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**Renal dopamine receptors: Characterization, autoradiographic
localization, and functional studies**

Huo, Teh-Li, Ph.D.

City University of New York, 1990

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RENAL DOPAMINE RECEPTORS:

Characterization, autoradiographic localization, and functional studies

by

Teh-Li Huo

**A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements for the degree of Doctor of
Philosophy, The City University of New York.**

1990

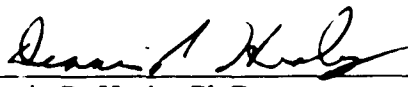
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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT**RENAL DOPAMINE RECEPTORS:
CHARACTERIZATION, AUTORADIOGRAPHIC LOCALIZATION, AND
FUNCTIONAL STUDIES**

by

Teh-Li Huo

Advisor: Professor Dennis P. Healy

Dopamine, a precursor of norepinephrine and epinephrine, has its own specific receptors in the kidney. Dopamine has profound physiological effects in the kidney such as vasodilatation, natriuresis, and diuresis via activation of renal dopamine receptors. The mechanisms involved in the receptor-mediated events or post-receptor signaling of renal dopaminergic system, however, have not been resolved. The renal DA₁ receptor has been reported to produce a renal vasodilatation and inhibition of sodium reabsorption in the proximal tubules by inhibition of Na⁺/K⁺ ATPase and the Na⁺/H⁺ antiporter. The role of the DA₂ receptors in the actions of dopamine in the kidney was unclear.

The present study was to characterize and localize DA₁ and DA₂ receptors and

to further determine the functions of the DA_2 receptors in the rat kidney. The localization of the DA_1 receptors was restricted to cortex, primarily in the proximal tubules. The results were compatible with a tubular site of action for dopamine in the kidney. The DA_2 receptors were localized primarily in the inner medullary collecting ducts with a lower density in the cortex. Radioligand binding and photoaffinity labeling experiments indicated that the medullary dopamine receptor may be a novel DA_2 receptor, because the renal DA_2 receptor exhibited an unique competition pattern but with similar receptor molecular weight compared with the central D_2 receptors. An endogenous dopaminergic system was found in the inner medullary collecting ducts (IMCD). IMCD cells have the capacity to synthesize dopamine from L-dopa locally via aromatic L-amino acid decarboxylase in the IMCD cells. Dopamine stimulated prostaglandin E_2 (PGE_2) synthesis via activation of the DA_2 receptors in cultured IMCD cells. The secondary messengers for dopamine-stimulated PGE_2 were unknown but activation of phospholipase A_2 and intracellular calcium were suggested. PGE_2 is known to be a potent inhibitor of vasopressin action. Dopamine was also able to inhibit vasopressin-stimulated cyclic AMP accumulation via activation of the α_2 -adrenoceptors in IMCD cells. Therefore, it is possible to speculate that dopamine in the inner medulla can inhibit vasopressin-mediated hydro-osmotic effect via two mechanisms: (a) directly via α_2 -adrenoceptor inhibition of adenylate cyclase activity, and (b) indirectly via synthesis of PGE_2 . The hypothesis is, therefore, that dopamine may be a functional antagonist of vasopressin in the renal medulla.

FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, several papers as chapters and a general discussion. Each chapter contains specific introductions, materials and methods, discussion and references. The references for the introduction and discussion follow the discussion. Copyright permission for chapter two has been obtained from the respective publisher.

DEDICATION

This, as with everything I do,
is dedicated to my wife, Amy, my daughter, Samantha, my parents and my brothers.

ACKNOWLEDGEMENTS

This thesis is the result of fruitful collaborative research. I owe a great deal of gratitude to many individuals for many things, especially my advisor:

Dennis P. Healy, Ph.D.

Chen-Shian Suen, Ph.D., Ravi Iyengar, Ph.D., Arcady Grenadar, Ph.D.,
Jack Peter Green, M.D., Ph.D., Sherwin Wilk, Ph.D., Thomas Mittag, Ph.D.,
Patrick Eggena, M.D., Shyan-Yih Chou, M.D., Detlef Schlondorff, M.D., and
Richard Premont, Ph.D.

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CHAPTER 1

OVERVIEW

Introduction

Dopamine, as well as being the precursor for norepinephrine and epinephrine, has specific receptors in central nervous system, peripheral nervous system, and several peripheral tissues such as pituitary, parathyroid gland, retina, gastrointestinal tract, and kidney (Kebabian and Calne, 1979; Stoof and Kebabian, 1984; Qu et al., 1989; Brown et al., 1977; Lefebvre, 1988; Lee, 1982; Goldberg et al., 1986). Serving as a neurotransmitter in the central nervous system, dopamine exerts its biological effects in motor function, behavior and psychiatric disturbance. Hypoactivity of nigrostriatal dopaminergic system results in Parkinson's disease. Hyperactivity of limbostriatal dopaminergic system may be associated with Schizophrenia. Other neuropsychiatric disorders such as Alzheimer disease and depression also were suggested to have some relationship with central dopaminergic system. Serving as a peripheral hormone, dopamine controls or modulates prolactin and melatonin release in pituitary, parathyroid hormone release in parathyroid gland, photoperceptive process in retina, motility in GI tract, and hemodynamic response as well as water and electrolyte balance in kidney. Unbalanced hypothalamo-pituitary dopaminergic system may cause hyperprolactinemia. Defect in renal dopaminergic system has been implied as one important etiological factor in essential hypertension.

The central D₁/D₂ receptor

The dopamine receptors in central nervous system and certain endocrine organs such as pituitary and parathyroid glands are classified into the D_1/D_2 subtypes based on the fact that the D_1 receptor is coupled to activation of adenylate cyclase and the D_2 receptor is not coupled or negatively coupled to adenylate cyclase (Kebabian and Calne, 1979). The prototype D_1 receptor is in the parathyroid gland and activation of D_1 receptors results in release of parathyroid hormone (Brown et al., 1977). The prototype D_2 receptor exists in the mammoth of the anterior lobe of the pituitary gland and activation of D_2 receptors results in inhibition of prolactin secretion (Kebabian and Calne, 1979). In the central nervous system, both D_1 and D_2 receptors are located postsynaptically. The D_2 receptor also exists presynaptically and may serve as an autoreceptor to inhibit dopamine release (Wolf and Roth, 1987). D_2 receptors play a major role in control of the behavior actions and D_1 receptors modulate the activity of D_2 receptor-mediated events (Arnt, 1987). Synergistic effects have been found between D_1 and D_2 receptors based on behavior and electrophysiological studies (Arnt, 1987; Walters et al., 1987), while the biochemical responses are opposed (positively and negatively linked to adenylate cyclase). Recently, striatal D_1 receptors expressed in *Xenopus* oocytes have been shown to couple to phosphoinositol cascade and Ca^{++} mobilization (Mahan et al., 1990). D_2 receptors, however, have been suggested to increase K^+ conductance (Freedman and Weight, 1988), inhibition of agonist-stimulated inositol phosphate formation (Vallar and Meldolesi, 1989), and inhibition of voltage-dependent Ca^{++} channels as well as Ca^{++} mobilization (Bigornia et al., 1988).

The peripheral DA_1/DA_2 receptor

Initially, the dopamine receptors outside the central nervous system has been classified into the DA₁ and DA₂ receptors based on *in vivo* physiological studies (Goldberg et al., 1986), while the classification of the central D₁ and D₂ receptor was based on radioligand binding and biochemical techniques (Kebabian and Calne, 1979). In general, the DA₁/DA₂ receptors represent the dopamine receptors in the cardiovascular and renal tissues, and the D₁/D₂ receptors include the dopamine receptors in the brain, pituitary gland, parathyroid gland and retina. The renal DA₁ receptor was characterized as being present in the renal vasculature based on the fact that dopamine- or DA₁ receptor agonists-mediated vasodilatation was blocked by DA₁ receptor antagonists (Goldberg et al., 1986; Kohli and Goldberg, 1987). Recently, by using ligand binding, receptor autoradiography, and biochemical techniques, the renal DA₁ receptor has been found primarily in the renal proximal tubules (Felder et al., 1988a and 1989c; Huo and Healy, 1989). The DA₂ receptor has been described in postganglionic sympathetic nerve terminals and vasculatures (Lokhandwala and DeFeo, 1988; Goldberg et al., 1986). The DA₁ receptor, like D₁ receptor, is located postsynaptically. The D₂ receptor, like D₂ receptor, is located both pre- and postsynaptically. The functions of DA₁ receptor, primarily, are renal vasodilation and natriuresis (Goldberg et al, 1986; Felder et al., 1989). The presynaptic DA₂ receptor, presumably, inhibits neurotransmitter release (Lokhandwala and DeFeo, 1988). The secondary messengers for DA₁ receptor, like D₁ receptor, are activation of adenylate cyclase and stimulation of phospholipase C (Felder et al., 1984a; Missale et al., 1988; Felder et al., 1989a and 1989d). The secondary messenger for DA₂ receptor, in some tissues, is inhibition of cAMP generation system (Missale et al., 1988; Amenta et al., 1990).

Comparison between the D₁/D₂ and the DA₁/DA₂ receptor

Ligand binding studies of the D₁/D₂ receptors using specific high affinity antagonists (for example, SCH23390 for D₁ and spiperone for D₂) showed that the dissociation constants (K_D) of these ligands were in the subnanomolar (0.03-1 nM) range. The half-maximal concentration of dopamine stimulation (EC_{50}) or inhibition (IC_{50}) of adenylate cyclase activity occurred at 1 - 10 μ M with maximal stimulation around 100 μ M (Kebabian and Calne, 1979). However, for the peripheral DA₁/DA₂ receptor, the K_D 's of the same ligands were in the nanomolar and submicromolar range (Felder et al., 1988b and 1988c; Huo and Healy, 1989), while the EC_{50} or IC_{50} for stimulation or inhibition of adenylate cyclase activity were similar or higher (less potent) when compared with the D₁/D₂ receptor. The reason for the discrepancies between the D₁/D₂ and DA₁/DA₂ receptors are unclear.

The D₁ receptor has been photoaffinity labeled to have a binding subunit of M_r = 72,000-74,000 (Niznik et al., 1988) compared with 74,000 for the DA₁ receptor in the proximal tubule (Felder et al., 1990). Two minor bands (M_r =50,000 and 32,000) were also present in the proximal tubules compared with two minor bands (M_r =62,000 and 51,000) in brain (Niznik et al., 1988). The D₁ receptor in bovine parathyroid gland, however, resided in a peptide of M_r = 62,000-64,000 (Niznik et al., 1989). After deglycosylation of the photolabeled D₁ receptors from the bovine brain and parathyroid gland, a single major labeled peptide of M_r =46,000 was found in both tissues (Jarvie et al., 1989). These data indicated that the protein backbone of the D₁ and DA₁ receptor

may display similar if not identical molecular weights (Jarvie et al., 1989).

The D_2 and DA_2 receptors also have been photoaffinity labeled with the same D_2/DA_2 ligand ^{125}I -NAPS (Amlaiky and Caron, 1985 and 1986; Huo and Healy, Chapter 3). Under stringent protease inhibitors condition a major labeled peptide of $M_r=120,000$ was found in striatum, pituitary and kidney inner medulla; however, under low stringency protease inhibitors condition, peptides of $M_r=94,000$ predominated in striatum and 38,000 predominated in rat pituitary and kidney inner medulla. In the porcine striatum, the D_2 receptor has been determined to be of $M_r=140,000/94,000$ (Jarvie et al., 1988) compared with 120,000-130,000 in the rat striatum (Huo and Healy, Chapter 3). Several low molecular weight peptide ($M_r=32,000-75,000$) sometimes occurred under low stringency or in the absence of protease inhibitors (Amlaiky and Caron, 1985 and 1986; Senogles et al., 1988; Jarvie et al., 1988; Huo and Healy, Chapter 3). Purified D_2 receptor from bovine anterior pituitary has been shown with the molecular weight of 120,000 (Senogles et al., 1988). Deglycosylation experiments of the D_2 receptor yielded a common unit of $M_r=44,000$ (Jarvie et al., 1988). Those studies indicated that the D_2/DA_2 receptors, like the D_1/DA_1 receptors, bear the structure similarities.

A dopamine- and cAMP-regulated phosphoprotein DARPP-32, which was supposed to be a protein phosphatase inhibitor, was found in high concentration in basal ganglia and was detectable in several peripheral tissue including kidney (Hemmings and Greengard, 1986; Hemmings et al., 1989; Meister et al., 1989). In the central nervous system, DARPP-32 has been shown to be localized to dopaminergic cells containing the D_1 receptor (Hemmings et al., 1989). It appears to serve as an

intracellular third messenger mediating the action of dopamine on the D_1 receptor (Hemmings et al., 1989). However, the major localization of this protein in kidney was in outer medulla (thick ascending limb of the loop of Henle) (Meister et al., 1989), whereas the DA_1 receptor is localized primarily in the cortex (Felder et al., 1988b; Huo and Healy, 1989). It is not clear that (1) whether the function of this protein in the kidney is only associated with the DA_1 receptor or may be associated with other receptors which are positively coupled to adenylate cyclase, or (2) whether it is a cAMP-regulated phosphoprotein in the kidney as in the central nervous system.

The synergistic effect of the D_1/D_2 receptor, as described earlier, in the central nervous system, was found in a similar way in the kidney that DA_1 or DA_2 agonists by themselves did not affect Na^+/K^+ ATPase activity in rat proximal tubule; however, the combination of DA_1 and DA_2 agonists did decrease Na^+/K^+ ATPase activity (Bertorello and Aperia, 1988a). The underlying biochemical mechanism is unclear for no synergistic effects of DA_1 -stimulated phospholipase C activity were found when DA_2 agonist was added (Felder et al., 1989a).

Dopamine and renal vasodilatation

Dopamine-induced renal vasodilatation has been demonstrated, in dogs, by low doses systemically or directly via the renal artery, and this effect could not be blocked by α or β adrenoceptor antagonists but could be blocked by dopamine receptor antagonists (Goldberg et al., 1986). Like other vasodilators, dopamine-induced renal vasodilatation is predominantly in cortico-medullary junction. The renal vasodilatation

induced by dopamine in animals such as dogs and rats, as well as humans is mainly due to activation of DA₁ receptors, because the effect persists in presence of α and β blockade, and is mimicked by DA₁ agonists and blocked by DA₁ antagonists (Bhat et al., 1986; Frederickson et al., 1985; Goldberg et al., 1986; Jose et al., 1987; Kohli et al., 1987; Hughes et al., 1988). The renal vasorelaxation effect of dopamine and DA₁ agonists, in rabbits and rats, had been shown in different sizes renal vessels (Edwards, 1986; Steinhausen, 1986). The role of DA₂ receptors in renal vasodilatation is not clear. In the report of Dupont et al. (1986), domperidone, a specific peripheral DA₂ receptor antagonist, was able to block apomorphine-induced vasoconstriction in rat hindquarters, renal and superior mesenteric vascular beds. However, in dogs, domperidone could block the femoral artery vasodilatation induced by dipropyl dopamine, a presumed DA₂ agonist, but could not block dopamine-induced renal vasodilatation (Kohli et al., 1983). Bromocryptine, a predominantly DA₂ agonist, did increase and LY171555, a selective DA₂ agonist, did not increase renal blood flow (RBF) in the rat and dog, respectively, and the specificity of their effect to the DA₂ receptor is not confirmed (Felder et al., 1988a; Stier et al., 1982; Jose et al., 1986). One possible mechanism of dopamine-induced vasodilatation that has been proposed is through presynaptic DA₂ receptor inhibition of norepinephrine release (Dupont et al., 1986; Missale et al., 1988). In humans, however, the vascular effect of dopamine may be mediated by calcium and prostacycline release (Manoogian et al., 1988). The discrepancies in vascular effects of DA₂ agonists and antagonists in vivo, in isolated vessels, and in isolated perfused kidney may be species or methods related (Kohli and Goldberg, 1987).

Dopamine and GFR

The effect of dopamine on glomerular filtration rate (GFR) is variable and may be dependent on experimental conditions. Dopamine increases GFR in hydropenic but not water loaded dogs (Imbs et al., 1981). The DA₁ agonist SKF38393 increased GFR in the denervated but not in the innervated rat kidney (Felder et al., 1988a). Baines and Drangova (1986) reported that neural, not tubular, dopamine increased GFR in isolated perfused rat kidney. DA₂ agonists have also been reported to increase superficial single-nephron GFR but not net total GFR (Stier et al., 1982). Pollock and Arendhorst (1987) have shown that SKF82526, a DA₁ agonist, increased single-nephron blood flow without affecting single-nephron GFR and glomerular pressure, which indicated that the ultrafiltration coefficient decreased. Barnett et al. (1986) showed that dopamine attenuated the contractile response to angiotensin II in isolated glomeruli and cultured mesangial cells. Felder et al. (1984a) suggested that the DA₂ receptor may modulate GFR based on the experiments of ligand binding and adenylate cyclase.

Tubular effects of dopamine

Although the renal vasodilator effect of dopamine and DA₁/DA₂ agonists contributes to the natriuretic and diuretic effects, the dopamine-induced changes in water and electrolyte excretion, however, can also occur in the absence of renal hemodynamic changes (Cuche et al., 1976; McGrath et al., 1985; Jose et al., 1987). Several lines of evidence demonstrated a direct tubular effect of dopamine on renal tubules. In the rat, microperfusion of peritubular capillaries with dopamine decreased fluid and bicarbonate transport in proximal convoluted tubules (Chan et al., 1986).

Dopamine also induced swelling of the isolated rat proximal convoluted tubule by decreasing sodium transport through inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (Aperia et al., 1987). In the rabbit, dopamine and the dopamine agonists epinine and 6,7-ADTN decreased fluid and sodium transport in the isolated perfused proximal straight but not convoluted tubule (Bello-Reuss et al., 1982). These effects were blocked by the nonselective dopamine antagonist haloperidol but not by α -adrenergic or serotonergic antagonists. The discrepancies in these studies may be due to species differences.

Recently, the mechanism underlying the natriuretic effect of dopamine has been explored. Bertorello and Aperia et al. (1988a) reported that dopamine could inhibit $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in rat proximal convoluted tubules and the combination of DA_1 agonist fenoldopam and DA_2 agonist LY171555 had similar effects. They also demonstrated that pertussis toxin-sensitive G proteins and protein kinase C may be the secondary messengers of dopamine-mediated inhibitory effect on $\text{Na}^+\text{-K}^+\text{-ATPase}$ in proximal tubules (Bertorello and Aperia, 1989a and 1989b). Felder et al. (1989b) reported that dopamine and the DA_1 agonist fenoldopam inhibited $\text{Na}^+\text{-H}^+$ antiport activity in renal proximal brush border membrane, and cAMP may be the secondary messenger for these effects. This effect was blocked by DA_1 antagonist SCH23390. They also revealed that dopamine and fenoldopam inhibited $\text{Na}^+\text{-K}^+\text{-ATPase}$ in proximal tubule basolateral membrane, and stimulation of phospholipase C and subsequent activation of protein kinase C may mediate these effects (Felder et al., 1989a and 1989d). In spontaneous hypertensive rat (SHR), a defective coupling of the DA_1 receptor and adenylate cyclase was found (Kinoshita et al., 1989).

There is also evidence that dopamine can influence water and electrolyte transport beyond the proximal tubule. Dopamine blocks the antidiuretic effect of vasopressin by inhibiting the hydro-osmotic effect of vasopressin in the toad bladder (Arruda and Sabatini, 1982) and in the rabbit cortical collecting tubule (Muto et al., 1985). Haloperidol, a nonselective DA receptor antagonist, blocked the latter effect. In water-loaded rats, dopamine inhibited the antidiuretic effect of vasopressin which could be blocked by haloperidol (Koyoma et al., 1985). Dopamine also antagonized the effects of DOCA, an analogue of aldosterone, on tubular transluminal potential difference in rabbit cortical collecting tubule (Muto et al., 1985). The receptor subtypes involved in the action of dopamine in the distal nephron are not clear. There is evidence that, on the one hand, dopamine might directly activate α_2 -adrenoceptors and inhibited the antidiuresis effect of vasopressin (Lehr et al., 1967; Baggio and Ferrari, 1981) or activate dopamine receptors (Koyoma et al., 1985; Muto et al., 1985); on the other hand, dopamine and bromocryptine, a D_1/DA_2 agonist, may increase PGE_2 secretion and indirectly inhibit the antidiuresis effect of vasopressin (see below).

Dopamine and renin

Dopamine may directly stimulate renin secretion via the DA_1 receptor. Dopamine and the DA_1 agonist fenoldopam increased renin secretion in enriched rat juxtaglomerular cells (Kurts et al., 1988) and rat cortical slices (Antonipillai et al., 1989). Both effects can be blocked by DA_1 antagonist SCH23390 but not propranolol.

Interactions of dopamine and other intrarenal hormones

There is evidence that exogenous and endogenous dopamine may have significant physiological effects in the kidney. Acute volume expansion with saline loading increased urinary dopamine secretion (Hegde and Lokhandwala, 1988). SCH23390, a DA₁ antagonist, can completely block fenoldopam-mediated natriuresis but only partially block renal responses to exogenous dopamine (Hegde and Lokhandwala, 1988). This suggests that DA-mediated effects can not solely be accounted for by DA₁ receptor activation. Baggio and Ferrari (1981) reported that dopamine and (3,4-dihydroxyphenylamino)-2-imidazoline (DPI) were able to produce diuresis and lowered urinary osmolality in rats, and that this effect could be blocked by α_2 -adrenergic antagonists yohimbine and phentolamine.

In the studies of Yoshimura et al. (1987), in the rat, urinary PGE₂ excretion was increased by treatment of bromocryptine, a DA₂ agonist, accompanied by augmented natriuresis suggesting a DA₂ receptor-mediated effect. The DA₁ agonist fenoldopam, however, failed to increase urinary PGE₂ secretion in dog (Jose et al., 1987). Urinary PGE₂ excretion was suppressed by treatment of carbidopa, a dopa decarboxylase inhibitor (Yoshimura et al., 1987). These results implied that renal dopaminergic system may be involved in the synthesis of PGE₂ via activation of the DA₂ receptor. In another study, lower urinary PGE₂ and dopamine excretion were found in essential hypertensive subjects, and dopamine infusion could significantly increase urinary PGE₂ excretion (Iimura et al., 1987). In humans, however, PGI₂ release may be the mediator of dopamine-mediated renal vasodilatation (Manoogian et al., 1988).

The natriuretic and diuretic effects of atrial natriuretic factor (ANF) may in part be due to dopaminergic mechanisms (Pettersson et al., 1986 and 1989; Hansell et al., 1987 and 1988; Martin-Crez, 1986; Katoh et al., 1986 and 1989). However, the mechanism is complicated. It has been suggested that the DA_1 receptor may mediate the action of ANF because the natriuretic effect of ANF were completely abolished by pretreatment of either haloperidol, R-sulpiride, or SCH23390 (Martin-Crez et al., 1986; Hansell et al., 1987; Katoh et al., 1989). The intact renal nervous system may or may not required for the effects of ANF. In the rat, the natriuretic and diuretic effects of ANF were not affected by denervation (Pettersson et al., 1989; Katoh et al., 1989). However, the renal response of ANF was significantly reduced by 6-hydroxy-dopamine pretreatment which destroys dopamine neurons (Martin-Crez et al, 1986). Pretreatment with reserpine which depletes both dopamine and norepinephrine in the kidney, also abolished the renal effects of ANF (Pettersson et al., 1989). Endogenously produced dopamine seems to be important for ANF actions, since pretreatment with carbidopa, a aromatic L-amino acid decarboxylase (AADC) inhibitor, reduced the natriuretic and diuretic effects of ANF (Katoh, et al., 1986 and 1989), but in the study of Pettersson et al. (1989), the effects of ANF were not changed with pretreatment of benserazide, another AADC inhibitor. In addition, the renal effects of ANF were independent of cGMP production (Katoh et al, 1989). Direct interaction of ANF with DA_1 receptors has been suggested based on the fact that ANF was able to compete ^3H -SCH23390 binding in whole kidney slices with $IC_{50}=10\text{ }\mu\text{M}$ compared with $6.3\text{ }\mu\text{M}$ for dopamine (Lokhandwala et al., 1990).

Endogenous dopamine

Several lines of evidence indicate that dopamine may be synthesized intrarenally and that endogenous dopamine may play an important role in the regulation of sodium excretion. Conversion of circulating L-dopa to dopamine has been proposed as a possible mechanism for dopamine synthesis in the kidney (Lee, 1982; Bell, 1987). Renal dopamine derives mainly from renal tubular dopamine production and to a lesser extent from dopaminergic nerves (Bell, 1988). Immunohistochemical staining of aromatic l-amino acid decarboxylase (AADC), the enzyme converts L-dopa to dopamine, was markedly high in the proximal tubules and less in distal tubule and papillary collecting ducts (Adam et al., 1986). Inhibition of AADC activity attenuated urinary dopamine and sodium excretion associated with acute or chronic sodium administration (Ball et al., 1977; Bertorello et al., 1988b). Activation of renal DA₁ receptors by endogenous dopamine was reported to contribute the natriuretic response to acute volume expansion by salt loading in rats (Hegde and Lokhandwala, 1988; Hegde et al., 1989a).

It has been suggested that circulating L-dopa is the main source of urinary dopamine (Brown and Dollery, 1981; Lee, 1982). This idea was supported by the observations that, in humans, urinary excretion of dopa also increased during dietary salt loading (Goldstein et al., 1989), and in dogs, urinary dopamine secretion could be accounted for completely by renal uptake and decarboxylation of circulating dopa (Zimlichman et al., 1988). In addition, in rats, renal nerves contributed to the early natriuretic and dopa and dopamine excretory responses, but in the absence of renal neuronal activity (> 5 days), salt-induced increases of urinary sodium, dopa, and

dopamine were maintained (Grossman et al., 1990). Extra-renal tissue such as muscle may be the source of L-dopa (Eldrup et al., 1989). Baines and Chan (1980) estimated from results of a micropuncture study that about 30% of urinary free dopamine was derived from plasma dopa, whereas Suzuki et al (1984) suggested that most, if not all, of urinary dopamine production by isolated, perfused rat kidneys was derived from plasma dopa.

Circulating conjugated dopamine has been suggested to be the source of urinary dopamine by Unger et al. (1980) because large amount of conjugated dopamine released from adrenal gland was present in the plasma and relatively abundant deconjugated enzymes such as β -D-glucuronidase and arylsulphatase were present in the kidney. However, this hypothesis was, not supported by Ball and Lee (1977). They observed that administration of carbidopa, a peripheral dopa decarboxylase inhibitor, reduced the urinary dopamine output, and parallel increases of urinary free and conjugated dopamine were found in oral salt loading rat (Lee, 1982). In addition, Akpaffiong (1981) demonstrated that, in rats with both adrenal gland removed, it was still possible to elicit a marked increase in urinary dopamine by oral salt loading suggesting that adrenal glands, a major source of circulating dopamine, do not contribute in an important way to the renal production of dopamine (Lee, 1982).

Other sources of urinary dopamine include release of dopamine or dopa from noradrenergic or dopaminergic nerve endings in the kidney (Dinerstein et al., 1979). Kambara et al. (1987) reported that renal denervation did not significantly attenuate urinary dopamine responses during dietary salt loading in rats. Baines and Drangova

(1984) noted that dopamine production in isolated perfused kidneys whether intact or denervated. It should be noted that most of the studies were done after denervation of one week. However, time course studies (Grossman et al, 1990) indicated that renal nerves contributed to the early dopamine and dopa excretory responses, but were not important after one week of denervation.

Dopamine receptors binding in the kidney

Dopamine receptors have been studied by homogenate binding with relatively nonselective radiolabeled dopamine antagonists (Felder et al., 1984a and 1984b). Recently, DA₁ antagonists ¹²⁵I-SCH 23982, ³H-SCH23390 and DA₁ agonist ³H-fenoldopam have been used in renal homogenate binding and autoradiographic studies (Felder and Jose, 1988b; Hegde et al., 1989b; Hughes and Sever, 1989; Huo and Healy, 1989). DA₁ ligand binding studies indicated that proximal tubules contained large amounts of DA₁ receptors (Huo and Healy, 1989) which is compatible with the physiological data. Interestingly, DA₁ receptors characterized by in vitro autoradiography in rats suggested that DA₁ receptors were either not present (Huo and Healy, 1989) or present at low density (Hegde et al., 1989b) on vascular elements. Less is known regarding the DA₂ receptors. Based on the binding of the nonselective radiolabeled dopamine antagonist ³H-haloperidol and inhibition of adenylate cyclase by dopamine, Felder et al. concluded that glomeruli expressed DA₂ receptors (Felder et al, 1984a). More recently, binding of the relatively selective DA₂ antagonist ³H-spiperone in the rat and sheep kidney has been reported (Hietala et al., 1988; Felder et al., 1988c and

1989). The possibility that ^3H -spiperone was labeling 5-HT₂ receptors in the kidney, as has been shown in brain (Creese and Snyder, 1978; Huff and Molinoff, 1982), was ruled out because the 5-HT₂ selective antagonist ketanserin was unable to compete for the ^3H -spiperone binding (Hietala et al., 1988; Felder et al., 1988c and 1989). We found that, by using in vitro autoradiography, ^3H -spiperone binding sites were highly concentrated on cells of the inner medulla collecting duct (Huo and Healy, Chapter 3). ^3H -spiperone binding in renal vasculature also has been shown (Brodde, 1981 and 1982).

Thesis plan

Since the intrarenal actions of dopamine are mediated via dopamine receptors, a better understanding of the distribution and function of dopamine receptors in the kidney is essential to understanding the renal dopamine system. This thesis was designed to answer the following questions: What is the localization of the DA₁ and DA₂ receptors ? What is the function of the DA₁ and DA₂ receptors ? Are the renal dopamine receptors similar to the central dopamine receptors ? Does dopamine interact with other intrarenal hormones ?

Several experimental techniques were used for studies. Radioligand binding and in vitro autoradiography were used to characterize and localize the DA₁ and DA₂ receptors. Photoaffinity labeling procedure was used to compare central and renal dopamine receptors. Primary cell culture and radioimmunoassay were used to study the functional relationship of dopamine, prostaglandins and α_2 -adrenoceptors. HPLC-

electrochemical detection and radioenzyme assay techniques were used to identify the existence of dopamine and dopamine-forming enzyme.

The thesis describes studies aimed at understanding the role of renal dopamine receptors in mediating the effect of dopamine in the kidney. Chapter 1, the overview, reviews the current knowledge of central and peripheral dopamine receptors and the physiological effects of dopamine in the kidney. Chapter 2 and 3 are studies which address the binding characteristics and localization of renal dopamine (DA_1 and DA_2) receptors. Chapter 3 compares the renal DA_2 dopamine receptor with the central D_2 receptors. Chapter 4 and 5 discuss the functions of renal DA_2 dopamine receptor and its interaction with renal prostaglandin and α_2 -adrenoceptor systems. Chapter 6 provides general conclusions and implications of these studies.

