Direct Inhibition of $I_h$ by Analgesic Loperamide in Rat DRG Neurons

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Submitted 10 August 2006; accepted in final form 18 March 2007

Vasilyev DV, Shan Q, Lee Y, Mayer SC, Bowly MR, Strassle BW, Kaftan EJ, Rogers KE, Dunlop J. Direct inhibition of $I_h$ by analgesic loperamide in rat DRG neurons. J Neurophysiol 97: 3713–3721, 2007. First published March 28, 2007; doi:10.1152/jn.00841.2006. Hyperpolarization-activated cyclic nucleotide–gated (HCN) channels are responsible for the functional hyperpolarization-activated current ($I_h$) in dorsal root ganglion (DRG) neurons, playing an important role in pain processing. We found that the known analgesic loperamide inhibited $I_h$ channels in rat DRG neurons. Loperamide blocked $I_h$ in a concentration-dependent manner, with an $IC_{50} = 4.9 \pm 0.6$ and $11.0 \pm 0.5$ μM for large- and small-diameter neurons, respectively. Loperamide-induced $I_h$ inhibition was unrelated to the activation of opioid receptors and was reversible, voltage-dependent, use-independent, and was associated with a negative shift of $V_{1/2}$ for $I_h$ steady-state activation. Loperamide block of $I_h$ was voltage-dependent, gradually decreasing at more hyperpolarized membrane voltages from 89% at −60 mV to 4% at −120 mV in the presence of 3.7 μM loperamide. The voltage sensitivity of block can be explained by a loperamide-induced shift in the steady-state activation of $I_h$. Inclusion of 10 μM loperamide into the recording pipette did not affect $I_h$ voltage for half-maximal activation, activation kinetics, and the peak current amplitude, whereas concurrent application of equimolar external loperamide produced a rapid, reversible $I_h$ inhibition. The observed loperamide-induced $I_h$ inhibition was not caused by the activation of peripheral opioid receptors because the broad-spectrum opioid receptor antagonist naloxone did not reverse $I_h$ inhibition. Therefore we suggest that loperamide inhibits $I_h$ by direct binding to the extracellular region of the channel. Because $I_h$ channels are involved in pain processing, loperamide-induced inhibition of $I_h$ channels could provide an additional molecular mechanism for its analgesic action.

INTRODUCTION

Loperamide, a potent μ-opioid receptor agonist, originally known as an anti-diarrheal drug, was recently shown to be an effective analgesic (DeHaven-Hudkins et al. 1999, 2002). Local injection of loperamide and its analogs resulted in antipruritic activity in a mouse model of itch, and caused potent antinociception, inhibiting late phase formalin-induced flinching (DeHaven-Hudkins et al. 2002). However, in the formalin test in rats, 10-fold higher doses of naloxone administered subcutaneously were needed to antagonize loperamide compared with the doses needed to antagonize morphine when the agonists were administered subcutaneously, suggesting that the effects of loperamide might be mediated in part by receptors different from those that mediate the effects of morphine (Shannon and Lutz 2002). In a thermal injury–induced rat model, loperamide was able to block thermal hyperalgesia in morphine-tolerant rats, indicating a nonopioid mechanism of action (Nozaki-Taguchi and Yaksh 1999). Loperamide-induced analgesia could be explained, at least in part, by its inhibitory action on ion channels involved in nociception. Loperamide is known to block several types of voltage-gated ion channels, including L-type Ca2+- channels (Church et al. 1994; Hagiwara et al. 2003; Reynolds et al. 1984), delayed-rectifier potassium channels (Yang et al. 2005), and N-methyl-D-aspartate (NMDA) receptors (Church et al. 1994).

