Electrophysiological properties of spinal dorsal horn neurons in vitro: calcium-dependent action potentials and actions of neuroactive peptides

Kazuyuki Murase
Iowa State University

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ELECTROPHYSIOLOGICAL PROPERTIES OF SPINAL DORSAL HORN NEURONS IN VITRO: CALCIUM-DEPENDENT ACTION POTENTIALS AND ACTIONS OF NEUROACTIVE PEPTIDES

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Electrophysiological properties of spinal dorsal horn neurons in vitro: Calcium-dependent action potentials and actions of neuroactive peptides

by

Kazuyuki Murase

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

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For the Graduate College

Iowa State University
Ames, Iowa

1983
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Bath Temperature Controller
Stimulus Signal Controller
Trace Shifter
Pressure Application Unit

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<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ADP</td>
<td>After depolarization</td>
</tr>
<tr>
<td>AHP</td>
<td>After hyperpolarization</td>
</tr>
<tr>
<td>c-AMP</td>
<td>Cyclic-adenosine monophosphate</td>
</tr>
<tr>
<td>C-MOS</td>
<td>Complementary metal-oxide semiconductor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D/A</td>
<td>Digital/Analogue</td>
</tr>
<tr>
<td>DALA</td>
<td>D-alanine&lt;sup&gt;2&lt;/sup&gt;-methionine&lt;sup&gt;5&lt;/sup&gt;-enkephalinamid</td>
</tr>
<tr>
<td>DC</td>
<td>Direct Current (d.c.)</td>
</tr>
<tr>
<td>ENK</td>
<td>Enkephalin</td>
</tr>
<tr>
<td>ENKLI</td>
<td>Enkephalin-like immunoreactivity</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>G&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Calcium conductance</td>
</tr>
<tr>
<td>G&lt;sub&gt;K&lt;/sub&gt;</td>
<td>Potassium conductance</td>
</tr>
<tr>
<td>G&lt;sub&gt;Na&lt;/sub&gt;</td>
<td>Sodium conductance</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>5HI</td>
<td>5-Hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>I&lt;sub&gt;A&lt;/sub&gt;</td>
<td>The transient outward current</td>
</tr>
<tr>
<td>I&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>The voltage-dependent calcium current</td>
</tr>
<tr>
<td>IC</td>
<td>Integrated circuit</td>
</tr>
<tr>
<td>I&lt;sub&gt;fir&lt;/sub&gt;</td>
<td>The fast inward-rectifying current</td>
</tr>
<tr>
<td>I&lt;sub&gt;K&lt;/sub&gt;</td>
<td>The fast potassium current (the delayed rectifier current)</td>
</tr>
</tbody>
</table>
\( I_{K(Ca)} \) The calcium-dependent potassium current

\( I_M \) The M-current

\( I_{Na} \) The fast sodium current

IPSP Inhibitory postsynaptic potential (i.p.s.p.)

\( I_Q \) The slow inward-rectifying current

\( I-V \) Current-voltage

LHRH Luteinizing hormone releasing hormone

ME Methionine-enkephalin

NA Noradrenaline

\( R_N \) Cell input resistance

S.D. Standard deviation

SP Substance P

SPLI Substance P-like immunoreactivity

SS Somatostatin

SSLI Somatostatin-like immunoreactivity

\( \tau \) Membrane time constant

TEA Tetraethylammonium

TTL Transistor-transistor logic

TTX Tetrodotoxin

\( V_m \) Membrane potential

\( V_r \) Reversal potential
INTRODUCTION

Explanation of Dissertation Format

This dissertation is written in an alternate format as permitted by the Graduate College. It includes an objective, a literature review, a rationale, an experimental part, a discussion, a summary and a list of literature cited. The experimental part is divided into four sections. Section I describes the details of the methods used in this series of experiments, sections II and III correspond to research papers already published, and section IV corresponds to a paper currently in press.

This dissertation presents the results of experiments performed by the author over three years under the supervision of his major professor, Dr. Mirjana Randić. A portion of the results included in section III was obtained by Dr. V. Nedeljkov to whom some credit belongs.

Objective

The purpose of this project is to study electrophysiological properties of neurons in the superficial parts of the spinal dorsal horn and determine how they are modified by the actions of several neuropeptides - substance P, methionine-enkephalin and somatostatin - by using intracellular recording from the neurons in a slice preparation of the immature rat spinal cord. We felt that this in vitro approach would provide new information about (1) electrophysiological properties of dorsal horn neurons, and (2) the neuronal elements substance P, methionine-enkephalin
and somatostatin act upon, their membrane actions and possible mechanisms of their synaptic actions.

Literature Review

Structural and functional organization of the dorsal horn

In this section, the organization of the dorsal horn is reviewed in order to provide the basic knowledge for the studies on the actions of peptides in the region.

**Primary afferent fibers** Primary afferent fibers arise from sensory receptors in skin, subcutaneous tissue, joints, skeletal muscles and viscera. They may be myelinated or unmyelinated. Their designation depends upon the conduction velocity. Major sensory receptors and primary afferent fibers associated with them are summarized in Table 1.

Cell bodies of primary afferent fibers are located in dorsal root ganglia although a few exist along the ventral root (Sherrington, 1894; Willis et al., 1967; Yamamoto et al., 1977). The majority of the cells are unipolar. About 30-40% of the unipolar cells have large cell bodies (60-120 µm) giving rise to large myelinated fibers, while the rest of them have diameters of 14-30 µm giving off small myelinated or unmyelinated axons (Ranson, 1912). About 3% of the cells in the ganglia are not of unipolar type. Some of them may have axon collaterals terminating on cells in the ganglia, others may have dendrite-like processes terminating near the cell body and form synapses (Kayahara et al., 1981). Little is known about their functions (Ranson, 1912; Willis & Coggeshall, 1981).
Table 1. Major sensory receptors and the types of primary afferent fibers associated with the various receptors (reconstructed from Willis & Coggeshall, 1981)

<table>
<thead>
<tr>
<th></th>
<th>Myelinated fibers</th>
<th>Unmyelinated fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>conduction velocity</td>
<td>Aαβ: 30-100 m/s</td>
<td>Aδ: 4-30 m/s</td>
</tr>
<tr>
<td></td>
<td>Group I: 72-120 m/s</td>
<td>III: 6-23 m/s</td>
</tr>
<tr>
<td></td>
<td>II: 24-71 m/s</td>
<td>IV: &lt;2.5 m/s</td>
</tr>
<tr>
<td>cutaneous mechanoreceptors</td>
<td>Type I &amp; II</td>
<td>D hair</td>
</tr>
<tr>
<td></td>
<td>G1 &amp; G2 (ST) hair</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Field</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Meissner's corpuscle</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Krause's end-bulb</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pacinian corpuscle</td>
<td>-</td>
</tr>
<tr>
<td>nociceptors</td>
<td>-</td>
<td>Aδ mechanical</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>C mechanical</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Aδ heat</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>C cold</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Aδ cold</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>C polymodal</td>
</tr>
<tr>
<td>thermoreceptors</td>
<td>-</td>
<td>Cold (monkey)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Cold (rat, cat)</td>
</tr>
<tr>
<td>muscle</td>
<td>Group I &amp; II;</td>
<td>-</td>
</tr>
<tr>
<td>mechanoreceptors</td>
<td>primary and secondary</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>endings of muscle</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>spindles,</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Golgi tendon organ,</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pacinian corpuscle</td>
<td>-</td>
</tr>
<tr>
<td>nociceptors</td>
<td>-</td>
<td>Group III</td>
</tr>
<tr>
<td>Joint mechanoreceptors</td>
<td>Ruffini ending</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Golgi tendon organ</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Paciniform ending</td>
<td>-</td>
</tr>
<tr>
<td>nociceptors</td>
<td>-</td>
<td>Aδ nociceptor</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>C nociceptor</td>
</tr>
<tr>
<td>visceral mechanoreceptors</td>
<td>Aδ mechanical</td>
<td>C mechanical</td>
</tr>
<tr>
<td>nociceptors</td>
<td>-</td>
<td>C nociceptor</td>
</tr>
</tbody>
</table>
The majority of primary afferent fibers enter the spinal cord through the dorsal roots, yet, some fibers, primarily unmyelinated fibers innervating visceral and to a lesser degree, cutaneous receptors, enter the spinal cord through the ventral roots (Coggeshall et al., 1974; Coggeshall & Ito, 1977).

Upon entering the spinal cord afferent fibers bifurcate and give off ascending and descending branches. The branches move medially in their ascent and descent and give rise to collaterals which enter the dorsal horn laterally and arborize (Brown, 1981). There is a general agreement on the segregation of primary afferent fibers in the dorsal horn according to the diameters of the fibers although some overlap may exist (see below for references). Table 2 is the summary of the termination of various types of primary afferent fibers.

**Cytoarchitecture of the dorsal horn: Rexed's scheme**  
Based upon the cytoarchitecture of Nissl-stained sections of spinal cord from adult cats and kittens, Rexed (1952) subdivided the spinal cord into nine cell layers (laminae), in which the upper six laminae (laminae I-VI) make up the dorsal horn. Although Rexed's scheme originally dealt with the cytoarchitectonic organization of the spinal cord, that is, the shapes, sizes, densities and distribution of neuronal somata, recent studies have shown that there is a good agreement between Rexed's scheme and the orientation of dendritic trees of neurons, axonal projections and terminations or synaptotological organization in the spinal cord (Brown, 1981). The scheme, therefore, has been used universally to describe the
Table 2. Terminations of primary afferent fibers in the spinal dorsal horn. The number of x refers to the density of the terminals in the lamina (reconstructed from Brown, 1981).

<table>
<thead>
<tr>
<th>Lamina</th>
<th>Cutaneous</th>
<th></th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aβ/β</td>
<td>α</td>
<td>I &amp; II</td>
</tr>
<tr>
<td>I</td>
<td>xxx</td>
<td>xx</td>
<td>xxx</td>
</tr>
<tr>
<td>IIO</td>
<td>x</td>
<td>xxx</td>
<td>x</td>
</tr>
<tr>
<td>III</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>III</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>xx</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>xxx</td>
<td>xx</td>
<td>x</td>
</tr>
<tr>
<td>VI</td>
<td>x</td>
<td>xxx</td>
<td></td>
</tr>
</tbody>
</table>


Morphology of each lamina has been studied intensively with the Golgi-staining method (Ramon y Cajal, 1909; Scheibel & Scheibel, 1968) and recently with intracellular staining using the horseradish peroxidase technique (Bennett et al., 1979; Light et al., 1979). From these studies, some major conceptual generalizations on the characteristics of the neurons and the organizations of fibers in each lamina have been established (Brown, 1981; Willis & Coggeshall, 1981).

Lamina I: Marginal zone of Waldeyer Lamina I is the most dorsal and the thinnest (12-20 μm: Hunt et al., 1980) of the layers covering the dorsal surface and the lateral half of the dorsal part of the gray matter.
It contains small, medium and large neurons, and the cell bodies of the neurons have a primarily horizontal arrangement. The larger cells in this lamina, called the marginal cells (Ramon y Cajal, 1909; Gobel, 1978a), have been characterized the best. Little is known about other smaller cells.

The cell bodies of marginal neurons are horizontally elongated (30-50 \( \mu \)m x 10-15 \( \mu \)m; Rexed, 1952), and the dendrites have a flattened disc shape with elliptic elongation in the rostro-caudal direction (500-1400 \( \mu \)m: Light et al., 1979; 500-680 \( \mu \)m: Price et al., 1979). The majority of the dendrites are confined to lamina I in the adult animal while the dendrites of the marginal neurons in the neonatal animal occasionally penetrate down to lamina II (Scheibel & Scheibel, 1968).

The axons of most marginal neurons seem to project to three regions: (1) to other segments of the spinal cord (some are more distant than two segments) via propriospinal pathways (Ramon y Cajal, 1909; Szentágothai, 1964; Burton & Loewy, 1976), (2) to the cerebellum via both the ipsi- and contralateral funiculi (Snyder et al., 1978), and (3) to the thalamus via the contralateral spinothalamic tract (Willis et al., 1974; Trevino and Carstens, 1975; Carstens & Trevino, 1978b).

The majority of primary afferent fibers terminating in this lamina are A\( \delta \)-fibers (LaMotte, 1977; Light & Perl, 1977a,b, 1979a,b; Grant et al., 1979; Ralston & Ralston, 1979), which appear to form synaptic contacts primarily on the fine dendrites of the marginal cells (Kerr, 1975).

Fine fibers originating from small cells in laminae II and III seem to terminate on the cell bodies of the marginal cells (Scheibel & Scheibel,
The third type of input to this lamina is from descending pathways originating in the raphe nuclei and the nucleus reticularis magnocellularis. These descending pathways lie in the dorso-lateral funiculus and appear to be inhibitory (Basbaum et al., 1978; Martin et al., 1978). Axons of some of marginal neurons located several segments away terminate in lamina I as well.

The plexus of fine fibers which surrounds and forms synaptic contacts with large neurons in this lamina is called the marginal plexus and the neuropil contains axodendritic, axosomatic, and axoaxonic synapses. There are also some complex synapses resembling the glomeruli of laminae II and III (see below).

**Lamina II: Substantia gelatinosa of Rolando** Lammina II is well-defined layer characterized by its pale appearance due to the absence of myelinated fibers and tightly packed small cells (5 x 5 μm to 10 x 10 μm: Rexed, 1952). It lies across the dorsal horn underneath lamina I and its lateral edge runs about half-way down the dorsal horn along the lateral part of lamina I.

On the basis of the dendritic organization and axonal projections of its neurons and afferent input to it, lamina II is subdivided into two layers, an outer region (lamina IIo: 30-40 μm thick) and an inner region (lamina IIIi: 40-50 μm thick) (Rexed, 1952; Beal & Cooper, 1978; Gobel, 1979; Ralston, 1979). Lamina IIo contains a large number of small perikarya while lamina IIIi contains fewer cell bodies. Gobel (1975, 1978b) has made an extensive Golgi-study of the cells in lamina II and has
described several types of cells. Among them, stalked cells and islet cells, which correspond to cellules limitrophes and cellules centralis of Ramon y Cajal (1909) respectively, appear to be the most characteristic of this lamina (Beal & Cooper, 1978; Price et al., 1979). While stalked cells are mostly found in lamina IIo, islet cells are predominantly located in lamina IIIi (Gobel, 1978b).

The stalked cells (border cells or limiting cells) are larger cells having the cell bodies at the outer edge of lamina II. The dendritic trees are cone shaped with the apex at the soma and are directed ventrally. The dendrites are sometimes within lamina IIo or sometimes extend to the outer part of lamina III. Occasionally, the dendrites enter lamina I. The axons appear to project into lamina I (Gobel, 1975, 1978b; Sugiura, 1975; Beal & Cooper, 1978).

The islet cells (central cells) are smaller in size having their cell bodies mainly in lamina IIIi. The dendrites are oriented cranio-caudally, somewhat flattened mediolaterally and confined primarily to lamina II and III (dorsoventral x craniocaudal x mediolateral; 50-200 x 200-500 x 15-20 μm; Scheibel & Scheibel, 1968; 150-230 x 200-250 x 80-100 μm; Sugiura, 1975).

On the basis of the axonal projections, islet cells can be further subdivided into two groups, the short-axeded cells and the funicular cells. The axons of short-axeded cells stay within lamina II while the axons of funicular cells project to ipsi- and contralateral lamina II of one or two segments away from the cell bodies via Lissauer's tract, the lateral propriospinal tract or the dorsal commissure pathway (Ramon y Cajal, 1909;
Szentágothai, 1964; Sugiura, 1975). Recent studies with horseradish peroxidase staining showed that some lamina II neurons including islet and stalked cells project to the contralateral thalamus (Willis et al., 1978) and to the lower brain stem (Giesler et al., 1978).

Unmyelinated fibers (C-fibers) predominantly terminate in lamina II (LaMotte, 1977; Réthelyi, 1977; Ralston & Ralston, 1979) while small myelinated fibers (Aδ-fibers) terminate in lamina IIo as well as lamina I (Light & Perl, 1977a,b, 1979b). Very few large myelinated fibers seem to project to the superficial laminae (laminae I and II). Descending fibers to lamina II originate from the raphe nuclei and the nucleus reticularis magnocellularis, which are the same pathways as those to lamina I. Axons from lamina II and III neurons located several segments away also terminate in lamina II (Matsushita, 1969, 1970; Mannen & Sugiura, 1975).

The neuropil in lamina II consists of numerous axodendritic synapses and a few axosomatic synapses (Ralston, 1968, 1977; Hunt et al., 1980), the latter comprise 1-2% of identified synaptic contacts in this lamina (DiFiglia et al., 1982). Few axoaxonic (.4%: Duncan & Morales, 1978; .8%: Ralston & Ralston, 1979; .1%: Zhu et al., 1981), dendrodendritic (Coimbra et al., 1974; Ralston, 1979) and dendroaxonic synapses (Gobel, 1974) are also found. From the total number of synapses in this lamina, a small portion degenerates following dorsal root rhizotomy, and the majority of the degenerated terminals are presynaptic terminals of axodendritic synapses (Ralston, 1977; Hunt et al., 1980).

Approximately 5% of the synapses in laminae I-III (Ralston, 1971) form a characteristic structure called the glomerulus. It consists of a
central axonal terminal and a group of peripheral processes (3-8 processes) forming a synaptic complex. Various schemes of the circuitry of glomeruli have been proposed. The central terminals seem to be primary afferent collaterals, probably of relatively large diameter primary afferent fibers (Ralston & Ralston, 1979) such as innocuous mechanoreceptive fibers (Ribeiro-de-Silva & Coimbra, 1982), yet, little is known about the peripheral processes (Rethelyi & Szentágothai, 1973; Coimbra et al., 1974; Gobel, 1974; Kerr, 1975).

**Lamina III**

Lamina III runs across the dorsal horn parallel with laminae I and II, and it is thicker (90-130 μm) than the first two laminae. The majority of cells in this lamina are slightly larger (7-8 x 10-12 μm) and less densely packed than those in lamina II.

The dendritic pattern of lamina III neurons is similar to that of the islet cells in lamina II although the dendritic fields tend to be larger (Scheibel & Scheibel, 1968). The axonal projections are also similar to those of lamina II neurons except that some axons travel widely through the deeper laminae before reentering the lamina II or III (Matsushita, 1969, 1970; Mannen & Sugiura, 1976). Some neurons in lamina III have very extensive dendritic trees that ramify in laminae I-III or IV and some axons travel in the spinocervical tract of the dorsal column (Mannen & Sugiura, 1976; Brown, 1981).

The primary afferent input to this lamina comes primarily from the coarse myelinated fibers such as Aδ-fibers (Sterling & Kuypers, 1967; Light & Perl, 1977a,b, 1979b; Rethelyi, 1977; Ralston, 1979). Some of the
fibers form flame-shaped arborizations in lamina III (Brown et al., 1977). The descending input to this lamina originates mostly from the contralateral sensory cortex via the corticospinal tract (Nyberg-Hansen & Brodal, 1963; Liu & Chambers, 1964; Petras, 1967; Coulter & Jones, 1977). The neuropil in this lamina can not be distinguished from that of lamina II except that a larger number of glomeruli may exist in lamina III than in lamina II.

Laminae IV-VI: Nucleus proprius. Laminae IV-VI are characterized by their wide range of cell sizes. Cells in lamina IV range from 7-9 x 10-12 μm up to 35 x 45 μm, and have round, triangular or star shapes. Lamina V contains cells from 8 x 10 μm to 30 x 40 μm and lamina VI from 8 x 8 to 30 x 35 μm with spindle or star shapes.

The dendritic trees of many neurons in lamina IV are predominantly oriented dorsally, medially and laterally but not much in the rostrocaudal direction. They penetrate up to lamina III or occasionally to lamina II (Szentágothai, 1964; Scheibel & Scheibel, 1968; Brown et al., 1976; Proshansky & Egger, 1977). Many neurons in lamina VI, on the other hand, have dendrites radiating from the cell body and confined to a transverse plane. The dendrites may reach lamina III dorsally or lamina VII ventrally, and extend mediolaterally across one-half to two-thirds of the dorsal horn (Scheibel & Scheibel, 1968; Mannen & Sugiura, 1976; Proshansky & Egger, 1977). The dendrites of other neurons in laminae IV-VI show intermediate patterns between the two extremes described above.

Axonal projections of neurons in these laminae are: (1) to the
lateral cervical nucleus from laminae III-IV, (2) to the thalamus from laminae V-VI, and (3) to the dorsal column and the medullary nuclei from laminae III-IV. Some neurons have local axon collaterals surrounding the cell bodies, which may reach ventrally as far as lamina VII and dorsally into lamina III (Ramon y Cajal, 1909; Matsushita, 1969, 1970; Willis et al., 1979; Mannen, 1975; Snyder et al., 1978).

The larger primary afferent fibers (Aδ-fibers) terminate in laminae III-VI (Sprague & Ha, 1964; Sterling & Kuypers, 1967) while collaterals of some smaller myelinated fibers (Aβ-fibers) terminate in lamina V as well as in lamina I (Light & Perl, 1977a,b, 1979b).

The descending fibers from the raphe nuclei (Brown, 1973; Basbaum et al., 1978) and from the nucleus reticularis magnocellularis (Nyberg-Hansen, 1965; Petras, 1967) terminate in lamina V as well as laminae I and II. The fibers from the sensory cortex terminate in laminae III-V and those from the motor cortex in laminae V-VI (Nyberg-Hansen & Brodal, 1963; Liu & Chambers, 1964; Petras, 1967; Coulter & Jones, 1977).

The neuropil of laminae IV-VI consists of axosomatic and axodendritic synapses, and a few axoaxonic synapses. The glomeruli, the characteristic feature of laminae I-III, can not be seen in these laminae.