CHAPTER 2

Autoradiographic Localization of Dopamine-1 (DA₁)

Receptors in Rat Kidney with ³H-SCH23390

(Am. J. Physiol. 257: F414-F423, 1989)

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ABSTRACT

Dopamine receptors in the rat kidney were characterized by homogenate binding and in vitro autoradiography using the DA₁-selective antagonist ³H-SCH23390. ³H-SCH23390 binding in cortical membrane preparations was saturable, stereoselective and competed for by DA agonists and antagonists with a rank order of potency consistent with the labeling of the DA₁ receptor. ³H-SCH23390 binding was best fit to a two-site model: a high affinity-low density site (K_{D1} 4.9 ± 1.4 nM, B_{max1} 31.4 ± 13.8 fmol/mg protein) and a low affinity-high density site (K_{D2} 86.4 ± 23.9 nM, B_{max2} 848.0 ± 227.4 fmol/mg protein). In vitro autoradiography indicated that ³H-SCH23390 binding sites were restricted to the cortex. High resolution autoradiography further indicated that ³H-SCH23390 binding sites were localized primarily on proximal tubules. Glomeruli and other vascular elements were devoid of ³H-SCH23390 binding sites. These results suggest that DA and DA₁ agonists may affect sodium excretion by a direct action on proximal tubule sodium reabsorption.

INTRODUCTION

Infusion of dopamine (DA) into experimental animals or man results in an increase in renal blood flow and sodium excretion (Lee, 1982; Lokhandwala and Barrett, 1982). Although the predominant physiological effect of DA appears to be on renal hemodynamics (Chapman et al., 1980; Frederickson et al., 1985; Hardaker and wechsler, 1973; Nichols et al., 1987; Schmidt and Imbs, 1980; Schmidt et al., 1987), the mechanism of the DA-mediated natriuresis is still controversial. The increase in sodium excretion by DA could be an indirect tubular effect, secondary to an increase in renal blood flow, or it could result from a direct tubular effect on renal tubular sodium transport (Carey and Hughes, 1987; Frederickson et al., 1985). Indeed, a direct tubular action by DA has been proposed to contribute to the DA-mediated natriuresis in a variety of species, including man (Bello-Ruess et al., 1982; Carey and Hughes, 1987; Wasserman et al., 1980).

Alterations in sodium intake are accompanied by parallel changes in urinary DA excretion in experimental animals and man (Bell, 1987; Lee, 1982). Several lines of evidence indicate that DA may be synthesized intrarenally and that endogenous DA may play an important role in the regulation of sodium excretion. Dopaminergic nerves have been identified in the vascular pole of glomeruli in the dog (Dinerstein et al., 1979), but in the rat the existence of these nerves has not been clearly established (Baines and Drangova, 1986; McGrath et al., 1983). In the rat, however, the levels of L-amino acid decarboxylase (AADC), the enzyme that converts L-dopa to DA, are high in the kidney cortex and AADC has been localized immunohistochemically to

proximal tubules (Bertorello et al., 1988). Conversion of circulating L-dopa to DA has been proposed as a possible mechanism for DA synthesis in the kidney (Bell, 1987; Lee, 1982). Inhibition of AADC in isolated perfused kidneys with carbidopa diminishes urinary sodium excretion, DA excretion and DA content (Baines and Drangova, 1986). DA or L-dopa inhibits $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in the rat proximal tubule and benserazide, an AADC inhibitor, reverses the effects of L-dopa (Aperia et al., 1987; Bertorello et al., 1988). In addition, rats on a high salt diet have depressed levels of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in proximal tubules and this activity can also be reversed by administration of benserazide (Bertorello et al., 1988). Thus, it has been proposed that endogenous DA might act as an intrarenal natriuretic hormone (Aperia et al., 1987; Bell, 1987; Lee, 1982).

DA receptors have been characterized extensively in the brain and have been subdivided into D_1 and D_2 receptor subtypes (Kebabian and Calne, 1979). A similar classification has been proposed for DA receptors in peripheral tissues, but because these receptors exhibit a somewhat different pharmacological profile than the brain receptors, they have been designated as DA_1 and DA_2 (Goldberg and Kohli, 1979). In general, DA_1 receptors appear to be postsynaptic and stimulate adenylate cyclase, whereas DA_2 receptors appear to be localized presynaptically on sympathetic nerve endings and inhibit the release of norepinephrine and are negatively linked, or unlinked, to adenylate cyclase (Goldberg and Kohli, 1979; Stoof and Kebabian, 1984). The effects of DA on the kidney appear to be mediated via the DA_1 receptor subtype since the DA_1 -selective agonist fenoldopam (SKF82526) increases renal blood flow in the dog (Hahn et al., 1982; Nichols et al., 1987) and the rat (Lokhandwala and Steenberg, 1984),

whereas the DA₁-selective antagonists SCH23390 and SKF83566 block the renal vasodilatory effects of DA or fenoldopam (Frederickson et al., 1985; Lokhandwala and Steenberg, 1984; Nichols et al., 1987; Schmidt et al., 1987). These effects are not blocked by DA₂ antagonists or α - and β -adrenergic antagonists. DA receptors have been studied by homogenate binding with relatively nonselective radiolabeled dopaminergic antagonists and by stimulation of adenylyl cyclase with dopaminergic agonists (Felder et al., 1984a and 1984b; Nakajima and Kuruma, 1980). Felder et al. (Felder et al., 1984a) concluded that in the rat DA₁ receptors were localized on cortical tubules and that DA₂ receptors were localized on glomeruli. Shultz et al. (Schultz et al., 1987) reported, however, that isolated mesangial cells grown in culture contain DA₁ receptors since fenoldopam and SKF38393, DA₁-selective agonists, increased cAMP accumulation. This effect could be blocked by SCH23390 but not by the DA₂-selective antagonist domperidone.

Further insights into the mechanism of action of DA in the kidney may be possible with a better understanding of the localization of DA receptors in the kidney. Since tissue isolation techniques have inherent problems of contamination from other cell types, an alternative approach would be to localize DA₁ receptors in the kidney using the *in vitro* receptor autoradiographic procedure. This method preserves the anatomical integrity of the tissue and has been utilized to localize a variety of receptors in the kidney (Healy and Fanestil, 1986; Healy et al., 1985). Therefore, in this report we first characterize DA₁ receptors in rat kidney with ³H-SCH23390 and selective DA agonists and antagonists using homogenate binding techniques followed by *in vitro* autoradiography of ³H-SCH23390 binding in kidney sections. Our results indicate

³H-SCH23390 binding sites are restricted to the renal cortex and are localized primarily on proximal tubules and not vascular elements.

MATERIALS AND METHODS

Preparation of Tissues

1. Homogenate binding

Male Sprague-Dawley rats (200-250g, Charles River) were housed on a 12 hour L/D schedule and allowed free access to food and water. Animals were decapitated and the kidneys removed quickly and placed on ice. All subsequent steps were carried out at 4°C. The kidneys were sliced longitudinally in half and the medulla excised. The kidney cortex was then homogenized with a Polytron (Brinkman) for 15 seconds at setting #4 in 10X volume of 25 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA and 10% sucrose. The homogenate was sieved through 5 layers of cheesecloth and centrifuged at 400 x g for 10 minutes. The pellet was discarded and the supernatant re-centrifuged at 20,000 x g for 20 minutes. The pellet was then resuspended with hypotonic buffer and centrifuged at 20,000 x g for 20 minutes. The final pellet was resuspended in isotonic Tris buffer and aliquots stored at -80°C, where they were stable a minimum of six months. Protein concentration was determined by the method of Lowry et al. (1951).

2. Autoradiography

Tissues were prepared for autoradiography as previously described (Healy and Fanestil, 1986; Healy et al., 1985). Briefly, male Sprague-Dawley rats (200-250g) were anesthetized with sodium pentobarbital (50 mg/kg, ip) and perfused transcardially with 200 ml 0.1% formalin in phosphate-buffered saline (PBS), pH 7.4. The kidneys were removed and frozen on microtome chucks with powdered dry ice. Fifteen μ m cryostat sections along the mid longitudinal axis were thaw mounted onto clean subbed microscope slides, dried and stored at -20°C until used (<one week).

Homogenate Binding Studies

DA₁ receptor binding assays were conducted using a modification of procedure of Savasta et al. (1986). The assay buffer was 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 100 μ M pargyline and 0.01% ascorbic acid with the final pH adjusted to 7.4 at 25°C. Assays were initiated by adding 50 μ l of crude membrane (250 μ g protein) resuspended in Tris buffer 50 μ l of dilute radioligand plus 50 μ l drug or vehicle in glass tubes on ice. Samples were incubated in a shaking water bath at 25°C for fifteen minutes. The reaction was terminated by adding 4 x 4 ml cold saline rinses and vacuum filtration through Whatman GF/C filters that had been presoaked with 0.3% polyethylenimine to reduce non-specific filter binding. The filters were placed in polypropylene scintillation vials, Aquasol-2 LSC cocktail (DuPont) added, and counted in a Beckman LS-3801 liquid scintillation counter with a counting efficiency of 62%.

For saturation binding experiments 0.25-420 nM ^3H -SCH23390 (Amersham, sp. act. 76-83 Ci/mmol) was used. Non-specific binding was defined as binding in the presence of 100 μM haloperidol, with a total:non-specific binding ratio of approximately 4:1. For competition studies, 5 nM ^3H -SCH23390 was used together with increasing concentrations of unlabeled DA agonists and antagonists.

Degradation of ^3H -SCH23390 during the incubation was monitored by thin-layer chromatography. Following incubation the protein was pelleted by centrifugation and a sample of the supernatant spotted on precoated plastic chromatography sheets (Polygram Sil G/UV254, mobile phase chloroform:ethanol, 15:1) and migration determined with excess unlabeled SCH23390 as the marker.

Autoradiography

The in vitro labeling of slide-mounted tissue sections with ^3H -SCH23390 was conducted using a similar procedure as reported previously (Healy and Fanestil, 1986; Healy et al., 1987) and incubation conditions similar to those used for the homogenate binding studies outlined above. In addition, since the kidney sections used for autoradiography are unfixed, we have noted that the quality of morphology for the high resolution autoradiographs is markedly improved if incubated at 4°C. Therefore, sections to be used for high resolution autoradiography were incubated at 4°C, otherwise sections were incubated at 25°C. There was no difference in the pattern of labeling in the tissues incubated at these two temperatures.

Slide-mounted kidney sections were thawed and pre-incubated for 15 minutes at 4°C or room temperature in incubation buffer minus radioactivity and then transferred to incubation buffer for 45 minutes at room temperature or for 90 minutes at 4°C. The incubation was stopped by placing the slides in cold buffer for 2 x 15 minute rinses. The sections were then dipped in water and dried. Sections were either wiped from the slides for measurement of radioactivity by liquid scintillation counting or apposed to emulsion for autoradiography (see below).

Competition studies were performed to insure that the slide-mounted tissue sections were labeled with ³H-SCH23390 in a manner consistent with labeling the DA₁ receptor. Kidney sections were incubated in buffer containing 2-3 nM ³H-SCH23390 and increasing concentrations of unlabeled DA agonists or antagonists. For localization studies, 10 nM ³H-SCH23390 was incubated alone or plus 100 µM haloperidol to define non-specific binding in adjacent sections, with a total:non-specific ratio of approximately 4:1.

Autoradiographic images from the radiolabeled kidney sections were generated using either low resolution LKB Ultrofilm or higher resolution emulsion-coated coverslips. Following the final drying step, radiolabeled slide-mounted kidney sections were placed in an X-ray cassette (Wolf Slimline) and a sheet of Ultrofilm placed over the sections and the cassette sealed. After exposure at 4°C for approximately 2 weeks the film was removed and developed. These same sections were then apposed with thin emulsion-coated coverslips that had been coated in NTB-3 (Kodak) liquid emulsion diluted 1:1 with water. After exposure the coverslips were developed and the section

counterstained with pyronin Y or processed for alkaline phosphatase histochemistry (Healy and Fanestil, 1986). Silver grains were counted over identified renal structures with a Zeiss microscope using a 100X oil-immersion objective and a 10X eyepiece with an ocular grid. Grain counts from multiple samples were averaged from each individual animal. A minimum of three animals were used for each group.

Data Analysis

Radioligand binding data was analyzed using the Munson and Rodbard LIGAND program (Munson and Robbard, 1980). All results are expressed as mean $\pm$ S.E.M.

Chemicals

The following agents were generous gifts from pharmaceutical companies: SCH23390 (Schering, Bloomfield, NJ); l- and d-sulpiride (Ravizza S.P.A., Milan, Italy); fenoldopam, (Smith Kline and French, Swedeland, PA); cis(Z)-flupentixol (Lundbeck, Copenhagen); and ketanserin (Janssen Pharmaceutica, Beerse, Belgium). SKF38393, SKF83566, SCH23388, (+) and (-)butaclamol, 6,7-ADTN and spiperone were purchased from Research Biochemical (Natick, MA). Other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Radioligand Binding

Binding of ^3H -SCH23390 to crude kidney cortex membranes reached equilibrium rapidly (less than 5 minutes) at 25°C and remained stable for up to 90 minutes (data not shown). Fifteen minute incubations were chosen for the remainder of the experiments. Specific binding at 5 nM ^3H -SCH23390 was 70-80% of total binding and was linear with protein concentration up to 0.3 mg/tube. Degradation of ^3H -SCH23390 during incubation with kidney cortex membranes was monitored by thin layer chromatography and there was no decrease in radioactivity even with incubations of one hour or more.

Specific binding of ^3H -SCH23390 to renal cortex membranes was saturable with increasing ligand concentration (Fig 1); non-specific binding was linear. Scatchard plots of the saturation isotherms ($n=4$) indicated that the data could best be fit to a high and low affinity binding component (Fig. 1, inset): K_{D1} 4.9 ± 1.4 nM, $B_{\text{max}1}$ 31.4 ± 13.8 fmol/mg protein, K_{D2} 86.4 ± 23.9 nM, $B_{\text{max}2}$ 848.0 ± 227.4 fmol/mg protein.

The specificity of binding of ^3H -SCH23390 to renal cortex membranes was determined by competition with unlabeled DA agonists and antagonists (Fig. 2 and Table 1). The rank order potency for agonists was SKF38393 > fenoldopam >> 6,7-ADTN $\geq$ dopamine and the Hill coefficient for agonist competition were all less than unity, except for dopamine. The rank order potency for antagonists was SCH23390 > SKF83566 > (+)butaclamol > cis(Z)-flupentixol > SCH23388 > (-)butaclamol > spiperone > haloperidol > d-sulpiride = l-sulpiride. The Hill coefficient for antagonist competition was close to unity except for SCH23390 and SCH23388 (Table 1). Further

analysis of SCH23390 with 12 point competition curves ($n=3$) indicated that binding was best fit to a two-site model; K_H 1.2 ± 0.6 nM ($23.4 \pm 5.6\%$) and K_L 245.9 ± 57.7 nM ($76.6 \pm 5.6\%$).

Competition studies for ^3H -SCH23390 binding to slide-mounted kidney sections with selected antagonists and agonists yielded a similar rank order of competition as in homogenate binding with SCH23390 > SKF38393 > l-sulpiride [IC_{50}s (μM) 0.3 ± 0.1 , 12.0 ± 7.9 , 79.4 ± 21.9 ($n=3$), respectively] (Fig. 3).

Autoradiography

Autoradiographic images of ^3H -SCH23390 binding to slide-mounted kidney sections indicated that the binding was restricted to the cortex, with the medulla devoid of labeling (Fig. 4A). DA agonists and antagonists ($100 \mu\text{M}$) competed for the binding to varying degrees (Fig. 4, B-F) reflecting their potencies as competitors (Table 1).

The anatomical localization of the ^3H -SCH23390 binding sites could not be determined with the low resolution film autoradiograms, although areas that were notably devoid of labeling were the pars recta, the thick ascending limb and medullary collecting ducts (outer and inner stripes of the outer medulla, and inner medulla, respectively). Higher resolution receptor autoradiography was performed using emulsion-coated coverslips in order to determine more precisely the localization of ^3H -SCH23390 binding sites in the cortex. The highest density of ^3H -SCH23390 binding sites was seen over tubules (Fig. 5). The grain density (^3H -SCH23390 binding sites)

was greater over alkaline phosphatase positive tubules (proximal tubules) than non-alkaline phosphatase positive tubules (distal tubules and cortical collecting ducts). Of particular interest was whether ^3H -SCH23390 binding sites were present on vascular elements. Glomeruli were devoid of labeling (Fig. 5). Likewise, small arterioles, including afferent arterioles and juxtaglomerular cells, did not exhibit grain densities above background (Fig. 5 and 6). Finally, large caliber vessels, arterial and venous, were also unlabeled (Fig. 6).

In order to determine whether the vascular elements contain the low affinity form of the DA_1 receptor, sections were incubated under conditions that would optimize the labeling of the low affinity receptor. Namely, sections were incubated with 100 nM ^3H -SCH23390 followed by the normal rinse procedure or with 10 nM ^3H -SCH23390 and only a 3 minute rinse in cold buffer. High resolution autoradiography was then performed and silver grains were counted over glomeruli or arterial vessels. There was no significant difference in grain density over the vascular elements between total and nonspecific sections (Table 2).

DISCUSSION

The benzazepine SCH23390 has been characterized extensively in the brain as a D_1 -selective antagonist and ^3H -SCH23390 has been used to characterize D_1 receptors in the brain (Billard et al., 1984; Hess et al., 1986; Reader et al., 1988; Savasta et al., 1986; Schultz et al., 1985). SCH23390 also blocks the natriuretic (Frederickson et al., 1985) and the vasodilatory effects (Lokhandwala and Steenberg, 1984) of exogenously

applied DA or DA₁ agonists in the kidney. Previous radioligand binding studies of DA receptors in the kidney have used the relatively nonselective radioligand ³H-haloperidol (Felder et al., 1984a and 1984b; Nakajima and Kuruma, 1980;). Thus our initial experiments sought to determine whether ³H-SCH23390 would be a useful radioligand for characterization of DA₁ receptors in the kidney. Binding of ³H-SCH23390 to kidney cortical membranes was saturable, stereoselective and specific. Scatchard analysis of the saturation binding isotherms from the kidney indicated that the binding could best be fit to a two-site model: a high affinity (K_D 4.9 nM), low density component and a low affinity (K_D 86.4 nM), high density component. DA agonists and antagonists competed for ³H-SCH23390 binding in the kidney with a rank order specificity consistent with labeling of the DA₁ receptor. The Hill slopes for dopaminergic antagonist competition of ³H-SCH23390 binding were not uniformly monophasic, with SCH23390, SCH23388, cis(Z)-flupentixol, and haloperidol the most divergent from unity. Also, agonist competition curves for ³H-SCH23390 binding were shallow with Hill slopes less than one. Further detailed competition studies with SCH23390 indicated that the competition curves could be best fit to a two-site model, with a high affinity component (K_H 1.2 nM) and a low affinity component (K_L 245.9 nM). The binding of ³H-SCH23390 in the kidney was stereoselective with the active enantiomers (+)butaclamol and SCH23390 being five-fold and ten-fold more potent than the inactive enantiomers (-)butaclamol and SCH23388, respectively, at competing for ³H-SCH23390 binding.

The binding of ³H-SCH23390 to kidney cortex membranes was qualitatively similar to the binding reported at D₁ receptors in brain and is consistent with the

labeling of the DA₁ receptor. The major difference appears to be that ³H-SCH23390 binds with lower affinity in the kidney than in the brain (subnanomolar). Likewise, the IC₅₀ values for DA agonists and antagonists in the kidney were higher than those reported for the brain receptor, but the rank order of competition was similar in both tissues. The binding of ³H-SCH23390 in the kidney was best fit to a two-site model, but the nature of this heterogeneous binding is unclear. In the brain ³H-SCH23390 has been characterized as labeling two binding sites (Reader et al., 1988) or as labeling a single receptor population with partially interconvertible high and low affinity agonist binding components (Hess et al., 1986). The D₁ receptor appears to stimulate adenylyl cyclase via coupling to a guanine-nucleotide binding protein and the high affinity agonist binding component can be shifted to a lower affinity state with the addition of GTP (Hess et al., 1986; Keabian and Calne, 1979; Schultz et al., 1985). An antagonist such as SCH23390 would not be expected to distinguish between affinity states of the receptor (Hess et al., 1986). Likewise, although ³H-SCH23390 is a highly selective D₁ antagonist in brain, binding to a second receptor population at high concentrations can not be ruled out. In the brain, at high concentrations ³H-SCH23390 binds to 5-HT₂ receptors (Hess et al., 1986). However, in our experiments, the 5-HT₂ specific antagonist ketanserin inhibited ³H-SCH23390 binding in the kidney only at very high concentrations (>100 μM). Also, the nonselective α-adrenergic antagonist phentolamine and the 5-HT_{1A} agonist 8-OH-DPAT were surprisingly potent competitors for ³H-SCH23390 binding, although still some ten to twenty-fold less potent than SCH23390. However, the α₁-selective antagonist prazosin, the α₂-selective antagonists yohimbine and rauwolscine, the β-adrenergic antagonist propranolol and the 5-HT_{1A} antagonist 5-CT were less potent competitors. Thus, although it appears unlikely, the

binding of ^3H -SCH23390 to two distinct receptors can not be ruled out.

The kidney cortex is a complex collection of heterogenous cell types and the localization of DA_1 receptors to a specific cell type would be important for further understanding the renal mechanisms of action of DA agonists. As discussed above, attempts to characterize DA receptors on isolated tissues have led to conflicting results (Felder et al, 1984a; Schultz et al., 1987). A possible problem with the tissue isolation experiments is the difficulty in insuring that the preparations are not contaminated by other cell types. An alternative approach that does not disrupt the anatomical integrity of the tissue is the localization of receptors to specific kidney elements by in vitro receptor autoradiography (Healy and Fanestil, 1986; Healy et al., 1985).

SCH23390, SKF38393 and l-sulpiride competed for ^3H -SCH23390 binding to slide-mounted sections of rat kidney with the same rank order potency as in kidney cortical membranes, indicating that the DA_1 receptor was being labeled using the in vitro approach. Autoradiography of in vitro ^3H -SCH23390 binding in kidney sections revealed a heterogenous pattern of labeling with binding sites concentrated in the cortex. Higher resolution autoradiography with the use of emulsion-coated coverslips indicated that the ^3H -SCH23390 binding sites were restricted primarily to tubules. Combination of autoradiography with alkaline phosphatase histochemistry further indicated that the heaviest tubular labeling was seen over proximal tubules. There was a notable absence of ^3H -SCH23390 binding sites over any portion of the vascular tree, including glomeruli. Likewise, under incubation conditions that would favor localization of the low affinity ^3H -SCH23390 binding site, there was still no detectable

specific binding over the vasculature.

Although there is general agreement that DA infusion produces a natriuresis in experimental animals and man, the mechanism of action has not been determined unequivocally. It is difficult to resolve this controversy because of the variety of experimental preparations that were employed, the possibility that species differences exist, and the possible involvement of both hemodynamic and tubular mechanisms. Most early reports indicated that DA infusion produced an increase in renal blood flow and that DA and DA₁ agonists produce a vasodilatation both in vivo and in vitro (Chapman et al., 1980; Hardaker and Wechsler, 1973; Lokhandwala and Steenberg, 1984; Schmidt and Imbs, 1980), consistent with a report that renal vessels contain DA receptors (Brodde, 1981). The effects of DA on glomerular filtration were less consistent (Carey and Hughes, 1987; Chapman et al., 1980; Wasserman et al., 1980), but Shultz et al. (1987) reported that mesangial cells contain DA₁ receptors. DA has also been reported to cause a redistribution of blood flow from outer glomeruli to juxtamedullary glomeruli (Chapman et al., 1980; Hardaker and Wechsler, 1973).

On the other hand, there is also strong physiological evidence which indicates that DA acts directly on renal tubules in man, cat and rabbit to promote natriuresis (Bello-Ruess et al., 1982; Carey and Hughes, 1987; Wasserman et al., 1980). Rabbit proximal tubules have been reported to contain DA₁ receptors (Felder et al., 1984b). The autoradiographic localization of DA₁ receptors on proximal tubules, as well as the absence of DA₁ receptors on the vasculature, as seen in the experiments reported here provides support for a tubular site of action for DA and DA₁ agonists in the rat.

Dopamine has also been implicated in the release of renin, but the findings have been controversial, largely due to the difficulty in distinguishing between a primary mechanism mediated directly by DA from a secondary mechanism resulting from a change in renal blood flow and sodium excretion (Lokhandwala and Barrett, 1982). Recently, juxtaglomerular cells grown in culture have been shown to secrete renin via a DA_1 -mediated mechanism (Cavero et al., 1987). However, we were unable to demonstrate ^3H -SCH23390 binding sites over juxtaglomerular cells by autoradiography. The lack of DA_1 receptors on juxtaglomerular cells is in marked contrast to the high density of β_1 -adrenergic receptors that these cells exhibit (Healy et al., 1985). These results would suggest that DA-mediated release of renin in vivo may involve secondary mechanisms and is not mediated directly by DA_1 receptors.

Recently it has been demonstrated that in the rat, DA or L-dopa inhibit Na^+ - K^+ -ATPase activity in proximal tubule segments from animals under normal sodium balance or from animals on a high salt diet (Aperia et al., 1987; Bertorello et al., 1988; Seri et al., 1988). The proximal tubules contain high levels of AADC and inhibition of AADC with benserazide or carbidopa abolishes the effects of L-dopa, indicating that locally generated DA is necessary for the inhibition of the Na^+ - K^+ -ATPase activity (Aperia et al., 1987; Bertorello et al., 1988; Seri et al., 1988). The existence of a high density of DA_1 receptors on proximal tubules is consistent with an action of locally generated DA as an autocrine or paracrine hormone to influence proximal tubule sodium reabsorption. However, Aperia et al. (1987) were unable to block the DA-mediated inhibition of proximal tubule Na^+ - K^+ -ATPase activity with the mixed DA

antagonist metoclopramide. These authors suggested that DA may inhibit $\text{Na}^+\text{-K}^+$ -ATPase activity via an intracellular mechanism (Aperia et al., 1987). Thus, the nature of the DA-mediated inhibition of proximal tubule sodium reabsorption and the relevance of DA_1 receptors on the proximal tubule cells remains to be established.

In summary, we have used radioligand binding studies of $^3\text{H-SCH23390}$ and in vitro autoradiography to characterize and localize DA_1 receptors in crude membranes and sections of rat kidney. The present study indicates that DA_1 receptors are concentrated primarily on proximal tubule cells and not vascular elements, results that support a tubular action for DA-mediated natriuresis.

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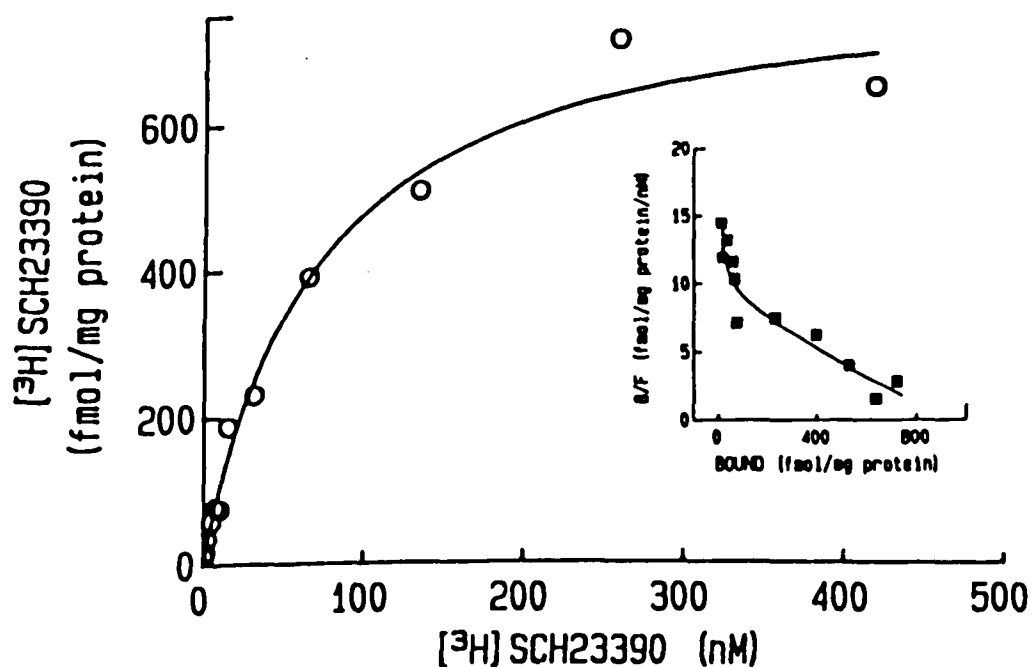


Figure 1. Saturation binding isotherm and Scatchard transformation (inset) of ^3H -SCH23390 binding to rat kidney cortical membranes. Increasing concentrations of ^3H -SCH23390 (0.25-420 nM) were incubated in the absence (total binding) or presence (non-specific binding) of 100 μM haloperidol. Shown is the specific binding (total minus non-specific) from a typical experiment. Transformation of the binding yielded a curvilinear plot that was best fit to a two-site model: $K_{D1} 4.9 \pm 1.4$ nM, $B_{\text{max}1} 31.4 \pm 13.8$ fmol/mg protein; $K_{D2} 86.4 \pm 23.9$ nM, $B_{\text{max}2} 848.0 \pm 227.4$ fmol/mg protein. Experiments were replicated four times with each point in triplicate.

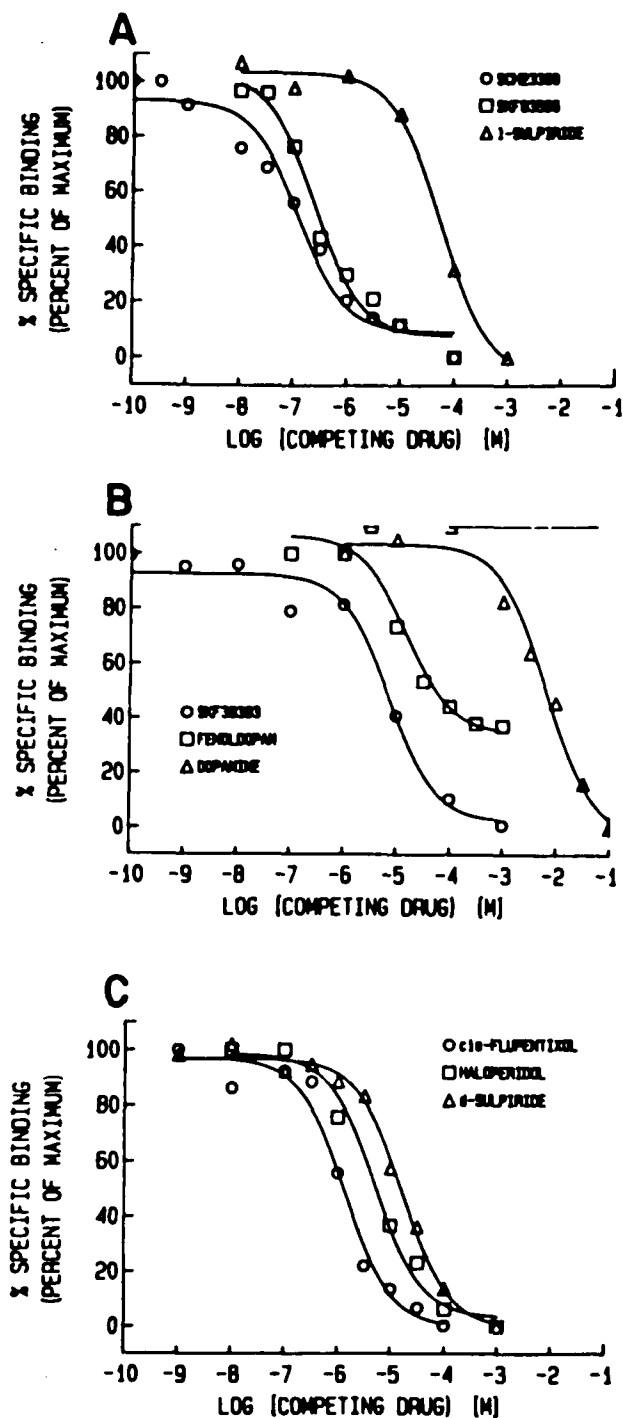


Figure 2. Competition by dopaminergic agonists and antagonists for ^3H -SCH23390 (5 nM) binding in rat kidney cortical membranes. Shown are typical competition curves for D_1 -selective (top panel, SCH23390 and SKF83566) and D_2 -selective (top panel, l-sulpiride) antagonists, agonists (middle panel) and mixed antagonists (bottom panel). Each curve was fit to a one-site model. Each experiment was repeated three times with each point in triplicate. (See Table 1 for IC_{50} values.)

