Inflammatory Mediators at Acidic pH Activate Capsaicin Receptors in Cultured Sensory Neurons From Newborn Rats

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INTRODUCTION

A distinct subpopulation of thinly myelinated and unmyelinated primary afferents serves to convey signals from nociceptive “free” nerve endings in peripheral tissue. It is reasonable to assume that a variety of membrane receptors and ion channels exist to transduce chemical or physical stimuli of potentially damaging intensity. Owing to their small size (<1 μM), these nerve endings are not suitable for measuring membrane currents. However the notion that the receptors and ion channels are proteins that may be incorporated in the plasma membrane of the cell body, suggests that a distinct subpopulation of sensory neurons in culture may serve for the study of transduction mechanisms involved in nociception (Baccaglini and Hogan 1983).

8-methyl-N-vanillyl-6-noneamide (Capsaicin), the pungent ingredient of the red pepper, induces burning pain in humans (Szolcsanyi 1991) and discharge activity in C polymodal nociceptors (Szolcsanyi et al. 1988). In ~80% of small dorsal root ganglion (DRG) neurons in culture, capsaicin induces an inward current that is carried by cations, including Ca²⁺, through channels of ~30 pS conductance (Bevan and Forbes 1987; Bevan and Szolcsanyi 1990; Vlachová and Vyklický 1993). This membrane current can be blocked by capsazepine, a competitive capsaicin antagonist that might also act on endogenous ligands acting at putative capsaicin receptors (Bevan et al. 1992). Because capsaicin is an agent foreign to the mammalian body, acting on a distinct class of DRG neurons, it seems likely that such endogenous mediator(s) exist.

Extracellular acidification produces nociceptron excitation in rat skin and burning pain in humans at pathophysiologically attainable proton concentrations (Steen et al. 1992; Steen and Reeh 1993). The underlying sustained membrane current induced by protons in DRG neurons (Bevan and Yeats 1991) exhibits many similarities to the capsaicin-induced membrane current. This led to the suggestion that protons may act as endogenous operators of the putative capsaicin receptor-channel complex (Bevan and Geppetti 1994).

Capsaicin-induced membrane current is greatly facilitated in acidic extracellular solution at a pH below threshold for evoking any membrane current by itself (Petersen and Lamotte 1993). Moreover there is evidence that that this facilitation is produced by protonation of capsaicin-gated channels rather than increased affinity of capsaicin for the receptor (Kress et al. 1996).

Recently, in capsaicin sensitive DRG neurons from adult rats cultured in the presence of nerve growth factor (NGF), it has been found that the inflammatory mediators (IM), bradykinin (BK), 5-hydroxytryptamine (5-HT), histamine (HIS), and prostaglandin E₂ (PGE₂) induce a marked inward current when applied together at acidic but not at neutral extracellular pH. These IM do not have such effects on neurons that are insensitive to capsaicin (Kress et al. 1997). This study is in agreement with previous findings in a skin-nerve preparation and with psychophysical studies demonstrating that the combination of protons together with inflammatory mediators (including BK, 5-HT, HIS, and PGE₂) had synergistic excitatory and algogenic effects (Steen et al. 1995, 1996).

The aim of the present study was to learn whether or not the sensitivity to different inflammatory mediator combinations parallels functional expression of capsaicin receptors...
in DRG neurons from newborn rats. We further examined whether or not the responses to inflammatory mediators can be blocked by capsazepine (Bevan et al. 1992). The similarity between inflammatory mediators and capsaicin in facilitating proton-induced currents (Kress et al. 1997) suggested that IM may act as endogenous ligands or operators at the capsaicin receptor that seems to be unique to nociceptive neurons.

