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Spinal neurons exhibiting a specific nociceptive response receive abundant substance P-containing synaptic contacts

(tachykinin/bispecific monoclonal antibody/dorsal horn/slow excitatory postsynaptic potential/horseradish peroxidase)

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ABSTRACT **Substance P has been implicated in nociceptive transmission in the spinal cord. However, evidence for a direct correlation between a specific nociceptive response in spinal dorsal horn neurons and substance P input is lacking. In this study, we combine intracellular recording from dorsal horn neurons *in vivo*, characterization of their nociceptive responses, intracellular labeling by injection of horseradish peroxidase, and immunocytochemical demonstration of substance P at the electron microscopic level. The results reveal that dorsal horn neurons that respond to noxious cutaneous stimulation with a slow, prolonged excitatory postsynaptic potential receive a preferentially high number of substance P fibers compared with nonnociceptive neurons, which scarcely receive any substance P input. Therefore, this study provides direct evidence of a structural–functional link for a substance P-mediated nociceptive response.**

Substance P (1) has been proposed to be a chemical mediator of nociceptive transmission in the dorsal horn (2). This proposal was based on a number of anatomical and physiological findings. Substance P occurs in high concentration in a subpopulation of fine-diameter primary sensory axons and nerve terminals (3, 4) in the regions of the dorsal horn (5–7) in which primary sensory nociceptors terminate (8–10). Substance P specifically excites nociceptive dorsal horn neurons (2, 11, 12), it is released in the spinal cord *in vivo* upon specific activation of nociceptive primary sensory fibers (13–17), and substance P receptor antagonists have been observed to block slow nociceptive responses in the dorsal horn *in vivo* (18, 19) and in the ventral horn *in vitro* (20). These different lines of evidence, however, do not directly demonstrate the synaptic association between substance P-containing boutons and the presence of such nociceptive responses. Therefore, we recorded intracellularly from dorsal horn neurons *in vivo* in the cat spinal cord, characterized each neuron on the basis of its response to natural stimulation of the cutaneous receptive field and to electrical stimulation of sensory nerves, injected the neuron with horseradish peroxidase (HRP), and processed the tissue to demonstrate the intracellularly injected HRP as well as substance P-like immunoreactivity. A bispecific anti-HRP/anti-substance P monoclonal antibody was used. As the bispecific antibody recognizes both the antigen (substance P) and the marker (HRP), a direct immunocytochemical protocol was used. Thence the relationship between the characterized dorsal horn neuron and the immunoreactive substance P was studied at both the light and electron microscopic levels.

METHODS

Methods for the preparation of the cats are described elsewhere (21). Briefly, 20 adult cats were anesthetized with α-chloralose (60 mg/kg i.v.). Recording was at the L_5–L_7 spinal level. The spinal cords were transected at the L_1 vertebral level to eliminate the influence of supraspinal structures. Cats were paralyzed with pancuronium bromide (Pavulon, Organon; 1 mg/kg i.v.) and ventilated artificially.

Glass micropipettes were filled with 4–8% HRP (Sigma type VI) in 0.5 M KCl or $K(C_2H_3O_2)$/0.05 M Tris buffer, pH 7.5 (resistances, 50–120 MΩ). Each neuron was functionally classified on the basis of the responses to natural cutaneous stimulation and electrical stimulation of the afferent nerves following a described scheme (18, 22). Natural stimuli used were movement of a single hair, noxious pinch with a serrated forceps, innocuous and noxious pressure, noxious radiant heat, and vibration using a feedback-controlled mechanical stimulator. Neurons were classified as nonnociceptive, wide dynamic range, and nociceptive specific. Special emphasis was made to characterize the after-response to noxious stimulation, as we have shown that this response is blocked by administration of a substance P (NK-1) receptor antagonist (18, 19). HRP was injected by intracellular iontophoresis by using 600-ms positive current pulses of 2–10 nA at 1 Hz for 10–30 min. One to three neurons were injected per cat, and a map of the dorsal surface of the cord was drawn to ensure exact localization and identification of the labeled cells.