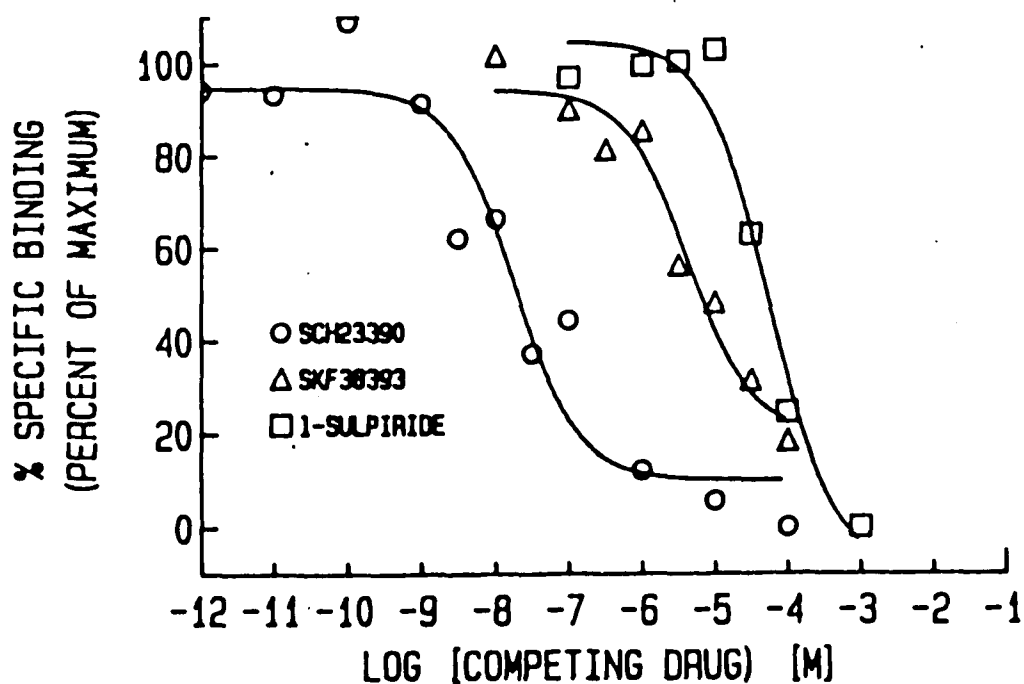


Figure 3. Competition by SCH23390, SKF38393 and l-sulpiride for ^3H -SCH23390 binding to sections of rat kidney. Slide-mounted sections of rat kidney were incubated with ^3H -SCH23390 (2-3 nM) and increasing concentrations of each drug. Sections were wiped from the slides and the amount of radioligand bound measured by scintillation counting. Each curve was fit to a one-site model, each experiment was repeated three times with each point in triplicate.

Figure 4. Darkfield autoradiograms of ^3H -SCH23390 binding to longitudinal sections of rat kidney incubated in the presence of 10 nM ^3H -SCH23390 alone (panel A) or with 100 μM competitor. Panel A. ^3H -SCH23390 binding (white area) was restricted to the cortex (C). The inner and outer stripes of the outer medulla (IS and OS, respectively) and inner medulla (IM) are devoid of labeling. Bar = 2mm. Panel B. ^3H -SCH23390 binding in the presence of 100 μM SCH23390. Note the complete absence of labeling in the cortex. Panel C. ^3H -SCH23390 binding in the presence of 100 μM fenoldopam. Note the reduction in labeling in the cortex. Panel D. ^3H -SCH23390 binding in the presence of 100 μM l-sulpiride. Labeling is reduced relative to total binding but less than with fenoldopam (panel C) and much less than with SCH23390 (panel B). Panel E. ^3H -SCH23390 binding in the presence of 100 μM haloperidol. Haloperidol competes for the majority of the binding but less effectively than SCH23390 (panel B). Panel F. ^3H -SCH23390 binding in the presence of 100 μM spiperone. Spiperone also effectively blocks ^3H -SCH23390 binding but somewhat less than SCH23390 (panel B).

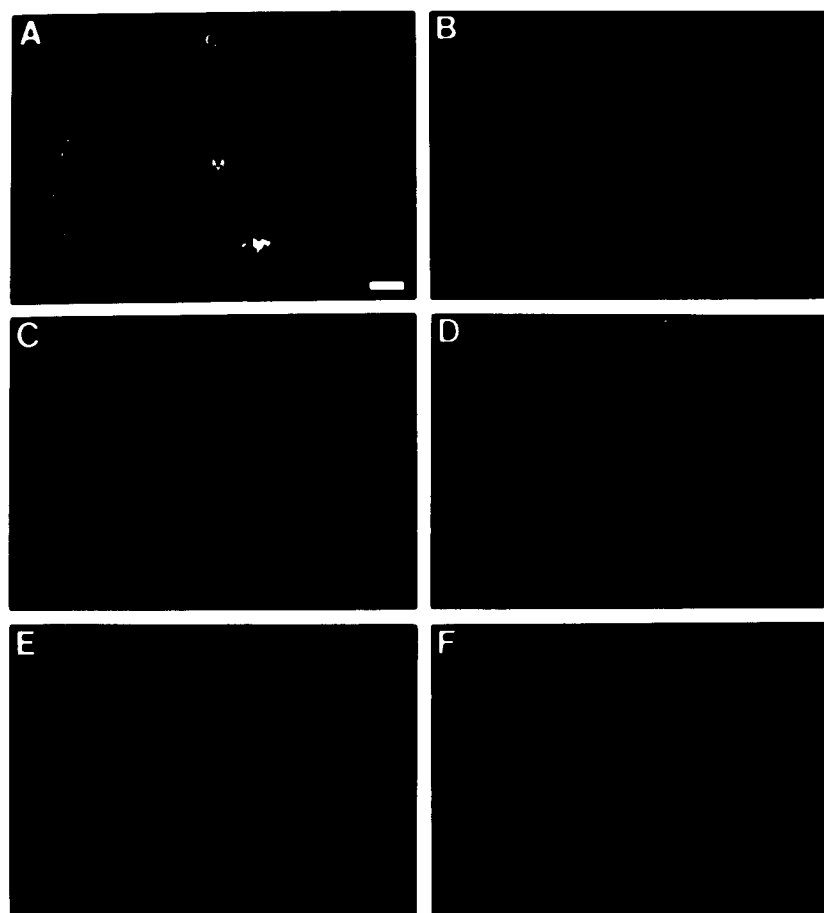


Figure 5. High resolution emulsion-coated coverslip autoradiography of ^3H -SCH23390 total binding (panels A-D) or non-specific binding (panel E and F) to slide-mounted sections of rat kidney. The photomicrographs were taken with the plane of focus either on the tissue (Panels A,C and E) or on the overlying coverslip in order to visualize the silver grains (panels B,D and F). Panel A and B. Photomicrograph of a section labeled with ^3H -SCH23390 and processed for alkaline phosphatase histochemistry which labels proximal tubules (p). Also seen is a glomerulus (g). In panel B, note that silver grains are concentrated over the proximal tubules. (See panel F for comparison of non-specific binding grain density.) Panel C and D. Photomicrograph of an afferent arteriole (small arrows) entering a glomerulus (g). Note the terminal portion of the afferent arteriole corresponding to the juxtaglomerular complex. In panel D, note that silver grains are concentrated over the proximal tubules but not over the vascular elements. Panel E and F. Non-specific binding of ^3H -SCH23390 in the presence of 100 μM haloperidol. Photomicrograph of an arteriole (a), proximal tubules (p) and a glomerulus (g). Note in panel F the diffuse pattern of the silver grains. Bar = 50 μm .

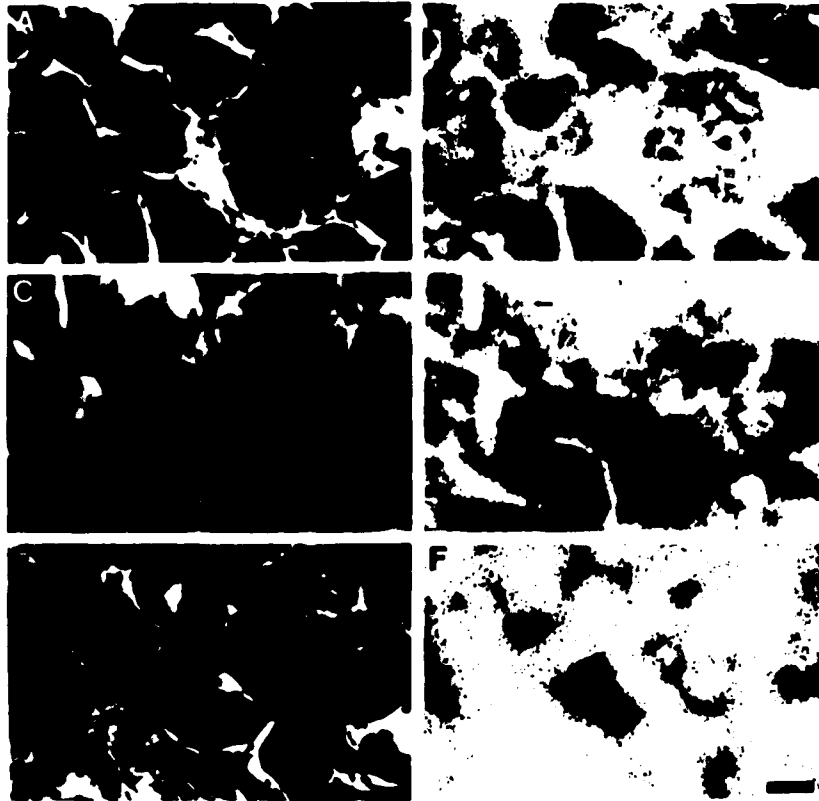


Figure 6. Autoradiography of ^3H -SCH23390 labeling in sections of rat kidney using emulsion-coated coverslips. Panel A and B. Photomicrograph of an arcuate artery (approximate diameter 250 μm , arrow) in cross-section and incubated with ^3H -SCH23390. In panel B note the overlying silver grains are concentrated over the tubules and not the blood vessel. (Compare to panel F for non-specific labeling.) Panel C and D. Photomicrograph of an interlobular artery (a) (approximate diameter 100 μm) with an arteriole branching off (arrows). In panel B note that the silver grains are concentrated over the tubules and not the vasculature. Panel E and F. Non-specific binding of ^3H -SCH23390 in the presence of 100 μM haloperidol. Photomicrograph of an interlobular artery (a) and an arteriole. In panel F note the diffuse pattern of the silver grains over the tubules and vasculature. Bar = 50 μm .

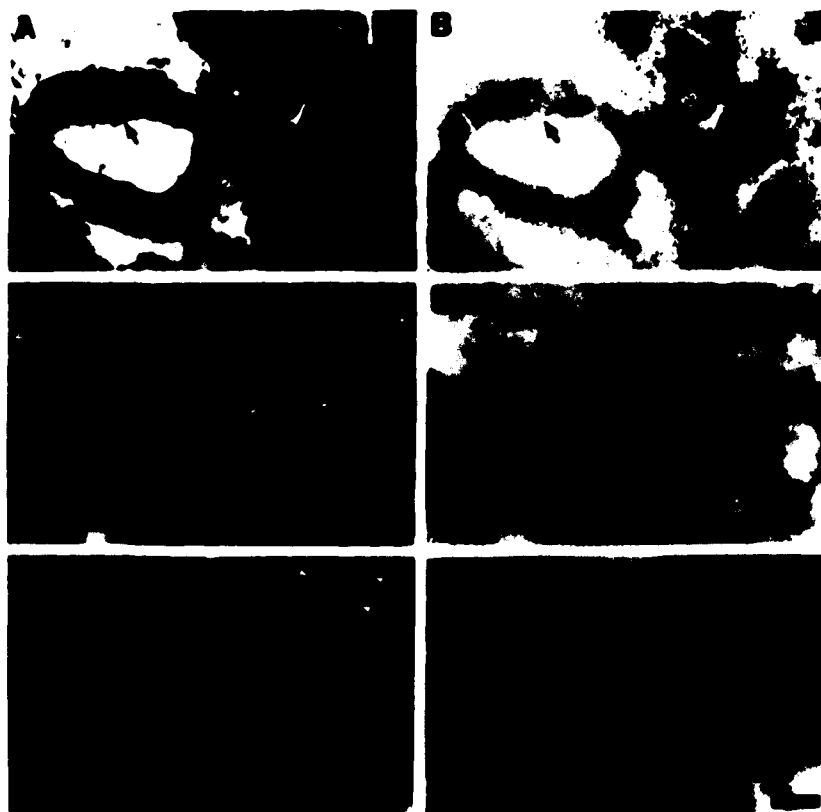


TABLE 1. *Inhibition of specific [³H]Sch 23390 binding in rat kidney cortical membranes*

	Subtype	IC ₅₀ , μM	Hill Slope
Dopamine antagonists			
Sch 23390	DA ₁	0.12±0.02	0.45±0.04
SKF 83566	DA ₁	0.23±0.06	1.01±0.13
(+) Butaclamol	DA ₁ /DA ₂	0.40±0.18	1.00±0.04
cis(Z)-Flupentixol	DA ₁ /DA ₂	1.23±0.21	0.82±0.03
Sch 23388	DA ₁	1.43±0.02	0.53±0.17
(-) Butaclamol	DA ₁ /DA ₂	2.28±0.66	1.03±0.08
Spiperone	DA ₁ /DA ₂	3.77±0.39	0.91±0.05
Haloperidol	DA ₁ /DA ₂	4.27±1.2	0.79±0.03
d-Sulpiride	DA ₁	37.2±11.5	0.86±0.03
l-Sulpiride	DA ₂	38.0±9.2	0.92±0.06
Dopamine agonists			
SKF 38393	DA ₁	13.5±2.8	0.51±0.04
Fenoldopam	DA ₁	22.9±7.8	0.54±0.01
6,7-ADTN	DA ₁ /DA ₂	>1,000	NA
Dopamine	DA ₁ /DA ₂	6,309±425	0.87±0.04
Other agonists and antagonists			
8-OH-DPAT	5-HT _{1A}	1.67±0.14	0.82±0.05
Phentolamine	α ₁ /α ₂	2.61±0.95	1.03±0.05
Prazosin	α ₁	5.79±0.90	1.04±0.05
Yohimbine	α ₂	40.3±9.1	1.08±0.10
Mesulergine	5-HT _{1C,2}	58.9±21.5	1.24±0.14
Rauwolscine	α ₂	85.4±17.6	1.05±0.10
Propranolol	β ₁ /β ₂	21.9±5.1	1.02±0.05
Ketanserin	5-HT ₂	122.7±13.7	1.06±0.08
5-CT	5-HT _{1A,1B,1D}	>1,000	NA

TABLE 1. Rat kidney membranes were incubated with ³H-SCH23390 (final concentration 5 nM) in the presence of unlabeled drug (8-10 concentrations) and the concentration of unlabeled drug that caused 50% inhibition of specific binding (IC₅₀) determined. The values are the means ± SEM of three experiments, each point determined in triplicate. Abbreviations: 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; 5-CT, 5-carboxyamido-tryptamine; 8-OH-DPAT, 8-hydroxy-2(di-n-propyl-amino)tetralin; n.a., not available.

TABLE 2. *Grain density over vascular elements from kidney sections incubated with 10 or 100 nM [³H]Sch 23390*

	[³ H]Sch 23390, 10 nM		[³ H]Sch 23390, 100 nM	
	Total	NSB	Total	NSB
Arterial vessels	485±26	490±34	1,074±93	945±65
Glomeruli	588±66	631±36	NA	NA

Values are grains ± SE per 1,000 μm²; *n* = 3 observations. There were no significant differences between total and nonspecific (NSB) sections. Slide-mounted sections of rat kidney were incubated under conditions to optimize labeling of the low-affinity [³H]Sch 23390 binding site; either 10 nM [³H]Sch 23390 and rinsed in cold buffer for 3 min only or with 100 nM [³H]Sch 23390 followed by the normal rinse procedure. Because the radiational spread of silver grains over labeled structures is related to the amount of radioactivity bound, structures chosen for counting were selected so as not to be immediately adjacent to the heavily labeled proximal tubules. At 100 nM [³H]Sch 23390 the intensity of labeling over proximal tubules was such that grain counts over adjacent glomeruli were unreliable and are thus not available (NA).

CHAPTER 3

**EVIDENCE FOR A NOVEL DA₂ DOPAMINE RECEPTOR
IN RAT KIDNEY MEDULLA**

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INTRODUCTION

Dopamine (DA) is known to have potent natriuretic properties in many species, including man (Bello-Reuss et al., 1982; Carey and Hughes, 1987; Wasserman et al., 1980). Although the mechanism of the DA-mediated natriuresis is still not fully understood, and may vary among species, it appears that the effects of DA are mediated via DA receptors (Lee, 1982; Felder et al., 1988a). In the dog, DA produces an increase in renal blood flow and a natriuresis that can be blocked by the DA₁ selective antagonist SCH 23390, but not by α -adrenergic, β -adrenergic or DA₂ antagonists (Frederickson et al., 1985; Nichols et al., 1987). Although there is some evidence for a direct vascular effect by DA in species other than the dog (Schmidt et al., 1987; Edwards, 1986; Steinhausen et al., 1986), a major component of the DA-mediated natriuresis appears to involve a direct effect on renal tubular sodium and water reabsorption (Cuche et al., 1976; McGrath et al., 1985; Jose et al., 1987). As with the dog, the predominant receptor mediating the DA effect on tubular sodium reabsorption appears to be the DA₁ subtype. However, there is some evidence that the DA₂ receptor subtype may also be involved. Domperidone, a selective peripheral DA₂ antagonist, inhibited the natriuresis caused by increased central blood volume in man (Bennett et al., 1982; Coruzzi et al., 1986). Bromocryptine, a predominantly DA₂ agonist, increased renal blood flow and superficial single-nephron GFR but not net total GFR in rat (Stier et al., 1982; Seri and Aperia, 1988). Bromocryptine also increased urinary PGE₂ excretion accompanied by augmented natriuresis in rat (Yoshimura et al., 1987).

There is evidence that dopamine can influence water and electrolyte transport in the distal nephron segments. Dopamine blocks the antidiuretic effect of vasopressin by inhibiting the hydro-osmotic effect of vasopressin in the toad bladder (Arruda and Sabatini, 1982) and the rabbit cortical collecting tubule (Muto et al., 1985). Haloperidol, a nonselective DA receptor antagonist, blocked the latter effect. In water-loaded rats, dopamine inhibited the diuretic effect of vasopressin which could also be blocked by haloperidol (Koyoma et al, 1985). Dopamine also antagonized the effects of DOCA, an analogue of aldosterone, on tubular transluminal potential difference in rabbit cortical collecting tubule (Muto et al., 1985). The receptor subtypes involved in the action of dopamine in the distal nephron are not clear.

Dopaminergic receptors have been studied in rat kidney using radioligand binding. DA₁ receptors have been characterized using selective antagonists (Felder and Jose, 1988b; Huo and Healy, 1989; Hughes and Sever, 1989) and agonists (Hegde et al., 1989). Renal DA₁ receptors have also been characterized by in vitro autoradiography in the rat. Interestingly, DA₁ receptors appear to be concentrated primarily on the proximal tubules and are either not present (Huo and Healy, 1989) or present at low density on vascular elements (Hegde et al., 1989). Less is known regarding the DA₂ receptor. Based on the binding of the nonselective radiolabeled DA antagonist ³H-haloperidol and inhibition of adenylate cyclase by DA, Felder et al. (1984a) concluded that glomeruli expressed DA₂ receptors. More recently Hietala et al. (1988) reported on the binding of the relatively selective DA₂ antagonist ³H-spiperone in rat kidney cortex. The possibility that ³H-spiperone was labeling 5-HT₂ receptors in the kidney, as has been shown in brain (Creese and Snyder, 1978; Huff

and Molinoff, 1982), was ruled out because the 5-HT₂ selective antagonist ketanserin was unable to compete for the 3H-sipiperone binding (Hietala et al., 1988; Felder et al., 1988c and 1989). In this report, we chose to utilize ³H-sipiperone and in vitro autoradiography in order to determine the regional localization of the DA₂ receptor in the rat kidney. We report that ³H-sipiperone binding sites are highly concentrated on cells of the inner medulla collecting duct. Further characterization of the ³H-sipiperone binding in the medulla by homogenate binding and photoaffinity cross-linking with ¹²⁵I-N-(p-aminophenethyl)sipiperone (¹²⁵I-NAPS), a analogue of sipiperone, revealed that sipiperone apparantly binds to a M_r=120,000 peptide consistent with labeling of the DA₂ receptor, but with a pharamcological profile that is unique from that reported for the DA₂ receptor in other tissues. These results suggest that the rat kidney medulla expresses a novel DA₂ receptor.

METHODS

Preparation of Tissues

1.Homogenate binding

Male Sprague-Dawley rats (200-250g, Charles River) were housed on a 12 hour L/D schedule and allowed free access to food and water. Animals were decapitated and the kidneys removed quickly and placed on ice. All subsequent steps were carried out at 4°C. The kidneys were sliced longitudinally in half and the inner medulla excised. The kidney inner medulla was then homogenized with a Polytron (Brinkman)

for 15 seconds at setting #4 in 10 X volume of 25 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA and 10% sucrose. The homogenate was sieved through 5 layers of cheesecloth and centrifuged at 400 x g for 10 minutes. The pellet was discarded and the supernatant re-centrifuged at 20,000 x g for 20 minutes. The pellet was then resuspended with hypotonic buffer and centrifuged at 20,000 x g for 20 minutes. The final pellet was resuspended in isotonic Tris buffer and aliquots stored at -80°C, where they were stable a minimum of six months.

2. Autoradiography

Tissues were prepared for autoradiography as previously described (Healy et al., 1985; Healy and Fanestil, 1986; Huo and Healy, 1989). Briefly, male Sprague-Dawley rats (200-250g) were anesthetized with sodium pentobarbital (50 mg/kg, ip) and perfused transcardially with 200 ml 0.1% formalin in phosphate-buffered saline (PBS), pH 7.4. The kidneys were removed and frozen on microtome chucks with powdered dry ice. Fifteen μ m cryostat sections along the mid longitudinal axis were thaw mounted onto clean subbed microscope slides, dried and stored at -20°C until used (<one week).

Homogenate Binding Studies

DA₂ receptor binding assays were done in the assay buffer consisting of 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 100 μ M pargyline and 0.01% ascorbic acid with the final pH adjusted to 7.4 at 25°C. Assays were

initiated by adding 50 μ l of crude membrane (50 μ g protein) resuspended in Tris buffer 50 μ l of dilute radioligand plus 50 μ l drug or vehicle in glass tubes on ice. Samples were incubated in a shaking water bath at 25°C for 30 minutes. The reaction was terminated by adding 4 x 4 ml cold saline rinses and vacuum filtration through Whatman GF/C filters that had been presoaked with 0.3% polyethylenimine to reduce non-specific filter binding. The filters were placed in polypropylene scintillation vials, Aquasol-2 LSC cocktail (DuPont) added, and counted in a Beckman LS-3801 liquid scintillation counter with a counting efficiency of 62%.

For saturation binding experiments 1-100nM ^{3} H-spiperone (Amersham, sp. act. 75-85 Ci/mmol) was used. Non-specific binding was defined as binding in the presence of 100 μ M haloperidol, with a total:non-specific binding ratio of approximately 4:1. For competition studies, 5 nM ^{3} H-spiperone was used together with increasing concentrations of unlabeled DA agonists and antagonists.

Autoradiography

The in vitro labeling of slide-mounted tissue sections with ^{3} H-spiperone was conducted using a similar condition to those used for the homogenate binding studies outlined above. In addition, since the kidney sections used for autoradiography are unfixed, we have noted that the quality of morphology for the high resolution autoradiographs is markedly improved if incubated at 4°C. Therefore, sections to be used for high resolution autoradiography were incubated at 4°C, otherwise sections were incubated at 25°C. There was no difference in the pattern of labeling in the tissues

incubated at these two temperatures.

Slide-mounted kidney sections were thawed and pre-incubated for 15 minutes at 4°C or room temperature in incubation buffer minus radioactivity and then transferred to incubation buffer for 60 minutes at room temperature or for 120 minutes at 4°C. The incubation was stopped by placing the slides in cold buffer for 2 x 30 minute rinses. The sections were then dipped in water and dried. Sections were either wiped from the slides for measurement of radioactivity by liquid scintillation counting or apposed to emulsion for autoradiography (see below).

For localization studies, 10 nM ³H-spiperone was incubated alone or plus 100 µM haloperidol to define non-specific binding in adjacent sections, with a total:non-specific ratio of approximately 4:1.

Autoradiographic images from the radiolabeled kidney sections were generated using either low resolution Hyperfilm (Amersham) or higher resolution emulsion-coated coverslips. Following the final drying step, radiolabeled slide-mounted kidney sections were placed in an X-ray cassette (Wolf Slimline) and a sheet of Hyperfilm placed over the sections and the cassette sealed. After exposure at 4°C for approximately 2 weeks the film was removed and developed. These same sections were then apposed with thin emulsion-coated coverslips that had been coated in NTB-3 (Kodak) liquid emulsion diluted 1:1 with water. After exposure the coverslips were developed and the section counterstained with pyronin Y.

Receptor photoaffinity labeling experiments

The procedures for photoaffinity labeling with ^{125}I -NAPS was similar to that described by Amlaiky and Caron (1985 and 1986) but modified as follows. Rats were decapitated and striatum, pituitary, and kidney inner medulla were rapidly removed and homogenized in 20 volumes (vol/wt) of ice-cold 25 mM Tris-HCl, pH 7.2 (25 °C), 250mM sucrose, and the following protease inhibitors: 10 mM EDTA, 10 mM EGTA, 15 $\mu\text{g/ml}$ benzamidine, 5 $\mu\text{g/ml}$ of soybean trypsin inhibitor, 5 $\mu\text{g/ml}$ leupeptin, 1 mM PMSF, 100 ng/ml pepstatin, 5 $\mu\text{g/ml}$ aprotinin, 1 mM phenanthroline. The homogenate was centrifuged at 500 X g for 10 min and the supernatant was recentrifuged again at 40000 X g for 20 min. The resulting pellet was resuspended in the above mentioned buffer and recentrifuged three times. The final pellet was resuspended in 25 mM Tris-HCl, pH 7.4 (25° C); 100 mM NaCl; 6mM MgCl_2 , in the presence or absence protease inhibitors to yield a final protein concentration of approximately 2-5 mg/ml. All operations were carried out at 0-4 °C and as rapidly as possible. Membranes (100-300 μg protein for all three tissues) were incubated in a volume of 1 ml with subnanomolar concentration of ^{125}I -N₃-NAPS (0.1-0.25 nM for striatum and 0.25-0.5 nM for kidney inner medulla) for 60 min at room temperature (in the dark) in the presence or absence of various agents as described. The membranes were centrifuged at 40000 X g for 20 min and the pellet resuspended in 25 mM Tris-HCl, pH 7.4 (25 C), 0.5 % bovine serum albumin, in the presence or absence of protease inhibitors; centrifuged again at 40000 X g for 20 min; and resuspended in 1 ml of the incubation buffer in the presence or absence of protease inhibitors. Photolysis was performed by exposing the incubation mixtures for 90 s at 12 cm from a high pressure mercury lamp. The

membranes were pelleted by centrifugation (12000 X g for 10 min) and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, pH 6.8; 10 % SDS; 10 % glycerol; 5 % β -mercaptoethanol; 0.003 % bromophenol blue) with or without protease inhibitors and allowed to stand 30-45 min at room temperature before being electrophoresed of a 10 % discontinuous SDS-polyacrylamide gel according to the method of Laemmli (1970). An equivalent amount of protein was loaded onto each lane. Following electrophoresis, the gels were dried and exposed to autoradiographic film for various periods of time at -70 °C.

Protein determinations were performed using the Bradford assay from Biorad.

Data Analysis

Radioligand binding data was analyzed using the Munson and Rodbard LIGAND program (Munson and Robbard, 1980). All results are expressed as mean $\pm$ S.E.M.

RESULTS

Radioligand binding

Binding of ^3H -spiperone to renal inner medulla membranes reached equilibrium after 30 min at room temperature and remained stable for up to 120 min (data not shown). 30 min incubations were used for the subsequent experiments. Specific binding at 4 nM ^3H -spiperone was 70-80% of total binding. Specific binding of ^3H -spiperone

to renal inner medulla membranes was saturable, specific, and of high affinity (Fig. 1); nonspecific was linear. Scatchard plot analyses yielded single binding site with the K_d of 17.2 ± 1.65 nM and B_{max} of 935 ± 83 fmol/mg ($n=4$).

In competition studies only a few ligands were able to compete for ^3H -spiperone binding effectively (Fig. 2 and Table 1). The rank order potency for antagonists was spiperone > haloperidol > (+)-butaclamol > cis(Z)-flupentixol > domperidone > (-)-butaclamol >> SCH23390, SKF83566 >> d-sulpiride, l-sulpiride. The Hill slope for antagonists competition were all close to unity, except for spiperone (Table 1). The only effective agonists for competition were 6,7-ADTN and dopamine, other agonists were ineffective with the concentration up to 300 μM (for fenoldopam, SKF38393, apomorphine, and norapomorphine) or 1 mM (for LY 171555). The α_1 - and α_2 -adrenoceptor antagonists, prazosin, and yohimbine, as well as ketanserin, a 5-HT₂ serotonergic receptor antagonist and isoproterenol, a β_1/β_2 adrenoceptor agonist, were unable to compete for ^3H -spiperone binding (Table 1).

Autoradiography

Autoradiographic images of ^3H -spiperone binding to slide-mounted kidney sections indicated that the binding was primarily located at inner medulla with diffuse labeling over cortex (Fig. 3). The labeling in the inner medulla was seen as longitudinally oriented structures that radiated outward from the tip of the papilla. High-resolution receptor autoradiography confirmed that this labeling pattern represented labeling of inner medullary collecting ducts. The diffuse labeling over cortex could not

be localized to any specific renal tubular structures. Glomeruli and vascular elements did not exhibit grain densities above background.