METHODS

Cell cultures

Primary cultures of DRG neurons were prepared in two steps as described previously (Guthrie et al. 1987). Hippocampi from newborn rats were removed, dissociated with trypsin and plated on collagen-coated glass coverslips in a nutrient medium composed of 90% Eagle’s minimum essential medium (MEM) and 10% fetal bovine serum. When the glial cultures became confluent, usually after 6–9 days, the medium was switched to nutrient-supplemented MEM with 5-fluoro-2-deoxyuridine and uridine and 10% horse serum to minimize cell division. In the second step, dorsal root ganglia were dissected from 2 to 3 day old Sprague-Dawley rats and incubated at 37°C in phosphate-buffered saline (PBS) containing 2% collagenase for 45 min and then in PBS with 0.3% trypsin for 10 min. The ganglia were then rinsed with calcium- and magnesium-free PBS and dissociated by trituration by using a fire-polished Pasteur pipette. DRG cells were plated on coverslips with the glial feeder layer cultures and grown in a medium composed of 90% MEM and 10% fetal calf serum and maintained at 37°C in a water-saturated atmosphere with 5% CO₂. This medium was replaced by a nutrient supplemented MEM containing 10% horse serum and 5-fluoro-2-deoxyuridine (15 mg/ml) and uridine (35 mg/ml) the next day (Guthrie et al. 1987). The nutrient supplement contained the following components added to 30 ml PBS: 2 ml bovine albumin (10 mg/ml PBS), 4 ml human transferrin (400 mg/4 ml PBS), 0.8 ml putrescine dihydrochloride (80 mg/ml PBS), 2 ml sodium selenite (1.04 mg/100 ml PBS), 0.2 ml triiodothyronine Na (1 mg/5 ml 0.01 N NaOH), 0.8 ml insulin (21.8 mg/ml 20 mM HCl), 0.2 ml progesterone [1 mg/8 ml absolute (abs) ethanol], and 0.04 ml corticosterone grade A (2 mg/ml abs ethanol). One milliliter of this supplement was added to 50 ml MEM. Neither antibiotics nor nerve growth factor (NGF) were added at any step of culturing because the glia monolayer supported the survival of DRG neurons. Under these conditions, the neurons had previously been observed to develop only short processes if at all (Vlachova and Vyklicky 1993).

Recording and perfusion techniques

The single-electrode patch-clamp technique was used to record whole-cell membrane currents employing an Axopatch 1D preamplifier, and pClamp 6 programs (Axon Instruments) with a laboratory PC for storing and evaluating the data. Electrodes were pulled from borosilicate glass and after fire polishing and filling they had resistances of 2–4 MΩ. The series resistance was usually <10 MΩ and was not compensated.

For drug application, a system for fast superfusion of the neurons illustrated in Fig. 1, the inflammatory mediators (IM), BK (10 μM), 5-HT (10 μM), and PGE₂ (1 μM), were tested singly, and in combination of all three at extracellular pH 7.3 and pH 6.1, in a sequence of 10-s applications separated by 20-s intervals of washing with control ECS. Fast application of acidic extracellular solution at pH 6.1 alone induced, in this set of neurons, a fast inactivating inward membrane current shown previously to be carried by sodium ions (Koh-
low pH or it was superimposed on its inactivating phase. The activation of the IM-induced membrane current resembled the capsaicin-induced response, the deactivation phase, after washout was as fast as that of the sustained proton-induced current. As expected the responses to capsaicin greatly increased at pH 6.1 (Fig. 1A). Corresponding to the magnitude of the membrane currents, depolarization of the neurons was observed in response to each of the stimuli (Fig. 1B). 

In all capsaicin sensitive DRG neurons, IM induced similar membrane currents. Figure 2 is a summary of the results. The magnitudes of the individual responses to capsaicin at pH 7.3 or 6.1 and to IM at pH 6.1 were positively correlated (CAPS pH 7.3 vs. IM pH 6.1: correlation coefficient $r = 0.99$; CAPS pH 7.3 vs. CAPS pH 6.1: $r = 0.81$; IM pH 6.1 vs. CAPS pH 6.1: $r = 0.82$), indicating possibly similar mechanisms of response generation. Large cells (>30 μM) and ~20% of the small size neurons were not sensitive to capsaicin or to inflammatory mediators at pH 6.1. In those cells, decreasing extracellular pH from 7.3 to 6.1 induced a fast inactivating current and a small sustained inward current with an increase of the membrane resistance ($n = 8$, data not shown).

