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Release of endogenous amino acids with putative neurotransmitter function from the rat spinal dorsal horn in vitro--modulation by neuropeptides

Ivan Kangrga
Iowa State University

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Kangrga, Ivan Milenko, Ph.D.

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Ann Arbor, MI 48106

Release of endogenous amino acids with putative neurotransmitter function from the rat spinal dorsal horn in vitro-- modulation by neuropeptides

by

Ivan Kangrga

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LIST OF ABBREVIATIONS

AHP	Afterhyperpolarization
Ala	Alanine
AP	Action potential
Asp	Aspartate
Asn	Asparagine
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CGRP-LI	Calcitonin gene-related peptide like immunoreactivity
CNS	Central nervous sytem
DC	Direct current (d.c)
DH	Dorsal horn
DR	Dorsal root(s)
DRG	Dorsal root ganglion
DYN	Dynorphine
EAA	Excitatory amino acids
EPSP	Excitatory postsynaptic potential
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
Glu	Glutamate
Gln	Glutamine
Gly	Glycine
h.p.	Holding potential
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IPSP	Inhibitory postsynaptic potential
I-V	Current-voltage
m	Mean
NKA	Neurokinin A
NKB	Neurokinin B
OPA	o-phthaldialdehyde
PSP	Postsynaptic potential
PSC	Postsynaptic current
Rn	Neuronal input resistance
S.E.M.	Standard error of the means
SD	Standard deviation
SEPSP	Slow excitatory postsynaptic potential
Ser	Serine
SP	Substance P
SS	Somatostatin
TEA	Tetraethylammonium
Thr	Threonine
TTX	Tetrodotoxin
VASO	Arginine-vasopressine
Vh	Holding potential
VIP	Vasoactive intestinal polypeptide
Vm	Membrane potential

GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation is written in an alternate thesis format, as permitted by the Graduate College. It includes experimental objective, a background and literature review, a rationale, an experimental part, a discussion, a summary, a list of references cited in the background and literature review and in the discussion. The experimental part has five sections. Sections I and II represent two research papers already published, sections III and V represent two manuscripts submitted for publication in the Brain Research, and section IV is a part of a published research paper.

The dissertation contains a large part of the experimental results obtained by the author during the course of his graduate study under the supervision of Dr. Mirjana Randić.

Research Objective

Anatomical and physiological data have provided a detailed description of the organization of afferent projections to the spinal cord and of the second order neurons in the dorsal horn. However, the identity of the neurotransmitter and neuromodulator substances at primary afferent synapses, and cellular mechanisms of their pre- and postsynaptic actions in the dorsal horn are still largely unknown. The objective of this research was to examine basal and depolarization-induced release of nine endogenous

amino acids, including glutamate and aspartate, from the spinal dorsal horn. The specific purpose of the conducted experiments was to study the characteristics of amino acid release in response to selective activation (electrical, chemical) of either primary afferent A- or C- fibers, the origin of the released compounds by using capsaicin as a probe of primary afferent C-fiber function, and the regulation of their basal and evoked release by peptides. The possibility of modulation of endogenous amino acids release by tachykinins (substance P and neurokinin A), calcitonin gene-related peptide, opioid peptides, and by the activation of B subtype of γ-aminobutyric receptors, has been investigated. The experiments used spinal cord slice-dorsal root ganglion *in vitro* preparation, high performance liquid chromatography with fluorimetric detection and intracellular recording from dorsal horn neurons.

Background and Literature Review

This section briefly outlines the projections of the primary afferent fibers, the cytoarchitectonic organization and the principal neurotransmitter systems in the spinal dorsal horn. The objective is to provide background information for the study of chemical neurotransmission in the spinal dorsal horn.

The spinal dorsal horn - structural and functional organization

Primary sensory neurons, primary afferent fibers and sensory receptors

Primary sensory neurons, located in the dorsal root ganglia (DRG), mediate the transfer of sensory information from the peripheral receptors

(cutaneous, muscle, visceral) to the spinal cord. The synaptic contacts formed by this heterogenous population of neurons with the dendrites or perikarya in the spinal cord represent the first synapse in the central nervous system (CNS) at which sensory information is processed and integrated. The major histological features of the cellular elements in the DRG were described by Ramòn y Cajal (1909) at the beginning of this century, and have been reviewed often since (Ranson, 1912; Lieberman, 1976; Tennyson and Gershon, 1984). Primary sensory neurons have been classified with regard to their perikaryal size, duration of somatic action potential, conduction velocity of nerve fibers, sensory modality, neurochemistry, chemosensitivity and distribution of cellular organelles (Yoshida et al., 1978; Yoshida and Matsuda, 1979; Belmonte and Gallego, 1983; Rambourg et al., 1983; Harper and Lawson, 1985a,b; Rose et al., 1986; Traub and Mendel, 1988; Sugiura et al., 1988). Two principal groups of neurons have been described in the rat DRG. Type A neurons are large cells (30-70 μm in diameter) that stain lightly with basic dyes or silver salts. Type B neurons are smaller (25-30 μM in diameter), having darkly stained perikarya (Andres, 1961). In L5 and L6 rat dorsal root ganglia, about 30 to 40% of the neurons are type A (Andres, 1961). Although the justification for this classification has been questioned (Kawamura and Dyck, 1978; Hato et al., 1980), the morphological heterogeneity is paralleled by a distinct functional heterogeneity. Large A neurons have short-duration action potentials (0.49-1.35 ms at the base) (Harper and Lawson, 1985) and give off large-diameter myelinated axons that conduct in the Aα (30-50 m/s) or Aβ (14-30 m/s) range. Small, type B neurons, are characterized by long-duration action potentials (0.5-8.0 ms at the base) and they are associated

with small-diameter unmyelinated or myelinated axons, that conduct in the C-fiber (<1.4 m/s) or Aδ range (2.2-8.0 m/s). The existence of an intermediate-sized group of cells has been suggested, associated with Aδ-fibers (Harper and Lawson, 1985).

The axons of primary sensory neurons divide into two branches that project in opposite directions. One branch innervates the sensory receptors and contributes to the peripheral nerve. The other, centrally directed branch, a primary afferent fiber, projects to the spinal cord via the dorsal roots (DR). The primary afferent fibers are classified on the basis of the presence of myelin, diameter, conduction velocity and sensory modality. The afferent fibers from the skin are designated as: Aα, Aβ, Aδ and C. The Aα and Aβ fibers are associated with type A sensory neurons. They constitute a group of large, myelinated, fast-conducting cutaneous afferents that enter the spinal cord via the medial division of the dorsal roots and project predominantly to laminae III to V (Brown 1981; Woolf, 1987). The terminals of these fibers generally contain round vesicles, make asymmetric synapses mainly with dendrites and spines of the second order dorsal horn neurons, and are postsynaptic to intrinsic spinal neurons (Réthelyi, 1983).

Aδ- and C-fibers are small-diameter, myelinated and unmyelinated fibers, respectively, associated with small type B or intermediate size cells in the DRG. They enter the cord through the lateral division of the dorsal roots and by way of Lissauer's tract, and project mainly to laminae I,II, and V, and to the region around the central canal (Christensen and Perl., 1970; Light and Perl, 1979a; Honda and Perl, 1985; McMahon and Wall, 1985; Willis, 1985; Chung, 1987). These fibers terminate in synaptic

glomeruli. They establish asymmetric axodendritic synapses and are postsynaptic to axonal and dendritic endings that contain flattened or pleomorphic vesicles (Maxwell and Réthelyi, 1987).

It is a general rule that the fast-conducting myelinated $A\alpha,\beta$ units have a low threshold of activation. Accordingly, these afferents innervate a variety of low-threshold mechanoreceptors. The slow-conducting $A\delta$ and C fibers are associated with high-threshold non-nociceptive and nociceptive function. The variability in the conduction velocity among the slow conducting units is considerable, with the tendency of the highest-threshold units to have the lowest conduction velocity.

A different nomenclature has been used for the afferents from the muscle: Group I (large myelinated), Group II (small myelinated), Group III (smaller myelinated) and Group IV (unmyelinated). Tables 1 and 2 present the alphabetical and numerical nomenclature for cutaneous and muscle afferents, and their respective diameters and conduction velocities.

It is necessary to consider two other recent findings in relation to primary afferent transmission. First, the possibility of synaptic modulation of sensory information in the DRG has been raised by the electron microscopic demonstration of the synaptic terminals and by the reported EPSPs in cat DRG (Kayahara et al., 1981; Miletić and Lu, 1988). Although the function of such putative synapses is as yet unknown, this finding implies that processing and modulation of afferent information may occur prior to entering the spinal cord. In addition, the concept of afferent transmission has further been complicated by the finding of afferent fibers in the ventral roots (Coggeshall et al., 1974, 1975; Maynard et al., 1977; Mawe et al., 1984). These fibers seem to emanate

Table 1. Types of peripheral receptors and afferent fibers active in various sensations

Receptor type	Fiber group	Modality
Hair follicle	Aβ	Tactile
Meissner corpuscle	Aβ	Tactile
Ruffini corpuscle	Aβ	Tactile
Merkel receptor	Aβ	Tactile
Pacinian corpuscle	Aβ	Tactile
Free nerve ending	Aδ,C	Pain and temperature sense
Muscle spindle	Aα,Aβ,	Proprioception
Joint receptors	Aβ	Extremes of joint angle; joint capsule pressure

Table 2. Fiber diameters and conduction velocities of cutaneous and muscle afferent groups

Muscle nerve	Cutaneous nerve	Fiber diameter (μm)	Conduction velocity (m/s)
I		13-20	80-120
II	Aβ	6-12	35-75
III	Aδ	1-5	5-30
IV[a]	C[a]	0.2-1.5	0.5-2.0

[a]Unmyelinated

from cutaneous, muscular and visceral receptors including nociceptors (Clifton et al., 1976). Their course and mode of termination is largely unknown.

Primary afferent fibers of cutaneous mechanoreceptors The mechanoreceptive input to the dorsal horn originates from the low-threshold, sensitive mechanoreceptors of the skin and is mediated by large and small myelinated Aβ and Aδ fibers, respectively (Table 1, 2) (Brown and Iggo, 1967). In some species, but not in humans, additional input is provided by unmyelinated C- fibers (Iggo, 1960). The large Aβ fibers, that are connected to sensitive mechanoreceptors, distribute their axons to some or all of laminae III to V, and the dorsal part of laminae VI. The small Aδ fibers that convey information from the hair-follicle mechanoreceptors terminate predominantly in lamina III, although some synaptic boutons can be found in laminae II and IV (Light and Perl, 1979b). By intracellular iontophoretic application of an immunocytochemical marker, Sugiura et al. (1986), were able to visualize central termination of functionally identified cutaneous C-fibers in the superficial dorsal horn. In addition, the type of cutaneous drive to the neurons in inner substantia gelatinosa (lamina II), point to this region as a likely projection area of C-fiber mechanoreceptive afferents (Light and Perl, 1979b).

Two types of dorsal horn neurons have been described based on their mechanoreceptive input. Neurons with an exclusively mechanoreceptive input from the skin are designated as class 1, low-threshold or lamina 4-type. Neurons that receive convergent excitatory input from several types of cutaneous receptors (i.e., mechanoreceptors, nociceptors and

thermoreceptors) are known as class 2, wide dynamic range or lamina-5 type. Mechanoreceptive neurons are located mainly in laminae III and IV and give off long axons through dorsal column pathway and spinocervical, spinoreticular and spinothalamic tracts. The function of the C-mechanoreceptor-driven "slow brush neurons" in substantia gelatinosa is not known (Light et al., 1979).

Primary afferent fibers of cutaneous nociceptors Nociceptive information from the skin enters the spinal dorsal horn via small myelinated Aδ- fibers (mechanonociceptors) and unmyelinated C-fibers (polymodal nociceptors). Aδ-fibers terminate mainly within the laminae I (the marginal zone), outer portion of laminae II (IIo) and send collaterals to lamina V and the zone around the central canal (Christensen and Perl, 1970; Light and Perl, 1979; Honda and Lee, 1985; Willis, 1985). C-fibers project to lamina II (the substantia gelatinosa), particularly to IIo (Light and Perl, 1979; Sugiura et al., 1986).

The majority of the neurons in lamina I of the dorsal horn respond to peripheral nociceptive stimuli, and it is generally accepted that the flat and relatively large marginal cells (the Waldeyer cells) are nocireceptive. Christensen and Perl (1970) have defined this region as a specialized sensory nucleus containing neurons important for nociception and for detection of thermal changes in the skin. Although some neurons in this lamina receive primary afferent input exclusively from nociceptors, others receive convergent input from a variety of peripheral receptors. (Willis et al., 1974; Price and Browe, 1975; Cervero et al., 1976; Price et al., 1976). Early studies from human material (Kuru, 1949) and studies in the cat (Willis and Coggeshall, 1978; Cervero et al., 1979; Widberg and

Blomquist, 1984) demonstrated that a proportion of lamina I neurons send their axons rostrally via the spinothalamic, spinoreticular and spinomesencephalic tracts. These projection neurons have been physiologically well characterized (Dilly et al., 1968; Willis et al., 1974; Giesler et al., 1976; Price et al., 1976; Hylden et al., 1989) and implicated in the transmission of sensory information from the skin that will eventually lead to the experience of cutaneous pain.

Primary afferent fibers of cutaneous thermoreceptors Information from cutaneous thermoreceptors (cold and warm receptors) is conveyed via small Aδ- and C-fibers (Iggo, 1969). The distribution of terminals of this class of primary afferents within the dorsal horn is largely unknown. Whereas some neurons, i.e. "cold units", located in the superficial dorsal horn seem to be driven exclusively by thermoreceptors, other neurons in the same region exhibit a great degree of convergence of different modalities. Such convergence has been demonstrated for thermo- and nociceptors (Christensen and Perl, 1970; Hellon and Misra, 1973).

Primary afferent fibers of muscle receptors Primary afferent fibers originating in the muscle convey proprioceptive information to the spinal cord. These include: group Ia axons (Aα) from the primary endings in muscle spindles, group II axons (Aβ) from secondary endings in the muscle spindle, and group Ib axons (Aα) from Golgi tendon organs. Group Ia axon collaterals project mainly to lamina VI and laminae VII and IX (the motorneuronal pool). Group II axons terminate in laminae IV to VII and those of group Ib send collaterals to a wide area between laminae V and VII (Brown, 1981).

Primary afferent fibers conveying information from visceral receptors

Another source of afferent input to dorsal horn neurons, especially in thoracic and sacral regions, is provided by central endings of visceral afferent fibers that originate from sympathetic and parasympathetic neurons. Visceral afferents are of Aδ and C fiber type. They terminate mostly in laminae I and V of the dorsal horn although synaptic boutons are occasionally found in the substantia gelatinosa and lamina III (Morgan et al., 1981). Although these fibers represent only a small proportion of the total afferent inflow to the spinal cord they can activate a large number of neurons in the spinal cord due to extensive functional divergence (Cervero, 1983a, 1984). For instance, destruction of almost 95% of afferent C fibers with neurotoxin capsaicin reduces the number of dorsal horn neurons driven by the surviving C fibers by only 50% (Cervero, 1984).

Dorsal horn neurons can be classified into two groups depending on the presence or absence of an excitatory visceral input. Somatic neurons are driven by mechanoreceptive input from their somatic fields and lack visceral input. They are distributed mainly throughout laminae II, III and IV. The second category are viscerosomatic neurons, that receive both somatic and visceral inputs (Pomeranz et al., 1968; Foreman and Ohata, 1980; Cervero, 1982, 1983a,b). These neurons are predominantly located in laminae I and V and also in the ventral horn (Cervero, 1986). Therefore, visceral and somatic pathways converge at the level of the spinal viscerosomatic neurons and project via somatosensory pathways, including spinothalamic and spinoreticular tracts (Cervero, 1983b). These findings present a strong experimental support for the "convergence-projection" theory of the referred visceral pain (Ruch, 1974).

Cytoarchitectonic organization of the dorsal horn: The Rexed's scheme

The Rexed's scheme (1952, 1954) was based on the study of neuronal somata from 100 μM-thick Nissl-stained sections of the spinal cord from kittens and adult cats. This scheme was cytoarchitectonic, concerned with shapes, sizes, densities and distribution of neuronal somata in the spinal cord, without taking into consideration dendritic trees or axonal projections and terminations. Rexed described nine cell layers (laminae) in the gray matter, and a region around the central canal (lamina X). The upper six laminae comprise the spinal dorsal horn.

Lamina I (Marginal zone) Lamina I was described originally as a thin (12-20 μm) veil of gray substance, forming the dorsal-most part and lateral side of the spinal gray matter (Rexed, 1952). Neurons in the marginal zone vary in size from small, to medium and large. Although large neurons comprise only a small fraction of the total number of cells in lamina I, these so called marginal cells have been characterized the best (Waldeyer, 1888; Ramon y Cajal, 1909). Their somata are large (10-15 x 30-50 μM) and located between the overlying white matter and the underlying lamina II (Waldeyer, 1888; Rexed, 1952). Marginal cells' dendrites are largely confined to lamina I. Their dendritic domains resemble a flattened disc with elliptic elongation in the rostro-caudal direction (500-1400 μm) (Ramon y Cajal, 1909; Scheibel and Scheibel, 1968; Light et al., 1979). Marginal cells are either local circuit or projection neurons. Two destinations of axonal projections of marginal cells have been proposed. The distant sites include the thalamus, via the spinothalamic tract (Willis et al., 1974; Trevino and Carstens, 1975; Giesler et al., 1979), the lateral cervical nucleus (Craig, 1978; Brown et al., 1980) and the midbrain

reticular formation (Trevino, 1976; Molenaar and Kuypers, 1978; Swett et al., 1985). As a part of propriospinal pathways, axons of marginal cells travel to other segments of the spinal cord (Burton and Loewy, 1976).

In terms of their functional properties, three groups of lamina I neurons have been described: nocireceptive specific neurons (NS), wide dynamic range (WDR, which respond both to nociceptors and low threshold mechanoreceptors) and thermoreceptive neurons that respond to warming and cooling.

Lamina II (substantia gelatinosa of Rolando) Due to the absence of myelinated fibers and the presence of densely-packed and mostly radially oriented neuronal somata, this lamina appears as an easily distinguishable pale band, just ventral to the marginal zone (Ramon y Cajal, 1909; Rexed, 1952,1954). On the basis of the dendritic arborizations and axonal projections of its neurons and afferent inputs, lamina II is subdivided into two regions, an outer region (LIIo: 30-40 μm thick) and an inner region (LIIi: 40-50 μm thick) (Rexed, 1952; Gobel, 1979; Ralston and Ralston, 1979).

Morphologically, two types of cells are present in lamina II (Beal and Cooper, 1978; Price et al., 1979). The larger (16-22 μm, Bennett et al., 1980), stalked cells (border cells or limiting cells), are mostly found along the outer edge of lamina II. Their dendritic trees are oriented ventrally, mostly confined to lamina II, and only occasionally project dorsally and enter lamina I. The axons of these cells project into lamina I (Gobel, 1975,1978; Sugiura, 1975; Beal and Cooper, 1978). The smaller, islet cells (5-10 x 5-10 μm), are found mainly in LIIi. Their dendritic trees are oriented primarily longitudinally and spread throughout laminae

II and III (Scheibel and Scheibel, 1968; Sugiura, 1975).

Functionally, the outer part of this lamina (LIIo) contains either NS or WDR neurons, whereas the inner part (LIIi) neurons respond only to innocuous stimuli.

Together, Rexed's laminae I and II, comprise the superficial dorsal horn. This is the main area of termination of small myelinated (Aδ) and unmyelinated (C) fibers from the skin, muscle and viscera (Christensen and Perl, 1970; Light and Perl, 1979; Cervero, 1983; Craig and Kniffki, 1985). Because of the nociceptive nature of the fine afferents that make the first synaptic relay in this region of the spinal cord gray matter, superficial dorsal horn has been considered an important area for transmission and modulation of the nociceptive information. Superficial dorsal horn contains more than a dozen of putative peptidergic neurotransmitters (e.g. SP-, NKA-, VAS-, OXY-, CCK-, SRIF-, VIP-, NT-, ENK-, NPY-, DYN-, GAL-, CGRP-, and TRH-LI).

Lamina III Lamina III is a broad band bordered medially by the white matter and laterally by the ventral bend of laminae I and II. The lighter appearance of this lamina in cytoarchitectonic stains is a result of the less dense packing of nerve cells. The neuronal somata are larger than those of lamina II (7-9 x 10-12 μm), spindle-shaped and are oriented vertically to the surface of the lamina. The dendritic pattern of the lamina III cells is relatively complex. Long dendrites extending through laminae I-IV and to the outer surface of the dorsal horn have been described (Mannen and Sugiura, 1976; Brown, 1981). Axons of lamina III cells take part in propriospinal pathways (Szentagothai, 1964; Scheibel and Scheibel, 1968). These axons travel widely throughout the gray matter of

the dorsal horn before passing into the white matter (Matsushita, 1969,1970). Upon returning to the gray matter, the axons project mainly to lamina II and III (Szentagothai, 1964). Some of the axons form synaptic junctions outside of this area, and the system may not be as closed as Szentagothai first proposed.

Lamina III neurons are driven by low-threshold mechanoreceptive fibers (Aβ) and are functionally regarded as inhibitory interneurons. The peptidergic content of these neurons includes SP-, ENK-, NT-, and TRH-LI.

Lamina IV Lamina IV is a relatively thick layer that extends straight across the dorsal horn. It is bordered medially with the white matter of the dorsal columns and laterally with the ventro-lateral bend of laminae I to III. One of the most striking features of this lamina is its neuronal heterogeneity (Rexed, 1952; Brown, 1981). The nerve cells of varying sizes (8 x 11 to 35 x 45 μm) and shapes are distributed relatively sparsely within a dense rostro-caudally oriented nerve fiber network. Although outnumbered by the smaller cells, the large, star-shaped neurons are the most prominent cells in the lamina IV. These cells have long, spine-studded dendrites that spread in all directions (Ramon y Cajal, 1909). The importance of the large cells in lamina IV (and V) is that their dorsally-directed dendrites penetrate into substantia gelatinosa and may represent the principal output from this still functionally little understood region (Szentàgothai, 1964).

Axons of lamina IV neurons project cranially via spinothalamic and spinocervical pathways, or contribute to propriospinal systems (Szentágothai, 1964; Réthelyi and Szentágothai, 1973, Willis and Coggeshall, 1978).

Lamina V This lamina occupies the narrowest part of the dorsal horn gray matter known as the neck of the dorsal horn. Medial part of this lamina is bordered distinctly by the dorsal funiculus, while its lateral border is obscure due to a mesh of passing myelinated fibers (Willis and Coggeshall, 1978).

The cell bodies of this lamina are of varying sizes (8x10 to 30x45 μm) and shapes, and cytoarchitectonically virtually indistinguishable from lamina IV cells (Rexed, 1952). Based on the arborization of their dendritic trees, a role for these neurons in conveying signals from substantia gelatinosa has been proposed. Axonal organization and projections of lamina V neurons are similar to laminae IV cells, i.e., their axons contribute to spinothalamic, spinocervical and propriospinal pathways (Coggeshall, 1978).

Lamina V is a major projection area of C afferents from the viscera, group IV fibers from the muscle and of Aδ fibers from ipsi- and contralateral skin nociceptors (Light and Perl, 1979; Craig and Mense, 1983; Cervero and Connel, 1984). At least eight peptides have been demonstrated in this lamina: SP-, CCK-, SRIF-, ENK-, DYN-, NPY-, CRF- and GAL-LI.

Organization of dorsal horn neurotransmitter systems

The dorsal horn of the spinal cord is the site of the first synapse in the central nervous system where peripheral somatic or visceral information is processed and integrated. As its function implies, the neural structure of the spinal dorsal horn is complex and rich in a variety of neurotransmitters and neuromodulators. Considerable efforts have been

directed towards establishing the identity of these neuroactive substances, their principal neuronal or non-neuronal sources in the dorsal horn, and their cellular mechanisms of actions. It is now clear that there is a precise laminar distribution of the neuroactive substances in the spinal cord, and that this distribution is dependent on the segmental level assayed.

A growing list of putative neuromediators in the spinal dorsal horn includes excitatory and inhibitory amino acids, bioamines, acetylcholine, purines and a number of peptides. It is noteworthy that many of these chemical mediators are particularly represented in the superficial laminae of the dorsal horn (Laminae I to III). Distribution and coexistence of some peptides in the primary sensory neurons, and axonal terminations of these neurons in the spinal dorsal horn are summarized in Table 3.

All putative messengers of chemical neurotransmission in the spinal dorsal horn derive from three principal neuronal sources. These are: primary afferent fibers, spinal interneurons and the descending pathways. They are briefly reviewed in this part of the thesis, with an emphasis on the experimental evidence for excitatory amino acids and neuropeptides as neurotransmitter or neuromodulator candidates of the primary afferent fibers.

Excitatory amino acids (EAA): L-glutamate and L-aspartate

Dicarboxylic amino acids, glutamate and aspartate, are the principal excitatory neurotransmitter candidates in the central nervous system, including spinal dorsal horn (Mayer and Westbrook, 1987; Evans, 1989; Rustioni and Weinberg, 1990). Available experimental evidence strongly supports the role for glutamate as a fast excitatory neurotransmitter of

Table 3. Neuropeptides in subsets of mammalian dorsal root ganglion neurons (Jessell and Dodd, 1989)

Neuropeptide	% DRG neurons	Lamina termination	Coexistence (rat)
Substance P (SP)	20	I, IIo	CCK, CGRP, NKA, VASO
Neurokinin A (NKA)	20	I, IIo	CCK, CGRP, SP, VASO, GRP
Cholecystokinin (CCK)	20	I, IIo	SP, CGRP, SK, VASO
Calcitonin gene-related peptide (CGRP)	30	I, IIo	SP, NKA, CCK, VASO
Vasoactive intestinal polypeptide (VIP)	5 (visceral)	I	?
Somatostatin (SS)	8-10	IIo	VASO
Dynorphin (DYN)	5 (visceral)	I	?
Enkephalin (ENK)	5 (visceral)	I	?
Corticotropin-releasing factor	?	I, IIo	?
Arg-vasopressin (VASO)	50-60	?	most other peptide-
Oxytocin	50-60	?	containing DRG neurons
Gastrin-releasing peptide (GRP)	10	I, IIo	SP
Angiotensin II	?	IIo	?
Galanin	10	I, IIo	?

the primary afferent fibers. Biochemical analyses in the spinal cord have demonstrated higher concentration of glutamate in the dorsal roots, as

compared to the ventral roots (Graham et al., 1967; Duggan and Johnston, 1970; Roberts et al., 1973), and receptor autoradiographic studies have shown a dense band of glutamate-binding sites in the superficial spinal dorsal horn (Greenamyre et al., 1984,1985). By using a glutaraldehyde-conjugated antibody for glutamate, it has been demonstrated that about 70% of both large and small dorsal root ganglion neurons are labelled for glutamate (Duce and Keen, 1983; Kvamme, 1983; Cangro et al., 1985; Wanaka et al., 1987; Battaglia and Rustioni, 1988), and consistent with this, glutamate-IR has been detected within a proportion of myelinated and unmyelinated primary afferent axons and terminals in the superficial laminae (I and II) of the dorsal horn (Duce and Keen, 1983; Miller et al., 1988; Westlund et al., 1989). Glutamate is synthesized in the brain from several precursors, including glucose, ornithine and glutamine (Fonnum, 1984), and the enzymes involved have been demonstrated in some DRG neurons with immunocytochemical methods (Cangro et al., 1985; Inagaki et al., 1986; Battaglia and Rustioni, 1988;). Ca^{2+}-dependent release of glutamate from electrically stimulated primary afferent fibers has been demonstrated both *in vivo* (Roberts et al., 1973; Roberts, 1974a,b) and *in vitro* (Kawagoe et al., 1986; Kangrga and Randić, 1990; Kangrga et al., 1990a,b). Glutamate has been demonstrated in vesicles within the nerve terminals (Nicholls and Sihra, 1986) and glutamate-accumulating vesicles were isolated from the central neurons (Naito and Ueda, 1983).

Electrophysiological studies provided first evidence for glutamate as an excitatory neurotransmitter candidate of the primary afferents. Glutamate excites and depolarizes almost all spinal dorsal horn neurons *in vivo* (Curtis et al., 1960; Galindo et al., 1967; Zieglgänsberger and Puil,

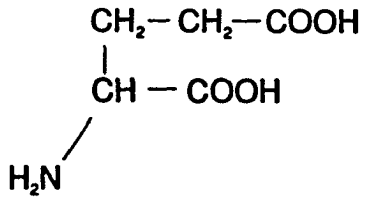
1973) and a proportion of dorsal horn neurons in vitro (Salt and Hill, 1983; Schneider and Perl, 1985, 1988). The reversal potential of glutamate-evoked depolarization is similar to that of the fast EPSP (Mayer and Westbrook, 1987; Gerber and Randić, 1989; Yoshimura and Jessell, 1990). Schneider and Perl (1985) found that, in the rat spinal dorsal horn in vitro, about one third of the neurons in the superficial laminae of the dorsal horn were responsive to iontophoretic application of glutamate. As these neurons were synaptically driven by activated C-fibers, it was proposed that glutamate and/or aspartate may act as neurotransmitter(s) of the small DRG neurons. Although there is evidence that primary sensory neurons mediating different modalities of afferent information may use glutamate, or a similar compound as neurotransmitter, there are also reports of the ineffectiveness of some broad spectrum amino acid antagonists, such as kynurenic acid, to block the low-threshold (Schneider and Perl, 1985) and the high-threshold (Yoshimura and Jessell, 1990) dorsal root stimulation-evoked EPSP.

Whereas glutamate has been long favored as a candidate neurotransmitter of the primary afferent fibers, anatomical and physiological evidence has recently emerged suggesting a neurotransmitter role for aspartate in various regions of the central nervous system (Collins et al., 1983; Hicks et al., 1985; Bliss et al., 1986; Perschak and Cuenod, 1990), including the rat medulla (Kihara et al., 1989; Kubo et al., 1990) and the spinal dorsal horn (Skilling et al., 1988; Kangrga et al., 1989, 1990a,b; Kangrga and Randić, 1990). Aspartate immunoreactivity has been demonstrated within both myelinated and unmyelinated primary afferent terminals in the spinal dorsal horn (Westlund et al., 1989a). It is of

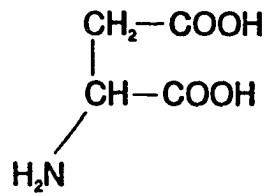
considerable interest that significantly more immunolabeled aspartate than glutamate, is present in small unmyelinated L4 dorsal root axons in the superficial dorsal horn (Westlund et al., 1989b), the finding indicating that aspartate may be a neurotransmitter for a certain population of small primary afferent neurons. Attempts to demonstrate the presence of a synthesizing enzyme of aspartate, aspartate-aminotransferase, were unsuccessful (Cangro et al., 1985).

Electrophysiological evidence for aspartate remains largely controversial. Whereas it has been demonstrated that aspartate does not depolarize cultured dorsal horn neurons (Jessell et al., 1986), about 85% of the acutely isolated dorsal horn neurons (Murase et al., 1989), and neurons recorded from spinal dorsal horn slices (Gerber et al., 1989) respond to the local application of aspartate and N-methyl-D-aspartate. Both NMDA and non-NMDA receptors appear to participate in the fast (Dale and Roberts, 1985; Dale and Grillner, 1986; Gerber and Randić, 1989a; Dickenson, 1990) and slow excitatory neurotransmission in the spinal dorsal horn (Gerber and Randić, 1989b). It has been suggested that aspartate is a putative endogenous ligand for NMDA receptors (Watkins and Evans, 1981).

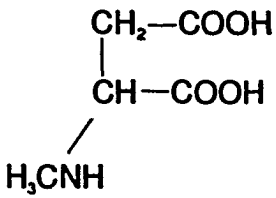
Based on their responsiveness to selective agonists and antagonists (Fig. 1), at least four distinct subtypes of ionotropic glutamate receptors have been identified. N-methyl-D-aspartate (NMDA), kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) are selective agonists for NMDA, kainate and quisqualate receptor subtypes, respectively. The fourth subtype of receptor is defined by the antagonistic action of 2-



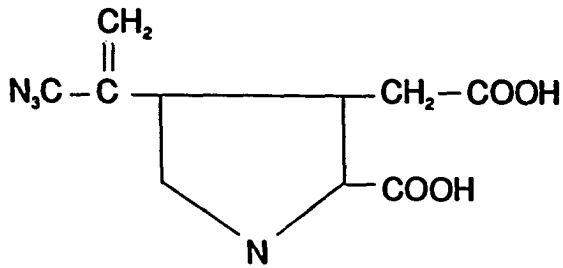
GLUTAMIC ACID



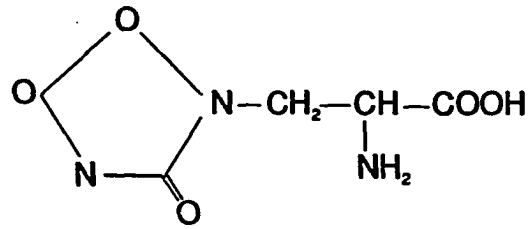
ASPARTIC ACID



N-METHYL ASPARTIC ACID



KAINIC ACID



QUISQUALIC ACID

amino-4-phosphonobutyrate (L-AP4). The present concept is that the multiple types of glutamate receptors are part of the same receptor-channel complex (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987).

NMDA is a selective agonist at NMDA receptor and its action can be effectively antagonized by D-2-amino-5-phosphonovalerate (D-AP5 or D-APV). Electrophysiological studies of NMDA receptors and associated ion channels have revealed several distinct properties of this system: 1) The NMDA channels are blocked by Mg^{2+} in a voltage-dependent manner (Mayer et al., 1984; Nowak et al., 1984; MacDermott et al., 1986); 2) The channels are permeable to Na^{+}, K^{+} and Ca^{2+} (Ascher et al., 1988; Ascher and Nowak, 1988); 3) The channels exhibit multiple conductance states (Ascher et al., 1988; Ascher and Nowak, 1988); 4) Glycine potentiates the response to NMDA by acting at strychnine-insensitive allosteric site(Johnson and Ascher, 1987) and, 5) The receptor has a micromolar affinity constant for glutamate (Olverman et al., 1984). Thus, the activation of NMDA receptors can induce Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by cell depolarization (Mayer and Miller, 1990).

KA and AMPA receptors are classified as non-NMDA receptors. KA and AMPA activate relatively voltage-insensitive conductances (MacDonald and Porietis, 1982). Quinoxalinediones (CNQX, DNQX and NBQX) are effective antagonists at non-NMDA receptors. Antagonist that can pharmacologically differentiate between KA and AMPA receptors has not been synthesized as yet.

Binding of quisqualic and ibotenic acid, to <u>metabotropic</u> receptors that are linked to phospholipase C, results in a G protein-mediated phosphoinositide hydrolysis and release of calcium from intracellular

stores. Thus, the activation of both ionotropic and metabotropic EAA receptors may result in elevation of $[Ca^{2+}]i$. The increase in intracellular free $[Ca^{2+}]$ has been implicated in the activation of other second messenger systems and in the processes of long-term changes in synaptic transmission (Malenka et al., 1989), neuronal excitability and gene expression (Quirion, 1988; Szekely et al., 1989).

A novel way of intercellular signaling by glutamate has been recently demonstrated in the cerebellum (Garthwaite et al., 1988). Glutamate, acting at NMDA receptor, can increase synthesis of cyclic GMP in the cerebellar neurons through a mechanism that involves formation of nitric oxide (NO) from the presynaptic structures, the granule cells being the likely generator of NO. The source of intracellular NO appears to be L-arginine. Thus, it appears that postsynaptic NMDA receptor activation may result in functional modification of the presynaptic terminal of the same cell, and that cyclic GMP may be of importance in mediating excitatory amino acid responses. The steps of the Ca^{2+}-dependent synthesis of NO are well understood, and the enzyme inhibitors are available. A sensitive enzymatic assay monitoring the conversion of [3H]arginine to [3H]citruline, which occurs stoichiometrically with the formation of NO, revealed that NO is widespread throughout the brain, being discretely localized in the neuronal and vascular elements (Bredt et al., 1990). Therefore, this pathway represents a promising site for presynaptic modulation of interneuronal signalling in the nervous system.

<u>γ-Aminobutyric acid (GABA)</u> The role of GABA as a principal inhibitory neurotransmitter in the mammalian central nervous system is more firmly established than for any other neurotransmitter (Krnjević, 1974,

1987; Nistri, 1984). Neurons utilizing GABA as a neurotransmitter are distributed throughout the neuroaxis and exhibit different morphology. The GABAergic neurons figure prominently as interneurons, and they are instrumental in defining and confining the response properties not only of single neurons, but also of large neuronal circuits. Perhaps the best understood electrophysiological actions of GABA are those described in the hippocampus (Newberry and Nicoll, 1983,1984a,b,1985) and cerebral cortex (Connors et al., 1988; McCormick, 1989) where GABA has been implicated in the mediation of fast and slow inhibitory postsynaptic potentials.

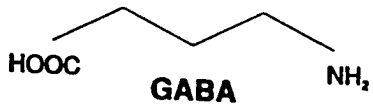
GABA acts in the nervous system at two clearly defined receptor subtypes, designated as $GABA_A$ and $GABA_B$ receptors (Bowery, 1980,1983). The distinction between these two receptor sites is based on numerous criteria including molecular, biochemical, pharmacological, and electrophysiological. $GABA_B$, but not $GABA_A$ receptors, are present at high concentration in the superficial dorsal horn (Bowery et al., 1983, 1987; Price et al., 1984). The higher concentration of $GABA_B$ than $GABA_A$ receptor subtype is unusual in the central nervous system and is found only in several regions (i.e., spinal trigeminal tract, globus pallidus, temporal cortex; Bowery et al., 1987). $GABA_B$ receptors are thought to be situated predominantly, but not exclusively, in the presynaptic membrane (Bowery et al., 1980; Price et al., 1984). Functional $GABA_B$ receptors have been demonstrated in the membrane of primary sensory neurons (Dunlap, 1981a,b; Dunlap and Fischbach, 1981; Desarmenien et al., 1984; Green and Cottrell, 1984; Dolphin and Scott, 1986), and electrophysiological and lesion studies suggest that a proportion of the primary sensory neurons exhibiting $GABA_B$ receptors, are small B neurons (Desarmenien et al., 1984;

Price et al., 1984).

