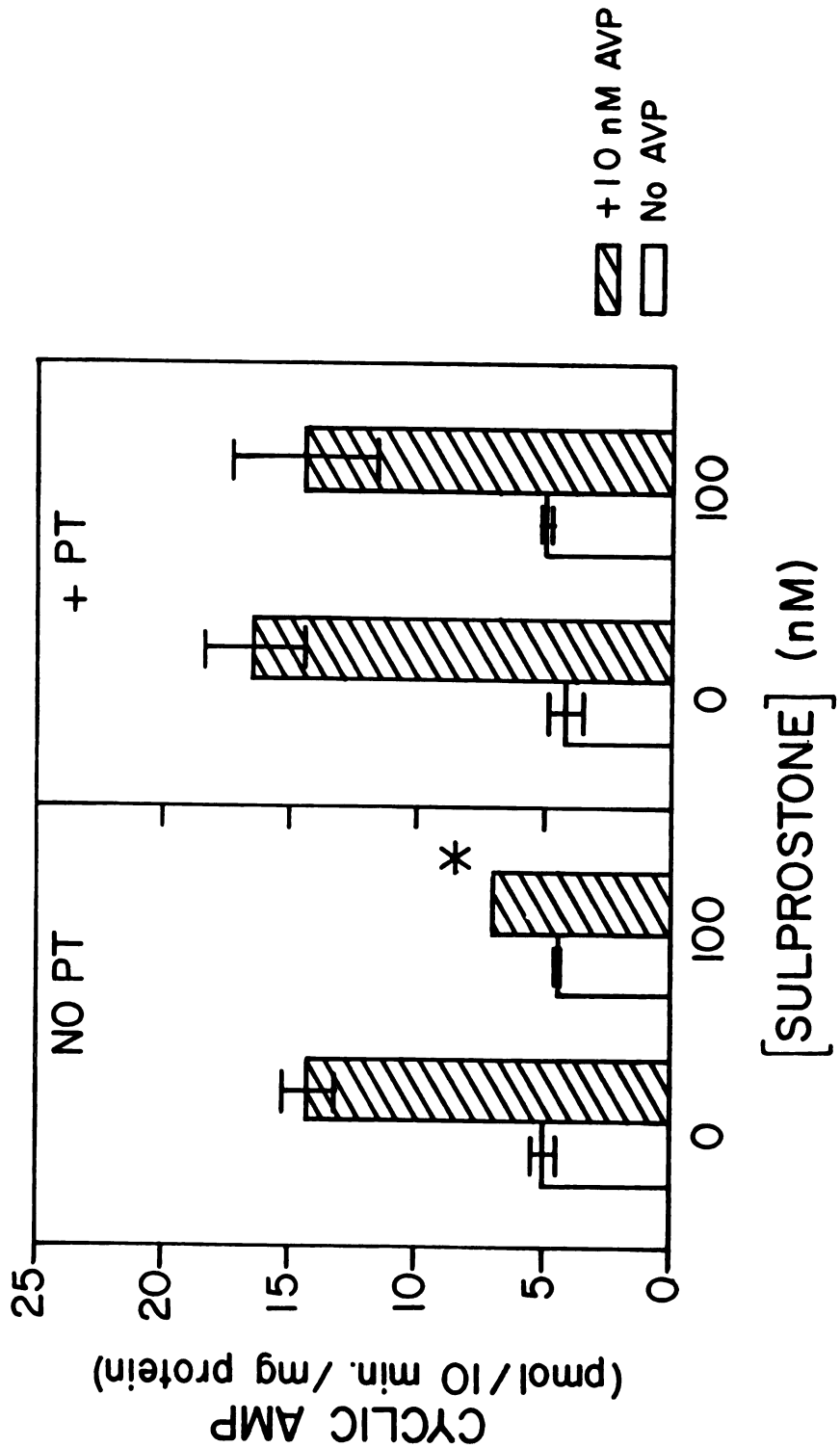
+ 10 nM AVP
No AVP
+ PT
NO PT
*
0
100
0
100
[SULPROSTONE] (nM)
CYCLIC AMP
(pmol / 10 min. /mg protein)
25
20
15
10
5
0

Figure 14.

The pertussis toxin sensitivity of the PGE_2/sulprostone effect suggested that these prostaglandin E derivatives act through G_i to inhibit adenylate cyclase. To test this concept, we measured adenylate cyclase activity directly in permabealized, freshly isolated RCCT cells. RCCT cell adenylate cyclase activity was stimulated two-fold using 100 nM AVP; half-maximal stimulation was obtained with 3 nM AVP (Fig. 15). Treatment of intact RCCT cells with 1 μM sulprostone inhibited subsequent AVP-stimulated adenylate cyclase activity 50 to 70 % (Fig. 15).

There is evidence suggesting that PGE_2 may activate a cAMP phosphodiesterase in microdissected collecting tubule segments from rat kidney (118,119). Therefore we determined indirectly whether PGE_2 or sulprostone may stimulate cAMP degradation. Treatment of RCCT cells with high concentrations of either RO 20-1724 or IBMX did not prevent PGE_2- or sulprostone-induced inhibition of AVP-stimulated cAMP synthesis (Fig. 16) and in fact, the inhibitory effects of both PGE_2 and sulprostone were somewhat greater in the presence of 1 mM RO 20-1724 than 10 μM RO 20-1724.

Effect of prostaglandins on cAMP metabolism in cultured RCCT cells. PGE_2 stimulates cAMP accumulation in cultured RCCT cells (Fig. 17) (89); moreover, sulprostone fails either to stimulate cAMP synthesis or to inhibit PGE_2-induced cAMP formation. These results are similar to those obtained with freshly isolated cells. However, PGE_2, at concentrations that maximally inhibit AVP-induced cAMP formation in freshly isolated cells, failed to block AVP-stimulated cAMP formation in cultured cells (Fig. 18).

Figure 15. Sulprostone inhibits AVP-stimulated adenylate cyclase activity in freshly isolated RCCT cells. RCCT cells were pretreated for 10 minutes with no prostaglandin (o) or 1 μM sulprostone (o) and frozen rapidly with dry ice. Permabealized cells were assayed for adenylate cyclase activity using reaction mixtures containing varying concentrations of AVP (0-100 nM, final). Each point represents the mean of triplicate determinations.

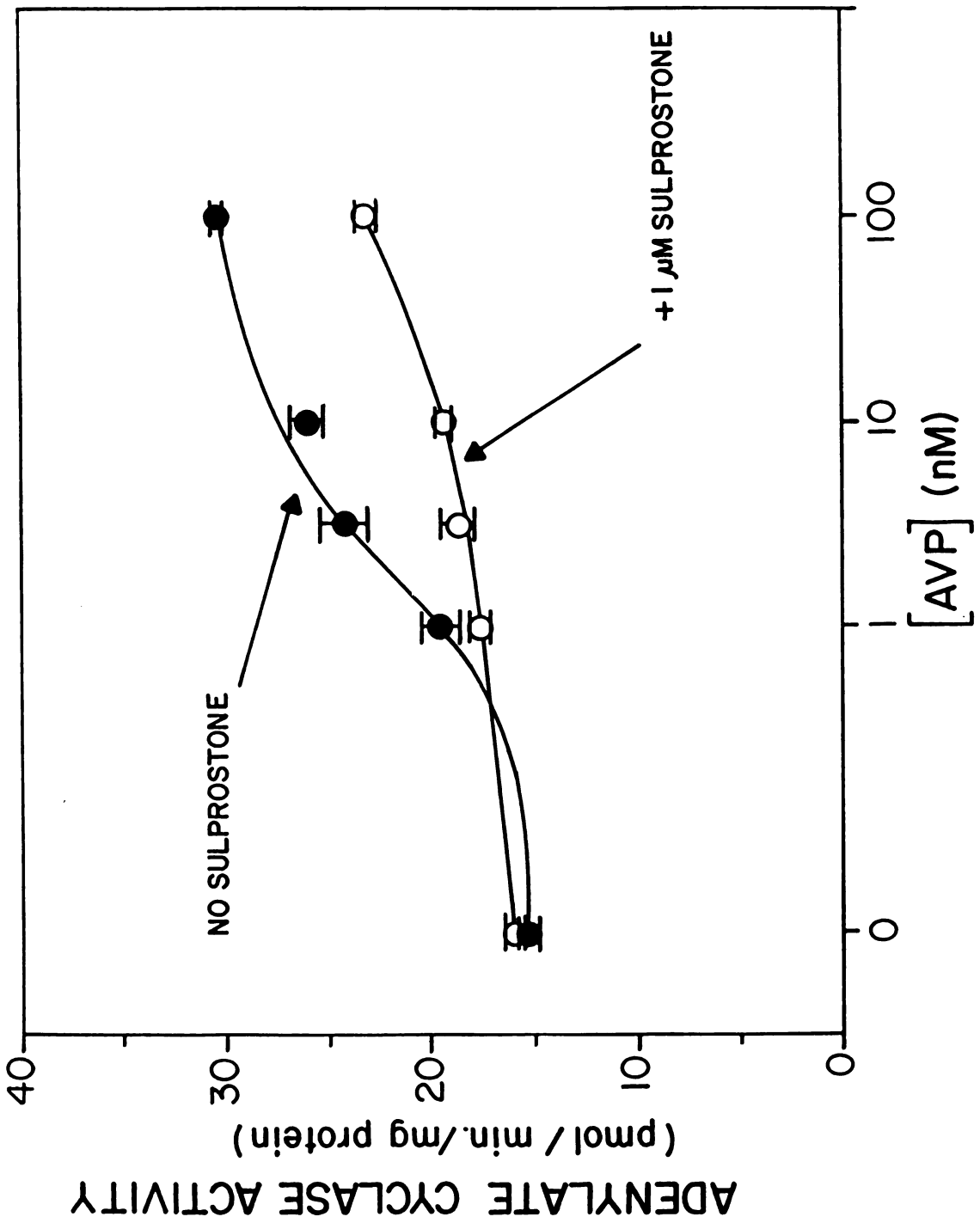
ADENYLATE CYCLASE ACTIVITY
(pmol / min./mg protein)
40
30
20
10
0
0
1
10
100
[AVP] (nM)
NO SULPROSTONE
+ 1 μM SULPROSTONE

Figure 15.