In an effort to expand our knowledge of hyperpolarization-activated cyclic nucleotide–gated (HCN) channel pharmacology, we screened a focused compound library composed of 175 known ion channel modulators (Wyeth Research, unpublished data). We found that loperamide produced a potent block of HCN channels at low micromolar concentrations. This observation was particularly interesting in the light of the involvement of HCN channels in regulating neuronal excitability, sensory processing, and the pathophysiology of pain (Chaplan et al. 2003; Hutcheon and Yarom 2000; Pape 1996; Yao et al. 2003). Indeed, prostaglandin E2–induced depolarization of membrane potential in dorsal root ganglion (DRG) neurons and dorsal horn neurons of the spinal cord involves cAMP-dependent induction of $I_h$ (Baba et al. 2001; Ingram and Williams 1994). Positive regulation of $I_h$ by prostanoids produced during inflammation may lead to membrane depolarization and facilitation of repetitive activity, thus contributing to the sensitization to painful stimuli. Additionally, HCN channels were shown to drive the frequency of ectopic discharges in Aδ- and C-fibers that is commonly associated with mechanical allodynia in rat models of neuropathic and postoperative pain. Peripheral administration of the HCN channel blocker ZD7288 significantly attenuated mechanical allodynia induced by partial sciatic nerve injury and hind-paw incision (Chaplan et al. 2003; Dalle and Eisenach 2005; Yao et al. 2003).

Thus considering the important role of HCN channels in nociception and pathological pain, the observed loperamide-induced inhibition of HCN channels could provide an additional molecular mechanism for its analgesic action. To further characterize this novel finding, we studied loperamide pharmacology on $I_h$ in rat DRG neurons.

METHODS

Primary culture of DRG neurons

All protocols involving animals were in accordance with National Institute of Health guidelines and approved by the Wyeth IACUS. Two- to 3-wk-old Wistar rats were anesthetized with halothane before two- to 3-wk-old Wistar rats for 2 wk. All experimental procedures were carried out in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
animals and incubated in DMEM (Sigma) buffer containing 0.5 mg/ml collagenase (Worthington, Lakewood, NJ) and 0.5 mg/ml trypsin for 25–30 min at 35°C. Subsequently, ganglia were washed for 10–20 min and dissociated by triturating with a fire-polished Pasteur pipette. Isolated neurons were plated on poly-d-lysine (Sigma)-coated glass coverslips and cultured at 37°C in a humified 5% CO2/air atmosphere in serum-free Neurobasal/B27 medium (Invitrogen, Carlsbad, CA).

**Electrophysiology**

Ih was recorded from the soma of visually identified small (22–30 μm), medium (32–40 μM), and large (50–65 μM soma diam) DRG neurons at 3–10 days in culture using standard whole cell recordings. Electropores were pulled from borosilicate glass capillaries (TW150F, World Precision Instruments, Sarasota, FL). Pipette resistance ranged between 2–3 (medium-to-large neurons) and 3–4 MΩ (small neurons) when filled with the intracellular solution (in mM): 100 K-glucuronate, 30 KCl, 1.5 MgCl2, 10 HEPES, 3 K2-ATP, and 0.5 cAMP; pH adjusted to 7.3 with Tris-OH. The series resistance (R_s) ranged between 5 and 11 MΩ and was compensated by 50–70%. Recordings with >25% R_s change were excluded from the analysis. Unless specified otherwise, electrophysiological recordings were made 10–15 min after establishing whole cell configuration to allow saline equilibration. Pipette solution was aliquoted and stored at –20°C; 10–15 min after establishing whole cell configuration to allow saline equilibration. Pipette solution was aliquoted and stored at –20°C; 10–15 min after establishing whole cell configuration to allow saline equilibration. Pipette solution was aliquoted and stored at –20°C; 10–15 min after establishing whole cell configuration to allow saline equilibration. Pipette solution was aliquoted and stored at –20°C; 10–15 min after establishing whole cell configuration to allow saline equilibration.

**RESULTS**

**HCN channel protein and functional expression in rat DRG neurons**

We used subunit-selective affinity-purified antisera and immunofluorescence to study HCN subunit immunoreactivity in DRG neurons (Fig. 1A). HCN1 immunoreactivity was highest in large DRG somata sections, present at a significant level in a subset of somata sections of medium size, and was generally low in small somata sections. HCN2 immunofluorescence was more broadly distributed in DRGs. The highest levels of HCN2 immunofluorescence were observed in large somata sections, with significant levels of immunofluorescence present in a certain subpopulation of small to medium somata sections. HCN4 immunofluorescence in DRGs was generally low. Ih was isolated from inward-rectifier potassium current based on the difference in sensitivity to external Ba2+.