The effects of a reduced combination of two inflammatory mediators (BK/PGE$_2$, BK/PGE$_2$, or 5-HT/PGE$_2$) were further tested on 31 capsaicin-sensitive neurons exhibiting a significant membrane current in response to all three IMs combined, similar to that shown in Fig. 1. A majority ($n = 23$; 74%) did not exhibit any significant current in response to any combination of two inflammatory mediators at pH 7.3 and 6.1. In eight of these neurons, the combination of any two mediators at pH 7.3 produced small inward membrane currents (~40 pA). However at extracellular pH 6.1, combinations of BK/5-HT, BK/PGE$_2$, and 5-HT/PGE$_2$ produced larger sustained currents than by acidic pH alone; increases were by $81 \pm 18\%$, $69 \pm 21\%$, and $86 \pm 34\%$, respectively.
INFLAMMATORY MEDIATORS AND CAPSAICIN RECEPTORS IN DRG NEURONS

The combination of all three inflammatory mediators together increased the pH 6.1-induced sustained membrane current by as much as 173 ± 61% in these capsaicin sensitive neurons. A further difference between the two groups of neurons was in the responses induced by rapid application of extracellular pH 6.1. The first group of 23 neurons exhibited a fast inactivating inward current followed by a very small sustained inward current (<50 pA) and was thus similar to the cell population described previously (Fig. 1). In the second group of 8 cells, lowering extracellular pH from 7.3 to 6.1 induced a marked sustained inward membrane current (316 ± 99 pA) comparable with that described by Bevan and Yeats (1991). Whether these two groups of capsaicin sensitive neurons differ in further respects representing distinct subpopulations of DRG neurons is not known.

Inhibition of responses to acidic inflammatory mediators by the capsaicin antagonist capsazepine

The effects of inflammatory mediators were observed exclusively in DRG neurons that were sensitive to capsaicin and a 100% overlap of capsaicin-sensitivity and sensitivity to IM at low pH was observed. In addition, the IM seemed to share one principle of action with capsaicin, that is to facilitate the pH-gated sustained inward current. Alternatively, membrane currents induced by the IM subthreshold at neutral pH, may have been facilitated by lowering extracellular pH. The alternatives are not mutually exclusive. If this action of the combined IM were the result of an effect on the putative capsaicin receptor, capsazepine, the capsaicin receptor antagonist, should block it (Bevan et al. 1992).

An example of the effects of capsazepine (10 μM and 3 μM) on the responses to the mixture of all three IM at extracellular pH 6.1 is shown in Fig. 4A. The record in Fig. 4B shows that capsazepine (6 μM) blocked the response induced by IM at pH 6.1 although it did not reduce the
large membrane currents induced by extracellular pH 5.5. Capsazepine appreciably inhibited the responses to capsaicin at pH 7.3, confirming previous findings in DRG neurons (Bevan et al. 1992). The column diagram in Fig. 4C summarizes the effects of 10 µM capsaicin on the responses induced by acidic IM in seven capsaicin-sensitive neurons. Capsazepine (10 µM) had no significant effect on the sustained inward membrane current induced by acidic ECS at pH 6.1. The suppressive effect of capsaicin on responses to acidic IM was observed in every single cell tested. In some experiments, the responses to acidic IM were reduced even more than was the control magnitude of sustained membrane current induced by pH 6.1 alone. This reduction may result from desensitization that was observed after repeated applications of capsaicin or acidic IM (see Fig. 4A).

**DISCUSSION**

Our results demonstrate that the combined inflammatory mediators, bradykinin, serotonin, and prostaglandin E2, together in acidic extracellular solution produce a marked inward membrane current in capsaicin-sensitive DRG neurons from newborn rats. The response is smaller or absent if any one of the four constituents is lacking. The data further suggest that the combination operates on the capsaicin receptor, because the responses to the acidic mixture of IM can be blocked by capsaizpine, a competitive capsaicin receptor antagonist.

The putative capsaicin receptor is a chemically gated non-specific cation channel expressed in a distinct class of small DRG neurons in mammals. It is permeable to Ca^{2+}, which triggers a cascade of intracellular processes that can desensitize and inactivate the channel from the intracellular side. Blockade of calmodulin-regulated phosphatase 2B, calcineurin, by cyclosporin A and cyclophillin A was found to prevent desensitization on repeated application of capsaicin, apparently by inhibiting dephosphorylation of the capsaicin receptor ion channel complex (Doeherty et al. 1996).

