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LOCAL ANESTHETIC INHIBITS HYPERPOLARIZATION ACTIVATED CATIONIC CURRENTS

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Running Title: lidocaine inhibits HCN channels

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Abbreviation: HCN, hyperpolarization-activated and cyclic nucleotide-gated; MAC, minimal alveolar concentration;

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ABSTRACT

Systemic administration of local anesthetics has beneficial perioperative properties and an anesthetic-sparing and antiarrhythmic effect, although the detailed mechanisms of these actions remain unclear. In the present study, we investigated effects of a local anesthetic, lidocaine, on HCN channels that contribute to the pacemaker currents in rhythmically oscillating cells of the heart and brain. Voltage clamp recordings were employed to examine properties of cloned HCN subunit currents expressed in Xenopus oocytes and HEK293 cells under control condition and lidocaine administration. Lidocaine inhibited HCN1, HCN2, HCN1-HCN2 and HCN4 channel currents at 100 μM in both oocytes and/or HEK293 cells; it caused a decrease in both tonic and maximal current (~30 to 50% inhibition) and slowed current activation kinetics for all subunits. In addition, lidocaine evoked a hyperpolarizing shift in half-activation voltage ($\Delta V_{½}$ of ~-10 to -14 mV), but only for HCN1 and HCN1-HCN2 channels. By fitting concentration-response data to logistic functions we estimated half-maximal (EC_{50}) concentrations of lidocaine of ~30 to 40 μM for the shift in $V_{½}$ observed with HCN1 and HCN1-HCN2; for inhibition of current amplitude, calculated EC_{50} values were ~50 to 70 μM for HCN1, HCN2 and HCN1-HCN2 channels. A lidocaine metabolite, monoethylglycinexylidide (100 μM) had similar inhibitory actions on HCN channels. These results indicate that lidocaine potently inhibits HCN channel subunits in dose-dependent manner over a concentration range relevant for systemic application. The ability of local anesthetics to modulate I_h in central neurons may contribute to CNS depression while effects on I_f in cardiac pacemaker cells may contribute to the antiarrhythmic and/or cardiovascular toxic action.

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INTRODUCTION

Local anesthetics have been widely used in surgical anesthesia and for acute and chronic pain management since they were first discovered by Koller in 1884 (Koller, 1928). Although typically used for regional anesthesia, where they are relatively safe, toxic systemic reactions have been a problem secondary to administration of an excessive dose (Brown et al., 1995). In addition, local anesthetics have also been used purposefully in systemic application, where low and moderate intravenous dose can produce beneficial actions (Kingery, 1997; Koppert et al., 2004). For example, it is well known that systemic administration of local anesthetics has antiarrhythmic (Pinter and Dorian, 2001), anesthetic-sparing and perioperative analgesic effects (Smith et al., 2004). Lidocaine is the most important class 1B antiarrhythmic drug; it is used intravenously for the treatment of ventricular arrhythmias (Trujillo and Nolan, 2000; Pinter and Dorian, 2001). Lidocaine reduces minimal alveolar concentration (MAC) of volatile anesthetics for suppression of responses to painful stimuli in animals by 20%-40% (DiFazio et al., 1976; Himes et al., 1977; Smith et al., 2004) and decreases the requirement of intravenous anesthetic propofol (Senturk, et al., 2002). The local anesthetic lidocaine can produce ~0.4 MAC (low dose systemic application) (DiFazio et al., 1976) and decrease the bispectral index (BIS) to 0 (higher dose systemic application) (Gaughen and Durieux, 2006). Perioperative intravenous lidocaine prevents postoperative and neuropathic pain and decreases postoperative morphine consumption (Kingery 1997; Koppert et al., 2004). Overall, these actions are generally considered to reflect cardiovascular and central neural systemic depressive actions of local anesthetics (Gaughen and Durieux, 2006).

