Functional Role of the Renal $\alpha_{2a/d}$-Adrenoceptor Subtype in Normotensive and Hypertensive Rats

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ABSTRACT

Clonidine is a mixed α_2-adrenoceptor/imidazoline receptor agonist. Previous studies showed that, in the anesthetized rat, clonidine increased urine flow rate by increasing both osmolar (solute) and free water clearance. These renal effects of clonidine and other α_2-adrenoceptor agonists were postulated to involve the stimulation of two distinct anatomical sites and/or receptors. The receptors mediating the renal effects of clonidine were investigated using male, uninephrectomized rats. Rats were anesthetized with pentobarbitone. Following a tracheotomy, the carotid artery and jugular vein were cannulated respectively for the measurement of blood pressure and saline infusion. The remaining kidney was exposed and the ureter was catheterized for the collection of urine. A 31 gauge needle was advanced into the renal artery for the infusion of the agonist of interest (or vehicle) directly into the kidney. If required, antagonists were administered as a slow intravenous bolus. A low infusion rate of clonidine (1.0 nmol/kg/min) increased urine flow rate by increasing free water and osmolar clearance. These renal effects were shown to be pharmacologically independent using prazosin (an α_1-antagonist with relative selectivity for the α_{2b}-adrenoceptor subtype) and naltrexone (an opioid receptor antagonist). The free water response to clonidine was prazosin-sensitive/naltrexone-insensitive whereas the osmolar response was prazosin-insensitive/naltrexone-sensitive. This was consistent with the renal effects of clonidine being mediated by two separable and distinct receptors. Based on the prazosin sensitivity of the increase in free water clearance, this response was postulated but not proven to involve the α_{2b}-subtype. The receptor mediating the clonidine-induced osmolar clearance was less clear. Moxonidine (an imidazoline receptor agonist) increased osmolar clearance but was insensitive to

naltrexone indicating that imidazoline receptors were not involved in the osmolar response to clonidine. UK-14,304 is an α_2-agonist that, at a low dose, selectively increased osmolar and not free water clearance. The osmolar response to UK-14,304 was also attenuated by naltrexone and unaffected by prazosin. Literature reports have speculated on the purported selectivity of UK-14,304 for the $\alpha_{2a/d}$-subtype over other α_2-subtypes. In the rat kidney, only the $\alpha_{2a/d}$- and α_{2b}-adrenoceptor subtypes have been identified. A clearly selective $\alpha_{2a/d}$-agonist, guanfacine, was used to test the hypothesis that the renal $\alpha_{2a/d}$-adrenoceptor subtype mediated osmolar clearance. Guanfacine increased osmolar clearance but not free water clearance. As with clonidine and UK-14,304, this response was naltrexone-sensitive and prazosin-insensitive. RX-821002, an $\alpha_{2a/d}$-selective antagonist, attenuated the osmolar response to guanfacine. These results supported the contention that the renal $\alpha_{2a/d}$-adrenoceptor subtype mediated osmolar clearance. Further studies examined the renal function of the $\alpha_{2a/d}$-adrenoceptor in hypertensive versus normotensive rats. Guanfacine consistently increased osmolar clearance when administered to the relevant, normotensive control rats. In contrast, the osmolar response to guanfacine was absent in a genetic model of hypertension (spontaneously hypertensive (SH) rats) while intact in an acquired model of hypertension (one kidney-one clip rats). These data suggested that the natriuretic function of the $\alpha_{2a/d}$-subtype was defective in SH rats and that this defect was not secondary to elevated blood pressure. Decreased natriuretic activity of the $\alpha_{2a/d}$-adrenoceptor subtype in SH rats would be consistent with this defect playing a causal role in the pathogenesis of hypertension.

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GENERAL INTRODUCTION

Historical Perspectives

Adrenergic receptors were initially subclassified into α-adrenoceptors and β-adrenoceptors by Ahlquist (1948). Five catecholamines including epinephrine, methylepinephrine, norepinephrine, methylnorepinephrine, and isoproterenol were studied in eight physiological assays. The aim was to determine the relative activities of these agents and rank them in order of potency to detect any variations. Two rank orders were detected: (E > NE > Me-NE > Me-E > I) and (I > E > Me-E > Me-NE > NE). These differences were attributed to two receptor systems which were termed α- and β-adrenoceptor, respectively.

Three subtypes of β-adrenoceptors have since been identified: β_1-, β_2-, and more recently, the β_3-subtypes (Emorine *et al.*, 1989). The original subtyping of β-adrenoceptors into β_1- and β_2-subtypes was based solely on pharmacological studies. The rank orders of potency of several sympathomimetic amines were examined for effects on fatty acid mobilization, cardiac stimulation, bronchodilation, and vasodilation. Again, differences were detected in the rank orders of potency for lipolysis and cardiac stimulation as compared to bronchodilation and vasodilation. This variation was attributed to two β-adrenoceptor subtypes, namely the β_1- and β_2-subtypes respectively (Lands, 1967). More recently, β-adrenoceptors other than the β_1- and β_2-subtypes were

proposed to exist. Emorine *et al.* (1989) screened a human genomic library with probes for the β_1- and β_2-subtype genes. A family of clones displayed approximately 50% homology to both probes. When expressed in Chinese hamster ovary cells, the "β_3-" protein responded to six β-adrenoceptor agonists with increased intracellular cAMP. The rank order of potency of these agonists differed from those of β_1- and β_2-subtypes. A third β-adrenoceptor subtype was thus identified as a result of both molecular biological and pharmacological techniques.

The definition of α-adrenoceptors into the α_1- and α_2-adrenoceptor subtypes was more complicated. Three premises (anatomical, functional, and biochemical) were initially proposed for the subclassification of α-adrenoceptors before the fourth, pharmacological subdivision, was finally adopted. Following the subclassification of α-adrenoceptors into α_1- and α_2-adrenoceptors, a combination of pharmacological and molecular biological techniques illuminated the existence of several subtypes of both classes.

The original anatomical distinction between α_1-adrenoceptors and α_2-adrenoceptors was based on their postulated post-synaptic and pre-synaptic locations respectively (Langer, 1974; 1977). However, exceptions to this postulate were identified rendering anatomical classification impractical. The key observations were that two post-junctional events were mediated by selective α_2-adrenoceptor agonists. These included the inhibition of isoprenaline-induced glycolysis and lipolysis in hamster epididymal adipocytes (Schimmel, 1976) and inhibition of melanin granule release in frog skin produced by α-melanocyte-stimulating-hormone (Pettinger, 1977). A second classification was therefore suggested where function was used to distinguish between α_1-

and α_2-adrenoceptors. The α_1-adrenoceptors were proposed to be excitatory where the prototype was the post-synaptic vascular smooth muscle receptor. The α_2-adrenoceptors were the inhibitory receptors with the key example being presynaptic receptors (Berthelson and Pettinger, 1977). This generalization was again too narrow and extensive overlap limited the usefulness of such functional classification. For example, in the rat, increasing doses of norepinephrine produced vasoconstriction. At maximal doses of norepinephrine, this response was abolished by both prazosin (α_1-adrenoceptor antagonist) and yohimbine (α_2-adrenoceptor antagonist) (Drew and Whiting, 1979). Norepinephrine was thus stimulating vasoconstriction by acting at α_2-adrenoceptors and α_1-adrenoceptors which were both post-junctional as well as both excitatory. Biochemical definition of the α-adrenoceptors was also proposed where α_1-adrenoceptors were coupled to phosphatidyl inositol turnover and elevated cytosolic calcium and α_2-adrenoceptors mediated decreased activation of adenylyl cyclase (Fain and Garcia-Sainz, 1980; Wikberg, 1979). This classification scheme was abandoned due to the limited knowledge of the mechanisms of action of α-adrenoceptors at the time. It is now known that activation of α_1-adrenoceptors does result in increased intracellular calcium concentrations. Usually, this is due to release of intracellular Ca^{2+} stores via G-protein-mediated phospholipase C activation (Minneman, 1988). Activation of voltage-gated Ca^{2+} channels also results from α_1-adrenoceptor stimulation (Tsujimoto *et al.*, 1989). As well as inhibiting adenylyl cyclase, α_2-adrenoceptors may also activate phospholipase C (Cotecchia *et al.*, 1990; Lanier *et al.*, 1996). These receptors have also been associated with activation of Gs proteins to stimulate adenylyl cyclase (Eason *et al.*, 1992; Eason *et*

al., 1994), potassium channels, phospholipase A_2, and Na^+/H^+ exchange (Isom *et al.*, 1987a; 1987b). Hence, the distinction between α_1- and α_2-adrenoceptors on a biochemical basis would still be too complicated.

The present classification scheme involves pharmacological distinction between α-adrenoceptor populations. In particular, at α_1-adrenoceptors, prazosin is more potent than yohimbine whereas at α_2-adrenoceptors, yohimbine is more potent than prazosin (Bylund and U'Prichard, 1983).

As a result of radioligand-binding studies and the advancement of molecular studies, the pharmacological definition of α_1- and α_2-adrenoceptors has broadened (Bylund *et al.*, 1995) to the identification of several α_1-adrenoceptor subtypes and α_2-adrenoceptor subtypes. In this thesis, the discussion will focus on the α_2-adrenoceptor subtypes particularly in the kidney and in the disease state, hypertension.

α_2-Adrenoceptor Subtypes

To date, four α_2-adrenoceptor subtypes have been identified. These include the α_{2a}-, α_{2b}-, α_{2c}- and α_{2d}-subtypes (MacKinnon *et al.*, 1994). α_2-Adrenoceptor heterogeneity was first speculated as a result of radioligand binding studies. Rauwolscine, yohimbine, and prazosin showed different affinities for α_2-adrenoceptors between human platelets and rat cerebral cortex (Cheung *et al.*, 1982) and neonatal rat lung (Latifpour *et al.*, 1982). The α_{2a}- and α_{2b}-subtypes were identified using prazosin which had low affinity (200-300 nM) and high affinity (5-10 nM) for these subtypes, respectively. These

studies also indicated that the human platelet possessed only the α_{2a}-subtype whereas the neonatal rat lung expressed predominantly the α_{2b}-subtype (Bylund, 1985).

Cell lines which contained only one of these two α_2-adrenoceptor subtypes were identified. The affinities of 34 drugs were determined in tissues which contain only one α_2-adrenoceptor subtype including the HT29 and NG108 cell lines, neonatal rat lung, human platelet, and human cerebral cortex tissue (Bylund *et al.*, 1988). Where the human platelet has been defined as carrying only the α_{2a}-subtype (Bylund *et al.*, 1985), similar rank orders of affinity were found for human cerebral cortex and HT29 cells. A different rank order of affinity was found to correlate between the NG108 cell line and the prototype tissue for the α_{2b}-subtype, neonatal rat lung (Bylund *et al.*, 1988). A third cell line, the opossum kidney or OK line, had an α_2-adrenoceptor with a pharmacological profile different than both the α_{2a}- and α_{2b}-subtype profiles (Murphy and Bylund, 1988). This novel α_2-subtype was later termed the α_{2c}-adrenoceptor subtype (Blaxall *et al.*, 1991). The fourth subtype, α_{2d}, was found in bovine pineal gland, rat submaxillary gland, and in the RINm5F cell line (Michel *et al.*, 1989; Remaury and Paris, 1992; Simonneaux *et al.*, 1991).

The existence of different α_2-adrenoceptor subtypes suggested the presence of several α_2-adrenoceptor genes (table 1.1). By analyzing somatic cell hybrids, the gene encoding the α_{2a}-subtype of the human platelet was identified on human chromosome 10 and therefore termed α_2-C10. Further analysis of human genomic DNA suggested two related genes existed on chromosomes 2 and 4 (Kobilka *et al.*, 1987). These genes have

since been cloned and similarly named α_2-C2 and α_2-C4 respectively (Regan *et al.*, 1988; Lomasney *et al.*, 1990; Weinshank *et al.*, 1990).

In the rat, three clones for the α_2-adrenoceptors have also been identified. The rat homologue of the human α_2-C2 gene is the α_2-RNG (Zeng *et al.*, 1990). Shared amino acid sequence identity was 82%. Lanier *et al.* (1991) have also identified the rat homologues of the α_2-C4 and the α_2-C10 genes, namely the RG10 and RG20 genes respectively. Both of these rat homologues share approximately 90% amino acid identity with their human equivalent. The correlation between the cloned α_2-subtypes and the pharmacologically identified α_2-subtypes has generally been established.

The α_{2a}-adrenoceptor subtype

The α_2-C10, which was isolated from the human platelet, clearly has the pharmacological profile of the α_{2a}-subtype with the rank order of affinity being rauwolscine > oxymetazoline > ARC-239 > prazosin (Kobilka *et al.*, 1987; Bylund *et al.*, 1994). The partial agonist oxymetazoline (Bylund, 1985) and more recently, the full agonist, guanfacine, (Uhlén and Wikberg, 1991) are selective ligands for the α_{2a}-adrenoceptor subtype. α_{2a}-Subtype selective antagonists include RX-821002 (Uhlén and Wikberg, 1991) and BRL 44408 (Bylund *et al.*, 1988; Young *et al.*, 1989).

The α_{2b}-adrenoceptor subtype

The expressed α_2-C2 and α_2-RNG clones have similar ligand-binding profiles; both have pK_i values for various antagonists that correlate with those of the rat neonatal lung

α_{2b}-adrenoceptor (Bylund *et al.*, 1994). Note the difference in rank order of affinities as compared to the α_{2a}-subtype: rauwolscine > ARC-239 > prazosin > oxymetazoline. These clones were detectable in tissues which contain the α_{2b}-subtype, namely, neonatal rat lung and adult rat kidney (Zeng *et al.*, 1990; Lomasney *et al.*, 1990). Several antagonists selective for the α_{2b}-subtype have been identified. These include prazosin, ARC 239, chlorpromazine and imiloxan (Young *et al.*, 1989; Michel *et al.*, 1990). Selective α_{2b}-subtype agonists have not yet been discovered.

The α_{2c}-adrenoceptor subtype

The α_2-C4 and RG-10 clones corresponded to the α_{2c}-subtype. The α_2-C4 gene hybridizes extensively with mRNA from OK cells from where the α_{2c}-subtype was identified (Lorenz *et al.*, 1990). Like the α_{2b}-subtype, the pharmacological profile of the α_{2c}-subtype was rauwolscine > ARC-239 > prazosin > oxymetazoline (Bylund *et al.*, 1992; Zeng and Lynch, 1991; Uhlén *et al.*, 1992). In fact, based on high affinities for prazosin, the α_2-C4 protein was initially thought to be the α_{2b}-subtype. The α_{2c}-subtype was distinguished from the other α_2-subtypes because the ratio of the affinities of prazosin and yohimbine was intermediate between the α_{2a}- and α_{2b}-subtypes. Two antagonists, BAM 1303 and WB 4104, also have selectivity for α_{2c}- over α_{2b}-adrenoceptors (for review, see Bylund *et al.*, 1994).

The α_{2d}-adrenoceptor subtype

The rat RG20 clone has posed some difficulties in association with an α_2-subtype. Structurally the α_2-C10 and the RG20 are certainly species homologues with high overall amino acid sequence homology (Lanier *et al.*, 1991). Like the α_{2a}-adrenoceptor, the α_{2d}-subtype has low affinities for prazosin, spiroxatrine, and ARC 239. Despite similar pharmacological binding profiles, several drugs have been identified which can discriminate between the α_2-C10 (α_{2a}-subtype) and the RG20. These include rauwolscine, yohimbine, BAM 1303, and SKF 104078. Most notable are the lower affinities of RG20 to rauwolscine and yohimbine as compared to those of the other three subtypes (Harrison *et al.*, 1991; Lanier *et al.*, 1991; Michel *et al.*, 1989; Simonneaux *et al.*, 1991). Furthermore, differences are apparent when the potency ratios for numerous antagonist pairs are compared (Simonneaux *et al.*, 1991). Based on the pharmacological studies, therefore, the RG20 has been designated as the α_{2d}-adrenoceptor subtype.

α_2-subtype		**clone**	
human	rat	human	rat
α_{2a}	α_{2d}	α_2-C10	RG20
α_{2b}	α_{2b}	α_2-C2	α_2RNG
α_{2c}	α_{2c}	α_2-C4	RG10

Table 1.1. Characteristics of α_2-adrenoceptor subtypes. (for review, Bylund *et al.*, 1994).

α_2-Adrenoceptors in the Kidney

In the rat kidney, autoradiographic binding studies using [^{3}H]-clonidine indicated that α_2-adrenoceptors were located in the renal medulla and cortex (McPherson and Summers, 1981). Using [^{3}H]-rauwolscine, others showed that α_2-adrenoceptors were concentrated in the renal cortex (Insel *et al.*, 1985; Muntz *et al.*, 1986) with lesser densities in the outer medulla. More specifically, α_2-adrenoceptors have been identified in the glomeruli (McPherson and Summers, 1983) as well as proximal tubules and arterioles in the cortex (McPherson and Summers, 1981; Insel *et al.*, 1985; Stephenson and Summers, 1985; Sundaresan *et al.*, 1987; Calianos and Muntz, 1990). Binding studies using epithelial cells from different sections of the rat nephron showed that α_2-adrenoceptors densely occupied the proximal tubules and decreased along the nephron (Zakieh *et al.*, 1993). Functional studies localized the ability of α_2-adrenoceptor stimulation to inhibit cAMP accumulation. Umemura *et* al. (1985) showed in rat isolated single nephron segments that α_2-adrenoceptors can inhibit parathyroid hormone or vasopressin-mediated cAMP accumulation in proximal convoluted tubules, cortical collecting tubules and medullary collecting tubules. However, no effect on adenylate cyclase was observed in cortical and medullary thick ascending limbs of Henle. Lower levels of α_2-adrenoceptors have also been identified on arterioles and the vasa recti with autoradiographic binding (Muntz *et al.*, 1986; Calianos and Muntz, 1990).

The localization of α_2-adrenoceptors in the rat kidney has recently concentrated on the subtypes of these receptors. *In situ* hybridization and reverse-transcriptase DNA amplification studies detected $\alpha_{2a/d}$-, α_{2b}-, and lower levels of α_{2c}-subtype mRNA in the rat

kidney (Meister *et al.*, 1994; Le Jossec *et al.*, 1995b; Wilborn *et al.*, 1996). Radioligand binding with [^{3}H]RX821002 labelled two populations of α_2-adrenoceptors. Of the renal α_2-adrenoceptors, 14% were the α_{2a}-subtype and 86% were the α_{2b}-subtype (Uhlén and Wikberg, 1991). Together these studies suggest that the $\alpha_{2a/d}$- and α_{2b}-subtypes exist in the rat kidney. Although the α_{2c}-subtype mRNA was detected at low levels in the rat kidney, binding studies have not indicated that this receptor is expressed (Uhlén and Wikberg, 1991).

In the human kidney, the mRNA for all α_2-adrenoceptor subtypes has been detected using reverse-transcription-PCR (Eason and Liggett, 1993) or RNAse protection (Perälä *et al.*, 1992; Berkowitz *et al.*, 1994) assays. Perälä *et al.* (1992) indicated that the α_2-C10 mRNA (α_{2a}) was detected more abundantly in the human renal cortex whereas the α_2-C4 mRNA (α_{2c}) was detected abundantly in both the renal cortex and medulla. Radioligand binding studies using various labelled α_2-adrenoceptor antagonists suggested that the α_{2a}-subtype is the preponderent α_2-subtype present in the human kidney with low densities of α_{2b}- or α_{2c}-adrenoceptors (Motomura *et al.*, 1989).

The identification of the renal α_2-adrenoceptor subtypes in other animal species has not yet been thoroughly pursued. However, in rabbit kidneys, the α_{2b}-subtype has been detected following radioligand binding studies (Mohuczy-Dominiak and Garg, 1993). The opossum kidney has been shown to express the α_{2c}-adrenoceptor subtype (Blaxall *et al.*, 1991).

α_2-Adrenoceptor Function in the Kidney: Hemodynamic and Tubular Effects

Hemodynamic Effects

In anesthetized rats, α_2-adrenoceptor agonists such as UK-14,304 and guanabenz failed to produce renal vasoconstriction at high doses approaching 50 μg/kg. This unresponsiveness was different than that observed with two α_1-adrenoceptor agonists (cirazoline and phenylephrine). Maximal renal vasoconstriction was observed at doses between 0.05 and 3.0 μg/kg (Wolff *et al.*, 1987). Thus, the α_1-adrenoceptors initially appeared to have a greater role in the modulation of vascular tone within the rat kidney as suggested by others (Schmitz *et al.*, 1981). Later studies suggested that anesthesia with sodium pentobarbital was confounding the renal vascular effects of α_2-adrenoceptor agonists. For example, in conscious, restrained rats, equipressor intravenous infusions of α_1- or α_2-adrenoceptor agonists resulted in equivalent decreases in effective renal plasma flow (Gellai and Ruffolo, 1987). In conscious, freely moving rats, norepinephrine decreased renal blood flow following intrarenal administration. The addition of α_1- and α_2-adrenoceptor antagonists (corynanthine and idazoxan, respectively) separated two components of this renal vascular response to norepinephrine. Rauwolscine at a dose which did not block the renal vasoconstrictor response to phenylephrine, also attenuated the decrease in renal blood flow produced by the α_2-agonist guanabenz (Wolff *et al.*, 1989). These studies suggested that renal α_2-adrenoceptors, along with α_1-adrenoceptors, contribute to the modulation of renal vascular tone.

Tubular effects

Although the α_2-adrenoceptors are known to affect renal salt and water reabsorption in the rat, the mechanisms involved remain unclear. Early studies using the α_2-adrenoceptor agonist, clonidine, showed increases in diuresis and albeit, less predictably, natriuresis (Biollaz *et al.*, 1979; Barr and Kauker, 1979; Miller, 1980; Roman *et al.*, 1979). These effects were postulated to involve the antagonism of vasopressin (Barr and Kauker, 1979; Roman *et al.*, 1979). In agreement with this hypothesis, studies using isolated rabbit cortical collecting tubules have shown that vasopressin induced water absorption in these cells (Krothapalli and Suki, 1984). Norepinephrine and clonidine inhibited vasopressin-mediated water reabsorption by a mechanism which was inhibited by yohimbine but not by prazosin. This suggested the involvement of the α_2-adrenoceptor. Since these α_2-mediated effects were abolished by 8-bromo-cAMP, they appeared to involve inhibiting vasopressin-induced cAMP production. The effects of vasopressin were also reversed by epinephrine (α_2-adrenoceptor agonist) and subsequently restored by yohimbine (α_2-adrenoceptor antagonist) in the isolated perfused rat kidney (Smyth *et al.*, 1985). These initial studies offered a potential renal role for α_2-adrenoceptors.

Renal activation of α_2-adrenoceptors has also been associated with mechanisms which would mediate the retention of sodium. For example, selective α_2-adrenoceptor agonists (guanabenz, UK-14,304, clonidine, guanfacine and BHT-933) activated the Na^+-H^+ exchanger in rat proximal tubule segments. The α_2-adrenoceptor antagonists, idazoxan and yohimbine, attenuated the effects of guanabenz and UK-14,304 on this antiporter

(Gesek *et al.*, 1989; Gesek and Strandhoy, 1990). The role, if any, of renal α_2-adrenoceptors in sodium reabsorption is still unclear.

The *in vivo* renal effects of clonidine in the rat have been investigated more extensively. Intrarenal infusion of this α_2-adrenoceptor agonist increased urine flow rate and sodium excretion. These effects were reflected by increases in osmolar and free water clearance (Blandford and Smyth, 1988a). Other α_2-adrenoceptor agonists such as BHT-933 (Gellai and Ruffolo, 1987; Stanton *et* al., 1987) and UK-14,304 (Smyth *et al.*, 1992a) have been shown to have similar effects on renal function. Clearly, renal α_2-adrenoceptor stimulation elicits increases in both water and sodium excretion. These renal responses to α_2-adrenoceptor stimulation apparently had physiological relevance. Yohimbine, an α_2-adrenoceptor antagonist, significantly decreased urine flow in euvolemic, conscious rats and in water-loaded rats undergoing moderate diuresis (Gellai, 1990). More specifically, in rats receiving a moderate saline infusion, sodium and water excretion were blocked by intrarenal infusion of yohimbine (Blandford and Smyth, 1988b).

Renal α_2-Adrenoceptors and Hypertension

Besides their normal physiological function in the kidneys, the α_2-adrenoceptors are of interest regarding their role in the development of hypertension. There is compelling evidence that the kidney contributes significantly to the pathogenesis of hypertension. In this disease state, there is also evidence that the α_2-adrenoceptors are altered.

Role of the kidney in hypertension

The role of the kidney in the origin of hypertension has been substantiated by several lines of evidence. The kidney plays a dominant role in the chronic regulation of blood pressure. In this thesis, only one potential etiology will be discussed, namely alterations in the mechanism involved which has been termed "pressure-natriuresis." Briefly, when blood pressure exceeds or falls below normal levels, the kidney responds by respectively increasing or decreasing water and sodium excretion thereby modulating blood volume and restoring normal arterial pressure (Guyton, 1991). The normal or equilibrium pressure, that is, the pressure at which the kidney maintains fluid output equal to fluid input, must be elevated for maintenance and/or development of hypertension. In fact, in several models of hypertension (for example, spontaneously hypertensive rats, one kidney-one clip Goldblatt rats) the equilibrium pressure is indeed elevated (Hall *et al.*, 1990).

The ability of anti-hypertensive drugs to shift the pressure-natriuresis system to maintain normal blood pressure has also been investigated. The premise behind these studies is that if hypertension necessarily involves alterations of pressure-natriuresis, then drugs used to treat hypertension should be shifting this system so adequate sodium and water excretion occurs at decreased blood pressure levels (for review, see Cowley and Roman, 1996). Calcium antagonists (diltiazem (Lu *et al.*, 1992) and nisoldipine (Fenoy *et al.*, 1992)), converting-enzyme inhibitors (captopril (Kline and Mercer, 1987) and enalapril (McLennan *et al.*, 1991)), angiotensin II receptor antagonists (losartan (Kline

and Liu, 1994)) and the vasodilator, hydralazine, (Kline and Mercer, 1987; Kline and McLennan, 1991) all have the ability to modify pressure-natriuresis.

Compelling evidence that renal dysfunction contributes to the development of hypertension has resulted from cross-transplantation studies. In humans and in rats, blood pressure has repeatedly followed the kidney in renal transplantation studies. In humans, normotensive recipients of a kidney from a hypertensive donor (Strandgaard and Hansen, 1986) or a donor with a familial history of hypertension (Guidi *et al.*, 1985) acquired elevated blood pressures and required more anti-hypertensive therapy as compared to those who received kidneys from a normotensive donor. The converse has also been demonstrated where normalization of blood pressure resulted from bilateral nephrectomy and transplantation of kidneys from normotensive donors to patients with hypertension and kidney failure (Curtis *et al.*, 1983). In rats, several genetic models of hypertension have been used to show that the kidney from a hypertensive donor produces hypertension in a normotensive recipient, the relevant control strains. These models include the spontaneously hypertensive rats (Kawabe *et al.*, 1978), spontaneously hypertensive-stroke prone rats (Rettig *et* al., 1989), Milan hypertensive rats (Fox and Bianchi, 1976) and Dahl salt-sensitive hypertensive rats (Morgan *et al.*, 1990).

The ability of a donor kidney to modify blood pressure in the recipient appears to be genetically determined. In rats, studies designed to investigate the genetic pre-determination of this observation have used the kidneys from hypertensive rats with controlled blood pressure or young rats (pre-hypertensive) to show that transplantation still produces hypertension in the normotensive recipient. Lifelong treatment with ramipril

(1 mg/kg/d) prevented hypertension in spontaneously hypertensive stroke-prone rats yet the kidneys from these rats when 20 weeks old still produced elevated blood pressure in the recipient rats (Rettig *et al.*, 1990). A separate study examined renal transplants between 5 to 6 week old spontaneously hypertensive stroke-prone rats and control Wistar-Kyoto rats. At this age, both strains had similar blood pressures. Donor kidneys were transplanted to bilaterally nephrectomized F_1 hybrid recipients. Hybrids were used to prevent immunological rejection of the transplanted kidney. The kidneys from the hypertensive strain but not control rats increased blood pressure in the recipient rats (Kopf *et al.*, 1993). These studies suggested that the genetic programming responsible, at least in part, for the development of hypertension is contained in the kidney.

Alterations of α_2-adrenoceptors in hypertension

As related to the kidney, the α_2-adrenoceptors have been implicated in the development of hypertension. In normotensive rats, radioligand binding studies have shown that the α_2-adrenoceptors represent at least two-thirds of all α-adrenoceptors in crude membrane fractions of rat kidney (Sanchez and Pettinger, 1981). In several models of genetic hypertension, the predominance of α_2-adrenoceptors in the rat kidney appeared enhanced. These models include the spontaneously hypertensive rat (Sanchez and Pettinger, 1981; Pettinger *et al.*, 1982), the Dahl salt-sensitive rat (Pettinger *et al.*, 1982), the Sabra hypertensive rat (Parini *et al.*, 1983; Diop *et al.*, 1984), the Milan hypertensive rat (Parini *et al.*, 1987), and the New Zealand genetically hypertensive rat (Smyth *et al*, 1992b). It should be noted that some investigators have not detected this increased α_2-

adrenoceptor density. For example, Michel *et al.* (1992) found that renal α_2-adrenoceptors had similar densities between spontaneously hypertensive and Wistar-Kyoto rats. Nevertheless, for two reasons, it was speculated that the proposed exaggerated expression of renal α_2-adrenoceptors in genetic models of hypertension likely represented a genetic abnormality. In several experimental forms of hypertension (Fukuda *et al.*, 1983) such as two kidney-one clip hypertension, one kidney-one clip hypertension, and deoxycorticosterone acetate-salt hypertension (Yamada *et al.*, 1980; Wilson, 1991), the α_2-adrenoceptor density was comparable to the relevant controls. The overexpression of the α_2-adrenoceptors has also been found in spontaneously hypertensive rats to precede the onset of hypertension (Saiz *et al.*, 1987; Takatori *et al.*, 1991). These findings suggested that the increase in renal α_2-adrenoceptors (due to depressed stimulation?) was not a consequence of hypertension but perhaps a causal factor in these models. To date, the α_2-adrenoceptor subtype(s) which is elevated in density in the rat kidney in genetic forms of hypertension remains unclear.

