Levo-tetrahydropalmatine (l-THP), a main bioactive Chinese herbal constituent from the genera Stephania and Corydalis, has been in use in clinical practice for years in China as a traditional analgesic agent. However, the mechanism underlying the analgesic action of l-THP is poorly understood. This study shows that l-THP can exert an inhibitory effect on the functional activity of native acid-sensing ion channels (ASICs), which are believed to mediate pain caused by extracellular acidification. l-THP dose dependently decreased the amplitude of proton-gated currents mediated by ASICs in rat dorsal root ganglion (DRG) neurons. l-THP shifted the proton concentration-response curve downward, with a decrease of 40.93% ± 8.45% in the maximum current response to protons, with no significant change in the pH0.5 value. Moreover, l-THP can alter the membrane excitability of rat DRG neurons to acid stimuli. It significantly decreased the number of action potentials and the amplitude of the depolarization induced by an extracellular pH drop. Finally, peripherally administered l-THP inhibited the nociceptive response to intraplantar injection of acetic acid in vivo, which for the first time provides a novel peripheral mechanism underlying the analgesic action of l-THP.
amiloride blocks peripheral ASICs and results in decreasing nociception during phases I and II of the formalin test (Rocha-Gonzalez et al., 2009). A recent study demonstrated that l-THP can effectively inhibit visceral and somatic persistent spontaneous nociception induced by injection of acetic acid and formalin, respectively (Cao et al., 2011). However, it is unclear whether the analgesic action of l-THP involves peripheral ASICs. This study shows that l-THP can inhibit the functional activity of ASICs in rat dorsal root ganglion (DRG) neurons.

**MATERIALS AND METHODS**

**Isolation of the DRG Neurons**

All experiments were approved by the Institutional Animal Care and Use Committee of Hubei University of Science and Technology (approval No.1094) and were carried out in strict accordance with the NIH Guide for the care and use of laboratory animals. Eight- to ten-week-old Sprague-Dawley male rats were anesthetized with ethyl ether and then decapitated. The DRGs were taken out and transferred immediately into Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO), pH 7.4. After removal of the surrounding connective tissues, the DRGs were minced with fine spring scissors, and the ganglion fragments were placed in a flask containing 5 ml of DMEM containing trypsin (type II-S; Sigma) 0.5 mg/ml, collagenase (type I-A; Sigma) 1.0 mg/ml, and DNase (type IV; Sigma) 0.1 mg/ml and incubated at 35°C in a shaking water bath for 25–30 min. Soybean trypsin inhibitor (type II-S; Sigma) 1.25 mg/ml was then added to stop trypsin digestion. Dissociated neurons were placed onto a 35-mm Petri dish and kept for at least another 60 min before electrophysiological recordings. The neurons selected for the electrophysiological experiment were 15–35 μm in diameter.

**Electrophysiological Recordings**

Whole-cell patch-clamp and voltage-clamp recordings were carried out at room temperature (22–25°C) with a MultiClamp-700B amplifier and Digidata-1440A A/D converter (Axon Instruments, Foster City, CA). Recording pipettes were pulled with a P-97 puller (Sutter Instruments, Novato, CA) and filled with internal solution containing KCl 140 mM, MgCl₂ 2.5 mM, HEPES 10 mM, EGTA 11 mM, and ATP 5 mM; the pH was adjusted to 7.2 with KOH, and the osmolality was adjusted to 310 mOsm/liter with sucrose. Cells were bathed in an external solution containing NaCl 150 mM, KCl 5 mM, CaCl₂ 2.5 mM, MgCl₂ 2 mM, HEPES 10 mM, and d-glucose 10 mM; the osmolality was adjusted to 330 mOsm/liter with sucrose, and the pH was adjusted to 7.4. The resistance of the recording pipette was in the range of 3–6 MΩ. A small patch of membrane beneath the tip of the pipette was aspirated to form a gigaseal, and negative pressure was applied to rupture it, establishing a whole-cell configuration. The series resistance was compensated by 70–80%. The adjustment of capacitance compensation was also performed before recording the membrane currents. The membrane voltage was maintained at −60 mV in all voltage-clamp experiments unless otherwise specified. Current-clamp recordings were obtained by switching to current-clamp mode after a stable whole-cell configuration had been formed in voltage-clamp mode. Only cells with a stable resting membrane potential (more negative than −50 mV) were used in the study. Signals were sampled at 10–50 kHz and filtered at 2–10 kHz, and the data were stored in an IBM-compatible PC for off- and online analysis in pClamp 10 acquisition software (Axon Instruments).

