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**Characterization of the vasoactivity of tachykinins in
isolated rat kidney: Functional studies and *in vitro* receptor
autoradiography**

Chen, Yuejin, Ph.D.

East Tennessee State University, 1994

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Ann Arbor, MI 48106

CHARACTERIZATION OF THE VASOACTIVITY OF TACHYKININS IN
ISOLATED RAT KIDNEY: Functional Studies and *in vitro*
Receptor Autoradiography

A Dissertation Presented to
the Faculty of the Department of Pharmacology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Science

by
Yuejin Chen, M.D.

May 1994

APPROVAL

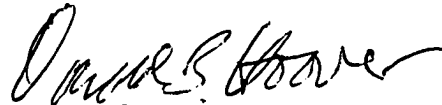
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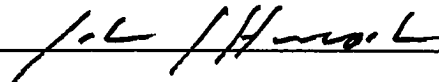
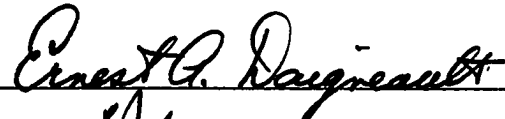
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The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.



Chairman, Graduate Committee



Associate Vice-President for Research
and, Dean, School of Graduate Studies

Signed on behalf of
Graduate Council

ABSTRACT

CHARACTERIZATION OF THE VASOACTIVITY OF TACHYKININS IN ISOLATED RAT KIDNEY: Functional Studies and *in vitro* Receptor Autoradiography

by

Yuejin Chen

Although tachykinins have potent vascular actions, their effect on renal resistance blood vessels is currently unknown.

The vasoactive properties of tachykinins and related analogs were assessed in isolated perfused rat kidney. At a basal perfusion pressure (PP) of 75 ± 6 mm Hg ($n=5$), bolus injections of substance P (SP) had no significant vasoactive effect. Following a sustained increase in baseline PP (134 ± 10 mm Hg) produced by phenylephrine ($1 \mu\text{M}$), SP evoked a dose-dependent increase in PP. The largest dose of SP increased PP by 60 ± 5 mm Hg. The vasoconstrictor response to SP was not blocked by phentolamine when angiotensin II was used to increase basal tone. Thus, the response to SP is not mediated by norepinephrine. Pressor responses to SP were not potentiated by peptidase inhibitors, captopril and thiorphan. SP(1-7) had no effect on PP, suggesting that the pressor response to SP is C-terminal dependent and tachykinin receptor mediated. The selective NK-1 receptor agonist, $[\text{Sar}^9, \text{Met}(\text{O}_2)^{11}] \text{SP}$, had no effect on PP. In contrast, both the selective NK-2 and NK-3 receptor agonists, GR-64349 and $[\text{MePhe}^7] \text{NKB}$, produced dose-dependent pressor responses (116 ± 8 and 134 ± 15 mm Hg increases in PP at 33 nmol, respectively) and were more potent than SP. Infusion of capsaicin (500 nM) produced an initial increase in PP following by a more prolonged decrease in PP. Clamping the renal vein produced a marked increase in PP.

The localization of NK-3 receptors in rat kidney evaluated by film autoradiography using ^{125}I - $[\text{MePhe}^7] \text{NKB}$ revealed a high density of specific binding sites on the proximal ureter and renal pelvis, moderate density in the renal vein and its large branches, and a low density in the inner strip of outer medulla, but no specific binding on the renal artery system and cortex. High resolution autoradiograms demonstrated ^{125}I - $[\text{MePhe}^7] \text{NKB}$ binding sites on the tunica media of the renal vein and tunica muscularis of renal pelvis and ureter. Specific binding of ^{125}I -BHSP was found in association with the renal artery and renal pelvis. No specific SP binding sites were associated with renal vein.

These data indicate that the pressor effect of tachykinins in the isolated rat kidney can be mediated by NK-2 and/or NK-3 receptors. The latter may be on the vascular smooth muscle of the renal vein.

DEDICATION

This is dedicated with love and deepest appreciation to my Mom, my Dad and my late sister, yuewei. Their words of encouragement and their genuine belief in my ability made this possible. Thank you.

献给：

亲爱的爸爸、妈妈
和妹妹，躍伟。

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LIST OF ABBREVIATION

ACE	Angiotensin converting enzyme
Ang	Angiotensin II
BHSP	Bolton and Hunter substance P
BSA	Bovine serum albumin
CGRP	Calcitonin gene-related peptide
DMSO	Dimethyl sulfoxide
Ele	Eledoisin
Kas	Kassinin
NEP	Neutral endopeptidase
NKA	Neurokinin A
NKB	Neurokinin B
NANC	Non-adrenergic non-cholinergic
NE	Norepinephrine
PHE	Phenylephrine
Phy	Physalaemin
PP	Perfusion pressure
PPT	Preprotachykinin
ROD	Relative optical density
SarSP	[Sar ⁹ ,Met(O ₂) ¹¹]SP
SP	Substance P
Senk	Senktide
VIP	Vasoactive intestinal polypeptide

CHAPTER 1

Introduction

The Problem

Statement of the Problem

The tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) belong to a family of peptides with similar biological actions and a common amino acid sequence at the C-terminus (Helke *et al.*, 1990).

Immunocytochemical study has demonstrated that SP and NKA are found in a subpopulation of primary sensory nerves that have a widespread distribution in the cardiovascular system (Wharton and Gulbenkian, 1989). Recently, considerable evidence has mounted supporting the concept that tachykinin-containing sensory neurons participate in regulation of vascular functions in many somatic and visceral tissues through their dual afferent and efferent actions. In addition to their potential role in the reflex control of the circulation via the actions at central release sites, tachykinins released from peripheral sensory nerve terminals also may exert an important local control over vascular effector functions (Maggi, 1991; Holzer, 1992). Pharmacological studies have revealed that the efferent functions of tachykinins on the vasculature are variable among different species and blood vessel studied and are dependent on different tachykinin receptors. Vasoconstrictor effects can occur through stimulation of any class of tachykinin receptors on vascular smooth muscle cells, while vasodilation

may result from stimulation of NK-1 receptors on the vascular endothelium and is mediated by endothelium-derived relaxing factor (Stewart-lee and Burnstock, 1989; Whittle *et al.*, 1989). Until recently, however, most studies on the vascular pharmacology of tachykinins have been performed on large vessels.

It is well established that the rat kidney is innervated by both sensory and postganglionic autonomic nerves (Barajas *et al.*, 1992). While much was known about the renal efferent sympathetic innervation, little was known about the renal afferent innervation until recently. Anatomical studies of rat kidney have identified SP-immunoreactive nerves that innervate the renal artery and its branches, and the renal veins (Ferguson and Bell, 1985; Knight *et al.*, 1987). Likewise, the presence of NKA and NKB in the rat kidney was also suggested based on the results of radioimmunoassays with reportedly selective antibodies (Tateishi *et al.*, 1990). Data from functional studies have shown that SP can increase the basal tone of rat renal conduit vessels (Bredy-Dobrevá *et al.*, 1988), relax rabbit renal arterial segments contracted by exposure to norepinephrine or phenylephrine (Malomvolgyi *et al.*, 1988), and increase total renal blood flow in the dog (Defelice and Brousseau, 1988). However, the effect of tachykinins on renal resistance vessels has received comparatively little attention. Moreover, no systematic studies have been performed to identify the classes of tachykinin receptors that mediate the responses of the renal vasculature to these peptides.

Thus, the present study was designed to characterize vasoactivity of tachykinins in isolated perfused rat kidney by using functional studies and *in vitro*

receptor autoradiography.

Significance of the Problem

Basic science advancements provide a foundation for the recognition of the physiological and pathophysiological significance of the tachykinins in various systems and possibly in various types of human diseases. Evidence supporting the involvement of SP in the pathology of a variety of diseases has been derived from studies of animal models and humans (Payan, 1989; Holzer, 1992). It is believed that SP or other tachykinins released from nerve endings by various stimuli can elicit diverse biological activities including smooth muscle contraction, vasoconstriction or/and vasodilation, plasma extravasation and protein secretion from distinct glands, all of which are referred to as local neurogenic inflammation (Payan, 1989). It was suggested that the release of SP in pulmonary tissues under certain circumstances may be involved in the pathogenesis of bronchial asthma (Frossard and Advenier, 1991). In a rat model of arthritis, it was found that joints that developed more severe arthritis were more densely innervated by SP-containing neurons than joints that developed less severe arthritis (Levine *et al.*, 1984). In addition, in colon tissue obtained from patients with chronic inflammatory bowel diseases, SP binding sites are present in higher than normal concentrations (1,000-2,000 times normal) in arterioles, venules, and lymph nodules in the inflamed tissue (Mantyh *et al.*, 1988). Therefore, quite similar considerations regarding the pathophysiological role of tachykinins in diseases of the renal vasculature or kidney could be made and remain to be examined. The

present study will provide a basis for understanding the potential role of tachykinins in the local control of the renal circulation and is an essential prerequisite for the subsequent evaluation of possible tachykinin involvement in the pathogenesis of diseases affecting the renal vascular bed and/or kidney such as nephritis and some types of hypertension.

Hypothesis

Based on previous work by other investigators as well as our preliminary experiments that have demonstrated that SP has a pressor effect in the isolated perfused rat kidney, the hypothesis to be tested in this study is that the pressor effect of tachykinins in isolated rat kidney is mediated by tachykinin receptors on the renal resistance vessels.

If tachykinin receptors are present in any segment of renal resistance vessels, then exogenous and/or endogenous tachykinins and their agonists should have vasoactive effects indicated by changes in perfusion pressure under appropriate experimental conditions. As mentioned above, it is anticipated that these responses would be dependent upon receptor type, vessel segment and species. Functional experiments using selective tachykinin agonists could demonstrate which type of tachykinin receptor is present in renal vasculature but could not identify the regional or cellular localization of these tachykinin receptors. However, if tachykinin receptors are present in the renal vasculature, and a vasoactive effects of tachykinin agonists could be proved by functional studies, it should be possible to determine their localization by receptor autoradiography

using selective radioligands. It is possible that any class of tachykinin receptor is present in vascular smooth muscle, but only NK-1 receptors would be expected to occur on vascular endothelium.

CHAPTER 2

Review of Literature

The Efferent Function of Sensory Nerves

It is now recognized that in addition to the classical adrenergic and cholinergic innervation, the cardiovascular system is also widely supplied by non-adrenergic, non-cholinergic (NANC) nerves, some of which utilize peptides such as SP and calcitonin gene-related peptide (CGRP) as their transmitters (Holzer, 1992). These perivascular NANC nerves are in fact primary sensory nerve fibers that are able to monitor the changes in their chemical and physical environment, convey this information to the central nervous system, and initiate autonomic homeostatic reflexes. In addition to their sensory roles, the peptidergic afferent neurons also have a distinct feature -- local effector functions via the release of neuropeptides from their peripheral terminals (Holzer, 1988; Maggi, 1991). As shown in Fig. 1, the sensory nerve fibers bifurcate in the periphery, one branch forming the sensory ending for reception of the irritant stimulus and the other supplying a blood vessel. When the sensory ending is activated, nerve impulses travel not only centrally but also pass antidromically to the blood vessel to cause vasodilation. Moreover, the neurotransmitters also may be released from the same terminal activated by the environmental stimulus (Maggi and Meli, 1988). This efferent function of sensory afferents provides a basis for speculating on a possible role of these nerves in regulating vascular tone.

Although the significance and mode of action of afferent neurons in the

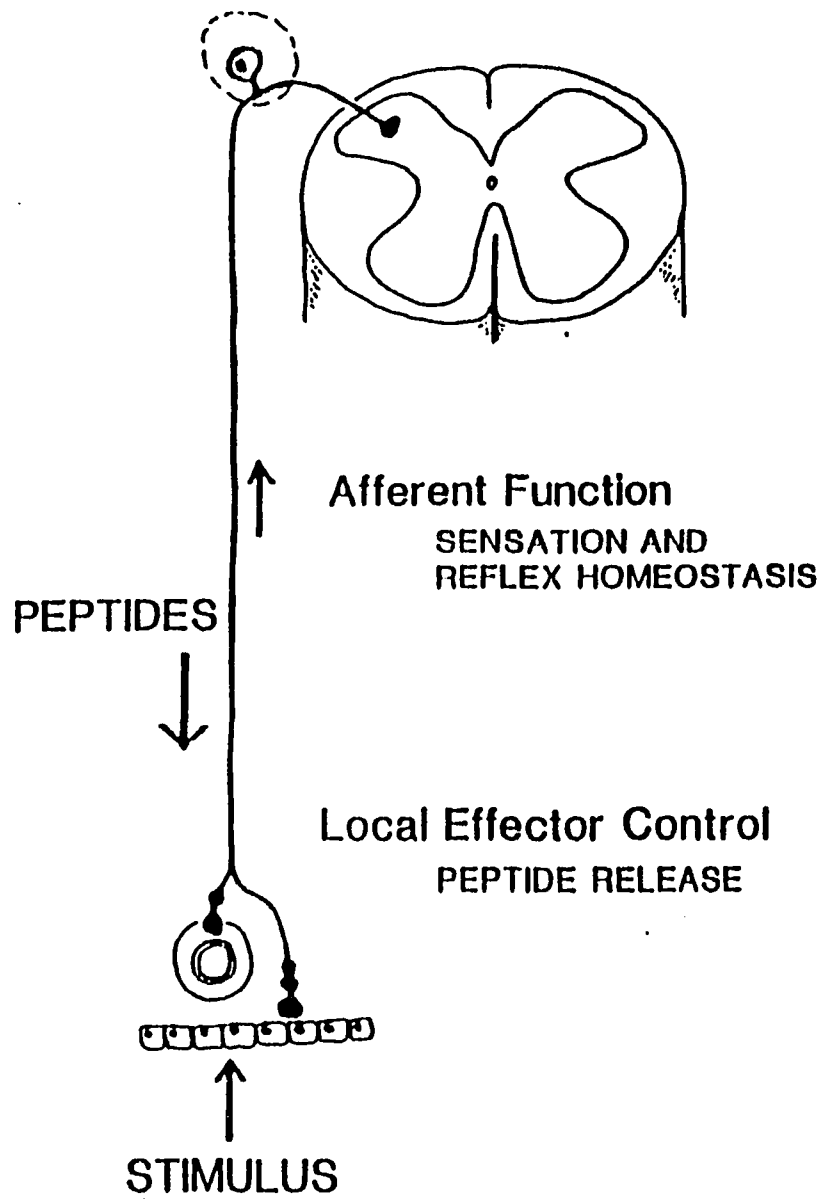


Figure. 1. Peptidergic Sensory Neurons in the Control of Vascular Function

control of vascular functions is only now being appreciated, the demonstration that certain sensory nerves release transmitters from their peripheral endings can be traced back to classic works of Stricker (1876) and Bayliss (1901) (see review by Maggi, 1991). Bayliss showed that the vasodilation produced by electrical stimulation of dorsal roots was unaffected by removal of the dorsal root ganglia and consequent degeneration of afferent nerves abolished it. On this basis, Bayliss concluded that the observed vasodilatation was produced by impulses conveyed along afferent fibers in an efferent direction (Maggi, 1991).

A major advance contributing to the elucidation of efferent functions of sensory neurons came with the discovery of the pharmacological properties of capsaicin by a Hungarian group headed by Jancso (Jancso *et al.*, 1968). Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a pungent chemical found in a wide variety of red peppers (Holzer, 1991). At doses very much higher than those used for spicing food, this compound has been found to act as a quite selective neurotoxin for primary afferent neurons. Low doses of capsaicin (i.e., the microgram per kilogram range) induce transient excitation of thin primary afferent neurons while systemic administration of high doses of drug (i.e., the milligram per kilogram range) causes long-lasting damage to these neurons (Holzer, 1991). Therefore, this compound has become an important probe for sensory neuron mechanisms.

Studies using immunocytochemistry and radioimmunoassay have revealed the presence of a variety of peptides, such as tachykinins, CGRP, and vasoactive

intestinal polypeptide (VIP) in primary afferent neurons (Holzer, 1988). They are released from the peripheral endings of afferent neurons by depolarizing stimulation and act on the receptors on the target cells to produce several biological actions that include vasodilation and constriction, increase in venular permeability (plasma protein extravasation), changes in non-vascular smooth muscle contractility, degranulation of mast cells and a variety of effects on leucocytes and fibroblasts. All these effects contribute to the neurogenic inflammation that occurs in response to tissue injury or exposure to irritants (Maggi and Meli, 1988; Holzer, 1988).

Mammalian Tachykinins

Tachykinins are closely related peptides characterized by sharing the common C-terminal sequence Phe-x-Gly-Leu-Met-NH₂ and exhibiting a similar spectrum of biological activities, such as exciting neurons and contracting visceral smooth muscles and certain blood vessels (Helke, *et al.*, 1990; Regoli and Nantel, 1991a). The primary structures of several tachykinins are listed in Table 1. SP is a representative peptide of this family, that was first described to be present in extracts of horse brain and intestine by Von Euler and Gaddum in 1931 (Pernow, 1983). The chemical structure of this peptide was not reported until four decades later (Pernow, 1983). The other two major tachykinins belonging to this family are neurokinin A (NKA, formerly called substance K) and neurokinin B (NKB, formerly called neuromedin K) which were isolated by Kimura *et al.* (1983) from extracts of porcine spinal cord. Also presented in Table 1. are structures of

TABLE 1.

Primary Structure of Tachykinins and Their Agonists

<u>Mammalian Tachykinins</u>	
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH ₂
<u>Nonmammalian Tachykinins</u>	
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH ₂
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH ₂
<u>Synthetic Agonists</u>	
[Sar ⁹ ,Met(O ₂) ¹¹]SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Sar-Leu-Met(O ₂) NH ₂
GR-64349	H ₂ N-(CH ₂) ₄ -CO-Phe-Phe-Pro-NMeLeu-Met-NH ₂
[MePhe ⁷]NKB	Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH ₂
Senktide	succinyl-Asp-Phe-MePhe-Gly-Leu-Met-NH ₂

physalaemin (Phy), eledoisin (Ele) and kassinin (Kas). These tachykinins are present in amphibians but not in mammals (Helke *et al.*, 1990; Regoli *et al.*, 1991b). However, they have been widely used in the pharmacological evaluation of tachykinin receptors.