The finding that α_2-adrenoceptors are elevated in hypertensive rats is not limited to the kidney. For example, dietary NaCl loading (8% NaCl) increased blood pressure in spontaneously hypertensive (SH) rats but not in normotensive, Wistar-Kyoto rats (Klangkalya *et al.*, 1988). In the anterior hypothalamic area of SH rats, a depressor region in the brain, α_2-adrenoceptor densities were increased prior to the elevation of blood pressure. The authors speculated that high sodium decreased norepinephrine release in the anterior hypothalamic region. This induced a compensatory increase in the α_2-

adrenoceptors. These data further associated decreased stimulation of α_2-adrenoceptors in the development of hypertension.

Purpose of these Studies

Throughout this thesis, the effects of various compounds on the kidney were investigated and compared based on their excretory effects. In these reports, we have elected to present urine flow rate additionally as its two components: osmolar clearance and free water clearance. Osmolar clearance represents the volume of plasma which is cleared of solutes per unit time. An increase in osmolar clearance reflects an increase in solute excretion. Free water clearance represents the clearance of water from the plasma. Negative free water clearance indicates that the urine is hypertonic as compared to plasma. Positive free water clearance indicates that the urine is hypotonic as compared to plasma.

The initial aim of these studies was to further explore the possibility that at least two receptors were involved in the renal effects of clonidine. An attempt to pharmacologically identify the two receptors was undertaken. Opioids were involved in the renal response to clonidine (Pan and Gutkowska, 1988). Therefore, the effects of opioid antagonism using naltrexone were determined. The preliminary observation that prazosin (α_1-adrenoceptor antagonist with relative selectivity for α_{2b}-adrenoceptors) attenuated the free water but not the osmolar response to clonidine (Blandford and Smyth, 1988a) was also going to be further investigated. As a result of the early experiments from both of these studies, it appeared that the actions of clonidine could be separated pharmacologically. Apparently, the clonidine-induced free water clearance was prazosin-

sensitive/naltrexone-insensitive. The clonidine-induced osmolar clearance was prazosin-insensitive/naltrexone-sensitive. Hence, the present studies focused firstly on systematically confirming the observation that the renal effects of clonidine could be dissociated pharmacologically.

The pharmacological dissociation of the renal effects of clonidine indicated that two receptors may have been involved. The next series of experiments was designed to determine the receptors involved, particularly the receptor mediating the osmolar response. Clonidine has been reported to be a mixed imidazoline receptor/α_2-adrenoceptor agonist. Consequently, the possibilities that the imidazoline receptor or the $\alpha_{2a/d}$-adrenoceptor subtype mediated the clonidine-induced osmolar clearance were investigated.

Following various pharmacological studies, it was determined that the $\alpha_{2a/d}$-adrenoceptor subtype mediated the increase in osmolar clearance produced by clonidine. There were several reports in the literature indicating that the $\alpha_{2a/d}$-subtype gene was altered in humans (Hoehe *et al.*, 1988) and in rats (Chun *et al.*, 1991). This second allele has correlated with hypertension in both species (Pettinger *et al.*, 1991; Lockette *et al.*, 1995; Svetkey *et al.*, 1996). We therefore investigated the osmolar function of the renal $\alpha_{2a/d}$-adrenoceptor subtype in two rat models of hypertension (spontaneously hypertensive and one kidney-one clip hypertensive rats).

2

Pharmacological Dissociation of the Renal Effects of Clonidine in the Anesthetized Rat

These data have been presented as an abstract at the XIIth International Congress of Pharmacology, Montréal, Québec, 1994 (Can. J. Physiol. Pharmacol. **72** (suppl. 1):562, 1994. These data have also been published: Intengan, H.D. and Smyth, D.D. Br. J. Pharmacol. **119**:663-670, 1996.

Summary:

Clonidine has been shown to increase urine flow rate in the anesthetized rat by increasing free water and osmolar clearance. These renal effects of clonidine were postulated to involve two anatomical sites and/or receptors. Preliminary experiments suggested that these actions of clonidine could be distinguished pharmacologically, where the free water response was prazosin-sensitive/naltrexone-insensitive and the osmolar response was conversely prazosin-insensitive/naltrexone-sensitive. This study was designed to determine if the renal effects of clonidine could be dissociated using prazosin and naltrexone. Male Sprague-Dawley rats were unilaterally nephrectomized 7 to 10 days prior to the day of the experiment. Clonidine (1.0 nmol/kg/min) infused into the renal artery of the anesthetized rats increased urine flow rate and sodium excretion. Osmolar and free water clearance were increased in response to clonidine administration. Following pre-treatment with prazosin (0.15 mg/kg, i.v.), the free water response was selectively attenuated. In contrast, naltrexone (3.0 mg/kg, i.v.) selectively blocked the osmolar but not the free water response to clonidine. Thus, the increase in osmolar and free water clearance following clonidine can be dissociated pharmacologically. These data were consistent with the postulate that the renal effects of clonidine were independent of each other.

Introduction

The *in vivo* administration of α_2-adrenoceptor agonists has been documented to increase urine flow rate by increasing osmolar and free water clearance (Strandhoy *et al.*, 1982; Gellai and Ruffolo, 1987; Stanton *et al.*, 1987; Blandford and Smyth, 1988a). It has since been postulated that these renal effects of α_2-adrenoceptor stimulation were mediated by two distinct anatomical sites and/or receptors (Strandhoy *et al.*, 1982; Blandford and Smyth, 1988a). Consistent with this hypothesis, preliminary experiments suggested that these effects could be dissociated pharmacologically.

We were firstly interested in the proposed role of opioids in the renal response to clonidine, an α_2-adrenoceptor agonist. Pan and Gutkowska (1988) had speculated that opioids mediated, at least in part, the clonidine-induced renal effects. Other effects of low doses of clonidine (for example, decreased blood pressure and heart rate) have also been blocked by opioid receptor antagonism using naloxone (Farsang and Kunos, 1979; Rhee and Lapp, 1988). Early experiments with naltrexone (non-selective opioid receptor antagonist) on the renal actions of clonidine showed attenuation of the osmolar but not the free water response. Concurrently, the preliminary observation that prazosin (α_1-adrenoceptor antagonist with relative selectivity for α_{2b}-adrenoceptors) attenuated clonidine-induced free water but not osmolar clearance (Blandford and Smyth, 1988a) was being investigated. These initial experiments suggested that the renal actions of clonidine could be separated pharmacologically where the free water response was prazosin-sensitive/naltrexone-insensitive and the osmolar response was conversely naltrexone-sensitive/prazosin-insensitive.

In the present study, the pharmacological dissociation of the renal responses to clonidine using naltrexone and prazosin was systematically investigated. The data indicated that two anatomical sites and/or receptors were involved in the renal actions of clonidine. These findings were consistent with the "two-site/receptor" hypothesis postulated in previous reports (Strandhoy *et al.*, 1982; Blandford and Smyth, 1988a).

Methods

Experimental Preparation

The general procedures have been described previously by Blandford and Smyth (1988b). Male Sprague-Dawley rats (200-225 g) were obtained from the University of Manitoba (Charles River Breeding Stock) and cared for according to animal care standards protocol (Canada Council on Animal Care). The animals were fed a standard rat chow diet with free access to tap water in cages at 22°C with a 12 h light/dark cycle.

Seven to ten days prior to the experiment, the animals were anesthetized with ether. A right flank incision was performed through which the right renal artery, vein, and ureter were occluded. The right kidney was removed. The incision was then sutured, and 2% lidocaine hydrochloride (Astra Pharma Inc., Mississuaga, Ontario) was administered topically.

On the day of the experiment, the rats were anesthetized with pentobarbitone (BDH Chemicals Ltd., Poole, England, 50.0 mg/kg, i.p.). Additional anesthetic was administered on return of the tail pinch or blink reflex in a bolus dose of 3.0 mg/kg, i.v. The rats were placed on a Harvard Animal Blanket Control Unit with a rectal thermometer probe, and the temperature was set for 37.5°C to maintain body temperature. Following a tracheotomy, the animals were allowed to breathe spontaneously. The left carotid artery was cannulated with polyethylene tubing (PE-50) and connected to a Statham pressure transducer (Model P23Dc) and a Grass model 5 polygraph for the monitoring of blood pressure. The left jugular vein was cannulated with polyethylene tubing (PE-160) for the continuous infusion of normal saline (0.9% NaCl) at 0.097 mL/min and the administration

of additional anesthetic as required. A left flank incision exposed the remaining kidney. A polyethylene catheter (PE-10) was inserted into the ureter to facilitate urine collection. A 31 gauge stainless steel needle was advanced into the renal artery and secured with glue (Super Glue; Mastercraft, Toronto, Ontario) for the infusion of the agonist of interest or vehicle with a Harvard syringe pump.

After surgery (time = 0 min), the continuous i.v. infusion (0.097 mL/min) of normal saline was initiated. The rats were allowed to stabilize for 45 min (time = 0 - 45 min). Antagonists were administered immediately following (prazosin) or 15 min into (naltrexone) the start of the stabilization period as a slow i.v. bolus (0.2 mL) over 1 min. Following the stabilization period, a 30 min control urine collection (time = 45 - 75 min) was obtained. After the control collection period, the intrarenal infusion (0.0034 mL/min) of clonidine or vehicle (0.9% saline) was initiated and maintained for the remainder of the experiment. Two subsequent urine collections were obtained (times = 75 - 105 and 105 - 135 min). Urine was collected into pre-weighed vials. Urine flow rate was determined gravimetrically.

Effects of prazosin or naltrexone on the renal effects of clonidine

Animals were randomly assigned to one of six study groups, each consisting of at least six rats. Group 1, the vehicle control group, received an intrarenal infusion of 0.9% saline at 0.0034 mL/min. Groups 2 and 3 received naltrexone (3.0 mg/kg) or prazosin (0.15 mg/kg) alone respectively. Group 4 received an intrarenal infusion of clonidine (1.0

nmol/kg/min). Groups 5 and 6 received pre-treatment with prazosin or naltrexone respectively followed by an infusion of clonidine.

Sample analysis

At the end of the experiment, a blood sample was collected through the carotid artery catheter. Lissamine green dye was injected through the renal artery line to confirm proper positioning of the needle. Creatinine levels in the urine and plasma were measured with a Beckman Creatinine 2 Analyzer. Urine and plasma osmolalities were determined with a Precision Systems Micro Osmometer. The sodium concentrations in urine and plasma were measured with a Nova Electrolyte Analyzer (System 13+).

Statistical analysis

Data are presented as the mean ± standard error. Data were analyzed by repeated measures of analysis of variance (ANOVA) using the SAS software, version 6.07. Significant interactions were further analyzed by a Fisher's least squared difference multiple comparison test. Significance is denoted in the figures with * representing $P<0.05$ and ** representing $P<0.01$.

Drugs

Clonidine, prazosin, and naltrexone were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A).

Results

Preparation controls

Data from the first collection period were analyzed to determine the similarity between groups following the surgery. Baseline values are shown in Table 2.1. Baseline levels of blood pressure, creatinine clearance, and heart rate did not differ as compared to control. Due to differences between baseline urine flow rate, sodium excretion, osmolar and free water clearance, data have been presented as the absolute difference between baseline and final collection values. Presenting the delta values highlighted the magnitudes of response between groups.

Effects of prazosin or naltrexone on the renal response to clonidine

A decrease and increase in blood pressure were observed when clonidine was administered with naltrexone or prazosin respectively. In all groups with clonidine, heart rate was also decreased. Creatinine clearance was unaltered by these interventions (figure 2.1). Naltrexone or prazosin pre-treatment alone had no effect on these parameters (data not shown).

Clonidine increased urine flow rate and sodium excretion (figure 2.2) as reflected by increases in osmolar and free water clearance (figure 2.3). Pre-treatment with prazosin selectively decreased the clonidine-induced free water response but had no effect on the osmolar response (figure 2.3). Pre-treatment with naltrexone attenuated the increase in urine flow rate and sodium excretion (figure 2.2) by selectively decreasing the osmolar response to clonidine (figure 2.3). In contrast with the effects of prazosin, naltrexone failed to alter the increase in free water clearance produced by clonidine.

	Veh (n = 9)	Clon (n = 9)	PZ (n = 6)	Clon + PZ (n = 6)	NX (n = 6)	Clon + NX (n = 6)
Blood pressure (mm Hg)	119 ± 5	119 ± 6	113 ± 4	106 ± 5	112 ± 3	124 ± 3
Creatinine clearance (mL/min)	1.7 ± 0.2	1.9 ± 0.2	1.6 ± 0.1	1.7 ± 0.3	1.8 ± 0.2	1.6 ± 0.1
Heart Rate (beats per min)	409 ± 14	387 ± 7	427 ± 12	397 ± 19	427 ± 12	413 ± 6
Urine flow rate (μL/min)	12 ± 1	22 ± 2**	8 ± 1	7 ±2	15 ± 3	20 ± 1*
Sodium excretion (μequiv./min)	1.3 ± 0.2	3.4 ± 0.6**	0.6 ± 0.1	0.4 ± 0.1	2.8 ± 0.9*	4.0 ± 0.5**
Free water clearance (μL/min)	-46 ± 4	-45 ± 6	-24 ± 4*	-34 ± 6	-49 ± 7	-66 ± 2*
Osmolar clearance (μL/min)	59 ± 6	66 ± 7	35 ± 5*	41 ± 7	65 ± 9	85 ± 3**

Table 2.1. Baseline values obtained before intrarenal clonidine or vehicle infusion. Veh, vehicle control; Clon, clonidine (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). These values represent the control collection following the stabilization/antagonist pre-treatment period.

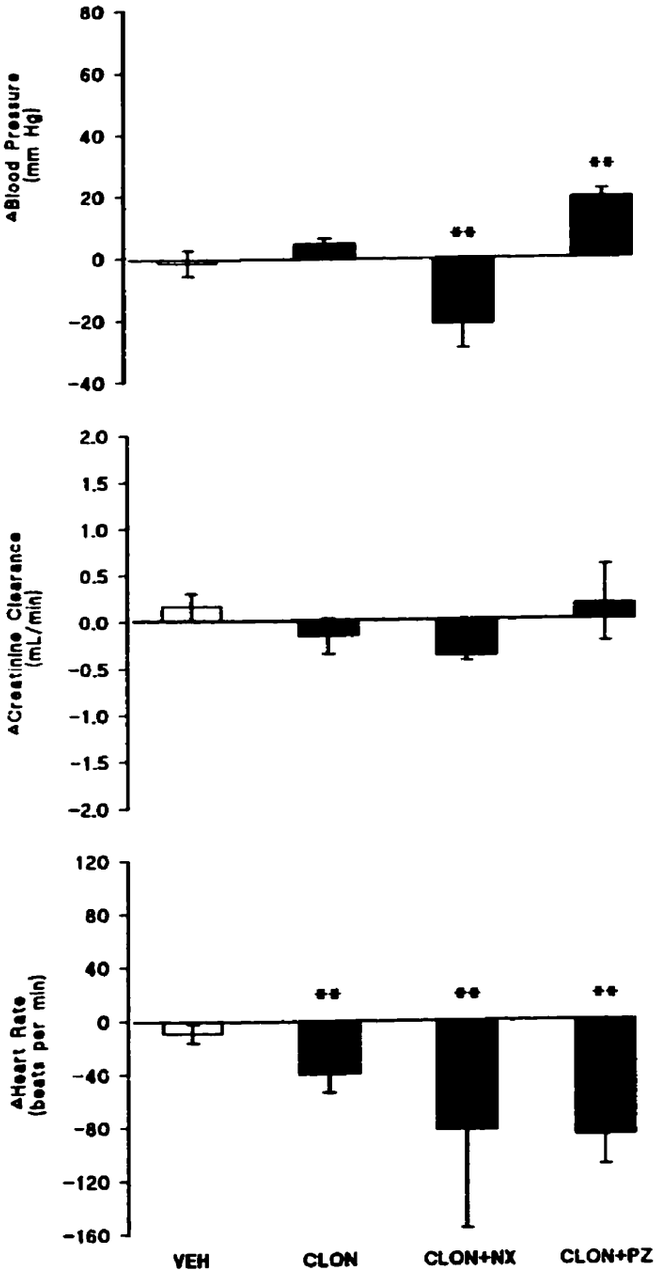

Figure 2.1. Effects of clonidine in the presence and absence of naltrexone or prazosin on blood pressure, creatinine clearance, and heart rate in the rat. VEH, vehicle control; CLON, clonidine (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments. $^{**}P<0.01$.

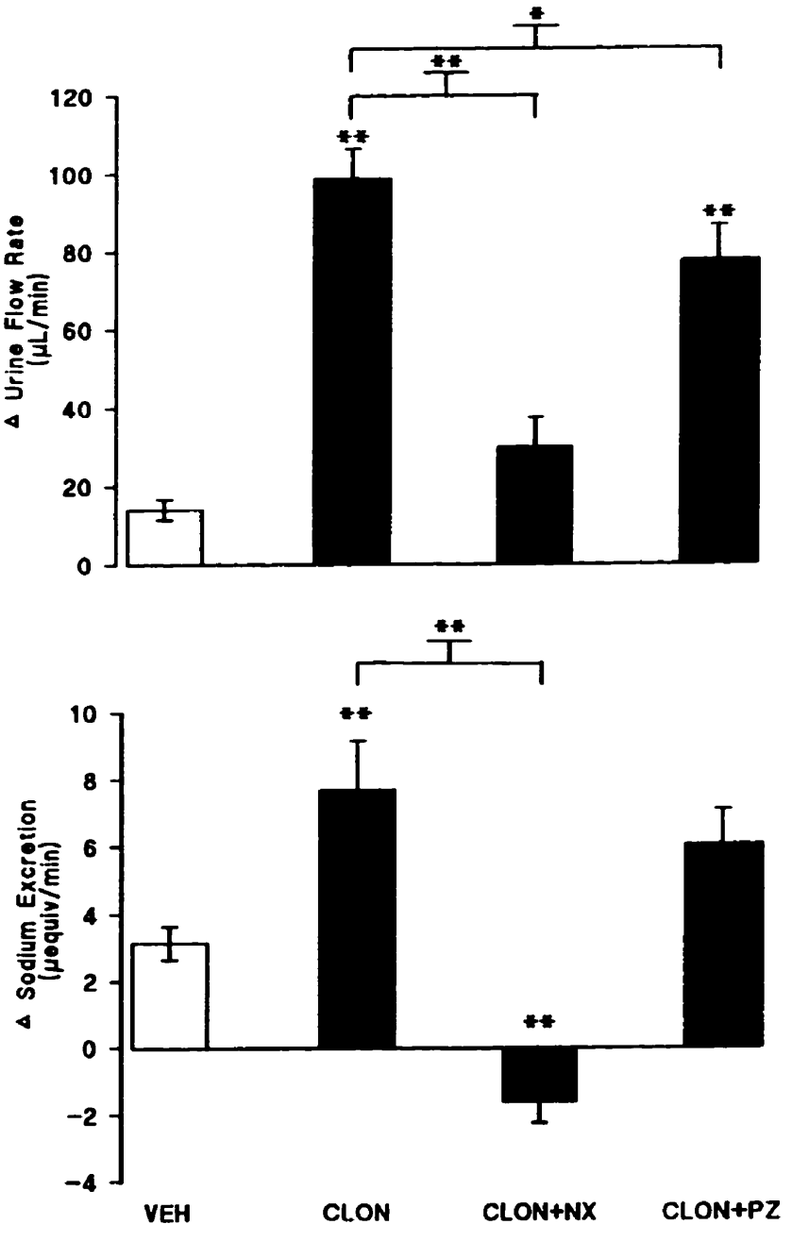

Figure 2.2. Effects of clonidine in the presence and absence of naltrexone or prazosin on urine flow rate and sodium excretion in the rat. VEH, vehicle control; CLON, clonidine (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

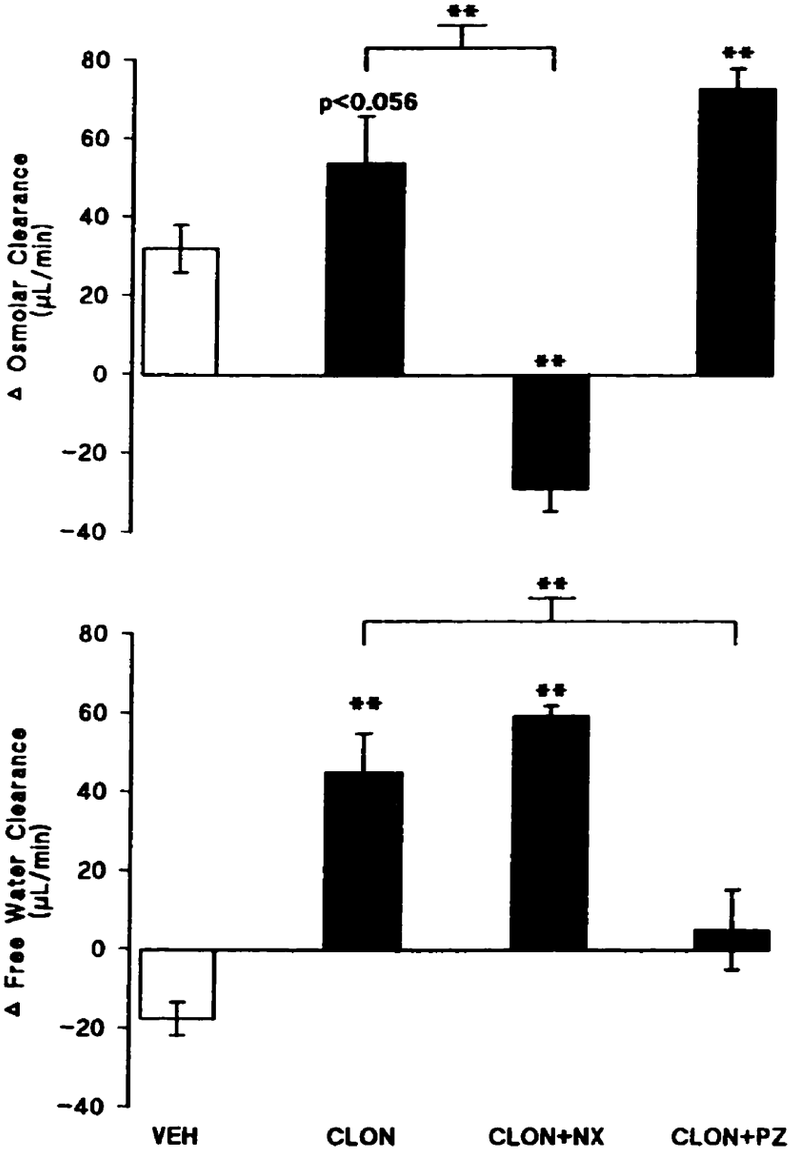

Figure 2.3. Effects of clonidine in the presence and absence of naltrexone or prazosin on osmolar and free water clearance in the rat. VEH, vehicle control; CLON, clonidine (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments. $^{**}P<0.01$.

Discussion

Clonidine has previously been reported to increase urine flow rate in the anesthetized rat by increasing free water and osmolar clearance (Blandford and Smyth, 1988a; 1991). In the dog, it was postulated that the renal effects of α_2-adrenoceptor stimulation with guanabenz were mediated by two anatomical sites and/or receptors (Strandhoy *et al.*, 1982). This was based on the observation that diuresis and natriuresis in response to intravenous administration of guanabenz were enhanced as compared to intrarenal infusion of this drug. Likewise, in the rat, both intravenous and intrarenal administration of clonidine produced increases in water and solute excretion (Blandford and Smyth, 1989). However, the intravenous infusion of clonidine was more potent in increasing sodium excretion. This suggested that the diuretic and natriuretic effects may have been mediated by an intrarenal and extrarenal site of action, respectively. The osmolar and free water effects of clonidine have also been dissociated by dose (Blandford and Smyth, 1988a) where low doses of clonidine produced an increase only in free water clearance. A higher dose was required to increase osmolar clearance indicating a second receptor with a lower affinity for clonidine may have been involved in this response. Studies with indomethacin pre-treatment were also consistent with this two-site/receptor hypothesis. Indomethacin potentiated the increase in osmolar clearance but had no effect or attenuated the increase in free water clearance (Blandford and Smyth, 1991).

The present study provided two further pharmacological lines of evidence which indicated that the renal effects of clonidine were independent of each other; that is,

dissociation by prazosin and dissociation by naltrexone. In a pharmacological study, such as this, consideration must be focused on the doses of the drugs utilized.

Previously in our laboratory, a dose response curve was determined for the pressor effects of clonidine (Blandford and Smyth, 1988a). The range of doses examined was 0.1 to 100 μg/kg/min of clonidine. A pressor response of approximately 25 mm Hg was observed with a clonidine infusion rate of 1.0 μg/kg/min. Blood pressure did not change in response to 0.3 μg/kg/min clonidine. Preliminary experiments showed that an infusion rate of 1.0 nmol/kg/min (equivalent to 0.27 μg/kg/min) produced increases in both osmolar and free water clearance. Since this dose produced the renal effects of interest without apparent blood pressure changes, further experiments were conducted with this infusion rate. Prazosin was administered as an intravenous bolus injection of 0.15 mg/kg. This dose was shown previously to have no effects on blood pressure or creatinine clearance in the same preparation (Penner and Smyth, 1994). The dose of naltrexone was selected after more circuitous research. In unrelated studies, doses as high as 10.0 to 50.0 mg/kg naltrexone (i.v.) have been utilized (Zeitune *et al.*, 1991; Trudeau *et al.*, 1991; Jessop *et al.*, 1989; Landymore and Wilkinson, 1988). In preliminary experiments, lower doses of naltrexone (1.0 and 3.0 mg/kg) were examined for effects on the renal response to clonidine. Whereas 1.0 mg/kg naltrexone had no apparent effect on the urine flow increase produced by clonidine, 3.0 mg/kg naltrexone appeared effective. In this preparation, it appeared that 10.0 mg/kg naltrexone was too high a dose as it became more difficult to maintain the rat in the anesthetized state. The moderate dose of 3.0 mg/kg naltrexone was therefore adopted for all further renal studies.

At these doses, prazosin significantly decreased the ability of clonidine to increase free water clearance without altering the osmolar response. In contrast, naltrexone attenuated the clonidine-induced osmolar response but failed to depress the increase in free water clearance. It was conceivable that two anatomical sites (for example, intrarenal *versus* systemic or alternatively, distal *versus* proximal segments within the nephron) were involved. Two different receptors at the same site could also have been involved. Finally, a combination of two anatomical sites as well as two receptors may have been responsible for the selective antagonism of these effects. In the following studies, irrespective of the anatomical sites involved, we considered the receptors which were involved in these effects, particularly in the osmolar response to clonidine.

Since prazosin is an α_1-adrenoceptor antagonist, it was rational to speculate that the increase in free water clearance, which was selectively blocked by prazosin, was mediated by α_1-adrenoceptors. However, the intravenous administration of an α_1-selective agonist, cirazoline, had no effect on urine flow rate, free water or osmolar clearance in the rat (Gellai and Ruffolo, 1987). It was then considered that prazosin may be blocking a different receptor. The α_2-adrenoceptors have been linked to decreased water permeability by antagonizing the renal effects of vasopressin (Krothapalli and Suki, 1984; Smyth *et al.*, 1985). Consistent with this suggestion, although clearly an α_1-adrenoceptor antagonist, prazosin has also displayed selectivity for the α_{2b}- and α_{2c}-adrenoceptor subtype over the $\alpha_{2a/d}$-subtypes (Bylund *et al.*, 1994). In fact, based on the low affinity of prazosin for the $\alpha_{2a/d}$-subtype, the initial distinction between $\alpha_{2a/d}$- and α_{2b}-subtypes was discerned (Bylund, 1985; Nahorski *et al.*, 1985). Since only $\alpha_{2a/d}$- and α_{2b}-

adrenoceptor subtypes have been detected in the rat kidney by radioligand binding studies (Uhlén and Wikberg, 1991; Le Jossec *et al.*, 1995a), the prazosin-sensitive free water response to clonidine was hypothesized to be mediated by the α_{2b}-subtype and independent of α_1-adrenoceptors. This contention has not yet been investigated further. Experiments with doxazosin may more conclusively exclude α_1-adrenoceptors from the clonidine-induced increase in free water clearance. Doxazosin is a selective α_1-adrenoceptor antagonist (K_i < 3.0 nM) with very low affinity for the α_{2b}-adrenoceptor subtype (K_i > 5000 nM) (Hieble *et al.*, 1995). It would also be useful to determine the dose-related effects of a selective α_{2b}-adrenoceptor agonist in the kidney in the presence and absence of naltrexone and prazosin. An increase in free water clearance would be anticipated without a concomitant change in osmolar clearance, thereby identifying a diuretic role of the α_{2b}-subtype. Such studies would hopefully also aid in excluding the α_{2b}-subtype from the osmolar response to clonidine. However, to our knowledge, such a selective α_{2b}-adrenoceptor agonist has not yet been identified. Currently in our laboratory, the effects of a selective α_{2b}-subtype antagonist, ARC-239 (Harrison *et al.*, 1991), on the renal effects of clonidine are being investigated.