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Because blockade of voltage-gated sodium channels by local anesthetics represents the main mechanism for inhibition of action potential propagation, inhibition of sodium channels by local anesthetics was believed to play an important role in producing local anesthetics systemic actions (Ragsdale et al., 1994). However, increasing evidence reveals that it is unlikely that blocking of sodium channel can account for the full spectrum of systemic actions of local anesthetics. For example, tetrodotoxin (TTX), a potent sodium-channel blocker and local anesthetic (Narahashi, 1972), is of special interest in this context as it is believed to induce no sedation by systemic application (Marcil et al., 2006), unlike lidocaine. Thus, the mechanisms mediating various systemic actions of local anesthetics remain unknown and molecular substrates other than sodium channels that contribute to those systemic actions should be sought.

In this study, we provide evidence that local anesthetic lidocaine potently inhibits the HCN channels that underlie the hyperpolarization-activated Na^+/K^+ current that has been designated I_f or I_h in cardiac myocytes and many central neurons (Biel et al., 1999). We find that lidocaine inhibits all HCN channels tested, including homomeric HCN1, HCN2 and HCN4 as well as heteromeric HCN1-HCN2 by decreasing tonic and peak current and slowing voltage-dependent channel activation; in addition, a hyperpolarizing shift in voltage dependence was observed for channels containing HCN1 subunits. We suggest that the ability of local anesthetics to modulate I_h in central neurons may contribute to the CNS depression seen with systemic administration while their effects on I_f in cardiac pacemaker cells may contribute to antiarrhythmic and cardiovascular toxic action.

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MATERIALS AND METHODS

Voltage clamp recording of HCN channels expressed in Xenopus oocytes

We obtained mHCN1, mHCN2 and mHCN4 from Drs. B. Santoro and S.A. Siegelbaum (Columbia Univ., NY) in pGHE or pcDNA3 expression vector and subcloned them into pcDNA3-HE3 for recording in Xenopus oocytes and HEK 293 cells. The concatemeric HCN1-HCN2 construct was made by using overlap extension PCR to produce a *PshAI-NheI* fragment that spliced the final leucine of HCN1 directly in frame with the initiating methionine of HCN2, as described previously (Chen et al., 2005b). To prepare RNA, in vitro transcription was performed with *NheI*-linearized DNA (HCN1), *SphI*-linearized DNA (HCN2), *XbaI*-linearized DNA (HCN1-HCN2) or *XbaI*-linearized DNA (HCN4) using T7 RNA polymerase (Promega Biotech Co., Ltd. Beijing). Xenopus laevis oocytes (Maosheng Biologic Science & Technology Development Co., Ltd, Shanghai) were injected with 46 nl of RNA (50–200 ng/μl) using a Nanoliter2000 microinjector (WPI). Following injection, oocytes were incubated at 17°C for 1-3 days in ND-96 solution, containing (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), pH 7.5 that was supplemented with 50 mg/l gentamycin sulfate. Whole cell currents were recorded from oocytes in solution, containing (mM): 107 KCl, 5 NaCl, 10 HEPES, 1 $MgCl_2$, and 1 EGTA, pH 7.3, with the two-microelectrode voltage clamp (TEVC) technique using a Warner OC-725B amplifier (Warner Instruments, Hamden, CT). An Ag–AgCl ground wire was connected to the bath solution by 2% agar salt bridge (in 3 M KCl) placed downstream of the oocyte. Recordings were obtained at room temperature (22–24°C). Voltage recording and current injecting electrodes were filled with 3 M KCl (1-3 MΩ).

