Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells

(trigeminal ganglion neurons/capsaicin/bradykinin/prostaglandin E_2/substance P)

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ABSTRACT   Sensory neurons were dissociated from trigeminal ganglia or from dorsal root ganglia of rats, grown in culture, and examined for expression of properties of pain sensory cells. Many sensory neurons in culture are excited by low concentrations of capsaicin, reportedly a selective stimulus for pain sensory neurons. Many are excited by bradykinin, sensitized by prostaglandin E_2, or specifically stained by an antiserum against substance P. These experiments provide a basis for the study of pain mechanisms in cell culture.

Pain sensory neurons have been identified as a distinct class of sensory neurons in mammals (1–4). Pain sensory endings are activated or sensitized by painful mechanical stimulation, by painful heat, and by compounds that are released locally in damaged tissue (1, 5, 6). Bradykinin, prostaglandins, and amines are among the compounds that have been shown to activate or sensitize pain endings and are thought to have a role in the pain associated with injury and inflammation (5, 6).

The action of these compounds on sensory endings has been studied in experimental animals (7–14), but the studies have encountered technical limitations. A major limitation is that the mechanisms that underlie excitation or sensitization of pain sensory endings are not accessible to biophysical measurements. Other limitations are that the concentration of bradykinin, prostaglandins, or amines at the sensory endings is not accurately known; and that each of these compounds produces inflammatory changes in the tissue as well as release of other mediators, so that its actions are not evaluated in isolation. These difficulties would be alleviated if differentiated pain sensory neurons could be studied in cell culture. This alternative approach would allow more detailed pharmacological, biophysical, and biochemical studies of pain sensory neurons.

As a first step toward the study of pain mechanisms in culture, we have tested whether some characteristics of pain sensory neurons are expressed by sensory neurons in culture. We find that sensory cells grown in the absence of other cells express sensitivity to capsaicin (8-methyl-N-vanillyl-6-nonenamide), a property restricted to unmyelinated pain sensory fibers in adult animals (15–18), and in addition express other properties (7, 8, 10, 11, 19) of differentiated pain sensory neurons.

METHODS

Cell Culture. The portion of the trigeminal ganglion associated with the mandibular nerve was dissected from newborn rats (CD strain; Charles River Breeding Laboratories) and the cells were dissociated by treatment with dispase (grade 2; Boehringer Mannheim) and collagenase (type I; Worthington). Cells were plated on islands of collagen less than 1 mm in diameter (20) and grown in a modified L-15-CO_2 growth medium (21)

from which methocel and bovine serum albumin were omitted and in which glucose, penicillin, and streptomycin concentrations were reduced by half. Cultures were treated with 10 μM 1-β-D-arabinofuranosylcytosine (cytosine arabinoside) during the 4 days after plating to minimize growth of non-neuronal cells.

Dorsal root ganglia from all spinal segments, and superior cervical ganglia, were dissociated and cultures were prepared by a similar procedure.

For histological experiments, sensory neurons were grown on a collagen substrate about 8 mm in diameter.

Electrophysiology. Neurons were studied after growth in culture for 10–35 days. Cultures were placed on the stage of a phase-contrast microscope and continuously perfused with Hepes-buffered medium at 35–37°C. The composition of the recording medium was as described (22), except that bovine serum albumin and $NaHCO_3$ were omitted; 5 mM Hepes, penicillin (100 units/ml), and streptomycin (100 μg/ml) were added; and pH was adjusted to 7.4 with NaOH. Conventional intracellular recording techniques were used.

Responses were displayed simultaneously on an oscilloscope and on a chart recorder. The action potentials and fast depolarizing potentials are attenuated in those traces reproduced from the chart record.

Drug Application. Capsaicin (Sigma) was dried from a stock solution in ethanol, redissolved at 10 μM in recording medium, and then further diluted. Capsaicin was applied from a micropipette by opening a solenoid valve connecting the micropipette to a reservoir of nitrogen at 4 psi gauge pressure (23). To ensure that capsaicin reached the cell soma and all the cell processes, micropipettes with tip outer diameters of 10–25 μm were used and capsaicin was delivered at several positions above the island. When responses to different concentrations were compared, in each case the capsaicin was applied from a micropipette with a tip outer diameter of about 20 μm.

Bradykinin triacetate (Sigma) in recording medium containing 0.1% bovine serum albumin (fraction V, fatty acid-free; Miles) was applied as described for capsaicin. There was no response when recording medium containing 0.1% bovine serum albumin was applied in this way.