**Immunofluorescence**

Immunofluorescence detection of HCN proteins was performed following previously published procedures (Vasileyv and Barish 2002) with minor modifications. In brief, DRGs were dissected and fresh frozen (–20°C on dry ice) in optimal cutting temperature compound (OCT, Sakura Finetek, Tokyo, Japan); 20-μm horizontal sections were cut on a cryostat (Leica, Nussloch, Germany). Sections were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20–30 min at 4°C, and rinsed (3 times, 15 min each) in PBS. Sections were permeabilized with 0.03% Triton X-100 (Sigma) in PBS containing 3% BSA and 5% normal goat serum for 1 h at room temperature. DRGs were incubated for 12–14 h at 4°C in primary antibody (Chemicon, Temecula, CA) diluted at appropriate concentrations (2 μg/ml for anti-HCN1 and anti-HCN2 and 4.0 μg/ml for anti-HCN4 antibodies) in PBS with 3% BSA. After rinsing in PBS (3 times, 20 min each), sections were incubated in fluorescein-conjugated goat antirabbit IgG (Zymed, South San Francisco, CA; diluted 1:100 in PBS containing 5% normal goat serum) for 1 h at room temperature. Finally, sections were rinsed in PBS (3 times, 20 min each) and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Controls included omission of primary antibody. Processed sections were visualized with a Zeiss Axiointer 135 microscope under a ×32 air objective and imaged using a Zeiss AxioCam MRm digital monochrome camera. To facilitate qualitative comparison of immunoreactivity, tissues were stained simultaneously using an identical protocol, and images were acquired using the same AxioCam digital camera parameters.
rectifier potassium current. Isolated in this way, \(I_h\) was 90% blocked by 100 μM ZD7288 (Fig. 1B) at test voltages positive to –100 mV. An apparent time-independent component was still present; however, it was not sensitive to 100 μM ZD7288, and thus was not included into analysis. A representative recording of \(I_h\) from a large-diameter DRG neuron is shown in Fig. 1B.

**Loperamide inhibits \(I_h\) in a concentration-dependent manner**

\(I_h\) was evoked by hyperpolarizing voltage steps to –90 mV delivered from a holding potential of –50 mV in the absence and presence of sequentially increasing concentrations of loperamide (in μM): 0, 1.2, 3.7, 11, 33, and 100 (Fig. 2, top). Loperamide blocked \(I_h\) in a concentration-dependent manner, with >90% of \(I_h\) being blocked at 100 μM. \(I_h\) steady-state current amplitudes recorded at different loperamide concentrations were normalized to the amplitude of \(I_h\) in control conditions, and the averages were plotted as a function of time (Fig. 2, middle) or drug concentration (Fig. 2, bottom). Fitting the concentration-response plots with Hill’s equation resulted in IC\(_{50}\) = 4.9 ± 0.6 μM, \(k\) = 1.7 ± 0.2 (\(n = 7\)) and IC\(_{50}\) = 11.0 ± 0.5 μM, \(k\) = 1.6 ± 0.1 (\(n = 6\)) for large and small neurons, respectively. The statistical analysis shows that the mean loperamide IC\(_{50}\) in small neurons was significantly different (\(P < 0.05\)) from the mean loperamide IC\(_{50}\) in large cells.

In the latter experiment with cAMP clamped at a high level because of intracellular dialysis, loperamide had no effect on \(I_h\) at a concentration saturating for opioid receptor activation. However, if cAMP levels were permitted to fluctuate, loperamide might indirectly affect \(I_h\) through activation of opioid receptors and the accompanying alterations of [cAMP], at least after forskolin exposure (Ingram and Williams 1994). Thus the relative contribution of opioid receptor–dependent versus opioid receptor–independent components to loperamide-induced \(I_h\) inhibition in DRG neurons in vivo needs further study.