**Physiological properties of dorsal horn neurons**

Much less is known at present about the organization of the dorsal horn in relation to different modalities of input. However, physiological findings obtained using natural stimulation seem to support, to a certain degree, some of the anatomical features already described for the dorsal horn.
On the basis of physiological properties of neurons in lamina I, Perl and his collaborators (Christensen & Perl, 1970; Kumazawa et al., 1975; Light et al., 1979) have described three principal categories of the cells, which have been generally accepted (Cervero et al., 1976; Price et al., 1979). These are cells being activated by: (1) noxious mechanical stimuli via Aδ-fibers, (2) noxious mechanical and noxious thermal stimuli via Aδ- and C fibers, and (3) noxious mechanical, noxious thermal and innocuous thermal stimuli via Aδ- and C fibers. In addition, some cells are excited by sensitive mechanoreceptors via C fibers (Price et al., 1979) and by sensitive thermoreceptors via C fibers (Kumazawa & Perl, 1978; Light et al., 1979).

The physiological properties of lamina II neurons are as follows (Light et al., 1979): (1) cells with dendrites in laminae I and II receive input from nociceptors via Aδ- and C fibers, (2) cells with dendrites in lamina II receive input from noxious mechanoreceptors or innocuous thermal receptors via C fibers, and (3) cells with dendrites in lamina III are excited by sensitive mechanoreceptors via C fibers. In addition, wide dynamic range neurons which respond to low threshold mechanoreceptors via Aβ-, Aδ- and C fibers and nociceptors via Aδ- and C fibers are present (Bennett et al., 1979). These neurons have dendrites reaching lamina III.

Lamina IV contains cells which are activated either by low threshold mechanical stimuli or by low and high threshold mechanical stimuli (Wall, 1960, 1973). Cells in lamina V receive input from high threshold muscle afferents and also visceral afferents, and cells in lamina VI from
proprioceptors (Hillman & Wall, 1969). Wide dynamic range neurons are found throughout the laminae V and VI (Price & Browe, 1973; Menetrey et al., 1977).

Another interesting observation concerning inhibitory mechanisms in the dorsal horn is the existence of "inverse class" neurons in lamina II. Iggo and his colleagues (Iggo, 1974; Handwerker et al., 1975) subdivided dorsal horn neurons into three classes; Class 1, neurons excited by sensitive cutaneous mechanoreceptors; Class 2, neurons excited by sensitive cutaneous mechanoreceptors and by nociceptors; Class 3, neurons excited only by nociceptors. In lamina II, they found that some neurons are inhibited by peripheral stimuli and they classified those in three inverse classes depending upon the types of natural stimuli which inhibit the neuronal activities (Cervero & Iggo, 1978; Cervero et al., 1979). Thus, Class 1 neurons are inhibited by sensitive cutaneous mechanoreceptors, Class 2 neurons by sensitive cutaneous mechanoreceptors and by nociceptors, and Class 3 neurons by nociceptors.

Peptides in the dorsal horn

Experimental evidence that substance P (SP), somatostatin (SS) and enkephalins (ENK) may be neurotransmitters and/or neuromodulators in the superficial parts of the spinal dorsal horn has been accumulated. However, the mode of action of the peptides in this area seems more complicated than previously thought. In this section, the current knowledge on sites and mechanisms of actions of SP, SS and ENK is reviewed.
**Substance P** An undecapeptide substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$) was discovered by von Euler & Gaddum (1931), purified by Chang & Leeman (1970), and synthesized by Tregear et al. (1971).

The distribution of SP-like immunoreactivity (SPLI) in the central nervous system (CNS) is uneven. High levels of SPLI have been found in dorsal roots and superficial layers of the spinal dorsal horn (Hökfelt et al., 1975; Chan-Palay & Palay, 1977; Cuello & Kanazawa, 1978; Barber et al., 1979), trigeminal nucleus, pars reticularis of the substantia nigra, hypothalamus and interpeduncular nucleus (Hökfelt et al., 1977, 1978).

The SPLI distribution in the spinal cord (Barber et al., 1979; Hunt et al., 1981; DiFiglia et al., 1982) and in the spinal trigeminal nucleus (Cuello & Kanazawa, 1978; DelFiacco & Cuello, 1980; Priestley et al., 1982) has been studied at the ultrastructural level. SPLI-positive terminals are predominantly found in laminae I and II, especially lamina IIo (Hunt et al., 1981; DiFiglia et al., 1982; Priestley et al., 1982), and in the lateral reticular region of lamina IV (DiFiglia et al., 1982). The majority of SPLI-positive axons are unmyelinated (Priestley et al., 1982) but some are thinly myelinated (DiFiglia et al., 1982). They are mostly of primary afferent origin (DelFiacco & Cuello, 1980; Nagy et al., 1981). In addition, efferent fibers from the nucleus raphe magnus seem to contain SP and serotonin (Gilbert et al., 1982).

Most of the SPLI terminals in lamina I form axodendritic synapses
with large dendrites presumably of marginal cells, and in lamina II with small dendrites and dendritic spines probably of islet cells (DiFiglia et al., 1982; Priestley et al., 1982). At the border of laminae IIo and III, large SPLI-positive terminals make contacts with several dendrites forming glomeruli (Priestley et al., 1982). Very few SPLI-positive terminals participate in axoaxonic contacts (Barber et al., 1979; DiFiglia et al., 1982). The majority of SPLI-positive terminals contain both small agranular and large granular vesicles (Chan-Palay and Palay, 1977; Barber et al., 1979; DiFiglia et al., 1982; Priestley et al., 1982).

SPLI-positive neurons in the spinal cord have been studied with an intensification of SPLI with colchicine (Hunt et al., 1981). SPLI-positive neurons are predominantly present within laminae IIo and V, and a few in laminae I, III and IV. The SPLI-positive neurons in laminae III and III have a single large dendritic profile directed into superficial layers. Some marginal neurons in lamina I (LaMotte & DeLanerolle, 1981) and some islet cells in lamina IIo (Barber et al., 1979; Hunt et al., 1981) seem to be SPLI-positive.

The concept that SP may be an excitatory transmitter released during activation of the nociceptive primary afferent fibers is further supported by the following findings. SPLI was found in 20% of the cells in the dorsal root ganglion and these cells had small somas and small probably unmyelinated or thinly myelinated axons (Hökfelt et al., 1975, 1977). Following a dorsal root ligation, the concentration of SPLI in the dorsal horn and dorsal root close to the spinal cord was decreased, whereas, the
concentration of SPLI on the ganglion side of the ligature was increased (Takahashi & Otsuka, 1975). These findings suggest that SP is transported from the ganglion to the dorsal horn via primary afferent fibers. A dorsal root ligation (Hökfeldt et al., 1977) or dorsal rhizotomy (Barber et al., 1979) markedly decreased SPLI-positive fibers in the substantia gelatinosa of the spinal cord. A chronic capsaicin treatment of newborn animals, known to produce degeneration in particular of the small-diameter primary afferent fibers in the spinal dorsal horn, and insensitivity to nociceptive stimulation (Jancsó et al., 1977; Yaksh et al., 1979; Hayes & Tyers, 1980; Nagy et al., 1980), decreased SPLI in lamina I & II (Gasparovic et al., 1964; Jancsó & Knyihar, 1975; Jessell et al., 1978) and abolished SPLI-positive terminals in the dorsal horn (Nagy et al., 1981). These capsaicin studies strongly suggest that SP may be a candidate for an excitatory neurotransmitter of the primary afferent fibers.

A Ca-dependent release of SP in response to the dorsal root stimulation or high-K has been demonstrated in the isolated rat spinal cord (Otsuka & Konishi, 1976) and in the cultures of dissociated dorsal root ganglion neurons (Mudge et al., 1979). Using a local perfusion of the rat spinal cord in vivo, Jessell et al. (1979a,b) demonstrated a release of SP by high-K or capsaicin as well as by sciatic nerve stimulation at the intensity which activates C- or Aδ-fibers.

Excitatory actions of substance P in the spinal cord have been reported. Correlative electrophysiological studies of SP excitation in relation to a specific modality of sensory input were attempted using iontophoretic application of SP in the spinal cord of intact animals. SP
excited approximately one-half of randomly selected neurons located in the deeper laminae (laminae IV-VI) of the dorsal horn (Henry et al., 1975), while about 85% of neurons classified as nociceptive and found in laminae I-III were excited by SP (Randić & Miletić, 1977).

While the slow and long-lasting nature of SP-induced depolarization of neurons (Otsuka et al., 1972; Konishi & Otsuka, 1974) is a common finding among different preparations, the membrane conductance change associated with the SP-induced depolarization is controversial. While an increase in the conductance was reported in motoneurons of the frog (Nicoll, 1976, 1978) and the newborn rat (Otsuka, 1978), no change in the conductance was observed in motoneurons and dorsal horn neurons of intact cats (Sastry, 1979a; Zieglgänsberger & Tulloch, 1979a). A decrease in the conductance was also reported in motoneurons of intact cats (Krnjević, 1977), mouse and rat spinal cord neurons in dissociated cell cultures (Hösli et al., 1981; Nowak & Macdonald, 1982), inferior mesenteric ganglia (Dun & Minota, 1981), sensory ganglia (Dray & Pinnock, 1982) and sympathetic ganglia (Adams et al., 1983).

The ionic mechanism of the SP-induced depolarization and the associated conductance decrease has been investigated in myenteric ganglia (Katayama et al., 1979), inferior mesenteric ganglia (Dun & Karczmar, 1979; Dun & Minota, 1981), cultured rat spinal neurons (Hösli et al., 1981), cultured mouse spinal neurons (Nowak & Macdonald, 1982) and sympathetic ganglia (Adams et al., 1983). In all cases, the SP-depolarization is thought to be predominantly due to a decrease in K conductance. A possibility that SP inhibits a particular voltage-
dependent K current, i.e., the \( I_M \) current (Brown & Adams, 1980; Adams et al., 1982a; see section on \( I_M \)), has been discussed (Nowak & Macdonald, 1982) and confirmed (Adams et al., 1983). However, a possible involvement of an increase of a Na-conductance in the excitatory response was also reported for cells of guinea-pig mesenteric ganglia (Dun & Minota, 1981) and some neuronal cell lines (Reiser et al., 1982).

In addition to the excitatory effect of SP, a depressant action of SP has been reported (Krnjević, 1977; Randić & Miletić, 1977; Sastry, 1979a; Davies & Dray, 1980). This finding is in a good agreement with the immunohistochemical data showing that some primary afferent fibers might converge on some ENK-containing interneurons especially marginal neurons of lamina I and islet cells of lamina II (Glazer & Basbaum, 1981; Hunt et al., 1981; Sumal et al., 1982), GABAergic neurons, probably islet cells (Barber et al., 1982) and stalked cells (Hunt et al., 1981) in lamina IIi, and SS-containing neurons in lamina III (Hunt et al., 1981).

Presynaptic actions of SP have been suggested in electrophysiological experiments. SP had a direct depolarizing effect on primary afferent fibers (Nicoll, 1976) and altered the excitability of primary afferent C- and A6-fibers in a complex manner (Randić et al., 1982). SP also modified the neurotransmitter release of spinal cord neurons in cell culture (Macdonald & Nowak, 1981) and depolarized rat dorsal root ganglion neurons, especially those having small cell bodies (Dray & Pinnock, 1982).

Somatostatin A tetradecapeptide somatostatin \((\text{H}_2\text{N-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH})\) was originally isolated from bovine hypothalami as a growth hormone release inhibiting factor.
Brazeau et al., 1973) and its wide distribution throughout the mammalian CNS has been demonstrated (Elde & Hökfelt, 1982). In dorsal root ganglia, the presence of SS-like immunoreactivity (SSLI) is found in 10% of the cell bodies of small dorsal root ganglion neurons. In the spinal cord, SSLI positive terminals are concentrated in lamina II (especially lamina IIo), Lissauer's tract and the adjacent areas of the lateral funiculus (Hökfelt et al., 1976; McQuillan, 1980; Hunt et al., 1981). SSLI-positive cell bodies are present mainly within lamina III (Hunt et al., 1981).

Voltage- and Ca-dependent release of SS from primary sensory neurons in cell culture (Mudge et al., 1977) and high-K evoked and Ca-dependent release of SS from primary afferent fibers of rats and cats in vivo (Jessell et al., 1979a) have been demonstrated.

The action of SS in the spinal cord seems inhibitory while an excitatory action of SS was reported in the cerebral cortex (Ioffe et al., 1978) and in the hippocampus (Dodd & Kelly, 1978; Olpe et al., 1980). SS depressed activities of nociceptive dorsal horn neurons in lamina I, II and V of intact cats (Randić & Miletić, 1978), and motoneurons and primary afferent fibers of frogs (Padjen, 1977). A bath application of SS also suppressed spontaneous activities of dorsal horn neurons in a rat spinal cord slice preparation bathed in a Ca$^{2+}$-free, Mg$^{2+}$-high solution, suggesting that the SS action is in part postsynaptic (Miletić & Randić, 1982). The membrane actions of SS on dorsal horn neurons in vivo are not known.
The SS-induced hyperpolarization of hippocampal CA1 neurons in vitro was found to be associated with a conductance increase (Pittman & Siggins, 1981). In cultured mouse spinal neurons, SS did not alter the membrane potential nor the input resistance but it modified the neurotransmitter release presynaptically (Macdonald & Nowak, 1981). SS was also found to decrease the voltage-dependent Ca-conductance of sensory ganglion neurons in cell culture, implying that SS might contribute to the presynaptic inhibition (Dunlap & Fischbach, 1981).

Enkephalin Pentapeptides methionine-enkephalin (Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (Try-Gly-Gly-Phe-Leu-OH) were first discovered in porcine and bovine brains (Hughes et al., 1975; Simantov & Snyder, 1976). Both immunoreactivities are found throughout the CNS and with a similar distribution pattern (Hökfelt et al., 1977; Sar et al., 1978). In the spinal cord, ENK-like immunoreactivity (ENKLI) is found in interneurons of the superficial laminae, particularly in marginal neurons of lamina I and islet cells of lamina II (Hunt et al., 1981; Sumal et al., 1982), and nerve terminals of laminae I, II, V and VII (Hökfelt et al., 1977; Hunt et al., 1980, 1981; Glazer & Basbaum, 1981). The latter are not of primary afferent origin (Micevych et al., 1983).

The concept of presynaptic site of ENK actions (Nicoll et al., 1980) is at present controversial. However, the following findings seem to indicate presynaptic actions of ENK. A high density of opiate receptors has been found in the substantia gelatinosa of the spinal cord (Atweh & Kuhar, 1977) and on the neurites of cultured dorsal ganglion neurons (Hiller et al., 1978). The number of the receptors in the spinal cord
was decreased by dorsal root section (LaMotte et al., 1976; Jessell et al., 1979b; Ninković et al., 1981). In addition, ENK inhibited the release of substance P from primary sensory neurons in culture and decreased the action-potential duration (Mudge et al., 1979). In contrast, morphological findings seem to suggest a postsynaptic site of ENK actions. Very few axoaxonic contacts have been found in the superficial layers of the dorsal horn (Duncan & Morales, 1978; Ralston, 1979; Zhu et al., 1981) and ENKLI terminals form predominantly axodendritic or axosomatic synapses with lamina I neurons (Glazer & Basbaum, 1981; Ruda, 1982), and with lamina II neurons (Hunt et al., 1980, 1981; Glazer & Basbaum, 1981; Sumal et al., 1982).

Electrophysiological results concerning the neuronal site of ENK actions are also conflicting. When ENK was applied iontophoretically in the substantia gelatinosa of the spinal cord, the activation of laminae IV and V neurons by noxious stimulation, but not by innocuous stimulation, was blocked (Duggan et al., 1977). Iontophoretically applied ENK in the substantia gelatinosa not only depressed the activity of the neurons in the region (Randić & Miletić, 1978) but also decreased the excitability of Aδ- and C primary afferent terminals (Sastry, 1978, 1979b). The above results suggest presynaptic site of ENK actions. However, postsynaptic site of ENK actions was also demonstrated. In an intracellular study of dorsal horn neurons of the intact cat, iontophoretically applied ENK did not modify the membrane potential nor the input resistance of dorsal horn neurons but reduced the efficacy of a putative excitatory transmitter l-glutamate postsynaptically (Zieglgänsberger & Tulloch, 1979b).
Spontaneous activities of dorsal horn neurons in a Ca\(^{2+}\)-free, Mg\(^{2+}\)-high solution were depressed by ENK (Miletic & Randic, 1982). ENK hyperpolarized myenteric neurons (North & Tonini, 1977; North et al., 1979) and locus coeruleus neurons (Pepper & Henderson, 1980) by increasing a K-conductance.

ENK actions on Ca conductances have been found and discussed in relation to its presynaptic action. ENK was found to decrease the SP release in the trigeminal nucleus (Jessell & Iversen, 1977) and in sensory neurons in cell culture (Mudge et al., 1979). This finding suggests the inhibition of the Ca-influx by ENK. In addition, ENK decreased Ca-spike duration of sensory ganglion neurons in a cell culture without affecting the membrane potential (Dunlap & Fischbach, 1978). The decrease in the Ca spike duration was due to a decrease of the voltage-dependent Ca-conductance (Dunlap & Fischbach, 1981).

**Voltage-dependent conductances and neurotransmitters**

Experimental evidence has revealed that some putative neurotransmitters modify cellular functions by means of acting on voltage-dependent conductances. In this section, voltage-dependent conductances identified in vertebrate neurons, and actions of putative neurotransmitters on voltage-dependent conductances are reviewed.

The ionic mechanism for the generation of action potentials in squid giant axons was first described by Hodgkin & Huxley (1952) using the voltage-clamp technique. The excitation of the membrane was explained in terms of two ionic currents, the fast sodium current (\(I_{\text{Na}}\)) and the fast
potassium current (the delayed rectifier current: $I_K$), and the behavior of each current was satisfactorily described by a kinetic model of the ionic conductance. The kinetics of an ionic conductance consists of two major steps, the activation and the inactivation. The voltage dependency and the time dependency of the activation constants and the inactivation constants characterize the ionic conductance. The advantages of the kinetic model are that it relates to the gating mechanism of ionic channels and that it allows one to predict the behavior of a neuron in unclamped conditions.

In vertebrate neurons, ten different ionic conductances ($I_{Na}, I_{K'}, I_{Ca}, I_{K(Ca)}, I_{A}, I_{M}, I_{Q}, I_{Fir}$, the slow Na conductance and the persistent slow Ca current) have been identified with the voltage-clamp technique and one additional conductance (the low threshold Ca conductance) has been proposed from current-clamp experiments. The parameters of each conductance, however, seem to differ among preparations and among neurons, and the differences of the parameters may define the "personality", i.e., the individual properties of the neuron or the functional role of a group of neurons (Adams, 1982).

$I_{Na}$ and $I_{K'}$ exist in all types of vertebrate neurons tested. $I_{Na}$ has a fast activation and a fast inactivation (Araki & Terzuolo, 1962; Barrett & Crill, 1980; Barrett et al., 1980; Kostyuk et al., 1981a), and is responsible for the fast rise of action potentials. Tetrodotoxin (TTX) was found to specifically block $I_{Na}$ (Narahashi et al., 1964).
A fast activation of $I_K$ is a common feature among different preparations and is responsible for the fast repolarization of action potentials and the fast afterhyperpolarization following the repolarization. An inactivation of $I_K$ exists in immature rat dorsal root ganglion neurons (Kostyuk et al., 1981c) and in frog sympathetic ganglion neurons (Adams et al., 1982a) while squid axons (Hodgkin & Huxley, 1952) and cat spinal motoneurons (Barrett et al., 1980) lack it. Tetraethylammonium (TEA) (Armstrong, 1971), aminopyridines (3-AP & 4-AP) (Llinás et al., 1976; Yeh et al., 1976) and a replacement of the intracellular K with caesium (Cs) block $I_K$. In cat spinal motoneurons in vivo a fast-activating K current was depressed by Ba or Ca-blocking divalent cations (Co, Mn & Ni), as well as by TEA and 4-AP (Schwindt & Crill, 1980b,c, 1981).

$I_{Ca}$ The voltage-dependent Ca current ($I_{Ca}$) has been demonstrated in invertebrate and vertebrate neurons (Hagiwara, 1973; Hagiwara & Byerly, 1981; Reuter, 1983). $I_{Ca}$ of dorsal root ganglion neurons in immature rats (Kostyuk et al., 1981b), cultured sensory neurons of chick embryo (Dunlap & Fischbach, 1981) and rat sympathetic neurons (Galvan & Adams, 1982) has been studied with the voltage-clamp technique. The activation of $I_{Ca}$ is slower than $I_{Na}$ and a very slow inactivation was observed (Dunlap & Fischbach, 1981; Kostyuk et al., 1981b). The inactivation of $I_{Ca}$ due to an increase of the intracellular Ca concentration, has been demonstrated in invertebrate neurons (Tillotson, 1979; Alkon et al., 1982; Plant et al., 1983) and in vertebrate sympathetic ganglion neurons (Adams et al., 1982c), and discussed for cultured sensory neurons of chick
embryo (Dunlap & Fischbach, 1981).

In current clamp experiments, after blocking $I_{Na}$ and $I_K$, $I_{Ca}$ appears as a distinct spike (the Ca spike) with a slow rise and a plateau. The Ca spike was demonstrated in frog motoneurons (Barrett & Barrett, 1976), CA1 and CA2 hippocampal pyramidal cells (Schwartzkroin & Slawsky, 1977; Wong et al., 1979), cerebellar Purkinje cells (Llinás & Sugimori, 1979), mouse spinal neurons in cell culture (Heyer et al., 1981; Heyer & Macdonald, 1982), inferior olivary neurons (Llinás & Yarom, 1981a), thalamic neurons (Llinás & Jahnsen, 1982), neocortical neurons (Connors et al., 1982) and motoneurons of neonatal rats (Harada & Takahashi, 1983).