The receptor mediating the naltrexone-sensitive increase in osmolar clearance following clonidine also remained unknown. Since the osmolar response to clonidine was intact following prazosin pre-treatment, this effect appeared to be independent of α_1-, α_{2b}-, and α_{2c}-adrenoceptors. It could have been hypothesized that naltrexone (opioid receptor antagonist) was competing for the same receptor as clonidine. However, preliminary radioligand binding studies showed that high millimolar concentrations of

naltrexone were required to displace [^{3}H]-rauwolscine from sites on rat renal membranes (data not shown). Clonidine showed effective displacement of [^{3}H]-rauwolscine at nanomolar concentrations (Smyth *et al.*, 1992a). For this reason, the hypothesis that clonidine and naltrexone were competing for the same receptor was not tested further.

Two possible receptors were considered as potential mediators of the osmolar response to clonidine. The first receptor considered was based on the premise that clonidine has been reported to be a mixed α_2-adrenoceptor/imidazoline receptor agonist (Ernsberger *et al.*, 1990). Renal imidazoline receptor stimulation has been shown to increase urine flow rate by increasing osmolar but not free water clearance (Allan *et al.*, 1993). Thus, imidazoline receptors may have been mediating the clonidine-induced osmolar clearance. Secondly, an α_2-adrenoceptor subtype other than the α_{2b}-receptor (for example, the $\alpha_{2a/d}$-subtype) may have been involved.

In conclusion, clonidine increased urine flow rate by increasing both free water and osmolar clearance. This response to clonidine was dissociated pharmacologically wherein the free water effect was prazosin-sensitive/naltrexone-insensitive and the osmolar effect was naltrexone-sensitive/prazosin-insensitive. These findings provided convincing evidence that the renal effects of clonidine were mediated by two distinct sites and/or receptors.

3

Clonidine-Induced Increase in Osmolar Clearance is Independent of Imidazoline Receptors

These data have been presented as an abstract at 38th Annual Meeting of the Canadian Federation of Biological Societies (CFBS). Saskatoon, Saskatchewan. These data have also been published: Intengan, H.D. and Smyth, D.D. Br. J. Pharmacol. **119**:663-670, 1996.

Summary:

We had established that the osmolar and free water effects of clonidine were mediated by two anatomical sites and/or receptors. We were interested in the receptor that mediated the increase in osmolar clearance produced by clonidine. Clonidine has been reported to be a mixed α_2-adrenoceptor/imidazoline receptor agonist. Therefore, in the next series of experiments, we tested the hypothesis that renal imidazoline receptors were mediating this naltrexone-sensitive osmolar response to clonidine. Male Sprague-Dawley rats were unilaterally nephrectomized 7 to 10 days prior to the day of the experiment. Moxonidine (3.0 nmol/kg/min) infused into the renal artery of the anesthetized rats increased urine flow rate and sodium excretion. Osmolar clearance increased as previously shown by others. Naltrexone pretreatment (3.0 mg/kg, i.v.) failed to attenuate the osmolar clearance increase elicited by moxonidine. This was in contrast to the clonidine-induced osmolar clearance which was abolished by naltrexone. These data suggested that the imidazoline receptor was not involved in the naltrexone-sensitive increase in osmolar clearance produced by clonidine.

Introduction

Having established that two anatomical sites and/or receptors were involved in the renal effects of clonidine, we were interested in the receptor which mediated the increase in osmolar clearance. It was firstly considered that clonidine was producing this osmolar response by stimulating a receptor other than an α_2-adrenoceptor - perhaps the imidazoline receptor.

The speculation that clonidine may stimulate a receptor other than the α_2-adrenoceptor originated from earlier studies which proposed the existence of "imidazoline" receptors. It was accepted that the hypotensive actions of some centrally acting compounds such as clonidine were attributable to α_2-adrenoceptor stimulation (Timmermans *et* al., 1981; van Zwieten *et al.*, 1983). Ensuing studies investigating the depressor effects of various compounds suggested that an additional site may have been involved. Bousquet *et al.* (1984) examined various α_1- and α_2-adrenoceptor agonists and their depressor effects when administered into the nucleus reticularis lateralis in the anesthetized cat. It was expected that the effects/non-effects of these drugs would be classifiable based on their α-adrenoceptor subtype selectivity. Instead, compounds with phenylethylamine moieties (i.e. α-methylnorepinephrine) had no blood pressure effects. Agonists with imidazoline moieties (i.e. clonidine, cirazoline) consistently decreased blood pressure irrespective of their α-subtype selectivity. These data supported the earlier postulate that imidazoline-preferring sites existed (Ruffolo *et al.*, 1977). The hypothesis was later tested that clonidine was exerting its hypotensive action in the rostral ventrolateral medulla by stimulating these putative imidazoline-preferring sites. Clonidine,

p-amino-clonidine, cimetidine, and imidazole-4-acetic acid (all compounds with an imidazole moiety) decreased blood pressure in the rat when administered into this brain region. Idazoxan, an imidazole, blocked the hypotensive effect of clonidine. SKF-86466, a non-imidazole α_2-antagonist, failed to have any effect on the depressor response to clonidine. The hypotensive activity of clonidine appeared to correlate with its affinity for the imidazoline-preferring site (Ernsberger *et al.*, 1990).

The finding that clonidine could stimulate a putative imidazoline-preferring site was consistent with radioligand binding studies. In bovine ventrolateral medullary membranes, [^{3}H]-p-aminoclonidine was only partially displaced by phenylethylamines such as norepinephrine. The remainder of the [^{3}H]-p-aminoclonidine bound to low-affinity "alpha-2" sites (30%) and was displaced by imidazole-containing compounds (Ernsberger *et al.*, 1987). A potentially endogenous ligand for this imidazoline-preferring site had also been isolated from rat brain (Atlas and Burstein, 1984). Based on its ability to displace clonidine from rat brain sites, the compound was termed "clonidine-displacing substance." This compound, a non-catecholamine, also displaced [^{3}H]-p-aminoclonidine binding in bovine ventrolateral medullary membranes but with a 30-fold selectivity for the low-affinity alpha-2 sites (imidazoline-preferring sites?). It was subsequently speculated that clonidine-displacing substance was an endogenous ligand for the imidazoline preferring sites (Ernsberger *et al.*, 1988).

The literature reports discussed here collectively suggested that clonidine may be a mixed α_2-adrenoceptor/imidazoline receptor agonist. Based on these reports, we hypothesized that clonidine may have increased osmolar clearance by stimulating

imidazoline receptors. In support of this hypothesis, moxonidine, a selective imidazoline receptor agonist, increased urine flow by increasing only osmolar clearance in the anesthetized rat (Allan *et al.*, 1993; Penner and Smyth, 1994).

In the present study, the hypothesis that imidazoline receptors were mediating the osmolar response to clonidine was tested using the imidazoline receptor agonist, moxonidine. If clonidine and moxonidine were increasing osmolar clearance by stimulating the same receptor, that is, the imidazoline receptor, then similar mechanisms of action could be expected. Thus, the osmolar response to moxonidine would be, as with clonidine, attenuated by naltrexone pre-treatment.

Methods

Experimental Preparation

The general procedures and statistical analysis have been described previously in Section 2 - Methods. The pre-experimental care, surgical preparation, and analyses were the same. Differences in the experimental procedures are summarized below.

Pharmacological Treatment

After surgery (time = 0 min), the continuous i.v. infusion (0.097 mL/min) of normal saline was initiated. The rats were allowed to stabilize for 45 min (time = 0 - 45 min). Naltrexone was administered 15 min into the stabilization period as a slow i.v. bolus (0.2 mL) over 1 min. Following the stabilization period, a 30 min control urine collection (time = 45 - 75 min) was obtained. After the control collection period, the intrarenal infusion (0.0034 mL/min) of moxonidine or vehicle (0.9% saline) was initiated and maintained for the remainder of the experiment. Two subsequent urine collections were obtained (times = 75 - 105 and 105 - 135 min). Urine was collected into pre-weighed vials. Urine flow rate was determined gravimetrically.

Effects of naltrexone on the renal effects of moxonidine

Animals were randomly assigned to one of four study groups, each consisting of six rats. Group 1, the vehicle control group, received an intrarenal infusion of 0.9% saline at 0.0034 mL/min. Group 2 received naltrexone (3.0 mg/kg) alone. Group 3 received an

intrarenal infusion of moxonidine (3.0 nmol/kg/min). Group 4 received pretreatment with naltrexone followed by an infusion of moxonidine.

Drugs

Moxonidine was a gift from Beiersdorf, AG (Hamburg, Germany). Naltrexone was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A).

Results

Preparation controls

Data from the first collection period were analyzed to determine the similarity between groups following the surgery. Baseline values of blood pressure, creatinine clearance, heart rate and others parameters of interest are shown in Table 3.1. Aside from differences in creatinine clearance between two of the groups, no other differences in baseline values were detected. Data have been presented as the absolute difference between baseline and final collection values. Presenting the delta values highlighted the different magnitudes of responses between groups.

Effects of naltrexone on the renal response to moxonidine

As compared to the control group, blood pressure increased in the moxonidine group (figure 3.1). However, blood pressure was not altered in the groups receiving naltrexone alone or naltrexone with moxonidine. Creatinine clearance decreased in the naltrexone group (figure 3.1). No changes in heart rate were observed. Moxonidine increased urine flow rate and sodium excretion (figure 3.2). This renal response was reflected by an increase in osmolar clearance and a decrease in free water clearance (figure 3.3). Pretreatment with naltrexone did not affect the ability of moxonidine to increase urine flow rate, sodium excretion, and osmolar clearance.

	Veh (n = 9)	Mox (n = 6)	NX (n = 6)	Mox + NX (n = 6)
Blood pressure (mm Hg)	113 ± 7	106 ● 3	107 ± 4	114 ± 5
Creatinine clearance (mL/min)	1.1 ± 0.1	1.7 ± 0.2**	1.8 ± 0.2**	1.4 ± 0.1
Heart Rate (beats per min)	417 ± 15	387 ± 15	413 ● 12	417 ± 11
Urine flow rate (μL/min)	15 ± 3	20 ± 4	17 ± 1	16 ± 2
Sodium excretion (μequiv./min)	2.4 ± 0.7	1.8 ± 0.4	2.2 ● 0.3	1.4 ● 0.3
Free water clearance (μL/min)	-50 ± 6	-45 ● 6	-63 ± 2	-38 ± 5
Osmolar clearance (μL/min)	65 ± 8	65 ● 8	80 ± 3	54 ± 7

Table 3.1. Baseline values obtained before intrarenal moxonidine or vehicle infusion. Veh, vehicle control; Mox, moxonidine (3.0 nmol/kg/min); NX, naltrexone (3.0 mg/kg, i.v.). These values represent the control collection following the stabilization/antagonist pretreatment period.

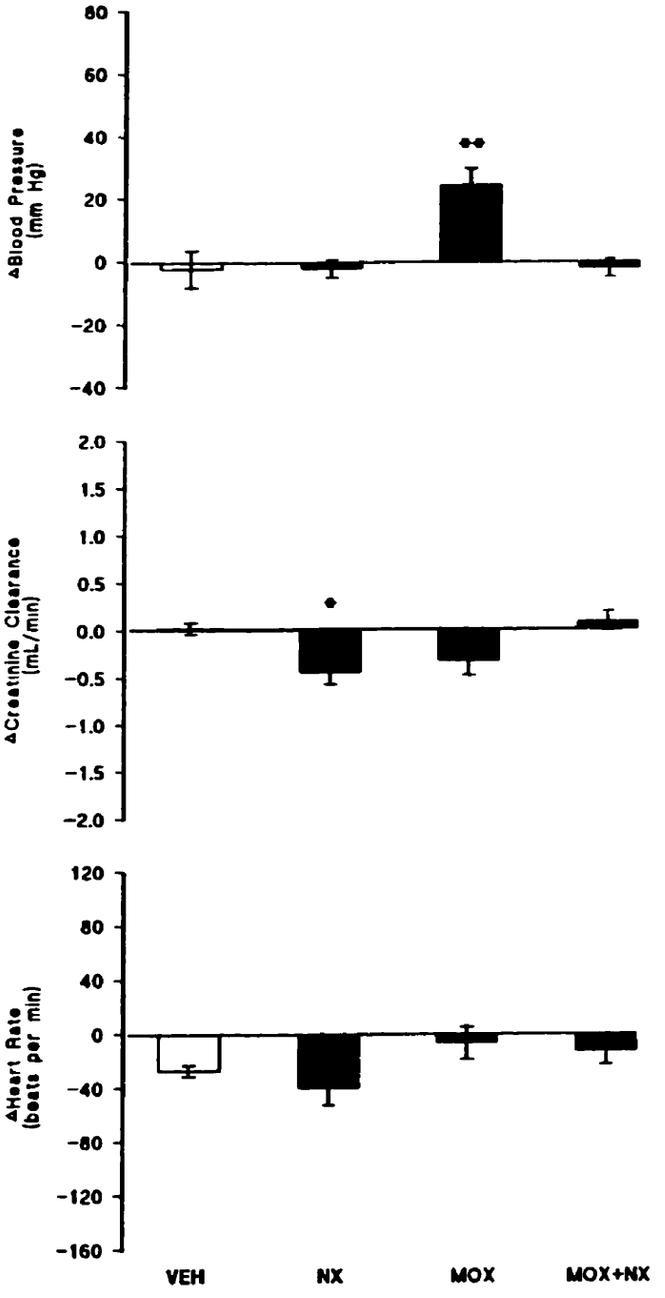

Figure 3.1. Effects of moxonidine in the presence and absence of naltrexone on blood pressure, creatinine clearance, and heart rate in the rat. VEH, vehicle control; MOX, moxonidine (3.0 nmol/kg/min); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of six experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

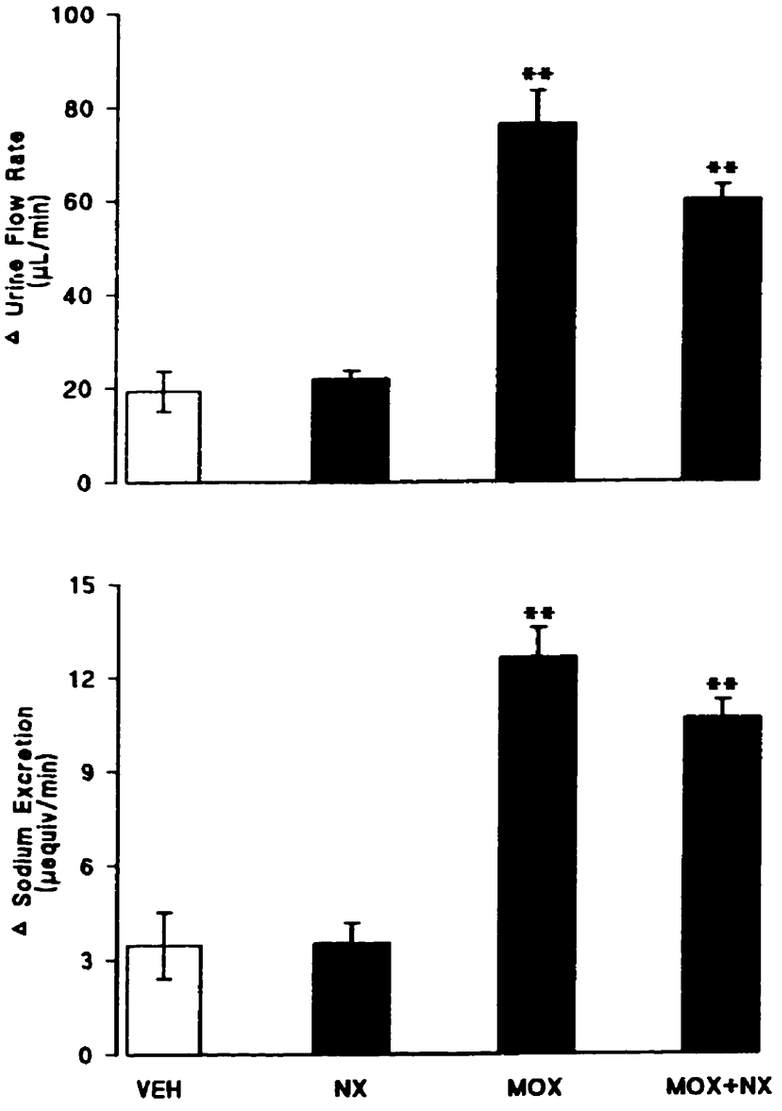

Figure 3.2. Effects of moxonidine in the presence and absence of naltrexone on urine flow rate and sodium excretion in the rat. VEH, vehicle control; MOX, moxonidine (3.0 nmol/kg/min); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{**}P<0.01$.

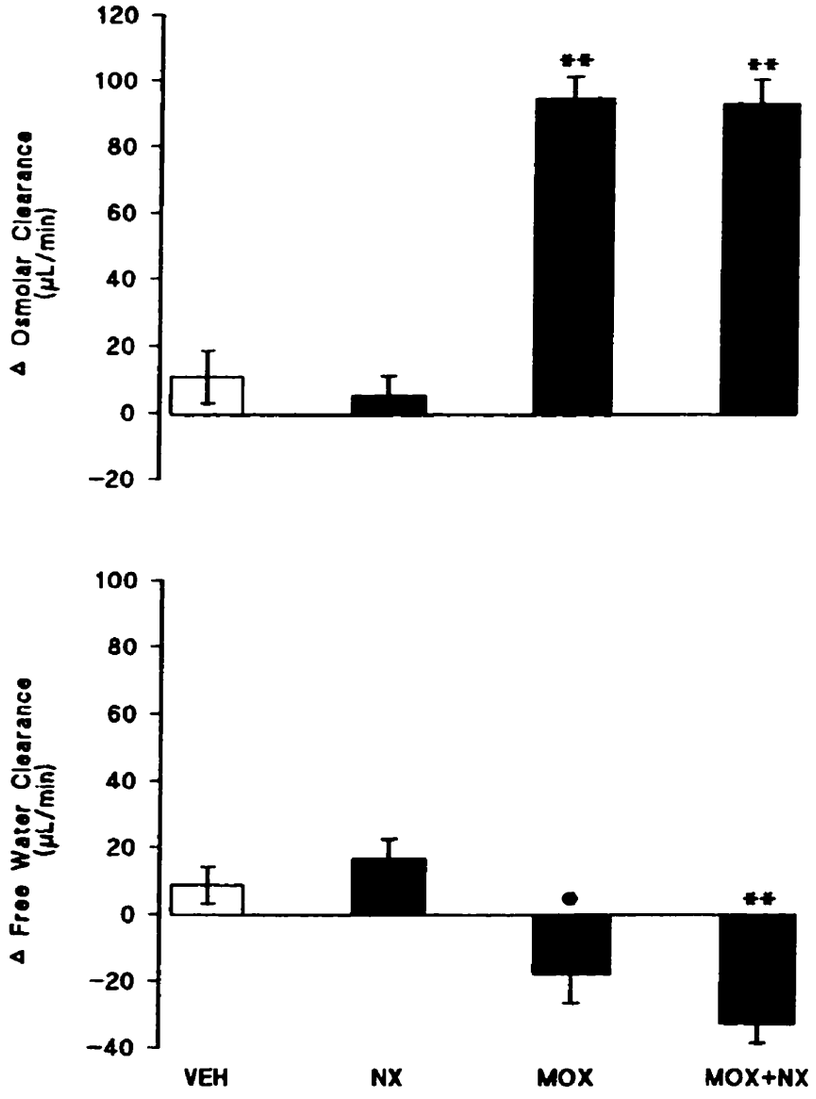

Figure 3.3. Effects of moxonidine in the presence and absence of naltrexone on osmolar and free water clearance in the rat. VEH, vehicle control; MOX, moxonidine (3.0 nmol/kg/min); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

Discussion

In this series of experiments, we tested the hypothesis that clonidine was increasing osmolar clearance by stimulating imidazoline receptors. To do this, we used the finding that the clonidine-induced osmolar response was naltrexone-sensitive. If imidazoline receptors were involved in this response, then an increase in osmolar clearance produced by imidazoline receptor stimulation would also be naltrexone-sensitive.

Experiments were designed so that imidazoline receptors and not α_2-adrenoceptors were stimulated in the rat kidney. For this purpose, the agonist, moxonidine, was utilized. Ernsberger *et al.*, (1993) have demonstrated that moxonidine has a 600-fold higher affinity for imidazoline receptors than for α_2-adrenoceptors, including both the $\alpha_{2a/d}$- and α_{2b}-subtypes which are found in the rat kidney (Uhlén and Wikberg, 1991). Previously in our laboratory, dose response curves (1.0, 3.0, and 10.0 nmol/kg/min) have been determined for the renal effects of moxonidine (Allan *et al.*, 1993). In these studies, an infusion rate of 3.0 nmol/kg/min moxonidine was used as this dose was previously shown to produce renal effects without significant effects on blood pressure or creatinine clearance. The dose of naltrexone utilized was that previously determined to attenuate the osmolar effect of clonidine.

As previously reported (Allan *et al.*, 1993; Penner and Smyth, 1994), moxonidine increased urine flow rate and sodium excretion. Osmolar clearance but not free water clearance was increased. Naltrexone pretreatment failed to attenuate the osmolar response to moxonidine. This was in contrast to the complete blockage of the osmolar response to clonidine by naltrexone. These data indicated that the naltrexone-sensitive

ability of clonidine to increase osmolar clearance could not have been mediated by imidazoline receptors. At some point, the mechanisms of action involved in the osmolar effects of clonidine and moxonidine were likely different. The clonidine-induced increase in osmolar clearance involved opioid receptors. The moxonidine-induced increase in osmolar clearance was independent of opioid receptors. The previously reported effects of indomethacin on the renal response to clonidine and moxonidine also suggested that distinct mechanisms were involved in the osmolar effects of these agonists. A dose of indomethacin (5.0 mg/kg) which attenuated the osmolar response to moxonidine (Darkwa and Smyth, 1995) instead potentiated the osmolar response to clonidine (Blandford and Smyth, 1991). The distinct mechanisms with respect to opioid receptors and prostaglandins which were stimulated by clonidine and moxonidine suggested that two different receptors were involved.

Although contrary to the hypothesis that imidazoline receptors were involved in the osmolar response to clonidine, these results were not completely unanticipated. Other reports have suggested the renal effects of clonidine were independent of tubular imidazoline receptors. One study used the antagonists idazoxan and rauwolscine at doses selective for imidazoline receptors and α_2-adrenoceptors, respectively. Idazoxan blocked the renal effects of moxonidine but not of clonidine. Rauwolscine attenuated the renal effects of clonidine but not of moxonidine (Allan *et al.*, 1993). Moreover, in one kidney-one clip hypertensive rats, the renal responses to imidazoline receptor agonists, moxonidine and rilmenidine were at least in part, attenuated (Li *et al.*, 1994). The renal response to clonidine, however, remained intact in these animals (Li and Smyth, 1993).

In conclusion, clonidine increased osmolar clearance in a naltrexone-sensitive/prazosin-insensitive manner. The hypothesized involvement of imidazoline receptors in this response was contingent on the osmolar effect of imidazoline receptor stimulation also being naltrexone-sensitive. This study demonstrated that naltrexone was unable to affect moxonidine-induced increases in osmolar clearance. These findings negated the postulate that imidazoline receptors were mediating the osmolar response to clonidine.

4

Renal Function of the $\alpha_{2a/d}$-Adrenoceptor Subtype

These data have been presented as abstracts at the XIth Scientific Meeting, The Inter-American Society of Hypertension, Montréal, Québec; 1995 Annual Meeting of the Royal College of Physicians and Surgeons of Canada, CSCI and participating Societies, Montréal, Québec; ASPET Colloquium: Alpha2-adrenergic receptors: structure, function & therapeutic implications, Nashville, Tennessee; British Pharmacological Society/ The Pharmacological Society of Canada/Canadian Society of Clinical Pharmacology Joint Meeting, Bath, UK.

Summary:

We had determined that the osmolar and free water responses to clonidine were mediated by two distinct anatomical sites and/or receptors. Subsequent to this finding, we considered two possible receptors which may have been mediating the osmolar response to clonidine: the imidazoline receptor and the $\alpha_{2a/d}$-adrenoceptor subtype. Having established that the imidazoline receptor was not involved in this effect of clonidine, we proceeded to test the hypothesis that the $\alpha_{2a/d}$-adrenoceptor subtype mediated osmolar clearance. To do these studies, we used two relatively selective $\alpha_{2a/d}$-selective agonists, UK-14,304 and guanfacine. Studies were conducted in anesthetized Sprague-Dawley rats that were unilaterally nephrectomized seven to ten days prior to the experiment. The infusion of UK-14,304 (1.0 nmol/kg/min) or guanfacine (3.0 nmol/kg/min) into the remaining renal artery increased urine flow rate and sodium excretion. These responses were secondary only to increased osmolar clearance. At these doses, no changes in free water clearance were observed. As with the osmolar response to clonidine, the osmolar responses to UK-14,304 and guanfacine were attenuated by naltrexone (3.0 mg/kg) but not by prazosin (0.15 mg/kg). We also determined the renal response to guanfacine in response to the selective $\alpha_{2a/d}$-adrenoceptor antagonist, RX-821002. RX-821002 (3.0 mg/kg) attenuated the ability of guanfacine to increase urine flow rate and osmolar clearance. These results were consistent with the $\alpha_{2a/d}$-adrenoceptor subtype in the rat

kidney mediating an increase in urine flow rate secondary to an increase in osmolar clearance.

Introduction

We had at this point established that two distinct mechanisms were involved in the osmolar and free water responses to clonidine. Two possible receptors were being considered as mediators of the osmolar effect of clonidine. The first, the imidazoline receptor, was excluded in part, on the basis that the osmolar response to moxonidine was naltrexone-insensitive. The hypothesis that the second possible receptor, the $\alpha_{2a/d}$-adrenoceptor, mediated osmolar clearance was tested in this next series of experiments.

The distribution of α_2-adrenoceptor mRNA in the rat kidney has been investigated by *in situ* hybridization. $\alpha_{2a/d}$- and α_{2b}-Adrenoceptor mRNAs were widely distributed whereas the α_{2c}-subtype mRNA was distributed to a much lesser extent (Meister *et al.*, 1994). Only the $\alpha_{2a/d}$- and α_{2b}-subtypes have been identified in the rat kidney by radioligand binding studies (Uhlén and Wikberg, 1991). If only the $\alpha_{2a/d}$- and α_{2b}-subtypes are found in the rat kidney, clonidine may have increased osmolar clearance by stimulating the $\alpha_{2a/d}$-adrenoceptor subtype. For this hypothesis to be supported, several criteria were required to be fulfilled. Firstly, selective stimulation of only the renal $\alpha_{2a/d}$-adrenoceptor subtype (and not the α_{2b}-subtype) would be expected to increase osmolar clearance and not change free water clearance. As with the osmolar response to clonidine, the osmolar effect of $\alpha_{2a/d}$-adrenoceptor stimulation would be attenuated by naltrexone but unaffected by prazosin pretreatment. It was also hypothesized that with a relatively selective $\alpha_{2a/d}$-subtype antagonist, the osmolar response to an $\alpha_{2a/d}$-subtype agonist would be attenuated.

As previously discussed, to more convincingly confirm the role of the $\alpha_{2a/d}$-subtype and non-involvement of the α_{2b}-subtype in mediation of osmolar clearance, the same studies would ideally be conducted with an α_{2b}-selective agonist. Selective stimulation of the α_{2b}-subtype would be expected to have no effect on osmolar clearance and to increase free water clearance. This free water effect would also be attenuated by prazosin but unaffected by naltrexone. Unfortunately, to date an α_{2b}-selective agonist has yet to be identified.

Nevertheless, we used the currently available selective pharmacological agents (table 4.1) to investigate the effects of $\alpha_{2a/d}$-adrenoceptor stimulation in the rat kidney. The purported selective $\alpha_{2a/d}$-agonist UK-14,304 (MacKinnon *et al.*, 1994) and the clearly selective $\alpha_{2a/d}$-agonist guanfacine (Uhlén and Wikberg, 1991) were administered in the presence and absence of naltrexone and prazosin pretreatment to anesthetized rats and observed for renal effects. The relatively selective $\alpha_{2a/d}$-adrenoceptor antagonist (RX-821002) co-administered with guanfacine was used to further assess the relationship between stimulation of this receptor subtype and osmolar clearance.

	K_d (nM) $\alpha_{2a/d}$	K_d (nM) α_{2b}
clonidine	84.4 ± 4.5	71.3 ● 1.4
UK-14,304	410 ± 63	636 ± 49
guanfacine	30.9 ± 3.7	1850 ± 179
prazosin	1360 ± 78	52.1 ± 3.0
RX-821002	0.68 ± 0.04	4.58 ± 0.20

Table 4.1. Pharmacological characteristics of adrenergic agents relevant to the $\alpha_{2a/d}$- and α_{2b}-adrenoceptor subtypes. Affinities for the $\alpha_{2a/d}$- and α_{2b}-adrenoceptor subtypes were determined simultaneously using [^{3}H]RX-821002 radioligand binding in the rat kidney. Data from Uhlén and Wikberg, 1991.

Methods

Experimental Preparation

The general procedures have been described previously in Section 2 - Methods. The pre-experimental care, surgical preparation, and analyses were the same. Differences in the experimental procedures are summarized below.

