CHARACTERIZATION OF *IN VITRO* RELEASE OF NEUROCHEMICALS FROM THE INTERMEDIATE AREA OF THE RAT THORACIC SPINAL CORD: REGULATION BY COEXISTING NEUROCHEMICALS AND PRESYNAPTIC AUTORECEPTORS

1995

YANG

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913

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Name of Candidate: Ling Yang
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Committee Chairperson	7/5/95 Date
Committee Member	6-16-95 Date
Committee Member	6-16-95. Date
Committee Member	6-16-95 Date
Committee Member	Date

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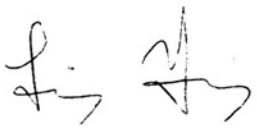

Ling Yang
Department of Pharmacology
Uniformed Services University of the Health Sciences

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Ling Yang
Department of Pharmacology
Uniformed Services University
of the Health Sciences

ABSTRACT

Serotonin (5-HT), substance P (SP), neurokinin A (NKA) and thyrotropin-releasing hormone (TRH) coexist in the ventral medullary neurons which project to the intermediolateral cell column (IML, site of sympathetic preganglionic neurons) of the thoracic spinal cord. It is not known whether these neurochemicals are released from the IML, interact with each other and thus regulate the activities of the sympathetic nervous system. In this thesis, the release of 5-HT, SP and NKA from the microdissected intermediate area (includes the IML) of the rat thoracic spinal cord is investigated using an *in vitro* superfusion system. Regulation of the release of 5-HT and SP by presynaptic autoreceptors and the other coexisting neurochemicals is also studied.

Potassium depolarization induces extracellular Ca^{2+}-dependent differential release of [^{3}H]5-HT, SP and NKA from the intermediate area. The release of [^{3}H]5-HT is induced by lower concentrations of K^+, while the release of SP and NKA requires higher concentrations of K^+. The release of [^{3}H]5-HT is negatively regulated by 5-HT through activation of presynaptic 5-HT_{1B} inhibitory autoreceptors and positively regulated by SP through activation of presynaptic NK_1 autoreceptors. The release of SP, comes at least partly, from serotoninergic nerve terminals, as

serotoninergic neurotoxin pretreatment decreased SP content and the absolute amount of SP released from the intermediate area. However, the release of SP is probably not under the influence of 5-HT because 5-HT and 5-HT agonists failed to change the percentage of fractional release of SP. The release of SP is possibly regulated by presynaptic NK_1 receptors, as an NK_1 receptor antagonist (GR 82334) dose-dependently increased the K^+-stimulated release of SP. Neither NKA, TRH nor their analogues altered the release of [^{3}H]5-HT or SP.

These data demonstrate for the first time that coexisting neurotransmitter (5-HT) and neuropeptides (SP and NKA) are differentially released from the intermediate area of the rat thoracic spinal cord, and the release of one neurochemical is regulated by presynaptic inhibitory autoreceptors as well as the other coexisting neurochemicals. These information are essential in understanding the spinal regulation of the sympathetic nervous system.

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by

Ling Yang

Dissertation submitted to
the Faculty of the Department of Pharmacology Graduate Program of the Uniformed Services of the Health Sciences
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DEDICATION

To My Loving Husband, Wei-Liang.

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List of Abbreviations

CA:	central autonomic nucleus
CNS:	central nervous system
5,7-DHT:	5,7-dihydroxytryptamine
5-HT:	5-hydroxytryptamine, serotonin
IC:	nucleus intercalatus spinalis
IML:	intermediolateral cell column
IMLf:	IML pars funiculars
IMLp:	IML pars principalis
IR:	immunoreactivity
MAP:	mean arterial pressure
NKA:	neurokinin A
NKB:	neurokinin B
NTS:	nucleus tractus solitarius
PGi:	nucleus paragiganto-cellular reticularis
PPR:	parapyramidal region
RIA:	radioimmunoassay
RMca:	nucleus reticularis magnocellularis pars alpha
RMg:	nucleus raphe magnus
RPa:	nucleus raphe pallidus
SP:	substance P
SPN:	sympathetic preganglionic neuron
TRH:	thyrotropin-releasing hormone
TK:	tachykinin
VM:	ventral medulla

A. INTRODUCTION

Neurons located in the ventral medulla (midline raphe and the parapyramidal region) project to the intermediolateral cell column (IML) of the thoracic spinal cord and thus, modulate sympathetic innervation to the cardiovascular system. Serotonin (5-HT), substance P (SP), thyrotropin-releasing hormone (TRH) and neurokinin A (NKA) coexist in these IML-projecting neurons. We propose that each of these colocalized neurochemicals (*i.e.*, 5-HT, SP, TRH and NKA) is released from the IML and affects the cardiovascular system either directly through interactions with IML neurons or indirectly by modifying the effects of other colocalized agents.

The primary objectives of this project are: (1) verify that 5-HT, SP, NKA and TRH are released from the intermediate area (which includes the IML); (2) assess presynaptic autoreceptor regulation of the release and (3) study the interactions of coexisting neurochemicals with presynaptic autoreceptors in modulating the release of these coexisting neurochemicals. The experiments will provide insights into the neurochemical modulation of transmitter release in the IML. This information may ultimately prove useful for therapeutic manipulations of sympathetic input to the cardiovascular system. In addition, the information will provide more general insights into the neurobiology of

colocalized neurochemicals.

In this thesis, the first section provides background information about the bulbospinal projection (ventral medulla and IML), characteristics (distribution, receptor binding and autonomic-related function) of coexisting neurochemicals (*i.e.*, 5-HT, SP, NKA and TRH) in the bulbospinal projection and general information about the release of neurotransmitter/neuropeptide and autoreceptor regulation of the release. The experiments on the release of [^{3}H]5-HT, endogenous SP and NKA, presynaptic 5-HT_{1B} /or possible NK_1 autoreceptors and other coexisting neuropeptides (NKA and TRH) modulation of 5-HT /or SP release in the intermediate area of rat thoracic spinal cord will then be described. Lastly, the data collected from the present experiments and their functional importance will be discussed.

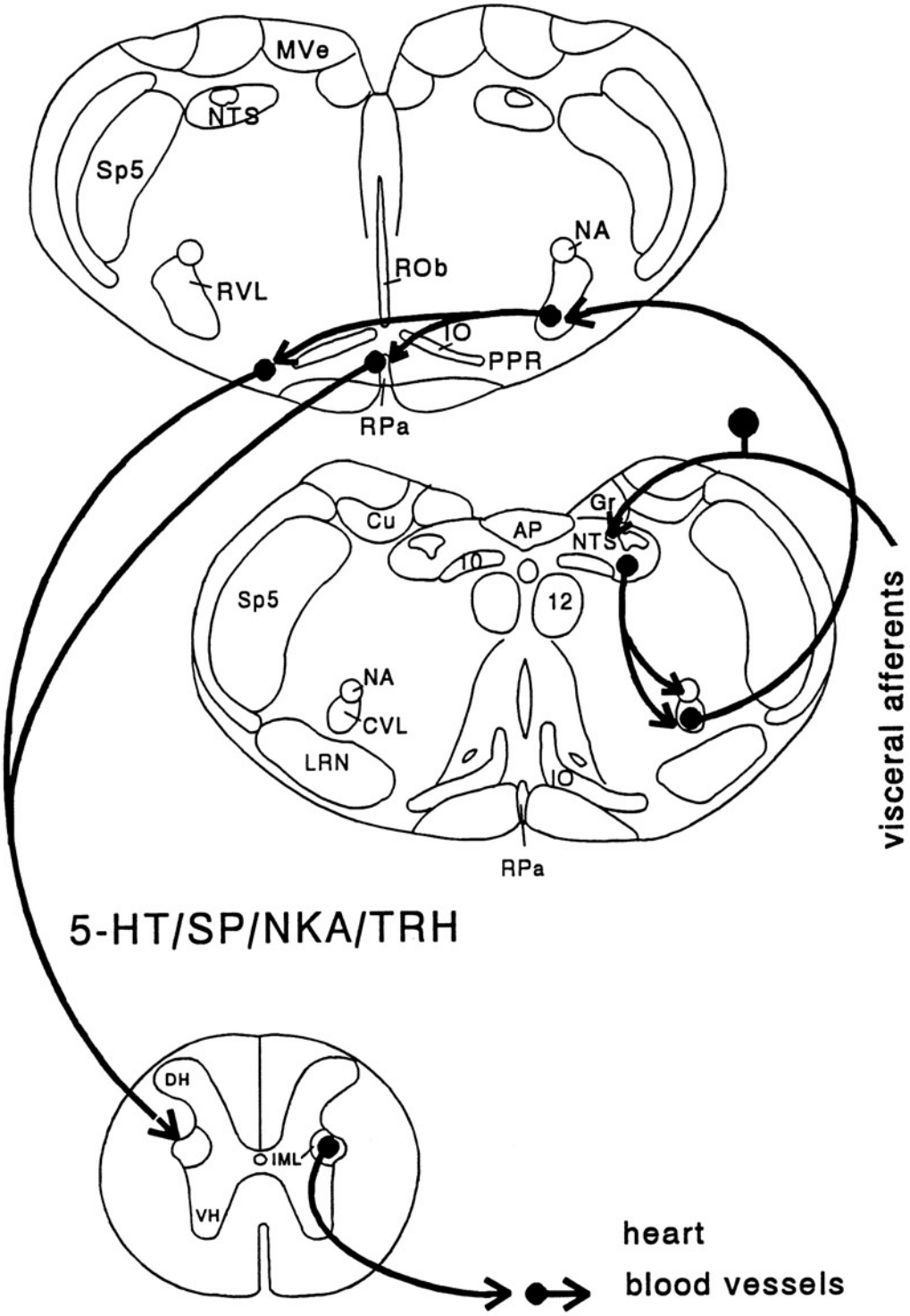
A. BULBOSPINAL PROJECTION: VENTRAL MEDULLA TO INTERMEDIOLATERAL CELL COLUMN

Axons of ventral medulla (VM) neurons course in the region of the dorsolateral and ventrolateral funiculi and terminate at the IML and the central autonomic area of the thoracic spinal cord (Ciriello *et al.*, 1986). This pathway is a bulbospinal projection (Fig. 1), which is involved in the central maintenance of vasomotor tone and regulation of mean arterial pressure (MAP) (Caverson *et al.*, 1983; McAllen *et al.*, 1982).

a. Intermediolateral Cell Column

The IML, a cell column in the region of the lateral horn in the thoracic and upper lumbar spinal cord segments (T_1-L_2), is the site of origin of sympathetic preganglionic neurons (SPNs). Petras and Cummings (1972) subdivided the IML into two subnuclei: the IML pars principalis (IMLp), which contains the largest number of SPNs in the thoraco-lumbar (T-L) cord, and the IML pars funicularis (IMLf), the part of IML which extends into the white matter. Small numbers of SPNs are also found in the nucleus intercalatus spinalis (IC): the transverse band of SPNs spanning the area

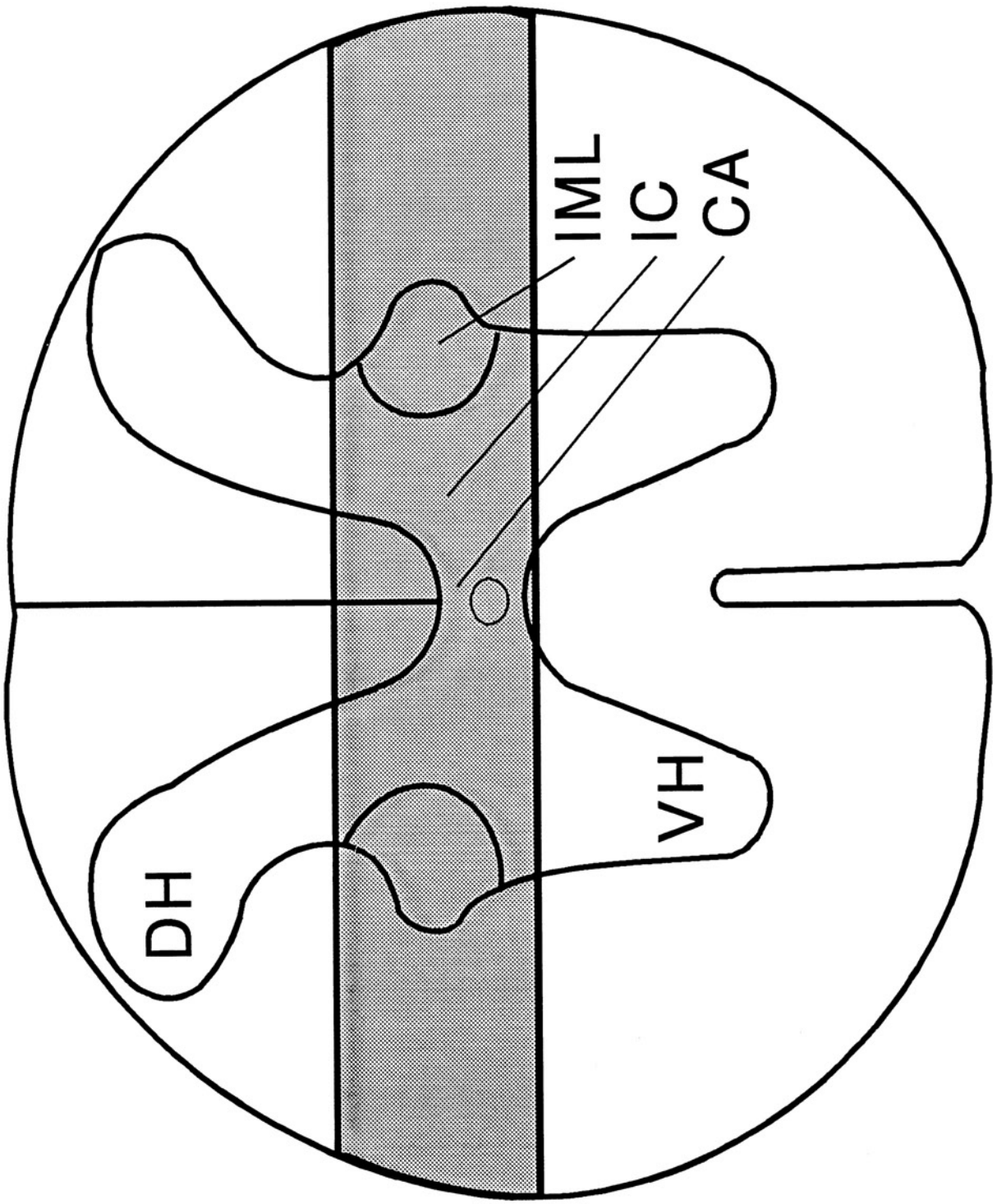
Fig. 1. Schematic of bulbospinal projections from the ventral medulla to the IML of the thoracic spinal cord. SP, NKA, 5-HT and TRH coexist in the bulbospinal pathway. The ventral medulla is innervated by the NTS, which receives the visceral afferents. Abbreviations: AP:area postrema; Cu:cuneate nucleus; DH:dorsal horn; Gr:nucleus gracilis; IML:intermediolateral cell column; LRN:lateral reticular nucleus; MVe:medial vestibular nucleus; NA:nucleus ambiguous; NTS:nucleus tractus solitarius; PPR:parapyramidal region; RPa:nucleus raphe pallidus; ROb:nucleus raphe obscuras; RVL:nucleus reticularis ventralis; Sp5:spinal nucleus of the trigeminal nerve; VH:ventral horn.


MVe
NTS
Sp5
NA
ROb
RVL
IO
PPR
RPa
Cu
AP
Gr
NTS
IO
12
Sp5
NA
CVL
LRN
IO
RPa
visceral afferents
5-HT/SP/NKA/TRH
DH
IML
VH
heart
blood vessels

between the IML and the central canal, and the central autonomic nucleus (CA): the region dorsal and dorsolateral to the central canal (Fig. 2). At each level, the majority of neurons are found in the IMLp, with the next-largest numbers in the IMLf and very few in the IC and CA (Petras and Cummings, 1972). The intermediate area of the rat thoracic spinal cord (Fig. 2) includes the IML, IC and CA.

Cells of the IML are described as bi- or multipolar cells and these cells have tonic or constant activities in the absence of external stimuli (Polosa *et al.*, 1979). The origin of sympathetic tone of SPNs in the IML is due to continuous excitatory and inhibitory synaptic inputs from both the peripheral and the central sources. Peripheral sensory input from somatic and visceral receptors, arterial baroreceptors and chemoreceptors initiates the synaptic activity of SPNs. The responsiveness of SPNs to peripheral sensory input is modulated by central synaptic input from supraspinal neural systems (Laskey and Polosa, 1988). The activity of SPNs, sympathetic ganglion neurons, and cardiovascular parameters such as MAP, heart rate, total peripheral resistance and cardiac contractility can be altered by increasing or decreasing the input to SPNs from neurons in the VM, the caudal portion of the pons, and hypothalamic nuclei by electrical or chemical stimulation, surgical or chemical lesions and pharmacological

Fig. 2. Schematic drawing of the intermediate area (includes the intermediolateral cell column, intercalated nucleus and central autonomic nucleus) of the rat thoracic spinal cord. Abbreviations: CA: central autonomic nucleus; DH: dorsal horn; IC: intercalated nucleus; IML: intermediolateral cell column; VH: ventral horn.


IML
IC
CA
VH
DH

manipulations of synaptic transmission (Laskey and Polosa, 1988).

Neurons in the IML give rise to sympathetic preganglionic fibers that emerge via the ventral root and accompany the somatic nerve trunks as they leave the spinal column. Shortly after, these thinly myelinated fibers branch off to form the white rami. Most of these fibers make synaptic connections in the lateral sympathetic ganglia, but some extend to the collateral ganglia before synapsing. Retrograde transport studies found that each sympathetic ganglion is innervated by SPNs located at several segments, although one segment is the principle source. However, the rostrocaudal origins of SPNs innervate different ganglia according to the relative rostrocaudal locations of the target ganglia (Strack *et al.*, 1988). The postsynaptic fibers arising from the sympathetic ganglia innervate different autonomic related organs: heart, gland, etc. (Laskey and Polosa, 1988).

The IML, with SPNs, is probably the final site at which major integration occurs in the control of cardiovascular system at rest, during exercise, in response to stress and of course in various pathological conditions. Multiple neurochemicals are known to modify the IML neurons responsiveness to input and thus, the function of sympathetic nervous system. The major portion of axon terminals in the IML are immunoreactive for dopamine β-

hydroxylase (an enzyme necessary for the synthesis of norepinephrine) (Loewy *et al.*, 1979). Serotonin-immunoreactivity (IR), SP-IR, NKA-IR and TRH-IR terminals have all been demonstrated surrounding identified SPNs of the IML and these terminals originate predominantly from the nuclei of the VM (Bowker *et al.*, 1982; Helke *et al.*, 1982; Holets and Elde, 1982).

b. Ventral Medulla

The central nervous system (CNS) control of the cardiovascular system involves the coordination of a series of complex neural mechanisms. The VM is the vasomotor center which integrates afferent information regarding cardiovascular variables from central structures and peripheral sensory receptors and produces control signals to effector organs (SPNs in the IML of the thoracic spinal cord) for physiological responses (Ciriello *et al.*, 1986; Dampney, 1994).

VM is a designation for a functionally identified area within the reticular formation of the ventral surface of the medulla oblongata that is involved in the autonomic regulation (Ciriello *et al.*, 1986; Dampney *et al.*, 1982). Terminology for defining different groups of nuclei in the VM is confusing. However, each group of nuclei can be distinguished on their morphological characteristics: size, shape, orientations and processes. In general, there are different groups of neurons: the rostral VM contains nucleus raphe magnus (RMg) and nucleus raphe pallidus (RPa); the rostral ventrolateral medulla contains nucleus paragigantocellular reticularis (PGi) and nucleus reticularis magnocellularis pars alpha (RMca); and parapyramidal region (PPR, neurons located close to the ventral surface and lateral to the pyramidal tract) (Newman, 1985; Sasek and

Helke, 1989).

Axons of these VM neurons project directly to the IML and intermediate area of the thoracic spinal cord. Retrograde labeling studies injecting rhodamine B into IML resulted in retrogradely labeled cell bodies within the VM (Charlton and Helke, 1987; Hirsch and Helke, 1988). Single unit recording techniques showed that electrical stimulation of the IML antidromically activated neurons within the VM (Caverson *et al.*, 1984). VM-IML projecting neurons were observed to respond orthodromically to selective excitation of arterial baroreceptors and peripheral chemoreceptors, and to electrical stimulation of the carotid sinus and aortic depressor nerves (Caverson *et al.*, 1984). These findings suggest that neurons in the VM project directly to the IML and these neurons are components of sympathoexcitatory or sympathoinhibitory pathways involved in the regulation of MAP.

IML-projecting neurons in the rostral VM receive catecholaminergic innervation from the lateral hypothalamic area, the nucleus tractus solitarius (NTS) at brainstem, the primary site of termination of cardiovascular afferent fibers (Fig. 1; Loewy and Burton, 1978; Tanaka *et al.*, 1994). It is clear that the VM is an important integrating station for peripheral and central cardiovascular afferent information.

Detailed information is available on the neuroactive

substances (e.g., 5-HT, SP, NKA, enkephalin and TRH) present within the VM IML-projecting neurons. For example, spinally projecting TRH-IR neurons are found in the VM reticular nuclei, nucleus interfascicularis hypoglossi and PGi (Helke *et al.*, 1986a; Hirsch and Helke, 1988). SP-IR neurons are located in the RPa, RMca and PPR (Charlton and Helke, 1987) and serotonin-IR neurons are contained in the RMg, RPa, RMca, PGi and PPR of the VM (Loewy and Mckellar, 1981). There are some VM IML-projecting neurons containing SP but not 5-HT (Helke *et al.*, 1982; Helke *et al.*, 1986b) and IML-projecting neurons containing only 5-HT but not SP (Lowey and McKellar, 1981). However, Sasek *et al.* (1990) suggested that most if not all SP in VM neurons coexists with 5-HT. Immunohistochemistry combined with retrograde studies demonstrated the co-existence of 5-HT, SP, NKA and TRH in the VM IML-projecting neurons (Nevin *et al.*, 1994; Sasek *et al.*, 1990).

B. 5-HT, SP, NKA AND TRH COLOCALIZATION IN THE BULBOSPINAL PROJECTION

a. Serotonin

Serotonin (5-hydroxytryptamine), a classical neurotransmitter in the CNS, was first identified as an endogenous vasoconstrictor in the serum (Rapport *et al.*, 1947). In the following years, the structure of serotonin was identified as 5-HT (Rapport, 1949) and was subsequently synthesized (Hamlin and Fisher, 1951). Shortly thereafter, the discovery of the presence of significant amounts of 5-HT in different regions within the mammalian brain led to the proposal of 5-HT as a CNS neurotransmitter (Twarog and Page, 1953).

Immunofluorescence studies found that 5-HT-IR cell bodies are restricted to cells lying in or near the midline or raphe region of the medulla, pons and upper brainstem (Steinbusch, 1984). These neurons are labelled as B_1-B_9 nuclei according to Dahlstrom and Fuxe (1964). The ventral raphe (B_1-B_4), which includes RPa, RMg, RMca and PGi, contains relatively high densities of serotoninergic cell bodies. The dorsal raphe (caudal B_5-B_6 and rostral B_7), the largest of the raphe nuclei, contains the highest density of serotoninergic cell bodies in the brain. The density of 5-HT neurons in the medial raphe (B_8) is much lower than in B_1-B_7 (Hillegaart, 1991; Steinbusch, 1984; Zagon, 1993). In

addition to the raphe 5-HT neurons, a small number (3-9 /spinal cord) of 5-HT neurons are detected in lamina VII and lamina X of rat thoracolumbar spinal cord (Newton and Hamill, 1988). These intraspinal 5-HT neurons in the intermediate area are thought to be involved in autonomic signal transduction, probably acting as interneurons.

Serotoninergic neurons have extensive projections: three major ascending and two major descending pathways have been described according to Steinbusch (1984). The three ascending pathways are a dorsal pathway to caudate/putamen, a medial pathway to substantia nigra, and a ventral pathway to the ventral tegmental area and medial forebrain bundle. The two descending pathways are a ventromedial pathway to the ventral horn and a lateral pathway to the central gray area of the spinal cord. Axons of the IML-projecting serotoninergic neurons descend in the lateral and dorso-lateral funiculi (the lateral descending pathway) and synapse on SPNs within the IML of the spinal cord (Coote, 1988; Vera *et al.*, 1990).

Serotoninergic nerve terminals are present in most parts of the CNS (Jacobs and Azmitia, 1992). High densities of 5-HT-IR terminals are located in the intermediate area of the thoracic spinal cord. In this area, IMLp and IMLf have the highest densities of 5-HT-IR terminals, while IC, CA and lamina X have relatively lower densities (Krukoff *et al.*, 1985; Wu *et al.*, 1993). The anatomical information provides

the evidence that 5-HT has a neurotransmitter role in the intermediate area.

Serotonin is involved in numerous physiological events and various pathological conditions in the CNS and the peripheral nervous system (for review see Zifa and Fillion, 1992). Multiple physiopathological events triggered by 5-HT depend on the presence of different specific 5-HT receptors. The existence of 5-HT receptors was first presented by Gaddum and Picarelli (1957), who described two types of receptors controlling the 5-HT-evoked guinea pig ileum muscle contraction: D receptors, for those blocked by dibenzyline, and M receptors, for those blocked by morphine. In the following 30 years, radioligand binding and physiological studies identified other different 5-HT receptor subtypes. It is apparent that 5-HT receptors can be classified into at least three, possibly up to seven classes although different nomenclatures and classifications are proposed (Bradley *et al.*, 1986; Zifa and Fillion, 1992). With the rapid recent progress made in molecular biology, many receptor genes have been cloned, and provide a definitive "fingerprint" of the different receptor subtypes. The three main criteria are operational (selective agonists, antagonists, ligand-binding affinities), structural (gene and receptor structure sequences) and transductional (intracellular transduction mechanisms) (Humphrey *et al.*, 1993). A new classification of 5-HT receptors is proposed

by the International Union of Pharmacology Classification of Receptors for Serotonin upon these criteria (Hoyer *et al.*, 1994) (Table 1).

Various 5-HT receptors are widely distributed in the CNS. In the spinal cord, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 binding sites have been demonstrated (Glaum and Anderson, 1988; Marlier *et al.*, 1991; Pluchino *et al.*, 1990). The localization of 5-HT_{1A}, 5-HT_{1B} and 5-$HT_{2A/2C}$ binding sites in the intermediate area (Thor *et al.*, 1993) suggests a role of 5-HT in the autonomic system.

