Regulation of firing frequency in nociceptive neurons by pro-inflammatory mediators

Aliakmal Momin · Peter A. McNaughton

Received: 4 December 2008 / Accepted: 12 February 2009 / Published online: 7 April 2009
© Springer-Verlag 2009

Abstract Nociceptive neurons generate trains of action potentials in response to painful stimuli, and the frequency of firing signals the intensity of the pain. Pro-inflammatory mediators such as prostaglandin E2 (PGE2) enhance the sensation of pain by increasing the frequency of action potential firing in response to a given level of painful stimulus. The mechanism by which the firing frequency is enhanced is discussed in the present review. One hypothesis proposes that the threshold for action potential initiation is lowered because the activation curve of a nociceptor-specific voltage-activated Na current, NaV1.8, is shifted to more negative values by PGE2. Recent measurements in our lab show, however, that the action potential threshold in fact changes little when AP firing is accelerated by PGE2. The enhanced firing is, however, abolished by a blocker of an inward current activated by hyperpolarisation, called Ih. The voltage sensitivity of Ih shifts in the positive direction in small nociceptive neurons when they are exposed to pro-inflammatory mediators, such as PGE2, which activate adenylate cyclase and therefore increase levels of cAMP. By this mechanism the inward current between the resting membrane potential and the threshold for firing of action potentials is enhanced, and the rate of depolarisation in the interval between action potentials is therefore increased. We conclude that the major mechanism responsible for increasing action potential firing following tissue damage or metabolic stress is the hyperpolarisation-activated inward current, Ih, and that other mechanisms play at most a minor role.

Keywords Pain · Sensory transduction · Action potential · Prostaglandin

Introduction

Pain is unique amongst sensory systems in its property of sensitisation, in which the intensity of the painful sensation increases with time of exposure to a painful stimulus. In other sensory systems the reverse happens; the perceived intensity of the stimulus declines with time, in the well-known and important process of adaptation, which is vital in enabling us to move among environments of widely differing ambient stimulus intensity levels. Sensitisation originates at least partly at the level of peripheral nerve terminals, because the phenomenon is present in recordings of action potentials from primary afferent nerve fibres (Belmonte and Giraldez 1981). However, sensitisation is not intrinsic to the sensory neurons themselves, because repeated stimulation causes no sensitisation in isolated neurons (Cesare and McNaughton 1996). Sensitisation is instead caused by the release of pro-inflammatory mediators from surrounding damaged or stressed cells, which act on specific receptors in the cell membrane of the nociceptive nerve terminal and activate intracellular signalling pathways which mediate sensitisation. A wide range of mediators are known to cause sensitisation. These can range from simple ions or molecules such as H+, NO or K+, through to small organic molecules such as ATP, lipid-derived messengers such as prostaglandin E2, peptide fragments such as bradykinin, which are released from larger precursor proteins by proteolytic enzymes released in tissue damage, and entire proteins such as nerve growth factor or members of the prokineticin family (reviewed by Dray 1995; Huang et al. 2006a).
Sensitisation at the level of the peripheral nerve terminal can occur by two broad mechanisms: a pro-inflammatory mediator can increase the generator current activated by a given level of painful stimulus; or it can increase the ease with which action potentials are elicited in the primary afferent nerve terminal. Examples of both are known, and in many cases a pro-inflammatory mediator acts by both mechanisms. One important sensitising action of bradykinin results from a direct action on the heat-activated ion current, carried by the heat and capsaicin-activated channel TRPV1, whose gating is enhanced via a pathway involving activation of the ε isoform of protein kinase C (Cesare and McNaughton 1996; Cesare et al. 1999; reviewed in Huang et al. 2006b). Prostaglandins also operate via a similar pathway to enhance activation of the heat-gated ion channel TRPV1 (Moriyama et al. 2005), but a second and probably more important action of PGE$_2$ is on the mechanism of action potential generation, rather than on the generator current, because PGE$_2$ causes potent sensitisation of action potential generation in response to a constant current stimulus (England et al. 1996). In this article we focus on this second mechanism of sensitisation.

