Direct Inhibition of \( I_h \) by Analgesic Loperamide in Rat DRG Neurons

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ABSTRACT

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 ± 0.6 µM and 11.0 ± 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 while concurrent application of equimolar external loperamide produced a rapid, reversible I_h inhibition. The observed loperamide-induced I_h inhibition was not due to the activation of peripheral opioid receptors since 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. Since 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 \( \mu \)-opioid receptor agonist originally known as an antidiarrheal drug was recently shown to be an effective analgesic (DeHaven-Hudkins et al. 1999; DeHaven-Hudkins et al. 2002). Local injection of loperamide and the 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, approximately 10-fold higher doses of naloxone administered subcutaneously were required to antagonize loperamide compared to the doses required 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 which 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 non-opioid 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 Ca\(^{2+} \) channels (Church et al. 1994; Hagiwara et al. 2003; Reynolds et al. 1984), delayed-rectifier potassium channels (Yang et al. 2005) and NMDA receptors (Church et al. 1994).

In an effort to expand our knowledge of HCN channel pharmacology we screened a focused compound library composed of 175 known ion channel modulators (Lee et al., in preparation). 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 E$_2$-induced depolarization of membrane potential in 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 prostaglandins 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 A$\delta$ 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 investigated 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. 2-3 week old Wistar rats were anesthetized with halothane prior to euthanization by decapitation. L4-6 DRGs were quickly dissected from 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 humidified 5% CO₂/air atmosphere in serum-free Neurobasal/B27 medium (Invitrogen, Carlsbad, CA).

Electrophysiology. Iₜ was recorded from the soma of visually identified small (22-30 µm), medium (32-40 µM) and large (50-65 µM soma diameter) diameter DRG neurons at 3-10 days in culture using standard whole-cell recordings. Electrodes were pulled from borosilicate glass capillaries (TW150F; World Precision Instruments, Sarasota, FL). Pipette resistance ranged between 2–3 MΩ (medium-to-large neurons) and 3-4 MΩ (small neurons) when filled with the intracellular solution (in mM): 100 K-Gluconate, 30 KCl, 1.5 MgCl₂, 10 HEPES, 3 K₂-ATP, 0.5 cAMP; pH adjusted to 7.3 with Tris-OH. The series resistance (Rs) ranged between 5-11 MΩ and was compensated by 50-70%. Recordings with more than 25% Rs change were excluded from the analysis. Unless specified otherwise electrophysiological recordings were made 10-15 minutes after establishing whole-cell configuration to allow saline equilibration. Pipette solution was aliquoted and stored at –20 °C; during voltage-clamp recordings the pipette
solution was kept on ice. The extracellular solution was HBSS (Gibco, Cat. No. 14025-092) (in mM): NaCl 137.9; CaCl$_2$ 1.3; MgCl$_2$ 0.5; MgSO$_4$ 0.4; NaHCO$_3$ 4.2; Na$_2$HPO$_4$ 0.3; KCl 5.3; KH$_2$PO$_4$ 0.4; Dextrose 5.6. Data were collected using a MultiClamp 700A amplifier and digitized using DigiData 1322A and pClamp9 software (all from Molecular Devices, Union City, CA). Current traces were filtered at 1–2 kHz and digitized at 4 kHz. Membrane voltages were not corrected for the +10mV junction potential between the pipette and bath solutions. Recordings were made at room temperature (21–23°C). All drugs were obtained from Sigma.

Data were analyzed using Clampfit (Molecular devices) and Origin 6.0 (OriginLab, Northampton, MA). Concentration-response data sets were fitted with Hill’s equation of the form $I/I_0 = 1/(1 + ([C]/IC_{50})^k)$, where [C] is the loperamide concentration, IC$_{50}$ is the loperamide concentration producing 50% block of $I_h$, and k is Hill’s coefficient. The G-V relationships were fitted with Boltzmann equation of the form $G/G_{max} = 1/[1 + \exp((V-V_{1/2})/k)]$, where $V_{1/2}$ is voltage for half maximal activation and k is the slope coefficient. The % reduction of $I_h$ steady-state activation was calculated for each cell according to the formula $100^\ast(1-([G/G_{max}]_{loperamide}/[G/G_{max}]_{control}))$ and subsequently averaged for every membrane voltage. Statistical significance was evaluated by Student’s t test using Origin 6.0. Statistical significance is indicated as follows: *p<0.05; **p<0.01; ***p<0.001. Unless specified otherwise, all data are means ± s.e.

