Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig

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1. The relationship between the afferent properties and substance P-like immunoreactivity (SP-LI) of L6 and S1 dorsal root ganglion (DRG) neuronal somata was examined in anaesthetized guinea-pigs. Glass pipette microelectrodes filled with fluorescent dyes were used to make intracellular recordings and to label DRG somata. The dorsal root conduction velocity (CV) and the afferent receptive properties of each unit were categorized according to criteria established in other species. Categories included a variety of low threshold mechanoreceptive classes, innocuous thermoreceptive and several nociceptive classes. Nociceptive units were further subdivided on the basis of CV and the locus of the receptive field (superficial cutaneous, deep cutaneous or subcutaneous).

2. SP-LI was determined using the avidin–biotin complex method and the relative staining intensity determined by image analysis. The possible significance of labelling intensity is discussed. Clear SP-LI appeared in twenty-nine of 117 dye-labelled neurones. All SP-LI positive units with identified receptive properties were nociceptive but not all categories of nociceptors were positive. The intensity of SP-LI labelling varied, often systematically, in relation to afferent properties. There was a tendency for nociceptive neurones with slower CVs and/or smaller cell bodies to show SP-LI.

3. Nineteen of fifty-one C fibre neurones showed SP-LI. Fewer than half the C polymodal nociceptors (CPMs) were positive. The most intensely labelled units were the deep cutaneous nociceptors and some of the CPMs in glabrous skin. C low threshold mechanoreceptors and cooling-sensitive units did not show SP-LI.

4. Ten of sixty-six A fibre neurones exhibited SP-LI, including eight of sixteen Aδ nociceptors and two of fifteen Aα/β nociceptors. A fibre neurones exhibiting SP-LI included seven of eight deep cutaneous mechanical nociceptors and some superficial cutaneous mechano-heat nociceptors of hairy skin. In contrast, none of twenty superficial cutaneous A high threshold mechanoreceptor units or the thirty-five A fibre low threshold units (D-hair and other units) showed detectable SP-LI.

5. We conclude that SP-LI labelling in guinea-pig DRG neurones is related to (a) afferent receptive properties, (b) the tissue in which the peripheral receptive terminals are located, (c) the CV and (d) the soma size.

A number of years ago it was established that a subpopulation of dorsal root ganglion (DRG) neurones express immunoreactivity to antibodies directed against substance P (SP) (Hökfelt, Kellerth, Nilsson & Pernow, 1974). In rat DRG neurones substance P-like immunoreactivity (SP-LI) is present in a subset of C fibre neurones and a limited number of Aδ fibre neurones (McCarthy & Lawson, 1989). However, primary afferent neurones with C and Aδ fibres have a variety of receptive characteristics and the relationship of the SP marker to these receptive properties remained unclear. An earlier study (Leah, Cameron & Snow, 1985) reported no correlation between broad categories of receptor properties and the SP-LI in neuronal somata of cat DRG neurones. In spite of its simplicity of concept, this type of study is subject to a number of technical difficulties. These problems, the broad categories of receptive properties used in that study, the small number of neurones involved and pre-treatment of the DRG with colchicine leave the results of this previous work open to question.

There are several reasons to believe that SP might be expressed by neurones with particular afferent properties. SP release in the spinal dorsal horn is evoked by noxious mechanical, but not by innocuous mechanical, cutaneous stimuli (Duggan, Hendry, Morton, Hutchison & Zhao, 1988;

Kuraishi, Hirota, Sato, Hanashima, Takagi & Satoh, 1989; Tiseo, Adler & Liu-chen, 1990). Release of SP in response to noxious heat stimuli is less consistently observed (Duggan *et al.* 1988, Kuraishi *et al.* 1989) and may depend upon the intensity or location of the applied heat. Unfortunately, studies of peptide release in the spinal cord do not distinguish between primary afferent fibres and intrinsic spinal cord neurones. Furthermore, they provide little data about the afferent properties of the primary afferent units and about the location of the activated receptive terminals.

For these reasons we undertook a re-examination of this question. The experiments were done on guinea-pigs because of the relative ease in this species of making stable intracellular recordings from the DRG somata of C fibre neurones. The observations provide information on the widely held assumption that SP presence marks nociceptive primary afferent neurones.

Preliminary reports of some of the data and of methodological problems encountered have been published in abstract form (Lawson, Crepps, Bao, Brighton & Perl, 1993, 1994; Lawson, Crepps & Perl, 1996).

METHODS

Young guinea-pigs (1- to 2-weeks-old; weight, 130–220 g; mean, 180 g) were used. Animals of this size are free ranging and exhibit a spectrum of motor and somatic afferent behaviour typical for the species. After anaesthesia with 50 mg kg^{-1} pentobarbitone (I.P.), the hair of the hindlimbs was clipped short, cannulae inserted in the trachea and in one carotid artery, and artificial ventilation initiated with continuous monitoring of end-tidal CO_2. A laminectomy was performed from L2 to S2 to expose the L6 and S1 DRGs. The vertebral column was stabilized with rigid support of the ileum and a clamp on the L1 vertebra. Dental impression material was used to create a large paraffin pool to protect the spinal tissues and exposed DRG. A small silver platform was placed beneath the DRG to stabilize it during recording. In most preparations the DRG surface was left undisturbed after removal of adipose tissue, to maintain a visible superficial blood supply.

Electrophysiological recording

The dorsal root of the DRG under study was sectioned close to the spinal cord and placed upon a bipolar electrode. Recording from this electrode initially established the receptive field of the ganglion during stimulation of skin and other peripheral tissues. Subsequently, these electrodes were used for stimulation during intracellular recordings. After surgical preparations, flaxedil (20 mg kg^{-1}) and supplementary pentobarbitone (10 mg kg^{-1}) was administered through the carotid cannula. Doses of both drugs were repeated at regular intervals throughout the experiment to maintain deep anaesthesia and areflexia. Typically 2–3 mg h^{-1} pentobarbitone was administered. Prior to supplementary flaxedil, the animal was tested for reflex reactions and pentobarbitone was always administered with additional flaxedil. Glass micropipette electrodes filled with a fluorescent dye, (Lucifer yellow CH (LY; 5 mg in 0·1 ml), ethidium bromide (EB; 6 mM in 1 M KCl), Cascade Blue (CB; 3% in 0·1 M LiCl), propidium iodide (PI; 1% in 1 M potassium acetate)) were used to record from DRG somata. Electrode impedances ranged between 250 and 500 MΩ for LY and CB or 80 and 120 MΩ for EB and PI. The microelectrode was advanced by a microdrive fitted with a stepping motor until a membrane potential (typically −40 to −70 mV) was seen and an action potential could be evoked by stimulation of the dorsal root with single 0·3 ms rectangular pulses. Stimulus intensity was then adjusted to twice threshold for A fibre units and suprathreshold for C fibre cells. Conduction velocity (CV) was calculated from the latency to the rise of the intracellularly recorded potential and the distance, (measured after the experiment) between the location of the dye-injected neurone in the ganglion and the stimulating cathode. Conduction distances were short (typically 4–7·5 mm) and the temperature of the liquid paraffin overlying the DRG was 28–31 °C. Estimation of CV was problematic for units conducting over about 11 m s^{-1} because the initial phase of the evoked action potential was distorted by the stimulus artifact. CVs were underestimated because utilization time was not considered. To establish the range of dorsal root CVs to be expected under these conditions, compound action potentials were recorded with separate pairs of stimulating and recording electrodes for the S2 dorsal root over a conduction distance of 6·5 mm.

Afferent receptive properties

The receptive properties of afferent units with evoked responses to dorsal root stimulation were examined with hand-held stimulators following previous classifications (Burgess & Perl, 1967; Bessou & Perl, 1969; Bessou, Burgess, Perl & Taylor, 1971; Burgess & Perl, 1973; Horch, Tuckett & Burgess, 1977; Shea & Perl, 1985). The small physical size of the animals, receptive fields in partially shielded locations, and the limited time for observation, particularly for C fibre units, precluded use of devices permitting detailed quantitative description of stimulus intensity. Light mechanical stimuli were produced by movement or pressure by a small brush, a blunt probe, or von Frey-type stimulators. Mechanical stimuli producing sensations of contact, light pressure and movement when applied to the skin of the experimenter were categorized as innocuous. Cooling was accomplished either by a brief application of ethyl chloride, contact with a cooled metal rod, or ice placed on the mechanically defined receptive field. Noxious mechanical stimuli were applied with fine forceps, a sharp needle, coarse-toothed forceps, or coarse flat forceps. Skin warming was produced by radiation from an infrared source or a heated glass rod. Noxious heat was applied by skin contact with a heated glass rod. Stimuli were considered noxious if they caused visible damage or were painful when applied to the skin of the experimenter. Regions of the hindlimb subjected to injurious stimuli were noted and subsequently avoided for classification of units. Figure 1 shows the sequence of afferent testing and the categories of the units classified in this fashion. Receptive fields of units in L6 and S1 DRGs were found on the whole of the foot including the toes and glabrous regions, the lateral, dorsal and part of the medial surface of the leg, the hairy skin over the left haunch and the region around the genitalia. Parts of the dorsal/anterior surface of the foot and lower leg that were used to support the leg were inaccessible.