Three mechanisms have been proposed to explain the enhancement of action potential generation by pro-inflammatory mediators such as PGE$_2$. The activation threshold for initiation of an action potential could be lowered by enhancing or changing the threshold of the voltage-dependent Na$^+$ current, making it easier to elicit an action potential. Secondly, a suppression of the voltage-sensitive K$^+$ current could depolarise the resting potential and increase the resting membrane resistance, again increasing the effect when translating a given level of generator current into action potentials. Finally, activation of a steady inward current at the resting potential would have much the same effect. Each theory has some evidence in its favour, as outlined below.

A second scenario in which repetitive firing of action potentials is important is neuropathic pain. Neuropathic pain is caused by partial damage to peripheral nerves, and is characterised by ongoing pain, by hyperalgesia (an enhanced response to normally painful stimuli) and by allodynia, a painful sensation in response to normally non-painful stimuli such as light touch or moderate cold. Neuropathic pain is encountered in a variety of pathological conditions, amongst which the most common are post-herpetic neuralgia, diabetic neuropathy, and neuropathic pain caused by cancer chemotherapy. It is a widespread and distressing clinical problem, poorly treated by current drug therapies (McQuay et al. 1996).

Continuous or burst firing is observed in afferent nociceptive nerve fibres in neuropathic pain states, and this continuous firing, which appears to be signalling a constant pain in peripheral tissues, is likely to be a causative agent in the continuous pain, alldynia and hyperalgesia which are characteristic of neuropathic pain states (Kajander et al. 1992; Sheen and Chung 1993). The origin of the continuous firing, in the sense of identification of the modified membrane conductances which underlie it, has not, however, been clearly established to date. Suspicion has fallen at various times on all of the candidates mentioned in connection with inflammatory hyperalgesia above.

**Mechanisms of sensitisation at the level of action potential generation**

It has been known for some time that nociceptive neurons express a unique nociceptor-specific high-threshold Na current which is insensitive to TTX (Elliott and Elliott 1993), in addition to the low-threshold voltage-gated Na$^+$ current commonly seen in other neurons. More recent work has cloned the high-threshold Na channel isoform responsible, Na$_{\mathrm{v}}$1.8, which is expressed only in nociceptive neurons (Akopian et al. 1996). An interesting feature of Na$_{\mathrm{v}}$1.8 is that the position of its activation curve as a function of membrane voltage is variable: the curve is shifted in the negative direction, and the magnitude of the peak current is enhanced, by phosphorylation by PKA and/or PKC (Gold et al. 1996, 1998; England et al. 1996; Fitzgerald et al. 1999). The mechanism for inflammatory hyperalgesia which is suggested by these studies, and now widely accepted in the field, is that inflammatory mediators such as PGE$_2$ activate adenylate cyclase and increase intracellular cAMP levels, leading to activation of PKA and phosphorylation of Na$_{\mathrm{v}}$1.8. The shift in the activation curve of Na$_{\mathrm{v}}$1.8 and the consequent lowering of the threshold for activation of Na current makes the cell more excitable, and as a result more action potentials are generated in response to a noxious stimulus. This mechanism seems unlikely to offer a complete explanation of either inflammatory hyperalgesia or neuropathic pain, however, because in Na$_{\mathrm{v}}$1.8$^{-/-}$ mice inflammatory hyperalgesia is reduced but not abolished, and neuropathic pain is unaffected (Akopian et al. 1999; Kerr et al. 2001). The repetitive firing observed in neuropathic pain also seems not to be driven by other Na channels because neuropathic pain was unaffected in mice in which the genes for Na$_{\mathrm{v}}$1.3, Na$_{\mathrm{v}}$1.7 or Na$_{\mathrm{v}}$1.9 had been deleted (Nassar et al. 2004, 2006; Priest et al. 2005; Amaya et al. 2006).

Secondly, a voltage-sensitive K$^+$ current has been shown to be inhibited by activation of PKA (England et al. 1996; Nicol et al. 1997; Evans et al. 1999). The K$^+$ channel isoform involved has not been identified but seems not to be K$_{\mathrm{v}}$1.1 (Jiang et al. 2003). The possible contribution of this mechanism has not been worked out in detail, but it seems unlikely to explain the marked acceleration of action potential frequency caused by (for example) PGE$_2$, for two reasons:
the effect is rather small, with less than a halving of the K\textsuperscript{\textast} current being observed; and because the voltage-dependent K current is largely de-activated at the resting membrane potential and so makes little contribution to membrane conductance in the crucial membrane voltage range between the resting potential and the action potential threshold.