$I_{Ca}$ can be blocked by Co, Mg, Mn, Ni, Cd, La, verapamil and D600 (Baker et al., 1973; Hagiwara & Byerly, 1981; Reuter, 1983), and Cd is said to be the most specific (Adams, 1982). Ba can move through the Ca channels faster than Ca (Werman & Grundfest, 1961; Hagiwara, 1973) and does not activate $I_K(Ca)$ (Krnjević et al., 1971; Eckert & Lux, 1976).

The regulation of $I_{Ca}$ in the excitable membrane may play an important role in many cellular functions. The intracellular free calcium modulates the metabolism of neurons and the membrane excitability by influencing the permeabilities of the other ionic channels. It also regulates the release of neurotransmitters from the nerve terminals (Hagiwara, 1973; Hagiwara & Byerly, 1981; Reuter, 1983).

A presynaptic modulation (increase) of $I_{Ca}$ by serotonin (5HT) was postulated in sensory neurons of Aplysia on the basis of observations that 5HT increased the c-AMP level in the cells and that an intracellular injection of c-AMP increased the duration and the amplitude of Ca spikes.
(Klein & Kandel, 1978; Pellmar & Carpenter, 1979, 1980). However, it was recently found that the increase in the duration of Ca spikes induced by 5HT was due to a decrease of a novel K conductance (S-current) (Klein & Kandel, 1980; Klein et al., 1982; Siegelbaum et al., 1982) which may regulate the concentration of the intracellular free calcium. In Helix neurons, an increase of the Ca-spike duration by 5HT, dopamine and an intracellular injection of c-AMP was also found to be due to a decrease of a voltage-dependent K conductance (Paupardin-Tritsch et al., 1981). c-AMP, thus, probably mediates the decrease of the voltage-dependent K conductance induced by 5HT and dopamine (Deterre et al., 1982). In addition, egg-laying hormone increased $I_{\text{Ca}}$ in Aplysia (Kaczmarek et al., 1982). A possibility of an increase of a Ca conductance by l-glutamate was discussed for frog motoneurons (Kudo & Oka, 1982).

Presynaptic inhibition by reducing the $I_{\text{Ca}}$ of the presynaptic terminal was reported in Aplysia (Shapiro et al., 1980). In sensory ganglion neurons maintained in cell culture, 5HT, noradrenaline (NA), gamma-aminobutyric acid (GABA), SS, and ENK decreased the Ca-spike duration and reduced the release of a putative excitatory neurotransmitter, SP (Dunlap & Fischbach, 1978; Mudge et al., 1979). A voltage-clamp study revealed that the decrease of the Ca-spike duration by the putative neurotransmitters (NA, GABA, and 5HT) was due to a decrease of $I_{\text{Ca}}$ (Dunlap & Fischbach, 1981). NA decreased the duration of Ca spikes of rat sympathetic neurons in vitro (Horn & McAfee, 1980) and decreased $I_{\text{Ca}}$ of frog sympathetic neurons in a voltage-clamp study (Galvan & Adams, 1982).
The Ca-dependent K current \( I_{K(Ca)} \) was originally described in molluscan cells in voltage-clamp experiments (Meech & Standen, 1975; Meech, 1978; Gorman & Thomas, 1980; Herman & Hartung, 1982) and later demonstrated in cat motoneurons (Barrett et al., 1980), frog sympathetic ganglion neurons (Adams et al., 1982c) and guinea-pig hippocampal neurons (Brown & Griffith, 1983). It is activated by an increase in the intracellular free calcium. In current clamp experiments, \( I_{K(Ca)} \) appears as a slow afterhyperpolarization (Godfraind et al., 1971; Barrett & Barrett, 1976; McAfee & Yarowsky, 1979; Grafe et al., 1980; Hotson & Prince, 1980; Schwartzkroin & Stafström, 1980; Hablitz, 1981; Wong & Prince, 1981; Harada & Takahashi, 1983). In Helix neurons, \( I_{K(Ca)} \) is an important determinant of the action potential duration (Molenaar & Spector, 1979).

\( I_{K(Ca)} \) can be blocked by replacing the intracellular K with Cs. Intracellular TEA (10^-2 M) reduced \( I_{K(Ca)} \) by 90% in rat dorsal root ganglion neurons but extracellular TEA (2x10^-2 M) did not affect it (Kostyuk et al., 1981c). However, extracellular TEA (2x10^-2 M) decreased the total outward current by approximately 95% in rat dorsal root ganglion neurons in cell culture (Dunlap & Fischbach, 1981), and extracellular TEA (5x10^-3 M) abolished \( I_{K(Ca)} \) of frog sympathetic ganglion neurons (Adams et al., 1982a) and guinea-pig hippocampal pyramidal neurons (Brown & Griffith, 1983). 4-AP (10^-3 M) exhibited no effect on \( I_{K(Ca)} \) in frog sympathetic ganglion neurons (Adams et al., 1982a) while \( I_{K(Ca)} \) in cat spinal motoneurons in vivo was depressed by iontophoretically applied extracellular 4-AP.
and intracellular TEA (Schwindt & Crill, 1980b,c).

An activation of $I_{K(Ca)}$ by ENK and clonidine, as a consequence of an increase in the intracellular free calcium but not an increase in $I_{Ca'}$ has been postulated for myenteric ganglion neurons (Tokimasa et al., 1981; Morita & North, 1982). Also, an increase of $I_{K(Ca)}$ by dopamine was reported in hippocampal pyramidal cells (Benardo & Prince, 1982).

$I_A$ and $I_M$ The transient outward current ($I_A$) is a fast-activating and fast-inactivating K current (Connor & Stevens, 1971; Adams et al., 1982a), while the M-current ($I_M$) is a slow-activating and noninactivating K current (Adams et al., 1982a). In contrast to $I_{Na'}$, $I_K$ and $I_{Ca'}$, which are predominantly activated at depolarized levels above the action potential threshold, $I_A$ and $I_M$ operate in the subthreshold range. In current-clamp experiments, a hyperpolarizing current pulse deinactivates $I_A$, exhibiting a notch on the repolarizing phase of the pulse response (Connor & Stevens, 1971).

$I_M$ was recently discovered in frog sympathetic ganglion neurons (Adams et al., 1982a; Akasu & Koketsu, 1982) and also found in mammalian sympathetic neurons (Constanti & Brown, 1981; Hashiguchi et al., 1982), guinea-pig hippocampal pyramidal cells (Adams et al., 1981; Halliwell & Adams, 1982) and cultured mouse spinal neurons (Nowak & Macdonald, 1983a,b).

$I_M$ is suppressed by muscarinic acetylcholine receptor agonists (Brown & Adams, 1980; Akasu & Koketsu, 1982; Brown et al., 1982), a
high concentration of TEA, external Ba (Constanti et al., 1981; Adams & Brown, 1982) and putative peptidergic neurotransmitters, such as luteinizing hormone release hormone (LHRH) (Adams & Brown, 1980), angiotensin (Constanti & Brown, 1981), uridine nucleotides (Brown et al., 1982) and SP (Nowak & Macdonald, 1982; Adams et al., 1983).

\( I^- \) and \( I^\text{fin} \). The slow inward-rectifying current (\( I^- \)) found in hippocampal pyramidal neurons (Adams & Halliwell, 1982; Halliwell & Adams, 1982) and the fast inward-rectifying current (\( I^\text{fin} \)) of olfactory cortex neurons (Constanti & Galvan, 1983) are activated at a more negative level than the resting membrane potential and are responsible for the anomalous rectification.

\( I^\text{fin} \) is a K current activated at a more negative level than \( I^- \), while \( I^- \) is a mixed Na/K current with the reversal potential of about -60 mV. Both are TTX-resistant. \( I^\text{fin} \) can be blocked by Cs and Ba but not by TEA or Cd, while \( I^- \) is blocked by Cs but not by Ba. A slow inward rectifying current, which could not be blocked by Ca-blocking divalent cations, Ba or intracellular Cl, has been found in cat spinal motoneurons and neocortical neurons (Barrett et al., 1980; Stafström & Schwindt, Physiology and Biophysics, University of Washington, personal communication). An anomalously rectifying K current in Aplysia, which could be blocked by Cs and Ba but not Cd, has been reported to be increased by 5HT (Benson & Levitan, 1983).
**Slow Na conductance, persistent slow Ca current and low threshold Ca spike**

These currents seem to be important for the bursting behavior in several types of mammalian neurons. The slow Na conductance and the persistent slow Ca current appear as a regenerative plateau in response to a subthreshold depolarization while the low threshold Ca spike is generated during the repolarizing phase of the afterhyperpolarization.

The slow Na current, found in cerebellar Purkinje cells, appears to be a slow-activating, noninactivating Na conductance that can be blocked by TTX but not by Co (Llinás & Sugimori, 1980a,b). A similar Na current was found in neocortical neurons of cats studied with the voltage clamp technique (Stafström et al., 1982).

The persistent slow Ca conductance has been described in hippocampal neurons as a TTX-resistant regenerative plateau carried by Ca ions and responsible for the occurrence of spontaneous bursts (Schwartzkroin, 1975, 1980; Prince & Schwartzkroin, 1978; Wong & Prince, 1978; Hablitz & Johnston, 1981). Recent voltage-clamp studies revealed its slow activating and noninactivating nature (Johnston et al., 1980; Brown & Griffith, 1983). A subthreshold persistent slow Ca current has been found to be responsible for the anomalous rectification and the bursting behavior of cat spinal motoneurons (Schwindt & Crill, 1980a,b,c).

The low-threshold Ca spike was found to be due to a fast-inactivating Ca conductance. This Ca conductance appears as a spike if the neuron is hyperpolarized and it is inactivated at the resting level (Llinás & Yarom, 1981a,b). The site of the low-threshold Ca conductance was found to be in the soma of the neuron and its functional role in maintaining
spontaneous firing was postulated. It was also found in thalamic neurons (Llinás & Jahnsen, 1982).

Intracellular recordings of electrical activities from spinal dorsal horn neurons

Intracellular recordings are necessary for examining the membrane actions of neurotransmitters. In this section, previous intracellular studies of spinal neurons \textit{in vivo} and attempts to develop \textit{in vitro} preparations of the mammalian spinal cord are reviewed.

\textbf{Dorsal horn neurons \textit{in vivo}}

Intracellular recordings from predominantly large dorsal horn neurons in intact animals have been achieved, and they have provided information on the electrophysiological properties of the neurons and the functional organization of the spinal dorsal horn (Frank & Fuortes, 1956; Haapanen et al., 1958; Hunt & Kuno, 1959; Eccles et al., 1960; Hongo et al., 1966). Frank & Fuortes (1956) reported that some interneurons in the cat discharged impulses with a rhythmic bursting pattern in the absence of applied stimulation. Action potentials of dorsal horn neurons were found to have a duration of .5 to 1.0 msec and to be followed by a brief post spike positivity but lacked the prolonged afterhyperpolarization which is characteristic of motoneurons (Hunt & Kuno, 1959). No accurate measurements of the time constant, input resistance, membrane potential and action potential size could be made \textit{in vivo} because of the instability of the preparation.

Satisfactory recordings from small interneurons in the superficial parts of the spinal dorsal horn have not as yet been obtained (Cervero
et al., 1977; Yaksh et al., 1977; Hayashi et al., 1978; Bennett et al., 1979; Wall et al., 1979). The low quality of the in vivo recordings did not allow rigorous electrophysiological analysis of neurotransmitter actions (Cervero et al., 1977, 1979; Sastry, 1979a; Zieglgänsberger & Tulloch, 1979a,b).

Spinal cord in vitro Since the introduction of a hippocampal slice preparation in electrophysiology by Yamamoto & McIlwain (1966), the slice technique has been extensively used in electrophysiological studies of mammalian central neurons. The primary advantage of the technique is that it allows stable intracellular recordings for many hours, as well as the possibility of changing the microenvironment of the neurons, i.e., the modification of the ionic composition of the perfusing solution or the utilization of specific ionic channel blockers in the solution.

An in vitro preparation of the mammalian spinal cord was originally developed by Otsuka & Konishi (1974), who introduced the hemisected spinal cord of newborn rats (0-7-days-old). The dorsal root potentials evoked by stimulation of the dorsal roots were recorded. Also, intracellular recordings from the motoneurons were achieved (Konishi & Otsuka, 1974). This preparation was later utilized by other investigators (Fulton et al., 1980; Fulton & Walton, 1981; Harada & Takahashi, 1983).

A transversal slice preparation of the spinal cord from newborn rats (1-4-day-old) was first developed by Takahashi (1978). The motoneurons were visually identified with Nomarski optics and the activities were recorded intracellularly. Lately, single unit extracellular recordings from
the dorsal horn neurons were achieved in a transversal slice preparation of young rats (2-30 days-old) and the effects of several peptides were tested (Miletic & Randic, 1980a,b, 1982).

Isolated hemisected spinal cord preparations of the adult mouse (Bagust & Kerkut, 1979, 1980) and hamster (Bagust et al., 1982) have been developed and the field potentials and root potentials were satisfactorily recorded. In addition, transversal and sagittal slice preparations of adult rat spinal cords have been used to record field potentials and the single unit activities extracellularly in the dorsal horn (Dhanjal & Sears, 1981a,b). Recently, activities of neurons in the lateral horn of the spinal cord were recorded intracellularly in a transversal slice preparation of adult cats (Yoshimura & Nishi, 1982).

Small interneurons in the superficial parts of the vertebrate spinal dorsal horn had long resisted intracellular study both in vivo and in vitro. Our preliminary experiments using an immature rat spinal cord slice preparation were the first to demonstrate the possibility of obtaining a stable intracellular recordings from the neurons for as long as 5 hours (Murase & Randic, 1981).

Rationale

Intracellular recordings from predominantly large mammalian spinal dorsal horn neurons in the intact animals and spinal neurons in cell culture have provided important information about their electrophysiological properties and functional organization. However, the neurons of the
superficial parts of the spinal dorsal horn have long resisted electrophysiological analysis due to the relatively small size of the cells and the technical difficulties of obtaining stable intracellular recordings in vivo. Successful development of an in vitro preparation of the spinal cord should satisfactorily solve the stability problems, and allow us to study the electrophysiological properties of the dorsal horn neurons such as the passive membrane time constant, the cell input resistance, the resting membrane potential, the action potential duration and height, the threshold, etc. A particular advantage of the slice preparation is that the extracellular environment may be modified, thus, allowing a more thorough analysis of the biophysical properties of the neurons, i.e., their voltage-dependent conductances.

The investigation of voltage-dependent conductances of central neurons is of considerable interest because they characterize the neuronal activities, and several putative neurotransmitters seem to alter the neuronal functions by modulating specific voltage-dependent conductance(s). Although a rigorous identification of voltage-dependent conductances requires voltage-clamp experiments, valuable information about some ionic conductances can be obtained in current-clamp experiments by utilizing agents known to block specific voltage-dependent ionic conductances. The voltage-dependent Ca conductance, which appears as the Ca spike in current-clamp conditions when Na- and K-channel blockers are used, seems worth investigating because of its prominent influence on cellular functions such as the synaptic transmission mechanism, the firing behavior
and the cellular metabolism - the functions which peptides might influence.

The concept that substance P (SP), somatostatin (SS) and enkephalin (ENK) act as neurotransmitters and/or neuromodulators in the superficial laminae of the spinal dorsal horn has been strongly supported by a number of histochemical studies. However, electrophysiological evidence for this hypothesis has been confined to indirect observations obtained from extracellular recordings in laminae I-III and intracellular recordings from neurons in the deeper laminae of the dorsal horn in intact animals, or in a dissociated culture of mammalian spinal neurons using intracellular recordings. Development of an in vitro preparation of the spinal cord will allow us to explore not only the sites of the peptide actions but also the biophysical mechanisms of their actions on the neuronal membrane. In addition, bath application of the peptides into the perfusing solution allows dose-response analysis and circumvents the frequently encountered "blocking" problem of electrodes used for microiontophoresis.

Isolated preparations could be effectively utilized to resolve much of the debate concerning sites of peptide actions in the spinal dorsal horn. For instance, by perfusing the slice with a Ca$^{2+}$-low, Mg$^{2+}$-high solution or a TTX-containing solution, the postsynaptic effects of the peptide could be isolated from the presynaptic or interneuronal interactions. It would be of a particular interest to determine whether the inhibitory effects of ENK and SS are presynaptic, and whether SP exhibits any indirect (synaptically-mediated) inhibitory effects. The questions regarding the peptide-elicited changes in the cell input resistance
accompanying the changes in membrane polarization could also be answered in the isolation from the indirect effects.

We chose to investigate ionic mechanisms of SP actions because SP has been the most extensively studied neuroactive peptide and presently is the best established candidate for an excitatory neurotransmitter of small primary afferent neurons. By utilizing several specific ionic channel blockers in the perfusion media in conjunction with the current-clamp technique, the ionic basis of the depolarizing effect of SP was investigated. Furthermore, the analysis of possible changes in the profile of the Ca spike elicited by SP provided valuable information regarding the effects of SP on voltage-dependent conductances of dorsal horn neurons.
SECTION I. SPINAL CORD SLICE PREPARATION AND THE INTRACELLULAR RECORDING SYSTEM

To achieve satisfactory recordings from the small neurons in the superficial parts of the spinal dorsal horn, we have made the following attempts: (1) Since the interneurons in the laminae I and II have dendrites predominantly oriented in a cranial-caudal direction, in order to maximally preserve the neuronal integrity the spinal cord was sectioned horizontally, (2) To impale the small neurons, a new cell penetrator was designed. In this section, the spinal cord slice preparation and the equipment designed for the experiments are described.

Horizontal Slice Preparation of the Spinal Cord

Experiments were performed on 9 to 35-day-old Sprague-Dawley rats. An animal was initially anesthetized with ether and cooled by immersing the thorax and abdomen in ice chilled water. When the body temperature fell to about 22°C and the animal reached the stage of hypothermic anesthesia, a laminectomy was performed to expose the lower thoracic and lumbosacral spinal cord. Following the laminectomy, the lumbosacral spinal cord with attached dorsal rootlets was quickly excised and immersed into aerated (95% O₂ and 5% CO₂) Ringer solution at 24°C. The composition of the solution was (mM): NaCl, 124; KCl, 5; KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; glucose, 10; pH 7.4. After removal of the pia mater on the lateral aspects of the spinal cord, a segment of the lumbosacral spinal
cord was manually cut and the ventral side was glued with cyanoacrylic
glue to the bottom of a chamber on the Oxford Vibratome. The chamber of
the Vibratome was filled with the Ringer solution at 24°C, the blade
of the Vibratome was positioned 300 μm below the dorsal surface of the
spinal cord and the spinal cord was sectioned to yield one horizontal
300 μm thick dorsal horn slice having dorsal rootlets. The duration of
the entire procedure from the removal of the spinal cord until the slice
was made usually did not exceed 5 min. After incubation at 35°C for
about an hour, the slice was transferred into a recording chamber. The
recording chamber was located under a microscope (100x) to allow clear
visual identification of the dorsal horn. The capacity of the chamber
was 0.5 ml and the slice was continuously perfused with modified oxygenated
Ringer solution (NaCl, 127; KCl, 1.9; \( \text{KH}_2\text{PO}_4 \), 1.2; CaCl_2, 2.4; MgSO_4,
1.3; NaHCO_3, 26; glucose, 10) at a flow rate of 2 ml/min. The tempera­
ture of the chamber was continuously monitored with a small thermistor
located in the chamber and controlled to 33°C by a feedback circuit and
a heating coil mounted on the chamber.

Synaptic activation of the dorsal horn neurons was obtained with
co-axial stainless-steel electrodes positioned on the dorsal roots. The
peptides were applied either by switching the Ringer solution to the
solution containing a known concentration of the peptide or by pressure
micro-injection. For the latter purpose, a micropressure application
unit was designed.
Intracellular Recording System

Intracellular recordings were performed with 3M K-acetate filled glass micropipettes having DC resistances of about 100 MΩ. The recordings were made by means of a high input impedance bridge amplifier (WPI Instruments, M707) allowing current injections on the order of 0.05–3.0 nA through the recording electrode. In order to determine the threshold of the action potentials, a stimulus signal controller, which can generate ramp signals and pulse currents with known amplitude as well as DC currents, was designed. The recorded signals were displayed on the screen of an oscilloscope and were either photographed or stored on floppy diskettes of a digital oscilloscope (Nicolet Instruments, M4094) for further analysis of the data using the programs of the oscilloscope (such as differentiation and filtering). The membrane potential was continuously monitored on a pen recorder (Gould, M2200). In order to analyze the neuronal responses when different stimulus parameters were used, a plug-in trace shifter for the Tektronix storage oscilloscope (M5113) was designed.

Cell Penetrator

Although manual tapping on the recording electrode or the experimental set-up has been employed to penetrate large neurons, small neurons can hardly be penetrated by this technique. In order to penetrate small neurons several investigators have developed precise mechanisms involving a fast displacement for a few micrometers or a small vibration of the
electrode. Jolting of the recording chamber by an electromagnetic mechanism was used for recording from small neurons in the retina (Tomita, 1965). Tapping of the electrode by electromagnetic mechanisms (Frank & Becker, 1964; Van der Pers, 1980), magnetostrictive mechanisms (Weiler & Zettler, 1976) or piezoelectric devices (Pascoe, 1955; Ellis, 1962; Lassen & Sten-Knudsen, 1968; Rikmenspoel & Lindemann, 1971; Chen, 1978; Fromm et al., 1980; Hengstenberg, 1981), and improving the speed of a microdrive (Brown & Flaming, 1977; Marshall & Klyce, 1981) are alternative procedures that have been utilized for penetration of a variety of small neurons.