**Immunofluorescence.** Immunofluorescence detection of HCN proteins was performed following previously published procedure (Vasilyev and Barish 2002) with minor modifications. In brief, DRG 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 then rinsed (three 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 hr at room temperature. DRG were then incubated for 12–14 hr 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 (three 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 hr at room temperature. Finally, sections were rinsed in PBS (three 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 Axiovert 135 microscope under a 32x 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.
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 DRG. 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 immunoreactivity in DRG was generally low.

$I_h$ was isolated from inward-rectifier potassium current based on the difference in sensitivity to external $Ba^{2+}$. Addition of 0.5mM $Ba^{2+}$ does not significantly affect $I_h$ (van Welie et al. 2005; Vasilyev and Barish 2002) while blocking inward-rectifier potassium current. Isolated in this way, $I_h$ was 90% blocked by 100 $\mu$M ZD7288 (Fig. 1B) at test voltages positive to -100mV. An apparent time-independent component was still present, however, it was not sensitive to 100 $\mu$M 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 $\mu$M): 0, 1.2, 3.7, 11, 33, and 100 (Fig. 2, top panel). Loperamide blocked $I_h$ in a concentration-dependent manner, with >90% of $I_h$ being blocked at 100 $\mu$M. $I_h$ 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 panel) or drug concentration (Fig. 2, lower panel). Fitting the concentration-response plots with Hill’s equation resulted in $IC_{50} = 4.9 \pm 0.6 \mu M$, $k = 1.7 \pm 0.2$ (n=7) and $11.0 \pm 0.5 \mu M$, $k = 1.6 \pm 0.1$ (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, then 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 vs. opioid receptor-independent components to loperamide-induced $I_h$ inhibition in DRG neurons in vivo requires further investigation.

**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 $\mu M$ loperamide were normalized to the maximal current amplitude recorded at a test voltage of –120 mV in control saline (Fig. 3A, lower panel). Bath-applied loperamide blocked $I_h$ amplitude in a voltage-dependent manner with a percent inhibition (at 3.7 $\mu M$) ranging from 88.7 ±
4.2% at –60 mV to 4.1 ± 8.7% at –120 mV (Fig. 3A-B), and was accompanied by an apparent deceleration of \( I_h \) activation kinetics (Fig. 3A, upper panel). The percent reduction of \( I_h \) amplitude and steady-state activation due to 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 investigated 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 \( \mu \)M loperamide (a concentration producing about 50% inhibition of \( I_h \)) were calculated from tail current amplitudes measured 50-70 ms after repolarization (Fig. 4, upper panel, 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 mV in control vs. -60 to -65 mV in the presence of 3.7 \( \mu \)M loperamide, respectively) as presented in Fig. 4, lower panel. The following results of the G-V fitting by the Boltzmann equation were obtained: \( V_{1/2} = -73.2 \pm 0.8 \) mV, 8.5 ± 0.7 mV; and \( V_{1/2} = -83.3 \pm 0.4 \) mV, k=7.9 ± 0.4 mV in control and after application of 3.7 \( \mu \)M loperamide, respectively. Bath-applied loperamide (3.7 \( \mu \)M) 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 when 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 2-fold (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 1131.3 ± 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 vs. external side of the membrane. Further, we investigated whether the effect was occurring via a c-AMP-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. Since 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 legend to Fig. 2). Subsequently, the holding potential was changed from \(-50 \text{ mV}\) to 0 mV (the threshold for \( I_h \) activation was about \(-55 \text{ mV}\), thus at 0 mV holding potential \( I_h \) channels are fully deactivated), and 3.7 \( \mu \text{M} \) 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 \( \mu \text{M} \) loperamide for 2 minutes resulted in the reduction of \( I_h \) amplitude by 40 ± 8% (n=5), a value similar to that observed with repeated activation (\( I_h \) amplitude was reduced by 38 ± 7%, n=6, with 3.7 \( \mu \text{M} \) loperamide) (Fig. 2, Fig. 7A). Subsequently re-establishing the voltage-stimulating protocol did not induce any additional use-dependent component of \( I_h \) block (Fig. 7A). Control recordings obtained with the same voltage protocol demonstrated no change of \( I_h \) in the absence of loperamide (Fig. 7A).

