Depressor and Diuretic Effects of Imidazoline Receptor Stimulation in the Paraventricular Nucleus of the Hypothalamus

Heather Mueller

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In Partial Fulfillment of the Requirements for the

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Department of Pharmacology and Therapeutics

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Depressor and Diuretic Effects of Imidazoline Receptor Stimulation in the Paraventricular Nucleus of the Hypothalamus

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Abstract

Imidazoline receptors and α_2 adrenergic receptors located within specific cardiovascular regulatory centres in the brain have been proposed to play a role in the regulation of blood pressure and renal function. To date, the blood pressure and renal responses to central injection of moxonidine have not been decisively attributed to either receptor class or to any specific area of the brain alone. Part of this ambiguity exists because moxonidine, which was developed as an α_2 agonist, is a specific agonist of the imidazoline receptor. We therefore investigated the response to intracerebroventricular (ICV) moxonidine with the specific purpose of locating a region of the brain responsible for the blood pressure and renal effects, and linking these effects to either the I_1 imidazoline receptor or the α_2 adrenergic receptor. Male Sprague-Dawley rats were unilaterally nephrectomized and allowed to recover for 7 to 10 days. Under pentobarbital anesthesia, a tracheotomy was performed and the carotid artery and jugular vein cannulated to monitor blood pressure and infuse saline respectively. The remaining kidney was exposed and the ureter cannulated for the collection of urine throughout the experiment. The rat was fitted into a stereotax and a hole drilled through the skull for later ICV injection. Moxonidine or saline was injected into the left cerebral ventricle using a Hamilton syringe according to predetermined coordinates. Following ICV injection of moxonidine, depressor and diuretic effects were observed. We proposed that the diuretic effect may involve an inhibition of vasopressin. Accordingly, we next investigated the paraventricular nucleus of the hypothalamus (PVN). The PVN is known to be involved in cardiovascular regulation, and also regulates the synthesis of vasopressin. We hypothesized that the depressor and diuretic responses may be separable

in the PVN. While injection of moxonidine into the PVN produced both a depressor and a diuretic effect, only a diuretic effect was observed following the same dose of guanfacine injected into the PVN. We found that moxonidine has a depressor effect that is not separable from the diuretic effect, while guanfacine (α_{2a} agonist) at the same dose is not capable of eliciting a depressor effect. This additional effect of moxonidine may be due to its imidazoline receptor specificity. Further to this experiment, we investigated the role of γ-aminobutyric acid (GABA) in the response to PVN moxonidine. Bicuculline methiodide or saline was injected into the left cerebral ventricle prior to moxonidine injection into the PVN in an attempt to block the depressor response to moxonidine. Although the depressor effect of moxonidine was not attenuated by bicuculline, we cannot rule out the role of GABA in the regulation of blood pressure by the PVN.

In summary, the PVN may play an important regulatory role in coordinating cardiovascular output. This regulatory pathway may involve adrenergic control of vasopressin release, and imidazoline receptor-mediated blood pressure control, which may be further regulated by GABA.

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Abbreviations

AI	Angiotensin I
AII	Angiotensin II
ACE	Angiotensin Converting Enzyme
cAMP	Cyclic Adenosine Monophosphate
E	Epinephrine
GABA	γ-aminobutyric Acid
I	Isoproterenol
ICV	Intracerebroventricular
meE	Methylepinephrine
meNE	Methylnorepinephrine
NE	Norepinephrine
NMDA	N-Methyl-D-Aspartate
NTS	Nucleus Tractus Solitarius
PC-PLC	Phosphatidylcholine Selective Phospholipase C
PVN	Paraventricular Nucleus of the Hypothalamus
RSNA	Renal Sympathetic Nerve Activity
RVLM	Rostral Ventrolateral Medulla
SFO	Subfornical Organ
SH	Spontaneously Hypertensive
WKY	Wistar-Kyoto

General Introduction

The pivotal role of both the kidney and the central nervous system in hypertension has been well documented. Daily blood pressure control is dependent on neural mechanisms; the baroreceptor response is invoked to ensure a rapid response, and hormone release is altered for longer duration of response. In a normotensive individual, neural mechanisms maintain a moderate degree of vasoconstriction. By so doing, the central nervous system can respond immediately to rapid changes in blood pressure by either increasing or decreasing vessel tone through similar neural connections. The tone of these resistance vessels is regulated by sympathetic neurons in the intermediolateral (IML) cell column of the spinal cord (Sun, 1995). These neurons in turn receive input from various supraspinal structures, including the rostral ventrolateral medulla (RVLM) (Dampney, 1994).

Generating Peripheral Sympathetic Tone

Three theories have been proposed by which the RVLM may generate impulses to stimulate the IML neurons and maintain the tone of resistance vessels. Neurons in the RVLM are sensitive to changes in local pO_2, pCO_2, and pH. Hypercapnia or hypoxia can stimulate neurons in the RVLM to cause an increase in sympathetic nerve discharge and arterial pressure (Richerson, 1995; Sun and Reis, 1995b; Sun and Reis, 1995c). According to this theory, as arterial pressure decreases, perfusion to the RVLM is compromised to produce a modest hypoxia or hypercapnia. In response, the RVLM increases sympathetic tone to correct the change in blood pressure.

Another theory is that of a global oscillating system in the brain that is monitored and regulated by the RVLM. This theory stems from the observation of low frequency discharge from various nuclei throughout the pons and medulla oblongata. These include the RVLM, the caudal ventrolateral medulla, the caudal raphe nuclei, the lateral tegmental field, the pontine parabrachial nucleus, and the Kolliker-Fuse nucleus (Huang *et al.*, 1988; Zhong *et al.*, 1993; Guyenet *et al.*, 1989). Due to the similar frequency of firing in these regions, it has been proposed that they comprise a "network oscillator", which generates impulses relayed through the RVLM to produce sympathetic tone (Gebber and Barman, 1989; Kocsis *et al.*, 1990). This theory has been widely debated, and there exist arguments for and against it (reviewed by Sun, 1995 and Barman and Gebber, 1989).

The third theory explaining the mechanism by which sympathetic tone may be generated is the RVLM pacemaker theory. The RVLM has been shown to have intrinsic pacemaker activity in both intact brain and in slice preparation, specifically in the neurons projecting to the spinal cord (Sun *et al.*, 1988). The observation that in a slice preparation automaticity was abolished by hyperpolarization and restored on repolarization suggests that the rhythmic depolarization is intrinsic to the RVLM and does not originate in other brain structures (Sun *et al.*, 1988). This pacemaker activity, which is propagated to the IML cell column, may account for the tonic vasoconstriction observed in resistance vessels.

Regardless of which theory will prove correct, the RVLM and other midbrain structures contain important projections to the spinal cord which are involved in the

generation of sympathetic tone. The RVLM is inhibited to give depressor effects by the caudal ventrolateral medulla (CVLM) (for review see van-Zwieten and Chalmers, 1994).

Baroreceptor Response

As well as tonic vasoconstriction, there are mechanisms by which this sympathetic tone can be adjusted. The baroreceptor response is the first regulatory mechanism to respond to a change in arterial pressure. An increase in blood pressure will stretch the vessels and excite baroreceptors in the carotid sinus and aortic arch. Consequently, these receptors will increase their firing rate and, via central and spinal nerve connections, dilate blood vessels, reduce the rate and force of contraction of the heart, and reduce vasopressin release from the posterior pituitary gland. Similarly, these neural connections also mediate vasoconstriction, increased heart rate and force of contraction, and release of vasopressin in response to a decrease in arterial pressure. The primary point of termination of these afferent neurons is in the nucleus tractus solitarius (NTS), and lesions to this area of the brain completely abolish the baroreceptor response, immediately increasing blood pressure (Colombari *et al.*, 1996). In 1985, it was reported that although the NTS receives input from the carotid sinus baroreceptors, aortic arch receptors, and vagus nerve, the majority of NTS neurons can only respond to stimulus from one source (Donoghue *et al.*, 1985). This finding indicates that the NTS is an important relay and integration center in the baroreceptor response, and is specifically designed with highly organized synaptic connections for this purpose. Neurons from the NTS project to the CVLM, which in turn project to the RVLM. These three centers act to regulate blood pressure as information is received from baroreceptor afferents, and final

output is onto the IML column of the spinal cord (for review, van-Zwieten and Chalmers, 1994).

Renal Regulation of Blood Pressure

Long term regulation of blood pressure is mediated by renal mechanisms to adjust urine concentration and volume as necessary. The renal-fluid volume mechanism for pressure control was first proposed by Arthur Guyton nearly thirty years ago (Guyton *et al.*, 1972). The kidney is capable of responding to stimuli by controlling solute and water excretion through several mechanisms.

One example is the renin-angiotensin system, which is activated in situations of decreased blood pressure or volume. The macula densa cells in the distal tubule of the nephron are sensitive to salt concentration. When glomerular filtration rate is low, the macula densa cells will stimulate renin release from the adjacent juxtaglomerular cells. The afferent arteriole is also sensitive to mechanical stretch and is capable of stimulating renin release. Renin acts on angiotensinogen, a protein produced primarily by the liver, to reduce it to angiotensin I (AI). The cleavage of angiotensinogen to AI is the principal regulatory step in this pathway. Angiotensin converting enzyme (ACE) is a zinc metalloproteinase which further cleaves AI to angiotensin II (AII). AII is the active peptide, which is responsible for mediating various physiological effects. AII corrects the decrease in blood pressure or volume by causing vasoconstriction, increased rate and force of heart contraction, and increasing release of aldosterone. Aldosterone will

promote sodium and water retention to correct the decrease in blood volume. These responses comprise the hormonal mechanism of blood pressure regulation.

Renal Function Curve

As previously mentioned, the kidney is also capable of altering extracellular fluid volume. This is explained by the isolated kidney renal function curve (Figure 1.1). The point on the graph where net intake meets the renal function curve (point A) is termed the equilibrium pressure. At this arterial pressure, the fluid output exactly equals the net intake to maintain this equilibrium pressure. If blood pressure changes, the kidney will compensate, either increasing or decreasing its fluid output to adjust blood pressure accordingly (Guyton et al, 1990). For example, an increase in arterial pressure to 140 mm Hg will result in the kidney excreting more fluid until blood pressure returns to normal. Conversely, if blood pressure is decreased, the kidney will decrease its excretion to accumulate fluid until the pressure rises to normal. Far more important than the fluid accumulation is the intake or loss of salt. As more salt is consumed, thirst will be stimulated and vasopressin released to promote water retention.

Due to the intrinsic ability of the kidney to coordinate both the renal fluid volume system and the renin-angiotensin system, the role of the kidney in the development, maintenance, and treatment of hypertension has been widely investigated.

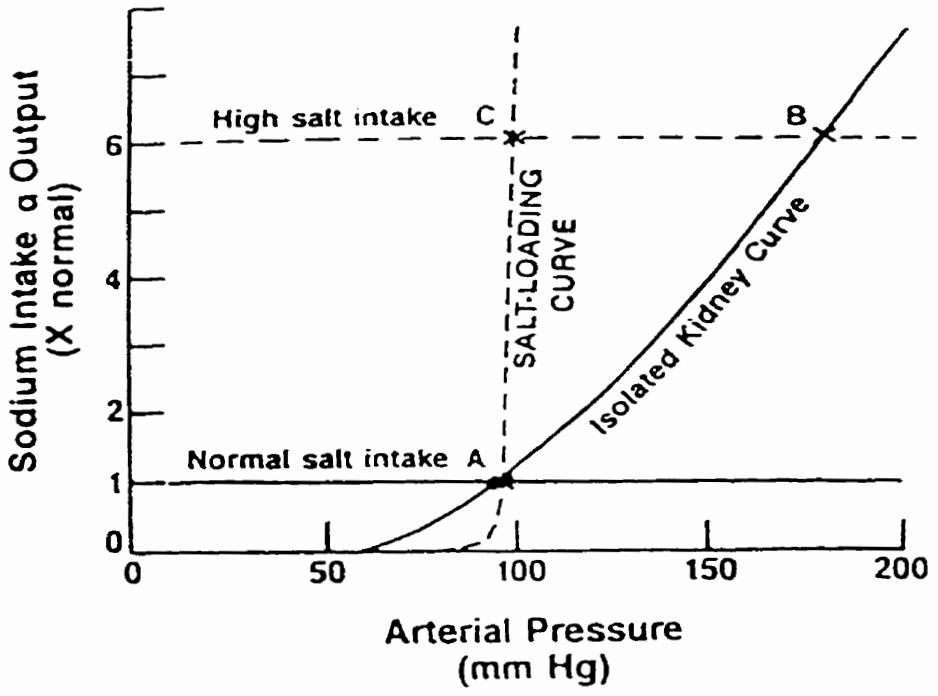

Figure 1.1: The isolated kidney and salt loading renal function curves. (from Guyton & Hall 1990)

The most compelling evidence implicating the kidney in the development of hypertension comes from renal transplantation studies (Dahl and Heine, 1975) (Bianchi *et al.*, 1974). In these experiments it was discovered that transplantation of a kidney from a hypertensive animal to a normotensive animal resulted in the recipient normotensive rat developing hypertension. The reverse was also found true, in that a hypertensive rat receiving a normotensive kidney would subsequently have a normal blood pressure. Similar observations have been recorded in humans (Strandgaard and Hansen, 1986). Transplant patients receiving a kidney from a hypertensive donor are at higher risk for developing hypertension following the transplant than patients who receive a kidney from a normotensive donor.

Hypertension

Although not a disease in itself, hypertension is a risk factor for many cardiovascular illnesses, such as stroke, myocardial infarction, angina, heart disease, and nephropathy. For this reason, the Joint National Committee on Hypertension recommends that blood pressure be maintained at a systolic pressure of less than 140, and a diastolic pressure of less than 90 (JNC, 1997). When hypertension develops, nonpharmacological treatment consists of salt reduction, decreased alcohol intake, increased aerobic activity, refrain from smoking, reduction of dietary saturated fat and cholesterol, and maintenance of adequate dietary potassium and calcium. Salt reduction effectively reduces blood pressure, and the mechanism of this reduction may be explained by the renal function curve. In Figure 1.1 it appears as though when salt intake is increased, arterial pressure also increases as shown by the curve. In reality, as salt

intake is increased, blood pressure is maintained at the equilibrium pressure as shown by the salt loading renal function curve also in Figure 1.1. This result can be explained by the various hormonal responses outlined earlier. As salt intake increases, there is a concurrent decrease in renin release from the kidney, resulting in a low concentration of AII in the blood. Consequently, there will be less vasoconstriction, less aldosterone, and less vasopressin released. These measures may effectively maintain blood pressure at an acceptable level. If the renin-angiotensin system were faulty or nonexistent, the blood pressure would increase to extremely high levels as predicted by the isolated kidney renal function curve. The actual renal function curve of a patient with hypertension is identical to the salt loading renal function curve, but is shifted to the right. Thus, for any given pressure, the hypertensive individual will have a decreased output of sodium, indicating an inability of the kidney to adequately excrete sodium (Guyton *et al.*, 1972). This solute is retained in the blood and contributes further to the increased blood pressure. For this reason, some patients with high blood pressure are recommended to maintain a restricted sodium diet. This increased salt load may account for the increased equilibrium pressure observed in hypertension, and may cause the kidney to reset blood pressure and maintain it at a high level. If this is the case, reducing salt intake would also be beneficial, as the only physiological mechanism of altering the renal function curve is by altering salt intake. If salt reduction, exercise, and relaxation techniques are not effective, pharmacological techniques are employed to reduce blood pressure to an acceptable level. Agents recommended for the treatment of hypertension include diuretics, ACE inhibitors, angiotensin receptor blockers, vasodilators, α_1 adrenergic receptor antagonists,

β adrenergic receptor antagonists, α_2 adrenergic receptor agonists, or calcium channel antagonists (JNC, 1997).

Adrenergic Receptors

If sympathetic tone is generated by the brainstem, a mechanism must exist by which the nerve impulse is converted into active constriction of resistance vessels. This transduction occurs via the release of catecholamines from the nerve terminal onto smooth muscle, where it binds adrenergic receptors to exert an effect. In 1948, an experiment was performed in which the responses to five catecholamines (epinephrine (E), norepinephrine (NE), isoproterenol (I), methylnorepinephrine (meNE), and methylepinephrine (meE)) were compared pharmacologically with respect to eight known physiological functions (Ahlquist, 1948). The design of these studies was novel in that historically, catecholamines had been studied based only on excitation or inhibition. Studying these compounds to determine their pharmacology resulted in the identification of two separate receptor systems. Ahlquist noted that for five of the assays (vasoconstriction, uterus/ureter excitation, contraction of the nictitating membrane, pupil dilation, and gut inhibition), the compounds had an order of affinity from most to least potent as follows: E, NE, meNE, meE, I. Conversely, for vasodilation, uterus relaxation, and myocardial stimulation, the compounds had a different order of potency: I, E, me-E, me-NE, NE. Due to their obvious diversity, Ahlquist concluded that two types of catecholamine receptors must exist, which he temporarily termed α and β respectively.

Subclassification of Adrenergic Receptors

The first subclassification of the α adrenergic receptor was proposed by Delbarre and Schmitt in 1973 based on the dissimilar ability of various adrenergic agonists to produce sedation in the chicken (Delbarre and Schmitt, 1973). Further subclassification of the α adrenergic receptor was hampered by seemingly contradictory effects in various studies. Although stimulation of a presynaptic nerve produced norepinephrine release, previous observations had documented that an α adrenergic receptor antagonist further increased the amount of norepinephrine in the synaptic cleft (Brown and Gillespie, 1957). It was later recognized that a mechanism of autoregulation existed at the synaptic level. Following release, norepinephrine was known to be bound by α adrenergic receptors on the postsynaptic cell, and also on presynaptic α adrenergic receptors to inhibit its own release (Kirpekar and Puig, 1971; Farnebo and Hamberger, 1971). Subclassification of the α adrenergic receptor proceeded rapidly on a presynaptic versus postsynaptic basis. In 1977, the two subtypes were named α_1 and α_2, for the post and presynaptic receptor respectively (Berthelsen and Pettinger, 1977). Over the course of the next 20 years, both subtypes were fully characterized, and it was discovered that the previous definition of pre/postsynaptic receptor was incomplete, if not inaccurate. It is currently recognized that the diversity between the two major subtypes of α adrenergic receptors, α_1 and α_2, is as great as that between the α and β adrenergic receptors (Bylund *et al.*, 1995). Not only does agoist potency differ between the three groups, but second messenger coupling and molecular sequence also necessitate the organization of adrenergic receptors into three groups: α_1, α_2, and β. Each of these groups can be further subclassified as detailed in the following sections.

β adrenergic receptors

Identification of β adrenergic receptor subtypes began in 1967, when subtypes 1 and 2 were separated on a functional basis (Lands *et al.*, 1967). β_2 adrenergic receptors were found to be primarily localized in smooth muscle, producing vasodilation and bronchodilation, while β_1 adrenergic receptors were found in the adipose tissue to stimulate lipolysis, and in the heart to increase both rate and force of contraction. Currently available antagonists as well as binding studies have substantiated this classification (Strosberg and Pietri, 1996). Further confirmation of β adrenergic receptor diversity surfaced with the advent of cloning techniques. Although subclassification proceeded on a pharmacological basis, it was not until 1986 that the first β adrenergic receptor was cloned and sequenced by Dixon and colleagues (Dixon *et al.*, 1986). This group isolated the β_2 adrenergic receptor from hamster lung and generated a peptide map by cleaving the protein with cyanogen bromide followed by reverse-phase HPLC analysis. The fragments were then sequenced from the N terminus and the sequence confirmed with anti-peptide antibodies. Cloning of the β_1 adrenergic receptor soon followed (Frielle *et al.*, 1987). In the late 1980's, several unexpected observations in fat and muscle tissue led to the discovery and cloning of a third subtype of β adrenergic receptor (Emorine *et al.*, 1989). This β_3 adrenergic receptor is localized in adipose tissue and is involved in lipolysis. The three β adrenergic receptors share common intracellular signalling mechanisms (stimulation of adenylate cyclase) and all potently bind the synthetic ligand isoproterenol. However, contrary to β_2, the β_3 adrenergic receptor has a

higher affinity for E than NE, and the β_1 adrenergic receptor has approximately equal affinity for both catecholamines. Despite many similarities in function and agonist potency, the three metabotropic transmembrane receptors are strikingly diverse in their amino acid sequences (Strosberg and Pietri, 1996; Molenaar *et al.*, 1997a). The most recent development in this classification scheme is the identification of a "putative β_4-adrenergic receptor" (Molenaar *et al.*, 1997b). This receptor, found in the heart, has proved pharmacologically unique to any currently classified β receptor, although it has not yet been cloned.

α_1 adrenergic receptors

The α_1 adrenergic receptor was first subclassified based on pharmacological distinctions observed by Han (Han *et al.*, 1987). This diversity was later confirmed by cloning and expression of three types of α_1 adrenergic receptor, α_{1a}, α_{1b}, and α_{1c} (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991). The distribution of α_1 adrenergic receptors varies greatly among tissues (Weinberg *et al.*, 1994), but is predominant in the urinary tract of man (Ford *et al.*, 1996), and is involved in sodium retention and contraction of prostatic smooth muscle. The α_{1C} adrenergic receptor was found predominantly in liver and heart, but also concentrated in prostate, lung, kidney, and spleen to lesser degrees. The aorta was the only tissue in which the α_{1C} adrenergic receptor was not the most dominant. The α_{1A} adrenergic receptor was dominant only in aorta, and was not detectable in heart or liver. Also in 1994 came the isolation of the α_{1d}

adrenergic receptor, which was cloned from rat brain and discovered to have a unique sequence. To complicate the situation, in 1995, it was discovered that the α_{1c} adrenergic receptor was actually the α_{1A} adrenergic receptor, so the name was removed, leaving the class of receptors as α_{1A}, α_{1B}, and α_{1D}. In addition, an α_{1L} adrenergic receptor has been proposed in human prostate, although not cloned (Ford *et al.*, 1996).