**Extracellular loperamide shifts \(I_h\) steady-state activation**

The percent inhibition of \(I_h\) current amplitude superimposed on the percent reduction of \(I_h\) steady-state activation at the corresponding membrane voltages is shown in Fig. 3. \(I_h\) current-voltage (I-V) relationships in control and after application of 3.7 μM loperamide were normalized to the maximal current amplitude recorded at a test voltage of –120 mV in control saline (Fig. 3A, bottom). Bath-applied loperamide blocked \(I_h\) amplitude in a voltage-dependent manner with a percent inhibition (at 3.7 μM) ranging from 88.7 ± 4.2% at –60 mV to 4.1 ± 8.7% at –120 mV (Fig. 3A, A and B) and was accompanied by an apparent deceleration of \(I_h\) activation kinetics (Fig. 3A, top). The percent reduction of \(I_h\) amplitude and steady-state activation caused by bath application of loperamide was calculated from a single pool of recordings, and these averages were plotted against the corresponding test voltages (Fig. 3B). The loperamide-induced reduction of \(I_h\) amplitude was generally equivalent to the reduction of \(I_h\) steady-state activation (see METHODS for details) at all studied membrane voltages (Fig. 3B; \(P > 0.05\) at all membrane voltages).

The conductance-voltage (G-V) relationships for \(I_h\) steady-state activation in control and in the presence of 3.7 μM loperamide (a concentration producing ~50% inhibition of \(I_h\)) were calculated from tail current amplitudes measured 50–70 ms after repolarization (Fig. 4, top, inset), because deactivation of \(I_h\) is relatively slow. The conductance ratios \(G/G_{\text{max}}\) show that loperamide shifted the threshold for \(I_h\) activation about –10 to –15 mV (~50% in control vs. –60 to –65 mV in the presence of 3.7 μM loperamide, respectively) as presented in Fig. 4 (bottom). The following results of the G-V fitting by the Boltzmann equation were obtained: \(V_{1/2} = –73.2 ± 0.8\) mV, \(k = 8.5 ± 0.7\) mV and \(V_{1/2} = –83.3 ± 0.4\) mV, \(k = 7.9 ± 0.4\) mV.
mV in control and after application of 3.7 μM loperamide, respectively. Bath-applied loperamide (3.7 μM) produced a statistically significant shift in the average $V_{1/2}$ (−10 mV, $P < 0.001$) of $I_h$ in large neurons and did not significantly change the slope coefficient ($P > 0.05$).

Concurrently, $I_h$ activation kinetics was significantly slower in the presence of bath-applied loperamide compared with control. In the double-exponential model for $I_h$ activation in large neurons, loperamide increased the value of the slow and fast time constants of activation by twofold (Fig. 5). In control recordings, values of fast ($\tau_{fast}$) and slow ($\tau_{slow}$) time constants for $I_h$ activation were voltage dependent, becoming progressively faster toward negative voltages (Fig. 5), and differed by almost an order of magnitude (in control at −110 mV, $\tau_{fast}$ was 91.4 ± 10.8 ms and $\tau_{slow}$ was 605.9 ± 105.9 ms; $n = 9$). These time constants were significantly slower in the presence of loperamide compared with control (in the presence of 3.7 μM loperamide: $\tau_{fast}$ at −110 mV was 188.7 ± 31.3 ms and $\tau_{slow}$ was 1,131.3 ± 152.2 ms; $n = 9$). A direct, model-independent comparison of the rate of $I_h$ activation (by measurement of $I_h$ half-activation time) in control and after loperamide exposure confirmed the respective results for the $I_h$ kinetics deceleration obtained using the two-exponential model for $I_h$ activation. A 2.4-fold change (272.5 ± 20.1 ms in control vs. 646.7 ± 77.5 ms in the presence of 3.7 μM loperamide) of $I_h$ half-activation time (at a test voltage of −90 mV) in small neurons was not significantly different from the 2.9-fold change (181.4 ± 21.5 ms in control and 500.7 ± 64.4 ms in the presence of 3.7 μM loperamide) measured in large cells (Fig. 6). To address the molecular mechanism of $I_h$ inhibition and identify the position of the loperamide binding site on the cellular membrane, we studied the efficacy of loperamide applied to the internal versus

