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Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels

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ABSTRACT

Previous studies have shown that hyperpolarisation-activated cyclic nucleotide-gated (HCN)–2 ion channels regulate the firing frequency of nociceptive sensory neurons and thus play a central role in both inflammatory and neuropathic pain conditions. Here we use ivabradine, a clinically approved anti-anginal agent that blocks all HCN channel isoforms approximately equally, to investigate the effect on inflammatory and neuropathic pain of HCN ion channel block. We show that ivabradine does not have major off-target effects on a sample group of Na, Ca, and K ion channels, and that it is peripherally restricted because it is a substrate for the P-glycoprotein multidrug transporter that is expressed in the blood–brain barrier. Its effects are therefore likely to be due to an action on HCN ion channels in peripheral sensory neurons. Using patch clamp electrophysiology, we found that ivabradine was a use-dependent blocker of native HCN channels expressed in small sensory neurons. Ivabradine suppressed the action potential firing that is induced in nociceptive neurons by elevation of intracellular cAMP. In the formalin model of inflammatory pain, ivabradine reduced pain behaviour only in the second (inflammatory) phase. In nerve injury and chemotherapy models of neuropathic pain, we observed rapid and effective analgesia as effective as that with gabapentin. We conclude that both inflammatory and neuropathic pain are rapidly inhibited by blocking HCN-dependent repetitive firing in peripheral nociceptive neurons.

1. Introduction

Many painful conditions are poorly treated by current therapies, and the drive to discover novel targets and therapies is consequently high. Therapeutic interventions directed at peripheral sensory neurons may offer an approach with fewer side effects than those directed at targets expressed in the central nervous system. The validity of approaches aimed at peripheral targets is controversial, however, because both inflammatory and neuropathic pain are known to be augmented by a poorly understood process of central sensitization [23,44], with the result that both centrally and peripherally acting drugs can deliver effective analgesia [4,22]. One major aim of the present study is to investigate whether a peripherally restricted intervention can alleviate inflammatory and neuropathic pain. Another unresolved question is whether central sensitization is a short-lasting state, maintained by continuous input from peripheral nociceptors, or whether it is long-lasting and persists independent of activity in peripheral nociceptors. A second aim was therefore to investigate whether long-established neuropathic pain can be rapidly reversed by a peripherally restricted intervention.

The family of hyperpolarisation-activated cyclic nucleotide-gated (HCN) ion channels has recently emerged as a potential analgesic target [9,14,15,24,25,29,39,43]. The HCN channel family comprises 4 members, HCN1 to HCN4. Of these, HCN1 and HCN2 are the dominant isoforms expressed in sensory neurons

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[1,9,29,30,43], whereas HCN4 is preferentially expressed in pacemaker regions of cardiac tissue [19,31]. The voltage-dependence of HCN2 and HCN4 is modulated by cAMP, whereas HCN1 and HCN3 are relatively cAMP-insensitive [31]. Electrophysiological experiments show that HCN2 is expressed in approximately half of small somatosensory neurons, where it plays an important role in the regulation of firing frequency in response to inflammatory stimuli [14]. The hyperpolarization-activated current in larger diameter neurons is dominated by HCN1, though there is also evidence for expression of HCN2 [14,29]. Immunocytochemical data using polyclonal antibodies is in broad agreement, although prominent HCN2 staining is detected in some large-diameter neurons (muscle spindle afferents, A-fiber nociceptors and non-nociceptors) [1,9,43].

Deletion of HCN2 from the $Na_V1.8$-expressing subpopulation of dorsal root ganglion (DRG) neurons prevents the development of inflammatory and neuropathic pain [14], suggesting that HCN blockers may be analgesic. The pan-HCN inhibitor ZD7288 is analgesic in inflammatory and neuropathic pain [9,14,24,25,27,39]; however, in view of the known off-target actions of ZD7288 [10,34], the use of a more specific blocker is desirable. In the present study, we investigated the effect of the clinically approved HCN blocker ivabradine, used in the treatment of angina [20,40,41], which causes bradycardia by blocking HCN4 ion channels in the sinoatrial node (reviewed by Heusch et al. [20]). Ivabradine blocks all 4 HCN isoforms with approximately equal potency [37], but has minimal effects on other ion channels (see Results). Ivabradine is a P-glycoprotein substrate (see Results) and so does not cross the blood–brain barrier. It is therefore an attractive tool to test the hypothesis that analgesia may result from peripheral HCN inhibition. We show here that ivabradine is an effective analgesic acting against both inflammatory and neuropathic pain.