α_2 adrenergic receptors

As stated previously, the α_2 adrenergic receptor was originally identified as an inhibitory receptor localized on presynaptic neurons at the nerve terminal to regulate norepinephrine release. Although this method of classification is not definitive, the classification served to identify the two receptor subtypes for further study. The effects of the α_1 adrenergic receptor are mediated through an increase in intracellular calcium to increase inositol triphosphate turnover. Conversely, signal transduction of the α_2 adrenergic receptor occurs through inhibition of adenylate cyclase to prevent the production of cAMP and the influx of calcium (Robison, 1970; Clarke and Garg, 1993).

Separation of the α_2 adrenergic receptor into subtypes became necessary in 1992. Bylund explained that 4 subtypes existed based on differences in their pharmacological affinities for various agonists and antagonists (Bylund, 1992). The α_{2a} adrenergic receptor, as expressed in human platelets, HT29 cells, and human cerebral cortex, had a high affinity for oxymetazoline, and low affinity for prazosin, spiroxatrine, and ARC-239 (Bylund *et al.*, 1988). This finding was in contrast to the α_{2b} adrenergic receptor in

neonatal rat lung and NG-108 cells, which had a high affinity for prazosin, spiroxatrine and ARC-239, but low affinity for oxymetazoline. The α_{2c} adrenergic receptor was expressed in opossum kidney cells (Murphy and Bylund, 1988) and natively in HepG2 and SKN-MC cell lines (Schaak *et al.*, 1997). This receptor has a high affinity for rauwolscine, BAM1303, and WB4101, but low affinity for oxymetazoline. Finally, the α_{2D} adrenergic receptor is found in bovine pineal gland and rat salivary gland, and has moderate affinity for rauwolscine and yohimbine, and low affinity for BAM1303, and mianserin (Simonneaux *et al.*, 1991).

Three distinct human α_2 adrenergic receptor genes have been isolated, cloned and sequenced. These include α_2-C10 (Kobilka *et al.*, 1987), α_2-C2 (Weinshank *et al.*, 1990), and α_2-C4 (Regan *et al.*, 1988), which are the α_{2A}, α_{2B}, and α_{2C} adrenergic receptors, respectively. In addition, another receptor has been cloned and isolated from rat brain which has similar pharmacology, but different sequence, to the α_{2A} adrenergic receptor (Chalberg *et al.*, 1990). This adrenergic receptor has been termed α_{2D}, as its pharmacology is identical to that of the α_{2D} adrenergic receptor characterized in bovine pineal gland (Lanier *et al.*, 1991). Interestingly, only 3 subtypes are found in any species. The α_{2B} and α_{2C} adrenergic receptors are present in all species studied; however the α_{2D} adrenergic receptor is present in rat, bovine, mouse, and guinea pig species, and appears to be an ortholog of the human α_{2A} adrenergic receptor (Bylund *et al.*, 1995).

Physiological effects of the α_2 adrenergic receptor

Stimulation of α_2 adrenergic receptors in various tissues will produce a variety of physiological effects. Postsynaptic α_1 and α_{2A} and α_{2B} adrenergic receptors mediate a pressor response to catecholamines released locally and in circulation. Norepinephrine released presynaptically binds α_1 adrenergic receptors, resulting in vasoconstriction, while the pressor effect of iv norepinephrine is mediated by α_{2B} adrenergic receptors (Doda, 1997). In addition, circulating catecholamines also cause an increase in blood pressure; however this response is mediated by α_{2A} adrenergic receptors. These findings support the use of peripherally acting α_1 antagonists in therapy for hypertension.

In the kidney, α_1 and α_2 adrenergic receptors are both found postsynaptically. These receptors alter vascular resistance to adjust glomerular filtration rate and renal blood flow, regulate the absorption of sodium, water, and urea, affect gluconeogenesis to acquire sufficient energy for tubular transport, and alter the release and/or action of hormones such as renin, erythropoietin, prostaglandins, parathyroid hormone, and vasopressin (Schmitz *et al.*, 1981).

An additional function of α_2 adrenergic receptors has been their role in the handling of sodium and water by the kidney. Stimulating α_2 adrenergic receptors increases sodium and water excretion. This may occur by α_2 mediated inhibition of vasopressin release in the brain (Armstrong *et al.*, 1982), or by direct antagonism of vasopressin at the cortical collecting duct (Krothapalli *et al.*, 1983; Krothapalli and Suki,

1984; Clarke and Garg, 1993). The physiological effects of vasopressin include decreasing free water excretion by increasing the permeability of the collecting tubule to water, involving the aggregation of aquaporin-2 at the apical membrane (reviewed by Nielsen *et al.*, 1998; Deen and Knoers, 1998). This effect results primarily in increased water reabsorption with a secondary increase in sodium concentration of the urine in vivo. In the isolated perfused kidney, vasopressin increases the reabsorption of both water and sodium. Administration of α_2 adrenergic receptor agonists in the isolated perfused kidney reversed the effects of vasopressin on water and sodium reabsorption (Smyth *et al.*, 1985a). This observation indicated that α_2 adrenergic receptors directly inhibit the effects of vasopressin on sodium and water reabsorption by inhibiting the adenylate cyclase dependent pathway of vasopressin signal transduction. In the rat cortical collecting tubule, vasopressin increases cAMP production, which can be inhibited by α_2 agonists to attenuate the effects of vasopressin (Umemura *et al.*, 1985). Although this finding appears to lend further support to the use of α_2 agonists in hypertension, as signal transduction of the effects of vasopressin will be blocked (Shockley *et al.*, 1993), it is interesting to note that α_2 mediated inhibition of vasopressin occurs only in the rabbit and rat, and is not present in dogs or primates. However, α_2 agonists in hypertension remain useful due to their inhibition of central norepinephrine release. Central α_2 adrenergic receptors will mediate a decrease in sympathetic nerve activity due to inhibition of norepinephrine-mediated neurotransmission (Gottschalk, 1979; DiBona and Sawin, 1982). Basal sympathetic tone will be decreased, resulting in decreased RSNA, and therefore, increased sodium excretion due to lack of stimulation of α_1 adrenergic receptors and decreased renin release. When administered subcutaneously,

α_2 adrenergic receptor agonists, such as guanabenz in the dog and guanfacine and clonidine and guanabenz in the rat, produce a dose dependent diuresis (Strandhoy *et al.*, 1982; Shockley *et al.*, 1993). This effect may attributed to their direct action on central α_2 adrenergic receptors to decrease sympathetic nerve activity and renin release (Strandhoy, 1985; Smyth *et al.*, 1987).

As sympathetic activity is modulated to maintain homeostasis, α adrenergic receptors respond to alter sodium and water retention. As previously explained, when sympathetic nerve activity is increased in response to decreases in arterial pressure, the renin-angiotensin system will be activated to stimulate sodium and water retention. In addition to this mechanism, α adrenergic receptors in the kidney tubules are stimulated by changes in renal sympathetic nerve activity. In the isolated perfused kidney, which is independent of hormonal and other regulatory mechanisms, the α_1 adrenergic receptor is stimulated by renal nerve activity to decrease sodium excretion (Smyth *et al.*, 1985b). The α_2 adrenergic receptor has also been shown to inhibit renin release, possibly mediating regulation of the renin angiotensin system (Pettinger *et al.*, 1976; Smyth *et al.*, 1987).

Following subcutaneous administration, α_2 adrenergic receptor agonists such as clonidine, guanabenz, and guanfacine produce a dose dependent diuresis due to their direct action on α_2 adrenergic receptors (Strandhoy *et al.*, 1982; Shockley *et al.*, 1993) and reviewed by (Smyth *et al.*, 1987). Based on this and other similar observations (Goldberg *et al.*, 1983; Intengan and Smyth, 1997a), studies have been performed in order to determine the involvement of α_2 adrenergic receptor subtypes in this response.

Direct administration of clonidine, an α_2 adrenergic receptor agonist, into the renal artery of the rat produces increases in both free water excretion and sodium excretion which appeared to be mediated by two distinct subtypes (Blandford and Smyth, 1991; Smyth *et al.*, 1992). These responses were later separated and attributed to α_2 adrenergic receptor subtypes (Intengan and Smyth, 1997a). It was determined that the sodium response was mediated by the $\alpha_{2A/D}$ adrenergic receptor, while the α_{2B} adrenergic receptor was responsible for the free water response (Intengan and Smyth, 1997b). These studies explained the inability of α_2 agonists to inhibit vasopressin in primates, as this function is attributed to α_{2b} adrenergic receptors, which are abundant in rat kidney, but not predominant in human kidney (Motomura *et al.*, 1989). Radioligand binding assays have determined that the α_{2C} adrenergic receptor is not present in the rat kidney (Uhlen and Wikberg, 1991).

The RVLM, which has been implicated in the generation of sympathetic tone, can be stimulated by α_2 agonists to decrease sympathetic flow and reduce arterial pressure (reviewed by Ruffolo *et al.*, 1993). This action would be accompanied by a decrease in renal sympathetic nerve activity (Head and Burke, 1998), and an increase in sodium excretion. These observations explain, in part, the use of α_2 agonists, such as clonidine and methyldopa, in the treatment of hypertension.

Involvement of the α_2 adrenergic receptor in hypertensive models

Study of the α_{2A} adrenergic receptor has led to investigation of its role in hypertension. Alterations of the α_{2A} adrenergic receptor in both man and rat strains have been correlated with increased blood pressure (Pettinger *et al.*, 1982; Graham *et al.*, 1982; Lockette *et al.*, 1995; Svetkey *et al.*, 1996). Several rat models of hypertension have been developed which can be used to study this issue. The spontaneously hypertensive (SH) rat was created by breeding the normotensive Wistar-Kyoto (WKY) rats with the highest blood pressure. This breeding was continued until a population of rats emerged that spontaneously develop hypertension at an early age (Okamoto and Aoki, 1963). These animals have been found to have an increased density of renal α receptors (Pettinger *et al.*, 1982). This increased density of renal α_2 adrenergic receptors has also been reported in other rat models of hypertension, including Milan rats (Parini *et al.*, 1987), and Dahl salt sensitive rats (Pettinger *et al.*, 1982).

Previous studies in our laboratory have shown that the natriuretic response to α_{2A} adrenergic receptor agonists, such as guanfacine, is absent in SH rats (Intengan and Smyth, 1997b). In addition, it was found that the natriuretic response to guanfacine was intact in one kidney, one clip (1K-1C) rats, indicating that the development of hypertension in the SH rat may be due, in part, to α_{2A} adrenergic receptor alteration. The defective α_{2A} adrenergic receptor in SH rats causes an inability to excrete sodium in response to guanfacine. This work is supported by additional studies indicating altered α_2 adrenergic receptors in SH rats (Pettinger *et al.*, 1982; Sanchez *et al.*, 1986). In this animal model, the α_2 adrenoceptor appears to be non-functional, resulting in increased

expression of the defective protein, which accounts for the increased levels of α adrenergic receptors detected in the kidney of SH rats.

The Sabra salt sensitive rat, contrary to the Sabra salt resistant rat, has borderline high blood pressure early in life, and becomes hypertensive following consumption of a high salt diet. In this rat model of hypertension, differences in α_{2A} adrenergic receptor distribution in the renal cortex were found to exist using gene amplification and Northern blot analysis (Le-Jossec *et al.*, 1995). The α_{2B} adrenergic receptor was the only subtype detected in the renal cortex; however mRNA for both the α_{2A} and the α_{2B} adrenergic receptor was detected, indicating a post-translational defect preventing the expression of α_{2A} adrenergic receptors in the renal cortex.

A unique study that specifically abolished the α_{2A} adrenergic receptor in mice demonstrates the importance of the α_{2A} adrenergic receptor in the hypotensive response to α_2 adrenergic receptor agonists (MacMillan *et al.*, 1996). A point mutation was introduced into the mouse genome through the use of gene targeting techniques. In these animals, basal blood pressure was not altered, indicating that α_{2A} adrenergic receptors are not involved in regulating basal blood pressure, or that other mechanisms have compensated for the loss of the α_{2A} adrenergic receptor in these animals. When blood pressure lowering doses of α_2 adrenergic receptor agonists are infused into the carotid or femoral artery, the depressor effect is nonexistent, demonstrating the importance of α_{2A} adrenergic receptors in the hypotensive response to α_2 agonists.

A similar study was performed to determine the role of the α_{2B} and α_{2C} adrenergic receptors in hypertension produced by subtotal nephrectomy and salt loading (Makaritsis *et al.*, 1999). The use of knockout mice for both the α_{2B} adrenergic receptor and the α_{2C} adrenergic receptor demonstrated that a functional α_{2B} adrenergic receptor was necessary for the development of hypertension following salt loading. Knockout of the α_{2C} adrenergic receptor did not affect the development of hypertension in this model.

These observations together indicate that the development of human hypertension may involve either decreased α_{2A} adrenergic receptor expression in the brain, or decreased expression of the α_{2A} adrenergic receptor in the kidney. The role of the α_{2B} adrenergic receptors appears to have importance in the development of hypertension in rats; however more study is required to elucidate the possible interaction of these processes in the pathophysiology of hypertension.

Relevant Clinical Observations

As hypertension is a powerful predictor of future cardiovascular disease, a common factor has been sought to link patients who develop high blood pressure. The advent of genetic screening has enabled detection of an α_{2A} gene polymorphism which has been correlated with hypertension (Freeman *et al.*, 1995; Lockette *et al.*, 1995). This polymorphism is an intermediate form of the α_{2A} adrenergic receptor gene which, when combined with external environmental factors, may contribute to the development of hypertension. These studies identified two forms of the α_{2A} adrenergic receptor, one 6.3-

kb, and one 6.7-kb in length. Individuals with either form of the α_{2A} adrenergic receptor were subjected to testing of their baroreceptor response, platelet aggregation, and urinary sodium measurements, all of which are dependent on α_{2A} adrenergic receptors. Although no differences in baseline parameters were observed, the group of individuals with at least one 6.3-kb (mutant) α_{2A} adrenergic receptor allele had increased baroreceptor response, increased epinephrine-induced platelet aggregation, and decreased natriuresis following water immersion (Freeman *et al.*, 1995). In addition, the presence of the 6.3-kb allele has been correlated with an increased incidence of hypertension in African American individuals (Lockette *et al.*, 1995). This evidence indicates that the 6.3-kb allele may not be translated into a protein, or may produce a protein that is unable to adequately excrete sodium.

As evidence mounts demonstrating the role of α_{2A} adrenergic receptors in the development or maintenance of hypertension, the use of α_2 adrenergic receptor agonists in treatment must also be emphasized. Clonidine, guanfacine, and α-methyldopa have long been studied as α_2 agonists, and are currently used as treatment for elevated blood pressure. Clonidine and guanfacine act directly on central α_{2A} adrenergic receptors, while α-methyldopa is converted into the α_{2A} adrenergic receptor agonist α-methylnorepinephrine, to decrease the release of norepinephrine from presynaptic nerves. These agents comprise the first generation of centrally acting antihypertensive agents; however they are associated with several unpleasant side effects, and their use is rapidly declining for this reason. These effects include sedation, dry mouth, and impotence. Sedation is produced by the binding of α_2 adrenergic receptor agonists to adrenergic

receptors in the locus coeruleus of the brain (Mizobe *et al.*, 1996; Lakhlani *et al.*, 1997; Hunter *et al.*, 1997;), and dry mouth by α_2 adrenergic receptors in the salivary glands (van-Zwieten, 1997). Impotence is produced by a decrease in sympathetic stimulation, which prevents ejaculation. Less frequent effects may include dizziness, constipation, orthostatic hypotension, and headache (Oster and Epstein, 1991). Insomnia, anxiety, paresthesia, vision disorders, hypertriglyceridemia, somnolence, and fatigue have also been reported (Wilson *et al.*, 1991). As the majority of these effects are directly related to α_{2A} adrenergic receptor stimulation, no new α_2 agonist can be developed which will overcome these effects.

Due to the frequency and severity of adverse effects associated with first generation antihypertensive agents, a second generation was developed. This group of α_2 adrenergic receptor selective compounds was generated in order to find an agent with higher affinity for central blood pressure lowering sites and low affinity for the central and peripheral sites associated with side effects. Moxonidine and rilmenidine were developed and included in this class of compounds. Subsequent testing resulted in the discovery of a selective effect on blood pressure assumed due to selective targeting of central α_{2A} adrenergic receptors.

The Imidazoline Receptor

In 1984, the first paper was published indicating that second generation antihypertensives may not be selective for the α_2 adrenergic receptor. This study by

Bousquet, Feldman, and Schwartz described the unique property of imidazoline agents such as clonidine, cirazoline, and ST 587 to elicit hypertension after their injection into the RVLM (Bousquet *et al.*, 1984). This effect could not be produced with α-methylnorepinephrine, an α_2 adrenergic receptor agonist. From these observations it was suggested that agents with an imidazoline structure may stimulate imidazoline preferring sites in the RVLM which are different from those which bind α_2 adrenergic receptor agonists.

Further pharmacological evidence for this theory surfaced through binding studies, which showed that α_2 adrenergic receptor antagonists rauwolscine and idazoxan had different binding distributions in the rat brain (Boyajian and Leslie, 1987). These distributions were confirmed by autoradiographic binding studies (Boyajian *et al.*, 1987). These studies indicated that there exists "a heterogeneous population of α_2 adrenergic receptors within rat brain, labeled entirely by [^{3}H]idazoxan, and only in part by [^{3}H]rauwolscine (Boyajian and Leslie, 1987). Further, autoradiographic binding of clonidine to rat brain resembles the distribution of idazoxan, indicating the similarity between these two compounds. The common link between idazoxan and clonidine is their imidazoline structure, and thus, the sites bound by these agents were termed the "imidazoline site/receptor". Moxonidine and rilmenidine, second generation antihypertensives and imidazoline derivatives, also bind these imidazoline sites. Both agents, when injected into the central nervous system of the rabbit or rat, produce a dose dependent decrease in arterial pressure. This effect can be reversed by the relatively selective I_1 imidazoline antagonists efaroxan and idazoxan (Chan and Head, 1996).

These observations indicate that imidazoline compounds selectively bind a site separate from the α adrenergic receptor to decrease blood pressure.

When administered to conscious SH rats, renal-hypertensive rats, dogs, or cats, moxonidine decreased blood pressure without causing sedation. In addition, the decrease in salivary volume was not as great following moxonidine treatment as compared with clonidine treatment (Armah *et al.*, 1988). These initial studies verified the differential action of imidazoline agonists and α_2 agonists on these parameters. As evidenced by these studies, the imidazoline derivatives may not produce the sedation and dry mouth experienced by patients taking first generation antihypertensive medications.

Although the imidazoline receptor has not yet been cloned, it can be separated into subtypes based on pharmacological studies. The I_2 imidazoline receptor is expressed in brain, and may be involved in insulin release and monoamine systems (Parini et al, 1996). The I_1 imidazoline receptor, of present interest, has localization similar to that of the α_{2A} adrenergic receptor but is thought to play a role in cardiovascular regulation (Head, 1995). Due to the lack of an amino acid sequence, antibody, or clone of either imidazoline receptor, its status remains questioned, despite mounting pharmacological evidence of its existence.

A blood pressure-selective alternative to α adrenergic agents

To further assess the central role of imidazoline receptor agonists on known pathways affecting blood pressure, rilmenidine was injected iv or into the RVLM. As

previously mentioned, the RVLM lies along the baroreceptor pathway and mediates final output onto the IML cell column, ultimately regulating basal sympathetic tone. Rilmenidine produced a hypotensive effect similar to that seen with clonidine, and the direct injection into the RVLM was 50x more potent than iv administration (Bousquet *et al.*, 1992). Further, antagonism by idazoxan was more effective than antagonism with yohimbine, indicating the imidazoline selectivity of idazoxan. This response to RVLM injection of imidazoline compounds has also been documented by others (Haxhiu *et al.*, 1994; Head and Burke, 1998). Activity of RVLM neurons is also inhibited by imidazoline agonists in vitro (Sun and Reis, 1995a), indicating the selective action of imidazolines on the pacemaker neurons within the RVLM.

The NTS, also involved in blood pressure regulation, has been injected with imidazolines. NTS rilmenidine treatment produced a dose dependent decrease in arterial pressure, heart rate and renal sympathetic nerve activity (RSNA). These effects were completely reversed by NTS idazoxan and efaroxan (imidazoline antagonists), and by 2-methoxyidazoxan (α_{2a} antagonist) (Head and Burke, 1998). In contrast, the non-imidazoline α_{2a} agonist α-methylnorepinephrine decreased arterial pressure following injection into the NTS, but did not have a significant effect on either heart rate or RSNA. The decrease in blood pressure was reversed by pretreatment with 2-methoxyidazoxan. Conversely, the imidazoline antagonists potentiated the effect of α-methylnorepinephrine on RSNA and heart rate at high doses (Head and Burke, 1998), possibly acting as partial imidazoline agonists at these doses.