Another method utilizes the passage of pulse (Zettler & Järvilehto, 1971; Llinás & Sugimori, 1980a) or high frequency (DeVoe, 1975) electrical currents through the recording electrode. Electrical current penetrators seem to have advantages over the tapping methods because it does not require a precise mechanical system and it allows control of the duration and strength of the electrical pulse in relation to the cell properties and the conditions of the tissue.

A cell penetrator utilizing the electrical current method was designed for our experiments (Fig. 1). It can generate a positive or negative current pulse, or a high frequency oscillation current with arbitrary duration and strength, characteristics necessary to accommodate the properties of a variety of cells and tissue conditions.

When the "PENETRATION" switch is depressed, the recording electrode is disconnected from the recording amplifier, and connected to the output of a high voltage pulse generator. After a short duration pulse is
Figure 1. Cell penetrator
delivered to the recording electrode, the electrode is again connected to the recording amplifier. The duration and the amplitude of the pulse may be chosen between 0.05 to 200 msec and 0 to 45 volts, respectively. The pulse may be selected by the "OUTPUT MODE" knob to be positive, negative or a train of smaller duration pulses. For dye injection with high voltage, the "EXT. INPUT" can be used.

When the "PENETRATION" button is pushed, a relay is turned on by a one-short multivibrator for a period of time during which the electrode is connected to the output of a high voltage operational amplifier (IC7). The input of the operational amplifier is connected to positive and negative voltage sources (IC5 & IC6) through C-MOS switches (IC8). The combination of the conditions of the C-MOS switches defines the mode of the output of the operational amplifier; positive, negative or oscillation of smaller duration pulses. The C-MOS switches are controlled by the output of multivibrators triggered with a delay after the "PENETRATION" switch is depressed.

Bath Temperature Controller

The primary purpose of this unit is to maintain a constant temperature (33°C) of the recording chamber. This is accomplished through a thermistor located in the chamber, a heating coil mounted on the chamber and a feedback on-off control circuit. An additional feature of this unit is that calibration can be achieved by a simple procedure.

The circuit diagram is in Fig. 2. The temperature of the chamber is detected by a thermistor, which is one arm of a bridge circuit, and the
Figure 2. Bath temperature controller
desired bath temperature is set by a potentiometer (TEMP. SET), which makes up another side of the bridge. If the bath temperature is lower than the temperature set by the "TEMP SET" knob, a comparator is activated, a relay is turned on, and DC current to heat the chamber is supplied to the heating coil.

A high-sensitivity DC current meter is connected across the bridge so that the temperature of the chamber can be monitored continuously. The meter deflects from 30°C to 40°C. The calibration is achieved with "30°C CAL." and "40°C CAL." knobs at the positions c and d of the switch SW1. At these positions, dummy resistors, which have identical resistances to the thermistor at 30°C and 40°C, respectively, are connected to the bridge instead of the thermistor. The "30°C CAL." knob adjusts the zero balance of the bridge by varying one arm of the bridge and the "40°C CAL." knob sets the full scale of the meter by changing the total current to the bridge.

**Stimulus Signal Controller**

The function of this instrument is to modify the square pulse output of a Grass stimulator (S88) into current command potentials necessary for the use of an intracellular bridge recording amplifier (WPI, M707). This instrument supplies three different signals; ramp, square pulse, and DC, to the amplifier. A ramp wave can be generated from a square pulse input and it may be used for testing the threshold of action potentials. Square pulses can be attenuated accurately and used for I-V curve measurements etc. DC current may be used to depolarize or hyperpolarize the
Figure 3. Stimulus signal controller
neurons. These three kinds of command signals can be superimposed in this unit if desired.

The circuit diagram is shown in Fig. 3. A ramp wave can be generated by an integrator (IC2) when the "GATE INPUT" is high (TTL level). The slope of the ramp wave can be adjusted by the "RAMP SLOPE" knob which changes the DC potential supplied to the integrator.

Square pulses supplied from an external isolated source to the "SQUARE PULSE INPUT" terminal and an internal DC potential can be superimposed on the ramp wave. The polarity and the amplitude of the square pulses and the DC potential can be adjusted by the corresponding knobs.

Trace Shifter

This plug-in unit for the storage oscilloscope (Tektronix 5100 series) allows one to display multiple traces on the screen from a single source of signal by shifting the trace vertically at each trigger pulse given to the "TRIG. INPUT". The number of traces can be chosen to be either 20, 10 or 4 by the "NUMBER OF TRACES" knob. After displaying the selected number of traces, the screen can be automatically erased and the next trace starts from the top of the screen if the "AUTO ERASE" is in the "ON" position.

The circuit diagram is illustrated in Fig. 4. The input signal is amplified by an operational amplifier (IC1) and supplied to the positive input of the oscilloscope. The trigger pulses given to the "TRIG. INPUT" are transmitted to the trigger input of the oscilloscope, and also the number of trigger pulses is counted by a series of counter IC's (IC5 & 6).
Figure 4. Trace shifter
The content of the counters is converted to a DC potential by a D/A converter (IC3), and the output of the D/A converter is delivered to the negative input of the oscilloscope. When the number of the counts reaches the value preset with the "NUMBER OF TRACES" knob, or when the "RESET" knob is depressed, the counters are reset to zero and the screen is erased by activating a relay and a photo-coupler connected to the erasing circuit of the oscilloscope.

Pressure Application Unit

The function of this instrument is to apply a small volume of a drug solution from a micropipette into the recording chamber by opening a solenoid air valve for a short period of time and applying a positive air pressure to the glass pipette.

The opening time of the solenoid valve is controlled by an one-shot multivibrator (Fig. 5). The period can be selected from 1 msec to 100 sec by the combination of the positions of two knobs (SW1 & SW2). When the "SET" knob is depressed, the one-shot multivibrator is triggered and the solenoid valve is turned on for the selected period of time. During the "on" period, if the "RESET" knob is pressed, the circuit is reset and the valve is shut off.
Figure 5. Pressure application unit
SECTION II. ELECTROPHYSIOLOGICAL PROPERTIES OF RAT SPINAL DORSAL HORN NEURONES IN VITRO: CALCIUM-DEPENDENT ACTION POTENTIALS

Summary

1. The electrophysiological properties of dorsal horn neurones have been investigated in the immature rat in vitro spinal cord slice preparation.

2. Intracellular recordings from dorsal horn neurones show that direct or orthodromic stimulation generates action potentials followed by a brief after-hyperpolarization. Synaptic potentials were elicited by the activation of primary afferent fibres in the dorsal root.

3. Input resistance for dorsal horn neurones ranged from 48 to 267 MΩ, and the membrane time constant was in the range of 4-19 msec.

4. In response to strong depolarizing currents, dorsal horn neurones perfused with TTX and TEA frequently exhibit a slow regenerative depolarizing potential followed by a slow after-hyperpolarization. The depolarizing potential probably results from an influx of Ca. It is blocked by low concentration Ca, Co or Mn, and enhanced by high levels of extracellular Ca.

5. There is, in addition, a low-threshold Ca-dependent response which is activated at membrane potentials more negative than -65 mV and has a maximum rate of rise at the polarization level of about -80 mV.

6. The addition of Ba or TEA to the perfusing medium provided support for the Ca-dependence of the low- and high-threshold responses, and the lack of fast inactivation of the high-threshold Ca potential.

Introduction

Intracellular studies of predominantly large mammalian spinal dorsal horn neurones in intact animals (Frank & Fuortes, 1956; Haapanen, Kolmodin & Skoglund, 1958; Hunt & Kuno, 1959; Eccles, Eccles & Lundberg, 1960; Hongo, Jankowska & Lundberg, 1966) and spinal neurones in cell culture (Ransom, Neale, Henkart, Bullock & Nelson, 1977; Heyer, Macdonald, Bergey, & Nelson, 1981) have provided important information about their electrophysiological properties and functional organization. However, the neurones of the superficial parts of the spinal dorsal horn have long resisted electrophysiological analysis due to the relatively small size of the cells and the technical difficulties in obtaining stable intracellular recordings in vivo (Cervero, Molony & Iggo, 1977; Yaksh, Wall & Merrill, 1977; Hayashi, Price, Dubner & Ruda, 1978; Kumazawa & Perl, 1978; Bennett, Hayashi, Abdelmoumene & Dubner, 1979). We have, therefore, developed an in vitro rat spinal cord slice preparation in order to achieve stable recordings and more thoroughly analyse the biophysical properties of the nerve cells in the superficial laminae of the spinal dorsal horn. Our initial studies have demonstrated the viability of this preparation (Miletic & Randic, 1980a,b; Miletic & Randic, 1982) and that satisfactory intracellular recordings could be maintained for
several hours enabling us to analyse the membrane actions of several neuropeptides known to be concentrated in the superficial parts of the spinal dorsal horn (Murase & Randić, 1981; Murase, Nedeljkov & Randić, 1982).

In this paper we will describe the ionic nature of action potentials of neurones in the superficial parts of the spinal dorsal horn, as recorded in the immature rat slice preparation in vitro, and the effects of using agents known to block specific voltage-dependent ionic conductances. We will provide additional evidence in support of the present concept that action potentials in mammalian spinal neurones are generated by voltage-dependent conductance increases to Na and Ca ions (Heyer et al., 1981). Two distinct types of Ca spikes are probably present in immature rat dorsal horn cells. A preliminary report has appeared (Murase & Randić, 1982).

Methods

Preparation

Experiments were performed on 9-18 day-old Sprague-Dawley rats. The animals were anaesthetized with ether and cooled by immersing the thorax and abdomen in an ice-water slurry. During the period of cooling, close attention was paid to respiration. In the majority of animals, respiration continued for at least 10 min following the onset of cooling, when it became shallow and regular, by which time the skin temperature had fallen to 20-22°C. The dissection was then started and a laminectomy
performed to expose the lower-thoracic and lumbosacral spinal cord together with dorsal roots. Following laminectomy, about 1-1.5 cm long segment of lumbosacral spinal cord, with attached dorsal rootlets, was quickly excised and immersed into aerated (95% O$_2$ and 5% CO$_2$) Ringer solution at approximately 24°C. The composition of the solution was (mM): NaCl 124; KCl 5; KH$_2$PO$_4$ 1.2; CaCl$_2$ 2.4; MgSO$_4$ 1.3; NaHCO$_3$ 26; glucose 10; pH 7.4. After the removal of the pia mater on the lateral aspects of the spinal cord, the spinal segment was cut manually into 5 mm blocks, and one of the blocks fixed with cyanoacrylic glue (Borden, Inc.) to the bottom of a Plexiglass cutting chamber of an Oxford Vibratome. The bath of the Vibratome was filled with the aerated Krebs solution, maintained at 24°C. The blade of the Vibratome was positioned 300 μm below the dorsal surface of the spinal cord, and the spinal segment sectioned to yield one horizontal 300 μm thick dorsal horn slice. The duration of the entire procedure from the removal of the spinal cord until the slice was made rarely exceeded 5 min. The slices were incubated in Ringer solution at 35°C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Ringer solution (NaCl 127 mM; KCl 1.9 mM; KH$_2$PO$_4$ 1.2 mM; CaCl$_2$ 2.4 mM; MgSO$_4$ 1.3 mM; NaHCO$_3$ 26 mM; glucose 10 mM) at 33 ± 1°C at a flow rate of about 2 ml/min. The recording chamber had a capacity of 0.5 ml. Variations in the composition of the perfusing solution are indicated in the Results.
Intracellular recording technique

Intracellular recordings were performed with micropipettes filled with 3M-K acetate having d.c. resistances of 90-110 MΩ. Stable intracellular recordings from single dorsal horn neurones could be maintained during multiple solution changes for as long as 5 h. Electrical properties of dorsal horn neurones were determined by means of a high-input impedance bridge amplifier (WP Instruments, M707) allowing current injections of the order of 0.05-3.0 nA through the recording electrode. Synaptic activation of the dorsal horn neurones was obtained with co-axial stainless-steel stimulating electrode positioned on the dorsal roots.

Results

The results described in this paper were obtained from forty-six neurones located in the superficial parts of the spinal dorsal horn. The various parameter estimates for immature rat dorsal horn neurones are given in Table 1. For forty-six studied units the resting potential varied, between -54 to -86 mV, with the mean value of -70.3 mV ± 6.3 (S.D., n = 46).

Passive membrane properties

The passive electrical properties of dorsal horn neurones were investigated using intracellularly injected depolarizing and hyperpolarizing current pulses. The input resistance was calculated for sixteen cells from the slope of the current-voltage relationship. The mean values for
Table 1. Passive and active membrane properties of immature rat dorsal horn neurones

<table>
<thead>
<tr>
<th>Property</th>
<th>n</th>
<th>Mean ± S.D.</th>
</tr>
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<tbody>
<tr>
<td>Input resistance ($R_n$)</td>
<td>16</td>
<td>98.1 ± 58.7 MΩ</td>
</tr>
<tr>
<td>Membrane time constant ($T$)</td>
<td>17</td>
<td>10.4 ± 5.1 ms</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>46</td>
<td>70.3 ± 6.3 mV</td>
</tr>
</tbody>
</table>

Na spike

<table>
<thead>
<tr>
<th>Property</th>
<th>n</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overshoot</td>
<td>24</td>
<td>25.0 ± 7.8 mV</td>
</tr>
<tr>
<td>Spike duration</td>
<td>25</td>
<td>1.4 ± 0.5 ms</td>
</tr>
<tr>
<td>Threshold</td>
<td>24</td>
<td>25.8 ± 8.4 mV</td>
</tr>
</tbody>
</table>

input resistance and time constant are given in Table 1. The measured values for the input resistance (range: 48-267 MΩ) and the time constant (range: 4-19 ms) varied widely from cell to cell.

Direct and synaptic activation

Fig. 1 illustrates examples of intracellularly recorded action potentials from the soma of a dorsal horn neurone of a 14-day-old rat following direct (Fig. 1A-E) and orthodromic (Fig. 1F) stimulation. In Fig. 1A a single action potential of approximately 70 mV amplitude and a duration of 1.4 ms is initiated. Further increase in the current strength (Fig. 1B-E) reduced the latency of the first action potential and evoked repetitive discharge. The repetitive firing usually occurred from a slow
Fig. 1. Direct and synaptic activation of two dorsal horn neurones in a spinal cord slice preparation of a 14-day-old rat (A-E) and a 12-day-old rat (F). Resting membrane potential was -71 mV (A-E) and -66 mV (F). Upper traces in all figures show applied current pulses, lower traces voltage response. In A intracellular responses to depolarizing (upward) and hyperpolarizing (downward) current pulses applied in 0.05 nA steps across the cell soma are shown. In B-E depolarizing square current pulses of increasing strength injected in 0.1 nA steps evoked repetitive firing. The repetitive discharge occurred from a slow plateau-type depolarizing local response. In F synaptic and action potentials recorded from another dorsal horn neurone following stimulation of a dorsal root. Synaptic and action potentials superimposed on intracellularly injected current pulses. Note marked increase in amplitude of synaptic potentials with hyperpolarizing pulses (downward traces) and appearance of repetitive discharge with depolarizing pulses (upward traces).
plateau-type depolarizing local response (Fig. 1C-E). In Fig. 1E, the maximal firing frequency calculated for the first interspike interval was 165/s, and was reduced for the second interspike interval to 110/s. As reported previously (Haapanen et al., 1958; Hunt & Kuno, 1959) the spike is often followed by a brief hyperpolarization relative to the firing level. In Fig. 1F the responses of another dorsal horn cell in a 12-day-old rat to a single dorsal root afferent volley are shown. An excitatory synaptic potential (e.p.s.p.) is evoked which was subthreshold for initiation of an action potential (third trace from above). The properties of this potential were examined by using hyperpolarizing (-0.2 to -1.2 nA) and depolarizing (0.2-0.4 nA) square current pulses of increasing strength and about 80 ms duration injected in 0.2 nA steps across the cell soma. With hyperpolarizing currents, as seen in Fig. 1F, the amplitude of the synaptic potential was elevated, while depolarizing current pulses evoked repetitive action potentials.

The high-threshold Ca action potential

Addition of tetraethylammonium chloride (TEA) to the Ringer solution slowed the rate of repolarization of the action potential in a dorsal horn neurone of a 12-day-old rat slice, prolonged the spike duration, and enhanced the after-hyperpolarization (Fig. 2B). Following the addition of tetrodotoxin (TTX) to the TEA-containing medium, in order to block the Na conductance, the fast action potential shown in Fig. 2B was completely blocked. However, the cell responded to the larger depolarizing
Fig. 2. Effect of TTX, TEA and Ca-free Ringer solution on action potentials evoked by depolarizing current pulses of increasing intensity injected in 0.2 nA (A-B), 0.4 nA (C-D) and 0.5 nA (E) steps across the cell soma of a dorsal horn neurone of a 12-day-old rat. Passive responses to subthreshold depolarizing and hyperpolarizing current pulses also shown. Initial resting potential was -66 mV. A, control response to direct stimulation in normal Ringer solution. B, action potential recorded after addition of $10^{-3}$M-TEA. C-D, TTX-resistant action potentials recorded following addition of $10^{-6}$M-TTX to the TEA-containing medium. Note higher threshold and slower rate of rise of TTX-resistant potentials if compared with a control. E, TTX-resistant action potentials disappeared when Ca was removed from TEA + TTX-containing Ringer solution.
current pulses (range: 1.7-2.0 nA) with a slow regenerative depolarization followed by a slow hyperpolarization. An example of two slow regenerative spikes is shown in Fig. 2C-D. These slow spikes were all-or-none in character and were seen in almost all tested cells. The latency of onset and duration of these spikes varied with the amount of stimulus current and the frequency of stimulation. Their latency shortened as current intensity was raised. These slow action potentials could be distinguished from the fast action potentials by their higher threshold, smaller amplitude, and longer spike duration and after-hyperpolarization (Table 2).

The Ca-dependence of the slow TTX-resistant action potentials is illustrated in Fig. 2E where it can be seen that removal of Ca from the bathing medium containing TTX and TEA made depolarizing current pulses, which triggered the slow action potentials shown in Fig. 2C-D, ineffective. Furthermore, bath application of a Ringer solution containing $5 \times 10^{-3}$ M-Ca potentiated the high-threshold Ca spike.

Blockade of the high-threshold spike (Fig. 3C or D) by Co ions in a dorsal horn neurone of a 18-day-old rat is illustrated in Fig. 3E. Upon washing the slice with a Co-free medium, a slow regenerative depolarizing response could again be elicited (Fig. 3F). After removal of TTX and TEA from the perfusing solution a partial recovery of the Na spike is seen in Fig. 3G.
Table 2. Some characteristics of the high-threshold and the low-threshold Ca action potentials<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean ± S.D.</th>
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<tr>
<td>High-threshold spike</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane potential</td>
<td>9</td>
<td>62.2 ± 7.4 mV</td>
</tr>
<tr>
<td>Spike height</td>
<td>9</td>
<td>32.7 ± 11.1 mV</td>
</tr>
<tr>
<td>Spike duration</td>
<td>7</td>
<td>13.1 ± 7.7 ms</td>
</tr>
<tr>
<td>Threshold</td>
<td>9</td>
<td>49.8 ± 8.9 mV</td>
</tr>
<tr>
<td>Magnitude of after-hyperpolarization</td>
<td>8</td>
<td>26.6 ± 7.6 mV</td>
</tr>
<tr>
<td>Duration of after-hyperpolarization</td>
<td>7</td>
<td>114.0 ± 83.0 ms</td>
</tr>
<tr>
<td>Low-threshold spike</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane potential</td>
<td>4</td>
<td>73.3 ± 2.4 mV</td>
</tr>
<tr>
<td>Spike height</td>
<td>4</td>
<td>13.8 ± 3.1 mV</td>
</tr>
<tr>
<td>Spike duration</td>
<td>4</td>
<td>26.5 ± 4.0 ms</td>
</tr>
<tr>
<td>Threshold</td>
<td>4</td>
<td>22.4 ± 2.9 mV</td>
</tr>
</tbody>
</table>

<sup>a</sup>All measurements done in a Ringer solution containing TTX + TEA.
Fig. 3. Blockade of TTX-resistant spikes by cobalt ions in a dorsal horn neurone of an 18-day-old rat. Resting potential was -62 mV. A, control response to direct stimulation in normal Ringer solution. B, action potential following addition of $2 \times 10^{-3}$ M-TEA. C-D, TTX-resistant action potentials following addition of $4 \times 10^{-6}$ M-TTX. E, TTX-resistant spike disappeared when Co chloride ($2 \times 10^{-3}$ M) was added to bathing medium. F, partial recovery of TTX-resistant spike after removal of cobalt. G, partial recovery of Na spike following removal of TTX and TEA from perfusing solution.
The low-threshold Ca action potential

Besides the high-threshold Ca-dependent spike already described, the neuronal membrane of dorsal horn cells with resting potentials more negative than 65 mV appears to be capable of generating a low-threshold TTX-resistant action potential having some properties similar to that recently described in the inferior olivary neurones by Llinás and Yarom (1981a,b). As shown in Fig. 4 direct stimulation of a dorsal horn cell in an 11-day-old rat by a depolarizing current pulse of 0.2 nA and 60 ms duration evoked two Na spikes which were preceded by a slow depolarization (Fig. 4A). Although addition of TTX into the bath abolished Na spikes, the low-threshold membrane electroresponsiveness remained (Fig. 4B). The rate of rise of this all-or-none low-threshold depolarizing potential is usually increased following membrane hyperpolarization.