Low threshold A fibre mechanoreceptive units were differentiated by their responses to moving brush, skin contact and light pressure with blunt objects, light tap, tuning forks vibrating at 100 or 256 Hz and pressure with calibrated von Frey-type stimulators. Subcutaneous units were differentiated from cutaneous elements by the stability of the response when the skin was lifted from subcutaneous structures and moved. Several subcutaneous units were classified as muscle spindles if they showed ongoing discharges at a regular frequency and had very low thresholds to gentle pressure against muscle tissue. A fibre units classified as high threshold mechanoreceptors (AHTMs) had receptive fields in the epidermis with punctate, typically multiple separated spot-like

regions from which responses could be evoked by strong mechanical stimuli. Some of the AHTM units were classed as moderate pressure because they responded with one or a few action potentials in response to blunt pressure but gave a much more vigorous discharge to pressure with a sharp object (e.g. needle) or pinching with fine forceps (see Burgess & Perl, 1967). Those high threshold mechanically excitable A fibre units that responded promptly to a single application of noxious heat were categorized as A mechano-heat (AMH) type.

The few C fibre units that were responsive to the most gentle mechanical stimulation of the skin and excited by sudden cooling, were classified as C low threshold mechanoreceptors (CLTMs). C fibre units that required strong mechanical stimulation of receptive regions in the superficial cutaneous tissue for excitation that also produced prompt, vigorous responses to noxious heat were classified as C polymodal receptors (CPMs). Neurones with C fibres that were excited by both strong mechanical and noxious heat of deep cutaneous tissues were categorized as C mechano-heat units (CMH). C fibre units excited by strong mechanical stimuli of cutaneous tissue but lacking prompt responses to noxious heat were considered as C high threshold mechanoreceptors (CHTMs). Units excited by cooling of the skin or subjacent tissue were only partially categorized because controlled thermal probes could not access the limited space available for many receptive fields. Units excited by moderate cooling, with ongoing discharge at room temperatures (20–25 °C), and showing cessation of discharge with slight warming were considered the thermoreceptive cooling type. Elements excited by marked cold but not by innocuous mechanical stimuli and lacking background discharge were classified as putative cold nociceptors. However, without controlled thermal probes it was not always clear to which group some neurones belonged. Nociceptors responsive to noxious heat without mechanically sensitive fields would not have been identified in most cases since noxious heat stimuli were rarely used as a primary search stimulus. Radiant warming stimuli were used for search only in later experiments.

Receptive tissue

For units with elevated mechanical thresholds, the tissue locations of the receptive fields were defined as follows. Units were classified as superficial cutaneous based upon responses to needle pressure and pinch with fine forceps; a major criterion was whether responses could be evoked by stimuli that lifted superficial tissue away from deeper layers. These units were thought to have receptive terminals in the epidermis or in the dermis at the junction between dermis and epidermis. Units unresponsive to stimulation of the superficial layers of the skin but excitable from soft, deeper, connective tissue, including the dermal tissue and possibly superficial fascia, were classified as deep cutaneous units. Units requiring a squeeze or pressure to underlying tissues (e.g. muscle, joint or bone) for activation were defined as subcutaneous; elements responsive to stimulation of deep fascia would have been included in this group. A few units with clearly subcutaneous receptive fields were tentatively

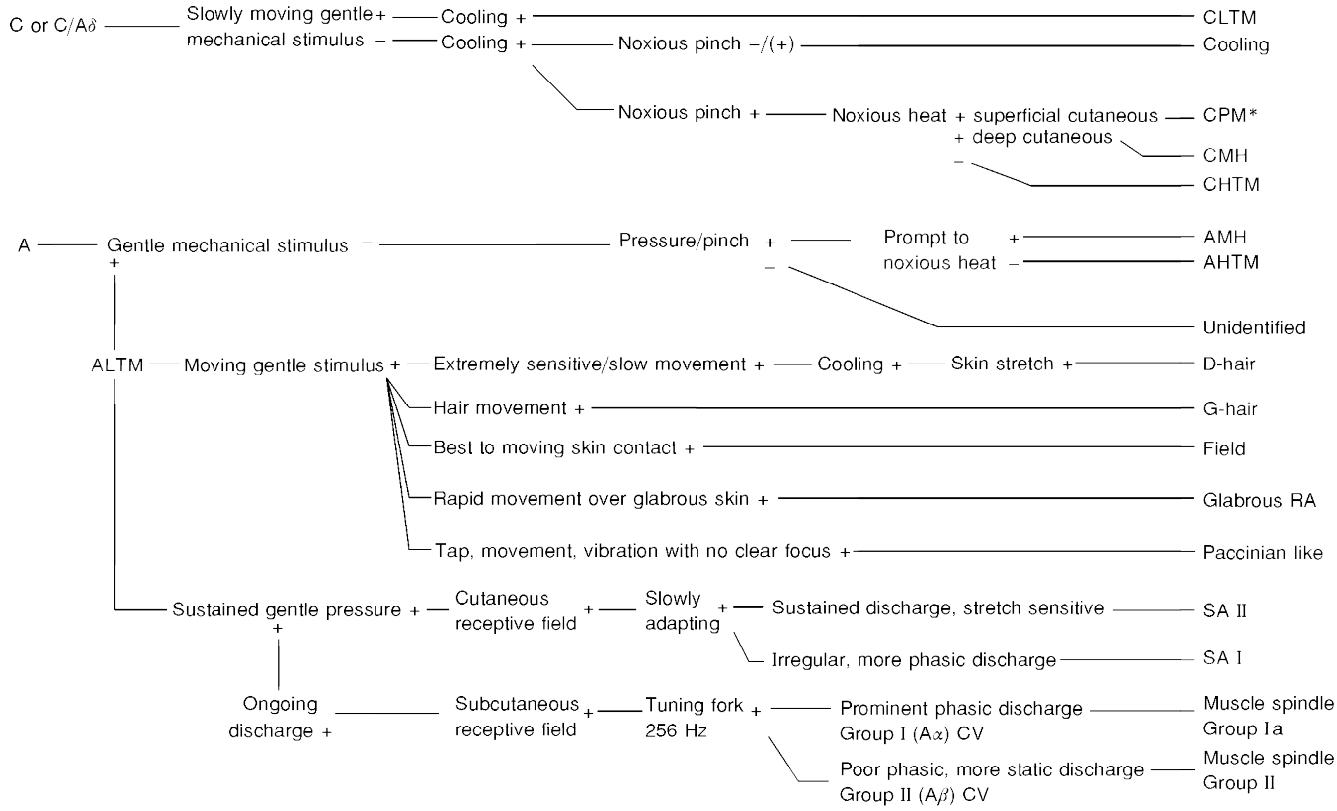

Figure 1. Flowchart of methods used to classify sensory receptor properties

+ indicates that there was a response to that test, and − indicates no response. Abbreviations: CLTM, C low threshold mechanoreceptor; Cooling, cooling- or cold-responsive neurone; CPM, C polymodal nociceptor (* subdivided into units in hairy skin or glabrous skin); CMH, C mechano-heat unit with deep cutaneous receptive field; CHTM, C fibre high threshold mechanoreceptor; AMH, A mechano-heat unit; AHTM, A fibre high threshold mechanoreceptor; ALTM, A fibre low threshold mechanoreceptor; RA, rapidly adapting; SA, slowly adapting.