Mammalian tachykinins are the products of two distinct genes (Helke *et al.*, 1990). SP and NKA are derived from the preprotachykinin (PPT) gene-A. Alternative RNA splicing of the primary transcript gives rise to three different mRNAs encoding distinct tachykinin precursor proteins: α -PPT-A, which codes only for SP and β -PPT-A and γ -PPT-A which code for both SP and NKA. NKB mRNA is synthesized from a separated gene, the preprotachykinin gene-B. Variable splicing of the primary transcript yields two different mRNAs, both of which code only for NKB. However, PPT-A and PPT-B genes exhibit a marked structural similarity, suggesting that they evolved from a common ancestor gene by duplication events (Ohkubo and Nakanishi, 1991). The preprotachykinin mRNAs and peptide products are differentially distributed throughout the nervous system and periphery (Helke *et al.*, 1990). NKA coexists with SP in primary sensory neurons (Dalsgaard *et al.*, 1985; Couture and Kerouac, 1987; Tateishi *et al.*, 1990).

Tachykinin Receptors and Agonists

It is clear that the presence or absence of postsynaptic receptors is critical in determining the response to released transmitter. Pharmacological, biochemical and histochemical studies have lead to the proposal that there are at least three distinct tachykinin receptors, namely NK-1, NK-2 and NK-3. SP, NKA and NKB

are the natural agonists for these receptors. SP has the highest affinity among the natural peptides for the NK-1 receptor, while NKA and NKB have highest affinities for NK-2 and NK-3 receptors, respectively. However, none of these endogenous tachykinins are highly selective for a given receptor and all can act on each of the tachykinin receptors at high enough concentration (Regoli and Nantel, 1991a). Fortunately, there are synthetic tachykinin analogues (see Table. 1) that display considerable receptor selectivity. For instance, [Sar⁹,Met(O₂)¹¹]-SP is a selective NK-1 receptor agonist with negligible activity at NK-2 and NK-3 receptors (Regoli *et al.*, 1988), GR-64349 (Hagan *et al.*, 1991) is a selective NK-2 receptor agonist, while succinyl-[Asp⁶,MePhe⁸]-SP₆₋₁₁ (Senktide) and [MePhe⁷]-NKB (Regoli *et al.*, 1988) are selective NK-3 receptor agonists. The proposed presence of an additional type of tachykinin receptor, such as the NK-4 (Mcknight *et al.*, 1988; Burcher *et al.*, 1991) and further tachykinin receptor subtypes such as NK-1A, NK-1B remain to be proven (Burcher *et al.*, 1991).

The molecular identification of three tachykinin receptors from rat, mouse, guinea pig, bovine and human has been recently accomplished by cDNA cloning. Rat NK-1, NK-2 and NK-3 receptors consist of 407, 390 and 453 amino acid residues, respectively (Gerard *et al.*, 1993). The overall homology of these receptors within a single species (rat) is approximately 60% and their core sequences are highly conserved. The overall homology of each type of receptor among species is greater than 85-90% (Nakanishi, 1991; Burbach and Meijer, 1992; Ohkubo and Nakanishi, 1991). All tachykinin receptors have seven

hydrophobic domains with extracellular amino termini and cytoplasmic carboxyl termini and share a significant sequence similarity with other G-protein coupled receptors. Thus, all three receptors belong to the family of G-protein coupled receptors (Nakanishi, 1991; Krause *et al.*, 1993). It was observed that stimulation of tachykinin receptors caused activation of phospholipase C, catalyzing the hydrolysis of phosphoinositides into inositol 1,4,5-trisphosphate and diacylglycerol. These second messengers are then available for the mobilization of calcium from internal reticular stores via the inositol 1,4,5-trisphosphate receptor and for the activation of protein kinase C (Ohkubo and Nakanishi, 1991; Guard *et al.*, 1988; Guillemain *et al.*, 1992; Gerard *et al.*, 1993). In some preparations, tachykinins also enhanced synthesis of cyclic AMP (Narumi and Maki, 1978; Yamashita *et al.*, 1983). Therefore, tachykinin receptors are thought to act through multiple G-proteins to activate phosphatidylinositol- Ca^{++} and/or cAMP system (Gerard *et al.*, 1993).

The distribution of tachykinin receptors in the central nervous system and peripheral tissues has been studied extensively by biochemical and autoradiographic analysis of tachykinin binding sites (Mantyh *et al.*, 1989; Otasuka and Yoshioka, 1993). The distribution of the three tachykinin receptor mRNAs in various tissues of the rat has also been investigated by RNA blot hybridization using each of the three receptor cDNAs (Ohkubo and Nakanishi, 1991). It was found that the NK-1 receptor is widely distributed both in the central nervous system and in peripheral tissues. The NK-3 receptor is also distributed in various

tissues, but it is expressed more in the central nervous system than in peripheral tissues. In contrast, NK-2 receptor is predominantly expressed in peripheral tissues (Mantyh *et al.*, 1989; Ohkubo and Nakanishi, 1991; Otasuka and Yoshioka, 1993). Thus, the three receptors and their mRNAs are expressed differently in the central nervous system and peripheral tissues.

Effects of Tachykinins on the Vasculature

Immunocytochemical evidence has demonstrated that SP-containing primary sensory nerve fibers are widely distributed in the cardiovascular system in a variety of mammals, including humans (Wharton and Gulbenkian, 1989). These immunoreactive nerve fibers are generally sensitive to capsaicin and have been identified in heart, around both large conducting arteries and veins, and in small vessels supplying many vascular beds (Barja and Mathison, 1982; Furness *et al.*, 1982; Brodin and Nilsson, 1981). In addition to SP, these nerves contain NKA and CGRP (Wharton and Gulbenkian, 1989).

It has been shown that SP and CGRP are released from both the arterial and venous mesenteric vasculature by capsaicin and antidromic nerve stimulation, and a significant reduction in the quantity of these peptides was observed in the same tissue from capsaicin-pretreated animals (Manzini *et al.*, 1991). Stimulation of sensory nerve endings in cerebral vessels is also associated with a release of SP (Holzer, 1988). These observations suggest that the local release of sensory neuropeptides from capsaicin-sensitive nerve endings might be important for the regulation of vessel tone.

On the other hand, SP binding sites have been identified by *in vitro* autoradiographic mapping in endothelium of blood vessels from guinea pig and human lung and from dog carotid artery (Wharton and Gulbenkian, 1989; Stephenson *et al.*, 1986). The presence of NK-2 receptors in rat portal vein has also been suggested based on work with selective agonists (Chassaing *et al.*, 1991).

The pharmacological effects of tachykinins on the vasculature have been evaluated extensively. It was found that SP had a vasodilator effect through NK-1 receptors on the endothelium of dog isolated carotid artery and guinea pig coronary vessels precontracted with NE and vasopressin, respectively (Regoli, *et al.*, 1988; Hoover and Hossler, 1993). In contrast, SP elicited a vasoconstrictor response mediated by the same receptor located on smooth muscle in the rabbit jugular and mesenteric veins (Regoli *et al.*, 1984; Nantel *et al.*, 1990). In rabbit pulmonary artery, SP and NKA evoke relaxation through NK-1 receptors on endothelium and contractions by stimulating NK-2 receptors on the vascular smooth muscle (Regoli *et al.*, 1984). It has been reported that NKB and NKA contract the rat portal vein by activating a large population of NK-3 receptors and a minor population of NK-2 receptors presumably located on the smooth muscle (Mastrangelo *et al.*, 1986; Chassaing *et al.*, 1991). In a recent study, Orleans-Juste *et al.* (1991) reported that the arterial and venous mesenteric vascular bed of the rat are insensitive to SP, yet the venous side had a constrictor response to NKB and was even more sensitive to [MePhe⁷]-NKB, a selective NK-3 agonist. The

latter findings suggest that tachykinin receptors in the mesenteric vasculature of rats are restricted to the venous side and are solely the NK-3 type. Therefore, tachykinins may stimulate or inhibit a variety of vascular smooth muscles by direct or indirect mechanisms and may thereby affect peripheral blood flow. The vascular response to tachykinins can vary between blood vessels and species. While vasoconstrictor effects can occur through stimulation of any class of tachykinin receptors on vascular smooth muscle cells, vasodilation has only been observed after stimulation of NK-1 receptors on the vascular endothelium and is mediated by endothelium-derived relaxing factor (Stewart-lee and Burnstock, 1989; Whittle *et al.*, 1989). Moreover, all three tachykinins can increase vascular permeability and induce plasma protein extravasation (Gamse and Saria, 1985; Couture and Kerouac, 1987; Jacques *et al.*, 1989).

Termination of Tachykinin Actions

Since reuptake into nerve terminals does not occur with neuropeptides, diffusion and degradation by peptidase are the main mechanisms by which their action is terminated (Couture and Kerouac, 1987; Holzer, 1992). Biochemical studies have shown that several peptidases such as neutral endopeptidase 24.11 (NEP) and angiotensin converting enzyme (ACE) are potentially involved in the degradation of tachykinins in both the central nervous system and periphery (Turner, 1987; Frossard *et al.*, 1989). NEP is a membrane-bound enzyme present in a variety of tissues including the vascular system. It has been demonstrated that the actions of SP and NKA on the guinea pig isolated urinary bladder and

ferret trachea and that of SP on rat portal vein were significantly potentiated by thiorphan, an endopeptidase 24.11 inhibitor (Rouissi *et al.*, 1990; Maggi *et al.*, 1990). However, NEP is not active in all systems. For example, relaxation of rat gastric fundus induced by SP was not influenced by two endopeptidase inhibitors, thiorphan and phosphoramidon (Smits and Lefebvre, 1991). In addition, it was found that captopril, an ACE inhibitor, significantly potentiated the maximal response of dog carotid artery and rabbit pulmonary artery to SP, NKA and NKB, and of rat portal vein to SP (Rouissi *et al.*, 1990), suggesting ACE can play a significant role in terminating the action of tachykinins in some tissues. On the other hand, responses to NKA and NKB were not affected by ACE inhibition in rat portal vein and lung (Hooper and Turner, 1986; Rouissi *et al.*, 1990). These results indicate that tissue and species differences are important factors in regard to tachykinin degradation by peptidases.

Tachykinins in the Kidney and Renal Vasculature

Neural regulation of the renal circulation is important in adjusting vessel tone that determines the distribution of blood flow within the organ and ultimately regulates blood pressure. While much is known about the renal efferent sympathetic innervation, little is known about the renal afferent innervation until recently. The intrarenal localization of sensory nerves has been investigated with the help of immunocytochemical methods for SP and CGRP which are generally regarded as markers for afferent fibers. Accumulated evidence supports the view that SP and CGRP coexist in a subpopulation of sensory nerve fibers that supply

the renal vasculature. By using a highly specific anterograde nerve tracing technique with wheat germ agglutinin-horseradish peroxidase, Marfurt and Echtenkamp (1991) found that labeled afferent fibers were seen around branches of the renal artery, interlobar arteries and arcuate arteries, and sparsely innervated the renal vein. Anatomical studies of the rat kidney have identified SP-immunoreactive nerves that innervate the arcuate arteries, interlobular arteries and afferent arterioles (Knight *et al.*, 1987), and the wall of the renal artery and vein where they enter the renal hilus (Ferguson and Bell, 1985), suggesting that SP might affect the renal vasculature at several sites. Although there has been no report about the presence of NKB or NKA in SP/CGRP containing sensory nerve innervation in renal vasculature, biochemical experiments using selective antibodies have detected the presence of NKA- and NKB-like immunoreactivities in extracts of rat kidney (Tateishi *et al.*, 1990). Furthermore, Tsudhida *et al.* (1990) reported the presence of mRNA for NK-3 receptors in rat kidney based on blot-hybridization and RNase-protection analyses using tachykinin receptor cDNAs.

In pharmacological studies, previous investigators have reported that SP can increase the basal tone of rat renal artery and cause relaxation of rabbit renal arterial segments contracted by exposure to norepinephrine, elevated potassium levels, prostaglandin $F_{2\alpha}$ or phenylephrine (Bredy-Dobrevá *et al.*, 1988; Malomvolgyi *et al.*, 1988). Infusion of SP into the renal artery of anesthetized dogs causes a dose-dependent increase in total renal blood flow, indicating that

the peptide has a relaxant effect on renal resistance vessels in this species (Defelice and Brousseau, 1988). Moreover, specific SP binding sites with NK-1 receptor characteristics were subsequently identified in several blood vessels of the dog kidney, including the afferent arterioles (Stephenson *et al.*, 1987). The effect of tachykinins on renal resistance vessels had not been evaluated in other species.

CHAPTER 3

Materials and Methods

Functional Studies

Isolated Perfused Rat Kidney Preparation

Male Sprague-Dawley rats (350-450 g) from our breeding colony were used. Viral antigen-free breeders were purchased from Charles River Laboratories (Raleigh, NC). Animals were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.). The abdomen was opened by a midline incision. The aorta was ligated above and below the left renal artery after the surrounding connective tissue was carefully removed *in situ*. With the aid of a stereomicroscope, a small polyethylene tube (PE 20) was inserted into the renal artery as described by Schwartz and Malik (1989) and flushed with 1 ml heparinized saline (200 units/ml). The kidney was removed, placed in a perfusion apparatus (Fig. 2) and perfused with a modified Krebs-Ringer bicarbonate buffer containing (millimolar): NaCl, 127.2; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.2; NaHCO₃, 24.9; MgSO₄, 1.2; sodium pyruvate, 2.0; dextrose, 5.5 and 0.1% bovine serum albumin (BSA). The perfusion fluid was prewarmed and maintained at 37° C and continuously gassed with a mixture of 95% O₂ and 5% CO₂. Kidneys were perfused at a constant flow rate of 3 ml/min/g of contralateral kidney using a Masterflex peristaltic pump (Cole Parmer, St. Louis, MO). The renal perfusate was allowed to flow through the cut ends of the renal vein and ureter and not recirculated. Perfusion

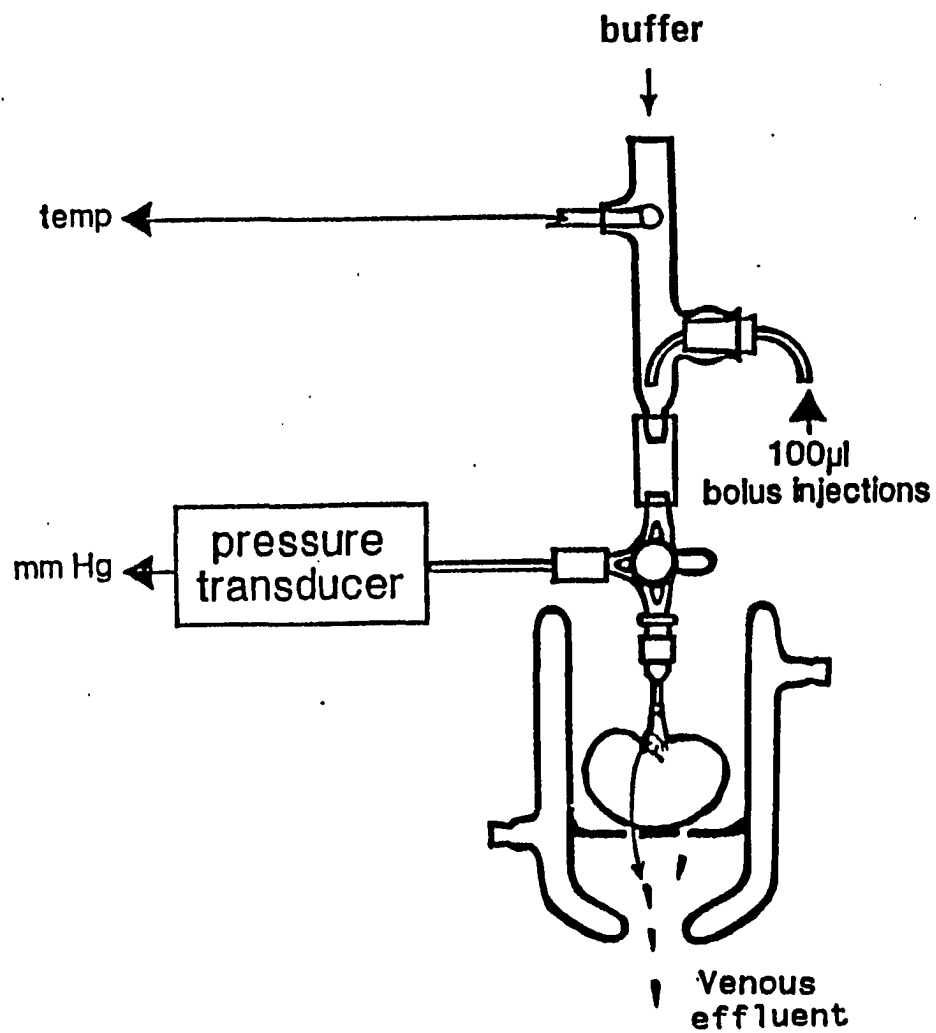


Figure 2. Isolated Perfused Kidney Preparation

pressure (PP) was measured with a Statham-Gould P23ID pressure transducer (Gould, Cleveland, OH) attached to a three-way stopcock at the end of the renal artery cannula to monitor changes in vascular tone with a Gould 2400S recorder (Gould, Cleveland, OH). SP and other tachykinins were dissolved in 0.1% BSA/saline or other diluent and administered by bolus injection of 100 μ l into the perfusing solution via an injection port close to the three-way stopcock (Fig. 2). In all experiments, the PP was allowed to return to baseline before the administration of a subsequent dose of agonist to avoid desensitization. The minimum time between injections was 5 min. All injections caused a small injection artifact in the PP record. Vehicle injection did not have a significant effect on PP. Each dose-response curve was constructed using data obtained from 4-9 kidneys.