Effects of 5-HT in the central regulation of the sympathetic activity to the cardiovascular system are complicated (Coote, 1988; McCall and Clement, 1994). Activation of medullary IML-projecting serotoninergic neurons either increased or decreased sympathetic nerve activity and MAP (Coote and MacLeod, 1974; McCall and Clement, 1989). Various studies on the spinal cord showed sympathoexcitatory and/or sympathoinhibitory effects of serotoninergic agents applied to the IML. For example, microiontophoresis of 5-HT onto identified SPNs in the IML most frequently produced a sympathoexcitatory action (Gilbey and Stein, 1991). However, the firing rate of a small

Table 1. Classification of 5-HT Receptors

Receptor Type	Subtype	Second Messenger	Comments
5-HT_1	5-HT_{1A}	↓cAMP K^+ Channel↑	
	5-HT_{1B}	↓cAMP	Rodent equivalent of $5\text{-HT}_{1D\beta}$
	5-HT_{1D}	↓cAMP	$5\text{-HT}_{1D\alpha}$ and $5\text{-HT}_{1D\beta}$
	5-ht_{1E}	↓cAMP	
	5-ht_{1F}	↓cAMP	
5-HT_2	5-HT_{2A}	IP_3/DG	Previous 5-HT_2
	5-HT_{2B}	IP_3/DG	Previous 5-HT_{2F}
	5-HT_{2C}	IP_3/DG	Previous 5-HT_{1C}
5-HT_3		Cation channel	
5-HT_4		cAMP↑	
5-ht_{5A} and 5-ht_{5B}		-	
5-ht_6		cAMP↑	
5-ht_7		cAMP↑	

5-ht: Indicates the receptor whose gene has been cloned but function in the intact tissue is not known.

percentage of SPNs was decreased by 5-HT (Coote *et al.*, 1981; Gilbey and Stein, 1991).

Further information on 5-HT release and regulation of 5-HT release from the intermediate area will be essential to understand the modulatory role of serotonin in the cardiovascular system.

b. Tachykinins

SP, NKA and neurokinin B (NKB) are members of the tachykinin (TK) family, a family of peptides sharing a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X represents an aromatic (Phe, Tyr) or a branched (Val, Ile) amino acid. TK is named for its relatively rapid initiation of smooth muscle contraction. TKs are widely distributed in both the central and peripheral nervous system and can evoke a variety of biological activities as neurotransmitters and/or neuromodulators in different tissues.

Neurons are the major sources of TKs both in the CNS and the peripheral tissues. The synthesis of TKs (as well as other neuropeptides) occurs in the ribosomes within the neuron cell body and afterwards they are transported axonally to terminal endings for final enzymatic processing (Maggio, 1988). Molecular cloning studies revealed that mammalian TKs are derived from large precursors encoded by two different, but related genes (Nawa *et al.*, 1983). The preprotachykinin A (PPT-A) gene codes for three mRNAs, α-, ß-, τ-PPT-A as a result of alternative RNA splicing events. SP is encoded by all three mRNAs, whereas NKA is encoded only by ß- and τ-PPT mRNAs (Krause *et al.*, 1987). NKB is encoded by the preprotachykinin B (PPT-B) gene (Nakanishi, 1987). Regulation of TKs synthesis may occur at the level of gene transcription, transcript slicing, transport of PPT

mRNAs, mRNA translation and specific posttranslational mechanisms (Helke *et al.*, 1990). The structure of PPT genes suggests a similar distribution of SP and NKA, while the distribution of NKB does not overlap with that of the other TKs (Helke *et al.*, 1990). For example, PPT mRNA-containing cells in the spinal cord are prominent in laminae I and II and in IML, whereas NKB precursor mRNA is prominent in lamina III and is absent from IML (Warden and Young, 1988). However, another product of the PPT-B, NKB peptide-2 containing fibers and terminals have been identified in the intermediate area of the thoracic spinal cord and the source of these NKB peptide-2 terminals are mainly the hypothalamic neurons (Zhuo and Helke, 1993).

TKs are highly susceptible to hydrolysis by tissue-specific peptidase, and experiments with TKs are usually carried out in the presence of peptidase inhibitors.

TKs exert a variety of biological actions. An important effect of TKs is neuronal stimulation, such as stimulation of autonomic responses and facilitation of neurotransmitter release (Maggi *et al.*, 1993). For example, TKs are thought to regulate cardiovascular responses at the level of the VM, NTS and spinal cord (Helke *et al.*, 1991b; Takano *et al.*, 1990).

The various biological activities exhibited by TKs are generated by the existence of diverse types of TK receptors. Members of the TK family bind to three different

neurokinin (NK) receptor subtypes: NK_1, NK_2 and NK_3. These receptors are characterized by their preferred affinity for SP, NKA and NKB, respectively. The NK_1/and NK_2 receptors will be discussed together with SP/and NKA in the following sections.

The NK_3 receptor has a high affinity for NKB and low affinities for NKA and SP (Maggi *et al.*, 1993). The cloned NK_3 receptor (from a rat brain cDNA library) has 452 amino acid residues with a high degree of homology with that of NK_1 and NK_2 receptors (Shigemoto *et al.*, 1990). The receptor has a wide distribution in the CNS and a few peripheral tissues such as the portal vein. In the spinal cord, NK_3 receptors are concentrated in the outer layer of the dorsal horn with lower levels throughout inner dorsal and ventral horns (Hunter *et al.*, 1987). Selective agonists are senktide and [MePhe^7]-NKB (Maggi *et al.*, 1993). Little progress has been made in the development of selective NK_3 antagonists, and the precise involvement of NK_3 receptors in the biological actions of TKs must await the development of high affinity NK_3 antagonists.

Specific interests of TKs of the present study are SP and NKA, which coexist with TRH and 5-HT in the bulbospinal projection pathway.

1. SP

SP, the most widely known TK peptide, was first isolated by Von Euler and Gaddum (1931) from intestinal tissue and was purified as an undecapeptide with the sequence: H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Chang *et al.*, 1971). Subsequent studies have revealed a marked regional distribution of SP in the brain and spinal cord. SP-IR containing cell bodies are localized in most parts of the CNS, in the central and peripheral components of autonomic control system including the VM and spinal cord (Ljungdahl *et al.*, 1978; Ogawa *et al.*, 1985). In the VM, SP-IR cells are located in the RMg, RPa, PPR and the lateral reticular nucleus. SP-IR neurons and cells containing mRNAs encoding SP in the spinal cord are quite limited and are mainly located in the IML, lamina I, II and V (Ljungdahl *et al.*, 1978; Warden and Young, 1988). Widespread networks of SP-IR nerve terminals are observed in the brain and spinal cord. In the thoracic spinal cord, IML has a dense network of SP-IR fibers running towards the central canal (Cuello and Kanazawa, 1978) and these IML SP-IR nerve terminals originate from the VM (Charlton and Helke, 1987; Helke *et al.*, 1982) as well as intra- and/or intersegmental neurons within the spinal cord (Davis *et al.*, 1984). It is possible that SP acts as a neurotransmitter or neuromodulator in the intermediate area of the thoracic spinal cord.

Multiple neurophysiological effects induced by SP are mediated through the activation of NK_1 receptors. SP is the endogenous ligand for NK_1 receptor (Maggi *et al.*, 1993). The NK_1 receptor cloned from the rat brain cDNA library is made up of 407 amino acid residues and has a molecular weight of 46,364 (Yokota *et al.*, 1989). It contains seven putative membrane-spanning domains and has a sequence similar to that of the members of G protein-coupled receptors. Activation of NK_1 receptors results in hydrolysis of phosphatidylinositols and increased cAMP levels (Nakajima *et al.*, 1991). Radioligand binding studies and *in situ* hybridization histochemistry have localized NK_1 receptors in the brain and spinal cord (Charlton and Helke, 1985; Elde *et al.*, 1990; Helke *et al.*, 1986b; Maneo *et al.*, 1993). The density of NK_1 receptor in the spinal cord is the highest in laminae I-II, IML and lamina X. The intermediate area has a very dense labeling of NK_1 binding sites (Charlton and Helke, 1985; Helke *et al.*, 1986b) and NK_1 receptor mRNA is expressed by IML neurons (Elde *et al.*, 1990). The NK_1 binding sites in the IML appear to be primarily located on SPNs because unilateral injections of the suicide transport agent, ricin, into the superior cervical ganglion reduced SP binding and cholinesterase-stained SPNs in the IML (Helke *et al.*, 1986). However, the possibility of the presence of presynaptic autoreceptors can not be completely ruled out from the study because the

remaining of the SP binding sites may be located presynaptically. The localization of NK_1 receptors in the spinal autonomic area suggests a role of SP in autonomic neurotransmission.

The most well known action of SP is involved in sensory mechanisms, especially related to pain (Fleetwood-Walker *et al.*, 1990). SP could also be an excitatory transmitter or modulator in the CNS (Stucky *et al.*, 1993). SP is thought to regulate cardiovascular responses at levels of the VM and spinal cord. Intrathecal administration of SP and agonists into the IML directly excited SPNs (Gilbey *et al.*, 1983) and caused an increase in MAP by activating sympathetic outflow to the adrenal and the vasculature (Helke *et al.*, 1987a; Pham and Couture, 1993). Moreover, intrathecal administration of NK_1 antagonists and more selective antagonists (CP 96345 and RP 67580) blocked the increase in MAP and tachycardia resulting from injection of SP (Helke *et al.*, 1987b; Pham and Couture, 1993; Picard *et al.*, 1994). These data indicate that SP has an excitatory effect on autonomic systems due to the activation of NK_1 receptors in the IML.

To examine the biological function of SP, highly selective and metabolically stable NK_1 receptor agonists and antagonists have been developed. Selective NK_1 agonists include SP methyl ester, $[Sar_9,Met(O_2)^{11}]SP$ and GR 73632. GR 73632 is a potent and selective agonist without any

antagonist activity at NK_1 receptors, and is resistant to degradation by peptidase (Hagan *et al.*, 1991). Selective and potent antagonists include L-668,169, GR 82334 (Maggi *et al.*, 1993). GR 82334 is not only a selective NK_1 receptor antagonist *in vitro*, but also displayed *in vivo* NK_1 receptor antagonist activity without any partial agonist activity (Hagan *et al.*, 1991). Recent discovery of new selective nonpeptide antagonists (e.g., CP 96345, RP 67580 and CP 99994) will give great momentum to the research of SP.

2. NKA

NKA (also known as substance K, neurokinin α, or neurokinin L), was first isolated from porcine spinal cord and then sequenced with the structure: H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 (Kimura *et al.*, 1983; Nawa *et al.*, 1983). After its discovery, studies of the regional distribution of NKA in various tissues have been carried out and compared with SP. The distribution of NKA in the central and peripheral nervous system exhibits nearly the same pattern as that of SP, although the amount of NKA in the CNS tissue is relatively small (Arai and Emson, 1986; Kanazawa *et al.*, 1984). In the VM, NKA-IR was found to coexist with SP-IR in both the PPR and raphe pallidus IML-projecting neurons (Nevin *et al.*, 1994). NKA accompanies SP all along the spinal cord and its concentration was only 1/2 to 1/4 that of SP (Moussaoui *et al.*, 1992; Takano *et al.*, 1986). The distribution of NKA-IR in the spinal cord is similar to that of SP; heavy labeling in the dorsal horn and the intermediate area, and moderate labeling in the ventral horn (Helke *et al.*, 1990). NKA and SP content in the IML were 1.70 ± 0.13 and 2.95 ± 0.26 pg/μg protein, respectively (Takano *et al.*, 1986) and the molar ratio of NKA to SP was 0.69. Although the IML contains a lower amount of NKA, it is still possible to detect the release of NKA and assess the function of NKA.

NKA is the endogenous ligand for NK_2 receptors with lower affinities for NK_1 and NK_3 receptors (Maggi *et al.*, 1993). The cloned NK_2 receptor from rat stomach (Sasai and Nakanishi, 1989) has 48% amino acid sequence homology with that of the rat NK_1 receptor. Compared to the clearly demonstrated expression in peripheral tissues, the presence of NK_2 receptors in the rat brain and spinal cord has been questioned. Radioligand binding studies showed very limited NK_2 binding sites in the spinal cord compared to the peripheral tissue (Hagan *et al.*, 1993; Mantyh *et al.*, 1989). These data are consistent with the absence of mRNA encoding for the NK_2 receptors in adult brain tissue (Poosh *et al.*, 1991; Sasai and Nakaniski, 1989). NK_2 binding sites in the IML have shown to be very limited (Mantyh *et al.*, 1989), although NKA is present in the nerve terminals of IML and functional studies suggest the NK_2 receptor mediated spinal cord responses. For example, NK_2 agonists are involved in the regulation of spinal nociceptive reflex, blood pressure and heart rate (Picard *et al.*, 1994; Takano and Kamiya, 1991). However, this is not always the case (Hassessian *et al.*, 1988). Thus, the involvement of the NK_2 receptor in spinal autonomic systems is unclear. One possibility is that the cardiovascular responses of NKA are mediated by NK_1 receptors, since NKA also has some low affinities to NK_1 receptors (Pham and Couture, 1993). Another possibility is that NKA acts on a receptor distinct from the classic NK_2

receptor subtype. Evidence obtained from different labs and using various ligands indicated the existence of heterogenous NK_2 receptor subtypes (NK_{2A} and NK_{2B}) in different species (Maggi *et al.*, 1993; Williams *et al.*, 1988). However, the exact mechanism of NKA in the spinal regulation of the autonomic system is not fully understand.

Highly selective and potent agonists for NK_2 receptors include [ß-Ala^8]NKA and GR 64349, while highly selective antagonists are L-659,877, SR 48968, MEN-10,376 and MDL 29,913 (Maggi *et al.*, 1993). Further work with the newly developed potent and selective agonists and antagonists will further elucidate the roles of NKA and NK_2 receptors in normal and abnormal CNS functions.

c. TRH

TRH, the tripeptide (H-Glu-His-Pro-NH_2), was first isolated from hypothalamus in 1969, and its synthesis was achieved soon after (Boler *et al.*, 1969). The discovery of TRH started the modern era of neuroendocrinology, because it is the first characterized hypothalamic releasing hormone involved in the regulation of hormone secretion from the anterior pituitary gland. Although the neuroendocrine role of TRH is to initiate the secretion of thyroid stimulating hormone (TSH) and prolactin from the anterior pituitary, TRH also exists in several CNS regions outside the hypothalamus to act as a neurotransmitter /or neuromodulator (Zabavnik *et al.*, 1993).

TRH-IR cell bodies and fiber systems are located in widespread areas of the brain (Lechan *et al.*, 1983). In the medulla oblongata, TRH-IR neurons are concentrated in the midline raphe nuclei and in the ventral reticular formation (Lechan *et al.*, 1983). Retrograde transport studies in combination with immunocytochemistry located TRH-IR in the ventrolateral and ventromedial medulla IML-projecting neurons. TRH-IR neurons include RPa, RMg, PGi, RMca and the superficial medullary surface (Helke *et al.*, 1986a; Hirsch and Helke, 1988; Sasek *et al.*, 1990). The localization of TRH in these neurons are of functional significance because the importance of the VM in the CNS regulation of the

cardiovascular system.

In the spinal cord, TRH-IR neurons are mainly located in laminae II-III in the dorsal horn (Coffield *et al.*, 1986). TRH-IR fibers are primarily found surrounding motoneurons in the ventral horn (Lechan *et al.*, 1983). In the intermediate area, the distribution of TRH-IR fibers is similar to that of SP-IR and 5-HT-IR fiber: relatively higher densities in the IML and around the central canal (Hirsch and Helke, 1988; Poulat *et al.*, 1992). It is possible that TRH is released from TRH-IR nerve terminal in the intermediate area and activates postsynaptic TRH receptors.

TRH receptor (which has been cloned) is a membrane-bound protein with the characteristic seven transmembrane domain structure with extracellular and intracellular regions (Straub *et al.*, 1990). TRH receptors are heterogeneously distributed throughout the CNS. In the spinal cord, high densities of TRH receptors and TRH receptor mRNA are found mainly in lamina II, with low densities in the ventral horn and the intermediate area (Manaker *et al.*, 1985; Zabavnik *et al.*, 1993). Spinal cord TRH receptors have similar binding affinities and pharmacological profiles to TRH receptors in the brain and pituitary. In addition, local depletion of TRH could up-regulate TRH receptor numbers in the spinal cord (Sharif and Burt, 1983). These data suggest that the spinal cord TRH

binding sites are true TRH receptors and are capable of mediating the spinal autonomic effects of TRH.

It is well known that TRH plays an important modulatory role in the autonomic nervous system. Peripheral administration of TRH increased MAP and plasma catecholamine levels (Koskinen and Bill, 1984; Kunos *et al.*, 1984). Intrathecal administration of TRH analog (MK-771), activating thoracic spinal TRH receptors, presumably in the IML, increased MAP and sympathetic tone to the vasculature and the adrenal (Helke *et al.*, 1988). Furthermore, iontophoresis of TRH into IML directly excited identified SPNs (Backman and Henry, 1984). Although the excitation induced by TRH in motoneurons is due to the suppression the K^+ conductance at resting membrane potential (Fisher and Nistri, 1993), however, the precise mechanism of TRH affecting the excitability of SPNs in the IML is still unknown.

TRH has a short half-life in the tissue due to its rapid metabolism, which makes it difficult to use TRH as a tool for research (Griffiths and Bennett, 1983). Metabolically stable TRH analogues (for example, MK-771, DN-1417 and TA-0910) have been synthesized to prolong drug action with similar endocrine effects. MK-771 is a relatively specific and stable drug as a replacement for TRH in many studies. In the rat spinal cord, the K_i value for TRH and MK-771 is 13.9 ± 0.8 and 190 ± 23.4 nM, respectively

(Sharif, 1993). The half-life ($t_{1/2}$) of TRH and MK-771 in the rat brain are 9 and 108 min, respectively (Metcalf, 1983). However, studies of TRH are hard to complete because specific antagonists for TRH are not yet available.

d. Serotonin, SP, NKA and TRH Colocalization

Immunohistochemistry studies combined with retrograde transport demonstrated the coexistence of 5-HT, SP, NKA and TRH in the VM IML-projecting neurons (Nevin *et al.*, 1994; Sasek *et al.*, 1990). Similar distribution patterns of 5-HT-, SP- and TRH-IR terminals within the IML (Davis *et al.*, 1984; Poulat *et al.*, 1992) and the coexistence of SP, TRH and 5-HT in the IML varicosities (Wessendorf and Elde, 1987b) supports the assumption of the coexistence of these neurochemicals in the IML nerve terminals. It is not known whether these agents are contained in the same or different vesicles in the IML. Studies on the ventral horn indicated the presence of SP and 5-HT in the same presynaptic boutons (Sakamoto and Atsumi, 1991) and the same large dense-core vesicles (Pelletier *et al.*, 1981).

Colocalization of peptides and classical transmitters in nerve endings makes it possible that both interact in chemical transmission. Cotransmission often involves synergistic actions of the cotransmitters, each transmitter having a facilitory effect on the action of the other transmitter on the postjunctional neurons (Lundberg and Hokfelt, 1983). Often the peptide seems to be responsible for a component of the response characterized by slow onset and long duration, whereas classical transmitters generally cause rapid and short-lasting effects (Lundberg and Hokfelt,

1983). Peptides principally subtly modify and amplify the action of the classical transmitter on the postsynaptic cell (Lundberg and Hokfelt, 1983). Studies on interactions of 5-HT, SP and TRH in the CNS regulation of adrenocortical secretion found that 5-HT and TRH serve to stimulate adrenocortical secretion, but SP exerts an inhibitory influence that is capable of overcoming of the stimulatory effects of 5-HT (Saphier *et al.*, 1994).

Several studies have been designed to examine the interaction of these coexisting putative neurotransmitters in the bulbospinal pathway. Inhibition or reduction of 5-HT synthesis increased the TRH mRNA and the mRNA encoding for SP precursors in medullary raphe IML-projecting neurons (Riley *et al.*, 1993). Serotoninergic denervation decreased TRH content (Lighton *et al.*, 1984) and the sympathetic outflow induced by TRH (Assadian *et al.*, 1991). Coadministration of SP and 5-HT_{1A} agonists to the IML modified the cardiovascular responses elicited by each other (Gradin, 1990). Although difficult to interpret, these complex results clearly indicate discrete functional interactions between coexisting neuropeptides and 5-HT in the IML.

To assess the significance of a neurochemical in transmitting neuronal activity to a target site (e.g. IML), it is important to determine whether the agent is released at the target site. An agent that is present in a neuron

but not released is likely to have a considerably different role in neurotransmission than one which is released. Studies on the corelease of 5-HT, SP, NKA and TRH and regulation of their release from the intermediate area are essential in understanding the function of these neurochemicals in the cardiovascular system.

C. RELEASE OF COLOCALIZED NEUROCHEMICALS AND AUTORECEPTOR MODULATION

a. Release Mechanism

Neurotransmitter release is necessary for communication of information between nerve cells and also the transfer of signals from nerve to muscles and glands. The term "stimulated (or evoked) release" is used for the passage of transmitter molecules from presynaptic vesicles across the neuronal membrane into the extracellular space through exocytosis at the nerve terminal. This process involves a Ca^{2+}-dependent secretion evoked by membrane depolarization induced by action potentials or high extracellular K^+ concentrations (Betz, 1992; Starke *et al.*, 1989).

Generally, the resting membrane potential in the presence of several ions is quantitatively described by the Goldman Equation: $Vm = (RT/F)\ \ln(P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{in} / P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{out})$ (R = gas constant; T = absolute temperature; F = Faraday's constant). In the case when the permeability to one ion is exceptionally high, the Goldman equation reduces to the Nernst equation for that ion. The membrane potential of a nerve cell at rest, with a high intracellular potassium concentration, is negative according to the Nernst equation: $E = 2.3\ (RT/F)\ \log$

($[K^+]out / [K^+]in$). Intracellular recording measured the resting membrane potential in a typical neuron at about -60 to -70 mV (Shepherd, 1988). Increases in extracellular K^+ concentration, with concomitant reduction in Na^+ concentration, depolarizes the cell membrane and induces an action potential (Shepherd, 1988). In the present study, the resting membrane potential of tissue slices under basal condition (buffer containing 5 mM K^+) is calculated to be -81 mV according to Nernst equation (calculated at room temperature, 18°C, the constant 2.3 RT/F= 58 mV; the intracellular K^+ equals 124 mM). The membrane potentials of the tissue slices during K^+-evoked depolarization are -65 mV and -48 mV, respectively for 30 mM and 60 mM K^+ concentrations.

Membrane depolarization opens presynaptic calcium channels, thereby increasing internal calcium concentrations near membrane transmitter-releasing sites (Mulkey and Zucker, 1991). Neurons possess several different types of voltage-sensitive calcium channels that differ considerably in their biophysical and pharmacological properties. L-type Ca^{2+} channels are located on the soma and N-type Ca^{2+} channels are located on presynaptic nerve terminals (Miller, 1987). The evoked release of neurotransmitter appears to be dependent on Ca^{2+} influx through N-type Ca^{2+} channels. Increased intraneuronal Ca^{2+} triggers a transient release of a quantity of neurotransmitter, apparently by facilitating

the fusion of synaptic vesicles with the presynaptic plasma membrane (Cooper *et al.*, 1986; Smith and Augustine, 1988). Classical neurotransmitters and peptidergic neurotransmitters are released in the form of small quantal packages and this process is thought to be the result of exocytotic secretion of the individual synaptic vesicles upon arrive of an action potential (Buma, 1989).

In addition to the stimulated release evoked by nerve depolarization, there is a small but continuous release of neurotransmitter from nerve terminals which is referred to as basal release (Nicholls, 1989). Compared to the stimulated release, the basal release is the non-quantal release under resting conditions, independent of external Ca^{2+} and results in a steady leakage of transmitter (Levi and Raiteri, 1993). The basal release may be carrier-mediated release (e.g., mediated by a specific membrane carrier) although neither the mechanism nor physiological implications of basal release is clear.

Termination of the classical neurotransmitter signal occurs through uptake of the transmitter back into the presynaptic terminals (Bennett *et al.*, 1974). Reuptake is an active process which assures constant and high levels of neurotransmitter in the neuron and low concentrations in the cleft (Hedgvist and Stjarne, 1969). Monoamine transporters for reuptake are located in the presynaptic cell membranes as well as membranes of storage vesicles in the presynaptic

terminals (Kanner 1983). The *in vitro* release of amine transmitters (e.g., 5-HT, dopamine and norepinephrine) is generally studied with the radiolabeled transmitter. That is, transporters in the presynaptic cell membrane will transport the labeled transmitter present in the synaptic cleft into the presynaptic cytoplasm, and transporters in the membrane of storage vesicles will further move the labeled transmitter into storage vesicles. Radiographic studies found that small vesicles appeared to be the site of intense of [^{3}H]5-HT labelling (Beaudet and Descarries, 1987). The labeled transmitter can be released from the vesicles during nerve depolarization. In addition to recycling the released transmitter, the transporter could also mediate the release of neurotransmitter as a reverse of transport during depolarization.

b. Presynaptic Autoreceptor Modulation of Release

The release of a neurotransmitter is modulated not only by specific nerve activity (action potential), but also by various factors influencing the membrane potential of the presynaptic neuron. Activation of presynaptic autoreceptors is one way in which the strength of synaptic transmission is regulated by altering the release.

The presynaptic autoreceptor is a presynaptic receptor site where a transmitter, or ligand present in excess in the synaptic cleft can bind and change its further Ca^{2+}-dependent evoked release (Starke and Langer, 1979). The presence of a presynaptic autoreceptor was first described by Koelle in 1961, when he found that acetylcholine could act at presynaptic terminals and regulate its own release. Since then, many studies have focused on the presynaptic autoreceptors. To demonstrate the presence of presynaptic inhibitory autoreceptors in a particular preparation requires that a number of criteria be met: (1) The release of the transmitter induced by membrane depolarization should be inhibited by the transmitter itself added to the system. (2) Agonists of the presynaptic autoreceptor should inhibit the release of the transmitter. (3) The inhibitory effects of the transmitter and agonists should be counteracted by specific antagonists of the presynaptic autoreceptor (Cerrito and Raiteri, 1979;

Thompson, 1993; Starke *et al.*, 1989).

