Physiol. Res. 45: 1–9, 1996

EDITORIAL

Can Sensory Neurones in Culture Serve as a Model of Nociception?

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Received July 13, 1995
Accepted August 1, 1995

Summary

Nociceptors belong to Aδ and C afferents that are equipped in the periphery with receptors for detecting potentially damaging physical and chemical stimuli. This review summarizes experimental evidence that these receptors represented by ionic channels are also functionally expressed on the cell bodies of sensory neurones in short-term cultures. The nociceptors belong predominantly to the small and medium size DRG neurones in which algogens such as weak acids, capsaicin, bradykinin and serotonin produce inward currents that can generate impulse activity. It seems likely that the neurones which are not sensitive to algogens but to GABA, ATP or glutamate, agents not producing pain in humans, belong to other categories of DRG neurones equipped for detecting other modalities of sensation. New techniques for physical stimulation of DRG neurones in culture may be of great help in the search for complementing the criteria for distinguishing nociceptors among other neurones in culture. It is suggested that such an *in vitro* model will be useful for studying cellular mechanisms of nociception.

Key words

Sensory neurones – Short-term culture – Nociception – Membrane currents – Algogens

1. Introduction

It is generally accepted that primary afferent neurones conveying pain producing signals (nociceptors) extend processes that belong to the groups of small myelinated Aδ and unmyelinated C fibers. There are several classes of nociceptors that differ in their sensitivity to physical and chemical stimuli (Lang *et al.* 1990, Schmidt *et al.* 1994, Kress *et al.* 1992). This diversity may account for the differences in the character of pain (McMahon and Koltzenburg 1990, Cervero and Laird 1991). A substantial part of Aδ and C fibers does not represent nociceptors, and serve other qualities of sensation, i.e. warmth, cold or pressure.

Nociceptors form free endings in the periphery which are not accessible to modern electrophysiological techniques that allow studying biophysical mechanisms of excitation. However, by analogy deduced from studies on central neurones, it can be assumed that free nerve endings possess in their plasma membrane ionic channels that are controlled electrically or chemically. In addition, nociceptors are able to transduce physical stimuli, such as heat or pressure, into physiological signals that finally appear encoded in nerve fibres as spike trains. The proteins that form ionic channels are products of a complicated process of gene expression in the nucleus and the cell body, from where they are transported and finally incorporated into the plasma membrane of the soma or processes.

It was suggested that dissociated neurones from the dorsal root ganglia (DRG) in culture may serve as a suitable model for studying the biophysical mechanisms of nociception and evidence supporting this idea has been presented (Baccaglini and Hogan 1983). There are at least two objections that can be raised against this idea. First, DRG neurones in culture represent cell bodies of sensory neurones that *in vivo* serve various stimulus modalities, and it is difficult to

distinguish which of them may have belonged to nociceptors. Secondly, it cannot be excluded that functional expression of ionic channels is modified under conditions of culturing.

Evidence is rapidly cumulating that DRG neurones appreciably differ in respect to their morphology and functional expression of ionic channels. Although no synapses are formed on the cell body of DRG neurones *in vivo*, some exhibit sensitivity to classical transmitters such as GABA, glutamate, serotonin and ATP in short-term culture. Some DRG neurones are also sensitive to low pH, and to several peptides such as bradykinin which plays a role as an algogen in inflammation, or capsaicin for which no endogenous analog has as yet been found.

This review gives an abbreviated account of the functional expression of ionic channels in dissociated DRG neurones in short-term cultures, focussed on the differences which may be useful in distinguishing nociceptors from other neurones equipped for detecting other modalities of sensation.

2. The size of DRG neurones in short-term cultures

According to the diameter of the cell body, DRG neurones of the rat in short-term culture (1–3 days) can be divided into three groups: A, large (>50 μm), B, medium (30–50 μm) and C, small (<30 μm). As neurones in culture are mostly ellipsoid, the cross-sectional area calculated from the measurements of the longest and the shortest diameter, was considered a better criterion for quantitative evaluation of their size (Petersen and La-Motte 1991). In other studies, the surface area was calculated from the capacitance of the neurone in whole cell mode of recording, assuming a specific capacitance of 1 μF/cm^2 (Campbell 1992, Pearce and Duchen 1994). It seems that all the techniques of measuring the size of DRG neurones give satisfactory results.