Photoaffinity labeling with ^{125}I -NAPS

Photoaffinity labeling studies with ^{125}I -NAPS in kidney inner medulla membranes in absence of protease inhibitors revealed labeling of a $M_r=38,000$ peptide that could be competed for by spiperone (a D_2 receptor antagonist) and haloperidol (a D_1/D_2 receptor antagonist) but not by SCH23390 (D_1 receptor antagonist). In the striatum, ^{125}I -NAPS labeled a major protein with $M_r=94,000$ and a minor protein with $M_r=38,000$ and labeling of both peptide could be competed for by haloperidol and spiperone (Fig. 4). In the pituitary, a similar $M_r=38,000$ peptide was labeled in the same fashion but no labeling was seen of any higher molecular weight proteins (Fig. 4). In all three tissues, a nonspecific 32 Kd peptide band was observed. The kidney inner medulla and the striatum, however, contained several nonspecific bands that the pituitary did not.

The photoaffinity labeling in presence of protease inhibitors showed different pattern. In all three tissues, the major labeling was a diffuse $M_r=120,000$ -130,000 protein that could be competed by spiperone and haloperidol but not by SCH23390 (Fig. 5). The $M_r=38,000$ peptide was not observed under these conditions.

DISCUSSION

Spiperone has been characterized extensively in the central nervous system as a D₂ antagonist and ³H-spiperone has been used to characterize D₂ receptors in the brain and pituitary (Creese et al., 1977; Frey et al., 1982). In cerebral cortex, however, ³H-spiperone labeled serotonin receptors (Creese and Snyder, 1978, Huff and Molinoff, 1982).

In the kidney, spiperone has been shown to block the ability of dopamine to increase renal blood flow, sodium excretion, and renal tissue adenylate cyclase activity (McGrath et al., 1985, Nakajima et al., 1977). Renal ³H-spiperone binding has been demonstrated in rabbit renal artery (Brodde, 1981; Alessandrini et al., 1984), sheep renal homogenate (Felder et al., 1988c), dog renal brush border and basolateral membrane (Felder et al., 1989), rat renal homogenate (Galbusera et al., 1988) and rat renal cortex (Hietala et al., 1988). We report here that the ³H-spiperone binding sites is highly concentrated in the rat kidney inner medulla. Binding of ³H-spiperone on isolated and cultured inner medullary collecting duct cells (IMCD) also supported that IMCD cells contain ³H-spiperone binding sites (data not shown). The possibility of labeling the 5-HT₂ serotonergic receptors has been ruled out since ketanserin, a 5-HT₂ receptor antagonist, could not compete the ³H-spiperone binding effectively with the concentration of 100 μ M.

Binding of ³H-spiperone to the kidney inner medullary membranes was saturable, stereoselective and specific. Scatchard analysis of the saturation binding isotherms from the kidney indicated that the binding could be fit to one site model with the K_D of 17.2 $\pm$ 1.65 nM and the B_{max} of 935 $\pm$ 83 fmol/mg protein. Antagonists and agonists

competed for ^3H -spiperone binding in the kidney inner medulla with a rank-order specificity consistent with the dopamine receptor. However, there were some differences for ^3H -spiperone binding in the kidney inner medulla compared with the ^3H -spiperone binding in the brain or the pituitary: (1) the affinities of dopaminergic ligands in the kidney was relative low (in μM range) when compared with the affinities in the brain or the pituitary (in nM range) (Creese et al., 1977; Frey et al., 1981); (2) the competition profile for receptor subtype classification in this study favored DA_2 dopamine receptor but was not the typical D_2/DA_2 dopamine receptor competition profile since the D_2 receptor-selective antagonist l-sulpiride and the D_2 receptor-selective agonist LY171555 did not compete for ^3H -spiperone binding effectively at concentrations up to 1 mM. The binding data of ^3H -spiperone in previous reports (Brodde, 1981; Alessandrini et al., 1984; Felder et al., 1988c and 1989; Hietala et al., 1988, Katayama et al, 1989) are comparable with ours. The reported K_D values for ^3H -spiperone binding ranged from 5.1 nM to 21.2 nM, and the B_{max} values ranged from 134 fmol/mg protein to 310 fmol/mg protein in different renal tissues. The B_{max} of the kidney inner medulla membranes in this study is apparently three to six times higher than other renal tissues (rat and sheep renal cortex, dog proximal tubules, rabbit renal arteries). Although the competition profile from our and those studies indicated that ^3H -spiperone labeled a DA_2 receptor in the kidney, the lack of biochemical evidence, however, can not rule out the possibility of labeling of a non-receptor site. In order to compare the biochemical properties between central and renal ^3H -spiperone binding protein, we chose to compare the molecular weights of the kidney spiperone binding site with that of the striatum and pituitary using ^{125}I -NAPS, an analogue of spiperone and a photoaffinity radiolabeled D_2 receptor ligand which successfully labeled the D_2

dopamine receptors in the striatum and pituitary (Amlaiky and Caron, 1985 and 1986; Senogles et al., 1988; Jarvie et al., 1988).

Tissues membranes of rat striatum, pituitary and kidney inner medulla were prepared and incubated with ^{125}I -NAPS under low or high stringency protease inhibitor conditions. Protease inhibitors were routinely included for membrane preparation. Removal of protease inhibitors (low stringency) in the subsequent steps, especially during the incubation, resulted in labeling of a $M_r=38,000$ peptide in all three tissues with the D_2/DA_2 receptor pattern. A diffuse protein band with an apparent $M_r=94,000$ was observed in the striatum. A similar labeling has been described in previous reports by Amlaiky and Caron (1985 and 1986). Several nonspecific bands were observed in the kidney and the striatum while the background of the pituitary was rather low. If protease inhibitors were included throughout the entire experimental procedures, a $M_r=120,000$ peptide became the predominant peptide labeled by the probe ^{125}I -NAPS in all three tissues. The absence of the $M_r=38,000$ and $M_r=94,000$ peptide under high stringency protease inhibitor condition suggests that the $M_r=38,000$ peptide in renal medulla and the $M_r=94,000$ peptide in striatum were the degradation products of the $M_r=120,000$ peptide. Previous attempts to photoaffinity label the renal DA_2 dopamine receptor using the same probe was unsuccessful (Amlaiky and Caron, 1986). In those experiments, protease inhibitors were not included in all the experimental steps suggesting that protease inhibitors must be present in all experimental procedures in order to label the renal DA_2 dopamine receptor. The molecular weight of D_2 dopamine receptor binding site in membrane preparations of various tissues determined by radiation inactivation technique ranged from $M_r=95,000$ to $M_r=135,000$ (Lilly et al.,

1983; Bouvier et al., 1986, 1987, and 1990). Purified D_2 dopamine receptor from bovine anterior pituitary has been shown with the molecular weight of 120,000 (Senogles et al., 1988) while the porcine striatal dopamine D_2 receptor has been determined to be a $M_r=140,000/94,000$ peptide (Jarvie et al., 1988). Low molecular weight peptides ($M_r=32,000-75,000$) sometimes occurred under low stringency or in the absence of protease inhibitors (Amlaiky and Caron, 1985 and 1986; Senogles et al., 1988; Jarvie et al., 1988). Our results indicate that the ^3H -spiperone binding site in the rat kidney inner medulla, photoaffinity labeled by ^{125}I -NAPS, is similar to the D_2 dopamine receptors in the striatum and the pituitary, thus suggesting that kidney protein is a dopamine receptor. The unique pharmacological profile, however, suggests that the kidney inner medulla receptor may represent a novel DA_2 receptor.

Two D_2 dopamine receptors from rat brain was recently cloned, sequenced, and expressed (Bunzow et al., 1988; Monsma et al., 1989; Giros et al., 1989; Grandy et al., 1989). The second D_2 dopamine receptor isoform differs only by an additional 29 amino acids in the putative third cytoplasmic loop. Both were pharmacologically indistinguishable and encoded by a single gene and produced by alternative RNA splicing. Evidence for a heterogeneity of D_2 dopamine receptors was recently presented based on binding of ^3H -spiperone to human retina (Mcgonigle et al., 1988). These data indicate that sulpiride, a D_2 receptor-selective ligand, and butaclamol, a D_1/D_2 receptor antagonist, discriminate among sites that have identical affinities for ^3H -spiperone. A D_2 dopamine receptor with genetic identity of brain from human retina was cloned and expressed (Stormann et al., 1990). Interestingly, retinal photoreceptors in the outer nuclear layer as well as inner and outer segments contain a high concentration of

functional D_2 dopamine receptors but no significant hybridization was detected with probes derived from rat brain D_2 dopamine receptor cDNA (Stormann et al., 1990) suggesting multiple forms of the D_2 receptor may exist in the retina. Our data support the existence of the heterogeneity of D_2/DA_2 dopamine receptors among the brain, pituitary, and the kidney. First, in our study l-sulpiride did not compete for ^3H -spiperone binding (Table 1). Second, the relative affinities for the stereoisomers of butaclamol in the kidney were not as disparate (10 X) as usually reported in other tissues (1000 X). A similar finding was recently presented that a D_2 dopamine receptor subtype was expressed with the properties of coupling to increase of intracellular calcium, pertussis toxin-insensitive and not coupling to G proteins (Todd et al., 1989). The difference between the affinities of (+)- and (-)-butaclamol was only 5-10 fold (Todd et al., 1989). It is not clear what the relationship of the D_2 receptors reported previously and the renal DA_2 receptor reported here is, but the DA_2 receptor that we have characterized in inner medulla is like those that have less affinity difference of (+)- and (-)-butaclamol.

The function of the putative renal DA_2 receptor is still unknown. Very recently, Amenta et al (1990) reported that DA_1 and DA_2 receptors both existed in rat renal medulla based on stimulation or inhibition of cAMP production, respectively. Significant amounts of dopamine in rat renal medulla and a dopaminergic innervation of renal medullary blood vessels in human kidney have been demonstrated (Petrovic and Bell, 1986; Bell et al., 1989). However, renal DA_2 receptors labeled by ^3H -spiperone in the rat may not exist presynaptically but postsynaptically based on the fact that denervation had no effect on the affinity and maximum binding capacity of

³H-spiroperone binding sites in rat renal cortex (Katayama et al., 1989). Based on the observation of the unique localization of the DA₂ receptor in the inner medulla, it is interesting to speculate the possible functions of the DA₂ receptor in the kidney. The target tissue of vasopressin in the kidney are collecting duct which is the terminal part of the nephron. The major effect of vasopressin in collecting duct is water, urea, and sodium reabsorption. The secondary messenger of the hydro-osmotic effect of vasopressin is activation of adenylate cyclase. It is reasonable to speculate that dopamine may inhibit vasopressin-induced cAMP response in collecting duct because dopamine inhibits cAMP generation in brain and pituitary and the diuresis effect of dopamine is opposite to the vasopressin action (antidiuresis) in the kidney. However, this speculation awaits further studies.

In summary, we have demonstrated the existence of a high density binding site on inner medullary collecting ducts. Competition profile of ³H-spiroperone in renal inner medulla is similar, but not identical to the pattern of the D₂ dopamine receptors in the brain and the pituitary. Photoaffinity labeling experiments suggest that similar size proteins are labeled in the kidney as in the brain and the pituitary. These results suggest the existence of a novel DA₂ receptor in the rat inner medulla. The physiological significance of renal medullary DA₂ dopamine receptors is currently under investigation.

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3H-SPIPERONE BINDING TO KIDNEY MEDULLA SATURATION BINDING

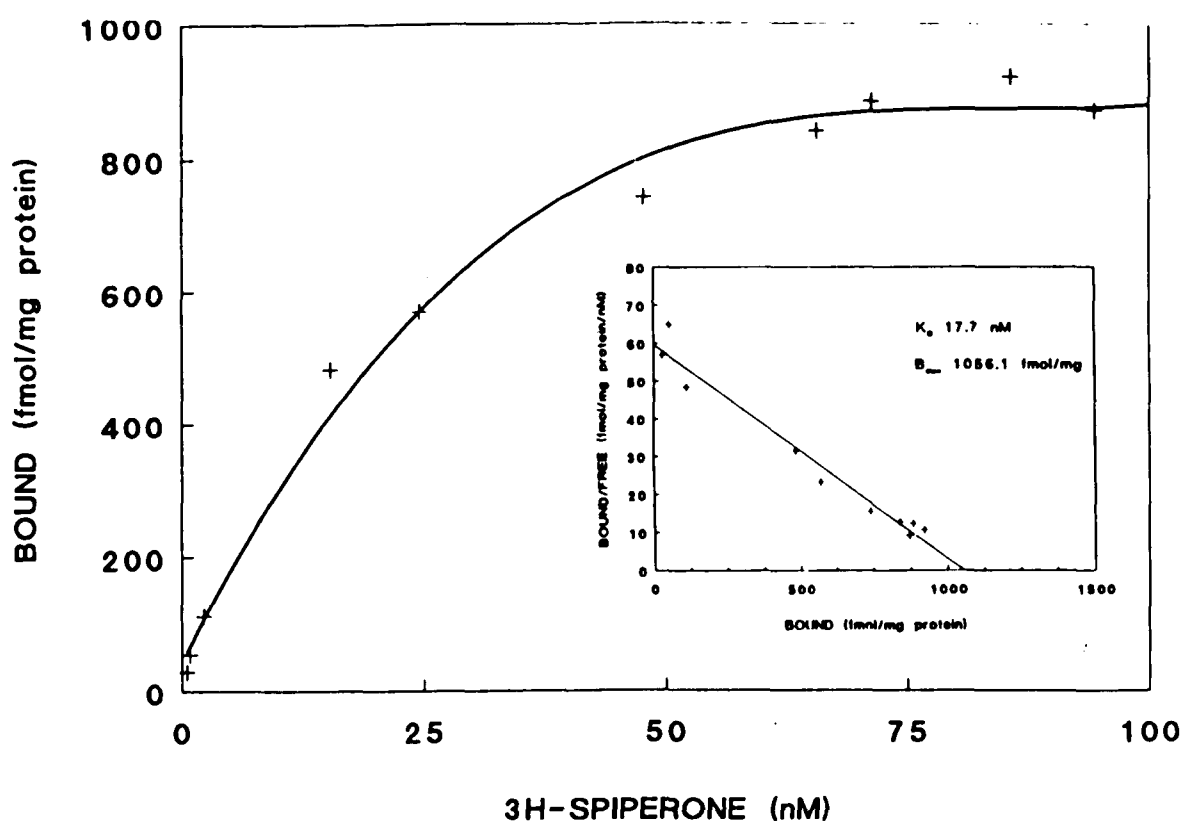


FIG. 1. Saturation binding isotherm and Scatchard transformation (inset) of ³H-spiperone binding to rat kidney inner medulla membranes. Increasing concentrations of ³H-spiperone (1-94 nM) were incubated in absence (total binding) or presence (nonspecific binding) of 100 μ M haloperidol. Shown is specific binding (total minus nonspecific) from a typical experiment. Transformation of binding was best fit to single binding site with a K_d 17.7 nM and a B_{MAX} 1056 fmol/mg protein. Experiments were replicated 4 times with each point in triplicate.

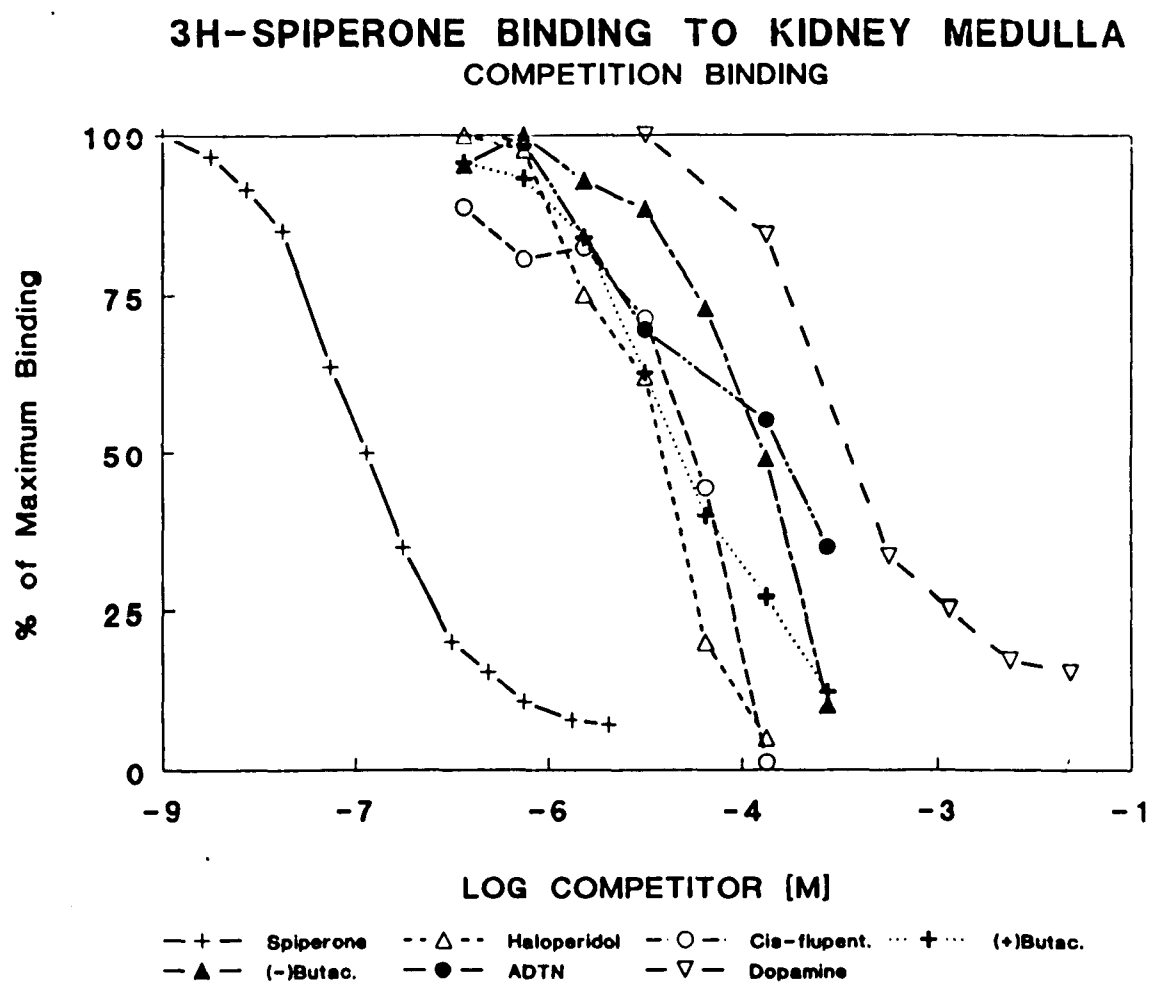


FIG. 2. Competition by dopaminergic agonists and antagonists for ³H-spiroperone (5 nM) binding in membranes from rat kidney inner medulla. Shown are typical competition curves for D₂-selective antagonist (spiroperone), D₁/D₂ mixed antagonists (haloperidol, cis-flupentixol, (+)-butaclamol and inactive enantiomer (-)-butaclamol), and dopamine agonists (ADTN and dopamine). Each curve was fit to a one-site model. Each experiment was repeated 3 times with each point in triplicate.

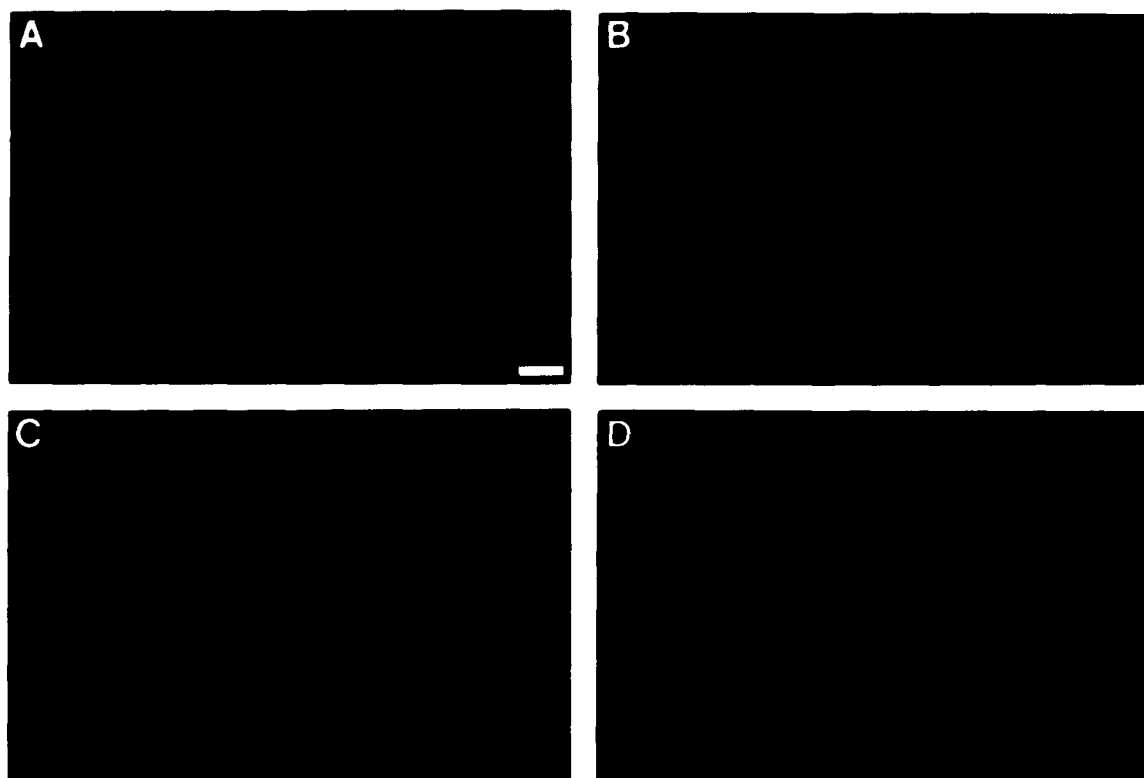


FIG. 3. Autoradiograms of ^3H -spiperone binding to longitudinal sections of rat kidney incubated in presence of 10 nM ^3H -spiperone alone (A) or with 100 μM spiperone (B) or with 100 μM ketanserin (C). A: ^3H -spiperone was concentrated to inner medulla. Cortex binding was less dense and diffuse. B: ^3H -spiperone binding in presence of 100 μM spiperone. Note complete absence of labeling in inner medulla and cortex. C: ^3H -spiperone binding in presence of 100 μM ketanserin (5-HT₂ receptor antagonist). Note that ketanserin was unable to compete ^3H -spiperone binding. D: close-up view of A. Note the longitudinally oriented tubular structure that radiated outward from the tip of the papilla.

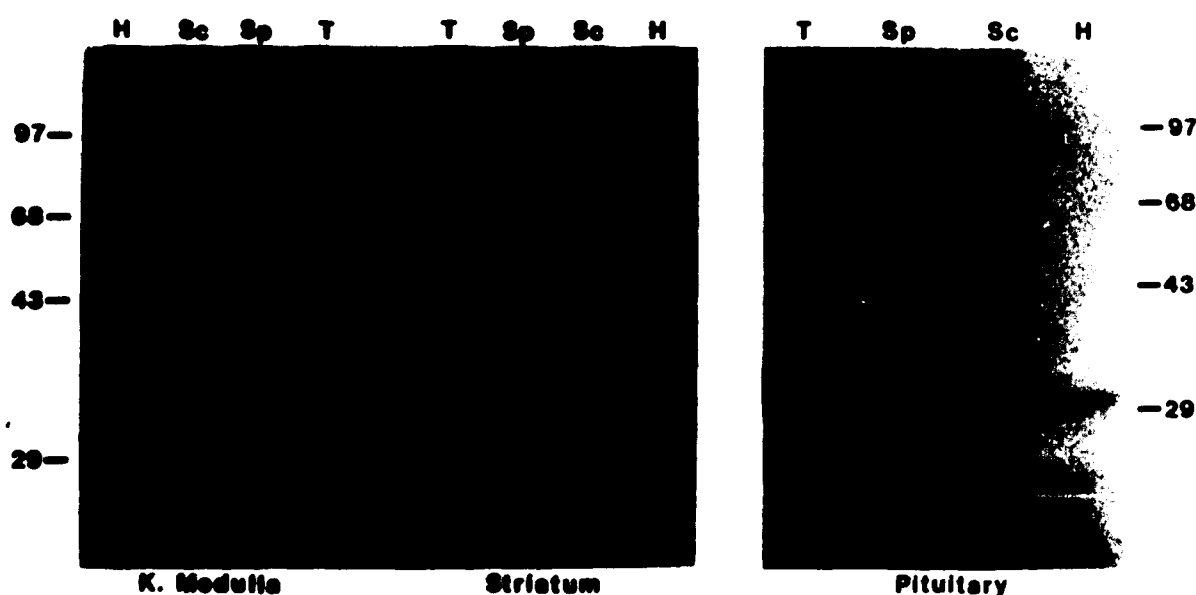


FIG. 4. Photoaffinity labeling of the kidney inner medulla, the striatum, and the pituitary membranes with ^{125}I -NAPS under low stringency protease inhibitors condition. Rat kidney inner medulla, striatum and pituitary membranes were freshly prepared in the presence of protease inhibitors described in Methods, but the incubation was without protease inhibitors. Note that a 94 Kd protein band in the striatum membranes could be competed by spiperone and haloperidol while SCH23390 was ineffective. Also note that in kidney inner medulla and pituitary, a 38 Kd protein showed a similar pattern of labeling. A faint 38 Kd protein band was also present in the striatum membranes. The numbers to the left or right of panels represent the relative migration of proteins of known M_r standards. T, total; Sp, spiperone; Sc, SCH23390; H, haloperidol.

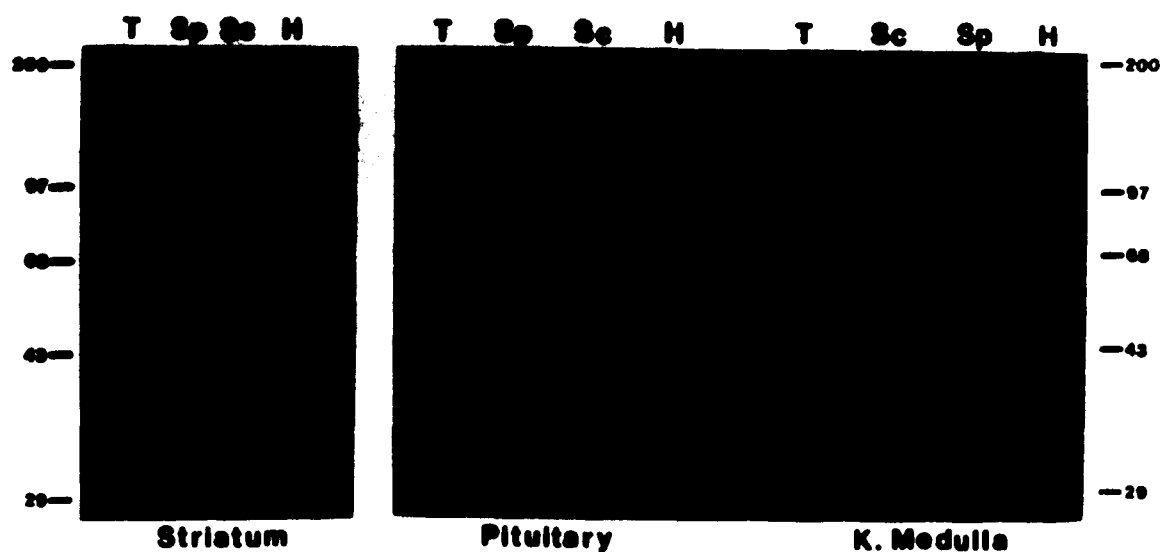


FIG. 5. Photoaffinity labeling of the kidney inner medulla, the striatum, and the pituitary membranes with ^{125} -NAPS under high stringency protease inhibitors condition. Rat kidney inner medulla, striatum and pituitary membranes were freshly prepared and incubated in the presence of protease inhibitors as described in Methods. Note that a diffuse 120-130 Kd protein band in all three tissues could be competed by spiperone and haloperidol but not by SCH23390. Also note that the 38 Kd protein band in Fig. 4 was no more present. The numbers to the left or right of panels represent the relative migration of proteins of known M_r standards. T, total; Sp, spiperone; Sc, SCH23390; H, haloperidol.

TABLE 1

Inhibition of specific ^3H -spiperone binding in rat kidney inner medulla membrane

	Subtype	K_i (μM)	Hill Slope
Dopamine Antagonists			
Spiperone	DA_2	0.035 ± 0.01	0.56 ± 0.05
Haloperidol	DA_1/DA_2	12.2 ± 4.7	0.92 ± 0.14
(+)Butaclamol	DA_1/DA_2	38.3 ± 23.2	1.19 ± 0.20
cis(Z)-flupentixol	DA_1/DA_2	43.3 ± 21.0	1.09 ± 0.11
Domperidone	DA_2	118.5 ± 17.5	1.21 ± 0.21
(-)Butaclamol	DA_1/DA_2	128.2 ± 5.5	1.30 ± 0.20
SCH23390	DA_1	>300	
SKF83566	DA_1	>300	
d-sulpride	DA_2	>1000	
l-sulpride	DA_2	>1000	
Dopamine Agonists			
6,7-ADTN	DA_1/DA_2	189.5 ± 34.1	0.54 ± 0.12
Dopamine	DA_1/DA_2	533.7 ± 169.7	0.60 ± 0.08
Fenoldopam	DA_1	>300	
SKF38393	DA_1	>300	
Apomorphine	DA_1/DA_2	>300	
Norapomorphine	DA_1/DA_2	>300	
LY171555	DA_2	>1000	
Other Agonists/Antagonists			
Prazosin	α_1	>100	
Yohimbine	α_2	>100	
Ketanserin	5-HT $_2$	>100	
Isoproterenol	β_1/β_2	>300	

Values are means $\pm$ SEM of three experiments, each point determined in triplicate. Rat inner medulla membranes were incubated with ^3H -spiperone (final concentration 5 nM) in presence of unlabeled drug and the concentration of unlabeled drug that caused 50% inhibition of specific binding (IC_{50}) was determined and was converted to K_i values as described by Cheng and Prusoff (1973).

CHAPTER 4

ENDOGENOUS DOPAMINERGIC SYSTEM IN RAT KIDNEY INNER MEDULLA:

**Dopamine stimulates prostaglandin E₂ synthesis
via activation of the DA₂ dopamine receptors**

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INTRODUCTION

Dopamine is known to be a potent renal vasodilator and natriuretic stimulant (Goldberg et al., 1986). Dopamine-mediated natriuresis has been attributed to, at least in part, the interaction of dopamine with DA_1 dopamine receptors in proximal tubules. Dopamine inhibited Na^+/K^+ ATPase activity in proximal tubule via DA_1 receptor-mediated activation of phospholipase C and protein kinase C (Bertorello and Aperia, 1989a and 1989b; Felder et al., 1989a and 1989d). It was also reported that dopamine inhibited Na^+/H^+ antiport activity via DA_1 receptor-mediated activation of cAMP generation system (Felder et al., 1989b). Beyond the proximal tubule, there is also evidence that dopamine can influence water and electrolyte transport. Dopamine blocked the antidiuretic effect of vasopressin by inhibiting the hydro-osmotic effect of vasopressin in rabbit cortical collecting tubule (Muto et al., 1985) and in toad bladder (Arruda and Sabatini, 1982). In water loaded rats, dopamine inhibited the diuretic effect of vasopressin which could be blocked by haloperidol, a dopamine receptor antagonist (Koyoma et al., 1985).