These observations indicate the efficacy of imidazoline compounds in decreasing blood pressure, specifically in the NTS and RVLM, which are the two important blood pressure regulatory nuclei.

Further evidence for the identity of imidazoline receptors unique from α_2 adrenergic receptors was recently demonstrated in conscious rabbits by iv injection of moxonidine and rilmenidine. The decreases in blood pressure and heart rate following these imidazolines was reversed by fourth ventricular efaroxan (an imidazoline antagonist), but not by 2-methoxyidazoxan (an α_2 antagonist) (Chan and Head, 1996). Conversely, the decreases in arterial pressure and heart rate induced by clonidine were equally reversed by 2-methoxyidazoxan and efaroxan, as the doses of both antagonists were standardized to produce equal reversal of α_2 effects. These results have been duplicated using other routes of administration with similar results (Chan *et al.*, 1996)

Additional support for the postulate that renal imidazoline receptors function independently from α_2 adrenergic receptors is their lack of antagonism by indomethacin. The effects of renal α_2 adrenergic receptor stimulation are potentiated by indomethacin (Smyth *et al.*, 1995; Blandford and Smyth, 1991), a cyclooxygenase inhibitor. However, the effects of intrarenal moxonidine are antagonized by indomethacin (Darkwa and Smyth, 1995). Again, we have further support for the independence of imidazoline from α_2 adrenergic receptors in the kidney.

According to the proposed selectivity of imidazoline compounds at I_1 imidazoline receptors, these agents should not produce sedation, as they would be expected not to bind α_2 adrenergic receptors in the locus coeruleus which mediate the sedative effects of

first generation agents. The sedation displayed by first generation antihypertensive medications is likely due to their affinity for α_{2D} adrenergic receptors, which mediate sedation in the locus coeruleus (Szabo *et al.*, 1996). Consequently, membrane preparations from human RVLM (for imidazoline binding capacity) and cortex (for α_2 binding mediating sedation) were studied to determine binding affinities of rilmenidine as compared to clonidine for the two receptor types. In RVLM, rilmenidine affinity was similar to that of clonidine, and guanfacine (a non-imidazoline α_2 agonist) exhibited weaker binding in the RVLM (Bricca *et al.*, 1989). In human cortical preparations, rilmenidine had weaker binding affinity when compared to clonidine and guanfacine, indicating that rilmenidine preferentially binds to imidazoline receptors in the RVLM without displaying a high affinity for sedative α_2 adrenergic receptors in the cortex. In fact, there is evidence to suggest that not only are imidazoline agents unable to bind α_2 adrenergic receptors in the locus coeruleus, but functional imidazoline receptors cannot be found in this region (Szabo *et al.*, 1996). This finding supports the lack of imidazoline binding documented in this nucleus, and explains the absence of sedative effects with imidazoline treatment.

Renal effects of imidazoline derivatives

The imidazoline receptor appears to be concentrated in the RVLM where it mediates a decrease in sympathetic activity following stimulation by various agonists. Further, the NTS, although predominantly a site of α_2 adrenergic receptor mediated

action, also responds to imidazoline agonists to decrease RSNA, a function which cannot be duplicated by α_2 agonists.

Of particular interest is whether administration of these agents affects renal handling of sodium and water. As previously stated, α_2 adrenergic receptors play an important role in the adequate excretion of sodium, and absence or alteration of this receptor has been implicated in the pathogenesis of hypertension in man and animal models (Pettinger *et al.*, 1982; Graham *et al.*, 1982; Sanchez *et al.*, 1986; Lockette *et al.*, 1995; Freeman *et al.*, 1995; Svetkey *et al.*, 1996).

In order to study the effects of imidazoline agonists on renal function, doses of these agents must be chosen which will not affect blood pressure. A decrease in blood pressure would alter renal perfusion pressure and thus sodium excretion; so these responses must be avoided to prevent complication of the data. Previous studies in our laboratory have explored this issue, and discovered that intracerebroventricular (ICV) administration of moxonidine elicits a dose dependent increase in urine excretion at doses that do not alter heart rate, blood pressure, or creatinine clearance. This increase in urine volume is secondary to an increase in solute excretion, specifically, sodium excretion (Penner and Smyth, 1994b; Penner and Smyth, 1995). These studies also demonstrate antagonism of this effect by ICV idazoxan. Thus, the effect of central injection of imidazoline agonists, similar to α_{2A} agonists, increases urine excretion secondary to an increase in solute excretion. This response was later shown through denervation studies to require an intact sympathetic nervous system (Penner and Smyth, 1995; Penner and Smyth, 1997). Thus, renal effects following central imidazoline administration are

mediated by a decrease in sympathetic nerve activity. An interesting difference was found, however, between the two agonists, moxonidine and rilmenidine. ICV moxonidine increased urine volume secondary to an increase in osmolar clearance, which was attenuated by renal denervation. Rilmenidine, in contrast, did not increase urine volume, but increased in free water clearance, which was not blocked by denervation. It must also be noted that rilmenidine only altered renal function at doses which decreased blood pressure. Consequently, this decrease in pressure may have obscured any increase in osmolar clearance, confounding results. Thus, moxonidine may be a more potent and selective agent to use in studies involving renal function parameters, as changes in urine flow and sodium excretion can be observed at low doses not altering arterial pressure.

After observation of altered renal function following ICV moxonidine or rilmenidine, further studies documented a direct effect of imidazolines on sodium and water excretion, indicating a peripheral binding site in addition to the well documented central sites. In fact, moxonidine has an affinity for imidazoline receptors in the rat kidney which is 600 times greater than that found for the α_{2B} adrenergic receptor (Ernsberger *et al.*, 1992). Therefore, the question arose as to whether moxonidine infused directly into the renal artery would affect renal function via a different mechanism than did α_2 adrenergic receptor agonists. Moxonidine increased urine volume secondary to an increase in sodium excretion without altering free water clearance (Allan *et al.*, 1993; Smyth *et al.*, 1995). This response was not due to antagonism of the effects of vasopressin (Smyth *et al.*, 1995), and was blocked by idazoxan but not rauwolscine (α_2 adrenergic receptor antagonist) (Allan *et al.*, 1993; Allan *et al.*, 1996). The sodium response following intrarenal infusion was duplicated for

rilmenidine (Smyth and Penner, 1995). Conversely, α_2 adrenergic receptor stimulation produces increases in free water clearance due to stimulation of α_{2B} adrenergic receptors and antagonism of vasopressin, and modest increases in osmolar clearance mediated by the α_{2A} adrenergic receptor (Intengan and Smyth, 1997a).

These effects, central imidazoline versus peripheral imidazoline, can be separated using prazosin. Intravenous prazosin completely attenuates the response to ICV moxonidine, but only partially attenuates the response to intrarenal moxonidine. Further, intravenous idazoxan completely attenuates the response to intrarenal moxonidine without altering the ICV response to moxonidine (Penner and Smyth, 1994a). This observation indicates that moxonidine has separate effects at central and renal sites to increase urinary excretion of sodium. A third site of action has also been identified, as activation of imidazoline receptors in the heart induces the release of atrial natriuretic peptide (Mukaddam *et al.*, 1997).

One unifying hypothesis has been suggested to tie in the central and renal effects of moxonidine. ICV moxonidine acts directly on I_1 imidazoline receptors to decrease sympathetic nerve activity, resulting in decreased RSNA, which would inhibit α_1 adrenergic receptors in the kidney and result in increased solute excretion (Smyth and Penner, 1999). The renal α_1 adrenergic receptor causes sodium retention when stimulated; so decreasing the RSNA would be expected to increase sodium excretion (Smyth *et al.*, 1985b). This pathway from I_1 to α_1 may involve many of the same mechanisms or structures as are stimulated by α_{2A} agonists, and in fact, each may require the action of the other in order to effect normal changes in sympathetic activity.

Endogenous Ligands and Signal Transduction

The endogenous ligand for the imidazoline receptor was first termed "clonidine-displacing substance" following the high affinity displacement of clonidine from its receptor (then thought to be the α_2 adrenergic receptor) by a substance purified from brain tissue (Atlas and Burstein, 1984). The identity of this substance has been widely debated, and several substances have been proposed to be the endogenous ligand for I_1 imidazoline receptors. The ligand initially isolated bound α_2 adrenergic receptors in addition to imidazoline receptors, as evidenced by the observed inhibition of platelet aggregation (Diamant *et al.*, 1987) and contraction of rat vas deferens (Diamant and Atlas, 1986). This partially purified compound also induced a depressor response following injection into the RVLM of rats (Meeley *et al.*, 1986).

Another suggested identity of the endogenous imidazoline ligand is agmatine (guanido butylamine). While not an imidazoline compound, agmatine binds with high affinity and specificity to both the I_1 and I_2 imidazoline receptor, and the α_2 adrenergic receptor (Li *et al.*, 1994). This mechanism was fully explained in a review by Regunathan and Reis (for review, Regunathan and Reis, 1996). Despite its high affinity for, and localization with imidazoline and adrenergic receptors, neither ICV agmatine nor RVLM agmatine alters sympathetic nerve activity (Sun *et al.*, 1995). Thus the debate remains as to whether imidazoline receptors are unique from adrenergic receptors and, if unique, whether the systems are independent.

As the imidazoline receptor has not been cloned, the study of its signal transduction has not attracted much attention. It has been discovered, however, that moxonidine, in addition to affecting signal transduction mechanisms attributed to the α_2 adrenergic receptor (decreased cAMP production), increases the hydrolysis of phosphoinositide to inositol-1-phosphate (Regunathan and Reis, 1994). Further study has shown that moxonidine may be coupled to and activate phosphatidylcholine-selective phospholipase C (PC-PLC) to produce diacylglycerol and phosphocholine (Separovic *et al.*, 1997). In addition, arachidonic acid release has been documented following imidazoline receptor stimulation (Ernsberger, 1998a). The proposed signal transduction mechanism is as follows: Activation of the imidazoline receptor stimulates PC-PLC to cleave phosphatidyl choline to phosphocholine and diglyceride. Diglyceride is then cleaved to release arachidonic acid, and also serves to activate further signal transduction cascades (Ernsberger, 1998b).

Relevant Clinical Observations

Following the development and initial study of moxonidine in 1988 it was determined that moxonidine decreased sympathetic nerve activity via its action on receptors in the brain (Armah *et al.*, 1988). Developed by BDF Research Laboratory for treatment of hypertension, clinical trials proceeded soon after. Moxonidine has proven effective in the management of hypertension without producing the high incidence of sedation and dry mouth experienced with central α_2 agonists (Wenzel *et al.*, 1998; Kirch *et al.*, 1990). Moxonidine treatment also decreased heart rate in normotensive subjects,

but not significantly in hypertensive subjects (Wenzel *et al.*, 1998). Norepinephrine levels in the plasma were decreased, but no change in levels of epinephrine or renin was observed. Similarly, levels of HDL, LDL, and total cholesterol were not affected by moxonidine treatment at the recommended daily dose of 0.4 mg.

In contrast, in 1991, a study was performed in which 0.4 mg of moxonidine was administered and found to significantly decrease plasma levels of norepinephrine and renin within 4 hours of administration (Mitrovic *et al.*, 1991). Moxonidine produced a significant fall in blood pressure, was well tolerated with very few reports of mild dry mouth, and the peak plasma concentration was observed approximately 1 hour after the dose.

The earliest support for the use of moxonidine in hypertension originates from clinical trials comparing moxonidine to currently available antihypertensive medication. Two studies by Vera Planitz (Beiersdorf) compare moxonidine to clonidine in order to examine both the efficacy and incidence of side effects of these two agents. One was a crossover study, and the other used two parallel groups of patients for the two arms of the study (Planitz, 1984; Planitz, 1987). Both double-blinded trials discovered that moxonidine and clonidine were equipotent; however, patients taking clonidine consistently reported a greater incidence of side effects than those taking moxonidine. In addition, withdrawal from clonidine produced a steeper rise in blood pressure than was observed following moxonidine withdrawal. Neither drug produced sodium or water retention (Planitz, 1984), indicating a lack of sympathetic response to the decrease in blood pressure.

Proposed Studies

The importance of the imidazoline receptor in the regulation of blood pressure remains an exciting and relatively new field of research. Although the NTS and RVLM have been shown to be sites of action of moxonidine, inputs to these areas have not been well studied, and may contribute to the effects of ICV moxonidine. In order to elucidate the role of imidazoline receptors in the regulation of sympathetic activity, it is necessary to examine all sites of action and determine their interaction. The mechanism by which moxonidine interacts with the adrenergic pathway, if at all, remains unknown. These two receptor systems may or may not be completely independent, or both systems may be regulated by a separate central regulatory pathway to respond to changes in blood pressure. The following studies investigate these issues via site specific injection of various compounds into the rat brain, and the new developments revealed by these studies are discussed.

Initially, the renal response to ICV moxonidine was confirmed, which implicated the involvement of vasopressin. This observation led to the investigation of the paraventricular nucleus of the hypothalamus (PVN) as a site of moxonidine action. Site specific injection of moxonidine into the PVN resulted in a differential action of the imidazoline agonists from the α_{2A} agonist guanfacine. Following this study, we investigated the role of GABA in the renal response to PVN moxonidine.

The experiments outlined investigate our hypothesis that imidazoline receptor and adrenergic receptor function are separable in the PVN, and may be mediated by mechanisms involving GABA.

General Methods

The experimental protocol has been described previously (Blandford and Smyth, 1988). Male Sprague-Dawley rats (175-225 g) were obtained from the University of Manitoba Central Breeding Facility and housed according to a protocol approved by the Protocol Management and Review Committee (Bannatyne Campus, University of Manitoba). All protocols are approved based on guidelines of the Canadian Council on Animal Care. Animals were housed in groups of two or three per cage with free access to Purina rat chow and tap water. The room was maintained at 22°C, and a 12 hour light/dark cycle maintained (7:00 a.m.-7:00 p.m.). The day after their arrival at the housing facility, animals had their right kidney removed by flank incision under ether anesthesia (Mallinckrodt). The muscle was stitched with 4-0 silk (Stevens and Sons) and the skin secured with Michel suture clips. This was followed by a subcutaneous injection of buprenorphine (3 μg). Following nephrectomy all animals were housed overnight in individual cages to facilitate their recovery. Animals were allowed to recover for 7 to 13 days prior to experimental study.

On the day of the experiment, rats (260-320 g) were anesthetized with 50 mg/kg pentobarbital (BDH Inc.). The left flank and scalp were shaved and a rectal thermometer inserted which was connected to a Harvard Homeothermic Animal Blanket and Control Unit on which the rat was placed to maintain body temperature at 37.5°C. The trachea was isolated and a tracheotomy performed with Clay Adams polyethylene tubing (PE-240) to allow spontaneous breathing and attachment of a Harvard rodent ventilator model

683 if necessary. Following the tracheotomy, the jugular vein was located and cannulated (PE-160) for the infusion of saline and administration of additional anesthetic as necessary. The carotid artery was then clamped and cannulated (PE-50) for the measurement of blood pressure and heart rate. Following left flank incision and cauterization of any bleeding, the kidney was lifted out by its fat and laid towards the dorsal side of the rat to expose the ureter. The ureter was carefully isolated, and was cannulated with PE-10 tubing. The animal was then placed in a stereotactic apparatus and a scalp incision was made. The scalp was cleared and a hole drilled (Foredom power tools, 1 mm drill bit) according to precise stereotactic coordinates for the site to be studied (Paxinos and Watson, 1986). After completion of surgery, the carotid line was connected to a pressure transducer (Cobe) which was, in turn, connected to a Grass polygraph (model 5D) to record and monitor blood pressure, heart rate, and time elapsed. At time zero, the infusion of saline into the jugular vein was initiated using a Sage syringe infusion pump. Based on previous studies, a flow rate of 0.097 ml/min was chosen to produce a modest diuresis. The animal was allowed to stabilize for 45 minutes in which no intervention was performed other than the infusion of saline, which was continued for the duration of the experiment. Also, via the jugular infusion line, additional bolus injections of anesthetic were given (0.1-0.3 ml of 6 g/100 ml) as necessary based on tail pinch or eye blink reflex.

Following the 45 minute stabilization period, the first 30 minute urine collection period was initiated from time 45 to 75 minutes. During this time, a pre-weighed 1.5 ml Eppendorf centrifuge tube was placed under the ureteral catheter to collect the urine excreted during this time period. This first urine collection was taken without any

intervention present and served as a control collection by which to compare the baseline renal function of all animals. Previous studies in our laboratory have indicated that the normal range for control urine collections is between 3 and 30 ul/min; therefore any animals excreting less than 3 ul/min or more than 30 ul/min during this collection period were excluded from the final analyses. The blood pressure and heart rate were recorded at the halfway point of the first urine collection, time 60 minutes.

The second 30 minute urine collection was initiated immediately following the first; thus a new Eppendorf tube replaced the first under the ureteral catheter. Central injection of the agent to be studied took place at time 75 minutes, at the start of the second urine collection. All agonists and antagonists were administered using a Hamilton syringe, attached to the stereotax, which could be directly inserted into the desired brain region based on previously published coordinates (Paxinos and Watson, 1986). The syringe remained inserted in the brain throughout the remainder of the experiment. The third urine collection was obtained in a similar manner, and blood pressure and heart rate measurements were recorded at the halfway point of each collection.

Following the third urine collection, at time 145 minutes, a blood sample was collected into a borosilicate tube containing one drop of heparin (Leo laboratories) to prevent clotting. The blood was centrifuged to separate and collect the plasma. Correct placement of the syringe into the injection site was confirmed histologically. Following the experiment all animals were euthanized by an overdose of pentobarbital. Eppendorf tubes containing the urine collections were weighed and urine volume determined gravimetrically. All samples, plasma and urine, were analyzed to determine creatinine

concentration (Beckman Creatinine Analyzer 2), and osmolality (Precision Systems MicroOsmette). These results were used to calculate creatinine clearance, free water clearance, and osmolar clearance. All of the following results are presented as the difference in value between the third urine collection and the control urine collection. It should be noted that the control urine collection is also referred to as the first urine collection, and should not be confused with the control treatment group, which denotes the injection of saline rather than drug treatment. Changes in urine flow rate are expressed as changes in osmolar clearance and free water clearance. An increase in osmolar clearance would indicate that the kidney has cleared more solute from the plasma during the three urine collections. Similarly, an increase in free water clearance suggests that more water has been cleared from the plasma over the three collections. These parameters are regulated independently and can be separated pharmacologically, as shown previously in our laboratory (Intengan and Smyth, 1997b). This experiment demonstrated that while increases in free water clearance following clonidine treatment could be attenuated by prazosin but not naltrexone, increased osmolar clearance following guanfacine treatment could be attenuated by naltrexone but not by prazosin. Consequently, changes in urine flow rate are dependent on changes in both osmolar and free water clearance and have been presented as such.

Significance is denoted throughout this thesis by *, which represents a p value of $p<0.05$, ** which represents $p<0.01$, and *** which represents $p<0.001$. All results were analyzed by repeated measures analysis of variance (ANOVA) followed by a post-hoc test to identify significant differences. Special thanks are given to Mary Cheang, who performed all statistical analyses presented.

Diuresis Following Intracerebroventricular Moxonidine

Introduction

Moxonidine, although originally thought to be an α_2 agonist, has been found to have a high affinity for the recently described imidazoline receptor (Bousquet *et al.*, 1984). In fact, this selectivity for the I_1 receptor over the α_2 adrenergic receptor ranges from 40 fold in bovine RVLM to 600 fold in rat renal medulla (Ernsberger *et al.*, 1992). This selectivity is common to other compounds with an imidazoline ring structure, such as rilmenidine (Bricca *et al.*, 1989). Imidazoline binding sites have been identified in the NTS and RVLM, nuclei which play an integral role in the maintenance of sympathetic tone (Ernsberger et al 1987, 1990; van-Zwieten and Chalmers, 1994; Colombari *et al.*, 1996; Dampney, 1994). Previous studies from our laboratory have demonstrated a dose dependent natriuresis following ICV moxonidine (Penner and Smyth, 1994b; Smyth and Penner, 1999). This effect was accompanied, at high doses, by a decrease in blood pressure and heart rate. Therefore, we initially sought to reproduce these results before attempting to locate specific nuclei that may be involved in these responses.

Methods

A detailed description of the general procedures has been documented previously in the general Methods section. Briefly, male Sprague-Dawley rats were unilaterally nephrectomized under ether anesthesia and allowed to recover for 7 to 13 days. On the day of the experiment, animals were anesthetized with sodium pentobarbital. A tracheotomy, and carotid arterial and jugular venous cannulation were performed as described to allow spontaneous breathing, monitoring of heart rate and blood pressure, and infusion of saline respectively. Following a 45 minute stabilization period, an infusion of saline was initiated via the jugular vein and maintained throughout the remainder of the experiment. A 30 minute control urine collection was obtained, and moxonidine (10 nmol/5 ul, Beiersdorf, AG, Hamburg, FRG) or saline (5 ul) was then injected over 2.5 minutes into the left cerebral ventricle of the brain. The stereotactic coordinates used, as determined from the atlas of Paxinos and Watson, were 0.3 mm posterior, 1.5 mm lateral, and 3.8 mm ventral to bregma (Paxinos and Watson, 1986).

Two 30 minute urine collections followed the ICV injection of moxonidine or vehicle. Heart rate and blood pressure were monitored, and urine flow rates determined gravimetrically. A plasma sample was obtained after completion of the experiment and stored at 4°C for up to a week until analysis.