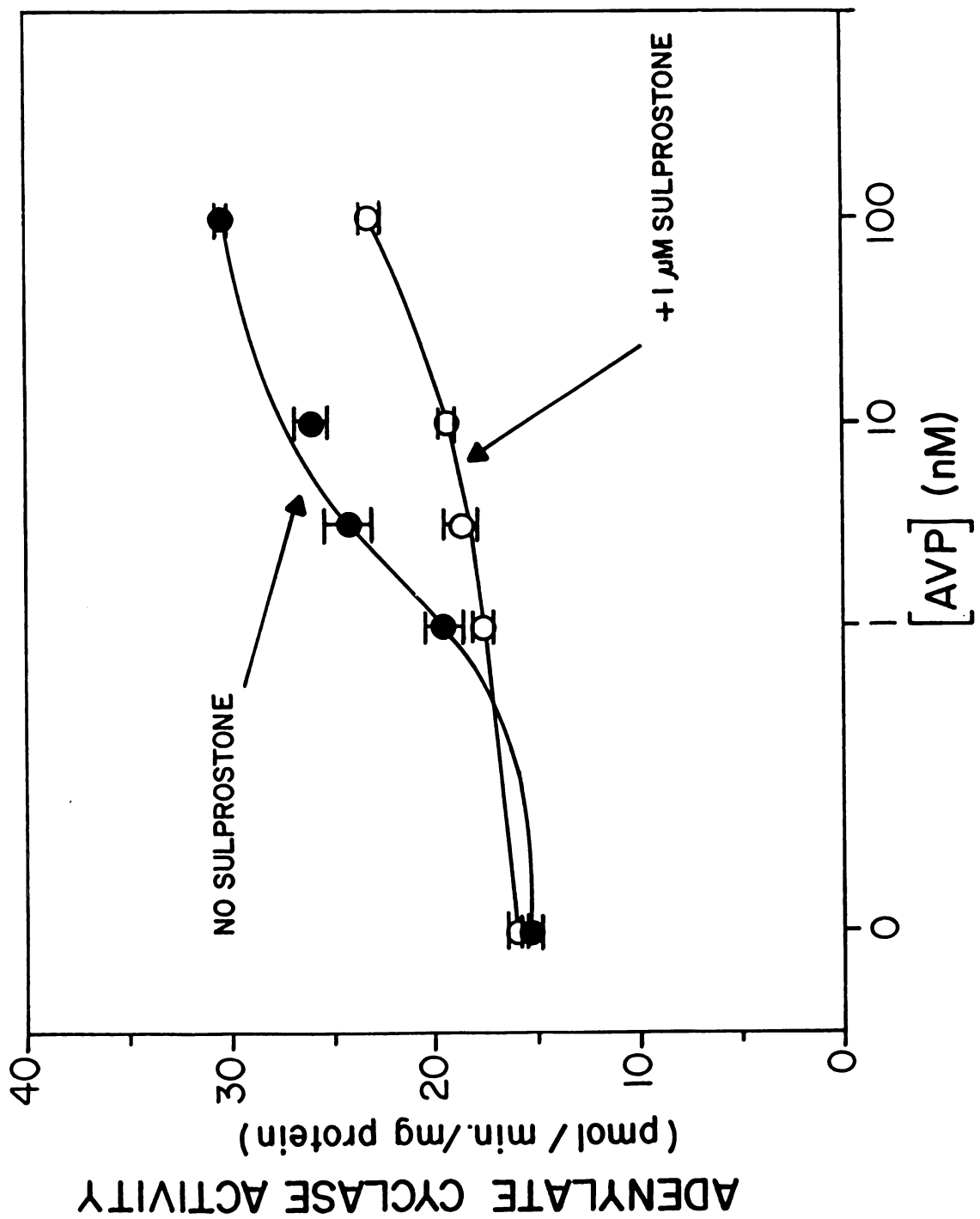
ADENYLATE CYCLASE ACTIVITY
(pmol / min./mg protein)
40
30
20
10
0
0
1
10
100
[AVP] (nM)
NO SULPROSTONE
+ 1 μM SULPROSTONE

Figure 15.

Figure 16. Reduction of cAMP phosphodiesterase activity fails to block inhibition of AVP-induced cAMP accumulation by either PGE_2 or sulprostone in freshly isolated RCCT cells. RCCT cells were treated with no prostaglandin, PGE_2 or sulprostone in the absence or presence of AVP (10 nM) for 10 minutes at room temperature. The treatment buffers contained either RO 20-1724 (0.01 or 1 mM) or IBMX (3 mM). Protein and cAMP were assayed as described in the text. Each value represents the mean of triplicate determinations. *Significantly different from the control value (i.e., 10 nM AVP alone)($P<0.05$).

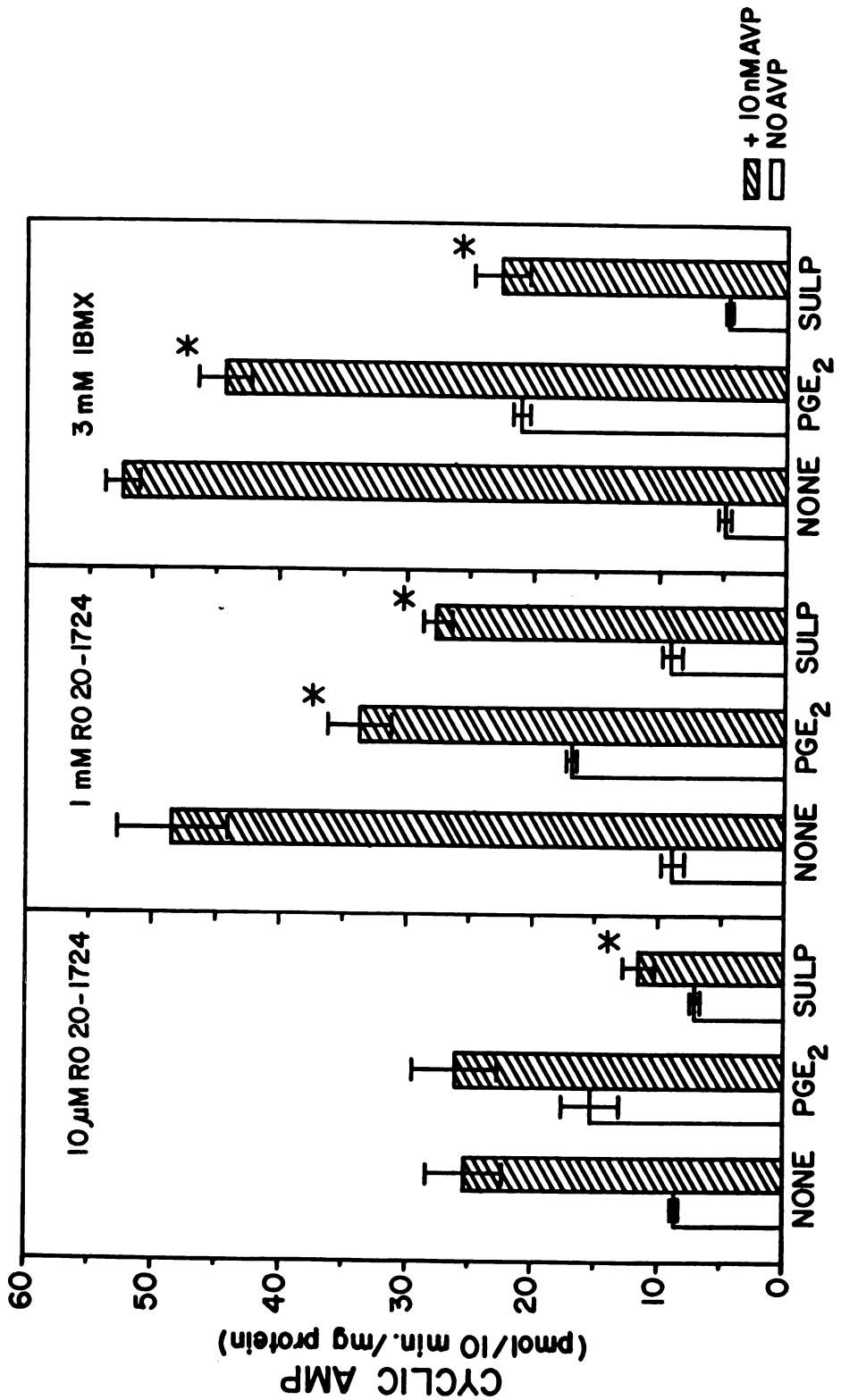
+ 10nM AVP
NO AVP
3 mM IBMX
1 mM RO 20-1724
10 μM RO 20-1724
NONE
PGE2
SULP
CYCLIC AMP
(pmol/10 min./mg protein)
0
10
20
30
40
50
60
*

Figure 16.

Figure 17. Effect of sulprostone on basal and PGE_2-stimulated cAMP accumulation in cultured RCCT cells. Cultured cells were treated with no prostaglandin or sulprostone (100 μM) alone or in combination with 1 μM PGE_2 for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each value represents the mean of triplicate determinations. *Significantly different from the control value (i.e., 10 nM AVP alone) ($P<0.05$).

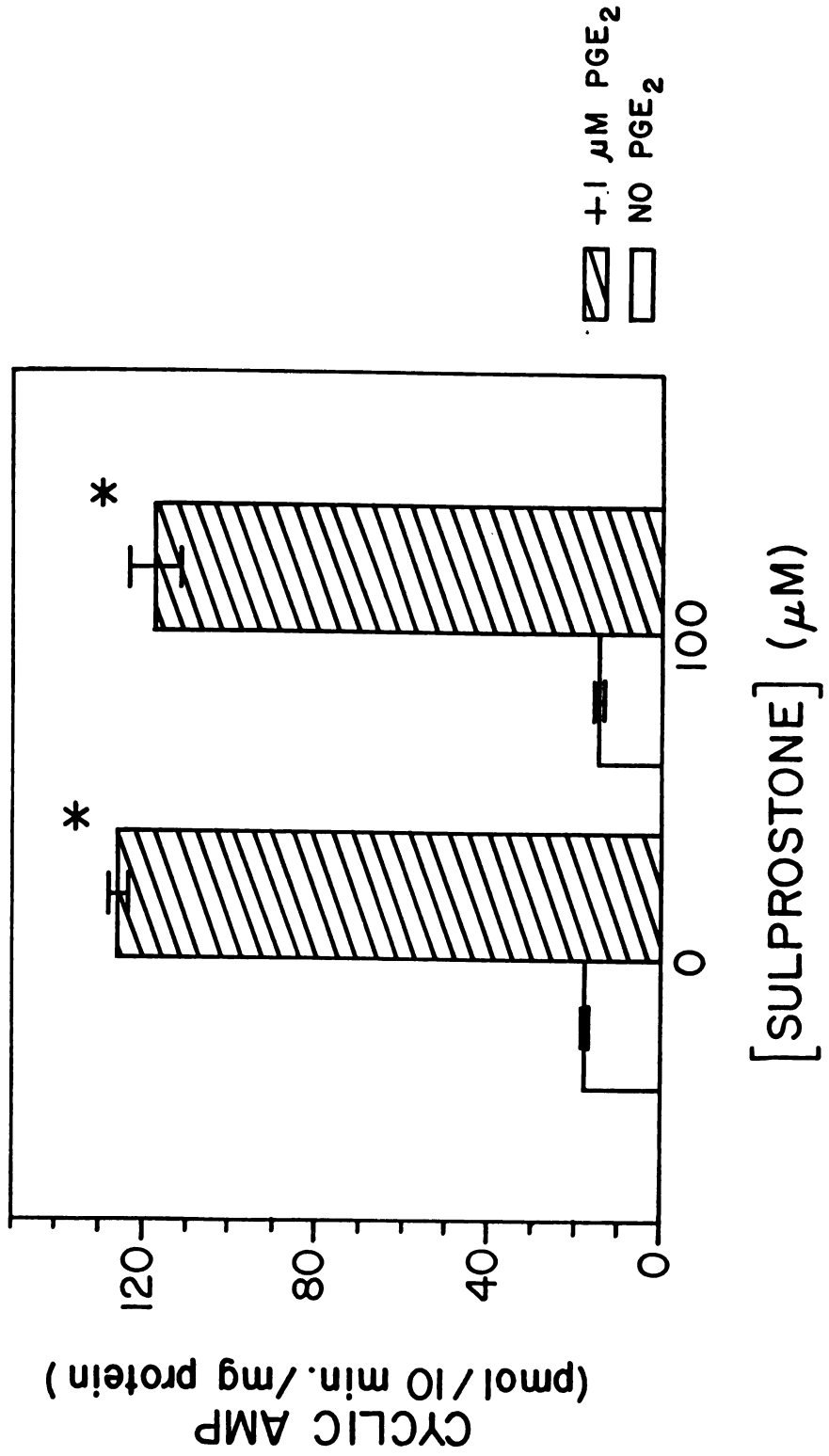
+1 μM PGE2
NO PGE2
*
*
0
100
[SULPROSTONE] (μM)
0
40
80
120
CYCLIC AMP
(pmol / 10 min. /mg protein)