Prostaglandin E_2 (Sigma) was dried from a stock solution in ethanol, redissolved at 10 μM in recording medium, and then further diluted.

The composition of the recording medium containing 50 mM K^+ was the same as that of normal recording medium, except for equimolar substitution of KCl for NaCl to increase the K^+ concentration.

Statistical Analysis. The mean number of action potentials elicited by the test stimulus in the presence of prostaglandin E_2 was compared with the mean number of action potentials elicited in normal recording medium by using the t statistic in a one-tailed test; $P < 0.05$ was required for significance.

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Immunologic Staining. Cultures were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.3 and stained by using the unlabeled antibody peroxidase-antiperoxidase method (24, 25). Sera used were rabbit anti-substance P (Rd2 pooled; provided by S. Leeman), goat anti-rabbit IgG (Sternberger–Meyer), and rabbit peroxidase-antiperoxidase complex (Sternberger–Meyer). Details of the procedure have been described (26).

RESULTS

Neurons were dissociated from sensory ganglia and grown on collagen islands <1 mm in diameter, each island having from a few to a few dozen neurons. Intracellular recordings from the neurons were made with standard microelectrode techniques. Both trigeminal ganglion cells and dorsal root ganglia cells in culture usually were quiescent under our recording conditions.

Capsaicin. To determine whether capsaicin sensitivity was expressed in culture, we tested trigeminal ganglion neurons with capsaicin at concentrations less than 0.1 μM. Capsaicin dissolved in recording medium was applied from a micropipette by a brief pulse of pressure. Many cells responded to capsaicin with action potentials and a few cells responded with a slow depolarization (Fig. 1). Frequently the responses also included fast depolarizing potentials whose amplitudes ranged from <1 mV to 30 mV (Fig. 1b). Because experiments described below
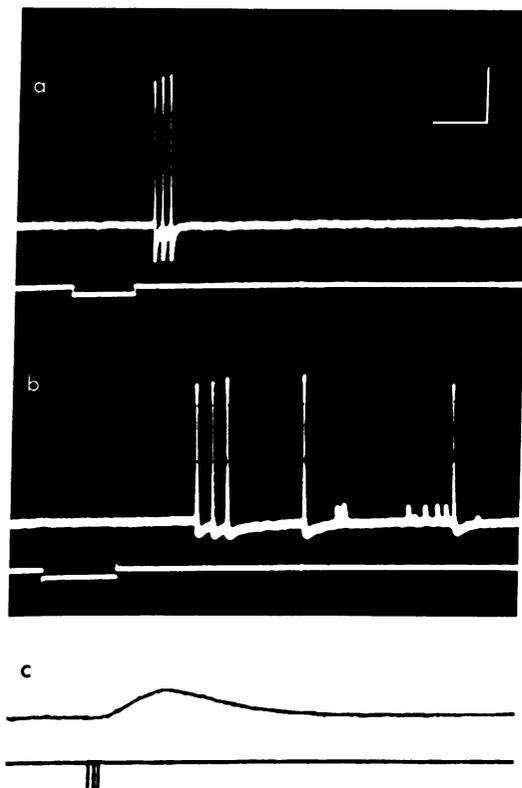
FIG. 1. Responses of three different neurons in cell cultures of rat trigeminal ganglion to application of 30 nM capsaicin. The upper trace in each panel is an intracellular recording from the neuron; the lower trace monitors application of capsaicin. (a) Brief train of action potentials. Twenty days in culture; resting membrane potential, −58 mV. (b) Train of action potentials and fast depolarizing potentials. The complete response lasted 17 sec from application of capsaicin. Only the initial portion is shown. Fifteen days in culture; resting membrane potential, −57 mV. (c) Slow depolarization. Thirteen days in culture; resting membrane potential, −61 mV. Each stimulus pulse in the lower trace of c is 200 msec in duration. Calibration: a and b, 20 mV, 0.5 sec; c, 20 mV, 8 sec.

gave no evidence of synaptic interactions in these cultures, it is likely that the fast depolarizing potentials were action potentials arising in the processes that failed to propagate into the cell body. Often the responses to capsaicin were mixtures of action potentials and fast depolarizing potentials (Fig. 1b) or of action potentials, fast depolarizing potentials, and slow depolarization. Our observations are consistent with the presence of a single class of responsive cells that depolarize when exposed to low concentrations of capsaicin and in which action potentials may arise as a result of depolarization in the cell body or in the processes.