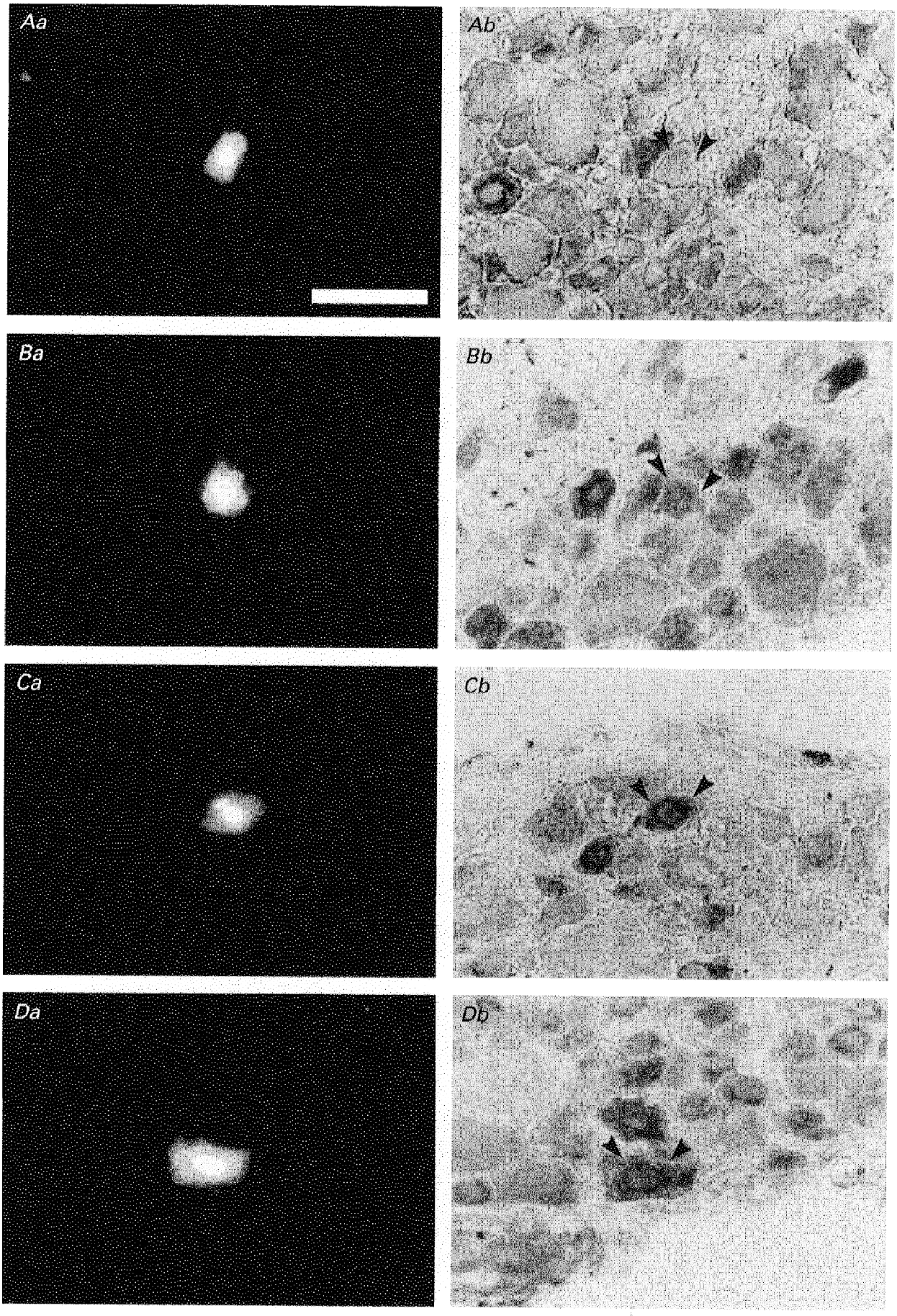
Aa
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Table 1. Fate of dye-injected neurones

	Number of neurones labelled					
	LY	EB	CB	PI	Total cells	Total (%)
1. Injected iontophoretically	239	21	31	2	293	100
A. Not found	90	0	11	0	101	34
B. Rejected: too many cells found	14	4	3	1	22	8
C. Rejected: wrong position	10	1	0	0	11	4
Available for immunocytochemistry	125	16	17	1	159	54
2. SP immunocytochemistry carried out	100	10	15	1	126	100
Poor immunocytochemistry	7	1	1	0	9	7
Successful immunocytochemistry	93	9	14	1	117	93

A, those not found. B, too many cells found dye labelled, and it was impossible to unequivocally identify the studied cell. C, labelled cell was in the wrong position (depth or position in the DRG). Most cells were later used for SP immunocytochemistry. LY, Lucifer Yellow; EB, ethidium bromide; CB, Cascade Blue; PI, propidium iodide.

identified as muscular in origin because they were excited by mechanical pressure applied across the belly of a muscle (gastrocnemius or hamstring) but not to tissues lying over the muscle belly. Many units of the subcutaneous type were found in tissues of the foot and toes. These general classifications were usually readily made except in the case of glabrous skin where it was harder to distinguish between the layers, due to the thick epidermis, the thin dermis and the close adherence of the skin to underlying structures.

Neuronal labelling

After characterization of receptive properties, dye was electrophoretically ejected from the electrode by pulses of current (0·6 nA rectangular 500 ms at 1 Hz) for periods up to 5 min, checking membrane potential every 30 s. The currents passed were negative for LY (0·9–1·5 nA min^{-1}) and made positive for EB (0·3–1 nA min), CB (1·2–1·5 nA min^{-1}) and PI. Initially, only one cell was marked with any one dye in each DRG. In later experiments, two neurones at opposite ends of the ganglia were injected with LY along with a third neurone located between the LY cells which was marked with EB.

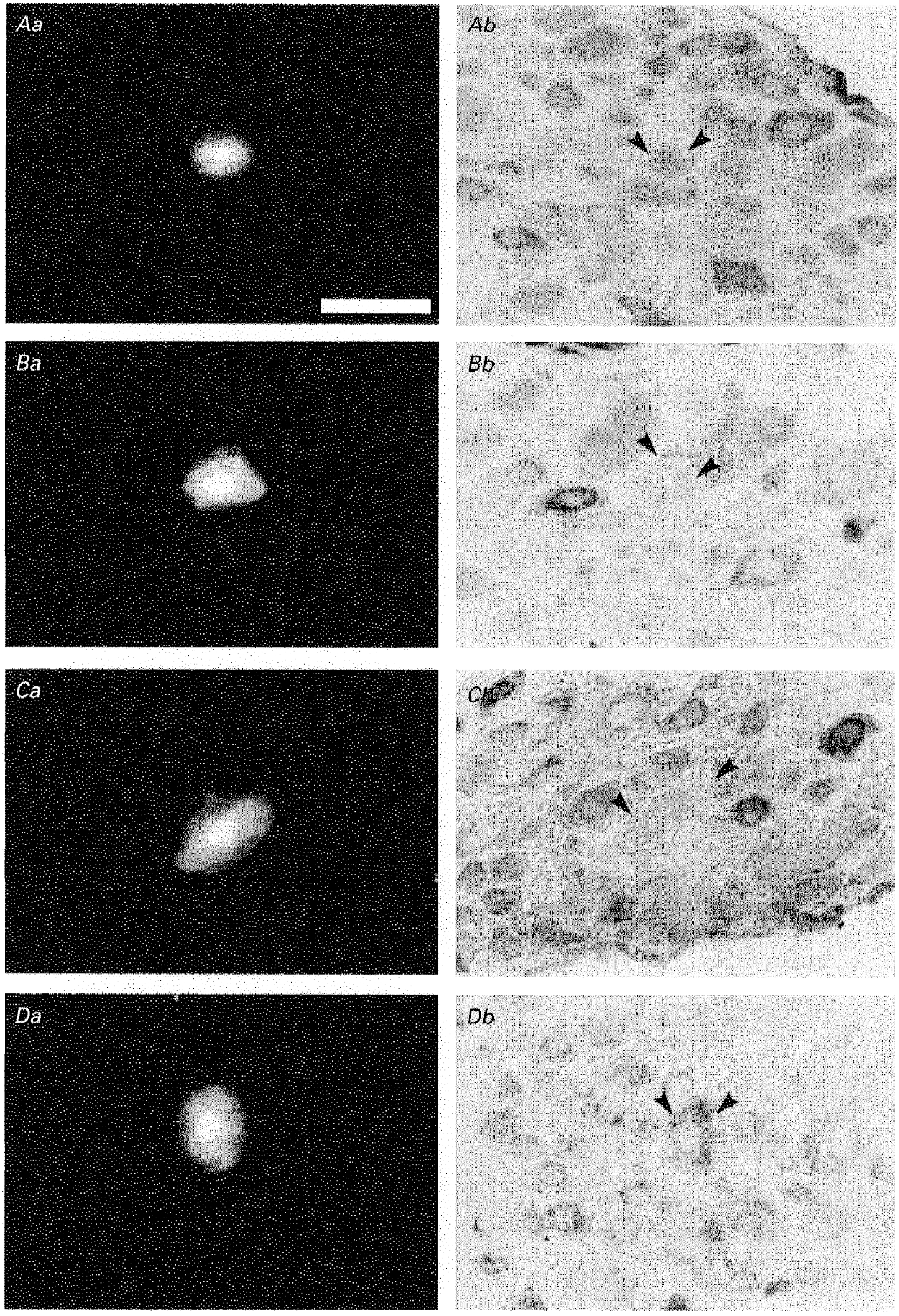
The greatest failure in data collection was the inability to find some of the dye-injected cells. In addition, leakage from the electrode could occur with all of the dyes used and occasionally more dye-labelled neurones were found than there had been deliberate attempts to label neurones. In this respect, EB was the worst of the dyes used and apparently was readily ejected from the electrode by brief high frequency pulses of current commonly used to cause penetration of the membrane. The combined failure to label some neurones and the occasional evidence of dye leakage meant that merely finding the right number of labelled cells was not adequate proof that they were the cells into which dye had been intentionally ejected. Exclusion of inappropriately labelled cells therefore proved essential. For the latter purpose, detailed records were kept of the location of the penetration on the surface of the DRG for each electrode track, the depth of every cell for which a membrane potential was obtained, the CV of every unit if available, whether the cell sustained a prolonged penetration and the size and duration of any iontophoretic current passed into the cell. The depth, rostrocaudal and medio-lateral position of every dye-filled cell recovered later was compared with these notes. Units were rejected from the data set if location and depth did not match or if more cells than expected were labelled (see Table 1). Considering all neurones for which marking by dye was attempted, 34% were not found and 12% were rejected because of a discrepancy in location or in the number of cells labelled.