Pharmacological Treatment

After surgery (time = 0 min), the continuous i.v. infusion (0.097 mL/min) of normal saline was initiated. The rats were allowed to stabilize for 45 min (time = 0 - 45 min). Prazosin was administered immediately following the surgery (t = 0 min) as a slow intravenous bolus (0.2 mL) over 1 min. Naltrexone or RX-821002 were likewise administered 15 min following the start of the stabilization period (t = 15 min). Following the stabilization period, a 30 min control urine collection (time = 45 - 75 min) was obtained. After the control collection, the intrarenal infusion (0.0034 mL/min) of vehicle (0.9% saline) or agonist (guanfacine or UK-14,304) was initiated and maintained for the remainder of the experiment. Two subsequent urine collections were obtained (time = 75 - 105 and 105 - 135 min). Urine was collected into pre-weighed vials. Urine flow rate was determined gravimetrically.

Renal response to UK-14,304; effects of naltrexone or prazosin pretreatment

Animals were randomly assigned to one of six study groups, each consisting of at least six rats. Group 1 received an intrarenal infusion of the vehicle (0.9% saline). Groups

2 and 3 respectively received intravenous injections of naltrexone (3.0 mg/kg) or prazosin (0.15 mg/kg) only. Group 4 received an intrarenal infusion of UK-14,304 (1.0 nmol/kg/min). Groups 5 and 6 received prazosin or naltrexone respectively, followed by an infusion of UK-14,304.

Renal response to guanfacine; effects of naltrexone or prazosin pretreatment

Animals were randomly assigned to one of six study groups, each consisting of six rats. Group 1, the vehicle control group, received an intrarenal infusion of the vehicle (0.9% saline). Groups 2 and 3 received naltrexone (3.0 mg/kg) or prazosin (0.15 mg/kg) alone, respectively. Group 4 received an intrarenal infusion of guanfacine (3.0 nmol/kg/min). Groups 5 and 6 received pretreatment with prazosin or naltrexone followed by guanfacine.

Renal response to guanfacine; effects of RX-821002

Animals were randomly assigned to one of four study groups, each consisting of six rats. Group 1 received an intrarenal infusion of the vehicle, 0.9% saline. Group 2 received RX-821002 (3.0 mg/kg, i.v.). Group 3 received an intrarenal infusion of guanfacine (3.0 nmol/kg/min). Group 4 received pretreatment with RX-821002, followed by intrarenal administration of guanfacine.

Statistical Analysis

Data are presented as the mean ± standard error (s.e.m.). Data were analyzed by repeated measures of analysis of variance (ANOVA) using the SAS software package, version 6.07 (UK-14,304 studies) or the Systat software package, version 5.0 (guanfacine studies). Significant differences were further located by Fisher's least squared difference multiple comparison test (UK-14,304 studies) or by the Tukey multiple comparison test (guanfacine studies). Significance is denoted with * representing $p<0.05$ and ** representing $p<0.01$.

Drugs

Prazosin and naltrexone were obtained from Sigma Chemical Co., St. Louis, MO. Guanfacine (Wyeth-Ayerst), RX-821002 (Research Biochemicals International) and UK-14,304 (Pfizer Central Research) were also used in the present studies.

Results

Preparation Controls

Data from the first collection period were analyzed to assess the similarities between groups following surgery. Baseline levels are shown in Tables 4.2, 4.3, and 4.4. Due to differences between baseline levels, the data were presented as the difference between baseline and third collection values. Presenting the data in this manner highlighted the magnitude of responses between groups.

Renal response to UK-14,304; effects of naltrexone or prazosin pretreatment

In this set of experiments, blood pressure, creatinine clearance, and heart rate were unaltered by experimental intervention (Figure 4.1). Intrarenal infusion of UK-14,304 increased urine flow rate and sodium excretion (Figure 4.2). This response was reflected solely by increased osmolar clearance. Free water clearance was unaltered by UK-14,304 (Figure 4.3). Whereas prazosin pretreatment had no effect on the renal response to UK-14,304 (Figures 4.2 and 4.3), naltrexone attenuated completely the effects of UK-14,304 on urine flow rate, sodium excretion (Figure 4.2) and osmolar clearance (Figure 4.3).

Renal response to guanfacine; effects of naltrexone or prazosin pretreatment

Again, blood pressure, creatinine clearance, and heart rate were unaltered by pharmacological intervention (Figure 4.4). Guanfacine increased urine flow rate and sodium excretion (Figure 4.5) by increasing only osmolar clearance and not free water clearance (Figure 4.6). Prazosin pretreatment failed to alter the renal response to

guanfacine (Figures 4.5 and 4.6), whereas naltrexone completely abolished the increases in urine flow rate, sodium excretion, and osmolar clearance produced by guanfacine (Figures 4.5 and 4.6).

Renal response to guanfacine; effects of RX-821002

Blood pressure and creatinine clearance were not affected by guanfacine and/or RX-821002. However, in the group that received guanfacine alone, heart rate was decreased (Figure 4.7). Guanfacine increased urine flow rate and sodium excretion although the latter did not reach statistical significance (Figure 4.8). This lack of significant effect is in contrast to the sodium excretion increase observed in the guanfacine group within the prazosin/naltrexone studies (see above). This discrepancy may be due to the apparently high control sodium excretion in this series. We examined the raw data and it appeared that this high control level was due to increased baseline values in two experiments of the six conducted. As expected, guanfacine did not increase free water clearance. The increased urine flow rate was due solely to increased osmolar clearance (Figure 4.9). Pretreatment with RX-821002 attenuated the guanfacine-induced urine flow rate (Figure 4.8) and osmolar clearance (Figure 4.9).

	Veh (n = 9)	UK (n = 8)	PZ (n = 9)	UK + PZ (n = 8)	NX (n = 6)	UK + NX (n = 6)
Blood pressure (mm Hg)	118 ± 5	123 ± 3	98 ± 5**	114 ± 4	118 ± 4	116 ± 2
Creatinine clearance (mL/min)	1.5 ± 0.2	1.3 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
Heart Rate (beats per min)	416 ± 15	398 ± 7	398 ± 8	395 ± 9	403 ± 11	397 ± 13
Urine flow rate (µL/min)	15 ± 2	20 ± 3	9 ± 1*	9 ± 2	17 ± 4	11 ± 1
Sodium excretion (µequiv./min)	1.6 ± 0.3	2.2 ± 0.3	0.7 ± 0.2*	0.9 ± 0.2	1.8 ± 0.6	0.7 ± 0.1
Free water clearance (µL/min)	-34 ± 3	-42 ± 3	-34 ± 4	-40 ± 5	-41 ± 4	-41 ± 3
Osmolar clearance (µL/min)	49 ± 5	61 ± 4	43 ± 5	50 ± 6	57 ± 5	52 ± 3

Table 4.2. Baseline values obtained before intrarenal UK-14,304 or vehicle infusion. Veh, vehicle control; UK, UK-14,304 (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). These values represent the control collection following the stabilization/antagonist pre-treatment period.

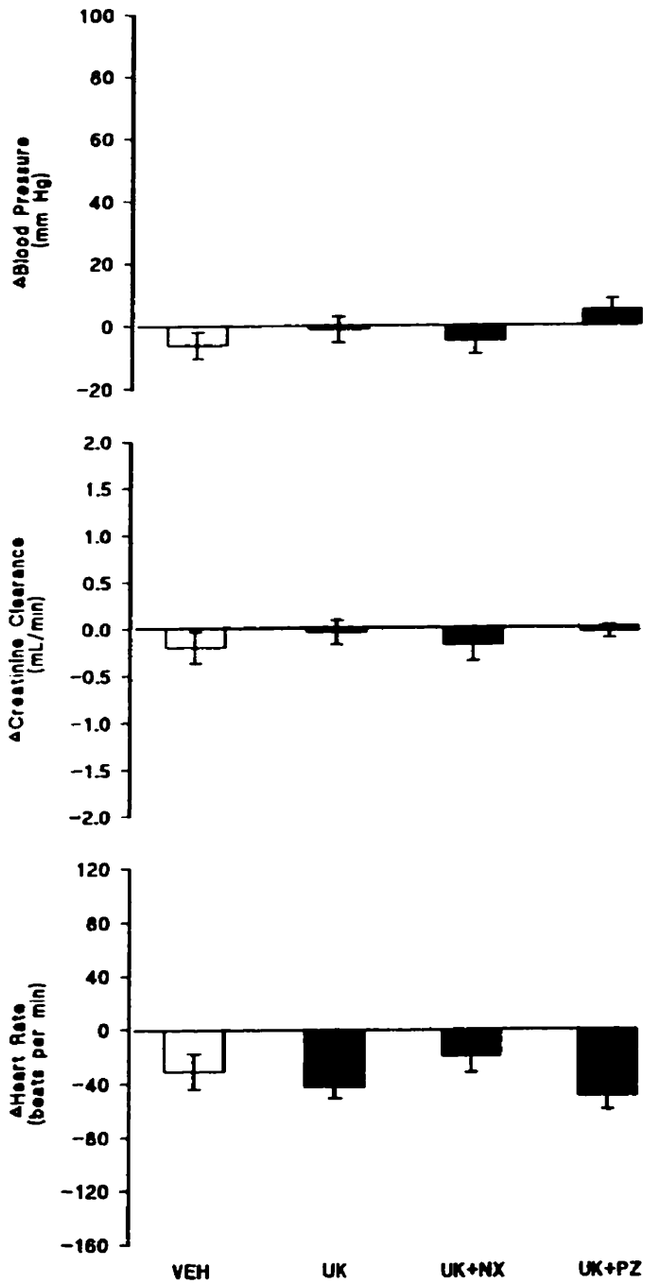

Figure 4.1. Effects of UK-14,304 in the presence and absence of naltrexone or prazosin on blood pressure, creatinine clearance, and heart rate in the rat. VEH, vehicle control; UK, UK-14,304 (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments.

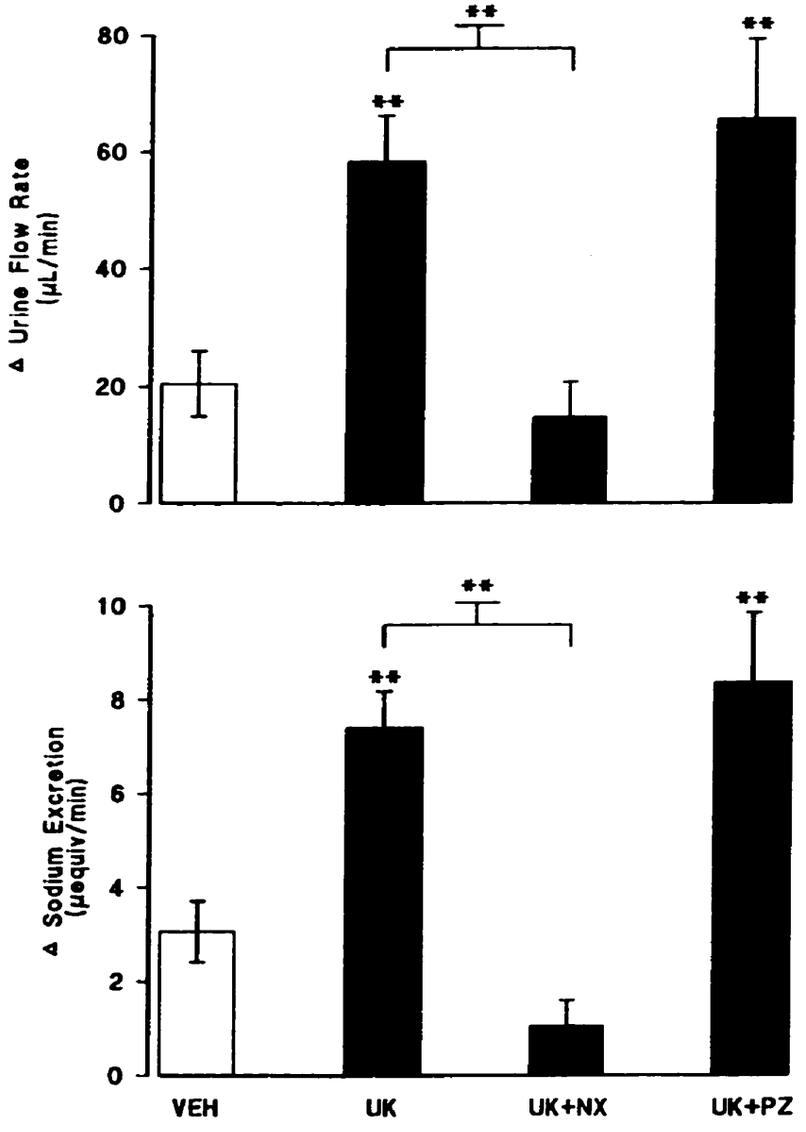

Figure 4.2. Effects of UK-14,304 in the presence and absence of naltrexone or prazosin on urine flow rate and sodium excretion in the rat. VEH, vehicle control; UK, UK-14,304 (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments. $^{**}P<0.01$.

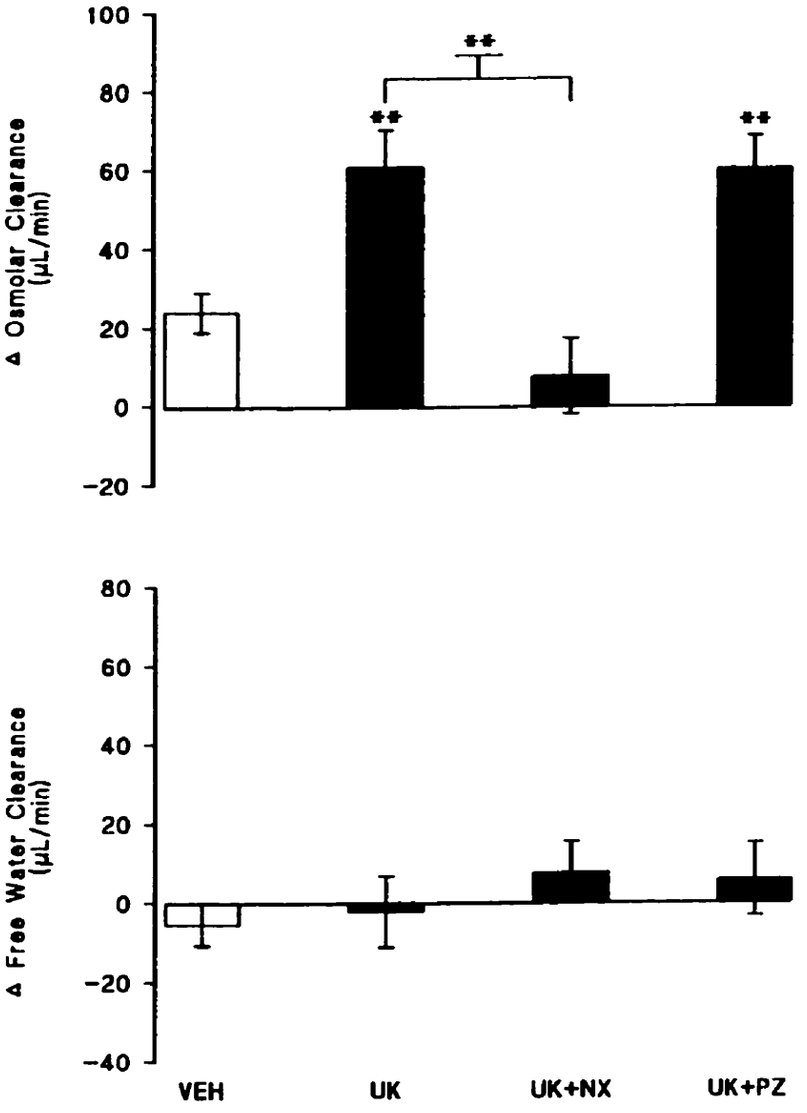

Figure 4.3. Effects of UK-14,304 in the presence and absence of naltrexone or prazosin on osmolar and free water clearance in the rat. VEH, vehicle control; UK, UK-14,304 (1.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of at least 6 experiments. $^{**}P<0.01$.

	Veh (n = 6)	GF (n = 6)	PZ (n = 6)	GF + PZ (n = 6)	NX (n = 6)	GF + NX (n = 6)
Blood pressure (mm Hg)	122 ± 8	120 ± 4	95 ± 2*	100 ± 8	111 ± 4	105 ± 3
Creatinine clearance (mL/min)	1.8 ± 0.3	1.4 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	1.6 ± 0.2	1.7 ± 0.3
Heart Rate (beats per min)	413 ± 4	423 ± 21	403 ± 14	440 ± 9	392 ± 4	393 ± 13
Urine flow rate (μL/min)	14 ± 2	11 ± 2	8 ± 1	13 ± 3	21 ± 3	17 ± 3
Sodium excretion (μequiv./min)	1.3 ± 0.6	1 1± 0.02	0.4 ± 0.1	1.3 ± 0.4	1.1 ± 0.2	2.1 ± 0.5
Free water clearance (μL/min)	-40 ± 4	-30 ± 8	-30 ± 4	-32 ± 4	-38 ± 9	-46 ± 7
Osmolar clearance (μL/min)	54 ± 4	41 ± 4	37 ± 5	45 ± 7	59 ± 10	63 ± 9

Table 4.3. Baseline values obtained before intrarenal guanfacine or vehicle infusion. Veh, vehicle control; GF, guanfacine (3.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). These values represent the control collection following the stabilization/antagonist pre-treatment period.

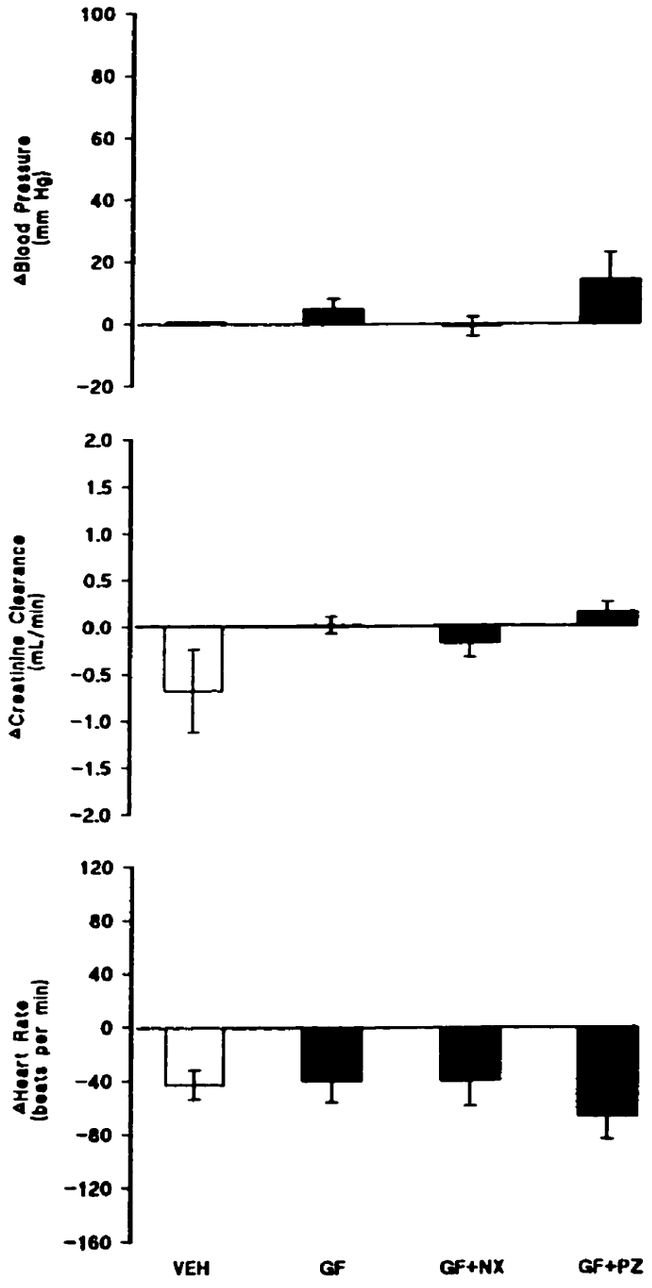

Figure 4.4. Effects of guanfacine in the presence and absence of naltrexone or prazosin on blood pressure, creatinine clearance, and heart rate in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ● s.e. of the difference between final collection and baseline values of 6 experiments.

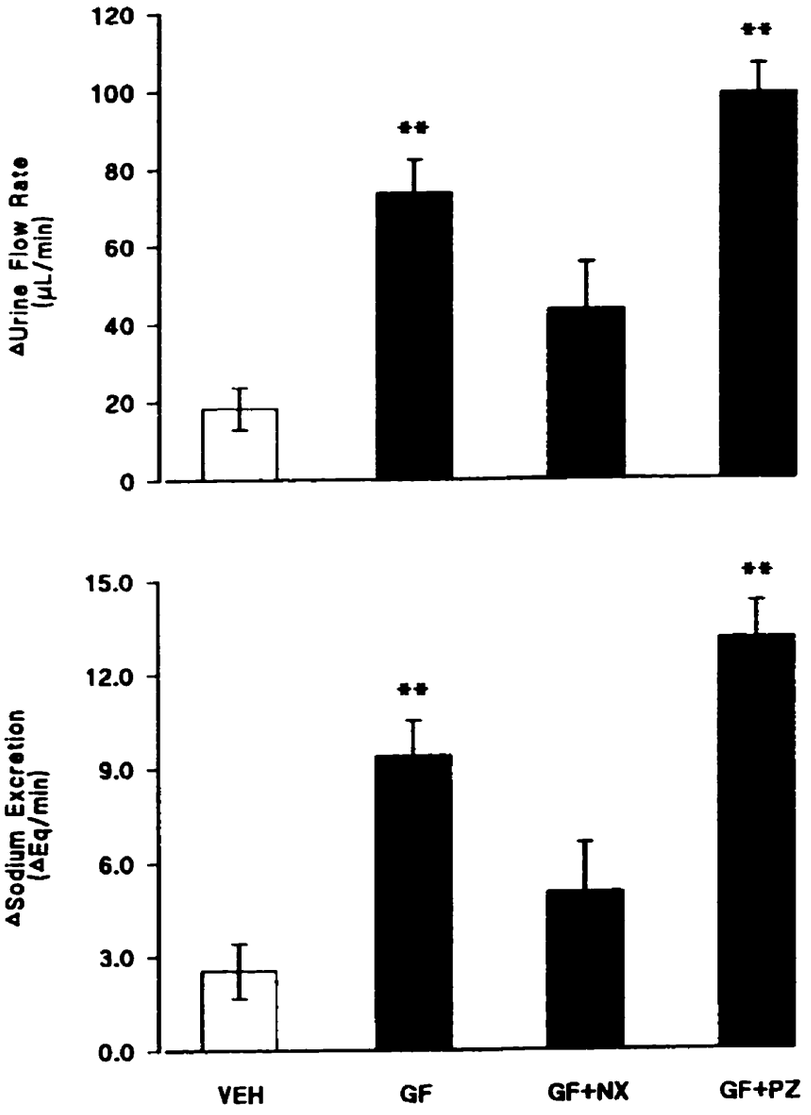

Figure 4.5. Effects of guanfacine in the presence and absence of naltrexone or prazosin on urine flow rate and sodium excretion in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{**}P<0.01$.

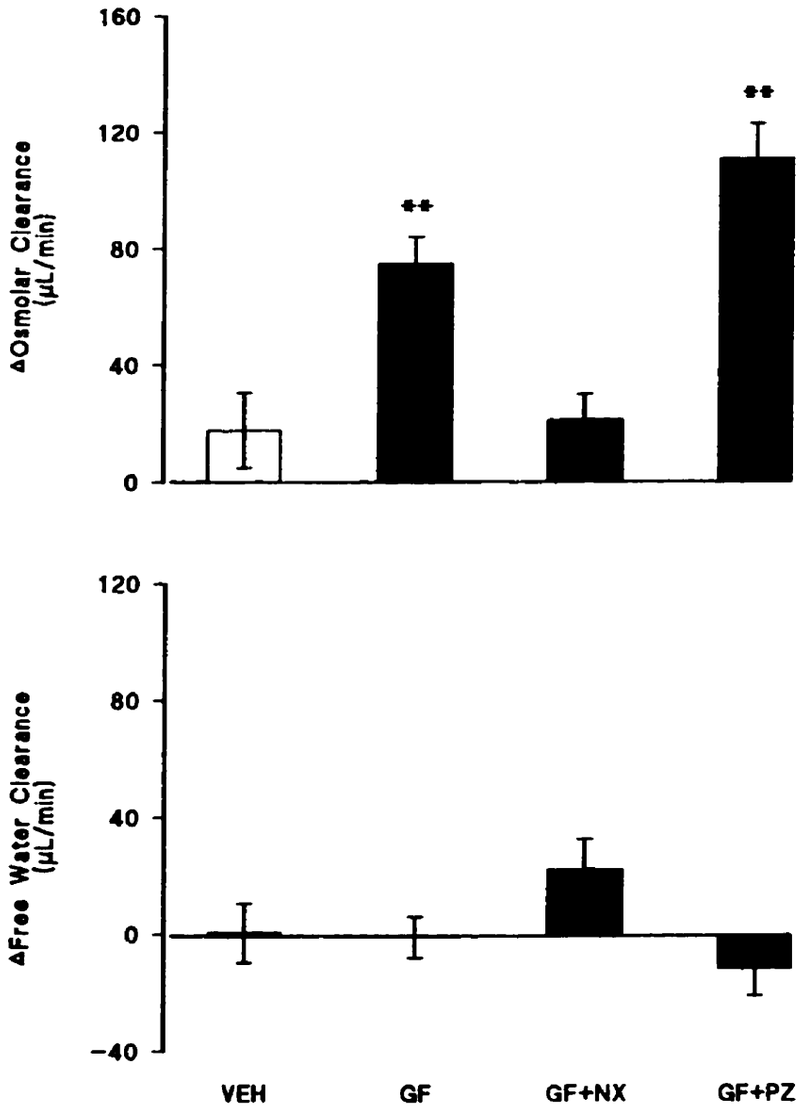

Figure 4.6. Effects of guanfacine in the presence and absence of naltrexone or prazosin on osmolar and free water clearance in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); PZ, prazosin (0.15 mg/kg, i.v.); NX, naltrexone (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{**}P<0.01$.

	Veh (n = 6)	GF (n = 6)	RX (n = 6)	GF + RX (n = 6)
Blood pressure (mm Hg)	107 ± 5	120 ± 5	108 ± 5	114 ± 7
Creatinine clearance (mL/min)	1.3 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.3 ± 0.1
Heart Rate (beats per min)	393 ± 15	406 ± 8	443 ± 19	457 ± 12*
Urine flow rate (μL/min)	10 ± 1	20 ± 1*	10 ± 1	12 ± 1
Sodium excretion (μequiv./min)	0.7 ± 0.3	1.4 ± 0.3	0.8 ± 0.2	1.7 ± 0.6
Free water clearance (μL/min)	-31 ± 3	-38 ± 6	-38 ± 4	-43 ± 6
Osmolar clearance (μL/min)	40 ± 4	55 ± 6	48 ± 5	55 ± 6

Table 4.4. Baseline values obtained before intrarenal guanfacine or vehicle infusion. Veh, vehicle control; GF, guanfacine (3.0 nmol/kg/min); RX, RX-821002 (3.0 mg/kg, i.v.). These values represent the control collection following the stabilization/antagonist pretreatment period. *$p<0.05$.

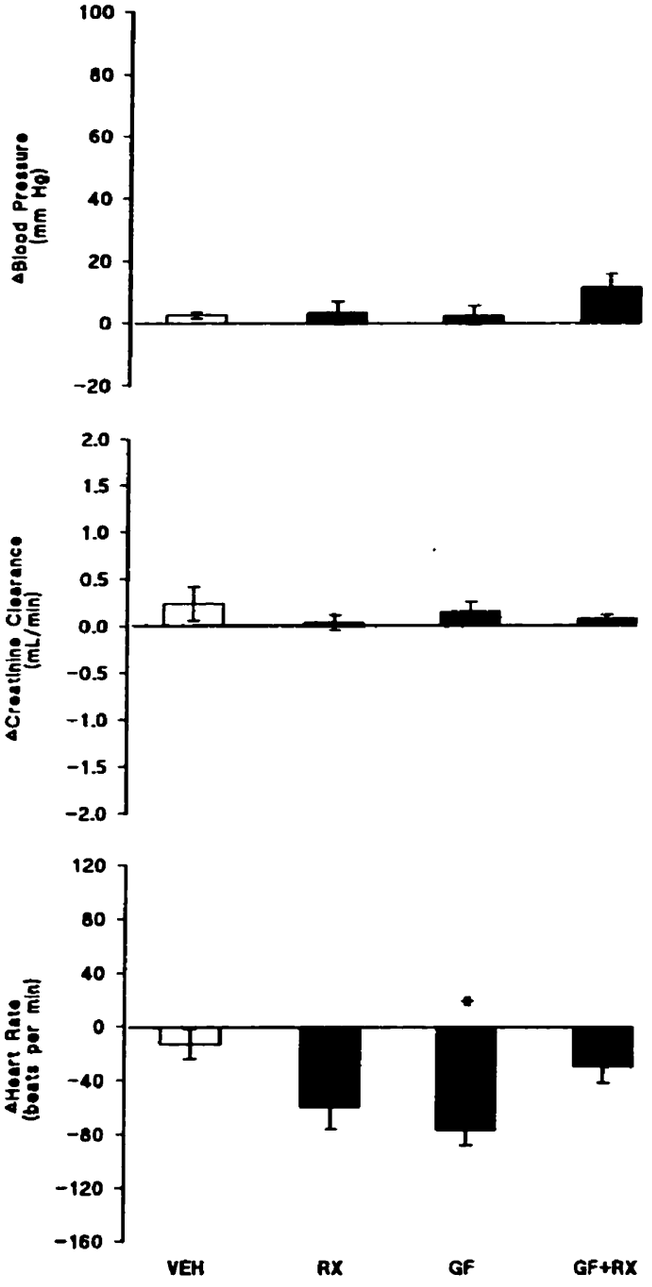

Figure 4.7. Effects of guanfacine in the presence and absence of RX-821002 on blood pressure, creatinine clearance, and heart rate in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); RX, RX-821002 (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments.