A high proportion (1,214/1,748) of trigeminal ganglion neurons in culture were excited by 0.1 μM capsaicin. The proportion of cells responding would be greater than the proportion of cells sensitive to capsaicin if some cells were excited synaptically, but two lines of evidence suggest that most of the responses to capsaicin were not synaptic potentials. We found that the excitatory responses to capsaicin persisted in recording medium in which the Ca^{2+} concentration was reduced to 0.28 mM and the Mg^{2+} concentration was increased to 10 mM. We also tested directly 39 pairs of neurons for synaptic interactions by eliciting short trains of action potentials at 0.5, 5, and 10 Hz in one of the cells of each pair. Action potentials, fast depolarizing potentials, and depolarization were never recorded in the second neuron, although 24 of these same neurons were excited by 0.1 μM capsaicin. Therefore, it seems likely that most of the cells excited by capsaicin in trigeminal ganglion cultures were excited directly.

Higher concentrations of capsaicin did not elicit action potentials in a larger fraction of the cells. We tested 30 neurons with increasing concentrations of capsaicin, from 1 nM to 10 μM. Eighteen of these cells responded with action potentials or fast depolarizing potentials, and all 18 responded at concentrations of 0.1 μM or lower. Depolarization became increasingly prominent as the amount of capsaicin increased; in almost all (17/18) cells it exceeded 30 mV at high concentrations. A typical response to low concentrations was a burst of action potentials and fast depolarizing potentials; a typical response to high concentrations was a burst of action potentials on the rising phase of a prolonged depolarization (Fig. 2). Two neurons did not respond to capsaicin with action potentials but had relatively large slow depolarizations which increased with the amount of capsaicin applied. Because depolarizing responses to capsaicin without action potentials are common during the first week in culture, these may have been cells that were maturing more slowly than most of the cells in the cultures.

Cells Insensitive to Capsaicin. The same experiment—testing neurons with capsaicin at concentrations from 1 nM to 10 μM—also examined the behavior of cells that did not respond with action potentials to low concentrations of capsaicin. Twelve of the 30 cells studied were insensitive by this test. Except for the two cells already described, which may have been responsive to capsaicin but electrically immature, these cells were also relatively unresponsive to high concentrations of capsaicin. Three of the cells did not respond at any concentration of capsaicin, and the other cells had only small slow depolarizing responses which did not increase appreciably with increasing concentration of capsaicin. All of these cells had depolarizations <4 mV when 10 μM capsaicin was applied. The insensitive cells were not desensitized to capsaicin, because the desensitization which may occur after exposure to high concentrations of capsaicin was avoided in this experiment by testing only one cell in each island.

An apparent insensitivity could have resulted from damage or from the inability of the processes to respond to depolarizing stimuli. Damage by the recording electrode seems an unlikely
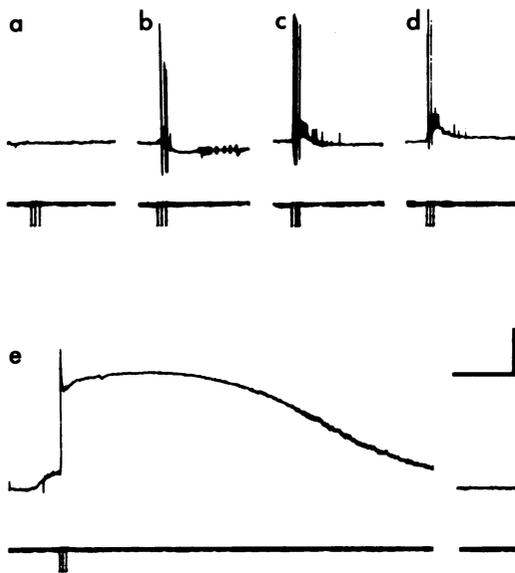
FIG. 2. Responses of a single neuron to capsaicin at concentrations from 1 nM to 10 μM. The upper trace in each panel is the intracellular recording; the lower trace monitors application of capsaicin. (a) At 1 nM. (b) At 10 nM; the disturbance occurring several seconds after the response is electrical noise caused during repositioning of the capsaicin micropipette. (c) At 0.1 μM. (d) At 1 μM. (e) At 10 μM. In e the response begins as the pipette is positioned. A part of the prolonged depolarization has been omitted, but the recovery of membrane potential 100 sec after the application of capsaicin is shown. Each pressure pulse is 200 msec in duration. Eighteen days in culture; resting membrane potential, -55 mV. Calibration: 20 mV, 10 sec.

explanation because, in our total sample of 1,748 cells, the proportion of cells that responded did not vary with the resting membrane potential. An inability of the processes to respond to a depolarizing stimulus also cannot account for the insensitive cells because some of these cells gave action potentials when medium with an increased K$^+$ concentration was applied to their processes. We conclude that some cells in trigeminal ganglion cultures are relatively insensitive to capsaicin.