Figure 17.

Figure 18. PGE_2 fails to inhibit AVP-induced cAMP accumulation in cultured RCCT cells. Cultured cells were treated with either no prostaglandin or PGE_2 (10 nM) alone or in combination with 10 nM AVP for 10 minutes at room temperature. The treatment buffers contained 10 μM RO 20-1724. Protein and cAMP were determined as described in the text. Each value represents the mean of triplicate determinations. *Significantly different from the control value (i.e., 10 nM AVP alone) ($P<0.05$).

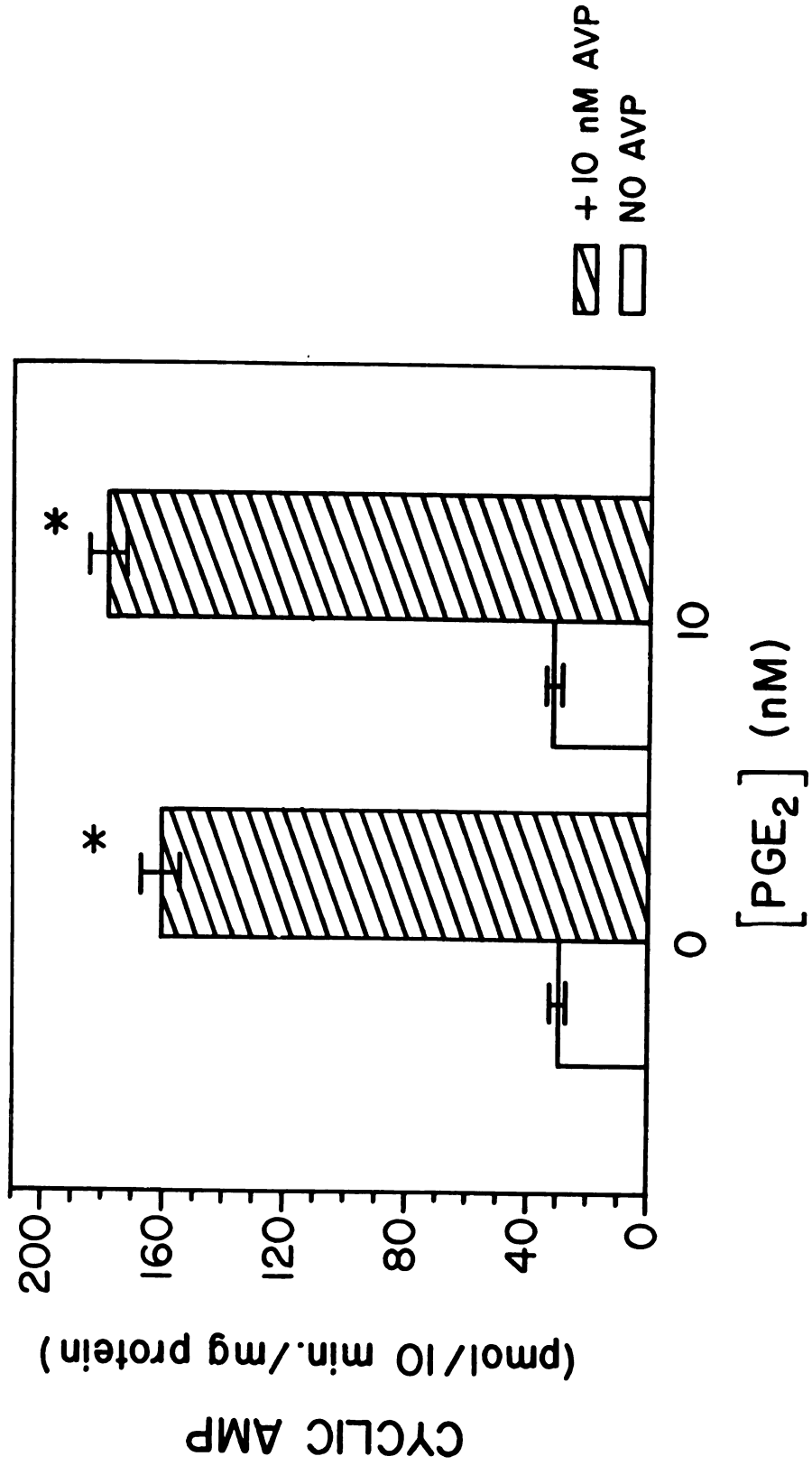
+ 10 nM AVP
NO AVP
CYCLIC AMP
(pmol/10 min./mg protein)
200
160
120
80
40
0
*
*
0
10
[PGE2] (nM)

Figure 18.

α_2-adrenergic agents inhibit AVP-induced cAMP formation in microdissected rabbit cortical collecting tubule segments (160,161). Therefore, to determine if inhibitory regulation of adenylate cyclase mediated by G_i was defective in cultured RCCT cells, we examined the response of the cells to α_2-adrenergic stimulation. Epinephrine in combination with propranolol (α_2-adrenergic stimulation) inhibited AVP-stimulated cAMP formation (Fig. 19); moreover, pertussis toxin pretreatment blocked this inhibitory α_2-adrenergic action, indicating that cultured RCCT cells do retain a functional G_i.

Figure 19. Pertussis toxin treatment blocks inhibition of AVP-induced cAMP formation by epinephrine in cultured RCCT cells. Cultured cells were pretreated for 4 hours with either vehicle or pertussis toxin (μg/ml) at 37°C, followed by treatment with either no effector or epinephrine (10 μM) alone or in combination with 10 mM AVP for 10 minutes at room temperature. The treatment buffers contained 10 μM propanolol and RO 20-1724 (10 μM). Protein and cAMP were determined as described in the text. Values represent the mean of triplicate determinations. *Significantly different from the control value (i.e., 10 nM AVP alone) ($P<0.05$).

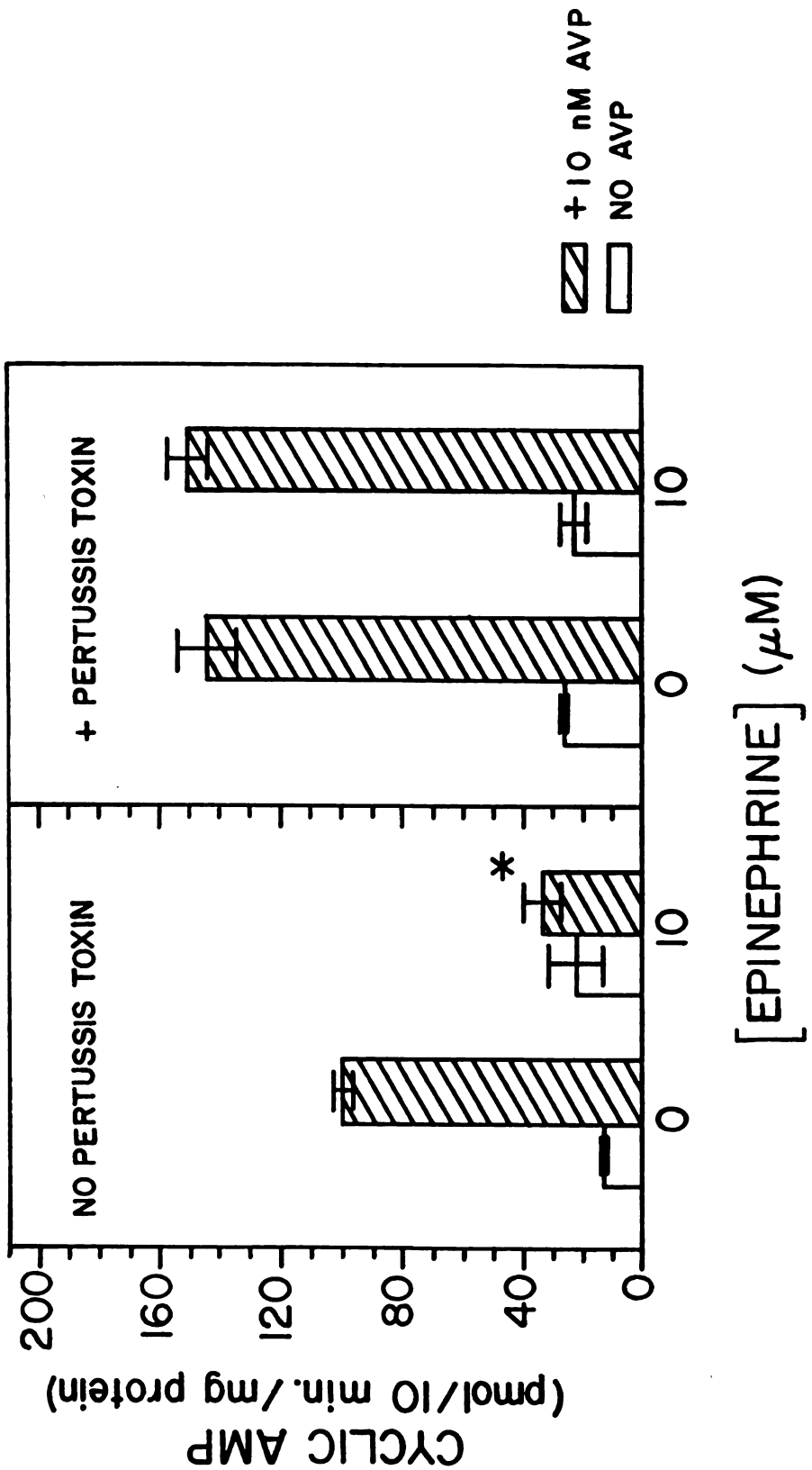
+ 10 nM AVP
NO AVP
NO PERTUSSIS TOXIN
+ PERTUSSIS TOXIN
*
0
10
0
10
[EPINEPHRINE] (μM)
CYCLIC AMP
(pmol/10 min./mg protein)
200
160
120
80
40
0

Figure 19.

