Voltage sensitive Ca2+ conductances mediating modulatory effects of neuropeptides in rat spinal dorsal horn neurons

Pan Dong Ryu

Iowa State University

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Voltage sensitive Ca\(^{2+}\) conductances mediating modulatory effects of neuropeptides in rat spinal dorsal horn neurons

Ryu, Pan Dong, Ph.D.
Iowa State University, 1989
Voltage sensitive Ca$^{2+}$ conductances mediating modulatory
effects of neuropeptides in rat spinal
dorsal horn neurons

by

Pan Dong Ryu

An Abstract of
A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1989
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Voltage-sensitive Ca\(^{2+}\) currents in vertebrate sensory neurons

Voltage-sensitive Ca\(^{2+}\) currents in rat spinal dorsal horn neurons and the effect of substance P

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- Ba\(^{2+}\) dependence

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Comparison of time constants of inactivation

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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CGRP-LI</td>
<td>Calcitonin gene-related peptide like immunoreactivity</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current (d.c)</td>
</tr>
<tr>
<td>DH</td>
<td>Dorsal horn</td>
</tr>
<tr>
<td>DR</td>
<td>Dorsal root</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>E10</td>
<td>Embryonic day 10</td>
</tr>
<tr>
<td>e.p.s.p.</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>h.p.</td>
<td>Holding potential</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>I-V</td>
<td>Current-voltage</td>
</tr>
<tr>
<td>m</td>
<td>Mean</td>
</tr>
<tr>
<td>NKA</td>
<td>Neurokinin A</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>P10</td>
<td>Postnatal day 10</td>
</tr>
<tr>
<td>p.s.p.</td>
<td>Postsynaptic potential</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>SS</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>Vh</td>
<td>Holding potential</td>
</tr>
<tr>
<td>t.p.</td>
<td>Test potential</td>
</tr>
<tr>
<td>Vm</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
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</table>
GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation is written in an alternate thesis format as permitted by the Graduate College. It includes an experimental objective, a background and literature review, a rationale, an experimental part, a discussion, a summary, a list of references cited in the background and literature review and in the discussion. The experimental part has four sections. Section I corresponds to the manuscript submitted for publication in the Journal of Physiology, and Sections II, III and IV represent three research papers already published.

This dissertation contains a large part of the experimental results obtained by the author during his graduate study under the supervision of his major professor, Dr. Mirjana Randić. A portion of the results included in Section II was obtained by Dr. Garbor Gerber to whom some credit belongs.

Research Objective

The purpose of this research is to characterize the $\text{Ca}^{2+}$ conductances underlying the synaptic modulation of neuropeptides in the dorsal horn of rat spinal cord with an emphasis on the excitatory effects of calcitonin gene-related peptide. The properties of $\text{Ca}^{2+}$ currents in dorsal horn neurons and the effects of calcitonin gene-related peptide on the neuronal membrane and synaptic transmission were studied in spinal cord slice with
or without attached dorsal root ganglia by using intracellular recording and single microelectrode voltage-clamp techniques.

Background and Literature Review

In this section the sensory inputs from primary afferent fibers and the cytoarchitectonic organization of dorsal horn are briefly reviewed in order to provide a background for the study of voltage dependent $\text{Ca}^{2+}$ conductances and the actions of calcitonin gene-related peptide (CGRP) in dorsal horn of the rat spinal cord.

Structural and functional organization of the dorsal horn

**Sensory receptors and their primary afferent fibers**  Sensory neurons within dorsal root ganglia have been differentiated by their perikaryal size, duration of somal action potential, conduction velocity of nerve fiber, sensory modality and distribution of cellular organelles (Yoshida et al., 1978; Yoshida and Matsuda, 1979; Belmonte and Gallego, 1983; Rambourg et al., 1983; Harper and Lawson, 1985a,b; Rose et al., 1986; Traub and Mendell, 1988; Sugiura et al., 1988). In general large neurons (60-120 $\mu$m in diameter) have short-duration action potentials (0.49-1.35 ms at base), large-diameter myelinated axons with fast conduction velocity (14-55 m/sec), whereas small neurons (14-30 $\mu$m in diameter) have long-duration action potentials (0.5-8.0 ms at base), small-diameter unmyelinated or myelinated fibers with slow conduction velocities (< 8 m/sec). The axons in both cell types divide and give off two processes that distribute in opposite directions. The central branch enters the dorsal root and
terminate in the dorsal horn, while the peripheral branch supplies sensory receptors.

Primary afferent fibers (axons from dorsal root ganglion neurons) are classified on the basis of their conduction velocity which is related to the diameter of the nerve fibers. The afferent fibers from skin are alphabetically coded as: Aα, Aβ, Aδ and C. Aα and Aβ fibers are known to mediate non-nociceptive sensation, and Aδ and C fibers are known to have either nociceptive or non-nociceptive receptive fields (Perl, 1968; Light and Perl, 1979b; Martin, 1985). A different nomenclature has been used for the afferent from muscles: Group I (large myelinated), Group II (small myelinated), Group III (smaller myelinated) and Group IV (unmyelinated). Each group of fibers mediates the sensory information from one or more of sensory receptors. Table 1 shows receptor types active in various sensations (Martin, 1985). In table 2, the numerical systems for muscle afferents and the alphabetical systems for cutaneous afferents are compared, and the diameter and conduction velocity of each fiber group is given (Martin, 1985).

Cutaneous nociceptors and their primary afferents

Nociceptive information from skin reaches the spinal cord via small myelinated Aδ fibers and unmyelinated C fibers. The former distribute their terminal arborizations within lamina I (the marginal zone) and send collaterals to lamina V (Light and Perl, 1979b). The latter probably terminate within the substantia gelatinosa (lamina II), especially in outer portions of this dorsal horn region (Light and Perl, 1979a; Sugiura et al., 1986). Nociceptive neurons are preferentially, but not exclusively, located in or around the marginal zone (lamina I). A portion of lamina I neurons sends
Table 1. Receptor Types Active in Various Sensations

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

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

<table>
<thead>
<tr>
<th>Muscle nerve</th>
<th>Cutaneous nerve</th>
<th>Fiber diameter (μm)</th>
<th>Conduction velocity (m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>13-20</td>
<td>80-120</td>
</tr>
<tr>
<td>II</td>
<td>Aβ</td>
<td>6-12</td>
<td>35-75</td>
</tr>
<tr>
<td>III</td>
<td>Aδ</td>
<td>1-5</td>
<td>5-30</td>
</tr>
<tr>
<td>IVa</td>
<td>Ca</td>
<td>0.2-1.5</td>
<td>0.5-2</td>
</tr>
</tbody>
</table>

*Unmyelinated.
their axons to supraspinal regions (Cervero et al., 1979), notably through the spinoreticular and spinothalamic tracts (Willis and Coggeshall, 1978).

**Cutaneous thermoreceptors and their primary afferents**

Cutaneous thermoreceptors (cold and warm receptors) are connected to small Aδ and C fibers (Iggo, 1969). Little is known about the pattern of termination of identified thermoreceptors within the dorsal horn. Some of the neurons located in the superficial dorsal horn appear to be exclusively driven by thermoreceptors, particularly cold receptors ("cold units"), whereas other neurons in this region show convergence from thermo- and nociceptors (Christensen and Perl, 1970; Hellon and Misra, 1973).

**Muscle receptors and their primary afferents**

Muscle afferent fibers that convey proprioceptive information to the spinal cord include a group Ia axons (Aα) arising from the primary endings in muscle spindles, group II axons (Aβ) from secondary endings in muscle spindles, and Group Ib axons (Aα) from Golgi tendon organs. Group Ia axon collaterals terminate in laminae VI and VII and lamina IX (the motor neuronal pool); group II axon collaterals are found in laminae IV, V, VI, VII and group Ib axon collaterals terminate in a wide region between laminae V and VII (Brown, 1981).

Small myelinated fibers from muscle (Aδ or group III) connected to muscle nociceptors, provide afferent terminals to laminae I and V with a similar pattern of projection to that of the cutaneous mechanoreceptors connected to Aδ afferents (Mense et al., 1981). However, unlike the pattern of cutaneous C fibers, muscle C fibers appear to terminate within laminae I and V of the dorsal horn, but do not send projections to the substantia gelatinosa (Craig and Mense, 1983). Some neurons in laminae I
and V activated by fine muscle afferents (Group IV) send their axons through spinothalamic tract (Willis and Coggeshall, 1978). These neurons are supposed to be involved in the signalling and integration of muscle pain.

**Visceral receptors and their primary afferents**

Dorsal horn neurons in thoracic and sacral segments of spinal cord receive a projection from sympathetic and parasympathetic neurons. Although these visceral afferents represent a very small portion of the total afferent inflow to the spinal cord (Cervero et al., 1984), they can activate a large number of neurons in the spinal cord through extensive functional divergence (Cervero, 1983b). Anatomic studies have shown that visceral afferent fibers terminate in laminae I and V of the dorsal horn with little or no projection to laminae II, III and IV (Morgan et al., 1981; Cervero and Connell, 1984).

Dorsal horn neurons can be classified into two groups depending on the presence or absence of an excitatory visceral input. Some neurons are not driven by visceral afferent fibers and can only be excited from their somatic receptive fields (somatic neurons). Other neurons have, in addition to their somatic input, an excitatory visceral drive (viscerosomatic neurons) (Pomeranz et al., 1968; Guilbaud et al., 1977; Foreman and Ohata, 1980; Cervero, 1982, 1983a,b). Thus visceral sensation can only be mediated through convergent signal via somatosensory pathways. Somatic neurons are mainly located in laminae II, III and IV, whereas viscerosomatic neurons are located in laminae I and V, and in ventral horn (Cervero, 1986). The majority of somatic cells are mechanoceptive, whereas viscerosomatic neurons are driven by nociceptive Aδ and C fibers.
Viscerosomatic neurons project through pathways in the ventral funiculus of the cord, including the spinothalamic and spinoreticular tracts (Hancock et al., 1975; Foreman and Weber, 1980; Cervero, 1983b).

**Multireceptive neurons in the dorsal horn** Many neurons in the dorsal horn can be activated by several modalities of cutaneous sensory stimulation. These neurons are termed class 2, convergent, lamina 5 type, wide dynamic range, or multireceptive neurons. Multireceptive neurons receive convergent inputs from several categories of cutaneous receptors (i.e., mechanoreceptors and nociceptors) and in some cases from several peripheral organs (i.e., skin, muscle and viscera). They tend to be located in or around lamina V, but their presence has also been described in other dorsal horn laminae (Cervero, 1986). All ascending pathways from spinal cord contain a portion of axons from multireceptive neurons (Willis and Coggeshall, 1978).

**Cytoarchitecture of the dorsal horn: Rexed's scheme** Based on the study of the neuronal somata from Nissl-stained sections of spinal cord of adult cats and kittens, Rexed (1952, 1954) proposed the anatomic division of the spinal cord gray matter into ten regions or laminae oriented along the dorsal axis of spinal cord, designated by Roman numerals I to X (Fig. 1A). The upper six laminae (Laminae I to VI) make up the dorsal horn.

Rexed's scheme originally dealt with the cytoarchitectonic organization of spinal cord, that is, the shapes, sizes, densities and distribution of neuronal somata. For some time there has been a debate among investigators of the superficial dorsal horn whether lamina II of Rexed or both laminae II and III constitute substantia gelatinosa, the area near the apex of the dorsal horn that has a distinctly more gelatinous and
less fibrous texture than the rest of the gray matter. Following Rexed's (1952) work, many investigators argued that his laminae II and III were structurally similar and therefore should both be considered as substantia gelatinosa (Szentágothai, 1964; Wall, 1967; Scheibel and Scheibel, 1968; Kerr, 1975; Price and Dubner, 1977). However, the data obtained in the Golgi studies showing distinct structural differences between laminae II and III (Réthelyi and Szentágothai, 1969) as well as the reports of differential input of primary afferent fibers into laminae II and III probably justify their separate consideration (Light and Perl, 1979a,b; Ralston and Ralston, 1979; Cervero and Iggo, 1980). Later 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 synaptological organization in the spinal cord. Thus Rexed's scheme has now become an universally accepted nomenclature, extrapolated without objections from the cat to practically every mammal studied (Cervero, 1986). Some major conceptual generalizations on the characteristics of the neurons and organizations of fibers in each lamina have been established (Brown, 1981; Willis and Coggeshall, 1978, see also Molander et al., 1984).

Fig. 1 shows Rexed's scheme in the cat (Rexed 1952) and rat lumbar spinal cords (Paxinos and Watson, 1982) and a picture of the transverse spinal cord slice (L6) taken without staining.

**Lamina I: Marginal zone of Waldeyer** Lamina I, as defined by Rexed (1952), is the thinnest layer (12-20 μm, Hunt et al., 1980) forming the dorsal-most part and lateral side of the dorsal horn. The neuronal cell bodies in lamina I vary in size being small, medium and large. The
Fig. 1. Rexed's laminae of spinal cord gray matter. Diagrammatic representations of the cytoarchitectonic scheme of Rexed are shown in A (segment L 7 of the cat spinal cord, Rexed, 1952) and B (segment L 5 of the rat spinal cord, Paxinos and Watson, 1982). The ten layers of Rexed in the spinal cord are indicated by the numerals. A transverse slice of a 15-day-old rat spinal cord (300 μm, segment L 5) used in this work is shown in C. This is an unstained preparation, and white matter appears as a dark area surrounding the gray matter. Notice the transluscent area at the apex of dorsal horn.
large cells, called marginal cells (Ramon y Cajal, 1909; Gobel, 1978a), have been characterized the best. Although most of the cells are much smaller, measuring about 5x5 μm (Molander et al., 1984), little is known about the smaller cells.

Marginal neurons have horizontally-elongated cell bodies (30-50 x 10-15 μm, Rexed, 1952; 15x5 μm, Molander et al., 1984). Marginal cells send their dendrites out over the surface of the dorsal horn. Their dendritic domains resemble a flattened disc with elliptic elongation in the rostro-caudal direction (500-1400 μm, Light et al., 1979; 500-680 μm, Price et al., 1979) and confines in lamina I (Scheibel and Scheibel, 1968).

Many marginal cells have axons that project to distant sites. Retrograde horseradish peroxidase studies have demonstrated projections to the lateral cervical nucleus (Craig, 1978; Brown et al., 1980), the medullary and midbrain reticular formation and probably the dorsal column nuclei (Trevino, 1976; Molenaar and Kuypers, 1978, Swett et al., 1985), the thalamus (Trevino and Carstens, 1975, Willis et al., 1974; Carstens and Trevino, 1978, Giesler et al., 1979; Willis et al., 1979, Craig and Burton, 1981) and also to the cord a segment or two away from the cell body, including segment caudal to it (Burton and Loewy, 1976).

Lamina I is the major terminating site of small myelinated primary afferent (Aδ) fibers from skin (mechanical nociceptors) (LaMotte, 1977; Light and Perl, 1977a,b, 1979a,b; Grant et al., 1979; Ralston and Ralston, 1979, Sugiuara et al., 1986), which appear to form synaptic contacts primarily on fine dendrites of the marginal cells (Kerr, 1975). In addition to Aδ fibers, some small unmyelinated afferent (C) fibers (polymodal nociceptive and mechanical cold nociceptive) terminate in lamina
I (Sugiura et al., 1986). Muscle C-fibers (Group IV) (Craig and Mense, 1983) and visceral afferent fibers (Morgan et al., 1981; Cervero and Connell, 1984) are also known to terminate in lamina I. The other two inputs to lamina I are from the neurons of laminae II and III (Scheibel and Scheibel, 1968) and from the descending pathways originating from the raphe nuclei and the nucleus reticularis magnocellularis (LaMotte and de Lanerolle, 1983). These descending pathways lie in the dorso-lateral funiculus and appear to be inhibitory (Basbaum et al., 1978; Martin et al., 1978).

**Lamina II: Substantia gelatinosa of Rolando**

Lamina II is equated by Rexed (1952, 1954) to the substantia gelatinosa of Rolando (1824, quoted from Ramon y Cajal, 1909). It is well characterized by its pale appearance due to the absence of myelinated fibers and tightly packed small cells (5x5 to 10x10 μm, Rexed, 1952). It lies across the dorsal horn ventral to lamina I and its lateral edge runs about half-way down the dorsal horn along the lateral part of the lamina I (Fig. 1). Lamina II is subdivided into two layers, an outer region (lamina IIo: 30-40 μm thick) and an inner region (lamina I: 40-50 μm thick), on the basis of the dendritic arborizations and axonal projections of its neurons and afferent inputs to it (Rexed, 1952; Beal and Cooper, 1978; Gobel, 1979, Ralston and Ralston, 1979).

Two types of neurons in lamina II, stalked cells and islet cells, are well characterized (Beal and Cooper, 1978; Price et al., 1979). The stalked cells (border cells or limiting cells) are larger and mostly found along outer edge of lamina II (lamina IIo). Their dendritic domains are cone-shaped with the apex at the soma and directed ventrally. The
dendrites are confined within lamina IIo or extend to the outer part of lamina III. Occasionally, the dendrites enter lamina I, and the axons primarily project into lamina I (Sugiura, 1975; Gobel, 1975, 1978b; Beal and Cooper, 1978).

Islet cells are predominantly found in inner layer of lamina II (lamina IIIi) and are smaller in size (5x5 - 10x10 μm). Their dendritic trees are oriented longitudinally, somewhat flattened mediolaterally and confined primarily to laminae II and III (Scheibel and Scheibel, 1968; Sugiura, 1975).

Unmyelinated primary afferent (C) fibers terminate predominantly in lamina II (LaMotte, 1977; Réthelyi, 1977; Ralston and Ralston, 1979) while small myelinated (Aδ) fibers terminate in lamina IIo as well as lamina I (Light and Perl, 1977a,b, 1979a,b). Lamina II also receives sensory input from somatic neurons in viscera (Cervero, 1986) but very few input from large myelinated fibers. Descending fibers from the raphe nuclei (Ruda et al., 1982; LaMotte and de Lanerolle, 1983) and locus ceruleus (Fuxe, 1965; Nygren and Olson, 1977; Satoh et al., 1982) heavily terminate in lamina II.

The most prominent structures in neuropil of lamina II are the glomeruli which make up approximately 5% of the synapses in laminae I-III (Willis and Coggeshall, 1978). Each glomerulus consists of a central relatively dark axonal terminal and a group of peripheral processes, most of which are in synaptic contact with central terminal, bouton "en passant" (Ralston, 1971; Knyihar and Gerebtzoff, 1973). Kerr (1975) regards the central terminal as arising from a primary afferent, but other investigators consider from a large pyramidal neurons between laminae III-IV as a more likely source (Réthelyi and Szentágothai, 1969, 1973). For
the synaptic contacts of peripheral processes axo-dendritic, axo-axonic, dendro-dendritic and dendro-axonic synapses are proposed by various investigators (see Willis and Coggeshall, 1978).

**Lamina III** Lamina III is a broad band across the dorsal horn (Fig. 1). It borders on the white matter medially, but laterally it is covered by laminae I and II. The border between lamina II and lamina III can be defined by the occurrence of transversely cut myelinated fibers in lamina III (Snyder, 1982; Molander et al., 1984). The nerve cells in this lamina are spindle shaped, slightly larger (7-9 x 10-12 μm) and less densely packed than those in lamina II. The dendritic pattern of lamina III cells is similar to that of lamina II cells, although it tends to be larger (Scheibel and Scheibel, 1968). The axonal projections are similar to those of lamina II neurons that would be regarded as propriospinal in that they return to the substantia gelatinosa after they leave the white matter, or they never leave the substantia gelatinosa and enter directly into synaptic relations with the neurons there (Ramon y Cajal, 1909, Szentágothai, 1964; Scheibel and Scheibel, 1968). However, some neurons in lamina III have very extensive dendritic trees that ramify in laminae I-IV, and some axons travel in the spinocervical tract of the dorsal column (Mannen and Sugita, 1976; Brown, 1981).

**Lamina IV** Lamina IV is thicker than previous laminae. It extends from the white matter of the dorsal columns medially to where the ventral bend of laminae I-III runs down the lateral side of the dorsal horn laterally. It is the first layer not to show the lateral bend (Fig. 1). The distribution of cells is less dense than in lamina III due to the increasing numbers of nerve fibers that pass through and into it (from
below as well as from above) and to the increased variability in cell size. Cell bodies are rounded, triangular or star-shaped, the different shapes being associated with small, medium and large cells respectively. Cells range from 7-9 µm x 10-12 µm up to about 35x45 µm. The very large cells are conspicuous because of their size but they are few in number (Rexed, 1952; Brown, 1981). These cells have long, spine-studded dendrites that pass dorsally, laterally, and ventrally and an axon that passes to the lateral white matter (Ramon y Cajal, 1909). The dorsal dendrites of the large cells in lamina IV penetrate the substantia gelatinosa, thus allowing axons from the central cells of the gelatinosa to contact the dendrites (Szentágothai, 1964).

Cells in lamina IV belong to the spinocervical system, the spinothalamic system, or the propriospinal systems (Szentágothai, 1964; Matsushita, 1969, 1970; Réthelyi and Szentágothai, 1973; Willis and Coggeshall, 1978).

The synaptic architecture of lamina IV is quite different from that of laminae I-III, but it is indistinguishable from that of laminae V and VI (Ralston, 1968, 1971; Kerr, 1970, 1975). The synapses in lamina IV are axo-somatic, axo-dendritic and axo-axonal (Ralston, 1968; Kerr, 1970). The axo-somatic synapses, which are almost never seen in laminae II and III, are moderately common in the lamina IV, and the glomeruli that characterize laminae I-III are not present (Ralston, 1968; Kerr, 1970).

**Lamina V** Lamina V extends as a rather thick band across the narrowest part of the dorsal horn (Fig. 1). It occupies the zone often called the neck of the dorsal horn. It has a sharp boundary on the dorsal funiculus, but its lateral boundary is indistinct because of the many
bundles of myelinated fibers coursing through this area. Lamina V can be subdivided into a medial and a lateral zone, the lateral zone usually occupies about one-third of the lamina. The medial zone does not have as many myelinated fibers, so its appearance is more uniform than that of the lateral part (Willis and Coggeshall, 1978).

Lamina V can be distinguished cytoarchitectonically from lamina IV because the cells are even more varying in size and shape than the lamina IV cells. The sizes of cell bodies range from the smallest (8-10 μm) to the largest (30-45 μm). However, most of the cells fall in the range of 10-13 x 15-20 μm) (Rexed, 1952).