Further evidence that the action of capsaicin is selective was obtained in studies of neurons from rat superior cervical ganglion. These neurons were grown in culture under the same conditions, and 20 neurons were tested with 10 μM capsaicin. No superior cervical ganglion cell responded with action potentials, fast depolarizing potentials, or depolarization when capsaicin was applied.

Bradykinin. A second physiological marker for some pain neurons is sensitivity to low concentrations of bradykinin. Bradykinin dissolved in recording medium was applied to trigeminal ganglion neurons in the same way as described for capsaicin. A majority (22/39) of the cells tested were excited by application of 0.1 μM bradykinin. A slow depolarization, usually lasting for 30–150 sec, was a characteristic response. In most cases, trains of action potentials occurred with the depolarization (Fig. 3). We cannot exclude that some of the other cells were sensitive because we have not fully investigated the characteristics of the response to bradykinin.

Prostaglandin E$_2$. A third physiological marker for some pain neurons is enhancement of the response to test stimuli by low concentrations of prostaglandin E$_2$. To study sensitization of trigeminal ganglion neurons, a test stimulus of recording medium containing 50 mM K$^+$ was applied to the processes. The number of action potentials elicited by K$^+$ in repeated control responses was nearly constant. When prostaglandin E$_2$ was
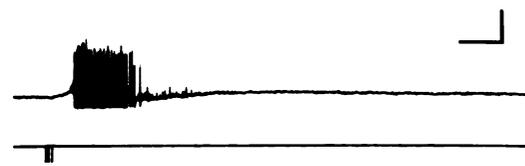
FIG. 3. Response of a neuron in a culture of rat trigeminal ganglion to 0.1 μM bradykinin. The upper trace is the intracellular recording; the lower trace monitors application of bradykinin. The response consists of a train of action potentials and a few fast depolarizing potentials. The underlying slow depolarization, about 2 mV in amplitude, lasts for a minute. Each pressure pulse is 200 msec in duration. Thirteen days in culture; resting membrane potential, -61 mV. Calibration: 20 mV, 10 sec.

added to the recording medium at a concentration of 0.1 μM or less, there was a statistically significant increase in the number of action potentials elicited by K$^+$ in about half (9/21) of the neurons. The number of action potentials returned to control levels after return to normal recording medium. Spontaneous action potentials were recorded in some cells during exposure to prostaglandin E$_2$.

Substance P. A histochemical marker for some pain neurons is staining with antiserum to substance P. In trigeminal ganglion cultures stained with an antiserum to substance P (unlabeled antibody peroxidase-antiperoxidase method), large numbers of neurons were stained consistently (Fig. 4). For three platings (in each of which more than 1,000 neurons were scored as stained or unstained) stained neurons accounted for 43%, 45%, and 41% of the total. When the antiserum was absorbed with substance P or when a nonimmune serum was substituted, there was no staining. We conclude that many trigeminal ganglion cells in culture contain substance P or a related antigen.