Once a neuron had been functionally classified and injected with HRP, the anesthetized animal was perfused through the left ventricle with 4% (wt/vol) paraformaldehyde/0.5% glutaraldehyde/0.1 M phosphate buffer, pH 7.4. The relevant segments of the spinal cord were sectioned parasagittally at 50 μm in a Vibratome, treated for the demonstration of HRP (23), and examined under the light microscope. The intracellularly injected neuron was photographed at this stage. The sections containing the cells were then processed for electron microscopic immunocytochemistry and flat-embedded in Epon as described (23). The antibody used was an anti-substance P/anti-HRP bispecific monoclonal antibody from the rat (P4C1; Medicorp, Montreal). The use of this antibody provides the advantage of a single-step immunocytochemical protocol, homogenous immunoreaction across the entire 50-μm-thick section, and, hence, the correlation of light and electron microscopic immunocytochemistry (24). The neurons as observed in flat-embedded slices were drawn with a *camera lucida* and photographed. After reembedding, 4-μm-thick plastic sections were obtained serially, photographed, and compared with the original drawings for identification of the parts of the intracellularly injected cell in each section. The 4-μm sections were then reembedded and sectioned for electron microscopy. The substance P-immunoreactive varicosities apposed to the dendritic processes and cell body of the intracellularly labeled cells were counted in sections containing different portions of the cell body and dendrites for each neuron. In an attempt to obtain a representative sampling of each cell, a total of three ultrathin

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Abbreviations: HRP, horseradish peroxidase; EPSP, excitatory postsynaptic potential.