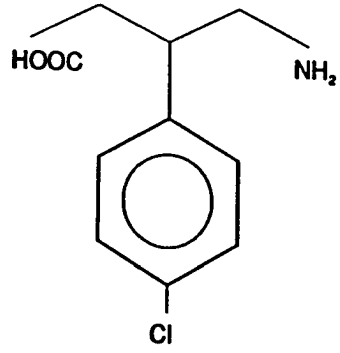
The structural requirements for $GABA_B$ receptor activation seem to be very stringent. To date, the only selective synthetic agonist at $GABA_B$ receptors is (β-p-chlorophenyl)-GABA (baclofen, Fig. 2). Baclofen has a binding affinity for brain $GABA_B$ sites of about 1nM (similar to that of GABA) and has been a particularly useful tool for investigating the function of $GABA_B$ receptors in the nervous system (Bowery et al., 1980). Two optical isomers of baclofen exist, (-)-baclofen being approximately two orders of magnitude as potent as (+)-baclofen. Recently, three baclofen analogs with antagonistic properties at $GABA_B$ receptors have been synthesized. 2-Hydroxy-saclofen (2-OH-S) (Kerr et al., 1988), a sulfonic derivative, and phaclofen (Kerr et al., 1987), a phosphonic analog of baclofen, are both selective and competitive antagonists at $GABA_B$ receptors. 2-OH-S (pA_2=5.0) appears to be more potent than phaclofen (pA_2=4.0) in displacing $GABA_B$ binding in the nervous system. In addition more recently, the biochemical, electrophysiological and pharmacological properties of a new $GABA_B$ receptor blocker, CGP 35348, have been reported (Olpe et el., 1990)

$GABA_B$ receptors are coupled to pertussis toxin (PTX)-sensitive guanosine triphosphate (GTP)-binding proteins and their activation results in inhibition of adenylate cyclase activity in the brain slices (Woczik and Neff, 1984; Hill et al., 1985; Karbon and Enna, 1985). This action does not appear to be linked to the changes in membrane conductance caused by $GABA_B$ receptors.

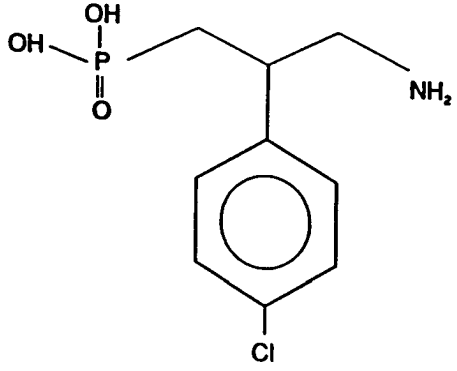
The $GABA_A$ receptors are believed to be situated mainly on the postsynaptic membrane, where they directly control membrane chloride



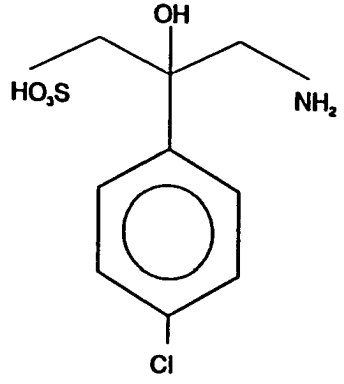
GABA



baclofen



phaclofen



2-hydroxysaclofen

channels. Binding of GABA to $GABA_A$ receptors results in opening of Cl^- channels, presumably via allosteric modification of the receptor channel proteins (Olsen , 1982). $GABA_A$ recepor is a part of a larger molecular complex having, in addition to the GABA recognition site, three other ligand-binding sites. Picrotoxin-like convulsants and bicuculline have antagonistic, while benzodiazepins and barbiturates have agonistic properties at this receptor. It has been demonstrated recently that in the acutely dissociated hippocampal neurons, the function of $GABA_A$ receptor can be modulated at an intracellular site by phosphorylation of the receptor or a closely associated regulatory molecule (Chen et al., 1990). The $GABA_A$ receptor consists of two α- and two β-subunits ($\alpha_2\beta_2$) whose primary sequence and cDNA have been determined (Barnard et al., 1987). A functional $GABA_A$ receptor has been demonstrated in the Xenopus oocyte expression system. After injection of putative $GABA_A$ mRNA, large chloride conductance across the oocyte membrane was elicited by GABA.

Biochemical (Graham and Apprison, 1969; Miyata and Otsuka, 1972) and immunocytochemical (McLaughlin et al., 1975; Barber et al., 1978, 1982; Kaduri et al., 1987; Magoul et al., 1987) studies of the mammalian spinal cord, including that of human spinal cord (Waldvogel et al., 1990), have shown that both GABA and the GABA synthesizing enzyme, glutamate decarboxylase (GAD), are present in the highest concentrations in the superficial laminae of the dorsal horn. The GABA-LI interneurons are evenly distributed throughout laminae I-III, the islet cells being the main neuronal source (Todd and McKenzie, 1989). The demonstration of GABA-LI (Kaduri et al., 1987; Magoul et al., 1987) and GAD-LI (McLaughlin et al., 1975; Barber et al., 1978) terminals establishing axo-axonic contacts with

primary afferent fibers, and axo-dendritic and axosomatic contacts with dorsal horn neurons, has provided a morphological evidence for the hypothesised role of GABA in pre- and postsynaptic inhibition in the dorsal horn.

Much of the present knowledge of the function of $GABA_B$ receptors in the central nervous system is based on experiments that used a selective agonist at these receptors, baclofen (Bowery et al., 1980, 1984, 1989). Baclofen inhibits neurotransmission at peripheral (Peng and Frank, 1989) and central excitatory and inhibitory synapses (Lanthorn and Cotman, 1981; Ault and Nadler, 1982; Inoue et al., 1985a; Howe et al., 1987; Connors et al., 1988), including the spinal cord (Pierau and Zimmermann, 1973; Fox et al., 1978; Jeftinija et al., 1986, 1987; Kangrga et al., 1987; Allerton et al., 1989). The primary mode of action of baclofen seems to be inhibition of neurotransmitter release, as demonstrated for excitatory amino acids (Potashner et al., 1979; Johnston et al., 1980; Collins et al., 1982; Huston et al., 1990), noradrenaline and dopamine (Bowery et al., 1980; Gray and Green, 1987) and peptides (Bowery, 1989). This presynaptic inhibitory action is believed to involve a reduction in calcium entry (Dunlap, 1981; Dunlap and Fischbach, 1981; Cherubini and North, 1984; Desarmenien et al., 1984; Heinemann et al., 1984; Schlichter et al., 1984; Dolphin and Scott, 1986; Green and Cottrell, 1988). In addition, by increasing conductance for potassium ions, baclofen exerts a direct hyperpolarizing effect on central (Gäwhiler and Brown, 1985; Inoue et al., 1985b; Newberry and Nicoll, 1985; Howe et al., 1987; Lacey et al., 1988) and peripheral neurons (Newberry and Gilbert, 1990).

First experimental evidence of the actions of baclofen in the spinal

cord supported its presynaptic site of action (Pierau and Zimmermann, 1973). Iontophoretically or intravenously applied baclofen potently depresses spontaneous and evoked discharge of motoneurons and interneurons (Pierau and Zimmermann, 1973; Curtis et al., 1974, 1981, 1985; Fox et al., 1978; Davies et al., 1981; Henry, 1982; Henry and Ben-Ari, 1982) in the cat spinal cord without altering their passive membrane properties or responsiveness to application of SP or glutamate (Pierau and Zimmermann, 1973; Fox et al., 1978). Consistent with this presumably presynaptic action of baclofen, are the findings that baclofen, or GABA acting at $GABA_B$ receptors, decrease the duration of Ca^{2+}-dependent action potentials and inhibit voltage-dependent calcium currents in the primary sensory neurons (Dunlap, 1981; Dunlap and Fischbach, 1981; Cherubini and North, 1984; Desarmenien et al., 1984; Schlichter et al., 1984; Dolphin and Scott, 1986; Green and Cottrell, 1988). In addition, a direct hyperpolarizing effect of baclofen on neurons in the dorsal horn (Jeftinija et al., 1986; Kangrga et al., 1987; Allerton et al., 1989; Yoshimura and Jessell, 1989) and spinal motoneurons (Wang and Dun, 1990) has been reported.

The two membrane effects attributed to $GABA_B$ receptor activation, increase in K^+ conductance and reduction in Ca^{2+} conductance, have been associated with pre- and postsynaptic actions, respectively. Whilst it was possible that these two effects are linked, the recent evidence suggests that they are not. For instance, the reduction in Ca^{2+} conductance produced in some cells, such as sensory neurons giving rise to $A\delta$ and C primary afferents, is not attenuated by inhibitors of K^+ conductance. One possible explanation for the difference between the two mechanisms is that the channels have different locations, Ca^{2+} channel predominating at sites

of presynaptic terminals and K^+ channel coupling being more important at somatic sites (Bowery, 1989). A second possibility, namely that different subtypes of $GABA_B$ receptors regulate the pre- and postsynaptic actions, has been recently proposed. Discriminative sensitivity of pre- and postsynaptic $GABA_B$ ionic mechanisms to pertussis toxin (PTX) (Dutar and Nicoll, 1988) and to a selective blocker at $GABA_B$ receptors, phaclofen, in the hippocampus (Dutar and Nicoll, 1988) and spinal cord (Kerr et al., 1987; Wang and Dun, 1990) have been reported. One should bear in mind that all known actions of baclofen are pharmacological, and it remains to be determined what the physiological role of $GABA_B$ receptors is (Bowery, 1989).

Tachykinins: Substance P and neurokinin A The tachykinins comprise a family of closely related peptides that participate in regulation of diverse biological processes. The known mammalian tachykinins (Table 4) currently include substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), neurokinin B (NKB) and neurokinin γ. SP and NKA are derived from the same preprotachykinin (PPT) gene, which explains the similar distribution of these two peptides in the CNS (Krause et al., 1987). Differential splicing of the PPT gene gives rise to two mRNAs, αPPT mRNA and βPPT mRNA. After translation, the αPPT-polypeptide opens up to release a 11-amino acid peptide, SP, whilst the βPPT-polypeptide is cleaved to give both SP and NKA. Tachykinin precursor synthesis can be regulated at a number of levels (i.e. SP/NKA gene transcriptional activation, primary transcript splicing etc.). The regulation of the expression of multiple tachykinin peptides in the nervous system has not been well understood.

Table 4. Amino acid sequences of mammalian tachykinins and calcitonin gene-related peptide

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-PHE-Phe-GLY-LEU-MET-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-PHE-Val-GLY-LEU-MET-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-PHE-Val-GLY-LEU-MET-NH_2
Neuropeptide K	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-PHE-Val-GLY-LEU-MET-NH_2
Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His Lys-Arg-His-Lys-Thr-Asp-Ser-PHE-Val-GLY-LEU-MET-NH_2
Calcitonin gene-related peptide	Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

SP and NKA are present in a proportion (35-50%) of small and medium-sized dorsal root ganglion and trigeminal neurons (Tuchscherer and Seybold, 1985) that give rise to small unmyelinated and myelinated fibers (Hökfelt et al., 1981; Nagy et al., 1981). Consistent with these findings, SP has also been demonstrated within axon terminals of the primary afferent fibers in laminae I, II and V of the spinal dorsal horn (Hökfelt et al., 1975,1981; Barber et al., 1979; Ruda et al., 1986), and dorsal root rhizotomy results in a marked reduction of the SP immunoreactivity in the superficial dorsal horn (Hökfelt et al., 1975; Yaksh et al., 1982; Schrøder, 1984). Similar effect is produced by neonatal capsaicin treatment, which causes a degeneration of 95% of unmyelinated C and about 30-40% of small myelinated Aδ fibers (Yaksh et al., 1982; Franco-Cereceda

et al., 1987). Thus, there seems to be a close relationship between the presence of SP and the integrity of small diameter C and Aδ primary afferent fibers. Whereas the primary afferents represent the principal source of SP in the dorsal horn, both intrinsic spinal neurons and descending terminals contribute to SP-LI in the dorsal horn (Gibson et al., 1981; Hökfelt et la., 1981; Nagy et al., 1981; Gilbert et al., 1986). SP-containing terminals form axodendritic and axosomatic synapses and they are also postsynaptic to small vesicle-filled axonal and dendritic profiles (Chan-Palay and Palay, 1977; Bresnahan et al., 1984). Within the central terminals of the primary sensory neurons, SP is confined to the large, dense-core vesicles (DeBiasi and Rustioni, 1988). In addition, colloidal double-labelling techniques have demonstrated within the central terminals the coexistence of the dense-core vesicles with small, clear vesicles stained for glutamate. SP and NKA are released by depolarizing concentrations of K^+ from the <u>in vitro</u> spinal cord preparations (Otsuka and Konishi, 1976; Gamse et al., 1979; Akagi et al., 1980; Hua et al., 1986; Saria et al., 1986) and from cultured primary sensory neurons (Mudge et al., 1979) in a Ca^{2+}-dependent manner. In addition, the release of SP-LI and NKA-LI within dorsal horn following noxious cutaneous and high-intensity electrical stimulation of the primary afferents (Yaksh et al., 1980; Kuraishi et al., 1985; Brodin et al., 1987; Duggan et al., 1987) has been demonstrated. An original study was conducted by Duggan and his associates, who used SP- and NKA-antibody-coated microprobes to investigate the spatial and temporal patterns of stimulus-dependent release of SP in the dorsal horn (Duggan et al., 1987,1990). The results of their work strengthen the claim that these two tachykinins have a neurotransmitter or

neuromodulator role in the spinal dorsal horn. Although the direct experimental evidence is still lacking, the fact that the SP and NKA synthesis is encoded by the same gene suggests that these two peptides are present in the same vesicles (Nawa et al., 1983).

Electrophysiological evidence has demonstrated that SP modulates neuronal excitability in the dorsal horn (Konishi and Otsuka, 1974; Henry, 1976; Randić and Miletić, 1977; Otsuka et al., 1982) by activating multiple ionic conductances (Murase et al., 1984, 1986, 1989). The cellular mechanisms underlying the neuromodulatory function of SP in the dorsal horn, however, have not been fully understood. Several lines of evidence suggest that the slow excitatory synaptic transmission in the spinal dorsal horn may be peptidergic. Thus, high-intensity repetitive stimulation of the dorsal roots elicits a slow excitatory postsynaptic potential (sEPSP) in about half of tested dorsal horn neurons (Urban and Randić, 1984). The time course of this potential, the associated conductance changes, and the sensitivity of the sEPSP to synthetic SP analogs having antagonistic properties, and to mono- and polyclonal antibodies to SP, suggest that the sEPSP may be mediated by SP, or a related tachykinin (Urban and Randić, 1984; Randić et al., 1986; Randić et al., 1987). Moreover, the depletion of SP-LI from the spinal dorsal horn by a neonatal capsaicin treatment resulted in a loss of the slow excitatory neurotransmission in the dorsal horn (Urban et al., 1985). Although this evidence suggests the involvement of SP, perhaps released from the capsaicin-sensitive population of DRG neurons, in the mediation of the sEPSP, it should be noted that the neonatal capsaicin treatment besides depleting SP-LI results also in a decrease of the immunoreactivity for NKA, somatostatin, cholecystokinin,

calcitonin gene-related peptide, vasoactive intestinal peptide and galanin.

Three distinct, G protein-coupled tachykinin receptors have been identified in the rat peripheral and central nervous system. The three receptors interact differentially with the tachykinin peptides and are uniquely distributed in the nervous system, including the spinal dorsal horn. Endogenous peptides SP, NKA and NKB, are preferential agonists at NK-1, NK-2 and NK-3 receptors, respectively (Cotman and Iversen, 1987). Advances in molecular cloning techniques have contributed to better understanding of the tachykinin receptors. SP-activated NK-1 receptor is the best studied neurokinin receptor whose primary structure has been recently determined (Hershey and Krause, 1990). This receptor is expressed by the neurons and glia in the CNS, but also by endothelial cells, fibroblasts and several circulating inflammatory and immune cells. Its activation results in increased hydrolysis of inositol phospholipids in the brain (Mantyh et al., 1984; Torrens et al., 1986). In addition, single cDNA clone for the NKA receptor has been isolated that is capable of inducing electrophysiological response in the Xenopus oocyte expression system (Masu et al., 1987).

Although all three types of tachykinin receptors and their endogenous ligands, SP, NKA and NKB, are present in the spinal dorsal horn, the functional significance of multiple neurokinin receptors was not addressed until recently. Fleetwood-Walker et al., (1990) performed a series of experiments designed to test the effects of iontophoretic application of selective tachykinin agonists and antagonists to the region of the substantia gelatinosa, on somatosensory responses of identified cat spinocervical tract (SCT) neurons. The results from this study implicate

NK-2 receptors, and its endogenous ligand NKA, in mediating or facilitating the expression of thermal nociceptive inputs to substantia gelatinosa. The activation of NK-1 receptors reduced the non-nociceptive responses of SCT neurons. No role for NK3 receptors has been proposed as yet.

Calcitonin gene-related peptide (CGRP) CGRP (Table 4) is a 37 amino acid peptide formed in the neural tissue by alternative splicing of the primary mRNA transcript of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). CGRP is widely distributed throughout the brain (Gibson et al., 1984; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985; Franco-Cereceda et al., 1987) but its function remains largely obscure. In the spinal cord, CGRP-immmunoreactive fibers and terminals are present at all spinal levels. Dense immunoreactivity is observed particularly in the areas of the dorsal horn where small primary afferent fibers terminate (Lissauer's tract, Laminae I-II), and in the ventral horn. About 40 to 50% of rat lumbar DRG neurons are immunoreactive for CGRP (Gibson et al., 1984; Lee et al., 1985) and this is considered the principal source of CGRP in the dorsal horn. No CGRP-LI has been detected in the dorsal horn neurons (Gibson et al., 1984). In addition, neonatal capsaicin treatment reduced CGRP-LI in the superficial dorsal horn only by 60% (Franco-Cereceda, 1987). Release of CGRP-LI was demonstrated from rat primary sensory neurons in response to capsaicin, high K^+ or electrical stimulation (Franco-Cereceda, 1987) and also in the spinal cord slices (Saria et al., 1986; Oku et al., 1987). Dense representation of high-affinity CGRP binding sites has been demonstrated in the superficial dorsal horn (Inagaki et al., 1986).

Double immunostaining techniques have shown that virtually all small

SP-LI neurons in the DRG are immunoreactive for CGRP (Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984; Franco-Cereceda, 1987). Although this interesting finding suggests a potentially important interaction between the two peptides stored and likely to be released from the same primary afferent terminals, further studies are needed to clarify the cellular mechanisms underlying this phenomenon. Similar to SP, CGRP has a slow depolarizing effect on the dorsal horn neurons, increases voltage-dependent Ca^{+} currents (both N- and L-type) in the DRG neurons and enhances excitatory synaptic transmission in the spinal dorsal horn (Randić and Miletic, 1977; Murase and Randić, 1984; Murase et al., 1986, 1989: Ryu et al., 1988a,b). The time course of the depolarizing action of CGRP, however, is more prolonged than for SP, and this peptide has been implicated in the spinal sensory integration processes (Ryu et al., 1988a). CGRP produces also a prolonged excitation of wide-dynamic range and low-threshold mechanoreceptive dorsal horn neurons (Miletić and Tan, 1988). Intrathecally administered SP and CGRP were reported to synergistically modulate the nociceptive flexor withdrawal reflex in the rat (Woolf and Wiesenfeld-Hallin, 1986). At present, the role for CGRP in the sensory function is not known.

Opioid peptides Largely due to their clinical significance and to recent technological advances, the opioid peptides are among the best understood peptide systems in the CNS. The precise chemical structures of opioids are known and their genes, mRNAs and precursors (Roberts and Herbert., 1977; Gubler et al., 1982; Kakidani et al., 1982) have been described. The opioid receptors have been characterized (Robson et al., 1983) and the release of opioid peptides from nervous tissue (Akil et al.,

Table 5. Amino acid sequences of opioid peptides

Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu-OH
Met-Enkephalin	Tyr-Gly-Gly-Phe-Met-OH
β-Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

1978) has been well documented. There are three major classes of opioid peptides represented by enkephalins (ENK), β-endorphin and dynorphin (DYN) (Table 5). They are synthesized independently and have distinct distributions in the mammalian CNS, including the spinal cord (Elde et al., 1976; Glazer and Basbaum, 1981; Hunt et al., 1981). Leucine-ENK is present in a few primary sensory neurons and dorsal roots of rats (Senba et al., 1982; Putney et al., 1984) but spinal terminations of these afferents are unknown. ENK-LI is present in laminae I to V in the dorsal horn (Ruda et al., 1986). In lamina I, local circuit neurons, but not spinothalamic projection neurons contain ENK-LI, whereas in lamina II both stalked and islet cells contain ENK-LI (Glazer and Basbaum, 1981; Bennett et al., 1982).

Dynorphin A appears to be located in low concentrations in the primary sensory neurons (Botticelli et al., 1981; Sweetnam et al., 1982). Dynorphin A (Basbaum and Glazer, 1986), and its translation system (Sweetnam et al., 1986), are particularly represented in the dorsal root

ganglia of the sacral region. Local circuit and projection neurons exhibiting DYN-LI have been demonstrated in high densities in laminae I and V (Basbaum and Glazer, 1986).

Opioid peptides exert their actions via opioid receptors of which at least 3 major types exist: μ, δ and κ. Synthetic compound, (Tyr-D-Ala-Gly-Me-Phe-Gly-ol)-enkephalin (DAGO) and morphine are relatively selective ligands for μ-opioid receptors (Handa et al., 1981). [D-Pen 2,5]-enkephalin (DPDPE) is a selective ligand for δ-receptors. Synthetic arylacetamides (U50488H and PD117302) are selective agonists, whereas nor-binaltorphimine is a selective κ-receptor antagonist (Takemori et al., 1988). There is convincing evidence that dynorphin A_{1-17} and related peptides, encoded by the pro-dynorphin gene, are endogenous ligands at κ-receptors. Endogenous ligands for μ and δ receptors have not been identified as yet.

The role of opioid receptors in spinal transmission has been recently reviewed (Evans, 1989; Fields and Basbaum, 1989; Jessell and Dodd, 1989; Yaksh and Aimone, 1989). μ-, κ- and perhaps also δ-receptors have been identified on subpopulations of cultured immature DRG neurons (Mudge et al., 1979) and adult DRG cells in <u>situ</u> (Williams and Zieglgänsberger, 1982). Whereas the inhibitory effect of opioids on the release of SP from the spinal cord was clearly demonstrated (Jessell and Iversen, 1977; Yaksh et al., 1980), recent evidence implies that this effect is much more complex. It has been shown that δ-receptor agonists inhibit the release of SP whereas μ-receptor agonists increase it. The finding (Pochl et al., 1989) that activation of μ- and δ- receptors attenuates the release of SP-LI from C-fibers of the knee joint suggests the presence of functional

opioid receptors on the C primary afferents (Yaksh and Aimone, 1989). Both spinal interneurons (Murase et al., 1982; Yoshimura and North, 1983) and primary afferent terminals (Jessell and Iversen, 1977; Carstens et al., 1979; Fields et al., 1980) may be involved in the μ-receptor depressant effects of opioids.

Recent studies on the cultured DRG neurons have shown that low concentrations of specific μ, δ- and κ-receptor ligands (1-10 nM) can evoke naloxone-reversible prolongation of the action potential in about 80% of tested cells. When applied in higher concentrations (about 1μM), the same agonists cause a reduction in the action potential duration (Crain and Shen, 1990). Increase in the action potential duration to μ- (DAGO) or δ- (DPDPE) opioid receptor agonists seems to be due to a decrease in a voltage sensitive K^+ conductance(s), whereas the action of κ-receptor agonist (U50488H) appears to involve an increase in a voltage-sensitive Ca^{2+} conductance (Crain and Shen, 1990). Crain and Shen (1990) have also suggested that the excitatory effects of opioids on DRG neurons are mediated by opioid receptors positively coupled via a G_S-like protein to adenyl cyclase and cAMP-dependent voltage-sensitive ionic conductances. By contrast, the inhibitory effect appears to be mediated by opioid receptors linked to Gi/Go. Since the cultured DRG neurons are devoid of synaptic contacts, the effects of opioids are likely to be a consequence of a direct action. Some of the excitatory effects of opioids in the brain and spinal cord were explained by disinhibiting mechanisms (Zieglgänsberger et al., 1979).

Lower concentrations of μ- or κ-receptor agonists facilitate the C-fiber-evoked nociceptive responses, whereas higher concentrations result in

inhibition (Knox and Dickenson, 1987). Spinal antinociceptive action of opioids has been demonstrated (Fleetwood-Walker et al., 1986, 1988). Inhibitory effects of opioids on dorsal horn neurons have been shown to be mediated by an increase in a Ca^{2+}-dependent K^{+} conductance resulting in a hyperpolarization of membrane potential (Yoshimura and North, 1983).

Release of Putative Endogenous Amino Acids Neurotransmitters in the Rat Spinal Dorsal Horn.

Secretion of specific chemical messengers by cells is a fundamental physiological process, central to our understanding of cell and tissue interactions in all multicellular organisms. Chemical neurotransmission represents the primary form of intercellular communication in the nervous system, yet relatively little is known about the identity of the chemical messengers and molecular processes involved.

A set of experimental criteria has been developed for identifying possible neurotransmitters and neuromodulators in the nervous system, as shown in Table 6 (reproduced from Gainer and Brownstein, 1981). One of the criteria of rigorous scientific identification of the neurotransmitter for a given central neuronal connection, requires the demonstration of release of the proposed transmitter from presynaptic nerve endings in response to stimulation of the nerve fibers.

The release and uptake of excitatory amino acids

The discovery of quantal neurotransmitter release (Fatt and Katz, 1952) and the subsequent morphological identification of the acetylcholine-

Table 6. Steps in identification of neurotransmitters (Gainer and Brownstein, 1981)

1. ***Anatomical***: presence of the substance in appropriate amounts in presynaptic processes.
2. ***Biochemical***: presence and operation of enzymes that synthesize the substance in the presynaptic neuron and processes, and remove or inactivate the substance at the synapse.
3. ***Physiological***: demonstration that physiological stimulation causes the presynaptic terminal to release the substance, and that iontophoretic application of the substance to the synapse in appropriate amounts mimics the natural response.
4. ***Pharmacological***: drugs that affect the different enzymatic and biophysical steps have their expected effects on synthesis, storage, release, action, inactivation, and reuptake of the substance.

containing synaptic vesicles (De Robertis, 1954) led to the concept that one quantum ofneurotransmitter corresponds to the neurotransmitter stored in one synaptic vesicle released by exocytosis (Del Castillo and Katz, 1956). Although challenged (Dunant, 1986), this hypothesis has been confirmed many times over the years by isolation of a highly pure preparation of synaptic vesicles (Whittaker et al., 1966), demonstration of exocytotic profiles
(Heuser et al., 1979), incorporation of vesicular antigens into the plasma membrane upon stimulation (Von Wedel, 1981) and uptake of extracellular markers by recycling synaptic vesicles (Zimmermann, 1979).

The present concept is that in the mammalian CNS, dicarboxylic amino acids, glutamate and aspartate, are the major excitatory transmitters whereas GABA and glycine are the major inhibitory neurotransmitters which

undergo Ca^{2+}-dependent exocytosis after stimulation (Fonnum, 1984; Krnjević, 1984). Use of brain synaptosomes has proven to be the best model for the studies of the release of glutamate from intact nerve terminals (Nicholls and Attwell, 1991). Release of glutamate is measured indirectly, by adding glutamate dehydrogenase and $NADP^+$ to the synaptosomal incubation medium, and measuring the increase in fluorescence as a result of a conversion of glutamate and $NADP^+$ into 2-oxoglutarate and fluorescent $NADPH^+$ (De Belleroche et al., 1977). Using this assay it has been demonstrated that about 15% of the total glutamate content of cerebral cortical or hippocampal synaptosomes can be released in a Ca^{2+}-dependent manner by prolonged depolarization with KCl (Nicholls, 1989). The Ca^{2+}-dependent pool of glutamate exchanges much more slowly with added glutamate than does the glutamate in the cytoplasm, the finding consistent with the possibility of release occurring from a non-cytoplasmic, presumably vesicular compartment (Wilkinson and Nicholls, 1989). The release of glutamate is dependent on the maintenance of high energy levels and may be substantially inhibited by a decrease in ATP/ADP ratio.

The Ca^{2+}-dependent release of endogenous aspartate from isolated nerve terminal preparation is about 10% of that seen for glutamate (McMahon and Nicholls, 1990). In apparent contradiction to this result are results obtained using brain slices (Szerb, 1988; Kihara et al., 1989; Cuenod, 1990; Kangrga and Randić, 1990, 1991; Kangrga et al., 1989, 1900a,b; Kubo et al., 1990) and <u>in vivo</u> perfused rat spinal cord (Smullin et al., 1988), where a significant release of aspartate has been reported. Nicholls and Attwell (1991) proposed two possible explanations for this apparent paradox. First, there may be a selective inactivating effect of the

synaptosomal preparation on the aspartatergic neuronal population. Second, the function of the plasma membrane amino acid carrier may be compromised in the brain slices and a non-vesicular release of the amino acid may occur.

Two transport pathways are integral to the ability of glutamate to function as a neurotransmitter: a powerful uptake carrier located in the cellular membrane, and a more specific transporter capable of packaging glutamate into a subpopulation of synaptic vesicles. The two transport mechanisms maintain the concentrations of glutamate in the extracellular space, the presynaptic cytoplasm and glutamatergic synaptic vesicles of the order of 1 μM, 10 mM and 100mM, respectively (Maycox et al., 1990).

Glial cells and neurons possess a similar plasma membrane glutamate (acidic amino acid) carrier which helps termination of the postsynaptic actions of glutamate and normally keeps its concentration below levels that damage neurons (Rothman and Olney, 1987). Although the most of radioactive glutamate applied to the brain is taken up by the glia, the neuronal uptake transporters are placed ideally in the presynaptic membrane to quickly remove glutamate after it has acted on the postsynaptic membrane. This carrier system is not specific for glutamate since it also transports L- and D- aspartate. Radiotracing studies have shown that the uptake of each ion of glutamate into a cell is driven by the movement of two Na^+ down the membrane electrochemical gradient (Stallcup et al., 1979). Recently, whole-cell patch-clamp technique revealed that both increase and decrease of intracellular K^+ inhibit glutamate uptake as does membrane depolarization (Barbour et al., 1988). In addition, a potent inhibitory effect of arachidonic acid on the glial glutamate uptake system has been

demonstrated (Barbour et al., 1989). This may contribute to the failure of glutamate uptake system observed in anoxia.

It has been shown that isolated synaptic vesicles, in the presence of an ATP-regenerating system necessary to prevent leakage of glutamate from the vesicles, retain high concentrations of glutamate (Burger et al., 1989). The vesicular glutamate transporter is readily distinguished from the plasma membrane acidic amino acid carrier since it is Na^+-independent and displays a millimolar rather than micromolar affinity for glutamate, consistent with the expected concentration of glutamate in the cytoplasm (Maycox et al., 1990). This carrier is specific for glutamate and does not appear to transport aspartate (Naito and Ueda, 1985).

In addition to aspartate and glutamate, considerable evidence has surfaced supporting the neurotransmitter role of sulfur-containing analogs of glutamate, homocysteine, sulfinic acid and homocysteic acid (HCA) in the brain (Cuenod et al., 1990). Although the release of these acids has not been demonstrated at the level of synaptosomes, studies in the cortical and hippocampal slices have clearly shown a Ca^{2+}-dependent release of HCA (Do et al., 1986). These findings are supported by those of the strong depolarizing effect of HCA on the neurons in the cerebral cortex, hippocampus, striatum, cerebellum, retina and spinal cord (Gähwiler, 1981; Zeise et al., 1988; Patneau and Mayer, 1990). These actions of HCA seem to be mediated by NMDA receptors. Immunocytochemical studies have indicated that in rat cortex, hippocampus, cerebellum and retina HCA is located predominantly in the glial elements (Cuenod et al., 1990). While release, uptake, and neural activity of HCA are compatible with an excitatory transmitter role, the localization of HCA in glial elements raises a

problem.

The presynaptic Ca^{2+} channel

Calcium clearly plays a pivotal role as an intracellular messenger for a variety of cellular responses, including contraction and secretion (Hille, 1984; Kennedy, 1989). When the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]i$) rises into the micromolar range many important cellular events are initiated. This transient increase of $[Ca^{2+}]i$ can be initiated in two ways: 1. Calcium can be released from intracellular storage sites associated with the endoplasmic reticulum, and 2. The increase in Ca^{2+} may be due to opening of Ca^{2+} channels in the plasma membrane through which Ca^{2+} can pass into the cytoplasm, down its electrochemical gradient.

With recent knowledge of the diversity of voltage-sensitive Ca^{2+} channel types (Nowicky et al., 1985; Tsien et al., 1990) several studies have attempted to identify the channels present in the presynaptic terminals. Several classes of compounds have been shown to interact with voltage-sensitive calcium channels. Particularly useful pharmacological tools are the dihydropyridines (DHP's), typified by agonists BAY K 8644 and antagonists, nitrendipine and nifedipine. While these compounds modulate selectively L, but not N and T, Ca^{2+} channels the other Ca^{2+} blockers such as phenylalkylamines, diltiazem or bepridil, have lower specificities and affinities for Ca^{2+} channels. The release of SP from cultured rat (Perney et al., 1986) and chick (Rane et al., 1987) primary sensory neurons is extremely sensitive to modulation by DHP's, the finding implicating involvement of L-type channels. By contrast, N-type channels seem to be responsible for neurotransmitter release from rat brain nerve terminals

(Reynolds et al., 1986). In rat sympathetic neurons both L- and N-type channels may be involved in release under certain experimental conditions (Nowicky et al., 1985). In *Aplysia* sensory neurons in culture, a slowly inactivating, DHP-sensitive Ca^{2+} channel (comparable to the L-type channel) has been implicated in modulation of transmitter release by tonic depolarization, whereas a rapidly inactivating, DHP-insensitive component, (resembling the N-type channel) seems to modulate neurotransmitter release evoked by transient depolarization, presynaptic facilitation and inhibition, and homosynaptic depression (Edmonds et al., 1990). Thus, it appears that different types of Ca^{2+} channels regulate transmitter release at different synapses, and this may have important functional consequences for transmitter release. One general feature of all presynaptic Ca^{2+} channels is that they inactivate slowly, perhaps as a means of permitting sustained release of transmitters during prolonged depolarization.

Location of clustered presynaptic Ca^{2+} channels in the nerve terminals within the site specialized for neurotransmitter secretion, called the active zone, has been possible with the use of a fluorescent Ca^{2+} indicator, fura-2 (Smith et al., 1988). The association of the active zone with fast, direct-acting transmitters stored in clear-core vesicles suggests its role in rapid signalling. It has been proposed that N-type Ca^{2+} channels (with approximate channel conductance of 13 pS) may be involved in triggering transmitter release from the active zones.

Although the mechanism of neuropeptide release is thought to be also vesicular, this process is slow and spatially diffuse, and appears to occur without any discernible active zone. L-type Ca^{2+} channels (25 pS) may regulate exocytosis of large, dense-core, peptidergic vesicles. Although

both peptides and fast-acting neurotransmitters may be released from the same nerve terminal, there is evidence that high frequency stimulation may be required to initiate peptide secretion (Lundberg et al., 1983). If peptides are indeed secreted at presynaptic regions distant from the active zones, this differential frequency sensitivity could be due to the fact that Ca^{2+} enters the presynaptic terminal at the active zones, but only extends beyond the active zone during the massive Ca^{2+} entry that accompanies high frequency stimuli and activation of L-type channels.

Release of glutamate and aspartate in the spinal dorsal horn

The available experimental evidence indicates that there are two major classes of putative excitatory neurotransmitters and neuromodulators that are released in the spinal dorsal horn during activation of primary sensory neurons. L-glutamate, or a related compound, has been implicated in fast excitatory transmission (Mayer and Westbrook, 1987; Evans, 1989), whereas neuropeptides, such as tachykinins, CGRP and opioid peptides, are considered as modulators of the excitatory neurotransmission in the dorsal horn (Urban and Randić, 1984; Jeftinija et al., 1986; Ryu et al., 1988a,b, 1989). Although there are good morphological (Battaglia and Rustioni, 1987; DeBiasi and Rustioni, 1988; Westlund et al., 1989a,b) and functional indications (Mayer and Westbrook, 1987; Schneider and Perl, 1988; Evans, 1989) that glutamate and aspartate may serve as neurotransmitters of the primary afferent fibers, the evidence of release of these amino acids from defined neuronal elements has been difficult to obtain.

Roberts and Mitchell (1972) were the first to examine the release of excitatory amino acids, glutamate and aspartate, from isolated amphibian

hemicord in response to electrical stimulation of the dorsal roots. Although this first study failed to support the proposed role of amino acids in primary afferent transmission, Roberts (1974) demonstrated that electrical stimulation of the rat dorsal column tract fibers in vivo, produced a Ca^{2+}-dependent increase in the efflux of glutamate and GABA from the superfused dorsal column nuclei. Although Osborne and Bradford (1973) demonstrated that, in addition to glutamate and GABA, aspartate and glycine are released by electrical- or high potassium-stimulation from crude synaptosomal preparations of the rat spinal cord and medulla, the source of aspartate and glycine in the spinal cord was thought to be primarily interneuronal (Davidoff et al., 1967, Homma, 1979). Studies in the isolated amphibian (Takeuchi et al., 1983; Kawagoe et al., 1985) and newborn rat spinal cord (Kawagoe et al., 1986) demonstrated the release of glutamate, but not aspartate, from the spinal dorsal horn in response to electrical stimulation of the dorsal roots, the findings favoring a role for glutamate as a primary afferent transmitter candidate. Potashner and Tran (1984) reported that in the isolated guinea pig spinal cord dorsal rhizotomy was associated with decreased uptake (by 20-30%) and release of D-aspartate (by 50%) evoked by electrical field-stimulation. D-aspartate was used as a non-metabolizable marker for L-Glu and/or L-Asp, as it is accumulated and released by neurons thought to use these amino acids as transmitters (Cuenod et al., 1982). Although these findings supported the role of excitatory amino acids as primary afferent transmitters they could not discriminate between glutamate and aspartate. Masters et al., (1989) demonstrated that, in in vivo, regionally superfused rat spinal cord, depolarization with KCl significantly increased the levels of glutamate,

glycine and taurine. This effect appeared to be specific since the levels of other 17 amino acids were not changed. Thus, glutamate was largely favored over aspartate as an amino acid transmitter candidate of primary afferent fibers. However, the possible source of released glutamate with regard to the class of primary sensory neurons, was not addressed in any of these studies.