The ionic nature of this low-threshold TTX-resistant potential was further investigated by adding Ba to the medium containing TTX and TEA (Fig. 4C-D). It is known that Ba moves through the Ca channel more rapidly than Ca (Werman & Grundfest, 1961; Hagiwara, 1973; Hagiwara, Fukuda & Eaton, 1974) and does not activate the Ca-dependent K conductance (Krnjević, Pumain & Renaud, 1971; Eckert & Lux, 1976). Fig. 4C illustrates the low-threshold depolarizing responses elicited in a dorsal horn neurone by depolarizing current pulses applied at resting level (-64 mV) and at increasing levels of membrane hyperpolarization (-64 to -82 mV). As hyperpolarization increases the amplitude and the rate of rise of the low threshold Ba spikes are increased. Estimation of the rate of rise of these low threshold spikes as a function of membrane hyperpolarization
Fig. 4. The low-threshold TTX-resistant spike and TTX-resistant Ba-action potentials in a dorsal horn neurone of an 11-day-old rat. Initial resting potential was -77 mV. A, control responses to direct stimulation. B, addition of $3 \times 10^{-6}$M-TTX into the bath abolished fast spikes and uncovered a low-threshold electroresponsiveness. In C-D, TTX-resistant action potentials following addition of Ba ($10^{-3}$M) into the perfusing solution containing TTX ($3 \times 10^{-6}$M) and TEA ($5 \times 10^{-3}$M). C, low-threshold Ba-action potentials recorded at resting level (-64 mV) and at increasing levels of membrane hyperpolarization (range: 64-82 mV). Note increased rate of rise and amplitude of these potentials with increasing hyperpolarization. D, co-activation of low-threshold and high-threshold Ba potentials. Note that the two spikes can be generated during the same stimuli. The presence of a Ba local response is also evident.
shows that plateau is reached in this cell at about -80 mV. The co-
activation of the low-threshold and the high-threshold Ba spikes and a slow
plateau phase following the low-threshold action potential are seen in
Fig. 4D. In the presence of Ba, the high-threshold spike was of longer
duration, while the decay phase of the low-threshold spike was not modified
by Ba.

The co-existence of two types of Ca-dependent action potentials in
a dorsal horn neurone of a 10-day-old rat slice preparation perfused
with normal Ringer solution containing TTX and TEA is shown in Fig. 5B.
Both responses were abolished if the slice was bathed in a 5mM-cobalt-
containing medium (Fig. 5C). When cobalt was removed, both potentials,
although not fully recovered, could again be evoked (Fig. 5D).

Recovery and co-existence of both the high-threshold and the low-
threshold Ca-dependent potentials 66 min after perfusion with a Co-free,
TTX- and TEA-containing Ringer solution are illustrated in Fig. 5E. Note
the striking difference in the time course of the two potentials: whereas,
the low-threshold potential has a relatively fast repolarizing phase even
under conditions of a reduction of the voltage-dependent K conductance, the
high-threshold electroresponsiveness is prolonged. Similar behavior of two
types of Ca spikes in the inferior olivary neurones in the presence of TEA
has been attributed to the inactivation properties of the low-threshold
Ca conductance and the lack of fast inactivation for the high-threshold Ca
spike (Llinaș & Yarom, 1981b).
Fig. 5. Both, high-threshold and low-threshold TTX-resistant spikes are blocked by cobalt. 10-day-old rat. Resting potential was -62 mV. A, control response. B, co-existence of both, low-threshold and high-threshold potentials in a medium containing TTX (5 x 10^{-6} M) and TEA (5 x 10^{-3} M). C, both responses are abolished by Co (5 x 10^{-3} M). D, E, recovery following removal of cobalt from TTX and TEA-containing Ringer solution. Note also in E (66 min since removal of Co) the difference in the time course between the low-threshold and the high-threshold potentials.
Discussion

The data presented illustrate that satisfactory intracellular recordings from immature rat dorsal horn neurones in vitro could be maintained for as long as 5 h, the time often required when testing the influence of various ionic environments on cell membrane properties. This is in itself an encouraging finding, since obtaining stable recordings from the superficial parts of the spinal dorsal horn in vivo has been technically very difficult (Cervero et al., 1977; Yaksh et al., 1977; Hayashi et al., 1978; Kumazawa & Perl, 1978; Bennett et al., 1979). This in vitro preparation, in addition has provided new information about the ionic mechanisms underlying some of the electrophysiological properties of dorsal horn neurones.

Passive membrane properties

Large variation was found in the input resistance ($R_N$), and the membrane time constant ($\tau$) of dorsal horn neurones of 9-18-day-old rats. In addition, it is significant that the $R_N$ values for the rat dorsal horn cells cover a higher range (48-267 M$\Omega$) than has been reported for mouse spinal cord neurones in primary dissociated cell culture (Ransom et al., 1977) and cat dorsal horn neurones, as recorded in vivo (Ziegglänsberger & Tulloch, 1979a). This generally higher $R_N$ value of immature rat dorsal horn neurones may be attributed, at least in part, to the small size of cells and the recording temperature of 33 ± 1°C, although possible differences in actual membrane properties (specific membrane resistivity) and developmental characteristics of dendritic trees should be also considered.
Anatomical studies of the superficial parts of the spinal dorsal horn have shown the preponderance of small-sized neurones (Rexed, 1952; Róthelyi & Szentágothai, 1973; Sugiura, 1975; Beal & Cooper, 1978; Gobel, 1979; Bennett et al., 1979). In addition, our preliminary investigation of immature rat dorsal horn neurones intracellularly labelled with horseradish peroxidase in vitro (Nedeljkov & Randić, 1982) has confirmed that the majority of cells are small with fewer dendritic branches. This morphological finding is consistent with our present physiological findings of high cell input resistance and of effective electrical accessibility to dendritic synaptic sites from a somatically positioned micro-electrode.

The values calculated for the membrane time constant of developing dorsal horn neurones (range: 4-19 ms) are somewhat higher than the values of 5-9.5 obtained for mouse spinal cord neurones in cell culture (Ransom et al., 1977).

Voltage-dependent action potentials

In rats 9-18 days old, all dorsal horn neurones tested could generate fast action potentials in response to direct and orthodromic stimulation. In comparison with the data obtained in adult animals, where intracellular recordings were performed in vivo, (Haapanen et al., 1958; Hunt & Kuno, 1959; Eccles et al., 1960; Cervero et al., 1977), the action potentials of rat dorsal horn neurones in vitro showed the mean value for "overshoot" of about 25 mV and the mean amplitude of approximately 69 mV in relation to the firing level. These values for the spike height and "overshoot" are larger than those observed in vivo,
possibly reflecting the more stable recording conditions in \textit{vitro}. However, the spike duration of approximately 1.4 ms obtained in immature dorsal horn cells is longer than the values of 0.5-1.0 ms recorded in adult dorsal horn neurones. The latter result may be accounted for by several factors, such as immaturity of the spike generation mechanism, and the lower recording temperature.

The finding that the action potentials were reliably blocked by tetrodotoxin indicates that a voltage-dependent Na conductance, as in other neurones, plays an important role in generation of fast action potentials evoked in immature dorsal horn neurones either by direct or orthodromic stimulation. The latter finding is in agreement with the observations made on mouse spinal cord neurones in primary dissociated cell culture (Ransom et al., 1977; Heyer et al., 1981).

\textbf{Voltage-dependent Ca conductances}

\textbf{High-threshold Ca spike} When the fast Na and K conductances are blocked by perfusion of the spinal cord slices with TTX and TEA, depolarizing current pulses of long duration often elicit a slow regenerative depolarizing potential followed by a slow after-hyperpolarization. The mechanism underlying the generation of this response appears to be a voltage-dependent Ca conductance change because this regenerative inward current is TTX-resistant, it requires the presence of extracellular Ca, and it is blocked by Co and Mn, the ions known to block Ca conductances. Since the appearance of these Ca-dependent action potentials requires that the membrane of the dorsal horn neurones be depolarized to a
higher level (range: 35-58 mV from rest) than is necessary to evoke Na spike (range 14-38 mV), the term "high threshold Ca spike", as recently proposed by Llinás and Yarom (1981a) is adopted in this paper.

Ca-dependent action potentials of similar properties have been recently demonstrated in mouse spinal cord neurones in cell culture (Heyer et al., 1981), and also in frog motoneurones (Barrett & Barrett, 1976), cerebellar Purkinje cells (Llinás & Hess, 1976; Llinás & Sugimori, 1980a,b), CA1 and CA3 hippocampal pyramidal neurones (Schwartzkroin & Slawsky, 1977; Wong, Prince & Basbaum, 1979) and inferior olivary neurones (Llinás & Yarom, 1981a,b). While we have no direct evidence for the site of generation of the high-threshold Ca potentials in the immature rat dorsal horn neurones, the dendritic localization in hippocampal pyramidal cells (Schwartzkroin & Slawsky, 1977; Wong et al., 1979), cerebellar Purkinje cells (Llinás & Sugimori, 1979, 1980b) and inferior olivary neurones (Llinás & Yarom, 1981a,b) has been demonstrated.

Low-threshold Ca electroresponsiveness

It has been recently reported that following membrane hyperpolarization above -70 mV, inferior olivary neurones may generate Ca-dependent potentials in response to direct or synaptic stimulation (Llinás & Yarom, 1981a). In immature rat dorsal horn neurones having resting potentials more negative than -65 mV a low threshold electroresponsiveness of similar properties to those of inferior olivary neurones following direct stimulation was observed. Because this low-threshold potential is reversibly blocked by Co, an agent known to block Ca conductance, and because it is
enhanced in the presence of Ba, an ion which moves easily through Ca channels (Hagiwara & Byerly, 1981), we suggest that the low-threshold spike may be generated by an inward Ca current. As in inferior olivary neurones this low-threshold Ca conductance exhibits inactivating properties as suggested by the perfusion experiments with Ba and TEA (Fig. 5E). However, a direct proof for the inactivation would require use of the voltage-clamp technique.

In several types of central neurones (cf. motoneurones: Barrett & Barrett, 1976; hippocampal pyramidal cells: Traub & Llinás, 1979; Wong et al., 1979; Purkinje cells: Llinás & Sugimori, 1980b; inferior olivary neurones: Llinás & Yarom, 1981b, and mitral cells: Mori, Nowycky & Shepherd, 1981) Ca current is believed to modulate a slow K conductance which in turn controls the repetitive firing. Thus, the presence of voltage-dependent Ca channels in developing rat dorsal horn neurones may be an important mechanism for regulating the normal and pathological firing behaviour of these cells. In addition, certain putative chemical messengers (adrenaline, noradrenaline, 5-hydroxytryptamine, γ-aminobutyric acid, somatostatin and enkephalins) have been found to modulate voltage-dependent Ca currents (Dunlap & Fischbach, 1978; Mudge, Leeman & Fischbach, 1979). This control of the Ca channel by neurotransmitters is potentially of great importance as a mechanism to explain plasticity and modulation at central synapses.
Plate 1. A-B, Golgi-Cox impregnated dorsal horn neurones in a 150 μm thick horizontal section of lumbosacral spinal cord of a 12-day-old rat.
Acknowledgments

We would like to thank Dr. E. Uemura for preparation of the Golgi-Cox material and to Drs. M. V. L. Bennett, W. E. Crill and M. Sugimori for helpful discussions and comments on the manuscript. This research was supported in part by Grants NS 17297 from the National Institute for Neurological and Communicative Disorders and Stroke and BNS 23871 from the National Science Foundation.
SECTION III. THE ACTIONS OF NEUROPEPTIDES ON DORSAL HORN NEURONES IN THE RAT SPINAL CORD SLICE PREPARATION: AN INTRACELLULAR STUDY

Summary

Responses of dorsal horn neurones to bath application of substance P, somatostatin and enkephalin were studied by intracellular recording in the neonatal spinal cord slice preparation. Substance P depolarized dorsal horn neurons and increased their excitability. The depolarization was most commonly associated with an increase in neuronal input resistance. Somatostatin and enkephalin hyperpolarized dorsal horn neurons and caused reduction or abolition of spontaneous firing. While the hyperpolarization produced by enkephalin was always associated with a fall in neuronal input resistance, in the case of somatostatin the similar effect was less consistently observed.

Introduction

Experimental evidence provides a support for the concept that substance P (SP), somatostatin (SS) and methionine-enkephalin (ME) may function as neurotransmitters and/or neuromodulators in synaptic transmission processes in the superficial layers of the spinal dorsal horn (McQuillan, 1980; Nicoll et al., 1980). However, the cellular mechanisms and the sites of action of these peptides on spinal dorsal horn neurons remain
uncertain. In intact animals, iontophoretically applied SP and enkephalin have been reported to produce no detectable change in resting membrane potential and input resistance of dorsal horn neurons (Sastry, 1979a; Zieglgansberger & Tulloch, 1979a,b). The membrane action of SS on dorsal horn cells in vivo is unknown. To further investigate the cellular mechanisms of actions of SP, SS and ME in the spinal dorsal horn, we have utilized intracellular recording from dorsal horn neurons in vitro in the neonatal rat spinal cord slice preparation (Miletić & Randić, 1982). Preliminary results of our findings have already been communicated (Murase & Randić, 1981).

Methods

Experiments were performed on 8-19-day-old Sprague-Dawley rats. The animals were anesthetized with ether, and a laminectomy performed to expose the lower-thoracic and lumbosacral spinal cord together with dorsal roots. Following laminectomy, about 1-1.5 cm long segment of lumbosacral spinal cord, with attached dorsal rootlets was quickly excised and immersed into aerated (95% O₂ and 5% CO₂) Krebs-Ringer solution at 37°C. The composition of the solution was (mM): NaCl 124; KCl 5; KH₂PO₄ 1.2; CaCl₂ 2.4; MgSO₄ 1.3; NaHCO₃ 26; glucose 10, pH 7.4. After the removal of the pia mater on the lateral aspects of the spinal cord, the spinal segment was cut manually into 5 mm blocks, and one of the blocks of tissue affixed with cyanoacrylic glue (Borden, Inc.) to the bottom of a Plexiglass cutting chamber of an Oxford Vibratome. The spiral cord block was positioned in the bath of the Vibratome with the dorsal surface of
the spinal cord facing the vibrating blade. The bath of the Vibratome had previously been filled with the Krebs solution, maintained at 37°C and bubbled with a mixture of oxygen and carbon dioxide. The blade of the Vibratome was positioned 300 μm below the dorsal surface of the spinal cord, and the spinal segment sectioned to yield two horizontal 300 μm-thick dorsal horn slices. The duration of the entire procedure from the removal of the spinal cord until the slice was made rarely exceeded 5 min. In a few experiments, transverse slices were used. The slices were incubated in Krebs-Ringer solution at 37°C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Krebs-Ringer solution (NaCl 127 mM; KCl 1.9 mM; CaCl₂ 2.4 mM; MgSO₄ 1.3 mM; NaHCO₃ 26 mM; glucose 10 mM, occasionally containing 0.001% H₂O₂) at 33 ± 1°C at a flow rate of about 1 ml/min. The recording chamber had a capacity of 0.5 ml.

Intracellular recordings were performed with micropipettes filled with 1-3 M potassium acetate having DC resistances of 60-100 MΩ. Good intracellular recordings from slices could be obtained for a period of up to 12 h after isolation of the spinal cord. A high-input impedance bridge amplifier (WP Instruments, M707) was used to inject current through the recording microelectrode. The amplitude of the recorded voltage produced by rectangular hyperpolarizing current pulses (0.1-0.3 nA, 50-100 ms duration applied at 0.2 Hz) was used as a measure of the membrane input resistance. SP, SS, ME and the stable enkephalin analogue D-Ala²-Met⁵-enkephalinamide (DALA, Beckman) were applied by bath perfusion in known
concentration.

Dorsal horn neurons were impaled up to 12 h after the slice was prepared. Stable intracellular recordings were made from single neurons for as long as 5 h. During the period of 5 min before peptide application control responses to constant current hyperpolarizing pulses and direct intracellular stimulation were monitored. Only cells which showed uniform responses and stable membrane potential during this period were used in further analysis. The average action potential amplitude was 75 mV (+ 11 S.D.), input membrane resistance 67 MΩ (+ 25) and membrane potential −67 mV (+ 8).

Results

The results of present experiments are based on data obtained from 38 dorsal horn neurons. In all of 13 tested cells, application of SP into the bathing medium (10⁻⁶ to 2 x 10⁻⁵ M for 1 min) produces depolarization that is slow both in onset and recovery (Fig. 1). The average size of the SP (10⁻⁵ M)-depolarizing response was 6.7 ± 3.6 mV. SP-induced depolarization is always accompanied by an increase in synaptic activities, as evidenced in Fig. 1A with an increase in baseline noise. In addition, the dorsal horn neurons frequently fired action potentials (Fig. 1A). The increased synaptic activity, but not the depolarization, is absent in a Ca²⁺-low (0.3-0.6 mM), Mg²⁺-high (10 mM) Krebs-Ringer solution (Fig. 1B). Application of SP increased the input resistance of 8 of 11 tested cells, and decreased resistance in 3 cells. The
Fig. 1. The effects of substance P (SP) on a dorsal horn neuron recorded with an intracellular microelectrode in a spinal cord slice preparation of 10-day-old rat. A, continuous recording of the membrane potential in a cell with a resting membrane potential of -72 mV. The downward vertical deflections represent membrane responses to constant hyperpolarizing current pulses (800 pA, 50 ms), applied through the recording electrode, in order to measure cell input resistance. Bath application of SP (10^{-5} M) for about a minute causes a depolarization that is slow both in onset and recovery. SP-induced depolarization is accompanied by an increase in numbers of synaptic (as evidenced with an increase in baseline noise) and action potentials (action potentials were truncated by the limited frequency response of the pen recorder). In addition, input resistance increased during the depolarizing action of SP. B, the increased synaptic activity and activity and action potential firing, but not the depolarization and increase in input resistance, are absent in a Ca^{2+}-free, Mg^{2+}-high (10 mM) Krebs-Ringer solution. This solution blocked synaptic transmission through the cord and reduced the amplitude of SP-induced depolarization.
input resistance was usually increased by 3-25% (mean 5.9 ± 2.1%) with SP applied at a concentration of $10^{-5}$ M. As illustrated in Fig. 1A, the earliest part of the SP application was occasionally accompanied by a hyperpolarization which lasted for up to 20 s.

Somatostatin applied into the bathing medium ($10^{-6}$ to $6 \times 10^{-5}$ M for 1 min) caused reversible, dose-dependent hyperpolarization (mean $-3.2 ± 1.8$ mV) in 15 of 18 dorsal horn neurons. In 3 cells, a depolarization, rather than a hyperpolarization, was observed. In 10 cells SS-induced hyperpolarization was associated with a decrease in membrane input resistance (mean $13.3 ± 7.2$%) and reduced numbers of spontaneous (Fig. 2) and SP-evoked action potentials, while in 5 cells increased input resistance was observed. SS-hyperpolarization persisted in low-calcium, high-magnesium containing Krebs-Ringer solution although its duration and amplitude were frequently reduced. In several cells, SS-hyperpolarization was preceded by a brief depolarization associated with increased membrane input resistance.

Bath application of DALA ($10^{-7}$ to $10^{-4}$ M for 1 min) and ME ($10^{-6}$ to $10^{-4}$ M) caused dose-related reversible hyperpolarization of the neuronal membrane in 21 of 24 cells, while 3 cells were depolarized. The amplitude of the hyperpolarization produced by 10 µM DALA varied from 2 to 9 mV among dorsal horn neurons (mean $-5.1 ± 2.8$ mV). The hyperpolarization began within 1 min of exposure of the tissue to enkephalin and the membrane potential reverted to the control value within 5-10 min when the enkephalin was washed out (Fig. 3).
Fig. 2. The effect of somatostatin (SS) on a dorsal horn neuron in a spinal cord slice preparation of 12-day-old rat. Resting membrane potential in this spontaneously active cell was -68 mV. Cell input resistance was measured by recording membrane responses to constant hyperpolarizing current pulses (200 pA). Bath application of SS (10^{-5} M for 96 s) causes hyperpolarization associated with a slight fall in membrane input resistance, reduction in synaptic activity and abolition of the spontaneous firing.
Fig. 3. The effects of DALA on 2 dorsal horn neurons in a spinal cord slice preparation of an 11-day-old rat. Resting membrane potential in these spontaneously active cells was -68 mV. Cell input resistance was measured by recording membrane responses to constant hyperpolarizing current pulses (200 pA, 50 ms). A, bath application of DALA (2 x 10^{-6}M) causes hyperpolarization accompanied by a fall in membrane input resistance, reduction in synaptic activity and abolition of the spontaneous firing. B (upper record), bath application of DALA (10^{-4}M) causes reversible hyperpolarization accompanied by a fall in neuronal input resistance. B (lower record), perfusion with a Ca^{2+}-low (1.3 mM), Mg^{2+}-high (10 mM) Krebs-Ringer solution blocked synaptic transmission through the cord and reduced the magnitude of DALA-induced hyperpolarization.
The hyperpolarization (DAIA, 10 μM) was always associated with a fall in neuronal input resistance (8.5 ± 4.9%), a decrease in synaptic potentials and a marked reduction or abolition of the spontaneous firing. In a few cells tested, the hyperpolarizing response was reversibly prevented by bath application of naloxone (1.2 × 10^{-6} M).

In 6 tested cells the enkephalin-induced hyperpolarization persisted in low-calcium (0.3–0.6 mM), high-magnesium (10 mM) Krebs-Ringer solutions, although its magnitude and associated fall in input resistance were reduced (Fig. 3B). In several cells tested with larger doses of enkephalin (10^{-5}–10^{-4} M), the hyperpolarization was preceded and followed by depolarizing responses. The late depolarization was frequently associated with appearance of the spontaneous firing.