In general, the dendritic patterns and the neuropil of neurons in lamina V do not differ greatly from those of lamina IV (Ramon y Cajal, 1909). However, Scheibel and Scheibel (1968) described the dendritic shapes of the cells in Lamina V as flattened disks, which contrast strongly with the longitudinal orientation of most of the neurons in lamina IV.

Lamina V receives primary afferent inputs from small myelinated (Aδ) fibers from skin (Light and Perl, 1979b), group IV fibers from muscle (Craig and Mense, 1983) and visceral afferent fibers (Morgan et al., 1981; Cervero and Connell, 1984). Axonal projection and axonal organization of the cells in lamina V are similar to those in lamina IV. Lamina V neurons project to the thalamus, the lateral cervical nucleus, and the gray matter of the spinal cord (Willis and Coggeshall, 1978).

**Neurotransmitter systems in the dorsal horn**

The dorsal horn of the spinal cord is endowed with neural structures rich in a variety of neurotransmitters and neuromodulators, including
Table 3. Identified neuropeptides in central and peripheral neurons that originate in, or project to, the dorsal horn of the spinal cord

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cellular origin</th>
<th>Dorsal horn Termination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Primary sensory neurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>-20% of small-diameter dorsal ganglion neurons</td>
<td>Laminae I,II &amp; V</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide</td>
<td>most of small- and some medium-sized sensory neurons</td>
<td>Laminae I-III, V &amp; X</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>-5-10% of small-diameter dorsal root ganglion neurons</td>
<td>Laminae I &amp; II</td>
</tr>
<tr>
<td>Cholecystokinin-like</td>
<td>Small-diameter dorsal root ganglion neurons (some may also contain substance P)</td>
<td>Laminae I &amp; II</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide</td>
<td>Scattered small cells in sensory ganglion</td>
<td>Lamina I</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>some sensory neurons</td>
<td>Lamina II</td>
</tr>
<tr>
<td>Dynorphin</td>
<td>Unidentified cells in dorsal root ganglia</td>
<td>Present in dorsal horn</td>
</tr>
<tr>
<td>Met-Tyr-Lys</td>
<td>Unidentified cells in dorsal root ganglia</td>
<td>not known</td>
</tr>
<tr>
<td><strong>II. Dorsal horn interneurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enkephalins</td>
<td>Laminae I-III</td>
<td>Laminae I,III &amp; V</td>
</tr>
<tr>
<td>Substance P</td>
<td>Laminae I-III</td>
<td>Laminae I,II &amp; V</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Lamina III</td>
<td>Lamina I &amp; II</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Lamina II</td>
<td>Laminae I &amp; II</td>
</tr>
<tr>
<td>Avian pancreatic polypeptide</td>
<td>Laminae I &amp; III</td>
<td>Laminae I &amp; II</td>
</tr>
<tr>
<td>Gastrin releasing peptide/bombesin</td>
<td>Laminae I &amp; II</td>
<td>Laminae I &amp; II</td>
</tr>
<tr>
<td><strong>III. Supraspinal origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>Midline brain stem nuclei</td>
<td>Lamina I, II?</td>
</tr>
<tr>
<td>Thyrotropin releasing hormone</td>
<td>Brain stem nuclei</td>
<td>Little, if any</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>Brain stem nuclei</td>
<td>not known</td>
</tr>
<tr>
<td>Cholecystokinin-like</td>
<td>Brain stem</td>
<td>Lamina I, II?</td>
</tr>
<tr>
<td>Oxytocin/vasopressin</td>
<td>Paraventricular nucleus</td>
<td>Lamina I</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Paraventricular nucleus</td>
<td>Lamina I</td>
</tr>
</tbody>
</table>
excitatory and inhibitory amino acids, catecholamines, serotonin and peptides. Many of these transmitters are concentrated in the superficial dorsal horn (laminae I-III). Three major sources of chemical mediators in the dorsal horn are primary afferents, spinal interneurons and descending systems. Table 3 shows the distribution of some neuropeptides in central and peripheral neurons that originate in, or project to, the dorsal horn of the spinal cord (Jessell, 1983; Gibson et al., 1984; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985b).

**Neurotransmitters from primary afferent fibers** Primary afferents are an important sources of excitatory amino acids, L-glutamate and L-aspartate. In addition a number of neuroactive peptides such as substance P (SP), neurokinin A (NKA), somatostatin (SS), cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and galanin derive mostly from the primary sensory neurons. The heavy immunohistochemical reactivity of these peptides in the superficial laminae of the spinal dorsal horn reflects their predominant origin from the small diameter primary afferent fibers. Indeed the presence of SP (Hökfelt et al., 1975b), SS (Hökfelt et al., 1975a), CCK (Dalsgaard et al., 1982), VIP (Lundberg et al., 1978) and CGRP (Gibson et al., 1984) in dorsal root ganglion neurons has been established by immunohistochemical methods. Dorsal root rhizotomy results in a significant reduction in the number of peptide-immunoreactive fibers and the immunoreactivities of these peptides in the superficial dorsal horn (Hökfelt et al., 1975b; Yaksh et al., 1982; Schröder, 1984). Capsaicin (8-methyl-N-vanillyl-6-nonenamide), when given neonatally to rats, causes a degeneration of 95% of unmyelinated primary afferent C fibers and at higher doses of about 30-40% of small myelinated
A5 fibers (Jansco et al., 1977). Cytochemical studies, in addition, have revealed a marked depletion of SP, CCK, CGRP and VIP (Jansco et al., 1981; Nagy et al., 1981; Franco-Cereceda et al., 1987) in primary sensory neurons and a partial loss in dorsal horn neurons. Although amino acids, L-glutamate and L-aspartate, were thought to be released as neurotransmitters during activation of large primary afferent fibers (Johnson and Aprison, 1970; Salt and Hill, 1983), the recent findings indicate that these substances may be the synaptic mediators of small afferent fibers (Cangro et al., 1985; Schneider and Perl, 1985, 1988).

Possible neuromodulator roles of the sensory peptides in the dorsal horn have been investigated by the Randić's group with an emphasis on SP and CGRP (Urban and Randić, 1983; Murase and Randić, 1984; Urban and Randić, 1984; Urban et al., 1985; Randić et al., 1986; Randić et al., 1987; Ryu et al., 1988a,b).

Single, low intensity electrical stimulus applied to the dorsal root (DR) elicits a fast excitatory postsynaptic potential in most dorsal horn neurons in a slice preparation of the rat spinal cord. Increasing the intensity and frequency of the DR stimulation elicits in about half of dorsal horn neurons a second type of excitatory synaptic potential that is slow in onset and of long duration. Similar slow depolarization can be produced by the application of SP to those dorsal horn neurons that exhibit a slow synaptically mediated depolarization (Urban and Randić, 1983; Murase and Randić, 1984; Urban and Randić, 1984; Randić et al., 1987). Synthetic analogues of SP with antagonistic properties block the slow depolarization of the dorsal horn neurons evoked either by DR stimulation or SP application (Randić et al., 1986), and after destruction of small diameter
primary afferent fibers by neonatal treatment of rats with capsaicin (Urban et al., 1985). These results suggest that SP, or a related tachykinin, is a strong candidate for the slow excitatory transmitter that is released upon activation of high threshold primary fibers. SP coexists with glutamate, the latter being the major neurotransmitter candidate for the fast excitatory synaptic potential (Schneider and Perl, 1985, 1988), in the terminals of primary afferent fibers in the superficial laminae of dorsal horn (DeBiasi and Rustioni, 1988) and in some primary sensory neurons (Battaglia et al., 1987).

**Calcitonin gene-related peptide (CGRP)**

CGRP is a 37 amino acid peptide formed in neural tissue by alternative splicing of mRNA of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). Immunocytochemical studies demonstrated wide distribution of the peptide within the peripheral and central nervous system (Gibson et al., 1984; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985b; Franco-Cereceda et al., 1987). Although in the spinal cord CGRP-immunoreactive fibers and terminals are present at all levels of the spinal cord, they were most numerous in the dorsal horn, where concentrated in Lissauer's tract and laminae I-III, V and X.

The presence of CGRP-like immunoreactivity (CGRP-LI) in primary sensory neurons was established by immunohistochemistry and radioimmunoassay in combination with high performance liquid chromatography (Gibson et al., 1984; Skofitsch and Jacobowitz, 1985b; Franco-Cereceda et al., 1987). In the dorsal root ganglia CGRP immunoreactivity was observed in most of the small and some of the intermediate sized cells. Substance P (SP) immunoreactivity, where present, co-existed with CGRP in a certain
proportion of the small cells. Dorsal rhizotomy caused a significant loss of CGRP-immunoreactive fibers from the dorsal horn of the spinal cord. On the basis of these results Gibson et al. (1984) suggested that the major origin of CGRP in the dorsal spinal cord is from primary afferent fibers deriving from the dorsal root ganglion cells. After capsaicin treatment the content of CGRP-LI was reduced by 60% in the dorsal part of the spinal cord (Franco-Cereceda et al., 1987). Thus the distribution of CGRP suggests a role in sensory transmission, and central administration of CGRP has recently been shown to have an antinociceptive effect (Bates et al., 1984). Using synaptosomal membrane preparations, high affinity binding sites for CGRP have been demonstrated in rat and human CNS (Rizzo and Goltzman, 1981; Tschopp et al., 1984; Goltzman and Mitchell, 1985; Tschopp et al., 1985). Release of CGRP-like immunoreactivity was demonstrated from cultured rat trigeminal ganglion cells (Mason et al., 1984) and also in response to capsaicin and high K⁺ from the slices of the rat spinal cord (Saria et al., 1986; Oku et al., 1987).

CGRP and SP coexist frequently in dorsal horn and in dorsal root ganglion neurons. Both peptides have excitatory effects on dorsal horn neurons (Randić and Miletić, 1977; Murase and Randić, 1984; Ryu et al., 1988a,b) and increase voltage dependent Ca²⁺ current (Murase et al., 1986, 1989; Ryu et al., 1988a). However, their time course of action is quite different. The depolarizing effect of CGRP occurred 10-15 min after the onset of the bath application of the peptide and the effect frequently outlasted the application for 30-60 min (Ryu et al., 1988b). In contrast, the excitatory effect of SP was usually observed within 1 min of bath application and recovered within 10 min of washout period (Murase and
Randić, 1984; Randić et al., 1987; Ryu et al., 1988b). Similar effect of CGRP was observed in the cat dorsal horn neurons in vivo (Miletić and Tan, 1988). The slow and prolonged action of CGRP suggests a novel type of neuromodulatory role for neuropeptides, one probably related to spinal sensory integration processes.

Neurotransmitters from spinal interneurons Although primary afferents represent a major source for SP, CCK and SS present in the dorsal horn, there are also contributions from the intrinsic neurons of the dorsal horn. SP-positive neurons (Gibson et al., 1981) and CCK-positive neurons have been found in laminae II - V (Schreder, 1983). SS-positive neurons are also present in laminae I - V (Dalsgaard et al., 1981; Hunt et al., 1981; Schröder, 1984).

Other neuroactive agents that primarily originate from intrinsic neurons include inhibitory amino acids (glycine, GABA) and peptides, enkephalin and neurotensin (LaMotte, 1986). Glycine-positive neurons appear to be located in lamina III (Aprison et al., 1969), whereas GABA-positive neurons are located more superficially. The heavy immunoreactivity of glutamate decarboxylase (GAD), a GABA synthesizing enzyme, was also reported in laminae I-III (McLaughlin et al., 1975; Barber et al., 1982). Enkephalin-positive interneurons are present in laminae I through V (Gibson et al., 1981; Glazer and Basbaum, 1981; Sumal et al., 1982). Neurotensin-positive cells and terminals have been found in laminae I - III of monkey, cat and rat with some species variation in the density of distribution within those laminae (Seybold and Elde, 1982, DiFiglia et al., 1984).
Neurotransmitters from descending fibers

Several neuropeptides present in the spinal dorsal horn have been identified in terminals and axons arising from descending fibers. SP-positive neurons in rat medullary raphe nuclei have been found to project to the spinal cord, with the ventral horn receiving a heavier projection than the dorsal horn (Bowker et al., 1981). These terminals may also contain serotonin, since SP and serotonin coexist in some cells of the raphe nuclei. CCK may coexist with SP in some terminals, including primary afferent (Dalsgaard et al., 1982) and descending fibers (Maciewicz et al., 1984). It is also known that small portions of SS or enkephalin in spinal cord originate from descending fibers (Bowker et al., 1981; Schröder, 1984).

Some neuromediators present in the spinal cord originate only from descending pathways. Those are serotonin, norepinephrine, and the peptides oxytocin and neurophysin. Serotonin terminals originate from the raphe nuclei of the midbrain and are present in the high density in laminae I, IIo IV and V (Fuxe, 1965; Ruda et al., 1982; LaMotte and de Lanerolle, 1983). Norepinephrine-containing terminals are found mostly in lamina II; they arise from the locus ceruleus. Dopamine-positive terminals originate from the periventricular region of the diencephalon (Anden et al., 1966; Bjorklund and Skagerberg, 1979). Immunopositive axons for oxytocin and neurophysin are found in the dorsal part of the lateral funiculus, and terminals in laminae I and X, and intermediolateral nucleus (Swanson and McKellar, 1979). These axons probably originate from the neurosecretory cell groups of the periventricular region of the hypothalamus (Hancock, 1976; Pittman et al., 1984).
Early-postnatal development of dorsal horn neurons

During early development of dorsal horn neurons there are two maturation periods, which involve two separate groups of dorsal horn neurons. Golgi type I projection neurons essentially mature prenatally while Golgi type II interneurons mature postnatally, and make up the largest portion of the neurons of the superficial dorsal horn (Bicknell and Beal, 1982; Beal and Bicknell, 1984). Similarly axonal and dendritic development of the Golgi-type-I projection and propriospinal neurons in laminae II and III occurs mostly before birth and attains adult appearances by postnatal day 10 (P10). During the early postnatal period, the Golgi-type-I projection and propriospinal neurons continue to increase in size and exhibit further dendritic branching. However, Golgi type II interneurons mature mostly during early postnatal days and attain adult morphology by P15 (lamina III) or P20 (lamina II) (Bicknell and Beal, 1984; Beal et al., 1988).

Maturation of synaptic glomeruli, the key structures in the synaptic organization of the neuropil of the dorsal horn neurons, occurs during early postnatal days. At P10, some varicosities and some central terminals of glomeruli contain neurofilament bundles. Peripheral axons and axo-axonic synapses appear for the first time. At P15, all glomeruli could be recognized as centered by their terminals, with or without neurofilaments, and lamina III became twice as thick as lamina IIo (at birth, the thickness of lamina III is half of lamina IIo) (Pignatelli et al., 1985).

The maturation of small dorsal root ganglion neurons, properties of action potentials and conduction velocities of C and Aδ fibers seem to be completed by P10 (Lawson, 1979; Bicknell and Beal, 1982; Fulton, 1987). C
fibers grow into dorsal horn later than A fibers, just before birth (Fitzgerald, 1987), and C fiber type terminals and glomeruli are not seen in substantia gelatinosa until P2 (Pignatelli et al., 1985). As the C fibers grow into substantia gelatinosa, their target interneurons are only beginning to mature, in contrast to the prenatal maturation of projection neurons (Bicknell and Beal, 1984). The perikaryal size and the spike duration of the large dorsal root ganglion neurons undergo changes during early postnatal days and reach the plateau level by P20 (Lawson, 1979; Fulton, 1987).

Some peptides and their receptors appear as early as embryonic day 15 (E15). SP-, CGRP- and SS-positive neurons were detected from E15-16, and their appearance precedes that of enkephalin and neurotensin (Senba et al., 1982; Gledic et al., 1988). SP-binding sites are wide-spread in the neonatal cord and only become specifically localized at P15 in the spinal dorsal horn (Charlton and Helke, 1986; see also Fitzgerald and Gibson, 1984).

**Voltage-sensitive Ca$^{2+}$ currents in vertebrate sensory neurons**

Ca$^{2+}$ channels control two important neuronal functions, neuronal excitability and transmitter release. Thus there has been great interest in the existence and properties of different types of Ca$^{2+}$ channels in neuronal tissue (Hagiwara and Byerly, 1981; Miller, 1987a,b; Tsien et al., 1988).

In vertebrate neurons, the existence of two types of Ca$^{2+}$ conductances, a low- and a high-threshold, was predicted on the basis of voltage recordings from cerebellar Purkinje cell (Llinas and Sugimori,
1980), inferior olivary neurons (Llinas and Yarom, 1981) and spinal dorsal horn neurons (Murase and Randić, 1982, 1983). Voltage-sensitive Ca$^{2+}$ currents have been characterized the best in cultured DRG neurons. In cultured sensory neurons, voltage-clamp studies demonstrated the existence of either two (Carbone and Lux 1984; Bossu et al., 1985; Fedulova et al., 1985) or three types (Nowycky et al., 1985) of Ca$^{2+}$ channels that exhibit different localization, kinetic, pharmacological and functional properties (Perney et al., 1986; Fox et al., 1987a,b; Gross and Macdonald, 1987; Huang, 1988; Madison et al., 1987; Miller, 1987a,b; Huguenard et al., 1988; Lipscombe et al., 1988; Tsien et al., 1988).

The three components of Ca$^{2+}$ currents, so-called, T (low-threshold or low-voltage-activated), N (high-threshold transient or high-voltage-activated transient) and L (high-threshold sustained or high-voltage-activated sustained) components, were distinguished on the basis of their kinetic and pharmacological properties of whole-cell macroscopic currents (Nowycky et al., 1985; Fox et al., 1987a) and also single-channel currents (Fox et al., 1987b). Unitary current recordings in cell-attached patches provide strong evidence for the co-existence of three types of voltage-activated Ca$^{2+}$ channels on the cell bodies of DRG neurons (Nowycky et al., 1985; Fox et al., 1987b). They are complementary to and mutually consistent with the data obtained from whole-cell recording.

As shown by Fox et al. (1987a, b), component L activates at relatively positive test potentials (t.p. > -10 mV) and shows little inactivation during a 200 ms depolarization. It is completely reprimed at a holding potential (h.p.) of -60 mV, and can be isolated by using a more depolarized h.p. (-40 mV) to inactivate the other two types of Ca$^{2+}$ currents. Single
L-type channels (in isotonic 110 mM Ba\textsuperscript{2+}) are distinguished by a large unitary slope conductance of 25pS, activation over the range of membrane potentials (Vm) between 0 and +40 mV, little inactivation over the course of a 136 ms depolarization, and availability for opening even at depolarized h.p. ( > -40 mV).

Component T can be seen in isolation with weak test pulses. It begins activating at potentials more positive than -70 mV and inactivates and completely during a maintained depolarization (time constant, 20-50ms). The current amplitude and the rate of decay increase with stronger depolarizations until both reach a maximum at approximately -40mV. Inactivation is complete at h.p. > -60 mV and is progressively removed between -60 and -95 mV. Single T-type channels (in isotonic 110 mM Ba\textsuperscript{2+}) are distinguished by a smaller unitary slope conductance (8 pS) and by activation and inactivation over relatively negative ranges of potential. Inactivation is complete by the end of 136 ms pulses to t.p. beyond -20 mV.

Component N activates at relatively strong depolarizations (t.p. > -20 mV) and decays with time constants ranging from 50 to 110 ms. Inactivation is removed over a wide range of holding potentials (between -40 and -95 mV). Single N-type channels (in isotonic 110 mM Ba\textsuperscript{2+}) are distinguished by an intermediate unitary slope conductance (13 pS), and by activation over a broad range of h.p. (-80 to -20 mV).

L type Ca\textsuperscript{2+} current component of both macroscopic and single channel currents is preferentially modulated by dihydropyridine Ca\textsuperscript{2+} channel agonist Bay K 8644 and antagonist nifedipine. T type Ca\textsuperscript{2+} currents are selectively sensitive to nickel ions and resistant to cadmium ions (20-50 \mu M) if compared with the L and N components.
Although a question has been raised regarding the presence of three types of Ca\textsuperscript{2+} channel currents (Swandulla and Armstrong, 1988), the results obtained from the analysis of unitary (Fox et al., 1987b; Hirning et al., 1988) and macroscopic Ca\textsuperscript{2+} currents (Fox et al., 1987a), the selective modulation of the three types of Ca\textsuperscript{2+} currents by neurotransmitters (Holz et al., 1986; Gross and Macdonald, 1987; Lipscombe and Tsien, 1987; Madison et al., 1987; Wanke et al., 1988; Anderson and Dunlap, 1988) and the selective involvement of the three components in the release of neurotransmitters (Perney et al., 1986; Rane et al., 1987; Hirning et al., 1988; Lundy et al., 1988) support the concept of the existence of three separate components of Ca\textsuperscript{2+} currents.

Voltage sensitive Ca\textsuperscript{2+} currents in rat spinal dorsal horn neurons and the effect of substance P

Spinal dorsal horn, especially laminae I-III, is the region where the processing and integration of sensory information from skin, muscle and viscera take place. This region has a highly developed synaptic organization (glomeruli) and multiple neurotransmitter systems arising from primary afferent fibers, descending fibers and spinal interneurons.

A number of putative neurotransmitter and neuromodulator substances, including peptides, have been found in high density in this region of the spinal cord and their functional role in afferent signal processing has been postulated. Although analysis of actions of neuroactive substances necessitates information about membrane currents, a limited amount of information for mammalian spinal dorsal horn neurons is available at present. In particular it would be important to learn more about the
presence of Ca\(^{2+}\) conductances in the rat dorsal horn neurons since neuronal Ca\(^{2+}\) channels are frequent targets for modulation by neurotransmitters and drugs. Distinguishing between multiple types of Ca\(^{2+}\) conductances is important for understanding their functional role both in electrophysiological (calcium spikes, bursting firing pattern) and chemical signalling (transmitter release, regulation of ion channel gating, cellular metabolism).

In immature rat spinal dorsal horn neurons, the presence of multiple types of Ca\(^{2+}\) currents was indicated by voltage (Murase and Randić, 1982, 1983) and current recordings (Huang, 1988). In voltage-clamped rat spinal dorsal horn neurons Dr. Randić and her colleagues described the presence of a Ca\(^{2+}\)-sensitive slow inward relaxation activated at membrane potentials close to the resting level and inward tail currents with repolarization to the holding potential (Murase et al., 1986). The slow relaxation can be recorded when dorsal horn neurons were held at the resting level (-65 to -70 mV) and subjected to 1 sec depolarizing commands to membrane potential between -55 to -35 mV.