A third possibility, which has been little considered in recent years, was first raised by the work of Ingram and Williams (1994, 1996). Many sensory neurons, in common with other neurons in the CNS and non-neuronal tissues such as cardiac muscle, express an inward membrane current which is unusual in the sense that it is activated by hyperpolarisation, rather than being activated by membrane depolarisation as is the case for other known voltage-activated membrane currents. The current is variously referred to as \( I_h \) (the “funny” current, reflecting the surprise of its discoverers that it is activated by hyperpolarisation—see DiFrancesco 1993), \( I_q \) (the “queer” current, for the same reason—see Pape 1996), or \( I_h \) (the current activated by hyperpolarisation—see McCormick and Pape 1990). We summarise below recent evidence from our own group that \( I_h \) is crucially involved in the regulation of action potential generation by pro-inflammatory mediators.

**The hyperpolarisation-activated current, \( I_h \)**

\( I_h \) was first studied in cardiac pacemaker cells (DiFrancesco and Ojeda 1980; DiFrancesco 1993). \( I_h \) is activated by hyperpolarisation in the range of membrane potentials from −60 to −90 mV, and is therefore well placed to modulate firing by altering the rate at which the membrane potential depolarises to the action potential threshold. A second important property of \( I_h \) is that the voltage dependence of activation is shifted to less negative membrane potentials by elevations in cAMP. The activation range of \( I_h \) is shifted in the positive direction by a direct binding of adenosine 3',5'-cyclic monophosphate (cAMP) to a cyclic-nucleotide binding region located in the C-terminal cytoplasmic domain (DiFrancesco and Tortora 1991; Kaupp and Seifert 2001), thereby increasing the inward current activated in the critical range between the resting potential and the action potential threshold. Thus \( I_h \) is not modulated by phosphorylation by protein kinase A (PKA), the normal downstream effector of the adenylyl cyclase/cAMP pathway, but is instead potently modulated by direct binding of cAMP, a difference which will form an important distinction in experiments to be described below. \( I_h \) is expressed in many neurons, where its presence can modulate the resting potential, action potential firing frequency and the efficiency of electrical transmission in dendrites (Pape and McCormick 1989; Pape 1996; Ludwig et al. 2003; Nolan et al. 2003; Chan et al. 2004; Tsay et al. 2007).

Four ion channel subunits responsible for \( I_h \) have been cloned and are termed hyperpolarisation-activated, cyclic nucleotide-gated (HCN1-4) channels (reviewed by Kaupp and Seifert 2001). HCN channels expressed as homomers differ in two main respects: the activation time constants are in the order of HCN1 < HCN2 < HCN3 < HCN4; and HCN2 and HCN4 are strongly modulated by elevations in cAMP, with the midpoint of the voltage-activation curve shifted in the positive direction by 12–20 mV, whereas HCN1 and HCN3 channels show little sensitivity to cAMP. These differences can be used as a guide to determining the molecular makeup of \( I_h \) in sensory neurons.

**Involvement of \( I_h \) in regulation of action potential firing**

Figure 1, taken from Momin et al. (2008), shows action potential firing in an isolated nociceptive neuron in response to the injection of a constant current pulse. The experiment therefore focuses on processes modulating action potential frequency *downstream* of the generator current, which is replaced by a constant current stimulus. The action potential frequency is seen to be substantially enhanced by application of PGE\(_3\), in agreement with observations both in vivo (Ferreira 1972; Dray 1995) and in vitro (England et al. 1996). The effect is due to an action of PGE\(_2\) via the adenylyl cyclase/cAMP pathway, because a similar acceleration is observed when the adenylyl cyclase activator forskolin is applied. Significantly, the resting membrane potential is depolarised by the application of PGE\(_2\) by a mean value of nearly 9 mV, an effect which is also visible in the work of England et al. (1996).