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Heterologous expression of HCN channel constructs in HEK293 cells

HEK 293 cells were cultured using standard procedures and transiently transfected with HCN channel constructs, together with a GFP plasmid (pEGFP; Clontech), using Lipofectamine 2000 reagent (Invitrogen). Recordings were obtained 1-2 days following transfection. Whole cell recordings were obtained at room temperature using 3-5 MΩ patch pipettes and an Axopatch 200B amplifier in a HEPES-buffered bath solution composed of (mM): 118 NaCl, 25 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.3 that was perfused continuously (~2 ml min^{-1}). Internal solution contained (mM): 120 KCH_4SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.3 GTP-Tris, pH 7.2 (Himes, et al., 1977). Stock solutions of lidocaine hydrochloride (Sigma) and the lidocaine metabolite, monoethylglycinexylidide (MEGX, Ryan Scientific, Inc. SC) were prepared in water and DMSO (100 mM) and brought to the indicated concentrations in HEPES-buffered bath solution, at pH 7.3.

Data acquisition and analysis

Data were acquired using pCLAMP software (Axon Instruments) and a Digidata 1322A or a Digidata 1200 digitizer (Axon Instruments). For voltage-clamp recording, time-dependent hyperpolarization-activated currents (I_h, HCN) were evoked with incrementing (Δ -10 mV) hyperpolarizing pulses (3-4 s) from a holding potential of -40 mV, followed immediately by a step to fixed potential (-90 mV) in order to obtain tail currents. Amplitude of voltage-dependent HCN currents were derived at each potential as the difference between 'instantaneous' current, measured immediately after the capacitive transient before time-dependent HCN activation, and the steady state current at the end of hyperpolarizing voltage steps; maximal available voltage-dependent current

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was determined at -120 mV (or -110 mV for some HCN1 channels). Input conductance at the holding potential was calculated from the slope of instantaneous *I*-V curves, with tonic (constitutive) I_h defined as the Cs^+-sensitive component of instantaneous current. Test pulses from -40 mV to -120 mV are a typical protocol to evaluate HCN channel function. The extreme hyperpolarization allows maximal voltage-dependent channel activation that is required to characterize maximal current amplitude and to normalize tail currents for analysis of voltage dependence of channel gating. Tail currents were normalized, plotted as a function of the preceding hyperpolarization step voltage and fitted with Boltzmann curves for derivation of half-activation voltage (V½) by using a least squares analysis and the 'Solver' add-in of Excel (Microsoft). Time constants (τ) were determined by fitting currents evoked during hyperpolarizing steps to a biexponential function (Clampfit).

Concentration-response relationships for lidocaine effects on V½ and maximal current amplitude were fitted in Prism 3.0 according to:

$$E/Emax = \{1 + ([lidocaine]/EC_{50})^n\}^{-1},$$

where fitted parameters were concentration of half-maximal effect (EC_{50}), Hill coefficient (n) and either the maximal shift of V½ or maximal inhibition of current amplitude by lidocaine (Emax). Results are presented as mean ± SEM. Statistical tests included two-way analysis of variance (ANOVA) or Student's *t* test, as indicated. Differences in mean values were considered significant if $P<0.05$.

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RESULTS

HCN1 and HCN2 channel subunits are differentially modulated by lidocaine

The HCN family of ion channels represents the molecular substrate for I_h in neurons and I_f in cardiomyocytes. HCN1 and HCN2 are abundant subunits expressed in brain and heart (Santoro et al., 2000, Biel et al., 2009). These two HCN subunits produce homomeric channels that differ markedly in activation properties; HCN2 currents activate more slowly and at more hyperpolarized potentials than HCN1 currents, and they are more sensitive to cAMP (Biel et al., 1999).