Dopaminergic receptors have been studied in rat kidney using radioligand binding. DA_1 receptors have been characterized using selective antagonists (Felder and Jose, 1988b; Huo and Healy, 1989; Hughes and Sever, 1989) and agonists (Hegde et al., 1989). Renal DA_1 receptors have also been characterized by in vitro autoradiography in the rat. Interestingly, DA_1 receptors appear to be concentrated primarily on the proximal tubules and are either not present (Huo and Healy, 1989) or present at low density on vascular elements (Hegde et al., 1989). Less is known

regarding the DA₂ receptor. Based on the binding of the nonselective radiolabeled DA antagonist ³H-haloperidol and inhibition of adenylate cyclase by DA, Felder et al. (1984a) concluded that glomeruli expressed DA₂ receptors. More recently Hietala et al. (1988) and Felder et al. (1988c) reported on the binding of the relatively selective DA₂ antagonist ³H-spiperone in rat and sheep kidney cortex. The possibility that ³H-spiperone was labeling 5-HT₂ receptors in the kidney, as has been shown in brain (Creese and Snyder, 1978; Huff and Molinoff, 1982), was ruled out because the 5-HT₂ selective antagonist ketanserin was unable to compete for the ³H-spiperone binding (Hietala et al., 1988; Felder et al., 1988c and 1989).

The kidney medulla synthesizes 10- to 100-fold more prostaglandins than the cortex in all species studied (Dunn, 1983). Both the medullary collecting duct cells and the medullary interstitial cells are able to synthesize prostaglandins (Dunn, 1983). PGE₂ is the most abundant prostaglandin produced in these cells, especially in inner medulla. PGE₂ synthesis in these cells can be increased by vasopressin, bradykinin, angiotensin II, and hyperosmolality state (Wuthrich et al., 1986b; Zusman and Keiser, 1977; Grenier et al., 1981). The mechanism of control of basal PGE₂ synthesis, however, is unclear in these tissues.

It is well established that renal prostaglandins, particularly PGE₂, regulate renal excretion of water and antagonize vasopressin-stimulated water reabsorption (Sato and Dunn, 1984). The relationship between dopamine and prostaglandins systems have been studied in vivo. Dopamine and the dopamine agonist bromocriptine increased PGE₂ urinary excretion in rats (Yoshimura et al., 1987) and in humans (Iimura et al., 1987),

and dopamine infusion could significantly increase urinary PGE₂ excretion in essential hypertensive subjects (Iimura et al., 1987). Lower amount of urinary dopamine and PGE₂ excretion was also found in hypertensive subjects (Iimura et al., 1987). Based on the observations that DA₂ dopamine receptors labeled by ³H-spiperone are apparently very dense in rat kidney inner medulla (Huo and Healy, Chapter 3) and the action of vasopressin is primarily in the same tissue, we sought to characterize further the DA₂ receptor in the inner medulla. First, ³H-domperidone, a peripheral DA₂-selective antagonist, was used to characterize the DA₂ receptor in cultured inner medullary collecting duct (IMCD) cells, a well characterized *in vitro* model (Grenier et al., 1982; Sato and Dunn, 1984; Taub and Sato, 1980). Second, the relationship between dopamine and prostaglandin E₂ synthesis was investigated using the same cells.

We report here that exogenous dopamine can increase PGE₂ synthesis via activation of DA₂ dopamine receptors and the response is blocked by concomitant DA₂ receptor blockade. An endogenous dopaminergic system may also control PGE₂ synthesis in inner medullary collecting duct (IMCD) cells. Characterization of DA₂ dopamine receptor in IMCD cells using ³H-domperidone confirmed that dopamine may exert important physiological functions as a autocrine or paracrine system in renal medulla.

METHODS

Isolation and culture of inner medullary collecting duct cells

IMCD cells were isolated from renal inner medulla of 200-250 g Sprague-Dawley rats according to the methods of Grenier et al (1982), which has been adapted by Sato and Dunn (1984), and by Wuthrich and Valloton (1986a and 1986b). The inner medulla tissue was dissected away from the outer medulla, finely minced with a tissue chopper (Brinkmann), and placed in a 1:1 mixture of DMEM and Ham's F-12 medium containing 1 mg/ml collagenase (Type I, Sigma). The minced tissue was incubated for 90 min at 37 °C in 5% CO₂/95% air with gentle pipetting up and down every 30 min. After collagenase dispersion of cells, distilled water was added to give a final osmolality of 120 mosmol/kg. The cellular mixture was immediately centrifuged at 400 g for 5 min, and the pellet containing intact cells was added on top of 10 ml of phosphate buffer saline solution containing 7.5% BSA. This solution was centrifuged for 5 min, and the pellet containing intact cells was resuspended in DMEM+F12 medium, then recentrifuged. The final cell pellet was resuspended in DMEM+F12 medium supplemented with 10% fetal calf serum, containing penicillin-streptomycin (1:100), 10 mM HEPES, 42 mM sodium bicarbonate. The cells obtained from 3-4 kidneys were seeded into 24 wells of a Falcon culture dish with 0.5 ml of media in each well. For AADC enzyme assay, cells pooled from 10 kidneys were seeded in 175 cm² culture flask. After 1 day in culture, the media were changed to a fully defined K-1 medium as described by Taub and Sato (1980). This hormone-supplemented serum-free medium contains 1:1 mixture of DMEM+F12, 10 mM HEPES, 42 mM sodium bicarbonate, penicillin-streptomycin (1:100), 5 µg/ml insulin, 50 nM hydrocortisone, 5 µg/ml transferrin, 5 pM triiodothyronine, 10 nM sodium selenite. This modified K-1 medium enhances kidney epithelial cell growth and retards proliferation of fibroblasts (Taub and Sato, 1980). The media were changed daily, and the IMCD cell cultures

were used for experimentation after 4 days. Morphological, biochemical, and physiological characteristics had been described previously (Taub and Sato, 1980).

³H-Domperidone binding on cultured IMCD cells

All binding experiments were done in triplicate in room temperature using 24 well culture dishes after 4 days of culture. The cells were rinsed twice with 1 ml incubation buffer consisting of the following components: Tris-HCl 50 mM, (pH 7.4), NaCl 120 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, pargyline 0.1 mM, and 0.01 % ascorbic acid. 150 µl incubation buffer containing different concentrations of ³H-domperidone (final concentrations: 2 nM to 200 nM) in absence or in presence of 10⁻⁶ M spiperone used to define nonspecific binding was incubated with cultured IMCD cells. Preliminary binding studies showed that maximal specific binding occurred at 10 min and remained constant for the subsequent 60 min (data not shown). Thus, a constant incubation time of 15 min was chosen in binding experiments. The ratio of total to nonspecific binding was 2:1 at the K_D of domperidone. 15 nM of ³H-domperidone and increasing concentrations of different classes of receptor antagonists and agonists were used for competition experiments. After incubation the cells of each well were washed three times with 1 ml of cold saline. Cells were dried by vacuum dehydration and dissolved with 1 N NaOH. The radioactivity was measured by liquid scintillation spectrometry.

Release of PGE₂ from culture IMCD cells

All experimental incubations of IMCD cells were in triplicate using 24 well culture dishes. The cells were rinsed with 1 ml DMEM+F12 media containing 100 μ M pargyline and 0.01% ascorbic acid before all experiments, and this media were used for subsequent experiments. Preincubation and incubation were done in 5% CO₂-95%air at 37 C. 0.5 ml of preincubation media containing vehicle, antagonist, or enzyme inhibitor were incubated for 30 min, then removed and replaced with 0.5 ml incubation media containing vehicle, agonists, antagonists, agonists in presence of antagonists, and agonists in presence of enzyme inhibitors. The incubation periods are indicated in the figure legend. After incubation, the media were aspirated and were frozen at -20 °C. PGE₂ content was measured by radioimmunoassay without extraction within 1 week. Cellular protein was measured after dissolved in 250 μ l 1 N NaOH and neutralized with 1 N HCl. Protein was determined using kits from Biorad.

PGE₂ assay

PGE₂ was measured by radioimmunoassay of extracellular media with antisera and standards purchased from Advanced Magnetics Inc. (Boston, MA). ³H-PGE₂ was purchased from New England Nuclear (Boston, MA), The range of the standard curve was 5-320 pg/tube. Dextran-coated charcoal was used for separation.

HPLC-Electrochemical detection of dopamine formation

The methods for measurement of dopamine formation was adapted from Blandina et al. (1989) and modified as follows. Freshly dispersed IMCD cells, after

being trypsinized briefly with 0.05% trypsin-EDTA, were washed twice with PBS, collected by centrifugation and resuspended in Hank's balanced salt solution (HBSS) consisting of the following composition: 136 mM NaCl, 5.3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 0.01 % ascorbic acid, 0.1 mM pargaline, 10 mM HEPES, pH 7.4. The reaction was in 0.9 ml HBSS solution containing 200 μM L-dopa, 0.1 mM pyridoxal phosphate and the IMCD cells pooled from 2-4 kidneys per sample. After selected incubation time at 37 °C. Perchloric acid (HClO_4 , 1 N, 0.1 ml) containing epinine (N-methyl-dopamine) as an internal standard was added to the incubation mixture. The mixture was centrifuged at 10,000 x g for 5 min and the supernatant was saved at -20 °C until assayed (within 3 days). For partial disrupted cell preparation, cells were frozen and thawed twice on dry ice before incubation. For preparation of cytoplasmic fraction, the cells were sonicated briefly at 4 °C and centrifuged at 10,000 x g for 5 min. The supernatant was used for reaction. DA was determined by high-performance liquid chromatography-electrochemical detection (Waters, Milford, MA) after alumina extraction (recovery 50-70 %). The samples were centrifuged in a Beckman Microfuge B Centrifuge for 3 min at 10,000 x g at 4 °C. Aliquots (750 μl) of supernatants were transferred to a micro column (Isolab Inc., Acron OH) containing 40 mg of activated alumina (Bioanalytical System, West Lafayette, IN), 20 μl of a solution containing (mM) Na_2CO_3 , 190; disodium EDTA, 4.5; $\text{Na}_2\text{S}_2\text{O}_5$, 5; and 1 ml of 1.5 M Tris-HCl buffer (pH 8.6) containing disodium EDTA (61 mM). After the microcolumns were shaken for 10 min (Fisher mixer model 346), the supernatant was discarded and the alumina was washed 3 times with 10 ml of 5 mM Tris-HCl buffer (pH 8.6) by means of a vacuum manifold (Baker-10 SPE). After elution with 200 μl of 0.1 N HClO_4 , 10-80 μl of the eluate were injected onto a reverse-phase column (Beckman Ultrasphere

Ion Pair, 5 μ m, 150 x 4.6 mm) at room temperature. The mobile phase had the following composition (mM): ClCH_2COOH , 111; NaOH , 100; disodium EDTA, 2; sodium octyl sulfate, 0.87; and methanol to 9 % (pH 3.8). The potential of the glassy carbon electrode was set at 0.6-0.8 V against the reference electrode. The flow rate was 0.8 ml/min. External standards (epinine) were always carried through the extraction with the biological samples. Peaks were identified by comparison of their retention times with those of the standards. Spiking the samples with DA increased the area of the appropriate peaks. Variation of the pH of the mobile phase failed to resolved further the peaks, suggesting that no additional substances were coeluting with the compound under investigation. The levels of DA in the extract were calculated by comparison of sample peak area with external standard peak area. The correlation of the peak area of DA divided by the peak area of the internal standard vs. the concentration of the analite was linear between 100 fmole and at least 1000 fmole ($R\text{-squared}=0.99$).

Enzyme assay of aromatic L-amino acid decarboxylase

Cultured IMCD cells were washed twice with phosphate buffered saline, treated with 0.05 % trypsin-EDTA for 3 min at room temperature, centrifuged at 400 x g for 5 min, disrupted by sonication, and recentrifuged at 10,000 x g for 5 min. The final supernatant (cytoplasmic fraction) was used for enzyme analysis. The activity of AADC was assayed by a modification of the method of Shirota and Fujisawa (1988). The assay was determined by measurement of the rate of $^{14}\text{CO}_2$ evolution from L- ^{14}C -dopa (L- ^{14}C -dihydroxyphenylacetic acid, New England Nuclear). The assay mixture contained, in a final volume of 0.5 ml, 100 mM sodium phosphate buffer (pH 7.0), 0.1 mM

pyridoxal phosphate, 0.1 mM EDTA, 0.01 % ascorbic acid, 0.1 mM pargyline, 0.1 μ Ci L- ^{14} C-dopa plus unlabeled L-dopa, and 50-100 μ g protein of cytoplasmic fraction. The reaction was carried out at 37 °C for an hour and terminated with 100 μ l of 50 % tetrachloroacetic acid. The mixture was kept on 37 °C for another 30 min to ensure the complete absorption of ^{14} CO $_2$ by the trapping agent hyamine (200 μ l). The trapping agent was counted in a Beckman scintillation counter.

RESULTS

^{3} H-Domperidone binding in cultured IMCD cells

Specific binding of ^{3} H-domperidone to cultured IMCD cells was saturable with increasing concentration; nonspecific binding was linear. The ratio of total to nonspecific binding was 2:1 at the concentration of K_D . Scatchard plots of the saturation isotherms indicated that the data could be fit into single binding site with K_D of 57.6 ± 10.5 nM and B_{max} of 14.9 ± 2.7 pmol/mg protein (Fig. 1, n=4). The specificity of binding of ^{3} H-domperidone to cultured IMCD cells was determined by competition with unlabeled DA agonists and antagonists (Fig. 2 and Table 1). The rank-order potency of antagonists was domperidone > spiperone > haloperidol = cis-flupentixol > eticlopride = (+)-butaclamol > SCH23390 > (-)-butaclamol >> l-sulpiride. The rank-order potency for agonists was norapomorphine > apomorphine = bromocriptine = fenoldopam >> 6,7-ADTN = dopamine = LY 171555. The Hill coefficients for antagonists competition were close to unity except for haloperidol and SCH23390 (Table 1). The Hill coefficient for agonist competition were close to unity

except for norapomorphine and apomorphine (Table 1).

Dopamine stimulates PGE₂ release in cultured IMCD cells

Time-course experiment (Fig. 3) showed that significant amounts of PGE₂ above basal level could be detected after 10 min incubation with dopamine. Maximal stimulation of dopamine occurred by 60 min and kept constant for subsequent 60 min. Basal PGE₂ release increase promptly in the initial 15 min and then increased slowly in the subsequent time period. Generally, the dopamine stimulation was 2 fold compared with the basal release after 60 min. The basal PGE₂ level was 10.6 ± 2.7 pg/mg at 10 min, and then went up to 18.9 ± 2.4 pg/mg at 60 min. The dopamine stimulated PGE₂ release was 18.7 ± 3.6 pg/mg at 10 min, and went up to 41.4 ± 3.9 pg/mg at 60 (n=3).

A 60 min incubation was selected for the rest of the experiments unless noted.

Dose-response experiments for dopamine (Fig. 4) was expressed as percent of stimulation compared with basal level due to different basal level in each preparation. The range of dopamine concentration was 3 μ M to 100 μ M. The concentration of dopamine for half-maximum stimulation (EC₅₀) was 11.1 μ M. (n=3).

The effects of other agonists or hormones is shown in Fig. 5. Compared with dopamine-stimulated PGE₂ synthesis (188 ± 8 % of basal level, n=12), the percentages of increase compared with basal level were: 10^{-4} M l-dopa, 171 ± 9 % (n=6); 10^{-3} M l-dopa, 204 ± 21 % (n=7); 10^{-5} M epinephrine, 210 ± 39 % (n=3); 10^{-4} M N-methyl-

dopamine (epinine), 173 ± 14 % (n=3); 10^{-5} M bradykinin, 164 ± 21 % (n=4); and 10^{-5} M arachidonic acid, 280 ± 42 % (n=4).

Antagonists inhibited basal and dopamine-stimulated synthesis of PGE₂

Several different classes of receptor-selective antagonists were screened for their ability to block dopamine-stimulated PGE₂ synthesis. Only the DA₂ receptor-selective antagonists domperidone and IBZM ((S)-(-)-3-Iodo-2-hydroxy-6-methoxy-N-[(1-ethyl-2-pyrrolidinyl)methyl]benzamine) were found to lower basal level and to antagonize the stimulation of dopamine effectively (Fig. 6). Domperidone (10^{-4} M) decreased basal synthesis to 28 ± 4.6 %, and completely blocked dopamine (10^{-4} M)-stimulated PGE₂ from 186.1 ± 7 % to 83.9 ± 11 % of basal synthesis (n=8). IBZM (10^{-4} M) lowered PGE₂ basal level to 61.7 ± 1.5 %, and blocked dopamine (10^{-4} M)-stimulated PGE₂ to 90.9 ± 8.8 % of basal synthesis (n=5). Other receptor antagonists were without effect: SCH23390, DA₁ dopamine receptor antagonist; prazosin, α_1 adrenoceptor antagonist; yohimbine and rauwolfia, α_2 adrenoceptor antagonists; propranolol, β adrenoceptor antagonist; ketanserin, mesulergine, and 5-carboxyamino-tryptamine, 5-HT₂, 5-HT_{1C2}, and 5-HT_{1A,1B,1C} serotonergic antagonists respectively (data not shown). It was shown that domperidone (3×10^{-5} M and 10^{-4} M) and IBZM (10^{-4} M) not only shifted the dose-response of dopamine to the right, but also lowered the basal synthesis of PGE₂ significantly (Fig. 7 and Fig. 8, n=2). The inhibition of basal PGE₂ synthesis by domperidone and IBZM were also dose-dependent (Fig. 9 and Fig. 10, n=3). The maximal inhibition for domperidone and IBZM was 67 % and 82 % lower than basal synthesis at the concentration of 10^{-4} M and 3×10^{-4} M, respectively.

PGE₂ synthesis was stimulated by L-dopa and basal PGE₂ synthesis inhibited by AADC inhibitors

Based on the observation that both DA₂ receptor antagonists, domperidone and IBZM, suppressed basal PGE₂ synthesis, attempts were made to identify the relationship of PGE₂ synthesis and a possible endogenous dopaminergic system in cultured IMCD cells. L-Dopa, the precursor of dopamine, like dopamine, was able to increase PGE₂ synthesis (Fig. 11, n=3). Preliminary time-course study indicated the stimulation of L-dopa was slower than dopamine, so L-dopa was preincubated for 30 min at 37 °C, removed and incubated with L-dopa for an hour. The maximal stimulation was about two fold of basal synthesis occurred at the concentration of 1 mM. 3-Hydroxybenzylhydrazine (HBH), an aromatic L-amino acid decarboxylase (AADC) inhibitor (Goodale and Moore, 1976; Carlsson, 1978), lowered and shifted the dose-response curve of L-dopa to the right at the concentration of 10⁻⁴ M (n=3). HBH inhibited basal PGE₂ synthesis as dose-dependent, shown in Fig. 12. The IC₅₀ was 50 μM (n=5). Carbidopa, another AADC inhibitor, lowered basal PGE₂ synthesis to 58 ± 13 % and 29 ± 1 % at the concentration of 10⁻⁵ M and 10⁻³ M, respectively (n=3). Indomethacin, a cyclooxygenase inhibitor, suppressed PGE₂ synthesis to less than 5% of basal level at the concentration of 10⁻⁵ M (n=3). Quinacrine (mepacrine), a phospholipase A₂ inhibitor (Flower and Blackwell, 1975), inhibited basal and dopamine-stimulated PGE₂ synthesis to 16 ± 5 % and 33 ± 4 % of basal level at the concentration of 10⁻⁴ M (Fig. 13, n=3). Nordihydroguaiaretic acid (100 μM), a lipoxygenase inhibitor (Yamamoto et al., 1983), was without effect (not shown). 8-

(Diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an intracellular calcium blocker (Malagodi and Chiou, 1974; Owen and Villereal, 1982), suppressed basal and dopamine-stimulated PGE_2 synthesis to $15 \pm 3 \%$ and $34 \pm 4 \%$ of basal level at the concentration of 10^{-4} M (Fig. 13, $n=3$). Neomycin (1 mM), a phospholipase C inhibitor (Lipsky and Leitman, 1982), and voltage-dependent calcium channel blockers, verapamil and diltiazem (100 μM), were without effect (not shown).

Local synthesis of dopamine in IMCD cells

Based on the observation that basal PGE_2 synthesis could be suppressed by DA_2 dopamine antagonists and dopa decarboxylase inhibitors, endogenous generation of dopamine in inner medullary duct cells appeared to be an important factor to explain the phenomena. Dopamine was barely detectable in freshly dispersed cell preparations. Dopamine formation in freshly dispersed cells was measured by incubating cells with 200 μM L-dopa for indicated time at 37 °C (Fig. 14c). The cytosol fraction of adrenal medulla was used as control (not shown). Dopamine formation was also detected in cytosol (14a), partially disrupted cells (14b). The local synthesis of dopamine was attenuated by treatment of aromatic L-amino acid decarboxylase inhibitors 3-hydroxybenzylhydrazine (HBH) and carbidopa (Fig. 14c). Usually 80-90 % dopamine synthesis was inhibited by treatment of 100 μM HBH or carbidopa when 200 μM L-dopa was used as substrate.

Attempts to identify the existence of dopa decarboxylase activity was also done in IMCD cells after four days of culture by using the more sensitive $^{14}\text{CO}_2$

radioenzymatic assay. Dopamine formation was linear up to 120 minutes (not shown). Eadie-Hofstee plot analysis showed biphasic enzyme kinetics with a higher affinity but lower capacity component ($K_{m1} = 0.351 \pm 0.066$ mM, $V_{max1} = 0.436 \pm 0.099$) and a lower affinity but higher capacity component ($K_{m2} = 30.1 \pm 10.4$ mM, $V_{max2} = 11.1 \pm 2.5$ nmol/mg/hr, Fig. 13, n=3).

DISCUSSION

Dopamine is known to regulate renal water and sodium excretion. However, the mechanisms of dopamine mediated effects are still not clear. Evidences have been accumulated in support of the view that dopamine acts directly on the proximal tubules to cause natriuresis via activation of DA_1 receptors (Felder et al., 1989c; Goldberg et al., 1986). The mechanism of dopamine-mediated diuresis effect was not known, but some studies suggested it may be due to either dopamine-mediated vasodilatation effect (Kohli and Goldberg, 1987) or inhibitory action on the hydro-osmotic effect of vasopressin, thereby increasing urinary excretion of water (Arruda and Sabatini, 1982; Muto et al., 1985; Koyoma et al., 1985). Base on the observation that there is a high density of DA_2 receptors in rat inner medullary collecting ducts (Huo and Healy, Chapter 3), in the present study, we further characterized and sought the function of the DA_2 receptors in those cells.

Domperidone has been characterized to be a peripheral specific DA_2 antagonist (Laduron and Leysen, 1979; Kohli et al., 1983). Domperidone blocked femoral vasodilatation elicited by apomorphine or dipropyldopamine, a dopamine receptor

agonist, in dog (Willems et al., 1981; Kohli et al., 1983), stimulated thyrotropin and prolactin release in man (Sowers et al., 1982), and abolished natriuresis produced by water immersion or lower-body positive pressure (Bennett et al., 1982; Coruzzi et al., 1986;). Binding studies of ^3H -domperidone used as D_2 receptor antagonist have been done in brain and retina while the nonspecific binding was so high that specific binding could not be detected in retina homogenate (Hamblin et al., 1984; Watling and Iversen, 1981). In preliminary studies, we also found a very high nonspecific binding that specific binding was not measurable in inner medullary membrane homogenate preparations, but the signal to noise ratio increased if using dispersed and cultured IMCD cells. So, by using cultured IMCD cells characterization of ^3H -Domperidone binding was done. The Scatchard analysis showed the affinity was high ($K_D = 57.6 \text{ nM}$) with a high density of receptor binding sites ($V_{\text{max}} = 14.9 \text{ pmol/mg protein}$). The rank-order potency from competition studies revealed that ^3H -domperidone binding in cultured IMCD cells was consistent with the labeling of a DA_2 receptor.

Since there is a high density of DA_2 receptors in rat inner medulla, an area also containing abundant of PGE_2 which is known to be a potent inhibitor of vasopressin (Dunn, 1983), the relationship of dopamine and PGE_2 synthesis was explored. 10^{-4} M Dopamine stimulated PGE_2 synthesis two fold in cultured IMCD cells. The time-course study revealed that significant increase of PGE_2 synthesis stimulated by dopamine was detected at 15 min after adding dopamine, however, the maximal response was at 60 min. The time course for dopamine stimulation was relatively slow compared with other hormone-stimulated PGE_2 synthesis such as bradykinin-stimulated PGE_2 synthesis in MDCK cells (Slivka and Insel, 1988) and rabbit papillary collecting duct cells (Grenier

et al., 1981), or angiotensin II-, vasopressin-, and bradykinin-stimulated PGE₂ synthesis in rat medullary tubular cells (Wurthrich et al., 1986). This may imply that it was an indirect effect or a slow response with complex secondary messenger systems. The dose-response study showed that the EC₅₀ for dopamine was 11.1 μ M, which was compatible with the finding that renal tissue concentration of dopamine was μ M range (Bell and Gillespie, 1981; Fernandez-Pardaland and Saavedra, 1982; Baines et al., 1985; Petrovic et al., 1986; Soares-da-silva, 1988). Interestingly, L-dopa could also stimulate PGE₂ synthesis. The effects of dopamine and L-dopa could be blocked by the selective DA₂ antagonists domperidone and IBZM but not other receptor antagonists suggesting DA₂ receptors mediated the response of dopamine. Aromatic L-amino acid decarboxylase inhibitors such as 3-hydroxybenzylhydrazine and carbidopa also attenuated the basal synthesis as well as the L-dopa-induced PGE₂ synthesis implying that an endogenously generated dopamine may control, at least in part, the synthesis of PGE₂ in cultured IMCD cells.

Aromatic L-amino acid decarboxylase (AADC) was reported to be present in rat papillary collecting ducts, although the immunostaining was less than in the proximal tubules (Adam et al., 1986). Direct generation of dopamine by AADC was measured by either HPLC-electrochemical detection or radioenzyme assay using L-¹⁴C-dopa as substrate. By using HPLC-electrochemical detection, dopamine formation could be detected in cytoplasmic fraction following incubation with L-dopa, partially disrupted and intact cells. The peak of dopamine was abolished by the AADC inhibitors 3-hydroxybenzylhydrazine or carbidopa confirming the specificity. In order to identify and quantitate the existence of AADC activity which was relatively lower in cultured IMCD

cells, a more sensitive radioenzyme assay was used. The enzyme kinetic studies revealed that saturation was not achieved at the concentration of 30 mM L-dopa suggesting a very high V_{max} of AADC (data not shown). Eadie-Hofstee plot showed biphasic enzyme kinetics with a high-affinity ($K_{m1} = 0.351$ mM), low-capacity ($V_{max1} = 0.461$ nmol/mg/hr) component and a low-affinity ($K_{m2} = 30.1$ mM), high-capacity ($V_{max2} = 10.1$ nmol/mg/hr) component. In previous reports, the range of K_m of AADC in hog kidney (Christensen et al., 1970), in rat kidney homogenate (Shirota and Fujisawa, 1988), or in rat proximal tubules (Hayashi et al., 1990) were 100-190 μ M when the substrate concentrations were up to 1 mM. However, the K_m was one log higher (3-5 mM) than usual when the concentrations of substrate were up to 60 mM (Seri et al., 1990). The concentrations of L-dopa used in the present studies were 30 μ M to 30 mM. It appeared that wide range of substrate concentration was important for determination of the K_m when the V_{max} of the enzyme is high. Whether the biphasic enzyme kinetics represented positive cooperativity or the existence of two isozymes is unclear. The possibility of two isozymes can not be ruled out, since a doublet was detected in partial purified bovine kidney preparation by Western blot analysis (Albert et al., 1987).

The secondary messenger system which might involved in DA_2 receptor-mediated PGE_2 synthesis was unclear. Neomycin, a phosphoinositol-specific phospholipase C inhibitor in the kidney (Lipsky and Leitman, 1982; Slivka and Insel, 1987), as well as verapamil and diltiazem, two voltage-dependent calcium channel blockers, had no effect on the basal and the dopamine-stimulated PGE_2 synthesis indicating these two pathways might not mediate the response. Phospholipase A_2

inhibitor quinacrine and intracellular calcium blocker TMB-8 not only suppressed the basal synthesis but also attenuated the dopamine-stimulated response suggesting activation of phospholipase A₂ and/or release of intracellular calcium might be involved in the DA₂ receptor-mediated response in cultured IMCD cells. Whether this DA₂ receptor directly coupled to activation of phospholipase A₂ or indirectly through intracellular release of calcium then to activate phospholipase A₂ will be investigated in future studies.

Several lines of evidence indicate that dopamine may be synthesized intrarenally and that endogenous dopamine may play an important role in the regulation of sodium excretion. Conversion of circulating L-dopa to dopamine has been proposed as a possible mechanism for dopamine synthesis in the kidney (Lee, 1982; Bell, 1987; Zimlichman et al., 1988; Grossman et al., 1990). Renal dopamine derives mainly from renal tubular dopamine production and to a lesser extent from dopaminergic nerves (Bell, 1988). Immunohistochemical staining of aromatic l-amino acid decarboxylase (AADC), an enzyme converting L-dopa to dopamine, was markedly high in the proximal tubules and less in distal tubule and papillary collecting ducts (Adam et al., 1986). Inhibition of AADC activity attenuated urinary dopamine and sodium excretion associated with acute or chronic sodium administration (Ball et al., 1977; Bertorello et al., 1988,) implying that endogenous dopamine may have significant physiological effects in the kidney. There are also evidences that dopamine-mediated effects can not solely be accounted for by DA₁ receptor activation. First, acute volume expansion with saline loading increased urinary dopamine secretion (Hegde and Lokhandwala, 1988). SCH23390, a DA₁ antagonist, can completely block fenoldopam-mediated natriuresis but

only partially block renal responses to exogenous dopamine (Hegde and Lokhandwala, 1988). Second, Baggio and Ferrari (1981) reported that dopamine and (3,4-dihydroxyphenylamino)-2-imidazoline (DPI) were able to produce diuresis and lowered urinary osmolality in rats, and that this effect could be blocked by α_1 -adrenergic antagonists yohimbine and phentolamine. Our evidence supports these findings. In freshly dispersed IMCD cells, dopamine and its agonist 6,7-ADTN were able to inhibit vasopressin-stimulated cAMP accumulation via activation of α_1 -adrenoceptor (Huo and Healy, Chapter 5). In present studies, however, we demonstrated additional cellular mechanisms of dopamine-induced physiological effects in the kidney.