When dorsal horn neurons were voltage-clamped at resting level, bath application of SP or NKA (10\(^{-7}\) to 10\(^{-5}\) M for 1 to 3 min) induced an inward shift of holding current lasting several minutes. The magnitude of this effect varied between 10 and 400 pA depending upon the concentration the peptides and the holding potential. During the tachykinin-induced inward shift in the holding current the inward relaxation and the tail current were augmented in a dose-dependent manner. The SP-induced augmentation of the slow inward relaxation and the tail current is likely to be due to the enhancement of the activation of the Ca\(^{2+}\) current, because the effects were
present, and even augmented in a zero-$\text{Ca}^{2+}$, $\text{Ba}^{2+}$-containing solution, it was reduced or completely abolished by zero-$\text{Ca}^{2+}$, $\text{Co}^{2+}$ or $\text{Mg}^{2+}$ containing solutions, and was largely independent of the changes in external $\text{Na}^+$, $\text{K}^+$ or $\text{Cl}^-$ ions (Murase et al., 1989). Moreover, in the presence of the $\text{K}^+$ channel blocker, tetraethylammonium (TEA), the effect is increased. We think that the enhancement of this current by SP may significantly contribute to generation of the SP-induced depolarization at membrane potentials more positive to $-60$ mV (Murase et al., 1986, 1987, 1989).

In addition to the inward current at potentials positive to $-60$ mV, SP induced an inward current at more hyperpolarized potentials, at which level the inward $\text{Ca}^{2+}$ current (Brown and Griffith, 1983b; Segal and Barker, 1986; Murase et al., 1986, 1987, 1989), $\text{Ca}^{2+}$ activated outward current and voltage-gated potassium current (Brown and Griffith, 1983a; Segal and Barker, 1986), and also the M-current (Adams et al., 1982, Murase et al., 1986) are not activated and therefore would have no effect. In addition SP did not change inward (anomalous) rectifier current, termed $I_Q$ (Halliwell and Adams, 1982; Mayer and Westbrook, 1983; Crepel and Penit-Soria, 1986) in rat dorsal horn neurons in difference to the depressant action reported in the cultured rat globus pallidus neurons (Stanfield et al., 1985).

The inward current at hyperpolarized potentials is frequently accompanied by a small but measurable increase in membrane conductance and appears to depend linearly on membrane potential. The inward current is reduced about half by lowering external sodium, the result suggesting that the current is in large part carried by sodium ions. Unlike the fast voltage-dependent sodium conductance that is responsible for Na-dependent action potentials, this current is not blocked by TTX. However, the
current is significantly reduced in a zero-Ca\textsuperscript{2+}, Co\textsuperscript{2+}-containing solution, the finding suggesting that it is a Ca\textsuperscript{2+}-sensitive current. Furthermore, substitution of Ba\textsuperscript{2+} for Ca\textsuperscript{2+} caused a decrease in the current, as expected for a Ca\textsuperscript{2+}-activated current. Thus ionic basis of this current resembles the properties of a calcium- and voltage-dependent nonspecific cationic conductance (Yellen, 1982; Swandulla and Lux, 1985; Zucker and Kramer, 1986). Non-selective, Ca\textsuperscript{2+}-activated cation channels, have been found in several cells including Helix (Swandulla and Lux, 1985) and Aplysia neurons (Zucker and Kramer, 1986). Since the current is relatively small in magnitude a more detailed analysis of the ionic basis of this conductance, including direct measurements of the reversal current, and its contribution to the SP-induced depolarization remains to be examined in the isolated rat dorsal horn neurons using whole-cell voltage or patch-clamp techniques as studied in neuroblastoma cells (Yellen, 1982).

Rationale

The neurons in the superficial dorsal horn of the spinal cord have for a long time resisted electrophysiological analysis because of the small size of the cells and the technical difficulties in obtaining the stable intracellular recordings in vivo. The development of slice preparation of the rat spinal cord in Dr. Randić’s laboratory allowed us to study the active and passive membrane properties of the small dorsal horn neurons and analyze the cellular mechanisms of action of a number of putative neuromediators present in the superficial dorsal horn. The slice preparation is technically relatively easy to use, and it is free from the
pulsations of heart beat and respiration. It allows investigators better visual access than the in vivo preparations, and application and removal of drugs is easily controlled. It preserves relatively well the local neuronal-environment, the latter is lost or drastically altered in cell- or organ-culture preparations. A particular advantage of the slice preparation is that the ionic microenvironment is easily changed allowing differentiation between direct and synaptically-mediated events (by use of TTX or low Ca$^{2+}$/high Mg$^{2+}$ solution) as well as determination of ionic basis of various voltage- and time-dependent membrane conductances and transmitter-specific mechanisms.

One important method for the investigation of voltage-dependent conductances of neuronal membrane has been the voltage-clamp technique. The membrane of dorsal horn neurons has several voltage- and time-dependent conductances and other methods such as constant current and single voltage recording techniques are inadequate for correct interpretation of experimental data. The voltage-clamp technique can electrically control the membrane potential of the cell and thus allows experimenter to set the membrane potential at a certain level and maintain that level despite the changes in membrane conductance that would tend to bring the membrane potential toward a new equilibrium level. From the relation between membrane current (I), conductance (g) and potential (V) i.e., $I = gV$, it is apparent that if the V is known and constant, and the I is determined by measuring the current necessary to hold the membrane potential at a given value, one can obtain measurement of the total conductance of the membrane as a function of voltage and time. To determine what portion of the total current and conductance change are due
to Na⁺, K⁺ or Ca²⁺ ions, it will be necessary to change (remove or elevate) their concentrations or replace an ion with another ion that cannot cross the membrane and repeat the measurement. In this work single-electrode (sample-and-hold) voltage-clamp method was used because the dorsal horn neurons are small and cannot be impaled with two microelectrodes as it is commonly done using the conventional voltage clamp technique in invertebrate neurons. Although sometimes there is difficulty in space-clamping the long neuronal processes, the single electrode voltage-clamp technique is presently the best available method for the analysis of the ionic conductances in the small dorsal horn neurons in the slice preparation (see Appendix for the basic principles of operation of the single-electrode voltage-clamp technique).

The spinal dorsal horn is one of the areas where primary afferent fibers terminate and the cutaneous sensory information is processed. A number of putative neurotransmitter and neuromodulator substances, including SP and CGRP have been found in high density in this region of the spinal cord, and their functional role in afferent signal processing has been postulated. Although analysis of actions of neuroactive substances necessitates information about membrane currents, a limited amount of information for mammalian spinal dorsal horn neurons is available at present. In particular it would be important to learn more about the presence of Ca²⁺ conductances in the rat dorsal horn neurons since neuronal Ca²⁺ channels are frequent targets for modulation by neurotransmitters and drugs. Distinguishing between multiple types of Ca²⁺ conductances is important for understanding their functional role both in electrophysiological (calcium spikes, bursting firing pattern) and chemical
signalling (transmitter release, regulation of ion channel gating, cellular metabolism).

In this work we have used the single electrode voltage-clamp technique and the spinal cord slice preparation in an attempt to characterize voltage-dependent Ca\(^{2+}\) conductances underlying the low- and high-threshold Ca\(^{2+}\) spikes. We provide evidence for the existence of three components of Ca\(^{2+}\) channel currents, i.e., low-threshold transient and high-threshold transient and sustained Ca\(^{2+}\) currents, that are distinguishable on the basis of their voltage- and time-dependent properties and their responses to organic and inorganic Ca\(^{2+}\) channel agonists and antagonists. We also provide some evidence for modulation of Ca\(^{2+}\) conductances by CGRP and SP.
SECTION I. LOW- AND HIGH-VOLTAGE-ACTIVATED CALCIUM CURRENTS IN RAT SPINAL DORSAL HORN NEURONS

Summary

1. Calcium currents in immature rat spinal dorsal horn neurons in transverse slices were studied with the single-electrode voltage-clamp technique. Using experimental conditions that minimized voltage-dependent Na\(^+\) and K\(^+\) currents, we distinguished low- and high-voltage-activated calcium currents on the basis of their voltage dependence and sensitivity to the Ca\(^{2+}\) channel agonist and antagonist drugs.

2. The low-voltage-activated transient calcium current is evoked with weak depolarizing voltage commands. It begins to activate at potentials positive to -70 mV and increases in the amplitude and the rate of decay with stronger depolarizations, the peak values being reached between -40 and -30 mV. The current is fully activated at holding potential of about -110 mV. Inactivation is complete at potentials in the range of -60 to -50 mV.

3. The transient component of the high-threshold calcium current activates at membrane potentials close to -40 mV and decays with time constants ranging from 100-600 ms. The amplitude of the current increases with more negative holding potentials (-100 to -40 mV).

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4. The sustained component of the high-threshold calcium current activates at potentials positive to -40 mV and exhibits little inactivation during 0.3-0.5s depolarizing commands. This component is readily isolated at more depolarized holding potentials (between -40 and -30 mV) that inactivate the transient components of the low- and high-threshold calcium currents.

5. A run-down of calcium currents was seen in dorsal horn cells. The time stability of the transient and sustained components of the high-threshold calcium current was lower than that of the low-threshold transient current. The latter current seemed to be insensitive for at least 1h.

6. Bay K 8644 (1-10 µM), a dihydropyridine agonist, enhanced the sustained component of the high-threshold calcium current but not the transient components of the low- or high-threshold calcium currents. The dihydropyridine antagonist nifedipine (5-50 µM) selectively reduced the sustained component of the high-threshold calcium current, while having little or no effect on the transient components of the low- and high-threshold calcium currents.

7. Cadmium ions (6-100 µM) and cobalt (2 mM) ions markedly reduced both components of the high-threshold calcium current and Cd$^{2+}$ only slightly decreased the low-threshold transient current. However, all three components are indiscriminately blocked by higher concentrations of Cd$^{2+}$ and Co$^{2+}$.

8. Nickel ions (200-400 µM) greatly reduced the low-threshold transient calcium current, but had little effect on the transient and sustained components of the high-threshold calcium current.
Introduction

The spinal dorsal horn is one of the areas where primary afferent fibers terminate and the cutaneous sensory information is processed. A number of putative neurotransmitter and neuromodulator substances, including peptides, have been found in high density in this region of the spinal cord, and their functional role in afferent signal processing has been postulated. Although analysis of actions of neuroactive substances necessitates information about membrane currents, a limited amount of information for mammalian spinal dorsal horn neurons is available at present. In particular it would be important to learn more about the presence of Ca$^{2+}$ conductances in the rat dorsal horn neurons since neuronal Ca$^{2+}$ channels are frequent targets for modulation by neurotransmitters and drugs. Distinguishing between multiple types of Ca$^{2+}$ conductances is important for understanding their functional role both in electrophysiological (calcium spikes, bursting firing pattern) and chemical signalling (transmitter release, regulation of ion channel gating, cellular metabolism).

In vertebrate neurons, the existence of two types of Ca$^{2+}$ conductances -a low- and a high-threshold - was predicted on the basis of voltage recordings (Llinas and Sugimori, 1980; Llinas and Yarom, 1981; Murase and Randić, 1982, 1983). Subsequent voltage-clamp studies demonstrated the existence of either two (Carbone and Lux 1984; Bossu, Feltz and Thomann, 1985; Fedulova, Kostyuk and Veselovsky, 1985) or three types (Nowycky, Fox and Tsien, 1984, 1985) of Ca$^{2+}$ channels in vertebrate sensory neurons that exhibit different localization, kinetic,
pharmacological and functional properties (Perney, Hirning, Leeman and Miller, 1986; Fox, Nowycky and Tsien, 1987a,b; Gross and Macdonald, 1987; Huang, 1987, 1988; Madison, Fox and Tsien, 1987; Miller, 1987a,b; Huguenard, Coulter and Prince, 1988; Lipscombe, Madison, Poenie, Reuter, Tsien, and Tsien, 1988; Tsien, Lipscombe, Madison, Bley and Fox, 1988). In immature rat spinal dorsal horn neurons, the presence of multiple types of $\text{Ca}^{2+}$ currents was indicated by voltage (Murase and Randić, 1982, 1983) and current recordings (Huang, 1987, 1988). We demonstrated that dorsal horn neurons generate high-threshold and low-threshold $\text{Ca}^{2+}$ spikes (Murase and Randić, 1982, 1983). In voltage-clamped rat spinal dorsal horn neurons we described the presence of a $\text{Ca}^{2+}$-sensitive slow inward relaxation activated at membrane potentials close to the resting level (Murase, Ryu and Randić, 1986) that is augmented by tachykinins (Murase, Ryu and Randić, 1986, 1989; Murase, Randić, Ryu and Usui, 1987).

In the present experiments we have used the single electrode voltage-clamp technique and the spinal cord slice preparation in an attempt to characterize voltage-dependent $\text{Ca}^{2+}$ conductances underlying the low- and high-threshold $\text{Ca}^{2+}$ spikes (Murase and Randić, 1982, 1983). We provide evidence for the existence of three components of $\text{Ca}^{2+}$ channel currents, i.e., low-threshold transient and high-threshold transient and sustained $\text{Ca}^{2+}$ currents, that are distinguishable on the basis of their voltage- and time-dependent properties and their responses to organic and inorganic $\text{Ca}^{2+}$ channel agonists and antagonists. In addition, this work presents one of the first attempts to characterize three types of neuronal $\text{Ca}^{2+}$ channel currents in a slice preparation. A preliminary report of some of these results has been made elsewhere (Randić and Ryu, 1989).
Methods

Experiments were performed on spinal cord slices from 2-4 week-old Sprague-Dawley rats, as described elsewhere (Murase and Randić, 1983; Murase et al. 1989). The rat was anaesthetized with ether, the lumbosacral spinal cord excised and cut transversely with a Vibratome at a thickness of 300 µm. The slices were incubated for one hour in a Krebs solution containing (mM): NaCl, 124; KCl, 5;KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; and glucose, 10, pH 7.4, aerated with 95% O₂ and 5% CO₂, at 31-33°C. The use of a high-K⁺-Krebs solution during cutting and incubation of the slices seemed to improve their viability. After the incubation, a slice was transferred into the recording chamber where it was continuously perfused at a rate of 3 ml/min with a Krebs solution containing (mM): NaCl, 127; KCl, 1.9;KH₂PO₄, 1.2; CaCl₂, 2.4, MgSO₄, 1.3; NaHCO₃, 26; and glucose, 10, aerated with 95% O₂ and 5% CO₂, and maintained at 32°C. When the composition of the Krebs solution was modified, the NaCl concentration was adjusted to maintain osmolarity and the pH was maintained at 7.4 by adding HCl.

To isolate Ca²⁺ current we used a modified recording medium (pH 7.4) which contained (in mM): NaCl, 107.3; BaCl₂, 2.0; CsCl, 2.0; MgCl₂, 1.3; KH₂PO₄, 1.2; glucose, 10; TEA, 20; and TTX, 0.5 µM. Ba²⁺ was used as a charge carrier for Ca²⁺ channel because it can permeate through Ca²⁺ channel without activating Ca²⁺-dependent currents such as Ca²⁺-activated K⁺ current or Ca²⁺-activated chloride current. In a Ca²⁺-free solution containing Ba²⁺ or Ca²⁺, HPO₄⁻ and SO₄²⁻ were replaced with Cl⁻.

Under visual control, a single electrode filled with 4M Cs⁺-acetate
having DC resistance between 70-110 Mohm was placed into the laminae II-V of the spinal dorsal horn. Approximate location of a neuron in the dorsal horn was roughly determined in relation to the substantia gelatinosa region. With transillumination under a binocular microscope, the substantia gelatinosa was identifiable as a translucent band across the dorsal horn. The neurons were impaled by oscillation generated by an additional circuit of the capacity compensation of the recording amplifier (Dagan 8100 or Axoclamp 2). After a successful impalement of a neuron, fast action potentials were blocked by TTX (0.5x10^-6 M) and Cs⁺ was iontophoresed by applying positive current pulses for 2-6 min (0.2-1.0 nA, 0.3 s, 0.3 Hz) until the appearance of the initial hump in voltage responses, which is corresponding to the presence of the low-threshold Ca²⁺ current (Figs. 1 and 2). For the voltage-clamp study a single-electrode voltage-clamp amplifier (Axoclamp 2) was used that switched between voltage sampling and current injection at 3-4 kHz with a 30% duty cycle. The limitations and pitfalls associated with the use of the sample-and-hold technique have been discussed previously in some detail (Adams, Brown and Constanti, 1982; Brown and Johnston, 1983; Finkel and Redman, 1984; see Appendix for the basic principles of operation). Voltage-clamped cells that exhibited the spike-like inward current during voltage commands, or large prolonged inward tail currents, the indications of inadequate space clamp (Brown and Griffith, 1983), were not included in analyses. The sampled membrane current responses were filtered with a low-pass filter (300 Hz) and current and voltage recorded on a Gould-Brush 2200S pen recorder or stored in diskette using Nicolet 4094 digital oscilloscope. Cells remained in a good condition for as long as 3 hours.
Since in the voltage-clamped dorsal horn neurons complex time- and voltage-dependent current relaxations and current tails were observed at membrane potentials close to the resting level, the "leak" component of the steady-state current responses was not subtracted in all of the illustrations presented in this paper. However, when membrane currents evoked by voltage commands were corrected for the steady-state leakage current, which was assumed to be a linear function of the voltage change in the membrane potential range studied (-100 to +10 mV), the membrane potential was usually clamped to the resting potential and the leakage conductance was determined by applying hyperpolarizing commands of the amplitude equal to depolarizing steps, and the responses digitally subtracted from the corresponding current responses to depolarizing commands.

Ni\(^{2+}\) (100-500 µM), Cd\(^{2+}\) (20-100 µM), Co\(^{2+}\) (2 mM), nifedipine (5-50 µM, Sigma) and Bay K 8644 (1-10 µM, Miles Pharmaceuticals) were added to the bath in known concentrations. Fresh solution of dihydropyridine agonists and antagonists were prepared from the aliquots of a stock solution of a drug originally dissolved in ethanol (10\(^{-2}\)M). Care was taken to protect dihydropyridines from light.

Results

A total of 95 dorsal horn neurons in laminae II to V of the spinal dorsal horn were successfully voltage-clamped in the presence of TTX/TEA/Ca\(^{2+}\)/zero-Ca\(^{2+}\)/Ba\(^{2+}\) and used in this analysis. The average resting membrane potential of these neurons was 66 ± 6.0 mV (m ± S.D.) and the
input resistance, measured by a 10-20 mV hyperpolarizing pulse command of 0.3-0.5 s duration from the membrane potential close to the resting potential, ranged from 40 to 260 Mohm (113 ± 60 Mohm).

**Depolarizing inward rectification in dorsal horn neurons-current clamp experiments**

When 0.5 s depolarizing current pulses were applied through Cs⁺-filled microelectrodes to the dorsal horn neurons, the membrane potential of which was hyperpolarized to -80 mV by applying negative DC current, an inward rectification invariably appeared in the voltage records as an initial hump in the transmembrane potential changes (Fig. 1A). With higher intensities of current pulses that produced voltage deflections above 20 mV, the cells responded with a transient depolarizing potential that gradually increased in the magnitude with increasing intensity of the current pulses (Figs. 1A arrow and 2A). The transient depolarizing potential reached the peak between 30 and 40 ms and had a maximal duration of about 100 ms. A similar transient depolarizing potential recorded in cat and rat neurons in vitro was thought to be due either to an interplay of an instantaneous activation of a voltage-dependent persistent Na⁺ current and delayed activation of outward K⁺ current(s) (Stafstrom, Schwindt, Flatman and Crill, 1984) or, in addition, to activation of a low-threshold Ca²⁺ current (Sutor and Zieglgansberger, 1987).

At voltage deflections larger than 40 mV, a second time-dependent depolarizing potential occurred that lasted more than 200 ms (Fig. 1A, arrowhead). These two time-dependent depolarizing potentials were absent when the membrane potential of the same cell was depolarized to -60 mV by
Fig. 1. Current-voltage (I-V) relationships of an intracellularly recorded caesium-loaded dorsal horn neurone of 17-day-old rat bathed in Krebs solution containing TTX (5x10^{-7} M). Inset shows approximate location of a cell in the spinal dorsal horn in relation to the transparent area of Laminae I-III. A. Depolarizing and hyperpolarizing current pulses of 0.5 s duration and of variable amplitudes were injected and the corresponding voltage responses measured at the time points indicated (◼, 37 ms; □, 490 ms). The consecutive recordings were superimposed. Upper traces represent the voltage responses and the lower traces the current signals. In this cell the membrane potential was clamped manually at -80 mV. B. Voltage responses recorded in the same neurone at a membrane potential of -60 mV. The voltage responses were measured 40 ms after the onset of the current pulse (▼). C. The I-V curves reconstructed from the data illustrated in A and B. Note the development of a low- (A-B, arrow) and high-threshold (A-B, arrowhead) Ca^{2+} spikes as indicated.
applying positive DC current through microelectrode (Fig. 1B). Instead, in most cells examined in a TTX-containing solution, hyperpolarizing responses elicited by negative current pulses were followed by two distinct rebounds of depolarizations (Fig. 1B arrow and arrowhead).

Current-voltage plots derived from the records in Fig. 1 A-B illustrate the magnitude of inward rectification recorded at the membrane potential levels between -60 and -20 mV by comparing I-V curves obtained at the peak of the transient depolarizing responses (Fig. 1C, filled squares) and at the steady-state level (Fig. 1C, open squares). As seen in Fig. 1C, the inward rectification (increase in slope resistance with depolarization) was present near resting potential and persisted at depolarized levels of membrane potential near to -20 mV.

Although the ionic basis of the transient depolarizing potential and steady-state inward rectification was not investigated in the present experiments (but see Murase and Randic, 1983) we found that nickel ions reduced both (Fig. 2 A-B). The effect of adding the Ca\(^{2+}\)-channel blocker Ni\(^{2+}\), an agent known to block preferentially T (transient) type of Ca\(^{2+}\) channel current (Carbone, Morad and Lux 1987; Fox et al. 1987a; Narahashi, Tsunoo and Yoshii, 1987; Crunelli and Pollard, 1988; Hagiwara, Irisawa and Kameyama, 1988) on the transient depolarizing potential evoked in another Cs\(^+\)-loaded cell bathed in a medium containing TTX (5x10\(^{-7}\)M) and 2.4 mM Ca\(^{2+}\) is shown in Fig. 2.