Although the molecular structure of the capsaicin receptor has not been determined, electrophysiological studies suggest that it possesses specific receptor recognition site(s) to which capsaicin binds tightly as suggested by the long (3.5 s) relaxation time constant (Vlachova and Vylicky 1993). The receptor-channel complex can be effectively modified by protons, which dramatically increase the capsaicin-induced, whole cell membrane current even at extracellular H^{+} concentrations that are subthreshold to induce any membrane current by themselves (Petersen and LaMotte 1993). The facilitation is apparently exerted by protonation of the capsaicin-gated ion channel and not by increasing the affinity of the receptor for capsaicin (Kress et al. 1996). This finding is in striking contrast to the effects of increased H^{+} on N-methyl-D-aspartate (NMDA) receptors that are allosterically blocked by lowering extracellular pH (Traynelis et al. 1990; Vylicky et al. 1990). It is also in contrast to the pH-dependent inactivation of various voltage-sensitive ion channels (see Hille 1992), which, in our results, may explain the increase in membrane resistance as a net-effect in all those neurons that exhibited only small sustained responses to low pH itself.

Capsaicin is a substance foreign to the mammalian body and efforts have been made to identify endogenous mediators that can activate the receptor under physiological or pathological conditions. As capsaicin is a well-recognized algogen (Fitzgerald 1983; Kress and Reeh 1996; Szolcsanyi 1993) the search for endogenous ligands has primarily been focused on agents that can play a role in signaling pain in inflammation. It has been suggested that H^{+} may play this role by activating capsaicin receptors (Bevan and Gepetti 1994), although not necessarily at the same recognition site (Rang et al. 1991). Indeed, pH in the exudates of inflammatory processes has been found to be below 5.5, which led to the idea that H^{+} might be the common principle of producing pain in inflammation (Lindahl 1962). Acidic extracellular solutions were found to induce a sustained inward current exclusively in small capsaicin-sensitive DRG neurons cultured with NGF added to the medium (Bevan and Yeats 1991). This current is likely to underlie pain induced by experimental acidosis in humans and excitation of nociceptors in skin nerve preparation or in the cornea (Belmonte et al. 1991; Steen et al. 1992; Steen and Reeh 1993).

In our DRG cells cultures on hippocampal glia, a pH 6.1-induced sustained current of small size and accompanied by increased input resistance was found in many capsaicin-sensitive neurons. The question remains as to whether extracellular pH 6.1 was still subthreshold for evoking a larger sustained membrane current or whether or not these cells represent a distinct subpopulation of the capsaicin-sensitive neurons. However, these neurons showed the facilitation by IM and by capsaizpine as did the subpopulation of capsaicin-sensitive neurons that exhibited a large pH response accompanied with decreased input resistance.

An obvious difference between the responses induced by capsaicin and acidic pH are relaxation times of the membrane currents after washout of the stimulants. Fast deactivation was observed with low pH (see Fig. 1), whereas that of capsaicin exhibited a time constant of 3.5 s as reported earlier (Kress et al. 1996; Vlachova and Vylicky 1993). This can be explained by differences in the strength of binding to the receptor, which is apparently very weak for protons, allowing their release at a speed exceeding the limits of solution exchange of the drug application system (~100 ms). In contrast, the long relaxation time of capsaicin-induced responses suggests tight binding to the receptor or slow diffusion of this lipophilic stimulant out from the lipid bilayer of the neuronal membrane.