Measurements of Pressor Responses to SP, NE and Tyramine at Basal and Elevated Perfusion Pressure

Kidneys were perfused with normal buffer for a 30 min equilibration period. Four doses of SP were given in order of ascending concentration (i.e., 1, 3, 10 and 33.3 nmol) and responses recorded. Then, the perfusion was switched to buffer containing 1 μ M phenylephrine (PHE), a selective α_1 -adrenergic agonist, to increase vascular tone. After another 30 min period for stabilization at a higher PP, a second set of SP dose-response data was obtained. Similar experiments were done with NE and tyramine except for using only one dose of each (100 pmol and 10 μ mol, respectively).

Effect of Phentolamine on Pressor Responses to SP and NE

The effect of phentolamine, an α -adrenergic antagonist, on the responses to SP and NE was evaluated in paired experiments in the presence of 0.22-0.25 μM angiotensin II (AII) to elevate vascular tone. Kidneys were perfused with normal buffer for 30 min, then AII was infused into the perfusion buffer at a site near the renal artery cannula by a Harvard syringe infusion pump (Harvard Apparatus, South Natick, MA). The infusion was started 15 min before beginning bolus injections of SP (33.3 nmol) and NE (333 pmol). After recovery from control responses to SP and NE, the AII infusion was stopped and perfusion was switched to buffer containing 1 μM phentolamine. After a 20 min equilibration period, the same protocol of AII infusion and bolus drug injections was repeated.

Effects of Thiorphan, Captopril and Indomethacin on Pressor Response to SP

After a 30 min perfusion of the isolated rat kidney with normal buffer, perfusion was switched to buffer containing 1 μM PHE plus either 10 μM thiorphan (endopeptidase inhibitor), 10 μM captopril (ACE inhibitor), or 5 μM indomethacin (cyclooxygenase inhibitor). Dose-response effects of SP on PP were assessed beginning at least 30 min after switching to the treatment buffer. Results were compared to those obtained in the presence of PHE alone.

Effects of Natural and Receptor Selective Tachykinins on Perfusion Pressure

Since preliminary experiments demonstrated that increased vascular tone was required for SP to elicit pressor responses, the vascular effects of all other

tachykinins were evaluated after increasing baseline perfusion pressure using 1 μM PHE. The perfusion was switched to buffer containing PHE after 30 min of perfusion with normal buffer. Injections of tachykinins and vehicle were started after another 30 min stabilization period and given in order of ascending dose (i.e., 1, 3.3, 10, 33.3 nmol).

Effect of *in vitro* Capsaicin Treatment

The kidney was initially perfused for 30 min with normal buffer. After another 30 min perfusion with 1 μM PHE buffer, the perfusion was switched to buffer also containing 0.01% ethanol (vehicle for capsaicin) for 20 min as control. This was followed by a 15 min perfusion using PHE buffer without ethanol. Then the kidney was perfused for 20 min with buffer containing capsaicin (500 nM, containing 0.01% ethanol).

Partial Occlusion and Clamping of the Renal Vein

The proximal renal vein was isolated from surrounding connective tissue prior to removal and perfusion of the left kidney as described previously. Kidneys were perfused with normal buffer and 1 μM PHE buffer each for 30 min. During continued perfusion with PHE buffer, a polyethylene cannulae (PE 60, external diameter 1.6 mm, internal diameter 1 mm) was inserted retrogradely into the renal vein (diameter about 2 mm) for a short of period time to determine the effect of partial venous occlusion on arterial PP. After removing the cannula and allowing PP to return to the baseline value, the renal vein was clamped for

several seconds by a hemostat to determine the effect of total venous occlusion on PP.

In vitro Receptor Autoradiography

Animal and Tissue Preparation

Male Sprague-Dawley rats (350-450 g body weight) were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.), and the kidneys were exposed through a midline abdominal incision. The kidneys and, in some experiments, the proximal part of the renal artery, renal vein and ureter were removed and carefully cleaned of surrounding fatty tissue prior to placement on brass microtome specimen plates and freezing with dry ice. Parasagittal, 20 μm -thick sections of kidney were cut on a microtome cryostat at -20°C and thaw mounted onto chrome alum/gelatin-coated microscope slides. Groups of three consecutive sections were collected serially. The first section of each group was stained with hematoxylin and eosin for identification of tissue structures. The remaining sections were used for autoradiography. The brain was also removed and sectioned as described for study as a positive control. The slides were transferred to plastic slide boxes on dry ice as soon as the tissue was dried and stored at -70°C until used.

Receptor Labelling

^{125}I -[MePhe⁷]NKB Slide-mounted tissue sections were brought to room

temperature and placed in a preincubation buffer (50 mM Tris-HCl, containing 0.005% vol/vol, polyethylenimine, pH 7.4) at room temperature for 10 min. Excess preincubation buffer was carefully removed with a gauze before adding the incubation buffer. To conserve radioligand, the incubation was accomplished by placing the slides on a horizontal surface in a humid chamber and covering the sections with about 500 μ l incubation solution per slide. Total binding was determined by incubation of slides for 60 min at room temperature in buffer containing 50 mM Tris-HCl (pH 7.4), 3 mM MnCl_2 , 0.02% (w/v) BSA, peptidase inhibitors (10 μ M phosphoramidon, 40 mg/L bacitracin, 2 mg/L chymostatin and 4 mg/L leupeptin) and 0.2 nM ^{125}I -[MePhe⁷]NKB according to Sadowski *et al.* (1993). To estimate non-specific binding, paired serial sections were incubated as described except that 1 μ M non-labelled [MePhe⁷]NKB, senktide, eledoisin or SP was also present in the incubation solution. Following incubation with radioligand, slides were transferred through four rinses (1 min each) in 50 mM Tris-HCl buffer (pH 7.4) at 4^o C and rapidly dipped in cold deionized water. Excess water on the slides was carefully removed with a gauze, and they were rapidly dried at room temperature with an electric fan.

^{125}I -BHSP For labelling with ^{125}I -BHSP (Substance P, [Na- ^{125}I -monoiodo-Bolton-Hunter]), the same protocol previously described was employed except that polyethylenimine was omitted from the preincubation and phosphoramidon was not included in the incubation buffer. Total binding was determined by using 0.1 nM ^{125}I -BHSP in the absence of unlabelled competitors while nonspecific binding

was estimated using 0.1 nM ^{125}I -BHSP in combination with 1 μM unlabelled SP or related compounds (Mantyh *et al.*, 1989).

Autoradiography

Film Autoradiogram To determine the overall localization of ^{125}I -[MePhe⁷]NKB or ^{125}I -BHSP binding sites in rat kidney, labelled sections were placed in close contact to Ultrafilm (Cambridge Ins., Nussloch, Germany) in light-tight X-ray cassettes at 4⁰ C. After six to fourteen days exposure, the films were developed in Kodak D-19 developer (1:1 dilution with water) at 20⁰ C for 5 min, rinsed in water, then fixed in Kodak Rapid Fixer for 5 min. The labelled sections were then stained with hematoxylin and eosin. These sections and the adjacent stained sections were used to identify labeled structures in the film autoradiogram. To quantitatively estimate the density of radiolabelled tachykinin binding sites, the relative optical density (ROD) of selected regions on the film were measured using a microcomputer-based imaging device (MCID, Imaging Research Inc., Ontario, Canada). Briefly, areas of interest were identified on a video image of the autoradiogram using a mouse, and the computer determined the corresponding ROD. For each area of interest, six to eight readings from each section were obtained and means were calculated. A total of two to three sections from each animal were viewed. Final mean values were calculated from each animal. In all cases, specific binding of SP or [MePhe⁷]NKB to selected regions of the kidney was calculated by subtracting the ROD for the nonspecific binding autoradiogram from the ROD on the total binding autoradiogram (both

corrected for film background). Nonspecific binding was defined as the binding which occurred in the presence of 1 μ M unlabelled peptide on a serially adjacent section.

Emulsion-coated Coverslip Autoradiogram When a higher degree of resolution was desirable, the same slide-mounted, labelled sections were processed by the emulsion-coated coverslip technique according to (Young and Kuhar, 1979). Briefly, acid washed coverslips (25 by 75 mm, Corning No. 0, Corning, New York) were dipped into Kodak NTB3 emulsion in the dark room under a safelight. The emulsion was diluted 1:1 with distilled water and liquified by heating to about 45 °C in a water bath. After dipping, the coverslips were placed vertically in a dark box and allowed to dry overnight. The emulsion-coated coverslips were attached tightly to the slides of labelled tissue sections in the dark with super glue which was applied to one end of the slide and coverslip so as to serve as a hinge. The assemblies were then placed in boxes and stored in the dark at 4°C. After an exposure period of four to six weeks, the coverslips were gently bent away from tissue at one end of the slide by inserting a Q-tip. The emulsions were developed in Dektol for 5 min, washed in distilled water, fixed in Kodak Fixer for 5 min and rinsed in distilled water for 20 min at 4 °C. The tissue sections were then stained with hematoxylin and eosin. The Q-tips were removed after the tissue dried, and coverslips were permanently attached with Eukitt mounting medium. Slides were examined with a microscope under bright-field and dark-field illumination.

Statistical Analysis

Functional Studies

Statistical differences among mean perfusion pressure responses to tachykinins were evaluated by one or two factor analysis of variance (ANOVA) and Dunnett's test for the pairwise comparison of multiple means or by a paired t test, where appropriate. The results are expressed as means \pm S.E. A $P < 0.05$ was considered significant.

Receptor Autoradiography

Grain densities on film autoradiogram were expressed as relative optical density (ROD) $\times 1000 \pm$ S.E. The different means of ROD between total and nonspecific binding or among different agonist treatments at selected regions were analyzed by three-way ANOVA, and differences for the pairwise comparison of multiple means were evaluated using Tukey's test with P values less than 0.05 being considered significant.

Drugs and Chemicals

Functional Studies

The peptides SP, NKA, kassinin, physaleamin, SP(1-7), [Sar⁹,Met(O₂)¹¹]SP and [MePhe⁷]NKB were purchased from Bachem Inc (Torrance, CA); GR-64349 from Research Biochemical (Natick, MA); and AII, PHE, NE, tyramine,

thiorphan, indomethacin and capsaicin from Sigma (St. Louis, MO). Phentolamine was obtained from Ciba Pharmaceutical Co (Summit, NJ). SP and related tachykinin agonists were dissolved in a solution of 0.9% NaCl and 0.01 N acetic acid (333 nmol peptide/100 μ l) and stored in small aliquots at -70° C until used. GR64349 was dissolved in 0.01 N acetic acid. [MePhe⁷]NKB was dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to produce a stock solution containing 333 nmol/100 μ l (10% DMSO). All stock solutions of tachykinin were diluted at the time of use with saline containing 0.1% BSA. Control injections showed that DMSO alone (maximum 1% final concentration) had no effect on PP or the response to agonists under study. Thiorphan was initially dissolved in 0.25 N NaHCO₃ and diluted with perfusion buffer at the time of use (pH 7.4). Indomethacin and capsaicin were dissolved in 100% ethanol. The maximum concentration of ethanol was 0.05% and had no significant effect on PP.

Receptor Autoradiography

¹²⁵I-BHSP (2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL); ¹²⁵I-[MePhe⁷]NKB (2200 Ci/mmol) from Dupont NEN Research Products (Boston, MA); SP, [MePhe⁷]NKB, senktide and eledoisin from Bachem (Torrance, CA). Phosphoramidon, bacitracin, leupeptin, chymostatin, polyethylenimine, MnCl₂, BSA and Tris-HCl (pH 7.4) were obtained from Sigma (St. Louis, MO). Dektol, Rapid fix, and NTB3 nuclear emulsion were purchased from Eastman Kodak, Rochester, N.Y. ¹²⁵I-BHSP was prepared by the reaction

of SP with N-succinimidyl 3-(4-hydroxy 5-[¹²⁵I] iodophenyl) propionate (Bolton and Hunter reagent). The lyophilized ¹²⁵I-BHSP was reconstituted by addition of 0.01 M acetic acid/ β -mercaptoethanol (50 μ Ci/500 μ l) and stored as 40 μ l aliquots in polypropylene tubes at -20 °C. [MePhe]NKB was labelled with ¹²⁵I on His and supplied in a stock solution containing acetonitrile:water (35:65), 0.05M β -mercaptoethanol, 0.13% TFA (trifluoroacetate salt), and 0.13% BSA by Dupont NEN Research. All peptidase inhibitors were dissolved in distilled water to produce stock solutions (0.2 μ mol phosphoramidon/100 μ l, 800 μ g bacitracin/100 μ l, 80 μ g leupeptin/100 μ l, 40 μ g chymostatin/100 μ l). These were stored in microtubes at -70° C until used.

CHAPTER 4

Results

Functional Studies

Pressor Responses to SP, NE and Tyramine in the Presence and Absence of PHE-induced Tone

Baseline PP was low in the absence of PHE, averaging about 74 mm Hg (Table 2), and bolus injections of 1 to 10 nmol SP had no effect on PP under this condition. A minor pressor response was generally noted with 33.3 nmol SP (Figs. 3 and 4), however, this did not achieve statistical significance when compared to baseline PP. During perfusion with 1 μ M PHE, the baseline PP was increased to about 136 mm Hg (Table 2), and SP elicited a dose-dependent pressor response. The largest dose of SP increased PP by 60 ± 5 mm Hg ($n=5$) under this condition (Figs. 3 and 4). A similar phenomenon of potentiation was observed with bolus injections of 100 pmol of NE and 10 μ mol of tyramine (Table 3). The pressor response to NE was approximately 3-fold larger in the presence of PHE compared with the control response. In the case of tyramine, no PP response occurred in the absence of PHE; however, a substantial pressor response could be evoked by the same dose of drug in the presence of PHE.

Effect of Phentolamine on the Pressor Response to SP

In order to determine if the response to SP was mediated by NE, AII was used

TABLE 2.

Baseline perfusion pressure in the isolated perfused rat kidney in the absence and the presence of phenylephrine.

Treatment	Baseline Perfusion Pressure (mm Hg)	
	- PHE	+ PHE
saline	74.2 ± 3.4	134.4 ± 5.9
1.0 nmol SP	73.8 ± 3.5	134.0 ± 7.1
3.3 nmol SP	73.4 ± 3.8	136.6 ± 8.1
10.0 nmol SP	74.2 ± 4.2	138.0 ± 7.6
33.3 nmol SP	75.2 ± 4.7	137.0 ± 6.3

The values indicated baseline PP before each bolus injection of SP and saline in the absence and the presence of 1 μ M phenylephrine (PHE). Data are expressed as mean \pm S.E. (n=5). No significant difference (P < 0.05) among each value in the absence or in the presence of PHE, respectively.

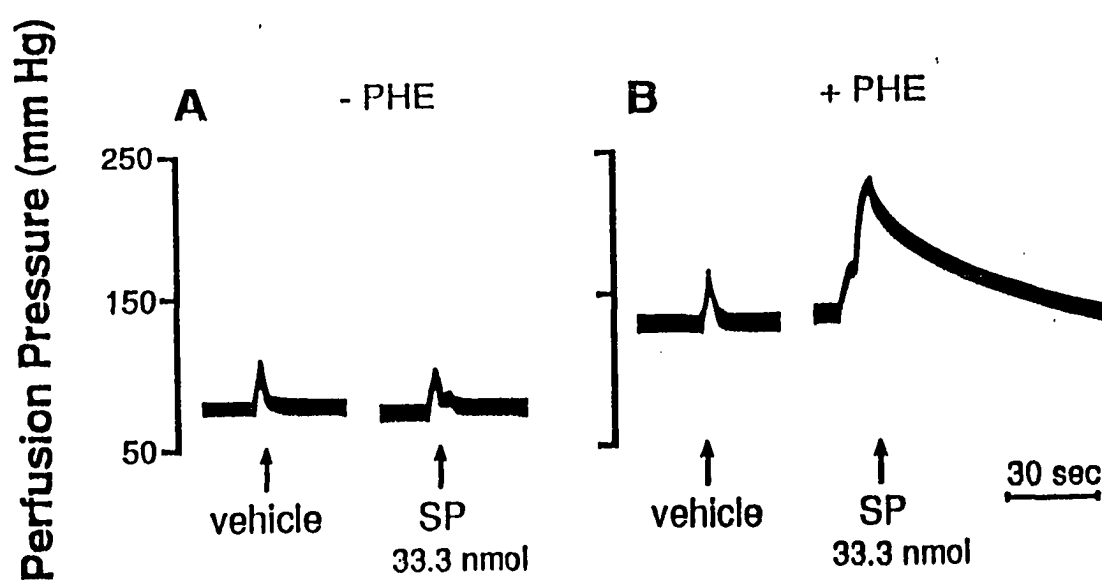


Figure 3. Recorder tracings showing perfusion pressure changes produced by bolus injections of SP and vehicle (0.1% BSA in saline) in the absence (A) and presence (B) of 1 μ M phenylephrine (PHE). Note that a brief injection artifact was produced by all bolus injections.