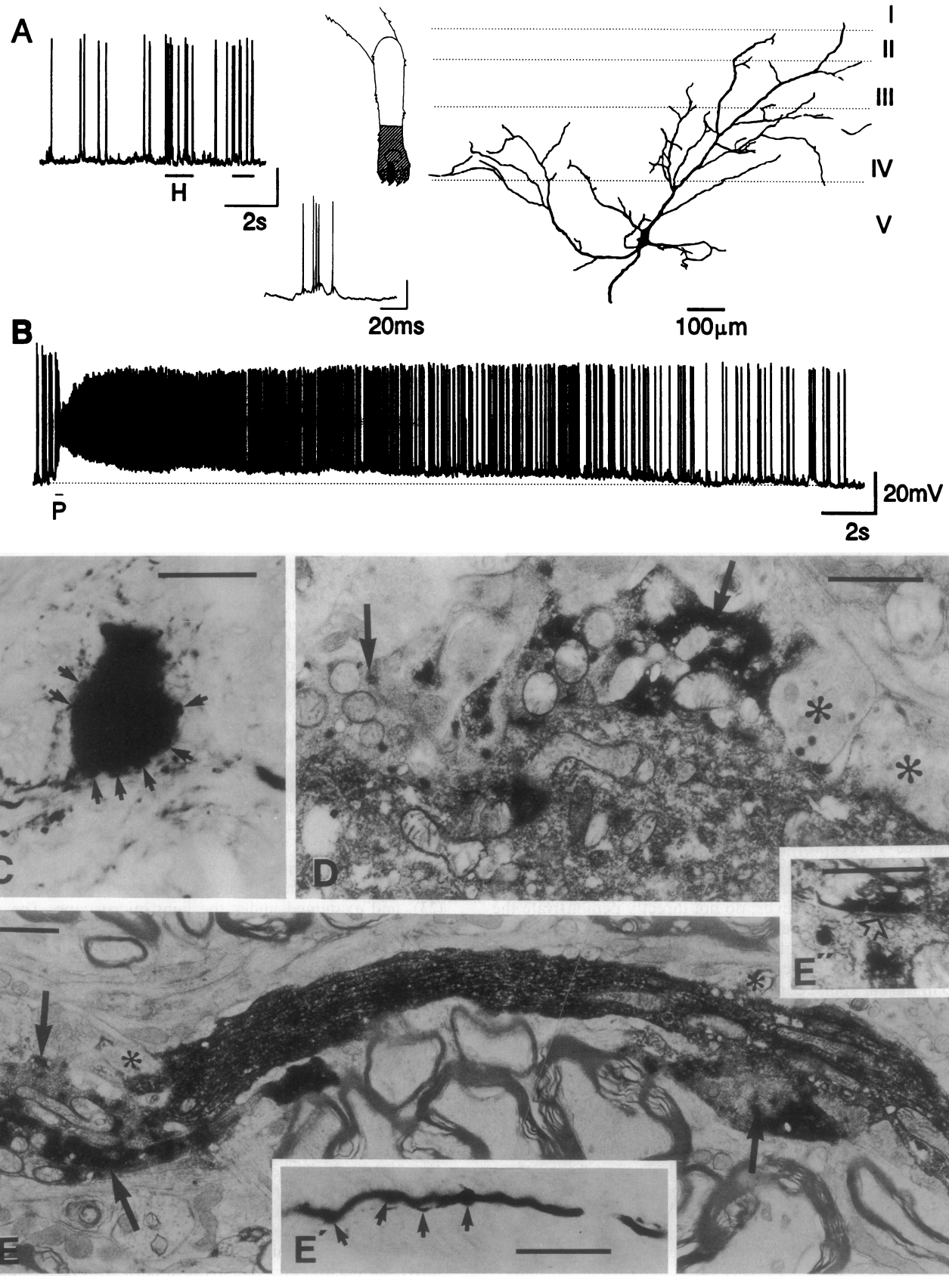

FIG. 1. Dorsal horn neuron responding with a slow, prolonged EPSP after noxious cutaneous stimulation. (*A*) (*Right*) *Camera lucida* reconstruction of the cell body, located in lamina V, that was associated with a discrete area of intense substance P immunoreactive fibers (see *C*). (*Left*) Response of the neuron to low-threshold mechanical stimulation (hair, H). The period of application of each of two stimuli is represented by the horizontal bars below trace. (*Inset*) Enlargement, on a faster time scale, of a typical burst of action potentials riding on EPSPs during the stimulus. (*Center*) The cutaneous receptive field of the neuron is represented on the schematic diagram: darkened area represents the low-threshold touch receptive field, and larger hatched area represents the high-threshold pinch receptive field. (*B*) Response of the neuron to high-threshold mechanical stimulation (pinch; P). The period of the stimulus is represented by the horizontal bar below trace. Note the marked

Table 1. Neurons associated with a slow EPSP to noxious stimulation possess higher numbers of contacts from substance P-immunoreactive varicosities

Cell type	Cell body localization	Slow EPSP	Substance P-like immunoreactive boutons, %	
			Cell body	Dendrite
Nociceptive-specific	Lamina I	+++++	39	38
Nonnociceptive	Lamina III–IV	−	2	0
Wide dynamic range	Lamina III	++	7	20*, 5†
Wide dynamic range	Lamina V	++++++	19	29

Data are expressed as % of total varicosities apposed to the membrane of intracellularly injected neurons; data are from one cell per type. Approximately 30% of such appositions displayed a synaptic specialization.
*Percentage of boutons located on dendrites located in lamina I–II.
†Percentage of boutons located on dendrites located in lamina III.

sections per portion—somata, proximal dendrites, and distal dendrites—was quantified. Each of these three ultrathin sections (total of 36 sections) was obtained after sectioning a reembedded 4-μm-thick semithin section. Data relative to the proximal and distal dendrites were pooled. Approximately 800 boutons were counted per cell.

RESULTS

The nociceptive response emphasized in the present study is a slow, prolonged excitatory postsynaptic potential (EPSP) following noxious mechanical stimulation of the skin. This is a specific nociceptive response, as it occurs after noxious but not after innocuous stimulation (see Fig. 1). In addition, this slow, prolonged EPSP differs from the brief EPSPs evoked by single stimuli to high-threshold afferents in that the former is blocked by substance P receptor (NK-1) antagonists (18, 26) and the latter are blocked by excitatory amino acid antagonists (27, 28). Therefore, the slow, prolonged EPSP appears to be the nociceptive response mediated by substance P. Hence, neurons were classified on the basis of the presence or absence of this slow, prolonged EPSP.

Eight of 30 neurons displaying fully characterized responses to natural stimulation were included in this study. All had a stable membrane potential (−50 to −65 mV), had spikes with overshoot (60–90 mV), were successfully labeled intracellularly with HRP, and showed an optimal ultrastructural preservation and immunocytochemical reaction for this type of investigation. These neurons represented prototypes of the functional and morphological classes of the neurons identified.