To further investigate the location of loperamide binding, we tested the efficacy of intracellularly vs. extracellularly applied loperamide. Inclusion of 10 \( \mu \text{M} \) loperamide into the pipette solution did not affect \( I_h \) kinetics and/or amplitude recorded immediately (20-30 seconds) after establishing whole-cell configuration for up to 10 minutes. Concurrent bath application of 10 \( \mu \text{M} \) loperamide produced a rapid and reversible \( I_h \) inhibition (Fig.
7B). Additionally, when applied via the patch-pipette, loperamide did not show any substantial effect on $I_h$ steady-state activation or activation kinetics as shown by a population analysis of $V_{1/2}$ and gating kinetics in loperamide filled versus control cells (Fig. 4-5), pointing to the extracellular location of the loperamide binding site.

The effect of loperamide on $I_h$ was measured in the presence of naloxone to test for the possible involvement of opioid receptors (Fig. 8). Application of 10 µM loperamide reduced $I_h$ amplitude by 77 ± 5% (n=6) and was not significantly different from the effect of equimolar loperamide when cells were pre-incubated for 5 min with 10 µM naloxone (loperamide-induced $I_h$ inhibition in the presence of naloxone was 78 ± 2%, n=3).
DISCUSSION

Physiological significance of loperamide-induced $I_h$ inhibition. The current 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; DeHaven-Hudkins et al. 2002; Menendez et al. 2003; Menendez et al. 2005; Nozaki-Taguchi and Yaksh 1999; Sevostianova et al. 2005). This effect appears to be manifested by activating peripheral $\mu$-opioid receptors (Menendez et al. 2003; Menendez et al. 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 required 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 at ipsi- and contra-lateral 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 liking 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; Hagiwara et al. 2003; Reynolds et al. 1984) and NMDA receptors (Church et al.
present both in CNS and in sensory fibers (Carlton et al. 1995; Coggeshall and Carlton 1998; Davidson et al. 1997; Kinkelín 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). Since PGE$_2$-induced depolarization of the membrane potential in 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), the mechanism of lowering pain threshold by prostaglandins released in the area of inflammation is thought to involve, at least 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 via µ- and/or δ-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 TTX-R 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 $IC_{50} = 4.9 \pm 0.6 \, \mu M$ 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; DeHaven-Hudkins et al. 2002), which in principle could result in a high-$\mu M$-range of loperamide in plasma. However, the pharmacokinetics of loperamide has not been reported in the above-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 mV and −60 mV; voltage for half maximal activation and the slope coefficient were $V_{1/2} = -73.2 \pm 0.8$ mV, $k = 8.5 \pm 0.7$ mV, respectively, which was similar to values reported by (Cardenas et al. 1999) ($V_{1/2} = -73.3$ mV, $k = 7.0$ mV).

The 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 via 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 since 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 vs. slow-gating HCN2-4 homomeric channel and/or heteromeric channel with a slow-gating (HCN2/4) stoichiometry (Biel et al. 1999; Kaupp and Seifert 2001; Ludwig et al. 1999; Santoro and Tibbs 1999; Vasilyev 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 due to opioid receptor-induced reduction of the intracellular cAMP (Ingram and Williams 1996) is unlikely since the cAMP level in this experiment was clamped by dialysis via the patch pipette, as the measurements involved were made 10-15 min after establishing the whole-cell configuration thus allowing time for stabilization of pipette solutions with a saturation level of 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 channel affected the activation kinetics about 16- and 3-fold, respectively (Ishii et al. 2001). Thus, it is reasonable to suggest that slowing of $I_h$ activation rate by
extracellularly-applied loperamide may be due to its interaction with HCN channel extracellular domain between S1 and S2, an observation supported by our finding that loperamide binding site appears 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) since accounting for the $-10$ mV shift of the $V_{1/2}$ was not sufficient to explain the loperamide-induced shift (about $-20$ 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 (two channels with two-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.
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LEGENDS TO FIGURES