Histochemical procedures

The animal was terminally perfused under deep anaesthesia through the carotid cannula with 0·9% saline followed by Zamboni's fixative (Stefanini, De Martino & Zamboni, 1967). Experimental time from the initial anaesthetic to tissue fixation

Figure 2. Appearance of SP-LI in identified C fibre neurones

On the left are fluorescence images of dye-injected neuronal profiles captured prior to immunocytochemistry (*Aa–Da*). In *Aa*, *Ca* and *Da* the dye is LY, and in *Ba* is ethidium bromide. *A*, *B* and *C* are from L6, *D* is from S1. All sections are through the nucleus. On the right in each case, the same section is shown after ABC immunocytochemistry to show SP-LI with the dye-labelled cell indicated by arrowheads (*Ab–Db*). *A*, CPM unit on the dorsum of the foot (hairy skin) with a CV of 0·35 m s^{-1}, which was negative for SP-LI. *B*, CPM unit on the side of the toe (glabrous skin) (CV, 0·48 m s^{-1}), which was weakly positive with clear spots of perinuclear immunoreactivity. *C*, a heat nociceptor (CV, 0·35 m s^{-1}) which responded to noxious but not non-noxious radiant heat on the foot, but for which no mechanically sensitive region could be found. This unit was strongly positive. *D*, CHTM unit with a deep cutaneous receptive field on the lateral thigh (CV, 0·56 m s^{-1}), which was strongly positive for SP-LI. Scale bar, 50 μm.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ranged from 7–9 h of which 3–5 h were spent in recording. The DRGs were removed, post-fixed for 1 h in Zamboni's fixative and stored overnight in 30 % sucrose buffer at 4 °C. Serial 7 μm cryostat sections of the experimental DRGs were mounted on ten slides so that each slide had every tenth section. The dye-labelled neurones were located under fluorescence microscopy and the position within the section of every profile of each dye-labelled neurone was recorded with camera lucida drawings to enable relocation of the marked cell after immunocytochemistry. Tissue sections were stored at −20 °C until immunocytochemistry was carried out. Only profiles through the centre of the cell were used for SP immunocytochemistry because the SP-LI reaction product was often closely perinuclear and sections through the periphery of the cell could give rise to an apparent weak or absent SP-LI staining.

Prior to immunocytochemistry, endogenous biotin-like activity was blocked using the Vector SP-2001 kit. Avidin–biotin immunocytochemistry (Vectastain Elite ABC kit, Vector Laboratories Burlingame, CA, USA: rabbit IgG, Vector PK-6101) was performed adding 0·3 % Triton X-100 to the washes, antibody and ABC steps. The primary anti-SP antibody was diluted 1 : 4000–1 : 8000 in tris-buffered saline with 0·3 % Triton X-100 for 15–25 h at 4 °C. Nine of 126 neurones located in tissue sections with generally weak SP-LI in all cells or with high background were discarded (Table 1).

The primary SP antibody was polyclonal (Incstar 20064; Incstar Corporation, Stillwater, MN, USA) raised in rabbit; it was reported by Incstar not to react with neurokinin A. Preabsorption tests were carried out as follows. SP antibody (1 : 6000) was incubated overnight at 4 °C with either SP or neurokinin A (NKA) at concentrations of 0, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. The preabsorbed antibody was used for ABC immunocytochemistry on DRG histological sections. Complete loss of immunoreactivity of the SP antibody occurred with pre-incubations of 10^{-4} and 10^{-5} M SP, almost complete loss at 10^{-6} M and partial loss of immunoreactivity at 10^{-7} M. With NKA preabsorption, none of the concentrations totally abolished immunoreactivity with the SP antibody; however, reduction in intensity appeared at NKA concentrations of 10^{-4} and 10^{-5} M. There was a greater staining intensity with the SP antibody after preabsorption with 10^{-4} M NKA than with 10^{-6} M SP.

Two techniques were used to score the immunocytochemical reaction product, although only the objective technique was used for the final compilation of the data. Dye-injected neurones were scored subjectively as negative (clearly unlabelled), positive (clearly labelled by the SP-LI) and borderline. A semi-quantitative method was eventually employed to assess the absorbance of light by the reaction product in each dye-labelled cell. The essence of the technique was to compare the absorbance of the marked profile with other cells in the same section. The absorbance in a profile was measured in 6·5 mm diameter circles centred over the neuronal cytoplasm using a high resolution CCD video camera (Optronics Dei-470T) and a digital computer fitted with Image Pro Plus (IPPLUS; Media Cybernetics, Silver Spring, MD, USA) software. For each section, the 0 % absorbance level (a) was taken as the mean absorbance of three neurones deemed to be unquestionably negative in the immediate vicinity of the labelled cell. The 100 % absorbance level (b) was the mean of three values from the most intensely stained cell in the section. The mean of three absorbance values from the dye-labelled cell provided the value c. The relative absorbance of the cell was calculated as $a - c/(a - b)$ and expressed as a percentage. If there was variability in staining intensity or background across the section a further measurement was taken. This was a 0 % absorbance level (a') calculated from three unquestionably negative cells in the vicinity of the most intensely labelled cell. The final calculation in these cases was $a - c/(a' - b)$.

Neurones with relative absorbance > 20 % were judged subjectively as clearly positive by all viewers. Those with absorbances of $\leqslant 15$ % were judged subjectively to be negative. Five neurones with absorbances of 15–20 % were classified as borderline; three were C fibre and two were A fibre units. To simplify description the term positive is used throughout to refer to neurones with relative absorbances > 20 %, and the term negative to refer to all units with relative absorbances < 20 %. For neurones with 15–20 % absorbances and classified as negative, the possibility that low levels of SP-LI were present cannot be excluded. Cell size was estimated from the cross-sectional area of the largest section (containing the nucleus) through each dye-labelled neurone.

Bias in selection of neurones

Several factors introduced bias into the selection of units studied. (1) Stable recordings are much easier to obtain from large somata than small ones. In part, this bias was compensated for in some experiments by rejection of units with rapidly conducting afferent fibres and low mechanical thresholds in order to search for elements with C or Aδ CVs. (2) Low threshold neurones are much easier to identify than high threshold neurones. Characterization of the latter takes longer and there is a greater chance that the neurone could be lost prior to identification or dye injection. (3) The search stimuli used initially can affect the population of neurones that are identified. Thus, warming-sensitive and purely heat-responsive nociceptive units would not have been identified in many experiments.