DISCUSSION

Prostaglandins of the D-, E- and I-series can stimulate cAMP formation in various sytems (1,131,162). PGE_2 has been shown to stimulate adenylate cyclase activity in microdissected segments of rat collecting tubule (131) and in cultured cells derived from the rabbit papillary collecting tubule cells (143). The present specificity studies with freshly isolated RCCT cells indicate that only prostaglandins of the E-series, excluding sulprostone, stimulate cAMP accumulation in these cells. Thus, there appears to be a PGE receptor (R_2, Fig. 20) coupled to adenylate cyclase activation in the rabbit cortical collecting tubule. The stimulatory effects of E-series prostaglandins were observed at relatively high concentrations -- half-maximal stimulation of cAMP accumulation occurred with 100 n<u>M</u> PGE_1. In the renal collecting tubule, water reabsorption is observed with 100 n<u>M</u> PGE_1, but not 1 n<u>M</u> PGE_1 (93). Since increases in intracellular cAMP are known to mediate water reabsorption (94), water reabsorption occurring in response to treatment of the perfused rabbit cortical collecting tubule with 100 n<u>M</u> PGE_1 (93) is probably due to stimulation of adenylate cyclase.

Suppression of prostaglandin synthesis <u>in vivo</u> with cyclooxygenase inhibitors actually leads to increased water reabsorption (154,155). Therefore, the physiological significance of PGE_2-stimulated cAMP synthesis and attendant water reabsorption in the collect-

Figure 20. A model describing the molecular basis for the physiological action of PGE_2 in the rabbit cortical collecting tubule. Abbreviations are: A.C., adenylate cyclase; cAMP-PDE, cAMP phosphodiesterase; G_s, stimulatory guanine nucleotide-binding regulatory protein; G_i, inhibitory guanine nucleotide-binding regulatory protein; R_s, stimulatory PGE_2 receptor; R_1, inhibitory PGE_2 receptor; V_1, "pressor"-like vasopressin receptor; V_2, adenylate cyclase-stimulatory vasopressin receptor.

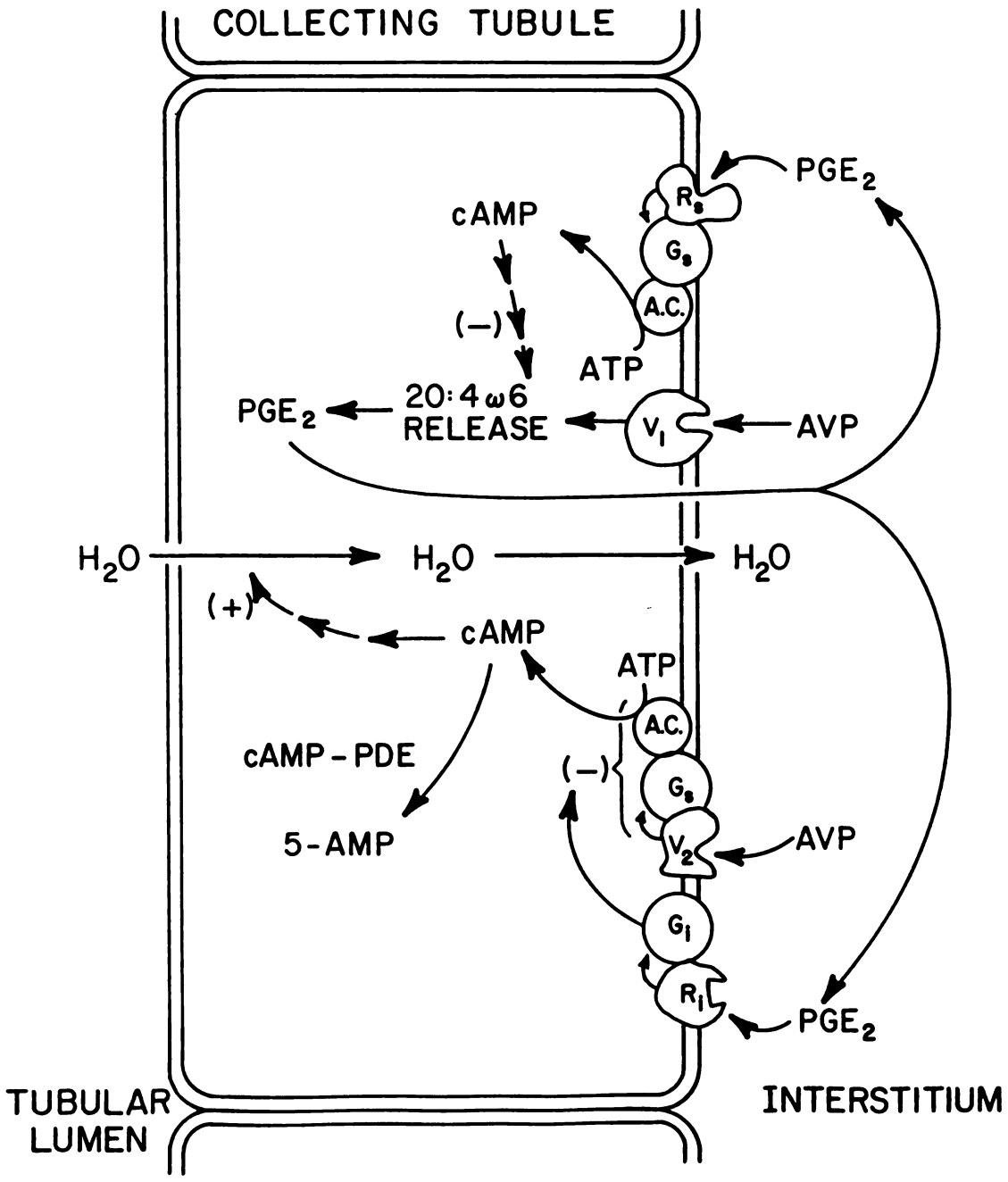
COLLECTING TUBULE
PGE2
Rs
cAMP
Gs
A.C.
(–)
ATP
PGE2
20:4ω6
RELEASE
V1
AVP
H2O
H2O
H2O
(+)
cAMP
ATP
A.C.
cAMP-PDE
(–)
Gs
5-AMP
V2
AVP
Gi
Ri
PGE2
TUBULAR
LUMEN
INTERSTITIUM

Figure 20.

ing tubule is unclear. One possibility suggested previously is that the stimulatory PGE receptor functions as part of a negative feedback loop regulating prostaglandin formation induced by AVP (acting via a putative V_1 vasopressin receptors; Ref. 91,92) in collecting tubule epithelia (135,136,164; Fig. 20).

Distinct from their cAMP stimulatory actions, E-series prostaglandins, including sulprostone, inhibited AVP-induced cAMP accumulation in freshly isolated RCCT cells. For each of the PGE derivatives, the inhibitory effect was detected at more than a 10-fold lower concentration than that required to stimulate cAMP formation. E-series prostaglandins were the most potent inhibitors although $PGF_{2\alpha}$ and carbacyclin at high concentrations also caused slight inhibition of AVP-induced cAMP formation. Our results suggest that inhibition by PGE_1 (93) and PGE_2 (95) of AVP-induced water reabsorption in the rabbit cortical collecting tubule occurs through suppression of AVP-induced cAMP accumulation (Fig. 20). Synthesis of cAMP induced by AVP is mediated by a V_2 receptor, presumably linked to G_s (94).

Pertussis toxin treatment of RCCT cells blocked the inhibitory effects of both PGE_2 and sulprostone. In addition, sulprostone had a direct inhibitory effect on AVP-stimulated adenylate cyclase activity of permabealized RCCT cells. Finally, the inhibitory effects of PGE derivatives on AVP-induced cAMP accumulation by intact cells occurred in the presence of concentrations of cAMP phosphodiesterase inhibitors which maximally block the detectable cAMP phosphodiesterase activity. We conclude from these results that the inhibitory effects of PGE derivatives on cAMP accumulation in RCCT cells result primarily from direct inhibition of adenylate cyclase mediated by the inhibitory

guanine nucleotide-binding regulatory protein (103,113), G_i, and not via activation of a cAMP phosphodiesterase. A corollary to this conclusion is that there is an inhibitory PGE receptor in cortical collecting tubule epithelia which is coupled to G_i (Fig. 20). In fact, a G_i-linked PGE receptor has been solubilized from canine renal outer medulla (124).

Curiously, the putative inhibitory PGE receptor of freshly isolated RCCT cells appeared to be lost when the cells are cultured. This conclusion is based on two observations. First, PGE_2 failed to block AVP-induced cAMP formation in cultured RCCT cells, and second, epinephrine acting via an α_2-adrenergic mechanism involving G_i (160,161) inhibited AVP-induced cAMP formation in cultured cells. These observations along with the fact that the cAMP stimulatory effect of PGE is retained by cultured RCCT cells also suggest that the inhibitory and stimulatory effects of PGE are mediated through different receptors.

Consistent with the concept that inhibitory and stimulatory PGE receptors are different proteins are the findings made with sulprostone. Sulprostone was a potent inhibitory agonist. But unlike the other prostaglandin E derivatives tested in this study, sulprostone failed either to stimulate cAMP synthesis or antagonize PGE_2-stimulated cAMP accumulation, even at concentrations of 0.1 m$\underline{M}$. To our knowledge, this is the first example of a PGE agonist being able to distinguish between inhibitory and stimulatory responses of prostaglandins on cAMP metabolism. Pharmacological studies have demonstrated that sulprostone can stimulate contraction in smooth muscle beds responsive to PGE_2 (122). However, in smooth muscle beds

where PGE_2 produces a relaxation, sulprostone has no effect (122). Smooth muscle relaxation is typically stimulated by increased levels of intracellular cAMP (122,165) and so our studies are consistent with the view that sulprostone does not interact with PGE receptors coupled to activation of adenylate cyclase.