Placement of the Hamilton syringe in the left cerebral ventricle was verified by injection of 0.1 ul Lissamine Green dye (1% aqueous solution) into the brain before the syringe was removed from the stereotactic apparatus. The rat was then sacrificed,

removed from the stereotax, and its brain was excised. The brain was then sectioned with a razor blade to verify the presence of dye in the ventricles.

Baseline values (first urine collection) for all parameters were compared. As minimal differences were observed between groups, all results are presented as the difference in value between the mean third urine collection and the mean control urine collection with standard error indicated. A change in urine volume can be mediated either by a change in osmolar clearance or by a change in free water clearance, as these functions are mediated by separate mechanisms. Therefore, we elected to present these values in addition to urine flow to investigate their regulation as affected by moxonidine. Results were analyzed by repeated measures analysis of variance (ANOVA) followed by a post-hoc test to identify significant differences. All significant differences are denoted by **, which represents a P value of $p<0.01$.

Measurement/Treatment	Pre-ICV saline 5 μl n=7	Pre-ICV moxonidine 10 nmol/5 μl n=5
Blood Pressure (mmHg)	128 ± 2	125 ± 4
Heart Rate (beats/min)	420 ± 8	408 ± 9
Creatinine Clearance (ml/min)	1.8 ± 0.3	2.2 ± 0.2
Urine Flow (ul/min)	10.5 ± 1.6	10.4 ± 1.3
Osmolar Clearance (ul/min)	58 ± 7	64 ± 5
Free Water Clearance (ul/min)	-48 ± 6	-53 ± 4

Table 2.1: Baseline values obtained from the first (control) urine collection before ICV injection of either saline or moxonidine. Results are presented as mean ± s.e.m.

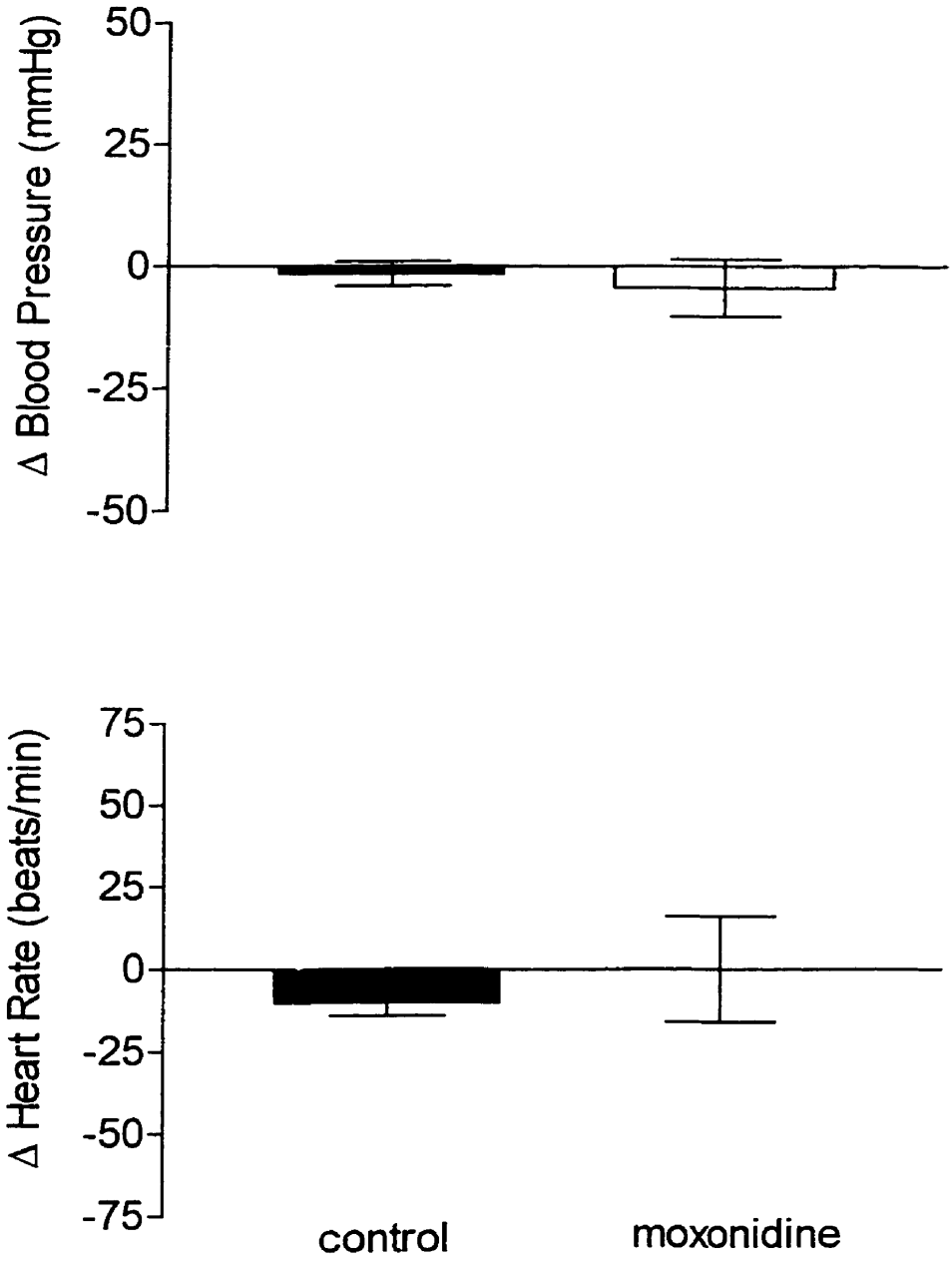

Figure 2.1: The effects of icv moxonidine on blood pressure and heart rate in the uninephrectomized rat. Moxonidine (10 nmol/5 ul) or saline was injected directly into the left cerebral ventricle over 2.5 minutes. Bars represent the mean ± s.e.m. of the differences between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 5 experiments. * represents a significant difference of $p<0.05$ vs control.

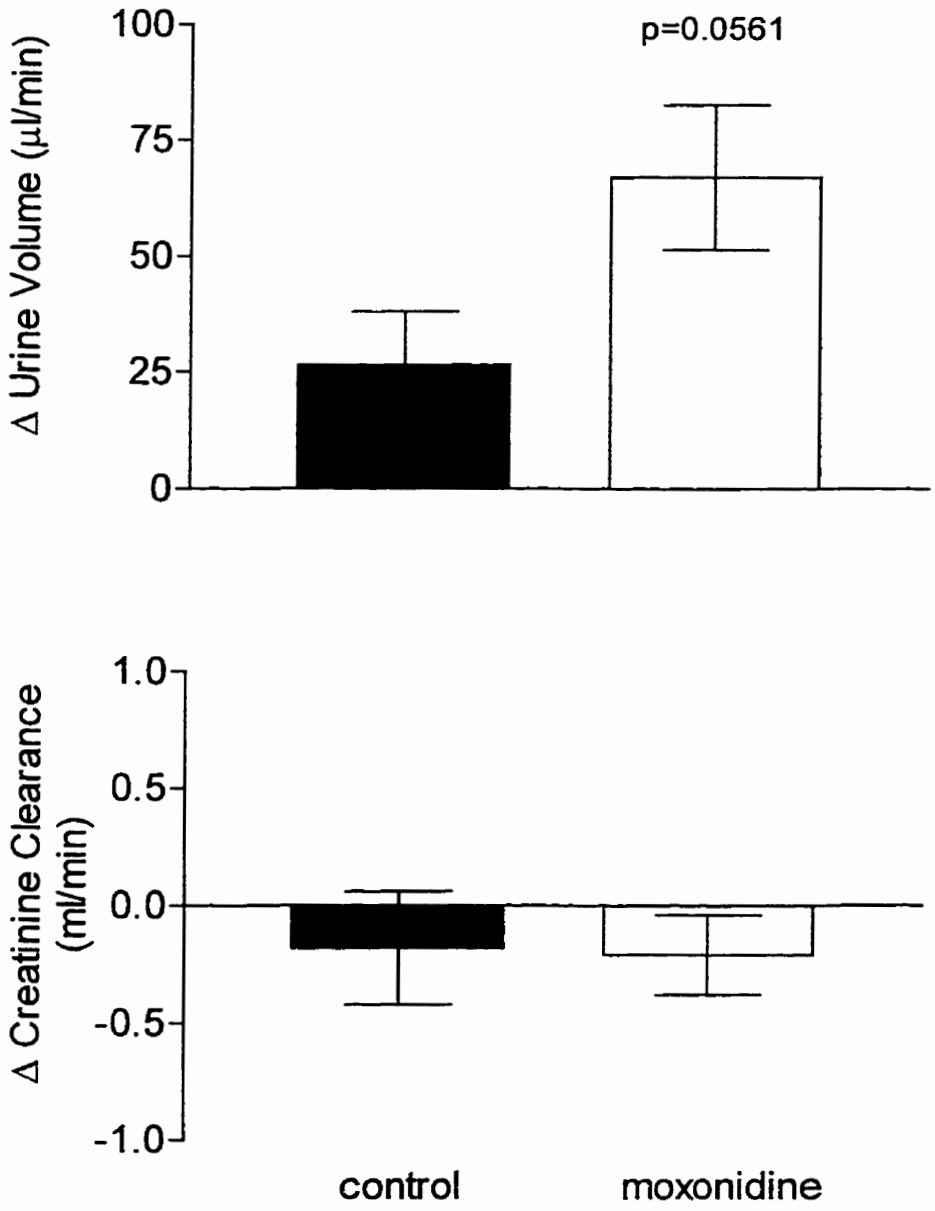

Figure 2.2: The effects of icv moxonidine on urine flow and creatinine clearance in the uninephrectomized rat. Moxonidine (10 nmol/5 ul) or saline was injected directly into the left cerebral ventricle over 2.5 minutes. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 5 experiments. * represents a significant difference with $p<0.05$ vs control vs control.

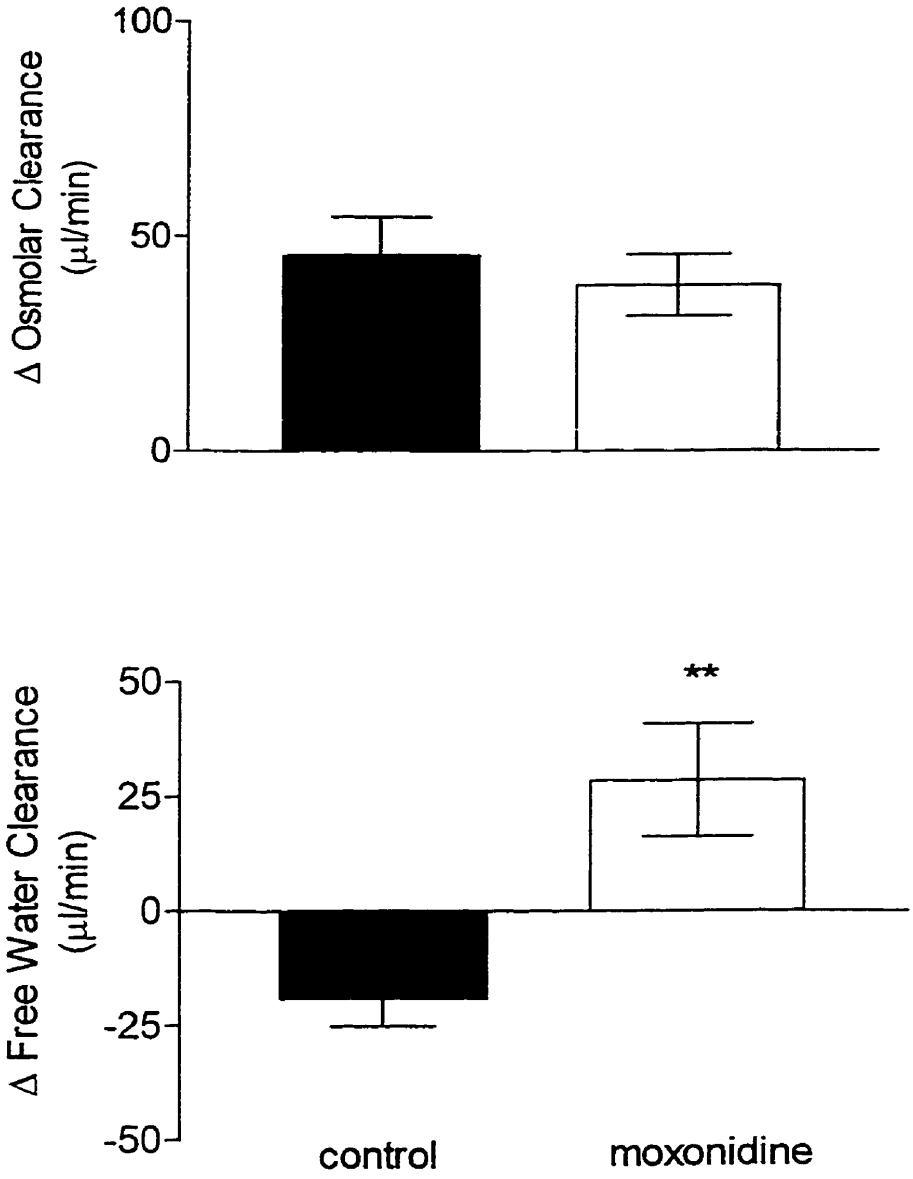

Figure 2.3: The effects of icv moxonidine on osmolar and free water clearance in the uninephrectomized rat. Moxonidine (10 nmol/5 ul) or saline was injected directly into the left cerebral ventricle over 2.5 minutes. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 5 experiments. * represents a significant difference with $p<0.05$ vs control. ** represents $p<0.01$ vs control

Results

Baseline values of data from the first urine collection are presented in Table 2.1. These values were obtained as controls before ICV injection of moxonidine (10 nmol/5 ul) or saline (5 ul). As shown in Table 2.1, no significant differences in any of the parameters exist between the two study groups. Presentation of all results as the difference between control and final (third collection) values further clarifies and emphasizes the treatment differences between the two groups.

The dose of moxonidine was chosen based on previous experiments in our laboratory to alter renal function without a change in blood pressure. It should be mentioned, however, that higher doses (30 nmol/5ul) of ICV moxonidine may decrease blood pressure by as much as 40 mmHg (unpublished observation). At the chosen dose of 10 nmol/5 ul, no change in blood pressure or heart rate was observed (Figure 2.1). Creatinine clearance was also not altered by experimental intervention (Figure 2.2).

Moxonidine treatment increased urine flow as compared to ICV injection of saline (Figure 2.2). This increase was not due to an increase in osmolar clearance, but an increase in free water clearance (Figure 2.3).

Discussion

In previous studies, ICV moxonidine was shown to increase urine volume secondary to an increase in osmolar clearance, more specifically, an increase in the fractional excretion of sodium (Penner and Smyth, 1994a; Penner and Smyth, 1994b; Smyth and Penner, 1999). In these studies, doses of 0.1 and 0.3 nmol were shown to increase urine flow without altering blood pressure. In the present study, a much larger dose (10 nmol) was required to obtain an increase in urine flow, and this increase was not due to an increase in osmolar clearance, but an increase in free water clearance. Further study in our laboratory has consistently duplicated the free water response to ICV moxonidine without an increase in osmolar clearance. The explanation for this change in potency and response is unclear.

The increase in free water clearance observed in the present study may involve inhibition of vasopressin release from the posterior pituitary in the hypothalamus, however this possibility requires further investigation. Increased sympathetic nerve activity has been shown to cause stimulation of the α_1 adrenergic receptor in the nephron to retain sodium. Moxonidine has been reported to decrease sympathetic nervous activity following ICV administration, which may decrease the level of stimulation of renal α_1 adrenergic receptors resulting in an increase in sodium excretion (Penner and Smyth, 1994a; Smyth and Penner, 1999). The response observed in the present study may represent a further function of the imidazoline receptor to inhibit vasopressin release. Another possibility is that the high doses required may produce binding at α_2 adrenergic

receptors, as norepinephrine, which binds presynaptic α_2 adrenergic receptors, has been implicated in the regulation of vasopressin release (Armstrong *et al.*, 1982).

From the present study we conclude that ICV administration of moxonidine produces diuresis. Further investigation is required to determine whether these effects are due to imidazoline receptor or α adrenergic receptor mediated mechanisms.

Separation of α_2 Adrenergic Receptor and I_1 Imidazoline Receptor Function in the Paraventricular Nucleus of the Hypothalamus

Introduction

In the previous study, we identified a diuretic effect following ICV injection of moxonidine. It was indicated that this function may occur through the inhibition of vasopressin release from the posterior pituitary. Therefore, we sought to confirm this hypothesis and determine whether this effect was due to stimulation of α_2 adrenergic receptors or I_1 imidazoline receptors. This function of the imidazoline receptor agonist moxonidine may be in addition to previously documented natriuresis, which occurs through decreased sympathetic nerve activity, resulting in decreased α_1 adrenergic receptor mediated sodium retention (Penner and Smyth, 1994a; Smyth and Penner, 1999).

Imidazoline receptors have been identified throughout the brain, in areas such as rat RVLM (Ernsberger *et al.*, 1992), rabbit brainstem (Bricca *et al.*, 1993), human ventrolateral medulla (Bricca *et al.*, 1994), and bovine ventrolateral medulla (Ernsberger *et al.*, 1993). It has not been specifically determined whether imidazoline receptors are present in the paraventricular nucleus of the hypothalamus; however, several observations led us to investigate this nucleus as a mediator of the aforementioned responses to ICV moxonidine.

Firstly, ICV moxonidine produced a diuresis that may have been mediated by an inhibition of vasopressin release. The PVN is responsible for the production of vasopressin and oxytocin, which are transported down the axon of the neuron for release onto capillaries in the posterior pituitary. If moxonidine inhibits the release of vasopressin, this effect would most likely be exerted along this pathway from the PVN. We propose that this inhibition of vasopressin release is not due to I_1 imidazoline receptor activation, but that of moxonidine acting at α_2 adrenergic receptors.

There are several documented examples of adrenergic inhibition of neurotransmitter and hormone release. The classical example of this regulation is the inhibition of norepinephrine release by presynaptic α_2 adrenergic receptors. When norepinephrine (NE) is released from the presynaptic neuron, NE binds adrenergic receptors on the postsynaptic cell, while at the same time binding α_2 adrenergic receptors to produce a negative feedback on further NE release. In similar manner, α_2 adrenergic receptors have been implicated in the regulation of vasopressin release. In 1982, the first paper directly implicating norepinephrine in the control of vasopressin release from the hypothalamus was published (Armstrong *et al.*, 1982). An organ culture system was created from explants of rat hypothalamo-neurohypophyseal tissue. Norepinephrine was applied to the tissue following stimulation by acetylcholine or sodium chloride, and vasopressin release was measured. Norepinephrine dose dependently inhibited both basal and acetylcholine induced release of vasopressin. This inhibition was blocked by phenoxybenzamine and phentolamine, but not by propranolol, indicating the α vs β adrenergic receptor specificity of this response.

Incidentally, α_2 adrenergic receptors have been located on the cell bodies of the neurosecretory neurons which extend from the PVN to the posterior pituitary, indicating the possibility of direct or indirect adrenergic control of vasopressin synthesis or release (Ruffolo *et al.*, 1991). Indirectly, activity of neurons that terminate in the PVN can be modulated under adrenergic control, inhibiting their firing rate to alter vasopressin release. In addition, the α_2 adrenergic receptors on neurosecretory PVN neurons may constitute direct adrenergic control of vasopressin release. The integration of these effects was best demonstrated through experiments by Khanna and colleagues in 1993, in which norepinephrine excited vasopressin neurons, but depressed their response to input from other areas of the brain (Khanna *et al.*, 1993). Another study involving stimulation of α_2 adrenergic receptors in the NTS demonstrated the integration and influence of other brain regions on vasopressin release. NTS injection of clonidine attenuated the release of vasopressin following volume depletion by polyethylene glycol injection (Iovino *et al.*, 1990).

The α_1 adrenergic receptor has also been identified on PVN neurons, and activation of this receptor has been correlated with increased neuron firing and increased plasma vasopressin levels (Armstrong *et al.*, 1986; Brooks *et al.*, 1986). Further, although ICV norepinephrine induces vasopressin release due to action at α_2 adrenergic receptors in the hypothalamus, ICV yohimbine also increases vasopressin release (Brooks *et al.*, 1986). It has been suggested that while α_1 adrenergic receptors are responsible for stimulating vasopressin release (supported by Armstrong *et al.*, 1986), α_2 adrenergic receptors may tonically dampen vasopressin release from that of α_1 adrenergic receptor activation alone (Ruffolo *et al.*, 1991).

The second line of evidence implicating the PVN in the response to ICV moxonidine involves the role of the PVN in the regulation of blood pressure. In a study involving eleven hypertensive patients, guanfacine (1 mg) was administered once daily for four weeks to determine the effect of the α_2 adrenergic agonist on plasma vasopressin levels (Berkman *et al.*, 1990). Inclusion of patients was primarily dependent on a high plasma vasopressin level as compared to a normotensive control group. Following four weeks of guanfacine treatment, plasma vasopressin was decreased in all patients, and was correlated with a decrease in blood pressure while not altering plasma osmolality or plasma renin activity. The authors speculate that the observed effect on vasopressin levels may be due to direct action of guanfacine on α adrenergic receptors to inhibit vasopressin secretion.