The origin of the acceleration of action potential firing is suggested by the last panel in Fig. 1A, where the specific \( I_h \) blocker ZD7288 was applied. Blocking \( I_h \) reversed the enhancement of firing, and also restored the resting membrane potential to its level before application of PGE\(_2\). In contrast, application of H89, which blocks PKA without affecting earlier stages in the adenylyl cyclase—cAMP—PKA pathway, had no effect on either action potential firing or on the resting membrane potential (Fig. 1B, right-hand panel). This finding supports an involvement of \( I_h \) because the HCN2 and 4 channel subunits are directly modulated by binding cAMP without the intervention of PKA (see above). Mechanisms involving modulation of Na\(_\text{v}\)1.8 or voltage-dependent K currents, by contrast, depend on phosphorylation of these ion channels by PKA and therefore should be blocked by the PKA inhibitor.

Figure 2 further investigates the basis of the effect of PGE\(_2\). It is evident from the traces that a major component of the effect of PGE\(_2\) is due to an acceleration of the rate of depolarisation in the pacemaker region between successive action potentials. If PGE\(_2\) caused a lowering of the voltage
threshold or an increase in the amplitude of \( \text{Na}_V \), then an effect on the action potential threshold or the rate of rise of the action potential (respectively) would be expected. In a similar way, an inhibition of voltage-dependent potassium currents by PGE\(_2\) should cause a decrease in the rate of fall of the repolarisation phase of the action potential. We estimated these parameters by calculating the first derivative of membrane potential, \( \frac{dV_m}{dt} \) (Fig. 2). Prior to exposure to PGE\(_2\), the action potential threshold was \(-34.7\) mV, while after PGE\(_2\) application it was \(-33.8\) mV, a difference found not to be significant. Furthermore, neither the maximum rate of rise nor the maximum rate of fall of the action potential (a, b in Fig. 2) was significantly affected by either PGE\(_2\) or by forskolin. These results are consistent with an involvement of \( I_h \) in the sensitisation caused by PGE\(_2\), but they argue against a major effect on either the voltage-dependent Na or the K currents.

Properties of \( I_h \) as a function of neuron size

Sensory afferent nerve fibres can be classified into three main categories, as follows: large-diameter, fast-conducting myelinated \( A_\beta \) fibres, which serve a variety of mainly

![Fig. 1](image1.png)

Enhancement of action potential firing frequency in small DRG neurons by PGE\(_2\) depends on \( I_h \). A Representative trace showing action potential firing produced by a 100 pA depolarizing current in control conditions (left), in the presence of PGE\(_2\) (10 \( \mu \)M, centre) and in the presence of PGE\(_2\) plus the specific \( I_h \) blocker ZD7288 (100 \( \mu \)M, right). B Same as A but in control (left), in the presence of PGE\(_2\) (10 \( \mu \)M, centre) and in the presence of PGE\(_2\) plus the specific protein kinase A blocker H-89 (10 \( \mu \)M, right). Reproduced with permission from Momin et al. (2008)

![Fig. 2](image2.png)

There is no significant change in action potential threshold, rate of rise or rate of fall when action potential frequency is accelerated by PGE\(_2\). A Action potentials at an expanded time scale, in control conditions (left) and in the presence of 10 \( \mu \)M PGE\(_2\) (right). The main effect of PGE2 is to accelerate the rate of depolarisation in the interval between action potentials. B Shows the first derivative (\( \frac{dV_m}{dt} \)) of the voltage trace shown above. Arrows show a: maximum rate of rise, b: maximum rate of fall. Dashed line shows the method of calculating the threshold from the rate of rise minimum immediately preceding the action potential upstroke. Note that the substantial acceleration of the rate of rise of the pacemaker potential between action potentials is not accompanied by a significant change in action potential threshold, rate of rise or rate of fall of the action potential. Reproduced with permission from Momin et al. (2008)
non-nociceptive functions; smaller and slower myelinated Aβ fibres, some of which are nociceptive and others non-nociceptive; and the smallest and slowest unmyelinated C-fibres, the majority of which are nociceptors (Lawson 2002). A correlation exists between cell body diameter and conduction velocity of the afferent fibre (Harper and Lawson 1985), so the class of a given sensory afferent can be at least approximately inferred from the diameter of its cell body. We separated neuronal cell bodies in adult mice into three categories, to correspond approximately with the three major classes of afferent fibres, as follows: large (>30 μm), medium (20–30 μm) and small (<20 μm).