RESULTS

The appearance of SP-LI staining was similar in most experimental and control ganglia. Most of the intense labelling was seen in neurones classified as small (< 25 μm diameter) or medium sized (25–35 μm diameter) (see Figs 2 and 3). The staining varied from very intense to weak diffuse granular labelling throughout the cytoplasm especially in the medium to large neurones. There were

Figure 3. Appearance of SP-LI in identified A fibre neurones

As in Fig. 2, dye-labelled profiles prior to immunocytochemistry are shown on the left (*Aa–Da*), and the same sections after ABC immunocytochemistry for SP-LI on the right with the dye-labelled cell indicated by arrowhead (*Ab–Db*). Again, all sections are through the nucleus. The dye is LY in *Aa*, *Ba* and *Da* and CB in *Ca*. Neurones are all from the S1 DRG. *A*, CLTM with receptive field on the upper thigh (CV, 0·81 m s^{-1}). This section includes only the edge of the nucleus. There is no clear SP-LI. *B*, a D-hair unit with a receptive field on the thigh. The soma is clearly negative for SP-LI (CV, 3·0 m s^{-1}). *C*, an Aδ fibre HTM unit (CV, 3·74 m s^{-1}), which had a punctate superficial cutaneous receptive field on the upper thigh, and which has no discernible SP-LI. *D*, an Aδ fibre HTM unit (CV, 1·88 m s^{-1}) with a deep cutaneous receptive field. This unit shows moderate SP-LI immunoreactivity. Scale bar, 50 μm.

some large neurones with very faint diffuse granularity that were not classified as positive in these counts.

This report is based on an analysis of 117 neurones (66 with A fibres and 51 with C fibres) that were successfully labelled, located in histological sections and for which there was acceptable staining of SP-LI in the tissue sections containing the marked neurone (see Table 1). Examples of typical positive and negative histochemical staining for SP-LI appear in Figs 2 and 3.

Conduction velocity (CV)

To gain a framework for the range of CVs expected in the dorsal roots under the condition of these experiments, a compound potential of a dorsal root (S2) was used as a reference (Fig. 4*A*). In this example, the Aα/β deflection involved fibres conducting over 4 m s^{-1}. As indicated, the prominent Aδ deflection reflected activity in fibres conducting between 2·5 and 1 m s^{-1}. The broad C wave involved fibres with CVs of 0·3 to slightly over 0·7 m s^{-1}. These CVs are low relative to those often quoted from work

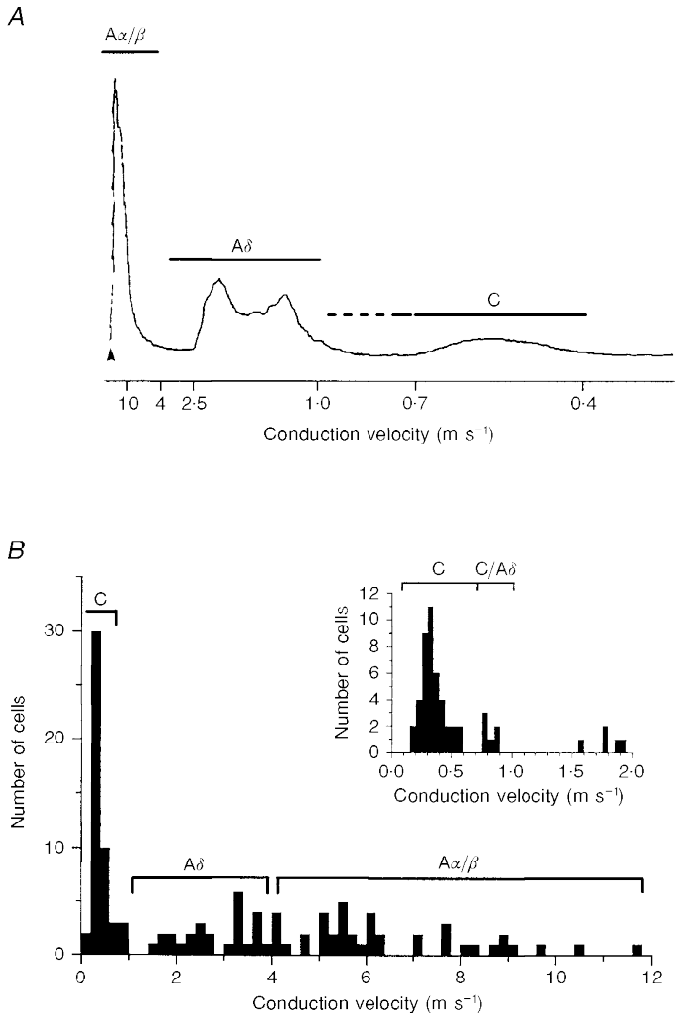

Figure 4. Dorsal root compound action potential and CVs of units

A, compound action potential recorded over a 6·5 mm length of an S2 dorsal root in a 200 g guinea-pig. The arrowhead shows the onset of the 0·3 ms stimulus. Approximate CVs (calculated by dividing distance by latency) are indicated below. These are approximate since distance is short and no allowance was made for utilization times. The onset of the A wave is masked by the stimulus artifact. The falling edge of the A wave includes CV values down to 4–5 m s^{-1}. A clear biphasic Aδ wave can be seen (CV range, 1–2·5 m s^{-1}), as well as a C wave (CV range, $<$ 0·4–0·7 m s^{-1}). The dashed line indicates C/Aδ CVs. *B*, frequency distribution histograms of dorsal root CVs of all units tested for SP-LI. From the compound action potential in *A*, the range of C, Aδ and Aα/β fibre CVs are shown above the histogram. The inset shows the slowly conducting units on an expanded scale, with (again from the data in *A*) the CV range of C fibres and of C/Aδ fibres.

on other species such as man, monkey, cat and even rat. This results from several factors: the species is small, the animals were young, temperature in the paraffin pool was low (28–31 °C), fibres in the dorsal roots conduct more slowly than those in peripheral nerves (Waddell, Lawson & McCarthy, 1989) and the calculated CVs, measured over short conduction distances, did not account for utilization time. Figure 4*B* matches the spectrum of CVs of the units studied to the compound potential of Fig. 4*A*. As this comparison indicates, units with CVs over 4 m s^{-1} are categorized as Aα/β, those with CVs of 1–4 m s^{-1} as Aδ and those with CVs under 1 m s^{-1} as C units. The few neurones with CVs between 0·7–1 m s^{-1} are labelled as C/Aδ in the inset of Fig. 4*B*; they include two CHTM units, the CLTMs and some spontaneously active cooling-sensitive neurones. The tendency for CLTM and cooling-sensitive units to be among the most rapidly conducting C fibre elements has been noted in other species (Bessou *et al.* 1971; Hensel & Iggo, 1971). In general, the categorization of unit type based upon receptive properties and the CVs relative to the overall CV range in these animals corresponded well with those of other species. The CVs of units classified as D-hair type (1·9–4 m s^{-1}) and as G-hair or other low threshold mechanoreceptive types (4·7–11·7 m s^{-1}) did not overlap. In contrast, as was noted originally in cat, the epidermal A fibre mechanical nociceptors (AHTMs) had CVs distributed over much of the A fibre range although the majority of the units had Aδ or lower Aα/β values (Burgess & Perl, 1967).

The proportions of neurones with SP-LI according to CV grouping are in the order C > Aδ > Aα/β (Fig. 5). Most positive C fibre units had CVs < 0·4 m s^{-1} and conversely most C fibre units with CVs > 0·4 m s^{-1} were negative. Thus, as previously documented in rat, there proved to be a greater tendency for more slowly conducting fibres to show SP-LI (McCarthy & Lawson, 1989).

Cell areas

Distributions of cross-sectional areas of all neurones studied showing those with unambiguous SP-LI labelling appears in Fig. 5, grouped according to the dorsal root CV category. The distributions of cross-sectional area roughly approximate normal patterns. The mean cell area range is Aα/β > Aδ > C, a pattern similar to that previously established in rat DRGs (Harper & Lawson, 1985). The majority of positive C fibre neurones had cross sectional areas < 400 μm^2. A fibre DRG neurones exhibiting SP-LI had cross-sectional areas < 1000 μm^2, placing them in the small to medium size range. As Fig. 5 documents, smaller neurones within both the A fibre group (Aδ plus Aα/β) and the C fibre group were the more likely to show SP-LI.