The nature of presynaptic inhibition of neurotransmitter release is thought to involve the receptor-mediated regulation of ion channels or regulatory proteins in the nerve terminals although the exact mechanism is unknown. For example, presynaptic receptors may directly inhibit the Ca^{2+} channels responsible for the Ca^{2+} influx that triggers action potential, coupling process and neurotransmitter release (Ryan *et al.*, 1985). Alternatively, the depolarization of the nerve terminal produced by an action potential may be modified so that the depolarization spike becomes less effective in activating Ca^{2+} channels. Opening K^+ channels (i.e., increasing presynaptic K^+ conductance) stabilizes the membrane potential, resulting in a briefer action potential which is less effective in depolarizing the terminal and therefore, results in a smaller Ca^{2+} influx (Bug *et al.*, 1986). Presynaptic autoreceptors, which are linked to the GTP-binding protein complex Gi, inhibit adenylate cyclase and decrease cAMP levels. A fall in cAMP can both reduce Ca^{2+} and promote K^+ current and hence depress transmitter release (Thompson, 1993; Starke *et al.*, 1989).

Presynaptic autoreceptors may offer great opportunities in the treatment of certain diseases as a result of modulating transmitter release. For example, selectively blocking presynaptic inhibitory receptors on

dopaminergic nerve terminals might relieve symptoms of Parkinson's disease (Thompson, 1993). More studies are conducted to clarify the nature of presynaptic autoreceptor as the result of its functional significance.

The study of presynaptic control of neurotransmitter release requires an accurate measurement of the transmitter outflow under controlled conditions. Using *in vitro* preparations containing only the nerve terminals, but not cell bodies containing the transmitter studied is the most commonly used method. Biogenic amines (e.g., 5-HT) are usually measured by the efflux of radiolabeled neurotransmitter previously taken up by the tissue. Peptides (e.g., SP) are usually measured by sensitive radioimmunoassay (RIA) in the presence of peptidase inhibitors. For both classical transmitters and putative transmitters, the spontaneous efflux is usually low and studies have been performed on electrical or high potassium evoked release (Chesselet, 1984; Starke *et al.*, 1989). Presynaptic autoreceptors for 5-HT, dopamine and norepinephrine have been widely studied, while presynaptic autoreceptors for neuropeptides have not received much experimental attention.

c. Release of 5-HT, SP, NKA and TRH

1. Release of Serotonin

Studies on the release of 5-HT and the presynaptic autoreceptor regulation of the release have been extended to most parts of the CNS. Electrical or K^+ depolarization stimulated, extracellular Ca^{2+}-dependent *in vitro* and/or *in vivo* release of endogenous and/or radiolabeled 5-HT from nerve terminals has been demonstrated from cerebral cortex, hippocampus, cerebellum and spinal cord (Gardier and Wurtman, 1991; Gray and Green, 1987; Matsumoto *et al.*, 1992; Monroe and Smith, 1985).

Most of the serotonin released is transported into serotoninergic terminals by 5-HT transporters located in the nerve terminals. 5-HT can also be transported into dopaminergic and adrenergic terminals by a different uptake mechanism (Steinbusch, 1984).

The release of 5-HT is regulated by presynaptic autoreceptors located on serotoninergic terminals. The existence of release-regulating presynaptic receptors in the rat CNS was originally established by several different groups (Cerrito and Raiteri, 1979; Farnebo and Hamberger, 1974; Gothert and Weinheimer, 1979). Presynaptic autoreceptors are also found in rabbit, guinea pig and human CNS tissues (Limberger *et al.*, 1986; Middlemiss *et al.*,

1988; Schlicker *et al.*, 1989). Now it is widely accepted that in the rat, 5-HT_{1B} receptors serve the role of nerve terminal autoreceptors (Engel *et al.*, 1986; Johanning *et al.*, 1992) although 5-HT_{1A} and 5-HT_3 receptor subtypes were previous candidates for autoreceptors (Sprouse and Aghajanian, 1987; Verge *et al.*, 1986). 5-HT autoreceptors in calf, pig, and human brain appear to be the 5-HT_{1D} subtype (Hoyer *et al.*, 1994), the species homologue of the rat 5-HT_{1B} receptor (Adham *et al.*, 1992).

In the rat whole spinal cord and undissected lumbar spinal cord, 5-HT_{1B} receptors are presynaptic autoreceptors modulating 5-HT release (Monroe and Smith, 1985; Murphy and Zemlan, 1988). However, neither depolarization-induced release of 5-HT nor the presence of presynaptic modulatory autoreceptors has specifically been described in the IML and other autonomic-related structures of the thoracic spinal cord.

2. Release of SP and NKA

Demonstration of a calcium-dependent release of an agent in response to depolarizing stimuli is a prerequisite to consider an endogenous substance as a neurotransmitter. Both SP and NKA meet this criterion in tissues where it has been studied.

Electrical stimulation or K^+-depolarization induced and extracellular Ca^{2+}-dependent *in vitro* release of SP from nerve terminals has been demonstrated in both peripheral and CNS tissues: trachea (Hua and Yaksh, 1992), cranial nerve sensory nuclei (Helke *et al.*, 1981), pituitary and hypothalamus (Calvo *et al.*, 1990; Iversen *et al.*, 1976). The release of SP could also be induced by electrical or chemical (capsaicin) stimulation of primary sensory afferent fibers (Helke *et al.*, 1981; Malcangio and Bowery, 1994). For example, local application of capsaicin (selectively exciting C-afferent neurons) induced the release of SP from the NTS and spinal trigeminal nucleus (Helke *et al.*, 1981).

Studies on the release of SP from rat spinal cord have focused on the dorsal horn where SP is present in primary afferent fibers within the dorsal root and plays a role in the transmission of nociceptive signals to the spinal cord (Duggan and Weihe, 1991). Superfusion of slices of the lumbar dorsal spinal cord detected the *in vitro* release of SP following nerve depolarization by electrical-,

K^+- or capsaicin-stimulation (Bourgoin *et al.*, 1993; Malcangio and Bowery, 1994; Takano *et al.*, 1993). Electrical, thermal or chemical (capsaicin) stimulated *in vivo* release of SP from dorsal spinal cord has been revealed using push-pull perfusion and microdialysis (Collin *et al.*, 1992; Tiseo *et al.*, 1990). Evoked release of SP from dorsal spinal cord is inhibited by σ receptor and α_2 adrenergic receptor activation (Chang *et al.*, 1989; Collin *et al.*, 1992; Mauborgne *et al.*, 1987; Takano *et al.*, 1993). It is possible that SP released at the spinal cord nerve terminals interacts at both presynaptic and postsynaptic sites and regulates sensory information transmission.

The facts that SP increased both *in vitro* and *in vivo* glutamate release from primary nerve terminals (Kangrga *et al.*, 1990; Smullin *et al.*, 1990) and that NK_1 receptor antagonists (RP 67850 and SR 140333) increased the electrically-induced release of SP from rat spinal cord (Malcangio and Bowery, 1994) suggest the presence of presynaptic NK_1 autoreceptors. However, the existence of presynaptic NK_1 autoreceptor in the IML was not supported by studies in which lesion of bulbospinal SP-containing neurons failed to alter the NK_1 binding in the IML (Helke *et al.*, 1986a). Furthermore, although noradrenergic and serotoninergic neurons also project to the IML, neither destruction of noradrenergic nor serotoninergic nerve terminals in the spinal cord reduced the SP binding sites in

the IML (Helke *et al.*, 1986a). These data suggested that NK_1 binding sites are not located presynaptically on SP, 5-HT or noradrenergic nerve terminals in the IML. However, the fact that unilateral injection of the suicide transport agent, ricin, into the superior cervical ganglion only reduced 50% of SP binding sites in the IML (Helke *et al.*, 1986) can not rule out the possibility that SP binding sites are located on interneurons or on projections from neurotransmitter systems not investigated or on presynaptic terminals that were not detected from the lesion studies. The failure of autoradiographic studies to detect presynaptic SP binding sites may be due to both the upregulation of postsynaptic receptors after SP denervation and/or a much lower presynaptic receptor number than the postsynaptic levels.

NKA and SP are encoded by the same gene and are colocalized in the nerve terminals, thus, it is likely that NKA and SP are coreleased by depolarizing stimuli. Electrical or high K^+ stimulated, extracellular Ca^{2+}-dependent corelease of NKA and SP had been detected in rat trachea (Hua and Yaksh, 1992), brain (Linderfors *et al.*, 1985) and spinal cord (Franck *et al.*, 1993; Saria *et al.*, 1986). The conditions which induced the release of NKA were the same as those used for SP, but a smaller amount of NKA was released compared to SP. Neither the release of NKA in the intermediate area of rat thoracic spinal cord nor the

presence of presynaptic autoreceptors for NKA has been studied.

3. Release of TRH

Previous studies on the release of TRH focused on the hypothalamus where TRH has a neuroendocrine role. *In vitro* perfusion studies using RIA detected K^+-depolarization induced and extracellular calcium-dependent release of TRH from rat intact hypothalamus, hypothalamic slices, synaptosome and fragments (Bennett, 1981; Charli *et al.*, 1978; Lewis *et al.*, 1987). For example, the release of TRH from the synaptosome-enriched fractions of hypothalamic homogenate was induced by depolarizing concentrations of K^+ (60 mM) in a Ca^{2+}-dependent manner (Warberg *et al.*, 1977). *In vivo* studies using microdialysis and antibody microprobes also detected the K^+ concentration-dependent stimulated release of TRH from the rat hypothalamus (Okuda *et al.*, 1990; Waterfall *et al.*, 1993).

TRH release from the spinal cord is not well studied because the low TRH content in the spinal cord limited the sensitivity of the assay. However, TRH content in the mouse spinal cord has been shown to be related to 5-HT: 2-methyl-5-HT (5-HT_3 agonist) increased, while 5,7-dihydroxytryptamine (5,7-DHT, selective serotoninergic neurotoxin) decreased TRH content (Endo *et al.*, 1993). This finding suggests the possibility that 5-HT regulates TRH turnover in the mouse spinal cord. It is not known whether this is true in the rat intermediate area, where TRH and 5-

HT coexist.

Studies on the cellular localization of TRH receptors indicated postsynaptic rather than presynaptic TRH receptors (Simasko and Horita, 1984). There is no published evidence supporting the autoreceptor regulation of TRH release or the presence of presynaptic TRH binding sites.

d. Corelease and Interaction of Coexisting Neurochemicals

Individual neurons can contain two or more neurochemicals including neuropeptide(s) and classical neurotransmitter(s) (e.g, 5-HT, SP, NKA and TRH). Accordingly, classical transmitters and neuropeptides can be released together from nerve terminals. Studies of the storage sites for 5-HT and SP by immunoelectron microscopy (Pelletier *et al.*, 1981) and by subcellular fractionation (Bucsics *et al.*, 1984; Fried *et al.*, 1989) indicate that SP and 5-HT are stored in different subcellular compartments in the ventral spinal cord, SP being stored in large, dense-cored vesicles and 5-HT mainly in small vesicles but also sometimes in large, SP-containing vesicles. The respective roles of small and large dense-cored vesicles are still poorly understood. Small agranular vesicles have been shown in radioautographic studies to be the site of intense [^{3}H]5-HT labelling and these vesicles are likely to be involved in the storage and the release of 5-HT, while the large vesicles also possess a preferential affinity for [^{3}H]5-HT and these vesicles are likely to be involved in the transport and metabolism of 5-HT (Beaudet and Descarries, 1987). However, the existence of separate vesicle populations and vesicles containing multiple putative neurotransmitters implies that coexisting neurosubstances can be coreleased or selectively released under different

conditions.

It is clear that the release of classical transmitters and neuropeptides differ in both the activation pattern and Ca^{2+} dependency (Stjarne, 1989). Classical neurotransmitters are released when low frequencies of stimulation induce a low increase of Ca^{2+} influx, whereas neuropeptides are released when repetitive action potentials induce a significant rise in the presynaptic intracellular Ca^{2+} (Verhage *et al.*, 1991; Zimmermann, 1990). This theory is confirmed in the rat ventral spinal cord by showing the differential release of endogenous 5-HT, SP and NKA in response to electrical stimulation. The release of 5-HT by electrical stimulation occurred at 2 Hz, whereas the release of SP and NKA required 20 Hz (Franck *et al.*, 1993).

Neurochemicals could also affect the release of other coexisting putative neurotransmitters and this regulation is likely to be mediated by presynaptic autoreceptors. The ventral spinal cord has been used as a model to demonstrate the interactions of 5-HT, SP, NKA and TRH on the regulation of their release, although there is no direct evidence of coexistence. 5-HT was shown to increase the K^+-evoked release of SP from slices of ventral spinal cord via a 5-HT_2 receptor (Iverfeldt *et al.*, 1989), while 5-HT increased the release of SP and NKA from dorsal cord slices via a 5-HT_3 receptor (Saria *et al.*, 1990). However, Bourgoin (1993) found that 5-HT_{1A} and 5-HT_3 receptors were not involved in

the control of the ventral spinal cord release of SP. SP was also reported to regulate the release of 5-HT from slices of ventral spinal cord. In one study, basal but not 20 mM K^+-evoked release of [^{3}H]5-HT was enhanced by SP (Tsai *et al.*, 1984). In a similar study, SP but not NKA, enhanced the evoked release of endogenous 5-HT (Franck *et al.*,1989). In another study using synaptosomes, no effect of SP on K^+-stimulated release of 5-HT was seen unless exogenous 5-HT was added, while TRH did not affect the release of [^{3}H]5-HT (Mitchell and Fleetwood-Walker, 1981). The above information obtained from ventral spinal cord suggests that the release of one transmitter is under the regulation of other neurochemicals. Corelease and regulation of the release of coexisting SP, 5-HT, NKA and TRH in nerve terminals of the intermediate area have not been established.

It is the goal of this project to study the *in vitro* release of 5-HT, SP, NKA and TRH and the regulation of their release by possible presynaptic autoreceptors and by the other coexisting neurochemicals in the intermediate area. Specific aims of this project are: (1) Demonstrate the *in vitro* release of each of the colocalized neurochemicals (5-HT, SP, NKA and TRH) in the intermediate area of rat thoracic spinal cord. (2) Examine the corelease of 5-HT, SP, NKA and TRH from same nerve terminals. (3) Determine

the presence of presynaptic autoreceptors that regulate the release of each of the coexisting neurochemicals. (4) Study the effects of the presence of one neurochemical on the release of the other coexisting neurochemicals.

K^+-depolarization-induced and Ca^{2+}-dependent *in vitro* release of 5-HT, SP, NKA and TRH from nerve terminals in many CNS regions has been demonstrated. It is also possible to detect the *in vitro* release of these individual neurochemicals from the intermediate area, which contains relatively high densities of SP-, TRH-, NKA- and 5-HT-IR nerve terminals. High K^+-depolarization stimulated *in vitro* release of transmitters from microdissected intermediate area will be studied in the present project. Extracellular Ca^{2+}-dependency will be assessed after detection of the release. These experiments will define the neurotransmitter roles of these neurochemicals in the sympathetic nervous system.

Similar distribution patterns of SP-, 5-HT-, NKA- and TRH-IR nerve terminals suggest the possibility of their corelease from same nerve terminals within the intermediate area. This hypothesis will be examined by studying the content and release of neuropeptides in rats whose serotoninergic nerve terminals are destroyed by serotoninergic neurotoxin pretreatment. These studies will provide evidence for coexistence of neuropeptides in serotoninergic nerve terminals in the IML.

Presynaptic inhibitory 5-HT_{1B} autoreceptors which regulate 5-HT release exist in the ventral and lumbar spinal cord and these autoreceptors may also exist in the intermediate area of the thoracic cord. Presynaptic autoreceptors for SP may also be present in the intermediate area. It is of interest to determine the presence of presynaptic autoreceptors in mediating the detected release. The effects of specific agonists and antagonists for possible 5-HT or SP autoreceptors will also be evaluated on the release of 5-HT or SP.

Interactions on corelease of 5-HT, SP, NKA and TRH in other systems (e.g., ventral horn) may indicate their functional interactions in the intermediate area where they do coexist. The release of one agent will be studied under the influence of the other coexisting neurochemicals, their analogues and antagonists. These information may provide further understanding of the functional importance of coexisting neurotransmitters in the bulbospinal pathway.

The hypotheses of the project are: Each of the coexisting neurochemicals (5-HT, SP, NKA and TRH) is released from nerve terminals of the intermediate area and activates postsynaptic receptors. Some of the neurochemicals are coreleased from the same nerve terminals. Presynaptic autoreceptors for 5-HT and SP exist to regulate their release. SP and 5-HT also can regulate the release of other colocalized agents by activation of presynaptic

autoreceptors. Thus, these coexisting neurochemicals in the IML affect the sympathetic activity to the cardiovascular system either directly through postsynaptic receptors or indirectly through presynaptic autoreceptors by modifying the effects of the other coexisting agents.

The present studies are aimed at expanding our knowledge of interactions of coexisting putative neurotransmitters in the intermediate area (which includes the IML) of the rat thoracic spinal cord. Such information may provide insights into a possible neurochemical basis for discrete modulation of sympathetic input to the cardiovascular system and is necessary in understanding the effects of transmitter alterations on cardiovascular regulation. In addition, this information may provide evidence relating functional interactions to neurochemical events.

METHODS

Animal Housing

Adult male Sprague-Dawley rats (220-250 g) were obtained from Taconic Farm (Germantown, NY). The rats were housed in a colony room with 12 hr light/12 hr dark cycle with food and water available ad libitum.

Reagents

Reagents used in this project were procured from the following sources: fluoxetine hydrochloride and nisoxetine (gifts) from Eli Lilly Pharmaceutical Corp. (Indianapolis, IN), [Lys^3,Gly^8-R-r-lactam-Leu^9]NKA(3-10) (GR 64349) and [D-Pro^9,(spiro-r-lactam)Leu^{10},Trp^{11}]physalamine(1-11) (GR 82334) (gifts) from Glaxo Group Research Co. (Hertfordshire, UK), L-Pro-2-aminoadipyl-histidyl-thiazolidine-4-carboxamide (MK 771) from Merck & Co. Inc. (Rahway, NJ), NKA and SP from Pennisula Lab Inc. (Belmont, CA), 1-(2-(bis(4-fluorophenyl)methoxy)ethyl)-4-(3-phenyl-propyl)piperazine (GBR-12909), σAva[L-Pro^9,N-$MeLeu^{10}$]SP(7-11) (GR 73632), 7-trifluomethyl-4(4-methyl-1-piperazinyl)-pyrolo [2,1-α] quinoxaline (CGS-12066B), (±)-2,5-dimethoxy-4-iodoamphetamine hydrobromide [(±)DOI], 2-methyl-5-hydroxytryptamine (2-methyl-5-HT), 1-(2-methoxyphenyl)-4(4-(2-phtalimido)butyl)piperazine (NAN-190), 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT) and methiothepin from

Research Biochemicals Inc. (Natick, MA), 5-methoxy-3-[1,2,3,6-tetrahydropyridin-4-yl)H-indole (RU 24969) (gift) from Roussel Uclaf (Romainvuie, France), pargyline hydrochloride from Saber Laboratories Inc. (Morton Grove, IL), Desmethylimipramine (DMI) from Revlon Health Care Group (Edison, NJ), p-chloromercuri-phenylsulfonic acid (PCMS), phenylalanylanine (Phe-Ala), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT) and 5-hydroxytryptamine creatinine sulfate (5-HT) from Sigma Chemical Co. (St. Louis, MO), 5-[1,2-^{3}H(N)]-hydroxytryptamine ([^{3}H]5-HT, 1.0 mCi/ml), [^{125}I]NKA (50 μCi/ml) and [^{125}I]Tyr^{8}-SP (86.9 μCi/ml) from New England Nuclear (Boston, MA).

Tissue Preparation

Immediately following decapitation, the thoracic spinal cord of the rat was rapidly expelled pneumatically from the vertebral column and placed on ice. The intermediate area of the thoracic spinal cord (400 μm horizontal section through the gray matter) (Fig. 2) was dissected from 2-3 mm coronal sections of the entire thoracic cord. In some experiments, the ventral horn of the thoracic spinal cord was also dissected. Dissected tissue was chopped into 250 x 250 μm pieces with a Sorvall TC-2 tissue chopper and suspended into modified Kreb's-HEPES

buffer (MKB) of the following composition (mM): NaCl, 127; KCl, 5; NaH_2PO_4, 1.3; $CaCl_2$, 2.5; $MgSO_4$, 1.2; HEPES, 15; glucose, 10. MKB also contains ascorbic acid (0.1 mM) and pargyline hydrochloride (0.01 mM) for the study of 5-HT release. To study the release of SP and NKA, MKB contains bacitracin (0.03%), Phe-Ala (100 μM) and PCMS (50 μM) to inhibit peptide degradation (Chen *et al.*, 1993) and bovine serum albumin (BSA; 0.2%) to reduce the absorption of SP to plastic and glass surfaces. The pH of MKB was adjusted to 7.4. In some experiments Ca^{2+}-dependency of the release was tested by omitting Ca^{2+}, substituting equimolar amount of Mg^{2+}, and adding 1 mM EGTA to MKB (for 5-HT release, this procedure was performed after the tissue was loaded with [^{3}H]5-HT).

In vitro Release of [^{3}H]5-HT

In vitro release of [^{3}H]5-HT and presynaptic autoreceptor modulation of the release of 5-HT from the intermediate area was assessed using the superfusion system. Following three washes with MKB, tissue was resuspended in MKB containing 100 nM GBR-12909 to inhibit uptake of [^{3}H]5-HT into dopaminergic terminals (Jacocks and Cox, 1992) and 100 nM nisoxetine to inhibit uptake of [^{3}H]5-HT into noradrenergic terminals. [^{3}H]5-HT (100 nM) was added for a 20 min incubation period (at 37°C), during which the tissue was gently bubbled with 95% O_2/5% CO_2. After three washes

with MKB, tissue was resuspended a final time in MKB and distributed in 275 μl aliquots between glass fiber filter discs into chambers of a BRANDEL superfusion apparatus (Model SF-188, Gaithersburg, MD). MKB, containing 1 μM fluoxetine to inhibit the reuptake of the released [^{3}H]5-HT, was gently bubbled with 95% O_2/5% CO_2 and superfused over the tissue at a flow rate of 0.5 ml/min. During an initial 30 min of superfusion, a low (less than 0.5%) stable baseline release was established. Subsequently, the tissue was exposed for 2 min to MKB containing an elevated concentration of K^+ and commensurate reduction in Na^+ or to MKB containing experimental agonist. Drugs, when tested with K^+-stimulation, were present during the last 20 min of superfusion prior to, and throughout K^+ stimulation. Then the tissue was superfused with MKB (5 mM K^+) for 10 min to allow the release to return to baseline and superfused with 0.2 N HCl for 40 min to extract the remainder of the radioactivity from the sample. Superfusates were continuously collected at 2 min intervals into scintillation vials. The tissue was collected into the final vial with the filter discs. Radioactivity was measured by liquid scintillation spectroscopy. Under these conditions, the radioactivity released is predominantly [^{3}H]5-HT (Murphy and Zemlan, 1988).

***In vitro* Release of SP and NKA**

Following three washes with MKB, tissue was resuspended a final time in MKB and distributed in 250 μl aliquots between glass fiber filter discs into chambers of the superfusion apparatus. MKB, was gently bubbled with 95% O_2/5% CO_2 and superfused over the tissue at a flow rate of 0.25 ml/min. During an initial 45 min of superfusion, a low stable baseline release was established. Subsequently, tissue was exposed for 4 min to MKB containing elevated K^+ (20-60 mM) and a commensurate reduction in Na^+. Experimental compounds, when tested, were present during the last 20 minutes of superfusion prior to, and throughout K^+ stimulation. The inflow was returned to MKB (5 mM K^+) for a period of 20 min. Superfusates (1 ml) were continuously collected at 4 min intervals into vials containing acetic acid (final concentration 2M) maintained at 0°C. Duplicate aliquots were dried (Speed Vac Concentrator) and stored at -70°C until the assay of peptide content. At the end of the superfusion, the tissue was collected into 1 ml ice-cold acetic acid (final concentration 2M), boiled for 10 min, sonicated, centrifuged and duplicate aliquots (50 μl) of supernatant were lyophilized and stored at -70°C until the assay of SP or NKA content. In preliminary experiments, the recovery of internal standards of SP added to superfusate samples averaged 94 ± 3%.

RIA for SP and NKA

The amount of SP-IR or NKA-IR in superfusates and tissue was quantified by RIA (Helke *et al.*, 1981). Briefly, the dried samples were resuspended in 200 μl of 0.1 M sodium phosphate buffer (pH 7.2) containing BSA (1.5%), Na azide (0.02%), and ethylenediaminetracetic (EDTA, 0.01 M). SP serum (SP3B3, 100 μl; 1/40,000 final dilution; Helke, *et al.*, 1982) or NKA serum (SK7, from Dr. Leeman, MA; 100 μl; 1/50,000 final dilution) was added. After 24 hr at 4°C, [^{125}I]Tyr^{8}-SP or [^{125}I]NKA (about 5000 CPM) was added and the incubation continued for 18 hr. Goat-anti-rabbit serum (200 μl of 5% solution; Pel-Freeze Biologicals, AK) and normal rabbit serum (25 μl of 20% solution) were added, the tubes were centrifuged at 3000 RPM (2323 Gram) at 4°C for 15 min, and the pellets were counted (Micromedic Gamma Counter).

In each experiment, RIA standard curves were constructed for SP or NKA by using known amounts of synthetic SP or NKA (5-200 pg per tube). Each time a reagent was added to the superfusion medium, a complete standard curve was drawn in the presence of this reagent at the same concentration as that used for the superfusion experiments. This allowed accurate determination of SP or NKA released in fractions containing inorganic chemicals, excess K^{+} and experimental compounds.

SP3B3 antibody recognizes the carboxyl terminal moiety of the SP molecule (Helke, *et al.*, 1982). Although NKA has C-terminal homology with SP, NKA was practically

devoid of immunoreactivity with SP3B3 (less that 0.01%). Other chemicals and compounds tested in the present experiments have negligible cross-reactivity with SP3B3 antibody. Assay sensitivity (10% replacement of the tracer) was 6 pg per tube with inter- and intra-assay variation 6.05 and 3.28%, respectively.

SK7 antibody recognizes NKA with negligible cross-reactivity to SP and NKB (Moskowitz *et al.*, 1987). Assay sensitivity was 2 pg per tube with inter-and intra-assay variation 4.34 and 2.60%, respectively.