It is generally assumed that nociceptors belong to the small and medium-size neurones and that the large neurones give rise to fast conducting afferents that supply muscle spindles, tendon organs and receptors for touch and pressure. Not all experimental evidence, however, supports this view. While a linear relationship between DRG cell body size and conduction velocities of their axons was reported by Yoshida and Matsuda (1979) and Cameron *et al.* (1986), Lee *et al.* (1986) reported a good correlation between cell size and conduction velocity for rapidly conducting axons, but no correlation for slowly conducting neurones. Correlation between the size of some sensory neurones and conduction velocity of their axons was also not found by some authors (Gallego and Eyzaguire 1978, Hoheisel and Mense 1986). Harper and Lawson (1985) demonstrated that the size of the cell body of DRG neurones correlates better with the conduction velocity of their axons than with their sensory function.

3. Voltage-dependent channels

Since the introduction of the patch-clamp technique to neurophysiology (Hamill *et al.* 1981), a great variety of voltage-dependent channels in DRG neurones was studied and characterized (see Hille 1992). The molecular structure of some is already known (see Hall 1992). Here, only some voltage-dependent channels are discussed which are thought to be relevant in the search of criteria for characterization of the nociceptors in culture.

Sodium channels: At least two types of voltage-dependent sodium currents that can generate impulse activity in acutely dissociated DRG neurones have been described in considerable detail. First, sodium channels sensitive to TTX (100 nM) which were predominantly found in large DRG neurones of type A. Second, sodium channels insensitive to TTX (1 μM) which relates to 25 % TTX-insensitive C-fibre terminals in rat skin (Kirchhoff *et al.* 1989) are present on small neurones of type C. On medium size neurones of type B, they usually coexist together with the sodium TTX-sensitive channels (Campbell 1992, Caffrey *et al.* 1992, Roy and Narahashi 1992).

Electrophysiological properties of TTX-resistant sodium channels on DRG neurones were first described by Kostyuk *et al.*(1981). Later, the finding of attenuation of the TTX-resistant sodium currents by calcium channel blockers raised the question of whether the channels underlying this current are similar to calcium channels (Bossu and Felz 1984). There was no doubt that the membrane current was carried by Na^+. However, the experimental condition profoundly decreasing the extracellular concentration of Ca^{2+} did not exclude the possibility that Na^+ was passing through transformed calcium channels (see Carbone and Lux 1988). Later evidence based on a more quantitative study of the permeabilities and blockade with different ions indicates that TTX-resistant sodium channels represent a distinct group of sodium channels (Ikeda and Schofield 1987) that have an epitope identical with the TTX-sensitive channels to which an antibody against brain-type of sodium channels (AP 7493) can bind (Caffrey *et al.* 1992). The functional meaning of the TTX-insensitive sodium channels remains obscure. The finding that they are predominantly found on small neurones implies that they are also present in nociceptors. However, a further study is needed to learn whether their functional expression is merely related to the size of neurones or to their function.

Calcium channels: Calcium channels of the T, N and L type were found on acutely isolated sensory neurones or in short-term culture (Nowycky *et al.* 1985, Fedulova *et al.* 1985), and their kinetics and pharmacological properties were elaborated in great

detail (see Hille 1992, p. 108, Table 1). T-type calcium currents were observed in small and medium size neurones, but not in large DRG cell bodies. It is of interest that the T-type of calcium current was not found in any DRG neurones which were sensitive to capsaicin (Petersen and La-Motte 1991). On the other hand, N-type calcium currents seem to be evenly distributed between the three size ranges. The L-current was found markedly larger in small diameter DRG bodies than in medium or large diameter DRG neurones (Scroggs and Fox 1992).

It was suggested that modulation of individual calcium currents could result in relatively selective modulation of different modalities of sensory transduction, however, as yet no direct evidence supporting this idea has been presented. The high threshold L-type channels seem to be a good candidate for regulation of transmitter and cotransmitter release.

Potassium channels: Potassium channels play a crucial role in determining the resting membrane potential and controlling excitability. Since the membrane potential at rest is always positive to the potassium equilibrium potential, activation of potassium channels results in cell hyperpolarization and decreased excitability, while opposite effects can be expected to occur when potassium channels are blocked. Evidence that potassium channel blockers, 4-aminopyridine and tetraethylammonium, unspecifically excite cutaneous sensory nerve endings in a skin-nerve preparation has been presented (Kirchhoff *et al.* 1992). There are at least six types of potassium channels in excitable and non-excitable cells that are voltage-dependent or are chemically controlled (see Kolb 1990, Hille 1992). Most of them are expressed in DRG neurones (Kostyuk *et al.* 1991).