Hyperpolarizing and depolarizing current pulses of variable intensities were applied from a membrane potential held at -80 mV (Fig. 2A-B) or -60 mV (Fig. 2C-D). After adding Ni\(^{2+}\) (4x10\(^{-4}\)M), the transient depolarizing potential (Fig. 2A, right records) and the inward
Fig. 2. The transient depolarizing potential (a low-threshold Ca$^{2+}$ spike) is depressed by Ni$^{2+}$ ions ($4 \times 10^{-6}$M). A and C show voltage responses of a Cs$^+$-loaded dorsal horn neurone of a 15-day-old-rat to a progressive series of depolarizing and hyperpolarizing current pulses of 300 ms duration recorded before (left traces) and 6 min after the onset of a perfusion with a Ni$^{2+}$-containing solution (right trace). Inset shows approximate location of a cell in the dorsal horn. The membrane potential was manually kept at -80 mV (A) or -60 mV (C) by applying DC current through the microelectrode. The voltage responses were measured 95 ms after the onset of a current pulse in the absence (O) and the presence of Ni$^{2+}$ ions (●). B and D, I-V plots constructed from the data shown in A and C. Control I-V curves (O) and I-V curves obtained during application of Ni$^{2+}$ at -80 mV (●) and -60 mV (▲) are shown. The arrows indicate the presence (C, left traces) and the blockade of the anodal break depolarizations by Ni$^{2+}$ (C, right traces).
rectification seen in the current-voltage plots obtained at -80 mV (Fig. 2B, open circles) were reduced (Fig. 2B, filled circles). In addition, anodal break excitations following hyperpolarizing pulses to potentials negative to -80 mV were abolished (Fig. 2C arrow, right records). In contrast, the I-V curves obtained at -60 mV were not modified by Ni$^{2+}$ (Fig. 2D). Taken together, the voltage dependence and the sensitivity to Ni$^{2+}$ of the initial depolarizing potential suggest that it is probably due to the activation of a low threshold transient Ca$^{2+}$ current de-inactivated by previous hyperpolarization of the cell (Llinas and Sugimori 1980; Llinas and Yarom 1981; Murase and Randić, 1982, 1983; Halliwell 1983; Carbone and Lux 1984; Jahnsen and Llinas 1984; Bossu et al. 1985; Fedulova et al. 1985; Crepel and Penit-Soria 1986; Sutor and Zieglgansberger 1987; Crunelli and Pollard 1988). In addition, the results suggest the presence of the low-voltage-activated and high-voltage-activated Ca$^{2+}$ channels in the membrane of the immature rat spinal dorsal horn neurons. To test this hypothesis single electrode voltage-clamp analysis was performed in dorsal horn neurons.

Multiple components of inward current in Cs$^+$-loaded dorsal horn neurons - voltage clamp experiments

Single-electrode, voltage-clamp recordings of membrane current were performed in 88 dorsal horn neurons in conditions devised to isolate Ca$^{2+}$ currents, as illustrated in Fig. 3. As a routine, 4 M Cs$^+$ acetate-filled microelectrodes and perfusing solution containing TTX/TEA/Ba$^{2+}$/zero-Ca$^{2+}$/Cs$^+$ were used in order to block fast voltage-dependent Na$^+$ current and minimize K$^+$ channel currents. Fig. 3 shows a representative example of
Fig. 3. The low-threshold transient inward current and the high-threshold transient and sustained components of inward current in two different Cs⁺-loaded dorsal horn neurones of 14-day (A) and 16-day-old rats (B). Inset shows approximate locations of the two cells in the dorsal horn. In A are shown inward current responses elicited by 300 ms depolarizing steps from a holding potential (h.p.) of -77 mV to test potentials of -36 mV (left trace) and -18 mV (right trace) in a medium containing TTX (0.5 μM), TEA (20 mM), Cs⁺ (2 mM), zero-Ca²⁺, and Ba²⁺ (2 mM). The same solution was used in the experiments illustrated in the remaining figures, if not stated. Current traces have been corrected for linear leak currents.

B, currents evoked with 300 ms depolarizing pulses from the holding potential of -40 mV (left and middle traces) and with 500 ms (right trace) from the holding potential of -80 mV (right trace) to test potential of -16 mV, as indicated. Leak current was not corrected. C, peak current-voltage relationships reconstructed from the records obtained at h.p. = -77 mV (●) and h.p. = -40 mV (▲). Current values have been corrected for leak current.
inward current responses recorded in the somatic membrane of two different dorsal horn neurons under these conditions.

A relatively weak depolarizing voltage step from a holding potential of -77 mV to test potential of -36 mV elicited a transient inward current that reached its peak between 10 to 30 ms and relaxed over the next 100 ms (Fig. 3A, left trace). This effect was observed in 59 out of 77 dorsal horn neurons (77%). The amplitude of this current varied from a few tenths of picoamperes up to 1 nA. The latter value was observed in several cells recorded in laminae II-IV (n=7). This current was relatively stable up to 1 hour.

With stronger depolarizing command to test potential of -18 mV a larger amplitude inward current was recorded in all successfully voltage-clamped cells. The current reached an initial peak within 6-15 ms then declined during the voltage step but still remained inward at the end of 0.3 s command (Fig. 3A, upper right traces). The inward current appeared to comprise two components, an initial rapidly decaying component (Figs. 3A, upper right traces) followed by a more sustained component (Fig. 3B, upper left and middle traces).

Ca$^{2+}$ and Ba$^{2+}$ sensitivity of the low-threshold transient and the high-threshold transient and sustained components of the inward current

Ca$^{2+}$ dependence When the perfusing fluid was changed to a zero-Ca$^{2+}$ solution (containing 10 mM Mg$^{2+}$) the low-threshold transient inward current (Fig. 4A) and the high-threshold transient and sustained components of the inward current were reversibly reduced (Fig. 4B). These findings led us to postulate the presence of three components of Ca$^{2+}$-sensitive
inward currents in the immature rat spinal dorsal horn neurons. The three components may correspond to the T, N and L currents first reported in chick sensory neurons (Nowycky et al. 1985; Fox et al. 1987a,b).

Since the presence of multiple Ca$^{2+}$ conductances has not been previously reported for the rat spinal dorsal horn neurons examined in the slice preparation, its ionic basis, kinetics of activation and inactivation, and pharmacological sensitivity were investigated in this work.

**Ba$^{2+}$ dependence** It is now well established that Ba$^{2+}$ can substitute for Ca$^{2+}$ as a charge carrier through Ca$^{2+}$ channels in a variety of excitable membranes (Hagiwara and Byerly, 1981) yet it suppresses Ca$^{2+}$-sensitive conductances.

Fig. 4 C-D shows the results obtained in two different cells having a low-threshold transient inward current (Fig. 4C) and the transient and sustained components of the high-threshold inward current (Fig. 4D). When either 2 mM (Fig. 4C) or 5 mM Ba$^{2+}$ (Fig. 4D) was substituted for 2.4 mM Ca$^{2+}$ in the external medium (n=2), the three components of the Ca$^{2+}$ current were present, and augmented in a barium-dependent manner, the result suggesting that Ba$^{2+}$ carried these inward currents. Concurrent with this, a small (about 0.1 nA) sustained inward shift of the holding current was recorded while the slope (leak) conductance at the resting level was reduced. Because of activation a delayed outward current during strong depolarizing commands it was difficult to quantitatively estimate the selectivity of Ca$^{2+}$ channels to Ba$^{2+}$ and Ca$^{2+}$ ions. However, our results suggest that the high-threshold current components have a higher selectivity for Ba$^{2+}$ than for Ca$^{2+}$ whereas the low-threshold transient
Fig 4. Ca$^{2+}$ and Ba$^{2+}$ dependence of low-threshold transient and high-threshold transient and sustained components of inward currents in three different Cs$^+$-loaded dorsal horn neurones of 14 (A and B) and 17 (C and D) day-old rats. Inset shows approximate location of tested cells in the spinal dorsal horn. In each panel superimposed are three inward current responses that show the control (Ca$^{2+}$, 2.5 mM in A-B; Ba$^{2+}$, 2 mM in C, 5 mM in D) (trace 1), the reduction in the inward currents after removal of external Ca$^{2+}$ (A, B) or Ba$^{2+}$ (C, D) from the perfusing solution (trace 2) and recovery (trace 3). In A a low-threshold transient inward current was isolated with Is hyperpolarizing prepulse to -114 mV from the holding potential of -50 mV. B, a high-threshold inward current comprising transient and sustained components was isolated with a test potential to +2 mV from the holding level of -60 mV. In C, a low-threshold inward current was elicited with Is hyperpolarizing repulse to -120 mV from the holding potential of -57 mV. In D, transient and sustained components of the high-threshold inward current evoked with a 0.5s depolarizing command to -27 mV from the holding potential of -90 mV.
current component appeared to discriminate less between these two ions (Data not shown).

**Characterization of the three components of \( \text{Ca}^{2+} \) currents**

Characterization of the three components of the \( \text{Ca}^{2+} \) channel current was made on dorsal horn neurons bathed either in a 2.4 mM \( \text{Ca}^{2+} \)-containing medium or in a zero-\( \text{Ca}^{2+} \), 2 mM \( \text{Ba}^{2+} \)-containing solution. In addition, perfusing solution contained TTX/TEA/Cs\(^+\) in order to minimize voltage-dependent \( \text{Na}^+ \) and \( \text{K}^+ \) currents. Although these conditions were devised to isolate \( \text{Ca}^{2+} \) currents, the separation of the individual components of the \( \text{Ca}^{2+} \) current was difficult because multiple components co-exist at a wide range of membrane potentials. However, the presence and the maximum and relative amplitudes of the various components are subject to great variations from one cell to another, a situation which provided an opportunity to study a component of \( \text{Ca}^{2+} \) current in a relative isolation in some cells.

**The low-threshold transient \( \text{Ca}^{2+} \) current**

This transient component of the calcium channel current can be evoked with weak depolarizations from a negative holding potential (-80 mV, or more). In a zero-\( \text{Ca}^{2+} \), 2 mM \( \text{Ba}^{2+} \) solution the current begins to activate at about -70 mV and its amplitude increases progressively with stronger depolarizations, as shown in Fig. 5C (right curve). The maximum current (about 1 nA) is reached between -40 and -30 mV, the time to peak was 20-60 msec and the duration 50-100 ms (Figs. 5B and 8B). The time-dependent run-down of the low-voltage-activated transient \( \text{Ca}^{2+} \) current component was much slower than for the transient and sustained components of the high-voltage-activated \( \text{Ca}^{2+} \) current.
Fig. 5. Voltage dependence of inactivation and activation of low-threshold transient current in two different dorsal horn neurones of a 19-day (A) and 17-day-old rat (B). Inset shows approximate locations of the examined cells. A, isolated low-threshold transient currents recorded after the offset of various 1 s hyperpolarizing commands to the potential of -45 mV as indicated. B, low-threshold transient currents elicited by depolarizing pulses from the holding potential of -88 mV to different test potentials as indicated. These records are from a cell illustrated in Fig. 8, and were obtained in the presence of 80 µM Cd2+. C,•: peak transient current amplitudes, normalized to the maximal current obtained from the holding potential of -109 mV, plotted against the holding potentials (from data illustrated in A). Data were fitted with a smooth curve derived from the Boltzmann relation $I/I_{\text{max}} = [1+\exp(V-V_{1/2})/k]^{-1} \text{ where } V_{1/2} = -86 \text{ mV and } k = 7.9 \text{ mV.}$ •: peak current amplitudes normalized to maximal current obtained at test potential of -25 mV, and plotted against the test potentials (from data shown in B). Data were fitted with a smooth curve $I/I_{\text{max}} = [1+\exp(-(V-V_{1/2})/k)]^{-1} \text{ where } V_{1/2} = -45 \text{ mV and } k = 7.0 \text{ mV}$
The transient component of the high-threshold Ca\(^{2+}\) current. This transient component is responsible for the additional increase in the magnitude of the low-voltage-activated transient Ca\(^{2+}\) current with stronger depolarizing commands from a negative holding potential (-80 mV). The threshold activation voltage for the current appears to be between -50 to -40 mV and the maximum current of 5 nA is reached at potentials between -20 and -10 mV (Fig. 3C, filled circles). Beyond -15 mV, the slope conductance increased steeply, possibly due to activation of outward currents incompletely blocked by internal Cs\(^+\) and external Cs\(^+\) and TEA.

The sustained component of the high-threshold Ca\(^{2+}\) current. This component is best isolated by using a more depolarized holding potential (between -40 and -30 mV) to inactivate the low- and high-threshold transient components of the calcium channel current. The current was activated at potentials positive to -40 mV (3C, filled triangles). The peak amplitude (~ 3 nA) is reached between -30 and -20 mV (Fig. 3C, filled triangles). The current is sustained over several hundred milliseconds with a very slow inactivation (Figs. 3B and 6B). In a number of dorsal horn cells an outward current can be detected that may simulate more rapid inactivation, despite the use of Cs\(^{2+}\)-filled microelectrodes and the presence of Cs\(^+\), TEA/Ba\(^{2+}\) in the perfusing solution.

The maximal and relative amplitudes of the low- and high-threshold Ca\(^{2+}\) current components are subject to great variation from one cell to another.
Comparison of time constants of inactivation

The time course of the low-threshold transient current and the transient and sustained components of the high-threshold Ca\textsuperscript{2+} current recorded in the immature rat dorsal horn neurons was different and each current component exhibited characteristic decay times over the whole range of potentials where it appeared (Fig. 6C).

The time course of inactivation for the three components of the Ca\textsuperscript{2+} channel current was examined at different test potentials (-70 to +10 mV), from the holding potential of about -85 mV for the low-threshold transient (Fig. 5B) and the high-threshold transient current components (Fig. 6A), and from -45 mV for the sustained component of the high-threshold current (Fig. 6B). Decay time constants were calculated from the slope of 150 ms segment (for transient and sustained components of the high-threshold current, Fig. 6A-B, arrowheads) or 20 ms segment (for low-threshold transient current, Fig. 5B), measured from the peak of each current response at different test potentials. In the range of membrane potentials between -60 mV and 0 mV, the time constants of decay of the low-threshold transient and the transient and sustained components of the high-threshold current varied between 10-50 ms, 100-600 ms and 300-4000 ms, respectively. Although the time constant of decay of each individual current component varied from one cell to the other, the rank order of the amplitudes always appeared to be: low-threshold > high-threshold transient > high threshold sustained. Under the conditions used the decay is unlikely to be due to the activation of outward K\textsuperscript{+} current since no evidence for a significant time-dependent outward current was obtained when the microelectrode contained Cs\textsuperscript{+} and the perfusing solution TEA (2-4x10^{-2} M) and zero-Ca\textsuperscript{2+},
Fig. 6. Voltage dependence of time constants of current decay for the low-threshold transient, and the transient and sustained components of the high-threshold Ca$^{2+}$ currents. A-B show current responses elicited in the same dorsal horn neurone by three depolarizing pulses of increasing strengths from the holding potentials of -85 mV (A) and -45 mV (B). Current traces have not been corrected for leak current. C, shows collected data for the time constants of current decay for the low-threshold transient (●), the high-threshold transient (●), and the high-threshold sustained (○), components of the inward currents from the cells illustrated in A-B (●,○) and Fig. 5B (●). Decay time constant was calculated from the slope of 150 ms segment (for the high-threshold transient and sustained components) and 20 ms segment (for the low-threshold component), measured from the peak of each current component. Note logarithmic scale of decay time constants.
2 mM Ba\(^{2+}\).

Fig. 6C shows collected data for the time constants of decay of the high threshold transient (filled circles) and sustained (open circles) components of the Ca\(^{2+}\) current obtained from the records illustrated in Fig. 6A-B, and for the low-threshold transient component (filled square) of the Ca\(^{2+}\) current from the cell illustrated in Fig. 5B. This plot indicates that: 1. The time constants of the various components of the Ca\(^{2+}\) current in the rat dorsal horn neurons are relatively well separated under the conditions used in our experiments. As shown in Fig. 6C, the time constants of decay of the transient and sustained components of the high-threshold Ca\(^{2+}\) current were 371 ms and 3460 ms respectively, at test potential about -20 mV. In contrast, a time constant of decay for a low-threshold transient current recorded at a similar potential in a different neuron (records shown in Fig. 5B) was only 10 ms. 2. The time constant of inactivation of each current component progressively decreases with stronger depolarizations, as has been observed by other investigators in the vertebrate sensory neurons (Bossu and Feltz, 1986; Carbone and Lux, 1987; Fox et al., 1987a).

**Voltage dependence of inactivation and activation of the low-threshold Ca\(^{2+}\) current**

To study the inactivation properties of the low-threshold transient Ca\(^{2+}\) current, the current was recorded after the offset of 1 s hyperpolarizing pulses of variable amplitudes applied to a constant level of -45 mV (Fig. 5A). The peak amplitude of the transient current was measured and plotted against holding potential (Fig. 5C, filled circles).
The experimental data were fitted with the Boltzmann equation. The inactivation curve shows strong voltage-dependence with a slope factor (k) of 7.9 mV and a mid-point voltage ($V_h$) of -86 mV. Thus the low-threshold transient component of the Ca$^{2+}$ current is fully de-inactivated at a holding potential negative to -110 mV and is completely inactivated at potentials between -60 and -50 mV.

The voltage dependence of activation of the low-threshold transient current was examined in another dorsal horn neuron by keeping a holding potential constant at -88 mV and varying the size of depolarizing test pulses (Fig. 5B). The transient and sustained components of the high-threshold Ca$^{2+}$ current were suppressed in this cell by adding 80 µM Cd$^{2+}$ into the bathing medium. A plot of peak current amplitude against the test potential (Fig. 5C, filled squares) gives a measure of the voltage dependence of activation. The activation curve obtained is strongly voltage dependent, with a slope factor and a mid-point voltage being 7.0 and -45 mV, respectively. Thus the low-threshold transient current appears to activate near -70 mV and its peak amplitude reaches a plateau between -40 and -30 mV. In agreement with the observations of Fox et al. (1987a) on vertebrate dorsal root ganglion neurons there is also an overlap between the inactivation and activation curves of the transient current in the rat dorsal horn neurons. This may lead to a steady inward flux of Ca$^{2+}$ ions and generation of a slow persistent current in a narrow range of potentials close to the resting membrane level (Murase et al. 1986, 1989).
Pharmacological separation of the three components of Ca$^{2+}$ currents

Inorganic Ca$^{2+}$ channel antagonists, such as cobalt, cadmium and nickel ions, and dihydropyridines, such as Bay K 8644 and nifedipine, are widely used in characterizing Ca$^{2+}$ channel currents. We here report the different sensitivity of the three components of Ca$^{2+}$ currents to these compounds. In the slice preparation, higher concentrations of these agents were needed to obtain the selective effects on the various components of the Ca$^{2+}$ current compared to the results obtained from the single cell preparations (Boll and Lux 1985; Nowycky et al. 1985; Carbone and Lux 1987; Fox et al. 1987a; Gross and Macdonald, 1987; Narahashi et al. 1987, Hagiwara et al., 1988).

Effects of dihydropyridines

1,4-Dihydropyridines bind specifically and with high affinity to voltage-sensitive Ca$^{2+}$ channels (Miller, 1987a,b). The ability of these agents to perturb Ca$^{2+}$ channel properties has been widely used to characterize the Ca$^{2+}$ current, especially the long-lasting component in the sensory neurons (Boll and Lux, 1985; Nowycky et al. 1985). In the present study the effects of the dihydropyridine Ca$^{2+}$ channel agonist Bay K 8644, and that of the dihydropyridine Ca$^{2+}$ channel antagonist nifedipine were studied on the low-threshold transient and high-threshold components of the Ca$^{2+}$ currents recorded from dorsal horn neurons. We report here that the high voltage-activated Ca$^{2+}$ current is selectively sensitive to these drugs.

Potentiation of the high-threshold Ca$^{2+}$ currents by Bay K 8644

In three out of six dorsal horn cells Bay K 8644 (10^{-6} - 10^{-5} M) doubled the amplitude of the high-voltage-activated Ca$^{2+}$ current. In the remaining cells the drug was without significant effect. In contrast, the
Fig. 7. Differential effects of Bay K 8644 and nifedipine on low-threshold and high-threshold Ca\textsuperscript{2+} currents in two different dorsal horn neurones of 17-day-old rats (A & B). In A, superimposed records obtained in a control run and following the addition of Bay K 8644 (10 \textmu M) to the bath (sweeps marked with *). Voltage protocols evoked low-threshold transient current (a), predominantly sustained component of the high-threshold current (c), and a combination of transient and sustained components of the high-threshold current (b). Amplitude of the sustained component of the high-threshold current almost doubled in the presence of Bay K 8644 (c). The combination current of both transient and sustained components of the high-threshold calcium current, and low-threshold transient currents were little or not affected, respectively. Current responses elicited by hyperpolarization from h.p. = -50 mV to test potential of -75 mV before and after the addition of Bay K 8644 (d). B, superimposed records obtained before and following the addition of nifedipine (20 \textmu M) to the bath (sweep marked with *). Voltage protocols evoked high-threshold current comprising either transient and sustained components (left traces) or predominantly a sustained component (middle traces). Nifedipine reduced the sustained current of the high-threshold current by 40%. Current responses elicited by hyperpolarizing pulse from h.p. = -36 mV to test potential of -55 mV before and after the addition of nifedipine (right traces).
low-voltage-activated Ca$^{2+}$ current was not modified by Bay K 8644.

Fig. 7A shows the different effects of Bay K 8644 on the different components of Ca$^{2+}$ current. The voltage protocols were chosen to isolate the low- and the high-threshold Ca$^{2+}$ currents. The results show that 10 µM Bay K 8644 produced little or no effect on the low-voltage-activated Ca$^{2+}$ current evoked from the holding level of -100 mV (Fig. 7Aa), while it increased the transient and sustained components of the high-voltage-activated Ca$^{2+}$ current evoked from the holding potential of -90 mV (Fig. 7Ab), and especially the sustained component clamped at -50 mV (Fig. 7Ac). Reversibility was not observed within 10 min of a wash-out period. Bay K 8644 caused no change of the "leak" current evoked during hyperpolarizing command steps (Fig. 7Ad). These results indicate that the high-voltage-activated Ca$^{2+}$ current, in particular the sustained component, appears to be more sensitive to Bay K 8644.