We expressed HCN1 and HCN2 homomeric channels in both Xenopus oocytes and HEK293 cells and found that they also differed in their modulation by local anesthetic lidocaine (**Figs. 1 & 2**). Lidocaine effects on HCN channels were fast (4.5 ± 0.3 min in HEK293 cells, n=23) and reversible (data not shown). In oocytes expressing HCN1 subunits, lidocaine (100 μM) caused a hyperpolarizing shift in voltage dependence of activation (ΔV½ of -12.9 ± 1.5 mV) and a decrease in maximal current amplitude of 35.5 ± 4.9 % (*upper panels* in **Fig. 1A, 1B**). Lidocaine also decreased HCN2 current amplitude (32.5 ± 9.5 %) but did not substantially change the voltage range of HCN2 activation (ΔV½ of -3.9 ± 2.0 mV, *middle panels* in **Fig. 1A, 1B**). Note that the initial V½ of HCN1 (-79.3 ± 9.1 mV, n=27) was substantially more depolarized than that of HCN2 (-100.7 ± 1.3 mV, n=25), as expected for these cloned channels (Biel et al., 1999). It is now clear that HCN subunits can form heteromeric channels (Biel et al., 2009). In order to test effects of lidocaine on heteromeric HCN channels, we expressed a linked HCN1-HCN2 construct in oocytes; this HCN1-HCN2 heteromeric channel produced hyperpolarization-activated currents with kinetic and voltage dependent properties

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intermediate to those of the constituent HCN1 and HCN2 subunits (initial V½ -89.9 ± 1.9 mV, n=16), as reported previously (Chen et al., 2005a). Effects of lidocaine on linked HCN1-HCN2 heteromeric channel currents were most like those on mHCN1, inducing a hyperpolarizing shift in V½ (ΔV½ of -11.3 ± 0.7 mV) and a decrease in maximal current amplitude (31.7 ±5.8 %, *lower panels* in **Fig. 1A, 1B**).

We repeated these studies in a mammalian heterologous expression system where we also found differential modulation of HCN1 and HCN2 channel currents by lidocaine (**Fig. 2**). Similar to un-injected oocytes (data not shown), un-transfected HEK293 cells expressed undetectable HCN currents (**Fig. 2A**), which indicated that all measured HCN currents were attributable to the transfected HCN channel construct. In HEK293 cells expressing HCN1 subunits, lidocaine (100 μM) caused a hyperpolarizing shift in voltage dependence of activation (ΔV½ of -11.8 ± 0.3 mV) and a decrease in maximal current amplitude of 30.9 ± 5.2 % (*left panels* in **Fig. 2A, 2B**). Again, lidocaine decreased maximal HCN2 current amplitude (46.6 ± 4.2 %) without substantially changing the voltage range of HCN2 activation (ΔV½ of -3.2 ± 1.5 mV, *central panels* in **Fig. 2A, 2B**). For the linked HCN1-HCN2 subunit currents, lidocaine induced a shift in V½ (ΔV½ of -10.1 ± 2.0 mV) and a decrease in maximal current amplitude (38.7 ± 3.2 %, *right panels* in **Fig. 2A, 2B**).

Please note that although we evaluated inhibition by lidocaine of current amplitude at -120 mV, a potential at which HCN channels are maximally activated, this should not be misconstrued to suggest that lidocaine actions occur only at these extreme potentials. Rather, as depicted in the *I*-V and activation curves, the effects of lidocaine on voltage-activated HCN channel currents are manifest in a smooth and continuous fashion

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over the entire voltage range examined, including at physiologically relevant membrane potentials **(Fig. 1A, 2A**). Moreover, as shown below, lidocaine also inhibits a tonic HCN current component that is active even at depolarized potentials.

Lidocaine inhibits HCN4 channel currents expressed in HEK293 cells

We also examined effects of lidocaine on HCN4 channel currents which unlike HCN1 and HCN2 are predominantly expressed in heart, with only limited expression in some brain areas. In HEK293 cells expressing the slowly-activating HCN4 subunits, lidocaine (100 μM) caused inhibition of maximal current amplitude of 30.4 ± 4.2 % (**Figs. 3A-B**), with little effects in voltage dependence of activation (**Figs. 3A-B**, ΔV½ of -2.6 ± 0.9 mV). Thus, the modulation of HCN4 by lidocaine was similar to that of HCN2 (i.e., decrease in current amplitude with essentially no effect on V½).