In summary, we have identified the DA_2 receptors and demonstrated that exogenous dopamine can stimulate PGE_2 synthesis via activation of the DA_2 dopamine receptors in cultured IMCD cells. An endogenous dopaminergic system, apparently, controls substantial basal PGE_2 synthesis in these cells. Based on the facts that (1) dopamine inhibited the hydro-osmotic effect of vasopressin in rats (Koyoma et al., 1985), in rabbits (Muto et al., 1985), and in toad bladders (Arruda and Sabatini, 1982) (2) dopamine increased urinary PGE_2 synthesis in rats (Yoshimura et al., 1987) and in humans (Iimura et al., 1987) (3) urinary PGE_2 excretion was suppressed by treatment of AADC inhibitor in humans (Yoshimura et al, 1987), the observations presented here and in Chapter 4 that activation of the DA_2 receptor and the α_1 -adrenoceptor by endogenous or exogenous dopamine resulted in stimulation of PGE_2 synthesis or inhibition of vasopressin-stimulated cAMP accumulation in rat IMCD cells suggest that dopamine may act, therefore, as a autocrine or paracrine hormone and may be an endogenous physiological antagonist of vasopressin in renal medulla.

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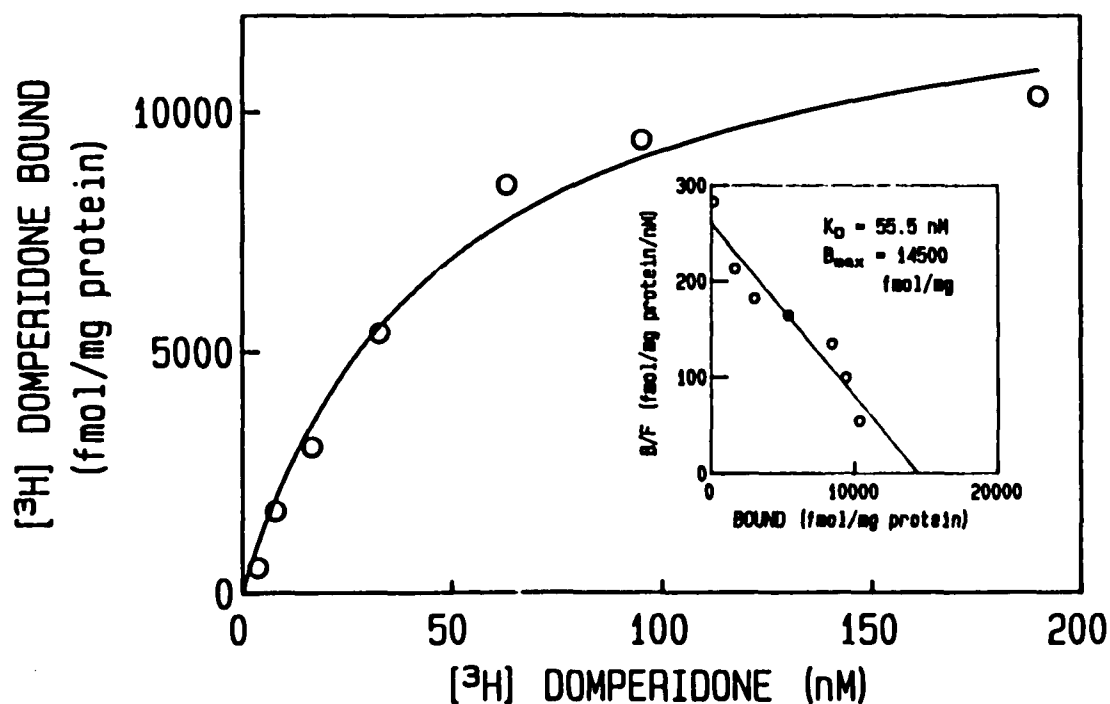


FIG. 1. Saturation binding isotherm and Scatchard transformation (*inset*) of ^3H -domperidone binding to rat kidney IMCD cells. Increasing concentration of ^3H -domperidone (2-200 nM) were incubated in absence (total binding) or presence (nonspecific binding) of 100 μM spiperone. Shown is specific binding (total minus nonspecific) from a typical experiment. Transformation of binding could be fit into one binding site with $K_D = 55.5 \text{ nM}$ and $B_{\text{max}} = 14500 \text{ fmol/mg protein}$. Experiments were replicated 4 times with each point in triplicate.

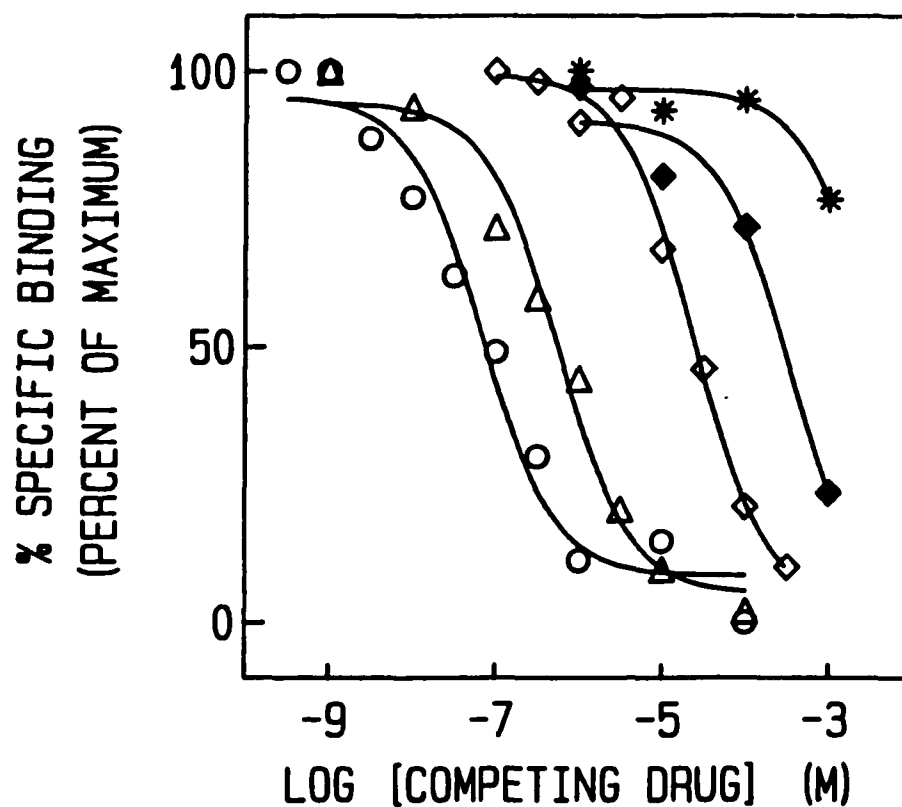


FIG. 2. Competition by dopaminergic antagonists for ^{3} -domperidone (15 nM) binding in rat IMCD cells. Shown are typical competition curves for DA_2 -selective antagonists domperidone (circle) and spiperone (triangle), stereoisomers (+)butaclamol and (-)butaclamol (unfilled and filled diamond, respectively), and l-sulpiride (asterik). Each curves was fit to one-site model. Each experiment was repeated 3 times with each point in triplicate. (see Table 1 for K_i values).

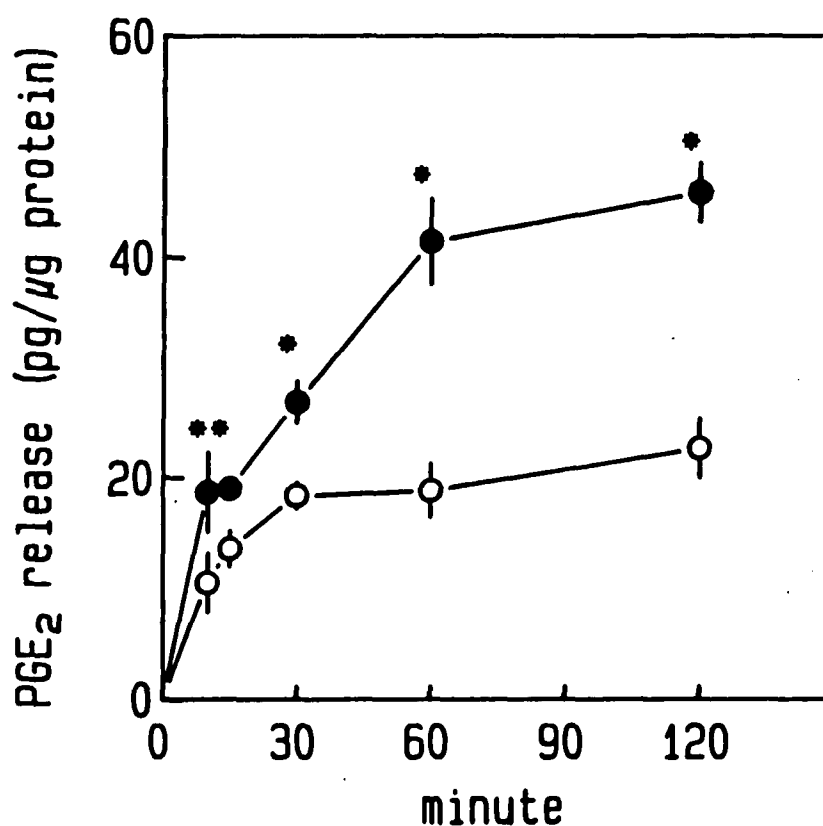


FIG. 3. Time-course study of dopamine stimulation of PGE₂ synthesis in cultured IMCD cells. Filled circle represented dopamine stimulation. Unfilled circle represented vehicle. Significant PGE₂ synthesis above basal level was detected at 10 min (18.7 ± 3.6 pg/μg protein vs. 10.6 ± 2.7 pg/μg protein) after incubation with dopamine (100 μM) and approached maximal stimulation after 60 min incubation (41.4 ± 3.9 pg/μg protein vs. 18.9 ± 2.4 pg/μg protein). All points of dopamine stimulation were higher than basal levels of each time interval (*, $P < 0.025$). Each point was the average of 3 experiments. Each experiment was in triplicates (3 wells).

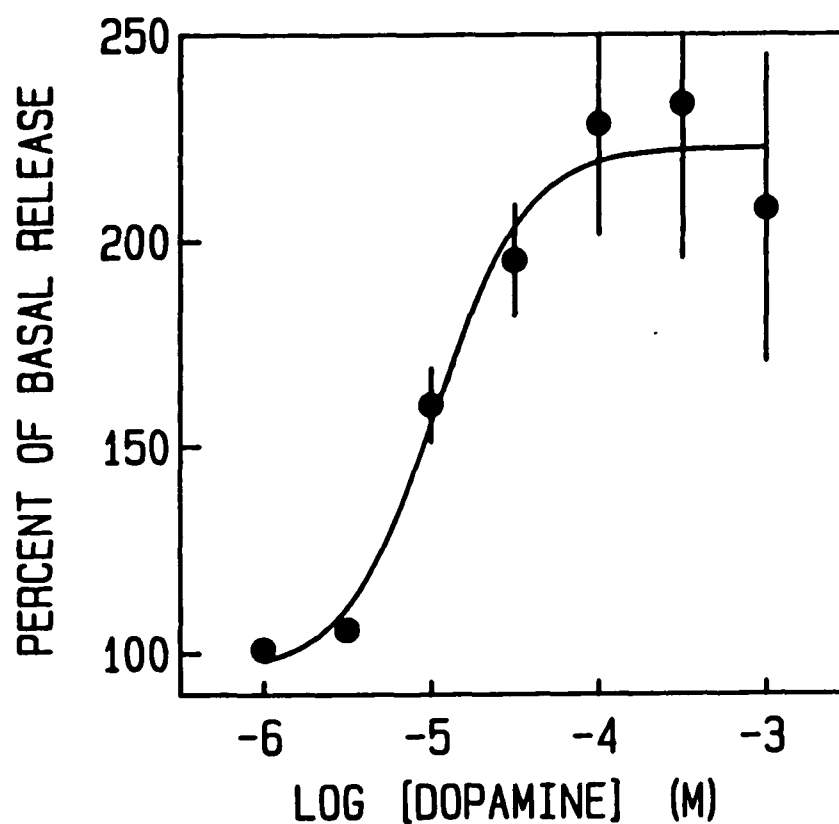


FIG. 4. Dose-response study of dopamine stimulation of PGE₂ synthesis in cultured IMCD cells. Data were expressed percent of basal PGE₂ synthesis (100 %). Dopamine increased PGE₂ synthesis as dose-dependent. The maximal stimulation by dopamine was 2 fold at the concentration of 10⁻⁴ M. The concentration of dopamine for half-maximum stimulation was 11.1 μ M. Each point was the average of 3 experiments. Each experiment was in triplicates (3 wells).

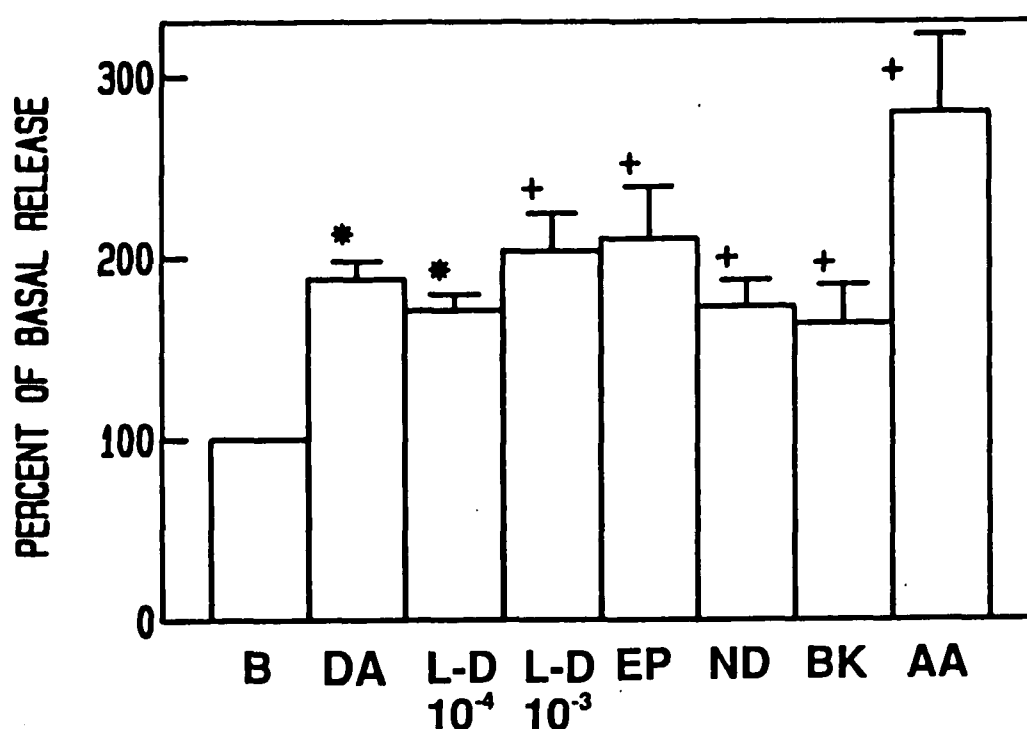


FIG. 5. Hormonal stimulation of PGE_2 synthesis in cultured IMCD cells. Data were expressed percent of basal PGE_2 synthesis (100 %). Hormonal stimulations were close to 2 fold of basal synthesis except arachidonic acid which was close to 3 fold (*, $P < 0.01$; +, $P < 0.05$). B, basal; DA, 10^{-4} M; L-D, 10^{-4} or 10^{-3} M L-dopa; EP, 10^{-5} M epinephrine; ND, 10^{-4} M N-methyl-dopamine (epinine); BK, 10^{-5} M bradykinin; AA, 10^{-5} M arachidonic acid. Each column was the average of 3 to 12 experiments. Each experiment was in triplicates (3 wells).

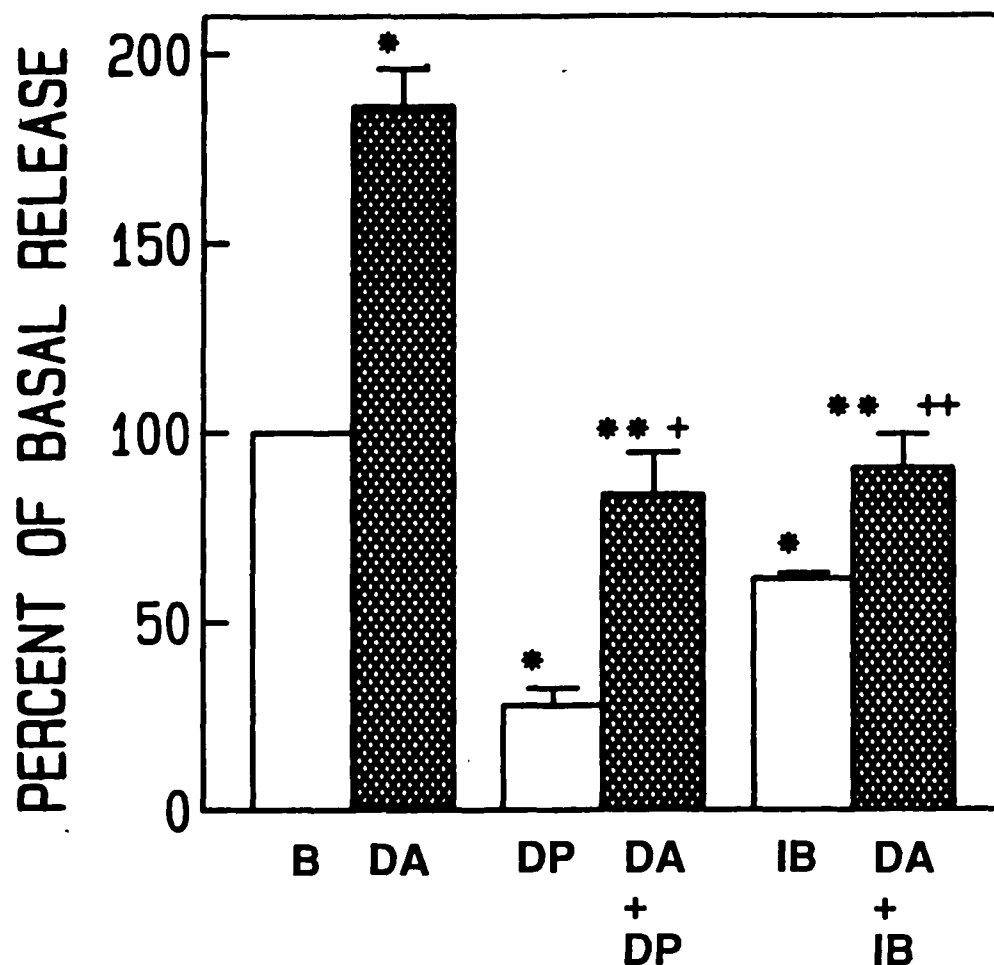


FIG. 6. Antagonists inhibited basal and dopamine-stimulated PGE_2 synthesis in cultured IMCD cells. Data were expressed percent of basal PGE_2 synthesis. Cells were preincubated with medium containing vehicle or antagonists for 30 min, aspirated away and replaced with medium containing dopamine with or without antagonists for an hour. Dopamine stimulation was 2 fold (*, $P < 0.025$, $n = 16$, compared with basal level). Domperidone or IBZM alone inhibited basal PGE_2 synthesis (*, $P < 0.025$, $n = 8$ and 5 , respectively, compared with basal level). Domperidone or IBZM inhibited dopamine-stimulated PGE_2 synthesis (**, $P < 0.01$, $n = 8$ and 5 , respectively, compared with dopamine stimulation), however, still higher than domperidone or IBZM alone (+, for domperidone, $P < 0.01$, $n = 8$; ++, for IBZM, $P < 0.025$, $n = 5$). B, basal; DA, 10^{-4} M dopamine; DP, 10^{-4} M domperidone; IB, 10^{-4} M IBZM. Each column was the average of 5 to 16 experiments. Each experiment was in triplicates (3 wells).

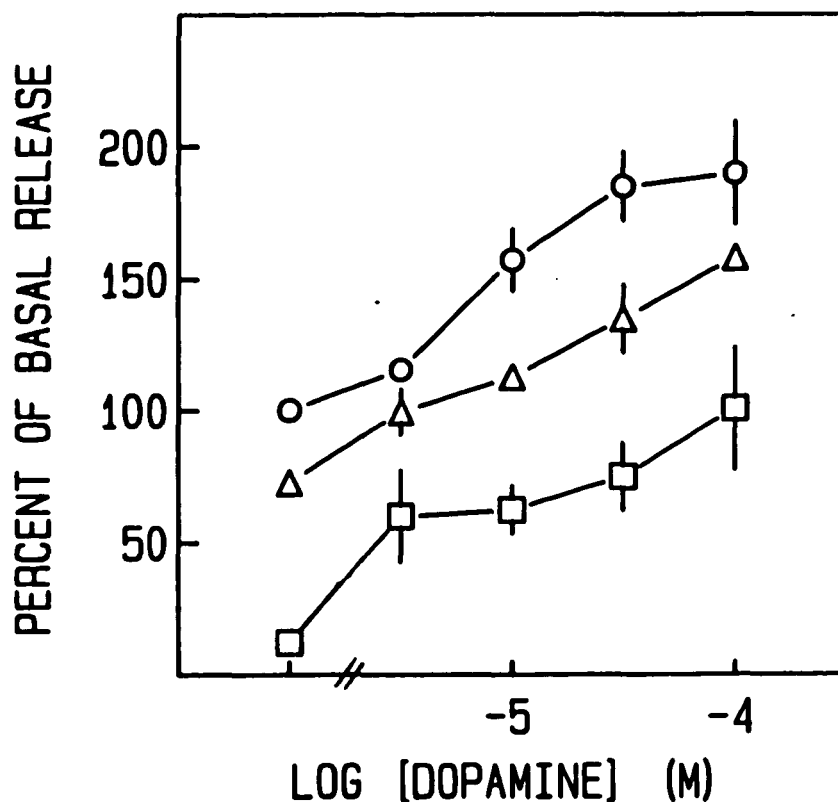


FIG. 7. Domperidone shifted the dose-response curve of dopamine-stimulated PGE₂ synthesis in cultured IMCD cells. Data were expressed percent of basal PGE₂ synthesis. Domperidone (triangle, 30 μ M; square, 100 μ M) lowered the dose-response curve of dopamine (circle, with domperidone). 30 μ M Domperidone inhibited PGE₂ synthesis to 73 ± 2 % of basal level. 100 μ M Domperidone alone inhibited PGE₂ synthesis to 12 ± 1 % ($n = 2$) of basal level (100 %). In the presence of 100 μ M domperidone, dopamine stimulation was lower than dopamine alone (101 ± 23 % vs. 190 ± 19 %, $n = 2$). Each point was the average of 2 experiments. Each experiment was in triplicates (3 wells).

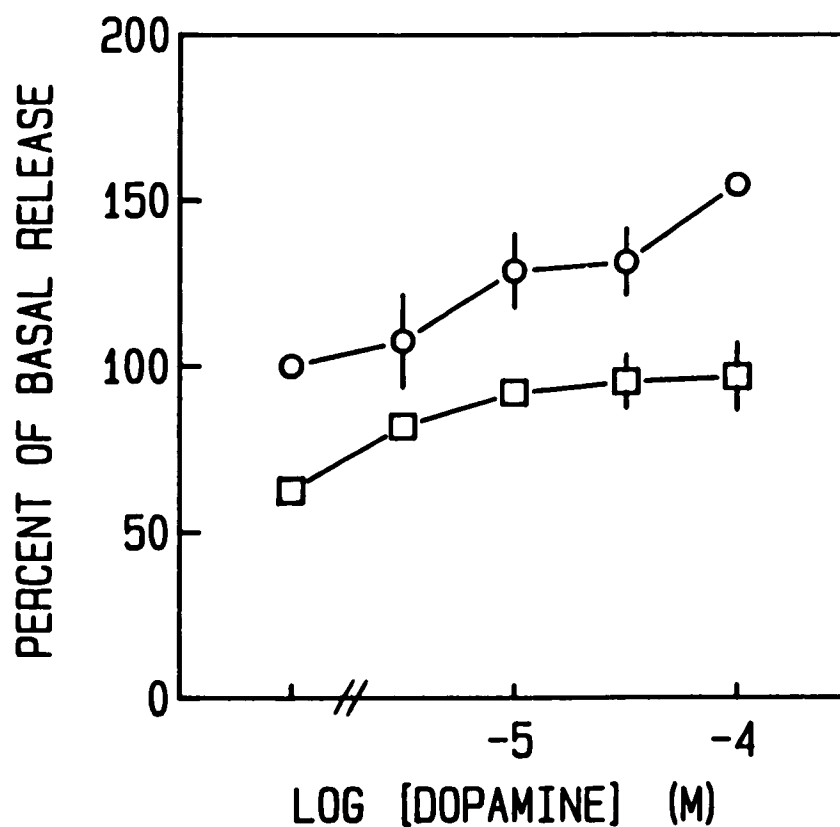


FIG. 8. IBZM shifted the dose-response curve of dopamine-stimulated PGE_2 synthesis in cultured IMCD cells. Data were expressed percent of basal PGE_2 synthesis. IBZM (square, $100 \mu\text{M}$) lowered the dose-response curve of dopamine (circle, without IBZM). $100 \mu\text{M}$ IBZM alone inhibited PGE_2 synthesis to $63 \pm 10 \%$ ($n = 2$) of basal level (100%). In the presence of $100 \mu\text{M}$ IBZM, dopamine stimulation was lower than dopamine alone ($97 \pm 10 \%$ vs $155 \pm 2 \%$, $n = 2$). Each point was the average of 2 experiments. Each experiment was in triplicates (3 wells).

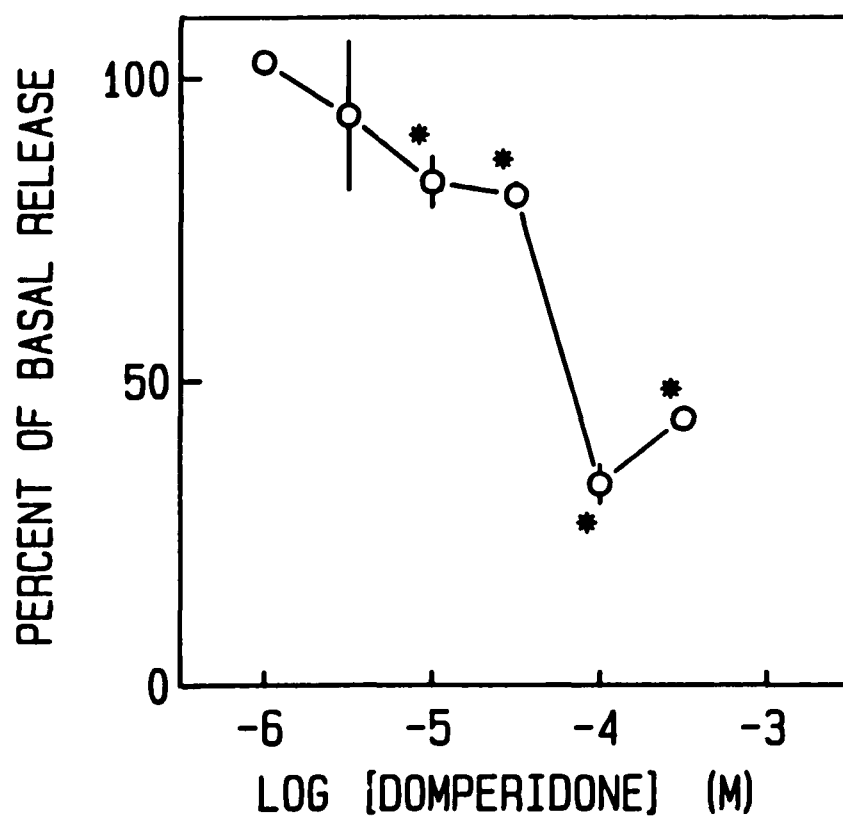


FIG. 9. Basal PGE₂ synthesis inhibited by domperidone in cultured IMCD cells. Data were expressed percent of basal PGE₂ synthesis. Domperidone inhibited basal PGE₂ synthesis as dose-dependent. 10⁻⁵ M Domperidone significantly reduced PGE₂ synthesis to 83 ± 4 % (n= 3) of basal level (100 %) and to 33 ± 3 % (n= 3) with 10⁻⁴ M domperidone (*, P < 0.05, n= 3). Each point was the average of 3 experiments. Each experiments was in triplicates (3 wells).

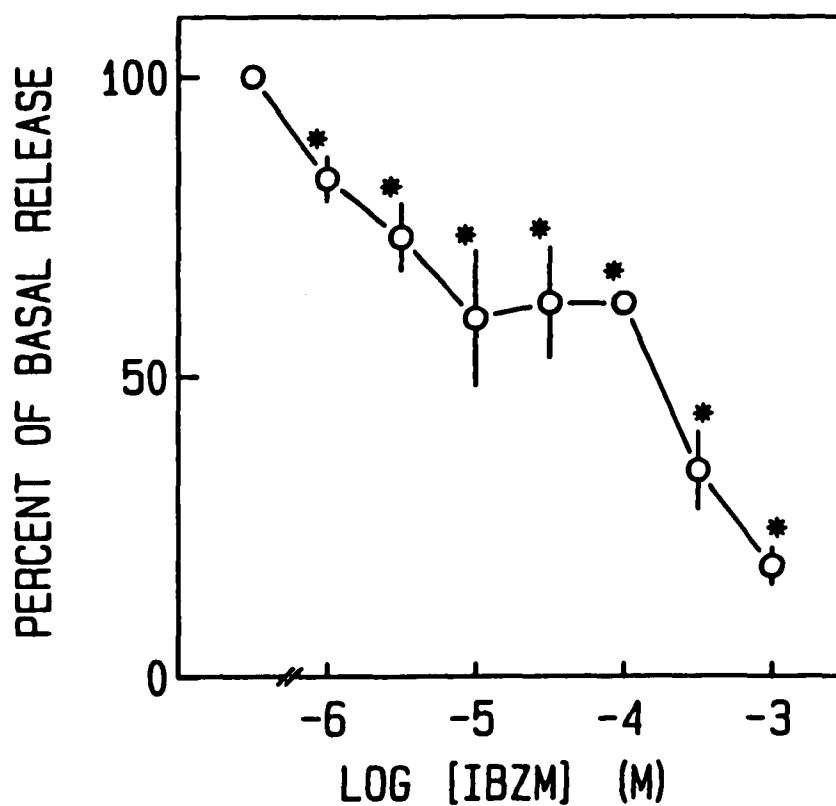


FIG. 10. Basal PGE₂ synthesis inhibited by IBZM in cultured IMCD cells. Data were expressed as percent of basal PGE₂ synthesis. IBZM inhibited basal PGE₂ synthesis as dose-dependent. 10⁻⁶ M IBZM significantly reduced PGE₂ synthesis to 83 ± 4 % of basal level (100 %) and to 62 ± 2 % (n=3) and 18 ± 3 % (n=3) with 10⁻⁴ M and 10⁻³ M IBZM (*, P < 0.025, n= 3). Each point was the average of 3 experiments. Each experiment was in triplicates (3 wells).