CHAPTER IV

IDENTIFICATION OF TWO PGE RECEPTORS FUNCTIONALLY COUPLED TO DIFFERENT GUANINE NUCLEOTIDE-BINDING PROTEINS IN RABBIT CORTICAL COLLECTING TUBULE CELLS

The studies described in Chapter III established that (a) prostaglandin E derivatives including the synthetic analogue, sulprostone, at low concentrations inhibit AVP-stimulated adenylate cyclase activity mediated by G_i only in freshly isolated RCCT cells, and (b) PGE derivatives except sulprostone, stimulate cAMP synthesis at high concentrations in either cultured or freshly isolated cells. Moreover, sulprostone failed to antagonize PGE_2-induced cAMP synthesis in both cultured and freshly isolated RCCT cells.

These results suggest that PGE_2 exerts these effects on cAMP metabolism by interacting with two different receptors specific for E-series prostaglandins: (a) a high affinity receptor expressed only in freshly isolated RCCT cells that binds PGE derivatives including sulprostone and (b) a low affinity receptor expressed in both cultured and freshly isolated RCCT cells that interacts with PGE derivatives excluding sulprostone.

This chapter describes the characterization of specific PGE-binding activities in freshly isolated and cultured RCCT cells.

METHODS

Materials. Reagents were obtained from the following sources: [5,6,8,11,12,14,15(N)-^{3}H]PGE_2 (160-200 Ci/mmol) from New England Nuclear; collagenase, RPMI 1640 and fetal bovine serum from Grand Island Biological Company (Gibco); PGE_2, PGE_1, $PGF_{2\alpha}$ and DM-PGE_2 from Cayman Chemical Company; PGD_2 from Upjohn Diagnostics; flurbiprofen and carbacyclin were gifts from Dr. John Pike of Upjohn; transferrin and guanosine-5'-(3-O-thio) triphosphate (GTPγS) from Boehringer Mannheim; GF/F microfibre glass filters (25 mm) from Whatman; Safety-Solve scintillation fluid from Research Products International; pertussis toxin from List Biological Laboratories; sulprostone was a gift from Berlex.

Isolation and culture of RCCT cells. RCCT cells were isolated by immunoadsorption as described in Chapter II using polystyrene culture dishs coated with a monoclonal antibody that reacts with an ectoantigen specific for the collecting tubule. Cells to be cultured were initially grown in RPMI 1640 medium supplemented with transferrin (5 μg/ml) and 3% fetal bovine serum until confluent (3-5 days). RCCT cells were subsequently cultured for 48 hours in the same medium without serum (156). All incubations were carried out in a 37°C incubator with a water-saturated 7% CO_2/air atmosphere. Each isolation (6 rabbit kidneys) routinely yielded 4-5 mg of membrane protein.

Preparation of membranes from RCCT cells. All buffers used to prepare membranes contained the cyclooxygenase inhibitor, flurbiprofen. Prior to preparing membranes, cultured cells were pretreated for 4 hours with flurbiprofen, changing the medium once after the first 30 minutes. RCCT cells (either freshly isolated or cultured) were scraped from the tissue culture plates using a rubber policeman, resuspended in 10 mM HEPES buffer, pH 7.5, and centrifuged for 15 minutes at 30,000 x g. The membranes were washed twice by resuspending the pellet in 10 mM HEPES, pH 7.5, containing 2 mM EDTA using a dounce homogenizer and centrifuged as described above. The final membrane pellet was resuspended in HEPES buffer, pH 7.5, containing 1 mM $MgCl_2$ using a dounce homogenizer, yielding a final protein concentration of 1.5 mg/ml. Membrane protein was determined by a modification (145) of the Lowry method (159).

PGE_2 binding assays. Total PGE_2 binding was determined by a method described previously (124). RCCT cell membranes (80-180 µg protein/assay) were incubated in a reaction mixture containing the following components: 50 mM Tris-maleate, final pH 5.5, 1 mM $MgCl_2$ and 2 nM [^{3}H]PGE_2; in a final volume of 200 µl. In some experiments, other additions were made and are noted in the text. Nonspecific binding was determined in parallel by incubating membranes in the same reaction mixture also containing 10 µM unlabeled PGE_2. Values for nonspecific binding were subtracted from values for total binding to yield specific PGE_2 binding. The total PGE_2 binding to cultured RCCT cell membranes was 10-20% of the total binding detected in membranes prepared from freshly isolated cells. As a result, nonspecific binding was 15% (range 10-20%) for freshly isolated cells, and 50%

(range 45-55%) for cultured RCCT cells. All values are expressed as specific binding except where noted in the text. For Scatchard analyses and competitive displacement studies, varying concentrations of unlabeled ligand were added to the reaction mixture. All reactions were carried out at 30°C. Using these assay conditions, PGE_2 binding was saturable, attaining equilibrium after 60 minutes. Therefore, incubations were carried out for 120 minutes to insure that equilibrium binding was achieved.

The reaction was stopped by adding 4 ml of ice cold 50 mM Tris-maleate, pH 5.5 and rapidly filtered through a Whatman GF/F filter. The filter was immediately washed three more times with 4 ml of Tris-maleate, pH 5.5. Each filter was placed into scintillation vials containing 10 ml of Safety-Solve and the bound radioactivity was quantitated by liquid scintillation counting (124).

Pertussis toxin treatments. Freshly isolated or cultured RCCT cells were pretreated with either vehicle or pertussis toxin (1 µg/ml, final) in RPMI 1640 medium for 4 hours at 37°C.

Scatchard analysis. The dissociation constants and maximum number of sites were estimated (166) graphically from Scatchard plots of specific PGE_2 binding, assuming that the data fit either a one-site (m = 1) or two-site model (m = 2) where the total specific PGE_2 binding is expressed as:

$$B = \sum_{i=1}^{m} ([Bmax_i] \times [PGE_2])/([PGE_2] + Kd_i),$$

where B = total PGE_2 specifically bound, $Bmax_i$ = maximum number of

sites i, [PGE_2] = concentration of free PGE_2, and Kd_i = dissociation constant of receptor i.

Statistical analyses. In some experiments, differences among treatment groups were determined using a completely random analysis of variance. Treatment means were compared using the lsd test or Student-Newman-Keul's test, where applicable ($P<0.05$).

RESULTS

PGE_2 binding characteristics in freshly isolated RCCT cells. In membranes prepared from freshly isolated RCCT cells, $[^3H]PGE_2$ specific binding was rapid and saturable (Fig. 21). Equilibrium binding was reached after 60 minutes at 30°C. Addition of 10 μM unlabeled PGE_2 rapidly reversed specific $[^3H]PGE_2$ binding. However, displacement of specific $[^3H]PGE_2$ binding was not complete (55% displacement) after a two-hour incubation period. In the absence of GTPγS, a Scatchard plot of specific PGE_2 binding revealed evidence for two independent classes of sites (Fig. 22A). Roughly 27% of the total number of PGE_2 binding sites consisted of a relatively high affinity class (R_1), whereas the remaining 72% comprised a slightly lower affinity class of binding sites (R_2). The estimated dissociation constants (Kd) and maximum number of sites (Bmax) for R_1 and R_2, respectively, were: Kd_1 = 9 nM, $Bmax_1$ = 700 fmol/mg protein; Kd_2 = 100 nM, $Bmax_2$ = 1900 fmol/mg protein. Addition of 100 μM GTPγS changed the specific PGE_2 binding characteristics to a single high affinity class of sites (R_3; Fig. 22B). The estimated values of the Kd and Bmax, respectively, for R_3 were 2 nM and 500 fmol/mg protein.

Effect of pertussis toxin on PGE_2 binding activity in the absence and presence of GTPγS. The observation that the stable GTP analogue, GTPγS changed the PGE_2 binding characteristics in RCCT cell membranes supports our contention that there may be two different PGE receptors

Figure 21. Time course and reversibility of specific [^{3}H]PGE_2 binding in RCCT cell membranes. Membranes were incubated in a reaction mixture containing 2 n$\underline{M}$ [^{3}H]PGE_2 as described in the text. Binding reactions were terminated at various time points by dilution and rapid filtration of the membranes with ice-cold 50 m$\underline{M}$ Tris-maleate buffer, pH 5.5. Values represent the mean of duplicate determinations.

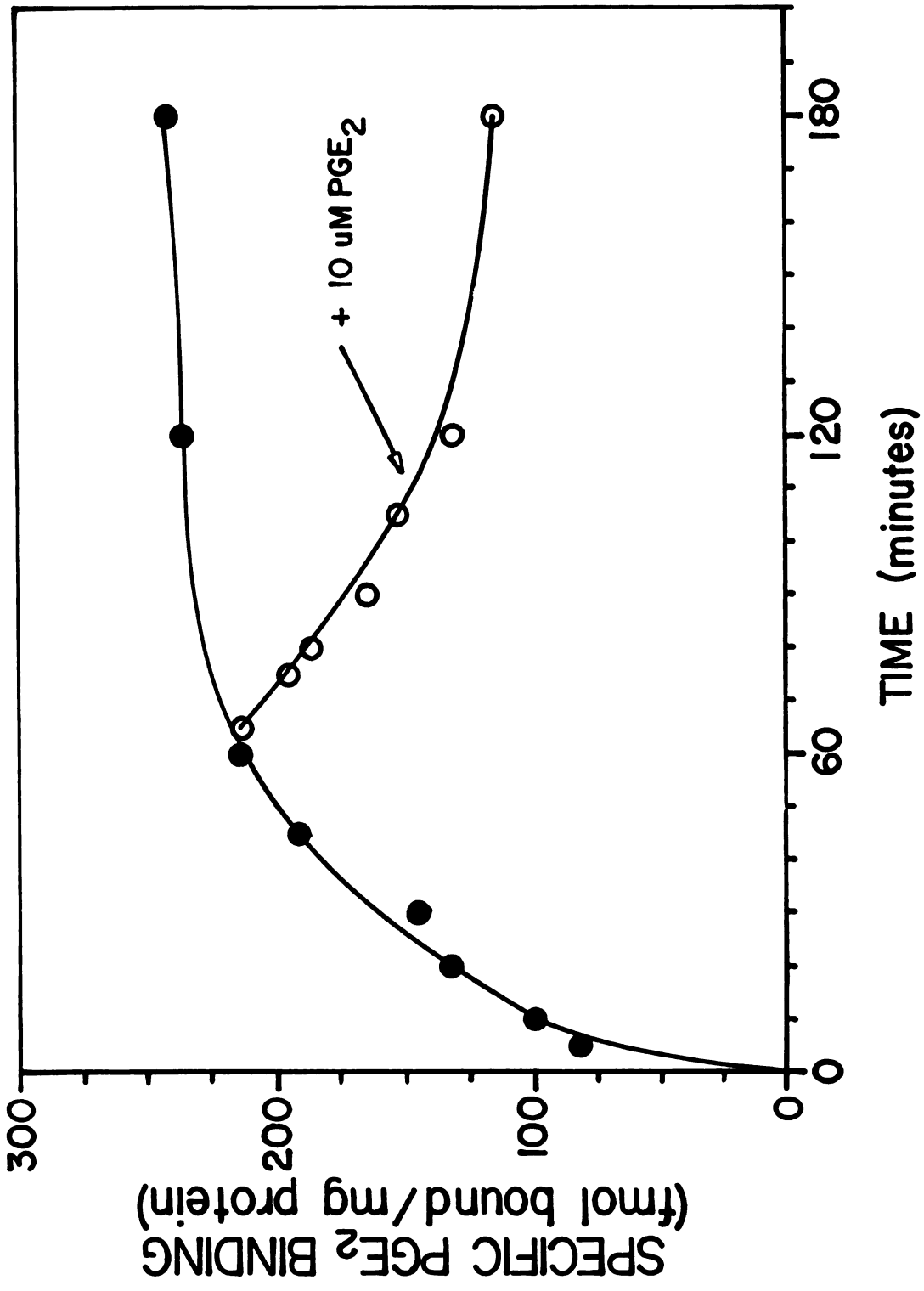
SPECIFIC PGE2 BINDING
(fmol bound/mg protein)
300
200
100
0
0
60
120
180
TIME (minutes)
+ 10 uM PGE2

Figure 21.