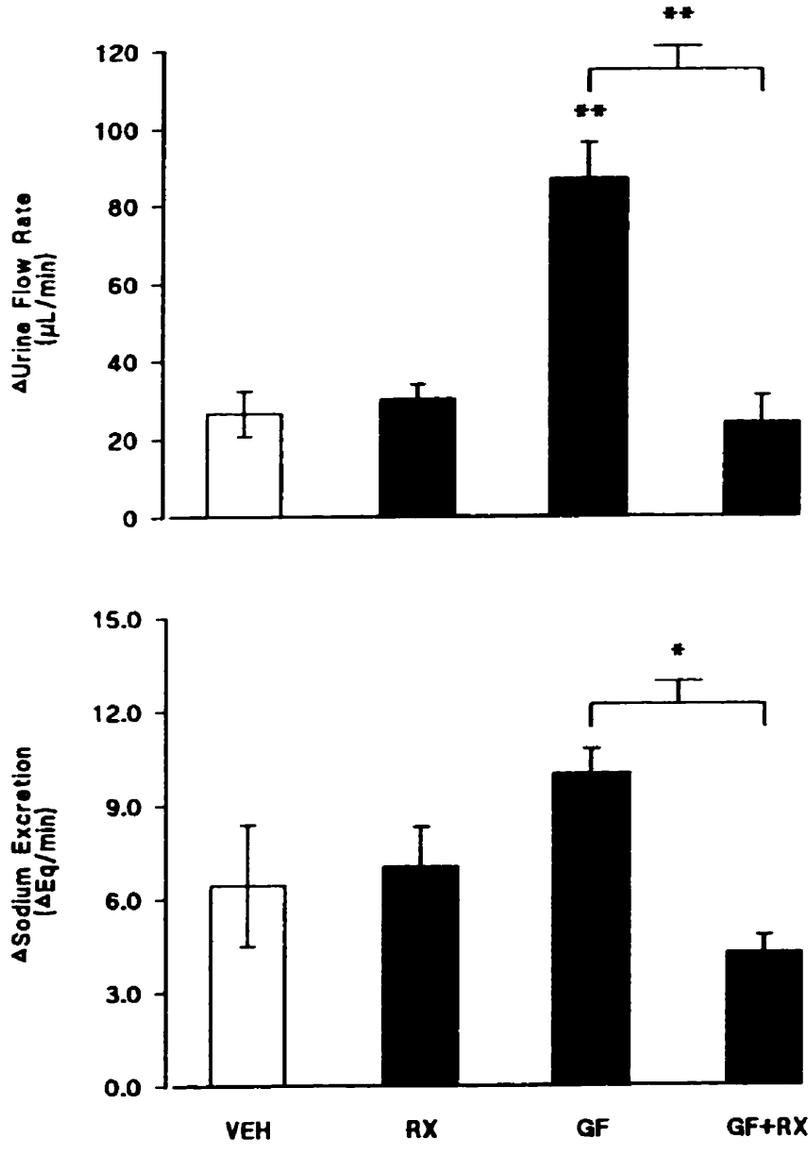

Figure 4.8. Effects of guanfacine in the presence and absence of RX-821002 on urine flow rate and sodium excretion in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); RX, RX-821002 (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

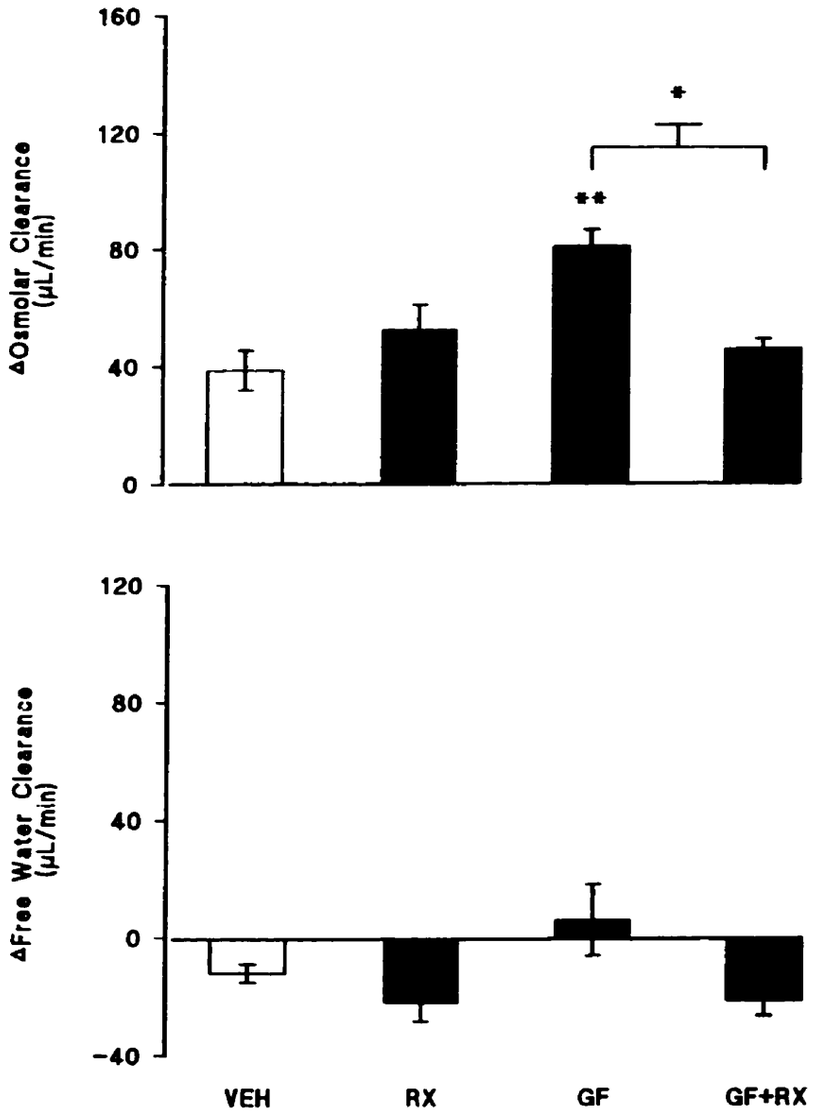

Figure 4.9. Effects of guanfacine in the presence and absence of RX-821002 on osmolar and free water clearance in the rat. VEH, vehicle control; GF, guanfacine (3.0 nmol/kg/min); RX, RX-821002 (3.0 mg/kg, i.v.). Each group represents the mean ± s.e. of the difference between final collection and baseline values of 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

Discussion

In the present study, the hypothesis was tested that the renal $\alpha_{2a/d}$-adrenoceptor subtype in the rat mediates osmolar clearance. The mechanism involved would be naltrexone-sensitive/prazosin-insensitive as was the osmolar response to clonidine. The doses of naltrexone and prazosin utilized were those previously determined to attenuate the renal effects of clonidine. Two agonists (guanfacine, UK-14,304) and one antagonist (RX-821002) with purported selectivity for the $\alpha_{2a/d}$-subtype were utilized in these studies. It was anticipated that stimulation of the $\alpha_{2a/d}$-subtype in the rat kidney would result in increased urine flow rate, sodium excretion, and osmolar clearance. These effects would be blocked by an $\alpha_{2a/d}$-selective antagonist (RX-821002).

The present data show that at these doses, UK-14,304 and guanfacine both increased urine flow rate, sodium excretion, and osmolar clearance. Free water clearance remained unaltered. As with the osmolar response to clonidine, the UK-14,304 and guanfacine-induced osmolar effects were attenuated by naltrexone and unaffected by prazosin. The osmolar effect of guanfacine was also attenuated by the selective $\alpha_{2a/d}$-subtype antagonist, RX-821002.

UK-14,304 has been previously reported as a relatively selective $\alpha_{2a/d}$-adrenoceptor agonist (MacKinnon *et al.*, 1994). Two cell lines which express the $\alpha_{2a/d}$-subtype (HT29) and α_{2b}-subtype (NG108) only, were used to investigate the ability of UK-14,304 to inhibit adenylate cyclase (Bylund and Ray-Prenger, 1989). UK-14,304 was 100-fold more potent at the $\alpha_{2a/d}$-subtype. Previously in our laboratory, a dose response curve was determined for the renal effects of UK-14,304 (Smyth *et al.*, 1992a).

Higher doses of UK-14,304 produced increases in both free water and osmolar clearance. However, based on the suggestion that UK-14,304 was selective for the $\alpha_{2a/d}$-subtype, we conducted preliminary experiments with a lower dose which we hoped would render this selectivity apparent. An infusion rate of 1.0 nmol/kg/min for UK-14,304 appeared to selectively increase osmolar clearance. Subsequent studies with UK-14,304 were conducted using this dose. When studied systematically, the renal effects of this dose of UK-14,304 included increases in urine flow rate, sodium excretion, and osmolar clearance. No effects on free water clearance were observed. The osmolar response was naltrexone-sensitive/prazosin-insensitive. This finding was in accordance with the hypothesis that stimulation of the renal $\alpha_{2a/d}$-adrenoceptor would produce increases in osmolar clearance but not free water clearance. However, the observation that UK-14,304 is selective for the $\alpha_{2a/d}$-subtype was debatable. In fact, radioligand binding studies indicated that UK-14,304 was either slightly selective (Gleason and Hieble, 1991) or non-selective between the $\alpha_{2a/d}$- and α_{2b}-subtypes (Uhlén and Wikberg, 1991). A second $\alpha_{2a/d}$-subtype agonist was subsequently chosen.

Guanfacine has clearly demonstrated selectivity for the $\alpha_{2a/d}$-adrenoceptor subtype over the α_{2b}-subtype. Radioligand binding studies, in fact, indicated a 60-fold difference in affinities (Uhlén and Wikberg, 1991). We conducted preliminary experiments with several doses (1.0, 3.0, and 10.0 nmol/kg/min) of guanfacine. All three doses had renal effects which qualitatively appeared dose-dependent. A moderate dose of 3.0 nmol/kg/min was subsequently chosen. Preliminary experiments also indicated that 3.0 mg/kg RX-821002 was the lowest effective dose. The intrarenal administration of guanfacine produced

increases in urine flow rate and osmolar clearance without changes in free water clearance. As with the osmolar response to clonidine and UK-14,304, this guanfacine-elicited effect was naltrexone-sensitive/prazosin-insensitive. Higher doses may have increased free water clearance, as well. However, the observation that a moderate dose of an $\alpha_{2a/d}$-selective agonist selectively increased osmolar clearance further supported the contention that the $\alpha_{2a/d}$-adrenoceptor was involved in modulating osmolar clearance.

In addition, the osmolar response to guanfacine was attenuated by pretreatment with RX-821002, an α_2-antagonist with selectivity for the $\alpha_{2a/d}$-adrenoceptor (Uhlén and Wikberg, 1991). The ability of RX-821002 to attenuate the osmolar response to guanfacine provided an additional line of evidence that the changes in osmolar clearance may be mediated by the $\alpha_{2a/d}$-subtype.

The RX-821002 data, although lending some support, did not prove or disprove the hypothesis that $\alpha_{2a/d}$-adrenoceptors mediated solute excretion. Difficulties arose when we attempted to attribute the increase in osmolar clearance produced specifically by clonidine to the $\alpha_{2a/d}$-adrenoceptors. Preliminary experiments showed that RX-821002 failed to attenuate the clonidine-induced osmolar effect (data not shown). Based on these early results, we theorized that perhaps the dose of RX-821002 was too high (3.0 mg/kg) and was occupying all α_2-adrenoceptors in the kidney. This effect would then promote the interaction of clonidine with imidazoline receptors resulting in an increase in osmolar clearance. Unfortunately, antagonism of imidazoline receptors with idazoxan also failed to attenuate the increase in osmolar clearance produced by clonidine in the presence of RX-821002 (data not shown). The discrepancy regarding RX-821002 blockade of increased

osmolar clearance was likely not a sufficient basis for discarding the hypothesis that the $\alpha_{2a/d}$-adrenoceptor subtype mediated increases in osmolar clearance. The selectivity of RX-821002 for the $\alpha_{2a/d}$- over the α_{2b}-subtype is approximately only 7-fold (Uhlén and Wikberg, 1991). To date, however, RX-821002 was the only available "$\alpha_{2a/d}$-selective" antagonist. The ability of guanfacine to increase osmolar clearance was compelling support for this hypothesis since this drug was 60-fold more selective for the $\alpha_{2a/d}$-subtype (Uhlén and Wikberg, 1991).

The ongoing development of novel techniques may help to confirm or refute our postulate that the $\alpha_{2a/d}$-adrenoceptor mediates osmolar clearance. A recent study used antisense technology to assert that the $\alpha_{2a/d}$-adrenoceptor subtype mediated the hypnotic response to dexmedetomidine in the locus coeruleus of the rat (Mizobe *et al.*, 1996). Based on the premise that antisense oligodeoxynucleotides inhibit receptor expression, the administration of these molecules should attenuate the effects of stimulation of the receptor for which they were targeted. A useful experiment would be to administer antisense oligodeoxynucleotides for the $\alpha_{2a/d}$-adrenoceptor subtype (or scrambled sequences/other receptor subtypes for controls) to the rat kidney. The osmolar response to clonidine, guanfacine, and UK-14,304, if mediated by the $\alpha_{2a/d}$-subtype, would then be absent or at least partially attenuated. Currently, investigations are in progress regarding appropriate delivery systems for oligodeoxynucleotides specifically to the kidney (Sawai *et al.*, 1996).

In conclusion, the relatively selective $\alpha_{2a/d}$-adrenoceptor agonists, guanfacine and UK-14,304, increased urine flow rate by increasing osmolar clearance without affecting

free water clearance. RX-821002 attenuated the increases in urine flow rate and osmolar clearance produced by guanfacine. As with clonidine, the osmolar responses to both UK-14,304 and guanfacine were attenuated by naltrexone but not by prazosin. These findings implicate the $\alpha_{2a/d}$-adrenoceptor subtype in mediating osmolar clearance. The present findings therefore describe a novel natriuretic function for the $\alpha_{2a/d}$-adrenoceptor subtype in the normotensive rat kidney.

5

Renal $\alpha_{2a/d}$-Adrenoceptor Subtype Function in Hypertension

These data have been presented as an abstract at the 29th Annual Meeting and Scientific Exposition of the American Society of Nephrology, New Orleans, Louisiana, 1996.

Summary:

Having shown that the $\alpha_{2a/d}$-adrenoceptor subtype in the rat kidney modulated solute excretion (osmolar clearance), we considered the potential relevance of this finding. The kidney plays a significant role in chronic regulation of blood pressure. Altered function of the kidney has also been suggested to a play a major role in the onset of hypertension. The association of an altered $\alpha_{2a/d}$-adrenoceptor gene with hypertension in rats and in humans led us to our next hypothesis: the osmolar response to $\alpha_{2a/d}$-adrenoceptor stimulation would be decreased or absent in spontaneously hypertensive (SH) rats. Based on the tentative assumption that such a non-responsiveness would be due to the altered $\alpha_{2a/d}$-adrenoceptor gene, it was further postulated that in an acquired model of hypertension where this gene is normal, the osmolar function of the $\alpha_{2a/d}$-subtype would be intact. The acquired model of hypertension that we used was the Wistar one kidney-one clip (1K-1C) rat. Male rats (Wistar and SH) were unilaterally nephrectomized under ether anesthesia. In the Wistar 1K-1C group, a silver clip (diameter 0.254 mm) was placed around the left renal artery. On the day of the experiment, rats were anesthetized with pentobarbitone (50.0 mg/kg, i.p.) The carotid artery and jugular vein were cannulated for blood pressure monitoring and saline infusion. The ureter was catheterized for urine collection. A 31 gauge needle was advanced into the renal artery for infusion of the $\alpha_{2a/d}$-selective agonist, guanfacine (0.0, 1.0, 3.0, and 10.0 nmol/kg/min in Wistar (control) and SH rats; 10.0 nmol/kg/min in Wistar 1K-sham (control) and 1K-1C rats). In Wistar rats, guanfacine dose-dependently increased urine flow rate and sodium excretion. Osmolar clearance was increased whereas free water clearance remained unaltered. In SH rats,

however, guanfacine failed to elicit these renal effects. In contrast, in both Wistar 1K-sham and 1K-1C rats, guanfacine (10.0 nmol/kg/min) increased urine flow rate, sodium excretion, and osmolar clearance. These data indicated defective $\alpha_{2a/d}$-adrenoceptor function in the kidney of spontaneously hypertensive (SH) rats. This unresponsiveness to guanfacine was likely not a consequence of the elevated blood pressure because the function of the $\alpha_{2a/d}$-subtype was intact in an acquired model of hypertension.

Introduction

As discussed in the general introduction (page 13), the kidney contributes significantly to the chronic regulation of blood pressure by modulating sodium and water excretion. Some change in the renal mechanism or set point of blood pressure control has been implicated in the pathogenesis of hypertension (Hall *et al.*, 1990; Guyton, 1991). In support of this hypothesis, kidney cross-transplantation studies found that post-transplant blood pressure in the recipient is determined by the donor kidney in rats (Kawabe *et al.*, 1978) and humans (Curtis *et al.*, 1983). The precise alteration(s) in renal function, however, remain(s) undetermined.

A potential link between the kidney and hypertension may be the $\alpha_{2a/d}$-adrenoceptor subtype. As discussed previously, the $\alpha_{2a/d}$-subtype in the rat kidney mediates osmolar clearance. A defect or alteration of the $\alpha_{2a/d}$-adrenoceptor may therefore result in defective modulation of solute excretion thereby predisposing the carrier to hypertension. So far in the literature, an alteration of the $\alpha_{2a/d}$-subtype gene has been identified in humans (Hoehe *et al.*, 1988) and rats (Chun *et al.*, 1991; Lockette *et al.*, 1995). Furthermore, in both species, this "hypertensive allele" has correlated with the incidence of hypertension (Pettinger *et al.*, 1991; Lockette *et al.*, 1995; Svetkey *et al.*, 1996). Aside from the gene itself, an alteration of function of the $\alpha_{2a/d}$-adrenoceptor, particularly in the kidney, had not yet been identified.

In the present study, the hypothesis was tested that the previously reported osmolar function of the renal $\alpha_{2a/d}$-subtype would be absent in SH rats. It was further postulated that this absent function of the $\alpha_{2a/d}$-adrenoceptor was not secondary to the

elevated blood pressure and therefore would be intact in an acquired (that is, non-genetic) model of hypertension, the 1K-1C rats. The dose-related effects of guanfacine (1.0, 3.0, 10.0 nmol/kg/min), a selective $\alpha_{2a/d}$-adrenoceptor agonist, on renal function were determined in Wistar (normotensive) and SH rats. Also, the renal effects of guanfacine (10.0 nmol/kg/min) in Wistar 1K-sham (normotensive) and Wistar 1K-1C rats were determined.

Methods

Experimental Preparation

The standard procedures have been described previously in Section 2 - Methods. Differences in the experimental procedures are summarized below.

Wistar and Spontaneously Hypertensive rats

Male Wistar and SH rats (8 weeks old) were used. Seven to ten days prior to the experiment, the right kidney was removed under ether anesthesia via a flank incision. The animals were then administered a subcutaneous injection of the post-operative analgesic, buprenorphine (0.015 mg/kg, s.c.).

Wistar one kidney-sham and one kidney-one clip rats

The standard procedures have been previously described by Li *et al.* (1994). Male Wistar rats (100-125 g) were separated into two groups. In the first group (1K-1C), both kidneys were exposed under ether anesthesia by an abdominal incision. A silver clip (diameter 0.254 mm) was placed around the left renal artery. The right kidney was then removed. In the second group (1K-sham), the surgery was identical to the 1K-1C rats except no clip was placed on the left renal artery. A subcutaneous injection of buprenorphine (0.015 mg/kg, s.c.) was then administered. The animals were maintained for at least 28 days after surgery before experimentation.

Pharmacological Treatment

Wistar and SH rats:

(a) Dose-related response to guanfacine

After surgery (time = 0 min), the continuous i.v. infusion (0.097 mL/min) of normal saline was initiated. The rats were allowed to stabilize for 45 min (time = 0 - 45 min) during which there was no pharmacological intervention. Following the stabilization period, a control urine collection (time = 45 - 75 min) was obtained. After this initial control collection, Wistar and SH rats received a continuous intrarenal infusion (0.0034 mL/min) of the vehicle (0.9% saline) or guanfacine (1.0, 3.0, and 10.0 nmol/kg/min) for the remainder of the experiment. Two subsequent urine collections were obtained (time = 75 - 105 and 105 - 135 min). Urine was collected into pre-weighed vials. Urine flow rate was determined gravimetrically.

The rats were randomly assigned to one of four groups, each consisting of 6 animals. Group 1 received an intrarenal infusion of vehicle only. Groups 2, 3, and 4 received 1.0, 3.0, or 10.0 nmol/kg/min guanfacine respectively.

(b) Renal response to furosemide

If a difference was detected between Wistar and SH rats in the renal response to guanfacine, it could be argued that this is a finding common to all natriuretic drugs. To confirm that not all natriuretic agents would be ineffective in SH rats, an extra urine collection was obtained following the end of the experiment in eight of the control rats -

four Wistar and four SH control rats which received intrarenal vehicle only. At the beginning of this collection, furosemide, a natriuretic drug unrelated to the $\alpha_{2a/d}$-adrenoceptor, was administered intravenously at a moderate dose, 0.3 mg/kg.

Wistar 1K-sham and 1K-1C rats:

Renal response to guanfacine

Following the control collection, 1K-sham and 1K-1C rats also received an intrarenal infusion (0.0034 mL/min) of 0.9% saline or guanfacine (10.0 nmol/kg/min). Again, during the intrarenal infusion, two 30 min experimental urine collections were obtained. Within the 1K-sham or 1K-1C groups, rats were assigned to one of two sub-groups, each consisting of 6 rats. Group 1 received vehicle. Group 2 received guanfacine.

Statistical Analysis

Data are presented as the mean ± standard error. Using Systat software version 5.0, data were analyzed by repeated measures of analysis of variance (ANOVA). Significant differences were further localized with Newman-Keul's multiple comparison test. The furosemide data (i.e. only the absolute values from the extra collection at the end of the control experiments) were analyzed with a Student's t-test. Significance is denoted as $^{*}P<0.05$ and $^{**}P<0.01$.

Data Presentation

(a) Guanfacine data

Similar levels of significance were determined for absolute values and delta values. Data have thus been presented as the difference between final collection and baseline values to delineate the different magnitudes of response between groups.

(b) Furosemide data

To avoid overlap in statistical analysis, only the absolute values from the extra, fourth collection were analyzed. Hence, data from each collection was not used for statistical analysis more than once. The furosemide data have therefore been presented as absolute values.

Results

Preparation Controls

To compare the baseline levels of measured parameters following surgical preparation, data from the pre-intrarenal treatment collection period were analyzed (tables 5.1 and 5.2). Variation was detected in free water and osmolar clearances in the Wistar rats whereas no variation was detected in baseline levels within the SH rats (table 5.1). Blood pressure appeared elevated in the SH *versus* the Wistar rats. Likewise, blood pressure was consistently elevated in the Wistar 1K-1C rats as compared to the Wistar 1K-sham rats. Heart rate was lesser in the Wistar 1K-sham rats which later received guanfacine. Baseline levels of other parameters of interest were otherwise similar (table 5.2).

Wistar and SH rats:

(a) Dose-related response to guanfacine

Blood pressure and creatinine clearance were unaltered by guanfacine as compared to respective controls in Wistar (figure 5.1) and SH (figure 5.4) rats. Heart rate was decreased in the SH rats by the higher dose of guanfacine (10.0 nmol/kg/min). In Wistar rats, guanfacine increased urine flow rate and sodium excretion significantly in a dose-related manner (figure 5.2). Increased osmolar clearance was observed whereas free water clearance was not altered (figure 5.3). The variation in baseline values of osmolar clearance in Wistar rats (table 5.1) was minor compared to the magnitude of increased osmolar clearance in rats after guanfacine. In SH rats, however, guanfacine had no effect

on urine flow rate or sodium excretion (figure 5.5) as well as osmolar and free water clearance (figure 5.6).

(b) Renal response to furosemide

In this fourth collection period (table 5.3), blood pressure still appeared elevated in the SH rats *versus* the Wistar rats. Creatinine clearance was similar between groups. Furosemide increased urine flow rate, sodium excretion, and osmolar clearance to statistically comparable levels in SH and Wistar rats. In both groups, free water clearance was not affected by furosemide.

Wistar 1K-sham and 1K-1C rats:

Renal response to guanfacine

Blood pressure and creatinine clearance were unaltered by guanfacine in 1K-sham and 1K-1C rats (figure 5.7). Heart rate was increased or decreased respectively by guanfacine in these two groups. Guanfacine increased urine flow rate but not sodium excretion in both groups (figure 5.8) secondary to increases in osmolar clearance only. Guanfacine did not affect free water clearance in these animals (figure 5.9).

	Wistar Pre-guanfacine				SH Pre-guanfacine			
	Vehicle (n=6)	**1.0 (n=6)**	**3.0 (n=6)**	**10.0 (n=6)**	**Vehicle (n=6)**	**1.0 (n=6)**	**3.0 (n=6)**	**10.0 (n=6)**
Blood Pressure (mm Hg)	127 ± 7	116 ± 4	122 ± 9	117 ± 6	181 ± 9	174 ± 8	185 ± 5	173 ± 5
Creatinine Clearance (mL/min)	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.1
Heart Rate (beats per min)	396 ± 10	400 ± 13	400 ± 16	410 ± 6	390 ± 11	393 ± 10	390 ± 8	383 ± 13
Urine Flow Rate (µL/min)	24 ± 3	20 ± 3	17 ± 3	16 ± 2	9 ± 1	8 ± 2	8 ± 1	11 ± 2
Sodium Excretion (µequiv./min)	3.0 ± 0.6	2.4 ± 0.5	1.6 ± 0.3	2.0 ± 0.3	1.0 ± 0.1	0.9 ± 0.3	1.1 ± 0.3	1.4 ± 0.3
Free Water Clearance (µL/min)	-39 ± 5	-54 ± 4**	-36 ± 4	-28 ± 3*	-27 ± 6	-28 ± 4	-22 ± 3	-33 ± 5
Osmolar Clearance (µL/min)	63 ± 7	74 ± 5*	53 ± 4*	44 ± 3**	36 ± 6	33 ± 11	31 ± 3	44 ± 5

Table 5.1. Baseline values obtained in Wistar and SH rats before intrarenal infusion of guanfacine (1.0, 3.0, and 10.0 nmol/kg/min) or vehicle (0.0 nmol/kg/min). These values represent the control collection following the stabilization/antagonist pre-treatment period.

	Pre-guanfacine			
	1K-sham		1K-1C	
	Vehicle (n=6)	GF (n=6)	Vehicle (n=6)	GF (n=6)
Blood Pressure (mm Hg)	124 ± 4	109 ± 5	178 ± 10**	168 ± 12**
Creatinine Clearance (mL/min)	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.1
Heart Rate (beats per min)	424 ± 14	373 ± 10**	420 ± 14	410 ± 6
Urine Flow Rate (µL/min)	13 ± 3	13 ± 2	11 ± 3	13 ± 4
Sodium Excretion (µequiv./min)	1.1 ± 0.3	1.1 ± 0.3	0.7 ± 0.1	1.3 ± 0.5
Free Water Clearance (µL/min)	-39 ± 5	-30 ± 4	-33 ± 3	-34 ± 5
Osmolar Clearance (µL/min)	52 ± 7	43 ± 3	44 ± 5	47 ± 9

Table 5.2. Baseline values obtained in Wistar 1K-sham and 1K-1C rats before intrarenal infusion of guanfacine (10.0 nmol/kg/min) or vehicle (0.0 nmol/kg/min). These values represent the control collection following the stabilization/antagonist pre-treatment period.

	Furosemide	
	Wistar (n=4)	**SH (n=4)**
Blood Pressure (mm Hg)	108 ± 7	173 ± 10**
Creatinine Clearance (mL/min)	1.1 ± 0.4	1.1 ± 0.1
Urine Flow Rate (µL/min)	123 ± 14	86 ± 4
Sodium Excretion (µequiv./min)	17 ± 1.8	13 ± 1.4
Free Water Clearance (µL/min)	-34 ± 6	-44 ± 11
Osmolar Clearance (µL/min)	157 ± 16	130 ± 11

Table 5.3. Absolute values obtained in Wistar and SH rats after intravenous administration of furosemide (0.3 mg/kg).