Discussion

The evidence presented in this paper shows that SP, SS and opioid peptides have distinct actions on the postsynaptic membrane of dorsal horn neurons in the neonatal rat spinal cord slice preparation, which may contribute to the excitatory (Henry et al., 1975; Henry, 1976; Randić & Miletić, 1977; Sastry, 1979a; Zieglgänsberger & Tulloch, 1979a), and depressant (Duggan et al., 1977; Randić & Miletić, 1978; Zieglgänsberger & Tulloch, 1979b) actions of these peptides observed on the dorsal horn neurons in vivo. Our results confirm the observations made
by other investigators that SP causes a dose-dependent, reversible depolarization (Nicoll, 1976, 1978; Krnjević, 1977; Otsuka, 1978; Sastry, 1979a; Zieglgänsberger & Tulloch, 1979a), which in our experiments was predominantly associated with an increase in neuronal input resistance. At present, controversy exists regarding the mechanism underlying the SP-induced depolarization in spinal neurons. Bath administration of SP to the spinal cord of the newborn rat (Otsuka, 1978) and to the amphibian spinal cord (Nicoll, 1976, 1978) produces a depolarization associated with a decrease in input resistance of the motoneuronal membrane. In the cat spinal cord in vivo, SP has been reported to produce either an increase in input resistance (Krnjević, 1977), or no change (Sastry, 1979; Zieglgänsberger & Tulloch, 1979a).

Our results indicate that hyperpolarization accompanied by a fall in membrane input resistance is the most common consequence of bath application of SS and opioid peptides to the dorsal horn neurons in vitro. Application of SS to the hemisected frog spinal cord causes increase in the ventral root potential obtained by dorsal root stimulation. However, in the presence of tetrodotoxin or low-calcium, high-magnesium solutions a direct small and variable hyperpolarization of ventral and dorsal roots was observed. In parallel with the latter direct action of SS, responses to glutamate were selectively depressed in both dorsal and ventral roots (Padgen, 1977). In mouse spinal cord neurons grown in culture, SS has been shown to modify neurotransmitter release by a presynaptic action. Membrane potential or input resistance of spinal neurons was usually not altered by SS (Macdonald & Nowak, 1981). In
relation to our results obtained with SS in the spinal dorsal horn it is of interest that in the hippocampal slice preparation, SS hyperpolarized the pyramidal cells by a direct, postsynaptic action, reduced the number of spontaneous and directly evoked action potentials and decreased their membrane input resistance (Pittman & Siggins, 1980). An excitatory action of SS on hippocampus has also been reported (Dodd & Kelly, 1978).

Our results show that the ME and its stable analogue, DALA, hyperpolarize dorsal horn neurons in vitro and increase their membrane conductance. This effect of ME is similar to that observed in locus coeruleus neurons (Pepper & Henderson, 1980) studied in vitro. Although a membrane hyperpolarization and the accompanying fall in neuronal input resistance may account for some of the reported depressant effects of enkephalin on neuronal firing (Duggan et al., 1977; Randić & Miletić, 1978; Zieglgansberger & Tulloch, 1979b), dorsal root potentials (Nicoll, 1978; Suzue & Jessell, 1980), and primary afferent excitability (Sastry, 1978, 1979a), this mechanism does not seem to be responsible for the effects of opioid peptides observed on cultured spinal neurons and dorsal root ganglion cells (Barker et al., 1978; Mudge et al., 1979).

The relevant question to be asked in relation to present data obtained with SS and ME is whether the membrane hyperpolarization and the associated increase in neuronal conductance is likely postsynaptic mechanism of the inhibition of firing of dorsal horn neurons (Duggan et al., 1977; Randić & Miletić, 1978; Zieglgansberger & Tulloch, 1979b). Although our results suggest an affirmative answer, the fact that we have observed a significant reduction of the peptide effects on dorsal
horn neurons in the low-calcium, high-magnesium Krebs-Ringer solution in several experiments, points out to an additional indirect presynaptic mechanism of action. Thus, the both direct and indirect inhibitory mechanisms may account for the depression of firing of dorsal horn neurons observed in vivo during local application of somatostatin and enkephalin.

Acknowledgments

We are grateful to Drs. M. Sugimori and R. Llinás, for sharing with us valuable information regarding the slice technique. This work was supported by the National Science Foundation (BNS 23871) and the United States Department of Agriculture.
SECTION IV. ACTIONS OF SUBSTANCE P ON RAT SPINAL DORSAL HORN NEURONES

Summary

1. The membrane actions of substance P (SP) and the effects on the Ca-dependent action potential of dorsal horn neurones have been investigated by means of intracellular recording techniques in the immature rat in vitro spinal cord slice preparation.

2. Bath-application of SP (2 x 10^-6 to 10^-5 M) induced a biphasic membrane response consisting of an initial hyperpolarization followed by a depolarization in about one-third of the cells examined. Initial hyperpolarization was not observed when synaptic activity was blocked by perfusing the slice with a tetrodotoxin-containing or low Ca/high Mg Ringer solution. This result is consistent with a presynaptic action of SP mediated through excitation of inhibitory interneurones. This interpretation was supported by recording of repetitive spontaneous inhibitory postsynaptic potential (i.p.s.p.)-like hyperpolarizing potentials during the initial hyperpolarization.

3. When Co ions were used to block voltage-dependent Ca conductance and possible indirect presynaptic actions, SP induced only a small depolarization of membrane potential. It seems, therefore, that Ca-conductance may have contributed to the depolarizing

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phase of the SP response, either through its mediation of synaptic transmission or through the direct effects as a charge carrier for inward current.

4. When tetrodotoxin was used, the SP-induced increase in neuronal input resistance was not modified, although depolarization was slightly diminished. In contrast, in medium containing tetrodotoxin and tetraethylammonium, the SP-depolarizing response was enhanced and accompanied by a small decrease in input resistance and firing of Ca spikes. These results suggest that SP-induced depolarization might be a consequence of a reduction in a voltage-dependent K conductance allowing Na and/or Ca conductances to dominate.

5. SP modified duration of Ca-dependent action potentials of dorsal horn neurones, the most consistent change being an initial dose-dependent and reversible decrease in the spike duration. The decrease in Ca spike duration was associated with a small reduction in the rate of rise and peak amplitude, and significant parallel increase in dV/dt of the falling phase of Ca spike.

6. Our data indicate that the initial decrease in Ca spike duration was not due to the depolarizing action of SP, although shunting of the membrane resistance, either through presynaptic or post-synaptic mechanisms has not been ruled out. Alternatively, these data are consistent with the possibility that SP
shortens the duration of the Ca spike by decreasing a voltage-sensitive inward Ca current and/or augmenting an outward K current.

7. Although the direct test of our interpretation can be accomplished only with a voltage-clamp analysis, the nature of our preparation does not seem to allow this study at present because of a probable dendritic origin of Ca spikes and a very high input resistance of dorsal horn neurones.

Introduction

Experimental evidence supports the concept that substance P (SP) may function as a neurotransmitter and/or neuromodulator in synaptic transmission between primary afferent fibres and neurones within the spinal cord (Nicoll, Schenker & Leeman, 1980). Electrophysiological data derived largely from extracellular recording showed that SP-induced excitation of dorsal horn neurones has a slow onset and decay (Henry, Krnjević & Morris, 1975; Henry, 1976; Randić & Miletić, 1977), and intracellular recordings demonstrated that SP produces slow neuronal depolarization associated with an increase in input resistance Krnjević, 1977; Murase & Randić, 1981; Murase, Nedeljkov & Randić, 1982) or no change (Sastry, 1979a; Zieglgänsberger & Tulloch, 1979a). In addition to the SP depolarization, an initial hyperpolarization associated with a decrease in membrane input resistance occurred in a smaller proportion of rat dorsal horn neurones (Murase et al., 1982).
Recent data obtained in cultured mouse and rat spinal cord neurones suggested that the post-synaptic excitatory action of SP was due to a decrease in a membrane K conductance ($G_K$) (Hösl, Hösl, Zehntner & Landolt, 1981; Nowak & Macdonald, 1982), possibly a voltage-dependent $G_K$, similar to the M-current in sympathetic ganglion neurones (Brown & Adams, 1980; Adams, Brown & Constanti, 1982a). Muscarine-sensitive K conductance has recently been demonstrated in cultured mouse spinal cord neurones (Nowak & Macdonald, 1981). However, nothing is known about the mechanism of the initial hyperpolarizing SP response.

In our experiments the cellular mechanisms underlying the initial hyperpolarization and ionic mechanisms of the SP depolarizing response of immature rat spinal cord neurones in the superficial parts of the dorsal horn were examined. Action potentials in these spinal neurones are generated by voltage-dependent conductance increases to Na and Ca ions (Heyer, Macdonald, Bergey & Nelson, 1981; Murase & Randić, 1982, 1983a) and two distinct types of Ca spikes are probably present in immature rat dorsal horn cells (Murase & Randić, 1982, 1983a). We have chosen to examine the effects of SP on Ca action potential properties since the control of the Ca channel by neurotransmitters is potentially of great importance as a mechanism for explaining plasticity and modulation at central synapses. Certain chemical messengers (catecholamines, 5-hydroxytryptamine, γ-aminobutyric acid) and the putative peptide transmitters (somatostatin and enkephalin) modulate voltage-dependent Ca currents (Dunlap & Fischbach, 1978, 1981; Klein & Kandel, 1978; Mudge,
Leeman & Fischbach, 1979; Werz & Macdonald, 1982). Preliminary results of our findings have already been communicated (Randić & Murase, 1983a).

Methods

Preparation

Experiments were performed on 10-15-day-old Sprague-Dawley rats. The animals were anaesthetized with ether and cooled by immersing the thorax and abdomen in an ice water slurry. During the period of cooling, close attention was paid to respiration. In the majority of animals respiration continued for at least 10 min following the onset of cooling, by which time the skin temperature had fallen to 20-22°C. The dissection was then started and a laminectomy performed to expose the lower-thoracic and lumbosacral spinal cord together with dorsal roots. Following laminectomy a segment of lumbosacral spinal cord about 1-1.5 cm long with attached dorsal rootlets, was quickly excised and immersed in aerated (95% O₂ and 5% CO₂) Ringer solution at approximately 24°C. The composition of the solution was (mM): NaCl, 124; KCl, 5; KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; glucose, 10; pH 7.4. After the removal of the pia mater on the lateral aspects of the spinal cord, the spinal segment was cut manually into 5 mm blocks, and one of the blocks fixed with cyanoacrylic glue (Borden, Inc.) to the bottom of a Plexiglas cutting chamber of an Oxford Vibratome. The bath of the Vibratome was filled with the aerated Ringer solution, maintained at 24°C. The blade of the Vibratome was positioned 300 µm
below the dorsal surface of the spinal cord, and the spinal segment sectioned to yield one horizontal 300 μm thick dorsal horn slice. The duration of the entire procedure from the removal of the spinal cord until the slice was made rarely exceeded 5 min. The slices were incubated in Ringer solution at 35°C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Ringer solution (NaCl, 127 mM; KCl, 1.9 mM; KH₂PO₄, 1.2 mM; CaCl₂, 2.4 mM; MgSO₄, 1.3 mM; NaHCO₃, 26 mM; glucose, 10 mM) at 33 ± 1°C at a flow rate of about 2 ml/min. The recording chamber had a capacity of 0.5 ml. Variations in the composition of the perfusing solution are indicated in the Results.

When MgCl₂ concentration (10⁻²M) was increased or when CoCl₂ (1-2 x 10⁻³M) was added to the bathing solution to eliminate synaptic activity, the NaCl concentration was adjusted to maintain osmolarity. The NaCl concentration was also adjusted when 4-aminopyridine (1-2 x 10⁻³M) or tetraethylammonium chloride (TEA, 2 x 10⁻³ to 2 x 10⁻²M, Kodak) was added to the bathing medium in order to block voltage-dependent K conductances (Armstrong, 1971; Yeh, Oxford, Wu & Narahashi, 1976). Fast voltage-dependent Na conductance was blocked with tetrodotoxin (TTX, 1-2 x 10⁻⁶M, Sankyo) (Narahashi, Moore & Scott, 1964). In a majority of experiments, TTX, rather than Co or Mg, was used in order to reduce spontaneous and synaptic activity, SP (Beckman, Peninsula Labs.) was applied by bath perfusion in known concentrations. It took about 25 s for SP to reach the slice in the chamber due to dead space between
the slice and the reservoirs containing drug solution. Stock solution of SP ($5 \times 10^{-3}$ M) was made by dissolving the peptide in ammonium acetate/acetic acid buffer (pH 5.5), which also contained in some experiments 0.1% bovine serum albumin. Aliquots (10 μl) of stock solution were pipetted into plastic tubes and frozen, until used in the experiment. The pH of all applied solutions was maintained between 7.2 and 7.4.

**Intracellular recording technique**

Intracellular recordings were performed with micropipettes filled with 3M K-acetate having d.c. resistances of 100-120 MΩ. Stable intracellular recordings from single dorsal horn neurones could be maintained during multiple solution changes for as long as 5 h. Electrical properties of dorsal horn neurones were determined by means of a high-input impedance bridge amplifier (WP Instruments, M707) allowing current injections of the order of 0.05-1.0 nA through the recording electrode. Data were recorded on a Gould-Brush pen recorder (model 2200) or stored in the disks of a Nicollet digital oscilloscope (model 4094) until processed and printed out onto an X-Y chart recorder.

**Results**

**Effects of SP on membrane potential: initial hyperpolarization**

Bath application of SP ($2 \times 10^{-6}$ to $1 \times 10^{-5}$ M) induced a biphasic membrane response consisting of an initial hyperpolarization followed by a depolarization in thirteen out of the forty-five dorsal horn neurones
Fig. 1. Biphasic membrane potential changes and inhibitory post-synaptic potentials evoked by SP. In A, bath application of SP (10^{-5}M) induced a biphasic response consisting of an initial hyperpolarization followed by a depolarization and spike discharges in a dorsal horn neurone of an 11-day-old rat. Resting membrane potential was -64 mV. In B, the initial hyperpolarization was blocked by perfusing the slice with a low Ca (1.3 x 10^{-3}M), high Mg (10^{-2}M) Ringer solution. The resting membrane potential was -65 mV. In C, a pressure microinjection of SP (3 psi, 2 x 10^{-2}M) evoked a burst of spontaneous hyperpolarizing events (arrow) in a different dorsal horn neurone of a 13-day-old rat. In D, higher gain and faster time scale records of the hyperpolarizing potentials are shown.
examined (Fig. 1A). However, as shown previously (Murase et al., 1982), the rest of the cells (n = 32) responded to the peptide with a monophasic response consisting of a slow depolarization frequently accompanied by an increase in synaptic activity and action potential firing.

The amplitude of hyperpolarization was small (-1.7 ± 1.0 mV, mean ± S.D., n = 13); it ranged from -0.5 to -4.0 mV and lasted from 20 to 150 s (64.0 ± 35.0 s, n = 13). However, the magnitude of the SP hyperpolarizing response was not easily reproducible from trial to trial within a single cell or among different cells. Thus, the variability of this phenomenon did not permit us to determine reliably dose-response dependence. The resting potential varied between -50 and -78 mV, with a mean value of -65.0 ± 9.06 mV (S.D.).

A typical biphasic response to SP in a dorsal horn neurone of a 10-day-old rat is illustrated in Fig. 1A. Here, within 30 s following addition of SP (10^-5 M), the neuronal membrane hyperpolarized by about 2.3 mV. This effect persisted for about 70 s. When the slice was perfused with a TTX-containing medium, or a low Ca (1.3 mM), high Mg (10 mM) Ringer solution (Fig. 1B) in order to reduce or eliminate synaptic activity, the initial hyperpolarization was not observed. The later finding suggested a possibility that the initial hyperpolarization was brought about by an indirect presynaptic action of SP. Thus, SP may have excited presynaptic inhibitory interneurones, which in turn induced inhibitory synaptic events in other dorsal horn neurones.

This hypothesis is supported by our finding that spontaneous hyperpolarizing potentials resembling inhibitory post-synaptic potentials (i.p.s.p.s.) were recorded in seventeen cells following application of SP.
(Fig. 1C and D) and in seven cells i.p.s.p.s. were associated with initial hyperpolarization. Amplitude of i.p.s.p.s. varied between -0.5 and -6.0 mV, with a mean value of -2.9 ± 1.7 mV (n = 12). The i.p.s.p.s. had a mean rise time of 7.8 ± 2.3 ms (n = 8), while on average, 30.1 ± 8.4 ms (n = 8) were needed for the membrane potential to decline to 37% of the maximum amplitude. Furthermore, we have observed in several cells that spontaneous activity is usually depressed during the initial hyperpolarization, and that membrane input resistance decreased (mean 6.2 ± 3.9%; n = 6). Reduction of i.p.s.p. amplitude could be produced with soma hyperpolarization. Since the hyperpolarizing response was only present in about 29% of all neurones examined, and because the response is relatively small in amplitude, an accurate determination of the reversal potential was not attempted.

Depolarization and firing of Ca-dependent spikes

In confirmation of previous observations (Murase et al., 1982; Nowak & Macdonald, 1982), bath application of SP produced a slow reversible depolarization in the immature spinal dorsal horn neurones, accompanied by an increase in synaptic activity and firing of action potentials (Figs. 2A and 3A). However, addition of CoCl$_2$ (10$^{-3}$M) to the bathing medium substantially reduced the level (Table 1) and duration of depolarization (Fig. 2B) following SP application. It seems, therefore, that Ca conductance (G$_{Ca}$) may have contributed to the depolarizing phase of the SP response, either through its mediation of synaptic transmission or through the direct effects as a charge carrier for inward current. It is
A typical SP-induced depolarization of a dorsal horn neurone in a 14-day-old rat and the effect of Co on this response. A, bath-application of SP (10^{-5}M) for 1 min produced a slow depolarization accompanied by an increase in synaptic activity and firing of action potentials (action potentials were truncated by the limited frequency response of the pen recorder). The insert showing slow depolarizing potentials and Na spikes was taken at the arrow in A. B, addition of Co (10^{-3}M) to the bathing medium substantially reduced the level and duration of the SP-induced depolarization (resting membrane potential, -82 mV).
known that perfusion of slices with Co-containing solution reduces or eliminates synaptic transmission. In addition, Co blocks voltage-dependent $G_{\text{Ca}}$ in rat dorsal horn neurones (Murase & Randić, 1982) and other neurones in the central nervous system. However, our result obtained with Co ions does not rule out participation of voltage-sensitive Na and K components in the slow SP-elicited depolarization (Nowak & Macdonald, 1982).

**TTX- and TEA-containing solutions**

As illustrated in Fig. 3, when the fast voltage-sensitive Na and K conductances were blocked by perfusion of the spinal cord slices with TTX ($10^{-6}$ M) and/or TEA ($2 \times 10^{-3}$ to $2 \times 10^{-2}$ M) a slow depolarizing response to SP could still be evoked although it was modified in its amplitude depending on the kind of solution used (Table 1). Thus, a slightly smaller depolarization was seen following SP application in the presence of TTX (Table 1) while the SP depolarizing response was increased in media containing TTX and TEA (Fig. 3C, Table 1). While Na-dependent action potentials were eliminated in a bathing medium containing TTX (Fig. 3B), SP depolarization in recording solution containing TTX and TEA was in four out of seventeen cells accompanied by firing of TTX-resistant spikes (Fig. 3C). As illustrated, in particular, in the insets of Figs. 3C and 6A, the TTX-resistant spikes were of two distinct amplitudes.

As shown in Fig. 4A-C, the depolarization recorded in TTX-containing solution was associated with an increase in neuronal input resistance ($13.3 \pm 1.7\%$, $n = 4$), and the mean reversal potential determined from
Fig. 3. Responses to SP of a dorsal horn neurone of a 10-day-old rat in solutions containing different ionic blockers. A, bath application of SP (3 x 10^{-6}M) induced a bursting firing pattern in a previously silent cell kept in normal Ringer solution. B, in the presence of TTX (10^{-6}M), sodium-dependent action potentials were eliminated while the SP-induced depolarization remained. C, in media containing TTX (10^{-6}M) and TEA (2 x 10^{-2}M) SP depolarizing response was significantly increased, and firing of TTX-resistant spikes was evoked. As illustrated in the insert (taken at the arrow in C), the TTX-resistant spikes were of two distinct amplitudes. The resting membrane potentials in A, B and C were -54, -56, and -53 mV, respectively.
Table 1. SP-induced depolarization

(A) SP-induced depolarization in the normal Ringer solution. The values are expressed as mean ± S.D.

<table>
<thead>
<tr>
<th>SP (M)</th>
<th>$V_m$ (mV)</th>
<th>$V_m$ (mV)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^{-6}$</td>
<td>3.0 ± 1.8</td>
<td>-65.0 ± 7.5</td>
<td>7</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>5.5 ± 4.8</td>
<td>-63.5 ± 10.4</td>
<td>14</td>
</tr>
</tbody>
</table>

(B) SP-induced depolarization in solutions containing ionic blockers (TTX $5 \times 10^{-7}$ to $2 \times 10^{-6}$M; TEA $2 \times 10^{-3}$ to $2 \times 10^{-2}$M; Co, 1-3 $\times 10^{-3}$M)

<table>
<thead>
<tr>
<th>Solution</th>
<th>% Control</th>
<th>$V_m$ (mV)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100</td>
<td>-64.5 ± 8.9</td>
<td>24</td>
</tr>
<tr>
<td>TTX</td>
<td>91 ± 41</td>
<td>-64.5 ± 8.9</td>
<td>9</td>
</tr>
<tr>
<td>TTX + TEA</td>
<td>170 ± 78</td>
<td>-64.5 ± 8.9</td>
<td>19</td>
</tr>
<tr>
<td>TTX + TEA + Co</td>
<td>57 ± 32</td>
<td>-64.5 ± 8.9</td>
<td>6</td>
</tr>
</tbody>
</table>

current-voltage curves was $-78.0 ± 6.6$ mV ($n = 4$). Reduction of depolarization could be effected by soma hyperpolarization in several of the cells tested, but inversion of the response to a hyperpolarizing response could not be achieved even at a membrane potential of $-104$ mV (Fig. 5A). The latter result suggested that the depolarization may be due to a decrease in a voltage-dependent $G_K$.