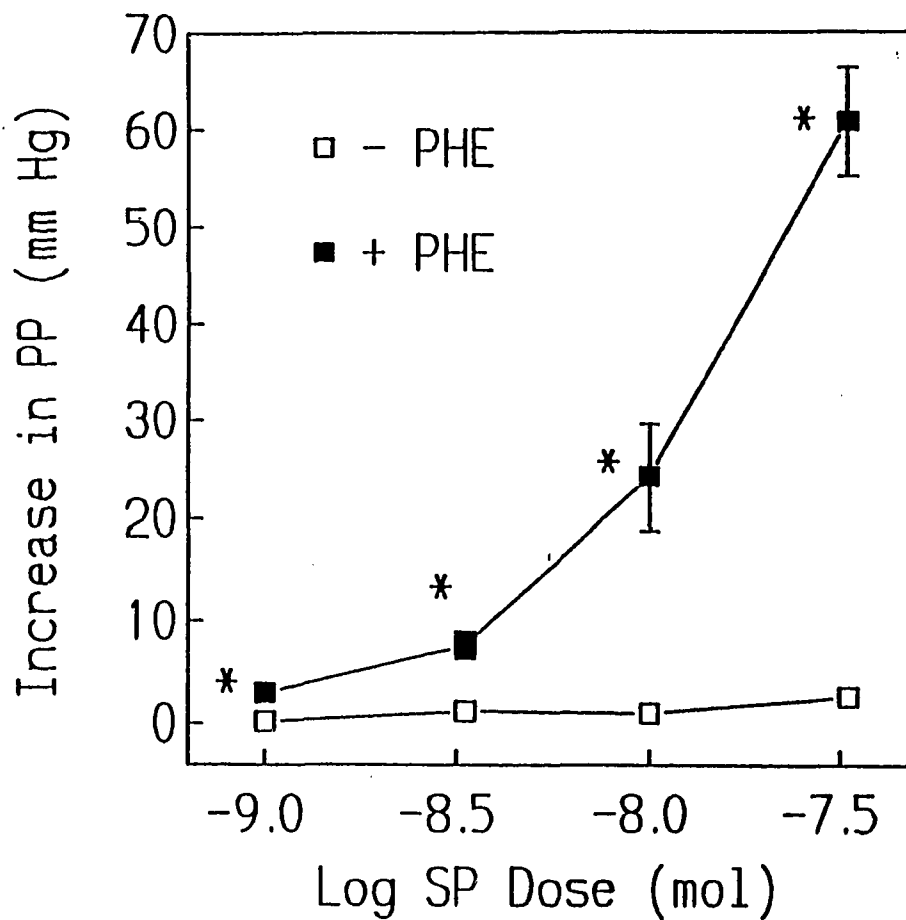


Figure 4. Dose-response curves for SP effect on perfusion pressure in the absence and presence of 1 μ M phenylephrine (PHE). Points represent the means and vertical bars the S.E. ($n=5$). *Significantly different from response to SP at same dose in absence of PHE.

TABLE 3.

Effect of phenylephrine to increase the pressor responses to NE and tyramine in the isolated perfused rat kidney

Treatment	Perfusion Pressure (mm Hg)			
	-PHE		+PHE	
	baseline	increase	baseline	increase
NE	60 ± 3	28.8 ± 7	126 ± 8*	96 ± 6.8*
Tyramine	57 ± 3	2.2 ± 0.5	121 ± 13*	42 ± 5*

Baseline PP and responses to bolus injections of 0.1 nmol NE and 10 μ mol tyramine were measured before and after adding 1 μ M phenylephrine (PHE) to the perfusion buffer. Data are expressed as mean \pm S.E. (n=5). *Significantly different from corresponding value in absence of PHE.

instead of PHE to increase vascular tone. Infusions of AII increased baseline PP to 124 ± 7 mm Hg (n=5). Bolus injections of 33 nmol SP and 333 pmol NE evoked pressor responses in the presence of AII, but only the response to NE was blocked by addition of $1 \mu\text{M}$ phentolamine to the buffer (Figs. 5 and 6). The pressor response to SP was slightly increased in the presence of phentolamine (Fig. 6).

Effects of Thiorphan, Captopril and Indomethacin on Pressor Response to SP

As shown in Table 4, the pressor responses to SP were not potentiated by a neutral endopeptidase inhibitor (thiorphan) or an ACE inhibitor (captopril). In contrast, at the highest dose of SP tested, the responses to SP were smaller than they were in the absence of the peptidase inhibitors. Indomethacin potentiated the responses to 3.3 and 10 nmol of SP significantly compared to control. Each of the inhibitors had no significant effect on PP at the concentrations used (data not shown).

Comparison of the Dose-dependent Pressor Responses to SP, NKA, Kassinin, Physaleamin and SP(1-7)

In the presence of $1 \mu\text{M}$ PHE, SP produced a dose dependent increase in PP. The amphibian tachykinins, physaleamin and kassinin had a similar pressor action to that of SP but caused a smaller response at the highest dose tested (Fig. 7). Although NKA could elicit a vasoconstrictor response, it was far less active than SP, physaleamin and kassinin (Fig. 7). The N-terminal fragment of SP, SP(1-7),

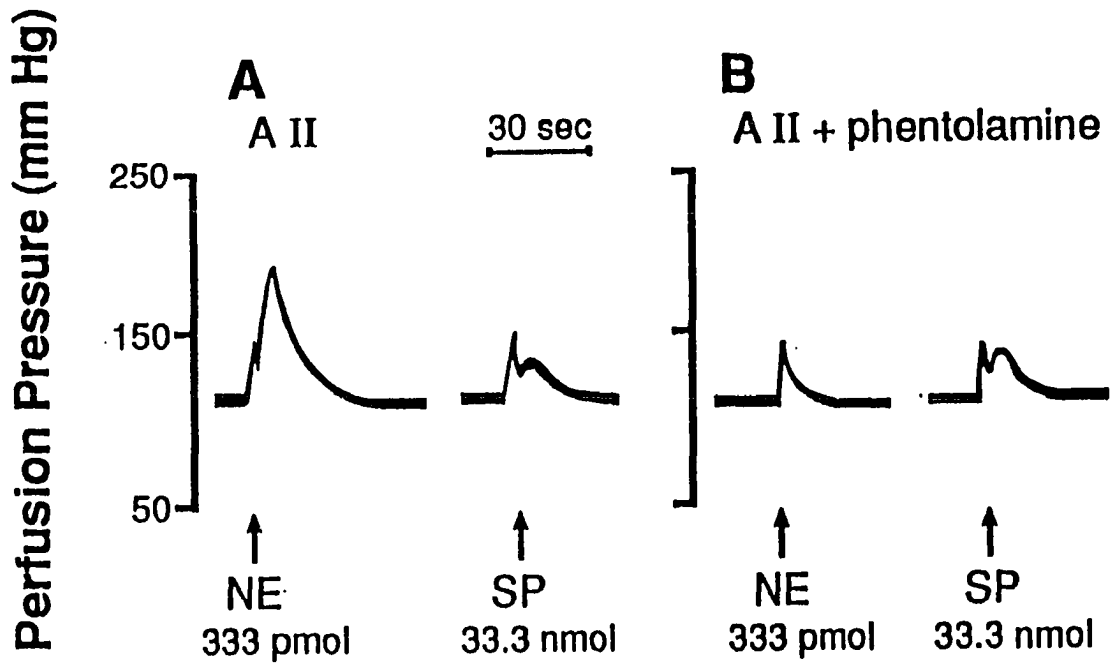


Figure 5. Recorder tracings showing perfusion pressure responses to NE and SP in the absence (A) and presence (B) of 1 μ M phentolamine. Angiotensin II (AII) was infused (0.22-0.25 μ M final concentration) to increase baseline perfusion pressure.

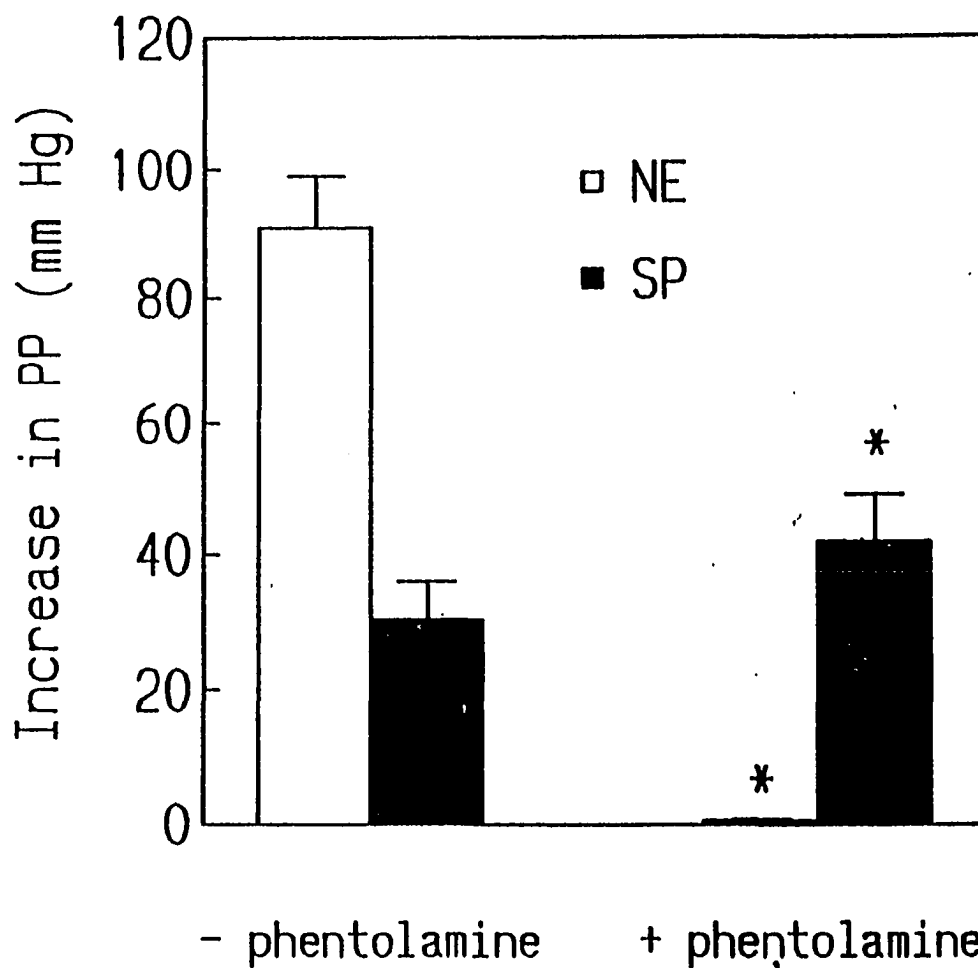


Figure 6. Effect of $1 \mu\text{M}$ phentolamine on perfusion pressure responses produced by bolus injections of SP (33.3 nmol) and NE (333 pmol). Angiotensin II (AII) was infused ($0.22\text{-}0.25 \mu\text{M}$ final concentration) to increase baseline PP. The bars represent mean values and vertical lines indicate S.E. ($n=5$). *Significantly different from response to same drug without phentolamine.

TABLE 4.
Effect of thiorphan, captopril and indomethacin on the vasoconstrictor responses to SP in the isolated perfused rat kidney

Treatment	Baseline (mm Hg)	SP dose (nmol)			
		1.0	3.3	10	33.3
Control	123 ± 9.4	3.3 ± 0.9	9.3 ± 1.5	16.7 ± 2.5	55.6 ± 6.7
Thiorphan (10 μM)	112 ± 4.0	5.7 ± 0.7	11.7 ± 1.3	21.5 ± 1.9	48.8 ± 8.7*
Captopril (10 μM)	125 ± 7.7	2.3 ± 0.8	7.2 ± 1.0	12.2 ± 1.5	36.5 ± 4.7*
Indomethacin (5 μM)	124 ± 9.7	8.3 ± 2.0	18.7 ± 2.8*	31.5 ± 2.3*	59.8 ± 7.7

Phenylephrine (1 μM) was used to increase baseline perfusion pressure in all groups. Treatment with inhibitors was started 30 min before injection of the first dose of SP. Values are means ± S.E. (n=6 for each group). *Significantly different from control response at the same dose of SP (P < 0.05).

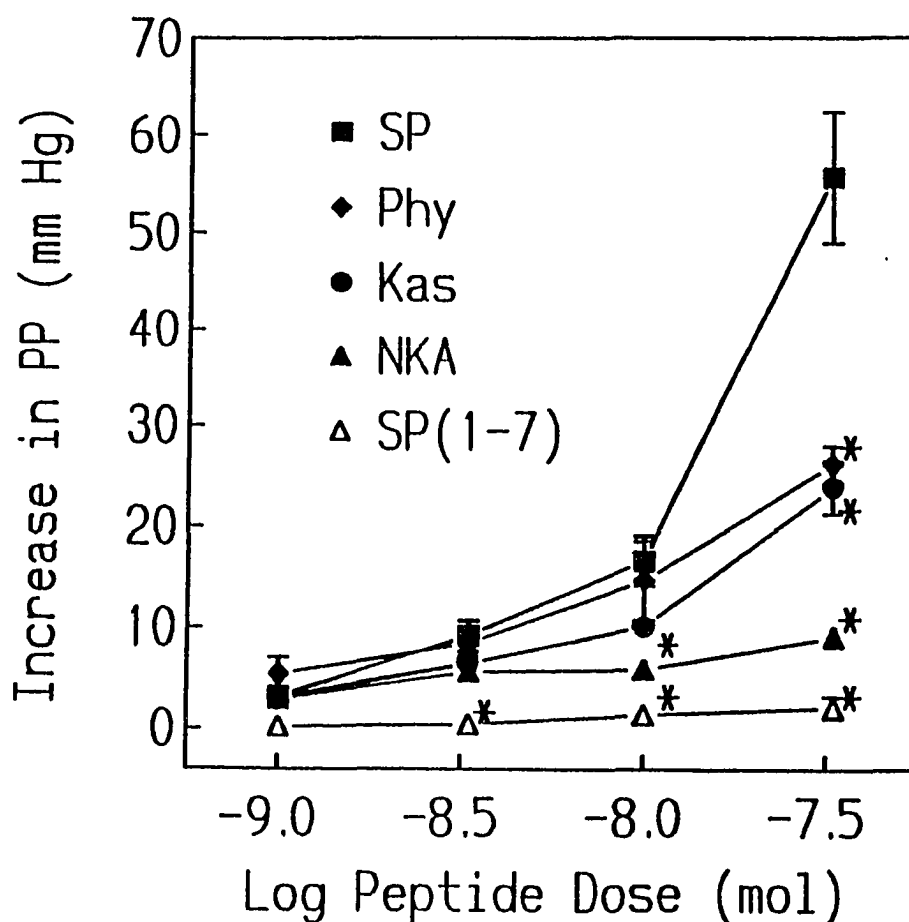


Figure 7. Dose-response curves for increase in perfusion pressure produced by bolus injections of SP, physaleamin (Phy), kassanin (Kas), neurokinin A (NKA) and SP(1-7). The baseline perfusion pressure was increased by including 1 μ M PHE in the perfusion buffer. The points represent the mean and vertical bars the S.E. ($n=4-6$ for each curve). *Significantly different from the response to SP at same dose.

was inactive in the same dose range tested for SP (Fig. 7).

Effect of Selective Tachykinin Receptor Agonists on Perfusion Pressure

Dose-response data for NK-1, NK-2 and NK-3 receptor agonists in the presence of PHE are shown in Figure 8. The selective NK-1 receptor agonist, [Sar⁹,Met(O₂)¹¹]SP had no significant effect on PP at doses from 1 to 33.3 nmol. By contrast, the selective NK-2 agonist, GR64349, and NK-3 receptor agonist, [MePhe⁷]NKB, both evoked dose-dependent pressor responses. The only significant difference between responses to GR64349 and [MePhe⁷]NKB occurred at the lowest dose tested and this was minor. Responses to NK-2 and NK-3 agonists were significantly greater than those to SP at doses of 10 and 33.3 nmol.

Biphasic Effect of Capsaicin on Perfusion Pressure

As shown in Figures 9 and 10, infusions of capsaicin (500 nM final concentration) in the presence of 1 μ M PHE, produced an initial increase in PP followed by a more prolonged decrease. The maximum and minimum PPs achieved in response to capsaicin were significantly different from the baseline PP. Vehicle (0.01% ethanol) infusion produced a slight increase in PP (maximum of 6 ± 3 mm Hg) with a slower onset compared to capsaicin. The maximum PP achieved during vehicle infusion was not significantly different from baseline PP.

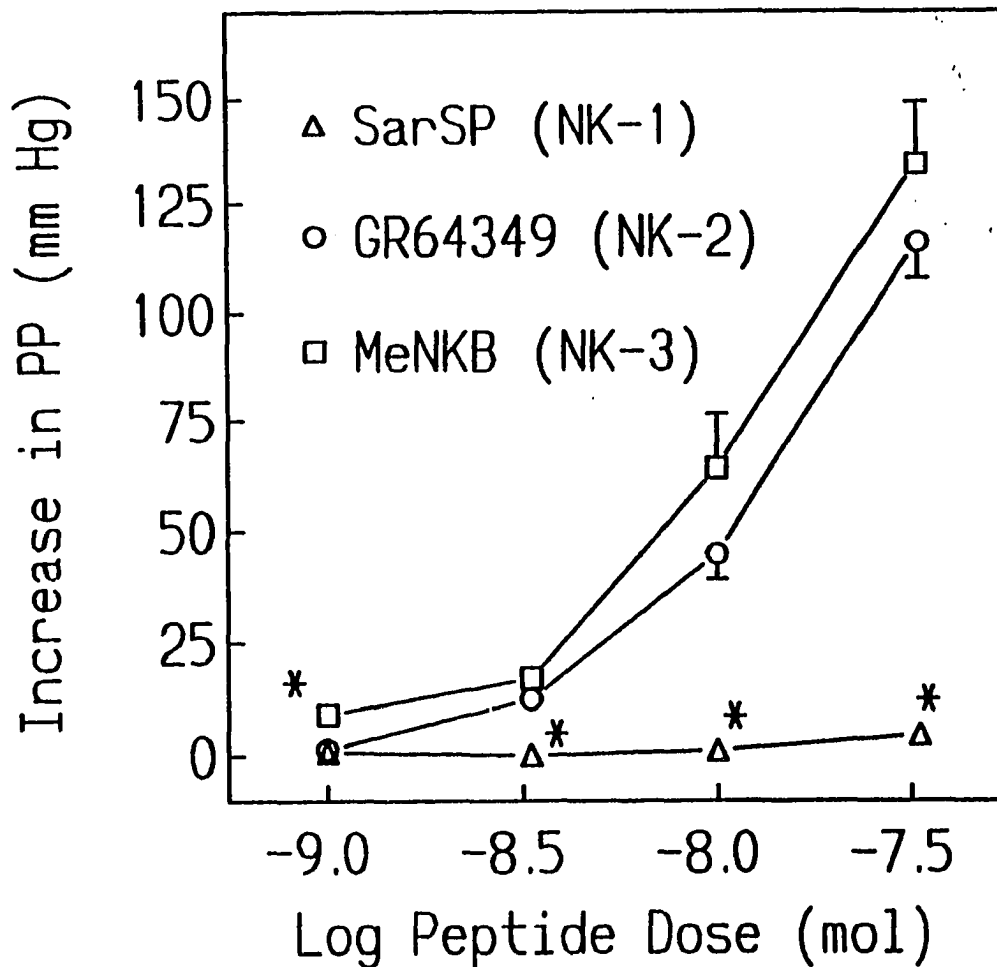


Figure 8. Dose-response curves for increase in PP produced by bolus injections of [MePhe⁷]NKB (MeNKB, n=9), [Lys³,Gly⁸-R γ -lactam-Leu⁹]NKA(3-10) (GR-64349, n=6) and [Sar⁹,Met(O₂)¹¹]SP (SarSP, n=4). The baseline perfusion pressure was increased by including 1 μ M PHE in the perfusion buffer. The points represent the mean and vertical bars the S.E. *Significantly different from the responses to other agonists at same dose.