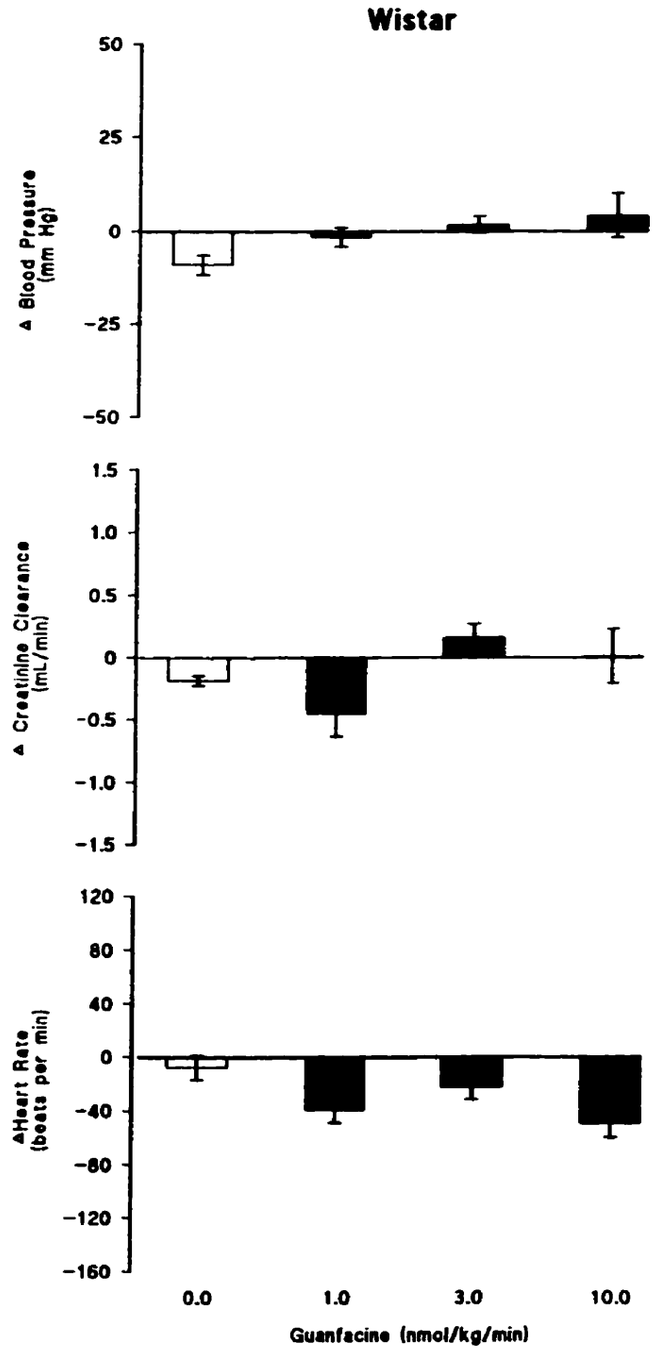

Figure 5.1. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on blood pressure, creatinine clearance, and heart rate in Wistar rats. Each group represents the mean ± s.e. of the difference the between the final collection and baseline values of 6 experiments.

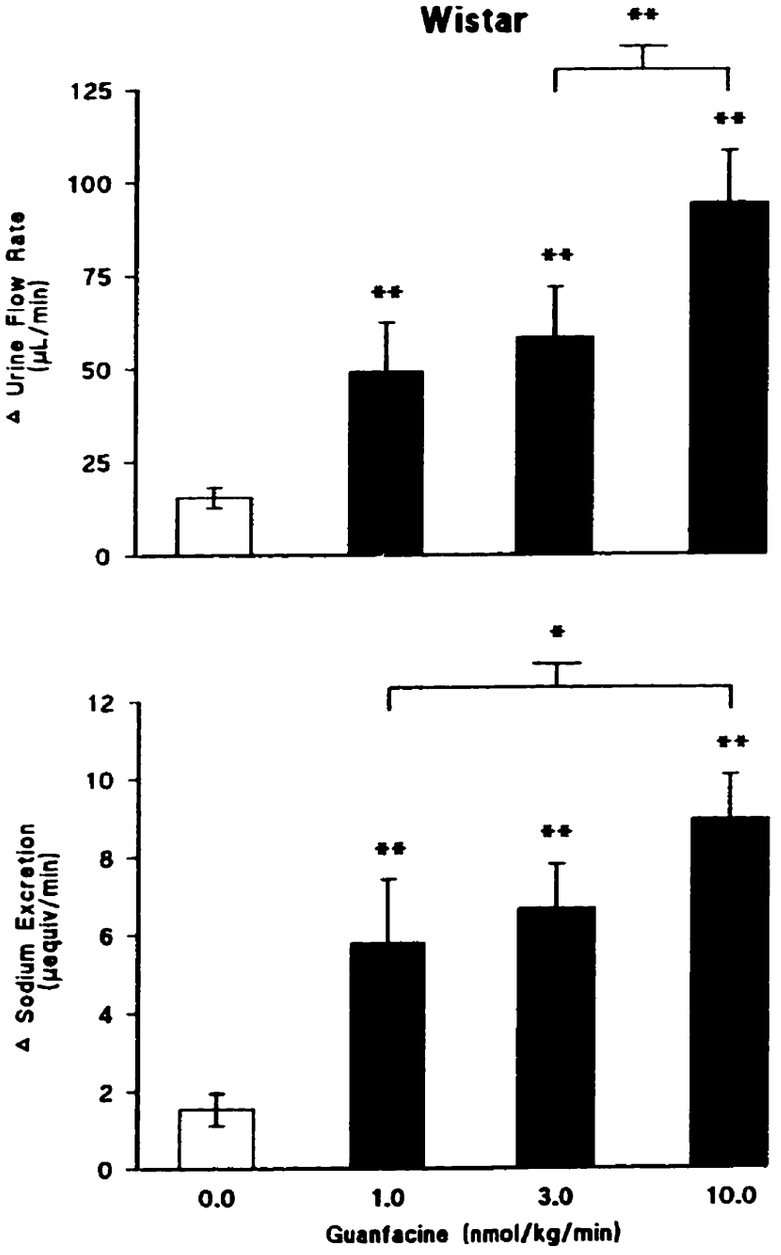

Figure 5.2. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on urine flow rate and sodium excretion in Wistar rats. Each group represents the mean ± s.e of the difference between the final collection and baseline values of 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

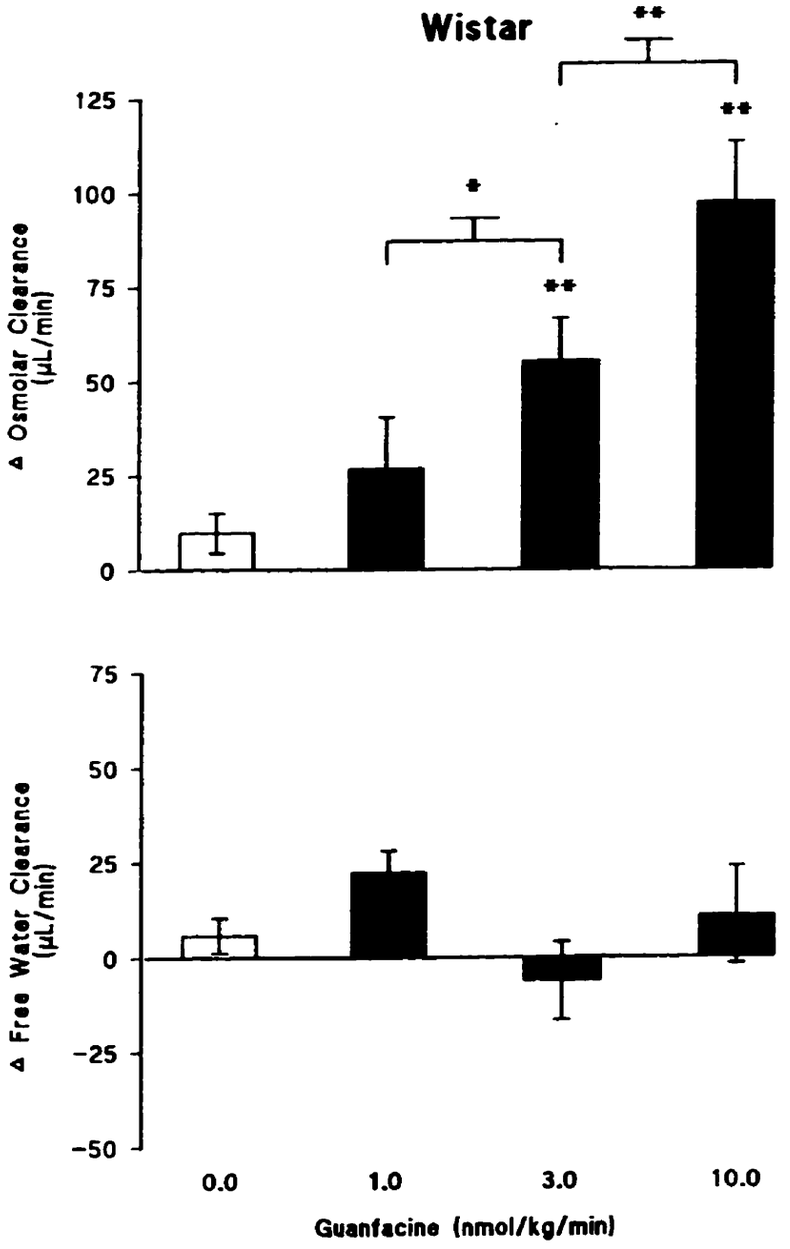

Figure 5.3. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on osmolar clearance and free water clearance in Wistar rats. Each group represents the mean ● s.e. of the difference between the final collection and baseline values of 6 experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

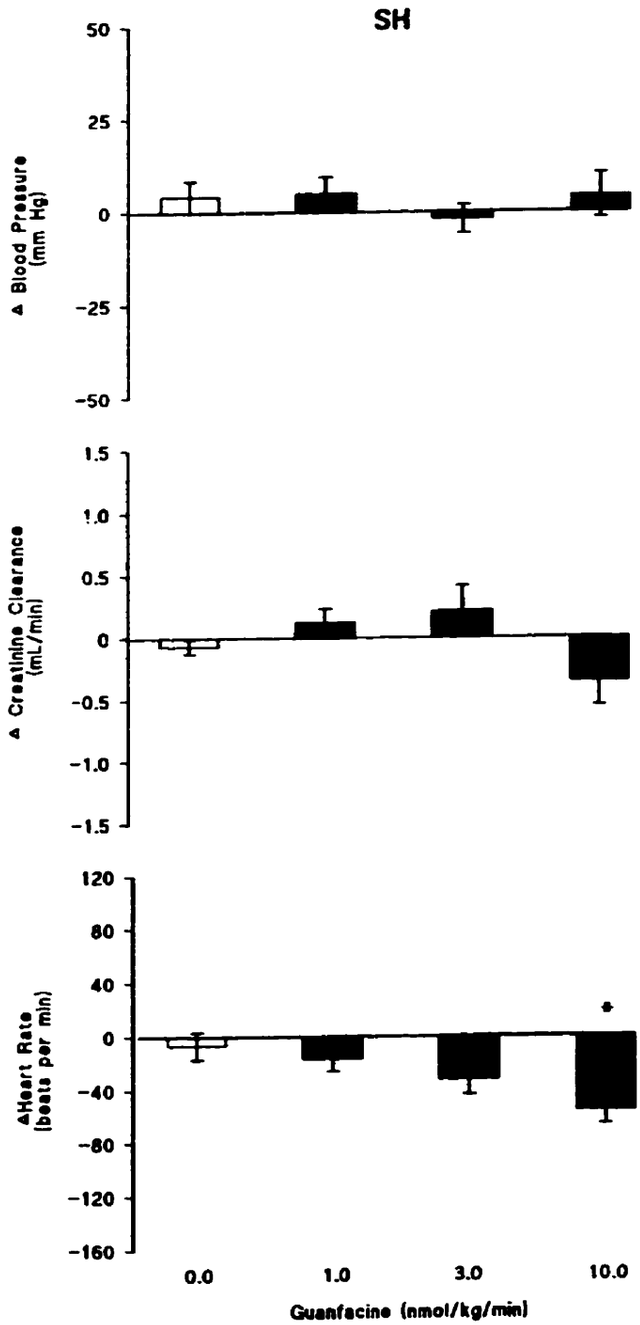

Figure 5.4. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on blood pressure, creatinine clearance, and heart rate in SH rats. Each group represents the mean ± s.e. of the difference between the final collection and baseline values of 6 experiments. $^{*}P<0.05$.

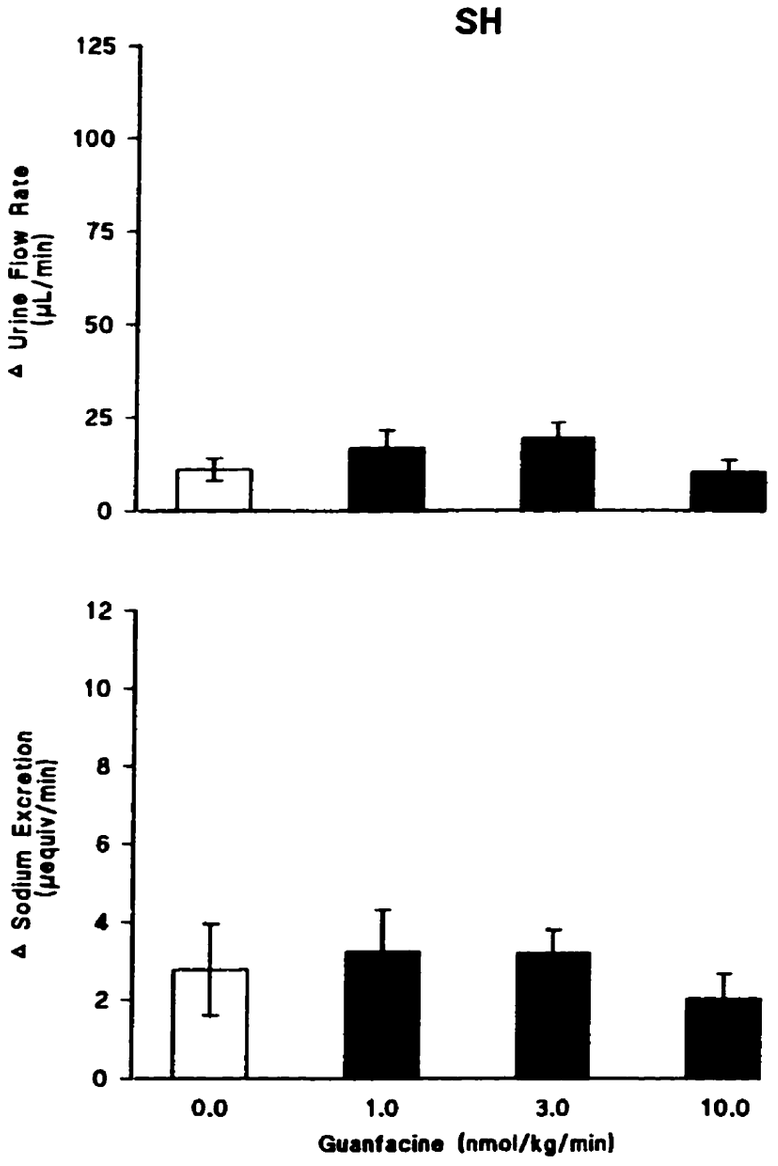

Figure 5.5. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on urine flow rate and sodium excretion in SH rats. Each group represents the mean ± s.e. of the difference between the final collection and baseline values of 6 experiments.

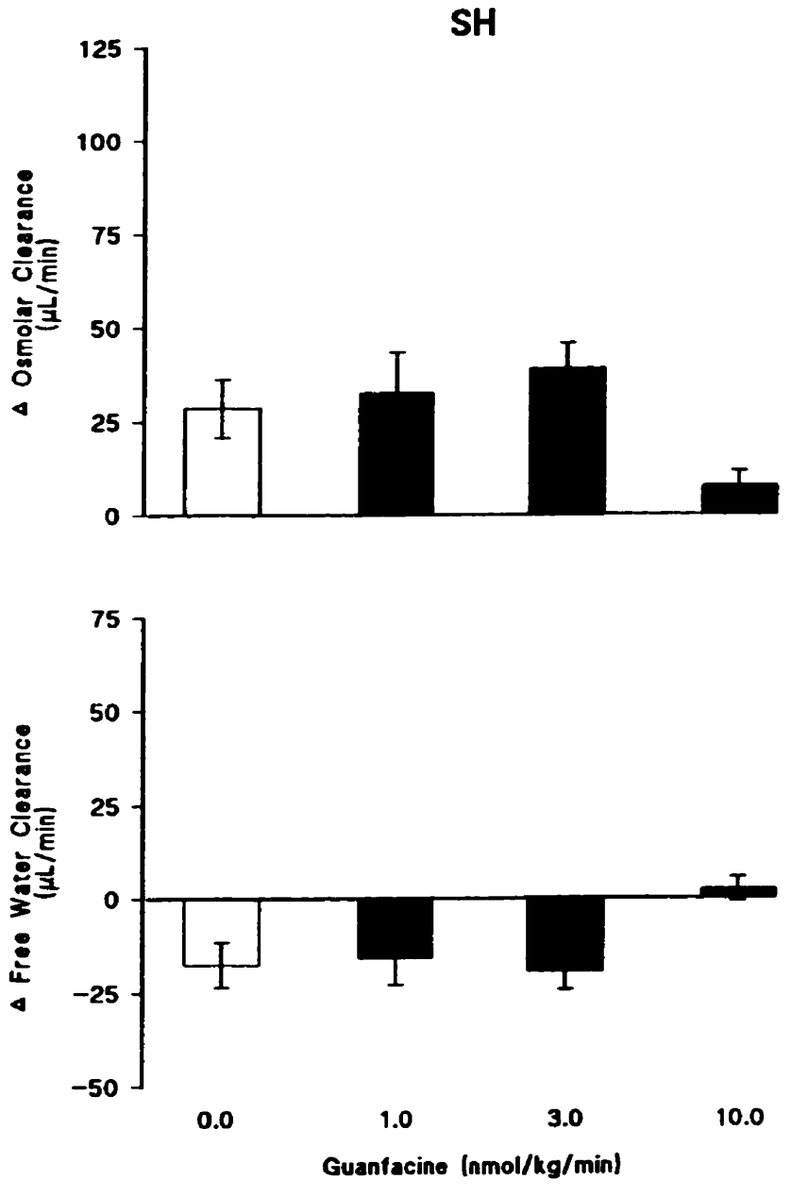

Figure 5.6. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (1.0, 3.0, 10.0 nmol/kg/min) on osmolar clearance and free water clearance in SH rats. Each group represents the mean ± s.e. of the difference between the final collection and baseline values of 6 experiments.

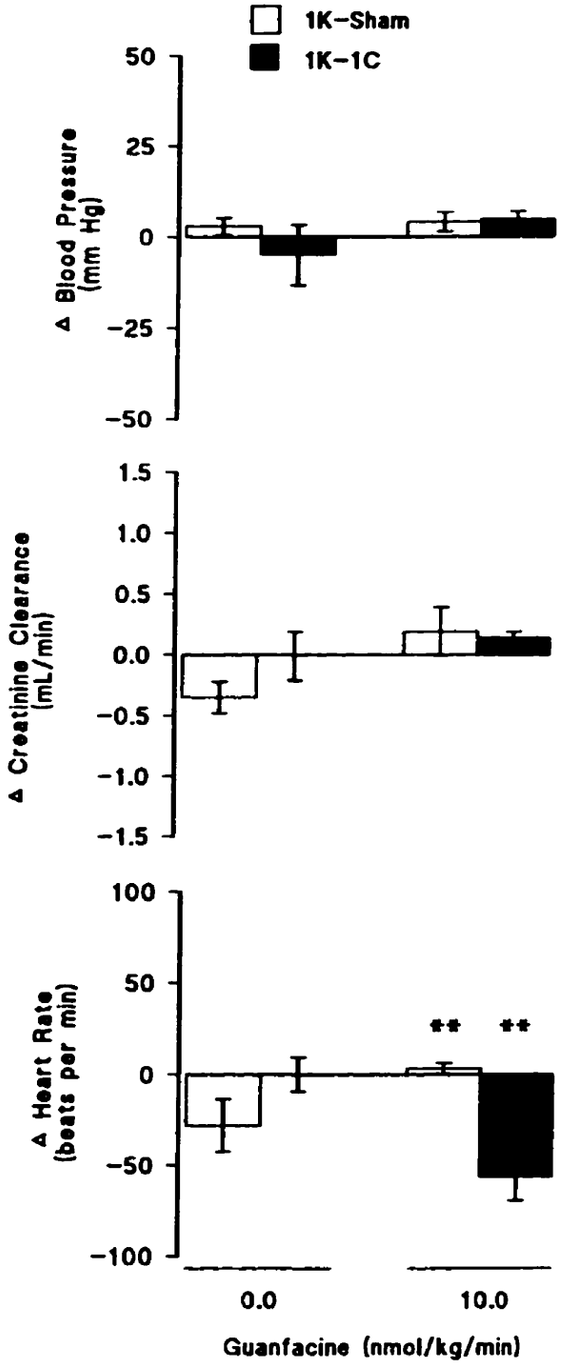

Figure 5.7. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (10.0 nmol/kg/min) on blood pressure, creatinine clearance, and heart rate in Wistar 1K-sham and 1K-1C rats. Each group represents the mean ± s.e. of the difference between the final collection and baseline values of 6 experiments. $^{**}P<0.01$.

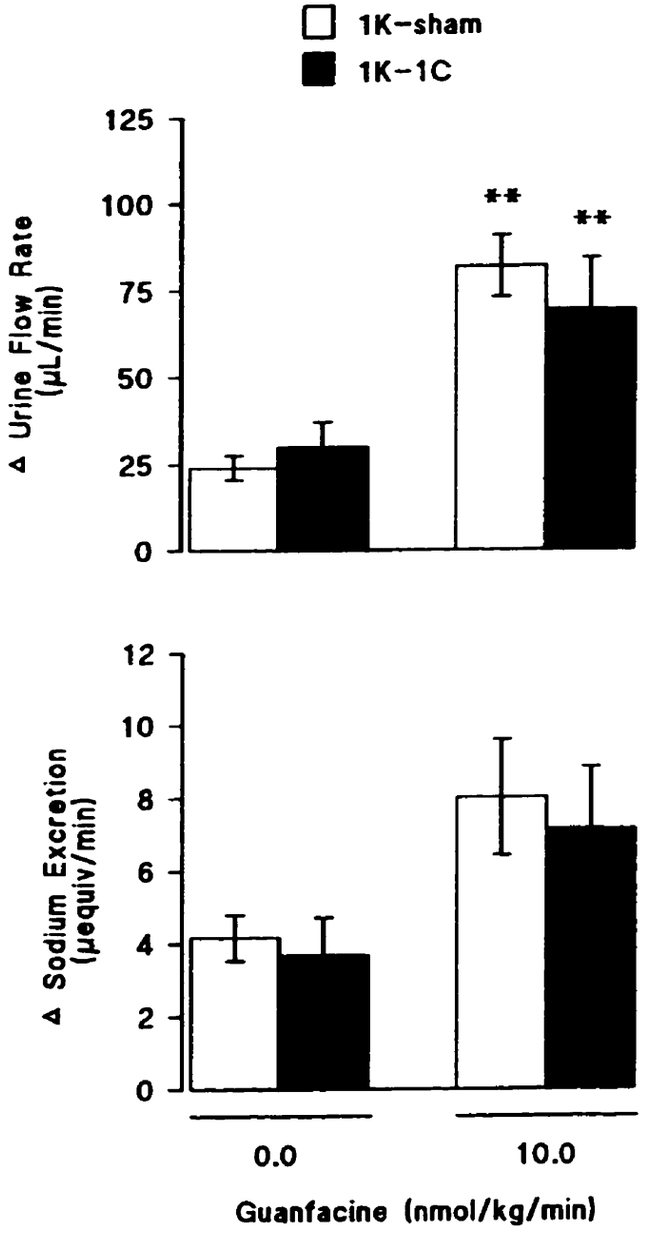

Figure 5.8. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (10.0 nmol/kg/min) on urine flow rate and sodium excretion in Wistar 1K-sham and 1K-1C rats. Each group represents the mean ± s.e of the difference between the final collection and baseline values of 6 experiments. $^{**}P<0.01$.

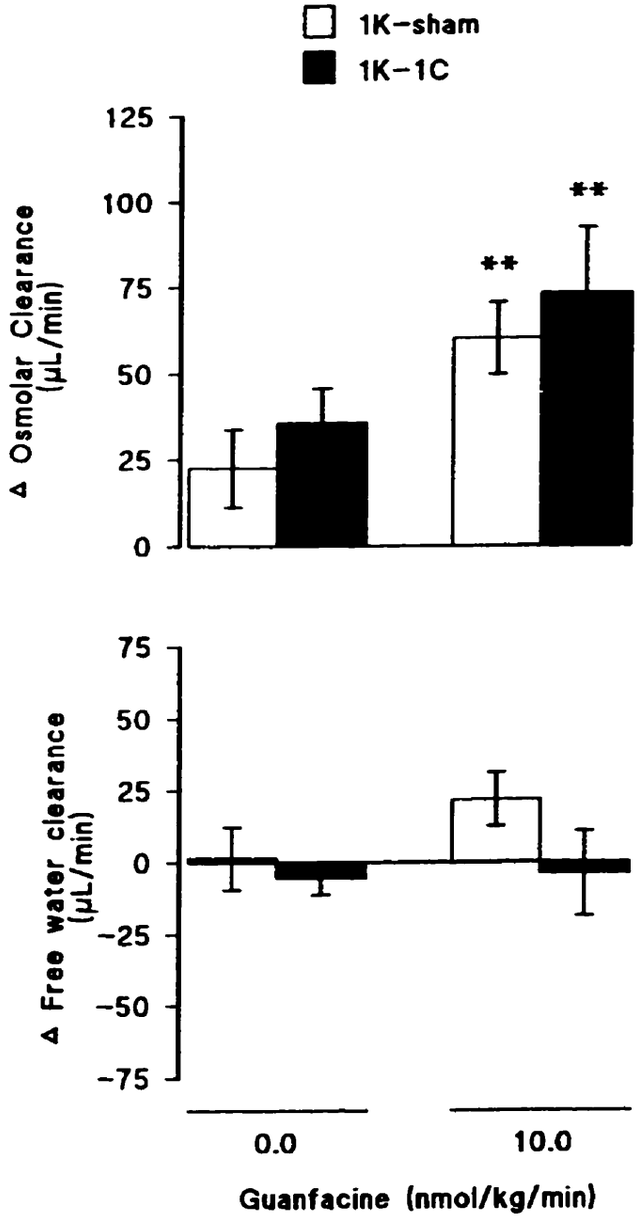

Figure 5.9. Effects of intrarenal infusion of vehicle (0.9% saline) or guanfacine (10.0 nmol/kg/min) on osmolar clearance and free water clearance in Wistar 1K-sham and 1K-1C rats. Each group represents the mean ± s.e. of the difference between the final collection and baseline values of 6 experiments. $^{**}P<0.01$.

Discussion

In this series of experiments, the function of the $\alpha_{2a/d}$-adrenoceptor in the kidney was determined in the SH rat which serves as a genetic model of hypertension. It was hypothesized that as in Sprague-Dawley rats, the $\alpha_{2a/d}$-subtype would mediate osmolar clearance in the normotensive control Wistar rats whereas this function would be absent in the hypertensive model.

Following selective stimulation of the $\alpha_{2a/d}$-adrenoceptor subtype with guanfacine in the kidney, increased urine flow rate, sodium excretion, and osmolar clearance were indeed observed in Wistar rats but not in SH rats. It is unlikely that this unresponsiveness to guanfacine was due to the elevated blood pressure. The 1K-1C rat, whose hypertension was acquired rather than genetic, still responded to guanfacine despite blood pressure levels which appeared comparable to those of the SH rats. Moreover, the unresponsiveness to guanfacine was likely not a non-specific outcome common to all natriuretic agents. The unrelated natriuretic drug, furosemide, induced renal responses in both Wistar and SH rats.

The decreased natriuretic activity in SH rats gives rise to the speculation that the alteration of the $\alpha_{2a/d}$-subtype gene detected in these rats may contribute to the pathogenesis of hypertension. As mentioned previously, in rats, Chun *et al.* (1991) identified an allele of the $\alpha_{2a/d}$-subtype gene using the restriction endonuclease Dde I and the human platelet α_2-adrenoceptor (i.e. the $\alpha_{2a/d}$-subtype) genomic probe. This 1.6 Kb allele cosegregated with elevated blood pressure in the F2 generation of cross-bred WKY and SH rats where heterozygotic and homozygotic carriers had intermediate and high

blood pressure levels respectively (Pettinger *et al.*, 1991). Recent studies with Sabra salt-sensitive and salt-resistant rats were consistent with the conjecture that this "hypertensive" allele contributes to salt-sensitive hypertension. In the renal cortices of Sabra normotensive salt-resistant and hypertensive salt-sensitive rats, the binding capacities for [3H]-yohimbine and [3H]-RX-821002 were significantly greater in the salt-sensitive rats as compared to the salt-resistant rats (Le Jossec *et al.*, 1995b). No nucleotide sequence was detected in the α_{2b}-adrenoceptor gene (Le Jossec *et al.*, 1995a). The mRNA for both $\alpha_{2a/d}$- and α_{2b}-subtypes were detected in the renal cortices of both the salt-sensitive and salt-resistant rats using reverse-transcription-cDNA amplification techniques (Le Jossec *et al.*, 1995b). However, only the α_{2b}-subtype was detected by radioligand binding studies in the salt-sensitive strain. In contrast, the $\alpha_{2a/d}$- and α_{2b}-adrenoceptors were both detectable in the renal cortical membranes of Sabra salt-resistant rats. The authors thus speculated that the absence of the $\alpha_{2a/d}$-subtype in these animals may be due to post-transcriptional or post-translational events since they detected the mRNA for both the $\alpha_{2a/d}$- and α_{2b}-subtypes in both strains. Based on the natriuretic function of the $\alpha_{2a/d}$-subtype which we have previously described in Wistar and Sprague Dawley rats, the absence of this receptor in the Sabra salt-sensitive rats may be contributing to the sodium sensitivity which pre-disposed these rats to hypertension.