![Figure 3](image-url)  
**FIG. 3.** Voltage-dependence of $I_h$ inhibition by loperamide. **A:** (top) representative $I_h$ records from a large cell obtained at 2 test voltages of −80 and −120 mV in control (left) and after bath application of loperamide (right). Voltage protocol was the same as described in Fig. 2. Note an obvious reduction of the rate of $I_h$ activation in the presence of 3.7 μM loperamide compared with control at the corresponding membrane voltages. **Bottom:** normalized $I_h$ current-voltage ($I$-$V$) relationships obtained in control (•, mean ± SE, $n = 10$) and after bath application of 3.7 μM loperamide (○, mean ± SE, $n = 10$). Current amplitudes were normalized to the amplitude of $I_h$ evoked by −120-mV test voltage step in control saline. **B:** percent reduction in $I_h$ amplitude (●, mean ± SE, $n = 10$) and $I_h$ steady-state activation (●, mean ± SE, $n = 9$) plotted against the corresponding test voltage. $I_h$ inhibition was strongly voltage-dependent, with fractional block being gradually decreased at more hyperpolarized test potentials. All recordings were made from large-diameter neurons.

![Figure 4](image-url)  
**FIG. 4.** Loperamide shifts $I_h$ activation toward more hyperpolarized potentials. **Top:** family of inward currents (in control) evoked by hyperpolarizing voltage steps delivered from a holding potential of −50 mV to different test voltages applied in −10 mV increments, followed by a voltage step to −70 mV to record $I_h$ tail current. **Bottom:** $I_h$ voltage conductance ($G/I_{max}$) relationships in control (●, $n = 14$) and in the presence of loperamide applied by bath (3.7 μM, ○, $n = 9$) or through patch pipette (10 μM, △, $n = 5$), respectively. $G/I_{max}$ was calculated from averaged tail current amplitudes (top, inset, shaded area) normalized to the maximal amplitude of the tail current (5-s-long voltage step to −130 mV). Application of 3.7 μM loperamide produced about −10 to −15 mV shifts in the $I_h$ threshold of activation, resulting in 50–90% reduction of $I_h$ steady-state amplitude at physiologically relevant membrane potentials. $G/I_{max}$ plots were fit with Boltzmann equations (solid lines superimposed on data points). The following fit parameters were obtained: control, $V_{1/2} = −7.3 \pm 0.8$ mV, $8.5 \pm 0.7$ mV; bath-applied loperamide, $V_{1/2} = −83.3 \pm 6.4$ mV, $k = 7.9 \pm 0.4$ mV; intracellularly applied loperamide $V_{1/2} = −74.1 \pm 4.0$ mV, $k = 7.4 \pm 0.4$ mV. The asterisks indicate the statistical comparison of the 2 data sets (control vs. bath-applied loperamide). Data obtained in the presence of intracellularly applied loperamide were not significantly different from the control. All recordings were made from large-diameter neurons.
Furthermore, we studied whether the effect was occurring through a cAMP-dependent (Ingram and Williams 1994) or cAMP-independent mechanism.

Loperamide directly inhibits $I_h$ in a use-independent manner that does not require activation of opioid receptors

If loperamide directly binds to the channel, the loperamide-induced slowing of $I_h$ activation kinetics could be explained by a number of molecular mechanisms, for example, by binding to the intracellular S6 bundle or by interaction with the cAMP-binding domain, two regions closely involved with HCN channel gating. Alternatively, loperamide might act extracellularly by binding to the S1-S2 extracellular loop, which has been shown to be an important determinant of the rate of HCN channel activation. Because binding to sites located in the pore-forming region might result in a use-dependent block, we first elucidated whether loperamide-induced $I_h$ inhibition depends on the application frequency of test pulses.

$I_h$ amplitude was monitored for 6 min using a standard voltage protocol (see Fig. 2). Subsequently, the holding potential was changed from $-50$ to 0 mV (threshold for $I_h$ activation was about $-55$ mV, thus at 0 mV holding potential, $I_h$ channels are fully deactivated), and 3.7 $\mu$M loperamide was added to the bath saline. During the first 2 min of loperamide application, cells were held at 0 mV, and no test voltages were applied, thus insuring a complete $I_h$ deactivation. Incubation with 3.7 $\mu$M loperamide for 2 min resulted in the reduction of $I_h$ amplitude by $40 \pm 8\%$ ($n = 5$), a value similar to that observed with repeated activation ($I_h$ amplitude was reduced by $38 \pm 7\%$, $n = 6$, with 3.7 $\mu$M loperamide; Figs. 2 and 7A). Subsequently re-establishing the voltage-stimulating protocol did not induce any additional use-dependent component of $I_h$ block (Fig. 7A).