The PVN has also been shown in animal studies to be neuronally connected to other sites in the brain which control blood pressure. For this reason, we proposed that moxonidine, in addition to inhibiting vasopressin release, may exert its hypotensive effect through direct activation of PVN neurons, possibly by action at imidazoline receptors. It has been well documented that both the NTS and RVLM project to the PVN (Sawchenko and Swanson, 1982; Swanson and Sawchenko, 1983; Weiss and Hatton, 1990), suggesting a possible role in regulation of or adaptation to changes in blood pressure. Rhodamine labeled microspheres (a retrograde tracing technique) have been injected into various areas of the brain, confirming that the NTS and RVLM are connected to the PVN (Petrov *et al.*, 1993, Krukoff *et al.*, 1992). In fact, there are PVN neurons with branching projections that make contact with both the NTS and RVLM, and these neurons have been identified as catecholaminergic (Petrov *et al.*, 1993). Thus, the NTS and RVLM

contain neurons that make contact with each other, and then project to the PVN, indicating a pathway of communication between these sites involved in blood pressure regulation. Further, norepinephrine appears to be the neurotransmitter in this pathway, which substantiates the role of α adrenergic receptors in its regulation.

Further support for the involvement of the PVN in blood pressure regulation arises from studies using *c-fos* as a marker of neuronal activation. Activated neurons will express Fos, which is the product of an immediate early gene and can be visualized on brain sections by immunocytochemistry techniques. In the conscious rat, following a hypotensive stimulus, the PVN is one of the most highly activated areas of the brain as measured by Fos expression, although it was not activated by a hypertensive stimulus (Krukoff *et al.*, 1995; Li and Dampney, 1994). It was noted that the activated neurons in the PVN were not immunoreactive for vasopressin (Li and Dampney, 1994), suggesting that the PVN responds to a direct hypotensive stimulus using mechanisms independent of vasopressin. This response mechanism may involve imidazoline receptors in the PVN.

The third line of evidence suggesting the role of the PVN in response to ICV moxonidine is that supporting a role of the PVN in renal function. Support for this pathway would indicate that the previously documented natriuresis observed following ICV moxonidine may be due to regulation by the PVN, more specifically, by imidazoline receptor activation in the PVN. The PVN has been implicated in the regulation of renal function following experiments identifying a descending neural pathway from the PVN to the kidney. As previously mentioned, decreases in sympathetic activity following ICV

moxonidine may result in decreased RSNA and α_1 adrenergic receptor-mediated natriuresis (Penner and Smyth, 1994a; Smyth and Penner, 1999).

Renal preganglionic neurons have been identified by retrograde tracing using the pseudorabies virus, which was injected into the kidney and allowed to migrate through the renal nerves to the brain (Schramm *et al.*, 1993). The PVN and rostral ventromedial medulla (RVLM) were densely labeled, as were the caudal medullary raphe, RVLM, and the A5 cell group, which are involved in the maintenance of renal sympathetic nerve activity and are stimulated by N-methyl-D-aspartate (NMDA). It has been demonstrated, however, that although electrical and chemical stimulation of the PVN produces renal vasoconstriction, the role of the PVN in cardiovascular control is neither altered by nor dependent on NMDA (Porter, 1993). This observation indicates that PVN regulation of blood pressure and renal function is mediated through another receptor system.

The descending pathway from the PVN to the kidney has also been implicated in the regulation of renal blood flow. Parvocellular lesions of the PVN with neurotoxin abolished the renal vasodilation observed following volume load (Lovick *et al.*, 1993). Therefore, the PVN is responsible for renal vasodilation through decreased RSNA in response to volume load. In addition, it has been recently demonstrated that the PVN is able to modulate RSNA, presumably through the descending nerve pathway to the kidney (Haselton and Vari, 1998; Zhang and Patel, 1998). The PVN may control blood pressure and sympathetic nerve activity through activation of imidazoline receptors, as seen with moxonidine, the imidazoline agonist. In support of this hypothesis, a recent study implicated imidazoline receptors in the PVN in the pressor response to angiotensin II injected into the subfornical organ (SFO) (Saad *et al.*, 1998). The SFO is involved with

sensing and responding to osmolar changes in the body, and is closely linked to blood pressure regulation. In this study, PVN clonidine and rilmenidine had additive effects in attenuating the increased arterial pressure following SFO injection of angiotensin II. Through the use of selective antagonists, it was determined that both the α_2 adrenergic receptor and the I_1 imidazoline receptor were involved in the increase in blood pressure following SFO injection of angiotensin II. This study demonstrated both the presence and a possible role of imidazoline receptors in the PVN.

These three lines of investigation together led us to hypothesize that moxonidine acts within the PVN to inhibit vasopressin release (resulting in increased free water clearance), through activation of α_2 receptors, and to decrease renal sympathetic nerve activity through the activation of imidazoline receptors in the PVN.

Methods

A detailed description of the general procedures has been documented previously in the general Methods section. Male Sprague-Dawley rats were unilaterally nephrectomized under ether anesthesia and allowed to recover for 7 to 13 days. On the day of the experiment, animals were anesthetized with sodium pentobarbital. A tracheotomy, carotid arterial and jugular venous cannulation were performed as described to allow spontaneous breathing, monitoring of the heart rate and blood pressure, and infusion of saline respectively. Following a 45 minute stabilization period, an infusion of saline was initiated via the jugular vein and maintained throughout the remainder of the experiment. A 30 minute control urine collection was obtained, and moxonidine (10 nmol/500 nl, Beiersdorf, AG, Hamburg, FRG), guanfacine (10 nmol/500 nl, Wyeth Ayerst) or saline (500 nl) was then injected over 2.5 minutes into the paraventricular nucleus of the hypothalamus. The stereotactic coordinates used, as determined from the atlas of Paxinos and Watson (Paxinos and Watson, 1986) were 1.9 mm posterior, 0.6 mm lateral and 7.8 mm ventral to bregma.

Two 30 minute urine collections followed the ICV injection of moxonidine, guanfacine, or saline. Heart rate and blood pressure were monitored, and urine flow rates determined gravimetrically. A plasma sample was obtained after completion of the experiment and stored at 4°C for up to one week until analysis.

Placement of the Hamilton syringe in the PVN was verified histologically. After the animal was sacrificed, the brain was removed and sectioned on a freezing microtome, each section 40 microns in thickness. The sections were mounted on frosted slides

(Fisher Superfrost Plus Microscope slides) and stained with thionin stain to visualize individual nuclei. The needle track was readily identifiable under a low power objective.

Following the completion of this study, we questioned whether an injection site adjacent to the PVN might provide different results than injection directly into the PVN, which may damage the site. We therefore conducted a series of experiments identical to those presently described, but with an injection site slightly rostral to the PVN. The coordinates, chosen based on a previous study (Bhatnagar, 1988) and according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986), were 0.9 mm posterior, 1.2 mm lateral, and 8.4 mm ventral to bregma. It has been shown in a recent paper that injection of norepinephrine into the PVN diffuses approximately 1 mm laterally (Itoi *et al.*, 1999), verifying that the coordinates chosen for this experiment were easily within the diffusional distance of the PVN.

All results are presented as the difference in value between the mean third urine collection and the mean control urine collection with standard error indicated. We again elected to present these values in addition to urine flow to investigate their regulation as affected by moxonidine. Results were analyzed by repeated measures analysis of variance (ANOVA) followed by a post-hoc test to identify significant differences. All significant differences are denoted by *, which represents a P value of <0.05, ** represents $p< 0.01$, *** represents $p<0.001$.

Measurement/Treatment	Pre-PVN Saline 500 nl n = 6	Pre-PVN Guanfacine 10 nmol/500 nl n = 10
Blood Pressure (mm Hg)	128 ± 2	136 ± 3
Heart Rate (beats/min)	400 ± 7	424 ± 9
Creatinine Clearance (ml/min)	1.4 ± 0.2	1.4 ± 0.1
Urine Flow (μl/min)	14.4 ± 2.1	12.9 ± 1.9
Osmolar Clearance (μl/min)	65 ± 5	59 ± 4
Free Water Clearance (μl/min)	-50 ± 3	-46 ± 3

Table 3.1: Baseline values obtained from the first (control) urine collection before injection of either saline or guanfacine into the PVN. Results are presented as mean ± s.e.m.

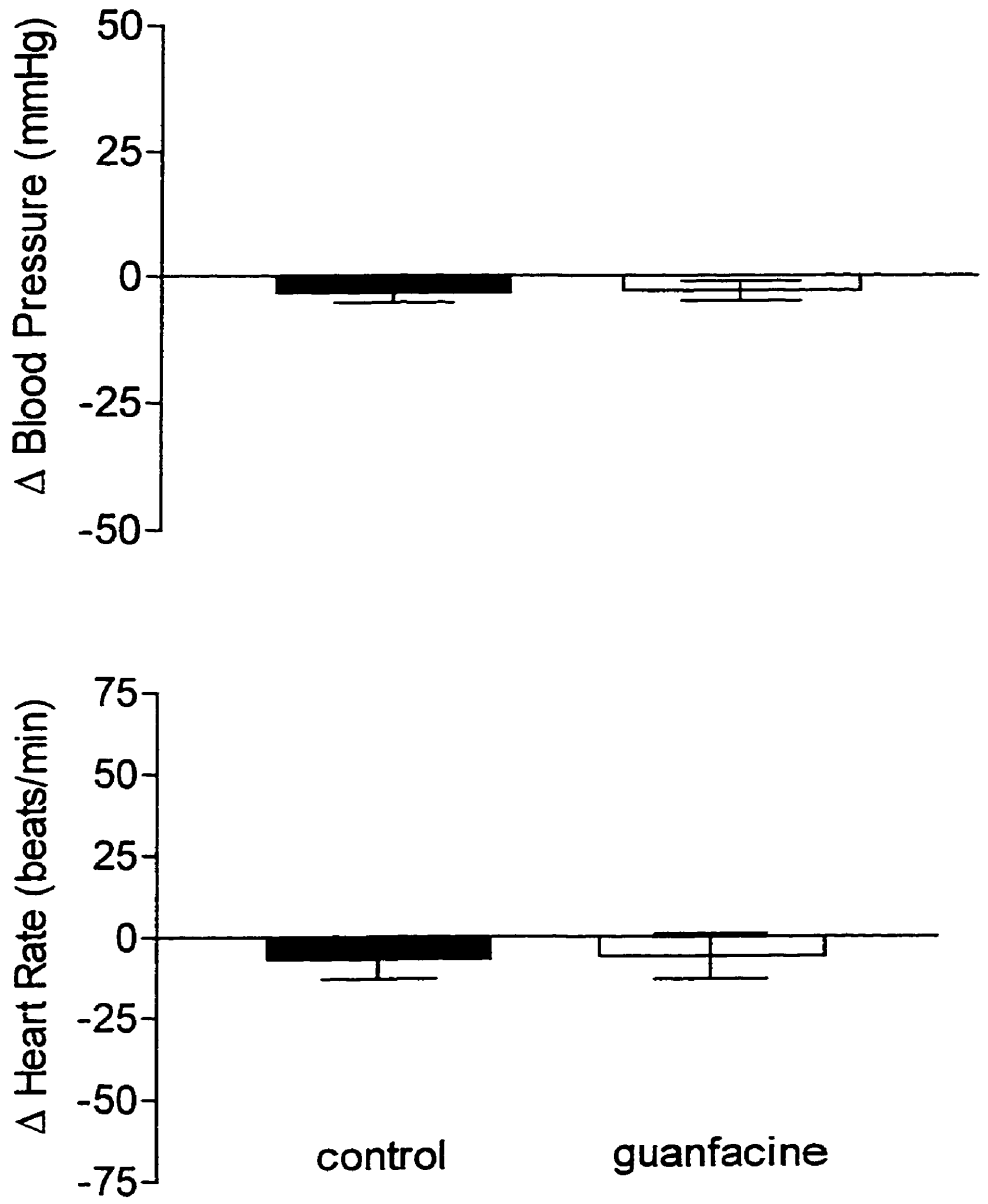

Figure 3.1: The effects of PVN guanfacine on blood pressure and heart rate in the uninephrectomized rat. Guanfacine (10 nmol/500 nl) or saline was injected directly into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with $p<0.05$ vs control.

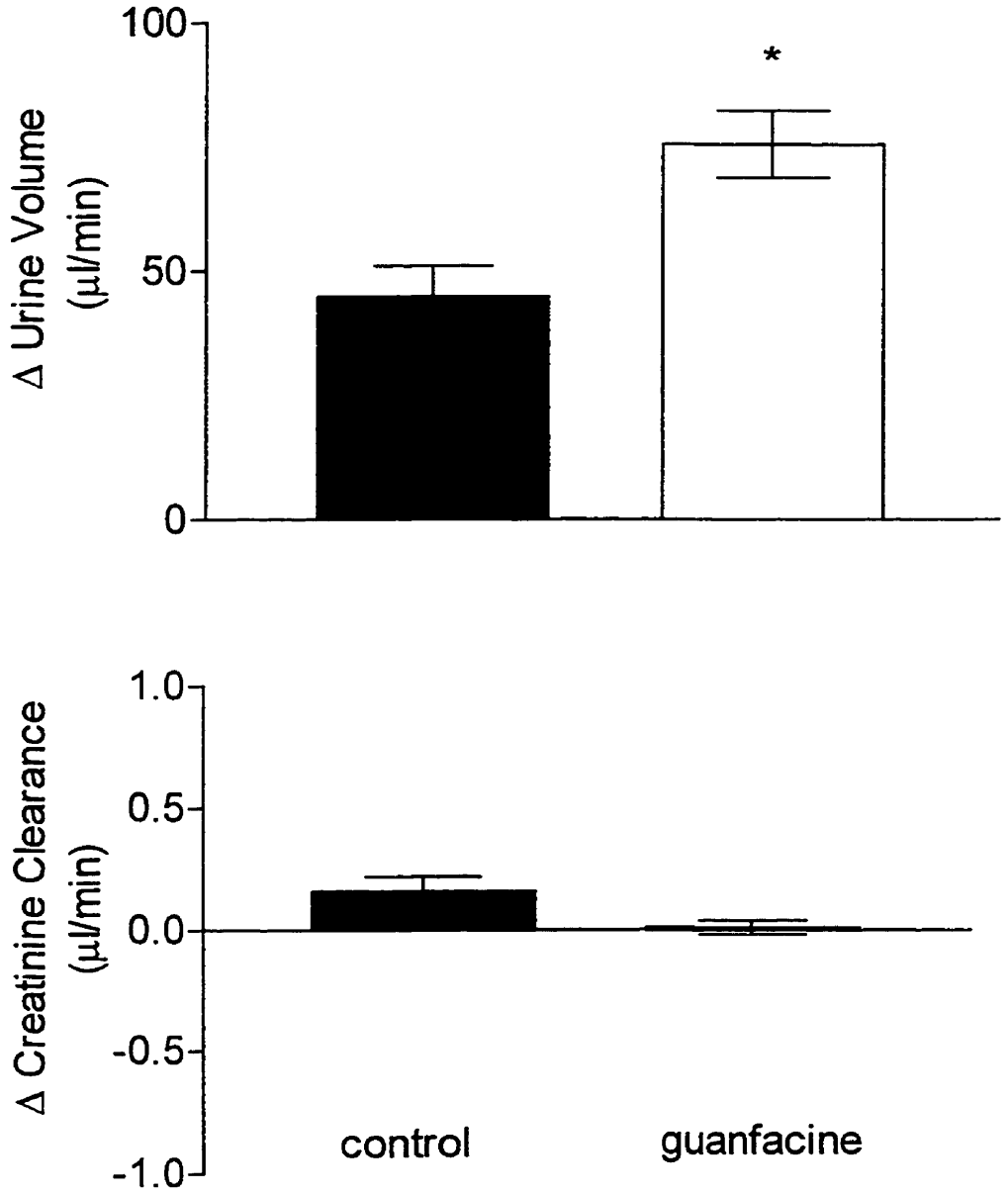

Figure 3.2: The effects of PVN guanfacine on urine flow and creatinine clearance in the uninephrectomized rat. Guanfacine (10 nmol/500 nl) was directly injected into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) urine collection. Each group contains at least 6 experiments. * represents a significant difference with $p<0.05$ vs control.

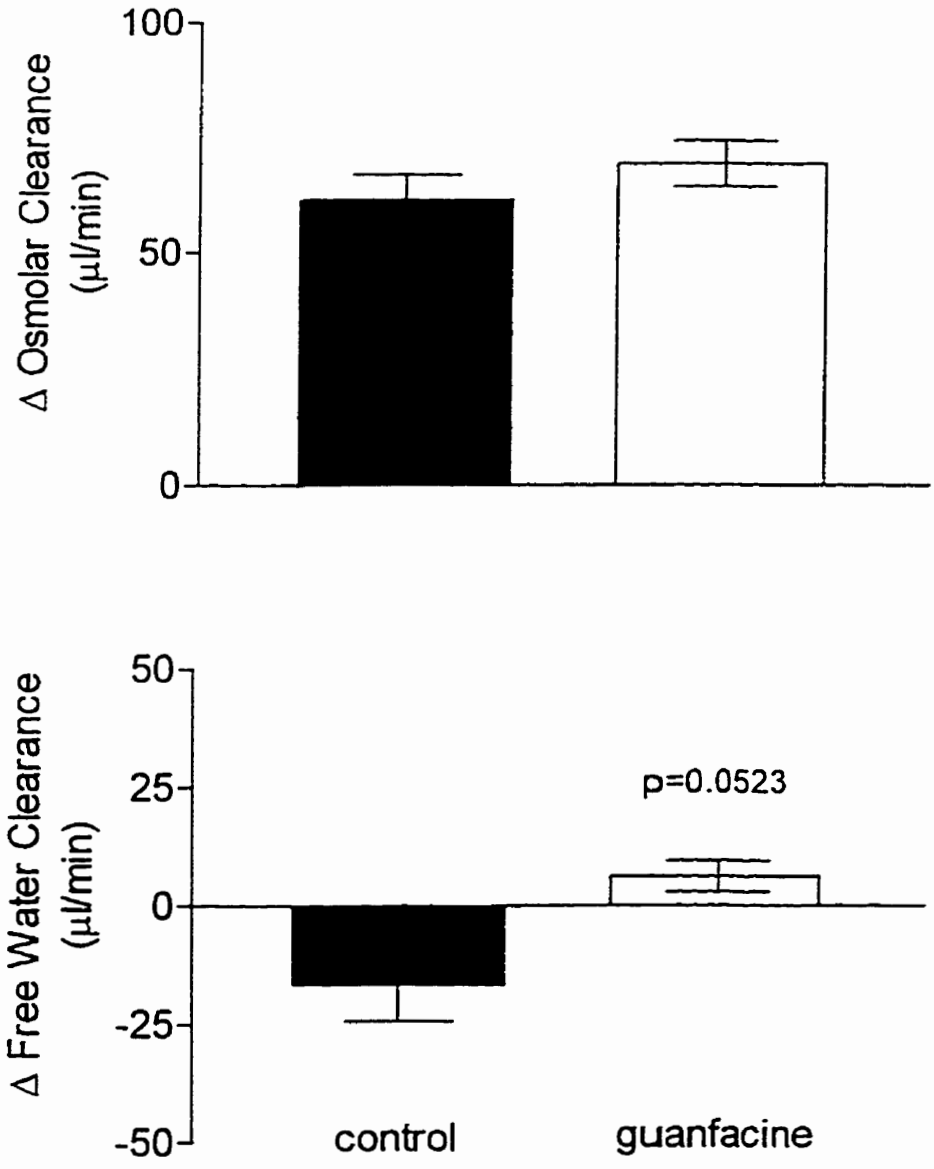

Figure 3.3: The effects of PVN Guanfacine on osmolar and free water clearance in the uninephrectomized rat. Guanfacine (10 nmol/500 nl) or saline (500 nl) was injected directly into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains at least 6 experiments. * represents a significant difference with $p<0.05$ vs control.

Measurement/Treatment	Pre-PVN Saline 500 nl n = 6	Pre-PVN Moxonidine 10 nmol/500 nl n = 6
Blood Pressure (mm Hg)	128 ± 2	126 ± 2
Heart Rate (beats/min)	420 ± 7	410 ± 14
Creatinine Clearance (ml/min)	1.6 ± 0.2	1.6 ± 0.1
Urine Flow (µl/min)	11.3 ± 1.3	10.9 ± 1.5
Osmolar Clearance (µl/min)	55 ± 5	52 ± 5
Free Water Clearance (µl/min)	-44 ± 4	-42 ± 4

Table 3.2: Baseline values obtained from the first (control) urine collection before PVN injection of either saline or moxonidine. Results are presented as mean ± s.e.m. * represents a significant difference with a p value < 0.05.