Blocking effect of nifedipine Fig. 7B shows that the dihydropyridine Ca$^{2+}$ channel antagonist, nifedipine (2x10$^{-5}$M), selectively reduces (49 ± 20%, n=6) the sustained component of the high-voltage-activated Ca$^{2+}$ current evoked from the holding potential of -36 mV, (Fig. 7B, middle traces), while producing little or no effect on the transient high-voltage-activated component evoked from -86 mV (Fig. 7B, left traces). Reversibility was not observed after a wash-out period of 10 min. The conclusion that may be drawn from this experiment is that nifedipine is less effective in inhibiting the transient than the sustained component of the high-threshold Ca$^{2+}$ current.
Effects of inorganic Ca$^{2+}$ channel blockers

Inorganic Ca$^{2+}$ channel antagonists such as Cd$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ are frequently used to block Ca$^{2+}$ channels.

**Block by Cd$^{2+}$ ions**

High-voltage-activated Ca$^{2+}$ current was more sensitive than low-voltage-activated transient Ca$^{2+}$ current. In the whole cell voltage-clamped chick sensory neurons Cd$^{2+}$ ions (20-50 $\mu$M) are shown to be more effective in blocking L and N types of the Ca$^{2+}$ channel current while leaving T type current relatively unaffected (Bossu et al. 1985; Nowycky et al. 1985). However, all three components were indiscriminately blocked by higher concentrations of Cd$^{2+}$ (Carbone and Lux, 1987; Fox et al. 1987a). In a general agreement with these data we found that Cd$^{2+}$ ions (60 - 100 $\mu$M) were more effective in blocking the high-voltage-activated Ca$^{2+}$ current (peak amplitude reduced by more than 50%) than in blocking the low-voltage-activated Ca$^{2+}$ current as illustrated in Fig. 8. The peak amplitude of the high-voltage-activated current evoked from the holding potential of -88 mV was decreased by 89% after 7 min of perfusion with Cd$^{2+}$ (8x10$^{-5}$M) (Fig. 8A, middle records), whereas the low-voltage-activated current decreased by 35% of the control value (Fig. 8A, left records). When the cell was clamped at a more positive level (-68 mV), where the low-threshold component is inactivated to a large degree, Cd$^{2+}$ completely abolished the high-threshold current (Fig. 8A, right records).

The corresponding I-V relationships for the high-voltage activated current in the presence and absence of cadmium for holding levels of -88 and -68 mV are shown in Fig. 8B. A typical I-V curve for the high-voltage-activated Ca$^{2+}$ current was obtained at a holding potential of -88 mV before
Fig. 8. Differential block of low-threshold and high-threshold currents by Cd$^{2+}$ (80 μM) in a dorsal horn neurone of a 17-day-old rat. Inset shows approximate location of a cell in the dorsal horn. A, left traces show superimposed low-threshold transient current responses and middle traces low- and high-threshold currents evoked by depolarizations from the holding potential of -88 mV to test potentials indicated, before and after the addition of Cd$^{2+}$ (arrowheads). Right traces show high-threshold currents evoked in relative isolation by depolarizing command to -32 mV from a holding potential of -68 mV before and in the presence of Cd$^{2+}$ (arrowhead). Current traces have been corrected for leak current. B, peak current-voltage relationships obtained at the holding level of -88 mV before (●), and during bath-administration of Cd$^{2+}$ (○). I-V plot obtained in the presence of Cd$^{2+}$ at h.p. = -68 mV is also shown (△)
A

-52

-88

-68

-34

-32

-88 mV

Control

Cd\(^{2+}(80 \, \mu M)\)

h.p. = -68 mV

100 ms

0.25 nA (a)

1.0 nA (b, c)

B

-80

-60

-40

-20

-0.5

-1.0

-1.5

-2.0 nA

10 mV

h.p. = -88 mV

Control

Cd\(^{2+}(80 \, \mu M)\)

h.p. = -68 mV

Cd\(^{2+}(80 \, \mu M)\)
adding Cd\(^{2+}\) to the bathing medium (Fig. 8B, filled circles). In the presence of Cd\(^{2+}\) the high-threshold current components were blocked and I-V curves showed only remaining low-threshold transient current (Fig. 8B, open circles). When the same neuron was held at a more depolarized level (-68 mV), however, Cd\(^{2+}\) completely blocked the high-threshold components (Fig. 8A, right traces), and since the low-threshold current was minimal at this level, the I-V curve approached zero current level (Fig. 8B, open triangles). Similar effects were observed in 7 dorsal horn cells. When a higher concentration of Cd\(^{2+}\) (millimolar) was used, or when application of Cd\(^{2+}\) was prolonged, a toxic effect on the dorsal horn neurons was observed which always resulted in an irreversible blockade of the current in agreement with the findings of Bossu et al. (1985) in rat sensory neurons.

**Block by Co\(^{2+}\)-ions** Inhibition of the high-voltage-activated Ca\(^{2+}\) current. The effect of another inorganic Ca\(^{2+}\) channel blocker, Co\(^{2+}\), on the high-voltage-activated Ca\(^{2+}\) current was also examined in 4 dorsal horn neurons.

Fig. 9 shows the effect of adding Co\(^{2+}\) ions (2 mM) on the transient and sustained components of the high-threshold Ca\(^{2+}\) current evoked in a Cs\(^{+}\)-loaded cell bathed in a medium containing TTX/TEA/Ba\(^{2+}\)/zero-Ca\(^{2+}\). Positive or negative jumps of 200 ms were applied from a holding potential of -60 mV (Fig. 9A-B, left records). After adding 2 mM Co\(^{2+}\), the depolarization-evoked inward current (Fig. 9A, middle trace) was markedly reduced and eventually reversed to outward (Fig. 9A, right trace), with little or no effect on the inward leakage current elicited by hyperpolarizing pulses (Fig. 9B, middle and right traces). On washout of Co\(^{2+}\) the effect was only in part reversible. These results indicate that
Fig. 9. The high-threshold current recorded in a dorsal horn neurone of 18-day-old rat and effect of Co^{2+}. Inset shows approximate location of a cell in the dorsal horn. A, in a Krebs solution containing TTX (0.5 μM), TEA (30 mM), zero-Ca^{2+}, Ba^{2+} (1 mM) depolarizing command to -15 mV (left trace) from the holding potential of -60 mV evoked high-threshold inward current (middle trace) that was reversed to outward in the presence of 2 mM Co^{2+} (right trace). B, little change in "leakage" conductance occurred during perfusion with Co^{2+}-solution (middle and right traces).
the transient and sustained components of the high threshold inward current recorded in the immature rat dorsal horn neurons are effectively blocked by Co²⁺ ions, as has been observed in other vertebrate neurons (Miller, 1987a,b). Co²⁺ blocks also the low-threshold transient component of the Ca²⁺ current (data not shown).

**Effects of Ni²⁺**ions

The low-threshold transient Ca²⁺ currents are more sensitive than the high-threshold Ca²⁺ currents. Ni²⁺ ions (10⁻⁴M) have been found to block T type of Ca²⁺ channel current in preference to L and N currents in the chick sensory neurons (Fox et al., 1987a). However, the low-voltage-activated and the high-voltage-activated Ca²⁺ currents were blocked reversibly by 5 mM Ni²⁺ in the rat dorsal root ganglion neurons (Carbone and Lux, 1987). Fig. 10 illustrates the effects of Ni²⁺ (2x10⁻⁴M) recorded 5 min after the onset of perfusion on the low-threshold and the high-threshold components of Ca²⁺ current in the rat dorsal horn neurons. As shown in Fig. 10A, Ni²⁺ ions greatly reduce the low-threshold transient Ca²⁺ current activated by a depolarizing command to -40 mV from the holding potential of -90 mV. Similar effect was seen in 4 cells examined (91 ± 12% block). In contrast, Ni²⁺ has little or no effect on the transient and sustained components of the high-threshold Ca²⁺ current isolated by a test pulse to -18 mV from the holding level of -70 mV (Fig. 10B).

**Substance P augments a low-voltage-activated Ca²⁺ current in rat spinal dorsal horn neurons**

A series of about thirty experiments designed to isolate Ca²⁺ currents were performed. Fast voltage-sensitive Na⁺ current was eliminated by block
Fig. 10. Selective block of low-threshold transient current by Ni$^{2+}$ (200 µM). Superimposed records obtained in a control run and after the addition of Ni$^{2+}$ to the perfusing medium (sweeps marked with *). Voltage protocols elicited low-threshold transient current (A) and high-threshold current comprising transient and sustained components (B).
of Na\(^+\) channels with TTX, and K\(^+\) channel current was minimized by using Cs\(^+\)-filled microelectrodes and external solution containing TEA/Ba\(^{2+}\)/zero-Ca\(^{2+}\). In confirmation of our previous data (Murase et al., 1986, 1987) we observed that under these conditions the prominent slow inward current relaxations and the inward current tails were recorded in rat dorsal horn neurons and they were increased by SP in a dose-dependent manner. A clear enhancement of net inward Ca\(^{2+}\)-sensitive current by SP is shown in Fig. 11.

The slow inward Ca\(^{2+}\)-sensitive current shares two common properties with the low-voltage-activated transient Ca\(^{2+}\) current described in Section I: 1. Both currents can be isolated with weak test pulses (Fox et al. 1987b; Murase et al., 1989). 2. Both Ca\(^{2+}\) currents can operate as a steady-state inward current at membrane potentials close to the resting level. The fact that there is a significant degree of overlap between the inactivation and activation curves of T-type current in embryonic chick DRG neurons (Fox et al., 1987a) and in rat dorsal horn neurons (Fig. 5) suggests that the low-voltage-activated transient Ca\(^{2+}\) channels may be capable of supporting a steady inward flux of Ca\(^{2+}\) ions over a narrow range of potentials close to the resting level. In cardiac sino-atrial node cells, Hagiwara et al. (1988) showed that Ni\(^{2+}\) is a relatively selective inhibitor of "T" channels and used that inhibition to show that T type channels participate in generating pace-maker depolarizations.

We have used a similar pharmacological approach to examine whether SP modifies the low-threshold transient Ca\(^{2+}\) current in the immature rat spinal dorsal horn neurons. As shown in Fig. 12, SP enhanced in a reversible manner the low-voltage-activated, Ni\(^{2+}\)-sensitive Ca\(^{2+}\) current in 6 out of 11 Cs\(^+\)-loaded dorsal horn neurons of the rat spinal cord. We do
Fig. 11. SP enhances an inward calcium conductance in a Cs⁺-loaded dorsal horn neuron. In A, are shown two superimposed current responses elicited by a depolarizing clamp step from a holding potential of -62 mV to a command potential of -58 mV in a medium containing TTX (0.5 μM) in a zero-Ca²⁺, 1 mM Ba²⁺, before (left trace), during the bath perfusion with SP (10⁻⁶ and 5x10⁻⁶ M, middle trace) and after the recovery (right trace). The response to higher concentration of SP is marked with a dot (•). In B, upon subtraction of the leakage current it is evident that the depolarizing voltage step of 4 mV in amplitude activated an inward current during application of SP and that this effect is concentration-dependent (middle trace). In C, are shown two superimposed current responses to a hyperpolarizing command to -66 mV.
Fig. 12. Effect of bath-applied SP (2x10^{-6} M) on the low-threshold transient Ca^{2+} current in a voltage-clamped spinal dorsal horn neuron of an 18-day-old rat. The current was isolated with a voltage command to -52 mV from a holding potential of -83 mV in a medium containing TTX (0.5 μM), TEA (20 mM) and Cs^+ (2 mM). The neuron was intracellularly loaded with Cs^+. In A, upper traces show the current responses obtained before (left), during application of SP (middle) and after the recovery (right). The arrowheads indicate the low-threshold Ca^{2+} current. Lower traces are the respective voltage commands. In B, the leak-subtracted current records shown in A are expanded.
A

SP 2x10^{-6} M

-83 -52

B

50mV 200pA

200ms

50pA

50ms
not know at present whether the SP-induced slow Ca\textsuperscript{2+}-sensitive currents (Fig. 11) represent two different types of Ca\textsuperscript{2+} current, or they are manifestations of a single type of Ca\textsuperscript{2+} channel current.

Discussion

This paper demonstrates the existence of multiple types of Ca\textsuperscript{2+} conductances in 2-4-week old rat spinal dorsal horn neurons studied in the slice preparation. Low voltage-activated and high-voltage activated Ca\textsuperscript{2+} conductances are characterized with regard to their voltage dependence and sensitivity to the Ca\textsuperscript{2+} channel agonist and antagonist drugs. These observations extend our previous studies that predicted the existence of two types of Ca\textsuperscript{2+} channels in the membrane of rat spinal dorsal horn neurons on the basis of voltage recordings (Murase and Randic, 1982, 1983).

The presence of two (Carbone and Lux, 1984; Bossu et al. 1985; Fedulova et al. 1985; Bossu and Feltz, 1986; Carbone and Lux, 1987) or three (Nowycky et al. 1985; Fox et al. 1987a,b) different types of Ca\textsuperscript{2+} channels have been shown in vertebrate sensory and other neurons. The question has been raised, however, whether N-type Ca\textsuperscript{2+} current is distinct from T and L components (Carbone and Lux, 1987; Leonard, Nargeot, Snutch, Davidson and Lester, 1987; Swandulla and Armstrong, 1988).
Distinguishing features of low-voltage-activated and high-voltage-activated Ca\(^{2+}\) currents

Voltage dependence of activation and inactivation

The activation voltage threshold and the peak voltage of the I-V curve are the most frequently used parameters to distinguish between different components of the Ca\(^{2+}\) channel currents. Although the range of activation for the low-threshold and the high-threshold components shows great variation in the immature rat dorsal horn neurons, which can be perhaps linked to various cell types or conditions of slice preparations, the low-threshold transient current and the sustained component of the high-threshold current can be isolated by working over a narrow voltage range. The transient component of the high-threshold current is the most difficult to study since it activates with strong depolarizations from the relatively negative holding potential.

Our results for the low-voltage-activated transient current obtained in the slice preparation (Fig. 5) are roughly in agreement with the values reported by Nowycky et al. (1985) for T component of the Ca\(^{2+}\) channel current studied in the cultured chick sensory neurons. However, there are marked differences between the values of threshold voltage and peak voltage in I-V curves of the high-threshold current components examined in the dorsal horn neurons and those reported for N and L types of Ca\(^{2+}\) current in the chick sensory neurons (Bossu et al. 1985; Nowycky et al. 1985; Carbone and Lux 1987). In our experiments, both components of the high-threshold Ca\(^{2+}\) current appear to activate at more negative levels, about -40 mV, and there is also a negative shift in the peak voltage on the I-V curve (Fig. 3). The negative shift of threshold and peak voltages of the high-
threshold components could be due to: (1) specific properties of the Ca$^{2+}$ channels of the immature rat spinal dorsal horn neurons (Jia and Nelson, 1986), (2) use of Ba$^{2+}$ as a charge carrier in most of our analyses (Kostyuk, Veselovsky and Fedulova, 1981), and (3) a residual outward potassium current escaped to blockade by intracellular Cs$^+$ ions and extracellular Cs$^+$ and TEA. However, the latter condition alone is not likely, because a similar observation was made recently in the acutely-isolated spinal dorsal horn projection neurons using whole cell voltage clamp method (Huang, 1988).

Although, the average amplitude of the high-threshold current in the immature rat dorsal horn neurons is larger than that of the low-threshold transient current, in some cells (see Fig. 10A) we found that the current carried by the low-voltage-activated channels is comparable in magnitude to that passing through the high-threshold channels. Similar observations were made by other investigators on cultured vertebrate sensory neurons (Carbone and Lux 1984; Bossu et al. 1985; Nowycky et al. 1985; Huguenard et al. 1988).

In all dorsal horn cells examined the low-threshold transient current and the transient and the sustained components of the high-threshold current inactivate with time. In each case the time constant of inactivation decreases with progressive depolarization (Fig. 6). The differences in time constants of decay for various current components are consistently present in all cells (Fig. 6). The value of time constant of decay of the low-threshold transient current is always the smallest, that of sustained component of the high-threshold current the largest and of the transient high-threshold current in-between. The voltage dependence of
steady-state inactivation of the low-threshold transient current component (Fig. 5) roughly corresponds to values reported for vertebrate sensory neurons (Fedulova et al. 1985; Carbone and Lux, 1987; Fox et al. 1987a). Time constants of the high-threshold components of Ca\(^{2+}\) current of dorsal horn neurons are larger than values reported for dorsal root ganglion neurons (Fox et al. 1987a; Gross and Macdonald, 1987). Although mechanism of inactivation of various components of Ca\(^{2+}\) channel currents were not studied in this work, it is of interest that inactivation of the low- and the high-threshold currents occurs when Ba\(^{2+}\), instead of Ca\(^{2+}\), is used as the external charge carrier (Fig. 4). In vertebrate sensory neurons inactivation of the low-voltage activated channels is not Ca\(^{2+}\)-dependent (Bossu and Feltz, 1986; Carbone and Lux, 1987) whereas inactivation of the high-voltage-activated channels appears to be dependent upon the rate of Ca\(^{2+}\) entry, similar as shown in other preparations (Eckert and Chad, 1984).

Pharmacological separation of low-threshold and high-threshold Ca\(^{2+}\) currents The different sensitivity of low-voltage-activated and high-voltage-activated Ca\(^{2+}\) channel currents is presently well documented. Our findings of high sensitivity of the low-threshold transient current to blocking action of Ni\(^{2+}\) ions (Fig. 10) and low sensitivity to dihydropyridine agonists and antagonists (Fig. 7), and low doses of Cd\(^{2+}\) ions (Fig. 8), are in general agreement with the previous reports made in other peripheral and central neurons (Boll and Lux 1985; Bossu et al. 1985; Fedulova et al. 1985; Nowycky et al. 1985; Carbone and Lux, 1987; Carbone et al. 1987; Crunelli and Pollard 1988; Huguenard et al. 1988). In comparison to the data obtained in isolated neuronal preparations, however, the potency of these agents was lower in the slice preparation, probably
due to the difficulty of drug access to a neuron buried in connective
tissue and tightly surrounded by other neuronal and non-neuronal elements
(see also, Crunelli and Pollard, 1988).

Although voltage dependence of activation and different rates of decay
of the transient and sustained components of the high-threshold Ca$^{2+}$
current suggest the presence of two populations of Ca$^{2+}$ channels in the
membrane of immature rat dorsal horn neurons, our present data do not
corroborate the conclusion in favor of two distinct types of Ca$^{2+}$ current
since the kinetic properties of the two components frequently overlap, and
there is also a possibility that incompletely blocked outward potassium
current may be responsible for the two components recorded. However, the
existence of two different types of the high-threshold Ca$^{2+}$ channel current
is suggested on the basis of pharmacological experiments. Our results show
that Bay K 8644 increases the sustained component of the high-voltage-
activated current but is relatively ineffective against the transient
component of this current (Fig. 7A). The differential responsiveness of
the transient and sustained components also extends to nifedipine (Fig.
7B). Our findings of a high sensitivity of the sustained component, and a
low sensitivity of the transient component to dihydropyridines are in
agreement with previous reports (Nowycky et al. 1985; Docherty and Brown
1986; Fox et al. 1987a; Rane, Holz and Dunlap 1987; Hirning, Fox,
McCleskey, Olivera, Thayer, Miller and Tsien, 1988).

Since both high-threshold components are insensitive to blocking
action of low concentrations of nickel ions (Fig. 10), but are virtually
eliminated by Cd$^{2+}$ ions (Fig. 8), a clear separation of the two components
of the high-voltage-activated current may still require extensive
structural and functional analyses with specific pharmacological tools, yet to be discovered.

Functional significance of multiple Ca\(^{2+}\) conductances

Functional significance of the multiple components of the Ca\(^{2+}\) current has not been well established yet. In mammalian neurons, low-voltage-activated Ca\(^{2+}\) conductance has been shown to regulate the neuronal excitability (Llinas and Sugimori 1980; Llinas and Yarom 1981; Murase and Randić, 1982, 1983; Halliwell and Scholfield 1984; Jahnsen and Llinas 1984, Crepel and Penit-Soria 1986; Sutor and Zieglgansberger 1987; Greene, Haas and McCarley 1986) and underlies the spike afterdepolarization in dorsal root ganglion neurons (White, Lovinger and Weight, 1988). Since there is a substantial overlap between the inactivation and the activation potentials for the low-voltage-activated transient conductance the channels underlying this current may support a steady inward flux of Ca\(^{2+}\) in the narrow range of potentials close to the resting level. Thus this current may be involved in the control of some intracellular Ca\(^{2+}\)-dependent processes near resting potential. It is known that in cardiac cells T-type channels participate in generation of spontaneous pace-maker depolarizations (Hagiwara et al. 1988). It is of interest that a slow persistent Ca\(^{2+}\) current was recorded at weak depolarizations in some dorsal horn cells (Murase et al. 1989).

Although the functional role of the low-voltage-activated transient current in acutely-isolated rat spinal dorsal horn neurons is the subject of another study, the presence of a relatively large current in some superficially located rat spinal dorsal horn neurons is of much interest
(Fig. 10) because this region is involved in processing and integration of sensory information, including pain.

While the low-voltage-activated Ca\textsuperscript{2+} conductance may play an important physiological role in near-threshold phenomena and in regulation of neuronal excitability, the slower high-threshold Ca\textsuperscript{2+} conductances corresponding to N and L currents, are evoked by strong depolarizations and functionally may be involved in generation of high-threshold Ca\textsuperscript{2+} spikes (Llinas and Sugimori, 1980; Llinas and Yarom, 1981; Murase and Randić, 1982, 1983) and neurotransmitter release (Miller, 1987a,b; Tsien et al. 1988). L or N types of Ca\textsuperscript{2+} channels can be involved in determination of the type of neurotransmitter released (Turner and Goldin, 1985; Reynolds, Wagner, Snyder, Thayer, Olivera and Miller, 1986; Thayer, Hirning and Miller 1987; Lipscombe et al. 1988). It is of interest that N current was coupled with the release of norepinephrine in sympathetic neurons (Perney et al. 1986; Hirning et al. 1988), while L current is coupled to the release of substance P in sensory neurons (Perney et al. 1986; Rane et al. 1987).