Figure 22. Scatchard analysis of specific PGE_2 binding to freshly isolated RCCT cell membranes in the absence (A) and presence (B) of 100 μM GTPγS. Binding assays were performed in duplicate at 30°C for 2 hours as described in the text in the presence of varying concentrations of PGE_2. Dashed lines represent estimated specific PGE_2 binding classes.

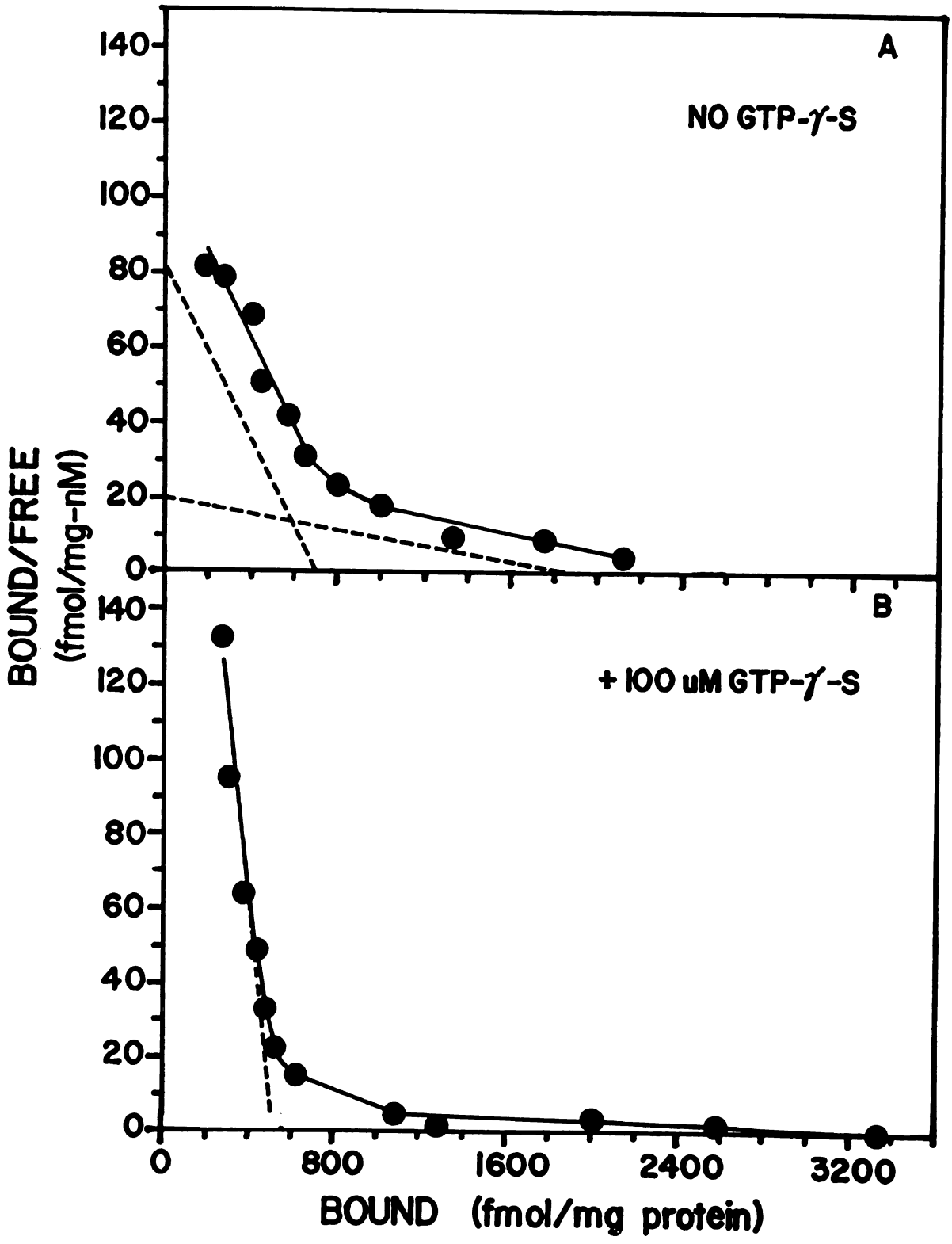
A
NO GTP-γ-S
B
+ 100 uM GTP-γ-S
BOUND/FREE
(fmol/mg-nM)
140
120
100
80
60
40
20
0
0
800
1600
2400
3200
BOUND (fmol/mg protein)

Figure 22.

functionally coupled to different guanine nucleotide-binding regulatory (G) proteins. To further test this hypothesis, we examined the effects of GTPγS on specific [^{3}H]PGE_2 binding activity in membranes prepared from freshly isolated RCCT cells pretreated with pertussis toxin.

In control RCCT cell membranes, 100 μM GTPγS stimulated specific [^{3}H]PGE_2 binding by 45%. However in pertussis toxin-pretreated membranes, addition of GTPγS slightly, but significantly decreased binding by 21% (Fig. 23).

Displacement of specific [^{3}H]PGE_2 binding by different prostaglandins in freshly isolated RCCT cells. To determine if PGE_2 interacts with distinct binding sites, the ability of different prostaglandins to displace [^{3}H]PGE_2 binding were compared with unlabeled PGE_2 in membranes prepared from freshly isolated RCCT cells (Fig. 24). Using 2 nM [^{3}H]PGE_2, 80% of the radioligand was specifically bound to the high affinity R_1 site, based on the Kd and Bmax values determined by Scatchard analysis. Therefore the displacement of [^{3}H]PGE_2 by the different prostaglandins tested under these conditions, reflects mainly the characteristics of the R_1 site. PGE_2 was the most potent; half-maximal displacement was achieved at 8 nM. Carbacyclin and $PGF_{2\alpha}$ were about 8-fold less potent than PGE_2 (EC_{50} = 54 nM and 72 nM, respectively), and 30-fold higher concentrations of PGD_2 than of PGE_2 were required to compete with [^{3}H]PGE_2 (EC_{50} = 263 nM).

Three different PGE derivatives were also compared with PGE_2 to displace [^{3}H]PGE_2 binding in freshly isolated RCCT cell membranes (Fig. 25). Sulprostone, PGE_1, and DM-PGE_2 were as potent as PGE_2 in

Figure 23. Comparison of the effect of GTPγS on specific PGE_2 binding to membranes from freshly isolated RCCT cells pretreated with or without pertussis toxin. Freshly isolated RCCT cells were pretreated for 4 hours at 37°C with vehicle (control) or pertussis toxin. Membranes were prepared and binding assays were performed in the absence or presence of 100 μM GTPγS for 2 hours at 30°C as described in the text. Values represent the mean of triplicate determinations ± S.E. *Significantly different from control values ($P<0.05$).

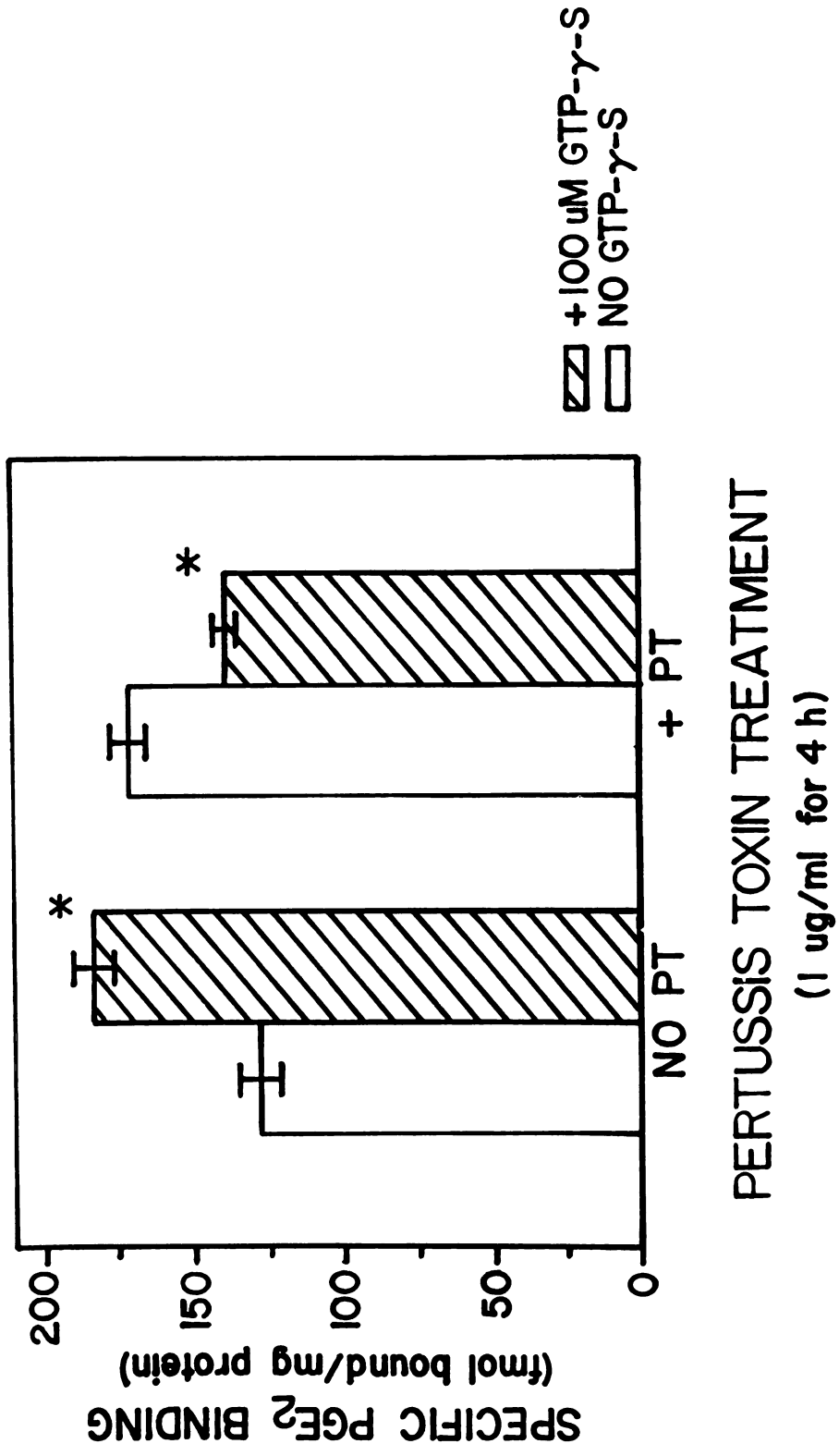
SPECIFIC PGE2 BINDING
(fmol bound/mg protein)
200
150
100
50
0
NO PT
+ PT
PERTUSSIS TOXIN TREATMENT
(1 ug/ml for 4 h)
+100 uM GTP-γ-S
NO GTP-γ-S
*
*

Figure 23.