Lidocaine metabolite MEGX inhibits HCN channel currents in HEK293 cells

It has been reported that a lidocaine metabolite, monoethylglycinexylidide (MEGX), can produce systemic actions similar to those of lidocaine (Halkin et al., 1975). Therefore, we examined effects of MEGX on HCN channel currents. In HEK293 cells expressing HCN1 subunits, MEGX (100 μM) caused a hyperpolarizing shift in voltage dependence of activation (ΔV½ of -9.3 ± 2.3 mV) and a decrease in maximal current amplitude of 54.7 ± 2.8 % (**Fig. 3C**). With HCN2 channels, MEGX decreased maximal current amplitude (59.3 ± 3.5 %) without substantially changing the voltage range of HCN2 activation (ΔV½ of 0.6 ± 0.5 mV, **Fig. 3C**), as we noted also for lidocaine. For HCN1-HCN2 heteromeric channel currents, MEGX induced a shift in V½ (ΔV½ of -3.5 ± 0.9 mV) and a decrease in maximal current amplitude (51.0 ± 7.7 %, **Fig. 3C**). Finally, MEGX caused inhibition of maximal s amplitude of HCN4 current (48.4 ± 6.6 %,

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left panel in **Figs. 3C**), with little effect on voltage dependence of activation (*right panel in* **Figs. 3C**, $\Delta V_{½}$ of -0.1 ± 2.3 mV). Thus, the modulation of HCN channels by MEGX was similar to that by lidocaine.

Lidocaine inhibits tonic currents of all HCN channels

A tonic component of current, in addition to the voltage- and time-dependent component, has been observed in recordings from cloned HCN channels (Proenza et al., 2002; Macri and Accili, 2004). This current component represents constitutive activation of HCN channels at holding potentials depolarized to the threshold for voltage-dependent activation, and at least for HCN2 channels, can represent ~10% of the total available current (Chen et al., 2005a). We therefore tested if lidocaine inhibits tonic currents from HCN channels, and if it does so in a subunit-specific manner. In HEK293 cells expressing HCN1, HCN2, HCN1-HCN2 and HCN4 channels we measured input conductance as the slope of *I*-V relationships from instantaneous currents (i.e., measured immediately after the capacitive transient and before development of time-dependent currents) evoked by hyperpolarizing voltage steps from a holding potential of -40 mV. As is evident in **Fig. 4**, instantaneous currents from cells expressing all four HCN constructs were inhibited by 3 mM CsCl, which completely blocks HCN channel currents (Biel et al., 2009); this Cs^+-sensitive instantaneous current component reflects tonic HCN current. Importantly, lidocaine inhibited tonically-active currents from all four HCN channels. When expressed relative to the Cs^+-sensitive, tonic HCN current, lidocaine (100 μM) inhibited 45.7% of tonic HCN1 current, 53.3% of tonic HCN1-HCN2 current, 54.3% of tonic HCN2 current and 40.6% of tonic HCN4 current. These data confirm earlier observations of constitutive activity of HCN channel currents at depolarized membrane potentials

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(Proenza et al., 2002; Macri and Accili, 2004), and they demonstrate that lidocaine inhibits tonic currents from HCN1, HCN1-HCN2, HCN2 and HCN4 channels.

Lidocaine causes a slowing of HCN current activation

In addition to inhibitory actions of lidocaine on current amplitude or V½ in HCN channels shown above, lidocaine also modulated HCN channel current kinetics. As shown in **Fig. 1, Fig. 2** & **Fig. 5** and consistent with previous reports, HCN channel currents differ in their activation properties, with HCN1 currents activating the fastest and HCN4 currents activating the slowest (Biel et al., 1999). Lidocaine caused a slowing of current activation (fast τ at -120 mV) for all HCN channels examined (HCN1: from 31.8 ± 4.8 ms to 99.3 ± 13.9 ms; HCN2: 217.8 ± 40.8 ms to 432.4 ± 57.9 ms; HCN1-HCN2: 98.3 ± 10.9 ms to 166.6 ± 31.8 ms; and HCN4: 1357.0 ± 227.9 ms to 1961.0 ± 240.3 ms).