In contrast, as evidenced from Fig. 4B and 4D, the current-voltage curve obtained in solution containing TTX and TEA showed that SP produced
Fig. 4. Typical changes in current-voltage (I-V) relationships of two dorsal horn neurones elicited by SP in media containing TTX (A, 13-day-old rat) or TTX and TEA (B, 10-day-old rat) media. The I-V curves were reconstructed from the records shown in C and D, respectively. A, an increase in the slope of a control I-V curve (•) was observed 2 min after the introduction of SP (2 x 10^{-6}M) into a bathing medium containing 10^{-6}M-TTX (●). Almost complete recovery of the slope occurred 10 min after stopping SP perfusion (■). As indicated with an arrow, the reversal potential (V_r) for SP response was about -83 mV. B, in media containing TTX (10^{-6}M) and TEA (2 x 10^{-2}M), SP (2.5 x 10^{-5}M) slightly decreased the slope (●, 3 min after SP) of a control I-V curve (■). Recovery of the slope to the control level occurred 13 min after SP (■). C and D, lower traces show applied current pulses, upper traces voltage response. Intracellular responses to depolarizing (upward) and hyperpolarizing (downward) current pulses applied in progressive steps across the cell soma are illustrated. D, depolarizing pulse that evoked a Ca spike was applied last in the stimulation sequence in order to avoid the post-spike excitability changes. Note, a decrease in Ca spike duration produced by SP (middle record) and a reversal of the after-depolarization to an after-hyperpolarization (AHP).
Fig. 5. The effects of soma hyperpolarization with a d.c. current on the amplitude of SP-induced depolarizations in two different dorsal horn neurones in media containing TTX (A) or TTX and TEA (B). A, in $10^{-6}$M-TTX the soma hyperpolarization decreased the SP ($5 \times 10^{-6}$M) response; 14-day-old rat. B, in solutions containing TTX ($10^{-6}$M) and TEA ($2 \times 10^{-2}$M) the hyperpolarization of the cell body did not alter the SP ($3 \times 10^{-6}$M) response; 13-day-old rat.
a decrease in input resistance and that the extrapolated reversal potential was much more positive than the resting membrane potential. In six cells the SP depolarization was associated with an average decrease in input resistance of $7.6 \pm 6.7\%$. The membrane hyperpolarization essentially did not modify the amplitude of SP-induced depolarization (Fig. 5B). These results suggest that the SP depolarization observed in neurones perfused with solution containing TTX and TEA is possibly due to an increase in $G_{Na}$ and/or $G_{Ca}$. In support of the concept of participation of $G_{Ca}$ in SP depolarization is our finding that the addition of Co either to the normal bathing medium (Fig. 2B) or that containing TTX and TEA (Fig. 6B, Table 1), produced a decrease in both amplitude and duration of the SP depolarization. The latter was possibly in part due to an increase in $G_{Ca}$.

**SP and generation of bursting activity**

In the normal bathing medium we observed that about one-quarter of immature rat dorsal horn neurones showed spontaneous activity, predominantly of a bursting firing pattern. The remaining cells were silent. SP evoked alterations in firing mode, with development of burst generation in the silent cells (Fig. 1A).

Furthermore, after a single SP application some of the silent and nonbursting neurones perfused with medium containing TTX and TEA became capable of prolonged depolarizations and burst generation (Fig. 6A). The SP-induced mode of burst firing could last for hours. Besides large-amplitude spike potentials, individual SP-evoked bursts contained a number.
Fig. 6. The effects of Co\textsuperscript{2+} on SP response of a previously silent dorsal horn neurone from a 12-day-old rat bathed in medium containing TTX (10\textsuperscript{-5}M) and TEA (2 x 10\textsuperscript{-2}M). A, SP (10\textsuperscript{-5}M) induced a depolarization and development of intermittent bursting of TTX-resistant spikes. Resting membrane potential was -57 mV. The records shown in the insert (taken at the arrow at a higher gain), illustrate three distinct amplitudes of TTX-resistant spikes. In B, addition of Co (10\textsuperscript{-3}M) to bathing medium containing TTX and TEA produced a marked decrease in both amplitude and duration of SP-induced depolarization. TTX-resistant spikes are completely blocked by Co, the result indicating Ca-dependency of the bursts (12-day-old rat).
of small spike-like potentials (Fig. 6A, insert).

Since the slow oscillations in the membrane potential which influence the total duration of the bursts are probably Ca-dependent, and since Ca entry is known to activate intracellular metabolic events which have a long time-course (Phillis, 1977), we have examined the nature and duration of SP-induced changes in resting and active membrane properties in slices perfused with Co. It is clear from Fig. 6B that when a Co, a Ca current blocker, is added to the bath, the rhythmic firing behaviour of this cell is blocked. As noted further in Fig. 6B, although a small SP depolarization still occurred, its time course was significantly reduced compared with the duration of changes in the membrane properties following SP application in normal medium.

Ca-dependent action potentials and after-potentials

When the Na and K conductances are blocked by perfusion of the spinal cord slices with TTX (5 x 10^-7 to 2 x 10^-6 M) and TEA (2 x 10^-3 to 2 x 10^-2 M) depolarizing current pulses applied across the cell soma of a dorsal horn neurone elicit a high-threshold Ca spike (Murase & Randić, 1982, 1983a). The spike duration varied from cell to cell presumably depending upon the degree of blockage of K conductances by TEA. Thus, the duration of Ca spikes recorded in dorsal horn neurones bathed in a solution containing 2-10 x 10^-3 M TEA was in the range of 5-15 ms (Fig. 7C). When a higher concentration of TEA (2 x 10^-2 M) was used, the spike duration ranged from about 15 to 400 ms (occasionally up to 12 s), and the repolarization phase of action potentials was marked by a distinct
The effects of membrane polarization and SP (10⁻⁵ M) on Ca spike duration in three different dorsal horn neurones bathed in solution containing TTX (10⁻⁶ M) and TEA (2 x 10⁻² M). Oscilloscope records of superimposed Ca-dependent action potentials are shown. Action potential duration was determined at half maximal amplitude. A, 14-day-old rat; B, 13-day-old rat; C, 10-day-old rat. A, resting membrane potential was -66 mV (trace 1). Membrane depolarization (trace 2; Vm = -50 mV) resulted in a marked increase in spike duration and a decrease in after-depolarization. Hyperpolarization in this cell (trace 3; Vm = -70 mV; trace 4; Vm = -77 mV) reduced the spike duration, and increased the magnitude of after-depolarization. B, SP induced a biphasic change in the spike duration (1: before SP) consisting of an initial decrease (2: 1 min after SP) followed by an increase (3: 3 min after, 4: 5½ min after SP). Almost full recovery of the spike duration occurred at about 20 min after stopping the SP applicator: (trace 5). Stimulus pulse: 1 nA, 20 ms. Vm = -51 mV. C, when Ca spike duration was less than 15 ms, SP elicited increase in the spike duration only was observed (1: before SP; 2: 2 min after SP, 3: 4 min after, and 4: 6½ min after SP). The spike broadening was accompanied by a reduction in the amplitude and duration of after-hyperpolarization. Stimulus pulse: 2 nA, 5 ms. Vm = -50 mV.
plateau (Fig. 7A).

The Ca spikes of short duration (< 15 ms) were frequently followed by afterhyperpolarization (Fig. 7C). When the Ca spike duration was longer (> 15 ms), the spike was followed by a prolonged after-depolarization (Fig. 7A).

The effects of membrane polarization on Ca-dependent action potentials of a dorsal horn neurone in a 14-day-old rat are shown in Fig. 7A. Membrane depolarization resulted in a marked increase in spike duration and a decrease in after-depolarization (Fig. 7A2). Hyperpolarization in this cell only slightly reduced spike duration, but the amplitude of after-depolarization was increased (Fig. 7A3-4).

The effects of SP on Ca spike

Bath application of SP (10^-5 M) modified the duration of Ca-dependent action potentials in a complex manner in fourteen out of twenty-two immature rat dorsal horn neurones examined. The remaining eight neurones showed no modification of the Ca spike duration despite the presence of membrane depolarization.

In general, it appears that the direction of a change in the Ca spike duration is highly dependent upon the initial control value. Thus, when the control Ca spike duration was approximately 15 ms, or more, the effect observed in about half of the cells examined was a biphasic modification of the spike duration consisting of an initial reversible decrease followed by an increase (Fig. 7B). However, when the spike duration was less than 15 ms, a monophasic increase only was observed
The SP-induced initial decrease in Ca spike duration was accompanied by a significant reduction in the magnitude of after-depolarization (Fig. 7B2). The SP-induced monophasic spike increase was usually associated with a decrease in the amplitude and duration of after-hyperpolarization (Fig. 7C).

The SP-elicited changes in Ca spike duration were not easily reproducible from trial to trial in a single cell; usually three consecutive applications of SP led to a reduction in the response. Thus, the desensitization of this response did not permit us to determine reliably dose-dependence in a single cell. It is of interest that during the desensitization of the Ca spike, the depolarizing response of SP remained.

To identify the component of the Ca spike responsible for the initial decrease in spike duration, the dV/dt of the Ca spike was utilized. As seen in Fig. 8B and C, in the cells showing an initial decrease in spike duration the dV/dt of the repolarizing phase of the spikes was significantly increased by SP (167% ± 13, n = 5). In contrast, only small reductions in dV/dt of the rising phase of Ca spikes (94.1% ± 7.9, n = 5) or in $V_{\text{max}}$ of Ca spikes were observed.

The initial decrease in Ca spike duration was not due to the depolarizing action of SP for two reasons: (1) the effect is present when resting membrane potential was re-established by passing adequate d.c. current, and (2) consistent increase in spike duration occurred as the membrane potential was made more positive than the resting membrane potential (Fig. 7A2).
Fig. 8. A and B, the membrane of a dorsal horn neurone of 13-day-old rat bathed in solution containing TTX ($10^{-6}$M) and TEA ($2 \times 10^{-2}$M) was depolarized by SP ($10^{-5}$M). During the depolarizing response a Ca spike was evoked with depolarizing current pulses (1 nA, 30 ms) applied across the cell soma at regular intervals of 1 min and the differentiation was calculated (C). B, the same records of Ca spikes as in A taken at a higher gain. Since the shape of the Ca spike at Ab was identical to one recorded at Aa, Aa was omitted from B and C. In Bc, SP produced a reversible decrease in spike duration which was associated with an increase in dV/dt of the repolarization phase of the spike. Also, the after-depolarization was almost completely abolished and the shunting effect on the after-depolarization lasted more than 20 s (Bc). Note, a late increase of slow after-depolarization (Ad and Ae).
SP-mediated inhibition

SP has depressant actions when applied iontophoretically near spinal dorsal horn neurones (Krnjević, 1977; Randić & Miletić, 1977; Sastry, 1979a; Davies & Dray, 1980). An initial hyperpolarization associated with a decrease in membrane input resistance was observed in some immature rat dorsal horn neurones following SP application (Murase et al., 1982). A transient depression or even abolition of spontaneous activity during the initial hyperpolarization was seen in the present experiments. This apparent inhibition of dorsal horn neurones by SP might have been brought about either by a direct post-synaptic action or by an indirect presynaptic mechanism involving excitation of inhibitory interneurones.

Although direct evidence for presynaptic excitatory effects of SP on spinal dorsal horn inhibitory interneurones is lacking, our results suggest that the initial inhibitory effect of SP is produced by an indirect presynaptic action rather than direct post-synaptic actions. This hypothesis is supported by our findings that the initial hyperpolarization was not observed when synaptic activity was reduced or eliminated by perfusion of the slice with a TTX-containing, or low Ca/high Mg, Ringer solution. In addition, spontaneous hyperpolarizing potentials resembling i.p.s.p.s. were recorded in dorsal horn neurones following application of SP, and in several cells i.p.s.p.s. were associated with hyperpolarization.

A presynaptic site of action for SP has also been suggested by other experimental data (Nicoll, 1976; Macdonald & Nowak, 1981; Randić, 1981;
Randić, Carstens, Zimmermann & Klumpp, 1982). In addition, immunocytochemical studies of localization and synaptic relationships of SP in the rat spinal dorsal horn (Barber, Vaughn, Slemmon, Salvaterra, Roberts & Leeman, 1979) and trigeminal nucleus (Priestley, Somogyi & Cuello, 1982) suggest several possible targets for SP-containing terminals, including the large lamina I projection neurones and two types of lamina II interneurones, one of which possesses presynaptic dendrites.

Depolarization and firing of Ca-dependent spikes

To investigate further the ionic mechanisms of SP-induced depolarization, we monitored changes in the magnitude of the depolarizing responses of dorsal horn neurones exposed to blockers of voltage-sensitive Na, K and/or Ca conductances (G_{Na}, G_{K} and G_{Ca}); we also investigated such changes as a function of membrane potential. The experiments utilizing the sequential blockage of G_{Na}, G_{K} and G_{Ca} in the same cell provide additional new data relevant to the nature of SP-induced depolarization. Thus, when voltage-sensitive Na and K conductances are blocked by perfusion of the spinal cord slices with TTX and TEA, respectively, the SP depolarizing response of dorsal horn neurones was enhanced and in about one-quarter of all cells examined was accompanied by firing of TTX-resistant spikes. The current-voltage curve showed that SP produced a small decrease in neuronal input resistance and that the extrapolated reversal potential for SP response was much more positive than the resting membrane potential. In addition, the membrane hyperpolarization essentially did not modify the amplitude of SP-induced depolarization. These findings do
not necessarily contradict the current concept that the principal ionic mechanism underlying SP-induced depolarization is a decrease in a voltage-dependent $G_K$ in the post-synaptic cell (Hösli et al., 1981; Nowak & Macdonald, 1982). However, the fact that the SP reversal potential was more positive than the $K$ equilibrium potential suggests that in addition to a decrease in $G_K$, SP may have increased positive ion conductances such as $G_{Na}$ and/or $G_{Ca}$. The ratio of the changes in these three conductances will ultimately determine the change of membrane resistance.

Furthermore, in support of the hypothesis of participation of $G_{Ca}$ in prolonged SP depolarization is our finding that the addition of Co either to the normal bathing medium or to that containing TTX and TEA produced a significant decrease in both amplitude and duration of the SP-induced depolarization. This result suggests that Ca entry might be primarily responsible for the prolonged depolarizations associated with SP excitation.

We are fully aware that the present conclusions about specific ion conductance changes produced by SP have been inferred indirectly by measurements of membrane potential, input resistance, and by use of specific conductance blockers, and that only voltage-clamp analysis of SP actions in dorsal horn neurones, in addition to changes of Ca and Na levels in the extracellular environment, will allow us to test our hypothesis directly.

**Modification of Ca spike duration by SP**

In this paper we have demonstrated that SP modifies the duration of Ca-dependent action potentials of the immature rat dorsal horn neurones, the most consistent change observed being an initial dose-dependent and
reversible decrease of spike duration.

In principle, the SP-induced decrease in Ca spike duration might be due to an effect on inward Ca current or outward K currents. Since we have observed a small decrease in the rate of rise and the peak amplitude of the Ca spike following SP application to dorsal horn neurones, and since the voltage-dependent conductance changes are probably occurring at dendritic sites, and the resting membrane of the cells is usually depolarized by SP, the possibility should be considered that SP shortens the duration of the spike by decreasing a voltage-sensitive inward Ca current. Employing voltage-clamp analysis Dunlap and Fischbach (1981) have shown that several neurotransmitters, including noradrenaline, γ-aminobutyric acid (GABA) and 5-hydroxytryptamine decrease the Ca conductance activated by depolarization of embryonic chick sensory neurones maintained in cell culture.

Our observation that in the cells showing an initial decrease in Ca spike duration the decrease was clearly paralleled by the increase in dV/dt of the falling phase of the Ca spike favours an alternative possibility, namely that the decrease may be due to an activation of an outward current(s). The latter interpretation is supported by two additional observations: (1) the after-depolarization was blocked, or even reversed into after-hyperpolarization during the initial decrease in Ca spike duration, and (2) the magnitude of the initial decrease is inversely proportional to the duration of the control spike. The direct test of our interpretations and identification of ionic current(s) involved are to be accomplished only with a voltage-clamp analysis.
Since the initial decrease in Ca spike duration of dorsal horn neurones was usually associated with SP-induced changes in membrane potential and resting membrane conductance, these effects of SP should be considered when discussing alternative mechanisms for explaining the decrease in Ca spike duration. Our data presented in the Results clearly indicate that the initial decrease in Ca spike duration was not due to the depolarizing action of SP. However, SP-modulation of voltage-sensitive ion channels involving shunting of the membrane resistance, either through post-synaptic or even presynaptic mechanisms (perhaps through activation of inhibitory interneurones releasing GABA or enkephalin) has not been excluded.

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DISCUSSION

The major points pertaining to the data obtained in this work have been already discussed in the Results section in detail. This chapter contains the summary of the discussions and some speculations on the specific topics presented in the Results.

Spinal Cord Slice Preparation and the Intracellular Recording

The results obtained in a slice preparation of the immature rat spinal cord illustrate that satisfactory intracellular recordings from dorsal horn neurons can be maintained for as long as 5 hours. Action potentials could be evoked by direct or orthodromic stimulation, and spontaneous or evoked synaptic potentials were present for many hours. These findings indicate that the neuronal circuitry of the dorsal horn is at least in part, intact in the spinal cord slice preparation. These are encouraging results since obtaining stable recordings from the superficial parts of the spinal dorsal horn in vivo has been technically very difficult (Cervero et al., 1977; Yaksh et al., 1977; Hayashi et al., 1978; Kumazawa & Perl, 1978; Bennett et al., 1979).

An important question which might be raised concerns the anatomical characteristics and localization of the neurons from which the intracellular recordings were made. Although intracellular labeling of electrophysiologically analyzed neurons was not done in this work, morphological analyses based upon Golgi-staining (Uemura, Veterinary Anatomy, Iowa State University, unpublished observation) and HRP-staining (Nedeljkov & Randić,
1982) of the neurons in the same preparation, and in intact animals (Gobel, 1975, 1978a,b) suggest that the recordings have been probably made from the neurons having morphological characteristics of the neurons described in the superficial parts of the dorsal horn. Recent studies with transversal immature rat spinal cord slices showed that the input resistance of neurons in the superficial laminae is considerably higher than that of neurons in the deeper laminae, the finding in agreement with the results obtained in this study (Urbán & Randić, 1983; Murase, unpublished observation) and supporting the thesis that our recordings were predominantly done in the small neurons of the upper dorsal horn.

Passive Membrane Properties

Large variation was found in the input resistance ($R_N$) and the membrane time constant of the dorsal horn neurons. In addition, $R_N$ values for the rat dorsal horn neurons covered a higher range than has been reported for mouse spinal cord neurons in primary dissociated cell culture (Ransom et al., 1977) and cat dorsal horn neurons in vivo (Zieglgansberger & Tulloch, 1979a). This generally higher $R_N$ values of the dorsal horn neurons may be attributed at least in part to the small size of the cells, the lower recording temperature ($33 \pm 1^\circ\text{C}$), stable recording conditions and differences in membrane properties and dendritic arborization between mature and immature neurons. In confirmation of the immaturity of the neurons, our Golgi-staining and a HRP-staining study (Nedeljkov & Randić, 1982) in this preparation showed fewer dendritic branches of
the neurons, and the equivalent cylinder length calculated for these neurons appeared to be less than 1.0 (Murase, unpublished data).

One interesting observation is that majority of the dorsal horn neurons recorded in this study did not exhibit significant anomalous rectification in contrast to other central neurons such as hippocampal pyramidal neurons (Adams & Halliwell, 1982; Halliwell & Adams, 1982), olfactory cortex neurons (Constanti & Galvan, 1983) or neocortical neurons (Stafström et al., 1982) studied in vitro. In motoneurons of cats in vivo, both anomalously rectifying and nonrectifying neurons were found (Nelson & Frank, 1967).

Voltage-Dependent Conductances

**Na spike**

The finding that the action potentials were reliably blocked by tetrodotoxin indicates that the fast voltage-dependent Na conductance, as in other central neurons including mouse spinal cord neurons in primary dissociated cell culture (Ransom et al., 1977; Heyer et al., 1981), plays an important role in generation of the fast action potentials in immature dorsal horn neurons. However, the spike duration of 1.4 msec obtained in immature dorsal horn neurons is longer than the values of 0.5-1.0 msec recorded in adult dorsal horn neurons (Haapanen et al., 1958; Hunt & Kuno, 1959; Eccles et al., 1960; Cervero et al., 1977). This result may be a consequence of the immaturity of the spike generation mechanism, the lower recording temperature and smaller size of the neurons studied.
High-threshold Ca spike

When the fast sodium and potassium conductances of dorsal horn neurons were blocked by perfusion of the spinal cord slice with TTX and TEA, depolarizing current pulses often elicited a slow regenerative depolarizing potential followed by a slow-afterhyperpolarization. The mechanism underlying the generation of this response appeared to be a voltage-dependent Ca conductance because this response was TTX-resistant, it required the presence of extracellular calcium, and it is blocked by cobalt and manganese. Since the appearance of this Ca-dependent action potential required that the membrane be depolarized to a higher level than was necessary to evoke Na spike, the term "high threshold Ca spike", as proposed by Llinás & Yarom (1981a) was adopted here.