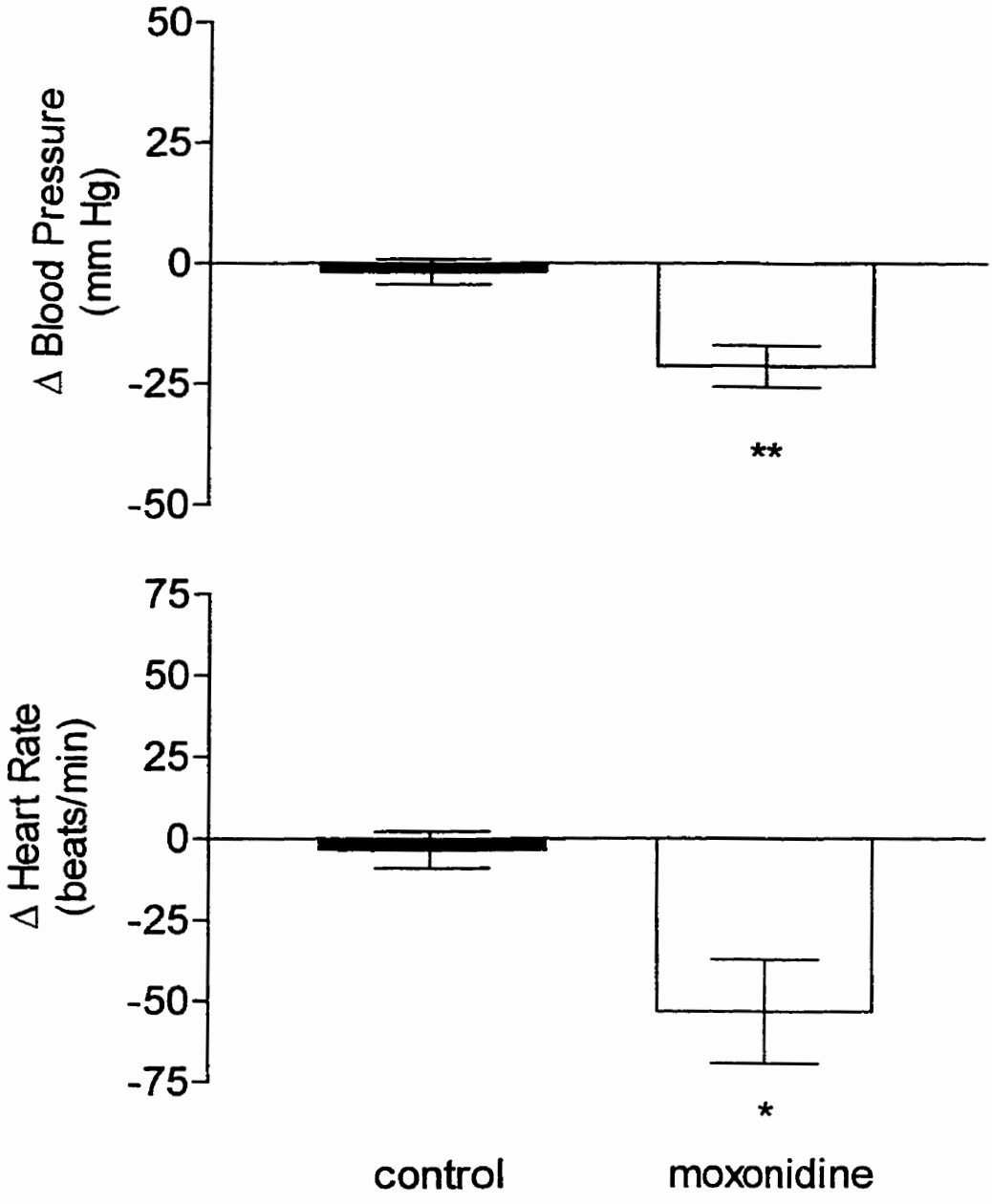

Figure 3.4: The effects of PVN moxonidine on blood pressure and heart rate in the uninephrectomized rat. Moxonidine (10 nmol/500 nl) or saline (500 nl) was injected directly into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains at least 6 experiments. * represents a significant difference with $p<0.05$ vs control. ** represents $p<0.01$ vs control

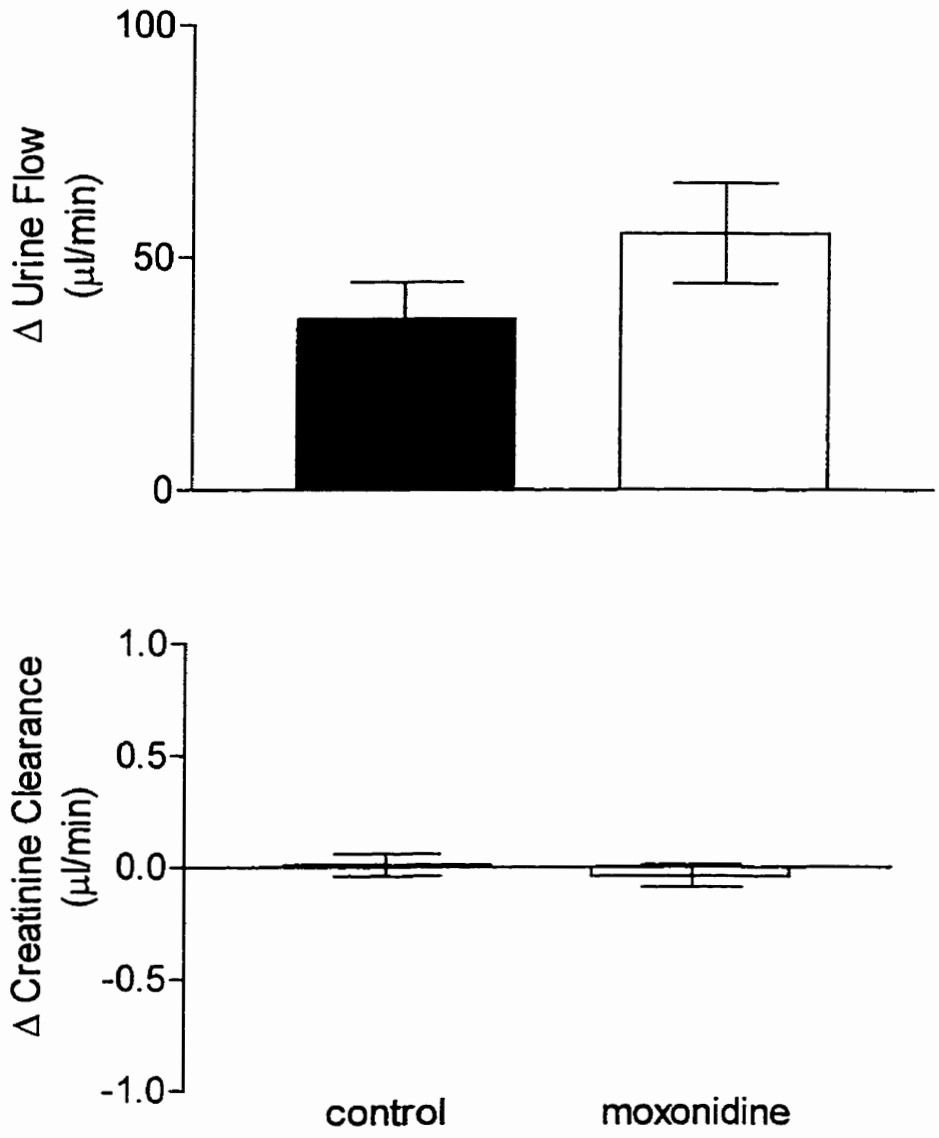

Figure 3.5: The effects of PVN moxonidine on urine flow and creatinine clearance in the uninephrectomized rat. Moxonidine (10 nmol/500 nl) or saline (500 nl) was injected directly into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains at least 6 experiments. * represents a significant difference with $p<0.05$ vs control.

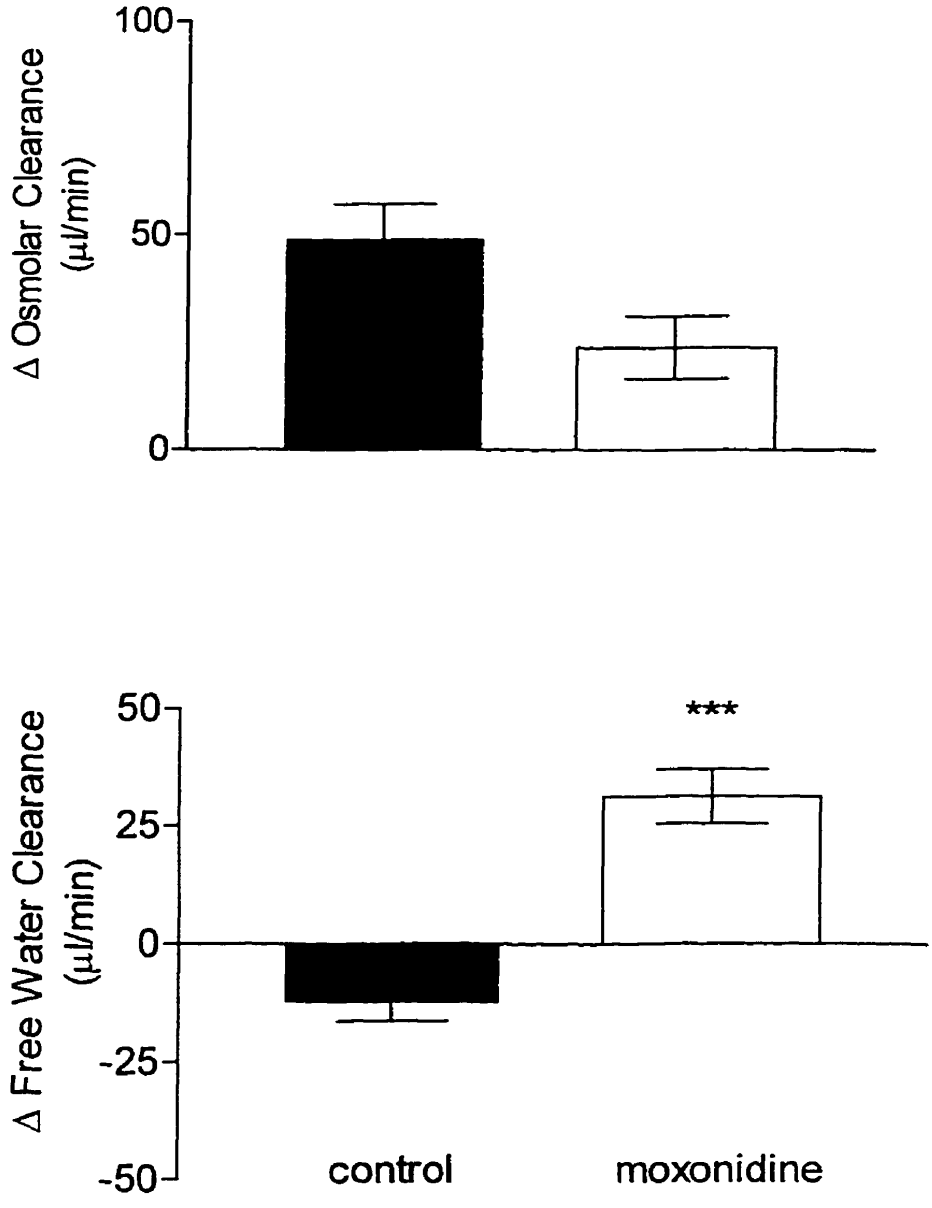

Figure 3.6: The effects of PVN moxonidine on osmolar and free water clearnace in the uninephrectomized rat. Moxonidine (10 nmol/500 nl) or saline (500 nl)was injected directly into the PVN. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains at least 6 experiments. * represents a significant difference with $p<0.05$ vs control. ** represents $p<0.01$ vs control *** represents $p<0.001$ vs control

Measurement/ Treatment	Pre-Rostral PVN Saline n=7	Pre-Rostral PVN Guanfacine n=6	Pre-Rostral PVN Moxonidine n=6
Blood Pressure (mm Hg)	121 ± 3	128 ± 4	125 ± 3
Heart Rate (beats/min)	397 ± 11	380 ± 9	383 ± 6
Urine Flow (μl/min)	13.0 ± 1.8	16.8 ± 1.0	16.4 ± 1.8
Creatinine Clearance (ml/min)	1.4 ± 0.2	1.7 ± 0.1	1.5 ± 0.1
Osmolar Clearance (μl/min)	64 ± 5	66 ± 3	65 ± 6
Free Water Clearance (μl/min)	-51 ± 4	-49 ± 3	-49 ± 5

Table 3.3: Baseline values obtained from the first (control) urine collection prior to injection of drug or vehicle. Results are presented as mean ± s.e.m.

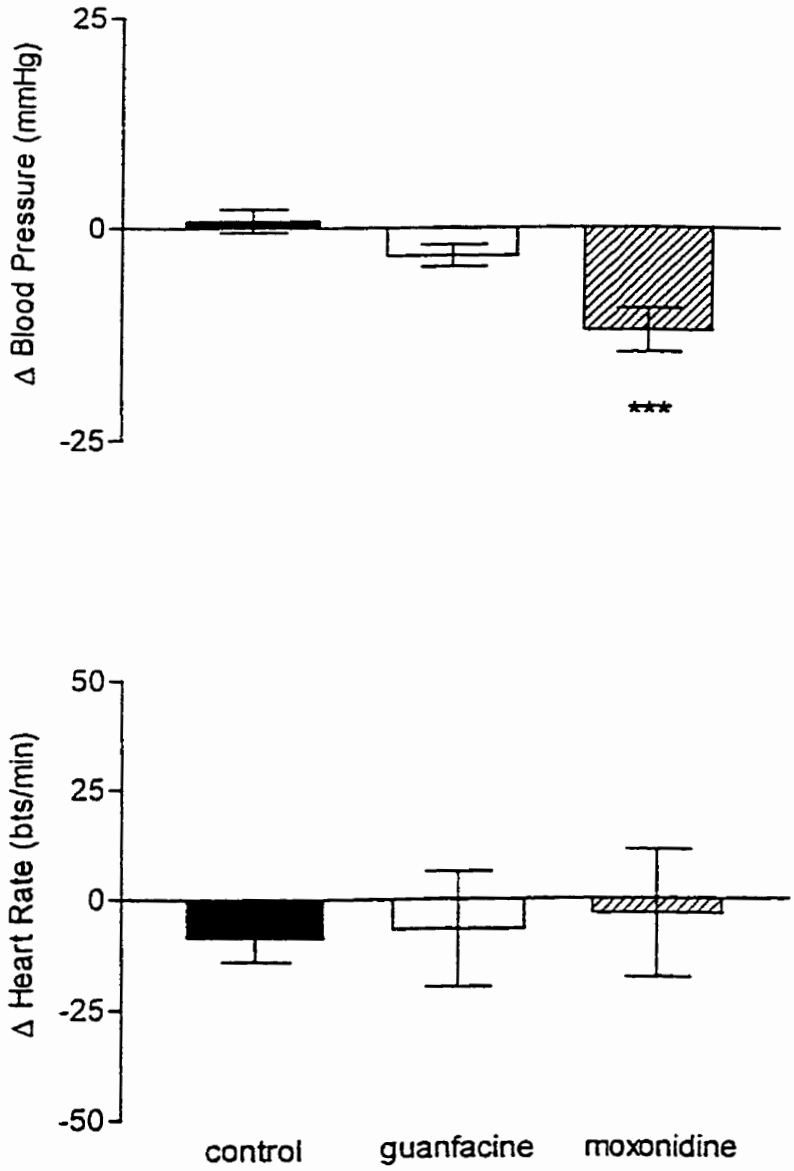

Figure 3.7: The effects of rostral PVN guanfacine or moxonidine on blood pressure and heart rate in the uninephrecromized rat. Moxonidine or guanfacine (10 nmol/500 nl), or saline (500 nl), was injected adjacent to the rostral PVN. Bars represent the mean ± s.e.m. of the differences between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. *represents a significant difference with $p<0.05$ vs control ** represents $p<0.01$ vs control *** represents $p<0.001$ vs control

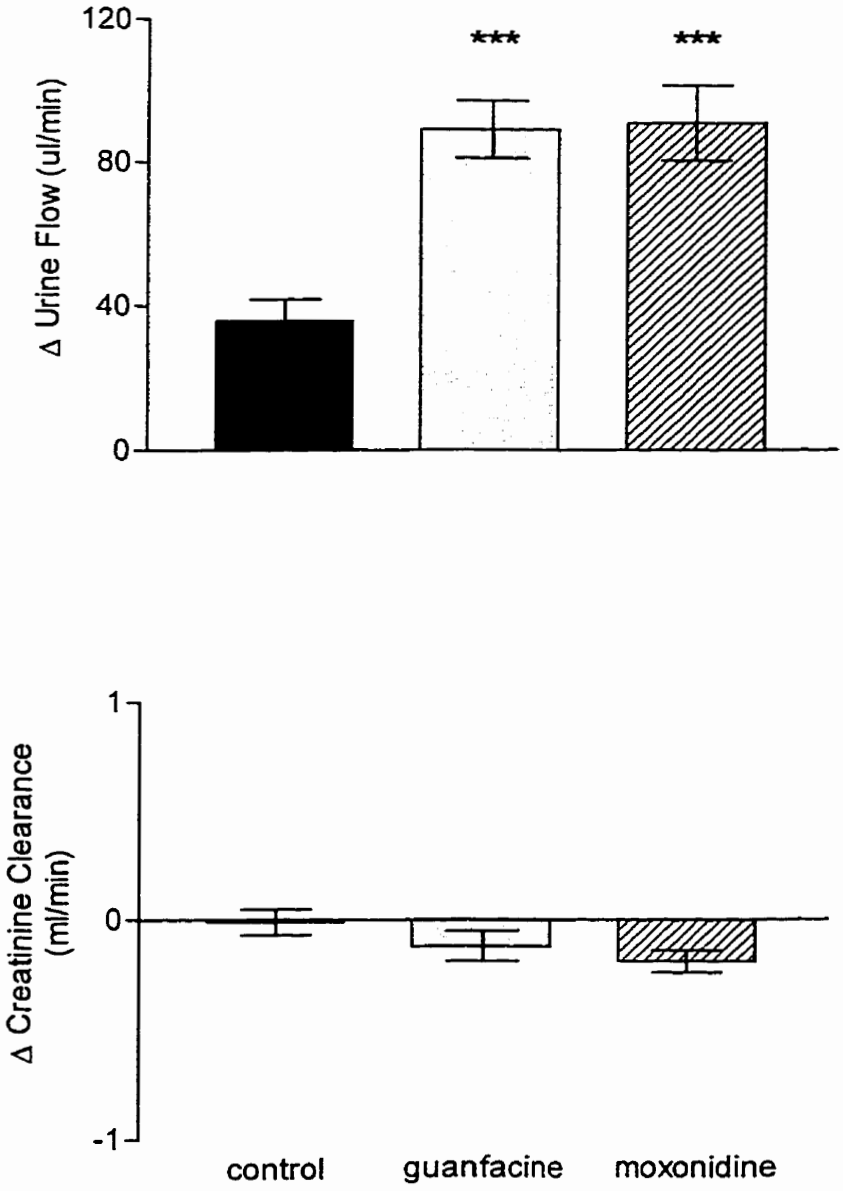

Figure 3.8: The effects of rostral PVN guanfacine or moxonidine on urine flow and creatinine clearance in the uninephrectomized rat. Guanfacine or moxonidine (10 nmol/500 nl), or saline (500 nl), was injected adjecent to the rostral PVN. Bars represent the mean ± s.e.m. of the differences between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with $p<0.05$ vs control ** represents $p<0.01$ vs control *** represents $p<0.001$ vs control

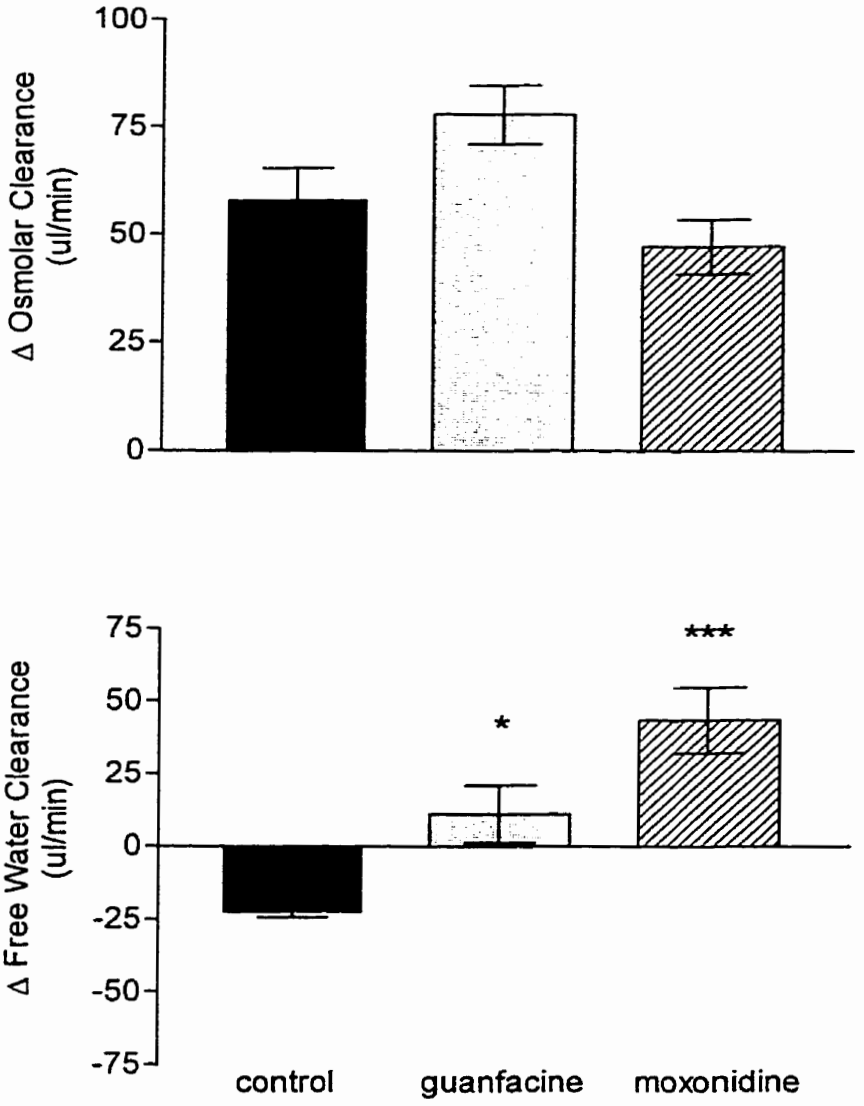

Figure 3.9: The effects of rostral PVN guanfacine or moxonidine on osmolar and free water clearance in the uninephrectomized rat. Guanfacine or moxonidine (10 nmol/500 nl), or saline (500 nl), was injected adjacent to the rostral PVN. Bars represent the mean ± s.e.m. of the differences between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with $p<0.05$ vs control ** represents $p<0.01$ vs control *** represents $p<0.001$ vs control