To further study the location of loperamide binding, we tested the efficacy of intracellularly versus extracellularly applied loperamide. Inclusion of 10 $\mu$M loperamide into the

FIG. 5. Loperamide slows kinetics of $I_h$ activation. Top: averages of normalized traces of $I_h$ (time-dependent component only, $n = 5$) evoked by hyperpolarizing pulses to $-90$ (left) and $-110$ mV (right) in control (+loper. in pip.) and in the presence of loperamide applied by bath (loper. in bath) or applied through patch pipette (+loper. in pip.). Bottom: computational analysis for the 2-exponential model of $I_h$ activation. Current traces were fitted with the sum of 2 exponential functions (excluding an initial delay) to describe the time-course of $I_h$ activation. In control recordings, values of fast ($\bullet$, $n = 9$) and slow ($\bullet$, $n = 9$) time constants were significantly smaller than the respective values in the presence of loperamide in bath saline ($\bigcirc$-fast and $\bigcirc$-slow time constant, $n = 9$), but were not significantly different from the respective time constants when loperamide was included in the pipette ($\bigcirc$, $n = 5$, $P > 0.05$ for all membrane voltages). Asterisks indicate level of statistical significance between values of slow and fast time constants in control vs. value of corresponding time constant in presence of loperamide. All recordings were made from large neurons.

FIG. 6. Loperamide-induced reduction in rate of $I_h$ activation is similar in small and large neurons. Top: representative current traces (normalized time-dependent component) evoked by a voltage step to $-90$ mV from a holding potential of $-50$ mV in control and after bath application of 3.7 $\mu$M loperamide. Dotted line represents $1/2$ $I_h$ amplitude. Computational analysis of half-activation time of $I_h$ in control and in the presence of 3.7 $\mu$M bath-applied loperamide is shown in the 2 bottom panels.

$J$ Neurophysiol • VOL 97 • MAY 2007 • www.jn.org
were preincubated for 5 min with 10 μM loperamide to test for the possible involvement of opioid receptors (Fig. 8). Application of 10 μM loperamide reduced I\textsubscript{h} amplitude by 77 ± 5% (n = 6) and was not significantly different from the effect of equimolar loperamide when cells were preincubated for 5 min with 10 μM naloxone (loperamide-induced I\textsubscript{h} inhibition in the presence of naloxone was 78 ± 2%; n = 3).

**DISCUSSION**

**Physiological significance of loperamide-induced I\textsubscript{h} inhibition**

This study revealed a novel pharmacology for the known analgesic loperamide that might provide an additional molecular mechanism of its analgesic action. Loperamide, originally known as an antidiarrheal drug, later was found to be active in several models of inflammatory, bone cancer, and acute pain (DeHaven-Hudkins et al. 1999, 2002; Menendez et al. 2003, 2005; Nozaki-Taguchi and Yaksh 1999; Sevostianova et al. 2005). This effect seems to be manifested by activating peripheral μ-opioid receptors (Menendez et al. 2003, 2005; Sevostianova et al. 2005; Shannon and Lutz 2002); however, the existence of an additional mechanism is supported by several findings. First, loperamide-induced analgesia could be antagonized with naloxone only at doses 10-fold higher than those needed to antagonize morphine administered by the same route (Sevostianova et al. 2005; Shannon and Lutz 2002); second, a significant antinociceptive effect of loperamide was found both ipsi- and contralateral to the thermal injury paw, with the effect at the contralateral site not being reversible with naloxone (Nozaki-Taguchi and Yaksh 1999); third, loperamide antagonized thermal hyperalgesia in morphine-tolerant rats, indicating a non-opioid mechanism of action (Nozaki-Taguchi and Yaksh 1999). Additionally, only high doses of loperamide were efficacious in reversal of the first phase of formalin-induced acute pain (paw licking and biting), but failed to produce antinociceptive effects in a model of acute thermal pain (Sevostianova et al. 2005).