The marked proton-induced facilitation of capsaicin responses suggests cooperativity between protons and capsaicin. Thus at capsaicin receptors, protons may play the role of a modulator, which exhibits some similarities to the role of glycine at NMDA receptors (Johnson and Ascher 1987). A difference, however, is that protons open the ion channels by themselves whereas glycine only supports the action of glutamate. If capsaicin receptors opened spontaneously, similar to nicotinic acetylcholine receptors (Brehm et al. 1984; Jackson 1984) or NMDA receptors (Turecek et al. 1997), it could be speculated that a high concentration of H^{+} may increase the probability of spontaneous openings and thus induce significant membrane current. The idea of identity of capsaicin and proton gated channels is further supported by the recent finding that the sustained proton induced membrane current is carried not only by monovalent cations but...
also by Ca\(^{2+}\), as is the case for the capsaicin-gated channel (Zeilhofer et al. 1996).

Apart from capsaicin, some inflammatory mediators can interact with protons and facilitate their effects in several experimental models. Bradykinin and serotonin have long been known as potent and cooperative allogens whose local application to the cannabidiin blister base evokes pain in human skin (Keele and Armstrong 1964). However loss of their excitatory potency during prolonged application cannot fully explain sustained pain (Kanaka et al. 1985; Kumazawa et al. 1987; Lang et al. 1990). Nevertheless, they are essential mediators of nociceptor sensitization, and combining BK and 5-HT with experimental acidosis, (in the presence of prostaglandin E\(_2\) and histidine) produces more than additive excitatory and algogenic effects (Kessler et al. 1992; Lang et al. 1990; Steen et al. 1995, 1996). The psychophysiological study, which demonstrated that IM induce a sustained sensitization to low pH stimulation (Steen et al. 1996), was recently corroborated by the finding that the combination of BK, 5-HT, PGE\(_2\), and HIS markedly increased the sustained membrane current induced by pH 6.1 (Kress et al. 1997). This analogy to the pH-capsaicin interaction leads to the idea that the IM combination may represent endogenous mediators acting directly or indirectly on the capsaicin receptor-channel complex.

Bradykinin and serotonin have been shown to activate specific ion channels, the majority of which are controlled by second messengers. In our experiments, we inconsistently observed small inward membrane currents (<50 pA) at neutral extracellular pH, which were induced by each of the IMs including prostaglandin E\(_2\). Because of their irregular occurrence, these currents were not analyzed in detail. Although very small, these currents may relate to the sensitization of nociceptors. The application of any combination of two IM facilitated the membrane currents induced by extracellular pH 6.1 only in some capsaicin-sensitive neurons but not in others. This diversity in the sensitivity to IM remains to be explained. It cannot be excluded that functional expression of capsaicin receptors may differ according to the conditions of culturing and may also contribute to the heterogeneity of capsaicin-induced currents reported in other studies (Liu and Simon 1996; Liu et al. 1996; Petersen et al. 1996). However, in all capsaicin-sensitive neurons, the combination of three inflammatory mediators, BK, 5-HT, and PGE\(_2\) at acidic extracellular pH, induced depolarizing membrane currents of magnitudes that are likely to induce spike activity in primary afferent nerve endings.

Capsazepine, the antagonist of capsaicin, was suggested as a tool in searching for endogenous mediators acting at the capsaicin receptor (Bevan et al. 1992). In contrast to capsaicin-induced responses, capsazepine did not block the sustained pH induced membrane current. However, as already pointed out by Rang et al. (1991), this finding does not exclude the possibility that capsaicin and protons act at the same receptor protein, because the ion channel could be gated from two recognition sites distinct for capsaicin and protons. Our results (Fig. 4) demonstrate that capsazepine, at concentrations that effectively inhibited responses to capsaicin, completely blocked the sensitizing effects of the inflammatory mediators on the pH responses but was ineffective in producing significant changes of the control responses induced by low extracellular pH alone. Although the specificity of capsazepine as an antagonist of capsaicin has been questioned because it was found in some preparations to antagonize the neuropeptide release by protons (see Bevan and Geppetti 1994), this does not contradict the original finding that capsazepine is a competitive antagonist at the putative capsaicin receptor, which is functionally expressed in small mammalian DRG neurons. These results together with the findings that acidic inflammatory mediators induce membrane current exclusively in capsaicin sensitive DRG neurons with capsaicin-like kinetics of activation, strongly support our view that the inflammatory mediators play a role as endogenous mediators at capsaicin receptor channels and produce excitatory membrane currents provided that the ion channels are protonized.

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