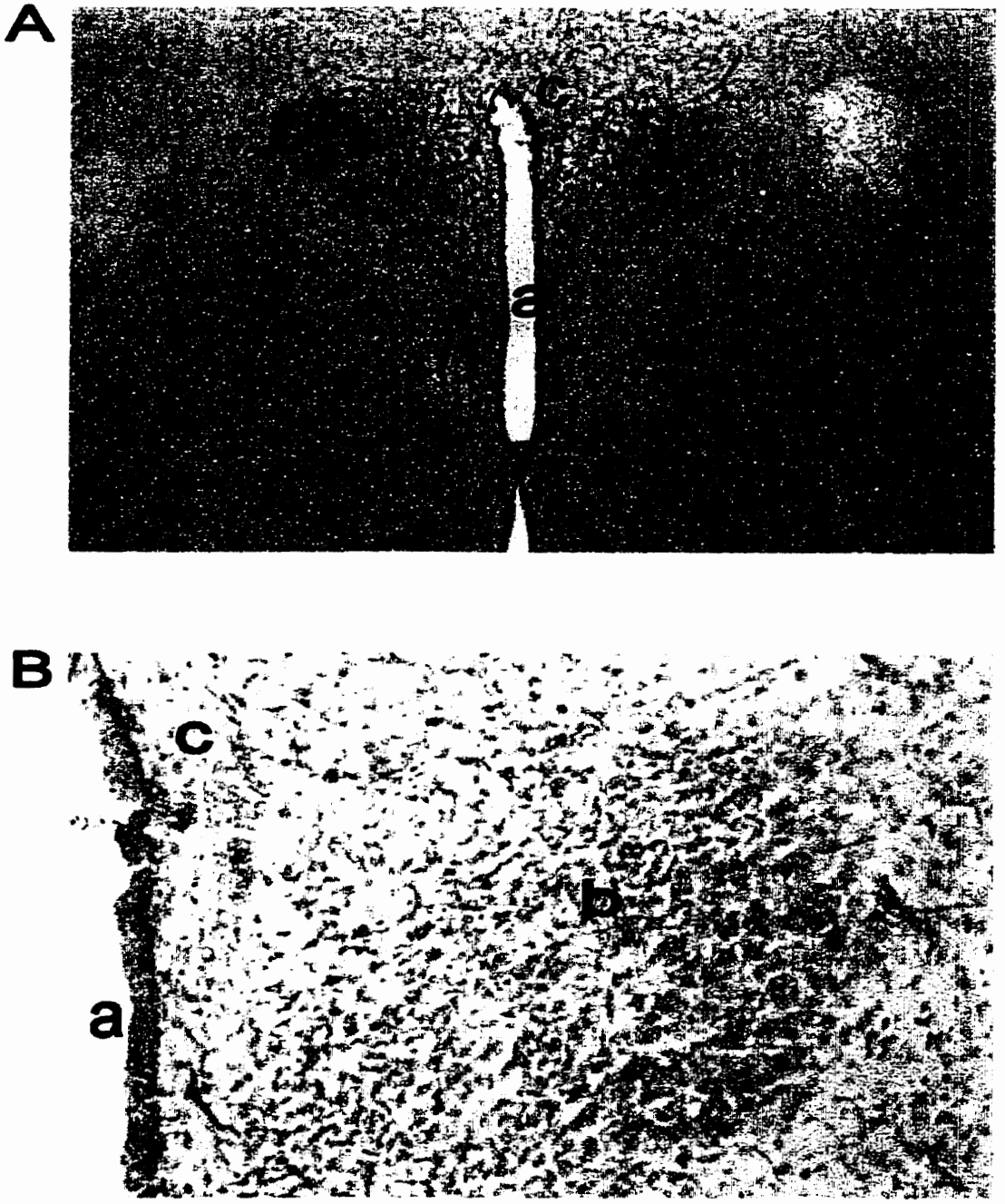

Figure 3.10: Photograph of a typical PVN placement. A scanning view is shown in **A**, with a higher power view shown in **B**. The third ventricle (**a**), PVN (**b**), and needle track (**c**) are indicated.

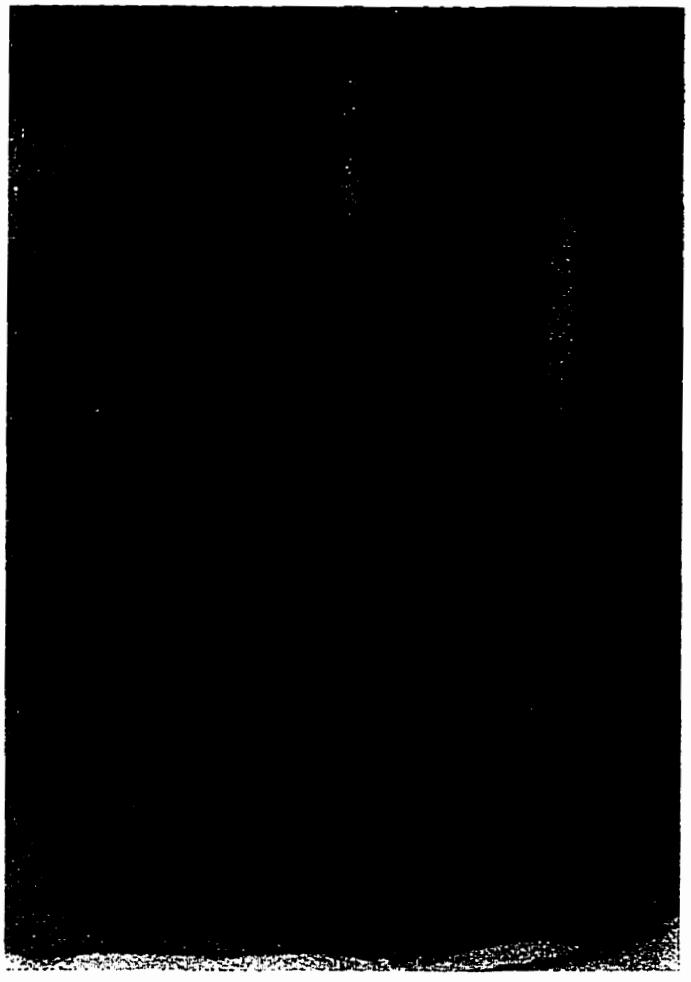

Figure 3.11: Photograph of a typical rostral PVN placement. The third ventricle (**a**), PVN (**b**), and needle track (**c**) are indicated.

Results

PVN guanfacine

Baseline values of data from the first urine collection are presented in Table 3.1. These values were obtained as controls before PVN injection of either guanfacine (10 nmol/500 nl) or saline (500 nl). As shown in the table, no significant differences exist between either of the groups for any of the parameters investigated.

Guanfacine injected directly into the PVN at a dose of 10 nmol/500 nl did not alter blood pressure, heart rate (Figure 3.1), or creatinine clearance (Figure 3.2). Urine flow was increased by guanfacine treatment (Figure 3.2), and this increase in urine flow was not secondary to an increase in osmolar clearance, but an increase in free water clearance. As seen in Figure 3.3, guanfacine treatment did not alter osmolar clearance, but increased the clearance of free water by the kidney.

PVN moxonidine

Baseline values of data from the first urine collection are presented in Table 3.2. These values were obtained as controls before injection of either moxonidine (10 nmol/500 nl) or saline (500 nl) into the PVN. As shown in the table, no significant differences exist between either of the groups for any of the parameters investigated.

Moxonidine injected directly into the PVN at a dose of 10 nmol/500 nl decreased both blood pressure and heart rate as shown in Figure 3.4. Neither urine flow rate nor creatinine clearance were altered by moxonidine treatment (Figure 3.5) as compared to

the saline control group. Although moxonidine did not appear to alter urine flow rate, this can be explained by the sum of both osmolar and free water clearance rates. Osmolar clearance was decreased following moxonidine treatment. This decrease is conceivably due to the decrease in blood pressure observed following PVN injection of moxonidine. Clearance of free water by the kidney increased following PVN moxonidine treatment, minimizing the apparent effect of moxonidine on urine flow rate.

Rostral PVN guanfacine/moxonidine

Baseline values of data from the first urine collection are presented in Table 3.3. These values were obtained as controls before PVN injection of either guanfacine or moxonidine (10 nmol/500 nl), or saline (500 nl). As shown, no significant differences exist between any of the groups for any of the parameters investigated.

Overall, the results following injection of guanfacine or moxonidine adjacent to the rostral PVN were similar to those obtained following direct injection of these agents at identical doses directly into the PVN. Guanfacine failed to alter blood pressure or heart rate, while moxonidine produced significant reductions in both blood pressure and heart rate. Both agents increased urine flow rate secondary to an increase in free water clearance. Although free water clearance was increased by either guanfacine or moxonidine injection, neither agent altered osmolar clearance.

Needle Placement

Needle placement was verified for both sets of coordinates used in these experiments. Representative pictures of these placements are shown for both direct PVN injection and rostral PVN injection in Figures 3.10 and 3.11 respectively.

Discussion

In the previous study we reported a diuretic effect following ICV injection of 10 nmoles of moxonidine. We speculated that this diuresis may be due to stimulation of receptors in the PVN, possibly resulting in the inhibition of vasopressin. The present study was designed in order to separate the diuretic effect from the depressor effect of moxonidine by injection directly into the PVN, a site associated with the regulation of vasopressin. Specifically, we hypothesized that moxonidine would act at imidazoline receptors to decrease blood pressure, and that moxonidine also binds α_2 adrenergic receptors at this dose to inhibit vasopressin. If the diuretic effect of moxonidine were due to α_2 adrenergic receptor binding, and the depressor effect due to imidazoline receptor binding, then it would follow that guanfacine, a specific α_2 agonist, would elicit a similar diuresis to moxonidine treatment without altering blood pressure.

Consistent with our hypothesis, moxonidine injected directly into the PVN produced both a decrease in blood pressure and an increase in urine flow secondary to an increase in free water clearance (consistent with vasopressin inhibition). As anticipated, guanfacine injection into the PVN produced only diuresis without altering blood pressure.

Following these results we questioned whether direct injection of drug into the PVN may damage the nucleus and provide a different result from one obtained using a site proximal to the PVN. A previously documented site, which did not involve advancement of the needle directly into the PVN, was therefore investigated. The new site was adjacent to the rostral end of the PVN. The results were similar to our original direct PVN injection results.

Although our results clearly identify a difference between PVN injection of guanfacine and moxonidine, presumably due to the imidazoline selectivity of the former, further experiments are required to fully separate roles of the α_2 adrenergic receptor and imidazoline receptor in the PVN. Specifically, the diuresis that we presume is due to an α_2 mediated inhibition of vasopressin must be confirmed through the use of selective vasopressin antagonists and selective α_2 adrenergic receptor antagonists, such as yohimbine or rauwolscine. These experiments could be combined with the measurement of vasopressin levels. Similarly, the depressor effect of PVN moxonidine must be investigated to determine whether this effect can be attributed to the imidazoline receptor, and whether this effect is mediated primarily by the PVN. This may be difficult due to the cross selectivity of imidazoline and α adrenergic antagonists. The primary involvement of the PVN in this response could be confirmed by an injection site-magnitude of response correlation. For example; the closer the site of injection to the PVN, the greater the magnitude of response. Another strategy would be to lesion the PVN or the descending sympathetic pathway from the PVN and determine whether the response to ICV and PVN moxonidine remains intact.

In conclusion, the injection of moxonidine either directly into or adjacent to the PVN results in diuresis and a depressor effect, which can be separated by comparison with a similar injection of guanfacine, an α_2 adrenergic receptor agonist. The imidazoline affinity of moxonidine may be responsible for its depressor effect, while the diuresis following moxonidine injection appears to be mediated by α_2 adrenergic receptors.

Role of GABA in the Depressor Response to PVN Moxonidine

Introduction

The paraventricular nucleus of the hypothalamus is responsible for the secretion of vasopressin to induce water retention. In the previous study, we demonstrated the diuretic role of the α_2 adrenergic receptor in the PVN. We also observed a depressor effect following PVN administration of moxonidine, which could not be duplicated by PVN injection of a similar dose of guanfacine, an α_2 adrenergic receptor agonist. As moxonidine is a relatively selective imidazoline agonist, we proposed that the depressor effect of moxonidine in the PVN resulted from the specific binding of moxonidine to imidazoline receptors in this nucleus. This response may also involve α_2 adrenergic receptors, as the activation of both imidazoline and adrenergic systems may be necessary for the depressor effect seen with moxonidine. In the present study, we chose to investigate the role of γ-aminobutyric acid (GABA) in the depressor response to PVN moxonidine.

GABA is released synaptically from GABAergic neurons, and its primary role is to inhibit neurotransmission. GABA binds either $GABA_A$ or $GABA_B$ receptors to promote influx of Cl^- or efflux of K^+ respectively (Costa, 1998). GABAergic inhibition is ubiquitous, such that when a pathway is found in which neurons express glutamic acid

decarboxylase (enzyme to synthesize GABA from glutamate), the pathway is assumed inhibitory.

The noradrenergic system and GABAergic pathways have been linked in several studies, indicating a role for GABA in the regulation of the sympathetic nervous system. In a preparation of rat spinal cord synaptosomes, it was shown that α_2 adrenergic receptors, and possibly imidazoline receptors, were involved in the inhibition of K^+ or Ca^+-evoked glutamate overflow (Kamisaki *et al.*, 1993). This observation indicates that adrenergic or imidazoline receptors may be involved in the presynaptic inhibition of glutamate release, possibly through stimulating GABA release. The IML cell column, which is responsible for relaying sympathetic impulses down the spinal cord, is dependent on GABA-induced inhibitory postsynaptic potentials to communicate inhibition to sympathetic preganglionic neurons (Inokuchi *et al.*, 1992).

A study by Pittaluga and colleagues reported a stimulatory effect of clonidine on GABA release in the NTS (Pittaluga *et al.*, 1991). Clonidine enhanced the release of GABA in synaptosomes prepared from NTS, but did not affect GABA release from cerebellum. Another in vitro study demonstrated that the clonidine induced hyperpolarization observed in RVLM was inhibited by bicuculline, implicating $GABA_A$ channels in this response (Sun and Reis, 1995a). These results indicate that the areas of the brain regulating blood pressure (NTS and RVLM) are capable of modulating GABA release in response to clonidine, indicating a role for the α_2 adrenergic receptor and possibly the imidazoline receptor in this process.

In addition, it has been suggested that α_2 adrenergic receptors may facilitate the release of both GABA and glutamate depending on the presynaptic input (Tingley and Arneric, 1990). Depending on the stimulus, adrenergic mechanisms either stimulate the pathway for glutamate mediated excitation, or GABA mediated inhibition of postsynaptic functions.

In whole animal experiments, spinal application of muscimol, a GABA agonist, has been shown to decrease blood pressure and heart rate, which can be completely reversed by bicuculline (Gordon, 1985). When muscimol is injected into the PVN, an increase in sympathetic activity is observed, including an increase in RSNA (Zhang and Patel, 1998). Bicuculline injection into the PVN attenuates the effects of GABA, and results in a decrease in RSNA (Haselton and Vari, 1998; Zhang and Patel, 1998). These observations, along with the finding that GABA is necessary for the inhibitory baroreceptor input to the PVN (Renaud et al, 1988), further rationalize the proposed study.

Clearly the mechanism of GABA mediated inhibition in the control of blood pressure is complex. The present study was undertaken to investigate the hypothesis that GABA is involved in the regulation of the depressor effect observed following the injection of moxonidine into the PVN.

Methods

A detailed description of the general procedures has been outlined in the general Methods section. Male Sprague-Dawley rats were unilaterally nephrectomized under ether anesthesia and allowed to recover for 7 to 13 days. On the day of the experiment, animals were anesthetized with sodium pentobarbital. A tracheotomy, carotid arterial and jugular venous cannulation were performed as described to allow spontaneous breathing, monitoring of heart rate and blood pressure, and infusion of saline respectively. Following a 45 minute stabilization period, an infusion of saline was initiated via the jugular vein and maintained throughout the remainder of the experiment. A 30 minute control urine collection was obtained, and bicuculline methiodide (10 nmol or 30 nmol/5 μl) or saline (5 μl) was injected over 2.5 minutes into the left cerebral ventricle, followed 10 minutes later by either moxonidine (3 nmol/500 nl) or saline (500 nl) injection into the PVN. The stereotactic coordinates used, as determined from the atlas of Paxinos and Watson (Paxinos and Watson, 1986), were 0.3 mm posterior, 1.5 mm lateral, and 3.8 mm ventral to bregma for ICV injections, and 1.8 mm posterior, 0.6 mm lateral, and 7.8 mm ventral to bregma for PVN injections.

Two 30 minute urine collections followed the PVN injection of moxonidine or vehicle. Heart rate and blood pressure were monitored, and urine flow rates determined gravimetrically. A plasma sample was obtained after completion of the experiment and stored at 4°C for up to one week.

Placement of the Hamilton syringe in the left cerebral ventricle was verified by injection of 0.1 μl Lissamine Green dye (1% aqueous solution) into the brain before the

syringe was removed from the stereotactic apparatus. The rat was then euthanized, removed from the stereotax, and its brain excised. The brain was then sectioned with a razor blade to verify the presence of dye in the ventricles. Stereotactic PVN coordinates were verified by preparing 40 micron thick sections of brain on a freezing microtome followed by thionin staining to visualize the needle track.

All results are presented as the difference in value between the mean third urine collection and the mean control urine collection with standard error indicated. As in the previous studies, we have also presented urine flow and clearance data in addition to blood pressure and heart rate. Results were analyzed by repeated measures analysis of variance (ANOVA) followed by a post-hoc test to identify significant differences. All significant differences are denoted by *, which represents $p < 0.05$, ** represents $p < 0.01$ versus the ICV control PVN control group.

Measurement/ Treatment	Pre-ICV Saline (5 µl) PVN Saline (500 nl) n = 8	Pre-ICV Bicuculline (10 nmol) PVN Saline (500 nl) n = 6	Pre-ICV Bicuculline (30 nmol) PVN Saline (500 nl) n = 6	Pre-ICV Saline (5 µl) PVN Moxonidine (3 nmol) n = 10	Pre-ICV Bicuculline (10 nmol) PVN Moxonidine (3 nmol) n = 7	Pre-ICV Bicuculline (30 nmol) PVN Moxonidine (3 nmol) n = 6
Blood Pressure (mmHg)	121 ± 3	115 ± 1	123 ± 5	120 ± 3	122 ± 2	123 ± 3
Heart Rate (beats/min)	388 ± 8	403 ± 9	407 ± 12	398 ± 7	403 ± 10	383 ± 9
Creatinine Clearance (ml/min)	2.5 ± 0.3	3.4 ± 0.2 *	2.5 ± 0.4	3.3 ± 0.2 *	3.6 ± 0.3 *	2.3 ± 0.3
Urine Flow (µl/min)	11.8 ± 2.3	8.4 ± 0.9	10.5 ± 1.6	11.1 ± 0.8	13.0 ± 1.3	9.1 ± 0.5
Osmolar Clearance (µl/min)	57 ± 7	48 ± 2	56 ± 7	60 ± 3	62 ± 4	59 ± 3
Free Water Clearance (µl/min)	-45 ± 5	-39 ± 2	-46 ± 5	-49 ± 2	-49 ± 4	-49 ± 3

Table 4.1: Baseline values obtained from the first (control) urine collection prior to ICV and PVN injection of saline or drug. Results are presented as mean ± s.e.m. * represents a significant difference with a p value <0.05 vs control.