**Drug Application**

Hydrochloric acid, amiloride, APETx2, capsazepine, and tetrodotoxin (TTX) were purchased from Sigma-Aldrich. l-THP was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Stocks of drugs were made up and diluted daily in the external solution at a minimum of 1:1,000 to a final working concentration. Next, they were held in a linear array of fused silica tubes (o.d./i.d. = 500 μm/200 μm) connected to a series of independent reservoirs. The application pipette tips were positioned ~50 μm away from the recorded neurons. The application of each drug was driven by gravity and controlled by the corresponding valve, and rapid solution exchange could be achieved within about 100 msec by shifting the tubes horizontally with a PC-controlled micromanipulator. Cells were constantly bathed in normal external solution flowing from one tube connected to a larger reservoir between drug applications. To characterize ASIC activity functionally, we used capsazepine (10⁻⁵ M) to block transient receptor potential vanilloid type 1 (TRPV1) in this study (Poirot et al., 2006).

**Nociceptive Behavior**

Male rats were maintained under a 12-hr light/dark cycle, with ad libitum access to food and water. Animals were placed in a 30 × 30 × 30 cm Plexiglas chamber and allowed to habituate for at least 30 min before nociceptive behavior experiments. After pretreatment with 10 μl capsazepine (100 μM) into the dorsal face of the left hind paw, a double-blind experiment was carried out. In rats, two intraplantar injections were made with a 30-gauge needle connected to a 100-μl Hamilton (Reno, NV) syringe. The experimenters coded and pretreated the animals with 20 μl external solution or different doses of l-THP into the left hind paw. After 5 min, the other experimenters subcutaneously administered 20 μl acetic acid solution (0.6%) into the same hind paw and observed nociceptive responses. Nociceptive behavior (number of flinches) was counted over a 5-min period starting immediately after the injection (Deval et al., 2008; Omori et al., 2008).

**Statistical Analysis**

Data were statistically compared by one-way ANOVA or Student’s t-test, followed by Bonferroni’s post hoc test. Statistical analysis of concentration–response data was performed in the nonlinear curve-fitting program ALLFIT. Data are expressed as mean ± SEM.

**RESULTS**

**l-THP Decreased ASIC Currents in Rat DRG Neurons**

Neurons isolated from rat DRGs in the range of 15–35 μm were used in the present study. To characterize
ASIC currents functionally, we measured proton-gated currents in the presence of capsazepine (10 M) to block proton-induced TRPV1 activation in the whole-cell patch-clamp configuration (Poirot et al., 2006). As we have previously reported (C.-Y. Qiu et al., 2012; F. Qui et al., 2012), a rapid reduction of extracellular pH from 7.4 to 5.5 for 5 sec evoked an inward current (IpH 5.5) in most native DRG neurons (73.08%, 95/130). Most (78.95%, 75/95) of these acid-evoked currents might be ASIC3-like currents, which can be characterized by a large transient peak current followed by fast inactivation and then a small sustained current with no or very slow inactivation (Wang et al., 2013). The ASIC3-like currents at pH 5.5 had a mean inactivation time constant of 1,563.8 ± 197.2 msec (n = 8). We mainly observed the ASIC3-like currents in this study (Fig. 1A). ASICs are the only known channels that mediate the proton-gated currents in the presence of the TRPV1 inhibitor capsazepine, inasmuch as ASIC3-like currents could be completely blocked not only by the broad-spectrum ASIC channel blocker amiloride (50 μM) but also by ASIC3 blocker APETx2 (3 μM) in six neurons tested (Fig. 1A).