Loperamide-induced inhibition of voltage-gated calcium channels (Church et al. 1994; Hagiwara et al. 2003; Reynolds et al. 1984) and NMDA receptors (Church et al. 1994) present both in CNS and in sensory fibers (Carlton et al. 1995; Coggeshall and Carlton 1998; Davidson et al. 1997; Kinkelin et al. 2000) is broadly consistent with its analgesic properties, considering the well-documented efficacy of selective antagonists of voltage-gated calcium channels and NMDA receptors in treating inflammatory and neuropathic pain symptoms (Chizh and Headley 2005; McGivern 2006). Our finding of loperamide-induced inhibition of HCN channels is also consistent with HCN channels involvement in the pathophysiology of pain (Chaplan et al. 2003; Hutcheon and Yarom 2000; Pape 1996; Yao et al. 2003). Because PGE\textsubscript{2}-induced depolarization of the membrane potential in DRG neurons and dorsal horn neurons of the spinal cord involves cAMP-dependent induction of I\textsubscript{h} (Baba et al. 2001; Ingram and Williams 1994), the mechanism of lowering pain threshold by prostaglandins released in the area of inflammation is thought to involve, at least
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in part, activation of HCN channels. Thus opioid inhibition of adenylyl cyclase and subsequent inhibition of $I_h$ might represent a mechanism by which opioids inhibit primary afferent excitability and relieve pain. The latter is supported by the fact that forskolin- and PGE$_2$-induced $I_h$ upregulation is inhibited by opioids acting through $\mu$- and/or $\delta$-receptors (Ingram and Williams 1994; Svoboda et al. 1999). However, our study showing a direct inhibition of HCN channels by loperamide does not support an exclusive mechanism of loperamide-induced analgesia through opioid receptor-induced $I_h$ inhibition, but is consistent with the possibility of both opioid receptor-dependent and -independent mechanisms. Moreover, additional mechanisms for the peripheral antihyperalgesic action of loperamide might include inhibition of voltage-gated calcium channels in sensory neurons and the inhibition of tetrodotoxin-resistant sodium channels, both shown to be involved in mechanisms of central and peripheral opioid antinociception (Gold and Levine 1996; Schroeder et al. 1991).

Loperamide blocked $I_h$ in a concentration-dependent manner with an $IC_{50}$ of $4.9 \pm 0.6$ and $11.0 \pm 0.5$ $\mu$M for large- and small-diameter neurons, respectively. These values are compatible to the reported $IC_{50}$ of loperamide-induced inhibition of voltage-gated calcium channels ($IC_{50}$ = 2.5 $\mu$M) (Church et al. 1994; Hagiwara et al. 2003; Reynolds et al. 1984) but are an order of magnitude smaller then the $IC_{50}$ reported for NMDA receptors ($IC_{50}$ = 73 $\mu$M) (Church et al. 1994). The reported efficacious doses of loperamide in vivo were as high as 3–10 mg/kg (DeHaven-Hudkins et al. 1999, 2002), which in principle could result in a high micromolar range of loperamide in plasma. However, the pharmacokinetics of loperamide has not been reported in the previously mentioned studies; thus drawing any conclusions about the efficacious loperamide concentration in blood plasma would be purely speculative. Our finding of loperamide-induced inhibition of $I_h$ channels is consistent with their involvement in the pathophysiology of pain (Chaplan et al. 2003; Hutcheon and Yarom 2000; Pape 1996; Yao et al. 2003).

HCN channels protein and functional expression

Four HCN channel subunits have been identified (Biel et al. 1999; Gauss and Seifert 2000; Kaupp and Seifert 2001; Ludwig et al. 1999; Monteggia et al. 2000; Santoro and Tibbs 1999) and expressed in heterologous systems. The observed immunofluorescence showing somewhat overlapping but independent expression patterns of HCN1 and HCN2 protein suggest the coexistence of different HCN channel isoforms in DRG neurons.

On the functional level, $I_h$ kinetics and pharmacology were close to those reported previously (Cardenas et al. 1999; Scroggs et al. 1994; Yagi and Sumino 1998). The threshold for $I_h$ activation ranged between $-55$ and $-60$ mV; voltage for half-maximal activation and the slope coefficient were $V_{1/2} = -73.2 \pm 0.8$ mV and $k = 8.5 \pm 0.7$ mV, respectively, which was similar to values reported by Cardenas et al. (1999) ($V_{1/2} = -73.3$ mV, $k = 7.0$ mV).