Dorsal Root Ganglia. We carried out similar experiments on cells dissociated from dorsal root ganglia and grown in culture. A large proportion (124/155) of these neurons responded to 0.1 μM capsaicin. The responses were excitatory and included action potentials, fast depolarizing potentials, and slow depolarization. When higher concentrations of capsaicin were applied, the same neurons responded with action potentials and with depolarizations >30 mV. Many cells in dorsal root ganglia cultures were excited by 0.1 μM bradykinin, and many were sensitized by prostaglandin E$_2$ (Fig. 5). The responses were similar to those seen in trigeminal ganglion neurons. In addition, many
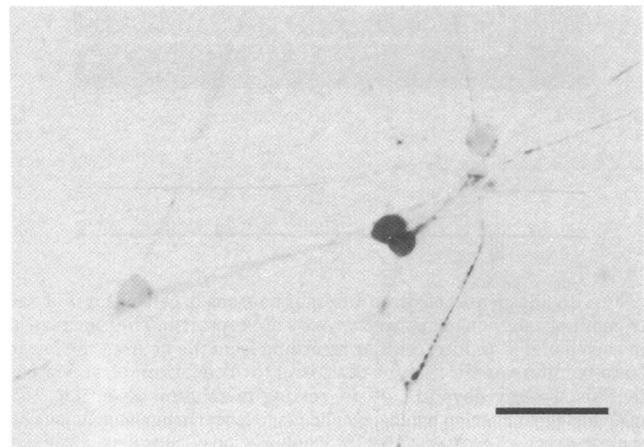
FIG. 4. Staining of trigeminal ganglion neurons in culture by using an antiserum to substance P. The cell bodies of two neurons are darkly stained, and some processes also are stained. Two neurons are not stained. Thirty days in culture. (Scale = 100 μm.)
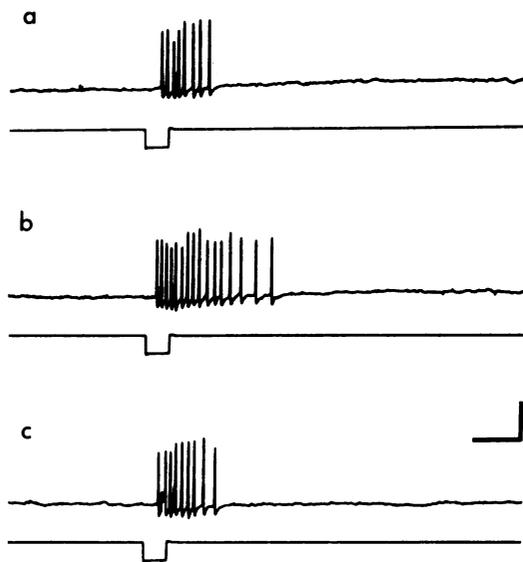
FIG. 5. Sensitization of a neuron in a culture of rat dorsal root ganglia during application of 0.1 μM prostaglandin E_2. The upper trace in each panel is the intracellular recording; the lower trace monitors application of the test stimulus. The test stimulus—recording medium containing 50 mM K^+—was applied every 5 min during the experiment. Only representative responses are shown. (a) Control response before application of prostaglandin E_2. (b) Increased response during perfusion with medium containing 0.1 μM prostaglandin E_2. (c) Control response after return to recording medium without prostaglandin E_2. Thirty-three days in culture; resting membrane potential, −51 mV. Calibration: 20 mV, 0.4 sec.

cells in dorsal root ganglia cultures stained with antiserum to substance P. Thus, it seems that these sensory ganglion cells do not differ from trigeminal ganglion cells in expression of the characteristics we studied.

DISCUSSION

The present study shows that many sensory neurons cultured from rat trigeminal ganglion and dorsal root ganglia are excited by low concentrations of capsaicin. A distinct smaller population of cells does not respond to capsaicin. In addition, many cells are excited by low concentrations of bradykinin, show enhanced responsiveness to stimuli in the presence of prostaglandin E_2, or stain with an antiserum to substance P.

Previous studies have provided evidence that sensitivity to capsaicin and to bradykinin, sensitization by prostaglandin E, and content of substance P are properties of pain sensory neurons. Capsaicin sensitivity is perhaps the most specific marker for unmyelinated pain sensory fibers. In rat and cat saphenous nerve, capsaicin excites only unmyelinated fibers (15, 16). Capsaicin applied to the skin excites only unmyelinated pain fibers, and not other unmyelinated fibers, in the rat saphenous nerve (17). Capsaicin applied to the skin or injected into the saphenous artery acts selectively on unmyelinated pain fibers, and not on unmyelinated mechanoreceptor fibers, in the cat saphenous nerve (16, 18).

An excitatory action of capsaicin on sensory neurons in culture has not been reported. One report described a hyperpolarization and a lengthening of the action potential plateau when 0.3 mM capsaicin was applied to rat and chicken dorsal root ganglia neurons in culture (27).

In contrast to the physiological studies, studies of the toxicity of capsaicin have led to the conclusion that it may not be useful as a selective marker for pain sensory neurons. When high con-

centrations of capsaicin are administered to newborn animals, there is a permanent decrease in the number of sensory ganglion cells, an almost complete loss of unmyelinated sensory fibers, and a loss of some myelinated sensory fibers (28, 29). The extent of damage to sensory fibers in these experiments implies that not only pain sensory neurons but also sensory neurons with other physiological functions are affected. Thus, both a selective physiological action of capsaicin in adult animals and a less selective toxicity of capsaicin in newborn animals are clearly established. The two results can be reconciled if, early in development, most unmyelinated sensory fibers are transiently sensitive to capsaicin and perhaps express other pain properties or if capsaicin toxicity and excitation by capsaicin involve separate mechanisms.