C fibre neurones

Positive SP-LI (> 20 % absorbance) appeared in nineteen of fifty-one C fibre units (37 %). All the C fibre units positive for SP-LI with receptive fields in somatic tissue were nociceptive (Fig. 6*A* and *B*, Table 2). Of those units with cutaneous (superficial or deep) receptive fields (excluding those in the 'miscellaneous' group) fourteen of thirty-seven (38 %) showed clear SP-LI. These included half the nociceptors (14/26) but no non-nociceptive units (0/10). Of the nociceptive units with subcutaneous receptive fields in

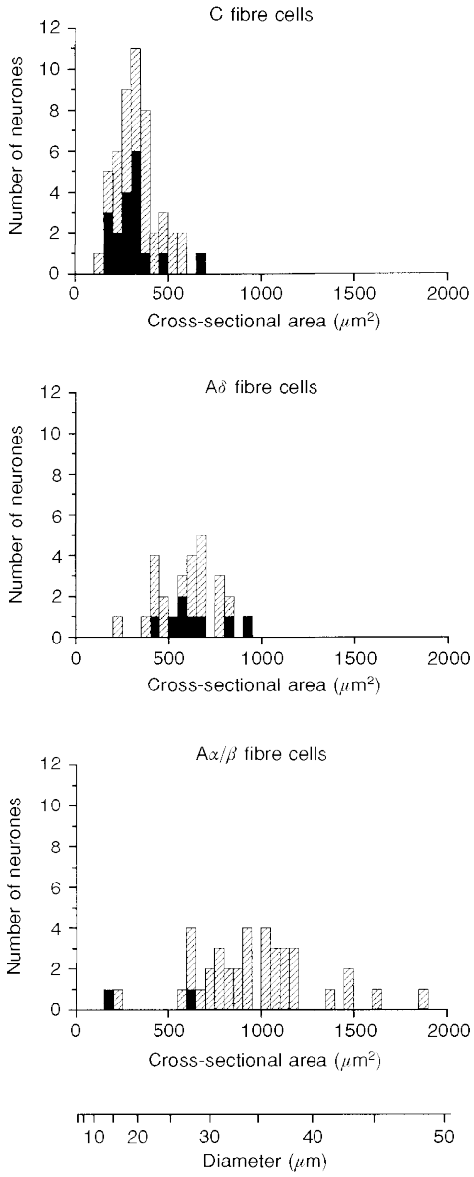

Figure 5. Cell sizes and SP-LI

Histograms of cross-sectional area of the largest section through each cell tested for SP-LI. These are subdivided into C (including C/Aδ) fibre neurones (upper histogram), Aδ fibre neurones (middle histogram) and Aα/β neurones (lower histogram). SP-LI positive neurones (i.e. those with an absorbance of > 20 %) are shown as filled columns, while the negative neurones are shown as hatched columns. The area of the largest section was not available for three cells (one C, one Aδ and one Aα/β fibre cell) and their areas are therefore not included.

the leg or foot, only one of six showed clear SP-LI. A possible visceral afferent unit was also positive. Overall, one half (16/33, 48%) of all the units classified as nociceptive, were clearly positive. Furthermore, the intensity of staining of the positive units varied substantially (Fig. 6*A*, Table 2).

The CPM units were particularly interesting in that only six of fifteen exhibited clear SP-LI. By definition, these all had superficial receptive fields. The proportion of CPM units with unambiguous SP-LI as well as the mean SP-LI intensity were both higher for glabrous units than for units from hairy skin (Table 2, Fig. 6*C* and *D*), although the numbers were not large enough to eliminate the possibility that these differences arose by chance. Images from a negative hairy skin unit and a positive glabrous skin unit are shown in Fig. 2*A* and *B*. The CMH units responding to noxious heat and to strong mechanical stimuli of deep cutaneous tissue or subcutaneous fascia exhibited intense SP-LI (Fig. 6*C*, Table 2).

Most cutaneous CHTM units exhibited SP-LI. Of these (Fig. 6*D* and Table 2) only three of five superficial cutaneous units showed weak SP-LI; the range of staining for this group was limited from just negative to just positive (Table 2, Fig. 6*C*). All three deep cutaneous CHTM units showed intense SP-LI (Fig. 6*C* and *D*, for example see Fig. 2*D*). Only one of five subcutaneous units (all of which were in the foot or toes) showed SP-LI and this positive unit was activated solely by squeezing laterally across the whole foot.

As shown in Fig. 6 and Table 2, all seven units that were excited by cooling or cold proved negative. This group included units with ongoing activity prior to cooling stimuli, as well as presumed cold nociceptive units that were also consistently excited by strong mechanical stimuli. We should have been unlikely to detect cold-sensitive neurones that required skin or tissue temperatures below 5 °C. In a few cases the position of the dye-labelled neurone and the position of a unit responding to skin cooling failed to match or were in question; that is, the dye-labelled cell failed to

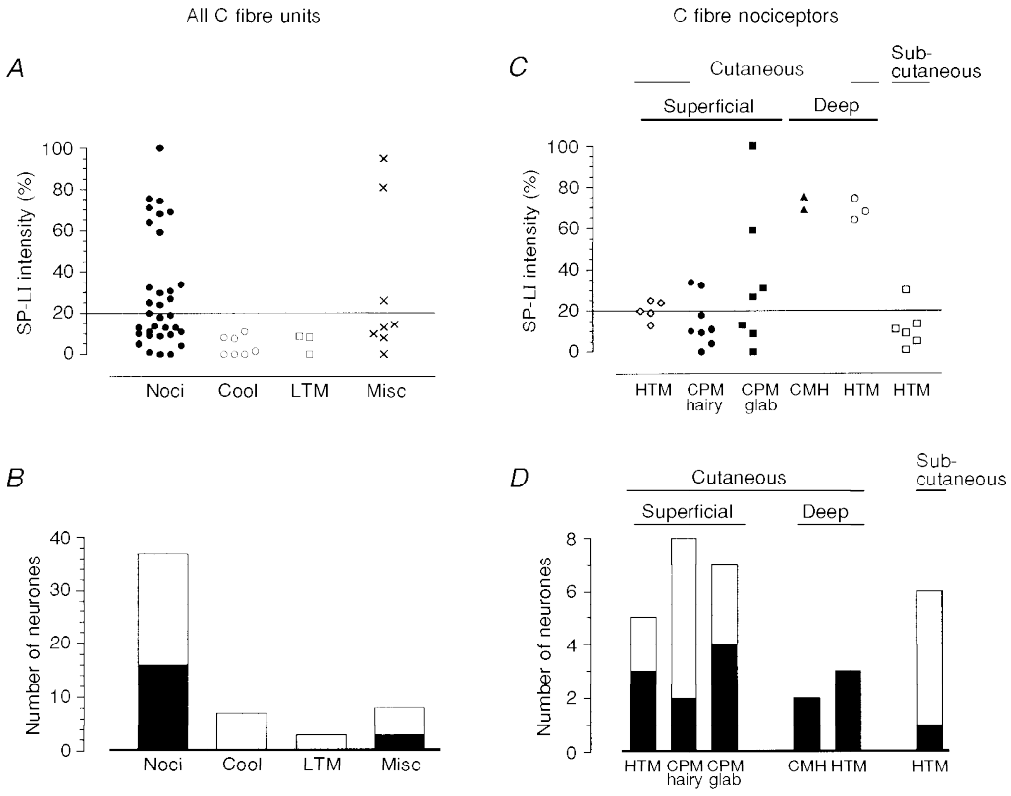

Figure 6. SP-LI in C fibre units

A and *B*, all C fibre units. Units are subdivided into Noci (all nociceptive units); Cool, units that responded to cooling; LTM, C low threshold mechanoreceptors; and Misc, a miscellaneous group, for details see text and Table 2. *C* and *D*, C fibre nociceptors. These units were subdivided according to their receptor properties, into those with superficial cutaneous, deep cutaneous and subcutaneous receptive field locations. HTM, high threshold mechanoreceptors; CPM, C polymodal nociceptors (by definition these have superficial cutaneous receptive fields). CPMs are subdivided into those in hairy skin (hairy) and those in glabrous skin (glab). MH, mechano-heat sensitive units with deep cutaneous receptive fields. Scattergraphs in *A* and *C* show SP-LI intensity (the relative absorbance of each cell as a percentage of the absorbance of the most intensely labelled cell in the section). Stacked bar charts in *B* and *D* show the proportions of units in *A* and *C* classified as positive for SP-LI (> 20% absorbance) as filled bars and those classified as negative for SP-LI (< 20% absorbance) as open bars.