Calcium-dependent action potentials of similar properties have been recently demonstrated in mouse spinal cord neurons in cell culture (Heyer et al., 1981), in frog motoneurons (Barrett & Barrett, 1976), cerebellar Purkinje cells (Llinás & Hess, 1976; Llinás & Sugimori, 1980a,b), hippocampal pyramidal cells (Schwartzkroin & Slawsky, 1977; Wong et al., 1979), inferior olivary neurons (Llinás & Yarom, 1980a,b) and thalamic neurons (Llinás & Jahnsen, 1982). While we have no direct evidence for the site of the generation of the high threshold Ca spikes in the immature rat dorsal horn neurons, the dendritic location in hippocampal pyramidal neurons (Schwartzkroin & Slawsky, 1977; Wong et al., 1979), cerebellar Purkinje cells (Llinás & Sugimori, 1979, 1980b) and inferior olivary neurons (Llinás & Yarom, 1981a,b) has been demonstrated.
Low-threshold Ca spike

It has been recently reported that following a membrane hyperpolarization above -70 mV, inferior olivary neurons and thalamic neurons may generate Ca-dependent potentials in response to direct or synaptic stimulation (Llinás & Yarom, 1981a; Llinás & Jahnsen, 1982). In immature rat dorsal horn neurons having the resting membrane potential more negative than -65 mV, a low threshold electroresponsiveness of similar properties was observed. Because it was reversibly blocked by cobalt and enhanced by barium, we suggest that the low threshold spike may be generated by an inward Ca current. As in inferior olivary neurons and thalamic neurons this low threshold Ca conductance exhibited inactivating properties as suggested by the experiments with Ba and TEA. However, a direct proof for the inactivation would require a series of voltage clamp experiments.

Presynaptic Effects of Substance P: SP-Mediated Inhibition

SP has depressant actions when applied near spinal dorsal horn neurons iontophoretically (Krnjević, 1977; Randić & Miletić, 1977; Sastry, 1979a; Davies & Dray, 1980). As we have described in the Results, an initial hyperpolarization associated with a decrease in membrane input resistance occurred in some immature rat dorsal horn neurons following SP application. Membrane hyperpolarization associated with a fall in membrane resistance was also observed following SP application to myenteric neurons of guinea-pig (Katayama & North, 1978; Katayama et al., 1979) and inferior
mesenteric ganglion cells (Dun & Minota, 1981).

A transient depression or even abolition of spontaneous activity during the initial hyperpolarization was seen in the present experiments. This apparent SP-inhibition of dorsal horn neurons might have been brought about by an indirect presynaptic mechanism involving excitation of inhibitory interneurons. Although direct evidence for presynaptic excitatory effect of SP on spinal inhibitory interneurons is lacking, our results suggest that the initial inhibitory effect of SP is produced by an indirect presynaptic mechanism of action of SP rather than direct postsynaptic actions. This hypothesis is supported by our finding that the initial hyperpolarization was not observed when synaptic activity was reduced or eliminated by a perfusion of the slice with a TTX-containing or a Ca^{2+}-low, Mg^{2+}-high Ringer solution. In addition, spontaneous hyperpolarizing potentials resembling IPSPs were recorded in dorsal horn neurons following application of SP, and in several cells, IPSPs were associated with a hyperpolarization.

A presynaptic site of SP action also has been suggested by the following data. SP has been reported to depolarize rat dorsal root ganglion neurons (Dray & Pinnock, 1982) and amphibian primary afferent fibers (Nicoll, 1976), to reduce synaptic efficacy at the Mauthner fiber-giant fiber synapse of hatchet-fish (Steinacker & Highstein, 1976), to have a strong presynaptic effect on transmission at frog neuromuscular junction (Steinacker, 1977), to modify neurotransmitter release in spinal cord neurons grown in culture (Macdonald & Nowak, 1981), and to modify electrical
excitability of single cutaneous primary afferent C- and Aδ-fibers in the cat spinal cord (Randić, 1981; Randić et al., 1982). SP-elicited increase in excitability of a few C-fibers was blocked by bicuculline, a well-known antagonist of GABA-ergic inhibitory interneurons (Raspantini & Randić, unpublished observations), thus, supporting the hypothesis that SP has indirect inhibitory effects mediated via excitation of possibly GABA-ergic inhibitory interneurons.

Immunocytochemical studies suggest that SPLI-positive terminals might terminate on inhibitory interneurons in the superficial parts of the spinal dorsal horn, SPLI-positive terminals originating from small primary afferent fibers have been found to make contacts predominantly with dendrites of marginal neurons in lamina I (Kerr, 1975; LaMotte & DeLanerolle, 1981; Priestley et al., 1982) and small neurons in lamina II, probably islet cells (Priestley et al., 1982). On the other hand, glutamic acid decarboxylase (GAD)-positive neurons were found to be most dense in lamina III and are probably identical with some of islet cells (Barber et al., 1982) and stalked cells whose axons arborize in lamina I (Hunt et al., 1981). In addition, in lamina I, some medium size (Hunt et al., 1981) and small neurons (Barber et al., 1982) are also GAD-positive (Hunt et al., 1981), ENKLI-positive somata are most dense in lamina II. Some islet cells (Glazer & Basbaum, 1981; Hunt et al., 1981; Sumal et al., 1982) and marginal neurons in lamina I are also ENKLI-positive (Glazer & Basbaum, 1981; Sumal et al., 1982), SSLI was found in small neurons of lamina III as well (Hunt et al., 1981).
Postsynaptic Effects of Substance P

Postsynaptic effects of SP were first examined in a TTX-containing solution in which Na-spikes were blocked. In TTX (10^{-6}M), SP depolarized dorsal horn neurons and increased their input resistance. The SP-induced depolarization was voltage-dependent, i.e., a membrane hyperpolarization decreased the SP-induced depolarization. These findings are in a good agreement with the data obtained in cultured spinal sensory neurons (Nowak & Macdonald, 1982) and in sympathetic ganglion neurons (Adams et al., 1983) where the postsynaptic excitatory action of SP was suggested to be due to a decrease of the M-current. Our finding that atropine, an antagonist of muscarinic receptors, depressed the SP-induced depolarization (Murase & Randić, unpublished data) supports this concept. However, the proof of this hypothesis will require the identification of the M-current in dorsal horn neurons with voltage-clamp experiments and tests of SP-effects on it. Possible alterations of the SP-effects by pharmacological agents such as Ba, muscarine etc. (Adams et al., 1982a) are also needed to be investigated.

In a TTX- and TEA-containing solution, the SP-induced depolarization of dorsal horn neurons was larger than one observed in a TTX-containing solution. In contrast to the increase of the resistance observed when only TTX-containing solution was used, SP slightly decreased the input resistance in TTX- and TEA-containing solution. The TEA concentration (2 x 10^{-2}M) used in our study was sufficient to reduce the total outward current by approximately 95% in cultured chick sensory neurons (Dunlap &
Fischbach, 1981) and to block $I_k$ and $I_{k(Ca)}$ almost completely in frog sympathetic neurons (Adams et al., 1982a). $I_M$ was also partially blocked by $5 \times 10^{-2} M$ of TEA (Adams et al., 1982a). The dorsal horn neurons bathed in a TTX- and TEA ($2 \times 10^{-2} M$)-containing solution seem to behave similar to the chick sensory neurons and the sympathetic ganglion neurons, i.e., $I_K$ and $I_{K(Ca)}$ appear to be almost completely blocked while $I_M$ is only blocked in part. The supporting facts for this statement are as follows. Ca spikes evoked in the presence of TTX ($10^{-6} M$) and TEA ($2 \times 10^{-2} M$) generally had a duration of over 100 ms and possessed an after-depolarization (ADP) rather than a slow afterhyperpolarization (AHP). However, the slow AHP was usually present in a solution containing less TEA ($< 10^{-2} M$). On the basis of these considerations, it appears that the mechanism underlying the SP-depolarization in a TTX-containing solution was blocked by TEA so that another mechanism contributing to the SP-elicited depolarization became dominant. The SP-elicited depolarization observed in a TTX- and TEA-containing solution appears not to be due to a decrease in K-current(s), but instead, may be due to an increase in positive ion permeabilities for two reasons: (1) the input resistance was decreased by the application of SP, and (2) the membrane hyperpolarization by DC current did not modify the SP-depolarization. Two candidate ions are Na and Ca. In this context, it is of interest that SP increased Na conductance in mesenteric ganglion neurons (Dun & Minota, 1981) and in neuronal cell lines (Reiser et al., 1982). Since a further addition of Co into TTX- and TEA-containing solutions
substantially reduced the SP-induced depolarization of dorsal horn neurons, a possibility that the SP depolarization might be carried by Ca ions, at least in part, should be considered. Although it has been reported that Ca might be a regulator or modulator of l-glutamate receptor sensitivity in frog spinal motoneurons (Kudo & Oka, 1982), it is possible that the actual current carrier might be Na. It would be necessary in a future work, to explore both possibilities by utilizing Na substitution experiments in the spinal cord slice.

Substance P Effects on Ca Spikes

The duration of Ca spikes in dorsal horn neurons bathed in TTX and TEA (5 x 10^{-3} - 2 x 10^{-2} M) varied from 5 ms to more than 1 sec, probably depending upon the degree of blockage of outward currents by TEA. We examined effects of SP on dorsal horn neurons with a variety of Ca spike durations, and found that the Ca-spike duration of a neuron was increased by SP when the control Ca-spike duration was shorter than 15 ms, while the Ca-spike duration was decreased by SP if the initial Ca-spike duration was longer than 15 ms.

The finding of the increase in the Ca-spike duration in the former group of neurons is not surprising. In this group of neurons, the outward potassium currents were only in part blocked by TEA as evidenced by the short duration of Ca-spikes, therefore, an additional decrease of the outward current by SP could increase the Ca-spike duration. A similar increase in the spike duration caused by SP was reported in spinal dorsal
ganglion neurons (Dray & Pinnock, 1982).

The decrease in the Ca-spike duration by SP, however, is an intriguing finding. From experiments done with a voltage-clamp technique in other preparations (Dunlap & Fischbach, 1981; Klein & Kandel, 1980), it seems that two possible mechanisms for the SP-induced decrease in Ca-spike duration should be considered. One implies that SP might decrease \( I_{Ca} \) and another that SP might activate a voltage-dependent outward current.

In sensory ganglion neurons in cell cultures, the Ca-spike duration was decreased by 5HT, NA, GABA, SS and ENK (Dunlap & Fischbach, 1978; Mudge et al., 1979) and later voltage clamp experiments revealed that the decrease of Ca-spike duration was due to a decrease of \( I_{Ca} \) by the putative neurotransmitters (Dunlap & Fischbach, 1981). In rat sympathetic neurons studied in vitro NA decreased the duration of Ca spikes (Horn & McAfee, 1980), and a voltage clamp study demonstrated a decrease of \( I_{Ca} \) by NA (Galvan & Adams, 1982). In both cases, however, the height of Ca spikes (Vmax) and the dV/dt of the rising phase of Ca spikes were decreased by the neurotransmitters. This contrasted with our results obtained with SP on dorsal horn neurons which showed very small and insignificant changes in the rate of rise or Vmax of Ca spikes.

Our observation that in the cells showing an initial decrease in the Ca-spike duration, the decrease was clearly parallel with the increase in dV/dt of the falling phase of Ca spike favours an alternative possibility, namely, that the decrease may be due to an activation of an outward current(s). This interpretation is supported by two additional
observations: (1) The afterdepolarization was blocked, or even reversed into afterhyperpolarization during the initial decrease in Ca-spike duration, and (2) The Ca-spike duration during the SP-effects tended to be 15 ms irrespective of the initial duration, the finding implying that the outward current might be TEA-resistant and that the activation time might be close to 15 ms.

A novel neurotransmitter sensitive outward current has been reported in invertebrate preparations. 5HT was found to increase the Ca-spike duration in *Aplysia* (Klein & Kandel, 1978; Pellmar & Carpenter, 1979, 1980) and in *Helix* neurons (Paupardin-Tritsch et al., 1981), and the voltage-clamp studies revealed that the increase in the Ca-spike duration is due to a decrease of a novel voltage-dependent K-current (Klein & Kandel, 1980; Deterre et al., 1982; Klein et al., 1982). Although the effect on the Ca-spike duration observed in these preparations was opposite to that of SP in dorsal horn neurons, a similar neurotransmitter sensitive outward current might exist in dorsal horn neurons and be modified by SP. Of course, a series of voltage-clamp experiments are needed to confirm this possibility.

Since the initial decrease in the Ca-spike duration of dorsal horn neurons was usually associated with SP-induced changes in membrane potential and resting membrane conductance these effects of SP should be considered when trying to discuss alternative mechanisms to explain the decrease in Ca-spike duration. Our data presented in the Results clearly indicate that the initial decrease in Ca-spike duration was not due to the depolarizing action of SP. However, SP-modulation of voltage-
sensitive ion channels involving a decrease in the membrane resistance, either through postsynaptic or even presynaptic mechanisms (perhaps through activation of inhibitory interneurons releasing GABA, or enkephalin), has not been excluded.

Effects of Enkephalin

Our results showed that methionine-enkephalin and its stable analogue D-alanine\(^2\) -methionine\(^5\) -enkephalinamide hyperpolarized dorsal horn neurons in vitro and increased their membrane conductance. These effects were present, although reduced, in a Ca\(^{2+}\)-low, Mg\(^{2+}\)-high containing solution, the findings suggesting that the effects were in part postsynaptic. This hyperpolarizing action of ENK may account for some of the depressant actions of ENK observed in the dorsal horn neurons in vivo (Duggan et al., 1977; Randić & Miletić, 1978) and in vitro (Miletić & Randić, 1982). The ENK-elicited membrane hyperpolarization and a reduction in the neuronal input resistance have been also observed in myenteric neurons (North & Tonini, 1977) and locus coeruleus neurons (Pepper & Henderson, 1980). Furthermore, it has been reported that ENK may activate Ca dependent K conductance by increasing intracellular free calcium in myenteric neurons (Tokimasa et al., 1981; Morita & North, 1982).

Postsynaptic action of ENK has been suggested by both electrophysiological and immunochemical findings. In a spinal cord slice preparation, ENK reduced spontaneous activity of dorsal horn neurons bathed in a Ca\(^{2+}\)-low, Mg\(^{2+}\)-high solution (Miletić & Randić, 1982).
Iontophoretically applied ENK did not modify the membrane potential nor the input resistance of spinal neurons of intact cats, but reduced postsynaptically, the efficacy of a putative excitatory transmitter, 1-glutamate (Zieglgänsberger & Bayerl, 1976; Zieglgänsberger & Tulloch, 1979b). Immunocytochemical data further support the electrophysiological findings, since ENK-positive terminals form almost exclusively axodendritic and axosomatic synapses with neurons in lamina I (Glazer & Basbaum, 1981; Ruda, 1982) and laminae II and III (Hunt et al., 1980, 1981; Glazer & Basbaum, 1981; Sumal et al., 1982). A very few axoaxonic or dendroaxonic synapses are present in the area (Duncan & Morales, 1978; Ralston, 1979; Ralston & Ralston, 1979; Zhu et al., 1981).

Since we have observed a significant reduction of the ENK-induced hyperpolarization in a Ca\(^{2+}\)-low, Mg\(^{2+}\)-high solution, it is likely that ENK may act presynaptically on dorsal horn neurons as well. Presynaptic effects of ENK have been already reported. ENK decreased the excitability of primary afferent terminals (Sastry, 1978, 1979b; Carstens et al., 1979) and hyperpolarized primary afferent fibers (Evans & Hill, 1978). These data are in agreement with other findings, i.e., opiate receptors were found on primary afferent fibers and the number of opiate receptors in the dorsal horn was decreased by dorsal root section (LaMotte et al., 1976; Macdonald & Nelson, 1978; Jessell et al., 1979b; Ninković et al., 1981). In addition, it is of interest that ENK did not modify the membrane potential nor input resistance of dorsal root ganglion neurons in cell culture, but decreased the voltage-dependent Ca conductance (Dunlap & Fischbach, 1979, 1981) and reduced the release of SP (Mudge
et al., 1979).

We have observed in several cells that ENK decreased the duration of Ca spikes. This effect may resemble the presynaptic action of ENK found in dorsal root ganglion neurons in cell culture (Dunlap & Fischbach, 1978). However, we have not analyzed whether the decrease of the Ca spike duration in the dorsal horn neurons produced by ENK is due to the hyperpolarization and the associated increase in outward current, or to a decrease of the voltage-dependent Ca conductance.

Effects of Somatostatin

Our results indicate that a hyperpolarization accompanied by a fall in the membrane input resistance is the most common consequence of bath application of SS to the dorsal horn neurons in vitro. These effects were somewhat reduced, but not abolished in a Ca$^{2+}$-low, Mg$^{2+}$-high solution, the result suggesting both presynaptic and postsynaptic sites of SS action. This postsynaptic hyperpolarizing action of SS is consistent with the following evidence. Iontophoretically applied SS depressed spontaneous activity of nociceptive dorsal horn neurons of intact cats (Randić & Miletic, 1978). In a slice preparation of neonatal rats, a bath application of SS suppressed spontaneous activity of dorsal horn neurons (Miletic & Randić, 1982). A similar SS-induced hyperpolarization associated with the conductance increase was found in hippocampal CA1 neurons in vitro (Pittman & Siggins, 1981). However, ionic mechanism of the response is not currently known.

Our observation that the SS hyperpolarization was reduced in a Ca$^{2+}$-
low, Mg\textsuperscript{2+}-high solution suggests that SS may act presynaptically as well. Presynaptic action of SS has been reported in several preparations. SS hyperpolarized primary afferent fibers of the frog (Padjen, 1977). The peptide increased the excitability of some cutaneous primary afferent C- and A\delta-fibers in intact cats (Jeftinija & Randić, 1981). SS also decreased the voltage-dependent Ca conductance of dorsal root ganglion neurons in cell culture (Dunlap & Fischbach, 1981) and modified the neurotransmitter release in cultured mouse spinal neurons (Macdonald & Nowak, 1981).

A few dorsal horn neurons tested in our experiments were depolarized by SS. From our limited amount of data, it is not certain whether this depolarizing effect of SS is a direct postsynaptic effect or it is mediated by interneuronal connections such as a disinhibitory circuitry. Excitatory effects of SS have been reported in spinal cord neurons in cell culture (Macdonald & Nowak, 1981), neurons of sensory motor cortex (Ioffe et al., 1978), hippocampal CA1 and CA2 pyramidal neurons (Dodd & Kelly, 1978), and neurons of hippocampus, neocortex and neostriatum (Olpe et al., 1980). However, the site of the excitatory action of SS is not clear at present.
SUMMARY

1. The electrophysiological properties of the dorsal horn neurons and actions of substance P (SP), somatostatin (SS) and enkephalin (ENK) have been investigated by intracellular recording in an immature rat spinal cord slice preparation.

2. The dorsal horn neurons in the slice preparation exhibited an average resting membrane potential of -70 mV. Action potentials with the average overshoot of about 25 mV were generated by direct and orthodromic stimulation. Spontaneous and evoked synaptic potentials were also recorded, the findings reflecting the viability of the slice preparation.

3. Input resistance for dorsal horn neurons ranged from 48 to 267 MΩ with the average value of 98 MΩ. The membrane time constant was in the range of 4-19 ms, the average being 10 ms.

4. In response to a strong depolarizing current, dorsal horn neurons perfused with TTX and TEA frequently exhibited a slow regenerative depolarizing potential resembling the Ca spike. The depolarizing potential seems to result from an influx of Ca since it was blocked by Co, Mn or low-Ca containing media, and enhanced by a high Ca- or Ba-containing media.

5. There was, in addition, a low-threshold Ca-dependent response in some dorsal horn neurons. It was activated at membrane potentials more negative than -65 mV and had a maximum rate of rise at about -80 mV.
6. Responses of dorsal horn neurons to SP, SS and ENK have been studied during the bath or pressure application.

7. SS and ENK hyperpolarized dorsal horn neurons and caused a reduction or abolition of spontaneous firing. The hyperpolarization produced by ENK was always associated with a fall in the neuronal input resistance, while in the case of SS the similar effect was less consistent.

8. The inhibitory effects of SS and ENK persisted in a low-Ca, high-Mg containing solution, the result suggesting that the effects are at least in part postsynaptic.

9. SP induced a slow depolarization most commonly associated with an increase in neuronal input resistance. In some cells, the depolarization was accompanied by an increase in excitatory-or inhibitory-like postsynaptic potentials and appearance of bursts of action potentials.

10. In about one-third of the examined cells, SP induced a biphasic membrane response consisting of an initial brief hyperpolarization followed by the slow depolarization. The initial hyperpolarization was not observed in a TTX- or Co-containing, or low-Ca, high-Mg solution. This finding is consistent with a presynaptic action of SP possibly mediated through excitation of inhibitory interneurons. The interpretation is supported by recording of repetitive spontaneous IPSP-like hyperpolarizing potentials during the SP-induced initial hyperpolarization.
11. The SP-induced depolarization was further studied with several ionic channel blockers. In TTX-containing media the depolarization was associated with an increase in the input resistance. In a TTX- and TEA-containing solution the depolarization was enhanced and accompanied by a small decrease in the input resistance. A further addition of Co to the TTX- and TEA-containing solution substantially reduced the SP-depolarization. These results suggest that the SP-depolarization might be a consequence of both a reduction in a voltage-dependent K-conductance and an increase in Na and/or Ca conductances.

12. SP modified the duration of the Ca spikes of dorsal horn neurons. The most consistent change was an initial dose-dependent and reversible decrease of the spike duration. The decrease in the spike duration was associated with a small reduction of the rate of the rise and the amplitude, and a significant parallel increase in dV/dt at the falling phase of the Ca spikes. The result of manual clamp experiments suggests that the initial decrease in the Ca spike duration is not due to the depolarizing action of SP.

13. These data are consistent with a possibility that SP shortens the duration of the Ca spike by decreasing the voltage-dependent Ca conductance and/or augmenting an outward K current(s), although shunting of the membrane either through pre- or postsynaptic mechanisms has not been ruled out.
14. Although the direct test of the interpretation is to be accomplished only with a voltage clamp analysis, the nature of the preparation does not seem to allow presently this study because of a probable dendritic origin of the Ca spikes and the very high input resistance of dorsal horn neurons.
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