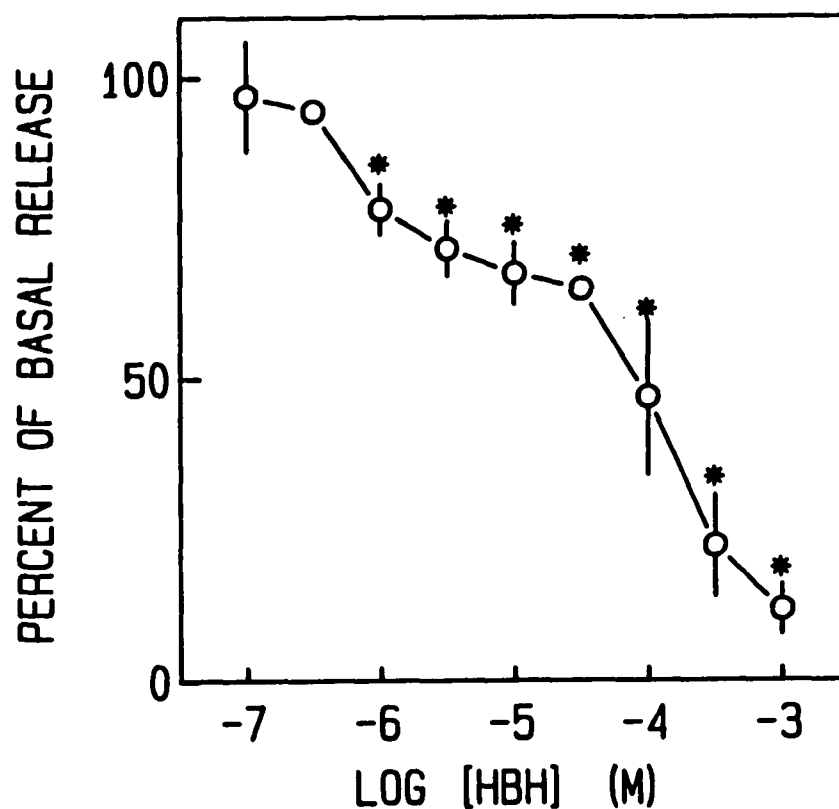


FIG. 11. Basal PGE₂ synthesis inhibited by aromatic L-amino acid decarboxylase inhibitor in cultured IMCD cells. Data were expressed percent of basal PGE₂ synthesis. 3-Hydroxybenzylhydrazine (HBH) inhibited basal synthesis as dose-dependent. 10⁻⁶ M HBH significantly reduced PGE₂ synthesis to 78 ± 4 % (n= 5) of basal level (100 %) and to 12 ± 4 % (n= 5) with 10⁻³ M HBH (*, P < 0.01, n= 5). Each point was the average of 5 experiments. Each experiments was in triplicates (3 wells).

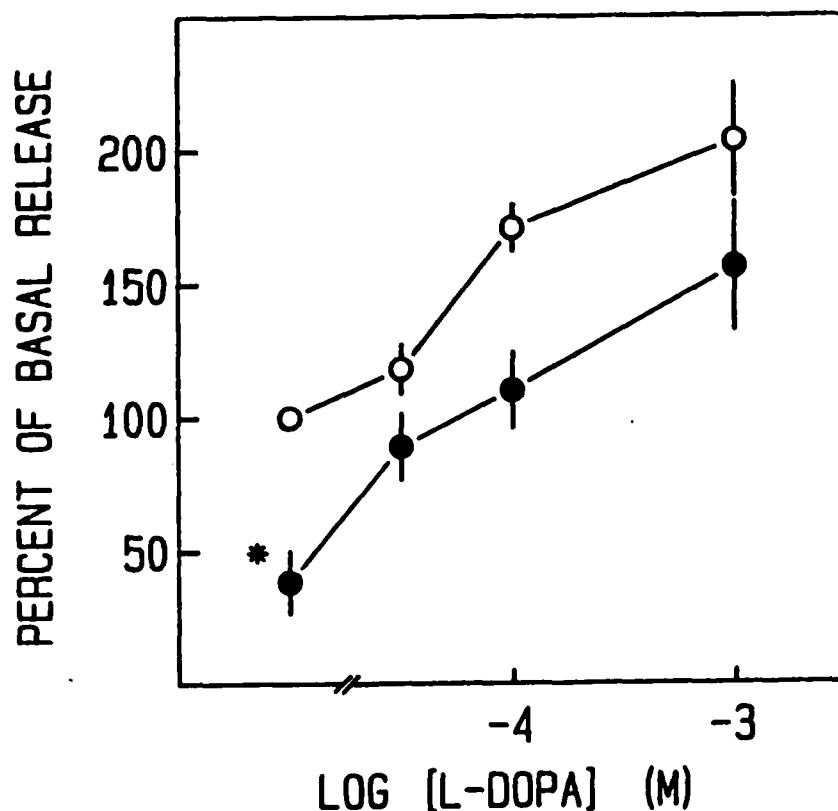


FIG. 12. Dose-response study of L-dopa stimulation of PGE_2 synthesis in cultured IMCD cells. Data were expressed percent of basal PGE_2 synthesis. L-Dopa alone (unfilled circle) stimulated PGE_2 synthesis as dose-dependent. Maximal stimulation was 2 fold with 1 mM L-dopa. 3-Hydroxybenzylhydrazine (HBH) (100 μM , filled circle) lowered dose-response curve of L-dopa. HBH alone inhibited PGE_2 synthesis to $38 \pm 12\%$ ($n = 3$) of basal level (100 %). In the presence of 100 μM HBH, L-dopa stimulation of PGE_2 synthesis was lower than L-dopa alone ($156 \pm 24\%$ vs. $204 \pm 21\%$, $P < 0.05$, $n = 3$). Each point was the average of 3 experiments. Each experiment was in triplicates (3 wells).

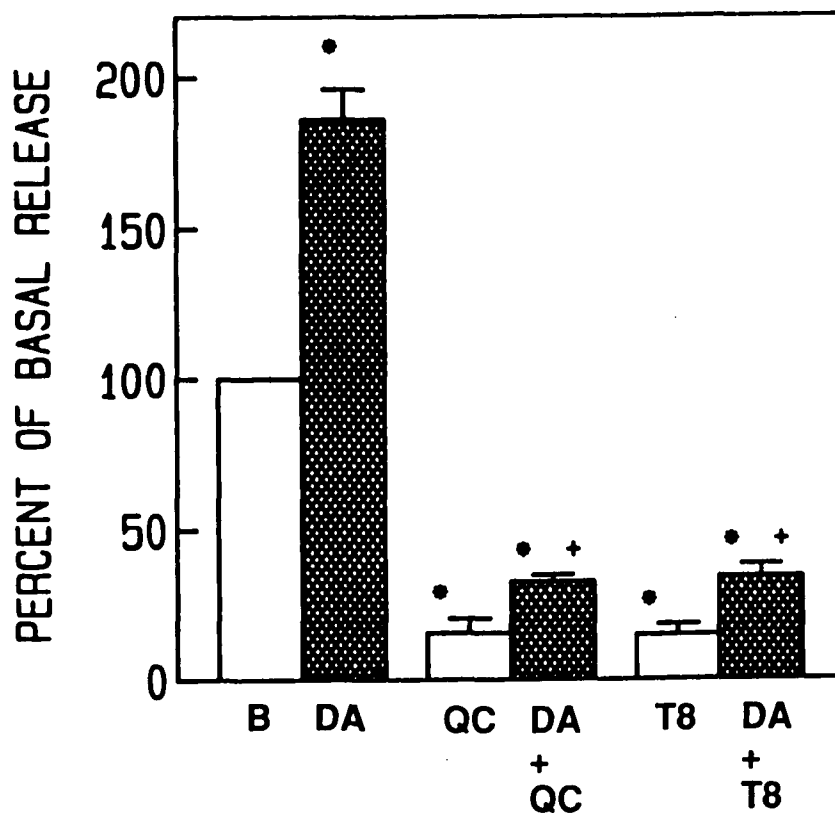


FIG. 13. PGE_2 synthesis was inhibited by quinacrine and TMB-8 in cultured IMCD cells. Data were expressed as percent of basal PGE_2 synthesis. Dopamine ($100 \mu\text{M}$) alone stimulated PGE_2 synthesis 2 fold. Quinacrine ($100 \mu\text{M}$) alone inhibited PGE_2 synthesis to $16 \pm 5 \%$ ($n=3$) of basal level (100%) and blocked dopamine stimulation ($33 \pm 2 \%$ vs $186 \pm 10 \%$, $n=3$). TMB-8 ($100 \mu\text{M}$) showed similar inhibition ($15 \pm 5 \%$ for TMB-8 alone vs. $34 \pm 4 \%$ for dopamine plus TMB-8, $n=3$). *, $P < 0.01$, compared with basal level (100%). +, $P < 0.01$, compared with dopamine stimulation ($186 \pm 10 \%$). Each column was the average of 3 experiments. Each experiment was in triplicates (3 wells).

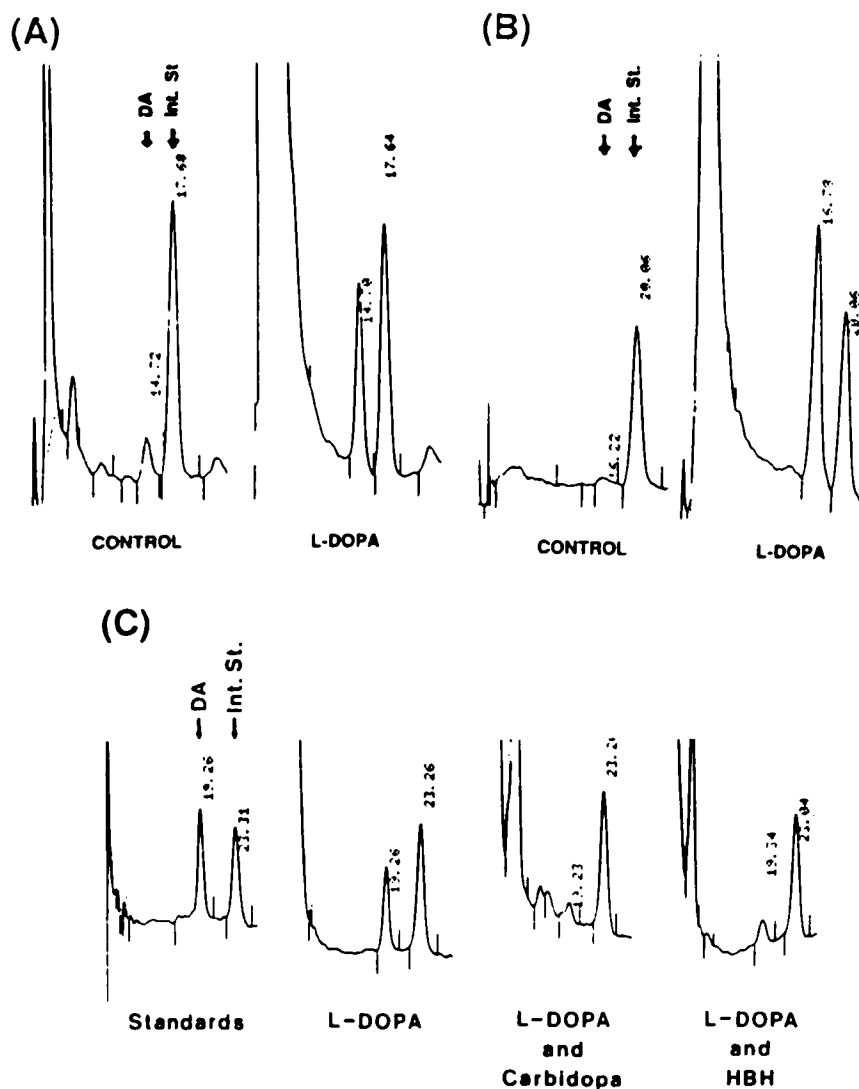


FIG. 14. HPLC-electrochemical detection of dopamine (DA) production by freshly dispersed IMCD cells. (A) Cytoplasm. (B) Partially disrupted cells. (C) Intact cells. Samples containing cytoplasm, partially disrupted cells, or intact cells were incubated without (control) or with 200 μ M L-dopa for 30 min at 37 $^{\circ}$ C. For intact cells (C) in presence of aromatic L-amino acid decarboxylase inhibitors, 100 μ M 3-hydroxybenzylhydrazine (HBH) or 100 μ M carbidopa were used. The media was then aspirated, alumina extracted and run on a HPLC with electrochemical detection. Epinine (0.4 pmole) was used as the internal standard (St.). Note that in the presence of HBH or carbidopa the DA peak was attenuated.

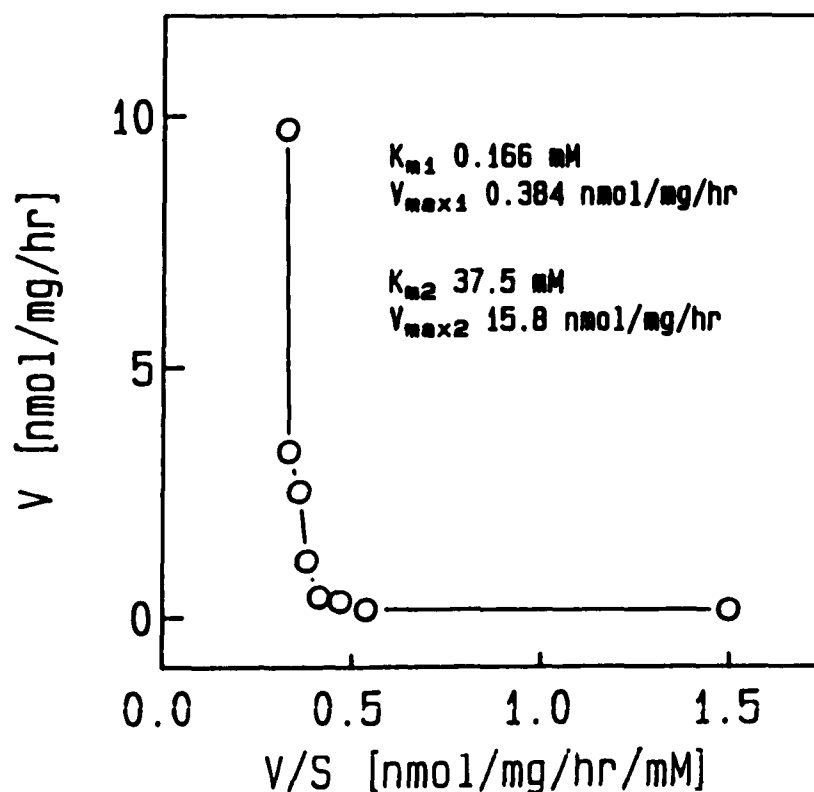


FIG. 15. Eadie-Hofstee plot of aromatic L-amino acid decarboxylase in cultured IMCD cells. Cells were used after four days of culture. Cytoplasm was prepared as described in Methods. Samples were incubated with 30 μ M - 30 mM L-dopa containing ^{14} C-L-dopa for one hour at 37 $^{\circ}$ C. The released ^{14} CO $_2$ was trapped by hyamine and counted. Shown is a representative experiment. Transformation of data yielded a biphasic enzyme kinetics with a higher affinity (K_{m1} = 0.166 mM) but lower capacity (V_{max1} = 0.384 nmol/mg/hr) component and a lower affinity (K_{m2} = 37.5 mM) but higher capacity (V_{max2} = 15.8 nmol/mg/hr) component. Experiments were replicated 3 times with similar results.

TABLE 1

Inhibition of specific ^3H -domperidone binding in rat cultured inner medullary collecting duct cells.

	Subtype	K_i (μM)	Hill Slope
Dopamine Antagonists			
Domperidone	DA_1	0.07 ± 0.02	0.86 ± 0.10
Spiperone	DA_2	1.75 ± 0.73	0.76 ± 0.11
Haloperidol	DA_1/DA_2	2.33 ± 0.57	0.68 ± 0.03
cis(Z)-flupentixol	DA_1/DA_2	2.30 ± 0.64	1.02 ± 0.20
Eticlopride	DA_1/DA_2	8.56 ± 2.45	0.99 ± 0.10
(+)Butaclamol	DA_1/DA_2	9.77 ± 4.2	0.99 ± 0.06
SCH23390	DA_1	21.6 ± 5.5	0.54 ± 0.11
(-)Butaclamol	DA_1/DA_2	176 ± 76	0.85 ± 0.05
l-Sulpiride	DA_2	> 1000	
Dopamine Agonists			
Norapomorphine	DA_2	9.66 ± 4.89	0.64 ± 0.04
Apomorphine	DA_1/DA_2	88.3 ± 7	0.59 ± 0.05
Bromocriptine	DA_2	100 ± 10	1.12 ± 0.15
Fenoldopam	DA_1	81.8 ± 9.4	1.17 ± 0.09
6,7-ADTN	DA_1/DA_2	>1000	
LY171555	DA_2	>1000	
Dopamine	DA_1/DA_2	3000	0.91 ± 0.05
Other Antagonists			
Ketanserin	5-HT_2	5.01 ± 2.71	0.54 ± 0.07
Propranolol	β_1/β_2	14.0 ± 3.37	0.66 ± 0.06
Phentolamine	α_1/α_2	17.22 ± 6.21	0.88 ± 0.07
5-CT	$5\text{-HT}_{1A,1B,1D}$	>1000	
Mesulergine	5-HT_{1C2}	>300	

Values are the means $\pm$ SEM of three experiments, each point determined in triplicate. Rat cultured inner medulla collecting duct cells were incubated with ^3H -Domperidone (final concentration 15 nM) in the presence of various concentrations of unlabeled drug and the concentration of unlabeled drug that caused 50% inhibition of specific binding (IC_{50}) was determined. 6,7-ADTN, ($\pm$)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; 5-CT, 5-carboxyamino-tryptamine.

CHAPTER 5

**INHIBITION OF VASOPRESSIN-STIMULATED CYCLIC AMP ACCUMULATION
BY DOPAMINE AND 6,7-ADTN VIA ACTIVATION OF
ALPHA-2 ADRENOCEPTORS IN DISPERSED INNER MEDULLARY
COLLECTING DUCT CELLS.**

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INTRODUCTION

Dopamine is known to be a potent renal vasodilator and natriuretic stimulant (Wasserman et al., 1980; Bello-Reuss et al., 1982; Goldberg et al., 1986; Carey and Hughes, 1987). Dopamine-mediated diuresis and natriuresis have been attributed to the activation of DA_1 dopamine receptors in renal vasculature or proximal tubules (Goldberg et al., 1986; Felder et al., 1989c). However, the renal effects of dopamine may not be only explained by activation of dopamine receptors and target cells of dopamine were not restricted to proximal tubules. Dopamine was shown to antagonize vasopressin action in the kidney (Lehr et al., 1967; Koyoma et al., 1985; Muto et al., 1985) or toad bladder (Arruda and Sabatini, 1982); and the diuretic effect of dopamine can be antagonized either by classical α -adrenergic blockers (Lehr et al., 1967; Baggio and Ferrari, 1981) or by dopamine receptor antagonists (Koyoma et al., 1985; Muto et al., 1985). More recently, it has been shown that the natriuresis effect of fenoldopam, a specific DA_1 receptor agonist, could be completely blocked by the specific DA_1 dopamine receptor antagonist SCH23390, while dopamine-induced natriuresis was only partially blocked by the same ligand (Hegde and Lokhandwala, 1989). These results indicate that dopamine may act on different nephron segments via activation of renal adrenergic or dopaminergic receptors.

α_2 -Adrenoceptors exist postsynaptically in the kidney and functional studies suggest that activation of renal α_2 -adrenoceptors results in modulation of vasopressin action (Krathopoli and Suki, 1984; Smyth et al., 1985; Gellai and Ruffolo, 1987; Stanton et al., 1987). More specifically, in isolated cortical, outer medullary, and

papillary segments of collecting ducts, α_2 -adrenoceptor agonists have been shown to inhibit vasopressin-stimulated cAMP levels which could be blocked by specific α_2 -adrenoceptor antagonists (Charbardes et al., 1984; Umemura et al., 1985; Edwards and Gellai, 1988).

The present study was designed to examine the effects of dopamine and dopamine receptor agonist 6,7-ADTN on vasopressin-stimulated cAMP accumulation in the inner medullary collecting ducts (IMCD) cells. The results confirmed that dopamine and 6,7-ADTN were able to inhibit vasopressin-stimulated cAMP accumulation as norepinephrine and these effects were blocked by α_2 -adrenergic but not dopaminergic antagonists.

METHODS

Isolation of inner medullary collecting duct (IMCD) cells.

IMCD cells were isolated from renal inner medulla of 200-250 g Sprague-Dawley rats according to the methods of Grenier et al (1981), which has been adapted by Sato and Dunn (1984), and by Wuthrich and Vallotton (1986a). The inner medulla tissue was dissected away from the outer medulla, finely minced with tissue chopper, and placed in HBSS-HEPES buffer consisting of (mM): NaCl 137, KCl 5.4, Na_2HPO_4 0.34, KH_2PO_4 0.44, CaCl_2 1.0, MgCl_2 0.5, MgSO_4 0.5, glucose 5.5, HEPES 20, and 0.1% BSA. 1 mg/ml collagenase (Type I, Sigma) and 10 $\mu\text{g/ml}$ Trypsin Inhibitor were added for collagenase treatment. The minced tissue was incubated for 90 min at 37 °C

in 5%CO₂-95% air with gentle pipetting up and down every 30 min. After collagenase dispersion of cells, distilled water were added to give a final osmolality of 120 mosmol/kg. The cellular mixture was immediately centrifuged at 400 x g for 5 min, and the pellet containing intact cells was added to 10 ml of phosphate buffer saline solution containing 7.5 % BSA. This mixture was centrifuged for 5 min. The intact IMCD cells were resuspended in HBSS-HEPES buffer and recentrifuged. The final cell pellet was resuspended in HBSS-HEPES buffer to make cell density around $0.5-1 \times 10^6$ /ml. The cell yield after hypotonic treatment was $0.5-1 \times 10^6$ per 2 kidney inner medulla. More than 95% of cells excluded trypan blue.

Incubation of IMCD cells.

Isolated IMCD cells (50 μ l) were preincubated for 5 min at 37 °C. After the preincubation period, another 50 μ l HBSS-HEPES buffer containing appropriate drugs, IBMX (final concentration 1 mM), propranolol (final concentration 20 μ M), Pargyline (final concentration 100 μ M) and ascorbic acid (final concentration 0.01%). For time-course experiment, various time points were selected. 5 min was chosen for other experiments. The incubation was stopped by the addition of 20 μ l of 50% trichloroacetic acid, and the mixture was extracted 3 times with 1 ml of water-saturated ether. The remaining aqueous phase was dried at 50 °C for 20 min, and the samples were stored at -80 °C. For experiments with antagonists, 25 μ l buffer containing appropriate concentrations of antagonists was added to 25 μ l buffer containing cells and the mixture was preincubated for 5 min at 37 °C. The samples were then incubated and processed as described above.

Measurement of cAMP accumulation.

Cyclic AMP was measured after appropriate dilution and acetylated by using radioimmunoassay kits (New England Nuclear, Boston, MA). The range of the standard curve was 2.5 fmol/tube to 160 fmol/tube. Results are reported as means $\pm$ S.E.M. Statistical analysis was performed using Student's t test or Dunnett's test for multiple comparisons. A value of $P < .05$ was considered statistically significant.

Materials.

Cyclic AMP radioimmunoassay kits were purchased from New England Nuclear (Boston, MA). The following drugs were used: Collagenase type I, dopamine, (-)-norepinephrine, 8-arg-vasopressin, yohimbine, Phentolamine, Haloperidol and prazosin (Sigma, St. Louis, MO); (+)-butaclamol, Spiperone, ketanserin and 6,7-ADTN (Research Biochemicals Inc., Natick, MA). Rauwolscine (Carl Roth, Karlsruhe, FRG).

RESULTS

Time-course of vasopressin-stimulated cAMP accumulation.

The concentration of 100 nM of Arg-vasopressin was chosen for its maximal stimulation. The total cAMP content increased as a function of time up to 8 min (Fig. 1). Usually, Arg-vasopressin stimulated 10 to 15 fold of basal cAMP accumulation after

5 min.

Dose-response of vasopressin-stimulated cAMP accumulation.

Fig. 2 showed the dose-response of cAMP accumulation to Arg-vasopressin. There was a significant increase in cAMP accumulation at 1 pM Arg-vasopressin. The maximum stimulation was observed at 1 nM. The half-maximal stimulation (EC_{50}) occurred at 30 pM.

Dose-response of inhibition of dopamine and 6,7-ADTN to vasopressin-stimulated cAMP production.

Submaximal stimulation concentration of vasopressin of 0.1 nM was chosen. The results, shown in Fig. 3, are expressed as the percentage of inhibition of VP-stimulated cAMP production. Over the concentration range of 3×10^{-6} M to 10^{-4} M, dopamine produced a significant ($P < 0.05$) inhibition of VP-stimulated cAMP production. Half-maximal inhibition occurred at 5×10^{-6} M of dopamine. Unexpectedly, the dopamine receptor agonist 6,7-ADTN had similar and more potent effect than dopamine. The half-maximal inhibition occurred at 10^{-6} M of 6,7-ADTN.

Antagonists effect on dopamine, 6,7-ADTN and norepinephrine inhibition to vasopressin-stimulated cAMP production.

To further characterize the receptors involved in dopamine- or 6,7-mediated inhibition of vasopressin-stimulated cAMP production in dispersed IMCD cells, several receptor-selective antagonists were tested for reversal of inhibition. At the concentration of 10^{-6} M, as shown in Fig. 4, α_2 -adrenoceptor antagonists rauwolscine, yohimbine and nonselective α -adrenoceptor antagonist phentolamine attenuated the inhibition produced by dopamine (10^{-6} M). α_1 -Adrenoceptor antagonist prazosin, dopamine receptor antagonists (+)-butaclamol, haloperidol, spiperone, and 5-HT₂ serotonergic antagonist ketanserin had no effect. All antagonists tested had no effect on basal cAMP production. For comparison, the effects of antagonists of different classes on the inhibition produced by 10^{-5} M norepinephrine were also examined. As shown in Fig. 5, 10^{-5} M yohimbine and phentolamine could attenuate the norepinephrine and dopamine inhibition of vasopressin-stimulated cAMP production. However, 10^{-4} M haloperidol and spiperone were without effect. The inhibitory effects of 10^{-5} M 6,7-ADTN, as shown in Fig. 6, was also blocked by 10^{-5} M yohimbine and rauwolscine, and were not blocked by 10^{-4} M haloperidol and spiperone.

DISCUSSION

The present study demonstrates that dopamine and dopamine receptor agonist 6,7-ADTN were able to inhibit vasopressin-stimulated cAMP production in freshly dispersed IMCD cells. Time-course and dose-response studies of vasopressin stimulation of cAMP were comparable with previous reports. Star et al. (1988) showed that vasopressin was able to increase intracellular of cAMP and calcium concentration in the isolated rat IMCD cells. Edwards and Gellai (1988) demonstrated that, in

microdissected rat papillary collecting ducts, α_2 -adrenoceptor agonists such as norepinephrine and B-HT 933 inhibited vasopressin-stimulated cAMP accumulation which was attenuated by α_2 -adrenergic blocker rauwolscine.

Dopamine inhibited vasopressin-stimulated cAMP as dose-dependent. The concentration of dopamine for half-maximal inhibition (IC_{50}) was 5 μ M *in vitro* which may be physiological significant since the tissue concentration of dopamine in the kidney is around μ M range (Bell and Gillespie, 1981; Fernandez-Pardal and Saavedra, 1982; Baines et al., 1985; Petrovic et al., 1986; Soares-da-Silva, 1988). It is reasonable to speculate, therefore, that dopamine may inhibit vasopressin-stimulated cAMP *in vivo*. 6,7-ADTN, a potent dopaminergic agonist, had similar effects but more potent than dopamine. The IC_{50} for 6,7-ADTN was 1 μ M. The effects of dopamine and 6,7-ADTN could be attenuated by α_2 -adrenoceptor antagonists yohimbine and rauwolscine as well as non-selective α -adrenoceptor antagonist phentolamine; and these effects were not blocked by other receptor antagonists such as α_1 -adrenoceptor antagonist prazosin, dopamine receptor antagonists haloperidol, spiperone, and (+)-butaclamol, as well as 5-HT₂ serotonergic receptor antagonist ketanserin. All experiments were done in presence of 20 μ M of propranolol to eliminate the possible activation of β -adrenoceptor. These results indicated that dopamine might interfere with the biochemical and physiological responses of vasopressin via activation of α_2 -adrenoceptor.

The dopamine receptor agonist ($D_2 > D_1$), 6,7-ADTN, a derivative of 2-aminotetrahydronaphthalene, antagonized the responses of vasopressin more potent than dopamine. DPI, a derivative of imidazoline and a supposed D_2 dopamine receptor

agonist (Boudier et al., 1975), had similar effect on activation of α_2 -adrenoceptors (Baggio and Ferrari, 1981). Why these dopamine receptor agonists activated α_2 -adrenoceptors is not clear. The possible explanation is that there might be some structural homology between α_2 -adrenoceptor and D_2/DA_2 dopamine receptor which both were with putative seven transmembrane domain and both were negatively coupled to adenylate cyclase activity. A D_2 dopamine receptor from rat brain and α_2 -Adrenoceptors from human platelet as well as from kidney were cloned (Bunzow et al., 1988; Regan et al., 1988; Kobilka et al., 1987). There was evidence that α_2 -adrenoceptor is not a homogeneous class of receptor, but can be divided into at least three subtypes, termed α_{2A} , α_{2B} , α_{2C} , and possibly, a fourth subtype, based on their pharmacological characteristics (Bylund et al., 1988). The subtype(s) involved in inhibition of vasopressin-stimulated cAMP accumulation await(s) further investigation.

Baggio and Ferrari (1981) had demonstrated that dopamine and (3,4-dihydroxyphenylamino)-2-imidazoline (DPI) were able to produce diuresis and to lower urine osmolality in rats, and these effects were antagonized by α_2 -adrenoceptor antagonists yohimbine and phentolamine. Dopamine also inhibited the hydro-osmotic effect of vasopressin in cortical collecting tubule in rat (Muto et al., 1985) and toad bladder (Arruda and Sabatini, 1982). Metoclopramide, a non-selective dopaminergic antagonist, could block the effects of dopamine in those experiments. It has been shown that dopamine could be formed and act locally in proximal tubules where L-dopa was taken up and converted to dopamine by aromatic L-amino acid decarboxylase (AADC) (Chan, 1976; Bell, 1987; Seri et al., 1988; Bertorello et al., 1988b). We also have evidence that dopamine is locally synthesized and controls PGE_2 synthesis in IMCD

cells (Huo and Healy, Chapter 5). It was noted that, in terms of tissue quantities, in the rat, cortical dopamine represented between 29 % and 52 % of total catecholamine content; in contrast, medullary dopamine levels were lower, only 12 % to 30 % of the total catecholamine content (Fernandez-Pardal and Saavedra, 1982). In the rat or dog inner medulla, the catecholamines including dopamine were almost entirely nonneuronal (Petrovic and Bell, 1986; Petrovic et al., 1986). It is not clear that what is (are) the physiological stimulant(s) to α_2 -adrenoceptor in IMCD cells and whether the dopamine formed in upstream nephron segments (proximal tubules) might act on downstream renal segments.

The present study provided the evidence that dopamine was able to antagonize vasopressin-induced cAMP accumulation via activation of α_2 -adrenoceptors in dispersed IMCD cells. In addition, the diuretic and natriuretic effects of dopamine in kidney might not be explained only by activation of renal dopamine receptors.