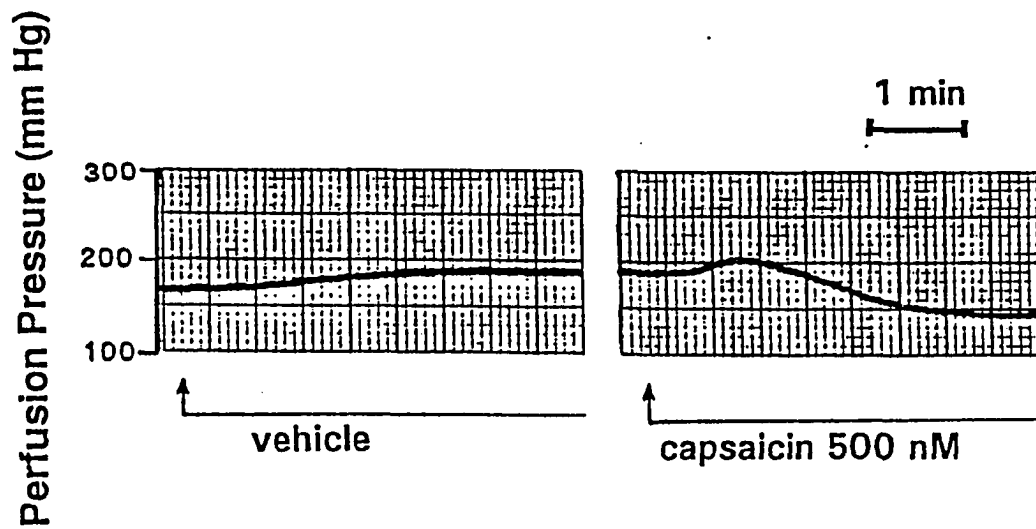


Figure 9. Original tracings showing the effects of vehicle (0.01% ethanol) and capsaicin (500 nM) infusions on the perfusion pressure in an isolated rat kidney in the presence of 1 μ M phenylephrine.

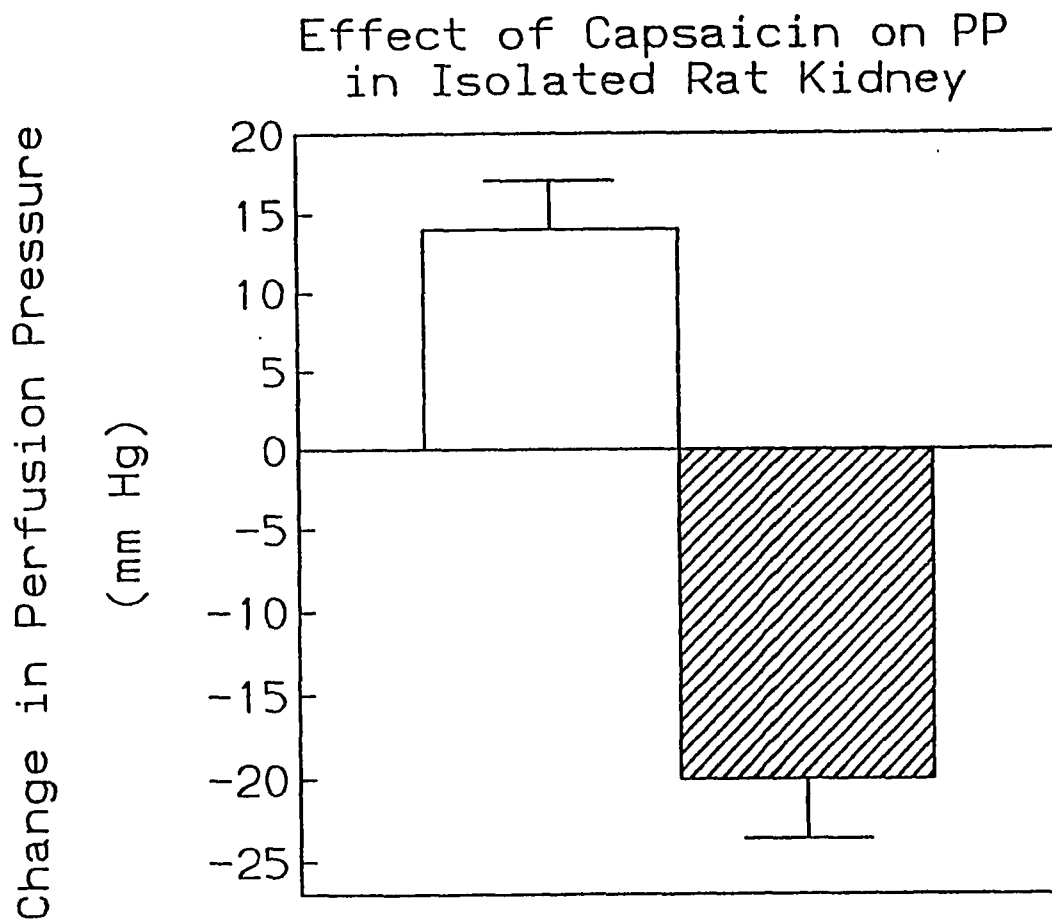


Figure 10. Biphasic changes of perfusion pressure in isolated rat kidney induced by infusion of capsaicin ($500 \mu\text{M}$) in the presence of $1 \mu\text{M}$ phenylephrine. The bars represent the mean and vertical lines the S.E. ($n=8$).

In vitro Receptor Autoradiography

Control Studies of the Rat Brain

^{125}I -[MePhe⁷]NKB Autoradiographic studies revealed that in the coronal sections of the rat brain, specific ^{125}I -[MePhe⁷]NKB binding sites are concentrated in cerebral neocortex, nucleus of the diagonal band and supraoptic nucleus. Much lower densities of specific binding are seen in other areas such as the caudate-putamen and paraventricular nucleus (Fig. 11a,b,c and d). This distribution of NK-3 receptor binding sites in rat brain, as visualized with ^{125}I -[MePhe⁷]NKB, is similar to that previously reported using another NK-3 selective ligand, [³H]senktide (Dam *et al.*, 1990; Stoessl and Hill, 1990; Quirion *et al.*, 1991).

^{125}I -BHSP The autoradiographic distribution of NK-1 receptors was visualized in rat brain using ^{125}I -BHSP. Specific binding sites were most densely distributed in the olfactory tubercle and caudate-putamen (Fig. 11e and f). This pattern is quite different from that observed with ^{125}I -[MePhe⁷]NKB but in accord with a previous report on ^{125}I -BHSP binding in the rat brain (Danks *et al.*, 1986).

Localization of Specific [MePhe⁷]NKB Binding Sites in the Rat Kidney

The localization of NK-3 receptors in rat kidney was subsequently evaluated by film and nuclear track emulsion autoradiography using ^{125}I -[MePhe⁷]NKB. Reverse image prints that were produced by using autoradiograms as negatives show ^{125}I -[MePhe⁷]NKB binding sites in rat kidney in Figures 12 and 13. A comparison of total binding and nonspecific binding reveals the presence of

specific radioligand binding sites in the renal vein, inner strip of the outer medulla, pelvis and proximal ureter. The localization of a specific binding to the interlobar veins was also visible at the cortical/medullary junction (Fig. 13d,e and f). No specific binding was detected in the renal artery or the intrarenal arterial system by film autoradiography, although a high amount of nonspecific binding to renal artery was observed (Fig.12 and 13). Likewise, specific binding was not detected in the renal cortex (Fig. 12). Based on the measurement of ROD by computer-assisted image analysis (n=5), a high density of specific binding sites for ^{125}I -[MePhe⁷]NKB was associated with the ureter and renal pelvis, a moderate density with the renal vein and a relatively low density with the inner strip of the outer medulla (Fig. 14). High resolution detection of ^{125}I -[MePhe⁷]NKB binding sites was obtained in a few specimens by using the emulsion coated coverslip method. It was found that specific silver grains (black on the prints) were associated with the tunic media of the renal vein (Fig. 15b), the tunic muscularis of renal pelvis (Fig. 15d) and the tunic muscularis of ureter (Fig. 15f). Since no successful observations of the inner strip of the outer medulla were obtained by high resolution autoradiography, further definition of the location of specific binding sites in this region was not possible.

Competition studies using unlabelled senktide, eledoisin and SP, each at 1 μM , were performed to further characterize the binding sites identified by labeled [MePhe⁷]NKB (Table 5). For most regions, it was found that both senktide and eledoisin were comparable to [MePhe⁷]NKB in ability to compete with

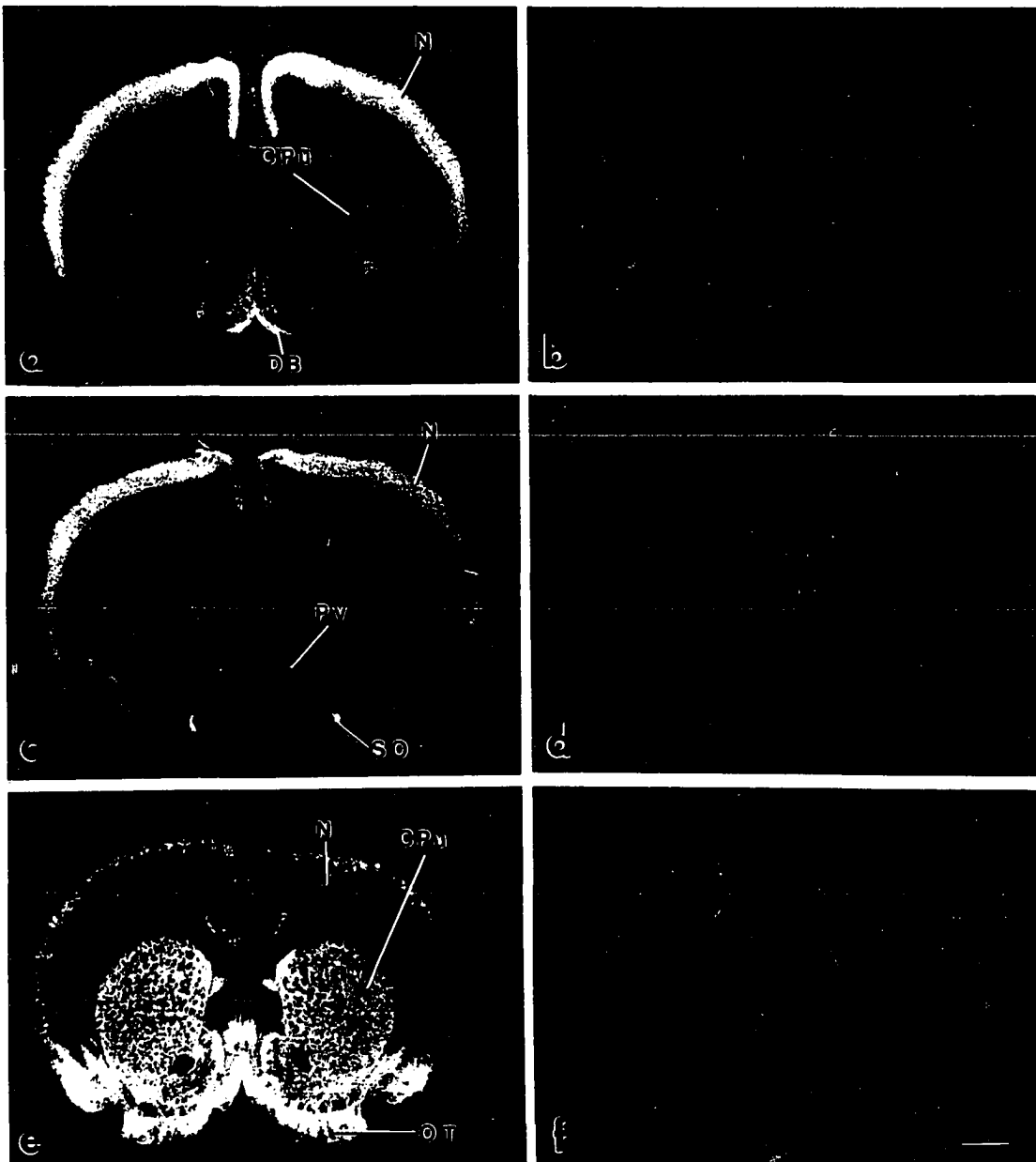


Figure 11. Autoradiographic visualization ($[^3\text{H}]$ Ultrofilm) of tachykinin binding sites in coronal sections of rat brain labeled by $0.2 \text{ nM } ^{125}\text{I}$ -[MePhe⁷]NKB (a,c) or $0.1 \text{ nM } ^{125}\text{I}$ -BHSP (e). Plate b, d and f show nonspecific binding to adjacent sections in the presence of $1 \mu\text{M}$ unlabelled ligands. Bar=2mm. CPu, Caudate-putamen; DB, nucleus of diagonal band; N, neocortex; OT, olfactory tubercle; PV, paraventricular nucleus; SO, supraoptic nucleus.

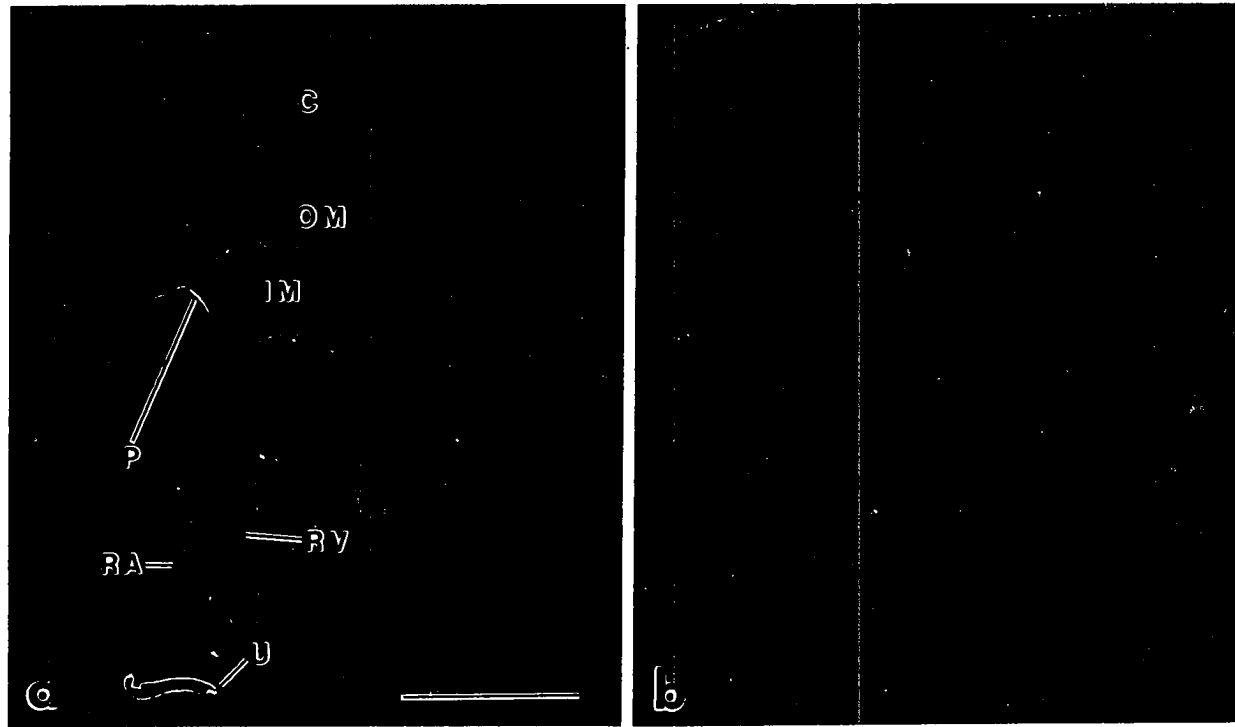


Figure 12. Autoradiographic visualization ($[^3\text{H}]$ Ultrofilm) of ^{125}I -[MePhe⁷]NKB binding sites in $20\ \mu\text{m}$ frozen sections of rat kidney. (a) Total binding to section incubated with $0.2\ \text{nM}$ ^{125}I -[MePhe⁷]NKB; (b) Nonspecific binding to adjacent section incubated with $0.2\ \text{nM}$ radioligand and $1\ \mu\text{M}$ unlabeled ligand. Photos are reverse images, so grains appear white on a black background. Note the specific binding of ^{125}I -[MePhe⁷]NKB to the inner strip of the outer medulla (OM), renal pelvis (P), renal vein (RV) and proximal ureter (U). High amount of nonspecific binding is seen on renal artery (RA). C, renal cortex; IM, inner medulla; Bar = 5mm.