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SECTION II. ACTIONS OF CALCITONIN GENE-RELATED PEPTIDE ON RAT SPINAL DORSAL HORN NEURONS

Summary

1. The membrane actions of calcitonin gene-related peptide (CGRP) and the effect on the Ca\textsuperscript{2+}-dependent action potential of dorsal horn neurons have been investigated by means of intracellular recording technique in the immature rat in vitro spinal cord slice-dorsal root ganglion preparation.

2. Bath application of CGRP (10^{-8}-10^{-6}M for 1-10 min) produced a slow reversible depolarization in about one-third of the cells examined. Biphasic membrane response consisting of an initial hyperpolarization followed by a late prolonged depolarization was seen in a smaller proportion of tested cells.

3. Both membrane responses were present, and even enhanced, when synaptic transmission and Na spikes were blocked by perfusing the slice with a TTX-containing Krebs solution. The CGRP-induced membrane changes were present in media containing TTX and TEA.

4. The CGRP-evoked depolarization was associated with an increase in the input resistance, and enhanced excitability in a majority of neurons tested.

5. CGRP, in addition, modified the duration of Ca\textsuperscript{2+}-dependent action potentials of dorsal horn neurons, the most consistent change

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being a prolonged increase in the spike duration.

6. Our results are consistent with a neurotransmitter or neuromodulator role for CGRP in the rat spinal dorsal horn.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide formed in neural tissue by alternative splicing of mRNA of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). Immunocytochemical studies demonstrated wide distribution of the peptide within the peripheral and central nervous system (Rosenfeld et al., 1983; Gibson et al., 1984; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985; Franco-Cereceda et al., 1987). Although in the spinal cord CGRP-immunoreactive fibers and terminals are present at all levels of the spinal cord, they were most numerous in the dorsal horn, where concentrated in Lissauer's tract and laminae I-III, V and X. The presence of CGRP-like immunoreactivity (CGRP-LI) in primary sensory neurons was established by immunohistochemistry and radioimmunoassay in combination with high performance liquid chromatography (Gibson et al., 1984; Skofitsch and Jacobowitz, 1985; Franco-Cereceda et al., 1987). In the dorsal root ganglia CGRP immunoreactivity was observed in most of the small and some of the intermediate sized cells. Substance P (SP) immunoreactivity, where present, co-existed with CGRP in a certain proportion of the small cells. Dorsal rhizotomy caused a significant loss of CGRP-immunoreactive fibers from the dorsal horn of the spinal cord. On the basis of these results Gibson et al. (1984) suggested that the major origin of CGRP in the dorsal
spinal cord is from primary afferent fibers deriving from the dorsal root ganglion cells. After capsaicin treatment the content of CGRP-LI was reduced by 60% in the dorsal part of the spinal cord (Franco-Cereceda et al., 1987). Thus the distribution of CGRP suggests a role in sensory transmission, and central administration of CGRP has recently been shown to have an antinociceptive effect (Bates et al., 1984). Using synaptosomal membrane preparations, high affinity binding sites for CGRP have been demonstrated in rat and human CNS (Rizzo and Goltzman, 1981; Tschopp et al., 1984, 1985; Goltzman and Mitchell, 1985).

Release of CGRP-like immunoreactivity was demonstrated from cultured rat trigeminal ganglion cells (Mason et al., 1984) and also in response to capsaicin and high K⁺ from the slices of the rat spinal cord (Saria et al., 1986, Oku et al., 1987). Pharmacological studies provided evidence for actions of CGRP on peripheral tissues, including vasodilation (Brain et al., 1985) and a positive inotropic action (Franco-Cereceda and Lundberg, 1985) possibly mediated through an increase in the influx of calcium (Satoh et al., 1986). However, little is known about actions of CGRP in the central nervous system presently. The purpose of the present work is to examine the membrane actions of CGRP on rat dorsal horn neurons in vitro by using intracellular recording techniques. These results have been presented in a preliminary form (Gerber et al., 1987; Ryu et al., 1987).

Methods

Slices from Sprague-Dawley rats, 16-21 days old were prepared as described by Murase and Randić (1983, 1984). The spinal segment was
sectioned to yield 1 horizontal or three 300-μm thick transverse spinal cord slices. The duration of the entire procedure from the removal of the spinal cord until the slices were made rarely exceeded 5 min. The slices were incubated in Krebs solution at 30 °C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Krebs 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 and a flow rate of about 3.0 ml/min. The recording chamber had a capacity of 0.5 ml.

Intracellular recordings were performed with micropipettes filled with 3 M potassium acetate (pH 7.2) having DC resistances of 80-120 MΩ. Stable intracellular recordings from single dorsal horn neurons could be maintained during multiple solution changes for as long as 5 h. Electrical properties of dorsal horn neurons were determined by means of a high-input impedance bridge amplifier (Dagan, 8100) 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 2200S) or stored in the disks of a Nicolet digital oscilloscope (model 4094) until processed and printed out onto a Digital plotter. Synaptic activation of the dorsal horn neurons was obtained with a co-axial stainless-steel stimulating electrode (o.d. of inner and outer electrodes being 25 and 200 μm, respectively, Frederick Haer, Co.) positioned on the dorsal root or dorsal root ganglia. Known concentrations of chemicals were added to the superfusate. We used rat CGRP from Cambridge Research Biochemicals and Peninsula Labs.
Results

A total of forty-six dorsal horn neurons in laminae I-V of the spinal dorsal horn was used in this investigation. The average resting membrane potential was 66.5 ± 6.7 mV (mean ± S.D.) and the input resistance averaged 87 ± 38 MΩ. Bath application of CGRP (10^{-8} to 2x10^{-6} M) produced a slow, reversible, dose-dependent membrane depolarization (6.1 ± 4.2 mV, n=13), increase in synaptic activity and occurrence of spontaneous action potentials, in about one third of the cells examined (Fig. 1 A-B). In a smaller proportion of examined cells (about 15%) the depolarization was preceded by a shorter-lasting hyperpolarization (-3.7±2.9 mV, n=7) as illustrated in Fig. 2 A-B. In eight cells only a small hyperpolarization was observed. The hyperpolarization occurred within 1-2 min from the onset of CGRP application, while 10-15 min were needed to reach the peak depolarization. The depolarizing response frequently persisted for 30-60 min. In the presence of tetrodotoxin (TTX, 3-5 x 10^{-7} M), used to block a fast voltage-dependent sodium conductance, Na-dependent action potentials and synaptic activity were eliminated (Figs. 1-2 C) while the CGRP-evoked depolarizing and hyperpolarizing responses remained and in some cells were even augmented (Fig. 2C). Both membrane responses were present in media containing TTX and tetraethylammonium (TEA, 2 x 10^{-2} M), the latter used to block voltage-sensitive potassium conductances (Fig. 2D). The CGRP-produced depolarization, measured in a normal Krebs solution, was associated with an increase in membrane input-resistance (about 20%, n=5). Enhanced excitability was manifested by an increased probability of action potential discharge, and by a significant increase in the number of spikes.
Fig. 1. Excitatory response to calcitonin gene-related peptide (CGRP) of a rat dorsal horn neuron. In A-B, bath application of CGRP (A, $10^{-8}$M; B, $5\times10^{-8}$M) for 3 min evoked a slow membrane depolarization, increase in synaptic activity and occurrence of spontaneous action potentials. C, in the presence of TTX ($5\times10^{-7}$M), Na-dependent action potentials and synaptic activity were eliminated, while the CGRP-induced depolarization remained. Insert shows approximate location of a dorsal horn neuron in the slice. The resting membrane potentials in A, B and C were -64, -66 and -58 mV, respectively. 17-day-old rat
Fig. 2. Biphasic membrane responses to CGRP of two rat dorsal horn neurons. Bath application of CGRP (A, $10^{-7}$M for 17 min; B, $10^{-6}$M for 1 min) induced a biphasic membrane response consisting of an initial hyperpolarization followed by a late appearance of a prolonged depolarization. C, both membrane responses were present, and even enhanced, when synaptic transmission and Na spikes were blocked by perfusing the slice with a tetrodotoxin (TTX) -containing Krebs solution. Resting potential -68 mV. 15-day-old rat. D, the CGRP-induced membrane changes were also present in media containing TTX ($5 \times 10^{-7}$M) and TEA ($2 \times 10^{-2}$M). Resting potential -60 mV. 16-day-old rat.
evoked in response to a constant current depolarizing pulse (Fig. 3). In addition anodal break spikes at the termination of hyperpolarizing pulse were frequently observed, also suggesting enhanced excitability. Similar effects of CGRP were observed in myenteric ganglia neurons of the guinea pig (Palmer et al., 1986). In contrast Twery and Moss (1985) observed that inhibition is the predominant effect of iontophoretically applied CGRP in the rat forebrain neurons. Few cells, however, were excited.

Since in the spinal dorsal root ganglion, immunoreactive CGRP coexists with immunoreactive substance P in some small cells (Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984; Lee et al., 1985) sensitive to capsaicin (Skofitsch and Jacobowitz, 1985), we have also examined chemical sensitivity of individual dorsal horn neurons to both peptides. As seen in Fig. 4, SP appears to be a more potent depolarizing agent than CGRP. In the peripheral nervous system CGRP has several actions differing from that of SP, for instance, increase in heart rate (Fisher et al., 1983; Mason et al., 1984) increase of sympathetic outflow (Fisher et al., 1983) or inhibition of gastric acid secretion (Hughes et al., 1984, Tache et al., 1984). However, it is of interest that spinal behavioral responses (biting and scratching of a hindlimb) are increased in intensity and duration with concomitant administration of SP and CGRP (Wiesenfeld-Hallin et al., 1984).

Discussion

We have recently reported that application of CGRP potentiates synaptic transmission measured intracellularly by recording spontaneous and
CGRP blocks accommodation of spike discharge in a dorsal horn neuron of a 16-day-old rat. Oscilloscope records of action potentials evoked by a current pulse of 0.4 nA and 1.5 s duration are illustrated. The left trace represents control response, the middle trace shows response obtained 3 min after onset of perfusion with CGRP, and the right trace represents response obtained 15 min after the removal of CGRP. Resting potential -58 mV
Fig. 4. Excitatory responses to substance P (SP, 10^{-7} M for 1 min) and CGRP (10^{-7} M for 3 min) in the same dorsal horn neuron of a 16-day-old rat. Resting potential -60 mV.
low and high threshold fast and slow excitatory postsynaptic potentials in the rat dorsal root ganglia-spinal cord slice preparation (Gerber et al., 1987; Ryu et al., 1987). The enhancement of synaptic transmission may be a consequence of modulation of calcium conductance (Nohmi et al., 1986), since we have also observed increased calcium action potential duration following administration of CGRP both in dorsal root ganglion cells and dorsal horn cells (Gerber et al., 1987).

In conclusion our experiments have revealed direct hyperpolarizing and depolarizing membrane actions of CGRP in neurons of the rat spinal dorsal horn. Our findings in agreement with reports of other neurochemical electrophysiological and behavioral effects of the peptide, indicate that CGRP, endogenous to the rat spinal cord, is capable of acting as neuromodulator both at presynaptic and postsynaptic sites.

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SECTION III. ACTIONS OF CALCITONIN GENE-RELATED PEPTIDE ON RAT SENSORY
GANGLION NEURONS

Summary

1. The membrane actions of calcitonin gene-related peptide (CGRP) and
the effect of CGRP on the Ca\(^{2+}\)-dependent action potential of rat
dorsal root ganglion (DRG) neurons have been studied by means of
an intracellular recording technique in isolated DRG of 2-3 week
old rats in vitro.

2. Bath application of CGRP (10\(^{-8}\)-10\(^{-6}\)M for 1-5 min) elicited a slow
reversible hyperpolarization and this hyperpolarizing effect was
still observed in medium containing TTX and TEA. However, about
half of the large cells, classified by duration of action
potential, were depolarized by CGRP.

3. These membrane effects of CGRP were associated with an increase in
membrane input resistance (about 20%). In addition, CGRP
increased the duration of Ca\(^{2+}\)-dependent action potentials.

4. Our results are consistent with the role of CGRP as an excitatory
neurotransmitter or neuromodulator in DRG-spinal cord.

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Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide encoded by the calcitonin gene and produced in neural tissue by specific RNA processing (Amara et al., 1982; Rosenfeld et al., 1983). Immunocytochemical studies have demonstrated the presence of CGRP-like immunoreactivity (CGRP-IR) in different areas of the central and peripheral nervous system (Lundberg et al., 1979; Rosenfeld et al., 1983; Gibson et al., 1984; Skofitsch and Jacobowik, 1985; Franco-Cereceda et al., 1987). In the dorsal root ganglia CGRP immunoreactivity was observed in most of the small and some of the intermediate sized cells. There is a considerable degree of co-existence between CGRP and substance P (SP) in the dorsal root ganglia (Gibson et al., 1984; Lee et al., 1985; Franco-Cereceda et al., 1987). Dorsal rhizotomy caused a significant loss of CGRP-immunoreactive fibers from the dorsal horn of the spinal cord. On the basis of these results Gibson et al. (1984) suggested that the major origin of CGRP in the dorsal spinal cord is from primary afferent fibers deriving from the dorsal root ganglion cells. After capsaicin treatment the content of CGRP was reduced by 60% in the dorsal part of the spinal cord (Franco-Cereceda et al., 1987). Thus the distribution of CGRP suggests a role in sensory transmission, and central administration of CGRP has recently been shown to have an antinociceptive effect (Bates et al., 1984). It is of interest that spinal behavioral responses (biting and scratching of hindlimb) are increased in intensity and duration on administration of SP and CGRP (Wiesenfeld-Hallin et al., 1984). However, inhibitory effects of CGRP have been reported in forebrain neurons (Twery
and Moss, 1985). Using synaptosomal membrane preparations, high affinity binding sites for CGRP have been demonstrated both in rat and in human CNS (Rizzo and Goltzman, 1981; Tschopp et al., 1984, 1985; Goltzman and Mitchell, 1985). Release of CGRP-like immunoreactivity was demonstrated from cultured rat trigeminal ganglion cells (Mason et al., 1984) and also in response to capsaicin or high K+ from slices of rat spinal cord (Saria et al., 1986; Oku et al., 1987).

Primary afferent neurons of mammalian dorsal root ganglia (DRG) can be classified on the basis of the conduction velocity of their peripheral axon branch as either C cells, which have small unmyelinated axons, or A cells, which have larger myelinated axons. By 14-day postnatal, the subclass of cells conducting at under 0.5 m/s constitutes the mature non-myelinated population (C cells) while the remaining cells are A cells, the axons of which have become myelinated to varying degrees. In spinal ganglia of adult rats the electrical properties of the soma membrane of A and C cells differ, especially with respect to the properties of their action potentials (Yoshida and Matsuda, 1979; Harper and Lawson, 1985; Rose et al., 1986). The membrane properties of dorsal root ganglia display a number of active electrical properties, including mixed sodium and calcium action potentials (Dichter and Fischbach, 1977) and anomalous and delayed rectification (Ito, 1957; Czeh et al., 1977). In the present work, the effects of CGRP have been examined on the passive and active membrane properties of 2-3 week old DRG neurons.
Methods

Sprague-Dawley rats, 2-3 weeks old, were anesthetized with ether and laminectomy was performed to expose the ganglia of the lower lumbar region. One to two ganglia without dorsal roots were removed and placed in a Krebs solution aerated with a mixture of 95% O₂ and 5% CO₂ for 1 hour. After incubation, ganglia were transferred to the recording chamber where they were continuously perfused with oxygenated Krebs solution of the following composition (mM): NaCl, 127; KCl, 1.9; KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; glucose, 10, at 33 ± 1 °C and a flow rate of about 3.6 ml per min. The recording chamber had a capacity of 0.5 ml. When tetraethylammonium chloride (TEA) was added (2×10⁻² M), the sodium chloride concentration was lowered to maintain constant osmolarity. Standard electrophysiological techniques were used for intracellular recording and stimulation via an electrometer with a bridge circuit that allowed current injection and voltage recording. Neurons were impaled at random throughout the ganglia with microelectrodes containing either 3 M potassium acetate (DC resistances, 80-100 MΩ) or 3 M caesium acetate (60-90 MΩ). Electrical properties of DRG cells were determined by means of a high-input impedance bridge amplifier (Dagan, 8100) 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 2200S) or stored on the disks of a Nicolet digital oscilloscope (model 4094) until processed and printed out onto a Digital plotter. We used synthetic rat CGRP from Cambridge Research Biochemicals or Peninsula Labs.
Results

The results are based on observations made on sixty-seven DRG neurons having mean resting membrane potential of \(-66 \pm 11.3\) mV, \((\text{mean} \pm \text{SD}, n = 33)\) for the cells with a short-duration action potential and \(-51.2 \pm 4.8\) mV \((n = 6)\) for the cells with a long-duration action potential. Input resistance values ranged from 30 to 103 MO; the mean input resistance of cells having a long-duration action potential was at least double \((70 \pm 23 \text{ MO})\) that of cells with a short-duration action potential. The mean amplitudes of the somatic action potential recorded were \(77.6 \pm 11.5\) mV \((n = 39)\) and \(90 \pm 11.2\) mV \((n = 6)\) for the cells with a short and a long-duration action potentials, respectively. We have differentiated cells by the characteristics of their action potentials, such as duration measured at the half amplitude of action potential and presence or absence of inflection on the repolarization phase. In our work two groups of cells were considered. One had slow action potentials \((\text{range} 3-6 \text{ ms})\) and displayed an inflection on the falling phase of the action potential; another had relatively fast action potentials \((\text{less than} 3 \text{ ms})\) and rarely displayed an inflection on the falling phase of the action potential. Slow action potentials have previously been reported in slowly conducting DRG neurons in rat \((\text{Williams and Zieglgansberger, 1981; Harper and Lawson, 1985})\). The inward calcium current has been proposed to underlie the presence of the inflection in cultured DRG neurons of the chick and mouse \((\text{Matsuda et al., 1976; Dichter and Fischbach, 1977; Yoshida et al., 1978; Heyer and Macdonald, 1982})\).

Bath-applied CGRP \((10^{-8} \text{ to } 2 \times 10^{-6} \text{M for 1-3 min})\) evoked a slow,
reversible, dose-dependent hyperpolarization in both small (-5.6 ± 2.1 mV), and large DRG cells (-2.1 ± 0.6 mV), as illustrated in Fig. 1A. In addition, several large DRG neurons were depolarized by CGRP (4.6 ± 1.5 mV). The CGRP-produced hyperpolarization was associated with reversible increase in membrane input resistance (121.7 ± 18.9%, n = 6) measured from the linear portion of current voltage curves. In the normal Krebs solution application of CGRP produced an increase in the duration of action potential (measured as time from peak to half decay) in two out of four small cells. Since the action potentials of small DRG cells are produced by increases in both sodium and calcium conductances (Heyer and Macdonald, 1982) and since transmitter release is thought to vary exponentially with the Ca^{2+} current, small changes in duration, without altering the spike amplitude, could have large effects on transmitter release (Hochner et al., 1986).

Action potentials in DRG neurons have a Ca^{2+} component which can be enhanced by the use of K^{+} channel blockers (Dichter and Fischbach, 1977; Dunlap and Fischbach, 1981). We examined action of CGRP on the calcium-dependent action potential since several putative neurotransmitters, including peptides, are known to modify the duration of calcium action potentials in chick and mouse DRG neurons (Dunlap and Fischbach, 1978; Werz and Macdonald, 1982). Ca^{2+} spikes were recorded in the presence of TTX (3x10^{-7} M) and TEA (2x10^{-2} M) to block sodium and potassium conductances, respectively. In addition we used caesium acetate-filled electrodes in the presence of TTX and TEA to block some of TEA-resistant potassium conductances. When DRG cells were filled with Cs+ by passing depolarizing current pulses (0.2-1 nA, 300 ms duration, 0.1 Hz
Fig. 1. A typical CGRP-induced hyperpolarization of a DRG neuron in a 14-day-old rat. A Ca\textsuperscript{2+} spike was evoked every 40 sec by applying a short depolarizing current pulse (1.4 nA, 5 ms) in a medium containing TTX (5x10\textsuperscript{-7} M) and TEA (2x10\textsuperscript{-2} M). Recording electrode was filled with 3 M caesium acetate. A, Bath application of CGRP (5x10\textsuperscript{-7} M for 3 min) hyperpolarized the neuronal membrane and markedly increased the duration of Ca\textsuperscript{2+} spike. V\textsubscript{m} = -51 mV. B, Initial five spikes taken before (a, b) and during (c, d and e) application of CGRP are shown at a faster time base.
CGRP $5 \times 10^{-7}$ M

20 mV

100 ms

1 min

20 ms
for 2-5 min) the duration of Ca\textsuperscript{2+} spikes was further prolonged and the afterhyperpolarization was blocked. The degree of prolongation depended on the frequency of stimulation and the membrane potential at which the cell was held (McBurney and Neering, 1985). However, in 5 cells in which calcium spikes were evoked every 60 to 90s and the resting membrane potential was more negative than -60 mV, CGRP (10\textsuperscript{-8} to 10\textsuperscript{-6} M) produced a significant, dose-dependent increase in the duration of Ca\textsuperscript{2+}-spike (on average 168 ± 41%, n=5), and also raised the height of the plateau phase of Ca\textsuperscript{2+} spike (Figs. 1A-B). The effect was only in part reversible. Similar increases in Ca\textsuperscript{2+}-spike duration have been reported in cat parasympathetic ganglia neurons (Nohmi et al., 1986).

Discussion

In principle, the CGRP-induced increase in Ca\textsuperscript{2+} spike duration might be due to an effect on inward Ca\textsuperscript{2+} current or outward K\textsuperscript{+} currents. It has been reported that the early phase of the DRG soma spike is dominated by a relatively large inward Na\textsuperscript{+} current, whereas plateau is due in large part to an inward Ca\textsuperscript{2+} current (Dichter and Fischbach, 1977). Thus an increase in spike duration by CGRP may involve an increase in Ca\textsuperscript{2+} entry. It is not clear, however, whether CGRP affects Ca\textsuperscript{2+} channels directly, since a decrease in outward K\textsuperscript{+} current could produce the same result. The direct test of these possibilities and identification of ionic current(s) involved are to be accomplished only with a voltage-clamp analysis.