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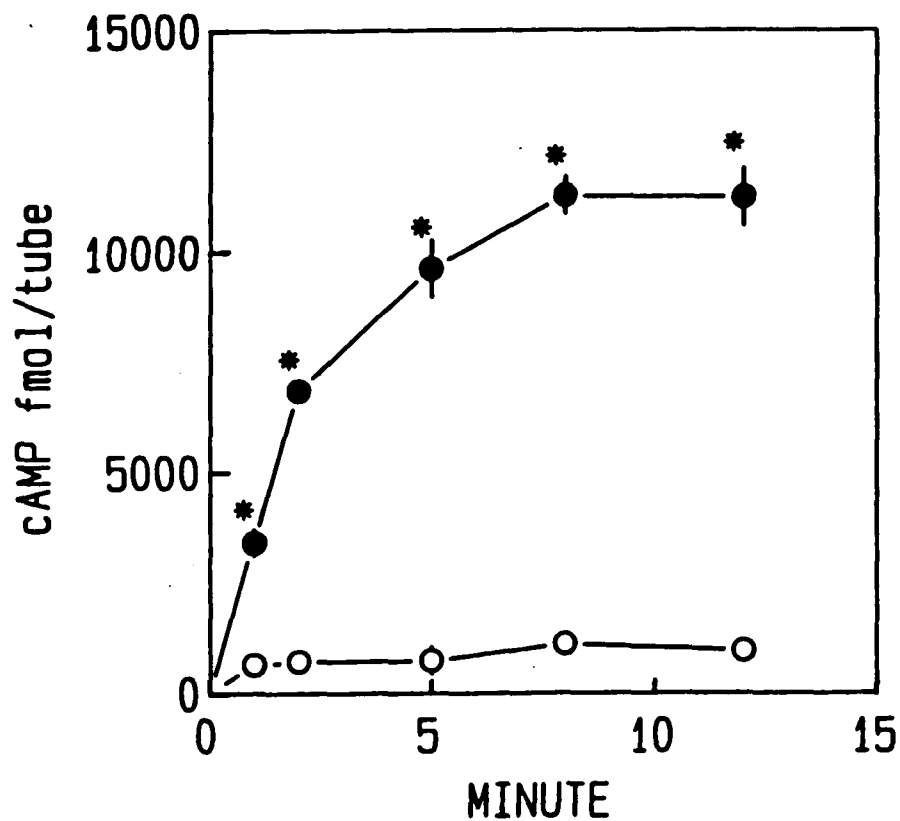


FIG. 1. Time-course study of Arg-vasopressin stimulation of cAMP accumulation. Arg-vasopressin (10^{-7} M, filled circle) significantly increased cAMP accumulation after 1 min of incubation and reach maximum between 5 to 8 min. The increase was 10 to 20 fold at 8 min compared with vehicle (11270 ± 420 fmol/tube vs. basal level of 1130 ± 130 fmol/tube, $P < 0.01$). The cell number of each tube was approximately 4×10^4 . Each point was the average of two experiments. Each time interval was in triplicates in each experiment. *, $P < 0.01$, compared with vehicles (open circle).

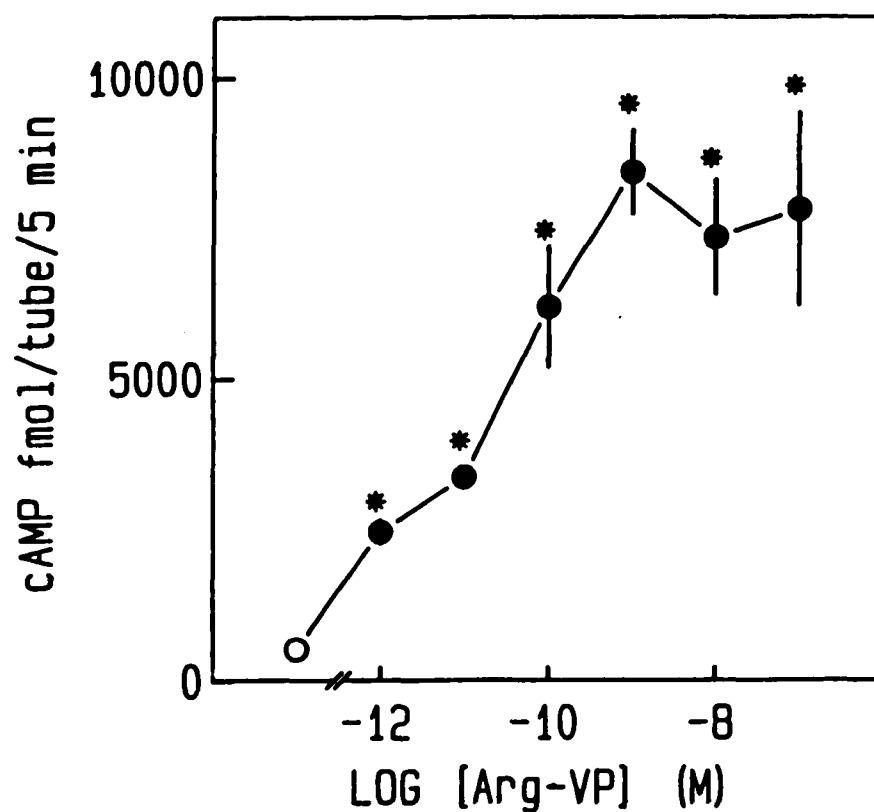


FIG. 2. Dose-response experiments of Arg-vasopressin stimulation of cAMP accumulation. Arg-vasopressin (filled circle) significantly stimulated cAMP accumulation at the concentration of 10^{-12} M. The maximal stimulation occurred at the concentration of 10^{-9} M of Arg-vasopressin (8430 ± 700 fmol/tube/5 min vs basal level of 520 ± 10 fmol/tube/5 min, $P < 0.01$). The half-maximum stimulation occurred at the concentration of 30 pM. Each tube contained approximately 2.5×10^4 cells. Each point was the average of two experiments. Each concentration was in triplicates in each experiment. *, $P < 0.01$, compared with vehicles (open circle).

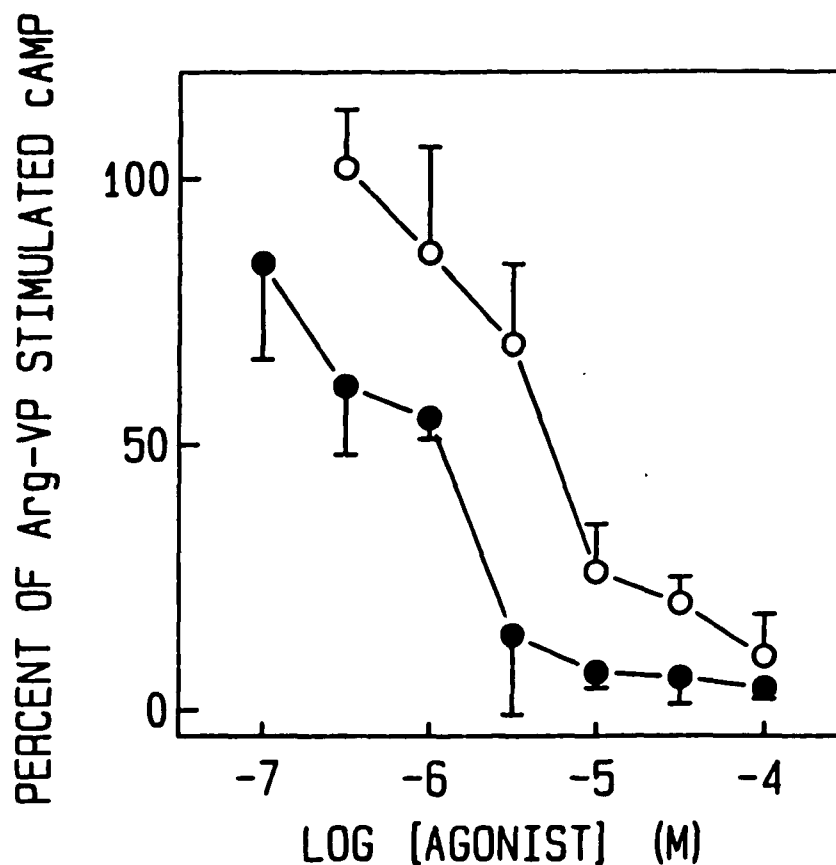


FIG. 3. Dose-response of inhibition of dopamine and 6,7-ADTN to vasopressin-stimulated cAMP accumulation. Arg-vasopressin (10^{-10} M) was used to stimulate cAMP accumulation (3500 ± 300 fmol/tube/5 min vs. basal level of 190 ± 50 fmol/tube/5 min, $P < 0.01$). Data were expressed percent of vasopressin-stimulated cAMP (the difference of stimulated and basal level). Dopamine (unfilled circle) significantly inhibited vasopressin-stimulated cAMP accumulation at the concentration of 3 to 100 μ M ($P < 0.05$) with half-maximum inhibition occurred at 5 μ M. 6,7-ADTN (filled circle) showed similar but more potent inhibition (0.3 to 100 μ M, $P < 0.05$) with half-maximum inhibition occurred at 1 μ M. Each tube contained approximately 1.5×10^4 cells. Each point was the average of two experiments. Each concentration was in triplicates in each experiment.

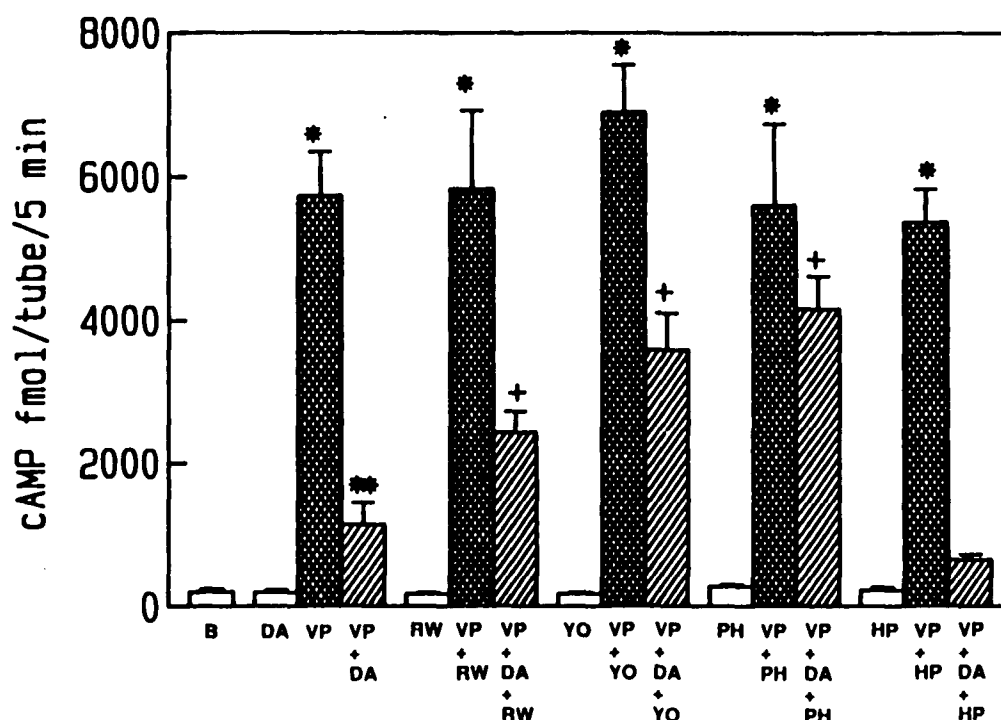


FIG. 4. Dopamine and antagonists effects on vasopressin stimulation of cAMP accumulation. Arg-vasopressin (10^{-10} M) was used to stimulated cAMP accumulation (5740 ± 610 fmol/tube/5 min vs. basal level of 200 ± 40 fmol/tube/5 min, $P < 0.01$). Dopamine ($100 \mu\text{M}$) inhibited approximately 83 % of vasopressin-stimulated cAMP accumulation (1150 ± 310 fmol/tube/5 min). Rauwolscline, yohimbine, and phentolamine (10^{-6} M for all three compounds) but not haloperidol (10^{-4} M) attenuated dopamine inhibition of vasopressin-stimulated cAMP. Each tube contained approximately 1.7×10^4 cells. *, $P < 0.01$, compared with basal level; **, $P < 0.01$, compared with VP alone; +, $P < 0.05$, compared with VP+DA. Each column was the average of two experiments. Each condition was in triplicates in each experiment. B, basal; DA, dopamine; VP, Arg-vasopressin; RW, rauwolscline; YO, yohimbine; PH, phentolamine; HP, haloperidol.

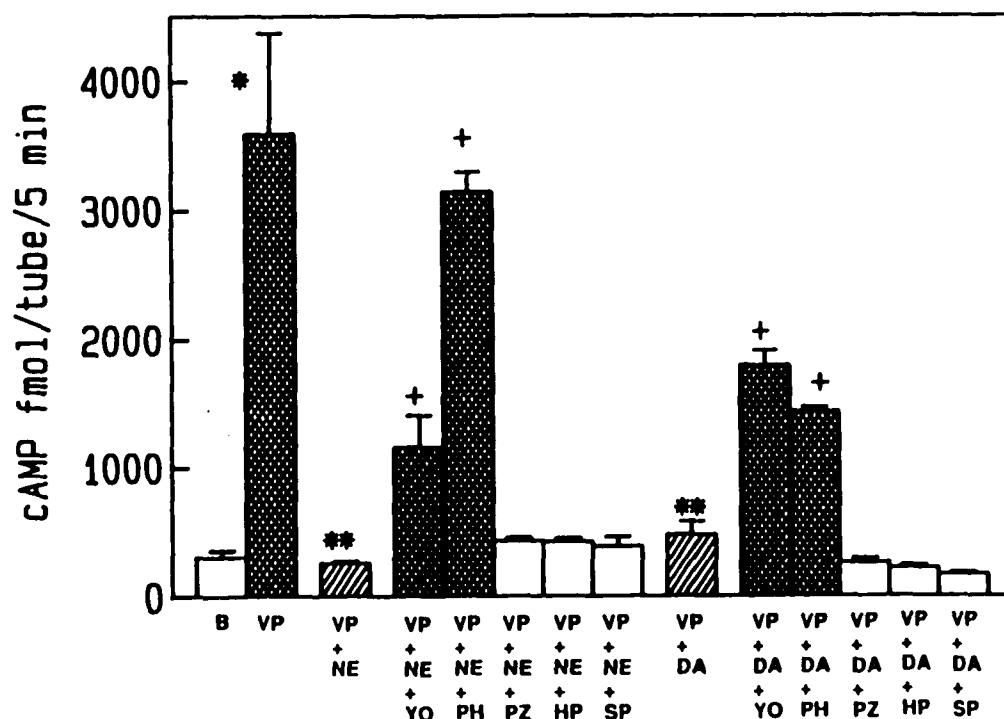


FIG. 5. Antagonists effects on dopamine or norepinephrine inhibition of vasopressin-stimulated cAMP accumulation. Arg-vasopressin (10^{-10} M) was used to stimulate cAMP accumulation (3590 ± 780 fmol/tube/5 min vs. basal level of 310 ± 50 fmol/tube/5 min). Norepinephrine (10^{-5} M) almost completely inhibited vasopressin-stimulated cAMP (260 ± 20 fmol/tube/5 min). Dopamine (10^{-4} M) inhibited approximately 95 % of vasopressin-stimulated cAMP (480 ± 100 fmol/tube/5 min). Yohimbine (10^{-3} M) and phentolamine but not prazosin, haloperidol, or spiperone attenuated norepinephrine or dopamine inhibition of vasopressin-stimulated cAMP. Each tube contained approximately 1×10^4 cells. *, $P < 0.01$, compared with basal level; **, $P < 0.01$, compared with VP alone; +, $P < 0.05$, compared with VP+NE or VP+DA, respectively. Each column was the average of two experiments. Each condition was in triplicates in each experiment. B, basal; VP, Arg-vasopressin; NE, norepinephrine; DA, dopamine; YO, yohimbine; PH, phentolamine; PZ, prazosin; HP, haloperidol; SP, spiperone.

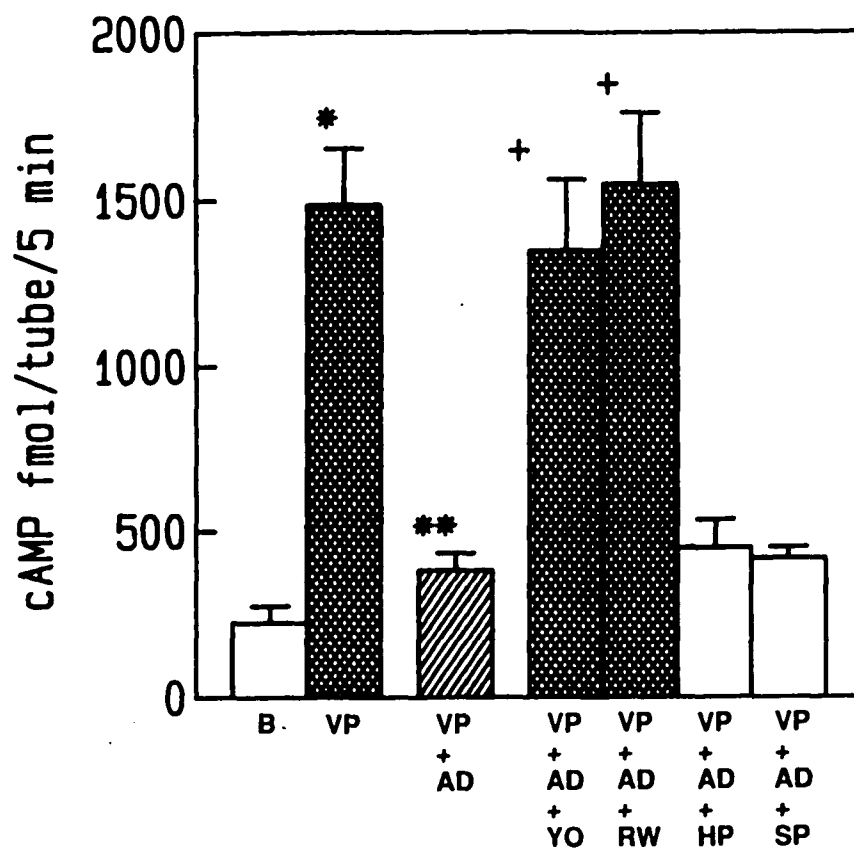


FIG. 6. Antagonists effects on 6,7-ADTN inhibition of vasopressin inhibition of vasopressin-stimulated cAMP accumulation. Arg-vasopressin (10^{-10} M) was used to stimulate cAMP accumulation (1490 ± 170 fmol/tube/5 min vs. basal level of 230 ± 50 fmol/tube/5 min). 6,7-ADTN (10^{-5} M) inhibited approximately 88 % of vasopressin-stimulated cAMP (380 ± 50 fmol/tube/5 min). Yohimbine (10^{-5} M) and rauwolscine (10^{-5} M) but not haloperidol or spiperone attenuated 6,7-ADTN inhibition of vasopressin-stimulated cAMP. Each tube contained approximately 0.8×10^4 cells. *, $P < 0.01$, compared with basal level; **, $P < 0.01$, compared with VP alone; +, $P < 0.01$, compared with VP+AD. Each column was the average of two experiments. Each condition was in triplicates in each experiment. B, basal; VP, Arg-vasopressin; YO, yohimbine; RW, rauwolscine; HP, haloperidol; SP, spiperone.

CHAPTER 6

DISCUSSION

DISCUSSION

In the 1960s and early 1970s, investigation of the unusual cardiovascular effects of dopamine by Goldberg and his colleagues led to the hypothesis of a specific dopamine receptor which mediated vasodilatation in the renal and mesenteric vascular beds of the dog (Goldberg, 1972). At the beginning, the localizations of dopamine receptors were restricted to the vasculature and sympathetic nerve terminals, namely, postsynaptic (DA_1) dopamine receptors which directly mediate the vasorelaxation effect on smooth muscle and presynaptic (DA_2) dopamine receptors which also mediated vasorelaxation but indirectly via inhibition of sympathetic nerve transmission (Goldberg and Kohli, 1979). In the late 1970 and early 1980s, specific dopamine receptors located in the renal tubules were found (Felder et al., 1984a and 1984b) but the specificities of the dopamine receptors were unresolved until specific D_1/DA_1 and D_2/DA_2 receptor ligands were synthesized and used to characterize the dopamine receptors (Felder, 1988b; Huo and Healy, 1989).

The studies I completed were designed to answer the following questions or address the following issues:

1. What are the pharmacological properties of DA receptors in the kidney? Do DA_1 and DA_2 receptor subtypes exist? Where are DA_1 and DA_2 receptors localized within the kidney?

2. Based on the high density of ^3H -spiperone binding sites in the renal inner medulla, I used freshly dispersed or primary cultures of inner medullary collecting duct cells to further characterize DA_2 receptors by radioligand binding and functional assays. These studies have addressed the following questions:
 - A. Is ^3H -spiperone and ^3H -domperidone binding to the DA_2 receptor?
 - B. Does dopamine inhibit vasopressin-stimulated increase in cAMP? Is this inhibition through DA_2 receptors?
 - C. Does dopamine stimulate PGE_2 synthesis through a DA_2 receptor mediated mechanism?
3. Based on the physiological effects of AADC inhibitors, do IMCD cells maintained in culture have the capacity to synthesize dopamine?

The studies presented in this thesis have hopefully resolved some of these questions. Based on previous physiological and pharmacological results, it has been known that DA_1 receptors exist in the proximal tubules and the renal vasculatures. However, the studies in Chapter 2 clearly demonstrated that, in rats, the renal vascular elements, including glomeruli, contained little or no DA_1 receptors. Hegde et al (1989b), however, showed low density of DA_1 receptors in renal vascular elements using ^3H -fenoldopam, a DA_1 receptor agonist. Both studies demonstrated that the DA_1 receptors were located primarily in the proximal tubules. It is not clear whether this

phenomenon is also present in other species such as dog in which most of the early studies of renal dopaminergic system were done. It is very possible that the major effects of dopamine are on proximal tubules rather than on renal vessels, because at low dose of dopamine, in rats and dogs, alterations in water and electrolyte excretion can occur in the absence of changes of renal blood flow (Cuche et al., 1976; McGrath et al., 1985). The dopamine-induced renal vasodilatation may be secondary to the activation of DA_1 receptors in the proximal tubules or directly activation of low-density DA_1 receptors in the renal vasculature. These discrepancies remained unresolved until further studies in various species are done.

Chapter 3 discussed the localization, pharmacological and biochemical characterizations of renal DA_2 receptors. The unusual pharmacological characteristics of DA_2 receptors in the renal medulla encouraged us to further compare the renal and the central D_2 / DA_2 receptors. From the photoaffinity labeling studies, it was concluded that the renal DA_2 receptors are similar to the central D_2 receptors if not identical. ^{125}I -NAPS bound to kidney, striatum, and pituitary membranes were found to be a protein of $M_r=120,000$ under high stringency protease inhibitors condition, but may have different binding subunits because under low stringency protease inhibitors condition, there were different patterns of labeling of low molecular weight proteins. The unique localization of DA_2 receptors in renal medulla also encouraged us to search for the functions of the renal DA_2 receptors. These studies were discussed in Chapter 4.

The medullary collecting duct is the terminal part of the nephron, and is the main target site of vasopressin which controls the final steps of water and electrolyte

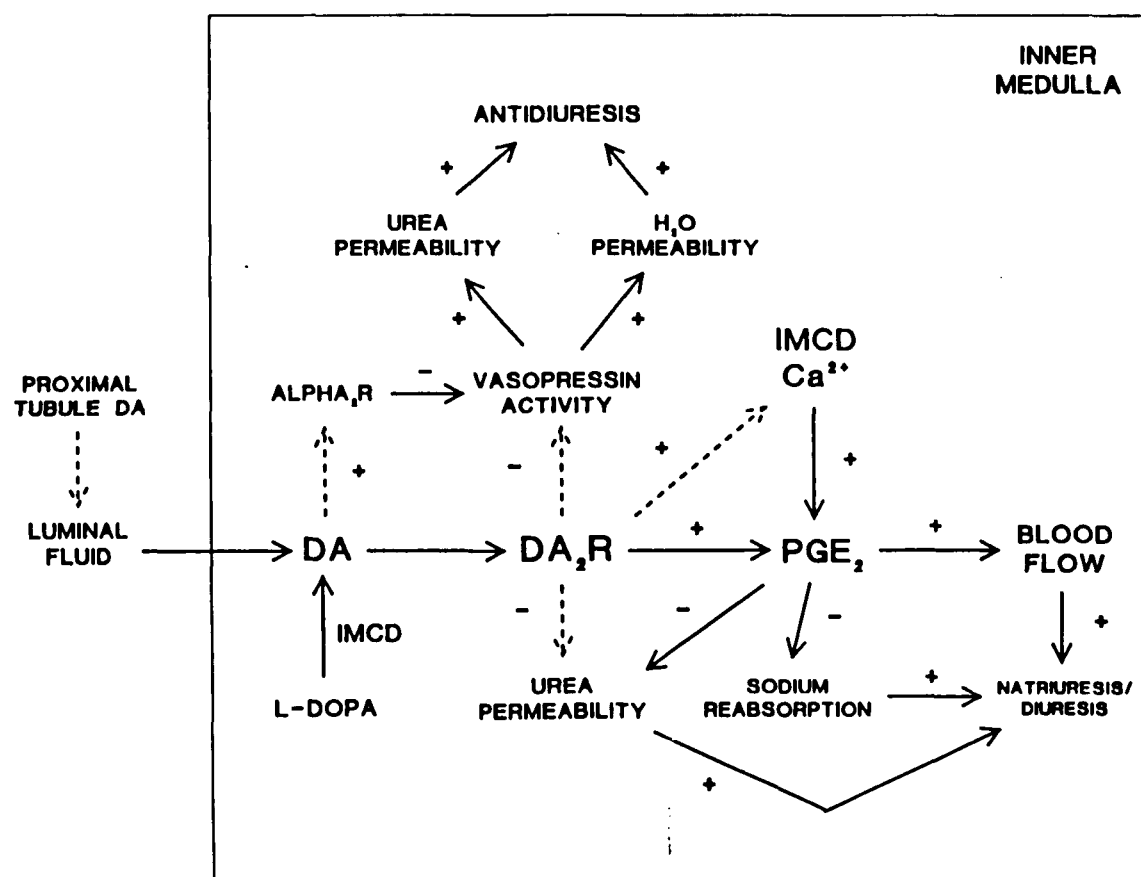
reabsorption. There is abundant prostaglandins, primarily PGE_2 which is known to be a potent inhibitor of vasopressin action (Dunn, 1983), and large amounts of dopamine in inner medulla (Fernandez-Pardel and Saavedra, 1982; Petrovic and Bell, 1986). Based on previous observations that dopamine may antagonize vasopressin action in vivo and in vitro (Koyoma et al., 1985; Muto et al., 1985; Arruda and Sabatini, 1982), the possibility of interactions between dopamine and PGE_2 was explored in chapter 4. Exogenous dopamine increased PGE_2 synthesis and this effect could be blocked by specific DA_2 antagonists. Interestingly, the DA_2 antagonists alone decreased PGE_2 synthesis. The dopamine precursor L-dopa, like dopamine, also increased PGE_2 synthesis, and the dopa decarboxylase inhibitors decreased the basal and blocked the L-dopa-induced PGE_2 synthesis suggesting an endogenous dopaminergic system controls PGE_2 synthesis in renal medulla. These hypothesis was supported by the experiments showing that intact inner medullary collecting duct cells contained dopamine and IMCD cells were able to convert L-dopa to dopamine and the conversion could be inhibited by dopa decarboxylase inhibitors. Existence of dopa decarboxylase in IMCD cells was further demonstrated by enzyme kinetic study. The existence of the DA_2 receptors was proved by radioligand binding study in the same cells. The secondary messengers of the DA_2 receptors are still unknown. However, the basal or dopamine-stimulated PGE_2 synthesis can be blocked by intracellular calcium blocker or phospholipase A_2 inhibitor and can not be blocked by voltage-dependent calcium blockers or phospholipase C inhibitor suggesting intracellular calcium or phospholipase A_2 may be the candidates of secondary messengers. Those important findings provided solid evidences that dopamine acts as an autocrine or paracrine hormone in the inner medulla, with at least, one of its function is in control of PGE_2 synthesis and may then, indirectly, antagonize

vasopressin action in renal medulla.

The secondary messenger of vasopressin (V_2) receptors is stimulation of adenylate cyclase activity. The secondary messenger of the central D_1 receptors is inhibition of adenylate cyclase activity. It was possible to speculate that the renal DA_2 receptors may also inhibit adenylate cyclase activity and then be able to antagonize vasopressin action. We tested this possibility for the DA_2 receptors in IMCD cells in Chapter 5. Indeed, dopamine inhibited vasopressin-induced cAMP accumulation, but this inhibition could only be reversed by α_2 -adrenoceptor antagonists, not by dopamine receptor antagonists. This finding raised a question that what is the endogenous stimulant of α_2 -adrenoceptor in IMCD cells ? There was evidence that the dopamine in inner medulla is non-neuronal origin because renal denervation studies showed little reduction of dopamine in inner medulla while the contents of other catecholamines did diminish in other renal tissues (Petrovic and Bell, 1986; Petrovic et al., 1986). Since norepinephrine and epinephrine are the prototype catecholamines, it is reasonable to expect these catecholamines exert biological functions in the renal medulla. However, the speculation that medullary nonneuronal dopamine which may be synthesized locally or transported downstream from proximal tubules acts as an important physiological regulator of vasopressin is highly possible based on the fact that dopamine is not only a natriuretic but also a potent diuretic hormone (Lee, 1982). The results of Chapter 5 that dopamine inhibit vasopressin-stimulated cAMP *in vitro* via activation of α_2 -adrenoceptors in IMCD cells support the hypothesis that dopamine may also antagonize vasopressin action *in vivo*. This is an important observation because vasopressin plays an significant role in several forms of essential hypertension (Share and Crofton, 1984),

and a defect in the renal dopaminergic system has been implied as one of the etiological factors of essential hypertension (Lee, 1982).

In conclusion, dopamine may exert its natriuresis effect in proximal tubules via activation of the DA_1 receptors. Less labeling of the DA_1 receptors in renal vasculature from autoradiographic studies supports the concept that proximal tubule is the main target site for dopamine-mediated DA_1 responses. The kidney inner medulla contains the DA_2 receptors. Dopamine may directly or indirectly antagonize vasopressin action via activation of the α_2 -adrenoceptors or the DA_2 receptors. Activation of the α_2 -adrenoceptors results in inhibition of vasopressin-stimulated cAMP which is the secondary messenger of hydro-osmotic effect of vasopressin. Activation of the DA_2 receptors controls the PGE_2 synthesis in IMCD cells. Dopamine is synthesized locally in IMCD cells and acts as an autocrine or paracrine hormone. A hypothetical model describing the effects of dopamine in IMCD cells is shown as follows.



MODEL: Hypothetical model of dopamine action in inner medulla. Solid arrows represent established results. Dash arrows are hypothetical. +, represents stimulation or enhancement. -, represents inhibition or attenuation.

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