In humans, the $\alpha_{2a/d}$-adrenoceptor may also be linked both to hypertension and sodium excretion. The $\alpha_{2a/d}$-subtype does comprise at least eighty percent of total renal α_2-adrenoceptors in the human kidney (Motomura *et al.*, 1989). As with rats, an RFLP for the $\alpha_{2a/d}$-subtype gene has been identified (Hoehe *et al.*, 1988) and correlated with

various sub-populations of hypertensive patients including African-American subjects (Lockette *et al.*, 1995) and Caucasian subjects (Svetkey *et al.*, 1996). Based on one report, a potential link may be established between this "hypertensive" allele and our proposed function for the renal $\alpha_{2a/d}$-adrenoceptor. Freeman *et al.* (1995) showed that individuals carrying at least one copy of the "hypertensive" 6.3 kb allele had decreased sodium excretion induced by immersion in thermal neutral water.

In conclusion, in humans, a second allele of the $\alpha_{2a/d}$-adrenoceptor gene correlated with both hypertension and with decreased sodium excretion. In the rat, a second allele of the $\alpha_{2a/d}$-adrenoceptor gene has also correlated with hypertension. However, until these data were reported, no functional correlate such as decreased sodium excretion had been identified. We have previously established the function of the $\alpha_{2a/d}$-adrenoceptor in the rat kidney as modulation of solute excretion. These studies showed that the osmolar response to stimulation of the $\alpha_{2a/d}$-adrenoceptor was absent in SH rats. This defective modulation of solute excretion in the SH rats was likely due to alteration of the $\alpha_{2a/d}$-adrenoceptor gene rather than elevated blood pressure or a global renal defect. Moreover, these data suggested that the altered $\alpha_{2a/d}$-subtype gene and defective function may contribute to the onset of hypertension.

6

GENERAL DISCUSSION

Summary

A summary of the findings in this thesis have been illustrated in figure 6.1. Initial experiments investigated the renal effects of clonidine. We had confirmed that in the uninephrectomized, anesthetized rat, clonidine increased urine flow rate and sodium excretion. Urine flow rate has two components: osmolar clearance and free water clearance. If a drug were to increase one of these components while decreasing the other, clearly change (or lack of change) in urine flow rate may not reflect these actions. Hence, we were interested primarily in osmolar and free water clearance when evaluating renal effects of various drugs. The increase in urine flow produced by clonidine was due to increases in both osmolar clearance and free water clearance.

The osmolar and free water effects of clonidine had been postulated to involve two anatomical sites and/or receptors. In support of this, we were able to pharmacologically distinguish between these effects. The osmolar response was naltrexone-sensitive, and the free water effect was prazosin-sensitive.

It was subsequently deduced (but not proven) that the free water response to clonidine was mediated by the α_{2b}-adrenoceptor subtype. This was based on the increased selectivity of prazosin for this α_2-adrenoceptor subtype. Thereafter, we were interested in the receptor which mediated the osmolar clearance induced by clonidine.

Two receptors were considered as possible mediators of this osmolar response to clonidine. The first possible receptor, the imidazoline receptor, was considered since clonidine has been reported to be a mixed α_2-adrenoceptor/imidazoline receptor agonist. Imidazoline receptor stimulation will also increase osmolar clearance. We postulated that clonidine was stimulating renal imidazoline receptors to increase osmolar clearance. It was expected then that if the selective imidazoline receptor agonist, moxonidine, was administered to the same experimental preparation, then the ensuing osmolar clearance would likewise be attenuated by naltrexone. Moxonidine did result in an increase in osmolar clearance. Naltrexone failed to block this response suggesting that imidazoline receptors were not involved in the osmolar response to clonidine.

Based on the limited α_2-adrenoceptor subtypes present in the rat kidney (that is, $\alpha_{2a/d}$- and α_{2b}-subtypes), we next considered the $\alpha_{2a/d}$-adrenoceptor subtype. Lower doses of two drugs with selectivity for the $\alpha_{2a/d}$-adrenoceptor subtype (UK-14,304 and guanfacine) increased urine flow rate and osmolar clearance. No effects on free water clearance were produced. As with the osmolar response to clonidine, these renal effects were attenuated by naltrexone. Prazosin failed to block these effects. RX-821002, an antagonist with relative selectivity for the $\alpha_{2a/d}$-adrenoceptor subtype, also attenuated the osmolar response to guanfacine. These data were consistent with the hypothesis that the renal $\alpha_{2a/d}$-subtype mediated osmolar clearance in the rat kidney.

Concurrently, reports in the literature indicated that in hypertension, the $\alpha_{2a/d}$-adrenoceptor gene was altered. One such alteration had been correlated with blood pressure in progeny from a cross between spontaneously hypertensive and Wistar-Kyoto

rats. Our finding that the renal $\alpha_{2a/d}$-adrenoceptor subtype mediated solute excretion led to the hypothesis that in spontaneously hypertensive (SH) rats, where this receptor may have been defective, the osmolar response to guanfacine would be absent. In Wistar rats (normotensive control), guanfacine dose-dependently increased urine flow rate secondary to increased osmolar clearance. In SH rats, however, guanfacine failed to elicit these effects. The unresponsiveness to guanfacine was not due to the elevated blood pressure in the SH rats. Despite the increased blood pressure in an acquired model of hypertension (Wistar one kidney-one clip rats), guanfacine still increased urine flow rate and osmolar clearance. The absence of the guanfacine-induced solute excretion in SH rats provided a functional link between a genetic alteration (the $\alpha_{2a/d}$-adrenoceptor gene) and hypertension.

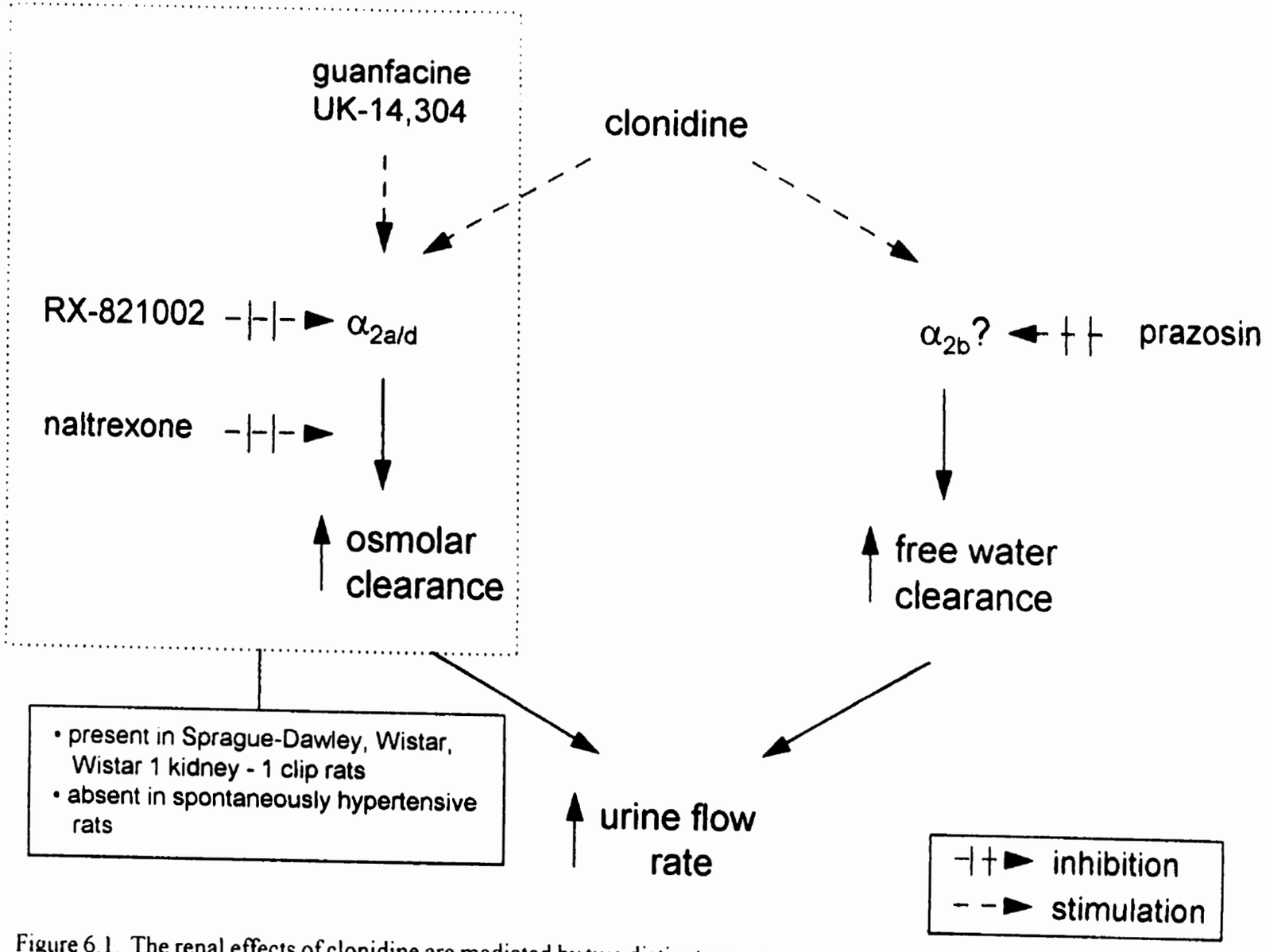

Figure 6.1. The renal effects of clonidine are mediated by two distinct receptors

Anatomical Localization of the Osmolar Actions of $\alpha_{2a/d}$-Adrenoceptors

The anatomical site(s) involved in the natriuretic effects of $\alpha_{2a/d}$-adrenoceptor stimulation was/were not determined by the experiments within this thesis. In general, there were three possibilities: central, extra-renal/peripheral, and intrarenal mechanisms may have been involved.

Intrarenal administration of clonidine and guanfacine produced decreases in heart rate (figures 2.1, 4.7, 5.4, and 5.7). Although not every group which received guanfacine displayed a bradycardiac response, this observation suggested that the natriuresis produced by these drugs was mediated, at least in part, centrally. The $\alpha_{2a/d}$-adrenoceptor agonists, albeit infused directly to the kidney via the renal artery, may have been reaching central sites, perhaps to decrease sympathetic nerve activity (Koepke and DiBona, 1986). The decrease in renal nerve activity and subsequent non-activation of post-junctional α_1-adrenoceptors would produce an increase in sodium excretion (Smyth *et al.*, 1985). Alternatively, the receptors involved may have been pre-synaptic within the kidney. $\alpha_{2a/d}$-adrenoceptors have been identified as the subtype which mediates terminal norepinephrine release from the renal nerves (Bohmann *et al.*, 1994). Stimulation of these receptors would again result in decreased norepinephrine release and non-activation of the post-synaptic α_1-adrenoceptors. This effective decrease in tonic activity of the renal sympathetic nerves would result in the observed increase in sodium excretion. If central mechanisms involving the renal sympathetic nerves, or systemically, stimulation of pre-synaptic $\alpha_{2a/d}$-adrenoceptors in the kidney, were involved in the natriuresis produced by α_2-adrenoceptor agonists, then pharmacological blockade of the post-junctional α_1-

adrenoceptors would antagonize these effects. In our studies, prazosin did not attenuate the increases in osmolar clearance produced by α_2-adrenoceptor stimulation. This suggested that central mechanisms involving the renal sympathetic nerves were not involved in these responses. The involvement or non-involvement of renal nerves could have been addressed using renal denervation experiments with intrarenal administration of the $\alpha_{2a/d}$-agonists. These studies have not yet been conducted.

Clonidine and guanfacine may also have been decreasing the central release of vasopressin as previously proposed (Barr and Kauker, 1979). Accordingly, antagonism of V_2-vasopressin receptors ablated the natriuretic effect of clonidine (Blandford and Smyth, 1990). These data suggested that the antagonism of vasopressin (either of the release or the tubular effects) was involved in the clonidine-induced increase in osmolar clearance. It has also been reported that α_2-adrenoceptor agonists did not affect plasma levels of vasopressin in the rat (Leander *et al.*, 1985) and the dog (Gellai and Edwards, 1988). Moreover, guanfacine increased osmolar clearance without a concomitant increase in free water clearance which would be expected if decreased plasma levels of vasopressin were mediating this response. To confirm that clonidine and guanfacine were not inhibiting the release of vasopressin, the renal effects of these drugs could be examined in a vasopressin clamp experiment where plasma vasopressin levels are maintained. If the responses to α_2-adrenoceptor agonists remain intact, then conceivably altering the release of vasopressin is not involved in the renal effects of these agonists. Vasopressin clamp studies would not, however, exclude the possibility that clonidine and guanfacine were reversing the tubular effects of vasopressin.

In the present thesis, the α_2-adrenoceptor agonists were administered directly to the kidney via the renal artery. Although the drugs were intended to reach the kidney only, their ability to affect heart rate and blood pressure indicated that low amounts may have reached extrarenal sites. In the dog, Strandhoy *et al.* (1982) reported enhanced natriuresis produced by α_2-adrenoceptor stimulation following an intravenous infusion as compared to intrarenal infusion of guanabenz. Likewise, in the rat, intravenous infusion of clonidine was more potent than intrarenal infusion in stimulating sodium excretion (Blandford and Smyth, 1989). It was speculated that this increased potency of these drugs following intravenous administration indicated the involvement of extrarenal mechanisms. In these studies, the possibility that extrarenal factors contributed to the natriuretic effects of $\alpha_{2a/d}$-adrenoceptor stimulation cannot be negated. However, it certainly seems plausible that intrarenal mechanisms were invoked by $\alpha_{2a/d}$-adrenoceptor stimulation. Preliminary studies in our laboratory have shown that the same dose of guanfacine administered intrarenally to elicit a renal response, when administered intravenously, failed to affect renal function. If central mechanisms were involved in the osmolar response to guanfacine following intrarenal administration, then intravenous administration of guanfacine would be expected to produce similar renal effects. Experiments with two-kidney rats may illuminate a role, if any, of extrarenal factors in $\alpha_{2a/d}$-mediated natriuresis. If extrarenal factors were not involved in these osmolar responses, then intrarenal infusion of guanfacine to the left kidney would increase sodium excretion ipsilaterally without altering contralateral (right) kidney excretory function. Moreover, intravenous administration of guanfacine would increase sodium excretion from both kidneys. Intrarenal infusion of an

$\alpha_{2a/d}$-selective antagonist to the left kidney would ablate the osmolar response ipsilaterally but would not alter the response of the contralateral kidney.

We have postulated that the $\alpha_{2a/d}$-adrenoceptor subtype in the rat kidney mediated osmolar clearance. The inability of prazosin to affect this response suggested that these receptors, if intrarenal, were extra-junctional in location. Speculation regarding the nephron segment which mediated the natriuretic effects of renal α_2-adrenoceptor stimulation has been controversial. BHT-933 increased water and solute excretion in Sprague-Dawley rats. Micropuncture experiments revealed that these effects were not mediated at the proximal tubule, loop of Henle, or distal tubule. It was surmised that the inhibition of Na^+ reabsorption was probably occurring at the collecting tubule (Stanton *et al.*, 1987). Sites beyond the distal tubule have also been speculated to mediate the increase in sodium excretion produced by clonidine (Barr and Kauker, 1979). In water loaded rats, infusion of clonidine failed to alter fluid reabsorption from the proximal tubule. This finding suggested that clonidine was not acting at a proximal site along the nephron to increase sodium excretion (Smyth *et al.*, 1992a).

Localization of the $\alpha_{2a/d}$-adrenoceptor subtype in the rat kidney may have illuminated the anatomical site of action of the $\alpha_{2a/d}$-adrenoceptor agonists. *In situ* hybridization localized $\alpha_{2a/d}$-adrenoceptor mRNA to the outer and inner zones of the renal medulla of Sprague-Dawley rats. Microscopic analysis showed labeling in collecting ducts (Meister *et al.*, 1994). More recent studies using reverse transcriptase-PCR also identified $\alpha_{2a/d}$-adrenoceptor mRNA in rat cortical collecting ducts (Wilborn *et al.*, 1996). This finding was in agreement with the speculation that clonidine was acting more distally

along the nephron to increase sodium excretion. These data also agreed with the reported ability of α_2-adrenoceptor agonists to decrease cAMP in cortical and medullary collecting ducts (Umemura *et al.*, 1985; Edwards and Gellai, 1988). α_2-Adrenoceptor stimulation has been reported to inhibit cAMP production by vasopressin only in the collecting tubules (Umemura *et al.*, 1985). α_2-Adrenoceptor stimulation in the collecting tubules would decrease cAMP thereby reversing the actions of vasopressin. Accordingly, antagonism of V_2-vasopressin receptors ablated the osmolar response to clonidine (Blandford and Smyth, 1990). Collectively, these reports suggested that the natriuretic effect of $\alpha_{2a/d}$-adrenoceptor stimulation was mediated at a distal site along the nephron.

It has, however, also been contended that the ability of clonidine to inhibit sodium reabsorption involved either the thin loop of Henle or the proximal tubule (Roman *et al.*, 1979). Preliminary experiments in our laboratory have also implicated the proximal segments in the natriuretic response to $\alpha_{2a/d}$-adrenoceptor stimulation. In water-loaded rats, a moderate dose (3.0 nmol/kg/min) and higher dose (10.0 nmol/kg/min) of guanfacine appeared to decrease fluid absorption from the proximal tubule (data not shown). These data implicated the proximal tubule in the sodium excretory response to guanfacine. Although some reports have identified mRNA for the $\alpha_{2a/d}$-adrenoceptor in the renal cortex (Le Jossec *et al.*, 1995b), others have found only the α_{2b}-subtype in the proximal tubules (Meister *et al.*, 1994). The possibility that proximal $\alpha_{2a/d}$-adrenoceptors exist and mediate natriuresis cannot yet be excluded.

Physiological role of the renal $\alpha_{2a/d}$-adrenoceptor subtype

Renal studies using selective α_2-adrenoceptor antagonists have suggested a physiological role for the α_2-adrenoceptors in renal function. In conscious, euvolemic rats, α_2-adrenoceptor blockade with yohimbine significantly decreased urine flow rate. Adrenal medullectomy resulting in undetectable levels of circulating epinephrine abolished these effects of yohimbine on renal function (Gellai, 1990). In anesthetized rats receiving an intravenous infusion of saline (0.097 mL/min), yohimbine also decreased sodium and water excretion (Blandford and Smyth, 1988b). Again, these effects of yohimbine were ablated by adrenalectomy. These results supported the contention that renal α_2-adrenoceptors were acting tonically to modulate renal function. They also suggested that circulating epinephrine (or another factor from the adrenal medulla) may be the endogenous $\alpha_{2a/d}$-adrenoceptor ligand whose actions were revealed by yohimbine.

In the experiments described here, this purported tonic activity of the α_2-adrenoceptors, specifically of the natriuretic $\alpha_{2a/d}$-subtype, was not apparent. RX-821002 failed to unmask a tonic natriuretic function of the $\alpha_{2a/d}$-adrenoceptors. The reason for this discrepancy in the anesthetized rat remained unclear. One possible explanation may be related to the comparative stress levels resulting from the surgical preparation. Blandford and Smyth (1988b) reported that in their experiments, the rats were artificially ventilated. It has been speculated that mechanical ventilation increases vasopressin release and decreases sodium excretion (Burchardi and Kaczmarchzyk, 1994). In these animals, the renal $\alpha_{2a/d}$-adrenoceptors may have been activated in an attempt to reverse or compensate for this effect. α_2-Adrenoceptor stimulation with epinephrine has been shown

to antagonize the effects of vasopressin on sodium excretion in the isolated perfused rat kidney (Smyth *et al.*, 1985). The apparent tonic natriuresis unmasked by yohimbine may therefore have been activity elicited by artificial respiratory stress. In the experiments presented here, following tracheotomy, this specific stress was not induced since the rats were allowed to breathe spontaneously. This may explain why RX-821002 did not unmask any endogenous activity of the $\alpha_{2a/d}$-adrenoceptor.

It has been speculated that circulating epinephrine rather than neuronally released norepinephrine is the endogenous ligand for the renal α_2-adrenoceptors (Smyth *et al.*, 1985). However, preliminary experiments in our laboratory have shown that in the intact, anesthetized rat, epinephrine infusion failed to affect urine flow rate (personal communication, D.D. Smyth). It has also been speculated that perhaps during stress, when circulating epinephrine is elevated, the concentrations of epinephrine are adequate to then stimulate the renal extra-junctional α_2-adrenoceptors (Pettinger *et al.*, 1987). This hypothesis seems contrary to the decreased sodium excretion in response to acute environmental stress which is mediated by increased activity of renal sympathetic nerves (Zimmerman and Frohlich, 1990; Koepke, 1989). Another possibility is that the actions of epinephrine alone are negligible but are revealed in the presence of a potentiator. For example, neuropeptide Y has been shown to potentiate epinephrine-induced constriction in segments of dog coronary arteries (Macho *et al.*, 1989). In conscious rats, neuropeptide Y also potentiated the pressor effect of epinephrine (Lopez *et al.*, 1989). Sodium excretion was increased by neuropeptide Y in anesthetized rats (Smyth *et al.*, 1989). It is possible that in these experiments, neuropeptide Y was increasing the ability of circulating

epinephrine to act on the renal $\alpha_{2a/d}$-adrenoceptor. If the hypothesis that the renal response to neuropeptide Y was mediated by epinephrine at the $\alpha_{2a/d}$-subtype were true, then α_2-adrenoceptor antagonism would abolish the neuropeptide Y-induced increase in sodium excretion.

Possible mechanisms of $\alpha_{2a/d}$-adrenoceptor mediated increases in natriuresis

The proposed mechanisms of increased solute excretion produced by stimulation of $\alpha_{2a/d}$-adrenoceptors have been illustrated in figure 6.2.

(1) *Link between α_2-adrenoceptors and opioids*

The ability of opioid receptor antagonism with naltrexone to block the osmolar response to $\alpha_{2a/d}$-adrenoceptor stimulation suggested an interaction between these two systems. The contention that opioids may have mediated the renal effects of α_2-adrenoceptor stimulation was not without basis. Several reports have implicated opioid receptors in the cardiovascular effects of clonidine. In spontaneously hypertensive rats, the hypotensive effects of clonidine were attenuated by naloxone (Farsang and Kunos, 1979; Farsang *et al.*, 1980) and naltrexone (van Giersbergen *et al.*, 1989). These responses to clonidine in Wistar-Kyoto rats were either attenuated (Farsang *et al.*, 1980) or unaffected (Farsang and Kunos, 1979; van Giersbergen *et al.*, 1989) by opioid receptor antagonism. Naloxone also prevented the hypotensive action of clonidine in anesthetized rabbits (Rhee and De Lapp, 1988). Inhibition of cardiovascular effects by opioid receptor antagonism was not limited to clonidine. The hypotensive actions of other α_2-

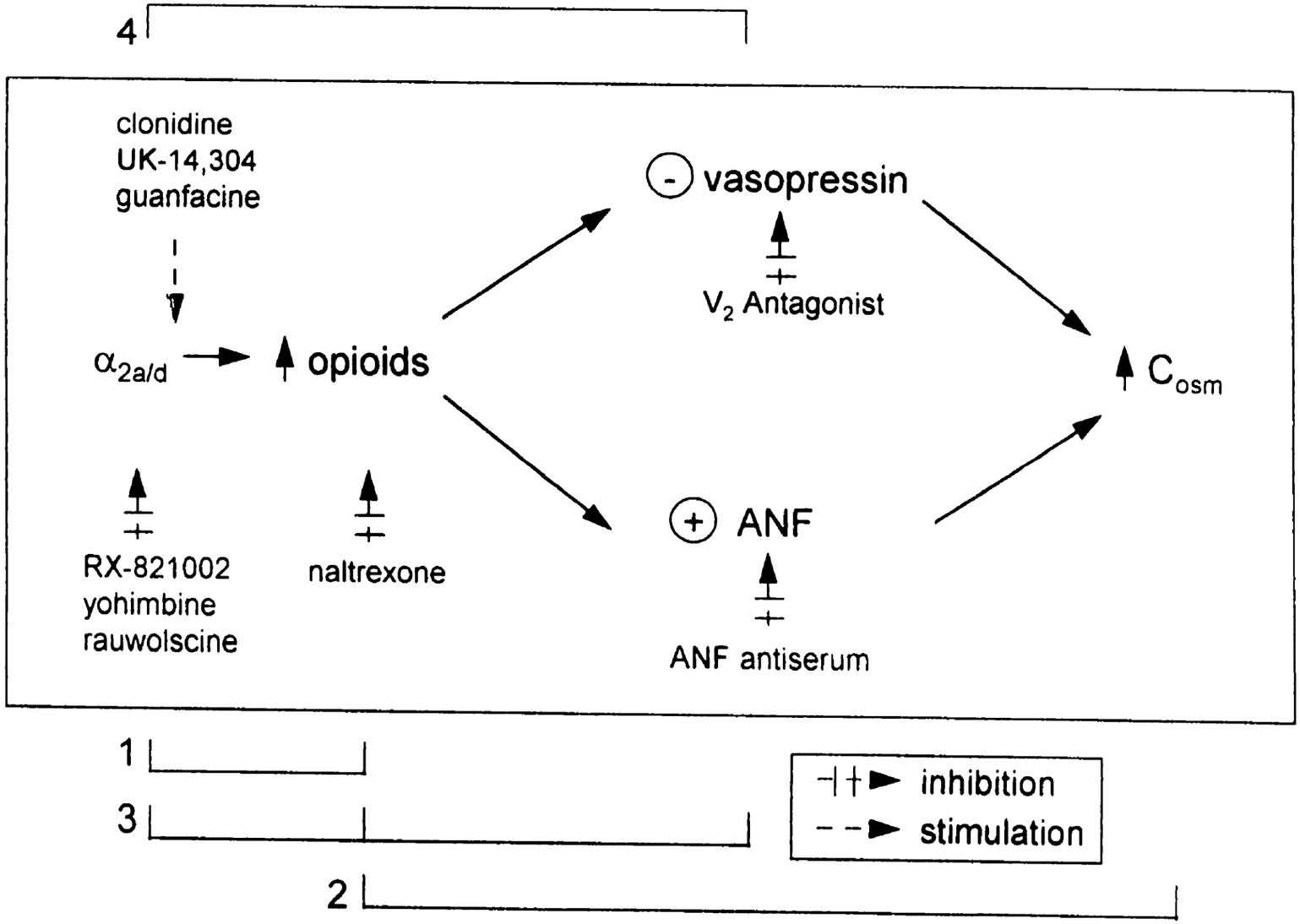

Figure 6.2 Proposed mechanisms of $\alpha_{2a/d}$-adrenoceptor mediated increases in natriuresis

adrenoceptor agonists including BHT-933 (Tackett and Laskey, 1987) and α-methylnorepinephrine (Petty and De Jong, 1984) were similarly blocked by naloxone.

Based on the strong evidence for interactions between the α_2-adrenoceptors and opioid systems and the naltrexone-sensitivity of clonidine, guanfacine, and UK-14,304-induced osmolar clearance, it is conceivable that opioids were involved in these responses to $\alpha_{2a/d}$-adrenoceptor stimulation.

(2) *Opioids and Solute Excretion*

Evidently, in response to $\alpha_{2a/d}$-adrenoceptor stimulation, opioids were somehow involved to later increase osmolar clearance. Several endogenous opioid ligands have been identified. More specifically, three opioid peptide families exist where the opioids arise from one of three precursor molecules. These include the beta-endorphin/ACTH precursor, the enkephalin precursor, and the dynorphin precursor (for review, see Akil *et al.*, 1984). Multiple opioid receptors including the μ-, δ-, κ-, σ-, and ε-subtypes have been identified (Akil *et al.*, 1984; Simonds, 1988). To date, knowledge is limited regarding the σ- and ε-subtypes. Although some overlap exists, all pro-enkephalin peptides show high affinity for δ-receptors. The dynorphins show κ-subtype preference. Beta-endorphin shows potency at both μ-and δ-receptor subtypes.

Several reports indicated that opioids play a role in renal function. The evidence supporting an anti-natriuretic role for opioids is extensive. Following sodium restriction in the rat, intracerebroventricular administration of naltrexone methylbromide (opioid receptor antagonist) resulted in a more negative sodium balance compared with control

animals. This suggested that central opioids are involved somehow in the retention of sodium (Kapusta and Obih, 1995a). For example, in spontaneously hypertensive rats, air stress decreased sodium excretion by a mechanism that was sensitive to central opioid receptor blockade. Peripheral opioid receptors were not involved (Kapusta *et* al., 1989a). Kapusta *et al.* (1990) also demonstrated that the decreased sodium excretion resulting from low frequency renal nerve stimulation was attenuated by naloxone.

The effects of μ- and κ-opioids on solute excretion have been studied extensively whereas the information regarding the δ-opioids is limited. The μ-opioids appear to be anti-natriuretic. Intravenous administration of morphine in conscious rats decreased sodium excretion by a mechanism dependent, at least partially, on renal nerves (Walker and Murphy, 1984; 1986). Intrarenal infusion of dermorphin (μ-selective agonist) also decreased sodium excretion. This response was abolished by naloxone and renal denervation. Central but not peripheral administration of dermorphin decreased sodium excretion in Sprague-Dawley rats independently of renal nerves (Kapusta *et al.*, 1991; 1993). The authors speculated that peripherally administered dermorphin may have been affecting nerve terminal release or the action of norepinephrine directly on the tubule (Kapusta *et al.*, 1991). The additional μ-opioid receptor mechanisms which mediated the central effects of dermorphin on renal sodium handling independently of intact renal innervation were unclear (Kapusta *et al.*, 1993).