Lidocaine inhibits HCN channels at physiological membrane potentials.

To this point, our data indicate that lidocaine inhibits current amplitude on HCN1, HCN2, HCN1-HCN2 and HCN4 channels, with a subunit-specific negative shift of voltage gating only in HCN1 subunit-containing channels (HCN1 and HCN1-HCN2). Lidocaine also inhibits tonic currents and slows current activation of all the four channels. At intermediate, physiologically relevant potentials (e.g. -70 mV), these effects together determine the degree of HCN inhibition by lidocaine. As shown in **Fig. 6A** for a cell expressing HCN1, a voltage step from -40 to -70 mV induces an instantaneous current followed by the voltage- and time-dependent component. The voltage- and time-dependent HCN1 current was strongly inhibited by lidocaine (by 89.0 ± 6.8%, **Fig. 6A & 6B**), reflecting both the negative shift of voltage gating and the decrease in

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maximum available current induced by lidocaine. In addition, lidocaine and Cs^+ significantly decreased instantaneous HCN1 current; lidocaine inhibited 60.3 ± 6.2% of the Cs^+-sensitive instantaneous (i.e., tonic) HCN1 current (**Fig. 6A & 6B**). For HCN2 channels, which have a more negative voltage range of activation, the tonic component accounts for most of the current at -70 mV and was significantly inhibited by lidocaine (74.4 ± 6.3%, **Fig. 6B**). For HCN1-HCN2 and HCN4 currents, lidocaine inhibits both voltage-dependent and tonic current components, as shown in **Fig. 6B**.

Lidocaine modulates HCN1 and HCN2 channel currents at clinically relevant concentrations

We tested effects of lidocaine on HCN1, HCN2 and HCN1-HCN2 heteromeric channels expressed in oocytes over a range of concentrations that encompasses those achieved clinically (20-200 μM). Lidocaine suppressed amplitude of heteromeric HCN channel currents (**Fig. 6C**, *left*) and induced a hyperpolarizing shift in the voltage dependence of activation (**Fig. 6C**, *right*) in a dose-dependent manner. By fitting amplitude inhibition data to logistic functions, we estimated half-maximal (EC_{50}) concentrations of lidocaine of, respectively, 67.6 ± 9.6 μM, 66.8 ± 15.3 μM and 51.6 ± 9.5 μM for HCN1, HCN2 and HCN1-HCN2, with corresponding maximal values of 43.8 ± 3.5%, 41.7 ± 5.2% and 32.4± 3.0% inhibition. For the shift in V½, calculated EC_{50} values were 34.0 μM and 41.3 μM for HCN1 and HCN1-HCN2, with corresponding maximal values of -13.9 mV and -12.2 mV. For both actions, effects of lidocaine were essentially maximal at 100 μM. These results indicate that lidocaine inhibits HCN channels in a dose-dependent manner over a relevant concentration range for various systemic actions.

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DISCUSSION

In the present study, we demonstrated that the local anesthetic lidocaine inhibits constitutive and voltage-dependent mouse HCN channel currents expressed in both oocytes and HEK293 cells systems. For channels containing HCN1 subunits (either homomeric HCN1 or heteromeric HCN1-HCN2), lidocaine caused a negative shift in V½, decreased tonic and maximal currents, and slowed activation kinetics. We also found that lidocaine modulated cloned HCN2 and HCN4 homomeric channel currents via a decrease in tonic and maximal current amplitude, but with no change in voltage dependence of activation. Our observations regarding effects of lidocaine on mouse HCN4 currents are generally consistent with an earlier report of rabbit HCN4 current modulation by lidocaine (Tamura et al., 2009). HCN channel inhibition was observed at concentrations that can be readily achieved during systemic administration of lidocaine (Edvardsson and Olsson, 1987; Estes et al., 1989; Heavner, 2002), whether purposeful or accidental. It is worth noting that effects of lidocaine on HCN channels were observed at physiologically relevant membrane potentials for neurons and cardiac cells; lidocaine inhibited the tonic current component at depolarized potentials and the voltage-and time-dependent component over the entire voltage range of activation. Therefore, these data suggest that inhibition of neuronal I_h or cardiac I_f currents could contribute to various beneficial and/or untoward systemic actions of local anesthetics, which remain poorly understood.