Bradykinin activates unmyelinated and thinly myelinated pain fibers in cutaneous nerves of the cat (7). It excites fibers with similar properties, which are probably pain fibers, in muscle nerves of the cat and the dog (8, 9). Myelinated sensory fibers from hair follicles, pacinian corpuscles, muscle spindles, and Golgi tendon organs are not appreciably excited by bradykinin (7, 8). However, bradykinin is somewhat less selective than capsaicin because it activates some myelinated mechanoreceptor fibers and some unmyelinated mechanoreceptor fibers in addition to pain fibers (7).

Prostaglandin E_2 sensitizes some unmyelinated fibers in cat plantar nerve to painful stimulation with noxious heat, and it sensitizes some unmyelinated fibers in cat muscle nerves to painful stimulation with bradykinin (10, 11). Prostaglandin E_1, which is structurally like prostaglandin E_2, has similar effects (10). Prostaglandin E_1 also sensitizes some thinly myelinated fibers in rat saphenous nerve to moderate mechanical stimulation (12). Some fibers that are sensitized by prostaglandin E_1 are slowly adapting mechanoreceptor fibers (12, 13). Thus prostaglandin E_1 and prostaglandin E_2, although important in sensitization of pain fibers, are probably not more selective than bradykinin.

Substance P has been proposed as a marker for some pain sensory cells. Substance P or a similar compound is present in some neurons located in the dorsal root ganglia and in the trigeminal ganglion (30). The processes of these neurons are unmyelinated and have a distribution similar to that of pain sensory fibers in the spinal cord and in the brainstem (30–33). Substance P is released in the spinal cord by stimulation of sensory nerves (34, 35), and substance P applied iontophoretically increases the activity of neurons in the spinal cord and brainstem that receive input from pain sensory neurons (36–38). The evidence that substance P is a transmitter for certain pain sensory neurons has been reviewed (19).

Substance P has been identified previously in chicken dorsal root ganglia cells in culture (39, 40).

Capsaicin sensitivity and content of substance P have been proposed as specific markers for unmyelinated pain fibers, but it is not known whether these markers are expressed together in all pain sensory cells. It can be estimated from fiber counts (41) and from physiological studies (4, 17, 42) that 30–50% of all sensory fibers in the rat are unmyelinated pain fibers and that most or all of these are sensitive to capsaicin (4, 17). In contrast, only about 20% of sensory neurons stain for substance P (30). In our cultures the fraction of cells sensitive to capsaicin also is larger than the fraction of cells staining for substance P. One interpretation of these results is that some unmyelinated pain fibers do not contain substance P. Another possible interpretation is that all unmyelinated pain fibers contain substance P but that immunologic staining is not sensitive enough to detect all cells in sensory ganglia which contain substance P. In favor of the second interpretation, all unmyelinated pain fibers

in the rat saphenous nerve release one or more compounds that can produce local inflammatory changes in the surrounding tissue (42). Substance P is currently considered the most likely mediator of this neurogenic inflammation (43–45). However, it remains possible that some unmyelinated pain fibers contain and release other peptides, either alone or together with substance P.

The fraction of cells in culture that are excited by capsaicin is larger than the fraction of sensory fibers in the rat that are excited by capsaicin. Likewise, the fraction of cells in culture that stain with antiserum to substance P is larger than the fraction that stain in the animal. This result suggests that a relatively large fraction of sensory neurons are able to express pain properties early in development, if it is assumed that the cells surviving in culture are a representative sample of the cells in the ganglion. An alternative possibility, however, is that pain sensory cells survive preferentially in our culture conditions.

We set out to determine whether characteristics of pain sensory neurons are expressed in culture. Previous studies indicated that capsaicin sensitivity is a marker for unmyelinated pain fibers. We have found that many neurons in rat trigeminal ganglion and dorsal root ganglia cultures are excited by low concentrations of capsaicin. Furthermore, in common with some pain sensory neurons, many neurons in culture are sensitive to low concentrations of bradykinin, are sensitized by prostaglandin E_2, or stain with an antiserum to substance P. These sensory neurons in culture which express characteristics of differentiated pain sensory cells are likely to be useful in studying the neuronal mechanisms involved in pain.

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