Four of the neurons responded to noxious stimulation with the slow, prolonged EPSP shown in Fig. 1. This EPSP outlasted the period of stimulation by 20 s to 1 min, peaking at an amplitude of 5–10 mV at 5–15 s after the end of the stimulus. We have recently shown that the electrophysiological and pharmacological properties of this slow, prolonged EPSP are consistent with the mediation via endogenously released substance P from activation of nociceptive primary sensory fibers (18). Of these cells, one was a nociceptive-specific neuron located in lamina I, and the remaining three were wide-dynamic-range neurons located in laminae III–VI. The lamina I neuron was multipolar, with elongated, scarcely branched dendrites virtually devoid of spines. Some dendritic branches penetrated the white matter of the dorsal columns, whereas others were directed ventrally, reaching as deep as the lamina II–III border. The somata of two additional cells were located in lamina V. The cell bodies were ovoid and gave rise to three to five main dendritic trunks. Most of the dendrites were oriented dorsally, with a few extending to lamina VI. Some of the dendritic trunks continued as very elongated dendrites, reaching as far dorsally as the lamina I–II border. The dendrites were virtually devoid of spines and were seldom postsynaptic to the central elements of synaptic glomeruli. The three neurons described above were surrounded by a considerable number of substance P-immunoreactive varicosities contacting both the cell body and dendrites (Fig. 1 and Table 1). About 30% of these substance P-immunoreactive contacts showed a distinct synaptic specialization (Fig. 1*E*″).

The cell body of the fourth neuron exhibiting the slow EPSP was located in lamina III and was stellate in shape. This cell body gave rise to profusely branched dendrites. Three of the dendritic trunks were oriented dorsally and reached into lamina I. The remainder of the dendritic tree was oriented ventrally and branched extensively in lamina III. Surprisingly, although most of the dendritic branches in lamina III had numerous spines, those in laminae I and IIo were virtually aspiny, and these were precisely those that were contacted by substance P-immunoreactive varicosities, although the percent of these varicosities was less than that of the three previous neurons (Table 1). Importantly, all four neurons responded with the slow, prolonged EPSP to noxious stimulation after the end of the noxious stimulus.

With the remaining four neurons, noxious stimuli to the skin and high-intensity electrical stimuli did not produce the slow, prolonged EPSP. These neurons showed brisk and brief responses to low-threshold hair input but showed only weak, if any, after-excitation to noxious stimulation. In these neurons the weak after-excitation was characterized by an increased discharge rate and increased, fast synaptic activity. However, this after-excitation was never associated with a

depolarization during the noxious stimulus associated with action potential inactivation. Note also the prolonged depolarization after the end of the stimulus associated with increased firing frequency. (*C*) Light microscopic photograph of a parasagittal, 4-μm-thick, Epon-flat-embedded section showing part of the neuronal cell body. Note the numerous substance P-immunoreactive profiles apposed to the cell body (arrows). The cell body was located within a region of intense immunoreactivity corresponding to one of the clusters of substance P-immunoreactive fibers commonly found in lamina V (25). (Bar = 20 μm.) (*D*) Electron microscopic photographs of an ultrathin section taken from the 4-μm-thick section shown in *C*. Note the two substance P-containing varicosities (arrows) apposed onto the cell body. Synapses between these varicosities and the cell body could be demonstrated in adjacent sections (data not shown). Asterisks indicate nonimmunoreactive axonal varicosities. (Bar = 1 μm.) (*E*) Electron micrographs of an ultrathin section taken from the 4-μm-thick section in *E*′. The portion of the dendrite shown in *E* corresponds to the curved portion of the dendrite shown in *E*′ (the two arrows on the left of *E*′ indicate the two boutons pointed to by two of the arrows in *E*). The immunoreactive profiles (arrows) belong to an axon that appears to wrap in a spiral-like fashion around the dendrite (arrows in *E*′) of the cell and make several contacts with the dendrite. Asterisks indicate nonimmunoreactive varicosities apposed onto the dendrite. (Bar = 1 μm.) (*E*″) Details of the synapse (open arrow) established by the profile on the right in *E* (right arrow in *E*), obtained from an adjacent ultrathin section. (Bars in *E*′ = 20 μm; in *E*″ = 0.5 μm.)

slow, prolonged EPSP. As substance P-mediated slow EPSPs are voltage sensitive and may be abolished at hyperpolarized potentials (26, 29, 30), care was taken to clamp the membrane potential at different levels (−70 to −50 mV) to ensure that