Figure 1. Characterization of HCN channels expression in DRG neurons. A, shown are horizontal sections of fresh-frozen tissue illustrating HCN subunit immunofluorescence in DRG neuron somata sections. Negative control represented by the omission of 10^0 ab is shown in insert. Abbreviations are as following: s, small somata sections, m, medium diameter somata sections of DRG neurons, L, large diameter somata sections of DRG neurons. Scale bar is 50 \( \mu \text{m} \). B, representative voltage-clamp recordings from a large diameter DRG neuron showing responses to hyperpolarizing 5-sec-long voltage steps delivered from a holding potential of –50 mV to voltages between –50 mV and –100 mV (in step of –10 mV), followed by a step to –70 mV to record \( I_h \) tail current. \( I_h \) (time-dependent component of inward current) recorded in this way was completely blocked by 100 \( \mu \text{M} \) of ZD7288 at voltages more positive then -100 mV.

Figure 2. Loperamide blocks \( I_h \) in a concentration-dependent manner. (Upper panel), \( I_h \) recorded from large DRG neuron in control and after cumulative applications of loperamide (in \( \mu \text{M} \)): 1.2, 3.7, 11.1, 33.3, and 100, respectively (5 min between sequential concentration changes). \( I_h \) was elicited once per 30 sec by hyperpolarizing 5-sec-long voltage steps delivered from a holding potential of –50 mV to the test voltage of –90 mV, followed by voltage step to –50 mV. (Middle panel), time course of averaged normalized \( I_h \) amplitudes (■, mean ± s.e., n=6) recorded from small neurons. A single representative recording from a small cell (○, n=1) is shown superimposed with the main graph. Arrows represent bath application of cumulative concentrations of loperamide (in \( \mu \text{M} \)): 0.04, 1.2, 3.7, 11.1, 33.3, and 100. (Lower panel), shown are normalized concentration-current relationships obtained from large (■, n=7) and from
small neurons (●, n=6) for loperamide. Amplitudes of Ih recorded in the presence of loperamide were normalized to the amplitude of Ih recorded in control saline at the same test voltage. The asterisks represent the level of statistical significance between two data sets obtained from small vs. large cells at the respective loperamide concentration.

**Figure 3.** Voltage-dependence of Ih inhibition by loperamide. A (top), representative Ih records from a large cell obtained at two 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 the legend to Figure 2. Note an obvious reduction of the rate of Ih activation in the presence of 3.7 µM loperamide when compared to control at the corresponding membrane voltages. A (bottom), normalized Ih current-voltage (I-V) relationships obtained in control (■, mean ± s.e., n=10) and after bath application of 3.7 µM loperamide (●, mean ± s.e., n=10). Current amplitudes were normalized to the amplitude of Ih evoked by −120 mV test voltage step in control saline. B, percent reduction in Ih amplitude (●, mean ± s.e., n=10) and Ih steady-state activation (■, mean ± s.e., n=9) plotted against the corresponding test voltage. Ih 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.** Loperamide shifts Ih activation toward more hyperpolarized potentials. (Upper panel), 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 Ih tail current. (Lower panel), Ih voltage-conductance (G/G_max) relationships in control (■, n=14) and in the presence of loperamide applied by bath (3.7 µM, ●, n=9) or via patch pipette (10 µM, ○,
n = 5), respectively. G/G_{max} was calculated from averaged tail current amplitudes (Upper panel, insert, shaded area) normalized to the maximal amplitude of the tail current (5-sec-long voltage step to −130 mV). Application of 3.7 μM loperamide produced about −10 to -15 mV shift in the I_h threshold of activation, resulting in 50-90% reduction of I_h steady-state amplitude at physiologically relevant membrane potentials. G/G_{max} plots were fit with Boltzmann equations (solid lines superimposed on data points, see text for details). The following fit parameters were obtained: control, \( V_{1/2} = -73.2 \pm 0.8 \) mV, \( k=8.5 \pm 0.7 \) mV; bath-applied loperamide, \( V_{1/2}= -83.3 \pm 0.4 \) mV, \( k=7.9 \pm 0.4 \) mV; intracellularly-applied loperamide \( V_{1/2}= -74.1 \pm 0.4 \) mV, \( k=7.4 \pm 0.4 \) mV. The asterisks indicate the statistical comparison of the two data sets (control vs. bath-applied loperamide). The data obtained in the presence of intracellularly-applied loperamide were not significantly different from the control. All recordings were made from large diameter neurons.