Likewise, κ-opioids have been reported to mediate anti-natriuresis. The increased sodium excretion produced by isotonic saline volume expansion was suppressed by central administration of U-50488H (κ-selective agonist). κ-subtype agonists were thus

speculated to have some anti-natriuretic activity (Kapusta and Obih, 1995b). In spontaneously hypertensive rats, peripheral κ-receptor stimulation also decreased sodium excretion. In contrast to the antinatriuresis produced by μ-subtype stimulation, this antinatriuresis was unaffected by renal denervation (Kapusta *et al.*, 1989b).

Natriuretic role of opioids?

There are accounts which demonstrated natriuresis mediated by opioids. Ribstein and Humphreys (1983) showed that acute reductions in the function of one kidney increased sodium excretion from the other kidney. This compensatory natriuresis was blocked by naloxone thereby implicating opioids in this response. In conscious Sprague-Dawley rats, D-Ala2-methionine enkephalinamide (an analog of methionine enkephalin) increased sodium excretion after intravenous administration. The confounding finding was that methionine enkephalin itself decreased sodium excretion. Both of these effects were attenuated by naloxone and was independent of renal nerves (Kapusta *et al.*, 1989c). More recently, the intravenous administration of a μ-selective tetrapeptide (TAPP or H-Tyr-D-Ala-Phe-Phe-NH_2) increased urine flow rate and sodium excretion. These effects, which were attributed to stimulation of μ-receptors in the periphery, were abolished by naloxone pretreatment (Gutkowska and Schiller, 1996). Sezen *et al.* (1996) recently reported that a selective delta opioid agonist BW373U86 increased natriuresis in conscious rats. These few studies allowed the hypothesis that opioids may be mediating the renal effects of clonidine, guanfacine, and UK-14,304.

(3) *Interaction between α_2-adrenoceptors, opioids, and atrial natriuretic factor*

The interaction between α_2-adrenoceptors and ANF has been established where peripheral administration of clonidine (subcutaneous or intravenous) increased plasma ANF levels (Vollmar *et al.*, 1987; Baranowska *et al.*, 1987). Anti-ANF antibodies also appeared to inhibit clonidine-induced urine flow in normally hydrated rats (Baranowska *et al.*, 1988) although the effects of anti-ANF antibodies alone were not documented. These data nevertheless implicated ANF in the renal response to clonidine. As with peripheral administration, the i.c.v. infusion of clonidine (10 μg/min, 45 min) increased plasma ANF without concomitant changes in blood pressure (Chen *et al.*, 1989).

ANF and the opioid system also appear to be related. A recent report by Gutkowska and Schiller (1996) indicated that the peripheral stimulation of μ-opioid receptors with TAPP increased natriuresis by a mechanism involving ANF. These findings were consistent with previous reports that μ-opioid receptor agonists such as fentanyl and morphine (administered centrally, though) increased plasma ANF levels (Vollmar *et al.*, 1987; Chen *et al.*, 1989).

Finally, a relationship between α_2-adrenoceptors, opioids, and ANF has been suggested although the mechanisms therein remain unclear. Naloxone attenuated the ability of high dose intravenous clonidine (50 μg) to increase plasma ANF in normally hydrated rats (Baranowska *et al.*, 1987) suggesting that opioids were somehow mediating this effect of clonidine on ANF. As with peripheral administration, the increase in plasma ANF in response to i.c.v. infusion of clonidine was partially attenuated by naloxone further implicating the opioid systems in these effects (Chen *et al.*, 1989).

(4) *Involvement of vasopressin in the osmolar effects of $\alpha_{2a/d}$-adrenoceptor stimulation*

The role of vasopressin in $\alpha_{2a/d}$-subtype mediated increases in solute excretion is unclear. V_2-vasopressin receptor antagonism blocked the clonidine-induced increase in sodium excretion. This strongly suggested that the antagonism of either the release or the tubular effects of vasopressin was involved in the clonidine-induced increase in osmolar clearance (Blandford and Smyth, 1990). It seems unlikely that clonidine or other agonists for the $\alpha_{2a/d}$-adrenoceptor were decreasing the release of vasopressin. α_2-Adrenoceptor agonists failed to affect plasma levels of vasopressin in the rat (Leander *et al.*, 1985) and the dog (Gellai and Edwards, 1988). Moreover, guanfacine increased osmolar clearance and not free water clearance. If guanfacine decreased plasma levels of vasopressin, an increase in free water clearance would be expected.

It is more conceivable that functional antagonism of vasopressin (for example, at the tubular level) was mediating the increased solute excretion produced by $\alpha_{2a/d}$-adrenoceptor stimulation. α_2-stimulation inhibited cAMP production by vasopressin only in collecting tubules (Umemura *et al.*, 1985) suggesting that this is the most likely site at which the $\alpha_{2a/d}$-adrenoceptors would be increasing osmolar clearance. In support of this, the $\alpha_{2a/d}$-adrenoceptor subtype mRNA has been detected in the collecting ducts by *in situ* hybridization (Meister *et al.*, 1994) and reverse transcriptase-PCR (Wilborn *et al.*, 1996). The difficulty lies in the ability of $\alpha_{2a/d}$-adrenoceptor stimulation to selectively increase osmolar clearance. If antagonism of vasopressin activity at the collecting tubules is responsible for clonidine-induced increases in both free water clearance and osmolar

clearance, then it seems unlikely that these effects could be dissociated pharmacologically as we have observed.

Studies have shown that vasopressin increases water permeability in rat kidney collecting ducts by translocating aquaporin water channels to the apical plasma membrane (Nielsen *et al.*, 1995). Both the distribution and the density of these water channels were regulated by vasopressin via the V_2-vasopressin receptor (Hayashi *et al.*, 1994). α_2-Adrenoceptor stimulation conceivably increased free water clearance by reversing the effects of vasopressin on these aquaporins. Since the free water and osmolar effects of clonidine can be attenuated by V_2-vasopressin receptor antagonism and dissociated by dose or pharmacologically, the mechanisms of these responses were clearly different in some way. Whether vasopressin modulates water and sodium excretion at the same site is unknown. Perhaps some members of the vasopressin-associated aquaporin family are permeable to ionic solutes whereas others are impermeable specifically to sodium.

Alternatively, the effects of renal α_2-adrenoceptors on vasopressin-mediated sodium retention may be indirect. For example, stimulation of V_2-vasopressin receptors in the rat renal medullary thick ascending limb of Henle's loop has been shown to stimulate Na^+/K^+-ATPase activity (Charlton and Baylis, 1990). Perhaps clonidine was stimulating the release of other factors (opioids or ANF) which in turn reversed the actions of vasopressin on Na^+/K^+-ATPase. The functional colocalization in collecting tubules of α_2-stimulation with decreased cAMP production by vasopressin (Umemura *et al.*, 1985) may therefore not necessarily mean that clonidine was increasing osmolar clearance in this

segment. Clearly, the interaction between $\alpha_{2a/d}$-adrenoceptors, vasopressin, and sodium excretion requires further investigation.

Possible mechanisms of α_2 -adrenoceptor mediated anti-natriuresis

(1) *Anti-natriuretic function of the α_2-adrenoceptor*

Despite extensive evidence that the α_2-adrenoceptors mediate natriuresis, possible mechanisms of α_2-adrenoceptor-mediated anti-natriuresis have been suggested (figure 6.3). It is conceivable that the renal α_2-adrenoceptors are dichotomous (natriuretic and anti-natriuretic) in function. Moreover, both functions may be present in concert, with the natriuretic activity being predominant so that the net effect is natriuresis. Blocking this natriuretic response to α_2-adrenoceptor stimulation may therefore unmask the anti-natriuresis. For example, in the presence of a V_2-vasopressin receptor antagonist (which abolished the natriuretic effect of clonidine), clonidine decreased sodium excretion (Blandford and Smyth, 1990).

In these studies, in the presence of naltrexone, clonidine (but not UK-14,304 or guanfacine) decreased sodium excretion. The inability of UK-14,304 or guanfacine to similarly decrease sodium excretion in the presence of naltrexone suggested that an α_2-adrenoceptor subtype other than the $\alpha_{2a/d}$-subtype was mediating the anti-natriuretic effect. Clonidine, being relatively non-selective between subtypes, was able to stimulate this decrease in sodium excretion. Assuming that the osmolar response to guanfacine is dependent on vasopressin, then in the presence of a V_2-vasopressin receptor antagonist, guanfacine would not be expected to increase sodium excretion. Furthermore, if the

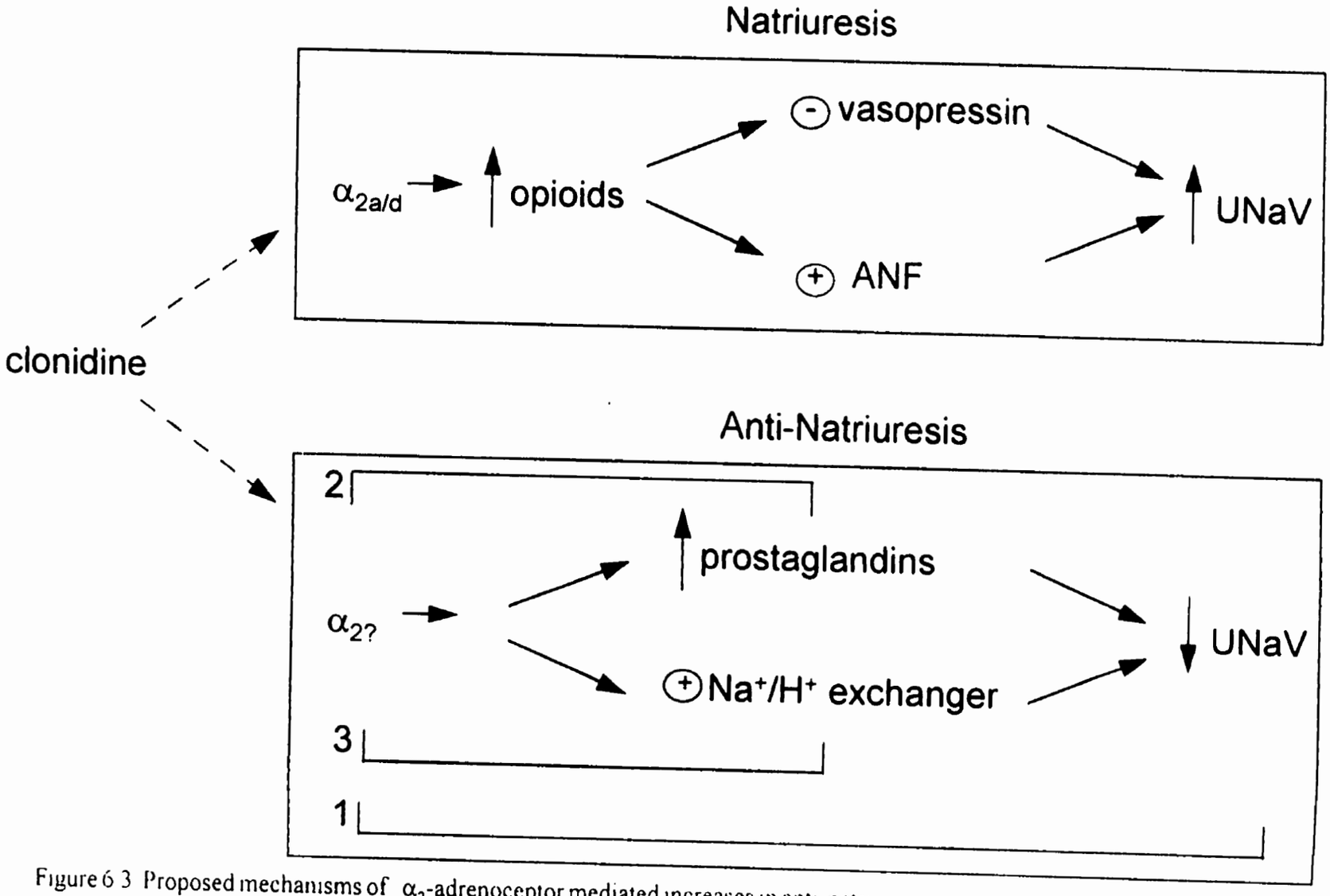

Figure 6 3 Proposed mechanisms of α_2-adrenoceptor mediated increases in anti-natriuresis

postulate that an α_2-subtype other than the $\alpha_{2a/d}$-adrenoceptor was mediating anti-natriuresis was true, then in the presence of a V_2-vasopressin receptor antagonist, guanfacine would also fail to decrease sodium excretion.

(2) *Link between α_2-adrenoceptors, prostaglandins, and decreased sodium excretion*

Indomethacin pretreatment potentiated the natriuretic effect of clonidine in the anesthetized rat (Blandford and Smyth, 1991). Intravenous infusion of prostaglandin E_2 reversed this effect. This report suggested that clonidine was increasing prostaglandin E_2 in the kidney which would then decrease sodium excretion. Likewise, infusion of prostaglandin E_2 into the renal interstitium of the rat decreased sodium excretion in the rat (Long *et al.*, 1990). Cooper and Malik (1985) reported modest but significant increases in prostaglandin E_2 in the isolated perfused rat kidney in response to the α_2-adrenoceptor agonists BHT-933 and guanabenz. Again, if the postulate that an α_2-subtype other than the $\alpha_{2a/d}$-adrenoceptor was mediating anti-natriuresis, then contrary to the results with clonidine, indomethacin pretreatment would fail to potentiate the osmolar response to guanfacine and UK-14,304.

(3) *α_2-Adrenoceptors and the Na^+/H^+-exchanger*

Renal α_2-adrenoceptors have also been associated with the Na^+-H^+ exchanger in rat proximal tubules (Gesek and Strandhoy, 1990). Using an amiloride analog inhibitor of this antiporter, ethylisopropyl amiloride (EIPA), the effects of α_2-adrenoceptor agonists specifically on the Na^+-H^+ exchanger could be identified. Guanabenz and BHT-933

increased the EIPA-sensitive activity of the Na^+-H^+ exchanger by a pertussis-sensitive mechanism. These findings were consistent with the earlier observation that α_2-adrenoceptor agonists activated the Na^+-H^+ exchanger in isolated proximal tubule cells of rabbits (Nord *et al.*, 1987). If activation of the Na^+-H^+ exchanger was involved in the anti-natriuretic effects of clonidine, then, in the presence of V_2-vasopressin receptor antagonism (to reveal the anti-natriuresis), EIPA pretreatment of the rat kidney would abolish the clonidine-induced decrease in sodium excretion. EIPA would also be expected to potentiate the osmolar response to clonidine as did indomethacin.

Candidate genes and hypertension

The problem with studying the pathogenesis of hypertension lies in the complexity of the disease. The cause of this disease is presumably polygenic in that several genes are responsible for blood pressure regulation. Moreover, environmental circumstances such as stress, diet, and physical activity may complicate the relationship between genotypic profile and phenotypic blood pressure.

Genes which contribute to the variability of blood pressure have been termed "candidate genes." The role of these genes in the onset of hypertension may be documented or merely suspected. In 1983, Rapp delineated criteria by which candidate genes may be defined. Functional relevance must be established. Certainly the gene products should be involved in blood pressure regulation. Alterations of these genes would result in some physiological/biochemical disorder which would accordingly

predispose to hypertension. The "hypertensive" allele of the candidate gene in question should also correlate with elevated blood pressure.

Several candidate genes for hypertension are presently being investigated. For example, various components of the renin-angiotensin-system have been studied extensively. The renin gene is a candidate gene with polymorphisms identified in humans (Webb *et al.*, 1989) and in rats (Rapp *et al.*, 1989). The hypertensive allele of the renin gene has co-segregated with elevated blood pressure in Dahl salt-sensitive rats (Rapp *et al.*, 1989). Transgenic rats called TGR(mREN2)27 have been produced which carry the mouse renin gene (ren-2) (Mullins *et al.*, 1990). Increased dosage of ren-2 resulted in higher blood pressure levels. In these animals, plasma renin levels were normal. The hypertension in these animals, in part, was mediated by increased adrenal renin levels which may interfere with adrenal steroid synthesis (Sander *et al.*, 1992). The renin gene then appeared to fulfil the criteria set out by Rapp (1983) for candidate genes. Where renin contributes physiologically to blood pressure regulation, alterations of the renin gene have cosegregated with hypertension in rats. Relevant functional defects may have also been identified to date, namely, altered regulation of steroid biosynthesis. Of concern, however, were several reports indicating that polymorphisms of the renin gene did not correlate with blood pressure in humans (Naftilan *et al.*, 1989; Morris and Griffiths, 1988; Webb *et al.*, 1989; Soubrier *et al.*, 1990).

Another candidate gene of hypertension is the SA gene. SA mRNA were initially identified by Iwai and Inagami (1991). SA mRNA was ten-fold more abundantly expressed in the kidneys of spontaneously hypertensive rats than of Wistar-Kyoto rats. In

the F2 population generated from crossbreeding SH and WKy rats, increasing dosage of the SA gene correlated with elevated blood pressure (Iwai and Inagami, 1992). The human counterpart of the SA gene was subsequently identified (Iwai *et al.*, 1994). A polymorphism of the SA gene was identified in humans and this allele also correlated with elevated blood pressure. In contending that the SA gene is a candidate gene for hypertension, many unanswered questions have remained. The product of this gene has not yet been identified. Although the gene does correlate with elevated blood pressure, the other criteria for candidate genes cannot be studied until this protein has been identified. Namely, functional relevance as well as some physiological disorder has not yet been linked to the SA gene.

Many candidate genes are under investigation for their role in the pathogenesis of hypertension. These include (not exclusively) the genes for angiotensinogen, angiotensin receptors, kallikrein, atrial natriuretic peptide and various ion exchange systems (for review, see Leckie, 1992).

Theoretical alterations of the $\alpha_{2a/d}$-adrenoceptor in hypertension

The information so far strongly supported the identification of the $\alpha_{2a/d}$-adrenoceptor gene as a candidate gene for hypertension. We found that the natriuretic function of the $\alpha_{2a/d}$-adrenoceptor was absent in spontaneously hypertensive rats. Aside from the molecular evidence suggesting the $\alpha_{2a/d}$-subtype gene is altered in hypertension, our data suggested that the function of the $\alpha_{2a/d}$-subtype is also different in this disease state. Since we demonstrated that the osmolar response to guanfacine was intact in an

acquired model of hypertension, the absence of response in spontaneously hypertensive rats was not a consequence of the elevated blood pressure. It was conceivable then that the defective renal function of the $\alpha_{2a/d}$-subtype was genetically determined.

The identification of polymorphisms of the $\alpha_{2a/d}$-adrenoceptor gene in humans (Hoehe *et al.*, 1988) and rats (Chun *et al.*, 1991) merely indicated the existence of a second allele. The correlation of these "hypertensive" alleles with elevated blood pressure (Pettinger *et al.*, 1991; Lockette *et al.*, 1995; Svetkey *et al.*, 1995) suggested that alterations of this gene may somehow be changing the function of the $\alpha_{2a/d}$-adrenoceptor. This modified function would then be contributing to the pathogenesis of hypertension. The precise mechanism of altered $\alpha_{2a/d}$-adrenoceptor function (in our case, absence of natriuretic function) has not yet been identified.

As there are several possible steps in the pathway of $\alpha_{2a/d}$-adrenoceptor stimulation leading to increased osmolar clearance, there are also several possible modifications of the $\alpha_{2a/d}$-adrenoceptor-related mechanisms. Firstly, regulation of expression of the $\alpha_{2a/d}$-subtype may be altered whereas the encoding sequence, when expressed, may produce the normal receptor. A workable example lies in the Sabra rats. As previously discussed, Le Jossec *et al.* (1995b) have shown that in renal cortex tissue from both Sabra salt-sensitive and salt-resistant rats, the mRNA for both the $\alpha_{2a/d}$- and α_{2b}-subtype were detectable. The $\alpha_{2a/d}$-subtype was found only in kidneys from salt-resistant rats by radioligand binding studies. Post-transcriptional or post-translational modifications of the $\alpha_{2a/d}$-subtype may have occurred in the salt-sensitive strain to affect expression of this receptor. Cell-specific regulation of transcription has recently been implicated for the rat $\alpha_{2a/d}$-adrenoceptor gene.

The -131/-92 region of this gene has recognition sites for Sp1, a zinc finger nuclear factor. Deletion of these Sp1 binding sites demonstrated a negative effect on transcription in HT29 cells and a positive effect in RINm5F cells. These data suggested that Sp1 positively and negatively causes transcription in these two cell systems respectively (Handy and Gavras, 1996). Perhaps in the Sabra salt-sensitive rats, the ability of Sp1 to bind to the -131/-92 region or to influence transcription initiation has been altered as compared to the Sabra salt-resistant rats. Alternatively, in the salt-resistant Sabra rats, the effect of Sp1 on transcription may be positive while exerting a negative effect in the salt-sensitive strain.

Alternatively, the effector system to which the $\alpha_{2a/d}$-adrenoceptor is coupled may be altered or "disconnected" as a result of the "hypertensive" allele of the $\alpha_{2a/d}$-subtype gene. The α_2-adrenoceptors have been associated with several effector systems. These include inhibition of adenylyl cyclase, stimulation of K^+ channels, and inhibition of voltage-sensitive Ca^{2+}-channels (Limbird, 1988). Mutation of the $\alpha_{2a/d}$-adrenoceptor gene at a conserved aspartate residue diminished allosteric regulation by sodium of the $\alpha_{2a/d}$-subtype (Horstman *et al.*, 1990). Interactions of the $\alpha_{2a/d}$-subtype with G-proteins were also disrupted by this mutation (Ceresa and Limbird, 1994). Surprenant *et al.* (1992) demonstrated that a mutation at the same aspartate residue dissociated the $\alpha_{2a/d}$-subtype from inwardly rectifying K^+ channels. This uncoupling partially attenuated inhibition of isoproterenol-stimulated ACTH secretion by UK-14,304. However, coupling of the $\alpha_{2a/d}$-subtype to adenylyl cyclase or Ca^{2+} channels were unaffected by this mutation. Some inhibitory actions of UK-14,304 remained intact because simultaneous modulation of

adenylyl cyclase and voltage-sensitive Ca^{2+}-channels also contribute to this effect of UK-14,304. These reports indicated that a point mutation can significantly alter the function of the $\alpha_{2a/d}$-adrenoceptor by affecting coupling to the relevant effector systems. An example of the effects of this mutation which was relevant to blood pressure regulation was reported recently. MacMillan *et al.* (1996) introduced this mutant $\alpha_{2a/d}$-subtype into mice. Wild-type mice responded to UK-14,304 administered into the carotid artery biphasically with a transient hypertensive response followed by a prolonged hypotensive response. In the mutant mice, the hypotensive response to UK-14,304 was absent. In hypertension, a similar, naturally-occurring mutation may in fact be responsible for modified $\alpha_{2a/d}$-adrenoceptor function.

A third possible variation in the mechanism of $\alpha_{2a/d}$-subtype function may be related to the link of this receptor to opioid receptors. In spontaneously hypertensive (SH) rats and normotensive Sprague-Dawley rats, naloxone inhibited the hypotensive and bradycardiac effects of clonidine in the nucleus tractus solitarius (Mosqueda-Garcia and Kunos, 1987). In the SH rats, δ-opioid receptor antagonism (but not μ-receptor antagonism) similarly attenuated the cardiovascular effects of clonidine. The converse was true in Sprague-Dawley rats. μ-Opioid receptor antagonism blocked the effects of clonidine whereas δ-opioid receptor blockade had no effect. In this report, it was shown that in Sprague-Dawley rats where hypertension was induced with deoxycorticosterone pivalate/salt, the opioid receptor mediating the cardiovascular effects of clonidine appeared to change to the δ-opioid receptor as in the SH rats. These results suggested that the blood pressure level and not genetic determination regulated the opioid receptor

subtypes associated with α_2-adrenoceptors. In our studies, the absence of response to $\alpha_{2a/d}$-adrenoceptor stimulation in SH rats may also have been due to hypertension-induced changes in the involved opioid receptor subtype. The available data however gave rise to the speculation that somehow the association between the $\alpha_{2a/d}$-adrenoceptor subtype and a specific opioid receptor subtype was determined by the $\alpha_{2a/d}$-subtype gene. In the above study, Sprague-Dawley and not Wistar-Kyoto rats were used because despite the normal blood pressure in these rats, several reports have documented the inability of naloxone to attenuate the cardiovascular effects of clonidine. Perhaps between the Wistar and SH rats, a fundamental genetic difference (possibly in the $\alpha_{2a/d}$-subtype gene) resulted in different interactions between the α_2-adrenoceptors and opioid receptors.

The few examples discussed above represent potential modifications which may be determined by the altered $\alpha_{2a/d}$-adrenoceptor gene which correlated with blood pressure. These or similar potential changes may translate into the absence of natriuresis in response to guanfacine which we observed in the spontaneously hypertensive rats.

Future Directions

The studies presented here have delineated several novel findings and in so doing, identified new questions.

1. *Opioids and renal $\alpha_{2a/d}$-adrenoceptor function*

We used the observation that naltrexone blocked the osmolar response to clonidine to distinguish it from the effect of clonidine on free water clearance. The opioid systems involved were not further investigated. Localization of the opioid system activated by $\alpha_{2a/d}$-adrenoceptor stimulation could be pursued pharmacologically. To determine whether central or peripheral opioid receptors were mediating the osmolar response to guanfacine, naltrexone methylbromide could be used. The quaternary structure of this naltrexone analog does not cross the blood-brain barrier. The effects of intracerebroventricular *vs.* intravenous administration of naltrexone methylbromide on the renal actions of guanfacine could be determined. If peripheral and not central opioids are involved, then naltrexone administered directly to the kidney and followed by guanfacine would determine whether the opioid receptors involved were intrarenal or extrarenal. Attempting to block the renal effects of guanfacine with selective μ-, δ-, κ-subtype antagonists would illuminate which opioid ligand and receptor subtype were involved in $\alpha_{2a/d}$-subtype mediated natriuresis. Furthermore, if the opioid receptor subtype was identified, the ability of selective agonists for that receptor to stimulate osmolar clearance in Wistar *versus* SH rats could be compared. Perhaps the opioid system rather than the $\alpha_{2a/d}$-

subtype was defective in our experiments which resulted in unresponsiveness to guanfacine.

2. *Interaction between $\alpha_{2a/d}$-adrenoceptors and atrial natriuretic factor*

The role, if any, of atrial natriuretic factor (ANF) in the renal effects of $\alpha_{2a/d}$-adrenoceptor stimulation have not been assessed. If the involvement of ANF was significant, then anti-ANF antibodies would be expected to abolish the osmolar response to guanfacine. ANF-mediated natriuresis is probably mediated by stimulation of guanylyl cyclase (Hamet *et al.*, 1984; Waldman *et al.*, 1984). Guanfacine would be expected to increase urinary excretion of cGMP. Also, if the opioid mediator of the renal responses to guanfacine were identified, then anti-ANF antibodies should also attenuate its osmolar effects.

3. *Anti-natriuresis and α_2-adrenoceptors*

As discussed previously, in the presence of a V_2-vasopressin receptor antagonist, clonidine decreased sodium excretion (Blandford and Smyth, 1990). Clonidine appears to be producing anti-natriuresis, in part, by increasing prostaglandin E_2 synthesis which decreased sodium excretion (Blandford and Smyth, 1991). Here, clonidine also appeared to be anti-natriuretic in the presence of naltrexone. Since guanfacine and UK-14,304 were not anti-natriuretic, it is conceivable that an α_2-subtype other than the $\alpha_{2a/d}$-subtype was mediating this decrease in sodium excretion. In the presence of a V_2-vasopressin receptor antagonist, then, guanfacine and UK-14,304 would not decrease sodium excretion as did

clonidine. Indomethacin would also have no effect on the natriuresis produced by these selective agonists in contrast to the potentiation observed with clonidine. Under the conditions where clonidine decreased sodium excretion (V_2-vasopressin receptor antagonist; naltrexone), indomethacin would in part, be expected to attenuate this effect.

Activation of the Na^+/H^+-exchanger has also been proposed to mediate α_2-adrenoceptor anti-natriuresis. If this were true, then an amiloride analog would potentiate the natriuretic effect of clonidine. The ability of clonidine to decrease sodium excretion in the presence of V_2-vasopressin receptor antagonism would also be attenuated at least in part by an amiloride analog.

Concluding remarks

We firstly confirmed that the renal effects of clonidine involved two distinct anatomical sites and/or receptors. The increase in osmolar clearance somehow involved opioid receptors whereas the free water response was independent of opioid receptors. The receptor mediating clonidine-induced increases in free water clearance remains to be identified. Secondly, a novel, potentially physiological role for the $\alpha_{2a/d}$-adrenoceptor subtype was identified. Inherent in the finding that these receptors mediated osmolar clearance was the new association between this α_2-adrenoceptor subtype and the opioid receptors. The nature of this relationship also remains to be elucidated. Finally, we identified a defect or absence of function of the $\alpha_{2a/d}$-adrenoceptor in the spontaneously hypertensive rat - the rat model for essential hypertension. Our implication of the $\alpha_{2a/d}$-adrenoceptor subtype in hypertension, based both on its normal natriuretic function and

the lack of such function in these hypertensive rats, will ideally lead to clearer insight into the mechanisms by which hypertension develops and is maintained.

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