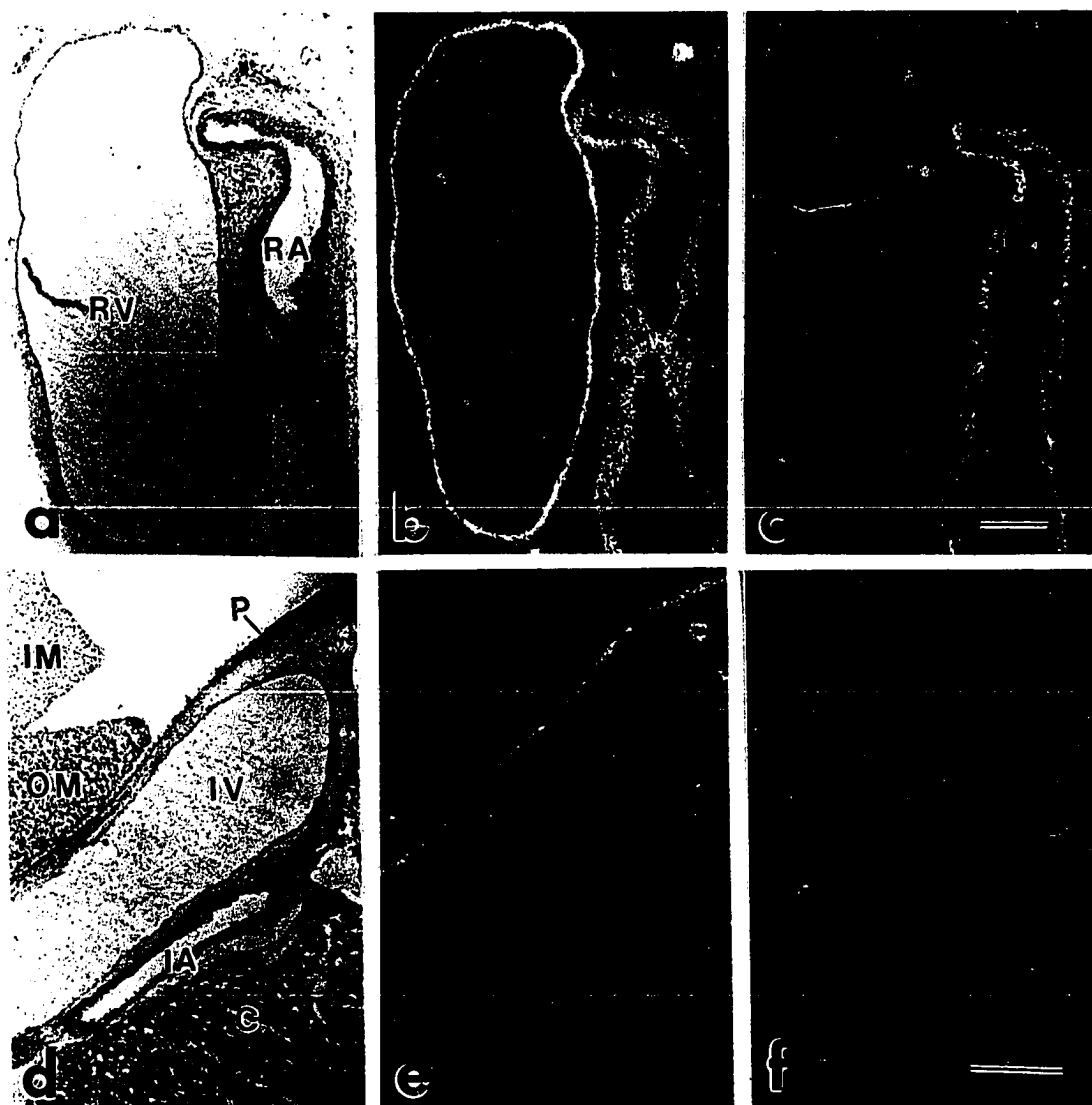


Figure 13. Higher magnification of autoradiographic localization ($[^3\text{H}]$ Ultrofilm) of ^{125}I -[MePhe 7]NKB binding sites in rat kidney. Images in (b) and (e) represent total binding while those in (c) and (f) represent nonspecific binding. (a) and (d) are Lightfield photomicrographs of adjacent sections of (b) and (e), respectively, stained with hematoxylin and eosin. C, renal cortex; IA interlobar artery; IM, inner medulla; IV, interlobar vein; OM, outer medulla; P, renal pelvis; RA, renal artery; RV renal vein; Bar = 50 μm .

^{125}I -(MePhe⁷)NKB Binding Sites
in Rat Kidney

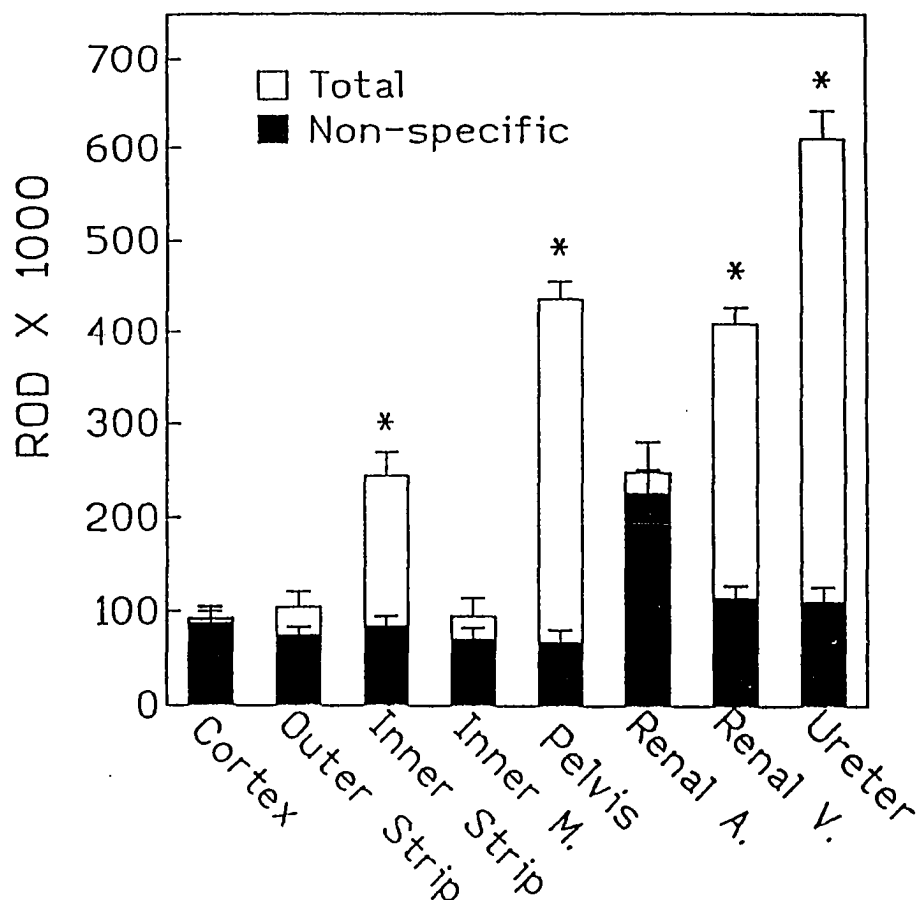


Figure 14. Evaluation of the regional density of ^{125}I -[MePhe⁸]NKB binding sites in rat kidney on film autoradiograms by computer-assisted image analysis. Values were expressed as mean relative optic density (ROD) \times 1000 \pm S.E (n=5). *significant difference between total and non-specific binding in the selected region. A P < 0.05 was considered significant. A, artery; M, medulla; V, vein.

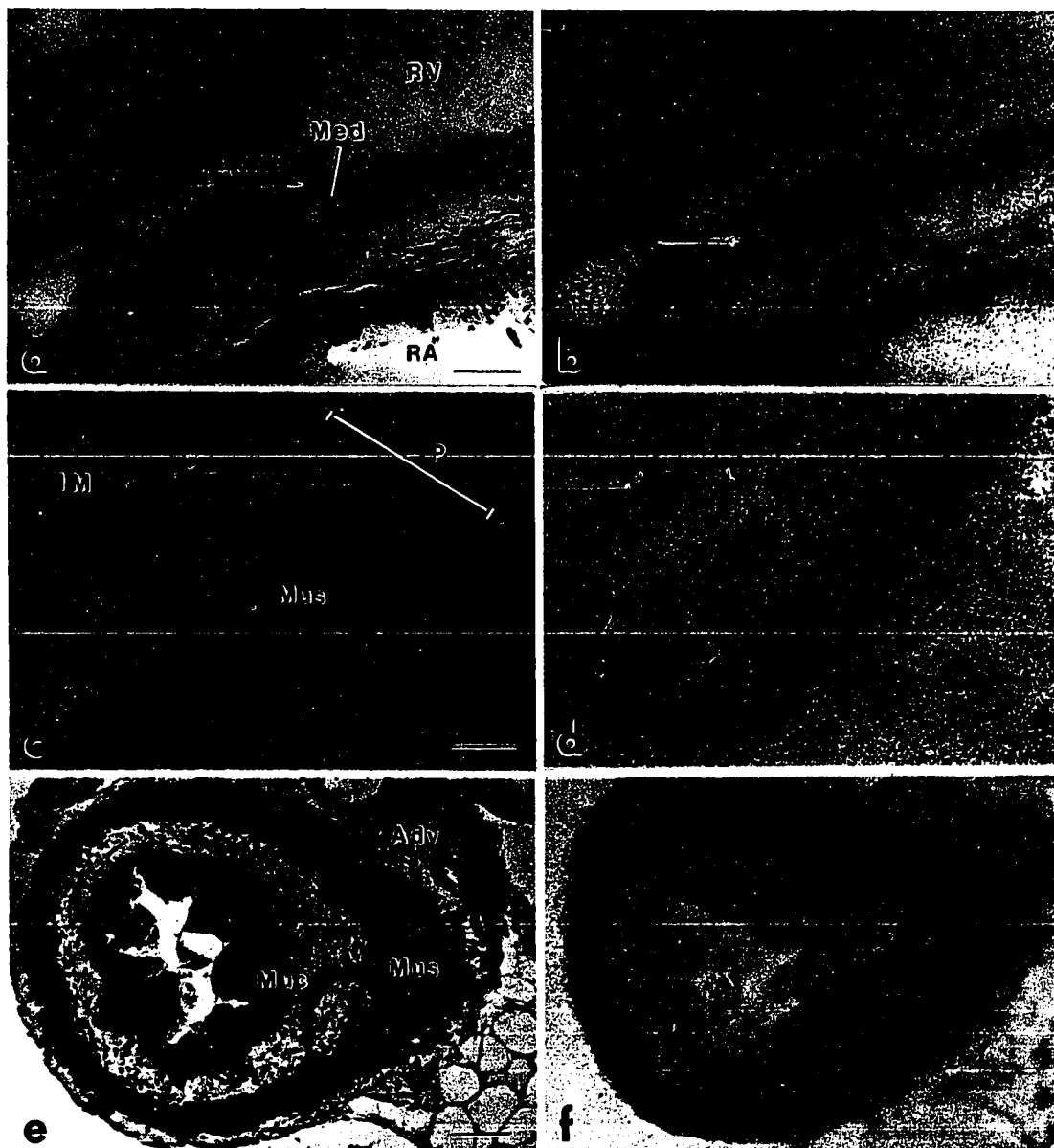


Figure 15. Light microscopic visualizations on emulsion-coated slides show ^{125}I -[MePhe⁷]NKB binding sites primarily over the tunica media (Med) of the renal vein (RV) in (b), the tunica muscularis (Mus) of renal pelvis (P) in (d) and ureter in (f). The microscope was focussed in the plane of the tissue (hematoxylin and eosin stain) for the photo in (a) while the focus was in the plane of the emulsion and silver grains in (b). Photographs in (c) and (e) show the hematoxylin and eosin staining pattern for sections corresponding to (d) and (f), respectively. Adv, tunic adventitia; IM, inner medulla; Muc, tunic mucosa; Bar = 50 μm in (a) and (c) and 1 mm in (e).

TABLE 5.
Autoradiographic Distribution and Characterization of ^{125}I -[MePhe⁷]NKB
Binding Sites in Rat Kidney

Region	Total binding	Nonspecific binding			
		MeNKB	Senk	Ele	SP
Cortex	92 ± 13	87 ± 13	42 ± 5 ^{*†}	79 ± 7	6 ± 2
Outer Stripe	94 ± 17	74 ± 9	34 ± 4 [*]	69 ± 9	73 ± 7
Inner Stripe	244 ± 25	84 ± 11 [*]	45 ± 8 ^{*†}	99 ± 15 [*]	124 ± 10 [*]
Inner Medulla	85 ± 19	70 ± 12	38 ± 6 ^{*†}	69 ± 8	71 ± 8
Pelvis	436 ± 19	67 ± 13 [*]	36 ± 3 ^{*†}	82 ± 12 [*]	183 ± 27 ^{*†}
Renal Artery	248 ± 32	226 ± 25	227 ± 20	220 ± 10	228 ± 21
Renal Vein	409 ± 18	114 ± 1 [*]	85 ± 7 ^{*†}	143 ± 10 [*]	221 ± 13 ^{*†}
Ureter	610 ± 31	110 ± 1 [*]	106 ± 9 [*]	227 ± 32 ^{*†}	379 ± 30 ^{*†}

Data are expressed as mean ROD X 1000 ± S.E.M (n = 5). * indicates significant difference from total binding and † indicates significant difference from nonspecific binding in the presence of the other agonists at selected region. Total binding was determined by using 0.2 nM ^{125}I -[MePhe⁷]NKB in the absence of unlabelled competitors while nonspecific binding was determined by using 0.2 nM ^{125}I -[MePhe⁷]NKB in combination with 1 μM unlabelled agonists. Ele, eldoisin; MeNKB, [MePhe⁷]NKB; Senk, senktide; SP, substance P.

radioligand for binding sites. However, eledoisin was less effective than [MePhe⁷]NKB and senktide at the ureter. In addition, senktide competed for radioligand binding at more sites than the other agonists at the concentrations tested. SP was the least effective at blocking the binding of ¹²⁵I-[MePhe⁷]NKB.

Localization of Specific SP Binding Sites in the Rat Kidney

Figures 16 and 17 illustrate the specific binding of ¹²⁵I-BHSP to selected regions of the rat kidney with reverse images from film autoradiograms and ROD measurements, respectively. Specific binding of SP was detected on the renal artery, where it seemed to be located at the media/adventitia border or in the adventitia. Likewise, specific SP binding sites were present in the interlobar arteries (data not shown) and the renal pelvis. No specific binding sites for ¹²⁵I-BHSP were seen in the renal vein and its branches, the renal cortex or the renal medulla (Fig. 17). Nonspecific binding of ¹²⁵I-BHSP in the presence of 1 μ M SP was negligible.

Effect of Partially Occluding or Clamping Renal Vein on PP

As shown in Figure 18, when the isolated rat kidney was perfused at a constant flow rate in the presence of 1 μ M PHE as described previously, cannulating the renal vein with a small diameter polyethylene tubing or closing the vein with a hemostat produced a marked increase in PP.



Figure 16. Autoradiographic visualization ($[^3\text{H}]$ Ultrofilm) of ^{125}I -BHSP binding sites in 20 μm frozen sections of rat kidney. (b) Total binding to section incubated with 0.1 nM ^{125}I -BHSP. (c) Nonspecific binding in adjacent section where 1 μM unlabelled SP was also added to the incubation medium. (a) Brightfield photomicrograph of adjacent hematoxylin and eosin stained section. Note that specific SP binding sites are associated with the renal pelvis (P) and renal arteries (RA) but not with the renal vein (RV). IM, inner medulla; Bar=1mm.

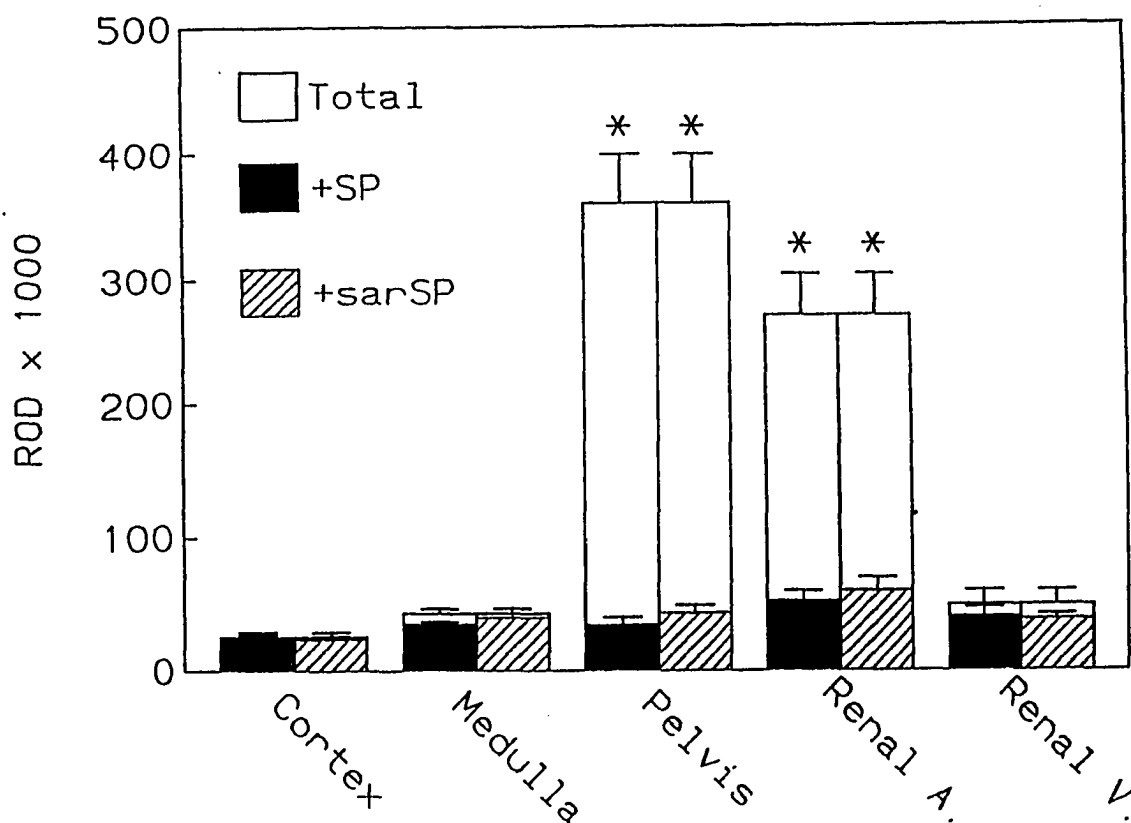
^{125}I -BHSP Binding Sites in Rat Kidney

Figure 17. Evaluation of regional density of ^{125}I -BHSP binding sites in rat kidney on film autoradiogram by computer-assisted image analysis. Values were expressed as mean relative optical density (ROD) $\times 1000 \pm \text{S.E}$ ($n=5$). Total bindings are determined with 0.1 nM labeled SP while nonspecific binding to adjacent sections was determined using 0.1 nM ^{125}I -BHSP plus 1 μM SP or [$\text{Sar}^9, \text{Met}(\text{O}_2)^{11}$]SP (sarSP). *significant difference between total and nonspecific binding in the selected region. A $P < 0.05$ was considered significant. A, artery; M, medulla; V, vein.