For the DRG neurons sensitive to acid stimuli, we observed that proton-evoked currents were inhibited by the administration of l-THP for 60 sec. l-THP (10 M) inhibited not only the peak phase but also the sustained component of ASIC3 currents; the relative amplitudes of peak and sustained currents decreased to 54.17% ± 5.50% and 76.13% ± 4.22% of control, respectively (Fig. 1A,B). The inhibition of proton-gated currents was dependent on the concentrations of l-THP. Figure 1C shows that the amplitudes of a representative ASIC current further decreased when the concentration of l-THP increased from 10 M to 10 M. Figure 1D shows the concentration–response curve for l-THP in the inhibition of ASIC peak currents. l-THP caused the maximum effect (51.74% ± 7.17%; n = 7) at a concentration of 10 M. The half-maximal response (IC50) value of the concentration–response curve for l-THP was 1.39 ± 0.44 × 10 M. The results indicate that l-THP inhibited ASIC currents in a concentration–dependent manner. In addition to ASIC3-like currents, other types of acid currents can also be inhibited by administration of 10 M l-THP for 60 sec.

**Effect of l-THP on the Concentration–Response Curve to Protons**

We then investigated whether the inhibition of l-THP was dependent on pH. Figure 2A,B shows that peak currents evoked by different pHs were inhibited by l-THP (10 M). First, treatment with l-THP produced a decrease of 40.93% ± 8.45% in the maximal current response to protons. However, the slopes or Hill...
coefficients of those two curves showed no significant difference in the presence (n = 1.28 ± 0.32) or absence (n = 1.54 ± 0.28) of l-THP (P = 0.55, Bonferroni’s post hoc test). Second, the pH_{0.5} values of both curves showed no statistical difference (pH_{0.5} of 5.96 ± 0.18 with l-THP treatment vs. pH_{0.5} of 5.98 ± 0.14 without l-THP treatment; P = 0.93, Bonferroni’s post hoc test). Third, the threshold pH values of both curves were essentially the same in the presence and absence of l-THP. These results indicate that l-THP decreased ASIC currents induced by different pH.

**l-THP Decreased Proton-Induced Membrane Excitability of Rat DRG Neurons**

Activation of ASICs by protons induces mainly sodium influx, resulting in membrane depolarization and neuronal excitation. Further experiments were performed to record DRG neuron excitability in the current-clamp model in the presence of capsazepine (10 μM) to block proton-induced TRPV1 activation. As shown in Figure 3A, a steep pH drop from 7.4 to 5.5 for 5 sec could trigger bursts of action potentials under current-clamp conditions in the tested neuron; the whole-cell inward current was also induced by pH 5.5 in the same cell with voltage-clamp recording. Pretreatment of l-THP (10^{-5} M) also decreased the number of action potentials evoked by acidosis. The mean number of action potentials decreased from 11.50 ± 2.79 of control condition to 6.20 ± 1.67 with pretreatment of l-THP in the ten neurons tested (P < 0.05, Bonferroni’s post hoc test; n = 10; Fig. 3C). After a washout of l-THP for 10 min, the mean number of action potentials evoked by acidosis was 10.67 ± 2.43, which was not significantly different from the control condition (11.50 ± 2.79; P = 0.83, Bonferroni’s post hoc test; n = 10; Fig. 3A,C).

It has been shown that TTX is not effective for blocking the proton-gated currents, although it blocks the majority of voltage-gated Na^+ currents (Lilley et al., 2004). We then observed the effect of l-THP on the acid-evoked depolarization of the membrane potential in the presence of capsazepine (10^{-5} M) and TTX (10^{-5} M).

**Fig. 2.** Concentration–response relationship for proton with or without l-THP. A: Sequential currents evoked by different pHs in the absence and presence of 10^{-5} M l-THP. B: The concentration–response curves for proton with or without 10^{-5} M l-THP treatment. The curves are a best fit of the data to the logistic equation I_{max}/[1 + (pH_{0.5}/C)]^n, where C is the concentration of protons, I is the normalized current response value, pH_{0.5} is the proton concentration that produced half the maximal current response to protons, and n is the Hill coefficient. The curves for protons without and with l-THP preapplication were drawn according to the equation described above. The concentration–response curve for proton with l-THP treatment shifted downward. Each point represents mean ± SEM of nine to 11 neurons. All current values were normalized to the current response induced by pH 4.5 applied alone (asterisk).