Figure 24. Inhibition of specific [^{3}H]PGE_2 binding by various prostaglandins in membranes prepared from freshly isolated RCCT cells. Binding assays were performed at 30°C for 2 hours in duplicate as described in the text in the presence of varying concentrations of PGE_2 (o--o), carbacyclin (o--o), $PGF_{2\alpha}$ (Δ--Δ), or PGD_2 (Δ--Δ).

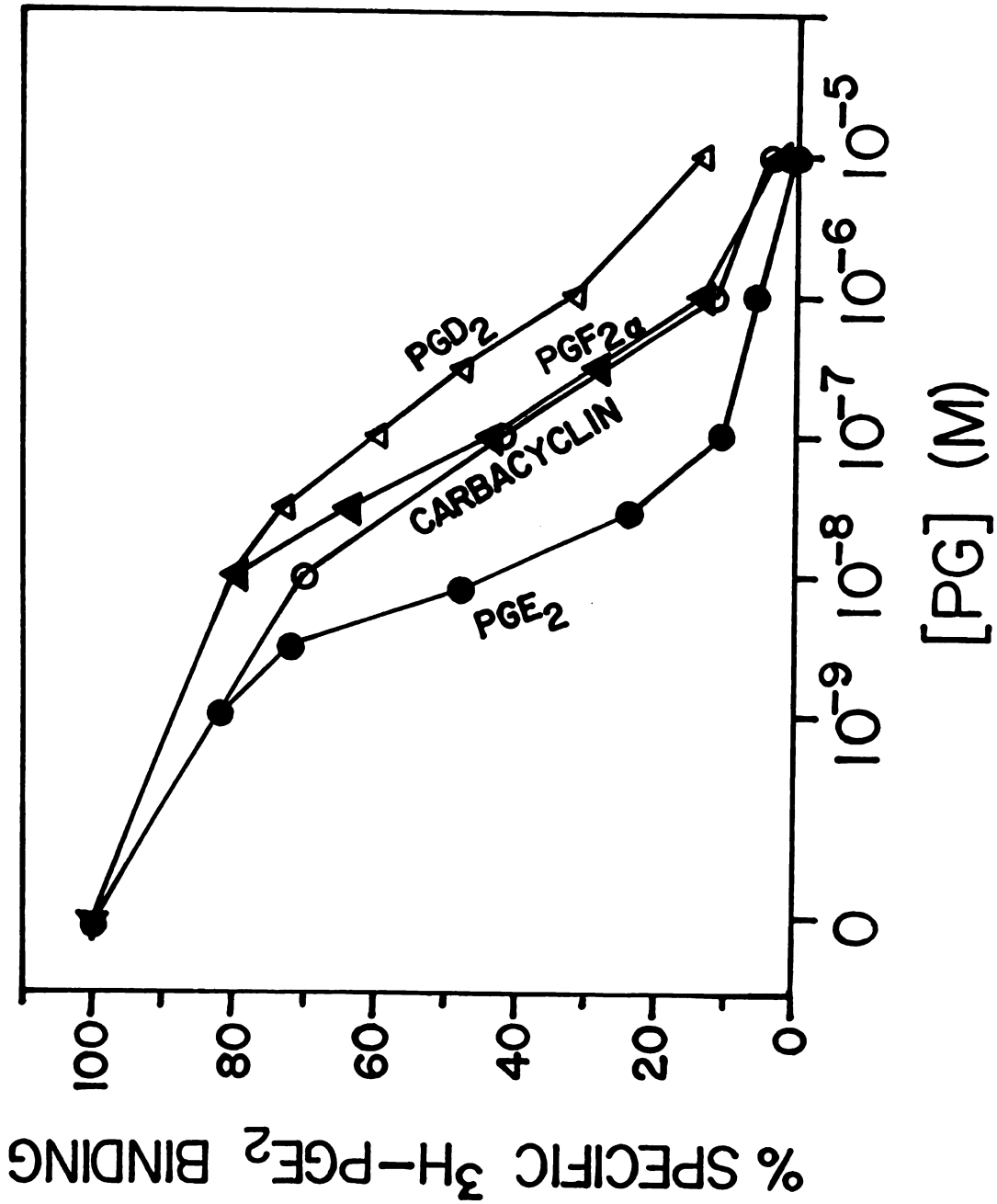
% SPECIFIC ^{3}H–PGE$_2$ BINDING
100
80
60
40
20
0
[PG] (M)
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
PGD$_2$
PGF2α
CARBACYCLIN
PGE$_2$

Figure 24.

Figure 25. Inhibition of specific [^{3}H]PGE_2 binding by various prostaglandins E derivatives to membranes prepared from freshly isolated RCCT cells. Binding assays were performed in duplicate at 30°C for 2 hours as described in the text in the presence of varying concentrations of PGE_2 (--), sulprostone, PGE_1, or DM-PGE_2.

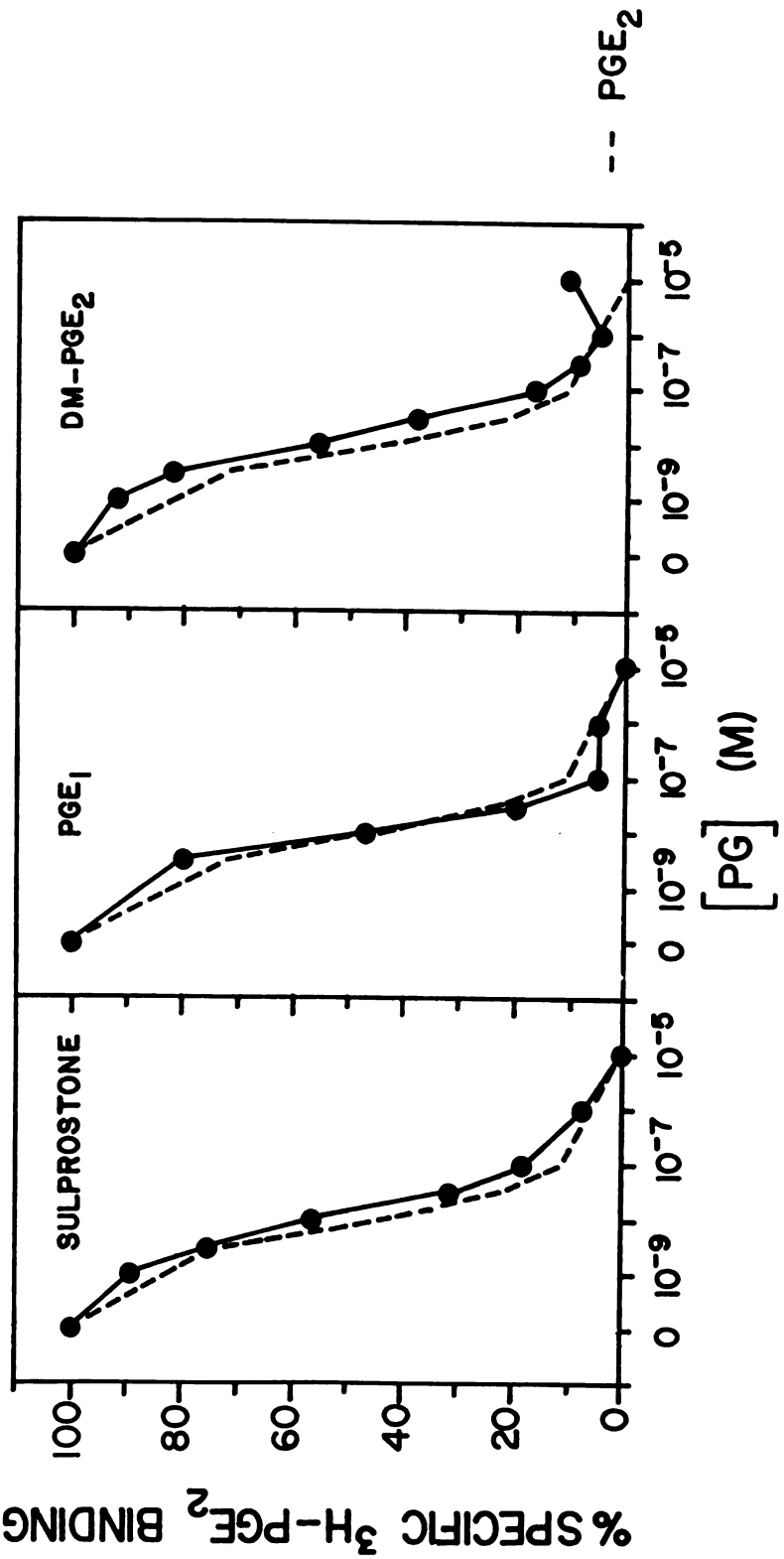
-- PGE2
DM-PGE2
PGE1
SULPROSTONE
0
10-9
10-7
10-5
[PG] (M)
0
20
40
60
80
100
% SPECIFIC 3H-PGE2 BINDING

Figure 25.

displacing [^{3}H]PGE_2 binding (EC_{50} values were 12 nM, 9 nM and 16 nM for sulprostone, PGE_1 and DM-PGE_2, respectively).