5,7-DHT Treatment

In halothane (2% in oxygen) anesthetized rats, 5,7-DHT (calculated as 200 μg free base in 20 μl of 0.01% ascorbic acid) was injected intracisternally (i.c.) 1 hr after DMI (25 mg/kg, intraperitoneally, i.p.). Vehicle-treated control rats received an i.c. injection of the ascorbic acid vehicle (1 mg/ml) solution 1 hr after DMI (25 mg/kg, i.p.). The rats were sacrificed by decapitation after 14 days. The thoracic spinal cord was removed, and the ventral horn and the intermediate area were rapidly microdissected. The intermediate area was used for the *in vitro* release studies and the ventral horn was used to assess the accumulation of [^{3}H]5-HT by serotoninergic nerve terminals. Protein content of the tissue was determined with the Lowry technique (Lowry *et al.*, 1951).

The cervical spinal cord was also removed and stored at -70°C for high performance liquid chromatography with electrochemical detection of 5-HT, dopamine and norepinephrine. Briefly, the cervical spinal cord samples were weighted and soaked into 400 µl of 100 mM perchloric acid. The tissue samples were then homogenized and centrifuged at 10,000 RPM for 15 min. The supernatant was placed in the autosampler (CMA Inc.) for subsequent analysis. The mobile phase (100 mM $NaHPO_4$, 1.5 mM sodium dodecylsulphate, 20 µM EDTA, 100 µl/l triethylamine, 15% acetonitrile and 12% methanol, pH 5.6) was filtered and degassed before pumping at a rate of 1 ml/min through a HR-80 column (ESA Inc.). Samples (20 µl) were injected to the column from the autosampler and electrochemical detection was performed using a colouchem detector (ESA Inc.).

Data Presentation and Statistical Analysis

The release of [^{3}H]5-HT was expressed as a fraction of total radioactivity contained in the tissue at the beginning of the release interval (percentage fractional release above baseline; Werling *et al.*, 1988). The release of SP or NKA from the superfusates was expressed as pg of SP/fraction of superfusate/µg tissue protein or as percent fractional release (superfusate content as a fraction of total SP content in the tissue at the beginning of the release interval). Data presented for K^+-evoked release of

5-HT (or SP and NKA) was the sum of transmitter released from fraction 3 to 5 (or fraction 4 to 6), while data presented for basal release of 5-HT (or SP and NKA) was calculated as the average of fractions 6 to 8 (or fraction 7 to 9). The monoamine contents of the cervical cord were expressed as ng/mg wet tissue weight. The statistical significance of the results was determined using a two-tailed Student's t-test for real data (pg or radioligand counts), or ANOVA and Tukey's protected T multiple comparison analysis on raw data or logarithmic transformation of percentage values. Values of $p < 0.05$ were considered significant.

RESULTS

A. Studies of [^{3}H]5-HT Release and Presynaptic Autoreceptor Regulation

Rationale: The following experiments were designed to measure K^+-stimulated release of preloaded [^{3}H]5-HT from the intermediate area, to show the presence of presynaptic inhibitory autoreceptors and determine the receptor subtype. Various K^+ concentrations were used to demonstrate the depolarization-induced release. In order to confirm release by vesicular mechanisms, Ca^{2+} free buffer containing EGTA was used. The effect of exogenous 5-HT on the release of [^{3}H]5-HT was studied to evaluate the presence of a presynaptic autoreceptor. The presynaptic autoreceptor subtype was determined by studying effects of 5-HT_{1A}, 5-HT_{1B} and 5-$HT_{2A/2C}$ agonists and antagonists on the release of [^{3}H]5-HT.

a. K^+-stimulated and extracellular Ca^{2+}-dependent release of [^{3}H]5-HT

Basal release of preloaded [^{3}H]5-HT was considered to be the release above baseline observed in the presence of 5 mM K^+. Basal release of [^{3}H]5-HT in the study was of a low level (< 0.5% of total tissue radioactivity accumulated), but measurable and stable. The basal release was not

calcium-dependent (Fig. 3).

The potassium depolarization-induced release of [^{3}H]5-HT was compared between the intermediate area and the ventral horn. The [^{3}H]5-HT efflux from both regions increased with increasing concentrations of K^+ (20-60 mM, with a commensurate reduction of Na^+) (Fig. 4). Thirty millimolar K^+ induced a fractional release of [^{3}H]5-HT in the intermediate area and the ventral horn of 3.8 ± 0.2% and 3.9 ± 0.2%, respectively (Fig. 4). The greatest fractional release of [^{3}H]5-HT was seen with 50 mM K^+ in both regions. Sixty millimolar of K^+ produced a smaller increase in the release than 50 mM K^+ (Fig. 4). Since the primary interest of the project was on structures related to autonomic outflow, subsequent experiments focused on the intermediate area only. When drug effects on K^+-stimulated release were examined, a concentration of 30 mM K^+ was chosen such that the release was reliably measurable and within the ascending portion of the dose-response curve. The K^+-induced release of [^{3}H]5-HT was dependent on the presence of calcium ions, as Ca^{2+}-free buffer containing EGTA resulted in K^+-evoked release of [^{3}H]5-HT which was not significantly different from the basal efflux (Fig. 3). Detection of the K^+-evoked and Ca^{2+}-dependent release is essential to characterize 5-HT as a neurotransmitter in the intermediate area.

Fig. 3. Effects of Ca^{2+} on K^+ (30 mM)-evoked release of [^{3}H]5-HT from the intermediate area of rat thoracic spinal cord. Ca^{2+} was present in the superfusion buffer at 1.2 mM or was replaced by equal molar of Mg^{2+} and with 1 mM EGTA. Values presented (% of fractional release above baseline) are the mean ± SEM of 6-10 sample. The baseline value was calculated as the average of fractions 6, 7 and 8. Although the tissue was stimulated with a high concentration of K^+ for only 2 min, effluent from the elevated K^+ period was at least partially collected in more than one fraction (fraction 3 to 5).

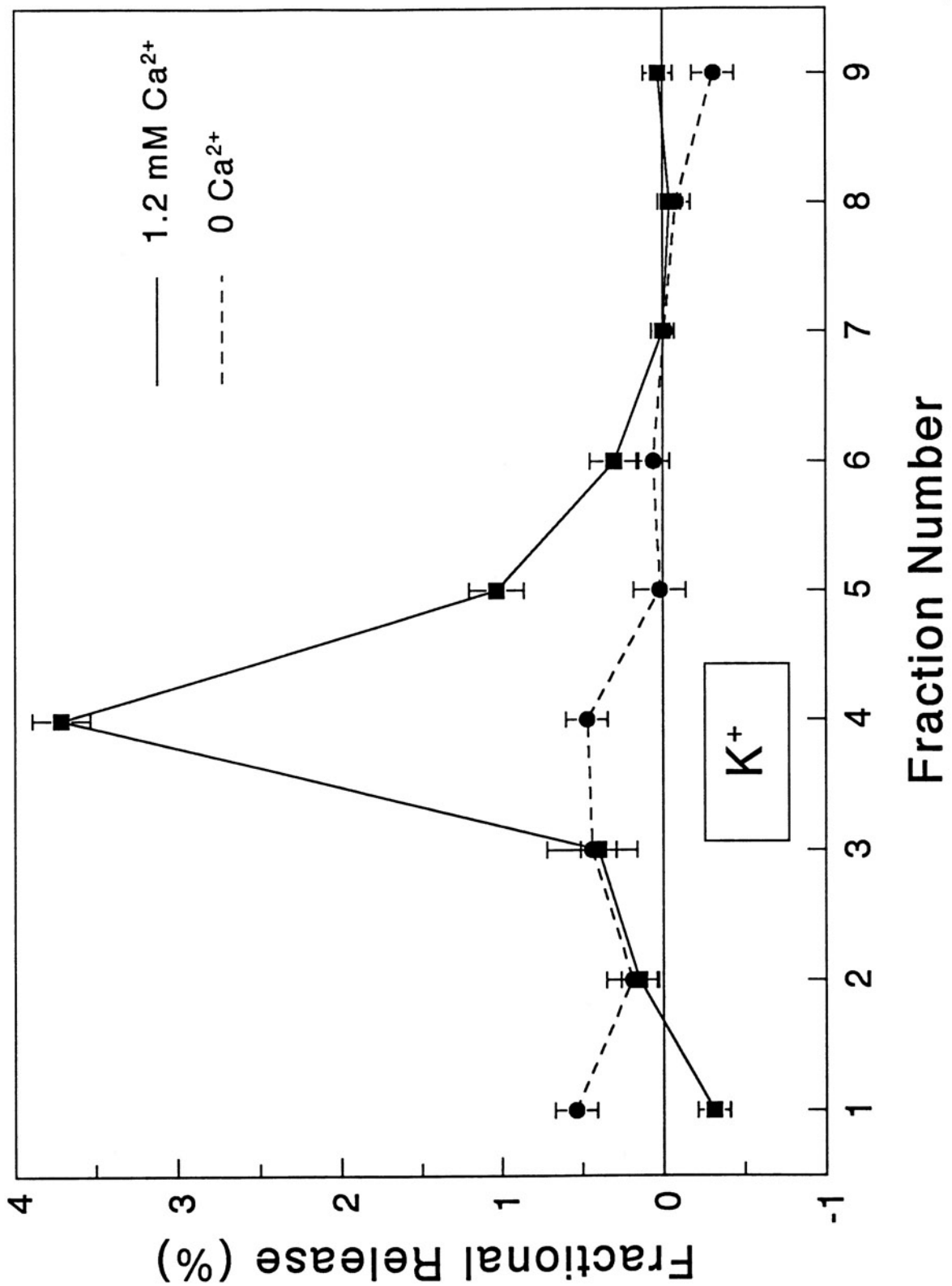
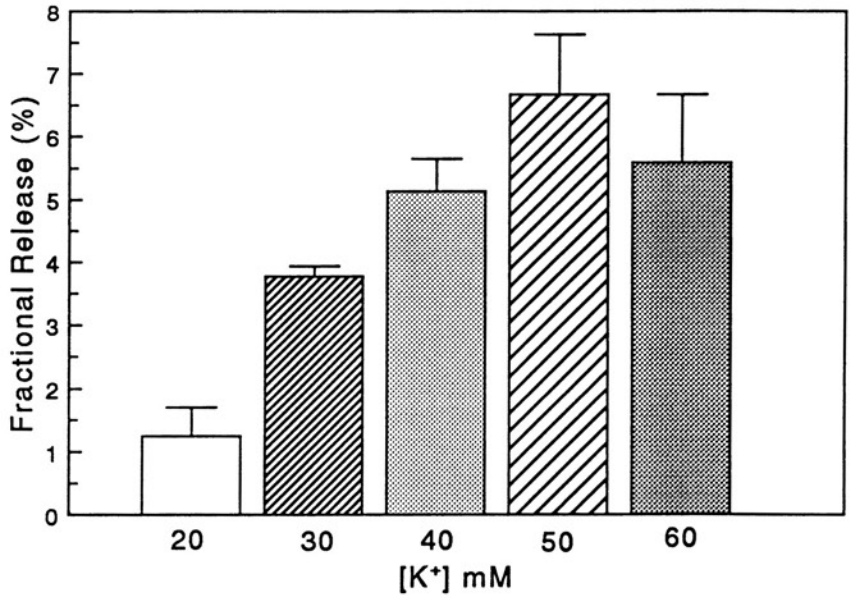
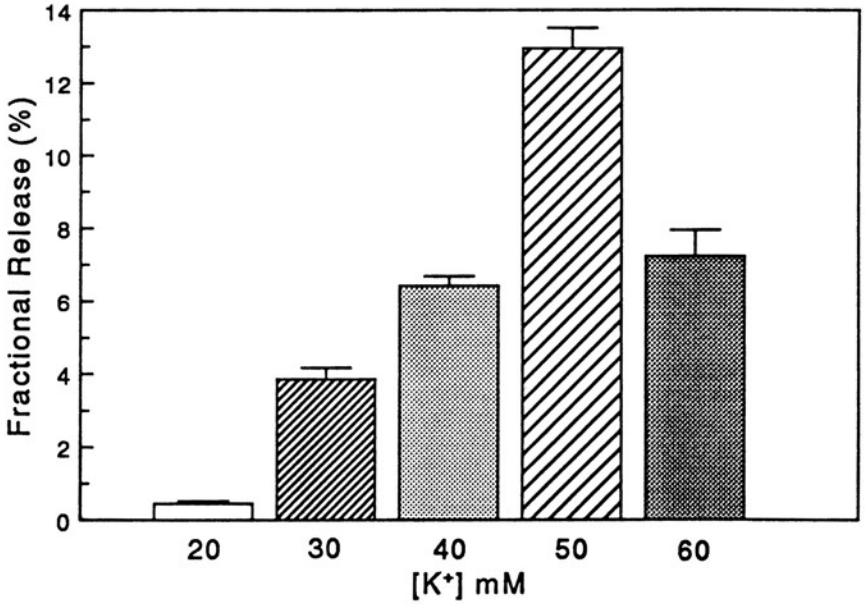

1.2 mM Ca2+
0 Ca2+
K+
Fractional Release (%)
Fraction Number
4
3
2
1
0
-1
1
2
3
4
5
6
7
8
9

Fig. 4. Effects of K^+ concentration on the amount of [^{3}H]5-HT released during stimulation period from (A) intermediate area and (B) ventral horn of rat thoracic spinal cord. Total tissue accumulation of [^{3}H]5-HT of the intermediate area was about 40,000 CPM with a baseline radioactivity contained in the superfusate of about 300 CPM. Values presented (% of fractional release above baseline) are the sum of [^{3}H]5-HT released from fraction 3 to 5 and are the mean ± SEM of 6-10 samples tested with each concentration of K^+.

A. Intermediate Area



B. Ventral Horn



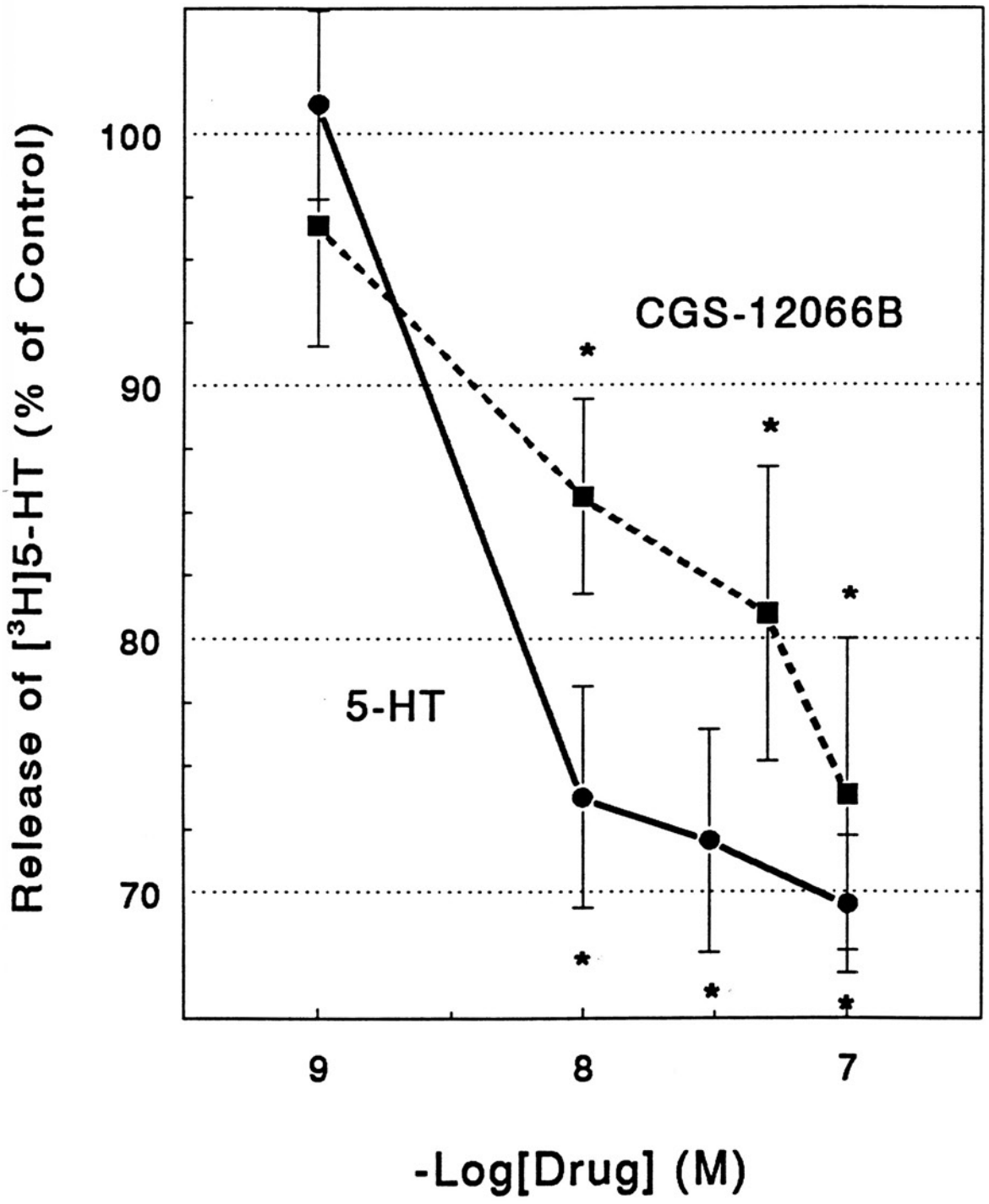
b. Effects of exogenous 5-HT on the release of [^{3}H]5-HT

Exogenous 5-HT was added to the superfusion system in an attempt to determine presynaptic autoreceptor regulation of the evoked release of [^{3}H]5-HT. Exogenous 5-HT inhibited the K^+-stimulated release of [^{3}H]5-HT from the intermediate area. A significant inhibition was produced by concentrations between 10^{-8} and 10^{-7} M) (Fig. 5). Basal release was not changed by exogenous 5-HT. Inhibition of 5-HT on its own release suggests the presence of presynaptic inhibitory autoreceptors in the intermediate area.

c. Effects of 5-HT_{1B} agonist and antagonist on the release of [^{3}H]5-HT

Because previous studies on the rat whole spinal cord suggested that presynaptic inhibitory autoreceptors appear to be of the 5-HT_{1B} subtype (Monroe and Smith, 1985), I examined the effects of 5-HT_{1B} receptor activation or inhibition on the release of [^{3}H]5-HT from the intermediate area of the thoracic spinal cord. CGS-12066B (10^{-9}-10^{-7} M), a 5-HT_{1B} agonist, caused a decrease in the K^+-induced release of [^{3}H]5-HT from the intermediate area (Fig. 5) without changing the basal release. RU 24969, a new and selective 5-HT_{1B} agonist, also dose-dependently decreased the release of [^{3}H]5-HT. The K^+-stimulated release of [^{3}H]5-HT

Fig. 5. Dose-response curves of the effects of exogenous 5-HT and of the 5-HT_{1B} agonist, CGS-12066B, on K^+-evoked release of [^{3}H]5-HT from the intermediate area. Control values were obtained from the K^+-stimulated release of [^{3}H]5-HT in the absence of 5-HT agonists. The fractional release (%) values of the controls ranged from 2.59% to 4.86% with the average of 3.7%. Drugs were present during the last 20 min prior to, and during K^+ stimulation. Each point represents the mean ± SEM of 6 determinations. * Significant difference compared with control ($p < 0.05$).


Release of [3H]5-HT (% of Control)
100
90
80
70
CGS-12066B
5-HT
9
8
7
-Log[Drug] (M)

decreased 39.12 ± 2.41 and 19.34 ± 3.57%, respectively in the presence of 10^{-8} and 10^{-7}M RU 24969.

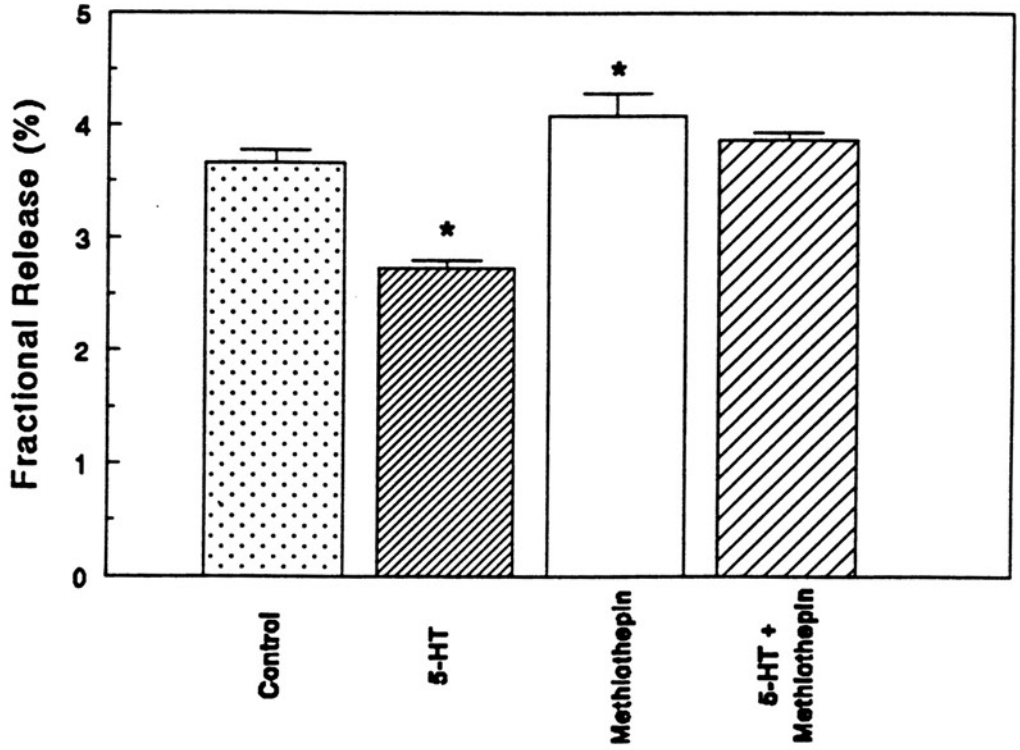
Methiothepin, a 5-HT_{1B} antagonist, at 10^{-6} M completely blocked the inhibitory effects of exogenous 5-HT (3 x 10^{-8} M) and of CGS-12066B (10^{-7} M) on the release of [^{3}H]5-HT from the intermediate area (Fig. 6A, 6B). Methiothepin did not change the basal release of [^{3}H]5-HT and produced a small enhancement (12%) of K^+-evoked release of [^{3}H]5-HT when used alone at 10^{-6} M (Fig. 6A). Both 5-HT and 5-HT_{1B} agonists decreased the stimulated release of [^{3}H]5-HT and the inhibition was blocked by the 5-HT_{1B} antagonist. Thus, the 5-HT_{1B} receptor appears to be the presynaptic autoreceptor involved in the regulation of 5-HT release from the intermediate area.

d. Effects of a 5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_3 agonist and antagonist on the release of [^{3}H]5-HT

5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_3 binding sites are located in the intermediate area (Thor *et al.*, 1993), so it is necessary to determine the effects of these receptors in regulating the release of [^{3}H]5-HT. 8-OH-DPAT, a highly selective 5-HT_{1A} agonist, did not alter the K^+-induced release of [^{3}H]5-HT when used at 10^{-7} M (Fig. 7A). NAN-190 (10^{-6} M), a potent competitive 5-HT_{1A} antagonist, had no

Fig. 6. Effects of methiothepin (10^{-6}M) on the (A) 5-HT-induced and (B) 5-HT_{1B} agonist, CGS-12066B-induced depression of K^+-evoked release of [^{3}H]5-HT. Methiothepin and 5-HT ($3x10^{-8}$M) or CGS-12066B (10^{-7}M) were present during the last 20 min prior to, and during K^+ stimulation. Values presented (% of fractional release above baseline) are the mean ± SEM of 6 samples. * Significant difference compared with control ($p< 0.05$).

A.


Fractional Release (%)
0
1
2
3
4
5
*
*
*
Control
5-HT
Methiothepin
5-HT + Methiothepin

B.