It has been recently demonstrated that, besides glutamate, aspartate may be released upon electrical stimulation in slices of the hippocampus and striatum (Bliss et al., 1986; Girault et al., 1986; Szerb, 1988; Cuenod et al., 1990) and medulla (Kihara et al., 1989; Kubo et al., 1990), and upon chemical stimulation (high potassium) from the cultured cerebellar neurons (Van Vilet et al., 1989). In addition, a variety of synaptosomal preparations have been shown to possess exocytotic pools of glutamate and aspartate (Nicholls and Talvinder, 1986; Nicholls, 1989).

Release of neurotransmitters can be modulated by activation of autoreceptors, heteroreceptors or receptors for the coexisting substances, such as neuropeptides (Lundberg et al., 1980; Bartfai et al., 1988). Although electrophysiological and radio-ligand binding studies support the existence of excitatory amino acid receptors on the primary sensory neurons (Davies et al., 1979; Evans, 1985; Agrawal and Evans, 1986), the putative feedback control of the release of excitatory amino acids from primary afferent fibers has not been demonstrated, as yet. The glutamate analog, AP4, has been reported to inhibit the release of glutamate from the hippocampal synaptosomes by acting at the presynaptic L-AP4 receptor (Gannon et al., 1989). Glutamate and kainate can regulate the release of glutamate by activating a presynaptic receptor controlling chloride

channels (Sarantis et al., 1988), and glutamate and quisqualate have been shown to inhibit neuronal calcium currents via a G protein-linked mechanism (Lester and Jahr, 1990) that may provide a negative feedback control of glutamate release.

Coexistence of excitatory amino acids and neuropeptides in the primary sensory neurons: physiological implication

Primary sensory neurons and dorsal horn interneurons represent complex coexistence systems. Glutamate and SP coexist in some small primary afferent neurons (Battaglia et al., 1987) and in their terminals in the superficial dorsal horn (DeBiasi and Rustioni, 1988). Neuropeptides SP, NKA and CGRP coexist in a proportion of capsaicin-sensitive DRG neurons (Nagy et al., 1981; Gibson et al., 1984; Franco-Cereceda et al., 1987; Diaz-Guerra et al., 1988). Thus, the primary sensory neurons contain multiple neuropeptides (i.e., SP, CGRP, GAL, SS), products of distinct genes, and low molecular weight "classical" neurotransmitters. This phenomenon of coexistence involves synchronized transcription and translation of several genes. Whereas it is clear that all neurons carry the genes for the neuropeptides, and for the enzymes that synthesize the classical low molecular weight transmitters, little is known about the mechanisms that govern the expression of these genes.

Modulation of release of glutamate by SP was studied using amphibian (Takeuchi et al., 1983) and newborn rat (Kawagoe et al., 1986) spinal cord *in vitro*. Perfusion with SP elicited an increase in the basal efflux of glutamate while the levels of aspartate were not changed. In the latter report the SP effect was Ca^{2+} sensitive and blocked in the presence of

tetrodotoxin (TTX).

In the in vivo experiments by Smullin et al. (1988, 1990), perfusion of the dorsal horn with SP (1 mM) increased the concentration of glutamate and aspartate in the spinal dialysis fluid. CGRP produced no changes in the release of endogenous glutamate, aspartate, glycine and taurine in this study.

Electrophysiological evidence suggests that SP, NKA and CGRP may modulate primary afferent transmission by acting at the postsynaptic site (Murase and Randić, 1984, Murase et al., 1989), and modulation of the responses to glutamate by SP has been directly demonstrated in acutely dissociated dorsal horn neurons (Randić et al., 1990). In addition, recent studies from our (Ryu et al., 1989; Kangrga et al., 1989, 1990a,b; Kangrga and Randić, 1990, 1991) and other laboratories (Kawagoe et al., 1986; Smullin et al., 1990) suggest the presynaptic site of action of neuropeptides. These findings are supported by the presence of peptide receptors on the primary sensory neurons (Mantyh, 1984; Henke et al., 1985) and modulation of Ca^{2+} channel currents by CGRP (Ryu et al., 1989) and SP (Murase et al., 1990) that are critical for the process of neurotransmitter release.

Rationale

Anatomical and physiological studies have provided a detailed description of the organization of afferent projections to the spinal dorsal horn and of the characteristics of the second order neurons. The data, in addition, have emphasized the central role of the primary afferent

synapse in the processing and integration of the cutaneous sensory information in the dorsal horn. However, the identity of neurotransmitters and neuromodulators at primary afferent synapses and cellular mechanisms of their pre- and postsynaptic actions have not as yet been resolved. The evidence indicates that there are two major classes of putative excitatory neurotransmitters and neuromodulators that are released during activation of primary sensory neurons in the mammalian spinal cord. The present concept is that glutamate and/or aspartate function as fast excitatory neurotransmitters. Tachykinins (substance P, neurokinin A), calcitonin gene-related peptide (CGRP) and opioid peptides are thought to be involved in the modulation of primary afferent neurotransmission.

The presence, and in some cases the co-existence of peptides (i.e., tachykinins, CGRP, opioid peptides) and glutamate, has been reported in primary sensory neurons. Chemical signal transfer via such neurons presents new aspects and complexities of presynaptic and postsynaptic regulation which have not been previously considered. Available data indicate that complex patterns of coexistence of multiple transmitters provide a new type of chemical coding that relates the chemistry of the neurons to their projections and functions. The finding of GLU-LI and SP-LI in a proportion of small primary sensory neurons (Battaglia and Rustioni, 1987), raises a possibility of chemically-coded and functionally distinct classes of GLU-containing small primary sensory neurons. The physiological implications of such plurichemical transmission in the spinal dorsal horn need to be elucidated.

The present study attempted to estimate the contribution of different classes of activated primary sensory neurons to the release of nine

endogenous amino acids (glutamate, aspartate, glutamine, asparagine, glycine, γ-aminobutyric acid, serine, threonine and alanine) in the spinal dorsal horn, the issue not addressed previously. Specifically, the experiments utilized selective electrical stimulation of the low-threshold (Aβ) and of both low- and high-threshold (A + C) primary afferent fibers. In addition, supporting evidence for the involvement of the small-diameter primary afferents was sought by using chemical stimulation of this class of primary afferents. In the latter experiments the dorsal root ganglia were perfused with capsaicin (8-methyl-N-vanillyl-6-noneamide) or resiniferatoxin, the agents known to selectively activate a subpopulation of small primary sensory neurons. Furthermore, the possibility of the modulation of the basal and the dorsal root stimulation-evoked release of the nine endogenous amino acids by tachykinins, calcitonin gene-related peptide, opioid peptides, and also by the activation of $GABA_B$ receptors, has been investigated.

We have used the <u>in vitro</u> superfused horizontal rat spinal cord slice - dorsal root ganglion preparation and high performance liquid chromatography for fluorimetric detection of amino acids in the spinal slice superfusate. The major advantages of the slice preparation are: 1. The slices are quickly prepared suffering less damage than, for instance, synaptosomes; 2. They provide a relatively well preserved primary afferent-dorsal horn neuronal circuitry; 3. The slice preparation allows for a fast application and removal of known concentrations of chemicals and for the alterations in the ionic microenvironment. However, there are also limitations to the slice preparation studies: 1. The complexity of the anatomical structure and the existence of several types of neuronal and

glial cells may complicate the interpretation of the results, and 2. The slices have an extracellular space and diffusion barriers and even in the superfusion experiments the re-uptake of neurotransmitters cannot be completely prevented.

In the present study we have provided evidence for the release of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro in response to the electrical activation of the low-threshold primary afferent fibers and also upon the electrical or chemical activation of the high-threshold primary afferents. In addition, our results indicate that tachykinins and CGRP may enhance the basal and the evoked release of endogenous glutamate and aspartate , whereas opioid peptides acting at μ-receptors, and the activation of $GABA_B$ receptors, suppress the evoked release of the two amino acids in the spinal dorsal horn. The interaction of the peptides and the excitatory amino acids at presynaptic sites may serve the purpose of increasing (tachykinins, CGRP) or decreasing (opioid peptides acting at μ-receptors) the capacity for sensory information transfer at the first incoming synapse into the CNS.

SECTION I. TACHYKININS AND CALCITONIN GENE-RELATED PEPTIDE ENHANCE RELEASE OF ENDOGENOUS GLUTAMATE AND ASPARTATE FROM THE RAT SPINAL DORSAL HORN SLICE[1]

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SUMMARY

The effects of dorsal root stimulation and of substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) on the basal release of nine endogenous amino acids have been investigated using the rat spinal cord slice-dorsal root ganglion preparation. The perfusate was analyzed for aspartate, glutamate, asparagine, glutamine, glycine, gamma-aminobutyric acid, serine, threonine and alanine using high performance liquid chromatography with fluorimetric detection. High intensity repetitive electrical stimulation of a lumbar dorsal root produced a Ca^{2+}-dependent increase in the basal release of aspartate, glutamate, glycine, serine and threonine. Low concentrations of SP ($2x10^{-7}M$) caused a selective increase in the rate of basal release of glutamate, whereas with higher concentrations ($1-5x10^{-6}M$), in addition to glutamate, an increase in the release of aspartate was observed. NKA ($5x10^{-7}$ to $10^{-6}M$), a related tachykinin that is co-expressed with SP in primary sensory neurons, enhanced the basal release of glutamate, aspartate and glycine. The enhancement of the basal release of glutamate by SP persisted in the absence of external Ca^{2+}, but the effect was blocked by (D-Arg^{1}, D-Pro^{2}, D-$Trp^{7,9}$, Leu^{11})-SP, a SP analogue claimed to be an antagonist of synthetic SP. CGRP ($10^{-7}M$) caused a significant, Ca^{2+}-independent increase of the basal release of glutamate and aspartate and a decrease of asparagine. SP and CGRP potentiated the electrically-evoked release of glutamate and aspartate. Neonatal capsaicin treatment did not markedly alter the basal efflux of nine endogenous amino acids from the spinal slices, but it prevented the dorsal root stimulation-evoked release of aspartate,

glutamate, glycine and threonine. In addition, the capsaicin treatment prevented the SP-induced increase in the release of glutamate, whereas the effect of CGRP was not significantly modified. These results indicate that tachykinins (SP and NKA) and CGRP are capable of modulating the basal and electrically-evoked release of endogenous glutamate and aspartate and these actions may provide an important mechanism by which the peptides contribute to the regulation of the primary afferent synaptic transmission. The enhancement of the basal and the dorsal root stimulation-evoked release of glutamate and aspartate by tachykinins and CGRP may have important physiological implications for strengthening the synaptic connections in the spinal dorsal horn.

INTRODUCTION

Immunohistochemical techniques have demonstrated that neurons contain multiple chemical substances that may act as neurotransmitters or neuromodulators. However, the physiological significance of this phenomenon, especially for information transfer in the nervous system, is not well understood.

Synaptic transmitters released during activation of primary sensory neurons in the dorsal horn of the rat spinal cord may elicit both fast and slow excitatory responses in a single neuron (Murase and Randic, 1983; Urban and Randic, 1984). Dicarboxylic amino acids, glutamate and aspartate, appear to be the major candidates for the fast excitatory neurotransmitters in the mammalian central nervous system (Watkins and Evans, 1981; Mayer and Westbrook, 1987), including the spinal dorsal horn (Galindo et al., 1967; Puil, 1983; Salt and Hill, 1983; Kangrga et al., 1988; Gerber and Randic, 1989a). Tachykinins, substance P (SP) and neurokinin A (NKA), and calcitonin gene-related peptide (CGRP), appear to be functionally involved in the slow primary afferent synaptic transmission (Urban and Randic, 1984; Randic et al., 1986; Yaksh, 1986; Ryu et al., 1988a, Gerber and Randic, 1989b).

Glutamatergic excitatory transmission at the terminals of primary sensory neurons has been indicated by several lines of evidence. The higher concentration of glutamate in the dorsal roots as compared to the ventral roots (Duggan and Johnston, 1970) and Ca^{2+}-dependent release of glutamate from electrically stimulated primary afferent fibers has been demonstrated (Roberts, 1974; Takeuchi et al., 1983; Kawagoe et al., 1986).

Immunocytochemical studies have shown that about 70% of both large and small DRG neurons are labeled for glutamate (Wanaka et al., 1987) and glutamate immunoreactivity has been detected within myelinated and unmyelinated primary afferent terminals in the superficial dorsal horn (Weinberg et al., 1987; Miller et al., 1988; Westlund et al., 1989a). ^{3}H-glutamate and ^{3}H-aspartate, are selectively taken up by a certain proportion of small and large dorsal root ganglia (DRG) neurons (Duce and Keen, 1983; Barbaresi et al., 1985). L-glutamate binding sites are found in high densities in the superficial laminae of the rat spinal dorsal horn (Greenamyre et al., 1984; Monaghan and Cotman, 1985). Glutamate was found to excite and depolarize almost all spinal dorsal horn neurons in vivo (Curtis et al., 1960; Watkins and Evans, 1981) and a proportion of dorsal horn neurons in vitro (Zieglgansberger and Puil, 1973; Schneider and Perl, 1985).

SP and NKA are also present in a proportion of small DRG neurons and in numerous terminals in the superficial laminae of the spinal dorsal horn (Hökfelt et al., 1975; Barber et al., 1979; Kanazawa et al., 1984; Dalsgaard et al., 1985). On electrical or chemical stimulation, Ca^{2+}-dependent release of SP and NKA from activated primary afferent fibers has been demonstrated both in vitro (Otsuka and Konishi, 1976; Gamse et al., 1979; Hua et al., 1986) and in vivo (Yaksh et al., 1980; Brodin et al., 1987). SP binding sites have been demonstrated autoradiographically in the spinal dorsal horn (Mantyh et al., 1984a; Ninkovic et al., 1985) and a correlation has been observed between the distribution of [^{3}H]-SP binding sites and the ability of SP to stimulate phosphatidylinositol turnover (Mantyh et al. 1984b). SP depolarizes dorsal horn neurons (Henry et al.,

1975; Randic and Miletic, 1977).

The presence of CGRP-like immunoreactivity (CGRP-LI) in rat DRG neurons and the spinal dorsal horn has been demonstrated (Gibson et al., 1984). Both CGRP binding sites (Henke et al., 1985) and CGRP-LI (Gibson et al., 1984) exist in high concentrations in the superficial layers of the spinal dorsal horn. Release of CGRP from rat primary sensory neurons in response to capsaicin (Franco-Cereceda et al., 1987; Diez Guerra et al., 1988) and electrical stimulation (Saria et al., 1986) has been shown.

There is evidence that SP, NKA and CGRP may modulate primary afferent neurotransmission by acting both at presynaptic (Kawagoe et al., 1986; Ryu et al., 1988a,b; Jinnai et al., 1989; Kangrga et al., 1989a,b) and postsynaptic sites (Murase and Randic, 1984; Murase et al., 1989a,b). Although the coexistence of SP and glutamate in some small primary afferent neurons (Battaglia et al., 1987) and their terminals in the superficial dorsal horn (DeBiasi and Rustioni, 1988) has been reported, and the coexistence of SP, NKA and CGRP in a proportion of capsaicin-sensitive DRG neurons (Nagy et al., 1981; Gibson et al., 1984; Franco-Cereceda et al., 1987; Diez Guerra et al., 1988), our understanding of physiological implications of this phenomenon is still unclear. An important, but as yet not systematically investigated site at which co-existent peptides could modulate excitatory amino acid function, and in this way contribute to primary afferent synaptic transmission, is found presynaptically in the control of basal and depolarization-evoked release of glutamate and aspartate. In an attempt to determine whether SP, NKA and CGRP modulate the release pattern of excitatory amino acids, we have investigated the efflux of nine endogenous amino acids, including glutamate and aspartate,

from the superfused spinal cord slices of the rat, in response to electrical stimulation of dorsal roots, administration of neuropeptides (SP, NKA and CGRP) and chronic treatment of rats with capsaicin.

Since the efflux of endogenous amino acids in the dorsal horn may derive from different neuronal sources, including some descending pathways (Stone 1979; Rustioni and Cuenod, 1982; Potashner and Tran, 1984; Potashner and Dymzyk, 1986) and intrinsic dorsal horn interneurons (Davidoff et al., 1967; Rustioni and Cuenod, 1982), we have tried to determine the contribution of the primary afferent fibers in the dorsal root to electrically-evoked and peptide-evoked release of amino acids by using neonatal capsaicin treatment. Sensory neurotoxin capsaicin (8-methyl-N-vanillyl-6-noneamide) (Jancso, 1968), when given neonatally causes the death of small DRG neurons, many of which are known to contain SP, NKA, and CGRP (Jancso et al., 1977; Nagy et al., 1981; Franco-Cereceda et al., 1987; Diez Guerra et al., 1988). Preliminary reports of some aspects of this work have been published (Kangrga et al., 1989, 1990).

METHODS

Horizontal slices were obtained from Sprague-Dawley rats of both sexes (23-45 days old) by using a technique that has been described in detail elsewhere (Murase and Randic, 1983; Gerber et al., 1989). Briefly, after the animal was anesthetized with ether a segment of the lumbosacral (L5-S1) spinal cord was dissected out and sectioned with a Vibratome to yield one 300-400 μm thick horizontal slice with dorsal rootlets and dorsal root ganglia attached. In some experiments a part of sciatic nerve was left in the contact with a dorsal root ganglion. The slice was incubated for 1 hr in oxygenated (95% O_2 + 5% CO_2) control solution (in mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; pH 7.4 at 30 ± 1°C. The use of a high K^+-solution during cutting and incubation of the slices seemed to improve their viability, as assessed electrophysiologically in the same preparation. After the incubation, a slice was placed in one compartment of the 2-compartment chamber and perfused with oxygenated modified Krebs solution (containing 1.9 mM KCl, all other salts were unchanged) at 0.5 ml/min. The dorsal roots and dorsal root ganglia were placed into the second compartment and immersed under the mineral oil. Lubriseal (Thomas Scientific) was used to ensure a leakproof and also electrical isolation between the 2 compartments. A lumbar dorsal rootlet was placed on the two pairs of bipolar platinum electrodes: the distal pair was used for electrical stimulation of primary afferent fibers and the proximal pair for recording of the compound action potentials. The stimulation parameters were selected to activate both low-threshold, fast-conducting myelinated fibers (Aβ) and the high-threshold, slower-conducting

myelinated (Aδ) and unmyelinated (C) fibers (25-30V, 0.02-1.0 ms at 3-10 Hz). The compound action potentials were monitored throughout the periods of stimulation and stored on diskettes of a digital oscilloscope (Nicolet, Model 4092). Samples of perfusate (0.5 ml) were collected at regular 5-10 min intervals before, during and after stimulation of the dorsal roots or application of tachykinins and CGRP. Samples were kept frozen at -80°C until derivatization and chemical analysis. Quantification of nine endogenous amino acids contained in the spinal perfusate was achieved by reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection following pre-column derivatization with o-phthaldialdehyde (OPA) 2-mercaptoethanol reagent (Lindroth and Mopper, 1979). OPA 2-mercaptoethanol derivatives were produced by taking 25 μl of OPA reagent solution and mixing with 25 μl of amino acid mixture (standards or sample). After 1 min, 150 μl of the mixture was injected onto the chromatographic column for analysis. Hydroxylysine (30 μM) was added to each sample as an internal standard. Chromatography was performed on a 15 cm, Adsorbasphere - OPA - HR column (Alltech Associates) using a pH 5.9 sodium acetate-tetrahydrofuran/methanol gradient. Fluorescence was detected with a Kratos FS 950 fluorimeter. The amino acids measured came off the column in the following order: aspartate (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), threonine (Thr), alanine (Ala) and γ-aminobutyric acid (GABA) followed by hydroxylysine. Results reported are the averages of duplicate runs with each run lasting 31 min. Substance P (10^{-7} to $5x10^{-6}$M, Cambridge Research Biochemicals, CRB) neurokinin A ($5x10^{-7}$ to 10^{-6}M, CRB) and rat CGRP (10^{-8} to 10^{-6}M, CRB) were applied into the slice perfusate for 5 min. The calcium

dependence of the dorsal root stimulation-evoked and peptide-evoked amino acid efflux was investigated by the removal of calcium ions from the Krebs solution. Statistical significance has been assessed relative to control conditions by use of either a paired or unpaired Student's t-test, as appropriate. Levels of significance are indicated as follows: * $p < 0.01$; ** $p < 0.005$.

Thirteen rats of both sexes taken from 2 different litters were injected subcutaneously with 50 mg/kg of capsaicin (Sigma) in vehicle (10% ethanol, 10% Tween (vol/vol) in 0.9% (wt/v) saline) 48 hr after birth. Eight control litter mates received equal volumes of vehicle alone. After a survival time of 23 to 45 days the animals were subjected to the experimental procedure described above.

For SP and CGRP immunohistochemistry lumbar spinal cords from vehicle-injected and capsaicin-treated rats were fixed with Zamboni's fixative. Serial, 50-μm-thick, transverse sections were cut through the spinal cord with a Vibratome. These sections were then processed for SP- or CGRP-like immunoreactivity with the Sternberger's peroxidase-antiperoxidase (PAP) method and commercially available SP (Incstar Corp.) or CGRP (Peninsula Labs.) antisera at a dilution of 1:7000 and 1:3000 respectively. Details of the PAP procedure have been published (Coffield et al., 1986). Following the PAP incubation, the sections were incubated for 7 to 10 min in 0.05% 3,3'-diaminobenzidine hydrochloride (DAB, Sigma) in 0.1M PBS and 0.01 hydrogen peroxide (H_2O_2) to obtain the specific immunoreactive label distinguished by a reddish-brown chromogen. The DAB-reacted sections were mounted on gelatin coated slides, dehydrated in ethanol, cleared in xylene and coverslipped with Permount for light microscopic analysis. As a

control for ligand specificity, spinal cord sections were processed without the addition of primary antisera. In this case no immunoreactivity was seen.

RESULTS

Basal release of endogenous amino acids from spinal dorsal horn slices

The efflux of nine endogenous amino acids-Asp, Glu, Asn, Gln, Gly, GABA, Ser, Thr and Ala-from the spinal cord slices into the Krebs perfusion-medium occurred, and the mean basal amino acid concentrations in the spinal cord slice perfusate are presented in Table 1. The nine amino acids studied are the most prominent ones to be detected in the spinal slice perfusate. The amount of Gln released was the highest, at least 5-6 times that of the next three relatively abundant amino acids, Ala, Gly and Ser. Glu, GABA and Thr were present in moderate amounts, whereas lower levels of Asp and Asn were found (Table 1).

No significant differences in the basal release of nine-endogenous amino acids were detected when the spinal slices were perfused with either nominally zero Ca^{2+}-containing medium (n=3; Table 1) or in those obtained from rats neonatally-treated with capsaicin (n=13, Table 1).

Effects of dorsal root stimulation

Electrical stimulation of a lumbar dorsal root produced a significant increase in the rate of the basal efflux of several endogenous amino acids (Fig. 1, Table 2). Thus, in six different slices, high intensity repetitive stimulation (25V pulses of 20-100μs duration applied at 3-5 Hz for 5 min) of a lumbar dorsal root produced a significant increase in the basal release of aspartate (to 160.7 ± 9.6%), glutamate (to 143.7 ± 9.5%), glycine (to 130.0 ± 9.6%), serine (to 145.3 ± 14.4%) and threonine (to 141.8 ± 8.5%), whereas the levels of asparagine, glutamine, gamma- Table 1.

Amino acid concentrations[a] in the perfusate of the horizontal spinal cord slices. The number of observations shown in parentheses (μM/ 5 min collection period)

Amino Acid	Control (n=12)	Zero-Ca^{2+} (n=3)	Capsaicin(n=13)
Aspartate	0.25 ± 0.04	0.21 ± 0.06	0.16 ± 0.03
Glutamate	0.51 ± 0.09	0.59 ± 0.15	0.66 ± 0.09
Asparagine	0.10 ± 0.02	0.21 ± 0.04	0.16 ± 0.03
Glutamine	7.20 ± 1.40	5.30 ± 2.80	6.94 ± 0.99
Glycine	1.21 ± 0.20	1.30 ± 0.17	1.20 ± 0.28
GABA	0.68 ± 0.15	0.61 ± 0.30	0.92 ± 0.15
Serine	1.10 ± 0.15	1.09 ± 0.21	1.39 ± 0.19
Threonine	0.60 ± 0.07	0.56 ± 0.15	0.89 ± 0.23
Alanine	1.29 ± 0.27	1.66 ± 0.60	1.82 ± 0.32

[a]Results are presented as mean ± S.E.M. of the basal efflux for n experiments conducted in duplicate.

aminobutyric acid and alanine were elevated only to smaller degrees (Fig. 1A). The stimulation-evoked increase in the basal release of amino acids was rarely maintained for more than one collection period of 5 min. The higher increase in the rate of stimulation-evoked release of aspartate than of glutamate, and the increase in the efflux of endogenous GABA, Gly, Ser, Thr, Ala and Asn in the spinal slice perfusate are reported here for the first time.

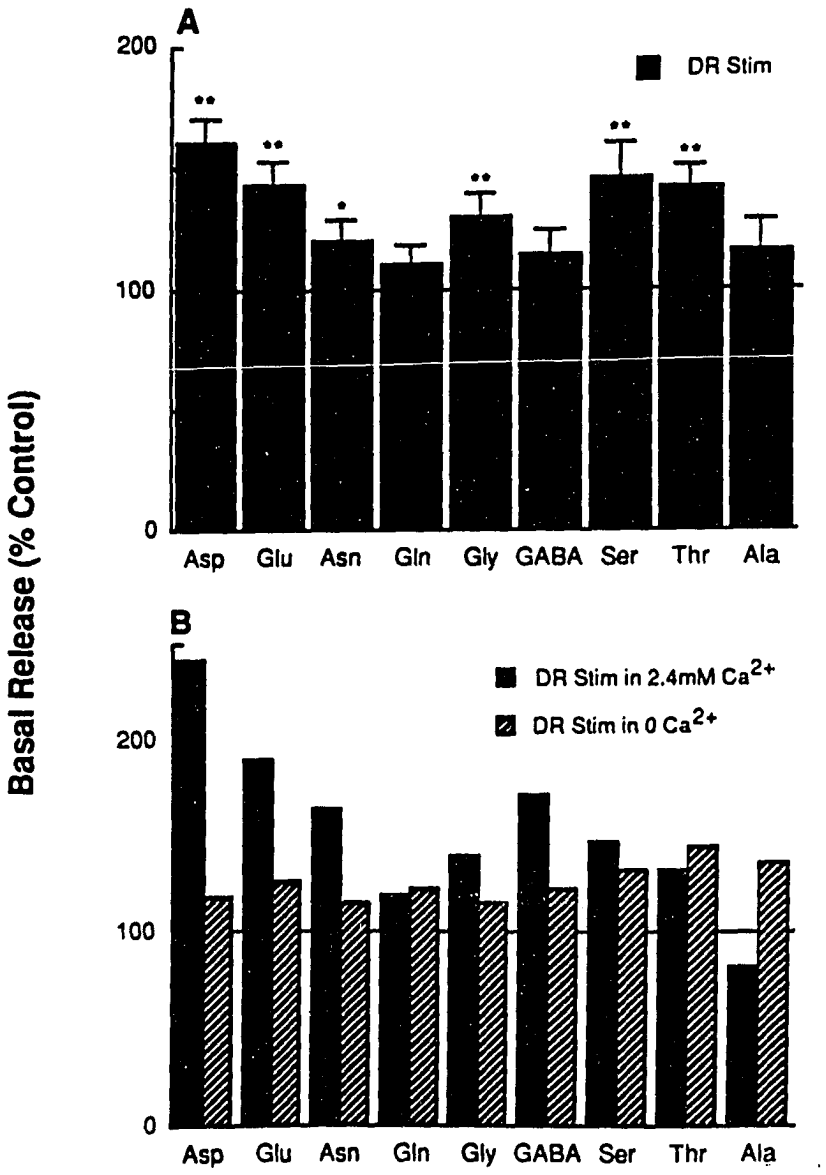
In order to evaluate whether the increased release of endogenous amino acids following activation of primary afferent fibers was likely to be of neuronal origin experiments were carried out with altered levels of Ca^{2+} ions in the perfusing medium. The dependence of the dorsal root electrically-evoked increase in the basal release of endogenous amino acids upon the presence of Ca^{2+} ions in the external medium was investigated by

perfusing the slices (n=3) with nominally zero-Ca^{2+} medium. The results obtained in one of those experiments are illustrated in Fig. 1B. When Ca^{2+} ions were omitted from the perfusing medium the synaptic transmission was blocked and the stimulation-evoked increase in the basal release of glutamate and aspartate was absent (Fig. 1B, Table 2).

SP and NKA modulate the basal and electrically-evoked release of glutamate and aspartate

Perfusion of spinal cord slices with lower concentrations of SP ($2x10^{-7}$M for 5 min) caused a selective and significant increase (to 254.3 ± 62.0%; n=8) in the rate of basal release of glutamate (Fig. 2A). With higher concentrations of SP (10^{-6} to $5x10^{-6}$M; n=3), in addition to glutamate, a significant and dose-dependent increase in the basal release of aspartate was observed (Fig. 2B). Thus, for instance in a single experiment, the increase in the basal efflux of aspartate elicited by 10^{-6} and $5x10^{-6}$M SP amounted to 150 and 270% of their control levels, respectively. With higher concentrations of SP the increased efflux of glutamate and aspartate was frequently maintained for two to three consecutive 5 min collection periods. The averaged values for the SP-caused increase in the release of glutamate and aspartate from spinal slices are summarized in Table 2. The levels of the seven other endogenous amino acids, determined in the spinal slice perfusate were not modified in a consistent manner by tachykinins (Fig. 2B).

Fig. 1. Effects of depolarizing stimuli and Ca^{2+} on the rate of basal efflux of 9 endogenous amino acids. Histogram of the release of 9 endogenous amino acids in response to electrical stimulation of a lumbar dorsal rootlet (25 V, 0.02-0.1 msec, 5 Hz, 5 min) relative to their basal efflux determined from first 2 collection periods prior to the first period of stimulation. The results are expressed as the mean percentage of the basal efflux ± SEM for 6 experiments conducted in duplicate. A, Electrical stimulation of a lumbar dorsal rootlet produced a significant increase in the concentrations of Asp (160.7 ± 9.6%), Glu (143.7 ± 9.5%), Gly (130.0 ± 9.6%), Ser (145.3 ± 14.4%), and Thr (141.8 ± 8.5%), whereas the levels of Asn, Gln, GABA, and Ala were elevated to a smaller degree. B, The stimulation-evoked increase in the basal efflux of Glu, Asp, Asn, Gly, and Ser (solid columns, n = 2) was reduced or blocked in a zero Ca^{2+} solution (hatched columns, n = 3). Statistically significant results in this and other figures are indicated: $^{*}p<0.01$; $^{**}p<0.005$. 23- to 33-day-old rats.


A
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Glu
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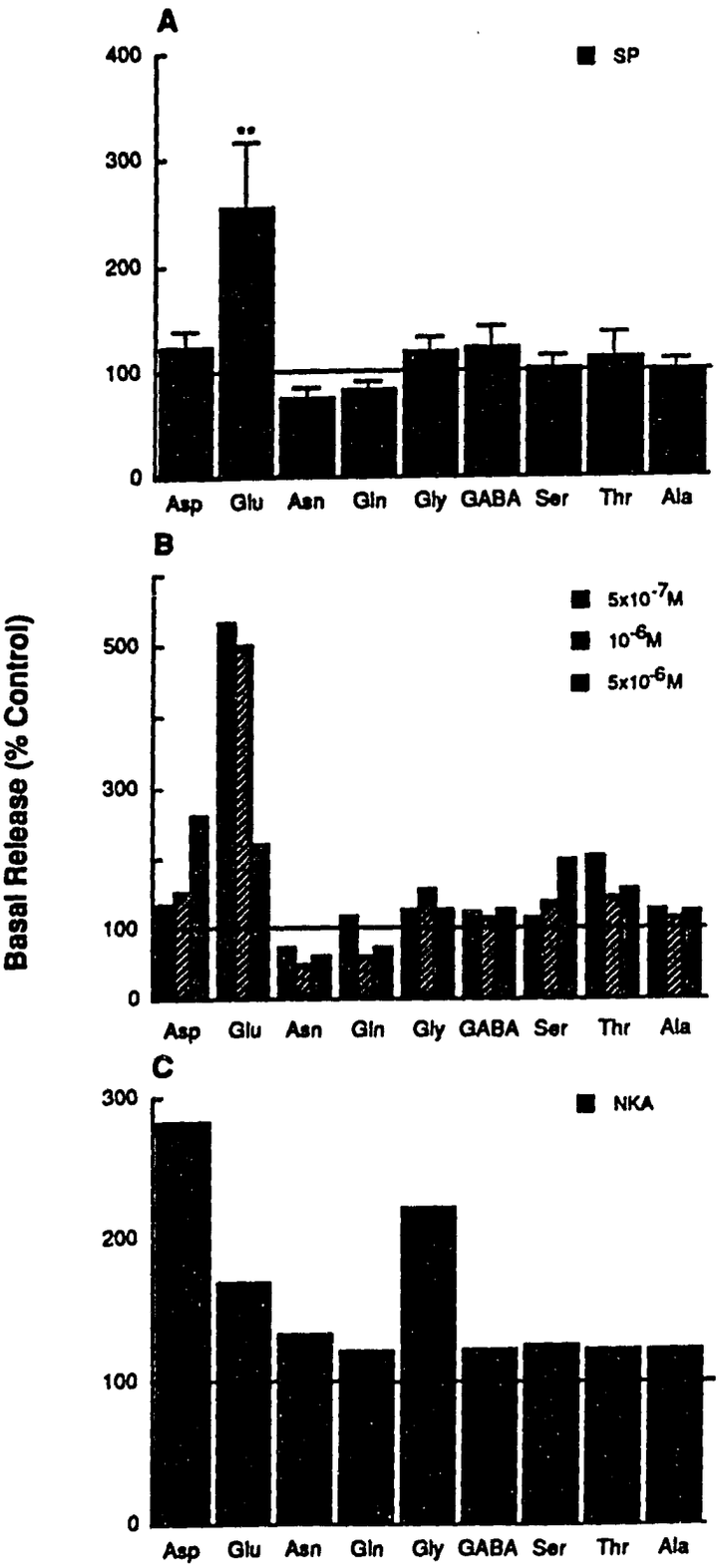
DR Stim in 2.4mM Ca2+
DR Stim in 0 Ca2+
Asp
Glu
Asn
Gln
Gly
GABA
Ser
Thr
Ala
Basal Release (% Control)

Fig. 2. SP and NKA enhance the basal efflux of endogenous glutamate and aspartate into the spinal perfusate. A, Perfusion of spinal cord slices with SP ($2\text{-}5\times10^{-7}$M for 5 min) produced a significant increase in the concentration of glutamate (254.3 ± 62.0%; n=8; $p<0.005$) in the perfusate, whereas the levels of other eight amino acids were not significantly altered. B, Histogram of the efflux of 9 endogenous amino acids, obtained in a single experiment in response to addition of three different concentrations of SP to the perfusing medium: solid columns, 5×10^{-7}M; hatched columns, 10^{-6}M; dotted columns, 5×10^{-6}M. Bath application of SP in concentration of 5×10^{-7}M markedly increased (533.6%) the basal efflux of glutamate, only. Higher concentrations of SP (10^{-6} and 5×10^{-6}M), in addition to glutamate, caused a significant and dose-dependent increase in the basal efflux of aspartate. A tendency for a decrease in the efflux of asparagine and glutamine was also observed. C, Addition of NKA (10^{-6}M, 5 min) to the perfusing medium produced a marked increase in the basal efflux of aspartate (282.7%), glycine (222.2%) and glutamate (170.3%), whereas the efflux of remaining six amino acids was not significantly changed. A, 24- to 33-day-old rats; B, 24-day-old rat. C, 28-day-old rat.