Two kinetically distinct voltage-dependent potassium channels have been reported in the frog DRG neurones that could be distinguished according to the rising phase of the potassium outward current. The fast type that resembles the A-type potassium current is dominant in large cells, while the slow type may represent a delayed rectifier and seems to be the only component of the potassium outward current in small DRG neurones. In the medium size neurones, a mixture of both components was found (Campbell 1992). Further correlation between expression of various types of potassium channels and the size of DRG neurones has recently been reported (Pearce and Duchen 1994, Scroggs *et al.* 1994).

4. Chemically-controlled ionic channels

Algogens

1. *Weak acids* (pH 6.1) applied to an exposed blister induce pain in man (Armstrong *et al.* 1953) and nociceptive reactions in all vertebrates. As marked decreases of pH were found in exudates produced by inflammatory processes, acidification of extracellular fluids was suggested to be an universal algogen (Lindhal 1962). Although there is no doubt about the algogenic action of acids, it has been demonstrated that there are many other chemicals that induce pain. The mechanisms involved in the action of low extracellular pH, however, remain in the focus of present neurophysiological research because multiple effects of hydrogen ions on the excitability of cultured neurones were reported (Gruol *et al.* 1980). Not only excitatory but also inhibitory effects are produced by acidification. For example, NMDA-gated channels which are probably involved in central transmission from nociceptors, profoundly decrease the probability of their opening when extracellular pH is decreased (Vyklický Jr. *et al.* 1990, Traynelis and Cull-Candy 1990, Tang *et al.* 1990).

Two types of inward current can be evoked in DRG neurones by a sudden decrease of extracellular pH (from 7.3 to 6.1). Firstly, a fast inactivating (τ = 1–2 s) inward current (Krishtal and Pidoplichko 1981) which is carried by Na^+ through protonized calcium channels (Konnerth *et al.* 1987). This current does not fulfil the criteria for inducing nociception because it becomes rapidly inactivated, while pain lasts during the whole period of acid application (Steen and Reeh 1993). In addition, this current can be elicited in about 80 % DRG neurones of all sizes and in central neurones isolated from various parts of the central nervous system. Secondly, a non-inactivating inward current which is carried unspecifically by monovalent cations. This membrane current was discovered by Bevan and Yeats (1991) who recorded it in about 45 % of predominantly small DRG neurones. The non-inactivating current induced by low extracellular pH fulfils the criteria expected for the generation of impulse activity in nociceptors (Steen *et al.* 1992).

Protons modify sodium and block calcium voltage-dependent channels (Hille 1992). There is, however, little information as to what extent potassium channels in DRG neurones are affected by lowering extracellular pH. Their blockade in low extracellular pH may represent an additional mechanism that underlies the increased excitability of nociceptors in acid solutions.

2. *Bradykinin* belongs to the group of peptides which are released during inflammation. In experimental and psychophysiological studies it represents one of the most studied algogens. Bradykinin induces pain or potentiates pain evoked by heat and chemical stimuli (Handwerker *et al.* 1976, Koppert *et al.* 1993, Steen *et al.* 1995) and has been shown to have excitatory effects on cutaneous (Lang *et al.* 1990), visceral (Kumazawa *et al.* 1991) and joint nociceptors (Schmidt *et al.* 1994). *In vivo,* the effects are not limited to a direct action on nociceptors. Bradykinin also activates endothelial cells, fibroblasts, mast cells, macrophages etc. which produce cytokinins and other inflammatory mediators that may sensitize or induce impulse activity in nociceptors.

Although bradykinin is an agent of great clinical interest, little is known about the molecular mechanisms of its action. In cultured DRG neurones, where direct effects can be studied, bradykinin induces a small inward current that is carried mainly by Na^+. There is evidence that bradykinin modifies ionic channels by activating several intracellular second messenger systems (Burgess *et al.* 1989, Allen *et al.* 1992, Bauer *et al.* 1993, Dray and Perkins 1993) and that G-proteins are involved in this process (McGuirk and Dolphin 1992).

A clue to a better understanding of bradykinin action in nociception may be provided by the studies on NG 108-15 neuroblastoma-glioma hybrid cells in which a dual response to bradykinin arising from opposing effects of two different species of membrane currents was observed. First, an early hyperpolarization is produced by an outward current arising from activation of Ca^{2+}-dependent, voltage-insensitive, potassium channels. Second, later depolarization primarily results from inhibition of a distinct class of voltage-gated K^+ current that can also be generated by the muscarinic type of acetylcholine receptor agonists (Higashida *et al.* 1986, Brown and Higashida 1988). Inhibition of Ca^{2+}-dependent potassium channels can be induced by forskolin, a direct activator of the catalytic subunit of adenylate cyclase, suggesting that bradykinin may control cyclic AMP-mediated processes (Weinreich and Wonderlin 1987).