We found a striking dependence of the speed of activation/deactivation of \( I_h \) on neuronal size (Fig. 3A). Large neurons, which mainly subtend Aβ fibres, all expressed a rapidly activating \( I_h \). Medium-sized neurons expressed two distinct values of activation rate. A fast class (“medium fast” neurons) had values indistinguishable from those seen in large neurons, and probably represents a “tail” of large neurons extending into the medium class. A slow class (“medium slow” neurons), which mainly subtend Aδ fibres, had time constants which were clearly separable from those of the fast class and are therefore likely to represent a functionally distinct subset of sensory neurons. Finally, small neurons, likely to be those subtending C fibres, and amongst which the majority of nociceptors are found, had a still slower time constant. Note also that \( I_h \) was present in only about half of small neurons, while all large neurons expressed \( I_h \).

The variation in time constant was not the only difference between the size classes of neurons. In large and medium fast neurons \( I_h \) was insensitive to elevations in cAMP, whether caused by application of PGE\(_2\), by direct activation of adenylate cyclase with forskolin, or by intracellular application of an elevated level of cAMP (see Fig. 4). These properties are consistent with expression of HCN1 or 3, both of which are insensitive to cAMP (see above), but not with expression of HCN2 or 4. In medium slow or small neurons, by contrast, \( I_h \) was strikingly sensitive to elevations of cAMP, with the membrane voltage at which the current is half-activated being shifted in the positive direction by c. 12–13 mV (see Fig. 4). These properties are consistent with expression of HCN2 or 4, both of which are sensitive to cAMP, in small and in the medium-sized neurons which express a slow \( I_h \).

Finally, we were able to identify a minor but clearly distinct subset of small neurons which express a fast, cAMP-insensitive \( I_h \) consistent with expression of HCN1. These neurons appear as the small number of grey bars in the fast group in Fig. 3A. The existence of the small neurons with fast-activating \( I_h \) was confirmed in both rat and mouse neurons. Significantly, this population was shown to be sensitive to menthol, consistent with co-expression of the cold-sensitive ion channel TRPM8 (McKemy et al. 2002). The existence of a subset of small neurons which co-express a fast-activating \( I_h \) and TRPM8 has also been commented on by Viana et al. (2002).
We confirmed the identification of HCN1 as the subunit responsible for \( I_h \) in large and medium fast neurons by creating an HCN1 knockout mouse. Following deletion of HCN1 the majority of large neurons showed no \( I_h \) at all (Fig. 3B), in contrast to the situation in wild-type animals, where all large neurons expressed a fast-activating \( I_h \). The \( I_h \) in small and medium neurons expressing a slowly activating \( I_h \) was unaffected by the deletion of HCN1, in confirmation of the proposal above that these neurons express HCN2 or HCN4.

Effect of deletion of HCN1 on behavioural responses to painful stimuli

Deletion of HCN1 caused no significant effect on behavioural responses to painful heat or mechanical stimuli, either in control animals or following the induction of an inflammatory pain state by injection of PGE\(_2\). This result is consistent with the observations above that HCN1 is expressed only in larger neurons, which typically do not serve a nociceptive function; and also with the fact that the position of the activation curve of HCN1 on the membrane voltage is unaffected by the elevations in cAMP caused by PGE\(_2\).

A similar observation might be expected in a neuropathic pain state, and for the same reasons. We found that mechanical allodynia, following the induction of neuropathic pain by partial ligation of a peripheral sciatic nerve, is largely unaffected by deletion of HCN1 (see Fig. 5A). However, the cold allodynia observed in response to application of a small drop of acetone to the plantar surface of the paw in which neuropathic pain has been induced was strikingly attenuated when HCN1 had been deleted (Fig. 5B). We think this observation may be related to the presence of HCN1 in a minor population of small neurons which express TRPM8 and are therefore sensitive to cool stimuli (see above). Deletion of HCN1 may reduce the ability of these neurons to fire repetitive trains of action potentials in response to the cold acetone stimulus, and therefore may reduce the intensity of neuropathic cold pain.