A
400
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SP
**
Asp Glu Asn Gln Gly GABA Ser Thr Ala
B
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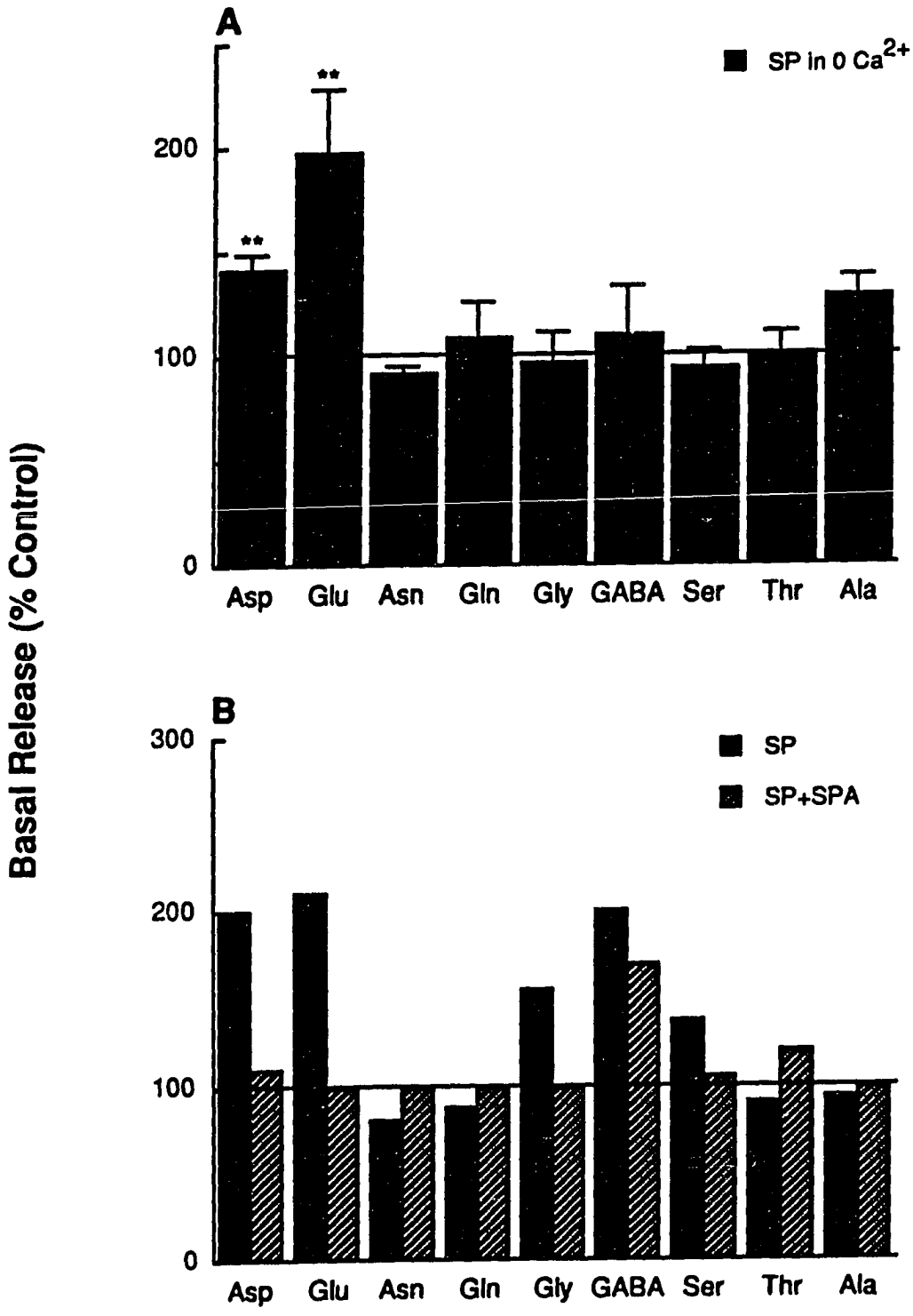
10-6M
5x10-6M
Basal Release (% Control)
Asp Glu Asn Gln Gly GABA Ser Thr Ala
C
300
200
100
0
NKA
Asp Glu Asn Gln Gly GABA Ser Thr Ala

The dependence of the SP-evoked increase in the release of glutamate and aspartate upon external Ca^{2+} ions was investigated by omission of this ion from the perfusing medium. When the slices (n=3) were perfused with a nominally Ca^{2+}-free medium, SP ($5x10^{-7}M$) still elicited a significant increase in the concentration of glutamate (to 198.2 ± 29.7%) in all 3 slices examined, whereas the aspartate increase (to 141.9 ± 7.0%) was present in 2 out of 3 slices (Fig. 3A, Table 2). The persistence of the SP effect did not appear to be a consequence of an inadequate removal of extracellular Ca^{2+} since the dorsal root-stimulation-evoked release of glutamate and aspartate was virtually abolished by the removal of external Ca^{2+} (n=3).

The effect of SP ($5x10^{-7}M$) was effectively blocked by a SP analogue, (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})-SP ($2x10^{-5}M$), a claimed antagonist of synthetic SP (Fig. 3B). This finding suggests that the effect of SP on the basal efflux of glutamate is a true tachykinin receptor-mediated response.

The possibility that NKA, a SP related tachykinin that is co-expressed with SP in primary sensory neurons (Dalsgaard et al., 1985), may regulate primary afferent transmission by modulating the basal release of glutamate and aspartate was investigated in five spinal slices. As shown in a single experiment illustrated in Fig. 2C, NKA ($10^{-6}M$) produced a marked increase in the rate of basal efflux of aspartate (to 282.7%), glycine (to 222.2%) and glutamate (to 170.3%), whereas the efflux of remaining six amino acids was not significantly altered. The summary of the effects of NKA ($5x10^{-7}$ to $10^{-6}M$) on the basal efflux of glutamate and aspartate is presented in Table 2.

Fig. 3. Enhancement of the basal efflux of aspartate and glutamate by SP is not Ca^{2+}-dependent, but is blocked by a claimed SP antagonist. A, Perfusion of the slices with nominally zero Ca^{2+} solution-containing SP ($5x10^{-7}M$, 5 min) produced a significant increase in the concentration of glutamate (198.2 ± 29.7%; n=3) and aspartate (141.9 ± 7.0%; n=3) in the spinal perfusate. The concentrations of remaining seven amino acids were not significantly different from the control. B, Perfusion of a spinal cord slice with SP ($5x10^{-7}M$, 5 min) in the presence of SP antagonist, (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})-SP ($2x10^{-6}M$, 10 min), failed to elicit an increase in the basal efflux of either aspartate or glutamate in the spinal perfusate (hatched columns). The data obtained during the perfusion with SP ($5x10^{-7}M$) alone are presented with the solid columns (the same slice). A, 26-33-day-old rats. B, 25-day-old rat.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300
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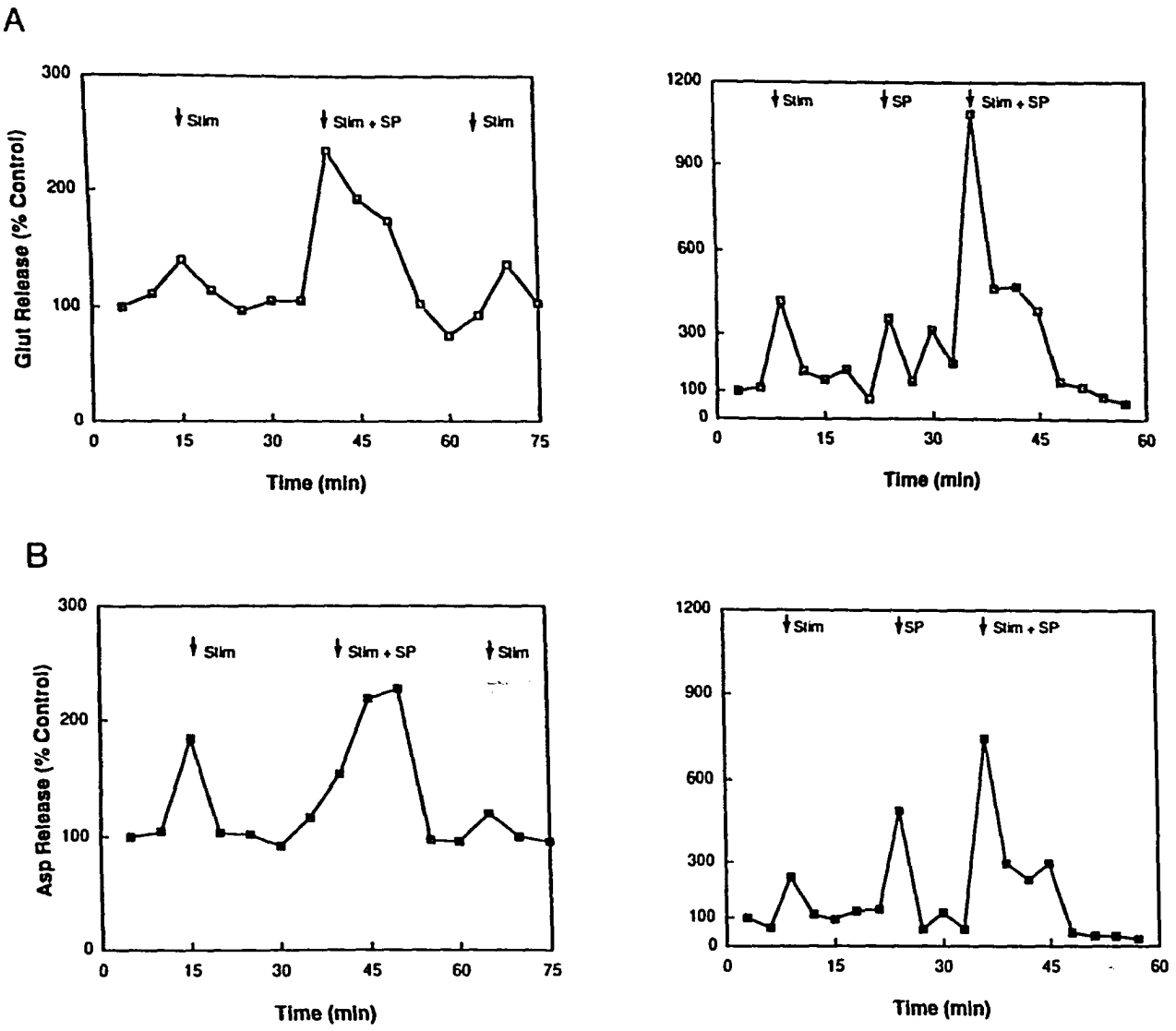
Asp
Glu
Asn
Gln
Gly
GABA
Ser
Thr
Ala
Basal Release (% Control)

Fig. 4. SP potentiates the dorsal root stimulation-evoked efflux of endogenous Glu and Asp. Interaction between the dorsal root stimulation- and the SP-evoked release of Glu and Asp was studied by using 2 different protocols (A and B). Left panels, Three periods of electrical stimulation (25V, 0.02 ms, 5 Hz for 5 min; onset of stimulation marked by arrowheads) were applied. During the first and third periods, the slice was perfused with a control Krebs solution, while during the second period, SP ($5x10^{-7}M$) was added to the perfusate. SP increased the electrically-evoked efflux of Glu from 141 to 236% (A) and that of Asp from 185 to 228% (B) of the basal value. Characteristically, the peptide prolonged the duration of the electrically-evoked efflux from 5 to 15 min. 29-day-old rat. Right panels, Electrical stimulation of the dorsal rootlet (30 V, 1 ms, 0.5 Hz for 3 min) produced about 4-fold increase in the basal release of Glu (A) and about 2.5-fold increase in Asp (B). Addition of SP ($5 \times 10^{-7}M$ from 3 min) to the perfusing medium produced a similar increase in Glu (359%) and somewhat higher increase of Asp (485%). However, the electrical stimulation of the primary afferents in the presence of SP resulted in a higher increase in the basal efflux of Glu (to 1091% of the basal release) and Asp (to 743%) during the first collection period if compared to the effects of either treatment alone. The SP-enhanced efflux of Glu and Asp lasted about 12 min. 27-day-old rat.


A
Glut Release (% Control)
Stim
Stim + SP
Stim
Time (min)
Stim
SP
Stim + SP
Time (min)
B
Asp Release (% Control)
Stim
Stim + SP
Stim
Time (min)
Stim
SP
Stim + SP
Time (min)

Moreover, we sought to determine whether, besides modulating the basal release, SP also modulates the dorsal root stimulation-evoked release of glutamate and aspartate from the spinal slices. Thus in four slices, the release during a stimulation period was compared with that measured during exposure of a spinal slice to SP (10^{-7} to 10^{-6}M) and electrical stimulation of a lumbar dorsal root. We found that in the presence of SP the electrically stimulated release of glutamate (Fig. 4A) and aspartate (Fig. 4B), was potentiated. Characteristically, unlike the response to electrical stimulation that was usually limited to the first collection period, the SP-evoked response was prolonged, lasting 10-15 min after the first exposure to the peptide (Fig. 4).

rCGRP modulates the basal and electrically-evoked release of glutamate and aspartate

We found that rat calcitonin gene-related peptide (rCGRP, 10^{-7}M) caused a significant increase of the basal efflux of glutamate (to 170.5 ± 30.6; n=4) and aspartate (to 159.5 ± 20.1; n=4), and a smaller increase in threonine (Fig. 5A). In addition, there was a significant decrease in the basal release of asparagine, whereas the levels of other five endogenous amino acids were increased only to a small degree. In contrast to the immediate elevation of glutamate and aspartate in response to electrical stimulation of primary afferents, the CGRP-caused increase of the basal release was delayed, frequently reaching a maximum 10 min after the first exposure of the slice to the peptide. At the second exposure to the same concentration of CGRP, the CGRP-effect was reduced, indicating the occurrence of desenzitization.

When calcium was removed from the perfusing medium, the CGRP-evoked enhancement of the basal release of Glu occurred in 2 out of 4 slices examined, whereas the increase of Asp was present, although reduced in magnitude, in 3 out of 4 slices. The average of 3 experiments where the effects of CGRP were tested in nominally zero Ca^{2+} medium is presented in Figure 5B.

We have recently shown that CGRP enhances calcium currents of rat dorsal root ganglion neurons and spinal excitatory synaptic transmission (Ryu et al., 1988a). As Ca^{2+} influx is intimately related to neurotransmitter release, a similar action of CGRP on voltage-sensitive Ca^{2+} channels at central terminals of primary sensory neurons, as shown for the somatic membrane of DRG neurons (Ryu et al., 1988a), could increase neurotransmitter release and facilitate excitatory synaptic transmission. In support of this hypothesis we found that in the presence of CGRP (10^{-7}M) the electrically-elicited release of glutamate (Fig. 6A) and aspartate (Fig. 6B) was increased. The CGRP-evoked response was prolonged, lasting about 30 min, and was oscillatory in character.

Fig. 5. A, CGRP enhanced the basal efflux of aspartate and glutamate into the perfusate of spinal slices. Perfusion of spinal cord slices with CGRP (10^{-7}M) produced a significant increase in the concentration of glutamate (to 170.5 ± 30.6%) and aspartate (to 159.5 ± 20.1%) and a decrease in asparagine (to 56.2 ± 29.2%) whereas the levels of the remaining six amino acids were not markedly changed. The results are expressed as mean percent of the basal efflux ± SEM. for 4 experiments. B, In 3 experiments the CGRP-evoked increase in the basal efflux of aspartate and glutamate (solid columns: before; dotted columns: after returning to 2mM Ca^{2+}-medium) was not significantly altered when the slices were perfused with a nominally zero Ca^{2+} medium (hatched columns). 27-31-day-old rats.

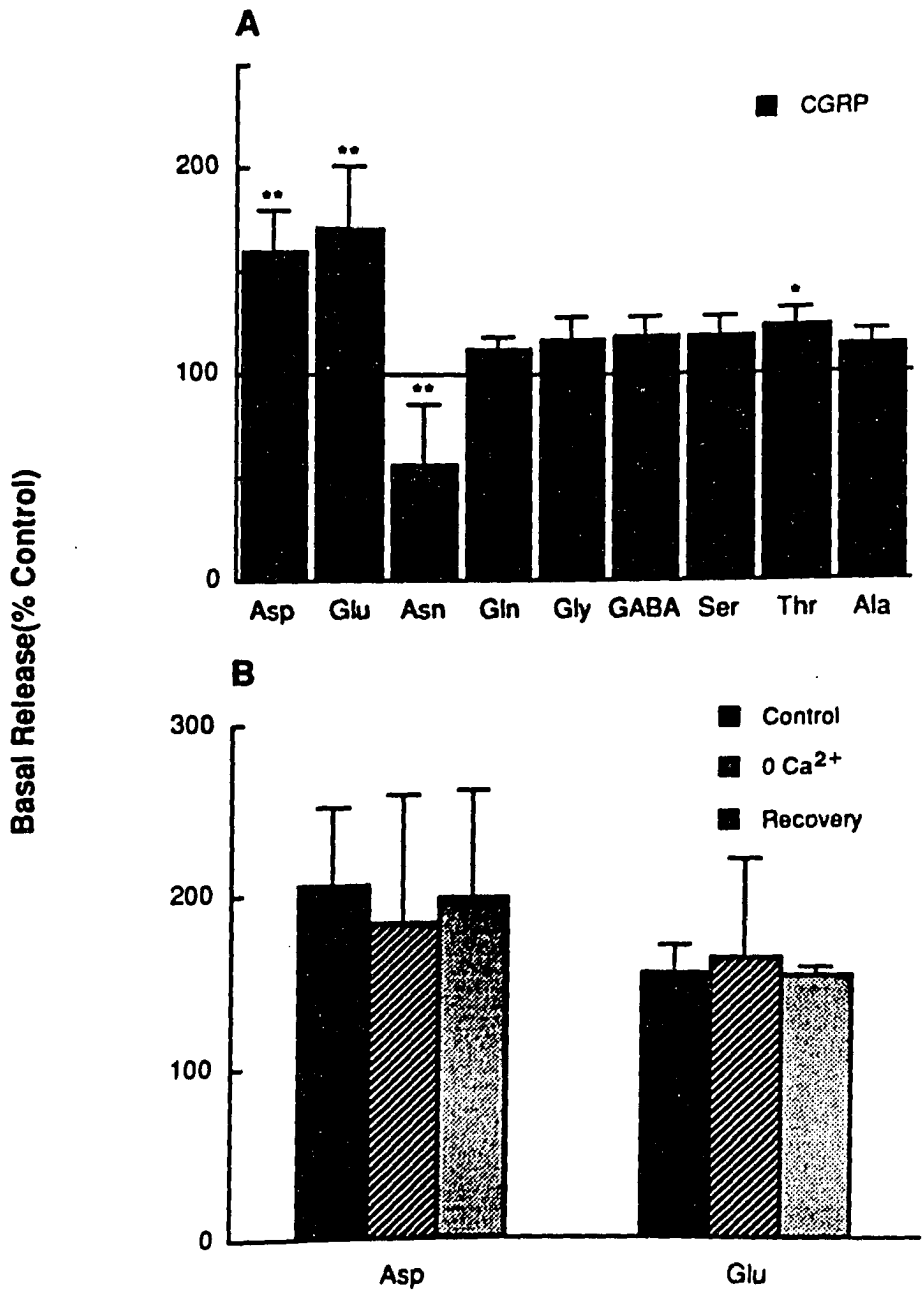
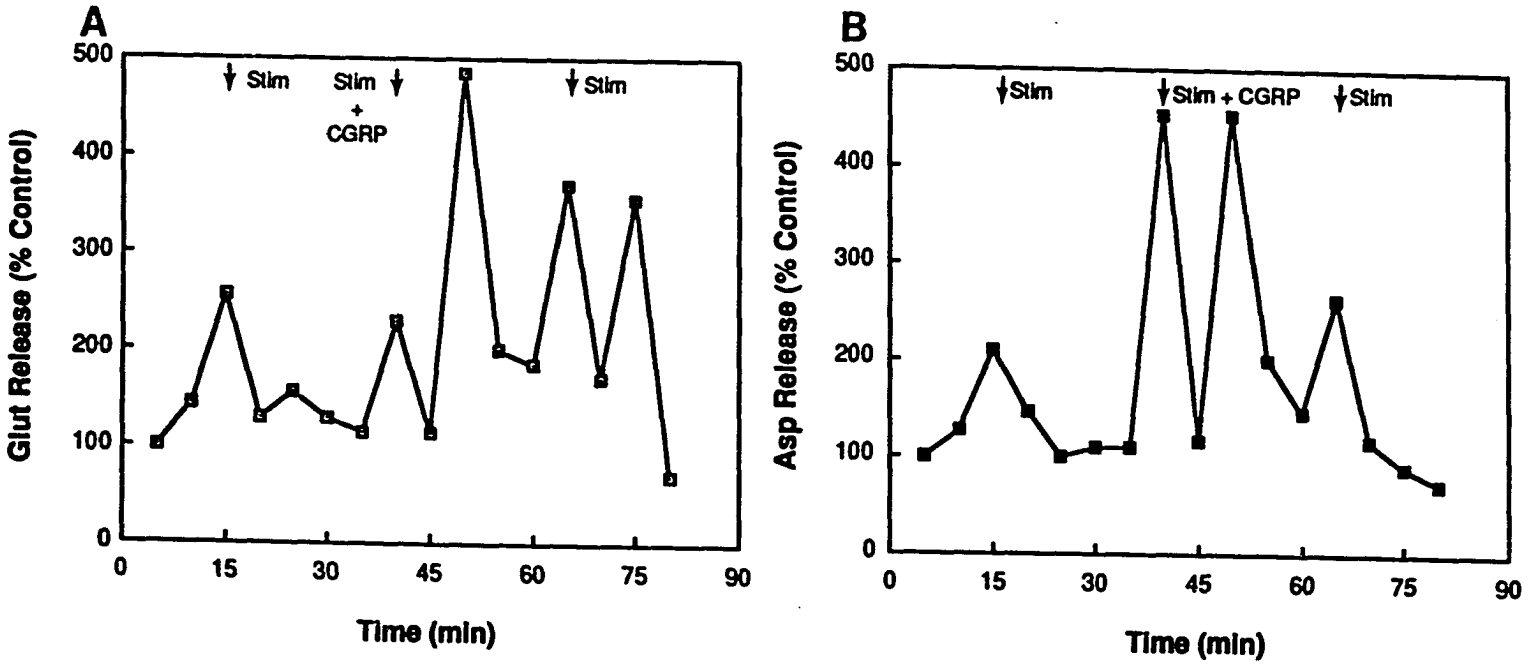

A
CGRP
200
100
0
**
**
**
*
Asp
Glu
Asn
Gln
Gly
GABA
Ser
Thr
Ala
B
Control
0 Ca2+
Recovery
300
200
100
0
Asp
Glu
Basal Release(% Control)

Fig. 6. CGRP enhances the dorsal root stimulation-evoked efflux of endogenous glutamate and aspartate. A, Bath addition of CGRP (10^{-7}M for 5 min) increased the electrically (25 V, 0.1 ms, 5 Hz for 5 min) -evoked efflux of glutamate from about 257 to 486% relative to control. Characteristically, the increase in the release oscillated and the oscillations lasted about 30 min. B, In the same slice, CGRP also increased the efflux of aspartate from 209 to 456% of control. Similar as with glutamate, the release was oscillatory in character and of prolonged duration. 29-day-old rat.


A
Glut Release (% Control)
500
400
300
200
100
0
Stim
Stim + CGRP
Stim
0
15
30
45
60
75
90
Time (min)
B
Asp Release (% Control)
500
400
300
200
100
0
Stim
Stim + CGRP
Stim
0
15
30
45
60
75
90
Time (min)

Effects of neonatal capsaicin treatment on the dorsal root- and peptide-evoked release of endogenous amino acids

The question of whether the enhanced release of glutamate and aspartate following electrical stimulation of dorsal roots and administration of SP, NKA and CGRP, reflects direct release from activated primary afferent fibers (axons and/or presynaptic endings) in the dorsal horn or whether it could reflect activation of secondary or tertiary cells in the primary afferent-dorsal horn neuronal pathways was investigated in slices obtained from capsaicin-treated rats. If capsaicin were to prevent the enhancement of the stimulation-evoked and peptide-evoked basal release of glutamate and aspartate, it is likely that a significant proportion of released amino acids would arise from sensory endings sensitive to capsaicin.

Although in the present study we found that neonatal capsaicin treatment did not markedly alter the basal efflux of 9 endogenous amino acids from the spinal slices, it prevented the dorsal root stimulation-evoked release of endogenous amino acids (Fig. 7). The stimulation-enhanced efflux of aspartate, glutamate, asparagine, glycine and threonine seen in vehicle-injected control animals (n=8) was significantly reduced in the slices obtained from the capsaicin-treated rats (n=13; Fig. 7, Table 2).

The effects of neonatal capsaicin treatment on the SP- and CGRP-enhanced basal efflux of glutamate and aspartate from the spinal slices of rats sacrificed at 3-6 weeks of age are shown in Figs. 8 and 9 and Table 2. The results obtained show that neonatal capsaicin treatment prevented the SP-induced increase in the concentration of glutamate in the spinal

perfusate (Fig. 8). This result suggests that intact unmyelinated (C) and perhaps small myelinated (Aδ) primary afferent fibers may be an important source of released glutamate following dorsal root stimulation or SP administration. In contrast to the SP-effect, the CGRP-induced increase in the basal levels of glutamate and aspartate was not prevented in the slices obtained from the capsaicin-treated rats (Fig. 9). It is of interest, however, that CGRP-caused reduction of the basal release of asparagine seen in the vehicle-treated animals appears to be abolished by capsaicin treatment. The blockade of the SP effect on glutamate release seen in the capsaicin-treated rats suggests that the peptide may regulate the release of the excitatory neurotransmitters by acting at presynaptic sites. On the other side, the persistence of the effect of CGRP in the capsaicin-treated rats may reflect the prevalent effect of CGRP on the release of glutamate and aspartate from the interneurons or descending afferents to the dorsal horn. However, since there is significant CGRP staining left after capsaicin treatment (Fig. 9A,B, see also Diez Guerra et al., 1988) this finding may imply that subcutaneous treatment with capsaicin is not effective to eliminate all the CGRP-like immunoreactive primary afferent input.

Fig. 7. Neonatally applied capsaicin reduced the dorsal root stimulation-evoked release of aspartate and glutamate. The dorsal root stimulation-evoked increase in the release of endogenous aspartate, glutamate, glycine and threonine from the horizontal spinal cord slices of intact rats (solid columns) was absent in the slices obtained from the rats treated neonatally with capsaicin (hatched columns). Rats were allowed to survive 3-6 weeks after injection of Tween 80/ethanol/saline (1:1:8) vehicle, or vehicle containing 50 mg/kg capsaicin. The results for control (n=6) and capsaicin (n=7) groups are expressed as mean percentages ± SEM of the respective basal values. Statistical difference between the release in intact and capsaicin-treated rats: $^{**}p<0.005$; $^{*}p<0.01$. 23-to 45-day-old rats.

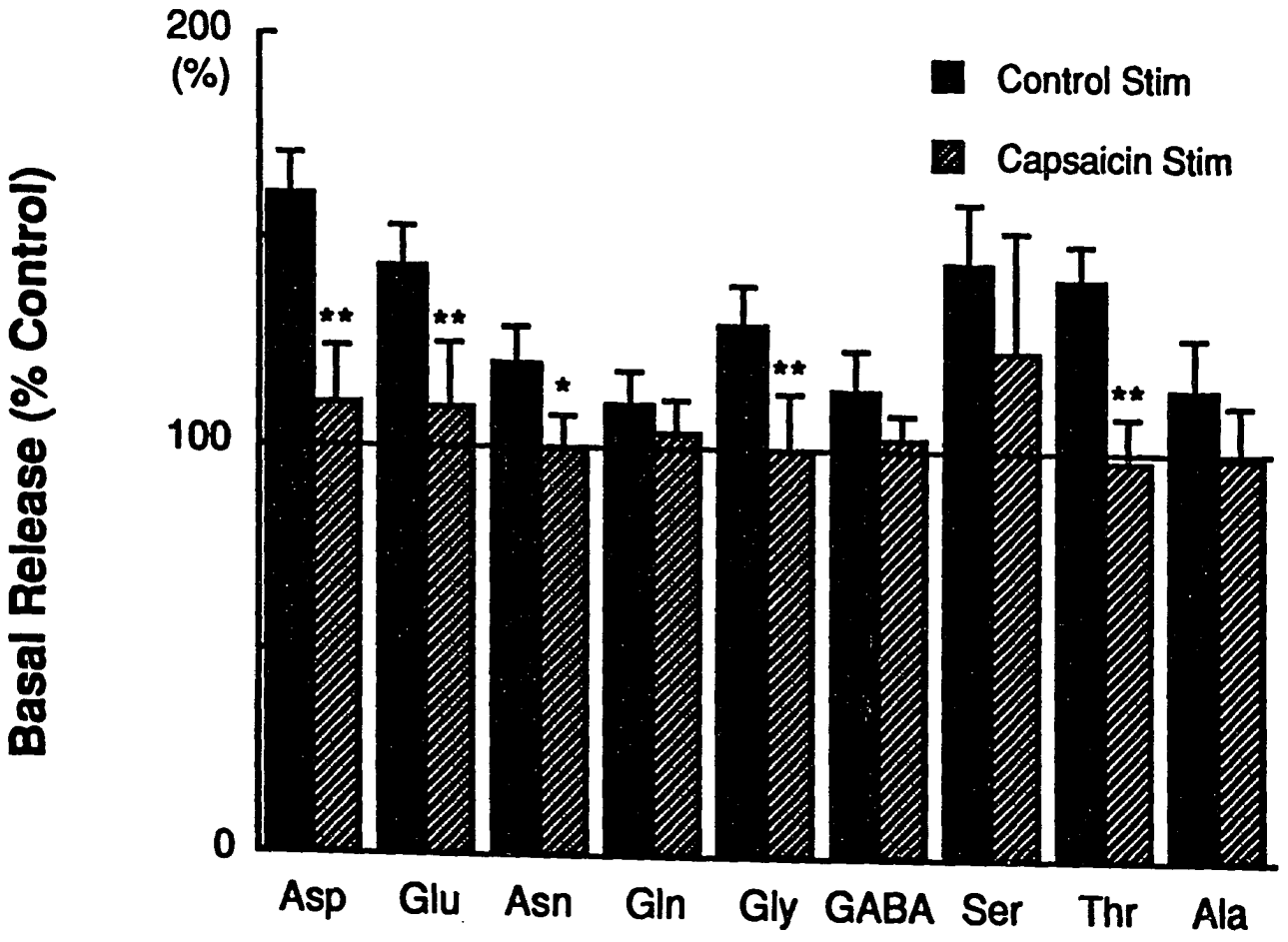

Basal Release (% Control)
200
(%)
100
0
Control Stim
Capsaicin Stim
**
**
*
**
**
Asp
Glu
Asn
Gln
Gly
GABA
Ser
Thr
Ala

Fig. 8. Immunoperoxidase staining of the rat lumbar spinal cord for SP-like immunoreactivity in control vehicle-injected rat (A) and neonatally capsaicin-treated rat (B). Scale bar, 100 μM . C, The SP-induced increase in the basal efflux of endogenous glutamate from the spinal cord slices of the intact rats (solid columns) was absent in the rats treated with capsaicin neonatally (hatched columns). The results for control (n=7) and capsaicin-treated animals (n=6) are presented as mean percentages ± SEM of their respective basal values. Statistical difference between the release in the intact and capsaicin-treated rats: $^{**}p<0.005$. 24- to 44-day-old rats.

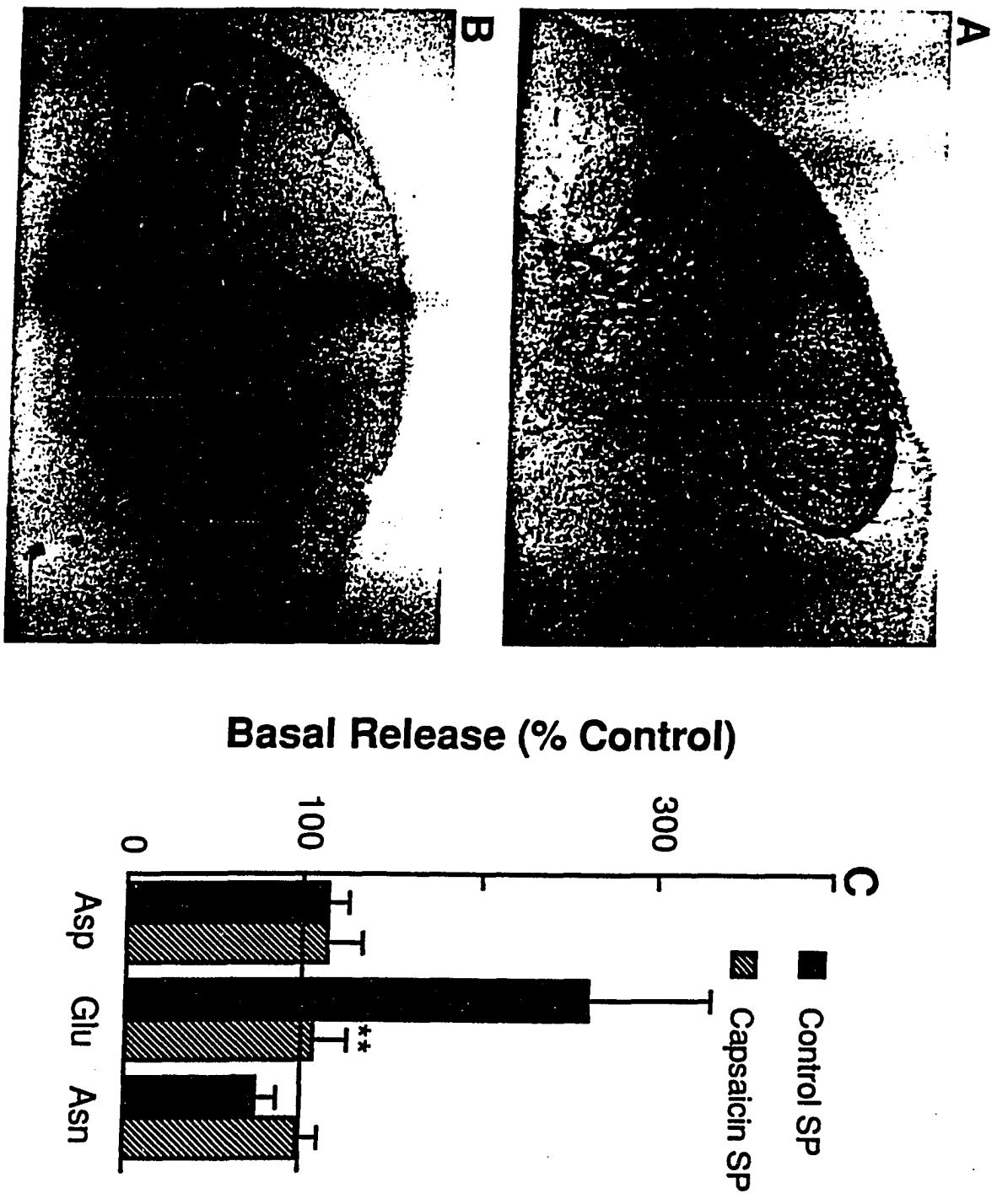

A
B
C
Basal Release (% Control)
0
100
300
Asp
Glu
Asn
**
Control SP
Capsaicin SP

Fig. 9. Immunoperoxidase staining of the rat lumbar spinal cord for CGRP-like immunoreactivity in control vehicle-injected rat (A) and neonatally capsaicin-treated rat (B). Scale bar, 100 μM. C, The CGRP-elicited increase in the basal efflux of endogenous glutamate and aspartate from the spinal cord slices of intact rats (solid columns) was present in rats treated with capsaicin neonatally (hatched columns). The results in control (n=4) and capsaicin-treated rats (n=4) are presented as mean percentages of their respective controls ± SEM 27-to-45-day-old rats.

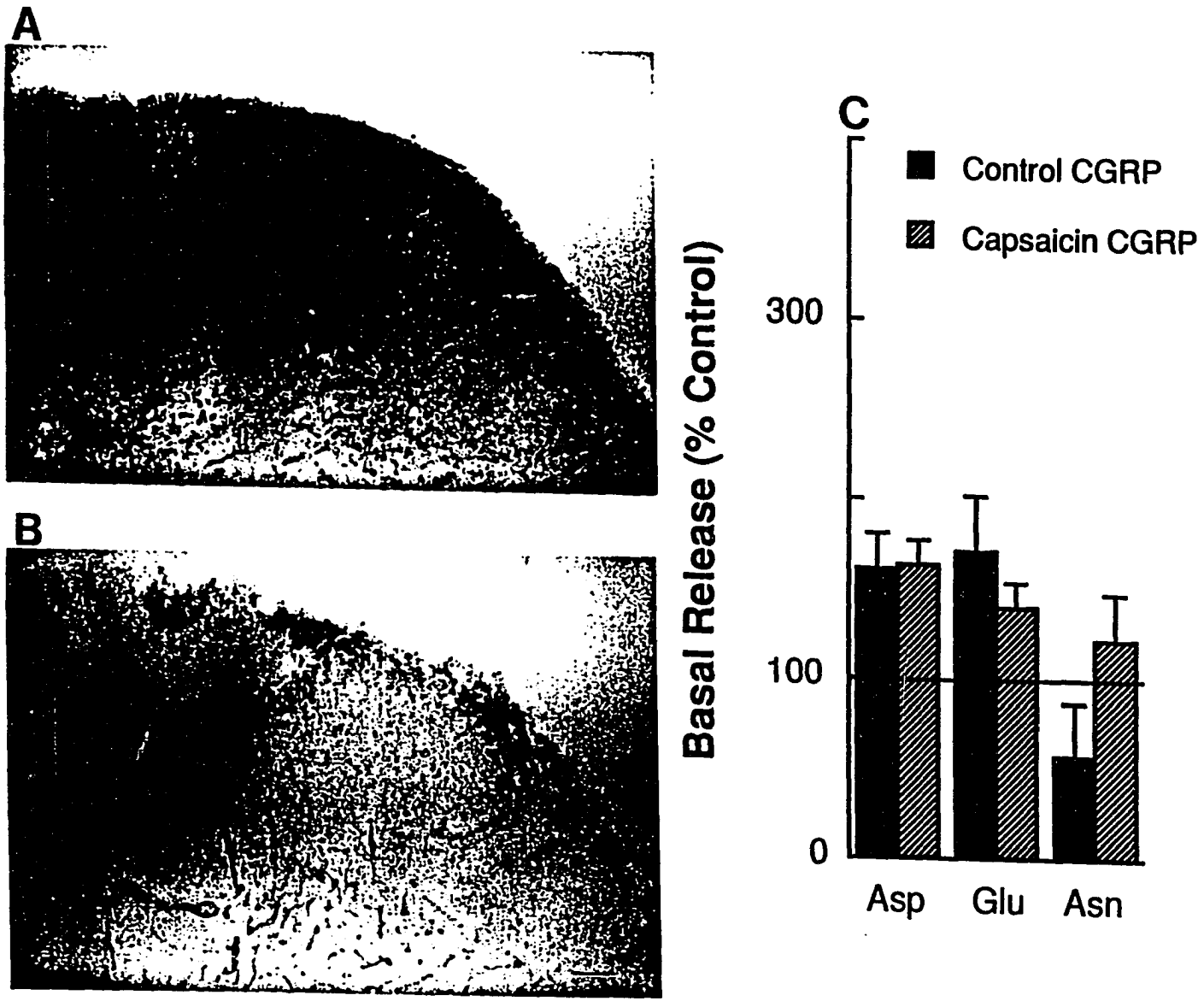

A
B
C
Control CGRP
Capsaicin CGRP
Basal Release (% Control)
300
100
0
Asp
Glu
Asn

Table 2. Summary of the effects of electrical stimulation of the dorsal rootlets and administration of SP, NKA, and CGRP on the levels[a] of endogenous aspartate (Asp) and glutamate (Glu) in the perfusate of the spinal slices

	Control			Capsaicin		
	Asp	Glu	n	Asp	Glu	n
A. Electrical stimulation						
Normal Krebs	209.2 ± 26.7	168.3 ± 14.0	(13)	109.8 ± 14.2	109.6 ± 14.8	(7)
Zero Ca^{2+} solution	94.7 ± 10.9	101.4 ± 7.8	(3)	-	-	
B. Peptides						
SP (2-5 x 10^{-7}M)	123.2 ± 15.3	254.3 ± 62.0	(8)	113.6 ± 18.7	104.9 ± 19.0	(6)
SP (1-5 x 10^{-6}M)	181.7 ± 40.1	339.3 ± 84.2	(3)	-	-	
SP (5 x 10^{-7}M in zero Ca^{2+})	141.9 ± 7.0	198.2 ± 29.7	(3)	-	-	
NKA (5 x 10^{-7} to 10^{-6})	179.1 ± 35.1	145.3 ± 16.1	(5)	-	-	
CGRP (10^{-7}M)	159.5 ± 20.1	170.5 ± 30.6	(4)	161.2 ± 15.0	138.8 ± 13.4	(4)

[a]Results are presented as mean percentages ± SEM of the basal efflux. Dorsal root stimulation: 25-30 V, 0.02-1.0 ms, 3-10 Hz for 5 min. Number of observations shown in parentheses (n represents the number of slices; each slice was obtained from a different animal).