3. *Serotonin* applied to the blister base induces pain in man (Armstrong *et al.* 1953) and potentiates impulse activity in many slow conducting afferent fibres that belong to the polymodal nociceptors (Handwerker *et al.* 1990). Although the molecular structure of many 5-HT receptors was analyzed in detail (see Weinshank *et al.* 1992), the studies on nociception are mostly oriented towards the subtypes of the 5-HT_3 and 5-HT_2 receptors. Serotonin induces a fast desensitizing inward current in frog DRG neurones which is carried by monovalent cations and also fulfils other criteria indicating activation of 5-HT_3 receptors (Philippi *et al.* 1995). This membrane current was exclusively observed in small neurones (60 %), however, its fast desensitization does not make it likely that 5-HT_3 receptors are significantly involved in generating impulse activity that produces pain. It can be speculated that *in vivo*, the 5-HT_3 receptors are located on central terminals of primary afferents, where serotoninergic descending pathways form their endings (Dahlstrom and Fuxe 1965) and account for presynaptic inhibition induced by stimulation of medial structures in the brain stem (see Basbaum and Fields 1979, Besson and Chaouch 1987, Cesselin *et al.* 1994).

In addition, serotonin was found to block high threshold calcium channels in mammalian central neurones (Penington *et al.* 1991) and in DRG neurones (Del Mar *et al.* 1994, Philippi *et al.* 1995). Although the mechanism involved is still not understood completely, the blockade of the high threshold calcium channels at the terminals of primary afferents in the spinal cord may represent another mechanism of presynaptic inhibition exerted by descending serotoninergic pathways.

With respect to nociception, the 5-HT_2 receptors may be of greater importance because it was found that their activation increases the input resistance of the small DRG neurones that is accompanied by depolarization (Todorovic and Anderson 1990). The mechanisms involved apparently include activation of second messengers.

4. *Capsaicin* (8-methyl-N-vanilyl-6-noneamide) represents the active substance in red pepper and is well known to induce burning pain when applied to a blister base (see Szolcsanyi 1993). After synthetization, it became a drug of priority interest in the investigation of nociception (see Dray 1992, Fitzgerald 1983). In young animals its long-lasting application leads to degeneration which affects preferentially, but not exclusively, small DRG neurones (Lawson and Harper 1984).

In some mammalian DRG neurones, capsaicin induces a membrane current carried by monovalent cations and Ca^{2+} through channels that exhibit a conductance of 40 pS (Bevan and Szolcsanyi 1990, Vlachová and Vyklický 1993). Repeated applications of capsaicin result in tachyphylaxis of unknown nature. Capsaicin receptors are present in mammals but apparently not in amphibians and birds. An endogenous ligand for capsaicin receptors has not yet been found. Although capsaicin receptors are predominantly expressed on small neurones (White 1990, Vlachová and Vyklický 1993) their presence on some larger neurones has also been demonstrated (Petersen and La-Motte 1991). As the DRG neurones in culture are devoid of axons, it remains uncertain to what category the large, capsaicin sensitive, DRG neurones belong.

5. *Other algogens.* There are many other chemicals of plant or animal origin, i.e. insect venoms and tachykinins, that could be of interest in the search which of the DRG neurones in culture represent nociceptors. Some of them may be of clinical importance. With respect to nociception, prostaglandins E_1 and E_2 represent a special challenge for studying peripheral mechanisms of pain (Khasar *et al.* 1994, Kumazawa *et al.* 1994), since their action can be prevented by antiinflammatory drugs.

Other chemical substances

6. *ATP*, in addition to its key role in energy metabolism, represents a fast mediator in a number of central neurones (see Benham 1992). It induces an inward current which is carried by monovalent cations in about 40 % of small DRG neurones in short-term

culture (Bean 1990). The idea that ATP might be an algogen was tempting because its intracellular concentration is high and made it likely that it could be released into the extracellular space after tissue damage. However, ATP (as well as ADP, AMP, AP-4-A and AP-3-A) does not excite nociceptors in the skin-nerve preparation, nor does it evoke pain in man when injected into the skin in up to millimolar concentrations (P. Reeh, personal communication). In addition, ATP, even at the highest possible concentration, does not elicit a wiping reflex in decapitated frogs, which is readily produced by weak acids (Vyklický, unpublished results). These results make it unlikely that ATP is an algogen by itself, though they do not exclude synergism with other mediators, and suggest that it acts on another type of small DRG neurones that serve other modalities of sensation (heat, cold, low-threshold mechanoreceptors).