**Figure 5.** Loperamide slows kinetics of I_h activation. (Upper panel), shown are averages of normalized traces of I_h (time-dependent component only, n=5) evoked by hyperpolarizing pulses to –90 mV (left) and –110 mV (right) in control (-loper. in pip.) and in the presence of loperamide applied by bath (loper. in bath) or applied via patch pipette (+loper. in pip.). (Lower panel), shown is a computational analysis for the two-exponential model of I_h activation. Current traces were fitted with the sum of two exponential functions (excluding an initial delay) to describe the time course of I_h activation. In control recordings values of fast (●, n=9) and slow (■, n=9) time constants were significantly smaller than the respective values in the presence of loperamide in bath saline (○-fast and □-slow time constant, n=9), but were not significantly different.
from the respective time constants when loperamide was included in the pipette (△, n=5, p>0.05 for all membrane voltages). The asterisks indicate the level of statistical significance between the values of slow and fast time constants in control versus the value of the corresponding time constant in the presence of loperamide. All recordings were made from large neurons.

**Figure 6.** The loperamide-induced reduction in the rate of I_h activation is similar in small and large neurons. (Upper panel), shown are 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 µM loperamide. The dotted line represents ½ I_h amplitude. The computational analysis of the half-activation time of I_h in control and in the presence of 3.7 µM bath-applied loperamide is shown in the two lower panels.

**Figure 7.** I_h inhibition by loperamide is use-independent and does not require drug access to the intracellular compartment. A (upper panel), shown are representative recordings of inward current evoked by a voltage step to −90 mV in control (left) and in the presence of 3.7 µM loperamide (right). The scale bars are 0.5 nA and 1 s, respectively. A (lower panel), during first 2 min after loperamide application cells (medium-to-large neurons) were held at 0 mV and no test voltages were applied to insure a complete deactivation of I_h. The 2-min-long bath application of 3.7 µM loperamide resulted in 46 ± 8 % (■, mean ± s.e., n=5) reduction of the initial I_h amplitude. No further I_h block was observed during re-establishment of a voltage-stimulating protocol. I_h amplitude in control recordings (●, n=3) was not significantly affected. Asterisks indicate the level of statistical significance between I_h amplitudes in
control and in the presence of loperamide at the respective time points. B (upper panel), \( I_h \) recordings obtained in the presence of 10 \( \mu \)M loperamide in the patch pipette in control extracellular saline, and in the presence of bath-applied 10 \( \mu \)M loperamide, and after washing-out of bath loperamide. B (lower panel), a summary of 5 experiments demonstrating \( I_h \) block in medium-to-large neurons by extracellularly vs. intracellularly applied loperamide, and the block reversal.

**Figure 8.** \( I_h \) inhibition by loperamide does not require activation of opioid receptors. (Upper panel), \( I_h \) recordings (medium-to-large neurons) in the presence of 10 \( \mu \)M naloxone in control and after bath application of 10 \( \mu \)M loperamide. \( I_h \) was evoked every 30s by voltage steps to –90 mV delivered from a holding potential of –50 mV. (Middle panel), the time course of averaged, normalized \( I_h \) amplitudes during control and after cumulative applications of the drugs. (Lower panel), shown is a bar graph demonstrating naloxone-independent \( I_h \) inhibition by loperamide. Bath-applied loperamide (10 \( \mu \)M) inhibited \( I_h \) by 77 ± 5% (mean ± s.e., n=6) and 78 ± 2% (mean ± s.e., n=3) in control and in the presence of 10 \( \mu \)M naloxone, respectively.
REFERENCES


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