DISCUSSION

Dorsal root stimulation-evoked release of endogenous amino acids and the effects of capsaicin

The findings of the present study confirm previous reports that dorsal root stimulation evokes a Ca^{2+}-dependent release of glutamate from the frog (Takeuchi et al., 1983) and the newborn rat spinal cord *in vitro* (Kawagoe et al., 1986). These earlier studies examined also the evoked release of aspartate and found it to be less regularly observed than for glutamate, and statistically insignificant. On the basis of these observations, the suggestion was made that glutamate is the principal transmitter released by dorsal root stimulation (Kawagoe et al., 1986). We have extended these original observations by characterizing the basal and electrically-evoked release of glutamate and aspartate in terms of stimulus-response relationship, its calcium dependence, and its modulation by tachykinins (SP and NKA), CGRP and neonatal treatment with capsaicin. In addition, the possibility as to whether, besides glutamate and aspartate (Roberts, 1974; Takeuchi et al., 1983), other endogenous excitatory and inhibitory amino acids were released *in vitro* was investigated. The results from this study clearly show that besides glutamate, several endogenous amino acids, including aspartate, asparagine, glycine, serine and threonine are released in significantly higher amounts upon high-intensity repetitive electrical stimulation of primary afferent fibers. It is worth noting that despite our finding that the basal efflux of aspartate is only about one-half that of glutamate, the stimulation-evoked increase in the basal release of aspartate appears to be similar, or even higher, than that of glutamate.

Two observations indicate that the electrically-evoked release of aspartate does not result from nonspecific changes in permeability of neuronal membranes. First, the release of aspartate was repeatable, and, following stimulation, baseline levels of released aspartate completely recovered to control levels. Second, the release was blocked by perfusing solution containing zero Ca^{2+}. This is as expected for a process of calcium-dependent exocytosis. We would like to suggest, therefore, that the stimulation-evoked release of aspartate is likely to be relevant for excitatory neurotransmission in the spinal dorsal horn. This suggestion is in agreement with a recent demonstration of aspartate-immunoreactive axons in normal rat L4 dorsal roots (Westlund et al., 1989b). Although it is well known that L-glutamate acts as a mixed agonist at both NMDA and non-NMDA excitatory amino acid receptors (Davies et al., 1982; Mayer and Westbrook, 1984) L-aspartate appears to be selective for NMDA receptors (Watkins, 1981; Mayer and Westbrook, 1984). Several recent <u>in vitro</u> studies, using either brain slices or isolated spinal cord preparations, have demonstrated that NMDA receptors can be activated during monosynaptic and polysynaptic transmission (Dale and Roberts, 1985; Forsythe and Westbrook, 1988; Gerber and Randic, 1989a).

As shown in Fig. 1A, electrical stimulation of dorsal roots also resulted in significant increases in the concentrations of glycine and serine in the spinal perfusate. This finding may have functional implications for spinal excitatory synaptic transmission and integration of sensory information incoming to the dorsal horn since it has been shown that responses of spinal neurons to the excitatory amino acid, N-methyl-D-aspartate (NMDA), are markedly potentiated by nanomolar concentrations of

glycine (Johnson and Ascher, 1987). This, together with the demonstration that the strychnine-insensitive glycine binding site is distinct from, but associated with, the NMDA receptor (Bonhaus and McNamara, 1988) has initiated considerable interest in glycine as a modulator of NMDA-receptor mediated synaptic transmission (Salt, 1989; Thomson et al., 1989). Although the mechanisms by which glycine acts on NMDA receptors are not well understood (Danysz et al., 1989), Mayer et al. (1989) have recently presented evidence indicating that at least part of the enhancement of NMDA responses by glycine occurs through acceleration of recovery from desensitization. D-serine (a glycine analog), is able to substitute for glycine in preventing desensitization (Mayer et al., 1989). In this context, it is noteworthy that we have recently observed that the responses of the rat dorsal horn neurons, either acutely isolated (Murase et al., 1989b) or in the spinal slice (Gerber et al., 1989), to NMDA, are augmented by 10^{-7} to 10^{-6}M of glycine. Therefore, the concentration of glycine (or serine) of about 10^{-6}M measured in the spinal perfusate after dorsal root stimulation, which probably reflects the elevated levels of glycine (and serine) in the extracellular space, may contribute to the enhancement of the NMDA receptor activity and excitatory synaptic potentials recently observed with glycine in a proportion of the rat spinal dorsal horn neurons examined in slices (unpublished observations). Because a high percentage (about 85%) of acutely isolated neonatal rat spinal dorsal horn neurons respond to NMDA (Murase et al., 1989b), variation in the concentration of endogenous glutamate, aspartate, glycine and serine could significantly influence excitatory synaptic transmission and integration of sensory information incoming to the spinal dorsal horn.

It has been shown that, besides the primary afferent fibers (Wheeler et al., 1966; Roberts, 1974; Takeuchi et al., 1983; Kawagoe et al., 1986), some descending pathways in the dorsal horn (Stone, 1979; Rustioni and Cuenod, 1982; Potashner and Tran, 1985; Potashner and Dymzyk, 1986) and some intrinsic dorsal horn interneurons (Davidoff et al., 1967; Rustioni and Cuenod, 1982) may use glutamate as a neurotransmitter. Therefore, the question of whether a proportion of the released glutamate and aspartate following electrical stimulation of primary afferent fibers derives from activation of primary sensory neurons, or whether it could reflect activation of secondary or tertiary cells in the dorsal horn pathways cannot be satisfactorily addressed by the experiments discussed above. In order to investigate the contribution of primary sensory neuronal glutamate and aspartate pools to the stimulation-evoked release of these amino acids we used neonatal treatment of rats with capsaicin, a neurotoxin known to cause degeneration of a large number of small "dark" sensory neurons (Jancso et al., 1977; Nagy et al., 1981). In this study, we show that capsaicin prevents the stimulation-evoked release of glutamate, aspartate, glycine and threonine, the results suggesting a possibility that a significant proportion of the release of these endogenous amino acids is likely to arise from primary afferent fibers sensitive to capsaicin. Our finding of capsaicin sensitivity of the stimulation-evoked glutamate release is in agreement with recent morphological data indicating that glutamate is preferentially localized in a subpopulation of small dorsal root ganglion cells (Cangro et al., 1985; Battaglia et al., 1987), unmyelinated dorsal root axons (Westlund et al., 1989a) and synaptic terminals in the superficial laminae of the spinal cord of rats, many of

which are likely to represent endings of unmyelinated (C) or small myelinated (Aδ) fibers (De Biasi and Rustioni, 1988). Thus, the morphological data and the results of the capsaicin release experiments reported in this work lend further support to the hypothesis that glutamate and/or aspartate is likely to be involved in the first order transmission of cutaneous information, particularly from C and Aδ-primary afferents (Schneider and Perl, 1985).

Enhancement of the basal and the dorsal root stimulation-evoked release of endogenous glutamate and aspartate by SP, NKA and CGRP

The results of the experiments reported here demonstrate that tachykinins (SP and NKA) and CGRP induce an apparently specific, predominantly Ca^{2+}-independent increase in the basal release of putative primary afferent transmitters, Glu and Asp. Although the enhancing and selective effect of SP on the release of endogenous Glu from the hemisected spinal cord of newborn rats, first observed by Kawagoe et al. (1986), has been confirmed, the different results were obtained in regard to the magnitude of the SP effect, its Ca^{2+}-dependence and the dose-related, SP-induced release of Asp. Kawagoe et al. (1986) found that the bath application of SP (5-10μM) caused an average increase in the basal release of glutamate of about 130%, the magnitude of the SP effect being comparable to the effect of dorsal root stimulation. The aspartate release induced by SP was small and statistically insignificant. In addition, they found that the release of glutamate, but not of aspartate, was decreased or abolished in the perfusing medium-containing low concentrations of Ca^{2+} or TTX. In contrast to the results of Kawagoe et al. (1986), we found, that perfusion

of spinal cord slices with lower concentrations of SP (10^{-7}M) was accompanied by a selective and marked (2- to 3-fold) increase in the rate of basal efflux of glutamate. With higher concentrations of SP (10^{-6}M), however, the basal release of aspartate was also augmented in a dose-dependent manner. The SP-caused increase in the release of glutamate was consistently larger than that produced by the dorsal root stimulation and the effect could be demonstrated in the absence of external Ca^{2+}. The inconsistencies between our results and those of Kawagoe et al.(1986), may be ascribed to methodological differences presented by their use of glucose-free perfusing solution, an amino acid uptake blocker (α-methyl-aspartate), and newborn-rat hemisected spinal cord preparation.

In relation to the results discussed above, it is noteworthy that Smullin et al. (1988), using dorsal horn dialysis probe in freely moving rats, observed a higher increase in endogenous Asp than Glu in response to 1 mM SP administration, and no increase in the basal concentration of Asp, Glu, Gly and taurine upon administration of 10 μm CGRP.

The data in Fig. 2C show that an SP related tachykinin, NKA, which occurs in primary sensory neurons, and in an even higher concentration than SP in the rat spinal dorsal horn (Kanazawa et al., 1984; Brodin et al., 1986), also increases the basal release of Glu, Asp and Gly from the rat spinal slices. Although in a few experiments the relative potencies of SP and NKA with respect to the stimulation of the basal release of Asp and Glu appear to be quantitatively similar, in 8 experiments SP appeared to increase the release of Glu more, whereas NKA preferentially increased the basal release of Asp (n=5). Since NKA also exhibits a potent excitatory action on spinal neurons that is depressed by a substance P antagonist (D-

Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-SP, it is likely that some of the physiological roles that have been attributed to SP in sensory neurotransmission are, in fact mediated by NKA, or both tachykinins.

The origin of the excitatory amino acids that are released and mechanism(s) underlying the enhancement of the release of glutamate and aspartate by tachykinins and CGRP have yet to be elucidated. In the present study, we have demonstrated that in difference to the electrically-evoked release of glutamate and aspartate from the spinal slice, which does not occur when Ca^{2+} is absent from the external medium, the enhancement of the basal release of the amino acids by SP and CGRP appears to be, in a large part, Ca^{2+}-independent. Since the Ca^{2+} independence of the releasing action of peptide receptors seems to exclude the involvement of Ca^{+}-mediated exocytosis, it is possible that peptide ligand-induced neurotransmitter release may be mediated by second-messenger systems rather than depolarization-induced calcium influx. There is evidence for the role of protein kinase C (Nishizuka, 1984; Nichols et al., 1987; Gerber et al., 1989) and the cyclic AMP system in neurotransmitter (Nestler and Greengard, 1983) and peptide release. In addition, it is of interest that CCK-8 evokes secretion of oxytocin and vasopressin from rat neural lobe, independent of external calcium, and that the CCK-8 action is blocked by an inhibitor of protein kinase C (Bondy et al., 1989). On the other hand, SP is known to increase hydrolysis of phosphoinositides in central neurons (Watson and Downes, 1983), whereas some actions of CGRP are thought to be mediated through activation of adenylate cyclase (Crossman et al., 1987; Wang and Fiscus, 1989). Two experimental results are of relevance for the possible second messenger mediation of the enhancement of the release of

glutamate by SP. First, Womack et al. (1988) have shown that in about one-third of cultured dorsal horn neurons, SP receptor activation increases cytosolic free Ca^{2+} via mobilization of intracellular Ca^{2+} stores, and a suggestion was made that the intracellular pathway for the action of SP may involve the generation of inositol phosphate intermediates. Second, we have recently demonstrated (Gerber et al., 1989) that perfusion of rat spinal slices with phorbol esters, the agents known to activate the calcium- and phospholipid-dependent protein kinase C (Nishizuka, 1984, 1986), produces an increase in the basal and electrically-evoked release of endogenous excitatory (glutamic, aspartic) and inhibitory amino acids (GABA, glycine).

Besides the neuronal source (axons and/or nerve terminals), the peptide-caused increase in the basal release of Glu and Asp from the glial cells should also be considered. The extracellular microenviroment of the central neurons is largely bounded by glial membranes, and glial cells have been postulated to influence the concentration of neurotransmitters in the synaptic cleft by a variety of mechanisms. Evidence exists that amino acids are released by high-K^+ solution in a Ca^{2+}-independent manner (Drejer et al., 1982, 1983) and that astrocytes may be intimately involved in neurotransmission processes, amino acid uptake (Hertz and Schousboe., 1986; Hösli et al., 1986), and they posses receptors for amino acid transmitters (Glu, Asp, GABA) and peptides (Bowman and Kimelberg, 1984; Kettermann and Schachner, 1985; Hamprecht, 1986; Torrens et al., 1986). Binding sites for SP were detected on glial cells of the spinal cord and SP enhances accummulation of labeled inositol phosphates in cultures of cortical glial cells from the mouse (Torrens et al., 1986). In addition, synthetic human

calcitonin raises intracellular concentration of cyclic AMP in rat astroglia cells (Hamprecht, 1986).

It is well established that the principal mode of inactivation of putative excitatory (glutamate, aspartate) and inhibitory (GABA, glycine) neurotransmitters released from nerve endings is by reuptake, using high-affinity ion- and membrane potential-dependent transport systems that are known to operate both in neuronal and glial membranes (Fonnum, 1984; Hösli, et al., 1986). Thus, another potential mechanism for the enhancement of the basal relase of glutamate and of aspartate by tachykinins and CGRP is via the electrogenic transport system, which is Ca^{2+}-independent and would be expected to "release" glutamate and aspartate whenever cells are depolarized. It is of interest that the Gly uptake system is inhibited by peptides, leu- and met-enkephalin (Rhoads et al., 1984).

It is well established that the release of classical neurotransmitters is controlled by autoreceptors, heteroreceptors, or receptors that are acted upon by co-localized substances, such as neuropeptides (Chesselet, 1984; Bartfai et al., 1988) and that this mechanism plays an important role in determining the amount of transmitter released per each stimulus. It was first demonstrated in the example of ACh/vasointestinal polypeptide (VIP) co-existence in the postganglionic neurons of the cat that muscarinic cholinergic autoreceptors inhibit the release of both ACh and VIP. VIP, on the other hand, enhanced the release of ACh (Lundberg et al., 1980). VIP enhancement of ACh-evoked salivation in the cat submandibular gland (Lundberg et al., 1980) was followed by demonstration of synergistic effects of ACh and VIP in promoting phosphatidylinositol turnover in the cerebral cortex, ie., at the sites of

coexistence of ACh and VIP. Synergistic effects of 5-HT, TRH and SP in the ventral spinal cord have also been observed (Iverfeldt et al., 1986; Tremblay et al., 1986).

The finding that glutamate and substance P coexist in primary afferent terminals in the superficial laminae of the rat spinal dorsal horn (De Biasi and Rustioni, 1988) coupled with the demonstrated potentiation of the basal and stimulation-evoked efflux of glutamate and aspartate by SP and NKA in this study, provide evidence for a role of tachykinins in the regulation of Glu and Asp release. Thus, in addition to the excitatory postsynaptic actions of tachykinins on the dorsal horn neurons (Murase and Randic, 1984; Murase et al., 1989a,b) and modulation of the Ca^{2+} conductances (Ryu and Randic, 1990), the tachykinins may also serve some important presynaptic function through the regulation of the release of coexisting primary afferent transmitters. It would seem that co-release of excitatory amino acids and tachykinins could serve to interact cooperatively to result in a potentiation of depolarizing action at postsynaptic sites on dorsal horn neurons. These pre- and postsynaptic mechanisms of action of tachykinins, and other sensory peptides, may have important physiological implications for strengthening the synaptic connections in the spinal dorsal horn. Such a dual role is consistent with our present knowledge about multiple pre- and postsynaptic actions of peptides in the peripheral nervous system (Lundberg et al., 1980).

Acknowledgements

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SECTION II. THE EFFECTS OF SUBSTANCE P AND CALCITONIN GENE-RELATED PEPTIDE ON THE EFFLUX OF ENDOGENOUS GLUTAMATE AND ASPARTATE FROM THE RAT SPINAL DORSAL HORN IN VITRO[1]

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SUMMARY

Bath applied SP (2×10^{-7} to 5×10^{-7}M) produced a significant increase in the concentration of glutamate in the spinal slice perfusate, whereas the efflux of aspartate increased only with a higher concentration of SP (5×10^{-6}M). The enhancement of the basal efflux of glutamate persisted in the absence of external Ca^{2+}, but the effect was blocked by (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-SP, a SP analogue claimed to be an antagonist of synthetic SP. Calcitonin gene-related peptide (CGRP, 10^{-7}M) produced a significant increase in the concentrations of glutamate and aspartate in the perfusate. Neonatal capsaicin treatment prevented the SP-induced increase in the release of glutamate. In contrast, the effect of CGRP was not significantly modified by the capsaicin treatment. These results indicate that SP and CGRP are capable of modulating the basal efflux of endogenous aspartate and glutamate and this modulation may represent one of the mechanisms by which these peptides contribute to primary afferent synaptic transmission.

INTRODUCTION

Excitatory amino acids (EAA), glutamate and aspartate, appear to be the major excitatory neurotransmitters in the mammalian spinal cord (Mayer and Westbrook, 1987). Peptides, substance P (SP) and calcitonin gene-related peptide (CGRP), seem to modulate primary afferent neurotransmission by acting both at presynaptic (Kawagoe et al., 1986; Ryu et al., 1988b; Smullin et al., 1988; Kangrga et al., 1989) and postsynaptic sites (Murase and Randić, 1984; Murase et al., 1989a,b). Glutamic acid is released upon stimulation of the primary afferents (Roberts, 1974; Kawagoe et al., 1986) and the Ca^{2+}-dependent release of SP from primary afferent fibers has also been reported (Otsuka and Konishi, 1977). Although co-existence of glutamate and SP in some small primary sensory neurons (Battaglia et al., 1987) and in primary afferent terminals in the superficial laminae of the rat spinal dorsal horn (DeBiasi and Rustioni, 1988), and of SP and CGRP in a proportion of small sensory neurons (Gibson et al., 1984), has been demonstrated by using histological methods, our understanding of the physiological significance of this phenomenon is still unclear.

The objective of this study was to investigate the possibility of a modulation of the basal efflux of nine endogenous amino acids, including glutamate and aspartate, from the superfused spinal slices of the rat, in response to SP, CGRP and neonatally-applied capsaicin. The results have been presented in a preliminary form (Kangrga et al., 1989).

METHODS

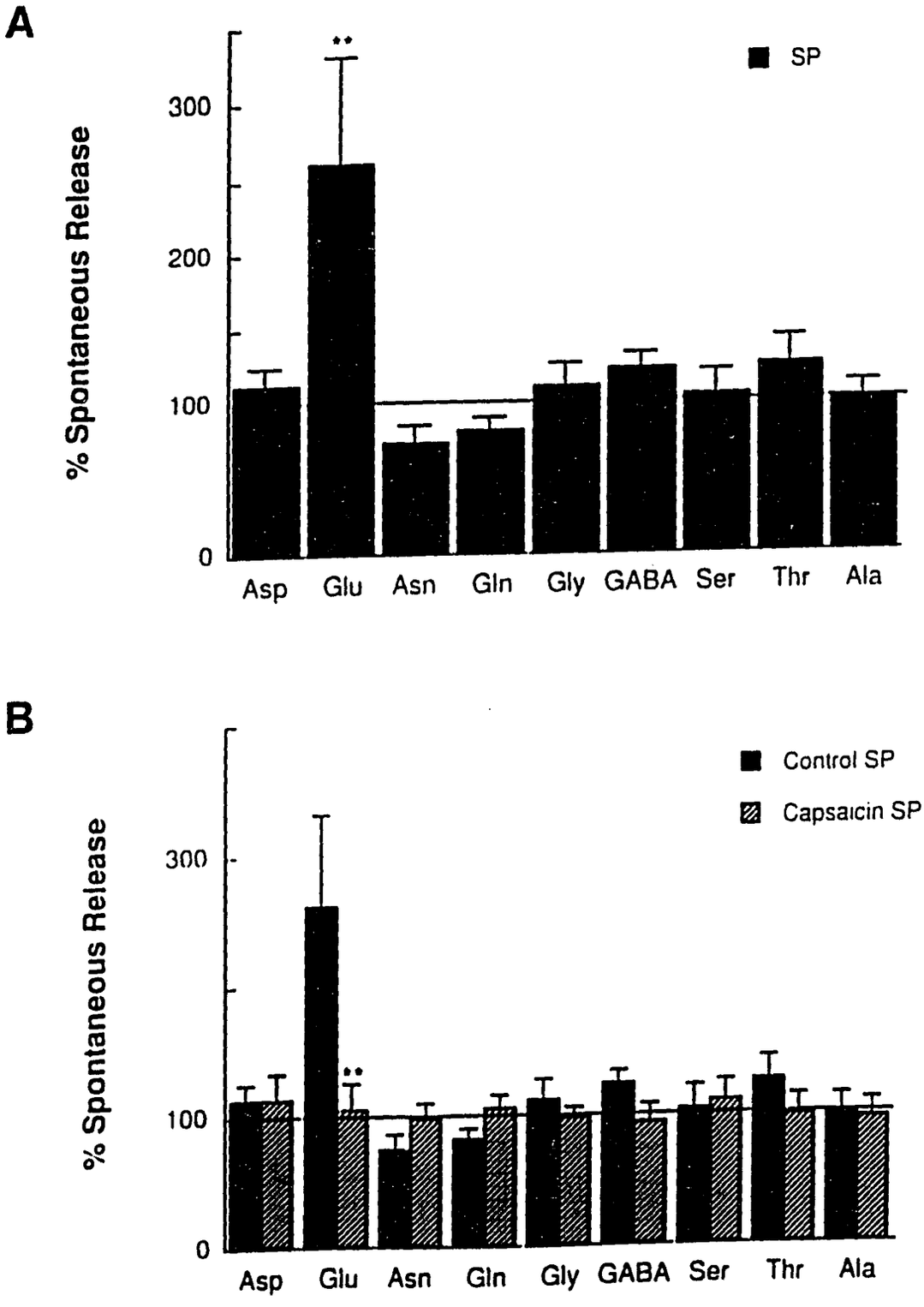
Experiments were performed on horizontal spinal cord slices, with dorsal roots and dorsal root ganglia attached, from 25 to 44-days old Sprague-Dawley rats, as described (Murase and Randić, 1984; Gerber et al., 1989; Murase et al., 1989a). Samples of perfusate (0.5 ml) were collected at regular 5 min intervals before, during and after peptide application and/or dorsal root stimulation. Samples were kept frozen at -80° C until derivatization and chromatographic analysis. SP and CGRP were bath-applied into the perfusate. Capsaicin (50 mg/kg) was s.c. administered to rats on the second day of age. The amino acid content in the samples was determined by high performance liquid chromatography (HPLC) with fluorescence detection (Lindroth and Mopper, 1979). Prior to injection, aliquots of the perfusates were derivatized with o-phthaldialdehyde (OPA) 2-mercaptoethanol reagent (Pierce). Hydroxylysine (30 μM) was added to each sample as an internal standard. Chromatography was performed on a 15 cm Adsorbasphere-OPA-HR column (Alltech Associates) using a pH 5.9 sodium acetate-tetrahydrofuran/methanol gradient. Fluorescence was detected with a Kratos FS 950 fluorimeter. Statistical significance has been assessed relative to control conditions by use of either a paired or unpaired Student's t test as appropriate. Levels of significance are indicated as follows: *$p < 0.01$; **$p < 0.005$.

RESULTS

Perfusion of spinal cord slices with SP ($2\text{-}5\times10^{-7}$M) was accompanied by a selective and a significant increase in the rate of basal efflux of glutamate (260.6 ± 71.2%, mean ± S.E.M., n=6), as illustrated in Fig. 1A. With a higher concentration of SP (10^{-6} to 5×10^{-6}M) the aspartate release was also increased (to 150% of basal efflux with 10^{-6}M SP and to 270% with 5×10^{-6}M of SP), whereas the levels of other seven endogenous amino acids were not significantly altered. The effect of SP was blocked by a claimed tachykinin receptor antagonist (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-SP. The latter finding indicates that the SP effect on the basal efflux of glutamate is probably a true receptor-mediated peptide effect. When the slices were perfused with a nominally Ca^{2+}-free medium, SP still elicited a significant increase in the concentration of glutamate (198.2 ± 29.7%) and aspartate (141.9 ± 7.0%, n=2). Womack et al. (1988) have reported that SP can produce a prolonged elevation in intracellular concentration of Ca^{2+} by mobilizing its release from intracellular stores. Thus SP-induced increase of glutamate efflux can perhaps be mediated by the mobilization of intracellular Ca^{2+}.

We have recently shown that CGRP enhances calcium currents of rat dorsal root ganglion neurons and spinal excitatory synaptic transmission (Ryu et al., 1988b). As Ca^{2+} influx is intimately related to neurotransmitter release, a similar action of CGRP on voltage-sensitive Ca^{2+} channels at central terminals of primary sensory neurons, as shown for the somatic membrane of dorsal root ganglion neurons (Ryu et al., 1988b), could increase neurotransmitter release and facilitate excitatory synaptic

Fig. 1. In A, perfusion of spinal cord slices with SP ($2\text{-}5 \times 10^{-7}$M for 5 min) produced a significant increase ($^{**}p < 0.005$) in the concentration of glutamate in the perfusate, whereas the levels of other eight amino acids were not significantly altered. The results are presented as mean percent of the basal efflux of nine endogenous amino acids from 7 experiments conducted in duplicate; S.E.M. shown by vertical lines. 24-to 33-day-old rats. In B, the SP-induced increase in the basal efflux of endogenous glutamate from the spinal cord slices of intact rats (solid columns) was absent in the neonatally-treated rats with capsaicin (hatched columns). The results for control (n=7) and capsaicin-treated animals (n=6) are presented as mean ± S.E.M. percent of their respective basal values. Statistical difference between the release of glutamate measured in the intact rats in the presence of SP compared with that which occurred in the rats neonatally treated with capsaicin: $^{*}p<0.005$. 24- to 44-day-old rats.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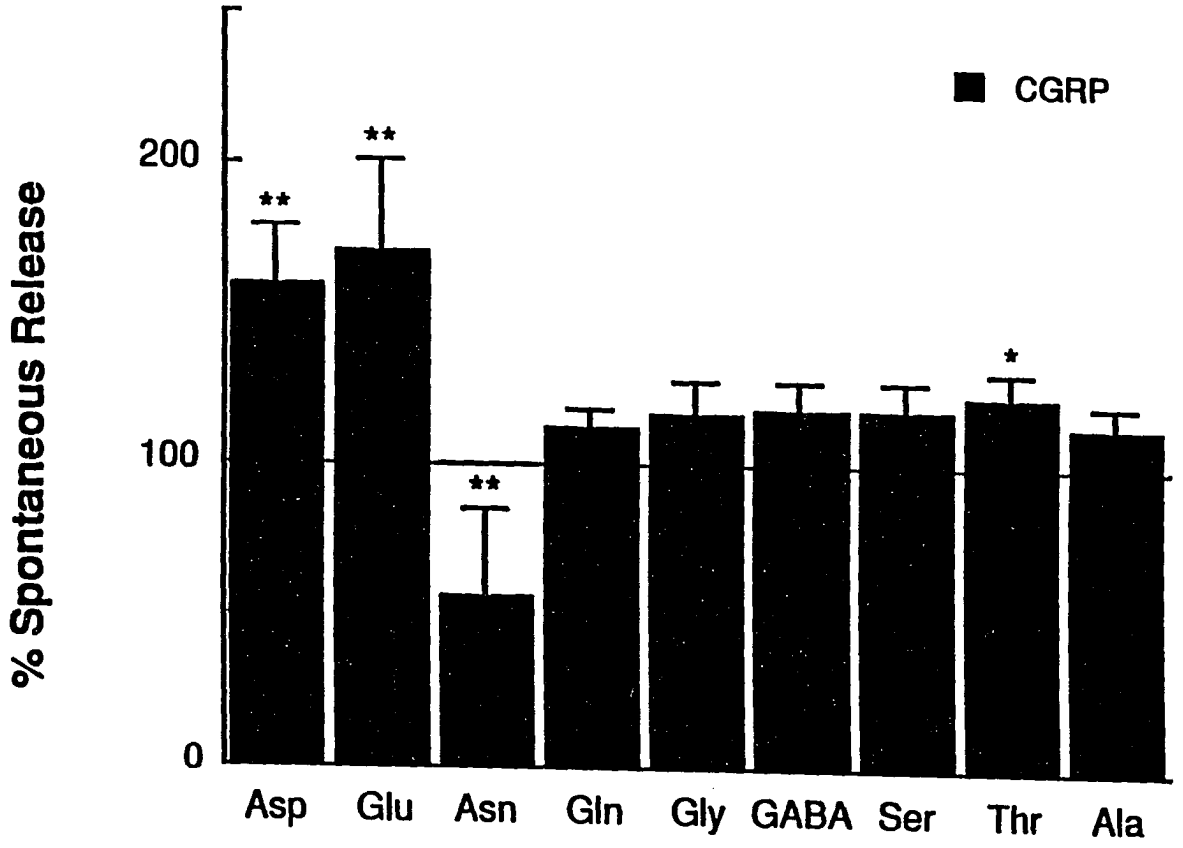
Ala

transmission. In support of this hypothesis we have observed in four different slices that CGRP (10^{-7}M) produced a significant increase in the basal efflux of glutamate (170.5 ± 30.6) and aspartate (159.5 ± 20.1) and a smaller increase in threonine (Fig. 2). In addition, there was a significant decrease in the concentration of asparagine (Fig. 2). The levels of the other five endogenous amino acids were not significantly altered

Capsaicin (8-methyl-N-vanillyl-6-noneamide) is known to cause the release of peptides present in C- and Aδ-primary afferent fibers, and when given neonatally causes the death of small dorsal root ganglion neurons (Jancso et al., 1977), many of which are known to contain peptides including SP and CGRP (Nagy et al., 1981; Franco-Cereceda et al., 1987). In the present study we found that the neonatal capsaicin treatment prevented the SP-induced increase in the concentration of glutamate in the spinal perfusate (Fig. 1B), as well as the dorsal root stimulation-evoked increase in the release of endogenous aspartate and glutamate from the spinal slices. However, the CGRP-induced increase in the basal efflux of aspartate and glutamate was not modified in the slices obtained from capsaicin-treated rats. These results suggest that unmyelinated and perhaps small myelinated, primary afferent fibers may be an important source of released glutamate and/or aspartate following dorsal root stimulation or SP application.

Fig. 2. Perfusion of spinal cord slices with CGRP (10^{-7}M) produced a significant increase in the concentration of glutamate and aspartate. In addition, there was a significant decrease in the concentration of asparagine. The results are expressed as mean percent of the basal efflux ± S.E.M. of 9 amino acids from 4 experiments conducted in duplicate; 27-to 31-day-old rats.


CGRP
% Spontaneous Release
200
100
0
**
**
**
*
Asp
Glu
Asn
Gln
Gly
GABA
Ser
Thr
Ala

DISCUSSION

The results of the experiments reported here demonstrate an apparently specific Ca-independent release of putative primary afferent transmitters, glutamate and aspartate, in response to administration of SP, the peptide known to influence excitatory afferent transmision in the rat spinal dorsal horn (Murase and Randić, 1984; Murase et al., 1989a). The question of whether the enhanced release of glutamate and aspartate by SP and CGRP reflects direct activation of primary afferent fibers, or whether it could reflect activation of local interneurons or descending fibers to dorsal horn cannot be satisfactorily addressed by the present methods. However, in view of the fact that the neonatal capsaicin treatment abolished the potentiating effect of SP on the basal efflux of glutamate, it is possible that glutamate derived from the primary afferent source. Conversely, since the enhanced basal efflux of glutamate and aspartate induced by CGRP is not modified by capsaicin, this finding would suggest that glutamate and aspartate may be derived from local interneurons or descending afferents to dorsal horn. In the latter context, it is of interest, that although the degree of depletion of CGRP and tachykinins content from the dorsal root ganglia by neonatal capsaicin is similar (Nagy et al., 1981; Franco-Cereceda et al., 1987; Diaz-Guerra et al., 1988), the relative change in the CGRP content of the dorsal horn is much less marked (Diaz-Guerra et al., 1988).

In this work the enhancing and selective effect of SP on the release of endogenous glutamate from the newborn rat spinal cord, first observed by Kawagoe et al. (1986), has been confirmed. The different results, however,

were obtained regarding Ca-dependence of the SP effect and the SP-induced release of endogenous aspartate.

Smullin et al. (1988) using the dorsal horn dialysis probe, in rats in vivo, found that perfusion with a high concentration of SP (10^{-3}M) produced a two-fold increase in the extracellular concentration of aspartate, but no increase in glutamate, asparagine or glycine. Whilst this technique does provide major advantages in that it allows the investigation of various deep neuronal structures in freely moving or anesthetized animals, the inevitable tissue damage associated with passing a steel probe through the spinal dorsal horn tissue produces a number of problems, notably considerable glial invasion of the damaged tissue.

The high concentration of SP in the laminae I and II, the area where small primary afferent fibers are known to terminate, and the demonstrated potentiation of the basal efflux of glutamate provide evidence for a role of SP, or a related tachykinin, in the regulation of the release of glutamate. Thus, in addition to the postsynaptic actions of SP on rat dorsal horn neurons (Murase and Randić, 1984; Murase et al., 1989a,b), the peptide may also have some important regulatory function in the release of primary afferent transmitters. Such a dual role is consistent with our present knowledge about the multiple actions of peptides in the peripheral nervous system. SP- and CGRP-produced enhancement of glutamate and aspartate efflux may have important functional implications at synapses where the possibility of multiple signalling exists, by yielding a high signal-to-noise ratio.

Acknowledgements

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SECTION III. OUTFLOW OF ENDOGENOUS ASPARTATE AND GLUTAMATE FROM THE RAT SPINAL DORSAL HORN IN VITRO BY ACTIVATION OF LOW- AND HIGH-THRESHOLD PRIMARY AFFERENT FIBERS - MODULATION BY μ OPIOIDS[1]

[1]Submitted to Brain Research as a research paper by Ivan Kangrga and Mirjana Randić. 1991. In press.

SUMMARY

Possible correlation of release of endogenous glutamate (Glu) and aspartate (Asp) with stimulation parameters used to activate primary sensory neurons was examined using the rat spinal cord slice - dorsal root ganglion preparation and high performance liquid chromatography with fluorescence detection. Selective activation of the low-threshold ($A\beta$) primary afferent fibers (PAF) resulted in a two-fold increase in the outflow of endogenous Asp and a smaller increase in the outflow of Glu from the rat spinal dorsal horn slices into the perfusing medium. Activation of both the low ($A\beta$)- and the high-threshold ($A\delta$+C) primary afferents elicited additional increase in the outflow of Asp and Glu, and a marked increase in the Asp/Glu outflow ratio. DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol-enkephalin), an agonist at μ-opioid receptors, reduced the dorsal root (DR) stimulation-evoked outflow of Asp and Glu in a naloxone-sensitive manner. Our results have provided further evidence in support of contention that Glu and Asp act as excitatory synaptic transmitters in the spinal dorsal horn. A role for μ-opioid receptors in modulation of spinal processing of somatosensory information is indicated.

INTRODUCTION

Excitatory amino acids (EAA), glutamate (Glu) and aspartate (Asp), are the principal neurotransmitter candidates in the central nervous system (Mayer and Westbrook, 1987; Kubo et al., 1990; Perschak and Cuenod, 1990) and spinal cord (Mayer and Westbrook, 1987; Evans, 1989; Rustioni and Weinberg, 1989). Although Glu has been long favored as a transmitter of the primary afferent fibers, anatomical and physiological evidence has recently emerged suggesting a role for Asp in the primary afferent neurotransmission. Asp has been detected by immunocytochemistry in approximately 15% of the unmyelinated and 4% of the myelinated axons in the rat L4 dorsal root (DR) (Westlund et al., 1989b), and electrically-evoked release of Asp has been demonstrated from the slices of medulla (Kubo et al., 1990) and spinal dorsal horn (Kangrga and Randić, 1990; Kangrga et al., 1990a,b). About 85% of acutely isolated rat spinal dorsal horn neurons are sensitive to NMDA (Randić et al., 1990) and both non-NMDA and NMDA receptors participate in the fast (Dale and Roberts, 1985; Gerber and Randić, 1989a; Dickenson, 1990) and slow excitatory transmission in the dorsal horn (Gerber and Randić, 1989b). Opioid peptides have been implicated in modulation of spinal segmental transmission (Jeftinija et al., 1986; Evans, 1989; Rustioni and Weinberg, 1989) and the presence of functional μ- and δ-receptors on C primary afferent neurons, capable of modulating release of SP, has been demonstrated (Crain and Shen, 1990; Go and Yaksh, 1987).

The objective of this study was to investigate the pattern of outflow of endogenous Glu, Asp and glutamine (Gln) from the superfused dorsal horn

slice in response to selective activation of the low-threshold or both the low- and high-threshold primary afferent fibers by electrical stimulation of lumbar dorsal roots. In addition, the possibility of a modulation of the DR stimulation-evoked outflow of Glu and Asp by a μ-opioid receptor agonist DAGO was examined. Some aspects of this work have been reported (Kangrga et al., 1990a,b).