**Fig. 3.** Effect of l-THP on proton-evoked membrane excitability of rat DRG neurons. A: Original current and spiking recordings from the same DRG neuron. Left panel shows that a pH-5.5 acid stimulus induced a cell spiking with current-clamp recording in the presence of the TRPV1 inhibitor capsazepine (10 μM). Holding potential was −60 mV. Right panel shows that the pH-5.5 acid stimulus produced a cell spiking with current-clamp recording in the same neuron. The treatment with l-THP (10^{-5} M) inhibited the acid-induced spiking activity. B: Original current and membrane potential recordings from the same DRG neuron. Left panel shows voltage-clamp recording of current induced by a pH-5.5 acid stimulus. Right panel shows current-clamp recording (I = 0 pA) of the depolarization evoked by the pH-5.5 acid stimulus from the same neuron. The treatment of l-THP (10^{-5} M) decreased the acid-induced membrane depolarization. No spiking was triggered by the membrane depolarization in the presence of capsazepine (10^{-5} M) and TTX (10^{-5} M) to block TRPV1 and Na^+ channel-mediated spiking, respectively. C,D: Graphs show the effect of l-THP on the number of spiking and membrane potential depolarization produced by pH 5.5. The acid-evoked depolarization and spiking recovered to control condition after washout of l-THP. *P < 0.05, one-way ANOVA, followed by Bonferroni’s post hoc test, compared with pH alone (n = 10).
to block TRPV1 and Na$^+$ channel-mediated action potentials, respectively. A pH-5.5 acid stimulus produced a depolarization of the resting membrane potential in a DRG neuron tested under current-clamp recording (I = 0 pA) conditions (Fig. 3B); it also induced an inward current in the same cell with voltage-clamp recording. In this particular cell, treatment with l-THP ($10^{-3}$ M) decreased this acid-evoked depolarization of the membrane potential (Fig. 3B). After exposure to pH 5.5, membrane potential depolarized from 66.38 ± 6.85 mV to 43.12 ± 4.56 mV in the 10 neurons tested. In contrast, l-THP decreased the magnitude of acid-induced depolarization from 23.27 ± 3.28 mV to 14.64 ± 1.53 mV (P < 0.05, Bonferroni’s post hoc test; n = 10). After a washout of l-THP for 10 min, the acid-evoked depolarization of membrane potential recovered to control conditions (21.45 ± 2.06 mV; P = 0.61, Bonferroni’s post hoc test; n = 10; Fig. 3B,D). Together, these results indicate that l-THP reversibly decreased proton-induced membrane excitability of rat DRG neurons.

**l-THP Decreased Nociceptive Response to Intraplantar Injection of Acetic Acid in Rats**

Finally, we observed the effect of l-THP on acidosis-induced pain behavior in vivo. Intraplantar injection of acetic acid elicited an apparent flinch/shaking response in rats (Deval et al., 2008; Omori et al., 2008). The flinch response occurred mainly during 0–5 min after injection of acetic acid. After pretreatment with the TRPV1 inhibitor capsazepine (10$^{-5}$ M), intraplantar injection of 20 μl acetic acid solution (0.6%) caused an intense flinch/shaking response. As shown in Figure 4A, administration of l-THP significantly decreased flinching behavior induced by acetic acid in a dose-dependent manner, with an IC$_{50}$ of 2.91 ± 0.36 μM. Quantitative analysis showed that l-THP decreased the number of flinches to 13.00 ± 1.35, 10.32 ± 0.69, 7.58 ± 0.43, and 7.18 ± 1.27 at doses of 0.1, 1, 10, and 100 μM, respectively, compared with vehicle treatment (14.20 ± 1.13; P < 0.05 or 0.01, Bonferroni’s post hoc test; n = 10). The acidosis-evoked pain might be mediated by ASIC3, inasmuch as it was potently blocked by treatment with 200 μM amiloride and 20 μM APETx2. Application of l-THP (100 μM) did not further decrease flinching behavior induced by acetic acid in the presence of ASIC inhibitors (Fig. 4B). In addition, we found that the same administration of l-THP (100 μM) had no effect on nocifensive response induced by intraplantar injection of capsaicin (6 μg/20 μl) in the presence of 200 μM amiloride (not shown). These results indicate that locally applied l-THP relieved acidosis-evoked pain.