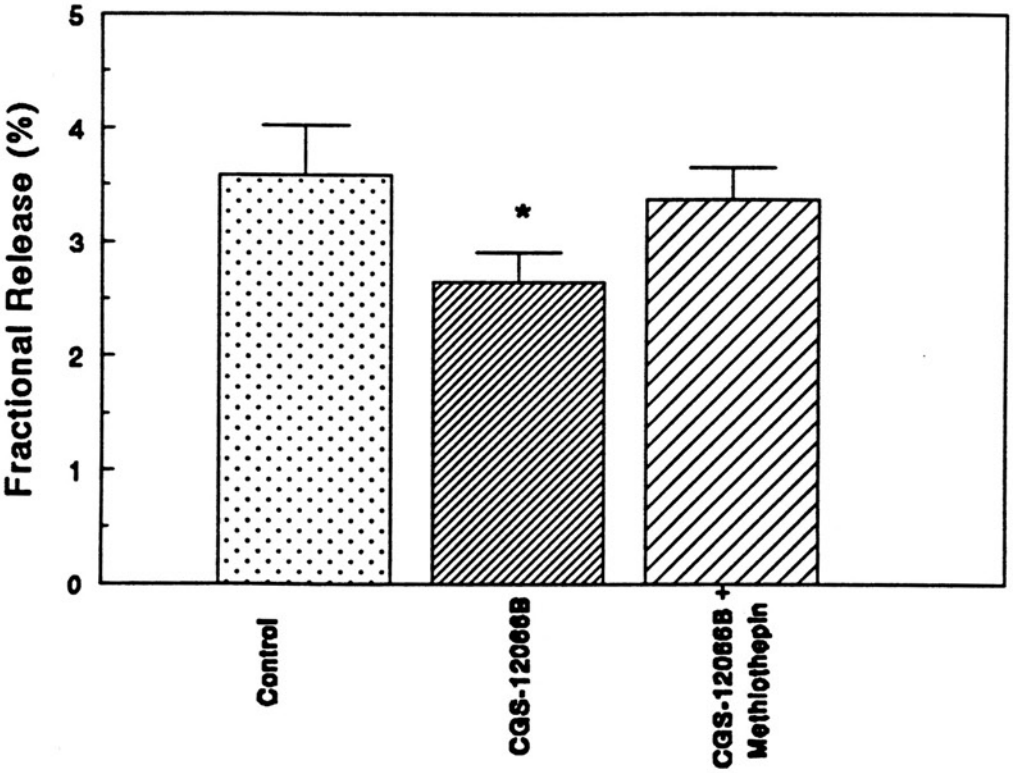
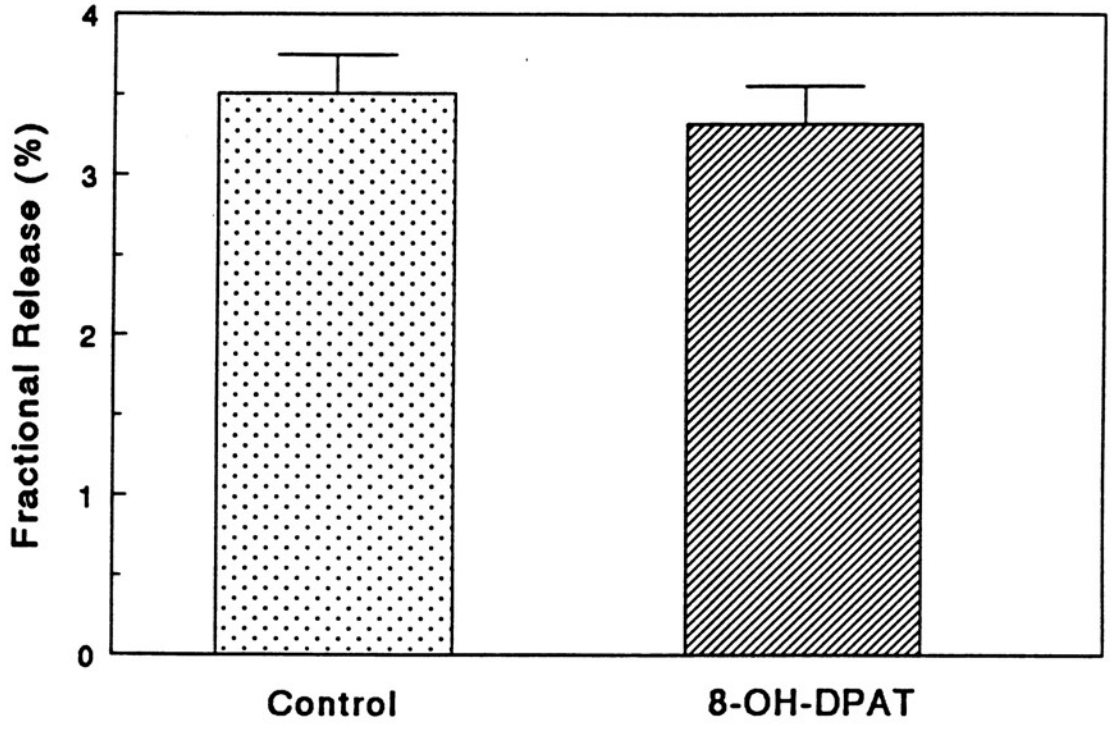
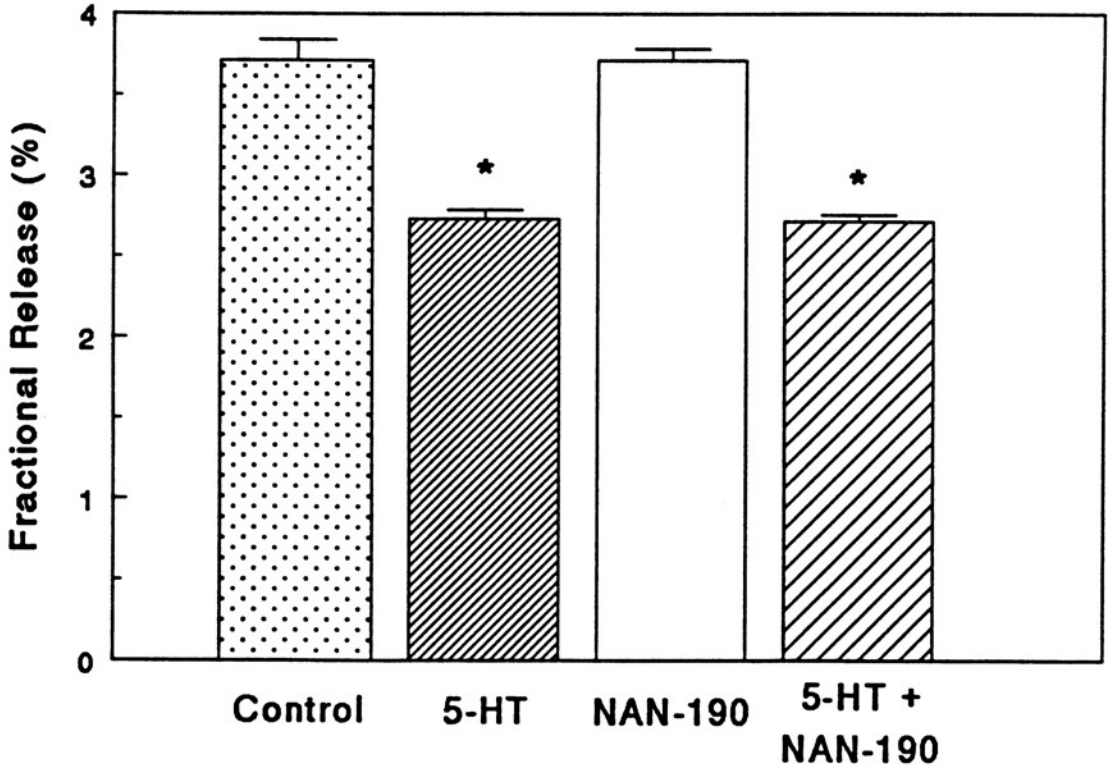

Fractional Release (%)
0
1
2
3
4
5
*
Control
CGS-12066B
CGS-12066B + Methiothepin

Fig.7. (A) Effect of 5-HT_{1A} agonist, 8-OH-DPAT (10^{-7}M), on K^+-evoked release of [^{3}H]5-HT. (B) Effect of 5-HT_{1A} antagonist, NAN-190 (10^{-7}M), on 5-HT ($3x10^{-8}$M)-induced depression of K^+-evoked release of [^{3}H]5-HT. Drugs were present during the last 20 min prior to, and during K^+-stimulation. Values presented (% of fractional release above baseline) are the mean ± SEM of 6 samples. * Significant difference compared with control ($p< 0.05$).

A.


Fractional Release (%)
0
1
2
3
4
Control
8-OH-DPAT


Fractional Release (%)
0
1
2
3
4
*
*
Control
5-HT
NAN-190
5-HT +
NAN-190

effect on the K^+-induced release of [^{3}H]5-HT, nor did it block the inhibitory effect of 3 x 10^{-8} M exogenous 5-HT (Fig. 7B). Neither 8-OH-DPAT nor NAN-190 had any effect on the basal release.

(±)DOI hydrochloride is a 5-$HT_{2A/2C}$ agonist (Appel *et al.*, 1990), and 2-methyl-5-HT is a 5-HT_3 agonist (Costall *et al.*, 1990). Neither 10^{-6} M (±)DOI nor 2-methyl-5-HT altered the K^+-stimulated release of [^{3}H]5-HT from the intermediate area. The K^+-stimulated release of [^{3}H]5-HT in the absence and presence of (±)DOI was 3.89 ± 0.29% and 3.78 ± 0.43%, respectively. The stimulated fractional release of [^{3}H]5-HT in the absence and presence of 2-methyl-5-HT was 3.21 ± 0.52% and 3.07 ± 0.65%, respectively. Neither of these drugs affected the basal release. These results suggest that 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors are not presynaptic autoreceptors regulating the release of 5-HT.

B. Studies of Coexisting Neurochemicals (SP, NKA and TRH) on the Regulation of [^{3}H]5-HT Release

Rationale: The following experiments were designed to study the effects of coexisting neurochemicals (SP, NKA and TRH) on the release of [^{3}H]5-HT. The effect of SP on the release of [^{3}H]5-HT was studied to examine the interaction between SP and 5-HT. In order to determine the mechanism and the receptor subtype involved, the effects of NK_1 agonist and antagonist were also investigated. NKA and TRH also coexist with 5-HT in the intermediate area, thus, it is also essential to evaluate the effects of NKA and TRH analogues on the release of [^{3}H]5-HT.

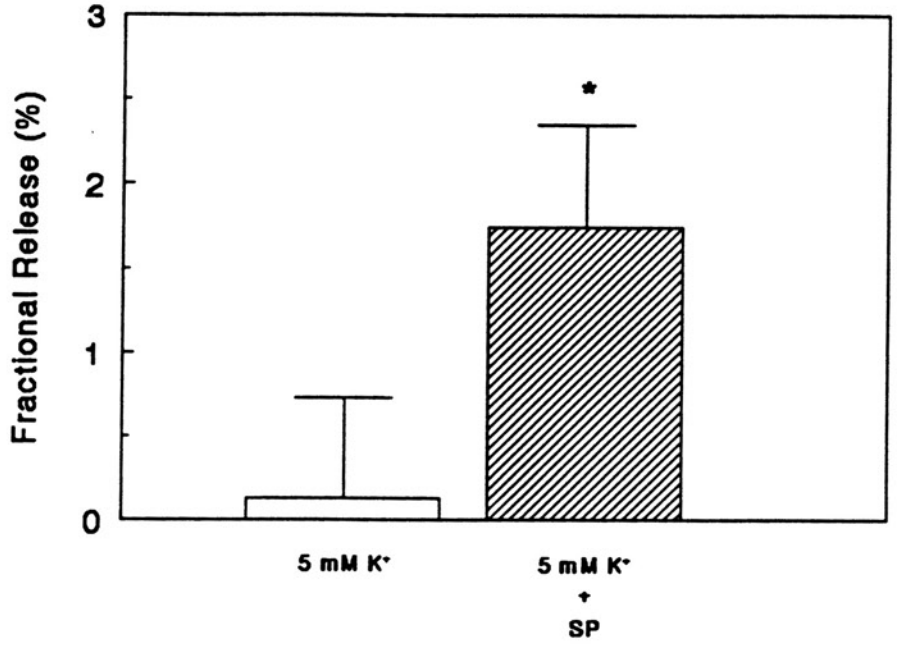
a. Effects of SP, NK_1 agonist and antagonist on the release of [^{3}H]5-HT

Both SP (10^{-6}M) and a selective NK_1 agonist, GR 73632 (10^{-6}M; Maggi *et al.*, 1993) significantly increased the basal release of [^{3}H]5-HT (Fig. 8A and 8B) without changing the K^+-stimulated (30 mM) release. The effect of GR 73632 on the basal release of [^{3}H]5-HT was dose-dependent from 10^{-8}M to 10^{-6}M. A lower concentration of GR 73632 (10^{-9}M) had no significant effect (Fig. 9).

A selective NK_1 antagonist, GR 82334 (10^{-8}-10^{-6}M; Maggi

Fig. 8. Effects of (A) SP (10^{-6}M) and (B) NK_1 agonist, GR 73632 (10^{-6}M) on basal (5 mM K^+) release of [^{3}H]5-HT from the intermediate area of rat thoracic spinal cord. SP or GR 73632 was added in the buffer for 6 min. Values presented (% of fractional release above baseline) are the mean ± SEM of more than 6 samples from 3 separate experiments. * Significant difference compared with the control (5 mM K^+) in the absence of SP or GR 73632 ($p< 0.05$).

A.


Fractional Release (%)
3
2
1
0
*
5 mM K+
5 mM K+
+
SP

B.

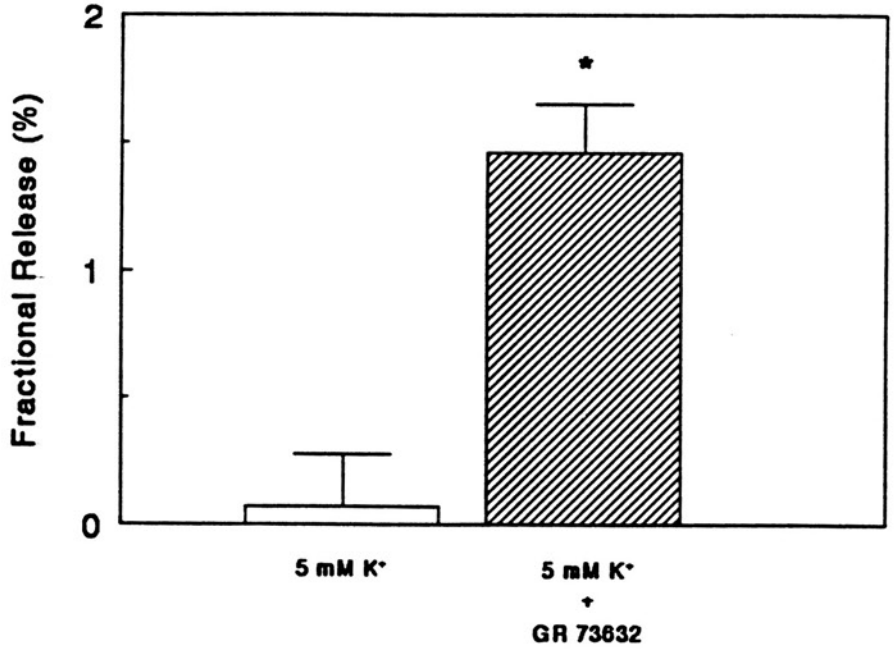
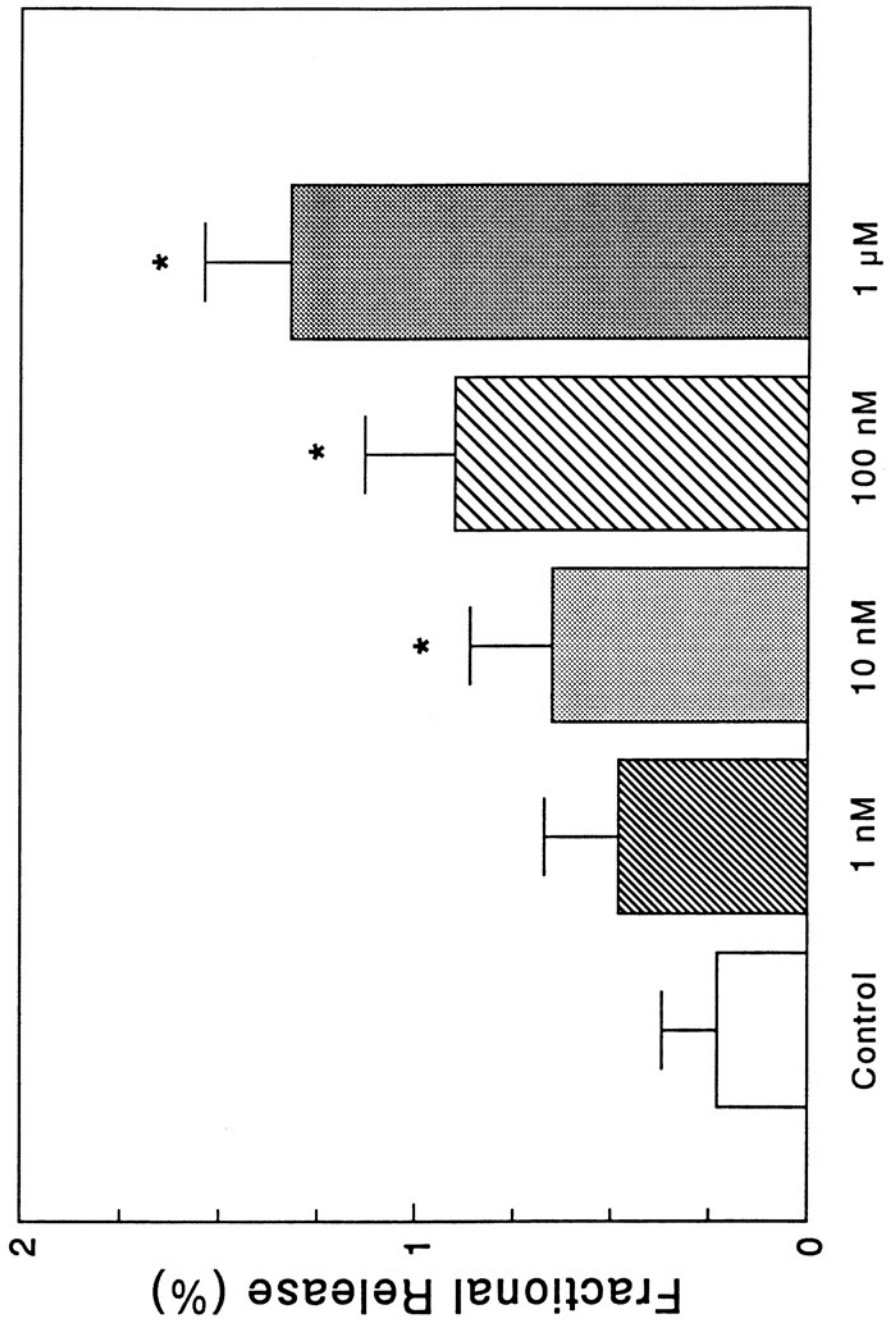

Fractional Release (%)
2
1
0
*
5 mM K+
5 mM K+
+
GR 73632

Fig. 9. Dose-response curve of the effect of the NK_1 agonist, GR 73632, on the basal release of [^{3}H]5-HT from the intermediate area. GR 73632 was added in the buffer for 6 min. Each point presents the mean ± SEM of more than 6 samples in 3 separate experiments. * Significant difference compared with control ($p < 0.05$).


Fractional Release (%)
0
1
2
Control
1 nM
10 nM
100 nM
1 µM
*
*
*

et al., 1993) dose-dependently reduced the effects of GR 73632 (10^{-6}M; Fig. 10) and SP (10^{-6}M; data not shown) to enhance the basal release of [^{3}H]5-HT. GR 82334 alone had no effect on the basal release of [^{3}H]5-HT. Basal release in the presence of 10^{-8}M, 10^{-7}M and 10^{-6}M of GR 82334 was 0.29 ± 0.13, 0.16 ± 0.09 and 0.31 ± 0.04%, respectively.

In Ca^{2+}-free buffer, GR 73632 (10^{-6}M) significantly increased the basal release to the same extent as it did in the presence of Ca^{2+} (Fig. 11). Therefore, the increased basal release of [^{3}H]5-HT induced by GR 73632 was not dependent upon extracellular Ca^{2+}.

Fig. 10. Effects of NK_1 antagonist, GR 82334 (10^{-8}-$^{-5}$M), on GR 73632 (10^{-6}M)-induced basal release of [^{3}H]5-HT. GR 82334 was added 20 min prior to, and during the addition of GR 73632. Values presented (% of fractional release above baseline) are the mean ± SEM of 6 samples in 3 separate experiments. * Significant difference compared with control (5 mM K^+) ($p< 0.05$).

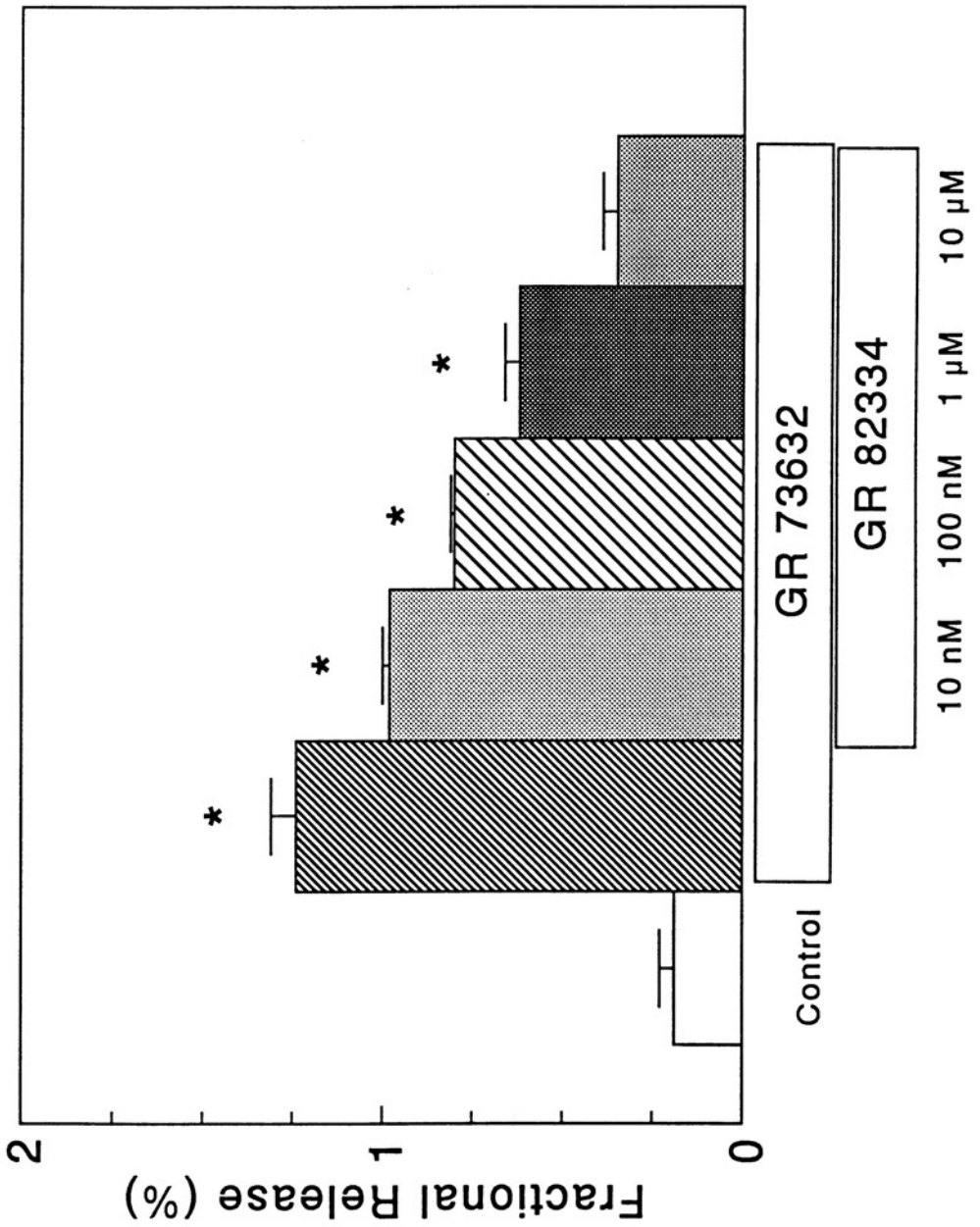
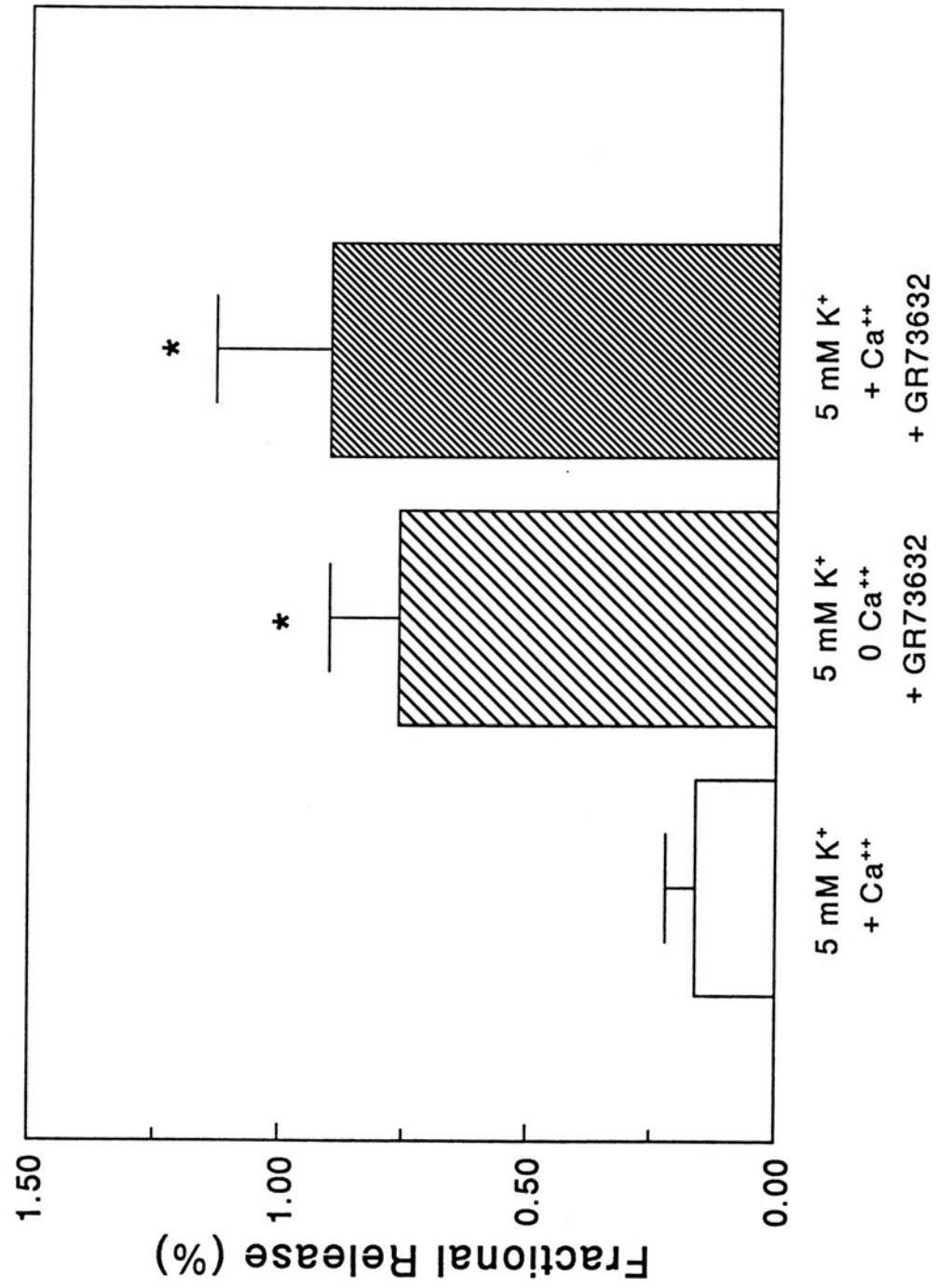

Fractional Release (%)
2
1
0
*
*
*
*
Control
GR 73632
GR 82334
10 nM
100 nM
1 µM
10 µM

Fig. 11. Effect of Ca^{2+} on GR 73632 (10^{-7}M) induced basal release of [^{3}H]5-HT from the intermediate area of rat thoracic spinal cord. Ca^{2+} was present in the superfusion buffer at 1.2 mM (Ca^{2+}) or was replaced by equal molar of Mg^{2+}, with 1 mM EGTA (0 Ca^{2+}). Values presented (% of fractional release above baseline) are the mean ± SEM of more than 6 samples in 3 separate experiments. * Significant difference compared with control (5 mM K^+ + Ca^{2+} without GR 73632) ($p < 0.05$).