There have been no previous studies detailing effects of lidocaine on the multiple HCN channels that could contribute to its systemic actions (e.g., antiarrhythmic, anesthetic-sparing or perioperative analgesic effects). The HCN family of channels underlie neuronal I_h and cardiac I_f currents (Biel et al., 1999). The four HCN channel

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transcripts and proteins are widely and variously distributed throughout the mammalian central nervous system (Santoro et al., 2000) and in cardiac sinoatrial node and Purkinje cells (Moosmang et al., 2001), where they display distinct but often overlapping patterns of expression: HCN1 and HCN2 have the broadest neuronal distribution while HCN3 and HCN4 expression is more restricted (Santoro et al., 2000). Each subunit makes functional homomeric channels with distinctly different voltage-dependence, kinetics and/or cyclic nucleotide sensitivity. Our results show that lidocaine inhibits HCN1, HCN2, HCN4 and the HCN1-HCN2 heteromeric channel currents, although the form of modulation is different: lidocaine caused both a hyperpolarizing shift in V½ and a decrease in current amplitude for HCN1 and HCN1-HCN2 but only a decrease in current amplitude for HCN2 and HCN4. Interestingly, we earlier found that inhalational anesthetics also differentially modulate voltage dependence and maximal amplitude of HCN1 and HCN2 channels (Chen et al., 2005b), an effect which could be attributed to difference in intrinsic allosteric inhibition of HCN channel gating that is conferred by distinct C terminal domains (Wainger et al., 2001; Chen et al., 2005b).

The current study extends previous work on HCN4 (Tamura et al., 2009) and demonstrates that HCN channel inhibition by lidocaine includes HCN1 and HCN2, the two other HCN subunits expressed in cardiomyocytes. These results could thus be relevant for both classic antiarrhythmic actions and cardiotoxic effects of systemic lidocaine (Trujillo and Nolan, 2000; Pinter and Dorian, 2001). For example, it is well known that I_f plays an important pacemaker role in cardiac cells (DiFrancesco, 1981; Irisawa et al., 1993) and indeed, lidocaine was reported to reduce an inward current activated by hyperpolarization in the rabbit sino-atrial node (Satoh and Hashimoto, 1984). In isolated

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sinoatrial node cells HCN1, HCN2 and HCN4 channels are known to contribute to cardiac pacemaking activity (Moosmang et al., 2001) whereas in ventricular myocytes HCN2 and HCN4 subunits are prominently expressed. These I_f-expressing cardiac cells that are located in regions other than the SA node (e.g., in atrioventricular tissues) can beat spontaneously and trigger abnormal automaticity (Cerbai et al., 1997; Hoppe et al., 1998a). Moreover, HCN channel expression is reportedly enhanced in extranodal areas under pathophysiological conditions, and it is possible that the corresponding increase in I_f may contribute to arrhythmogenesis (Hoppe et al., 1998b). Therefore, inhibition by lidocaine of pacemaker current in these extranodal areas may contribute to its well known antiarrhythmic actions. On the other hand, strong block of HCN currents at high doses could induce cardiac toxicity, e.g. by decreasing SA nodal function and lowering heart rate to dangerous levels.