The presence of calcium-dependent action potentials in DRG neurons may have relevance for the study of presynaptic mechanisms. Transmitter
release from synaptic terminals requires $\text{Ca}^{2+}$, and $\text{Ca}^{2+}$ influx into synaptic terminals has been demonstrated at the squid giant synapse (Katz and Miledi, 1967; Llinás et al., 1976). However, intracellular recordings cannot be made from most presynaptic terminals and thus the calcium entry and calcium conductance changes cannot be directly studied. Dunlap and Fischbach (1978) have shown that some putative neurotransmitters ($\gamma$-aminobutyric acid, norepinephrine and 5-hydroxytryptamine) and some neuropeptides (met-enkephalin and somatostatin) reduce the duration of the calcium component of the ganglion neuron action potentials, suggesting that these compounds may produce inhibition, at least in part, by reducing transmitter release. In addition, mouse and rat DRG neurons grown in primary dissociated cell culture have been used to study neurotransmitter and neuropeptide actions on $\text{Ca}^{2+}$-dependent action potentials and currents (Mudge et al., 1979; Forda and Kelly, 1981; Heyer and Macdonald, 1982; Werz and Macdonald, 1982; Fedulova et al., 1985; Dolphin et al., 1986).

Little is known, presently, of the nature of the CGRP receptors involved or the ionic mechanism of CGRP actions upon neurotransmitter release in the CNS. Increase in presynaptic $\text{Ca}^{2+}$ entry by CGRP would result in an increase in neurotransmitter release. In agreement with this possibility we have recently observed that CGRP increases $\text{Ca}^{2+}$ current in voltage-clamped DRG neurons and also causes an enhancement of excitatory synaptic transmission in rat dorsal horn (Gerber et al., 1987; Ryu et al., 1987).
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References


SECTION IV. CALCITONIN GENE-RELATED PEPTIDE ENHANCES CALCIUM CURRENT OF RAT DORSAL ROOT GANGLION NEURONS AND SPINAL EXCITATORY SYNAPTIC TRANSMISSION

Summary

1. The actions of calcitonin gene-related peptide (CGRP) were examined on Ca$^{2+}$-dependent action potentials and voltage-dependent Ca$^{2+}$ currents in rat dorsal root ganglion (DRG) neurons in vitro. In addition, we tested the effect of CGRP on excitatory synaptic transmission in the rat spinal dorsal horn.

2. CGRP produced a reversible increase in the amplitude and the duration of the Ca$^{2+}$ spike of DRG neurons and directly increased the voltage-dependent Ca$^{2+}$ current by enhancing both the transient and the sustained components of the current.

3. The increase in the Ca$^{2+}$ current is likely to be responsible for the increase in the Ca$^{2+}$ spike and facilitation of excitatory synaptic transmission.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide formed in neural tissue by alternative splicing of mRNA of the calcitonin gene (Rosenfeld et al., 1983). The presence of CGRP-like immunoreactivity

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(CGRP-LI) in rat dorsal root ganglion (DRG) neurons and the spinal dorsal horn has been demonstrated (Gibson et al., 1984). Both CGRP binding sites (Henke et al., 1985) and CGRP-LI (Gibson et al., 1984) exist in high concentrations in the superficial layers of the spinal dorsal horn, an area where primary afferent fibers are known to terminate. Dorsal root rhizotomy causes a significant loss of CGRP-LI from the dorsal horn, the finding suggesting that the major origin of CGRP in this area is from primary afferent fibers deriving from DRG cells (Gibson et al., 1984). These findings are consistent with the possibility that one of physiological functions of CGRP might be to modulate neurotransmitter release and synaptic transmission. However, little is presently known about the actions of CGRP in DRG neurons or spinal excitatory synaptic transmission. Here, using intracellular recording techniques, we report that CGRP augments Ca\(^{2+}\)-dependent inward current in immature rat DRG neurons and enhances excitatory synaptic transmission in the spinal dorsal horn.

Methods

Experiments were performed on isolated preparations of lower lumbar (L4-L6) dorsal root (spinal) ganglia attached to a length of dorsal roots (7-15 mm) and horizontal spinal cord slices with attached dorsal roots and ganglia prepared from 14-28 days-old rats (Murase and Randić, 1983; Ryu et al, 1988). Intracellular current-clamp and single-electrode voltage-clamp recordings were made from DRG neurons, having conduction velocities between 4.6 and 19.6 m/s, using an Axoclamp II amplifier. The recording methods
were the same as those described previously (Murase et al., 1986). The $\text{Ca}^{2+}$-dependent action potentials were elicited by direct stimulation through the recording electrode. Tetrodotoxin (TTX, $5 \times 10^{-7}$ M) was added regularly into the superfusate to suppress the fast voltage-dependent Na$^+$ current and tetraethylammonium (TEA, $2 \times 10^{-2}$ to $10^{-1}$ M) to reduce voltage-dependent K$^+$ currents. When TEA was added, the NaCl concentration was lowered to maintain constant osmolarity. In some experiments KCl in the Tris-buffered Krebs solution (containing 26 mM NaCl) was replaced with CsCl and DRG cells were regularly impaled with micropipettes filled with 3 M CsCl (d.c. resistances 30-50 MΩ) or 3-5 M caesium acetate (60-90 MΩ) to further inhibit voltage-dependent K$^+$ currents and thus predominantly isolate the $\text{Ca}^{2+}$ current. A co-axial stainless-steel stimulating electrode was placed on a DRG to orthodromically stimulate (20 V pulses of 0.5 ms duration) superficial dorsal horn neurons and spontaneous and evoked excitatory postsynaptic potentials (e.p.s.p.s) were recorded with microelectrodes filled with 4 M potassium-acetate. Synthetic rat CGRP (Cambridge Research Biochemicals, Peninsula Labs) was applied into the bath in known concentration.

Results

We first examined the effect of CGRP on $\text{Ca}^{2+}$-dependent action potentials in DRG neurons under conditions in which K$^+$ currents were minimized. CGRP ($10^{-8}$ to $10^{-6}$ M) increased the duration (Fig. 1A) and amplitude (Fig. 1B) of the $\text{Ca}^{2+}$ spike, the latter by about 70% (Ryu et al., 1988). The effect of CGRP was in part reversed by washing the preparation
Fig. 1. Effects of CGRP on Ca\textsuperscript{2+}-dependent action potentials and Ca\textsuperscript{2+}-dependent inward current in three different rat DRG neurons bathed in a TTX (5x10\textsuperscript{-7} M)- and TEA (2x10\textsuperscript{-2} M)-containing solution. A, CGRP (5x10\textsuperscript{-7} M for 2.3 min) reversibly increases both the duration and the plateau of the action potential. The action potentials were evoked by depolarizing current pulses (2.5 nA, 5 ms in duration, applied every 50 s as shown in insert) in the absence (1) and 1.5 min (2), 2.5 min (3) and 4 min (4) following CGRP. The resting membrane potential was -71mV. B, Faster speed records of the initial phase of the action potential shown in A indicate that CGRP increases the peak of the action potentials. C, In a voltage-clamped DRG neuron, both the initial transient and later sustained components of the inward current (upper records) evoked by 0.5 s depolarizing commands to -25 mV from the holding potential of -63 mV were augmented by CGRP (3x10\textsuperscript{-7} M for 3.3 min). D, Plot of peak inward current against command potential obtained from the same neuron shown in C. E, The effect of CGRP on the inward current is slow in onset and recovery. Voltage commands (200 ms) to -35 mV were given at 20 s intervals from a holding potential of -73 mV to evoke the inward current. The effect of a 2 min exposure to 10-7M CGRP is plotted as a function of time for the peak current. TTX (5x10\textsuperscript{-7} M), TEA (8x10\textsuperscript{-2} M), Tris-HCl (5x10\textsuperscript{-3} M) and Mg\textsuperscript{2+} (2.6x10\textsuperscript{-3} M)-containing solution
for 20 minutes.

We next analyzed the effect of CGRP on somatic membrane currents present in DRG neurones using voltage-clamp technique. Figure 1C (upper record) shows an example of the inward current recorded during depolarizing voltage-clamp pulses from -63mV (about resting level) to -25mV in a DRG cell impaled with Cs⁺-filled microelectrode and bathed in TTX/TEA solution in order to block voltage-dependent Na⁺ and K⁺ conductances. The inward current appeared to consist of two components, an initial rapidly decaying component followed by a more sustained component. This pattern is similar to that described in chick and rat DRG neurons (Carbone and Lux, 1984; Bossu et al., 1985; Fedulova et al., 1985; Nowycky et al., 1985). CGRP (5x10⁻⁸ to 5x10⁻⁷M) consistently increased the amplitude of both components of the inward current (Fig. 1C, middle trace) in 48% of examined cells (n=54). The amplitude of the transient component of the inward current increased by an average of 52% (± 11%, s.e.m., n=13). The effect of CGRP occurred within 90 to 120 s of the start of the bath application (Fig. 1E) and the inward current recovered to 117.0% (± 8.0%, n=13) of the control current amplitude within 20 min of the washout of the peptide. Enhancement of the inward current was accompanied by a small increase in "leak" current and by a slight inward shift in the holding current. In a few cells, however, a reduction in inward current by an average of 16% (± 2.8%, n=4) and an outward shift in holding current was observed. Responses to hyperpolarizing pulses were not modified by CGRP (Fig. 1C, lower records).

The current-voltage relationship illustrated in Fig. 1D suggests that CGRP may increase the inward current carried through Ca²⁺ channels. When CGRP (3x10⁻⁷M) was applied, there was no change in the hyperpolarizing
Fig. 2. Effect of CGRP on the membrane current recorded from a voltage-clamped DRG neuron bathed in a Ba$^{2+}$- or Co$^{2+}$-containing solution. To isolate the inward current flowing through the Ca$^{2+}$ channels, the neurons were bathed in a solution containing TTX (5x10$^{-7}$ M), TEA (10$^{-1}$ M), low Na$^+$ (26 mM Na$^+$ left, the rest replaced by Tris chloride), zero K$^+$ (K$^+$ replaced with 2 mM Cs$^+$), and in which Ca$^{2+}$ was replaced either with Ba$^{2+}$ (left two records) or Co$^{2+}$ (right two records). A, CGRP (5x10$^{-7}$ M for 2 min) increased the amplitude of both the transient and sustained components of the inward current (left two records). Co$^{2+}$ (2x10$^{-3}$ M) abolished the inward current; in this solution CGRP was ineffective. B, Current-voltage curves obtained in the same neuron before (open symbols) and after (filled symbols) the application of CGRP in Ba$^{2+}$- (circles) and Co$^{2+}$- (triangles) containing solutions. The Ba$^{2+}$ current component, represented by the difference I-V curve constructed by subtracting the I-V curve obtained in the Co$^{2+}$-containing solution from the I-V curve obtained in the Ba$^{2+}$-containing solution, was augmented by CGRP without alteration in the voltage dependence of the current activation. This finding suggested the direct action of CGRP on the Ca$^{2+}$ channels.
portion of the current-voltage curve but there was an increase in the inward current recorded at all voltages above -45mV.

Since the effect of CGRP on inward current in DRG neurons could be caused either by the enhancement of a voltage-dependent Ca\(^{2+}\) conductance or by the reduction of voltage- and/or Ca\(^{2+}\)-dependent K\(^{+}\)-conductance, we measured Ba\(^{2+}\) current in an attempt to identify the ionic mechanism underlying the effect of CGRP. In a TTX/TEA/Ba\(^{2+}\)/Cs\(^{+}\)/low Na\(^{+}\)-containing solution, CGRP (3x10\(^{-7}\)M) elicited an even greater increase in both transient (63.6 ± 23.0\%, n=9) and sustained components of the inward current (Fig. 2A). When the inward Ba\(^{2+}\) current was blocked by Co\(^{2+}\) (2-3 mM, n=4), a Ca\(^{2+}\) channel blocker, the remaining current was unaffected by CGRP (Fig. 2A). It can be seen that CGRP produced an increase in the peak amplitude of the Ba\(^{2+}\) current throughout the membrane potential range over which the current was recorded (Fig. 2B). Furthermore, the membrane potential at which the inward current became maximal in the presence of CGRP (3x10\(^{-7}\)M) was the same (between -15 and -10 mV) as in the absence of the peptide (Fig. 2B). These results are consistent with the possibility that CGRP acts to increase voltage sensitive Ca\(^{2+}\) current, and the increase in the Ca\(^{2+}\) current is likely to be responsible for the increase in the Ca\(^{2+}\)-spike.

As Ca\(^{2+}\) influx is intimately related to neurotransmitter release (Katz and Miledi, 1967), a similar action of CGRP on voltage-sensitive Ca\(^{2+}\) channels at central terminals of DRG neurons, as described for the somatic membrane of DRG neurons, could increase neurotransmitter release and facilitate excitatory synaptic transmission. In support of this hypothesis we observed that CGRP facilitates fast excitatory synaptic transmission
Fig. 3. CGRP increases excitatory synaptic transmission in the spinal dorsal horn. A, Spontaneous postsynaptic potentials (p.s.p.'s) recorded from a dorsal horn neuron, in the absence of electrical stimulation of DRG, are shown before and after CGRP at the times indicated on the records. CGRP (10^{-6} M for 1 min) reversibly increased the frequency and the amplitude of the spontaneous p.s.p.'s. Resting membrane potential was -58 mV, 15-day-old rat. B, In another dorsal horn neuron, CGRP (10^{-7} M for 14 min) reversibly augmented the amplitude of the e.p.s.p. The responses at 9, 20, 28 and 38 min after the application of CGRP are shown
Bath-application of CGRP (10^{-7} to 10^{-6}M) caused a pronounced and long-lasting increase in the amplitude of the fast excitatory synaptic potential (e.p.s.p.) recorded in a dorsal horn neuron in response to high intensity electrical stimulation of a lumbar dorsal root (Fig. 3B). The average increase in e.p.s.p. amplitude was 64.4 ± 11.7% (n=16). The onset of the CGRP effect varied from 5 to 20 min in the different dorsal horn neurons and the effect lasted up to 40 min. CGRP also caused a marked increase (n=8) in the frequency and amplitude of presumptive spontaneous e.p.s.p.s. (Fig. 3A).

Discussion

These results demonstrate that CGRP increases the voltage-dependent Ca^{2+} current, by a direct action. This effect of CGRP may be responsible for facilitation of excitatory synaptic transmission, although postsynaptic mechanism is not excluded.

The modulatory effect of several inhibitory neurotransmitters and neuropeptides on the Ca^{2+} current in DRG neurons is to decrease this current and, consistent with this effect, to decrease the duration of DRG Ca^{2+} spike and neurotransmitter release (Dunlap and Fischbach, 1978, 1981; Mudge et al., 1979; Dolphin et al., 1986; Gross and Macdonald, 1987). This paper is the first to describe a peptide-induced increase of Ca^{2+} current in either DRG neurons or any vertebrate neurons. The finding that the activation of CGRP receptors in the somatic membrane of immature rat DRG neurons enhances both the transient and sustained components of the Ca^{2+} current is of interest. By analogy with results from chick DRG cells, it
may be assumed that the transient component of the rat DRG current is due primarily to the activation of N channels, while the sustained component is probably due to the activation of L channels, with a small residual of a non-inactivating N current (Carbone and Lux, 1984; Bossu et al., 1985; Fedulova et al., 1985; Nowycky et al., 1985). However, the direct proof that any particular type of Ca\(^{2+}\) channel is being modulated by CGRP must await further single channel analysis.

Molecular mechanisms underlying the CGRP-induced enhancement of the Ca\(^{2+}\) current, and the facilitation of excitatory synaptic transmission, have yet to be elucidated. By analogy with β-adrenergic receptor-mediated increases in Ca\(^{2+}\) current in myocytes (Reuter, 1983) and in hippocampus (Gray and Johnston, 1987), the involvement of cyclic AMP-mediated phosphorylation of voltage-sensitive Ca\(^{2+}\) channels might be a possibility. In this context, it is of interest that several actions of CGRP in peripheral tissues and spinal motoneurons (J-P. Changeux, personal communication, Neurobiologie Moleculaire, Inst. Pasteur, Paris) are thought to be mediated through activation of adenylate cyclase (Crossman et al., 1987), and if a similar mechanism operates in the spinal and sensory neurons, it could explain some of the observed actions of CGRP. Although intracellular cyclic AMP has been shown to enhance Ca\(^{2+}\) current in rat DRG neurons (Fedulova et al., 1985), forskolin, an activator of adenylate cyclase, appears to prolong the Ca\(^{2+}\) spike by inhibiting K\(^{+}\) currents (Dunlap, 1985).

The CGRP-induced enhancement of voltage-sensitive Ca\(^{2+}\) current might be of physiological relevance for the well-known capability of chemical synapses to undergo plastic changes in synaptic effectiveness important in
the processes underlying transmission and integration of sensory information.

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References


DISCUSSION

The major points pertaining to the results obtained in this thesis have been already discussed in the Results section in detail. This chapter considers some technical limitations of single-electrode voltage-clamp technique in the slice preparation and offers some speculative ideas on specific data presented in the Results.

Single-Electrode Voltage-Clamp Technique in the Spinal Cord Slice Preparation

In order to evaluate the quality of the voltage-clamp data, we needed to assess first the degree to which the non-ideal conditions influence the interpretation of the measurements. Non-ideal conditions are always present when a voltage clamp is applied to a neuron with non-isopotential dendritic processes. Ideal voltage-clamp conditions imply that the voltage across the plasma membrane has a constant and known value. A perfect space-clamp is achieved when a voltage-clamp is applied to that portion of plasma membrane through which current is uniformly distributed (Cole, 1968; Carnevale and Johnston, 1982). Only under these conditions will the measured current be proportional to membrane conductance. Unfortunately, in few experimental situations, in particular in branched mammalian central neurons, are perfect voltage- and space-clamp conditions achieved. The important issue was then to identify as early as possible voltage- and space-clamp problems under our experimental conditions and try to estimate the magnitude of errors in the measurements.
We found that the ability of the Axoclamp-2 system to clamp the soma of a dorsal horn cell critically depended on the electrode resistance and the tuning of the electronic controls. From our data, it is evident that the recorded voltage steps follow the rectangular shape reasonably well. It is of interest to mention that the Dagan system (Model 8100 used in our early experiments) can point-clamp bullfrog ganglion cell body even in the presence of ionic currents (e.g., M current) 10- to 20-fold larger than those encountered in CA1 pyramidal cells (Halliwell and Adams, 1982) and spinal dorsal horn neurons (Murase et al., 1986). It is clear also that the CA1 cells were successfully clamped using microelectrodes of 60-100 MΩ, the values we also used in our experiments. In addition, because of the smaller size of dorsal horn cells (in comparison to CA1 cells), smaller currents were required to clamp their membrane potential.

We have examined passive electrical properties of the dorsal horn cells (Laminae I-III) and found that they are in a reasonable agreement with those expected for an isopotential "somatic" region connected to a short equivalent cable. We have estimated that the electronic length of the equivalent cable (see Rall, 1977) is about 0.9, which means that the dorsal horn cells of young rats (having smaller dendritic trees) are relatively compact electrically and that in the steady-state, most of the recorded current flows through the membrane which is close to the measured point. Although, of course, there will always be attenuation of voltages between the soma and the distal dendrites, even if the cable represented by the neuron is purely passive, this attenuation (acting as a filter) reduce the fidelity of the voltage clamp measurement, specially for high frequency events. Since the calcium currents in the rat dorsal root ganglion and
dorsal horn cells have time constants of several tens to hundreds of milliseconds, a reasonably accurate measurement was possible.

On the whole, our results obtained in the spinal slice preparation with single-electrode voltage-clamp technique illustrate that satisfactory qualitative analysis of voltage-dependent Ca\(^{2+}\) conductances can be achieved by a combined use of these techniques. Since the high-voltage activated Ca\(^{2+}\) conductances were recorded by voltage-clamp applied across the soma membrane of rat dorsal horn neurons having attached dendritic trees and axons, there is a real possibility that the Ca\(^{2+}\) currents somatically recorded are contributed by Ca\(^{2+}\)-dependent responses in non-clamped dendritic regions of the cell (Brown and Johnston, 1983; Johnston and Brown, 1983). This question could be resolved in a future by a demonstration of single Ca\(^{2+}\) channels which could account for the Ca\(^{2+}\) currents recorded at soma. Experiments with acutely-isolated rat spinal dorsal horn neurons using the patch-clamp and whole-cell voltage-clamp analysis (Murase et al., 1989) recently undertaken in Dr. Randić’s laboratory will hopefully resolve this question and improve the extent of quantitative kinetic analysis of the ionic current as well.

Low- and High-Voltage-Activated Ca\(^{2+}\) Currents

Based on the voltage- and time-dependent properties of activation and inactivation, and the sensitivities to organic and inorganic Ca\(^{2+}\) channel agonists and antagonists, we have demonstrated the presence of three components of Ca\(^{2+}\) current in the spinal dorsal horn neurons of young rats. Although our results on the kinetic properties of the three components of
Ca\textsuperscript{2+} current are roughly in agreement with those reported by Nowycky et al. (1985) and Fox et al. (1987a) in cultured chick sensory ganglion neurons, there are some important differences. As shown in Figs. 3 and 8 in Section I, the threshold and peak voltages in the current-voltage relationships of the low- and high-voltage activated Ca\textsuperscript{2+} currents appear to shift in a negative direction of the membrane potential range when compared to those obtained from chick sensory neurons (Fox et al., 1987a). The voltage-dependence of activation of a high-threshold transient Ca\textsuperscript{2+} current cannot be distinguished well from the voltage properties of a low-voltage activated Ca\textsuperscript{2+} current. At the membrane potentials positive to -40 mV, the high-voltage activated Ca\textsuperscript{2+} current was co-activated with the low-voltage activated Ca\textsuperscript{2+} current. The negative shift of the I-V curve and the absence of the "shoulder" in the I-V curve (Fig. 3 in Section I) were probably due to one or more of following: 1. The use of Ba\textsuperscript{2+} as a charge carrier for Ca\textsuperscript{2+} channel (Kostyuk et al., 1981), 2. The cellular properties of dorsal horn neurons such as the buffering capacity of intracellular Ca\textsuperscript{2+} ions (Jia and Nelson 1986), 3. The presence of a residual outward K\textsuperscript{+} current due to its incomplete suppression by internal Cs\textsuperscript{+} and external Cs\textsuperscript{+} and TEA. However, the third reason, alone, is not the likely possibility because a similar observation was made recently in acutely-isolated spinal dorsal horn projection neurons under whole-cell voltage-clamp condition (Huang, 1988).