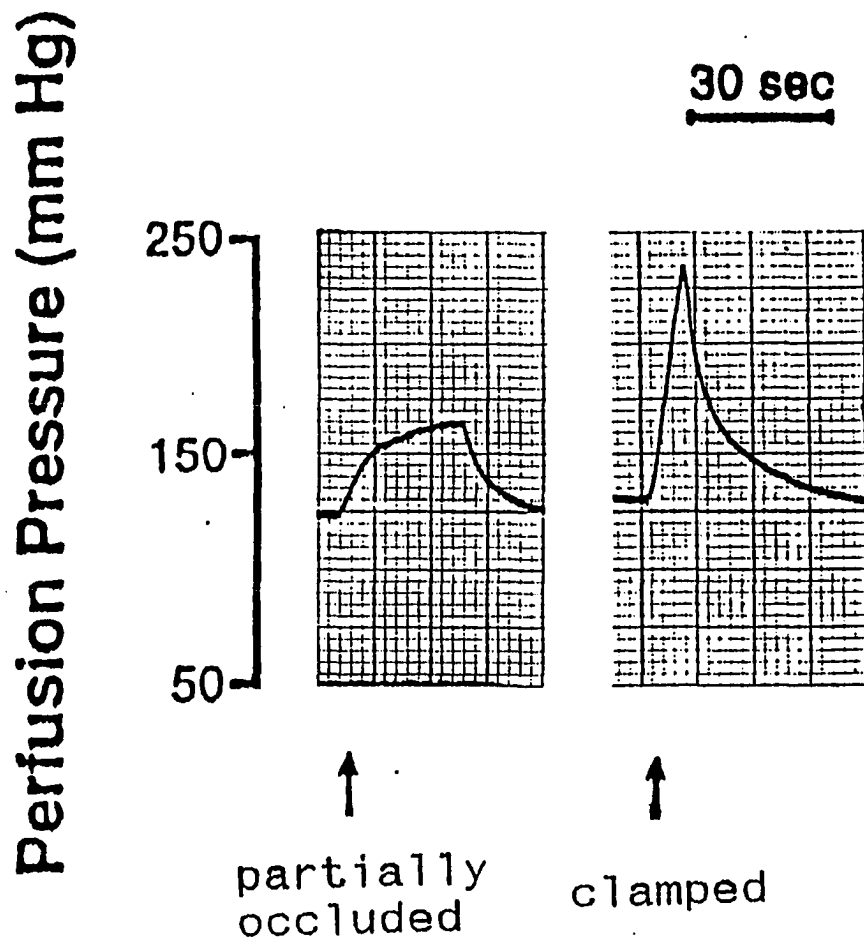


Figure 18. Recorder tracings showing the marked increase in perfusion pressure produced by cannulation of the renal vein with small diameter polyethylene tubing or closing the renal vein with a hemostat in the isolated rat kidney in the presence of $1 \mu\text{M}$ phenylephrine.

Chapter 5

Discussion

Functional Studies

Effect of Elevated Vessel Tone on Response to SP

SP had no effect on PP in the isolated perfused rat kidney when basal vascular tone was low but elicited a dose dependent vasoconstriction after baseline PP was increased by PHE or AII. A similar phenomenon was observed with single doses of NE and tyramine. These results indicate that, in the isolated perfused renal vascular bed of the rat, the magnitude of the response or the sensitivity of the renal blood vessels to vasoconstrictors depends upon the pre-existing level of vascular tone. The same phenomenon has also been observed for α_2 -adrenoceptor- and neuropeptide Y-mediated contractions in the isolated rat tail vascular bed with endothelin-1 used to generate tone (Maclean and Mcgrath, 1990), the constrictor response of cat cerebral arteries to serotonin with mechanical elevation of transmural pressure (Harder, 1988) and AII evoked contractions in rabbit femoral artery with NE used to adjust baseline tone (Prins *et al.*, 1992). Since the vascular response to multiple pressor agents acting through different receptors is enhanced by elevation of vessel tone, it seems that this kind of potentiation results from a common alteration in intracellular mechanisms which regulate the contractile process.

It has been reported that elevation of transmural pressure causes a significant

increase in inositol-triphosphate and diacylglycerol levels in isolated dog renal arteries (Harder *et al.*, 1992). This suggests that the enhanced response to vasoconstrictors observed under this condition might be related to increased phospholipase C activity. Other investigators have also shown that the amplification of AII-induced contractile responses by NE in rabbit femoral artery are attenuated markedly by nifedipine, a calcium channel antagonist, implicating calcium channel activation in this phenomenon (Prins *et al.*, 1992). In addition, Collins *et al.* (1992) have implicated protein kinase C activation in the contractile response that occurs to PHE in single vascular smooth muscle cells at constant intracellular free Ca^{++} concentration. It has been demonstrated that all three tachykinin receptors have been linked to the phosphatidylinositol - calcium transduction system (Nakanishi, 1991). Likewise, both α_1 -adrenergic and AII receptors are coupled to specific phospholipase C (Alexander *et al.*, 1985; Steinberg *et al.*, 1989; Takata *et al.*, 1990). Therefore, one possible explanation for enhanced vasoconstrictor responses to SP and NE in the present study is that PHE and AII increase phospholipase C activity and the sensitivity of calcium-dependent and -independent steps in the contractile process.

Other mechanisms may be involved as well, such as membrane depolarization upon elevation of transmural pressure. Electrophysiological experiments have demonstrated that elevation of transmural pressure causes depolarization of the vascular smooth muscle and a significant increase in vessel sensitivity to NE (Lombard *et al.*, 1990). Responses to tachykinins might also be potentiated under

this condition.

The Role of NE in the Pressor Response to SP

It has been demonstrated that some of the effects of SP and other tachykinins appear to involve interactions with sympathetic neuroeffector control (Rand *et al.*, 1990). Immunocytochemical study has established that SP is present in cerebral perivascular axons and these axon terminals are in close proximity to those containing NE in the cerebral blood vessels (Matsuyama *et al.*, 1986). Couture *et al.* (1989) suggested tachykinins can accelerate heart rate by release of catecholamine through activation of NK-1 and/or NK-2 receptors, on noradrenergic fibers and adrenal chromaffin cells. Likewise, Tousignant *et al.* (1987) have demonstrated that NKA facilitates the release of NE from sympathetic nerves in the rat vas deferens.

In rat kidney, anatomical studies have demonstrated that both the efferent sympathetic innervation and SP-containing nerve terminals reach many similar segments of the renal vasculature, including afferent and efferent arterioles, cortical capillaries, arcuate arteries and renal veins (Knight *et al.*, 1987; Barajas *et al.*, 1992). Stimulation of adrenergic nerves is known to cause a NE-mediated pressor response in the isolated perfused rat kidney (Schwartz and Malik, 1989). Therefore, it was possible that the pressor response to SP and the other tachykinins in the present study could have been an indirect effect mediated by NE released from sympathetic nerves. However, since phentolamine, an alpha-adrenergic receptor antagonist, blocked the pressor effect of NE but did not

inhibit the effect of SP in this preparation, the pressor response to tachykinins in rat kidney is not mediated by NE.

Effects of Peptidase Inhibitors and Indomethacin on Response to SP

Degradation of peptides by proteases may influence their effect. Biochemical studies have indicated that both neutral endopeptidase 24.11 (NEP) and angiotensin convert enzyme (ACE) are capable of inactivating SP and other tachykinins in many organs and in various animal species (Stephenson and Kenny, 1987; Turner, 1987; Frossard *et al.*, 1989; Rouissi *et al.*, 1990). It has been reported that in the rat kidney NEP and ACE are mainly located in the proximal tubular brush border (Stephenson and Kenny, 1987; Turner, 1987). These enzymes are also present in the endothelium of some arteries and veins (Turner, 1987; Ilorens *et al.*, 1992). In the present study, the pressor effect of SP was not potentiated by treatment with thiorphan to inhibit NEP or captopril to inhibit ACE, suggesting that significant degradation of SP by these peptidases does not occur in this experimental condition. However, at the highest dose of SP tested, the pressor responses were reduced. The reason for this is not clear. One possibility is that thiorphan and captopril may have increased the quantity of bradykinin in the renal vasculature. Bradykinin can produce vasodilation not only as an endocrine hormone but also as an autocrine/paracrine mediator (Oza *et al.*, 1990; Carretero and Scicli, 1991; Campbell *et al.*, 1993). Moreover, it is known that bradykinin is a potent dilator in the rat renal circulation (Fulton *et al.*, 1992) and can be produced in renal vessels from kallikrein-sensitive kininogens (Gardes

et al., 1990). It is also known that bradykinin can be inactivated by both NEP and ACE, and endogenous bradykinin is thought to participate in the antihypertensive and renal effects of ACE and NEP inhibitors (Rouissi *et al.*, 1990; Campbell *et al.*, 1993; Carretero and Scicli, 1991). Therefore, it is possible that intense vasoconstriction produced by the high dose of SP somehow stimulates the release of bradykinin from the renal vascular wall and that the peptidase inhibitors decrease its degradation. Consequently, a vasodilator effect from locally released bradykinin may partially counteract the vasoconstrictor response to SP.

Recently, Brelli *et al.* (1993) purified another enzyme from rat kidney that is also able to degrade SP. This enzyme, endopeptidase 24.16, is not sensitive to specific inhibitors of NEP 24,11 or ACE. It is not known if this enzyme has an impact on the response to SP in the isolated perfused kidney.

The pressor response to SP was not inhibited by the prostaglandin synthesis inhibitor, indomethacin, indicating that this response is not mediated by prostanoids. In contrast, the pressor response to SP was potentiated at 3 and 10 nmol doses in the presence of indomethacin, indicating that SP may stimulate the release of some relaxant prostanoids in rat kidney. Kopp and Smith (1993a) demonstrated that renal sensory receptor activation by substance P, capsaicin and bradykinin is dependent on intact renal prostaglandin synthesis and suggest that prostaglandins play a permissive role in renal pelvic sensory receptor activation by many stimuli. This finding indicates that SP can stimulate production of renal prostaglandins. Devillier *et al.* (1992) have demonstrated that SP causes

relaxation of rat tracheal smooth muscle and at same time induces a 6 fold increase in tracheal PGE₂ production. Preincubation with indomethacin abolished the relaxant effect of SP. It has also been reported that activation of tachykinin receptors is associated with the release of prostanoids, particularly PGE₂, from macrophages (Hartung *et al.*, 1986).

Effect of Capsaicin on Perfusion Pressure

SP has been found coexisting with other neuropeptides such as CGRP within a subpopulation of primary sensory neurons which are widely distributed at both the somatic and visceral level including the rat kidney and renal vasculature. These peptide containing neurons were found to be susceptible to the neurotoxic action of capsaicin (Wiessfeld-Hallin *et al.*, 1984; Manzini *et al.*, 1991). In a variety of tissues and organs, acute exposure to capsaicin induces excitation of SP/CGRP-containing primary afferents that leads to calcium-dependent neuropeptide release and can be followed by prolonged insensitivity to various stimuli, including capsaicin itself, and neuronal degeneration. Manzini *et al.* (1991) have demonstrated that superfusion of isolated rat mesenteric vascular bed with capsaicin produced a prompt and remarkable release of both SP-like and CGRP-like immunoreactivities from arteries and veins. It was also noted that in both tissues a second challenge with capsaicin resulted in no significant release of these peptides. In the present study, infusion of isolated rat kidney with capsaicin produced a biphasic effect on PP. It is possible that the pressor response might be through SP or tachykinins released from renal afferents, while vasodilation may

be mediated by CGRP. Anatomical studies have shown that CGRP coexists with SP in primary sensory nerve terminals in rat kidney (Knight *et al.*, 1991). There is also evidence that CGRP may have a vasodilator function in the renal vascular system (Geppetti *et al.*, 1989).

Duckles (1986) has demonstrated that cat cerebral artery and guinea pig thoracic aorta are locally regulated by sensory neuropeptides such as SP. Capsaicin transiently vasoconstricted these vessels before causing a vasodilation, but the vasoconstrictor effect in these vessels appeared to be a direct action not mediated by sensory neuropeptides. However, direct effects of capsaicin have not been observed in all kinds of vascular beds of different species. For example, in guinea pig isolated perfused heart, 1 μ M capsaicin caused a substantial release of SP and coronary vasodilation (Hoover, 1987). A second challenge with the same concentration of capsaicin did not release SP or affect perfusion pressure. These findings suggest that the effect of capsaicin on the guinea pig coronary vasculature is entirely mediated by sensory neuropeptides. Maggi and Meli (1988) proposed that desensitization of the acute responses to capsaicin is a useful functional marker to distinguish specific effects consequent to activation of sensory nerves from nonspecific effects of this substance. Therefore, further work will be needed for a conclusive determination regarding the ability of endogenous tachykinins to affect the renal vasculature.

Effects of Tachykinin Analogs on Perfusion Pressure

Amino-terminal Fragment of SP: SP(1-7) Structure-activity studies have

demonstrated that C-terminal fragments of tachykinins which are six amino acids in length or longer retain the biological actions of tachykinins, such as the contraction of smooth muscle. Amino-terminal fragments of tachykinins, on the other hand, lack activity at tachykinin receptors (Maggio, 1988). However, the N-terminus of tachykinins is involved in receptor-independent effects observed at high tachykinin concentrations. Jacques *et al.* (1989) have found that two positively charged amino acids (i.e., Arg and Lys) at the N-terminal of SP are essential for the triggering of prostaglandin and histamine release by this tachykinin. These responses appear to be mediated by the direct activation of a pertussis toxin sensitive G protein (Mousli *et al.*, 1990). The observation that SP(1-7) was essentially inactive in the present study suggests that the pressor response to tachykinins in isolated perfused rat kidney is C-terminal dependent and a tachykinin receptor-mediated event rather than a consequence of direct G-protein stimulation. The ability of other natural tachykinins (i.e., physaleamin, kassinin and NKA) to elicit a pressor response in rat kidney also supports the conclusion that tachykinin receptors are involved, since these peptides lack the structural features required for producing receptor-independent responses.

Selective Tachykinin Receptor Agonists Natural tachykinins have a very limited ability to discriminate among the three classes of tachykinin receptors. In the absence of selective tachykinin agonists and antagonists, the classification of tachykinin receptors in mammalian tissues has been based chiefly upon the relative order of potency of natural peptides and their analogues in various

bioassays or binding experiments (Lee *et al.*, 1986; Regoli *et al.*, 1987). Recently, several tachykinin analogues with a high degree of selectivity as agonists have been reported (Regoli *et al.*, 1988; Regoli *et al.*, 1991b). [MePhe⁷]NKB is a potent and highly selective agonist for the NK-3 receptor (Regoli *et al.*, 1988). In the present study, [MePhe⁷]NKB was shown to exert a dose-dependent vasoconstrictor responses that was significantly greater than that to SP. This suggests that NK-3 receptors contribute to the pressor action of tachykinins in rat kidney. Other investigators have provided evidence that NK-3 receptors are also present in the rat portal and mesenteric veins and likewise mediate vasoconstriction at these sites (Mastrangelo *et al.*, 1986; Orleans-Juste *et al.*, 1991).

In addition, the experiments described here also indicate that a population of NK-2 receptors is present in the renal vasculature of rat. This is based on the fact that the natural NK-2 agonist, NKA contracted renal blood vessels. Furthermore, the selective NK-2 agonist, GR64349 [(Lys³,Gly⁸-R- γ -lactam-leu⁹)NKA(3-10)] which has been shown to possess marked selectivity for NK-2 receptors over NK-1 and NK-3 receptors (Hagan *et al.*, 1991), caused a marked pressor effect that was essentially equal to that of the NK-3 agonist, [MePhe⁷]NKB.

In spite of the fact that NKA has a higher affinity than SP for NK-2 and NK-3 receptors (Regoli *et al.*, 1988), NKA was less potent than SP in evoking a pressor response in the rat kidney. This discrepancy may be due to degradation of NKA by peptidases in the kidney. Hagan *et al.* (1991) have demonstrated that the

potency of NKA in contracting guinea pig trachea was increased by over 100 fold in the presence of peptidase inhibitors. In contrast, our previous studies have demonstrated that the potency of SP in the isolated perfused rat kidney was not affected by peptidase inhibitors (Chen and Hoover, 1992).

During the course of this study, we also tried to determine the effect of two other selective NK-2 agonists (i.e., Nle¹⁰-NKA(4-10) and Ala⁸-NKA(4-10)) and the endogenous NK-3 receptor ligand, NKB. These experiments were not successful because of the limited water solubility of these compounds. This factor made it necessary to use DMSO as a vehicle, and DMSO affected PP substantially at the dilutions that would be required to achieve tachykinin concentrations in the effective range.

Using Met(O₂) as the C-terminal residue and replacing Gly⁹ with Sar (sarcosine), a potent and selective NK-1 receptor agonist was obtained that was three fold more active than SP on the NK-1 and inactive on the NK-2 and NK-3 receptor systems (Regoli *et al.*, 1988). In the present study, [Sar⁹,Met(O₂)¹¹]SP was inactive, suggesting that the pressor effect of tachykinins is not mediated by NK-1 receptors in rat kidney.