**DISCUSSION**

The present study shows that l-THP can exert an inhibitory effect on the functional activity of ASICs. l-THP decreased the amplitude of ASIC peak and sustained currents and acidosis-evoked membrane excitability in dissociated rat DRG neurons. Moreover, locally administered l-THP inhibited nociceptive response to intraplantar injection of acetic acid in vivo.

A rapid drop in the extracellular pH from 7.4 to 5.5 for 5 sec evoked an inward current in most native DRG neurons. The acid-evoked currents are involved in the activation of ASIC and TRPV1 channels (Bevan and Geppetti, 1994). The present acid currents were mediated mainly by ASICs in the presence of the TRPV1 inhibitor capsazepine, inasmuch as the acid currents were completely blocked by amiloride, a broad-spectrum ASIC channel blocker. Moreover, the blockade of the acid currents by the ASIC3 blocker APETx2 further supports the idea that they were ASIC3 currents. To date, at least seven ASIC subunits have been cloned in mammals (Deval et al., 2008). It has been shown that all other ASICs except for ASIC4 are present in primary sensory neurons of the DRG (Alvarez de la Rosa, 1994). The present acid currents were mediated mainly by ASICs in the presence of the TRPV1 inhibitor capsazepine, inasmuch as the acid currents were completely blocked by amiloride, a broad-spectrum ASIC channel blocker. Moreover, the blockade of the acid currents by the ASIC3 blocker APETx2 further supports the idea that they were ASIC3 currents. To date, at least seven ASIC subunits have been cloned in mammals (Deval et al., 2008). It has been shown that all other ASICs except for ASIC4 are present in primary sensory neurons of the DRG (Alvarez de la Rosa, 1994).
et al., 2002; Benson et al., 2002). The pH0.5 for activation of the native ASIC current was about 6.0 in this study, but the pH0.5 of ASIC3 homomeric channels was about 6.5. One possible explanation is that, because the application pipette tips were positioned ~50 μm away from the recorded neurons, the proton might have been diluted to some extent before it arrived at the cell surface. Thus, actual pH0.5 might be greater than about 6.0. In addition, the proton-induced currents might also be mediated by ASIC3 heteromeric channels. The present ASIC currents showed an inactivation time constant of 1.56 sec, which was longer than that of ASIC3 homomers and was close to that of ASIC1α/3 heteromers (Deval et al., 2008). Determining the exact ASIC subunits will require further study.

The present study shows that l-THP decreases not only the peak phase but also the sustained component of ASIC3 currents. l-THP decreased ASIC currents in a dose-dependent manner. l-THP induced a decrease in the maximum current response but with no significant change in the pH0.5 and threshold values. Thus, inhibition of l-THP was due to reduction of the efficacy but not the affinity of ASICs for protons. ASICs are extracellular pH sensors and are selectively permeable to cations (Wemmie et al., 2013). Activation of most ASICs by protons induces an inward current mediated by sodium ion, which in turn causes a depolarization of the resting membrane potential and triggers bursts of action potentials (Mamet et al., 2002). The current-clamp experiments showed that l-THP decreased the amplitude of the depolarization and the number of action potentials induced by extracellular acid stimuli. l-THP inhibition of ASIC current amplitude in voltage-clamp experiments correlated with its effect on acid-evoked neuronal excitability of DRG neurons. In agreement with the electrophysiological results, behavioral experiments showed that l-THP relieved pain induced by the intraplantar injection of acetic acid in a dose-dependent manner. The acid-evoked pain is mediated by ASICs in the presence of the TRPV1 inhibitor capsazepine, inasmuch as it is significantly blocked by amiloride, a broad-spectrum ASIC channel blocker (C.-Y. Qiu et al., 2012; F. Qui et al., 2012).