Fractional Release (%)
1.50
1.00
0.50
0.00
*
*
5 mM K+
+ Ca++
5 mM K+
0 Ca++
+ GR73632
5 mM K+
+ Ca++
+ GR73632

b. Effects of NKA and NK_2 agonist on the release of [^{3}H]5-HT

Neither NKA (10^{-8}-10^{-6}M) nor GR 64349 (10^{-8}-10^{-6}M, a selective NK_2 agonist, Maggi *et al.*, 1993) changed the basal or stimulated (30 mM K^+) release of [^{3}H]5-HT. The percentages of fractional basal release in the absence and presence of NKA (10^{-6}M) were 0.39 ± 0.13% and 0.42 ± 0.17%, respectively. The percentages of fractional basal release in the absence and presence of GR 64349 (10^{-6}M) were 0.43 ± 0.24% and 0.33 ± 0.19%, respectively. The percentages of fractional release induced by 30 mM K^+ in the absence and presence of NKA (10^{-6}M) were 4.88 ± 0.85% and 4.68 ± 0.88%, respectively.

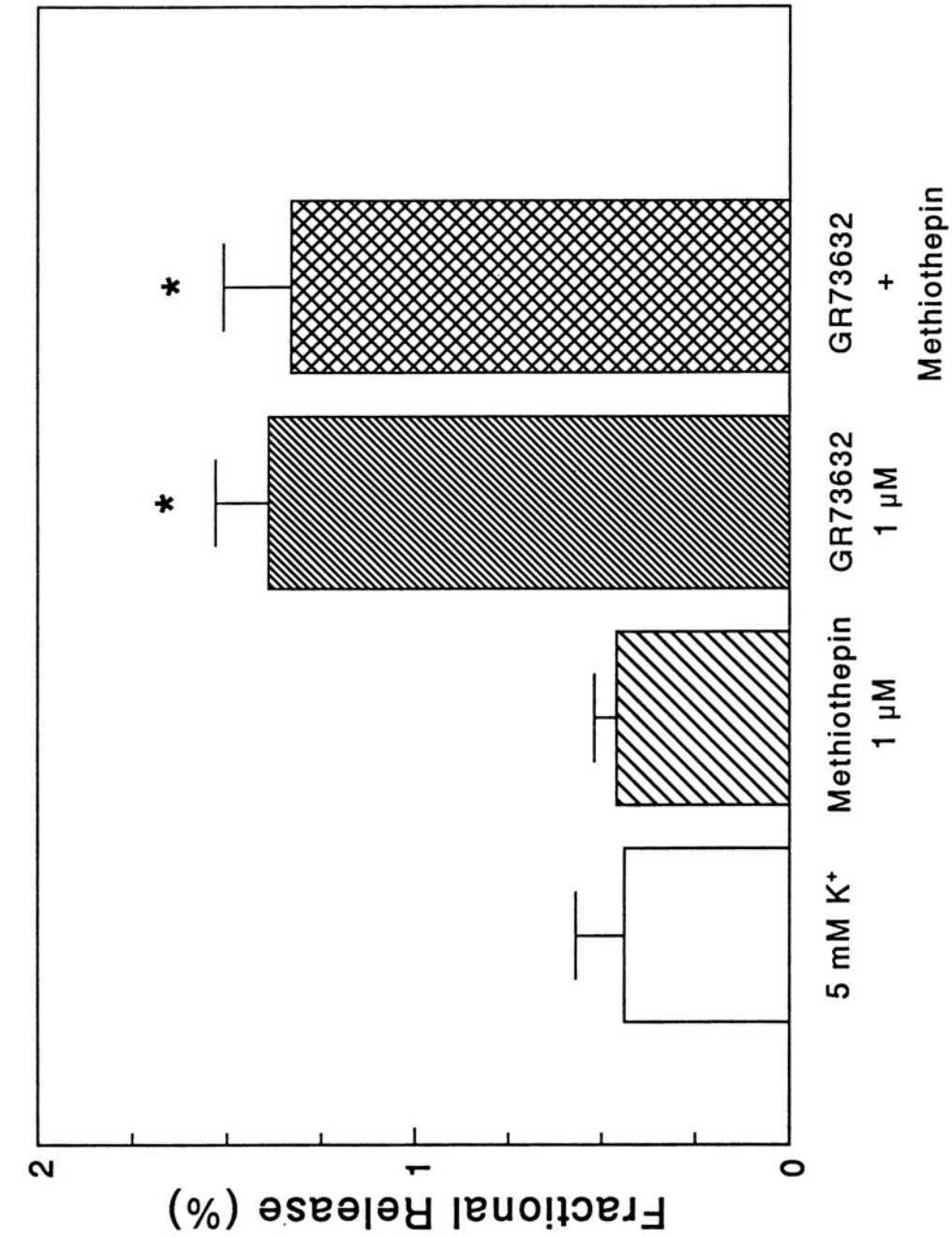
c. Effect of TRH analog on the release of [^{3}H]5-HT

MK-771 (10^{-6}M), a metabolically stable TRH receptor agonist (Sharif and Burt, 1983; Yarbrough and McGuffin-Clineschmidt, 1979), did not change the basal or stimulated (30 mM K^+) release of [^{3}H]5-HT. The percentages of fractional basal release in the absence and presence of MK-771 (10^{-6} M) were 0.37 ± 0.16% and 0.44 ± 0.21%, respectively. The percentages of fractional release evoked by 30 mM K^+-depolarization in the absence and presence of MK-771 (10^{-6}M) were 3.87 ± 0.44% and 4.24 ± 1.07%, respectively.

d. Interaction of NK_1 agonist with $5\text{-}HT_{1B}$ presynaptic inhibitory autoreceptor

$5\text{-}HT_{1B}$ receptor activation inhibited the K^+-evoked release of [^{3}H]5-HT and this suggests the presence of presynaptic inhibitory $5\text{-}HT_{1B}$ autoreceptor. It was of interest to evaluate the interaction of NK_1 agonists with $5\text{-}HT_{1B}$ autoreceptors. Blocking the presynaptic $5\text{-}HT_{1B}$ autoreceptor with methiothepin (10^{-6}M) did not alter the GR 73632-induced increase of basal release of [^{3}H]5-HT (Fig. 12).

Fig. 12. Effects of NK_1 agonist (GR 73632, 10^{-6}M) with $5\text{-}HT_{1B}$ antagonist (methiothepin, 10^{-6}M) on the basal (5 mM K^+) release of [^{3}H]5-HT. Methiothepin was added 20 min prior to and during the addition of GR 73632. Values presented (% of fractional release above baseline) are the mean ± SEM of more than 6 samples in 3 separate experiments. * Significant difference compared with control (5 mM K^+) ($p< 0.05$).


Fractional Release (%)
2
1
0
*
*
5 mM K+
Methiothepin 1 µM
GR73632 1 µM
GR73632 + Methiothepin

C. Studies on the Release of SP and Presynaptic Autoreceptor Regulation

Rationale: The following experiments were designed to study the release of endogenous SP and presynaptic regulation of the release of SP in the intermediate area of the thoracic spinal cord. Various concentrations of K^+ were used to evoke depolarization-induced release. Ca^{2+}-dependence was studied to confirm a vesicular release mechanism. In an attempt to determine whether SP is released from serotoninergic nerve terminals, SP content and the release of SP from the intermediate area were studied in rats pretreated with serotoninergic neurotoxin (5,7-DHT). The effects of exogenous 5-HT and a 5-HT_{1B} presynaptic autoreceptor activation were studied to evaluate their roles in regulating the release of SP. The effects of NKA and TRH on the release of SP were also studied by adding NKA, NK_2 agonist and a TRH analogue to the system.

a. K^+-stimulated and Ca^{2+}-dependent release of SP

The basal release of SP from the intermediate area was low (an average of 0.34 $\pm$ 0.19%), but measurable and stable (Fig. 13). The release of SP increased with increasing concentrations of K^+ (20-60 mM) (Fig. 14). The release of SP induced by 60 mM K^+ (an average of 4.59 $\pm$ 0.11%) was reliably measurable and was at the middle range

Fig. 13. Effects of Ca^{2+} on K^+ (60 mM)-evoked release of SP from the intermediate area of rat thoracic spinal cord. Ca^{2+} was present in the superfusion buffer at 1.2 mM or was replaced by equal molar of Mg^{2+} and with 1 mM EGTA. Although the tissue was stimulated with a high concentration of K^+ for only 4 min, effluent from the elevated K^+ period was at least partially collected in more than one fraction (fractions 4 to 6). Values presented (% of fractional release) are the mean ± SEM of 6-10 samples in each group.

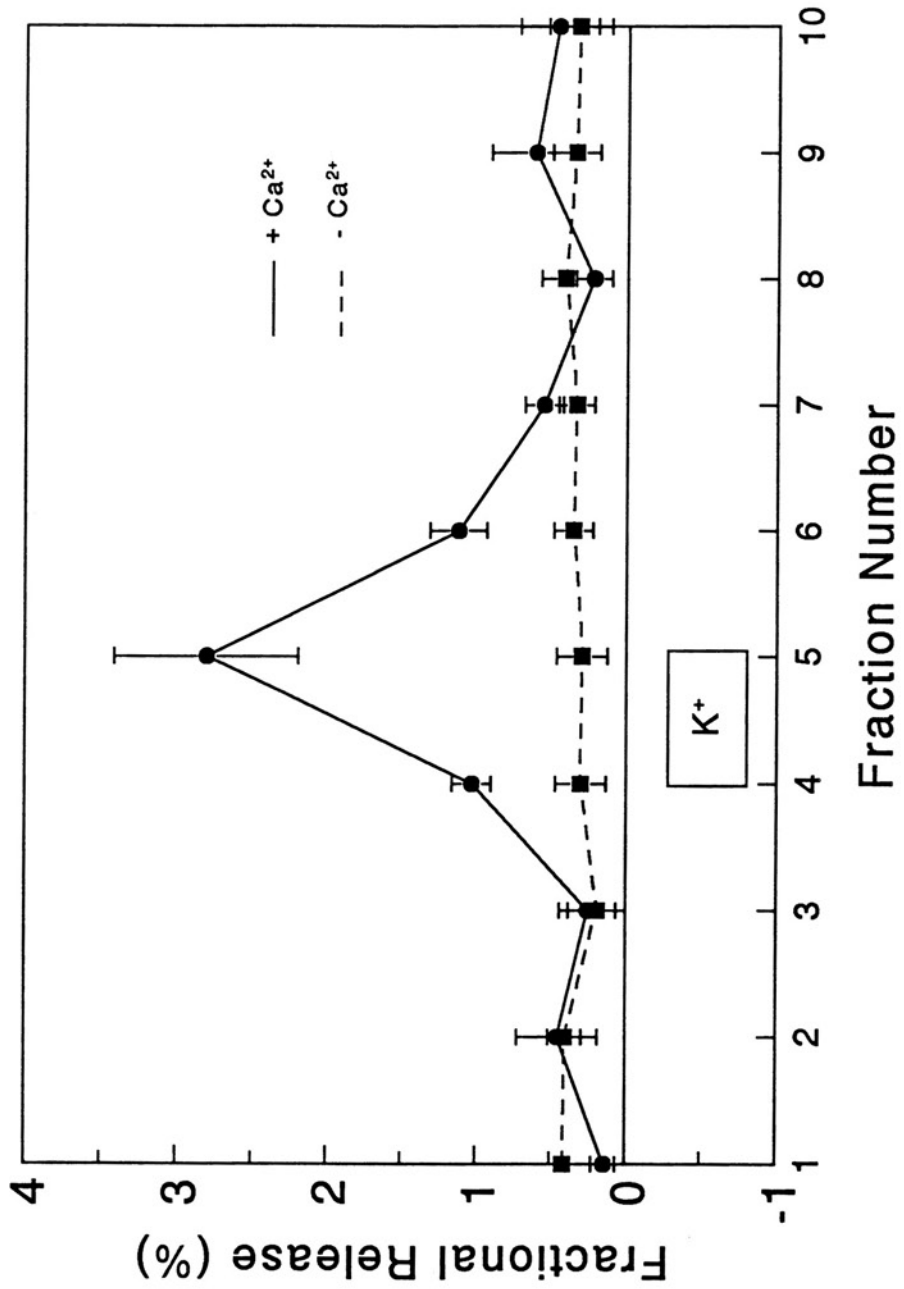
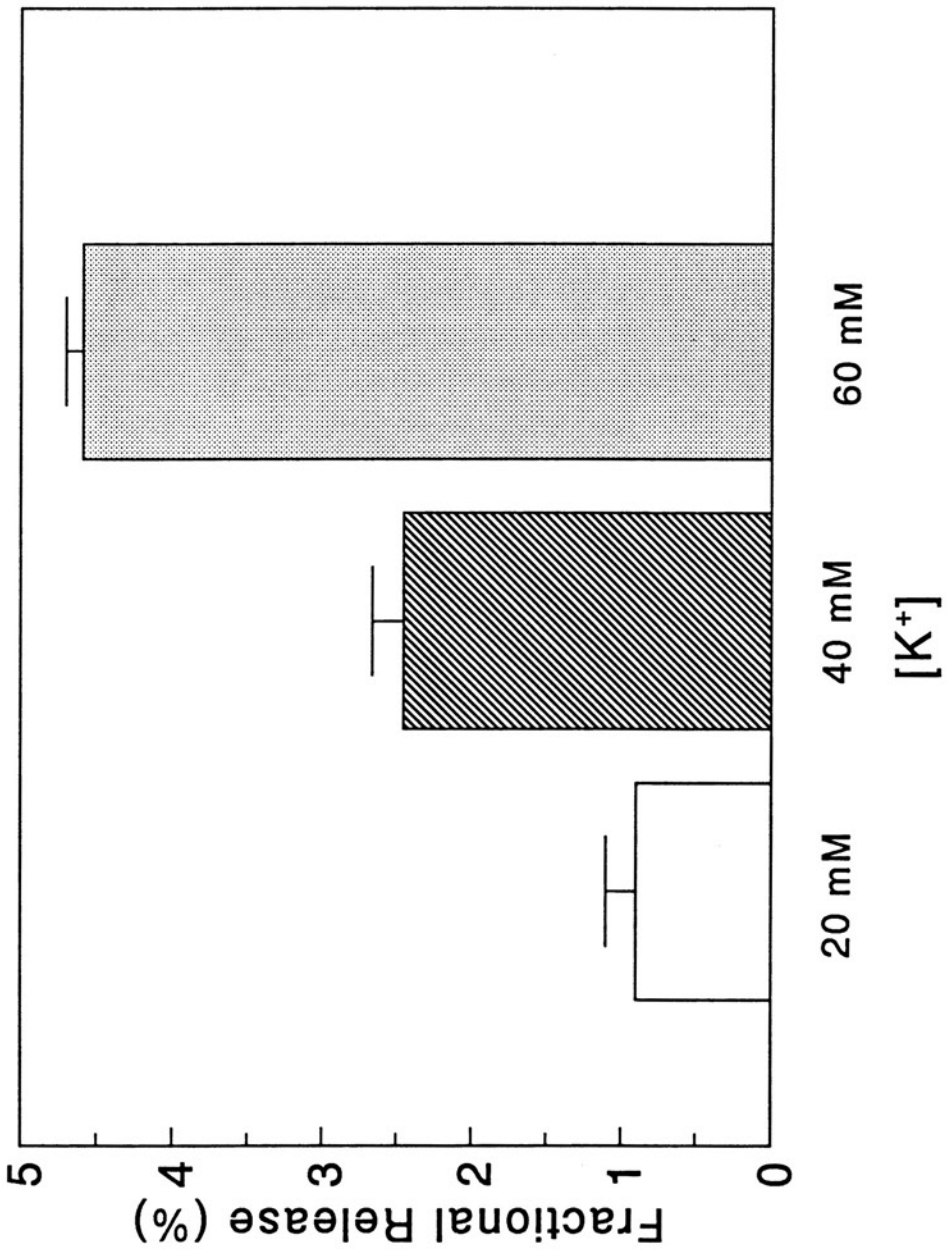

+ Ca2+
- Ca2+
K+
Fractional Release (%)
Fraction Number
4
3
2
1
0
-1
1
2
3
4
5
6
7
8
9
10

Fig. 14. Effects of K^+ concentration on the amount of SP released during stimulation period from the intermediate area of rat thoracic spinal cord. Values presented (% of fractional release) are the mean ± SEM of 6-10 samples tested with each concentration of K^+.


Fractional Release (%)
0
1
2
3
4
5
20 mM
40 mM
60 mM
[K+]

of the RIA standard curve. Thus, when drug effects on K^+-stimulated release were examined, a concentration of 60 mM K^+ was chosen. The K^+-induced release of SP was dependent on the presence of extracellular calcium, as Ca^{2+}-free buffer resulted in K^+-evoked release of SP which was not significantly different from basal efflux (Fig. 13). The basal release of SP was independent of extracellular Ca^{2+} (Fig. 13).

b. Effects of 5,7-DHT treatment on the release of SP

Pretreatment of rats with the selective serotoninergic neurotoxin, 5,7-DHT, resulted in a depletion of 5-HT content in the cervical spinal cord and decreased the accumulation of [^{3}H]5-HT by the thoracic ventral horn. The cervical cord 5-HT content of 5,7-DHT treated rats was reduced nearly 80% compared to that of the vehicle control rats (Table 2). Whereas, cervical spinal cord norepinephrine and dopamine contents were not altered by the 5,7-DHT treatment (Table 2). The accumulation [^{3}H]5-HT by thoracic ventral horn slices from 5,7-DHT treated rats was 36 ± 11% of the accumulation by slices from the vehicle control rats.

Table 2

Cervical spinal cord 5-HT, dopamine and norepinephrine content in 5,7-DHT treated and vehicle control rats.

Monoamine	Sample Concentration (ng/mg wet weight)	
	Vehicle Control	5,7-DHT Treated
Serotonin	0.044 ± 0.011	0.010 ± 0.004
Dopamine	0.025 ± 0.003	0.025 ± 0.005
Norepinephrine	0.243 ± 0.083	0.274 ± 0.044

The cervical spinal cord was removed 14 days after 5,7-DHT or vehicle treatment. Values presented are the mean ± SEM of more than 12 rats totally.

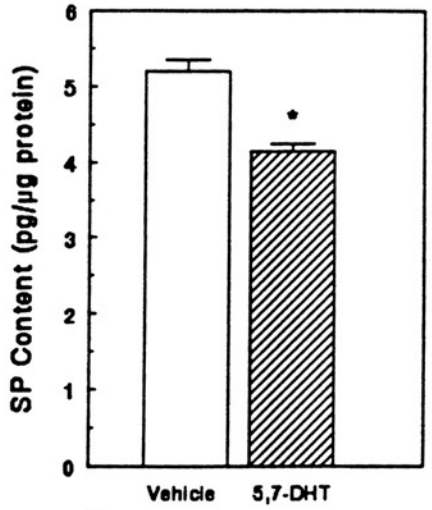
Pretreatment with 5,7-DHT decreased the SP-IR content, and the absolute amount of SP released from the intermediate area (Fig. 15A and 15B). Intermediate area SP-IR content was significantly reduced (21%) in 5,7-DHT treated compared to vehicle control rats (Fig. 15A). The amount of SP released (pg/fraction/μg protein) was also reduced (30%) in 5,7-DHT treated rats. However, the percent fractional release of SP (i.e., the fraction of the remaining tissue content of SP released by K^+-depolarization) from the intermediate area was not changed after 5,7-DHT treatment (Fig. 15C).

c. Effects of 5-HT and analogues on the release of SP

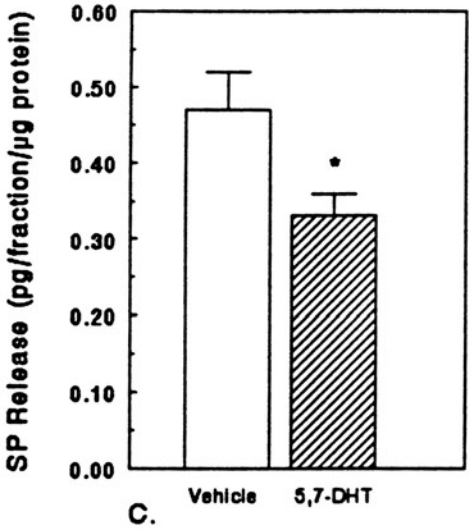
Because the release of [^{3}H]5-HT from the intermediate area is modulated by presynaptic 5-HT_{1B} autoreceptors, it is of interest to determine whether 5-HT or 5-HT_{1B} receptor agonists likewise regulate the release of SP. Neither serotonin ($3x10^{-8}$ and 10^{-6}M), nor the 5-HT_{1B} receptor agonists (CGS-12066B, 10^{-7}M; RU 24969, 10^{-8}-10^{-6}M) changed the basal or stimulated release of SP from the intermediate area (Table 3). Furthermore, a 5-HT_3 receptor agonist (2-methyl-5-HT, 10^{-6}M) failed to change the K^+-stimulated release of SP (Table 3).

Fig. 15. Effects of serotoninergic neurotoxin, 5,7-DHT treatment on (A) SP content (pg/μg protein), (B) absolute amount of SP released (pg/fraction/μg protein) and (C) percentage of SP release (fractional release, %) from the intermediate area. The release of SP was studied 14 days after 5,7-DHT treatment. Values presented are the mean ± SEM of more than 6 samples. * Significant difference compared with vehicle control ($p < 0.05$).

A.


SP Content (pg/µg protein)
0
1
2
3
4
5
6
*
Vehicle
5,7-DHT

B.


SP Release (pg/fraction/µg protein)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
*
Vehicle
5,7-DHT

C.

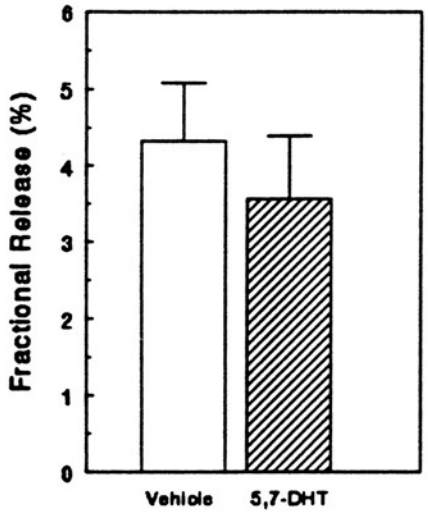

Fractional Release (%)
0
1
2
3
4
5
6
Vehicle
5,7-DHT

Table 3.

Effects of 5-HT, 5-HT_{1B} agonists (CGS-12066B and RU 24969), 5-HT_3 agonist (2-methyl-5-HT), NKA and NK_2 receptor agonist (GR 64349) and TRH analog (MK-771) on K^+-stimulated release of SP from the intermediate area.

Drug	[nM]	SP Release (% of control evoked release)
5-HT	30	94 ± 5
	1000	103 ± 8
CGS-12066B	100	92 ± 10
RU 24969	10	105 ± 5
	1000	102 ± 6
2-Methyl-5-HT	1000	108 ± 3
NKA	10	104 ± 4
	100	108 ± 3
GR 64349	100	100 ± 3
	1000	99 ± 3
MK-771	1000	97 ± 7

Drugs were perfused during the last 20 min prior to, and during K^+-stimulation. Values presented (% of control) are the mean ± SEM of more than 6 samples. Control values are the release of SP induced by 60 mM K^+ without agonists.

d. Effects of NK_1 agonist and antagonist on the release of SP

In an attempt to determine whether the amount of SP released by K^+ stimulation was regulated by presynaptic tachykinin autoreceptors, the tissue was superfused with the NK_1 receptor antagonist, GR 82334 (Maggi *et al.*, 1993), at concentrations between 10^{-9}-10^{-7}M. GR 82334 significantly increased K^+-evoked release of SP and this effect was concentration-dependent (Fig. 16). The dose-response curve to GR 82334 appeared to be "bell-shaped" in that 10^{-8}M of GR 82334 had the highest effect (Fig. 16). GR 82334 had no effect on the basal release of SP.

To further characterize NK_1 receptor modulation of SP release from the intermediate area, an attempt was made to investigate the effects of the selective NK_1 receptor agonist, GR 73632. GR 73632 (10^{-8}-10^{-6}M) significantly increased K^+-stimulated, extracellular Ca^{2+}-dependent (Table 4) and basal release (161.4 ± 2.4% of control) of SP in the presence of peptidase inhibitors. The effects of GR 73632 (10^{-8}M) on the release of SP was not antagonized by GR 82334 (up to 10^{-7}M) (Table 4). Another NK_1 agonist, [Sar^9,$Met(O_2)^{11}$]SP (10^{-9}-10^{-7}M) also significantly increased K^+-stimulated (Table 4) and basal release of SP and the effects were not antagonized by GR 82334. Basal release of SP in the presence of [Sar^9,$Met(O_2)^{11}$]SP (10^{-8}M) was 137.9 ±

Fig. 16. Dose-response curve of the effect of the NK_1 antagonist, GR 82334, on K^+-stimulated (60 mM K^+) release of SP from the intermediate area. GR 82334 was added to the superfusion buffer 20 min prior to, and during K^+-stimulation. Values presented (% of fractional release) are the mean ± SEM of more than 6 samples. * Significant difference compared with control ($p< 0.05$).