METHODS

Longitudinal slices (300-400μm), with dorsal roots (DR) and dorsal root ganglia (L4-L6) attached, were obtained from 27 to 57-day-old Sprague Dawley rats (Kangrga and Randić, 1990). After 1 hr of incubation at 30±1^{0}C a slice was transferred into one compartment of a 2 compartment-chamber and continuously perfused with oxygenated artificial cerebrospinal fluid at a flow rate of 0.3-0.5 mlmin^{-1}. The dorsal roots were led across a leak-proof partition of vaseline into the compartment filled with mineral oil and placed on two pairs of bipolar platinum electrodes. The distal pair was used for stimulation of the DR, and the proximal pair for recording and continuous monitoring of compound action potentials of primary afferent fibers. Conduction velocities, estimated from the distance between the centers of the two bipolar electrodes and the latency of the first negative deflection of a volley, were: Aα,β, 22.6±1.9 ms^{-1} and C, 0.32±0.02 ms^{-1}, (n=20). DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol)enkephalin, Cambridge Research Biochemicals), naloxone (Du Pont), capsaicin (Sigma) and resiniferatoxin (RTX) (Chemsyn Science Laboratories) were bath-applied in known concentrations. Samples of the perfusate were collected at regular intervals (3-5 min) prior to, during, and after the periods of DR stimulation and/or chemical application, and stored at -80^{0}C until chemical analysis. Solutions of drugs applied to the slice were tested for the content of Glu, Asp and Gln. Determination of the amino acid concentrations in the spinal perfusate was performed using high-performance liquid chromatography (HPLC) with O-phthaldialdehyde (OPA) precolumn derivatization and fluorescence detection (Lindroth and Mopper, 1979).

Results are expressed as mean percent ± S.E.M of the basal outflow determined as the average of first 3 samples collected prior to stimulation. The statistical difference was determined by ANOVA: $^{*}p<0.05$; $^{**}p<0.01$.

RESULTS

Basal concentrations of amino acids detected in 3 to 5 min-samples of spinal perfusate were in the nanomolar range and the absolute values (nM) were 39.4±8.0 for Asp and 488.6±58.6 for Glu (n=20). In eight different slices, selective activation of primary afferent Aβ-fibers (5-12V, 20μs, 3Hz, 180-300 pulses), elicited a significant increase in the rate of basal outflow of Asp (194.2± 15.6%, $^{**}P<0.01$) and Glu (148.4 ±13.8%, $^{*}P<0.05$) during the first collection period following the electrical stimulation of the dorsal roots (Fig. 1A). Higher intensity of electrical stimulation (25-30V, 0.4-1.0ms, 0.5-1.0Hz, 180-300 pulses) that recruited both primary afferent Aδ- and C-fibers increased the outflow of Asp (231.2±17.6%, $^{**}p<0.01$) and Glu (182.9±16.5%, $^{**}p<0.01$). However, the level of Gln was not significantly changed.

For better assessment of the outflow of Asp and Glu observed in response to the differential stimulation of primary afferents, the net amount of Asp and Glu released in consecutive samples following Aβ or A+C stimulation, was calculated for six experiments (Fig. 1B). Three results were noted: 1) Recruitment of both the low (Aβ)- and the low- and high-threshold (A+C) primary afferents resulted in a marked increase in the absolute amounts of Asp and Glu (Fig. 1B); 2) Although the absolute amount of Glu in the spinal perfusate significantly ($^{**}p<0.01$) exceeded that of Asp, the relative increase in the Aβ or A+C stimulation-evoked outflow of Asp was higher ($^{*}p<0.05$) than that of Glu (1C,D); 3) The greater increase in the amount of Asp relative to Glu produced by the electrical stimulation of A+C-afferents (720.0% and 216.3%, respectively, of that elicited by Aβ-

fiber stimulation), was reflected in an increased Asp/Glu outflow ratio. The Asp/Glu outflow ratio was 0.045 after stimulation of Aβ-afferents and 0.145 after stimulation of A+C-afferents.

In Fig. 1C,D, the increase in the outflow of Asp and Glu is presented as a function of time. Whereas the increase in the basal outflow of Asp and Glu, evoked by a selective low-threshold (Aβ) stimulation of PAF was present only during the first 3 min-collection period, the outflow elicited by a high threshold (A+C) stimulation was somewhat greater and needed a longer time to recover (Fig. 1C,D).

In order to examine whether the selective activation of small diameter afferents results in the increased outflow of Glu and/or Asp from the spinal slice, we used capsaicin (8-methyl-N-vanillyl-6-noneamide) and its potent analog, resiniferatoxin (RTX) (Maggi et al., 1990), as tools for selective activation of most of C afferent fibers. When the L4-L6 DRG were perfused with capsaicin (5-10 μM for 10 min, n=4) or RTX (0.1-1.0 nM for 3 min, n=4) an increase in the outflow of Asp and Glu was measured in the spinal slice perfusate (Table 1).

Superfusion of the spinal slices (n=4) with a μ-opioid receptor agonist DAGO did not have any consistent effect on the basal outflow of Asp and Glu. DAGO (5-10μM for 10 min), however, applied 5 min prior to and during the period of electrical stimulation of DRs, significantly reduced the outflow of Asp and Glu evoked by A+C PAF stimulation (30V, 1 ms, 1 Hz for 5 min) in 4 different slices (Fig. 2A,B). The depressant effect of DAGO was effectively reversed by naloxone (Fig. 2C). This finding suggests that the effect of DAGO on the stimulation-evoked outflow of Glu and Asp is a true μ-opioid receptor mediated response.

Fig. 1. A, selective activation of the low-threshold primary afferent fibers (5-12V, 20μs, 3Hz, 180-300 pulses) significantly increased the rate of basal outflow of Asp and Glu (solid columns) uring the first period following the stimulation. Recruitment of both low- and high-threshold PAF (25-30V, 0.4-1.0ms, 1Hz, 180-300 pulses) produced a greater increase in Asp and Glu (hatched columns), whereas Gln outflow was unaffected. The results are presented as mean percent of the basal outflow preceding each stimulation period ± S.E.M. for 8 experiments. 30- to 57-day-old rats. B, the average (n=6) amount of Asp (solid column) and Glu (hatched column) released in response to activation of Aβ or A+C afferents is presented. The basal outflow of Asp and Glu measured prior to each stimulation period has been subtracted. When A+C primary afferents were activated, a marked increase in the absolute amounts of Asp and Glu (702.0% and 216.3%, respectively, of that elicited by Aβ stimulation) in the spinal perfusate was observed. C and D, the time course of the outflow of Asp (C) and Glu (D) for data shown in A is presented. While activation of the low-threshold (Aβ) PAF (C-D, single arrows) produced a significant increase in the basal outflow of Asp and Glu during the first 3 min-collection period, the activation of both the low- and the high-threshold PAF (C-D, double arrows) resulted in a somewhat greater but characteristically prolonged increase in the outflow of both Asp and Glu. **:$p<0.01$; *:$p<0.05$.

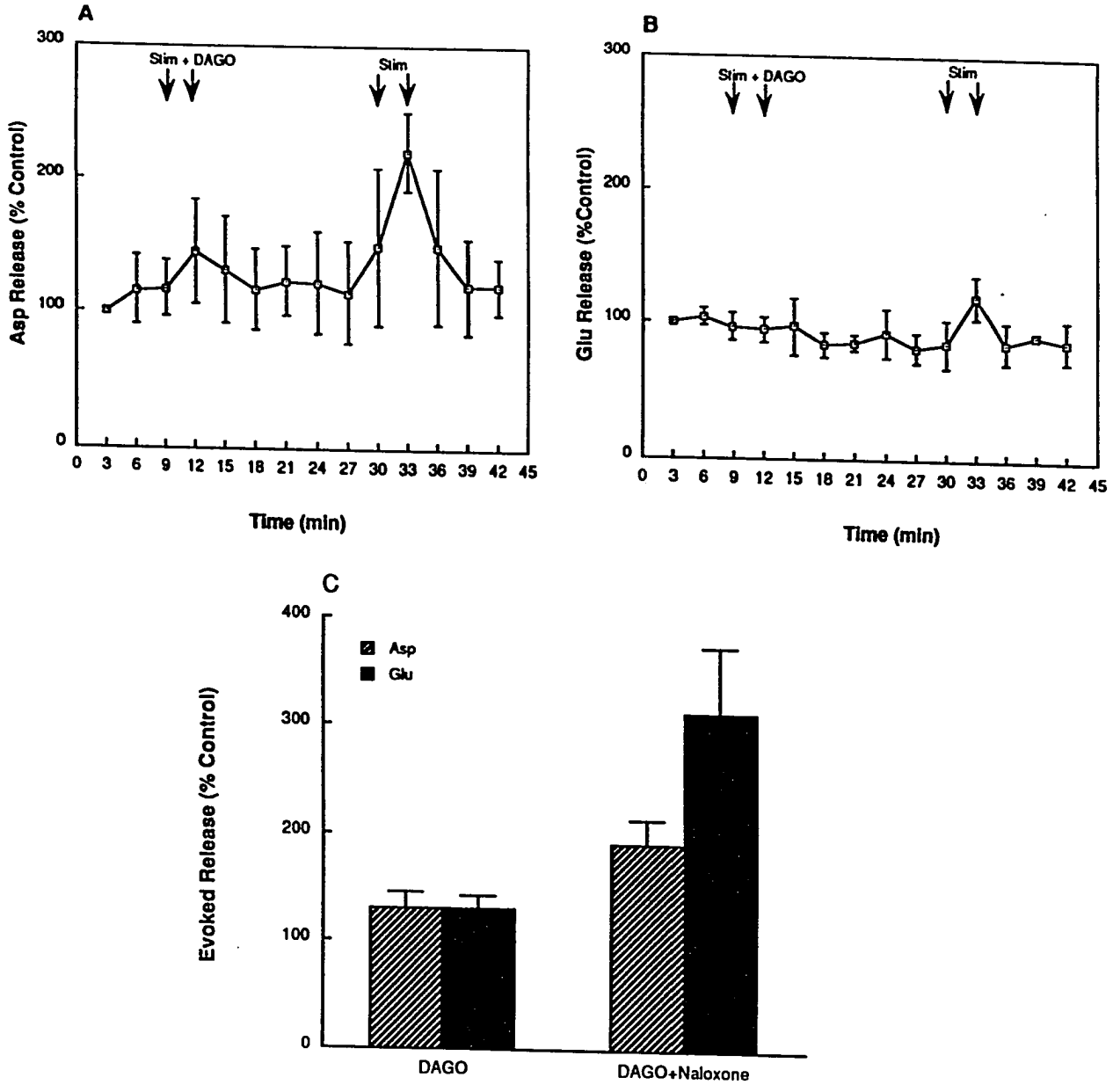
Basal Release (% Control)
A
A
A+C
Asp
Glu
Gln
pmols/3 min
B
Asp
Glu
A:3.0 Hz
A+C:0.5-1.0 Hz
Asp Release (%Control)
C
A
A+C
Time (min)
Glu Release (%Control)
D
A
A+C
Time (min)

Table 1. Summarized[a] effects of the perfusion of DRG (L4-L6) with capsaicin and RTX on the outflow of endogenous Asp, Glu and Gln from the spinal slices

	n	Aspartate	Glutamate	Glutamine
Capsaicin (5-10μM for 5-10 min)	4	229.5±45.1*	161.8±19.8*	104.5±28.3
RTX (0.1-1.0 nM for 3 min)	4	235.7±54.6	235.5±49.5**	191.2±57.6

[a]Results are presented as mean percentages ± SEM of the basal efflux. 30- to 37-day-old rats.

Fig. 2. DAGO blockade of the DR-stimulation evoked outflow of endogenous Asp and Glu is naloxone sensitive. Electrical stimulation of a lumbar dorsal rootlet (30V, 1ms, 1Hz for 5 min; second set of arrows) in four experiments elicited a significant increase ($p^{**}<0.01$) in the basal outflow of Asp (A) and Glu (B). The evoked outflow of Asp and Glu was reduced in the presence of DAGO (10μM for 10 min; first set of arrows). 33-to 34-day-old rats. C, in four experiments, electrical stimulation of a lumbar dorsal rootlet (30 V, 200 μs, 1 Hz for 5 min) in the presence of DAGO (5-10μM for 10 min, application began 5 min prior to stimulation and lasted for 10 min) elicited only a small increase in the basal outflow of Asp (hatched bars) and Glu (solid bars). However, during the combined administration of DAGO and naloxone (5-10μM for 15-20 min, application started 5-10 min prior to DAGO), the dorsal root-electrical stimulation evoked a marked increase in the outflow of Asp and Glu. 32-to 36-day-old rats.


A
Stim + DAGO
Stim
Asp Release (% Control)
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45
Time (min)
B
Stim + DAGO
Stim
Glu Release (%Control)
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45
Time (min)
C
Asp
Glu
Evoked Release (% Control)
400
300
200
100
0
DAGO
DAGO+Naloxone

DISCUSSION

The experiments described here demonstrate that the activation of both low- and high-threshold primary afferent fibers is accompanied by an increase in the amount of endogenous Glu and Asp released into the spinal slice perfusate (Kangrga et al., 1990a,b). An interesting finding is that significant release of physiologically active amino acids occurs at relatively modest rates of stimulation.

The possibility that Glu and/or Asp are transmitters of the primary sensory neurons has been frequently discussed (Rustioni and Weinberg, 1989; Westlund et al., 1989a,b Kangrga and Randić, 1990; Kangrga et al., 1990a,b), although available biochemical and physiological evidence favors Glu rather than Asp (Evans, 1989). The result from this study indicates, however, that Asp release appears preferentially mediated by small, slowly-conducting primary afferent fibers because of: 1. Differential release of Asp and Glu with the high-threshold-electrical stimulation; 2. The releasing effect of acutely-applied capsaicin and resiniferatoxin, the agents thought to selectively activate slowly-conducting, presumably unmyelinated fibers, and 3. Blockade of the electrically-evoked release of aspartate by neonatal treatment of rats with capsaicin (Kangrga and Randić, 1990). Thus, although the anatomical distribution of Asp and Glu in the primary sensory neurons (Westlund et al., 1989a,b) in conjunction with the results reported (Kangrga et al., 1990a,b) strengthens the claim of aspartate as a transmitter candidate for small-diameter primary afferent fibers, the interpretation of this work is not without difficulties. Before it can be concluded that a causal relationship exists between

increased Asp and/or Glu release and activation of a specific category of primary afferent fibers it is essential to establish in a future work that increased amounts of Asp and/or Glu in the spinal slice perfusate reflect an increase in the amount or probability of release from activated primary afferent terminals, rather than a decrease in uptake, or release from other neuronal or non-neuronal sources.

At the spinal level, the intrathecal administration of opiates and opioid peptides elicits behavioral analgesia in animals and man. Multiple opioid receptors which include μ-, δ- and κ- subtypes are present both on the primary sensory and dorsal horn neurons. Endogenous peptides with high affinity for those receptors are also present in the dorsal horn and descending neurons, as well as on the primary afferents where they appear to be preferentially associated with small diameter fibers. Available evidence indicates excitatory and inhibitory functions for opioids (Crain and Shen, 1990) in the rat spinal dorsal horn. Both presynaptic and postsynaptic sites of action have been suggested (Jeftinija et al., 1986; Go and Yaksh, 1987; Murase et al., 1982).

In the present experiments the μ- agonist DAGO caused a marked reduction of the DR stimulation-evoked outflow of Asp and Glu and this inhibitory effect of DAGO was modified by naloxone. It should be noted that the stimulation-evoked release of Glu after perfusion with DAGO and naloxone appears to be greater than after stimulation alone. Further studies should assess the significance of this observation. The results presented indicate that μ-opioid receptors can participate in the selective antinociceptive actions that opioids can exert upon somatosensory processing at the spinal level.

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APPENDIX. EFFECTS OF δ- AND κ-OPIOID RECEPTOR AGONISTS ON THE RELEASE OF ENDOGENOUS GLUTAMATE AND ASPARTATE FROM THE RAT SPINAL DORSAL HORN[1]

Introduction

The spinal cord is one of the major sites where opioids mediate nociception (Yaksh, 1984). Recent studies have established that the three principal types of opioid receptors, μ, δ and κ, are present in the spinal cord (Zarr et al., 1986; Traynor and Wood, 1987). Whereas the most numerous, μ-binding sites, are concentrated in substantia gelatinosa they are also observed throughout laminae III, IV, V and VIII. In contrast, the δ- and κ-binding sites seem to be confined to lamina I and substantia gelatinosa, respectively (Morris and Herz, 1986; Mansour et al., 1988; James et al., 1990). Since the relative concentration of receptors gives little information as to their functional importance, it should not be disconcerting that the density of δ- and κ-opioid receptors in the rat spinal cord is rather low when compared with μ-receptors. The differences in the discrete distributions of the three types of opioid receptors may contribute to different functional roles.

Whereas functional studies performed in the mammalian spinal cord support the involvement of δ-opioid receptors in spinal antinociception (Porecca et al., 1984; Rodriguez et al., 1986; Traynor et al., 1990), the

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[1]This part represents unpublished data.

role of κ-receptors is still not clear. Two factors have been decisive in advancing our knowledge of the significance of κ-receptors in the spinal afferent processing. First, highly selective ligands, arylacetamides (e.g., U50488 and PD117302) have become available (Takemori et al., 1988). Second, there is convincing evidence that dynorphin A_{1-17}, and related opioid peptides encoded by the pro-dynorphin gene, exert their effects via κ-receptors (Millan, 1986; Mansour et al., 1988). Dynorphin-containing neurons are located predominantly in substantia gelatinosa, but also in lamina I, IV and V (Basbaum et al., 1986). Thus, κ-receptors and dynorphin-containing neurons are strategically located for the modulation of nociception at the level of the spinal dorsal horn (Millan, 1990). Although functional evidence for a role of κ-receptors in the spinal afferent processing has been equivocal (Schmauss and Yaksh, 1984; Go and Yaksh, 1987; Leighton et al., 1988), a selective reduction in the nociceptive responses of cat dorsal horn neurons by $dynorphin_{1-13}$ and U50488H has been reported (Fleetwood-Walker et al., 1988).

δ- and κ-opioid receptors are differentially coupled to ion channels. Opioid ligands selective for δ-receptors hyperpolarized locus coeruleus neurons (North et al., 1987) and reduced the duration of Ca^{2+}-dependent action potential of mouse DRG neurons (Werz and Mcdonald, 1982). Both of these actions are due to the enhancement of potassium current. A selective agonist at κ-receptors, dynorphin A, reduced the duration of Ca^{2+}-dependent action potential of DRG (Werz and Macdonald, 1985) and myenteric neurons (Cherubini and North, 1985). The action of dynorphin was a result of a decrease of voltage-dependent Ca^{2+}-current. The peptide selectively affected the large, inactivating N-type Ca^{2+} channel current in the DRG

neurons (Gross and Macdonald, 1987).

Thus, functional δ- (Werz and Macdonald, 1982) and κ-opioid receptors (Gross and Macdonald, 1987) have been demonstrated on the DRG neurons, and both types of receptors have been implicated in mediating the effects of opioid peptides on the spinal afferent processing (Yaksh, 1984). In the present experiments we have examined the possibility of a modulation of the basal and dorsal root stimulation-evoked outflow of endogenous glutamate and aspartate from the spinal dorsal horn by δ- and κ-opioid receptor agonists.

Methods

The experiments were performed on 28- to 34-day-old Sprague Dawley rats. The procedures with regard to the preparation of horizontal spinal cord slices, the collection of spinal perfusate samples and amino acid determination by HPLC, were similar to those described in Sections I, II and III (Kangrga et al., 1990a,b; Kangrga and Randić, 1990). Selective δ-receptor agonists, (D-Ala^2,D-Leu^5)enkephalin (DALEA, Cambridge Research Biochemicals, CRB) and (D-Pen^2,D-Pen^5)enkephalin (DPDPE, CRB), and selective κ-receptor agonists U50488H (Upjohn Diagnostics) and dynorpnin$_{1-13}$ (CRB), were added to the bathing perfusion.

Results

Effects of a δ-receptor agonist, DALEA, were tested on the basal efflux of aspartate and glutamate. Superfusion of three different slices

with DALEA (0.5-5.0 μM for 3 min) produced no consistent change in the outflow of endogenous aspartate and glutamate.

Effects of electrical stimulation of lumbar dorsal rootlets (L 4 and 5) (25-30 V, 1 ms, 1 Hz, 300 pulses) on the efflux of endogenous glutamate and aspartate from the spinal slice were tested in the absence and the presence of DPDPE (10 μM for 6 min) in the superfusing medium. No significant effect of DPDPE on the dorsal root stimulation-evoked outflow of glutamate and aspartate was observed in three different slices.

The effect of a selective κ-receptor agonist U-50488H on the dorsal root stimulation-evoked outflow of glutamate and aspartate was examined in two slices. The increase in the basal rate of glutamate and aspartate outflow evoked by dorsal root stimulation (25-30 V, 1 ms, 1 Hz, 300 pulses) observed in the absence of U50488H was not altered when the agent (10 μM for 6 min) was present in the superfusing medium. The effects of DPDPE and U-50488H on the dorsal root stimulation-evoked outflow of glutamate and aspartate are summarized in Table 1.

Table 1. Summary[a] of the effects of superfusion of apinal cord slices with DPDPE and U50488H on the dorsal root stimulation-evoked outflow of endogenous glutamate and aspartate.

		Control		Peptide	
	n	Glutamate	Aspartate	Glutamate	Aspspartate
DPDPE (10 μM for 6 min)	3	154.8±19.5	204.5±50.2	125.3±9.5	244.6±47.9
U-50488H (10 μM for 6 min)	2	164.2±45.4	168.2±9.8	170.6±32.9	159.9±31.5

[a]Results are presented as mean percentages ± SEM of the basal efflux.

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SECTION IV. EFFECTS OF PHORBOL ESTERS ON THE BASAL AND EVOKED RELEASE OF PUTATIVE ENDOGENOUS AMINO ACID NEUROTRANSMITTERS FROM THE RAT SPINAL DORSAL HORN[1]

[1]This section represents experimental results obtained by I. Kangrga that constitute a part of a published research paper by G. Gerber, I. Kangrga, P. D. Ryu, J. S. A. Larew and M. Randić. 1989. J. Neurosci. 9: 3606-3617.

INTRODUCTION

It is presently an accepted idea that hydrolysis of membrane phosphoinositides is one means by which some neurotransmitters may mediate their actions at synapses (Nishizuka, 1984, 1986; Berridge, 1987). One of the products of inositol phospholipid metabolism is 1,2- diacylglycerol (DAG), which has been shown to activate protein kinase C (PKC) by increasing the affinity of the enzyme for Ca^{2+} ions and phospholipids in such a manner that it becomes fully active without a net increase in the intracellular concentration of Ca^{2+} (Takai et al., 1979; Kishimoto et al., 1980; Nishizuka, 1984, 1986). This action of diacylglycerol is mimicked by membrane-permeant, tumor-promoting phorbol esters (Castagna et al., 1982). Phorbol esters seem to cause a dramatic shift in the intracellular localization of PKC in a variety of cell types showing an increase in membrane-associated kinase activity and a decrease in the cytosolic and soluble fractions. There is strong evidence that the cellular receptor for the phorbol esters is C-kinase (Niedel et al., 1983; Sando and Young, 1983). When activated by diacylglycerol, or the phorbol esters, the C-kinase phosphorylates specific substrate proteins that contribute to various cellular processes including neurotransmitter release (Wu, et al., 1982; Gispen et al., 1985; Nichols et al., 1987) and receptor transducing mechanisms (Sorensen et al., 1981; Kristjansson et al., 1982; Rodnight and Perrett, 1986; Sladeczek, 1988). PKC is present in high concentrations in the mammalian brain (Inoue et al., 1977; Takai et al., 1979), where it shows differential regional and cellular localization, with high levels in presynaptic terminals (Girard et al., 1985; Wood et al., 1986; Worley et

al., 1986a,b; Mochly-Rosen et al., 1987). It is now clear that PKC is a family of closely related but distinct enzymes (Huang et al., 1986), found in neuronal and glial cells (Mochly-Rosen et al., 1987).

The finding that the spinal dorsal horn contains high levels of binding sites for phorbol esters (Mantyh et al., 1984) and that PKC is present in the rat spinal dorsal horn (Worley et al., 1986a; Mochly-Rosen et al., 1987) raised the possibility that PKC may play a functional role in sensory transmission both in the release of putative neurotransmitters, and also in the signal transduction at various subclasses of excitatory amino acid receptors. Since PKC activation can be mediated directly by phorbol esters, in the absence of phosphoinositide breakdown, we used these agents to examine the effects of the enzyme activation on basal and evoked release of endogenous excitatory (glutamate, aspartate) and inhibitory (gamma-aminobutyric acid, glycine) amino acids. Preliminary reports of some aspects of this work have been published (Gerber et al., 1988).

METHODS

Slices were obtained from Sprague-Dowley rats (14-32 days old) by using a technique that has been described in detail elsewhere (Murase and Randic, 1983, Urban and Randic, 1984). After the animal was anesthetized with ether a segment of the lumbosacral (L5-S1) spinal cord was dissected out and sectioned with a Vibratome to yield one horizontal slice, 300-400 μm thick, with attached dorsal roots and ganglia. After the incubation for one hour in oxygenated (95% O_2 + 5% CO_2) control Krebs solution (in mM): NaCl, 124; KCl,5; KH_2PO_4, 1.2; $CaCl_2$,2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26, glucose, 10, pH 7.4 at 30 ± 1 °C, a slice was placed in one compartment of the 2-compartment chamber where it was submerged beneath an oxygenated superfusing medium (total volume 1ml) containing lowered concentration of potassium ions (1.9 mM KCl). The dorsal roots with attached dorsal root ganglia were placed into the second compartment and immersed under the mineral oil. Lubriseal (Thomas Scientific) was used to ensure a leak-proof, and also electrical isolation between the two compartments. The dorsal roots were placed on two pairs of bipolar platinum electrodes; the distal pair was used for electrical stimulation and the proximal pair for recording of compound action potentials of the primary afferent fibers. The compound action potentials were monitored throughout the periods of stimulation and stored in a Tektronix (5113) oscilloscope and later photographed. Samples of perfusate (1 ml) were collected at regular 10 min intervals before, during and after stimulation of the dorsal roots and/or phorbol ester application. Samples were kept frozen at -80°C until the derivatization and chemical analysis. Phorbol esters were applied into the

slice perfusate for 10 min in known concentrations. The amino acid content in the samples was determined by high performance liquid chromatography (HPLC) with fluorescence detection (Lindroth and Mopper, 1979). Prior to injection, aliquots of the perfusate were derivatized with o-phthaldialdehyde (OPA) 2-mercaptoethanol reagent. Ethanolamine was added to each sample as an internal standard. Chromatography was performed on a 15cm Adsorbasphere-OPA-HR column (Alltech Associates, Deerfield, IL) using a pH 5.9 sodium acetate/tetrahydrofuran/methanol gradient. Fluorescence was detected with a Kratos FS 950 fluorimeter. The amino acids measured came off the column in the following order: aspartate, glutamate, asparagine, serine, glutamine, glycine, threonine, alanine and γ-aminobutyric acid (GABA). Results reported are the average of duplicate runs with each run lasting 31 minutes.

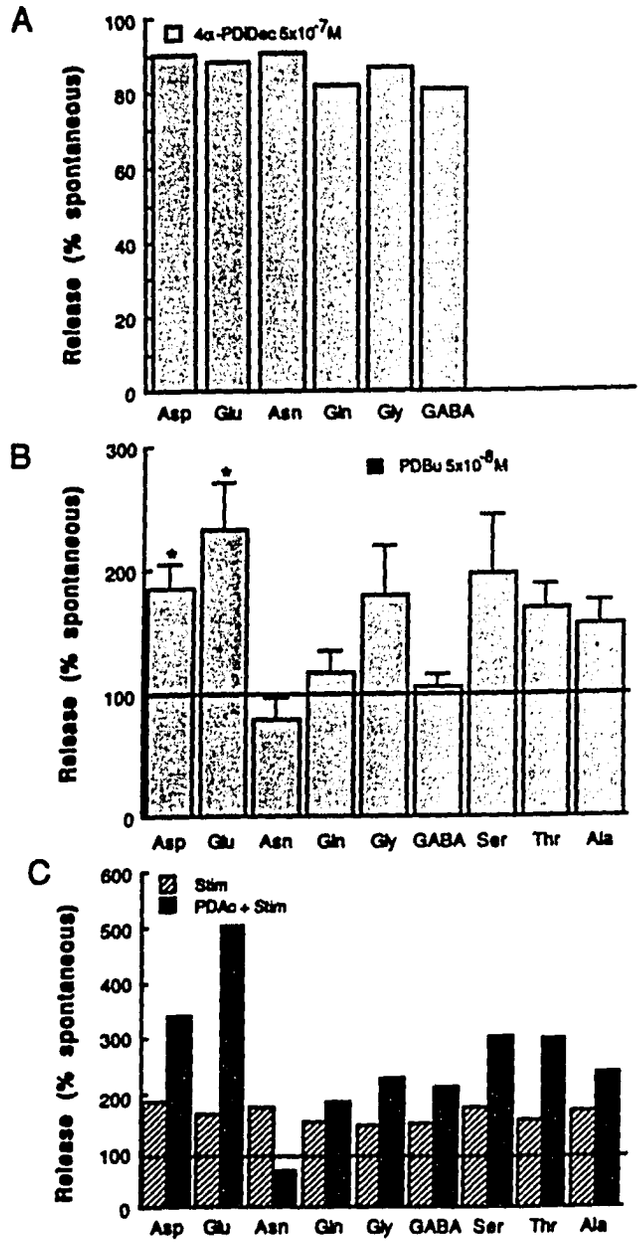
Stock solutions of phorbol esters (Sigma Chemical Co., St. Louis, Mo), 4β-phorbol-12, 13- dibutyrate (PDBu), and 4α-phorbol-12, 13-didecanoate (4αPDiDec) of 10^{-3}M were made in dimethyl sulfoxide, or in distilled water for 4β-phorbol-12-13-diacetate (PDAc), and then frozen in aliquots to be used in single experiments. The aliquots were diluted in oxygenated Krebs solution prior to bath administration. Results are expressed as the mean percent ± SEM of the basal outflow determined as the average of 3 samples collected prior to stimulation and/or phorbol ester application. The statistical difference was determined by paired Student's t test: $^{*}p<0.05$.

RESULTS

Presynaptic modulation of the release of endogenous amino acids by phorbol esters

The electrophysiological data indicate that phorbol esters can induce or increase the release of putative neurotransmitters in the rat spinal dorsal horn (Gerber et al., 1988, 1989). Since excitatory synaptic transmission is augmented in the dorsal horn by phorbol esters (Gerber et al., 1987, 1989), we examined the effects of phorbol esters on the release of nine endogenous amino acids, including putative excitatory neurotransmitters (glutamate, aspartate) and putative inhibitory neurotransmitters (gama-aminobutyric acid and glycine). Addition of $5x10^{-7}$M 4αPDiDec, a phorbol ester analog that does not activate PKC, had no effect on the basal release of six endogenous amino acids (Fig. 1A). However, in the presence of an active phorbol ester, PDBu ($5x10^{-8}$M), a significant but transient (10 min) increase in the basal release of endogenous glutamate and aspartate (Fig. 1B) was observed ($p>0.05$, n=4). In addition, the rates of release of glycine, serine, threonine and alanine were also elevated (n=4). Another active phorbol ester, PDAc (5×10^{-7}M), produced a marked but transient increase in the dorsal root stimulation-evoked release of endogenous glutamate and aspartate (Fig. 1C, n=2) from the spinal cord slice. The rates of release of glycine, GABA, serine, threonine and alanine were also elevated but to a smaller degree. Although the basal release of asparagine was little modified (Fig. 1B), the evoked release was reduced (Fig. 1C).

Fig. 1. Phorbol esters enhance both basal and evoked release of endogenous amino acids. Column graphs of amounts of rates of release of endogenous amino acids during administration of phorbol esters in the rat horizontal spinal cord slice preparation are expressed as percentages of the average values measured during the 3 rest periods preceding the treatment. A, Addition of 4α-PDiDec (5×10^{-7}M for 10 min), a phorbol ester analog that does not activate PKC, had no effect on the basal release of 6 endogenous amino acids. B, Bath applied PDBu (5×10^{-8}M for 10 min) enhances the resting release of glutamate (Glu) and aspartate (Asp) in the spinal cord slice. In addition, the rates of release of glycine (Gly) serine (Ser), threonine (Thr), and alanine (Ala) were also elevated. The bars represent the SEM (n=4). Significant ($p<0.05$) changes are marked with asterisks. C, Phorbol esters produced a marked but transient increase of the dorsal root stimulation-evoked release of endogenous glutamate and aspartate. The rates of release of glycine, GABA, serine, threonine and alanine were also elevated but to a smaller degree. A lumbar dorsal root was electrically stimulated (25 V, 40 μsec, 5 Hz for 5 min) either in the absence (hatched columns) or in the presence of bath- applied PDAc (5×10^{-7}M, solid columns). Amounts of rates of release of various amino acids obtained during the stimulation periods are expressed as percentages of the average values measured during the rest 3 periods preceding stimulation.


A
4α-PDDec 5x10⁻⁷M
Release (% spontaneous)
100
80
60
40
20
0
Asp Glu Asn Gln Gly GABA
B
PDBu 5x10⁻⁸M
Release (% spontaneous)
300
200
100
0
Asp Glu Asn Gln Gly GABA Ser Thr Ala
C
Stim
PDAc + Stim
Release (% spontaneous)
600
500
400
300
200
100
0
Asp Glu Asn Gln Gly GABA Ser Thr Ala

DISCUSSION

One mechanism that could account for the enhancement of excitatory synaptic transmission produced by phorbol esters in the spinal dorsal horn (Gerber et al., 1987, 1988, 1989) is that the nerve terminals increase secretion of neurotransmitters on PKC activation as do other secreting non-neural (Knight and Baker, 1983; Pocotte et al., 1985) and neural cells (Tanaka et al., 1984; Osses et al., 1986; Feuerstein et al., 1987; Haimann et al., 1987; Shapira et al., 1987; Versteeg and Florijn, 1987). In this study, we have presented neurochemical data showing that phorbol esters enhance the basal and the depolarization-evoked release of endogenous amino acids, including putative excitatory (glutamate and aspartate) and inhibitory (GABA, glycine) synaptic mediators from the rat dorsal horn slices. Enhancement in basal (Publicover, 1985; Eusebi, et al., 1986; Aniksztejn et al., 1987; Malenka et al., 1987) and evoked release of various putative transmitters (glutamate, acetylcholine, dopamine, norepinephrine, serotonin) caused by phorbol esters has been also reported (Zurgil and Zisapel, 1985; Allgaier et al., 1986, 1988; Allgaier and Hertting, 1986; Lynch and Bliss, 1986; Feuerstein et al., 1987; Shapira et al., 1987). Furthermore, oleoyl-acetylglycerol, another activator of PKC, enhances release of glutamate from synaptosomes derived from hippocampus (Lynch and Bliss, 1986). In agreement with these data are the findings that phorbol esters enhance the frequency of "spontaneous" EPSPS in the spinal dorsal horn neurons (Gerber et al., 1989), rat hippocampal neurons (Malenka et al., 1987), and of miniature end-plate potentials at the frog neuromuscular junction (Publicover, 1985; Eusebi, 1986).

The effects of phorbol esters on neurotransmitter release appear to be mediated by the activation of PKC since inactive phorbol analogs have no effect on synaptic transmission (Kuo et al., 1980; Castagna et al., 1982), however, an action of phorbol esters independent of PKC cannot be ruled out entirely (Bell et al., 1985; Hollingsworth et al., 1985; Fink et al., 1988). Modulatory effects of phorbol esters, such as blockade of the uptake systems for neurotransmitters or interference with the autoreceptor-mediated negative feedback circuits, have been reported.

Although, our results suggest that in the rat spinal dorsal horn protein kinase C may have a role in controlling the release of putative excitatory and inhibitory neurotransmitters, the source of amino acids release and the identity of endogenous substances participating in this effect is presently unknown. The increased release of endogenous amino acids is consistent with a presynaptic (terminal) site of action, but it also could be explained by enhanced interneuronal activity.

The precise biochemical mechanism underlying the enhancement of transmitter release produced by phorbol esters is as yet not clear. There is evidence that kinase C activation enhances the Ca^{2+}-sensitivity of the secretory process in non-neural cells (Knight and Baker, 1983; Pocotte et al., 1985), although it is not clear whether the enzyme is a mediator or modulator of secretion. It has been also shown in non-neural cells (Rink et al., 1983, Di Virgilio et al., 1984) that activation of PKC by phorbol esters or 1,2-oleoyl-acetylglycerol (OAG) can stimulate secretion without raising cytosolic Ca^{2+} levels. The evidence for the role of PKC in neurotransmitter release is less compelling. Augustine et al.(1986) suggested that the enhancing effect of kinase C activation at the squid

giant synapse may be due to a broadening of the presynaptic action potential, because of a decrease in K^+ conductance. Since the potentiation of synaptic transmission was absent when the presynaptic terminal was voltage-clamped it was suggested that kinase C is not a mediator, but a modulator, of a transmitter release. The spike broadening effect of phorbol esters seen in the rat dorsal horn cells in our work (Gerber et al., 1989), and also in the CA1 hippocampal pyramidal cells (Storm, 1987) may contribute to the enhancement of synaptic transmission caused by phorbol esters, provided a similar mechanism operates in the synaptic terminals of primary afferent fibers. Thus, a prolonged spike in the synaptic terminal will allow more influx of Ca^{2+} ions during the action potential and this will in turn lead to the increased release of transmitter. Other possible mechanisms involved include diminished sequestration of internal Ca^{2+}, or its release from internal stores (Nishizuka, 1986), increase of Ca^{2+} influx through voltage-dependent Ca^{2+} channels (De Riemer et al., 1985; Lipscombe et al. 1988; but see Rane and Dunlap, 1988) or changes in the properties of the Ca^{2+}-activated K^+-channels (Baraban et al., 1985; Malenka et al., 1986b).