Conclusion

The work described in this review has shown that \( I_h \) is likely to be critical in modulating the ability of pro-inflammatory mediators such as PGE\(_2\) to modulate nociceptor firing, in contrast to the proposals arising from previous studies, which had placed much more emphasis on a role for the nociceptor-specific Na channel, Na\(_v\)1.8.

\( I_h \) shows a striking variation with neuronal diameter, with large neurons expressing a fast, cAMP insensitive \( I_h \), while small neurons express a slower, cAMP sensitive \( I_h \). The expression of a fast-activating \( I_h \) in large neurons has also been commented on in other studies (Chaplan et al. 2003; Yao et al. 2003; Doan et al. 2004; Tu et al. 2004), and is supported by in situ hybridisation and immunohistochemistry data (Moosmang et al. 2001; Chaplan et al. 2003) showing high levels of HCN1 mRNA and protein expression in large and medium DRG neurons. By the use of an HCN1 knockout mouse model we have identified the HCN isoform expressed in large neurons as HCN1.

The isoform(s) associated with small neurons have yet to be conclusively identified, but the fact that \( I_h \) in small neurons was unaffected by deletion of HCN1 shows that it this isoform is not expressed to any significant extent. From the slow activation kinetics and sensitivity to cAMP the relevant small neurons isoform is likely to be HCN2 and/or
HCN4. In immunocytochemical and in situ hybridisation studies HCN2 has been found to be strongly expressed in DRG neurons, while HCN4 is expressed at a low level (Moosmang et al. 2001; Chaplan et al. 2003; Tu et al. 2004; Matsuyoshi et al. 2006; Luo et al. 2007; Obreja et al. 2008). These studies suggest, therefore, that HCN2 is the only isoform expressed in smaller sensory neurons, but this does not explain why the time constants of \( I_h \) activation differ in the “medium slow” and “small” groups of neurons (see Fig. 3). A clearer identification awaits the investigation of the effect of deletion of HCN2 on the phenotype of \( I_h \) in small neurons.

Voltage-clamp experiments showed that elevating cAMP levels, for instance by exposure of neurons to PGE\(_2\), shifted the voltage-activation curve of \( I_h \) in small nociceptive neurons, but not in large neurons, to more depolarised potentials. This shift has the important effects both of increasing the amplitude of inward current carried by \( I_h \), and of increasing its rate of activation when the neuronal cell membrane is hyperpolarised following an action potential. Both of these actions will increase the inward current following an action potential, and will therefore speed the rate at which the membrane potential depolarises to threshold for initiation of the following action potential. A third important effect is the steady depolarisation of the resting membrane potential caused by tonic activation of \( I_h \), which is equivalent to a constant current injection at the resting potential.

Finally, behavioural experiments on mice from which HCN1 had been deleted have uncovered a surprising impact of the gene deletion on one aspect of neuropathic pain, namely cold allodynia. The observation of reduced neuropathic pain in the absence of HCN1, at least insofar as cold allodynia goes, may be clinically important. Previous studies have suggested an important role for \( I_h \) in the initiation of action potentials in neuropathic pain (Chaplan et al. 2003). The \( I_h \) blocker ZD7288 reduces ectopic firing in sensory neurons and alleviates allodynia caused by nerve injury (Chaplan et al. 2003; Luo et al. 2007). In addition, a role for \( I_h \) in the hyper-excitability of damaged peripheral nerves has been proposed (Yao et al. 2003).

Cold allodynia, an unpleasant sensation in response to normally non-noxious cold stimuli, is a particular problem in some forms of cancer chemotherapy, and is often a limiting factor in patients’ ability to tolerate chemotherapy. The discovery that HCN1 is a critical factor in the induction of cold allodynia may be related to our observation that HCN1 is expressed in small, cold-sensitive neurons, where it is likely to play a modulatory role in action potential firing. This finding may act as a stimulus to drug discovery directed at the development of isoform-specific blockers of \( I_h \), which may be of value in the treatment of the cold allodynia associated with neuropathic pain and also in the treatment of the much more common condition of inflammatory hyperalgesia.

References


Lawson SN (2002) Phentype and function of somatic primary afferent nociceptive neurons with C-, Adelta- or Abeta-fibres. Exp Physiol 87:239–244