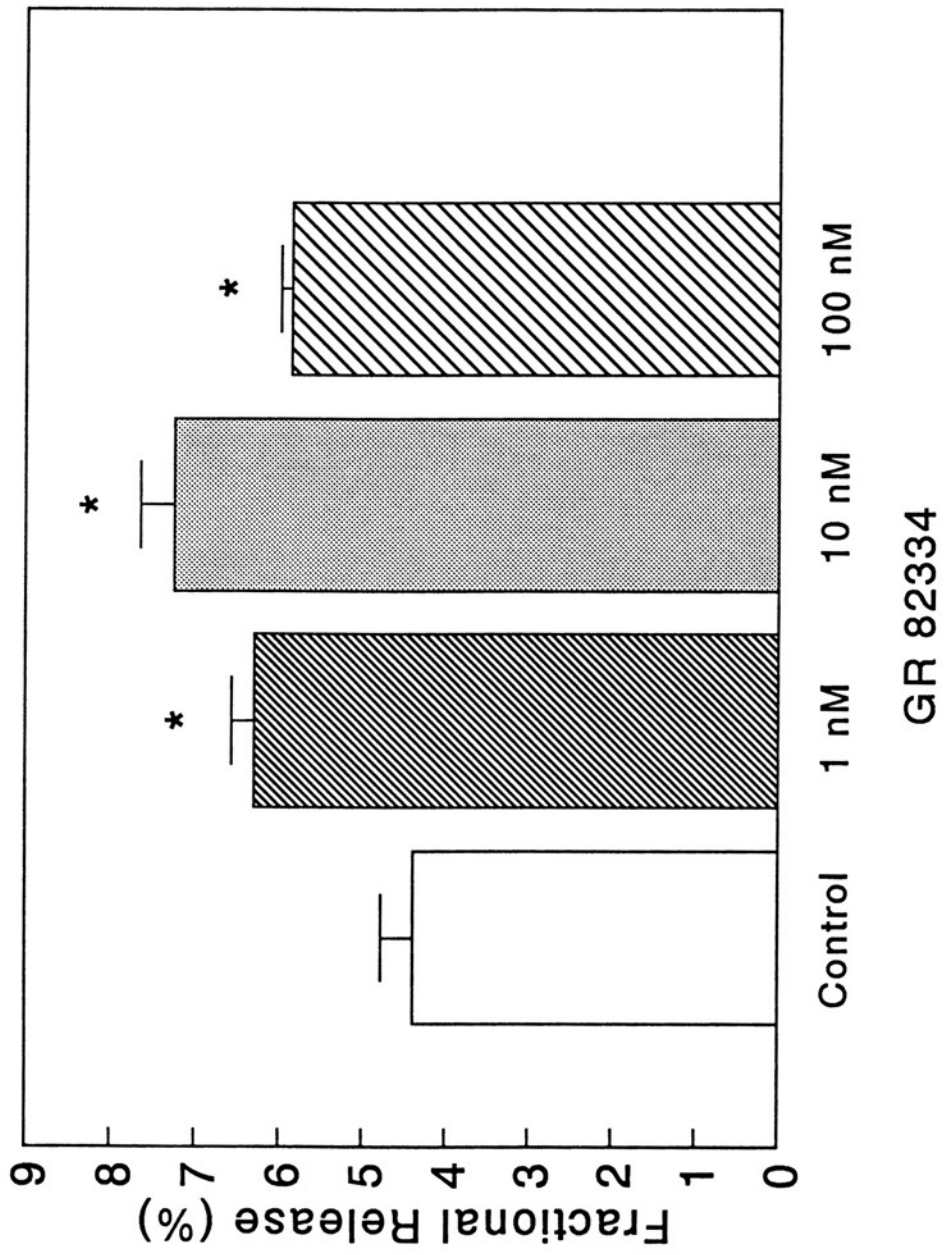

Fractional Release (%)
0
1
2
3
4
5
6
7
8
9
*
*
*
Control
1 nM
10 nM
100 nM
GR 82334

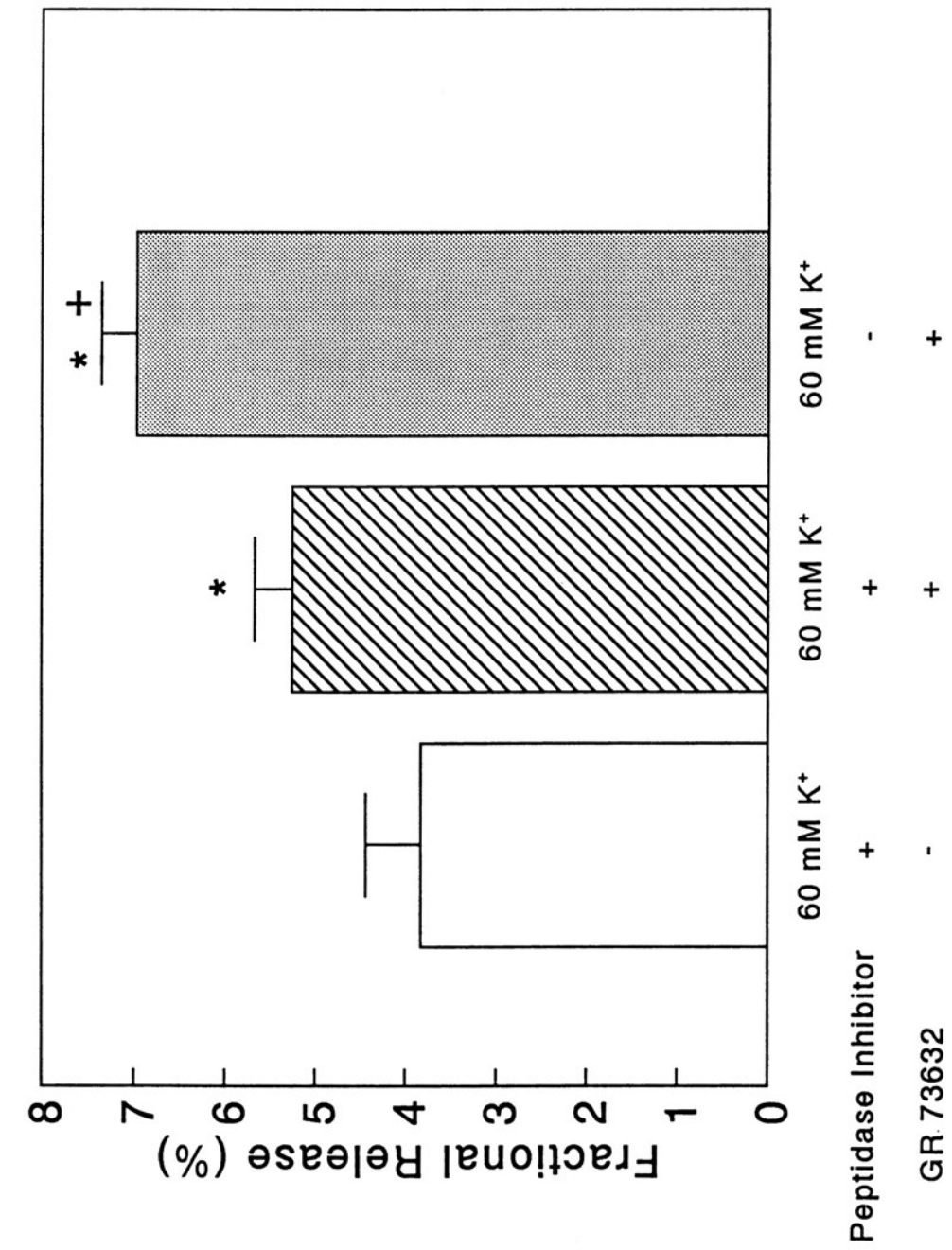
Table 4.

Effects of NK_1 agonists GR 73632 and [Sar^9,$Met(O_2)^{11}$]SP on K^+-stimulated release of SP and the effect of NK_1 antagonist (GR 82334, 100 nM) on GR 73632 (100 nM) increased release of SP from the intermediate area.

Drug	[nM]	SP Release (% of control evoked release)
GR 73632	10	147 ± 5
	100	156 ± 14
	1000	180 ± 16
GR 73632 (10 nM) + GR 82334	10	153 ± 7
GR 73632 (10 nM) + GR 82334	100	142 ± 3
[Sar^9,$Met(O_2)^{11}$]SP	1	139 ± 4
	10	175 ± 3
	100	191 ± 5

Agonists were added to the buffer 20 min prior to, and during K^+-stimulation and GR 82334 was added 10 min prior to the addition of GR 73632. Values presented are the mean ± SEM of more than 6 samples. * Significant difference compared with control ($p < 0.05$).

Fig. 17. Effects of the selective NK_1 agonist (GR 73632, 10^{-8} M) on K^+-stimulated release in the presence and absence of peptidase inhibitors. Values presented (% of fractional release) are the mean ± SEM of more than 6 samples. Control values are the release of SP induced by 60 mM K^+.


Fractional Release (%)
0
1
2
3
4
5
6
7
8
*
* +
60 mM K+
60 mM K+
60 mM K+
Peptidase Inhibitor
+
+
-
GR 73632
-
+
+

5.5% of control.

The maximal recovery of SP released from the intermediate area was obtained by exposing the tissue to the superfusion buffer which contains a combination of bacitracin (20 μM), Phe-Ala (100 μM) and PCMS (50 μM) (Chen *et al.*, 1993). However, findings of Malcangio and Bowery (1994) suggested that GR 73632 might reduce SP degradation by interacting with peptidase inhibitors. Therefore, I studied the effect of GR 73632 on the release of SP in the absence of peptidase inhibitors which are normally contained in the standard superfusion buffer. Similarly to findings of Malcangio and Bowery on the dorsal spinal cord (1994), the excitatory effect of GR 73632 on the release of SP was greater in the absence of peptidase inhibitors than in the presence of peptidase inhibitors (Fig. 17).

e. Effects of NKA and TRH on the release of SP

Because NKA and TRH coexist with SP in the intermediate area, the effects of NKA and TRH on the release of SP from the intermediate area were also studied. NKA did not change the basal or the stimulated release of SP at concentrations tested (10^{-8}M and 10^{-7}M). GR 64349, a selective NK_2 agonist (Maggi *et al.*, 1993), changed neither the basal nor 60 mM K^+-stimulated release of SP at concentrations up to 10^{-6}M (Table 3).

MK-771 (10^{-6}M), an analog of TRH, did not change the basal or stimulated (60 mM K^+) release of SP from the intermediate area (Table 3).

D. Studies of K^+-Stimulated and Ca^{2+}-Dependent Release of NKA

Rationale: The following experiments were designed to demonstrate the release of NKA from the intermediate area. K^+-stimulation and Ca^{2+}-dependence of the release were studied.

In preliminary experiments the recovery of internal standards of NKA added to superfusate samples was about 88.9 ± 4.72%. The basal release of NKA from the intermediate area was low (0.32 ± 0.07%), but measurable and stable (Fig. 18). The release of NKA increased with increasing concentrations of K^+ (20-60 mM) (Fig. 19). For example, 60 mM K^+-induced a fractional release of NKA in the intermediate area of 4.77 ± 0.41% (Fig. 19). The release of NKA induced by 60 mM K^+ was reliable measurable and thus, when Ca^{2+}-dependency of the K^+-stimulated release was examined, a concentration of 60 mM K^+ was chosen. The K^+-induced release of NKA was dependent on the presence of extracellular calcium, as Ca^{2+}-free buffer resulted in K^+-evoked release of NKA which was not significantly different from basal efflux (Fig. 18). The basal release of NKA was independent of extracellular Ca^{2+} (Fig. 18).

Fig. 18. Effects of Ca^{2+} on K^+ (60 mM)-evoked release of NKA from the intermediate area of rat thoracic spinal cord. Ca^{2+} was present in the superfusion buffer at 1.2 mM or was replaced by equal molar of Mg^{2+} and with 1 mM EGTA. Although the tissue was stimulated with a high concentration of K^+ for only 4 min, effluent from the elevated K^+ period was at least partially collected in more than one fraction (fractions 4-6). Values presented (% of fractional release) are the mean ± SEM of 6-10 samples in each group.

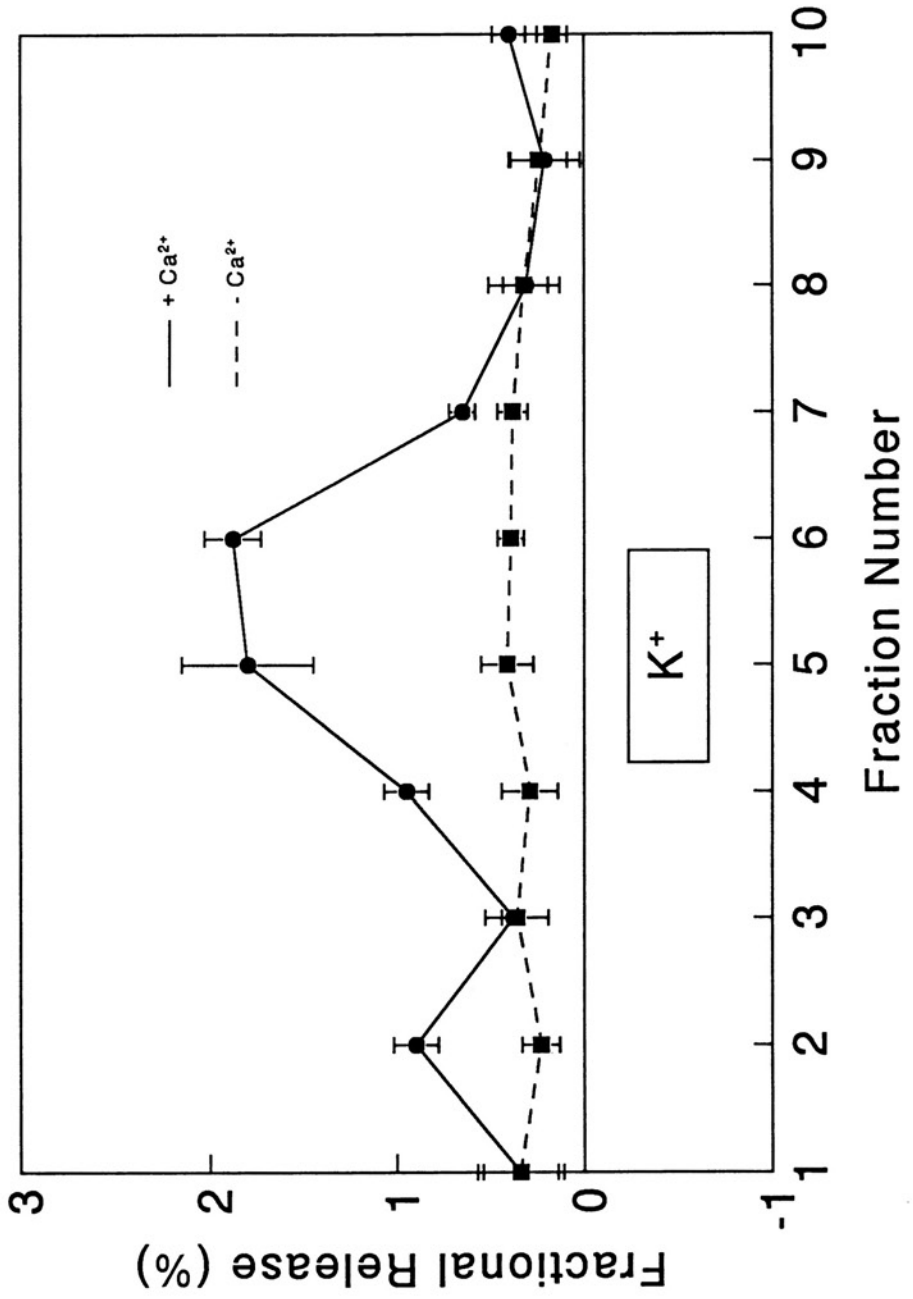
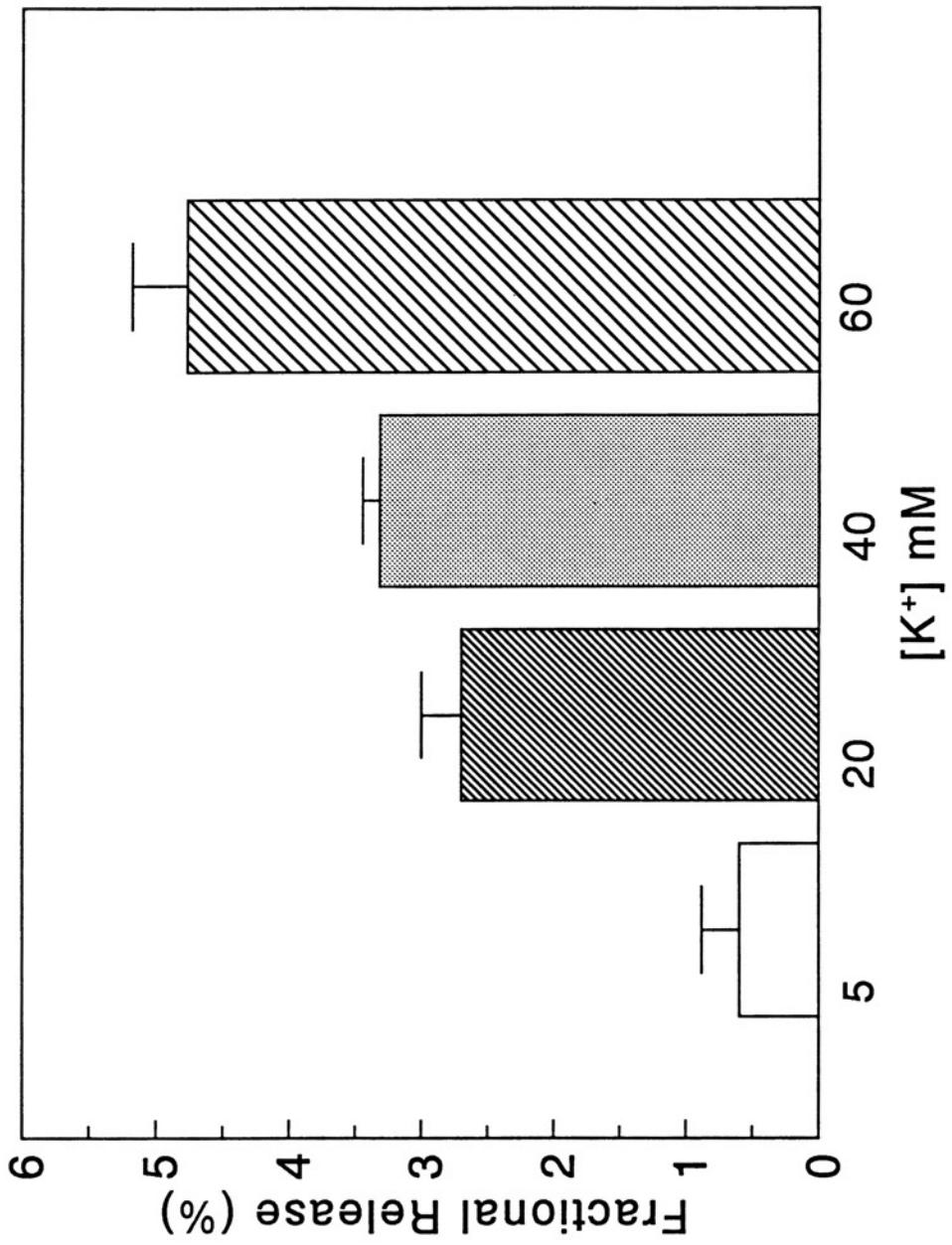

+ Ca2+
- Ca2+
K+
Fractional Release (%)
Fraction Number
3
2
1
0
-1
1
2
3
4
5
6
7
8
9
10

Fig. 19. Effects of K^+ concentration on the release of NKA during stimulation period from the intermediate area of rat thoracic spinal cord. Values presented (% of fractional release) are the sum of NKA release from fraction 4 to 6 and are the mean ± SEM of 6-10 samples tested with each concentration of K^+.


Fractional Release (%)
0
1
2
3
4
5
6
5
20
40
60
[K+] mM

DISCUSSION

This project studied the *in vitro* differential release of coexisting 5-HT, SP and NKA and the regulation of their release from the intermediate area of the rat thoracic spinal cord.

A. K^+-Stimulated and Extracellular Ca^{2+}-Dependent Differential Release of [^{3}H]5-HT, SP and NKA

The present project demonstrated the K^+-depolarization evoked and extracellular Ca^{2+}-dependent *in vitro* differential release of coexisting neurochemicals (5-HT, SP and NKA) of the bulbospinal pathway from the intermediate area. Although it is well known that 5-HT, SP, NKA and TRH coexist in the nerve terminals of the autonomic-related structures of the intermediate area of the thoracic spinal cord, it is the first time that the release of these neurochemicals and the regulation of their release have been demonstrated from the intermediate area.

The system used microdissected tissue from the intermediate area as a model. Studies of 5-HT, SP and NKA containing nerve terminals in the thoracic spinal cord indicated that 5-HT, SP and NKA fibers innervate regions of lamina VII (IML) and lamina X (which are included in the dissection), and that the IML receives the densest 5-HT, SP

and NKA innervation (Moussaoui *et al.*, 1992; Newton *et al.*, 1986; Steinbusch, 1981). It is likely that the greatest component of [^{3}H]5-HT, SP and NKA released from the intermediate area is coming from the IML. Other nuclei included in the intermediate area (i.e., the IC and CA) also contain 5-HT-IR, SP-IR and NKA-IR nerve terminals and SPNs (Hancock and Peveto, 1979; Petras and Cummings, 1972). Thus, the intermediate area is a valid and specific area to study the release of coexisting bulbospinal neurochemicals (5-HT, SP and NKA) and their functional importance in the regulation of the sympathetic nervous system.

For the study of 5-HT release, the radioactivity measured in the superfusate most likely represents [^{3}H]5-HT. The metabolism of 5-HT to 5-HIAA was inhibited with 0.01 mM pargyline hydrochloride, an inhibitor of monoamine oxidase. HPLC analysis of superfusate collected under similar conditions used in the study indicated that the radioactivity measured was [^{3}H]5-HT and not a metabolite of [^{3}H]5-HT (Jacocks and Cox, unpublished data). In addition, the evoked release of [^{3}H]5-HT most likely represents release from serotoninergic nerve terminals. Preliminary studies showed that addition of fluoxetine (1 μM), an inhibitor of the serotonin transporter, prevented the accumulation and subsequent release of [^{3}H]5-HT from the intermediate area. Moreover, GBR-12909 and nisoxetine were routinely added to the incubation buffer during the [^{3}H]5-HT

preloading incubation to prevent the accumulation of [^{3}H]5-HT in either dopaminergic or noradrenergic nerve terminals.

For the study of SP and NKA release, peptidase inhibitors were constantly present in the superfusion system to prevent the degradation of SP and NKA. Furthermore, the recovery of internal standards of SP or NKA added to superfusate samples was above 88%. Thus, it is likely that most of SP or NKA released from the tissue to the superfusates was recovered and analyzed without degradation. The SP (SP3B3) and NKA (SK7) antibodies used in the present study are specific antibodies for SP or NKA without any cross reactivity to other related peptides (Helke *et al.*, 1982; Moskowitz *et al.*, 1987).

The term "release" is used for the exocytotic extrusion of transmitter molecules across the neuronal membrane into the extracellular space. In the absence of releasing stimulator (high K^+ concentration), tissue from the intermediate area expressed efflux of [^{3}H]5-HT or endogenous SP or NKA at a rate which remained stable through the superfusion period. This basal release was independent of extracellular Ca^{2+}. The basal release of 5-HT, SP and NKA in the resting condition may play a role in the maintenance of neuronal activities of SPNs.

Demonstration of a calcium-dependent release in response to depolarizing stimuli is a prerequisite to consider an endogenous substance as a neurotransmitter.

Increasing the external K^+ concentration in the superfusion buffer with a concomitant decrease in Na^+ stimulates release of neurotransmitter. The K^+-stimulated release of [^{3}H]5-HT, SP or NKA studied herein required the presence of calcium, indicating Ca^{2+}-dependent secretion of neurotransmitter was evoked by nerve depolarization. These data provide additional evidence for the function of 5-HT, SP and NKA as neurotransmitters in regions of the thoracic spinal cord that is involved with regulation of the sympathetic nervous system.

The present study demonstrated the release of SP from the intermediate area and the release at least partly, from the serotoninergic terminals. Treatment with 5,7-DHT, as performed in the present study, has previously been shown to cause a long-lasting selective depletion of 5-HT caused by a degeneration of serotoninergic neurons (Baumgarten *et al.*, 1973). Treatment with 5,7-DHT reduced the tissue SP content and the absolute amount of SP released from the intermediate area. These data further confirm the fact that SP and serotonin coexist in the IML nerve terminals and that some of the SP is released from serotoninergic nerve terminals. 5,7-DHT may also reduce the tissue levels of norepinephrine and dopamine, presumably through uptake into noradrenergic or dopaminergic nerve terminals (Baumgarten *et al.*, 1973). However, in the present study, DMI was used to prevent such uptake and thus, the cervical spinal norepinephrine and

dopamine content was not changed.

Coexisting neurochemicals (e.g. 5-HT, SP and NKA) may be released in different proportions depending on the stimulation frequency. In some experiments, classical neurotransmitter (e.g. 5-HT) is released when the nerve terminal is activated by low-frequency stimulation, and the peptide (e.g. SP) is released when the nerve terminal is activated by high-frequency stimulation (Bartfai, *et al.*, 1986; Lundberg and Hokfelt, 1983). Differential release of 5-HT and SP had been demonstrated in the ventral spinal cord, (Franck *et al.*, 1993; Iverfeldt *et al.*, 1989) where the release of 5-HT by electrical stimulation occurred at 2 Hz, whereas the release of SP required 20 Hz (Franck *et al.*, 1993). A similar phenomenon appears also to occur in the intermediate area where the release of [^{3}H]5-HT was evoked by a lower concentration of K^+ than was the release of SP and NKA. These data support the idea that SP, NKA and 5-HT are differentially released from the intermediate area of the rat thoracic spinal cord.

Although TRH coexists with 5-HT, SP and NKA in the intermediate area and it is likely that TRH is also released from the intermediate area, TRH release is difficult to detect because the TRH content in the spinal cord is lower than the detection sensitivity of the assay. Generally, the sensitivity of RIA assay for detection of the *in vitro* release of TRH from the tissue is about 5 pg (Lewis *et al.*,

1987). In the rat, the whole spinal cord contains only 8.7 ± 0.7 ng of TRH and TRH concentration was 41.7 ± 0.7 pg/mg wet weight. The TRH content in the central canal (Kardon *et al.*, 1977) and the IML (Helke *et al.*, 1986a) are 0.96 pg/μg protein and 1.02 pg/μg protein, respectively. In addition, the low molecular weight of TRH makes it difficult to produce pure and highly specific antibody for TRH RIA. Furthermore, studies of TRH are hard to complete due to the lack of specific antagonists for TRH receptors. All of these limitations make the detection of TRH release from the intermediate area impossible at the present time.