Although voltage dependence of activation and inactivation of the three components of Ca\textsuperscript{2+} currents suggest the presence of three populations of Ca\textsuperscript{2+} channels in the membrane of immature rat dorsal horn neurons, our present data do not corroborate the conclusion in favor of three different
types of Ca$^{2+}$ current since the kinetic properties of the three components frequently overlap. The question about the identity of the Ca$^{2+}$ channels in the membrane of rat spinal dorsal horn neurons will be more adequately addressed in our future single-channel recordings of different types of Ca$^{2+}$ channels. It is of interest that the question of the identity of N-type Ca$^{2+}$ current has not been yet resolved even in sensory neurons (Leonard et al., 1987; Swandulla and Armstrong, 1988).

**Functional Significance of Multiple Ca$^{2+}$ Conductances**

Functional significance of the multiple components of the Ca$^{2+}$ current has not been well established yet. Some possibilities for their physiological involvement were discussed in the Discussion part of Section I. While the low-voltage activated Ca$^{2+}$ conductance may play an important physiological role in near-threshold phenomena and in regulation of neuronal excitability, the slower high-threshold Ca$^{2+}$ conductances corresponding to N and L currents, are evoked by strong depolarizations and functionally may be involved in generation of high-threshold Ca$^{2+}$ spikes (Llinas and Sugimori, 1980; Llinas and Yarom, 1981; Murase and Randić, 1982, 1983) and neurotransmitter release (Miller, 1987a,b; Tsien et al., 1988).

Under normal physiological conditions only certain neurons can fire Ca$^{2+}$-dependent action potentials. However, more frequently, neurons do not fire Ca$^{2+}$ spikes under normal conditions, although voltage-sensitive Ca$^{2+}$ channels are clearly present and can be revealed using various pharmacological means, as in this work. Influx of Ca$^{2+}$ through such
channels may play a key role in the regulation of $Ca^{2+}$-sensitive conductances.

We know that several kinds of ion channels can be regulated by changes in the intracellular levels of $Ca^{2+}$. These include various types of $Ca^{2+}$-sensitive $K^+$ conductances (Eckert and Lux, 1976; Barrett, Barrett and Crill, 1980; Schwindt and Crill, 1980a,b; Brown and Griffith, 1983a; Segal and Barker, 1986; Huang, 1987), $Ca^{2+}$ sensitive chloride conductance (Mayer, 1985; Owen et al., 1986) and a non-specific cationic conductance (Kramer and Zucker, 1985). The other observed phenomenon of an afterhyperpolarization (AHP) following a spike is the result of the activation by $Ca^{2+}$ of a type of $K^+$ channel (Llinas and Walton, 1980). Activation of such channels subsequent to $Ca^{2+}$ influx alters the state of excitability of the neurons and is an important factor in determining spike discharge rate.

Apart from the regulation of membrane excitability, the role of $Ca^{2+}$ as a trigger for neurotransmitter release is well known. L and N types of $Ca^{2+}$ channels can be involved in determination of the type of neurotransmitter released (Turner and Goldin, 1985; Reynolds et al., 1986; Thayer et al., 1987; Lipscombe et al., 1988). It is known that conventional transmitters such as norepinephrine and acetylcholine are localized in both small and large vesicles, but neuromodulators such as peptides are found only in large dense-core vesicles. Based on this morphological finding, Hökfelt et al. (1986) suggested that differential release of co-existing messenger molecules is possible if mechanisms exist allowing selective activation of two types of vesicles on arrival of nerve impulses. The selective activation of vesicles could be frequency coded -
at low frequency of nerve impulse flow small vesicles release the classical transmitter, whilst at higher frequencies larger vesicles release both peptides and classical neurotransmitters (Lundberg and Hökfelt, 1983; Bartfai et al., 1988). In conjunction with this hypothesis, it would be important to know whether different types of calcium channels, which provide intracellular calcium signal essential for the release of neurotransmitters (Parnas and Parnas, 1986; Zucker et al., 1986; Zucker and Haydon, 1988), are selectively involved in the activation of the different vesicles. It is of relevance that N current was coupled with the release of norepinephrine in sympathetic neurones (Perney et al., 1986; Hirning et al. 1988) and acetylcholine from chicken brain synaptosomes and slices (Lundy et al., 1988), while L current is coupled to the release of substance P in sensory neurones (Perney et al., 1986; Rane et al., 1987).

Assuming that multiple types of Ca\(^{2+}\) channel current are selectively involved in the activation of different types of synaptic vesicles which contain different types of transmitters, it is necessary to find the physiological signals that can recruit a specific type of Ca\(^{2+}\) current (Bartfai et al., 1988). The co-existence of high-voltage activated transient and sustained components of Ca\(^{2+}\) channel currents may allow certain impulse pattern to activate a particular type of Ca\(^{2+}\) channel selectively thereby producing a specific response. Such relationship may be seen in systems where the pattern of presynaptic activity can determine the type of neurotransmitter released (Ip and Zigmond, 1984).
Modulation of $\text{Ca}^{2+}$ Currents by Neurotransmitters and Peptides

One of the most important aspects of research in the area of neuronal $\text{Ca}^{2+}$ channels concerns their possible regulation by neurotransmitters and peptides. Such effects are of fundamental importance in any consideration of the regulation of interneuronal communication. It is by such mechanisms that neuromodulators may modify both their own release and the release of other neurotransmitters and generally modify neuronal excitability directly.

In recent years considerable data have been accumulated indicating that various neuromediators, including several peptides, can modulate voltage-dependent $\text{Ca}^{2+}$ channels. The first observation supporting this idea was made on cardiac muscle fibers where activation of $\beta$-adrenergic receptors by epinephrine resulted in an increase in $\text{Ca}^{2+}$ current (Reuter, 1983; Tsien, 1983). Patch clamp studies of cardiac muscle cells have shown that $\beta$-adrenergic agonists produce an increase in the average number of functional $\text{Ca}^{2+}$ channels and reduce the time course of their activation and inactivation (Bean et al., 1984). Norepinephrine, 5-hydroxytryptamine, dopamine, $\gamma$-aminobutyric acid, enkephalins, dynorphin and somatostatin are all found to decrease the duration of the somatic spike of cultured dorsal root ganglion cells (Dunlap and Fischbach, 1978; Dunlap and Fischbach, 1981; Galvan and Adams, 1982; Werz and Macdonald, 1982). The ionic mechanism of the opioid peptide actions appear to involve a reduction in $\text{Ca}^{2+}$ conductance (Gross and Macdonald, 1987).

Work in Dr. Randić's laboratory indicated that SP, NKA and CCK-8 cause a reversible decrease in the duration of $\text{Ca}^{2+}$ spike of dorsal horn cells,
Substance P modulates voltage-dependent Ca\(^{2+}\) Conductance

The principal action of substance P on central and peripheral neurons is to produce depolarization and to increase their excitability in such a way that repetitive firing is facilitated. Frequently neurons respond to SP by bursting type of discharge (Otsuka et al., 1975; Krnjevic, 1977; Otsuka and Konishi, 1977; Katayama and North, 1978, Katayama et al., 1979; Sastry, 1979; Ziegglansberger and Tulloch, 1979; Dun and Minota, 1981; Jan and Jan, 1982; Adams et al., 1983; Murase and Randić, 1984; Jones, 1985; Randić et al., 1987). However, conductance mechanism underlying the SP-induced depolarization appears to vary in different neurons. An increase in membrane input resistance in myenteric neurons in guinea-pig (Katayama and North, 1978; Katayama et al., 1979), and cat motoneurons and cuneate neurons (Krnjevic, 1977); a decrease in rat spinal motoneurons (Otsuka and Konishi, 1977), and a variable change in sympathetic neurons of the guinea-pig (Konishi et al., 1979; Dun and Minota, 1981) were observed.

Analysis of the ionic mechanism of the SP-induced depolarization in unclamped myenteric neurons of the guinea-pig led to the suggestion that the SP-induced depolarization is caused by an inactivation of a resting potassium conductance (Katayama and North, 1978; Katayama et al., 1979) or Ca\(^{2+}\)-sensitive K\(^+\) conductance (Galligan et al., 1986; Morita et al., 1986).

Interestingly, in inferior mesenteric ganglion of the guinea-pig, the SP-induced depolarization appears to result from the combination of
inhibition of potassium conductance and activation of a sodium conductance. In agreement with this finding, voltage-clamped experiments have revealed two different types of inward current following application of SP in these neurons (Brown and Griffith, 1984; Brown et al., 1985). In about one-third of the neurons the inward current was accompanied by an increase in input conductance and became larger with hyperpolarization, whereas in the rest of the cells there was a fall in membrane conductance and the inward current diminished with hyperpolarization. The authors suggested that the latter effect is probably due to inhibition of a time- and voltage-dependent $K^+$ current, the $M$ current, while the former effect of SP appeared to be a result of a reduction of time-independent "leak" current. However the nature of this current was not determined.

Similarly in bull-frog sympathetic neurons, beyond $M$-current inhibition the application of SP produces an additional inward current at hyperpolarized potentials (Jan and Jan, 1982; Brown et al., 1985; Jones, 1985; Jones and Adams, 1987). The inward current appears as a voltage-insensitive change in the instantaneous conductance and the effect operationally is equivalent to an increase in the leakage conductance since no voltage-dependent relaxations were seen. This current has not been studied in much detail, although several investigators have suggested that it is likely to be due to an increase in membrane conductance to sodium ions (Katayama and Nishi, 1982).

**Physiological consequences of substance P actions**

The small conductance increase that is often seen with SP application at hyperpolarized potentials (Murase et al., 1989) should not have a strong
excitatory effect on the neuronal activity. The enhancement of the voltage-sensitive Ca$^{2+}$ current seen in the present work, which can last for several minutes, provide a mechanism that increase the excitability to natural inputs.

Effects of Calcitonin Gene-Related Peptide

The slow onset and long-lasting duration of CGRP actions in dorsal horn neurons (Section II; Miletić and Tan, 1988; Ryu et al., 1988b) suggest a novel type of peptide action that may be functionally important in processing and integration of sensory information arriving from various peripheral receptors into the spinal dorsal horn. In our experimental conditions, no other peptide including SP, NKA, NKB, SS, physalaemin, eledoisin, kassinin, galanin and enkephalin, produced such slow and prolonged membrane effects, as was observed with CGRP. The biphasic membrane actions of CGRP, together with its slow time course of action, could indicate the involvement of the intracellular mechanisms possibly mediated by second messengers (Brown and Higashida, 1988). CGRP-like immunoreactivity of functionally identified DRG neurons (McCarthy and Lawson, 1987), conduction velocities of afferent fibers arising from CGRP-responsive DRG neurons (Ryu et al., 1988a) and their laminar termination in the dorsal horn (Gibson et al., 1984) indicate that CGRP is involved in the processing of sensory information carried by A$\delta$ and C fibers. Iontophoretic application of CGRP to cat spinal dorsal horn neurons showed that it is relatively selective for multireceptive neurons, i.e., those that receive information from mechanoreceptive, nociceptive and
thermoceptive afferents (Miletic and Tan, 1988).

Since CGRP is frequently co-localized with SP in DRG neurons and other cells (Wiesenfeld-Hallin et al., 1984; Lee et al., 1985; Lundberg et al., 1985; Skofitsch and Jacobowitz, 1985a; Hökfelt et al., 1986; Franco-Cereceda et al., 1987), the functional role of CGRP was examined when applied concurrently with SP. It has been shown that CGRP retards SP degradation (Greves et al., 1985), the effect that may explain the potentiation of the actions of SP by co-administered CGRP (Hökfelt et al., 1986; Woolf and Wiesenfeld-Hallin, 1986). Furthermore, CGRP is co-released with SP from the dorsal horn (Saria et al., 1986). It is also reported that SP, when injected with CGRP into human skin, converts the long-lasting vasodilation induced by CGRP into a transient response (Brain and Williams, 1988). However, similar interaction of CGRP and SP at the level of dorsal horn neurons was not observed in our experiments.

The increase of $\text{Ca}^{2+}$ current in DRG neurons by CGRP is likely to be responsible for the increase of duration and amplitude of $\text{Ca}^{2+}$-dependent action potential and facilitation of excitatory synaptic transmission (Section III; Ryu et al., 1988a). The results indicate that both transient and sustained components of $\text{Ca}^{2+}$ current are enhanced by CGRP. Molecular mechanisms underlying the CGRP-induced enhancement of the $\text{Ca}^{2+}$ current, and the facilitation of excitatory synaptic transmission, have yet to be elucidated. By analogy with $\beta$-adrenergic receptor-mediated increases in $\text{Ca}^{2+}$ current in myocytes (Reuter, 1983) and in hippocampus (Gray and Johnston, 1987), the involvement of cyclic AMP-mediated phosphorylation of voltage-sensitive $\text{Ca}^{2+}$ channels might be a possibility. In this context, it is of interest that several actions of CGRP in peripheral tissues and
spinal motoneurons are thought to be mediated through activation of adenylate cyclase (Crossman et al., 1987), and if a similar mechanism operates in the spinal and sensory neurons, it could explain some of the observed actions of CGRP. Although intracellular cyclic AMP has been shown to enhance Ca\textsuperscript{2+} current in rat DRG neurons (Fedulova et al., 1985), forskolin, an activator of adenylate cyclase, appears to prolong the Ca\textsuperscript{2+} spike by inhibiting K\textsuperscript{+} currents (Dunlap, 1985).
SUMMARY

1. Voltage-activated calcium currents in immature rat spinal dorsal horn neurons and their modulation by SP and CGRP have been investigated by using single-electrode voltage-clamp techniques in the transverse spinal cord slice preparation.

2. Using experimental conditions that minimized voltage-dependent Na\(^+\) and K\(^+\) currents, we distinguished low- and high-voltage-activated calcium currents on the basis of their voltage dependence and sensitivity to the Ca\(^{2+}\) channel agonist and antagonist drugs.

3. The low-voltage-activated transient calcium current is evoked with weak depolarizing voltage commands. It begins to activate at potentials positive to -70 mV and increases in the amplitude and the rate of decay with stronger depolarizations, the peak values being reached between -40 and -30 mV. The current is fully activated at a holding potential of about -110 mV. Inactivation is complete at potentials in the range of -60 to -50 mV.

4. The transient component of the voltage-activated calcium current activates at membrane potentials close to -40 mV and decays with time constants ranging from 100-600 ms. The amplitude of the current increases with more negative holding potentials (-100 to -40 mV).

5. The sustained component of the high-threshold calcium current activates at potentials positive to -40 mV and exhibits little inactivation during 0.3-0.5s depolarizing commands. This component is readily isolated at more depolarized holding potentials (between -40 and -30 mV) that inactivate the transient components of the low-
and high-threshold calcium currents.

6. A run-down of calcium currents was seen in dorsal horn cells. The time stability of the transient and sustained components of the high-threshold calcium current was lower than that of the low-threshold transient current. The latter current seemed to be stable for at least 1 hour.

7. Bay K 8644 (1-10 μM), a dihydropyridine agonist, enhanced the sustained component of the high-threshold calcium current but not the transient components of the low- or high-threshold calcium currents. The dihydropyridine antagonist nifedipine (5-50 μM) selectively reduced the sustained component of the high-threshold calcium current, while having little or no effect on the transient components of the low- and high-threshold calcium currents.

8. Cadmium ions (6-100 μM) and cobalt (2 mM) ions markedly reduced both components of the high-threshold calcium current and Cd$^{2+}$ only slightly decreased the low-threshold transient current. However, all three components are indiscriminately blocked by higher concentrations of Cd$^{2+}$ and Co$^{2+}$.

9. Nickel ions (200-400 μM) greatly reduced the low-threshold transient calcium current, but had little effect on the transient and sustained components of the high-threshold calcium current.

10. The actions of CGRP were examined on Ca$^{2+}$-dependent action potentials and high-voltage-activated Ca$^{2+}$ currents in rat DRG neurons in vitro using single-electrode voltage-clamp technique. In addition the membrane actions of CGRP and the effect on the Ca$^{2+}$ spike of dorsal horn neurons have been investigated by intracellular recording in an
immature rat spinal cord slice preparation.

11. Bath application of CGRP (10^{-8} to 10^{-6} M for 1-3 min) elicited a slow, reversible, dose-dependent hyperpolarization in both small and large DRG neurons that was associated with an increase in membrane input resistance. The hyperpolarizing effect persisted in the medium containing TTX and TEA. In addition, about half of the large DRG neurons tested were depolarized by CGRP.

12. CGRP produced a reversible increase in the amplitude and the duration of the Ca^{2+} spike of DRG neurons and directly increased the voltage-dependent Ca^{2+} current by enhancing both the transient and the sustained components of the current.

13. Bath application of CGRP (10^{-8}-10^{-6} M for 1-10 min) produced a slow reversible depolarization in about one-third of the dorsal horn neurons examined. Biphasic membrane response, consisting of an initial hyperpolarization followed by a late prolonged depolarization, was seen in a smaller proportion of tested cells. Both membrane responses were present, and even enhanced, when synaptic transmission and Na^+ spikes were blocked by perfusing the slice with a TTX-containing Krebs solution. The CGRP-induced membrane changes were also present in media containing TTX and TEA.

14. The CGRP-evoked depolarization was associated with an increase in the input resistance, and enhanced excitability in a majority of neurons tested.

15. CGRP modified the duration of Ca-dependent action potentials of dorsal horn neurons, the most consistent change being a prolonged increase in the spike duration.
16. Bath application of CGRP (10^{-7} to -10^{-6} M) caused a pronounced and long-lasting increase in the amplitude of the fast excitatory postsynaptic potentials recorded in dorsal horn neurons in response to electrical stimulation of a lumbar dorsal root.

17. The increase in Ca^{2+} current in DRG neurons is likely to be responsible for the increase in the duration of Ca^{2+} spike and the facilitation of excitatory synaptic potentials.

18. We have observed that SP enhanced a low-threshold, transient Ca^{2+} current in rat dorsal horn neurons. This effect may be responsible for the increase in excitability produced by SP.
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Principles of Operation of Single-Electrode Voltage-Clamp

The theory and the practical use of the single-electrode voltage clamp technique have been thoroughly discussed elsewhere (Wilson and Goldner, 1975; Finkel and Redman, 1984; Finkel and Redman, 1987). Here the principles of the technique are only briefly described.

A block diagram and a timing diagram that illustrate the principles of operation of the SEVC of Wilson and Goldner (1975) are shown in Figs. 1 and 2. A single microelectrode is used to penetrate the cell, and the recorded voltage \((V_m + V_e)\) is buffered by a high-speed amplifier (Fig. 1, amplifier A1), where \(V_m\) is the deviation of the membrane potential from the resting potential and \(V_e\) is the voltage developed through the microelectrode resistance and capacitance by current \(I_0\). At the beginning of the timing diagram (Fig. 2), \(V_e\) is almost zero. The sample-and-hold device (SH1) samples \(V_e + V_m\) at the time indicated by the arrow in Fig. 2, and the sampled voltage (Vms) is held for a complete cycle.

The sampled voltage is compared with command potential (Vc) in the differential amplifier (Fig. 1, amplifier A2), and the sample of membrane potential is more negative than required (Fig. 2). The semiconductor switch S1 is connected to the current-passing position and the output of amplifier A2 is applied to a controlled current source (CCS), causing it to generate a depolarizing current that is applied to the cell via the microelectrode. This current is directly proportional to the CCS input.
Fig. 1. Block diagram of single-electrode voltage clamp
Fig. 2. Switch S1 is closed during current passing and grounded during voltage recording. Microelectrode voltage (Ve) is shown charging exponentially during current-passing period and discharging exponentially during voltage-recording period. Sum of membrane potential (Vm) and Ve is sampled at times indicated by arrows. Output (Vms) of sample-and-hold device (SH1) only changes at time of each sample. Comparing Vms and command voltage (Vc) in amplifier A2 determines magnitude and sign of next current pulse. Vms, average value of Vms
voltage, regardless of the microelectrode resistance. During the currentpassing period (T1) the square pulse of the current passing through the microelectrode causes Vc to rise at a rate determined by the electrode resistance, the capacitance of the wall of the microelectrode to the surrounding tissue or solution, and the input capacitance of amplifier A1. The current pulse would normally last several electrode time constants, but it would be very much shorter than the time constant of the neuron. For this reason a linear change in membrane potential occurs during the current pulse (Fig. 2, Vm). For the present it is assumed that Ve can be described by a simple exponential charging and discharging curve, although for a number of reasons the microelectrode response is nonexponential.

At the end of the current-passing period, switch S1 changes to the voltage-recording position. In this position the input to the CCS is zero, thus its output is zero. The microelectrode potential decays toward zero while the cell membrane potential decays toward the resting membrane potential. The decay rates are determined by the respective time constants of the microelectrode and cell membrane, and it is assumed in this diagram that these time constants differ by several orders of magnitude. Before a new voltage sample is taken, sufficient time must be allowed for Ve to decay to within a fraction of a millivolt from zero. Because Ve may have reached several volts at the end of a current pulse, the time allowed for the decay of Ve (Tv) must be many (up to 10) microelectrode time constants (in Fig. 2, changes in Vm are magnified compared with changes in Ve). Another sample is taken at the end of the decay period, and the cycle is repeated.

Amplifier A2 (Fig.1) provides negative feedback to clamp Vms to a
value nearly equal to $V_c$. Under steady-state conditions, $V_{ms}$ moves in small increments about its average value, $\bar{V}_{ms}$. These changes in $V_{ms}$ are caused by system, electrode, and membrane noise sources. The difference between $V_{ms}$ and $V_c$ is the steady-state error of the voltage clamp. This difference arises because, to maintain stability, the open-loop transconductance ($G_t$) is finite. The open-loop transconductance is the product of the transfer conductance of the CCS and the voltage gain of amplifier $A_2$.

The CCS and $S_1$ can be used to implement a discontinuous current clamp (DCC) by switching $S_2$ to the current-clamp position (Fig. 2). In this mode the input to the CCS during the current-passing periods is a current command voltage $V_i$. The resulting current causes a shift in the membrane potential. Provided that the voltage of the microelectrode decays to almost zero during the interval between current pulses, $\bar{V}_{ms}$ is a reliable measure of the membrane potential.

Successful operation of an SEVC depends on the correct adjustment of those parameters that can be controlled. These are the open-loop transconductance ($G_t$), the switching period ($T$), and the duty cycle ($D$; fraction of cycle during which current is passed). The electrical characteristics of the microelectrode are also very important, and to some extent the tip resistance ($R_e$) and the microelectrode capacitance can be controlled by the experimenter. The operation of the clamp also depends on the resistance ($R_m$) and capacitance ($C_m$) of the neuronal membrane.