Table 2. C fibre neurones

Sensory receptor classification	Receptive region	SP-LI (+/−)	SP-LI intensity (%)
All C fibre units		19/51	
1. All nociceptive units	Superficial and subepidermal	16/33	29 (0–100)
Superficial cutaneous			
CPM in hairy skin	Hairy skin	2/8	15 (0–34)
CPM in glabrous skin	Glabrous foot and toes	4/7	34 (0–100)
CHTM	Hairy and glabrous skin	3/5	20 (13–25)
Deep cutaneous			
CMH	Dermis/fascia	2/2	72 (69–76)
CHTM (leg/foot)	Dermis/fat/fascia	3/3	70 (64–75)
Subcutaneous	Muscle/joint/deep fascia in foot	1/6	12 (1–30)
Incompletely characterized			
CHTM or CPMN	Superficial cutaneous, hairy	0/1	13
?Heat nociceptor	Foot	1/1	71
2. Cooling units	Not known	0/7	4 (0–11)
3. CLTM units	Superficial cutaneous	0/3	6 (0–9)
4. Miscellaneous units	Various	3/8	31 (0–95)
Not tested	Unknown	1/1	95
Spontaneous ?visceral	Lower abdominal	1/1	81
?Silent no response	Unknown	1/2	18 (10–26)
?Silent or very high threshold	Subepidermal/deep	0/2	7·5 (0–15)
LTM/HTM	?Superficial in hairy skin	0/1	8
?Warm receptor	Unknown	0/1	13

Categories of C fibre neurones are categorized according to receptive properties and receptive field locations. For each group the ratio of positive to negative units (+/−) is given, and in the final column the mean SP-LI relative intensity is given, with the minimum and maximum relative intensity for that group of neurones in parentheses.

meet the criteria set. Since two of these showed SP-LI, there remains the possibility that some cells excited by cooling or cold may express SP, despite the lack of direct evidence presented here.

Three CLTMs with C/Aδ CVs were negative for SP-LI (Fig. 6). An example of the immunocytochemical staining for this class appears in Fig. 3*A*.

One incompletely classified C fibre unit that responded to noxious mechanical stimulation of the skin in the thigh was negative (CHTM or CPM, Table 2). Another incompletely categorized unit that responded to noxious heat but not to noxious mechanical stimulation of the foot demonstrated intense SP-LI (see Fig. 2*C*, Table 2).

A miscellaneous group of eight units with afferent receptive properties that were not fully categorized is grouped together as 'Misc' in Fig. 6*A* and *B* and Table 2. One with strong SP-LI had sporadic low frequency bursts of action potentials and was excited by pressure on the lower abdomen; it was tentatively considered of visceral origin. Several units (Table 2) were classified as '?silent'. One showing SP-LI and one negative for SP-LI could not be excited by a full range of tests over the hindlimb and may have had inaccessible receptive fields. Two '?silent' units that responded with only one or a few action potentials to very intense mechanical stimuli (squeezing of tissues) were negative; they either had very high thresholds or were excited by direct activation of the nerve fibre in passage. One negative unit was not excited by noxious heat and had a response pattern to mechanical stimuli intermediate between typical CHTMs and CLTM units.

Additionally, a unit with ongoing activity that was suppressed by cooling and lacked a mechanically receptive region (possibly a warming receptor) was negative. One unit was not tested for its afferent receptor properties. It showed SP-LI.

There were apparent patterns in the proportion and relative intensity of SP-LI in different categories of DRG neurones with C fibres (Table 2, Fig. 6*A* and *C*). Most groups showed a fairly limited range of relative staining intensities, the notable exceptions being the CPMs. The most intense SP-LI staining was found in deep cutaneous C fibre nociceptors and in some CPMs of glabrous skin. There was weak or undetectable staining in superficial cutaneous CHTM units and most hairy CPM units. Surprisingly, SP-LI was absent in most of the subcutaneous nociceptors we encountered.

A fibre neurones

Overall, ten of sixty-six A fibre units showed SP-LI. Of these fifty-six were cutaneous units, seven of which showed clear SP-LI. None of the twelve D-hair units (Aδ afferent fibres) and none of a variety of the twenty-three other low threshold mechanoreceptors with Aα/β fibres (Fig. 7*A* and *B*, Table 3) showed detectable SP-LI. The sample of low threshold units included eight Aα/β units with receptive fields in muscle/tendon, and twenty-three with receptive fields in skin. The typical absence of immunocytochemical staining in a D-hair unit is shown in Fig. 3*B*.

As indicated in Fig. 7*A* and Table 3, SP-LI in both cutaneous and subcutaneous units was confined to nociceptive A fibre neurones. A higher proportion of the Aδ than the Aα/β nociceptive units showed SP-LI (Table 3, Fig. 7*A* and *B*). In HTM units, SP-LI was notably absent from AHTM units with superficial receptive fields. The negative superficial AHTM group had functional features typical of this class of mechanical nociceptors, exhibiting high mechanical thresholds and multiple punctate receptive fields in both hairy and glabrous skin. The staining of a typical SP-LI negative AHTM appears in Fig. 3*C*. The twenty negative AHTM units included five (3 Aδ and 2 Aα/β) that were most effectively excited by noxious mechanical stimuli but gave weak responses to less than noxious pressure; these appeared to be equivalent to units labelled moderate pressure receptors in other species (Burgess & Perl, 1967). In contrast to the negative superficial cutaneous AHTM units, the superficial cutaneous AMH units from hairy skin that we encountered were positive (3/3), as were nearly all A fibre nociceptors with deep cutaneous and sub-cutaneous receptive terminals; for an example see Fig. 3*D*.

DISCUSSION

The results of this study lead to two principal conclusions. Firstly, in guinea-pig DRG neurones with somatic receptive terminals, unquestionable SP-LI was a feature only of units that were adequately activated solely by noxious stimuli, that is, of nociceptive neurones. Secondly, while some

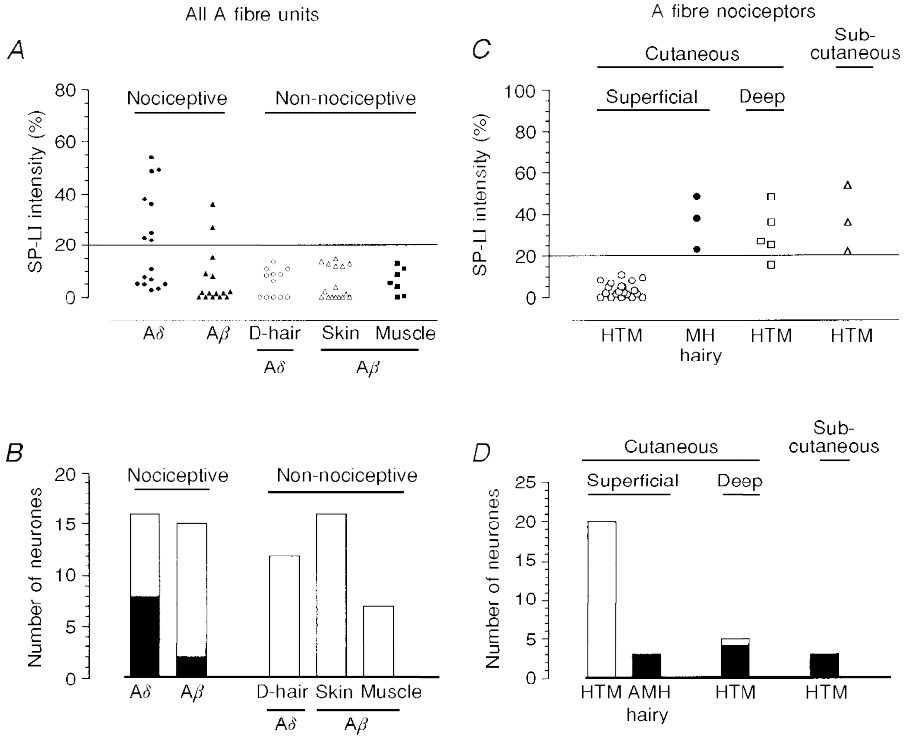

Figure 7. SP-LI in A fibre units

Scattergraphs in *A* and *C* show SP-LI intensity (the relative absorbance of each cell as a percentage of the absorbance of the most intensely labelled cell in the section). Stacked bar charts in *B* and *D* show the proportions of units in *A* and *C* that are classified as positive for SP-LI (> 20% absorbance) as filled bars and those classified as negative for SP-LI (< 20% absorbance) as open bars. *A* and *B*, all A fibre units. Units are subdivided into nociceptive and non-nociceptive groups (see Fig. 1) and subdivided into Aδ and Aα/β units. The non-nociceptive Aδ fibre neurones were all D-hair units, and the Aα/β fibre units are divided into two groups, Skin and Muscle according to the locations of their receptive terminals. *C* and *D*, A fibre nociceptors. Nociceptive units shown in *A* and *B* were subdivided into those with superficial cutaneous, deep cutaneous and subcutaneous receptive field locations. HTM, high threshold mechano-receptors; MH, mechano-heat sensitive units; hairy indicates that receptive fields were in hairy skin.