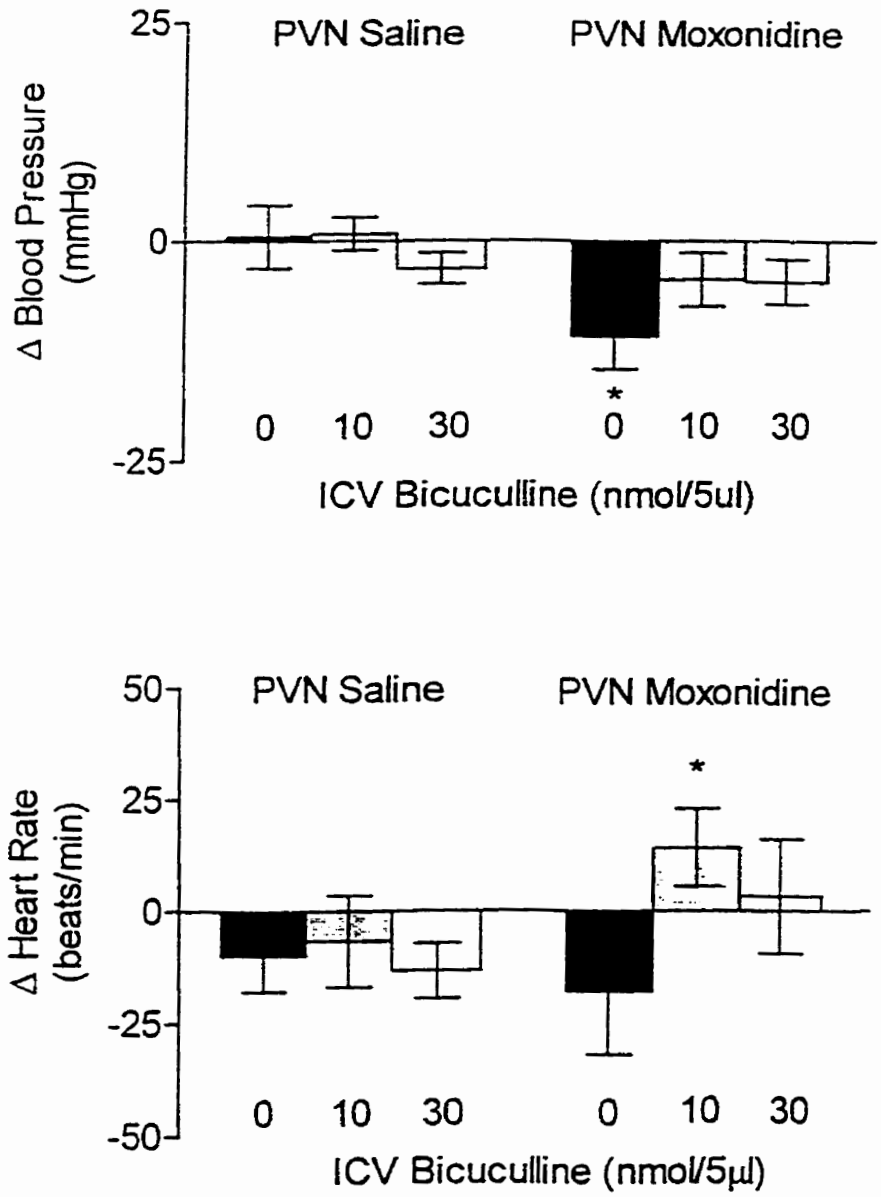

Figure 4.1: The effects of ICV pretreatment with bicuculline on the blood pressure and heart rate response following PVN moxonidine. Bicuculline was injected into the left lateral ventricle 10 minutes prior to PVN injection of moxonidine. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with a $p<0.05$ vs the control group.

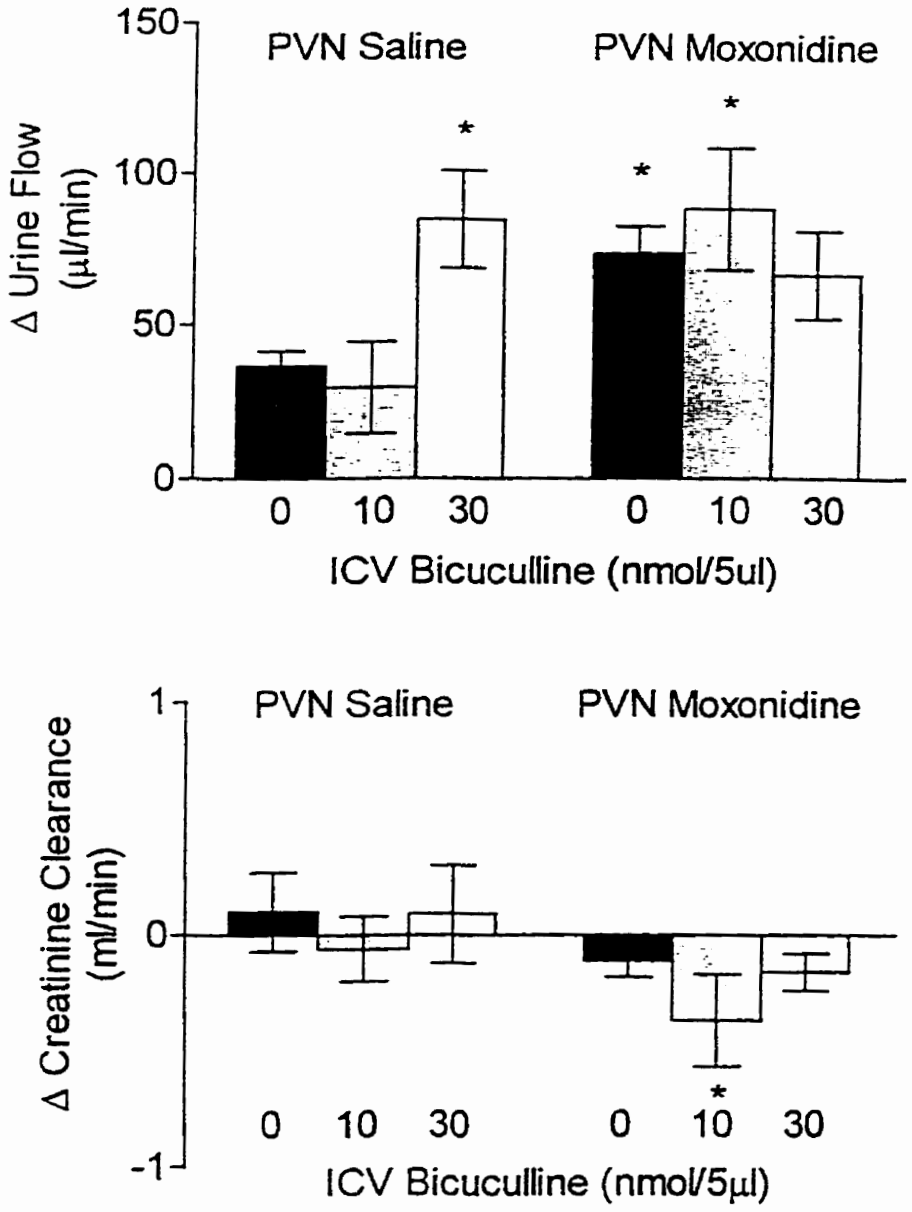

Figure 4.2: The effects of icv bicuculline pretreament on the urine flow and creatinine clearance response following PVN moxonidine. Bicuculline was injected into the left lateral venricle 10 minutes prior to PVN moxonidine treatment. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with $p<0.05$ vs the control group.

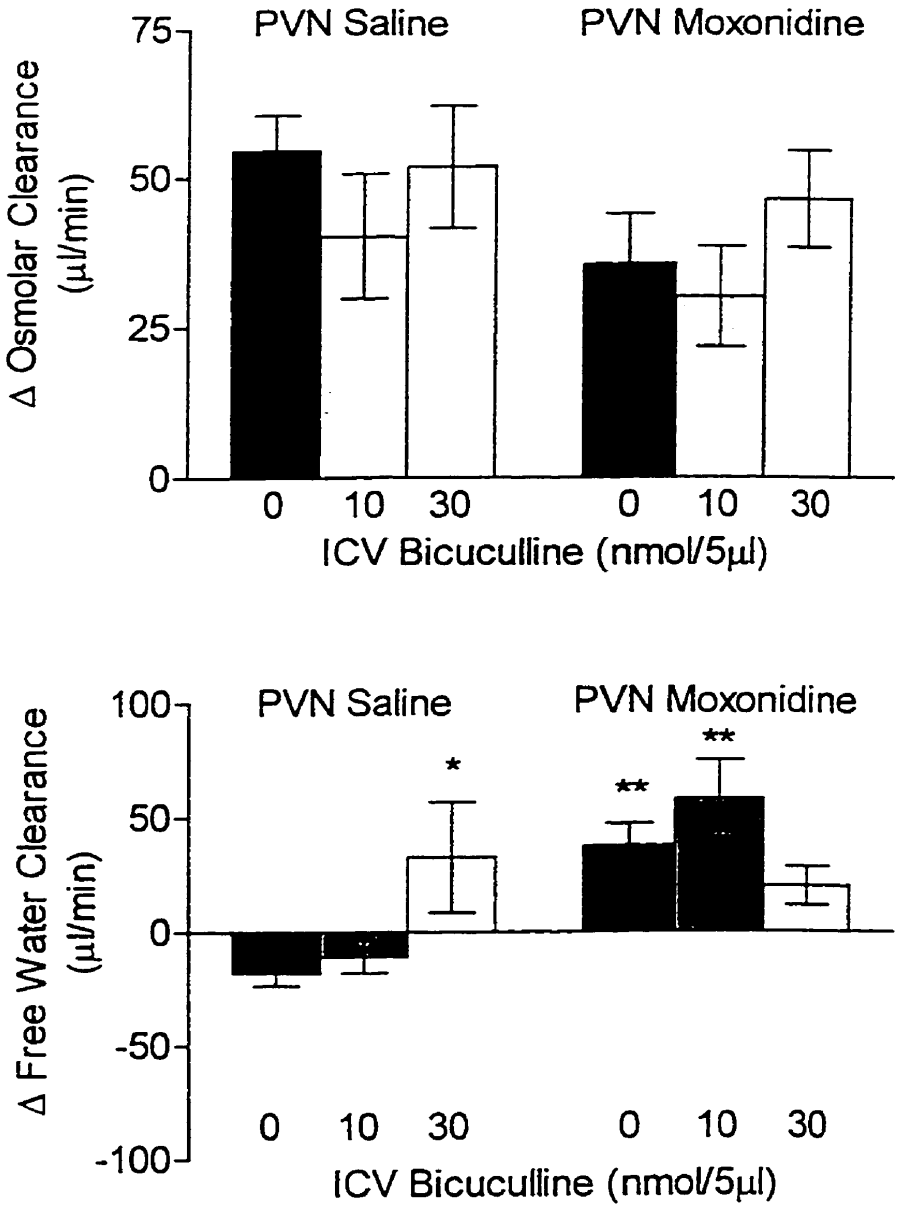

Figure 4.3: The effects of ICV bicuculline pretreatment on the osmolar and free water clearance response to PVN moxonidine. Bicuculline was injected 10 minutes prior to PVN moxonidine treatment. Bars represent the mean ± s.e.m. of the difference between the third 30 minute urine collection and the first (control) collection. Each group contains a minimum of 6 experiments. * represents a significant difference with $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$ vs the control group.

Results

Baseline values of data from the first urine collection are presented in Table 4.1. These values were obtained as controls prior to ICV injection of bicuculline or saline, or PVN injection of moxonidine or saline. As shown in the table, no significant differences were found between either of the groups for any of the parameters investigated other than creatinine clearance. The mean creatinine clearance measurement was skewed by only one animal in each group. All other animals had measurements clustered around a similar number to that seen in the control group.

Injection of bicuculline into the left cerebral ventricle alone (PVN saline) at either dose investigated, did not alter blood pressure, heart rate, or creatinine clearance (Figures 4.1 and 4.2). Urine flow rate was also not changed by injection of 10 nmol bicuculline; however, 30 nmol of bicuculline increased urine flow rate secondary to an increase in free water clearance (Figures 4.2 and 4.3). No change in osmolar clearance was observed (Figure 4.3)

Moxonidine injected into the PVN alone (ICV saline) decreased blood pressure (Figure 4.1) and increased urine flow rate (Figure 4.2). This increase in urine flow was secondary to an increase in free water clearance. No change in osmolar clearance was observed (Figure 4.3).

When moxonidine treatment was preceded by ICV bicuculline, no change in any of the parameters was observed as compared with moxonidine alone. The decreases in blood pressure and heart rate following PVN moxonidine were not reversed by either dose of ICV bicuculline (Figure 4.1). The moxonidine induced increase in free water

clearance was not attenuated by bicuculline pretreatment (Figure 4.3), and no change was observed in osmolar clearance. Therefore urine flow rate was not altered by bicuculline pretreatment (Figure 4.2). No significant changes in creatinine clearance were observed.

An alternative view of the data would be that PVN moxonidine alone decreased blood pressure, and this decrease was no longer significant with the addition of ICV bicuculline. From this perspective, ICV bicuculline may have attenuated the response to PVN moxonidine.

Discussion

In this study we investigated the role of GABA in the depressor response to PVN injection of moxonidine (3 nmol/5 μl). The dose of moxonidine was chosen based on previous experiments in our laboratory as the lowest dose resulting in a significant depressor effect. We investigated the role of GABA in this effect using the GABA antagonist bicuculline. If GABA release is activated or potentiated by moxonidine, bicuculline would be expected to attenuate the response to moxonidine. This hypothesis was drawn from and supported by various reports of adrenergic mechanisms interacting with GABA, as well as GABA activity in the PVN, as stated in the introduction to this study.

Moxonidine has been reported to be a selective imidazoline agonist; however, at high doses, or if imidazoline receptors are not concentrated as densely as α_2 adrenergic receptors at the site of action, moxonidine may also bind α_2 adrenergic receptors. Although PVN injection of moxonidine produces various effects in our preparation (decrease in blood pressure, decrease in heart rate, increase in free water clearance), not all of these effects can be attributed to the imidazoline receptor. In this study, we investigated the role of GABA in the response to PVN moxonidine despite this ambiguity, as compelling arguments exist for both the imidazoline receptor and the α_2 adrenergic receptor in mediating the depressor effect.

Our previous study demonstrated that the depressor effect and the free water effect of PVN moxonidine could be separated using the α_2 adrenergic receptor agonist guanfacine. These experiments identified that the selective α_2 agonist injected into the PVN produced a diuresis similar to that following PVN moxonidine. The depressor effect of PVN moxonidine was not observed following PVN guanfacine treatment, indicating that the depressor effect may be due to imidazoline receptor stimulation by moxonidine. Contrarily, there are several reports suggesting that cardiovascular regulation occurs through GABA dependent adrenergic mechanisms (Pittaluga *et al.*, 1991; Kamisaki *et al.*, 1993; Zhang and Patel, 1998; Haselton and Vari, 1998; Sun and Reis, 1995a). Taking into account these conflicting arguments, the possibility remains that these two receptor systems may be closely linked and interdependent, so the present study was undertaken without prejudice as to which receptor system was involved.

As previously observed, injection of moxonidine into the PVN produced a decrease in blood pressure and heart rate, and an increase in urine flow rate that was secondary to an increase in free water clearance. Although the addition of bicuculline treatment to PVN moxonidine did not significantly differ from moxonidine alone, the addition of bicuculline produced a trend towards reversal of the depressor and bradycardic effect of moxonidine by the low dose (10 nmol) of bicuculline and rendered the moxonidine response insignificant from the control treated group. The high dose of bicuculline (30 nmol) did not further reverse these effects; however this dose of bicuculline alone produced an increase in free water clearance. This observation may indicate that at this dose, bicuculline may produce nonspecific effects, complicating any GABA specific effects that may have been present.

We can not yet discard the possibility of GABAergic function in the PVN to regulate blood pressure through imidazoline or adrenergic receptors. As previously stated, the evidence from previous studies indicates such a role, and further investigation into this process is required.

General Discussion and Concluding Statements

Our initial study involved confirming the increase in osmolar clearance following ICV moxonidine, and resulted in an increase in urine flow. ICV injection of 10 nmol moxonidine in 5 μl, as anticipated, increased urine flow. Although previous studies have observed an increase in osmolar clearance associated with this increase in urine flow, in our experiments urine flow was increased by an increase in free water clearance. Osmolar clearance was not altered by moxonidine treatment. Our results indicate that ICV moxonidine binds receptors to which cerebrospinal fluid has direct access, to either directly alter, or initiate a cascade of events to alter free water clearance. This diuresis may be caused by inhibition of vasopressin, as vasopressin acts on the cortical collecting ducts of the nephron to increase water reabsorption. In addition, there is evidence to support a role of the α_2 adrenergic receptor in the regulation of vasopressin release. ICV moxonidine also elicits a depressor effect, which may be mediated by the imidazoline receptor. Moxonidine, although originally developed as an α_2 agonist, has a higher selectivity for the imidazoline receptor, so it is conceivable that both receptor systems are involved in the response to ICV moxonidine.

The paraventricular nucleus of the hypothalamus, which is located adjacent to the fourth ventricle and is also involved in vasopressin production, has been implicated in the regulation of cardiovascular response. Therefore, we injected moxonidine directly into

this nucleus to determine primarily whether both the depressor and diuretic effects of ICV moxonidine were present in the PVN. Secondarily, we also investigated whether the α_2 adrenergic receptor and imidazoline receptor responses were separable in the PVN. From these studies, it is clear that both the diuretic and depressor responses are present following injection of moxonidine into the PVN. Further, the diuretic effect of PVN moxonidine can be mimicked by another α_2 agonist (namely, guanfacine), but the depressor effect cannot be reproduced by guanfacine.

These observation led us to investigate whether the separable responses to ICV moxonidine can be selectively blocked using specific α_2 and imidazoline antagonists. These results were not presented in this thesis as no conclusions could be drawn from this data. Due to unknown factors, our animals were extremely unpredictable in this series of experiments, and our first collection urine flow rates ranged from 3 μl/min to 45 μl/min. Animals not given any drug produced third collection urine flow rates as high as 160 μl/min. These unpredictable values confounded our results, rendering them uninterpretable, however this experiment should be repeated in the future in order to test our hypothesis.

The investigation of the role of GABA in these responses stemmed from discussions with various researchers interested in the role of the PVN in renal function. As outlined earlier, PVN application of GABA agonists and antagonists affects both renal function and cardiovascular parameters. This observation led us to investigate whether GABA was involved in the response to PVN moxonidine. Although this experiment also did not conclusively define the role of GABA in the depressor response to PVN

moxonidine, this line of study is one that should be pursued. Again, our animals were unpredictable during this experiment, however not to the extent of exclusion from the study. Part of the difficulty in attempting to reverse the depressor effect induced by PVN moxonidine is the small depressor effect we observed. A dose of moxonidine must be chosen which gives an adequate decrease in blood pressure; however the dose must not be excessive in order to avoid nonspecific binding and inconsistent observations. The vasodepression observed with 3 nmol of moxonidine was adequate for these purposes. In these studies there was a trend towards a reversal in the depressor response to moxonidine; however, there was no attenuation of the response. Increased doses of bicuculline did not further attenuate the response. Future studies should further investigate the role of GABA in the response to moxonidine. This could be done by injecting moxonidine ICV, and bicuculline directly into the PVN, opposite to our study, or investigating the peripheral administration of moxonidine with ICV or PVN bicuculline. Another agent that could be used to confirm any findings is muscimol, the GABA agonist, which would lend further support to the results. A possible mechanism unifying the observations and future direction arising from these works is presented in Figure 4.4.

If our observations can be confirmed by further studies, the PVN may be recognized as an important regulatory site for cardiovascular changes, including blood pressure and solute and water clearance.

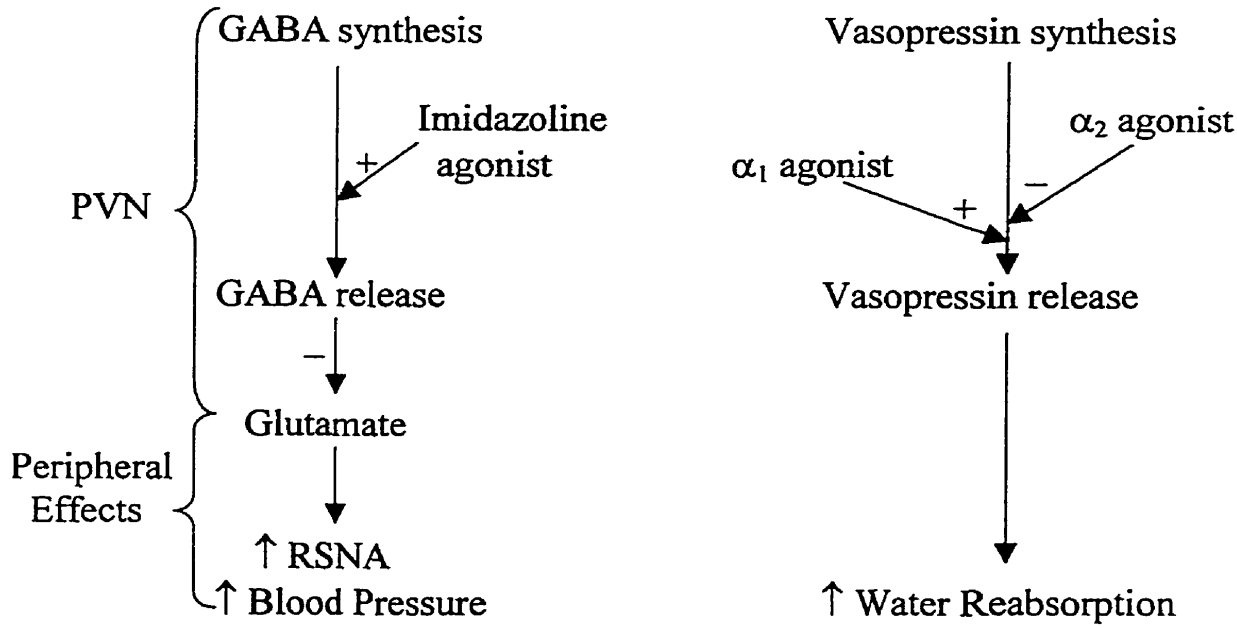

Figure 4.4: Proposed mechanism of interaction of the α_2 adrenergic receptor and the I_1 imidazoline receptor. A possible role of GABA is also indicated. GABA release is potentiated by imidazoline receptor activation in the PVN. GABA inhibits the release of the excitatory amino acid glutamate, eliminating the excitatory sympathetic stimulus. Vasopressin release is inhibited by α_2 adrenergic receptor, preventing water retention by the kidney. At the doses investigated in the PVN, moxonidine may stimulate both receptor types, resulting in a depressor and diuretic effect.

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