Comparison of PGE_2 binding activities in membranes from cultured and freshly isolated RCCT cells. The PGE_2 binding activity detected in cultured RCCT cell membranes was markedly different from the activity observed in membranes prepared from freshly isolated cells. In membranes prepared from cultured RCCT cells, the specific [^{3}H]PGE_2 binding activity was only 10-20% of that detected in freshly isolated cells (Fig. 26). As was observed in previous experiments, GTPγS stimulated specific [^{3}H]PGE_2 binding activity in freshly isolated RCCT cell membranes (Figs. 23 and 26). However, in membranes prepared from cultured RCCT cells, addition of 100 μM GTPγS decreased the PGE_2 binding activity by 65% (Fig. 26). Moreover, pertussis toxin pretreatment of cultured RCCT cells did not block the inhibitory effect produced by GTPγS on binding (data not shown).

In membranes prepared from freshly isolated RCCT cells, sulprostone displaced most of the [^{3}H]PGE_2 specific binding at concentrations greater than 100 nM (Figs. 25 and 26). However in membranes prepared from cultured RCCT cells, 1 μM sulprostone was ineffective in displacing specific [^{3}H]PGE_2 binding (Fig. 26).

Figure 26. Comparison of the effects of GTPγS and sulprostone on specific PGE_2 binding to membranes prepared from freshly isolated or cultured RCCT cells. Membranes from either freshly isolated (A) or cultured (B) RCCT cells were prepared and assayed for specific PGE_2 binding in the absence or presence of either 100 μM GTPγS or 1 μM sulprostone at 30°C for 2 hours as described in the text. Values represent the mean of triplicate determinations ± S.E. *Significantly different from control values ($P<0.05$).

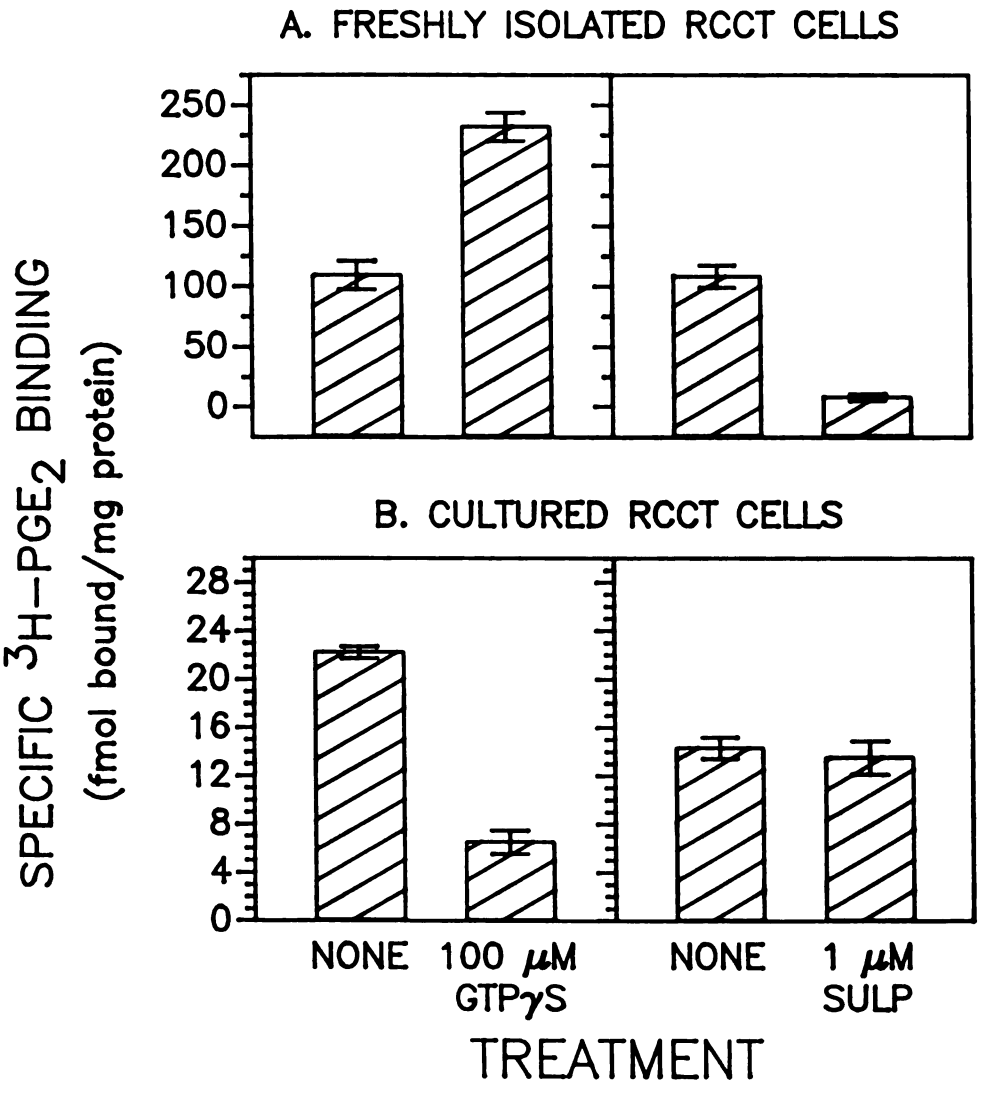
A. FRESHLY ISOLATED RCCT CELLS
250
200
150
100
50
0
B. CULTURED RCCT CELLS
28
24
20
16
12
8
4
0
SPECIFIC ^{3}H–PGE$_2$ BINDING
(fmol bound/mg protein)
NONE
100 μM GTPγS
NONE
1 μM SULP
TREATMENT

Figure 26.

DISCUSSION

In the rabbit cortical collecting tubule, PGE_2 and PGE_1 at low concentrations (1-100 nM) inhibit arginine-vasopressin (AVP)-induced water reabsorption (93-95), while 100 nM PGE_1 alone stimulates water reabsorption (93). These biphasic effects on water reabsorption have been attributed to the ability of E-series prostaglandins to differentially regulate cellular cAMP levels (94). In the previous chapter, we demonstrated that prostaglandin E derivatives have both stimulatory and inhibitory effects on cAMP metabolism in RCCT cells. At low concentrations (1-100 nM), E-series prostaglandins via a pertussis toxin-sensitive mechanism inhibit AVP-induced cAMP accumulation, and at higher concentrations ($\geq$100 nM) stimulate cAMP synthesis. The stimulatory effect was specific for E-series prostaglandins except the PGE_2 analogue, sulprostone. However, PGE derivatives including sulprostone were equally effective in inhibiting AVP-induced cAMP formation, suggesting that PGE produces these effects by interacting with two distinct types of receptors. In the present study we have attempted to correlate prostanoid binding to cAMP inhibitory and stimulatory actions in RCCT cells.

In membranes prepared from freshly isolated RCCT cells, $[^3H]PGE_2$ binding was rapid, saturable, reversible and specific for E-series prostaglandins. Our results indicate that there are two guanine nucleotide-sensitive classes of PGE_2 binding to membranes prepared

from freshly isolated RCCT cells. In the absence of GTPγS, the dissociation constant (Kd) of the high (R_1) and low (R_2) affinity sites were determined to be 9 and 100 nM, respectively. These Kd values correspond closely to the concentrations at which half-maximal inhibition of AVP-stimulated cAMP synthesis (3 nM), and PGE_2 stimulated cAMP formation (100 nM) were detected. Addition of GTPγS increased the affinity of R_1 approximately four-fold, and decreased the affinity of R_2 for PGE_2 to the extent that this binding class was no longer detectable. Pretreatment of freshly isolated RCCT cells with pertussis toxin blocked the ability of GTPγS to stimulate $[^3H]PGE_2$ binding to the high affinity site, suggesting that R_1 was coupled to the guanine nucleotide-binding regulatory protein (G_i) of adenylate cyclase. Moreover, GTPγS fails to stimulate $[^3H]PGE_2$ binding in cultured RCCT cells; in cultured RCCT cells PGE_2 does not inhibit AVP-induced cAMP formation. Lack of an inhibitory PGE_2-action is not due to the absence of a functional G_i-mediated transmembrane signaling pathway in cultured cells, since α_2-adrenergic stimulation inhibited AVP-induced cAMP accumulation, and pertussis toxin pretreatment blocked this effect (Chapter III). Therefore it is likely that cultured RCCT cells lack an inhibitory PGE receptor. In a previous study, Watanabe et al. solubilized a PGE_2 receptor from canine renal outer medulla (124). This receptor is physically coupled to a pertussis toxin-sensitive guanine nucleotide-binding protein. The guanine nucleotide and pertussis toxin sensitivity, as well as the pharmacological characteristics of the canine renal outer medullary receptor are similar to R_1 in freshly isolated RCCT cells.

Additional evidence supporting the notion that R_1 mediates inhibition of AVP-induced cAMP formation is based on the pharmacological properties of $[^3H]PGE_2$ binding to membranes prepared from freshly isolated and cultured RCCT cells. PGE_2, PGE_1, DM-PGE_2 and sulprostone were all equipotent in displacing $[^3H]PGE_2$ to membranes prepared from freshly isolated RCCT cells. Similarly, all of these E-series prostanoids were equally effective in inhibiting AVP-induced cAMP formation. In contrast to this observation, displacement of $[^3H]PGE_2$ specific binding did not correlate well with stimulation of cAMP synthesis by these prostaglandin E derivatives in freshly isolated RCCT cells. For example, DM-PGE_2 was one-tenth as potent as either PGE_2 or PGE_1 in stimulating cAMP synthesis. Moreover, sulprostone neither stimulated cAMP synthesis nor antagonized PGE_2-induced cAMP formation in freshly isolated RCCT cells, suggesting that this prostaglandin E derivative does not interact with R_2. Based on these results, we conclude that the R_1 binding class represents PGE/sulprostone receptors functionally coupled to G_i, which mediates the direct inhibition of adenylate cyclase in RCCT cells.

It is likely that the lower affinity R_2 binding site is coupled to activation of adenylate cyclase mediated by the stimulatory guanine nucleotide-binding regulatory protein (G_s) of adenylate cyclase. This notion is supported by the following observations: (a) the Kd for PGE_2 binding and the EC_{50} for PGE_2-stimulated cAMP formation are both approximately 100 nM; (b) GTPγS decreases the affinity for PGE_2 binding to R_2, suggesting that this class of binding represents a receptor functionally linked to a G-protein; (c) $[^3H]PGE_2$ binding in

cultured RCCT cell membranes is decreased by GTPγS, and this decrease is not sensitive to pertussis toxin; and (d) the amount of $[^3H]PGE_2$ specifically bound to cultured RCCT cell membranes and to R_2 sites in freshly isolated RCCT cell membranes (10-20% of total specific $[^3H]PGE_2$ binding at 2 nM $[^3H]PGE_2$) are approximately equal. Although these observations suggest that the R_2 sites in freshly isolated RCCT cells and the $[^3H]PGE_2$ binding detected in cultured RCCT cells represent PGE receptors linked to activation of adenylate cyclase mediated by Gs, it is conceivable that these low affinity receptors may be linked to other transmembrane signaling pathways (e.g. Ca^{++} mobilization).

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