Together the present results indicate that l-THP inhibits the functional activity of ASICs in primary sensory neurons.

As a traditional analgesic agent, l-THP has been found to alleviate pain in several models. In the tail-flick test, l-THP has been shown to increase tail-flick latency in rats in a dose-dependent manner (Xu et al., 1982). In the formalin test, intragastric administration of l-THP inhibited both phases of response (Wang et al., 2010; Cao et al., 2011). In a visceral pain model, l-THP decreased visceral nociception induced by intraperitoneal injection of acetic acid (Cao et al., 2011). The analgesic mechanism of l-THP does not depend on prostaglandins because l-THP does not change the level of prostaglandins (Xu et al., 1982). The opioid receptors also might not be the molecular targets of l-THP because l-THP does not exhibit any binding affinity for opioid receptors (Zhang et al., 1986). However, it has been shown to release endogenous opioid peptides such as β-endorphin, enkephalin, and dynorphin at brain and spinal levels (Chu et al., 2008). Thus, at least in part, endogenous opioid peptides might contribute to the antinociceptive action of l-THP. The central dopaminergic systems play an important role in regulating nociception (Taylor et al., 2003). It has been shown that l-THP is a nonselective antagonist of different dopamine receptors (Guo et al., 1997; Hu and Jin, 1999b; Mantsch et al., 2007). Antagonism of the central D2 dopamine receptor is thought to be involved in the antinociceptive effects of THP. Intraperitoneal injection of the D2 receptor agonist quinpirole antagonizes the l-THP-induced antinociception (Hu and Jin, 1999b). However, the D2 dopamine receptor antagonist spiperone can mimic the effect of l-THP (Chu et al., 2008). It appears that l-THP and its analogs enhance activity in brainstem descending pain modulation system neurons by blocking D2 dopamine receptors in the striatum and in the arcuate nucleus and subsequently inhibit inputs from peripheral pain afferents in the spinal cord (Hu and Jin, 1999a; Chu et al., 2008). Thus, the analgesic action of l-THP is mediated by blocking the supraspinal D2 receptors (Hu and Jin, 1999b; Chu et al., 2008).

Extracellular acidosis is a common feature in pain-generating pathological conditions, such as inflammation, tissue injury, ischemic stroke, infections, and cancer (Wemmie et al., 2013). ASICs, sensors for extracellular protons, have been found on cell bodies and cutaneous nociceptive sensory terminals (Price et al., 2001). Activation of these ASICs by acidic pH results in membrane depolarization and excitation of nociceptive primary sensory neurons, which are thought to trigger and to contribute to nociception (Leng et al., 2013). Indeed, previous studies have shown that pain induced by cutaneous injection of acid appears to be largely mediated through ASICs because the pain sensation was significantly attenuated by the ASIC blocker amiloride in human volunteers (Ugawa et al., 2002). The present study shows that intraplantar injection of acetic acid elicits an intense flinch/shaking response in rats, and the acidosis–evoked pain is mediated by ASICs in the presence of the TRPV1 inhibitor capsazepine. We found that locally applied l-THP can significantly relieve acidosis–evoked pain. The analgesic effect of l-THP appeared to be related to its inhibition on functional activity of ASICs in DRG neurons. As mentioned above, most work to date has focused on the central mechanism of l-THP. This study reveals a novel peripheral analgesic mechanism of l-THP by modulating native ASICs in the primary afferent neurons.

**CONCLUSIONS**

In summary, l-THP inhibits ASIC-mediated currents, membrane excitability, and pain. The present study provides a cellular and molecular basis for the peripheral analgesic action of l-THP for the treatment of pain. Thus,
l-THP could be further developed as a potential therapeutic drug for treating pain.

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