B. Presynaptic 5-HT_{1B} or NK_1 Autoreceptor Regulation of the Release of 5-HT or SP

The present project demonstrated the presence of presynaptic inhibitory 5-HT_{1B} autoreceptors and the possible NK_1 autoreceptors in regulation of 5-HT and SP release from the intermediate area of the rat thoracic spinal cord.

a. Presynaptic inhibitory 5-HT_{1B} autoreceptor regulation of the release of [^{3}H]5-HT

The 5-HT_{1B} receptor subtype appears to be involved in the regulation of the K^+-evoked release of [^{3}H]5-HT from the rat intermediate area. Exogenous 5-HT and the 5-HT_{1B} agonist, CGS-12066B were both capable of inhibiting K^+-stimulated release of [^{3}H]5-HT. The concentration used for each drug has affinity for 5-HT_{1B} binding site. The K_i value at 5-HT_{1B} receptor for 5-HT and CGS-12066B is 10 nM (Zifa and Fillion, 1992) and 114 nM (Schoeffter and Hoyer, 1989), respectively. Methiothepin, a 5-HT_{1B} antagonist (K_i=50 nM, Hoyer and Schoeffter, 1991) also has binding affinities for 5-HT_{1A} (K_i=89 nM, Gozlen *et al.*, 1983), 5-HT_{2C} (K_i=25 nM, Hoyer and Schoeffeter, 1991) and 5-HT_{2A} (K_i=6.5 nM, Hoyer and Schoeffter, 1991) receptors. In our study, methiothepin reversed the inhibition on the release of [^{3}H]5-HT by both 5-HT and 5-HT_{1B} agonist, CGS-12066B. Although the compounds

used were not absolutely specific for the 5-HT_{1B} receptor subtype, they each have affinity for the 5-HT_{1B} receptor at the concentration used in the present study. Likewise, the IC_{50} values for CGS-12066B are 51 nM and 876 nM at 5-HT_{1B} and 5-HT_{1A} binding sites, respectively (Neale *et al.*, 1987). Thus, the CGS-12066B concentrations (10^{-9}-10^{-7} M) found to inhibit the release of 5-HT support the contention that the autoreceptor is associated with the 5-HT_{1B} site. Moreover, a highly potent and relatively selective 5-HT_{1B} receptor agonist, RU 24969 (K_i=3.8 nM, Hoyer *et al.*, 1985), also dose-dependently (10^{-8}-10^{-6}M) decreased the K^+-stimulated release of [^{3}H]5-HT. The small (12%) facilitating effect of methiothepin on stimulated tritium overflow may be caused by a competitive blockade of the presynaptic inhibitory 5-HT receptors, thus, preventing their further activation by endogenous 5-HT released from the serotoninergic fibers.

Presynaptic serotonin autoreceptors in human and pig cortex are of the 5-HT_{1D} type (Galzin *et al.*, 1988; Schlicker *et al.*, 1989). Whereas 5-HT_{1D} receptor subtypes have been cloned and functionally described in the rat, the possibility that the 5-HT_{1D} receptor subtype also serves as the rat presynaptic autoreceptor is small. Pharmacological studies suggested that in the mouse and rat the autoreceptor is of the 5-HT_{1B} subtype, while in the pig, guinea pig, rhesus monkey, and human, the autoreceptor is of the 5-HT_{1D} subtype (Schipper, 1990). *In situ* hybridization studies

found that the distribution of 5-HT_{1D} receptor mRNA in the lateral horn of the rat spinal cord was more scarce compared to that of the 5-HT_{1B} receptor (Neumaier *et al.*, 1993).

Although 5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_3 binding sites are located in the rat thoracic spinal cord (Glaum and Anderson, 1988; Thor *et al.*, 1993), the present experiments support the conclusion that they do not function as presynaptic inhibitory autoreceptors in the intermediate area. Specifically, a highly selective 5-HT_{1A} agonist, 8-OH-DPAT (K_i=1.86 nM, Hoyer and Shoeffter, 1991), was ineffective in altering K^+-evoked release of [^{3}H]5-HT. Likewise, NAN-190, a potent competitive 5-HT_{1A} antagonist (K_i=1.3 nM, Rydelek-Fitzgerald *et al.*, 1990) was ineffective in reversing the effects of exogenous 5-HT on the release of [^{3}H]5-HT. Thus, 5-HT_{1A} receptors do not appear to be involved. In addition, the present study used 2-methyl-5-HT (5-HT_3 selective agonist; K_i=100-1000 nM, Zifa and Fillion, 1992) and (±)DOI (5-$HT_{2A/2C}$ selective agonist; K_i=6.45 nM, Zifa and Fillion, 1992). Neither of them affected the release of [^{3}H]5-HT from the intermediate area. These findings are consistent with the evidence that 5-$HT_{2A/2C}$ and 5-HT_3 receptors are not involved in the modulation of the K^+-evoked release of [^{3}H]5-HT in other regions (Martin and Sanders-Bush, 1982; Williams *et al.*, 1992).

5-HT_4 receptor in the CNS may facilitate

neurotransmitter release (Bockaert *et al.*, 1992). Although high densities of 5-HT_4 receptors are found in the nigro-striatal pathways (Grossman *et al.*, 1993), where 5-HT_4 agonists stimulated the release of dopamine from rat striatal slices (Steward and Barnes, 1994), there is no evidence that the 5-HT_4 receptor subtype regulates the release of 5-HT. Although the localization of 5-HT_4 receptors in the spinal cord has not been reported, the possibility of the involvement of 5-HT_4 receptors as presynaptic autoreceptors can not be completely excluded.

The autonomic effects of 5-HT in the spinal cord are complex (Coote, 1988). Various studies showed sympathoexcitatory and/or sympathoinhibitory effects of serotoninergic agents applied to the IML. Micro-iontophoretic administration of 5-HT onto SPNs in the IML generally increases sympathetic preganglionic activity (deGroat *et al.*, 1967; Gilbey and Stein, 1991; Kadzielawa, 1983; McCall, 1983). However, the firing rate of some SPNs was decreased by 5-HT (Gilbey and Stein, 1991). Data presented in this study suggest that in addition to the direct actions of 5-HT on preganglionic neurons, 5-HT (or 5-HT_{1B} agonists) can activate presynaptic receptors to decrease the release and thus, the sympathoexcitatory actions of endogenous 5-HT. The findings that RU-24969 (a 5-HT_{1B} agonist) reduces blood pressure (and presumably sympathetic outflow), following intrathecal administration

(Soloman and Gebhart, 1988) is consistent with this concept. However, the exact role that 5-HT, acting upon various 5-HT receptor subtypes, plays in the regulation of the sympathetic system is not known yet.

In conclusion, a 5-HT_{1B} presynaptic receptor is associated with the nerve terminal modulation of the evoked release of [^{3}H]5-HT from the intermediate area of the rat thoracic spinal cord.

b. Evidence for presynaptic NK_1 receptor regulation of the release of SP

It is possible that the NK_1 receptor is the presynaptic autoreceptor that regulates the release of SP from the intermediate area. In the intermediate area, NK_1 binding sites have been located with high densities in the IML (Helke *et al.*, 1986b). Although no direct anatomical evidence exists for the presence of presynaptic NK_1 binding sites in the intermediate area, the presence of a presynaptic NK_1 receptor in the intermediate area is possible because SP increased the basal release of [^{3}H]5-HT, the effect of which is mediated through activation of NK_1 receptors. These data suggest the presence of presynaptic NK_1 autoreceptors that regulate the release of 5-HT. In addition, the ability of a NK_1 antagonist (GR 82334) to dose-dependently increase the K^+-evoked release of SP from the intermediate area supports the concept of an autoreceptor (NK_1) mechanism through which SP modulates its own release from nerve terminals in the intermediate area. These data are consistent with the finding that NK_1 receptor antagonists (RP 67850 and SR 140333) increased the electrically-induced release of SP in the dorsal lumbar spinal cord (Malcangio and Bowery, 1994).

However, we could not demonstrate any inhibition of SP release under the current condition since the available

agonists all increased SP content in the perfusate by a mechanism which appeared not to be associated with the NK_1 receptor. Experiments with NK_1 receptor agonists, GR 73632 and [$Sar^9Met(O_2)^{11}$]SP, revealed that both greatly increased the basal and stimulated release of SP. The inability of a NK_1 antagonist to block the effect suggests that it is not a typical receptor-mediated phenomenon. Moreover, the fact that GR 73632 exerted its effect in the absence of peptidase inhibitors to a greater extent than in their presence, suggests that GR 73632 might inhibit peptidase activity and thus, reducing SP degradation. These data are consistent with findings of Malcangio and Bowery (1994) on dorsal spinal cord, where GR 73632 increased the basal and stimulated release of SP to a greater extent in the absence of peptidase inhibitors.

The autonomic effects of SP in the spinal cord are thought to be mediated by postsynaptic NK_1 receptors. Intrathecal administration of SP or a SP analog onto the IML caused an increase in MAP (Helke *et al.*, 1987a; Keeler *et al.*, 1985), which was blocked by intrathecal injection of selective antagonists (Helke *et al.*, 1987b; Pham and Couture, 1993). These data indicate that activation of postsynaptic NK_1 receptor has an excitatory effect on SPNs in the IML. The present data are consistent with the idea that SP is released from IML nerve terminals to activate postsynaptic NK_1 receptors, to increase the MAP. At the

same time, SP released from nerve terminals may activate presynaptic NK_1 inhibitory autoreceptors to reduce its release, thus prevent the excessive excitation of IML neurons.

In conclusion, a presynaptic NK_1 receptor is likely to be associated with the nerve terminal facilitation of potassium-evoked release of SP from the intermediate area of the rat thoracic spinal cord.

There is no published evidence showing the presence of presynaptic NK_2 receptor regulating the release of NKA. Moreover, there is a limited number of NK_2 binding sites in the spinal cord and the IML (Mantyh *et al.*, 1989). Thus, it is unlikely that presynaptic NK_2 autoreceptors present in the intermediate area regulating the release of NKA. The involvement of NKA in the spinal autonomic systems is still unclear. Intrathecal administration of NKA analogues failed to alter MAP in one study (Hassessian *et al.*, 1988), but increased heart rate and MAP in some other studies (Pham and Couture, 1993; Picard *et al.*, 1994). One possibility is that the cardiovascular response to NKA is mediated by NK_1 receptors, since NKA has a low affinity to NK_1 receptors (Pham and Couture, 1993). Another possibility is that NKA acts on a receptor distinct from the classic NK_2 receptor subtype (NK_{2A} and NK_{2B}) (Maggi *et al.*, 1993; Williams *et al.*, 1988). However, the exact mechanism of NKA in the spinal regulation of the autonomic system is not fully understand.

C. Regulation of the Release of 5-HT and SP by Other Coexisting Neurochemicals

The release (basal and stimulated) and function of a transmitter could be changed in the presence of another putative transmitter, possibly through the activation of presynaptic autoreceptors. Interactions on the release of 5-HT, SP and NKA from the ventral spinal cord has been demonstrated previously (Franck *et al.*, 1989; Iverfeldt *et al.*, 1989). Although 5-HT, SP, NKA and TRH coexist in the intermediate area of the thoracic spinal cord, interactions of these neurochemicals on their release have not been studied previously. The present study demonstrated that SP and the NK_1 agonist regulate the basal release of [^{3}H]5-HT from the intermediate area of the rat thoracic spinal cord, and that the effects maybe mediated by presynaptic NK_1 autoreceptors. Furthermore, studies with 5,7-DHT depletion of serotoninergic nerve terminals failed to change the K^+-stimulated percentage of fractional release of SP (i.e. the fractional release of SP of the remaining tissue) from the intermediate area and confirmed the concept that endogenous 5-HT does not regulate the release of SP.

a. Regulation of the release of 5-HT

Both SP (the natural ligand for NK_1 receptor) and a

NK_1 selective agonist (GR 73632, pD_2=6.23; Hall *et al.*, 1992) increased the basal release of [^{3}H]5-HT from the intermediate area, and the effect of the agonist was dose-dependently blocked by a NK_1 antagonist (GR 82334). These data suggest that the effects of SP on the basal release of 5-HT are mediated through a NK_1 receptor. In a similar study, using the ventral horn region of the entire spinal cord, Tsai and colleagues (1984) reported that basal but not K^+-evoked (20 mM) release of [^{3}H]5-HT was enhanced by SP (10-50 μM). However, in another study using rat cortex, both basal and stimulated release of [^{3}H]5-HT were enhanced by SP (10^{-6}M, Solti and Bartfai, 1987). The increased basal release of 5-HT may be a direct action of SP and the selective NK_1 agonist on a presynaptic NK_1 receptor as discussed previously. These data suggest that under physiological conditions, SP modulates the basal release of 5-HT and thus modulates the function of 5-HT on SPNs.

It is conceivable that activation of postsynaptic NK_1 receptors located on 5-HT interneurons (instead of 5-HT nerve terminals) in the IML could increase the release of 5-HT. Although 5-HT cell bodies (labelled as B_1-B_9 neurons) are predominantly found in the brain stem (Dahlstrom and Fuxe, 1964), intraspinal 5-HT neurons have been found among spinal autonomic nuclei (Newton and Hamill, 1988). It is not known whether there are NK_1 receptors located on these intraspinal 5-HT neurons. Thus, the possibility that the

elevated basal release of 5-HT in the intermediate area reported herein is due to the activation of postsynaptic NK_1 receptors located on 5-HT interneurons in the intermediate area can not be completely excluded.

The mechanism for the NK_1 receptor induced enhancement of basal 5-HT release in the intermediate area is unclear. Consistent with the findings that basal release of neurotransmitters is a spontaneous extracellular Ca^{2+}-independent outflow, the elevation of the basal release seen in the study did not depend-upon extracellular Ca^{2+}. However, activation of NK_1 receptors can increase intracellular Ca^{2+} secondary to an increase in phospholipase C and an increase in phosphoinositide turnover (Guard and Watson, 1991). In another study, Mochizuki-Oda *et al.* (1994) demonstrated that SP (10^{-6}M) induced both extracellular Ca^{2+} entry into the cell and intracellular Ca^{2+} mobilization through an interaction with $Ins(1,4,5)P_3$. Whether the increase of intracellular Ca^{2+} (without increasing the entry of extracellular Ca^{2+}) increased the basal release of [^{3}H]5-HT in the present study is not known.

Whereas an agent acting at a presynaptic $5\text{-}HT_{1B}$ autoreceptor can affect the release of 5-HT, $5\text{-}HT_{1B}$ receptor activation is not likely to be involved in the action of NK_1 agonists reported in this study. The modulatory effects of $5\text{-}HT_{1B}$ presynaptic receptors alter stimulated but not basal release of 5-HT, and are known to affect Ca^{2+}-dependent

release (Gothert, 1980; Starke *et al.*, 1989). Moreover, in the present study, blocking the presynaptic inhibitory 5-HT_{1B} autoreceptor with 10^{-6}M methiothepin (a 5-HT_{1B} antagonist, Hoyer and Schoeffter, 1991) did not change the excitatory effect of the NK_1 agonist on the basal release of [^{3}H]5-HT. Although methiothepin is not a specific 5-HT_{1B} antagonist, at the concentration used it has been shown to block the 5-HT_{1B} receptor (Hoyer and Schoeffter, 1991. Thus, it is unlikely that SP exerts its action through direct interaction with the presynaptic 5-HT_{1B} autoreceptor.

Another mechanism for the increased release of neurotransmitter is the carrier-mediated release. Agents such as amphetamine which use the monoamine transporter to access the nerve terminal, can displace transmitter in a Ca^{2+}-independent manner from the nerve terminal (Glowinsky and Axelrod, 1964; Levi and Raiteri, 1993). However, in the present study, the serotonin transporter was inhibited by fluoxetine (1 μM) and it is therefore unlikely that the NK_1 agonists increase the basal release of [^{3}H]5-HT as mediated by 5-HT transporter.

The functional interactions between SP and 5-HT on the autonomic system through activation of receptors in the spinal cord are complex. Intrathecal administration of a 5-HT_{1A} agonist partially reversed the cardiovascular responses induced by SP (Gradin, 1990). In another study, serotonin depletion by parachlorophenylalanine (PCPA) potentiated the

pressor response elicited by intrathecal application of SP suggesting an antagonistic interaction between SP and 5-HT in the spinal cord control of MAP (Hassessian *et al.*, 1993). Our data suggest that under physiological conditions, SP modulates the basal release of 5-HT and thus, modulates the function of 5-HT on cardiovascular regulation.

NK_2 agonists (NKA and GR 64349) did not change either the basal or K^+-stimulated release of [^{3}H]5-HT in the intermediate area. These data suggest that the NK_2 receptor is not involved in modulating the release of 5-HT from the intermediate area and is consistent with the sparse distribution of NK_2 receptors in the spinal cord (Buck *et al.*, 1986; Yashpal *et al.*, 1990).

Although TRH also coexists with SP and 5-HT in the VM IML-projecting neurons, and TRH binding sites were identified in the thoracic IML (Manaker *et al.*, 1985), the TRH analog (MK-771) did not change either the basal or stimulated release of [^{3}H]5-HT from the intermediated area. These data are consistent with previous work of Mitchell and Fleetwood-Walker (1981), who found that although SP increased the release of 5-HT in ventral lumbar spinal cord in the presence of exogenous 5-HT, TRH had no effect.

In conclusion, the basal release of [^{3}H]5-HT from the intermediate area of the rat thoracic spinal cord was regulated by SP acting through an presynaptic NK_1 receptor, but not by NKA and TRH. These findings demonstrate a role

for SP as a modulator of serotoninergic function, and suggest a functional interaction of SP and 5-HT in the intermediate area of the rat thoracic spinal cord.

b. Regulation of SP release

Although the release of 5-HT from the intermediate area is regulated by SP, it does not appear that endogenous 5-HT regulates the release of SP from the intermediate area. Although the released 5-HT from the intermediate area is regulated by presynaptic 5-HT_{1B} autoreceptors, neither 5-HT nor 5-HT_{1B} agonists (at the same concentrations shown to decrease the release of [^{3}H]5-HT) changed the release of SP. These data suggest that activation of presynaptic 5-HT_{1B} autoreceptors does not change the release of SP.

Activation of 5-HT_3 receptor in the regulation of the release of SP from the spinal cord is debatable. Saria *et al*. (1990) reported that activation of 5-HT_3 receptors in the spinal cord inhibited the release of SP. While Bourgoin *et al*. (1993) found that neither 5-HT nor selective 5-HT_{1A} and 5-HT_3 receptor agonists affected the spinal release of SP. However, the 5-HT_3 agonist studied in the present study failed to change the release of SP from the intermediate area.

The negative results of 5-HT, 5-HT_{1B} agonists and 5-HT_3 agonist on the release of SP correlate with the negative effect of 5-HT depletion with 5,7-DHT treatment on the percentage of fractional release of SP (5-HT depletion did not change the fraction of the remaining tissue content of SP released by K^+-depolarization), and thus, the release of

SP. Although SP and 5-HT coexist in nerve terminals of the intermediate area and SP regulates the basal release of 5-HT, endogenous 5-HT is devoid of any activities on the release of SP.

The fact that presynaptic 5-HT_{1B} autoreceptors regulate the release of 5-HT without change the release of SP indicates the differential regulation mechanism for the release of 5-HT and SP. This concept also supports the differential release of 5-HT and SP from the intermediate area. It is possible that two types of storage vesicles exist for 5-HT and SP in the intermediate area and each type of vesicle has a different regulation system. Studies of the storage sites for SP and 5-HT in the ventral spinal cord by immunoelectron microscopy (Pelletier *et al.*, 1981) and by subcellular fractionation (Bucsics *et al.*, 1984; Fried *et al.*, 1989) indicate that SP is stored in large, dense-cored vesicles and 5-HT is stored mainly in small vesicles but also sometimes in large, SP-containing vesicles. The respective roles of small and large vesicles are still poorly understood. Small agranular vesicles have been shown to be the site of intense [^{3}H]5-HT labelling and these vesicles are likely to be involved in the storage and release of 5-HT, while the large vesicles are likely to be involved in the transport and metabolism of 5-HT although they also possess a preferential affinity for [^{3}H]5-HT (Beaudet and Descarries, 1987). It is possible that in the

present study, the depolarization induced release of SP comes from large dense-cored vesicles, which contain both SP and 5-HT and, are not affected by 5-HT_{1B} autoreceptor inhibition, whereas, the release of 5-HT comes from small vesicles, which contain 5-HT but not SP, and are affected by 5-HT_{1B} autoreceptor inhibition.

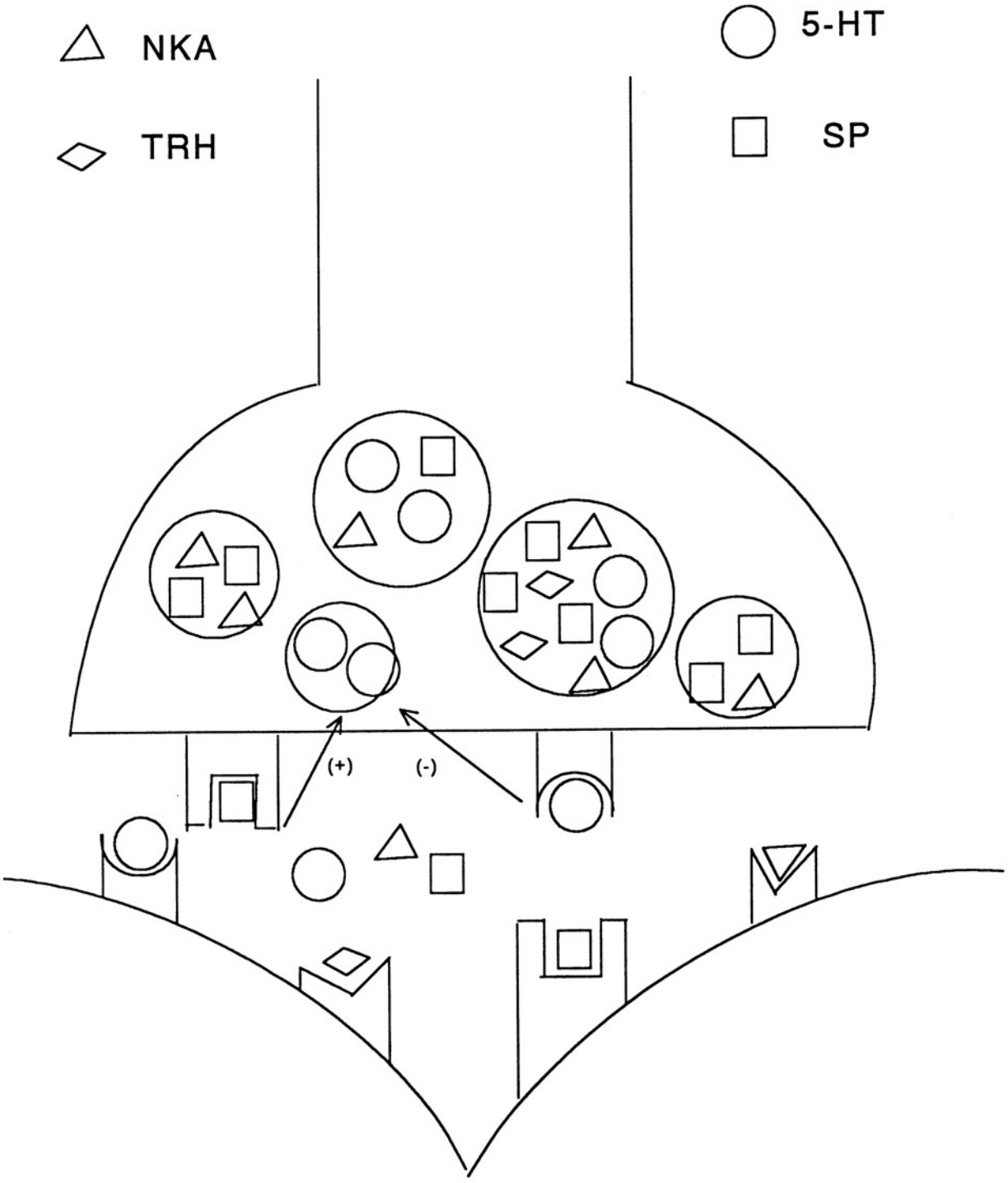
Although NKA (the endogenous agonist for NK_2 receptor) coexists with SP, very few NK_2 binding sites have been identified in the intermediate area (Mantyh *et al.*, 1989). Furthermore, neither NKA nor a NK_2 agonist (GR 64349) altered the release of SP in the present study. TRH also coexists with SP and 5-HT in the intermediate area and TRH binding sites are identified in the thoracic IML (Manaker *et al.*, 1985), however, the TRH analog (MK-771) did not change either the basal or stimulated release of SP from the intermediate area. Thus, it is unlikely that NKA and presynaptic NK_2 receptors or TRH regulate the release of SP from the intermediate area.

The physiological and pharmacological significance of the studies presented in this thesis requires comment. Combined with previous published studies, the present project suggests the following model system (Fig. 20). 5-HT, SP, NKA and TRH coexist in the nerve terminals of the intermediate area. At least some of the SP and NKA are in the same terminal with 5-HT. Coexisting 5-HT, SP and NKA in

the intermediate area are coreleased during nerve depolarization and upon their postsynaptic receptors (5-HT and NK_1) in regulating the activity of sympathetic preganglionic neurons. At the same time, the released 5-HT and SP may also activate presynaptic inhibitory $5\text{-}HT_{1B}$ and NK_1 autoreceptors to prevent further release of 5-HT and SP and thus, prevent the excessive excitation of the cardiovascular system. At resting conditions, SP leaked into the synaptic cleft binds to presynaptic NK_1 receptors, increases the basal outflow of 5-HT into the synaptic cleft and helps to maintain the persistent action of 5-HT on SPNs. Although the exact mechanism is not known the presynaptic NK_1 receptors probably only sensitize the 5-HT terminals which do not contain SP and NKA because NK_1 receptors activation did not change the basal release of SP.

This information will help us in further understanding the mechanism and interactions of coexisting neurochemicals in the central regulation of the sympathetic nervous system and may provide knowledge useful for pharmacological therapeutic management of cardiovascular diseases.

Fig. 20. Schematic of nerve terminals in the intermediate area showing the coexistence of 5-HT, SP, NKA and TRH and autoreceptor regulation. SP, NKA and 5-HT are released at nerve terminals and bind to postsynaptic receptors. 5-HT also binds to presynaptic 5-HT_{1B} autoreceptor to inhibit its further release. SP activates presynaptic NK_1 autoreceptor to increase basal 5-HT overflow.


NKA
5-HT
TRH
SP
(+)
(-)

APPENDIX: Drugs used in the study

Drugs	Characteristics
5-HT	Endogenous ligand for 5-HT receptors
CGS-12066B	5-HT_{1B} agonist
RU 24969	5-HT_{1B} agonist
Methiothepin	5-HT_{1B} antagonist
8-OH-DPAT	5-HT_{1A} agonist
NAN-190	5-HT_{1A} antagonist
(+/-)DOI	$5\text{-HT}_{2A/2C}$ agonist
2-methyl-5-HT	5-HT_{3} agonist
5,7-DHT	Serotoninergic neurotoxin
SP	Endogenous ligand for NK_1 receptor
GR 73632	NK_1 agonist
$[Sar^{9},Met(O_2)^{11}]SP$	NK_1 agonist
GR 82334	NK_1 antagonist
NKA	Endogenous ligand for NK_2 receptor
GR 64349	NK_2 agonist
MK-771	TRH analog

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