Although it has been proven that SP and [Sar⁹,Met(O₂)¹¹]SP are potent hypotensive agents in the rat *in vivo* (Couture *et al.*, 1989), these peptides caused no vasodilation in rat renal vasculature in the present study, even though the endothelium was intact, as demonstrated by the ability of acetylcholine to elicit a vasodilator response in this preparation (data not shown). The present finding

therefore suggests that NK-1 receptor activation is not involved in any detectable vasoactive response of the rat isolated kidney. Thus, this particular vascular bed may not contribute to the overall hypotensive effect of SP administered intravenously in the rat.

Studies of *in vitro* Receptor Autoradiography

NK-3 Receptors in Rat Brain

^{125}I -[MePhe⁷]NKB has been reported as a high affinity and selective ligand for the human and rat NK-3 receptors (Sadowski *et al.*, 1993). In the present study we used ^{125}I -[MePhe⁷]NKB to determine the presence and localization of NK-3 receptors in rat brain and kidney. In our control study using coronal sections of rat brain, the distribution of NK-3 binding sites visualized with ^{125}I -[MePhe⁷]NKB was very similar to that previously reported using another NK-3 selective ligand, [³H]senktide (Dam *et al.*, 1990; Stoessl and Hill, 1990; Quirion *et al.*, 1991). Furthermore, ^{125}I -[MePhe⁷]NKB binding to rat brain was completely blocked in the presence of excess unlabeled senktide. These data give further support to the conclusion that ^{125}I -[MePhe⁷]NKB is a highly selective ligand for the NK-3 receptor.

NK-3 Receptors on the Renal Vein

Film autoradiography with ^{125}I -[MePhe⁷]NKB demonstrated that NK-3 receptors are found on the rat renal veins and their large branches. Experiments with higher resolution emulsion autoradiography revealed the localization of these

NK-3 receptors on the vascular tunica media. Unexpectedly, no specific binding sites for [MePhe⁷]NKB were detectable in the renal artery system. Now the question has to be raised as to whether the [MePhe⁷]NKB binding sites shown in renal veins in autoradiography corresponded to NK-3 receptors identified in our functional studies. Generally, it is considered that in the physiological condition, the venous system makes little contribution to vascular resistance, especially in the renal vascular bed (Ulfendahl and Wolgast, 1992). However, the fact that clamping the renal vein significantly increased PP in the isolated rat kidney suggests that intense constriction of the renal vein following bolus injection of the tachykinins would be expected to increase PP when the isolated kidney is perfused at a constant flow rate. Indeed, SP and NKB are constrictors of isolated veins such as the rat portal vein and rabbit jugular vein (Mastrangelo *et al.*, 1986; Nantel *et al.*, 1990). Furthermore, neurokinins act on the mesenteric bed of the rat to increase the PP of the venous vasculature via activation of NK-3 receptors while NK-3 agonists are inactive on the arterial mesenteric vasculature (Orleans-Juste *et al.*, 1991). These results are in agreement with those we obtained in isolated rat kidney.

The study of venous function has always been second to that of arterial function. This is in part due to the assumption that the responses of arteries are more important than those of veins. It must also be because of the methodological problems involved in the study of the venous circulation and the responses of the veins have to be interpreted indirectly from other measures *in*

vivo. However, recently, with the double simultaneously perfused mesenteric arterial and venous preparation which allows the responses of the arteries and veins of a single perfused vascular bed to be compared, Warner (1990) has demonstrated that the vasoactive agents such as NE, AII and arginine vasopressin show pronounced arterial-venous selectivity and the venous portion of the vasculature is significantly involved in the responses of the intact circulation. Other investigators (Grega and Adamski, 1987) also indicate that large vein constriction is proportionately greater than the large artery constriction to NE and venous resistance may also contribute in an important manner to increase in total vascular resistance. Large vein constriction not only decreases vascular volume, promoting increases in venous return, but may provide 15-20% of the total resistance to flow during local infusions of NE, serotonin, or dopamine in the cutaneous vasculature.

Grega and Adamski (1987) intimate that patterns of neurogenic constriction may be as diverse as those produced by vasoconstrictor agents, and these differences may reflect the interplay between function and design to subserve specific physiological activities. Large vessels participate in vasoconstrictor responses triggered by stimuli that impose a severe stress on the circulation. Constriction of large arteries and large veins is, for example, a characteristic feature of circulatory shock states. In contrast, small vessels participate primarily in normal vascular adjustments required to maintain blood pressure at the set point. Certainly the physiological significance of the occurrence of NK-3

receptors on the rat renal veins remains to be elucidated. However, the local release of sensory neuropeptides such as tachykinin in renal venous bed might have functions more than changes of the renal capacitance and venous return by the control of venous tone. For example, they may be mediate the defense response through neurogenic inflammation or have a trophic role for tissue protection and repair (Maggi and Meli, 1988).

The doses of [MePhe⁷]NKB used in our perfusion study were relatively higher than that used in isolated mesenteric veins (Orleans-Juste *et al.*, 1991).

Differences in receptor density on the vessels may contribute to this difference in sensitivity. Additionally, the unique characteristics of the renal vascular bed may be another factor. Since NK-3 receptors were on the renal venous side and the tachykinins were administered from renal arterial side in functional studies, these peptides had to pass through partial filtration in glomeruli and partial exchange in second capillaries in renal medulla and possible enzyme inactivation along the way before reaching to receptor sites.

NK-3 Receptors on the Renal Pelvis and Ureter

In addition to renal veins, specific [MePhe⁷]NKB binding sites were also found on the renal pelvis and ureter. These results are consistent with some anatomic and functional studies. It has been demonstrated that the walls of the renal pelvis and ureter are rich in unmyelinated nerve fibers (Marfurt and Echtenkamp, 1991) and SP-immunoreactive nerves were found to innervate these sites (Knight *et al.*, 1987; Ferguson and Bell, 1985). NKB-like immunoreactivity has been found in

homogenate of rat kidney (Tateishi *et al.*, 1990), however, there have been no studies on the localization of NKB immunoreactive fibers in the rat kidney. Urine produced in the kidney is propelled toward the urinary bladder by ureteral peristalsis, which is a primarily myogenic phenomenon initiated by pacemaker cells in the proximal part of renal pelvis (Constantinous, 1974). Circular muscle strips of the guinea pig renal pelvis exhibit a spontaneous phasic contractile activity, which is augmented by NKA and SP (Maggi *et al.*, 1992). Electrical field stimulation produces a positive inotropic effect that is unaffected by atropine and guanethidine but abolished by *in vitro* capsaicin desensitization (Maggi *et al.*, 1992). Contractile effects of NKA and SP were also observed in the ureter (Hua *et al.*, 1987; Amann *et al.*, 1988). These data suggest that the release of tachykinins from primary afferents may produce local motor responses in the renal pelvis and ureter. This action may be a defensive mechanism which could facilitate removal of irritants present in the urine and protect the kidney during obstruction and ureteral antiperistalsis (Maggi *et al.*, 1992).

NK-3 Receptors on the Outer Medulla of Kidney

Film autoradiography has shown that ^{125}I -[MePhe⁷]NKB binds specifically in a lower density to the inner stripe of the outer medulla in rat kidney. However, the resolution of this method does not permit a discrimination among the various potential cellular sites for NK-3 receptors in these areas. It has been recognized that the renal medulla plays a fundamental role in maintaining body fluid and electrolyte homeostasis through the medullary microcirculation and through

urinary concentrating mechanisms (Wilhelm, 1981).

Anatomically, the renal medulla is divided into an outer medulla subdivided into an outer stripe and inner stripe, and an inner medulla. The renal medulla is composed of the loops of Henle and the collecting ducts and vessels. The inner stripe comprises descending thin limbs, ascending thick limbs and collecting ducts. The blood supply of the medulla in intact kidney is entirely postglomerular. The juxtamedullary efferent arterioles divide in the outer stripe to form the descending vasa recta which then descend through the medulla in cone-shaped bundle. At intervals a descending vasa rectum leaves the bundle to supply an adjacent capillary plexus. The physiological role of NK-3 receptors in the inner stripe of the outer medulla remains to be clarified. Several cell types could serve as the targets of tachykinins within this architecture.

It has been reported that intrarenal infusion of SP produces an increase in renal blood flow, urine volume and sodium excretion in dog (Pernow, 1983; Defelice and Brousseau, 1988). These effects result presumably from the endothelium dependent vasodilator action of SP on afferent glomerular arterioles of the kidney (Cairns *et al.*, 1991). Other investigators demonstrated that in anesthetized rats, infusion of SP at 50 pg/min into the abdominal aorta above the renal arteries produces diuretic and natriuretic effects which are considered to be responsible for the reduction of salt and water reabsorption by proximal convoluted tubule (Arendshorst *et al.*, 1976). Whether SP or other tachykinins have any effect such as modulation of sodium and water transport through tachykinin receptors in the

thick ascending limbs of the loop of Henle or other tubular component in the renal medulla is unknown.

In the renal medulla, interstitial cells, like the rungs of the ladder are transversely interposed between the longitudinally running tubules and vessels and are thought to adjust the distance between tubules and vessels (Wilhelm, 1981). They specifically produce prostaglandins (PGs) (Wilhelm, 1981; Kopp and Smith, 1993a), and stimulation of renal PG production by SP has been suggested in the present study and other investigations (Kopp and Smith, 1993a). Thus, SP/tachykinins might act on these cells to produce PGs, which in turn can have widespread influences on medullary structures. Prostaglandins can antagonize the effects of vasopressin on collecting ducts (Wilson, 1992), which may contribute to the diuretic actions of SP. PGs also inhibit sodium reabsorption by collecting tubules provoking natriuresis and diuresis (Iino and Imai, 1978; Stokes and Kokko, 1977).

Another possible site of tachykinin binding is the pericyte, a modified smooth muscle cell that replaces smooth muscle around the descending vasa recta (Wilhelm, 1981). Tachykinin acting here could influence medullary blood flow patterns, perhaps reducing the efficiency of countercurrent mechanisms and leading to natriuresis. Furthermore, if NK-3 receptors are on these cells, they may be involved in neurogenic inflammation of the kidney. Tachykinins have been found to enhance vascular permeability through a direct action on capillary-venular permeability, and NK-3 receptors have been proposed to mediate this

response (Couture and Kerouac, 1987).

Both senktide and eledoisin were able to compete with [MePhe⁷]NKB for binding sites in the rat kidney, and this was anticipated based on their known high affinities for the NK-3 receptor. However, it was not anticipated that senktide would be more effective than [MePhe⁷]NKB in competing with radiolabelled [MePhe⁷]NKB at selected sites. This suggests that specific binding sites in different regions of the kidney may differ regarding the relative affinities of these NK-3 agonists and hints at the possibility of receptor subtypes. As expected, SP was less effective than all other agonists in competing with radiolabelled [MePhe⁷]NKB for binding sites in rat kidney. This is consistent with the fact that SP binds to NK-3 receptors with a much lower affinity than at NK-1 and NK-2 receptors. Nevertheless, the autoradiographic results do document binding of SP to NK-3 receptors and support the suggestion that pressor responses to SP are due at least in part to effects at NK-3 receptors.

SP Binding Sites in Rat Kidney

¹²⁵I-BHSP is one of the ligands which has been used in many NK-1 receptor binding studies (Quirion *et al.*, 1991). In our control sections of rat brain, a high density of specific ¹²⁵I-BHSP binding sites was observed in the caudate-putamen and olfactory tubercle which is in agreement with previous reports (Mantyh *et al.*, 1989; Danks *et al.*, 1986).

Specific BH-SP binding sites in section of rat kidney were primarily confined to the renal artery and its large branches, where the grains appeared to be

localized to the media/adventitial border or the adventitia. Like ^{125}I -[MePhe⁷]NKB, specific binding of radiolabelled SP was also identified in the renal pelvis. Based on the known K_d values for SP at the three types of tachykinin receptor, relatively selective labelling of the NK-1 receptor was anticipated at the concentration of radiolabelled SP used in this study. Furthermore, binding of radiolabelled SP was completely eliminated in the presence of unlabeled SP and [Sar⁹,Met(O₂)¹¹]SP, a NK-1 selective agonist. Accordingly, the sites identified with radiolabelled SP are most likely to be NK-1 receptors.

Stephenson *et al.* (1987) have reported that ^{125}I -BHSP binding to dog kidney was localized over glomeruli and endothelium of intrarenal arteries and endothelium and adventitia of the renal artery. In this species, SP produces a vasodilator effect on dog kidney through stimulation of NK-1 receptors on vascular endothelium and release of EDRF. Although specific binding of BH-SP was also associated with the rat renal artery in the present study, these sites appeared to be associated with adventitia instead of intima.

There is strong evidence that capsaicin sensitive afferent neurons participate in afferent sensitization and the reflex regulation of renal functions in addition to the possible local effect on the tone of vessels, pelvis and ureter. Renal mechanoreceptors are mechanosensitive fibers that monitor hydrostatic changes within the kidney while renal chemoreceptors respond to alterations in the composition of the interstitial or pelvic environment (Kopp, 1993). Evidence that SP fibers may be involved in the transmission of afferent input from the kidney to

the central nervous system is provided by investigations that combined retrograde fluorescent dye labeling and immunolocalization methods. In studying the rat and guinea pig, Su *et al.* (1986) found a dense network of SP and CGRP fibers associated with the smooth muscle and epithelium of the renal pelvis, and in the interlobar branches of the renal artery and in the hilar connective tissue. Ferguson *et al.* (1986) also reported abundant SP-immunoreactive nerve fibers in the wall of the proximal ureter and renal pelvis and around the larger renal blood vessels. These findings emphasize the possible importance of SP and CGRP-positive fibers in renal afferent transmission. It has been shown that renal pelvic administration of SP increased ipsilateral afferent renal nerve activity and contralateral urine flow rate and urinary sodium excretion (Kopp and Smith, 1991). Furthermore, the NK-1 receptor antagonist CP-96,345 produced a competitive blockade of the increase in afferent renal nerve activity (Kopp and Smith, 1993b). These data suggested that the presence of functional SP receptors in the renal pelvic area, activation of which would result in a similar inhibitory contralateral renorenal reflex responses as increased ureter pressure. The results of our *in vitro* autoradiography using ^{125}I -BHSP showed the presence of NK-1 receptors in the walls of renal artery and renal pelvis of rat and may represent an anatomical correlate of the above referenced functional observations. Thus, the NK-1 receptors present in the rat renal artery and pelvis might have a function different from that of NK-3 receptors localized in renal vein and pelvis. Neuropeptides such as SP present in primary sensory nerve terminals may have

dual role through different tachykinin receptors in the maintenance of homeostasis. Stimuli such as increased ureteral pressure or renal ischemia may activate one kind of tachykinin receptors (e.g., NK-1) on afferents in renal blood vessel or renal pelvis and ureter, to transmit the perceived information to the central nervous system and initiates both voluntary and autonomic reflexes. Additionally, activation of another tachykinin receptor (e.g., NK-3) could lead to local tissue responses at the site of stimulation to promote resistance of the tissue against further damage and aid the repair of injury.

Summary

1. Tachykinins can cause a vasoconstrictor effect in the rat renal vascular bed which has some pre-existing tone produced by phenylephrine or angiotensin II.
2. The vasoconstrictor effect of SP is not blocked by phentolamine, indicating that norepinephrine does not mediate this response.
3. The pressor response to SP is not enhanced by thiorphan or captopril, indicating that neutral endopeptidase and ACE are not involved in SP degradation under the experimental conditions.
4. The pressor response to SP is not mediated by vasoconstrictor prostanoids, but SP may release some relaxant prostanoid in rat kidney.
5. The N-terminal fragment SP (1-7) had no effect on PP, indicating that the pressor response to SP is C-terminal dependent and tachykinin receptor

mediated.

6. The selective NK-1 receptor agonist, [Sar⁹,Met(O₂)¹¹]SP, was inactive and the selective NK-2 and NK-3 receptor agonists, [MePhe⁷]NKB and GR64349, resulted in much larger pressor responses than that of SP. These observations suggest that the vasoconstrictor effect of tachykinins in the isolated rat kidney is mediated by NK-2 and NK-3 receptors.
7. Capsaicin produced a biphasic change of renal vascular tone, indicating that endogenous sensory neuropeptide might be able to influence the renal vasculature.
8. By *in vitro* receptor autoradiography, specific NK-3 ligand binding sites were localized to the vascular tunica media of the renal vein, tunic muscularis of the renal pelvis and ureter and inner strip of the outer medulla, while NK-1 receptors were localized to the renal artery and pelvis.
9. Partially occluding or clamping the renal vein increased perfusion pressure in the isolated rat kidney.

Conclusion

Tachykinins have vasoconstrictor effects mediated by tachykinin receptors in the isolated perfused rat kidney. Experiments with selective tachykinin agonists established that these responses could occur through stimulation of NK-2 and/or NK-3 receptors. Activation of NK-3 receptors on smooth muscle of the renal vein can increase perfusion pressure in the isolated kidney perfused at a constant rate.

NK-2 receptors mediating pressor responses could be on smooth muscle of the renal resistance vessels and/or the renal vein. However, the localization of NK-2 receptors in the renal vasculature still needs to be determined by using selective radioligands.

Implications for Further Research

The present studies with synthetic tachykinins and their agonists have demonstrated the pharmacological effects of tachykinins in the isolated perfused rat kidney and provided some anatomical localization of tachykinin receptors within the kidney. However, the physiological significance of these findings is currently unknown and would be an interesting and important topic for future study. Results from preliminary work with capsaicin reported here could indicate that endogenous sensory neuropeptides are able to produce local effects in the kidney. However, the possibility that capsaicin may have a direct local constrictor effect unrelated to sensory neuropeptides needs to be eliminated.

In vitro film autoradiography of frozen kidney sections revealed a low density of NK-3 receptors in the inner strip of the outer medulla, but it lacked adequate resolution to localize receptors to specific cells. Further investigation by using improved high resolution light and electron microscopic autoradiography (Zhou *et al.*, 1992) will be needed to determine the cellular localization of these receptors.

Finally, studies are necessary to clarify the pathophysiological role of neuropeptides released from capsaicin sensitive nerve terminals in the renal

vascular bed and kidney. Hence, future work might be directed at related pathological animal models or some human disease such as certain types of hypertension to investigate possible tachykinin involvement.

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Abstracts:

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