The phosphorylation of nerve terminal proteins involved in exocytotic release, may be a mechanism of the PKC-augmenting action in the neurotransmitter release. The presence of a presynaptically located PKC (Girard et al., 1985), and the phosphorylation of several brain proteins, including an 87kDa substrate by PKC during depolarization has been demonstrated (Wu et al., 1982). Phosphorylation of the 87 kDa substrate by phorbol ester-activated PKC in synaptosomes occurs in parallel with the enhancement of stimulation-elicited neurotransmitter release. In contrast,

the inactive 4α-phorbol esters were without effect either on release or phosphorylation (Nichols et al., 1987). In addition, high-K^+-evoked increase in neurotransmitter release in the hippocampus correlates well with the degree of phosphorylation of B-50 protein (Versteeg and Florijn, 1987).

In summary, we would like to suggest that our finding of the enhancement of the basal and the stimulation-evoked release of putative excitatory and inhibitory synaptic mediators from rat dorsal horn slices caused by phorbol esters indicates that PKC may be involved in the presynaptic modulation of the basal and the depolarization-evoked neurotransmitter release.

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SECTION V. ACTIONS OF (-)-BACLOFEN ON RAT DORSAL HORN NEURONS[1]

[1]Submitted to Brain Research as a research paper by I. Kangrga and M. Randić. 1991.

SUMMARY

The actions of a $GABA_B$ agonist, (-)-baclofen, on the electrophysiological properties of neurons and synaptic transmission in the spinal dorsal horn (laminae I-IV) were examined by using intracellular recordings in spinal cord slice from young rats. In addition, the effects of baclofen on the dorsal root stimulation-evoked outflow of glutamate and aspartate from the spinal dorsal horn was examined by using high performance liquid chromatography (HPLC) with flourimetric detection. Superfusion of baclofen (5nM to 10μM) hyperpolarized, in a stereoselective and bicuculline-insensitive manner, the majority (86%) of tested neurons. The hyperpolarization was associated with a decrease in membrane resistance and persisted in a nominally zero-Ca^{2+}, 10mMg^{2+}- or a TTX-containing solution. Our findings indicate that the hyperpolarizing effect of baclofen is probably due to an increase in conductance to potassium ions. Baclofen decreased the direct excitability of dorsal horn neurons, enhanced accommodation of spike discharge, and reduced the duration of Ca^{2+}-dependent action potentials. Baclofen depressed, or blocked, both excitatory (EPSPs) and inhibitory postsynaptic potentials (IPSPs) evoked by electrical stimulation of the dorsal roots. Spontaneously occuring EPSPs and IPSPs were also reversibly depressed by baclofen. Whereas baclofen did not produce any consistent change in the rate of the basal outflow of glutamate and aspartate, the stimulation-evoked release of the amino acids was blocked. The present results suggest that baclofen, by activating $GABA_B$ receptors, may modulate spinal afferent processing in the superficial dorsal horn by at least two mechanisms: 1. Baclofen depresses

excitatory synaptic transmission primarily by a presynaptic mechanism involving a decrease in the release of excitatory amino acids, and 2. At higher concentrations, the hyperpolarization and increased membrane conductance may contribute to the depressant effect of baclofen on excitatory and inhibitory synaptic transmission in the rat spinal dorsal horn.

INTRODUCTION

Baclofen, a β-p-chlorophenyl analogue of γ-aminobutyric acid (GABA), is a highly selective agonist at $GABA_B$ receptors (Bowery et al., 1980, 1984; Bowery, 1989). Baclofen inhibits neurotransmission at peripheral (Peng and Frank, 1989) and central excitatory and inhibitory synapses (Lanthorn and Cotman, 1981; Ault and Nadler, 1982; Olpe et al., 1982; Inoue et al., 1985a; Howe et al., 1987; Connors et al., 1988), including primary afferent terminals in the spinal cord (Pierau and Zimmermann, 1973; Fox et al., 1978; Jeftinija et al., 1986, 1987; Kangrga et al., 1987). Pre- and postsynaptic inhibitory effects of baclofen (Bowery et al., 1980; Ault and Nadler, 1982; Olpe et al., 1982; Newberry and Nicoll, 1984a,b, 1985; Gähwiler and Brown, 1985) have been demonstrated. Presynaptically, baclofen is known to inhibit the release of neurotransmitters such as excitatory amino acids (Potashner, 1979; Johnston et al., 1980; Collins et al., 1982; Huston et al., 1990), noradrenaline and dopamine (Bowery et al., 1980), peptides (Bowery, 1989), as well as GABA, via GABA autoreceptors (Neal and Shah, 1989). The presynaptic inhibitory action may involve a reduction in calcium entry (Dunlap, 1981; Dunlap and Fischbach, 1981; Cherubini and North, 1984; Heinemann et al., 1984; Schlichter et al., 1984; Dolphin and Scott, 1986; Green and Cottrell, 1988). In addition, baclofen has a potent postsynaptic hyperpolarizing action (Misgeld et al., 1984; Newberry and Nicoll, 1984; Pinnock, 1984; Jeftinija et al., 1986; Kangrga et al., 1987; Osmanovic and Shefner, 1988) involving an increase in conductance for potassium ions (Gähwiler and Brown, 1985; Inoue et al., 1985b; Newberry and Nicoll, 1985; Howe et al., 1987; Lacey et al., 1988).

Available evidence supports the concept that excitatory amino acids, glutamate and aspartate, are the principal excitatory neurotransmitter candidates of the primary afferent fibers (Mayer and Westbrook, 1987; Evans, 1989; Kangrga et al., 1990a,b; Kangrga and Randić, 1991), whereas GABA, acting at $GABA_A$ and $GABA_B$ receptors, has been implicated in presynaptic and postsynaptic inhibitory transmission in the spinal cord (Eccles et al., 1963; Curtis et al., 1971, 1977; Randić, 1981; Nedeljkov and Randić, 1982; Bowery, 1989). Levels of GABA (Miyata and Otsuka, 1972; Hunt et al., 1981; Kaduri et al., 1987, Magoul et al., 1987; Todd and McKenzie, 1989) and the GABA synthesizing enzyme, glutamate decarboxylase (GAD) (Graham and Apprison, 1969, McLaughlin et al., 1975; Barber et al., 1978, 1982), are high in the superficial laminae (I-III) of the dorsal horn, an area where primary afferent fibers terminate (Light and Perl, 1979). Some GAD-positive terminals are presynaptic to primary afferent terminals (Barber et al., 1982) but others make axodendritic or axosomatic synapses with dorsal horn interneurons (McLaughlin et al., 1975; Barber et al., 1978, 1982, Magoul et al., 1987). Although a subpopulation of primary sensory neurons seems to contain GABA (Roy and Philippe, 1989), the major source of GABA- and GAD-immunoreactivity in the superficial dorsal horn is of interneuronal origin, the islet cells being the main neuronal source (Todd and McKenzie, 1989). High density of $GABA_B$ but not $GABA_A$ receptors has been demonstrated in the superficial dorsal horn (Bowery et al., 1987) and neonatal capsaicin treatment reduces the density of this receptor band by 40 to 50% (Price et al., 1984).

Early studies have demonstrated that intravenous or iontophoretic application of (-)-baclofen has a potent depressant effect on spontaneous

and evoked discharge of motoneurons and interneurons (Pierau and Zimmermann, 1973; Curtis et al., 1974, 1981; Henry and Ben-Ari, 1976; Fox et al., 1978; Ono et al, 1979; Davies, 1981; Henry, 1982; Curtis and Malik, 1985) in the cat spinal cord, without altering their passive membrane properties or responsiveness to substance P or glutamate (Pierau and Zimmermann, 1973; Fox et al., 1978). These results suggested a presynaptic action of (-)-baclofen and were supported by the findings that (-)-baclofen or GABA, acting at $GABA_B$ receptors, decrease the duration of calcium action potential (Dunlap, 1981, 1984; Dunlap and Fischbach, 1981; Desarmenien et al., 1984; Schlichter et al., 1984) and inhibit voltage-dependent calcium currents (Dunlap and Fischbach 1981; Dolphin and Scott, 1986; Green and Cottrell, 1988) in the primary sensory neurons. In addition, a direct hyperpolarizing effect of (-)-baclofen on neurons in the superficial (Yoshimura and Jessel, 1989) and deep dorsal horn (Jeftinija et al., 1986, 1987, Kangrga et al, 1987; Allerton et al., 1989), as well as in motoneurons (Wang and Dun, 1990), has been reported. Existence of two pharmacologically distinct types of $GABA_B$ receptors in the hippocampus (Dutar and Nicoll, 1988) and in the spinal cord (Kerr et al., 1987; Wang and Dun, 1990) has been recently suggested. A phosphonic derivative of baclofen, phaclophen, selectively blocked the (-)-baclofen-caused reduction of monosynaptic excitation of spinal interneurons (Kerr et al., 1987) and excitatory and inhibitory synaptic potentials of spinal motorneurons (Wang and Dun, 1990).

In the present experiments, we have attempted to further characterize the presynaptic and postsynaptic actions of baclofen in the rat spinal dorsal horn (laminae I-IV) by examining the effects of this agent on basal

and evoked release of glutamate and aspartate, on passive and active membrane properties of rat spinal dorsal horn neurons and on excitatory and inhibitory synaptic transmission. Some aspects of this work have already been communicated (Jeftinija et al., 1986, 1987; Kangrga et al., 1987).

METHODS

Horizontal spinal cord slices were obtained from 13- to 35-day-old Sprague Dawley rats by using a technique described previously (Gerber et al., 1989, Murase and Randić, 1983; Urban and Randić, 1984). After the animal was anesthetized, a segment of the lumbosacral (L3-S1) spinal cord was dissected out and sectioned along the longitudinal axis with a Vibratome to yield one 300 to 400μm thick horizontal slice with dorsal roots and/or dorsal root ganglia (DRG), and a length of the sciatic nerve, attached. After 1 hr incubation in oxygenated (95%O_2 + 5%CO_2) control solution (in mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; pH 7.4 at 30 ± 1^0 C, the slice was transferred into a recording chamber where it was continuously perfused at 3 to 4 $mlmin^{-1}$ with oxygenated modified control medium (containing 1.9 mM KCl, all other salts were unchanged). Conventional electrophysiological techniques were used for intracellular recording from and stimulation of neurons in the dorsal horn (laminae I-IV). Neurons were impaled with fiber-filled glass microelectrodes that contained 4 M potassium acetate (pH 7.2) and had DC impedances of 80-120 MΩ, by oscillating the capacity compensation circuit of the amplifier (Neurodata, IR 281). Dorsal horn neurons were activated either directly with a DC current injection (0.2 to 2 nA) via the bridge circuit, or synaptically by electrical stimulation of the dorsal rootlets (L4 to L6) with bipolar platinum electrodes. Data were recorded on a Gould Brush pen recorder (model 220) or stored on floppy discs by a Nicolet digital oscilloscope (model 4092) until processed and printed out onto a digital plotter.

In the release experiments, after the incubation, a slice was placed in one compartment of the 2-compartment chamber and continuously perfused with oxygenated modified Krebs solution (1.9 mM KCl) at 0.3-0.5 $mlmin^{-1}$. The dorsal roots were led across a leak-proof partition of vaseline into a compartment filled with mineral oil and placed on the 2 pairs of bipolar platinum electrodes: the distal pair was used for electrical stimulation of the primary afferent fibers, and the proximal pair for recording of the compound action potentials. Samples of perfusate were collected at regular 3 min intervals prior to, during, and after stimulation of the dorsal roots and/or bath application of (-)-baclofen. Samples were kept frozen at -80^0C until derivatization and chemical analysis. These experiments were performed in the presence of bicuculline methiodide (10 μM) in order to block activity at $GABA_A$ receptors. L-aspartic acid β-hydroxamate (50 μM) was used in two experiments in order to assess the contribution of neuronal and glial uptake to the outflow of glutamate and aspartate from the spinal slice. Quantification of endogenous glutamate and aspartate contained in the spinal perfusate was achieved by reverse-phase high-performance liquid chromatography with o-phthaldialdehyde-2-mercaptoethanol precolumn derivatization and fluorimetric detection (Lindroth and Mopper, 1979). The chromatography was performed using a Beckman liquid chromatograph with on-line analysis (System Gold). Results are presented as mean ± SEM. Statistical significance has been assessed relative to control conditions, by use of a paired Student's t test. Levels of significance are indicated as follows: $^*p<0.05$; $^{**}p<0.01$.

(-)- and (+)-baclofen (CIBA-GEIGY Corp.), bicuculline methiodide (Sigma), L-aspartate-β-hydroxamate, tetrodotoxin (Sigma) and

tetraethylammonium (Aldrich Chemical Co.) were applied by bath perfusion in known concentrations.

RESULTS

Effects of baclofen on membrane potential and neuronal input resistance

A total of 49 neurons in laminae I-IV of the spinal dorsal horn were examined in this study. The average resting membrane potential of these neurons was -66.7±0.8mV (mean±S.E.M.) and the input resistance measured by hyperpolarizing pulses (-0.2 to -0.8 nA of 100 ms duration) 74.3±5.2 MΩ.

Bath application of (-)-baclofen (5 nM to 10 μM for 1 min) produced hyperpolarization (-5.3±0.5 mV; range: -1 to -15 mV,) in 43 of 49 dorsal horn neurons. A complete recovery of the membrane potential following the application of baclofen took 5.9 ± 1.1 (n=20) min and was observed in a majority of tested neurons. The hyperpolarization to baclofen was a direct action on the postsynaptic membrane of a dorsal horn neuron since it could be elicited when synaptic transmission was blocked by TTX (n=7; Fig. 1A) or in a nominally Ca^{2+}-free, 10 mM Mg^{2+}-containing medium (n=2; Fig. 1B). In 13 cells the hyperpolarizing response to baclofen (0.1 to 1.0 μM) was followed by a prolonged (2-10 min) depolarization (1-5 mV).

We further examined the responsiveness of dorsal horn neurons to the (-) and (+) optical isomers of baclofen and to a $GABA_A$ receptor antagonist, bicuculline. In two dorsal horn neurons, bath application of 100 and 500 μM (+)-baclofen produced hyperpolarizations of similar magnitude to those evoked by 1 μM (-)-baclofen (Fig. 2A). This result indicates that the (-) isomer was about two orders of magnitude more potent than the (+) isomer in causing the hyperpolarizing response. Next, we tested the effects of the $GABA_A$ receptor antagonist, bicuculline (5 μM), on the baclofen-elicited hyperpolarizing response (n=2). As shown in Fig. 2B, the baclofen

Fig. 1. (-)-Baclofen hyperpolarizes spinal dorsal horn neurons. A, bath application of (-)-baclofen (10^{-6}M, 1min) reversibly hyperpolarized a dorsal horn neuron and depressed spontaneous synaptic activity and spike discharges (upper record). In the presence of TTX ($3x10^{-7}$M) sodium spikes and synaptic activity were eliminated while the baclofen-induced hyperpolarization remained (lower record). B, the hyperpolarizing effect of baclofen in another dorsal horn neuron (upper record) persisted when the slice was perfused with a nominally zero Ca^{2+}/high-Mg^{2+} (10mM) solution (lower record). A, 16-day-old rat, resting membrane potential (Vm) was -69mV. B, 13 day-old rat, Vm, -60 mV.

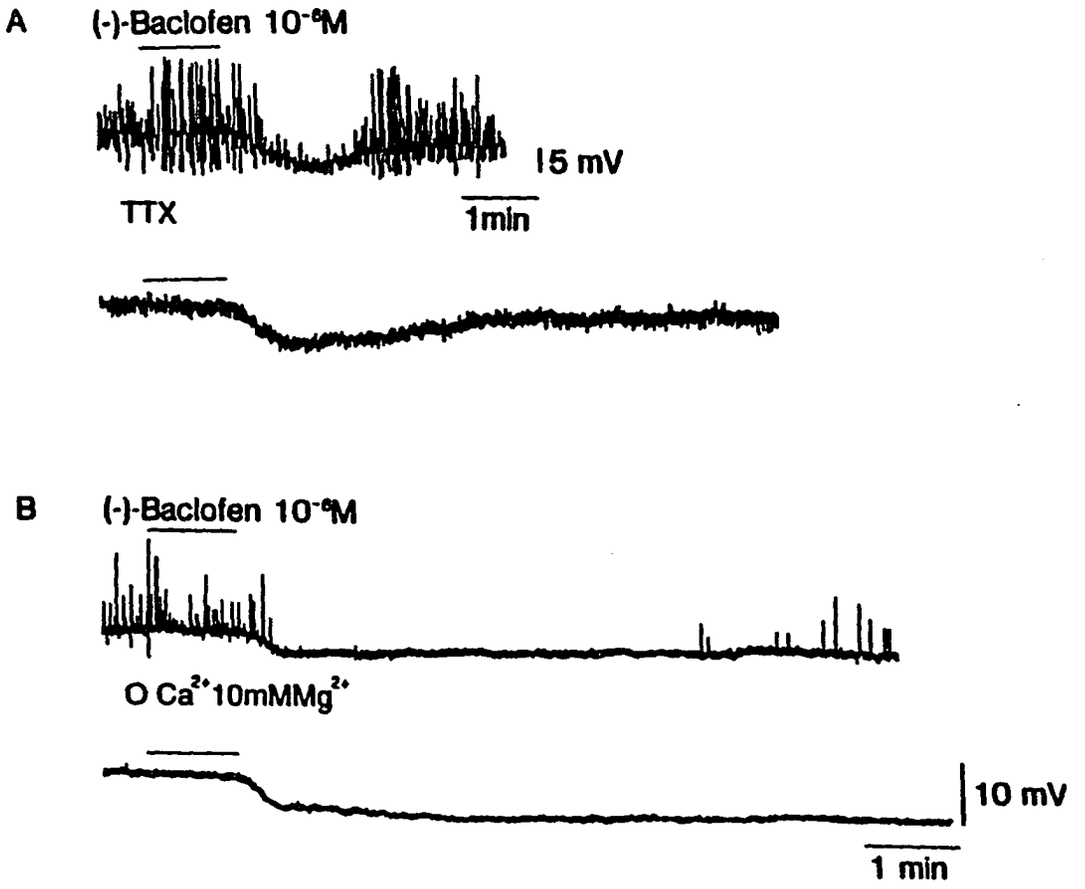

A
(-)-Baclofen 10⁻⁶M
15 mV
TTX
1min
B
(-)-Baclofen 10⁻⁶M
O Ca²⁺10mMMg²⁺
10 mV
1 min

Fig. 2. Baclofen-induced hyperpolarization is stereoselective and resistant to bicuculline. A, two segments from a continuous record comparing 1 min perfusions of the two isomers of baclofen: (-)-baclofen, 1 μM for 1 min; (+)-baclofen, 100 μM for 1 min. B, hyperpolarization of a dorsal horn neuron caused by (-)-baclofen (1 μM for 1 min) in a control medium (upper record) persisted in the presence of (-)-bicuculline methiodide (Bic, 5 μM) (lower record). Perfusion with bicuculline started 2 min before the application of (-)-baclofen. A, 14-day-old rat, Vm, -69 mV. B, 24-day-old rat, Vm, -71 mV.

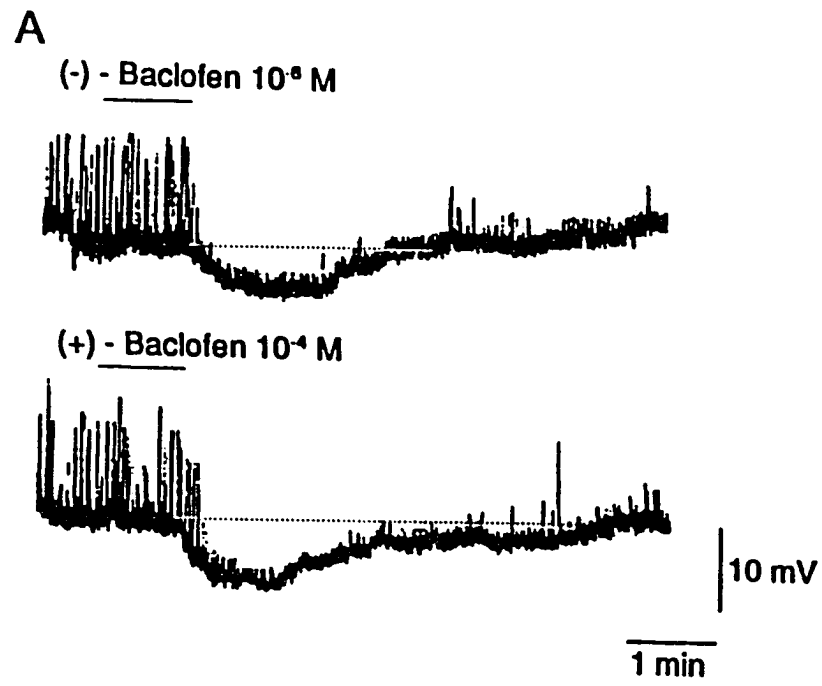
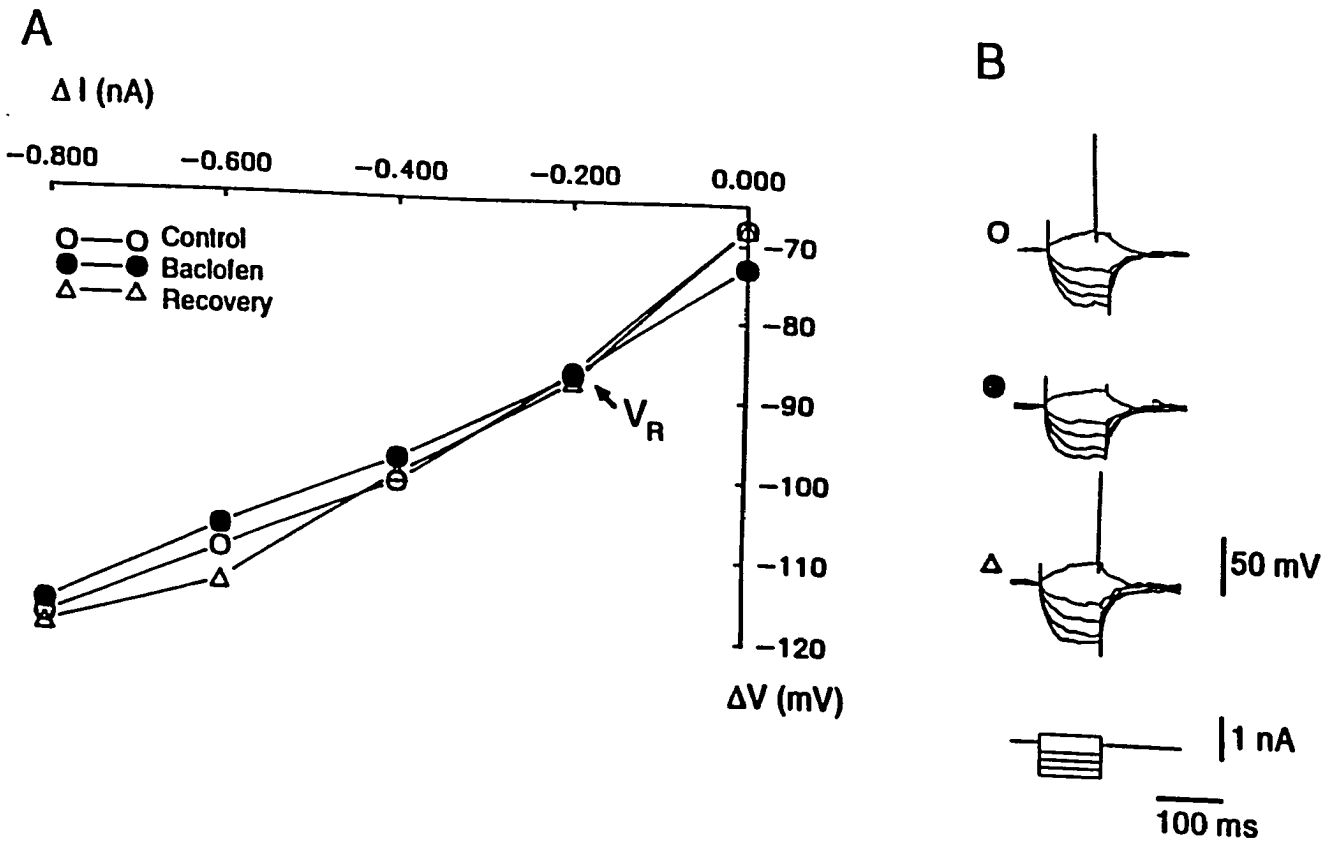

A
(-) - Baclofen 10⁻⁶ M
(+) - Baclofen 10⁻⁴ M
10 mV
1 min
B
(-) - Baclofen 10⁻⁶ M
(-) - Baclofen + Bic
5 mV
1 min

Fig. 3. Typical change in a current-voltage (I-V) relationship of a dorsal horn neuron elicited by (-)-baclofen. A, current-voltage relationship before (open circles), during (solid circles) and 4 min after (open triangles) application of (-)-baclofen (0.1 μM for 1 min). Continuous curves were fitted by eye through control (o), (-)-baclofen ( ) and recovery ( ) data points. As indicated with an arrow, the reversal potential (Vr) for the baclofen response was about - 87 mV. B, intracellularly recorded voltage transients elicited by depolarizing (upward) and hyperpolarizing (downward) current pulses (-0.8 to 0.2 nA of 100 ms duration) applied in progressive steps across the cell soma. 21-day-old rat, Vm, -64 mV.


A
Δ I (nA)
−0.800
−0.600
−0.400
−0.200
0.000
Control
Baclofen
Recovery
−70
−80
−90
−100
−110
−120
V_R
ΔV (mV)
B
50 mV
1 nA
100 ms

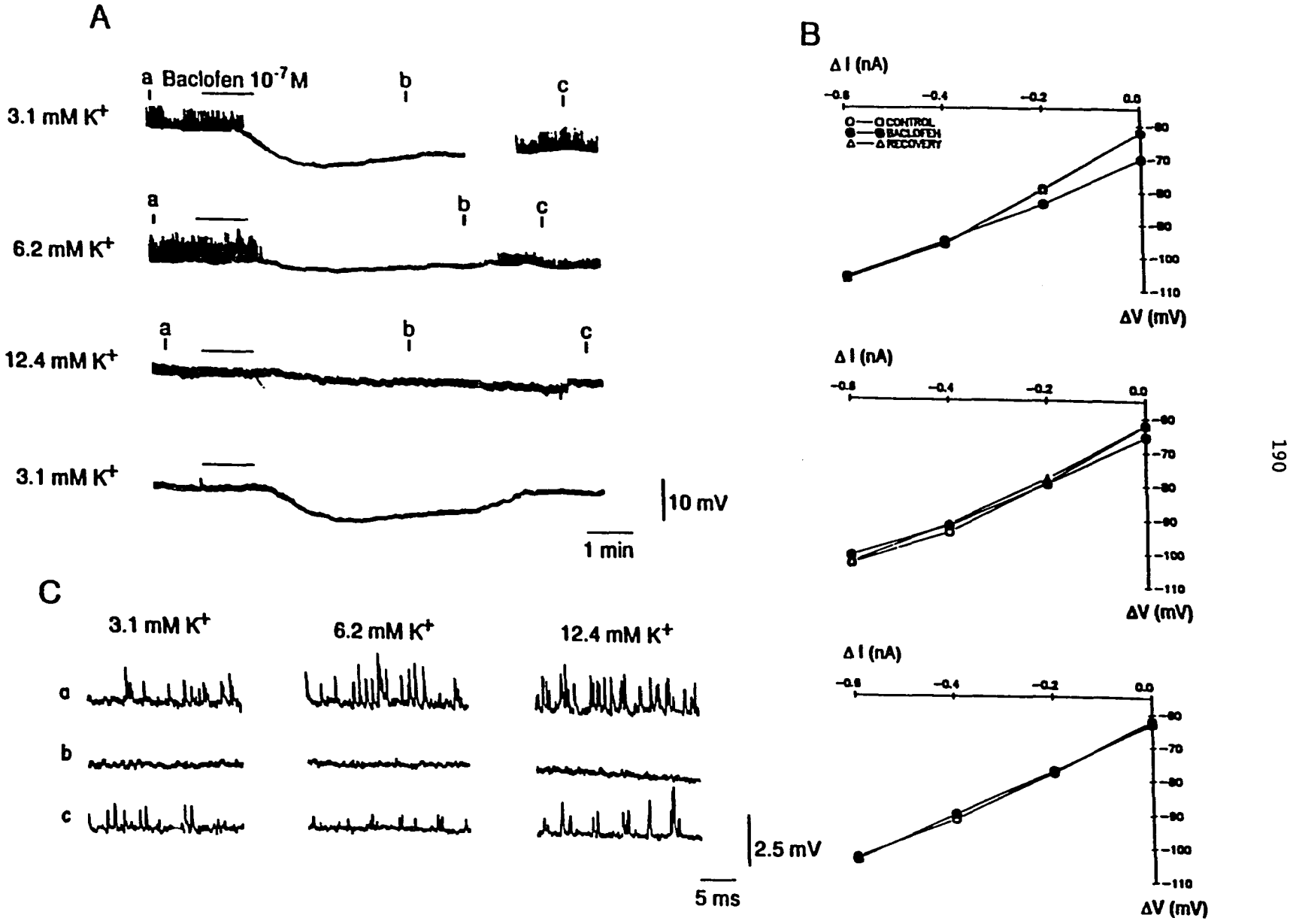
hyperpolarization had a similar time course in the absence and the presence of bicuculline.

Current-voltage relation determinations in control and baclofen-containing (0.01-1.0 μM) solutions were made in twenty-one neurons. In 18 (about 86%) of the examined neurons, a small to moderate decrease in membrane input resistance (12.6±6.4%; range: 5 to 30%) was recorded during the baclofen-induced membrane hyperpolarization (Fig. 3A-B). The observed changes in membrane resistance were not solely a result of the presence of inward rectification in these neurons (Murase et al., 1986), because a significant reduction in the input resistance remained when the membrane potential was shifted back to its pre-application value by injecting positive d.c. current through the microelectrode. Two other observations made can be explained by a reduction in membrane resistance: 1) Depolarizing d.c. injections were associated with a decreased probability of action potential discharge during perfusion with baclofen-containing medium; 2) Anodal break spikes, often observed at the offset points of the hyperpolarizing pulses, were usually blocked by baclofen.

Ionic mechanism

It has been well documented for hippocampal and neocortical cells that the ionic mechanism underlying the baclofen hyperpolarization is an increase in conductance to potassium ions (Gähwiler and Brown, 1985; Inoue et al., 1985b; Newberry and Nicoll, 1985; Howe et al., 1987). In spinal dorsal horn neurons, the mean reversal potential determined from the intersection point of current-voltage relation plots, measured in normal

Fig. 4. Potassium dependence of the baclofen response: dissociation of membrane and synaptic effects. A, hyperpolarizing responses of a dorsal horn neuron to repeated applications of baclofen (0.1 μM for 1 min) in 3.1- (upper record), 6.2- (second record from the top) and 12.4 mM potassium (third record from the top).Recovery (lower record) was recorded 20 min after returning to control medium (3.1 mM K^+). B, I-V curves for a single neuron shown in A, were reconstructed from the records obtained in 3.1- (upper graph), 6.2- (middle graph) and 12.4-mM-potassium (lower graph). A progressive decrease in the slope of the I-V curve occurred in response to baclofen (solid circles) with increasing the external potassium concentration. The estimated reversal potential (Vr) shifted from about -95 mV in 3.1-, to -80 and -71 mV in 6.2 mM- and 12.4 mM-potassium, respectively. 23-day-old rat, Vm, -63 mV. C, the effects of baclofen and altering the external potassium concentration on the spontaneous EPSPs recorded from the same neuron shown in A. The EPSPs were sampled in 3.1-(left records), 6.2-(middle records) and 12.4-mM potassium (right records), before (a), during peak baclofen response (b) and following the recovery (c), of the baclofen effect. Inhibitory effect of baclofen was observed irrespective of the concentrations of potassium used.


A
a Baclofen 10^{-7}M
b
c
3.1 mM K+
6.2 mM K+
12.4 mM K+
3.1 mM K+
10 mV
1 min
B
Δ I (nA)
O—O CONTROL
●—● BACLOFEN
Δ—Δ RECOVERY
ΔV (mV)
C
3.1 mM K+
6.2 mM K+
12.4 mM K+
a
b
c
2.5 mV
5 ms

(3.1 mM K^+), baclofen-containing medium, was -95.7±2.6mV (range: -87 to -107mV, n=7) (Fig. 3A), the finding indicating also the involvement of K^+ ions. We have examined the effects of altering the extracellular potassium concentration on the magnitude and the reversal potential of the baclofen response (Fig. 4). Bath application of baclofen (1 μM for 1 min) in the control medium (3.1 mM K^+) produced a prolonged (over 20 min) hyperpolarization (-8.5 mV) of a dorsal horn neuron (Fig. 4A). Increasing the K^+ concentration to 6.2 and 12.4 mM, markedly reduced and abolished the baclofen response, respectively, and caused a depolarizing shift in the reversal potential (Fig. 4B).

Effects of baclofen on sodium- and calcium-dependent action potentials

It was previously determined that immature rat dorsal horn neurons have both sodium- and calcium-dependent action potentials (Murase and Randic, 1983). In six of eight neurons examined, baclofen (0.05-1.0 μM for 1 min) increased (25-30%) the magnitude of the depolarizing current necessary to elicit an action potential (Fig. 5A). However, the threshold voltage, or the rise-time and duration of sodium-dependent action potentials, were not noticeably changed. Although this effect had a similar time course to the above described changes in the membrane potential and input resistance, it remained when constant current was injected to restore the membrane potential to its control value.

In response to a prolonged depolarizing current pulse (1s) dorsal horn neurons do not fire action potentials at a constant rate. The initial series of spikes is usually accompanied by a reduced rate of firing, i.e., accommodation (Fig. 5B, left trace). Baclofen markedly increased spike

Fig. 5. Baclofen decreases direct excitability and reduces repetitive firing. A, action potentials and synaptic potentials caused by intracellular depolarizing current pulses (0.6nA, 10ms) before (left trace), during the bath application of baclofen (middle trace) and 6 min after returning to control medium (right trace). Baclofen increased the magnitude of the depolarizing current necessary to evoke an action potential (middle trace, not illustrated). Although baclofen hyperpolarized the neuronal membrane (-4 mV in A, left trace), this change in the direct excitability was not solely the consequence of the hyperpolarization, because the effect remained in the cell (A, middle trace) when depolarizing d.c. current was injected to shift membrane potential to its pre-baclofen value. 15 day-old rat, Vm, -63mV. B, examples of repetitive firing in a rat dorsal horn neuron under control conditions (left trace), bath-applied baclofen (1 μM for 1 min, middle trace) and 5 min after the perfusion with baclofen had been discontinued (right trace). Action potentials were evoked by rectangular depolarizing current pulses of 0.8nA and 1s duration. The hyperpolarization caused by baclofen was annulled by passing direct depolarizing current through the recording electrode. 16 day-old rat, Vm, -60mV.

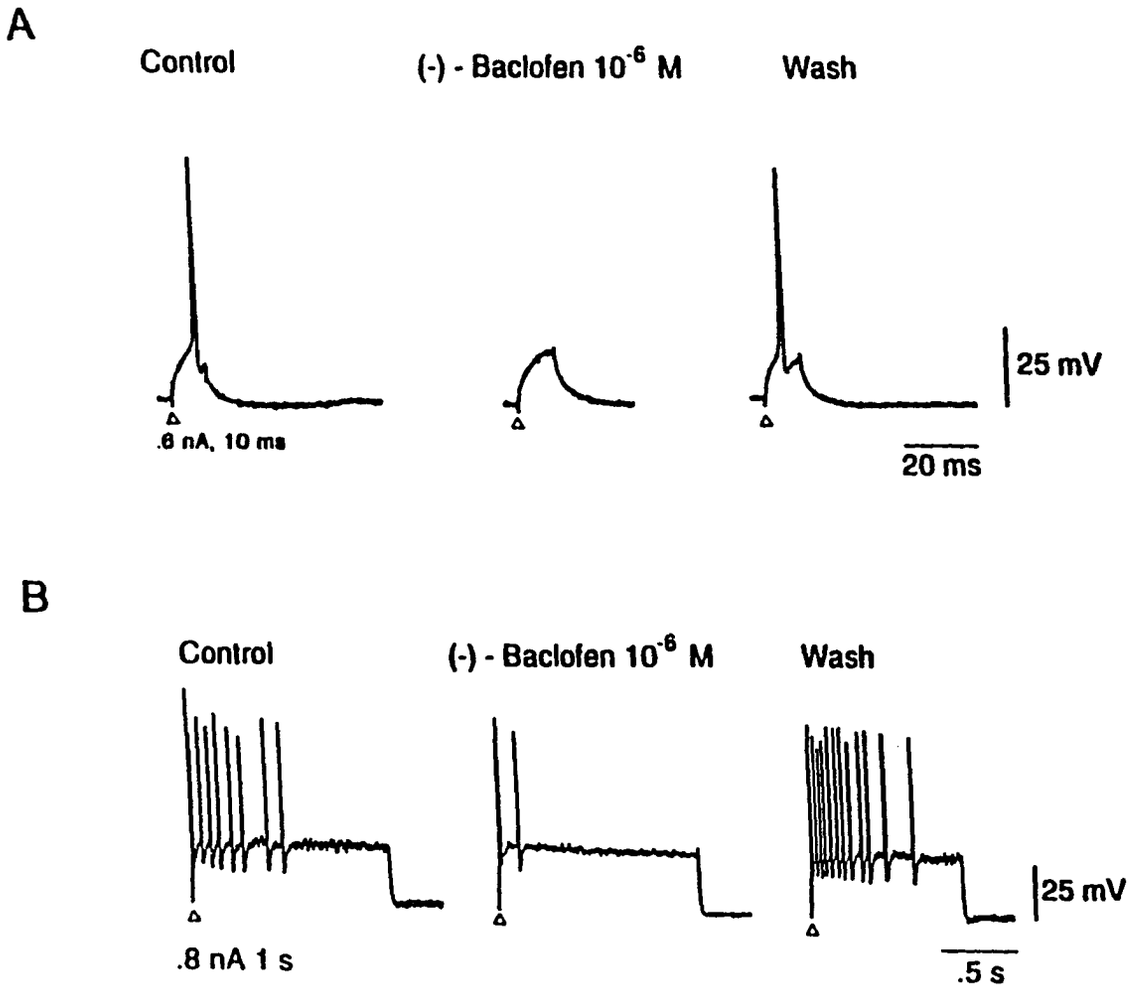
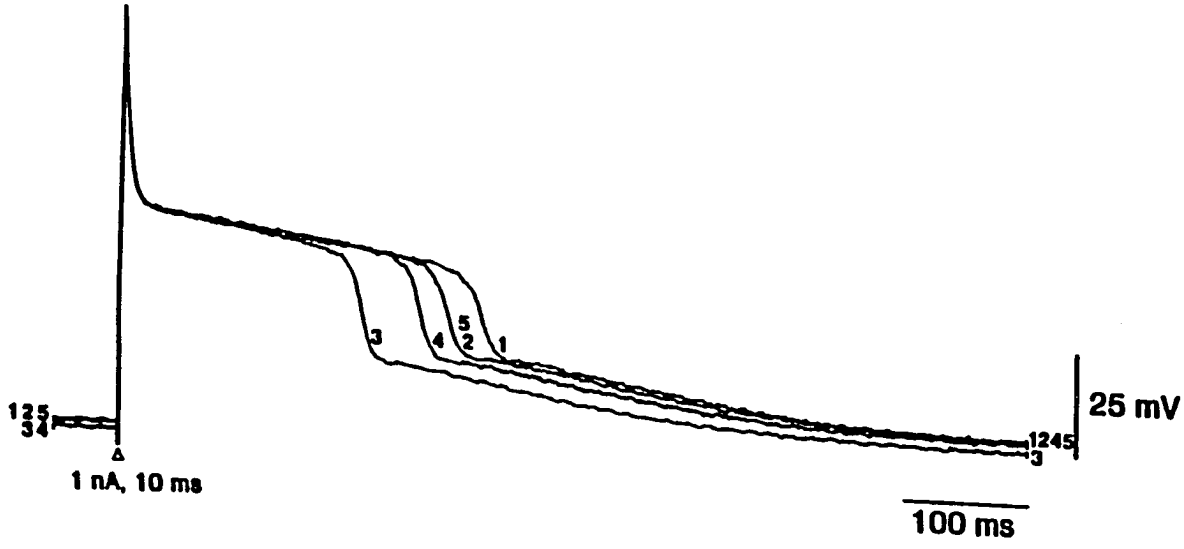

A
Control
(-) - Baclofen 10^{-6} M
Wash
25 mV
.6 nA, 10 ms
20 ms
B
Control
(-) - Baclofen 10^{-6} M
Wash
25 mV
.8 nA 1 s
.5 s

Fig. 6. Baclofen reduces the duration of the Ca^{2+}-dependent action potential and afterdepolarization in a dorsal horn neuron bathed in a medium containing TTX ($3x10^{-7}M$) and TEA ($2x10^{-2}M$). Oscilloscope records of superimposed Ca^{2+}-dependent action potentials taken at 1 min intervals; prior to (trace 1), and following (-)-baclofen (1 μM for 1 min) application (traces 2-5). The baclofen-induced hyperpolarization (-5mV) in this cell reduced the spike duration by 30% as determined at half maximal amplitude. Stimulus pulse 1nA, 10 ms. 16 day-old rat, Vm, -55mV.