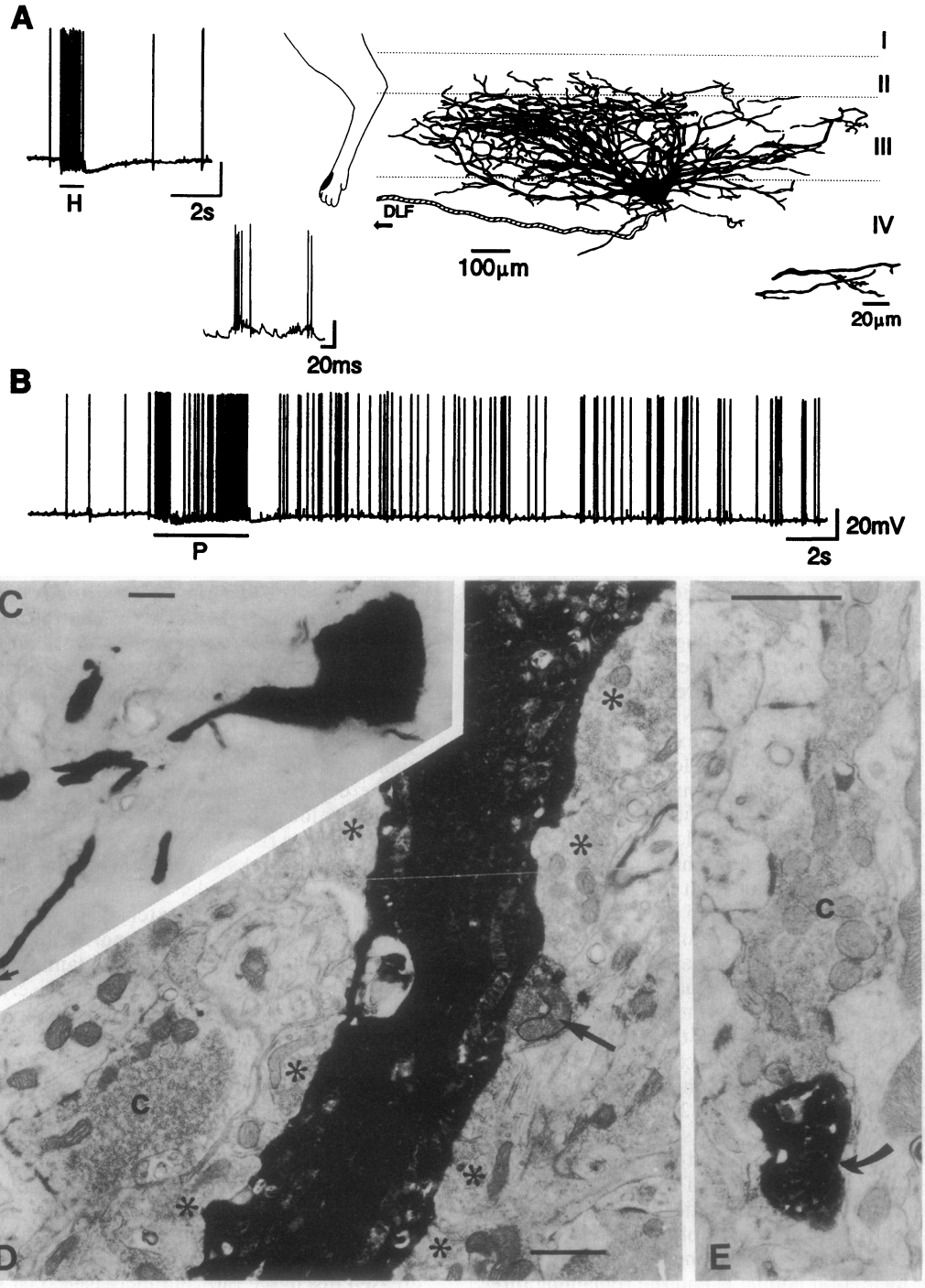

FIG. 2. Lamina IV neuron that did not respond with a slow, prolonged EPSP after noxious cutaneous stimulation. Very few substance P-immunoreactive varicosities were found in apposition to the cell body and the dendritic tree of this neuron. (*A*) (*Left*) Response of the neuron to low-threshold mechanical stimulation (hair; H). The period of stimulus is represented by the horizontal bar below trace. Note the hyperpolarization immediately after the end of the stimulus. (*Inset*) Enlargement, on a faster time scale, of typical bursts of action potentials riding on EPSPs during the stimulus. (*Right*) In the *camera lucida* reconstruction, note the extensive dendritic arborization in the lamina II–III border, a region where abundant hair afferents terminate (31). Note also the presence of axon-like dendritic spines (*Inset*), typical of spinocervical neurons (31). The receptive field of the neuron is shown by the darkened area on the schematic diagram of the hind leg. DLF, dorsolateral funiculus. (*B*) Response of the neuron to high-threshold mechanical stimulation (pinch; P). The period of the stimulus is represented by the horizontal bar below the trace. Note the on-and-off response similar to the response to hair stimulation in *A*. After the end of the stimulus, the rate of discharge of the neuron increased slightly, but no prolonged EPSP was observed, in contrast to the cell response in Fig. 1. (*C*) Light micrograph of a parasagittal, 4-μm-thick, Epon-flat-embedded section showing a portion of the cell body and proximal dendrites. Note the lack of substance P-immunoreactive profiles in the vicinity of the cell body of this neuron. (*D*) Electron microscopic photograph of an ultrathin section showing a portion of a proximal dendrite of the cell. Only one substance P-positive varicosity was found in contact with this dendrite. Asterisks indicate nonimmunoreactive terminals apposed onto the dendrite. (Bar = 1 μm.) (*E*) Dendrites of the neuron were frequently postsynaptic to the central varicosity (C) of synaptic glomeruli. (Bar = 1 μm.)