7. *Glutamate.* Evidence for the presence of many types of glutamate receptors, including NMDA receptors, was found in DRG neurones by employing immunocytochemical methods (Sato *et al.* 1993, Tohyama *et al.* 1994). A functional expression, however, was demonstrated only for the kainate type receptors (Huettner 1990). Glutamate and kainate induce membrane currents that are carried by monovalent cations in about 65 % of small DRG neurones. L-glutamate applied to exposed rat tail skin evoked nociceptive reflexes (ED_{50}=136 μM) in the isolated spinal-cord-tail preparations of the neonatal rat (Ault and Hildebrand 1993). The suggestion that L-glutamate represents an algogen has to be further explored because up to millimolar concentrations has not been found to excite nociceptors in a skin-nerve preparation or to evoke pain when applied into the human skin (P. Reeh, personal communication). Neither kainate in millimolar concentrations induced pain when applied to the basal epidermis after removing a blister produced by an accidental burn (Vyklický Jr., personal communication).

8. *GABA* (gamma-aminobutyric acid) represents a classical inhibitory transmitter. $GABA_A$ receptors are present *in vivo* at central terminals of primary afferents in the spinal cord where they play a crucial role in the mechanism of presynaptic inhibition. In the frog, $GABA_A$ receptors can be found in all acutely isolated DRG neurones (Hattori *et al.* 1984, Vyklický *et al.* 1993). In the rat, $GABA_A$ receptors can be detected on large and medium size neurones. In small neurones which are sensitive to capsaicin, responses to GABA are small or absent (White 1990, Vlachová and Vyklický, 1993). Some medium size DRG neurones in culture, however, are sensitive to both GABA and capsaicin. It can be speculated that these neurones represent *in vivo* nociceptors with axons of Aδ fibre conduction velocity. This view is substantiated by an earlier finding that Aδ fibre tooth pulp afferents exhibit classical primary afferent depolarization which can be produced by electrical stimuli applied to the trigeminal nerve (Davies *et al.* 1971). The finding that some small DRG neurones in short-term culture are not sensitive to GABA is of interest because, if this is also the case *in vivo,* this may suggest that another mechanism underlies the depolarization of C group primary afferents in the spinal cord rather than that of fast conducting afferents in which presynaptic inhibition was originally studied (see Eccles 1964).

5. Mechanical and thermal stimulation

In experimental studies on nociception, pressure and heat represent the most widely used stimuli. It is likely that pressure above a certain level activates stretch-induced channels (see Hamill *et al.* 1992). Mainly due to methodological difficulties in activating these channels, their physiological meaning still remains debatable (Gustin *et al.* 1991, Hamill *et al.* 1992). No doubt that further progress in techniques for the mechanical stimulation of neurones in culture will also be useful for deciding whether nociceptors have a different threshold for the activation of stretch-induced channels than the DRG neurones equipped for detecting other modalities of sensation. A technique for producing relatively rapid temperature changes that might be applicable to cultured neurones has already been reported (Lynch *et al.* 1988).

Conclusion

The aim of this commentary was to show that nociceptors are likely to be recognized among other sensory neurones in short-term cultures according to morphological, electrophysiological and pharmacological criteria. Immunocytochemical techniques for staining membrane-bound or intracellular particles can be expected to be helpful in guiding this search (Hokfelt *et al.* 1976, Lawson 1984, 1985, Price 1985). No doubt that such a cellular model will be useful for studying membrane mechanisms of nociception. It would, however, be unreasonable to assume that it has a perspective to become a substitute for animal models in studying nociception or in man for studying pain. A cellular model of nociception is promising to extend present facilities in the research aiming to understand better the mechanisms involved in the initiation of impulse activity in distinct groups of nociceptors that may account for various qualities of pain and in the search for a better understanding of the mechanisms involved in the action of analgesics.

Acknowledgements

This study was supported by the grants of EU CIPA-CT93-0226 and STAPRO Pardubice, CR. The authors thank Prof. P.W. Reeh for critical reading of the manuscript and many useful suggestions and Dr. P. Hník for helpful comments during its preparation.

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