Table 3. A fibre neurones

Sensory receptor properties	Receptive region	SP-LI (+/−)	SP-LI intensity (%)
All A fibre units		10/66	
1. Nociceptive units		10/31	14 (0–54)
A. Subdivided by CV category			
Aδ units		8/1621	(3–54)
Aα/β units		2/15	7 (0–36)
B. subdivided by receptive field location			
Superficial cutaneous			
AHTM units	Hairy and glabrous skin	0/20	3 (0–11)
AMH units	Hairy skin	3/3	37 (23–49)
Deep cutaneous AHTM units	Leg and foot	4/5	30 (15–49)
Subcutaneous AHTM units	Muscle, joint, deep fascia	3/3	38 (22–54)
2. Non-nociceptive units			
Aδ cutaneous			
D-hair	Skin	0/12	6 (0–14)
Aα/β cutaneous: all	Skin	0/16	5 (0–15)
G-hair (G1 and G2)	Skin	0/4	3 (0–12)
Field	Skin	0/4	3 (0–13)
Slowly adapting (SA I and SA II)	Skin	0/4	10 (2–14)
RA glabrous	Skin	0/3	6·2 (0–15)
Pacinian	Skin	0/1	0
Aα/β subcutaneous			
Muscle spindle (groups 1a and II)	Muscle	0/6	7 (0–13)
Probable Golgi tendon organ	Tendon	0/1	0

Categories of A fibre neurones: see legend to Table 2.

varieties of nociceptive neurones expressed SP-LI in their DRG somata, not all types of nociceptive DRG somata had demonstrable SP-LI. Thus, while it is possible to argue that the presence of SP in a primary afferent neurone projecting to somatic tissue may mark that cell as nociceptive, the absence of SP-LI does not preclude nociceptive function. Our observations also suggest a correlation between receptive features of primary afferent neurones and the immunoreactivity for SP. These conclusions are at odds with an earlier report that concluded there was a lack of relationship between functional characteristics and SP-LI in DRG neurones (Leah *et al.* 1985). The conditions under which our analyses and the earlier ones were carried out differ significantly and possibly explain the discrepancies.

The earlier work of Leah *et al.* (1985) on cat involved DRG treated with colchicine 1 day prior to the definitive experiment. Colchicine, a potent and neurotoxic agent, has been reported to induce expression of peptide mRNA in neurones (Rethelyi, Mohapatra, Metz, Petrusz & Lund, 1991). The functional classification of neurones used by Leah *et al.* (1985) represented very broad categories and less specific classification of the functional properties than were employed in our experiments. Moreover, their study presented data on only twenty neurones and mentioned no precautions to minimize inclusion of accidentally labelled neurones. We documented accidental labelling of neurones a number of times in our experiments. In addition Leah *et al.* (1985) employed very high iontophoretic currents (7–70 multiples of those we used). These and procedural distinctions, such as our use of only nuclear or central sections for examination of SP-LI, may account for the differences in results between that analysis and our present experiments.

There was a broad spectrum of staining intensities for SP-LI in the guinea-pig DRGs. Although we cannot totally exclude the possibility that some neurones with relative SP-LI intensities $< 20\%$ may have had a low level of peptide, the patterns clearly show much higher probabilities of clear or intense labelling in certain groups of neurones. The fraction of our neurones with positive SP-LI ($> 20\%$ absorbance) was 25% of the total sample; this included nearly 40% of the C fibre and 15% of A fibre cells tested. We found 22% of cutaneous afferent neurones to exhibit SP-LI, a value similar to that reported for cutaneous afferent DRG neurones in rat (18–28%; O'Brien, Woolf, Fitzgerald, Lindsay & Molander, 1989; Lawson, 1992). These comparable numbers lend circumstantial support to our labelling and adequacy of sampling. Also, given the possible influence of species and sampling, they suggest that probably, at most, the effects of experimental conditions on the detectability of SP-LI were minor.

A general presumption in peptide immunocytochemistry is that the presence of a peptide known to modify neuronal excitability or affect other cells is an indication that the host neurone releases the peptide and thereby modifies activity in local neurones or other cells with specific receptors. However, predictions of such actions of a peptide based on its presence in the neuronal soma are more tenuous than predictions based on its presence in neuronal processes and their terminals. Nonetheless, it seems reasonable to surmise that in DRG neurones the presence of SP in their somata represents a store of SP destined to be distributed to the central or peripheral branches. However, the biological significance of the intensity of staining for SP-LI in the neuronal somata remains uncertain. Does more intense staining in the soma indicate that the cell synthesizes and utilizes more SP than a cell that is lightly stained? Does a lightly stained soma represent a neurone that uses SP less for communication or could it result from greater active transport of SP from the soma. In addition the intensity of immunocytochemical staining is well known to be variable and did vary between sections and DRGs. The effects of this variability on our data were reduced by our use of relative staining intensities for SP-LI. Thus, it is possible that systematic differences in the staining in our sample of neurones according to categories of sensory properties may have functional significance.

The systematic differences in SP-LI labelling in relation to the functional properties of the different neuronal groups include the following. None of the DRG neurones with somatic receptive fields that were strongly excited by innocuous mechanical stimuli were SP-LI positive. Further, none of a substantial sample of AHTM units (mechanical nociceptors) with superficial receptive fields were SP-LI positive and none of those C and C/Aδ units that were most effectively excited by skin cooling and that fulfilled the acceptance criteria for location of dye-injected cells proved positive. In contrast nearly all A and C fibre nociceptors with deep cutaneous receptive fields were positive, those with C fibres showing intense labelling. It may be significant that units with receptive fields in deeper cutaneous tissues are more intensely labelled than most of those with superficial terminals. If DRG SP-LI is indeed related to the availability of SP for release from central terminals, excitation of deep nociceptive units may lead to greater SP release in the dorsal horn than afferent units with less SP-LI labelling.

Compared with other groups, the somata of CPM neurones were notable for their lack of consistent staining. Less than half this population showed convincing SP-LI, and only two of fifteen showed strong SP-LI. One possibility, that differences in SP staining reflects activity of the neurone cannot be excluded. However, total depletion has been shown not to occur in the time scale of these experiments, since proportions of rat DRG neurones with SP-LI were unchanged 3 h after prolonged nerve stimulation at 1 Hz at C fibre intensities for 2 h (McCarthy & Lawson, 1989). It therefore seems that the absence of SP-LI in most CPM neurones indicates that SP is not, or is only marginally, expressed. This raises the possibility that the CPM class of nociceptor may include subtypes, one expressing SP at detectable levels and another with no expression or very low, undetectable, expression.

SP is released by primary afferent neurones and possibly by intrinsic neurones in the dorsal horn of the spinal cord in response to peripheral stimulation. Noxious mechanical stimulation of skin (probably of both epidermis and dermis), but not innocuous mechanical stimulation, evoked detectable SP release in the spinal cord (Duggan *et al.* 1988; Kuraishi *et al.* 1989). These data can be considered consistent with our observations since units with low mechanical thresholds did not show detectable SP-LI, while nociceptors with receptive fields in deep cutaneous tissues consistently showed SP-LI, many with intense staining. SP release in the dorsal horn after noxious heat stimuli was more variable. Radiant heat, judged noxious, applied briefly to hairy skin of the hindlimb of the rabbit failed to produce detectable SP release (Kuraishi *et al.* 1989). However, immersing a cat hindpaw in hot water for up to 30 min did cause detectable release (Duggan *et al.* 1988). The latter experiments were more likely than the former to have activated both deep cutaneous heat-sensitive nociceptors and glabrous CPMs, which may account for the differences observed.

SP is released from peripheral terminals of certain primary afferent neurones where it can cause increased local blood flow and permeability leading to plasma extravasation (Lembeck, Donnerer, Tsuchiya & Nagahisa, 1992; Otsuka & Yoshioka, 1993). Bharali & Lisney (1992) reported that only the CPM type of primary afferent fibre is able to evoke such increases in permeability and plasma extravasation; however, only two-thirds (12/18) of their CPM fibres elicited vascular permeability changes. Assuming SP to be the only factor producing plasma extravasation, our observation that only part of the CPM population exhibited clear SP-LI agrees with their finding that only part of the CPM group provokes plasma extravasation. The relatively small number of elements involved in both studies suggests that it may be unwise to make an issue of the differences in the proportion of guinea-pig CPM units exhibiting SP-LI in their somata and the proportion of rat CPMs capable of eliciting plasma extravasation.

As a final comment, it is worth emphasizing that our observations and conclusions on SP only apply to primary afferent units with receptive terminals in somatic tissues. The one unit we studied with an apparent receptive field in the pelvic visceral region proved positive for SP-LI and raises the possibility that distributions of SP may appear in categories of visceral afferent neurones other than purely nociceptive types. This latter possibility is supported by the high proportion ($> 80\,\%$) of visceral afferent units in rat that show SP-LI (Lawson, 1992), a larger proportion than appears likely to represent solely nociceptive units.

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