2. Methods

2.1. Animals

All animals used were C57/BL6 wild-type mice (Charles River, UK). For neuronal cultures, neonatal animals of both sexes were used. For behavioural experiments, males 4 to 6 weeks of age at the start of the experiment were used, except for experiments in $Na_V1.8$-HCN2 mice, for which both males and females 4 to 6 weeks of age were used. Median weights did not vary systematically between different experimental groups and were all in the range of 20 to 26 g. All experiments were compliant with the guidelines of the Committee for Research and Ethical Issues of IASP, were approved by the Animal Welfare and Ethical Review Committee, University of Cambridge and by the Home Office (UK), and adhered to the legislation set out in the Animals (Scientific Procedures) Act 1986.

2.2. DRG neuron culture

Cultures of murine DRG neurons were prepared as described previously [14]. Briefly, neonatal mice (<P10) underwent cervical dislocation and decapitation, and DRG were exposed by performing a spinal laminectomy. DRG from all spinal levels were collected. Ganglia were treated for 1 hour in Dulbecco's modified Eagle's medium (DMEM) containing collagenase (Worthington; 2 mg/mL). Neurons were then dissociated by manual trituration and plated onto culture dishes pre-coated with poly-l-lysine/laminin. Electrophysiological recordings were made 24 to 48 hours after plating.

To assess whether developmental changes had any impact on the properties of I_h recorded from cultured neurons, we measured the half-activation voltage ($V_{½}$), the shift in $V_{½}$ on elevation of cAMP, and maximal current density of I_h in small, medium, and large neurons from both neonatal and adult mice (Table 1). Although neuronal size increased as the animals grew, there was no significant change in any of these 3 parameters between neonatal and adult mice.

A second possible complication may be changes in properties of I_h with time in culture. Our cultures did not contain NT3, which has recently been shown to affect the expression of HCN1 and HCN2 in large neurons but to have little impact on expression levels in small neurons [1]. Table 2 shows that no significant changes in properties of I_h in small (<20-m-diameter) DRG neurons were observed between 24 and 48 hours in culture.

2.3. Cell permeability

Caco-2 and MDCK cultured cell monolayers were used to assess directional transport of ivabradine. MDCK cells were stably transfected with the MDR1 gene which codes for P-glycoprotein. Cells were seeded at a density of 5×10^4 cells onto the BD Falcon multiwell insert plate with microporous polyethylene terephthalate (PET) membrane and cultured for 21 days before the assay. Ivabradine (1% in dimethyl sulfoxide [DMSO]) was added to either the basal or the apical cell surface for 60 minutes at 37°C with shaking, and the rate of transport to the transepithelial compartment was measured with LCMS/MS. The effect of inhibiting the multidrug P-glycoprotein transporter was tested by applying the specific inhibitor elacridar [2] to the apical cell surface. Transport assays were carried out by Argenta Inc (Spire Green, Harlow, UK).

2.4. Electrophysiology

2.4.1. Solutions

Manual patch clamp experiments were carried out using extracellular solution containing (in mmol/L) 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 glucose; pH was adjusted to 7.4 with NaOH, and osmolarity was 300 to 310 mOsm. The intracellular solution contained (in mmol/L) 140 KCl, 1.6 $MgCl_2$, 2.5 MgATP, 0.5 NaGTP, 2 EGTA, and 10 HEPES; pH was adjusted to 7.3, and osmolality was 300 to 310 mOsm. Drugs used were forskolin (FSK, 50 mmol/L stock in DMSO (Sigma-Aldrich, St. Louis, MO) and ivabradine (iva, 50 mmol/L stock in DMSO; Avachem, San Antonio, TX) and were diluted in extracellular solution on the day of the experiment.

2.4.2. Whole-cell patch-clamp

Small DRG neurons <20 μm in diameter were selected for electrophysiological recording. Whole-cell patch-clamp recordings were performed using an Axopatch 200B patch-clamp amplifier. Patch pipettes were pulled using a P-97 horizontal micropipette puller (Sutter Instruments, USA). All pipettes were fire polished with a microforge before use and had resistances ranging between 3.5 and 5.5 MΩ. Pipette offset was corrected before seal formation. Once a giga-seal was formed between the pipette and the cell surface pipette capacitance transients were cancelled before achieving the whole cell configuration. Whole cell series resistance was compensated by 75% to 90% with a lag time of 10 μs. Cells were held at −60 mV in the whole-cell configuration. Current-clamp protocols were performed using an I-Clamp fast mode. Junction potentials were calculated and corrected offline by −4.3 mV for all recordings. Whole cell recordings were low-pass Bessel filtered at 10 KHz and sampled at 20 KHz. Data were acquired and analysed using pClamp 9/10 software.

2.4.3. Experimental protocol

The voltage-clamp mode was used to investigate the voltage-dependence of channel activation. An initial holding potential of

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