1 2 5
3 4
1 nA, 10 ms
3
4
5
2
1
1 2 4 5
3
25 mV
100 ms

frequency accommodation in a manner similar to that shown in rat neocortical neurons (Connors et al., 1988). Thus in 8 of 11 tested neurons, baclofen (0.1 to 1 μM for 1 min) enhanced the neuronal accommodation of action potential firing (30.0±2.5%; range: 17-70%), as shown in Fig. 5B. In neurons hyperpolarized by baclofen this effect persisted when membrane potential was brought to its control value by passing depolarizing d.c. current through the microelectrode.

When the spinal cord slices were perfused with a control medium containing TTX (0.3 μM) to supress the fast, voltage-dependent sodium current, and tetraethylammonium chloride (TEA, 20 mM) to reduce voltage dependent potassium current(s), a depolarizing current pulse (0.8 to 1.0 nA for 10 ms) applied to the soma elicited high-threshold Ca^{2+}-dependent action potential (Murase and Randic, 1983). Baclofen (1 μM for 1 min) reduced the duration of Ca^{2+} spikes (41.8±11.8%, n=4; range: 17-80%) elicited at membrane potentials ranging from -65 to -55 mV (Fig. 6). No significant changes in the dV/dt of the rising phase or the amplitude of the Ca^{2+} spike were recorded. This effect was not associated with an increase in threshold of Ca^{2+} spikes. Although the peak effect and its recovery had a similar time course to the baclofen-induced hyperpolarization, the decrease in the duration of Ca^{2+} spike was present when the resting memebrane potential was re-established by passing adequate d.c. current through the microelectrode.

Depression of excitatory and inhibitory synaptic transmission by baclofen

Activation of $GABA_B$ receptors in the central nervous system is known to depress the amplitude of both excitatory postsynaptic potentials (EPSPs)

and inhibitory postsynaptic potentials (IPSPs) (Bowery et al., 1980; Howe et al., 1987; Kangrga et al., 1987; Connors et al., 1988). Bath application of baclofen (5 nM to 1 μM) to a slice (stimulation site on a dorsal root was not exposed to baclofen) caused a marked and prolonged decrease in the amplitude (n=10), or abolition (n=5), of mono- and polysynaptic EPSPs evoked in dorsal horn neurons by electrical stimulation of a lumbar dorsal root (Fig. 7). The depressant effect was present in almost all of tested cells (97%) where it infrequently occurred in the absence of, or after a minimal increase in membrane potential (<-1.5 mV, n=6) and decrease in input resistance. The average reduction in the EPSP amplitude, recorded in response to suprathreshold stimulation of a dorsal root (1-10V pulses of 20μs duration), amounted to 64.0±5.2.0% (n=15; range: 30 to 100%) of control. Full or partial reversibility of the baclofen's depressant effect on EPSPs was demonstrated in the great majority of the neurons. Although the maximal depressant effect on the excitatory synaptic transmission was usually concomitant with the peak membrane hyperpolarization, it often required a longer time to fully recover. By increasing the parameters of dorsal root stimulation, the EPSPs or dorsal root-evoked action potentials could still be elicited in some neurons during the maximal depressant effect of baclofen. The average increase in the magnitude of dorsal root stimulation, required to restore action potentials or the amplitude of EPSPs to the pre-baclofen values, was 256.2±46.6% (n=5) and 240.5±14.1 (n=12), respectively.

In addition, we found that (-)-baclofen abolished fast IPSPs recorded in two dorsal horn neurons in response to low intensity electrical stimulation of a dorsal root (1-3 V, 20 μS pulse duration), as shown in

Fig. 7. Baclofen depresses the intracellularly recorded compound EPSP. Electrical stimulation of the dorsal root (8V, 20μs) evoked an EPSP; first trace represents the control response, whereas the traces 2-6 represent the responses recorded at 1, 2, 6, 7 and 8 min after the onset of the (-)-baclofen (1 μM for 1min) application, respectively. The baclofen caused hyperpolarization (-1.5mV) in this cell was annulled by passing adequate depolarizing current. Vm, -64 mV. 16-day-old rat.

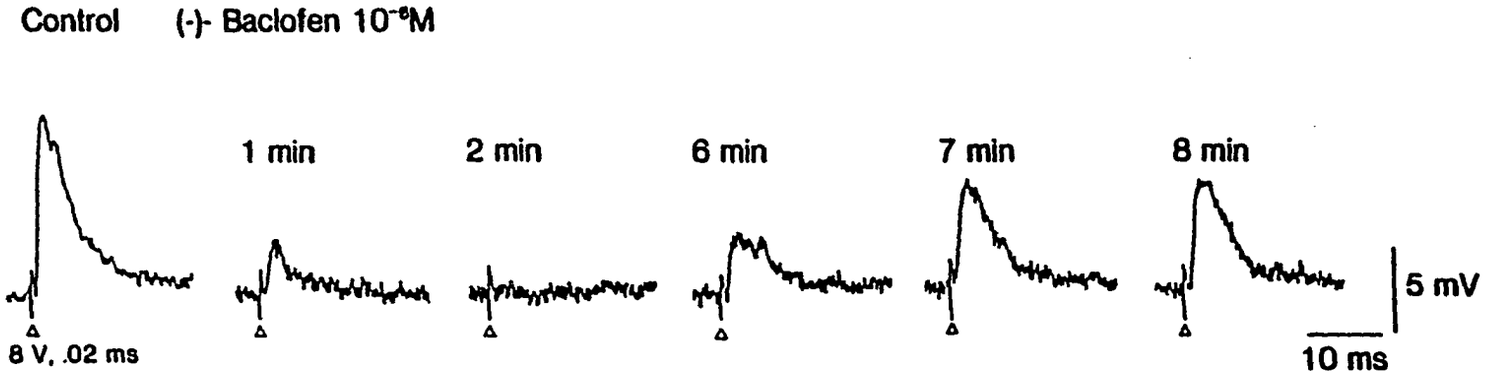

Control
(-)- Baclofen 10^{-6}M
1 min
2 min
6 min
7 min
8 min
8 V, .02 ms
5 mV
10 ms

Fig. 8. Baclofen depresses intracellularly recorded fast and slow IPSPSs. A, bath application of baclofen (10 nM for 1 min) did not produce any change in the neuronal membrane potential. B, IPSP (upper record) evoked by a lumbar dorsal root stimulation (3V, 20 μs) and voltage transients in response to rectangular hyperpolarizing current injections (0.2nA, 100ms) (lower record) were taken before (a), at 1 min (b), 2.5 min (c), at 7 min with the doubled intensity of stimulation (6V, 20 μs) (d), and at 10 min (e) after the application of baclofen. Note the gradual decrease in the amplitude of the IPSP following application of baclofen and the increased stimulation threshold (d). 23-day-old rat, Vm, -60 mV. Calibration: A, 10 mV, 1 min; B, top record, 10 mV, 20 ms; middle record, 25 mV, 100 ms; bottom record, 0.2 nA, 100 ms. C, a slow IPSP evoked by high-intensity repetitive stimulation (25 V, 0.5 ms, 20 Hz for 2 sec) of a lumbar dorsal root before (left record), during superfusion with baclofen (0.1 μM for about 5 min) (middle record) and 15 min after the offset of application of baclofen (right record). 20-day-old rat, Vm, -60 mV.

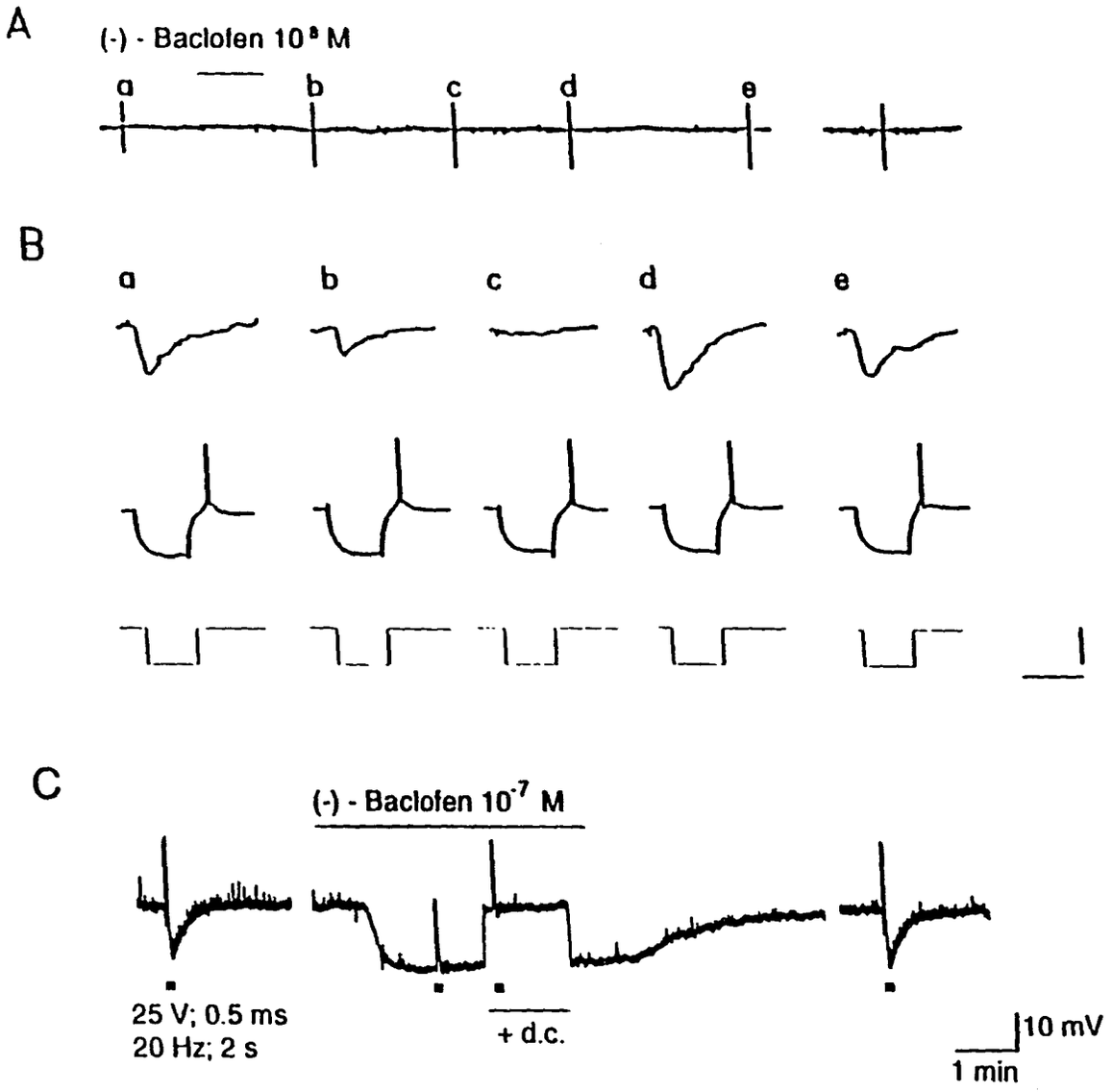

A
(-) - Baclofen 10⁻⁸ M
a
b
c
d
e
B
a
b
c
d
e
C
(-) - Baclofen 10⁻⁷ M
25 V; 0.5 ms
20 Hz; 2 s
+ d.c.
10 mV
1 min

Fig. 8B. Whereas this depressant effect of 0.1 μM (-)-baclofen was associated with membrane hyperpolarization (-6 mV) in one neuron, no apparent change in the membrane potential and input resistance was recorded in a neuron shown in Fig. 8, where 10 nM (-)-baclofen was applied. The IPSP in this neuron could still be evoked during the peak depressant effect of (-)-baclofen, by increasing the intensity of dorsal root stimulation to 200% of the control value.

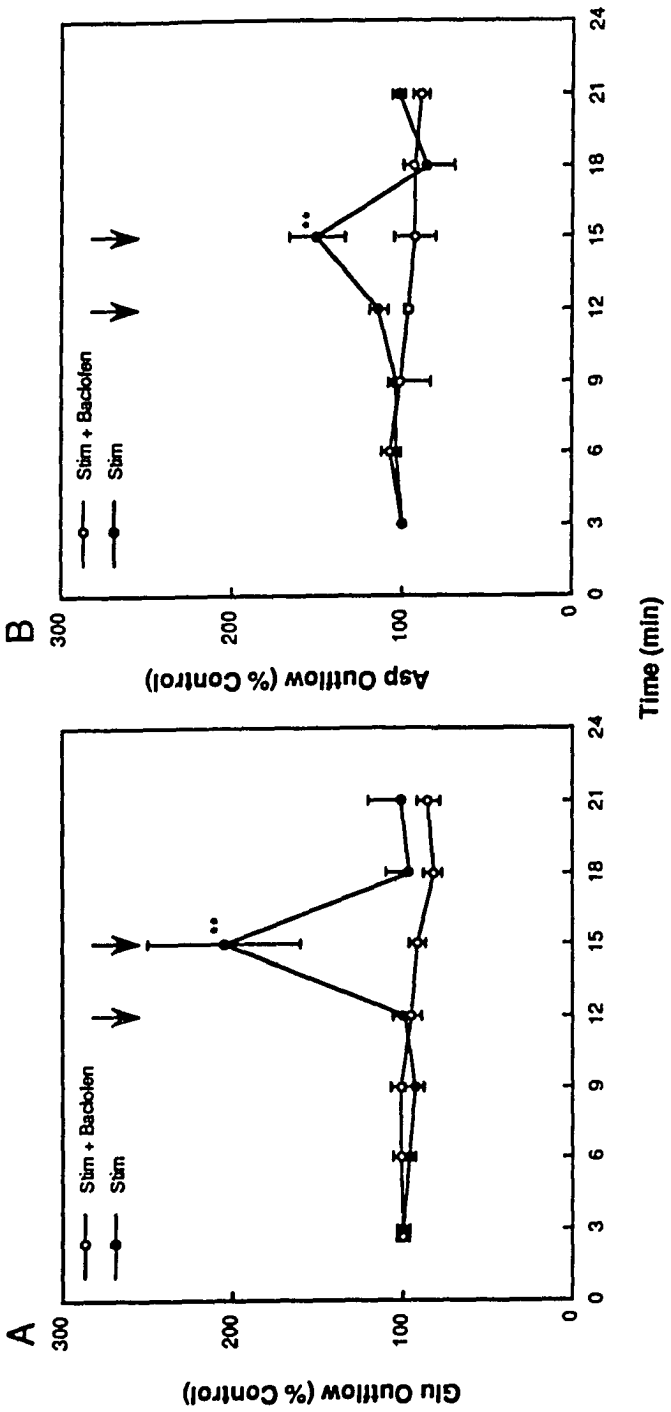
In all of 36 tested neurons, (-)-baclofen caused a reduction or abolition in the frequency and amplitude of presumptive spontaneous EPSPs and IPSPs (Fig. 4C). With lower concentrations of baclofen (<0.1 μM) the supression of the spontaneous EPSPs occurred in the absence of membrane hyperpolarization, whereas with higher concentrations (about 1 μM) the effect was always associated with membrane hyperpolarization. In addition, this effect of baclofen appeared to be resistant to changes in the extracellular potassium concentration. As shown in Fig. 4A-B, whereas the direct hyperpolarization evoked by baclofen in a control medium (3.1 mM K^+) was markedly depressed by increasing concentration of potassium ions in the extracellular medium (to 6.2 and 12.4 mM), the depressant effect of baclofen on spontaneous synaptic potentials was not affected (Fig. 4C). The depression of spontaneous and evoked synaptic activity was observed with both isomers of baclofen, and it was bicuculline insensitive.

Since the most common site responsible for alteration in synaptic strength has been thought to be the presynaptic terminal, we have examined the possibility of involvement of baclofen in the modulation of the basal and the dorsal root stimulation-evoked release of endogenous glutamate and aspartate.

Presynaptic modulation of the release of endogenous glutamate and aspartate

The depression of excitatory synaptic transmission observed in the present study suggested that (-)-baclofen could reduce the release of putative excitatory neurotranmsitters, glutamate and/or aspartate, in the rat spinal dorsal horn. To obtain a more direct evidence for possible inhibition of glutamate and/or aspartate release by the activation of $GABA_B$ receptors we have examined the effects of (-)-baclofen (0.1 to 10 μM for 3 to 6 min) on the spontaneous and dorsal root stimulation-evoked outflow of endogenous aspartate and glutamate from the spinal dorsal horn slices. All experiments were performed in the presence of bicuculline methiodide (10 μM) in the slice perfusate. In two experiments, L-aspartic acid -β-hydroxamate was employed in order to block the neuronal and glial uptake of the excitatory amino acids. Whereas perfusion of slices with (-)-baclofen (0.1 to 10 μM for 3 to 6 min, n=6) did not produce any consistent change in the rate of the basal outflow of glutamate and aspartate, the stimulation-evoked release of both amino acids was completely abolished by baclofen (1 μM for 6 min, n=5), as illustrated in Fig. 9.

Fig. 9. Baclofen blocks the DR stimulation-evoked outflow of endogenous glutamate (Glu) and aspartate (Asp) from the spinal cord slice. Electrical stimulation (30-35 V, 1 ms, 1 Hz, 180-300 pulses; set of arrows) of lumbar dorsal roots (L 4 and 5) in five slices elicited a significant ($^{**}p<0.01$) increase in the basal outflow of Glu (A) and Asp (B) in the absence of baclofen (solid circles). This effect, however, was absent when (-)-baclofen (1 μM for 6 min) was present in the perfusing medium. Bicuculline methiodide (10 μM) was present in all experiments and L-aspartic acid β-hydroxamate (50 μM) in 2 experiments. 31- to 35-day-old rats.


A
B
Glu Outflow (% Control)
Asp Outflow (% Control)
Stim + Baclofen
Stim
300
200
100
0
3
6
9
12
15
18
21
24
Time (min)

DISCUSSION

In agreement with earlier reports (Jeftinija et al., 1986; Kangrga et al., 1987; Allerton et al., 1989; Yoshimura and Jessel, 1989; Wang and Dun, 1990;), our results show that the $GABA_B$ receptor agonist, (-)-baclofen, has a direct slow hyperpolarizing action on the postsynaptic membrane of both superficial and deep spinal dorsal horn neurons. The direct action of (-)-baclofen in the present study is indicated by the persistence of the hyperpolarizing effect in the presence of TTX, or in a nominally zero Ca^{2+}/high Mg^{2+} perfusing medium, the procedures known to block the synaptic transmission. The baclofen-induced hyperpolarization, and a decrease in neuronal input resistance, were insensitive to blockade by bicuculline, the finding suggesting an interaction with $GABA_B$ receptors (Newberry and Nicoll, 1984a,b, 1985; Pinnock 1984; but see Inoue et al., 1985b). It is known that the $GABA_B$-mediated responses are most reliably defined by their insensitivity to bicuculline and their activation by the specific agonist baclofen. The hyperpolarizing action of baclofen in rat spinal dorsal horn neurons was more than 100-fold stereoselective; (-)-baclofen being more potent than (+)-baclofen. Quantitatively similar data were obtained for the stereoselectivity of baclofen actions in other studies (Ault and Nadler, 1982; Collins et al., 1982; Inoue et al., 1985b; Howe et al., 1987). The baclofen-induced hyperpolarization in spinal dorsal horn neurons is likely to be mediated by an increase in potassium conductance since its reversal potential, and the shift in the reversal potential, are modulated by the external potassium concentration similar to reports for other central neurons (Inoue et al., 1985; Newberry and Nicoll, 1985; Dutar

and Nicoll, 1988; Allerton et al., 1989).

Electrical stimulation of dorsal roots, besides fast and slow EPSPs (Urban and Randić, 1984; Gerber et al., 1989), and fast IPSPs, elicits a long-lasting IPSP in about 30% of dorsal horn neurons (Urban and Randić, 1984; Jeftinija et al., 1985, 1986, 1987). The latter response is bicuculline-insensitive and accompanied by a small increase in conductance (Jeftinija et al., 1985). Several other CNS structures have a long-lasting IPSP (Alger and Nicoll, 1982; Newberry and Nicoll, 1984a,b, 1985; Howe et al., 1987; Connors et al., 1988) that resembles in its properties to that described in the spinal dorsal horn (Jeftinija et al., 1985). Remarkably similar properties of the baclofen-induced hyperpolarization and the dorsal-root evoked long-lasting IPSP raise an intriguing possibility that in a physiological situation the released GABA acting on $GABA_B$ receptors, may mediate the long-lasting IPSP, as proposed in other CNS structures (Dutar and Nicoll, 1988; Soltesz et al., 1988). Although the role of the long-lasting IPSP in spinal dorsal horn function is still obscure, it has been implicated in modulation of excitability and repetitive firing behaviour of dorsal horn neurons (Urban and Randić, 1984).

Another significant effect of (-)-baclofen observed in the rat spinal dorsal horn neurons is the depression of spontaneous and evoked EPSPs and fast- and long-lasting IPSPs. These findings are consistent with reported actions of baclofen in other regions of the mammalian CNS (Pierau and Zimermann, 1973; Fox et al., 1978; Curtis et al., 1981; Blaxter and Carlen, 1985; Curtis and Malik, 1985; Inoue et al., 1985; Howe et al., 1987; Dutar and Nicoll, 1988; Harrison et al., 1988; Allerton et al., 1989; Yoshimura and Jessel, 1989; Wang and Dun, 1990). Our study attempted to address the

question of the site of inhibitory action of baclofen in the superficial spinal dorsal horn. In agreement with the majority of earlier reports, our results indicate that the primary site of the depressant action of baclofen is presynaptic, although at higher concentrations the increased membrane conductance may contribute to the effect of baclofen. Thus baclofen, when used at low concentrations, frequently depressed synaptic transmission at a time when little or no change in the membrane properties of spinal dorsal horn neurons was recorded. Although at higher concentrations the action of baclofen to increase potassium conductance of postsynaptic membrane of rat dorsal horn neurons is likely to contribute to the baclofen's depression of the stimulation-evoked synaptic potentials, the hyperpolarization and the depression of synaptic transmission had different time course. Whereas the maximal depression of the evoked synaptic potentials coincided with the peak hyperpolarizing response to baclofen, the effect on synaptic potentials usually had a longer time course and still persisted when the hyperpolarization and decreased input resistance were fully recovered. Furthermore, the degree of depression of synaptic potentials produced by a given dose of baclofen was much greater than could be accounted for by an increase in membrane conductance. These results suggest that the sites and the cellular mechanisms responsible for the baclofen-caused depression of synaptic transmission and hyperpolarization are likely to be distinct.

In support of the statement that baclofen depresses synaptic transmission predominantly by reducing transmitter release from nerve fibers presynaptic to dorsal horn neurons, are two major results. 1. The dorsal root stimulation-evoked release of glutamate and aspartate from the spinal dorsal horn slice was depressed by baclofen. 2. Both inhibitory

and excitatory synaptic potentials were abolished by (-)-baclofen in the absence of a marked hyperpolarization and the effect appears to be independent of the external concentration of potassium.

It is clear that in both peripheral and central neurons, including primary sensory neurons, many $GABA_B$ receptors are located on presynaptic nerve terminals (Dunlap, 1981; Čapek and Esplin, 1982; Desarmenien et al., 1984; Price et al., 1984), and that their activation inhibits transmitter release evoked by electrical (Potashner, 1979; Collins et al., 1982) or chemical (high-potassium or proveratridine) stimulation (Bowery et al., 1980; Johnston et al., 1980; Collins et al., 1982; Hill and Bowery, 1986; Gray and Green, 1987; Zhu and Chuang, 1987; Neal and Shah, 1989; Huston et al., 1990). However, the depolarization-evoked release of glutamate and aspartate from activated primary afferent fibers has not been examined until the present work. Our experiments clearly show that the $GABA_B$ agonist (-)-baclofen inhibits the dorsal root stimulation-evoked increase in the outflow of glutamate and aspartate from the superficial spinal dorsal horn slice (Kangrga et al., 1990a,b; Kangrga and Randić, 1991). An apparently greater potency of baclofen to inhibit the excitatory amino acid release from the spinal cord slice relative to other CNS structures (Potashner, 1979; Johnston et al., 1980; Collins et al., 1982; Kato et al., 1982; Zhu and Chuang, 1987; Neal and Shah, 1989) may be a consequence of the use of electrical stimulation of primary afferent fibers, the procedure resulting in the excitatory amino acid release from appropriate pools of nerve terminals (Potashner, 1979; Johnston et al., 1980). Since with the parameters of dorsal root stimulation used in this study both the low- and the high-threshold primary afferent fibers were activated, the specific

information regarding the type of primary afferents affected by (-)-baclofen cannot be deduced. It is conceivable, however, that the inhibition of the stimulation-evoked release of glutamate and aspartate by baclofen may affect both capsaicin-sensitive and capsaicin-insensitive primary afferent sources (Kangrga and Randić, 1991). Functional $GABA_B$ receptors have been demonstrated on the primary sensory neurons (Dunlap, 1981, 1984; Dunlap and Fischbach, 1981; Čapek and Esplin, 1982; Desarmenien et al., 1984) and in the superficial laminae of the spinal dorsal horn (Price et al., 1984). Moreover, the neonatal capsaicin treatment reduces the density of $GABA_B$ receptors in the superficial dorsal horn by 40 to 50% (Price et al., 1984). Further support for the concept that baclofen acts primarily at $GABA_B$ receptors located on primary sensory neurons should be sought out by using spinal dorsal horn synaptosomes.

In the present experiments we cannot rule out the possibility that baclofen is acting on a feedback interneuronal loop. If the inhibition of the somatic high-threshold Ca^{2+} spike of the dorsal horn neurons presented in this study can be extrapolated to the nerve terminals, the release of transmitters from synaptically activated interneurons is very likely modulated by baclofen.

The precise mechanism underlying inhibition of transmitter release by (-)-baclofen has yet to be elucidated. There is evidence that (-)-baclofen decreases Ca^{2+}-dependent action potential and Ca^{2+} current in the isolated dorsal root ganglia (Desarmenien et al., 1984; Dunlap, 1984; Schlichter et al., 1984; Dolphin and Scott, 1986) by acting at G protein-coupled $GABA_B$ receptors (Dolphin and Scott, 1986, 1987). (-)-baclofen, however, inhibits predominantly the N component of the Ca^{2+} current in the mouse and rat

cultured DRG neurons (Green and Cottrell, 1988).

Acknowledgements

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DISCUSSION

A more detailed account pertaining to the results presented in this thesis is given in the Results section. This chapter briefly outlines some of the major conclusions deriving from the presented work.

The dorsal horn of the spinal cord is the site of the first synapse in the central nervous system where peripheral somatic and visceral information is processed and integrated. Whereas considerable experimental data have accumulated describing the anatomical and physiological characteristics of the afferent projections to the spinal dorsal horn, the identity of neurotransmitters and neuromodulators at the primary afferent synapse has not been resolved, as yet. Dicarboxylic amino acids, glutamate and aspartate, have been implicated in the fast excitatory transmission, whereas neuropeptides (i.e., tachykinins, calcitonin-gene-related peptide, opioid peptides) are thought to be involved in the modulation of the primary afferent transmission.

In order to test the hypothesis that excitatory amino acids may function as neurotransmitters of the primary afferent fibers, this research has examined the basal and the dorsal root electrical stimulation-evoked release of nine endogenous amino acids, including glutamate and aspartate, from the spinal dorsal horn. In addition, the possibility of modulation of the basal and the stimulation-evoked release of the nine amino acids by tachykinins, CGRP, and by activation of μ-opioid and $GABA_B$ receptors, was investigated. The experiments utilized the <u>in vitro</u> rat spinal cord slice --dorsal root ganglion preparation, high performance liquid chromatography and intracellular recording from dorsal horn neurons.

Tachykinins and CGRP enhance the basal and the dorsal root stimulation--evoked release of endogenous glutamate and aspartate from the rat spinal dorsal horn

The effects of dorsal root stimulation and of neuropeptides substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP), on the basal and the dorsal root stimulation evoked-release of nine endogenous amino acids have been investigated using the rat spinal cord slice-dorsal root ganglion preparation. The perfusate was analyzed for amino acids aspartate, glutamate, asparagine, glutamine, glycine, gamma-aminobutyric acid, serine, threonine and alanine.

High intensity repetitive electrical stimulation of a lumbar dorsal root produced a Ca^{2+}-dependent increase in the basal release of aspartate, glutamate, glycine, serine and threonine. Low concentrations of SP ($2x10^{-7}M$) caused a selective increase in the rate of basal release of glutamate, whereas with higher concentrations ($1\text{-}5x10^{-6}M$), in addition to glutamate, an increase in the basal release of aspartate was observed. NKA ($5x10^{-7}$ to $10^{-6}M$), a related tachykinin that is co-expressed with SP in primary sensory neurons, enhanced the basal release of glutamate, aspartate and glycine. The enhancement of the basal release of glutamate by SP persisted in the absence of external Ca^{2+}, but the effect was blocked by (D-Arg^{1}, D-Pro^{2}, D-$Trp^{7,8}$, Leu^{11})-SP, a SP analogue claimed to be an antagonist of synthetic SP. CGRP ($10^{-7}M$) caused a significant, largely Ca^{2+}-independent increase of the basal release of glutamate and aspartate and a decrease of asparagine. SP and CGRP potentiated the electrically-evoked release of glutamate and aspartate. Neonatal capsaicin treatment did not markedly alter the basal efflux of nine endogenous amino acids from the spinal

slices, but it prevented the dorsal root stimulation-evoked release of aspartate, glutamate, glycine and threonine. In addition, the capsaicin treatment prevented the SP-induced increase in the basal release of glutamate, whereas the effect of CGRP was not significantly modified.

These results indicate that tachykinins (SP and NKA) and CGRP are capable of modulating the basal and the electrically-evoked release of endogenous glutamate and aspartate and these actions may provide an important mechanism by which the peptides contribute to the regulation of the primary afferent synaptic transmission. The enhancement of the basal and the dorsal root stimulation-evoked release of glutamate and aspartate by tachykinins and CGRP may have important physiological implications for strengthening the synaptic connections in the spinal dorsal horn.

Outflow of endogenous glutamate and aspartate from the rat spinal dorsal horn by activation of low- and high-threshold primary afferent fibers--modulation by μ-opioids

Our study attempted to estimate the contribution of different classes of primary sensory neurons to the release of putative amino acid neurotransmitters glutamate and aspartate. Our approach was to examine the possible correlation of release of endogenous glutamate and aspartate with stimulation parameters used to activate primary afferent fibers.

Selective activation of the low-threshold (Aβ) primary afferent fibers (PAF) resulted in a two-fold increase in the outflow of endogenous aspartate and a smaller increase in the outflow of glutamate from the rat spinal dorsal horn slices into the superfusing medium. The activation of both the low (Aβ)- and the high-threshold (Aδ+C) primary afferents elicited

an additional increase in the outflow of Asp and Glu, and a marked increase in the Asp/Glu outflow ratio.

The involvement of the small-diameter primary afferent fibers in the release of endogenous glutamate and aspartate in the spinal dorsal horn was further examined by using a selective chemical stimulation of a subpopulation of the primary sensory neurons. Superfusion of the dorsal root ganglia with capsaicin (8-methyl-N-vanyllyl-6-noneamide, 1-10 μM) or resiniferatoxin (0.1-1.0 nM), the agents known to selectively activate a subpopulation of small primary sensory neurons, resulted in a prolonged (10-15 min) two- to three-fold increase in the rate of outflow of glutamate and aspartate into the spinal superfusate.

DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol-enkephalin), an agonist at μ-opioid receptors, attenuated the dorsal root stimulation-evoked outflow of Asp and Glu in a naloxone-sensitive manner.

Our results have provided further evidence in support of the contention that glutamate and aspartate act as excitatory synaptic transmitters in the spinal dorsal horn. The results obtained with capsaicin and resiniferatoxin suggest that a population of small primary sensory neurons may be an important neuronal source of the depolarization-evoked release of glutamate and aspartate in the spinal dorsal horn. A role for μ-opioid receptors in modulation of spinal processing of somatosensory information is indicated.

Effects of phorbol esters on the basal and the dorsal root stimulation--evoked release of endogenous amino acids in the rat spinal dorsal horn

Protein kinase C (PKC) has been implicated in the process of interneuronal signalling within the central nervous system. Activation of

protein kinase C can be mediated directly by phorbol esters in the absence of phosphoinositide breakdown. We used phorbol esters as a probe of protein kinase C function in modulating the basal and the dorsal root stimulation-evoked release of endogenous amino acids in the spinal dorsal horn.

Phorbol esters, 4β-phorbol-12, 13-dibutyrate (PDBu) and 4β-phorbol-12, 13-diacetate (PDAc), produced a brief increase in the basal and the electrically-evoked release of endogenous excitatory (glutamate and aspartate) and inhibitory (glycine and GABA) amino acids. In addition the rates of release of serine, threonine and alanine were also elevated. By contrast, superfusion of the spinal slices with, 4α-phorbol-12, 13-didecanoate (PDiDec), a phorbol ester analog that does not activate protein kinase C, had no effect on the basal release of six endogenous amino acids.

Enhancement of the basal and the stimulation-evoked release of putative excitatory and inhibitory amino acid neurotransmitters caused by phorbol esters indicates that protein kinase C may be involved in the presynaptic modulation of the basal and depolarization-evoked neurotransmitter release.

Actions of $GABA_B$-receptor agonist baclofen in the rat spinal dorsal horn

The actions of a $GABA_B$ agonist, (-)-baclofen, on the electrophysiological properties of dorsal horn neurons and excitatory and inhibitory synaptic transmission in the spinal dorsal horn (laminae I-IV) were examined by using intracellular recordings in spinal cord slice from young rats. In addition, the effects of baclofen on the dorsal root stimulation-evoked outflow of glutamate and aspartate from the spinal dorsal horn was examined by using high performance liquid chromatography

(HPLC) with flourimetric detection.

Superfusion of baclofen (5nM to 10μM) hyperpolarized, in a stereoselective and bicuculline-insensitive manner, the majority (86%) of tested neurons. The hyperpolarization was associated with a decrease in membrane resistance and persisted in a nominally zero-Ca^{2+}, 10mMg^{2+}- or a TTX-containing solution. Our findings indicate that the hyperpolarizing effect of baclofen is probably due to an increase in conductance to potassium ions. Baclofen decreased the direct excitability of dorsal horn neurons, enhanced accommodation of spike discharge, and reduced the duration of Ca^{2+}-dependent action potentials. Baclofen depressed, or blocked, both excitatory (EPSPs) and fast and slow inhibitory postsynaptic potentials (IPSPs), evoked by electrical stimulation of the dorsal roots. Spontaneously occurring EPSPs and IPSPs were also reversibly depressed by baclofen.

Whereas baclofen did not produce any consistent change in the rate of the basal outflow of glutamate and aspartate, the stimulation-evoked release of the two amino acids was blocked.

The present results suggest that baclofen, by activating $GABA_B$ receptors, may modulate spinal afferent processing in the superficial dorsal horn by at least two mechanisms: 1. Baclofen depresses excitatory synaptic transmission primarily by a presynaptic mechanism involving a decrease in the release of excitatory amino acids, and 2. At higher concentrations, the hyperpolarization and increased membrane conductance may contribute to the depressant effect of baclofen on the excitatory and the inhibitory synaptic transmission in the rat spinal dorsal horn.

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