Mechanism of $I_h$ inhibition by loperamide

We showed that the mechanism of loperamide-induced $I_h$ inhibition is unrelated to the activation of opioid receptors and is reversible, voltage-dependent, use-independent, and is associated with a negative shift of $V_{1/2}$ for $I_h$ steady-state activation. The voltage dependence of $I_h$ activation has been shown to be modulated by forskolin, PGE$_2$, and opioids through a cAMP-dependent mechanism (Ingram and Williams 1994; Svoboda et al. 1999). Opioids had no effect on $I_h$ alone, but were shown to reverse the effect of forskolin on $I_h$. This effect was antagonized by a broad-spectrum opioid receptor antagonist naloxone (Ingram and Williams 1994). Involvement of opioid receptors in the reported loperamide-induced $I_h$ inhibition is unlikely because we did not observe any substantial $I_h$ inhibition by 1.2
μM loperamide, considering a low-nanomolar affinity of loperamide for the opioid receptors. Additionally, the loperamide effect was not antagonized by naloxone. Therefore we suggest a direct inhibition of HCN channel activity by loperamide, probably by binding to the extracellular region of the channel. Alternatively, lipid-soluble drugs such as loperamide (clogP = 4.9) can bind to the channel site embedded in the lipid bilayer; however, in this scenario, intracellularly applied loperamide should also block $I_h$.

### Slowing the rate of $I_h$ activation by loperamide

The observed shift of $I_h$ steady-state activation accompanied by the slowing of its activation kinetics in the presence of loperamide could be explained, at least in part, based on the preferential block of fast-HCN1 versus slow-gating HCN2-4 homomeric channels and/or heteromeric channels with a slow-gating (HCN2/4) stoichiometry (Biel et al. 1999; Kaupp and Seifert 2001; Ludwig et al. 1999; Santoro and Tibbs 1999; Vasiliev and Barish 2002). This idea is supported by our observation of preferential block of $I_h$ channels in large versus small DRG neurons, considering a slower gating kinetics of $I_h$ in small cells; however, equimolar loperamide reduced $I_h$ activation rate in small and in large neurons to a similar extent. The pharmacology of loperamide on recombinant HCN channels would answer this question in more detail.

The mechanism of $I_h$ activation kinetics slowing by bath-applied loperamide caused by opioid receptor–induced reduction of the intracellular cAMP (Ingram and Williams 1996) is unlikely because the cAMP level in this experiment was clamped by dialysis through the patch pipette (the measurements involved were made 10–15 min after establishing the whole cell configuration, thus allowing time for stabilization of the cAMP and the cytoplasm). Alternatively, loperamide might affect $I_h$ kinetics by binding to domains principally involved in regulating the rate of HCN channel activation. Two regions affecting HCN channel activation kinetics have been identified, one being S1 and S1–S2 and the other being S6–CNBD. The reciprocal replacements of the whole S1 and S1–S2 region between recombinant HCN1 and HCN4 channels affected the activation kinetics about 16- and 3-fold, respectively (Ishii et al. 2001). Thus it is reasonable to suggest that slowing of the $I_h$ activation rate by extracellularly applied loperamide may be caused by its interaction with HCN channel extracellular domain between S1 and S2, an observation supported by our finding that the loperamide binding site seems to be extracellular, located outside of the lipid bilayer. Additionally, the loperamide-induced reduction of $I_h$ activation rate could not be explained in terms of a simple two-state model (by loperamide affecting the forward and backward rate constants) because accounting for the $\sim$10 mV shift of $V_{1/2}$ was not sufficient to explain the loperamide-induced shift (about $\sim$20 mV) for the slow and fast time constants determined from a two-exponential model for $I_h$ activation. The latter observation is also consistent with the S1–S2 hypothesis proposed earlier (Ishii et al. 2001) for HCN1 and HCN4 channel gating (2 channels with 2 orders of magnitude difference in their activation rates, yet a similar $V_{1/2}$). The hypothesis of the loperamide binding site could be explored further with side-directed mutagenesis of the S1–S2 linker region in future studies.

### Acknowledgments

We thank M. Pangalos for support and critical reading of the manuscript.

### References