the presence or absence of such a response was not merely from differences in the neuronal resting membrane potential. Of these four neurons, two had morphological characteristics similar to spinocervical tract neurons (31): (*i*) they were multipolar neurons with the cell body located in lamina IV and dendritic trunks directed dorsally; (*ii*) their dendritic tree was elongated rostrocaudally, extending dorsally to lamina II, and possessed numerous axon-like spines; (*iii*) axons from these neurons could be traced to the dorsolateral funiculus. In a third cell, the soma was located in lamina III, and its numerous dendrites were oriented dorsally and displayed rather short spines. The axon of this cell projected in the dorsal column, suggesting that this cell may have belonged to the dorsal column postsynaptic pathway. The fourth neuron was located in lamina IIi, and its axon arborized locally. The cell was multipolar, with dendrites reaching lamina IIo and lamina III and possessing numerous elongated dendritic spines. With all four neurons, the cell body and most of the dendritic tree were surrounded by synaptic varicosities lacking substance P-immunoreactivity (Table 1). All had numerous spines (see Fig. 2 for representative example). In contrast to the previous group of neurons, as illustrated in Fig. 2*B* the dendritic processes were frequently postsynaptic to the central varicosities of synaptic glomeruli in laminae II and III (32–34). None of these central varicosities were immunoreactive for substance P.

DISCUSSION

As the slow, prolonged EPSP seen with the first group of neurons (Fig. 1) has been shown to be blocked by a specific NK-1 receptor antagonist (18), the striking observation here is that neurons associated with this EPSP are also associated with a relatively high percentage of substance P-immunoreactive terminals. This result contrasts with the lack of substance P contacts seen with neurons not exhibiting this slow, prolonged EPSP (Table 1).

In addition, neurons that responded with the slow, prolonged EPSP to noxious pinch stimulation possessed dendrites mainly devoid of spines, and the dendrites were not associated with synaptic glomeruli. Rather, these neurons were contacted by a relatively large number of substance P-containing varicosities. On the contrary, neurons that had brisk low-threshold hair responses had very spiny dendrites and dendritic processes that were often postsynaptic to the central varicosities of glomeruli; these neurons were essentially devoid of any type of substance P-immunoreactive input. Interestingly, an intermediate type of synaptic arrangement was observed in one of the wide-dynamic-range neurons included in this study. This cell responded with a slow EPSP to noxious stimulation, but this response was not as pronounced as that seen with the other nociceptive neurons. In addition, the neuron had a brisk response to hair stimulation similar to that seen with some neurons in the second group. The dendritic tree of this neuron was of both types—some parts lacking spines but rich in substance P input and yet other areas rich in spines and associated with synaptic glomeruli but poor in substance P input. These findings suggest a direct association of dendritic spines with hair afferent input and a postsynaptic localization in synaptic glomeruli. These findings also suggest an association of substance P input with dendritic processes poor in spines and unrelated to glomeruli. The functional significance of these associations remains to be elucidated, but we can speculate that these associations may reflect the difference in time course of the synaptic responses related to these two inputs.

In conclusion, this study provides direct evidence of an association of substance P-containing synaptic contacts with dorsal horn neurons exhibiting a specific, nociceptive response, consisting of a slow, prolonged EPSP to noxious stimulation. The converse evidence is also presented of the lack of such contacts on neurons that do not exhibit such a response. The direct action of substance P on the postsynaptic membrane of nociceptive dorsal horn neurons may thus account for the period of hyperexcitability after noxious stimulation. This period of hyperexcitability may be the neuronal substrate underlying the central component of hyperalgesia after noxious input.

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