Also of importance to this study, it has been shown that I_h is a prominent current near resting membrane potential in thalamocortical neurons and cortical pyramidal cells (McCormick and Pape, 1990, Spain et al., 1991). In cortical and hippocampal pyramidal cells, HCN1 and HCN2 expression appears predominant (Santoro et al., 2000) , whereas in thalamocortical cells, HCN2 subunits account for the majority of current (Ludwig et al., 2003). We have previously shown that general anesthetics like propofol and ketamine produce anesthesia partly through inhibition of I_h in thalamocortical circuit neurons (Chen et al., 2009). We, therefore, suggest that inhibitory actions of local anesthetics on I_h in thalamocortical cells and cortical or hippocampal pyramidal neurons may contribute to their central anesthetic-sparing actions. In addition, since inhibition of HCN channels in sensory neurons can reduce pain sensation (Chaplan et al., 2003), it is possible that

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anesthetic sparing (DiFazio et al., 1976; Himes et al., 1977; Senturk, et al., 2002) or analgesic actions in postoperative or neuropathic contexts (Kingery, 1997; Smith et al., 2004) could be due to effects of systemic local anesthetics on HCN1 or HCN2 subunits expressed in nociceptors (Chaplan et al., 2003).

We did not include the HCN3 subunit in these studies. There is limited expression of HCN3 in central nervous system and little to no expression of HCN3 in the cardiac conduction system or the myocardium (Biel et al., 2009). However, HCN3 transcripts have been detected in heart muscle (Biel et al., 2009) and it remains possible that lidocaine may have some effect on HCN3 which could also contribute to its systemic actions.

Any suggestion of a role for I_h inhibition in the clinical actions of lidocaine presupposes modulation of the channels over a concentration range that is achieved clinically. In this respect, circulating concentrations of lidocaine that produce antiarrhythmic actions are reported to be from 7 μM to 40 μM (Edvardsson and Olsson, 1987; Estes et al., 1989). However, the plasma concentration for significant systemic actions such as cardiovascular depressive actions can be as high as 120 μg/ml (417 μM) (Heavner, 2002). We showed in the current study that inhibitory effects of lidocaine on HCN channels occur within this concentration range, with IC_{50} values from 20 to 70 μM. An earlier report suggested an IC_{50} of ~274 μM for lidocaine inhibition of homomeric HCN4 current at -70 mV (Tamura et al., 2009). However, that analysis was based on a 3-point curve that did not establish a saturating concentration and did not consider effects on activation kinetics or on maximal or tonic current (Tamura et al., 2009). In this respect, inhibition of tonic current and slowing of current kinetics may be particularly

important for the slowly-activating HCN4 channel (fast tau > 1 sec) in rapidly firing heart tissue (~ 4-5 beats/sec in mouse). Moreover, exemplar data in that earlier report (Tamura et al., 2009) shows clear effects on maximal voltage-dependent HCN4 channel currents of only 30 μM lidocaine (see step to -140 mV in their Fig. 4, *top panel*), well within expected concentrations for antiarrhythmic actions of lidocaine.

We also found that MEGX, a lidocaine metabolite, inhibited all HCN channel subunit tested in a manner that was qualitatively identical to lidocaine-mediated inhibition. In the liver, lidocaine is nearly completely metabolized by CYP3A4 to MEGX (Wang et al., 2000), a pharmacologically-active metabolite that is almost as potent as lidocaine in terms of systemic toxicity (Halkin et al., 1975). Thus, our current results suggest that both lidocaine and its principal metabolite, MEGX, could contribute to HCN-channel mediated systemic actions of lidocaine.

In conclusion, these data suggest that I_h in neurons and I_f in cardiomyocytes are likely targets for systemic actions of local anesthetics. The ability of local anesthetics to modulate I_h in sensory and central neurons may contribute to anesthetic-sparing, analgesic and CNS depressive actions while their effects on I_f in cardiac pacemaker cells may contribute to the antiarrhythmic and cardiovascular toxic actions.

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Authorship Contribution:

Participated in research design: Xiangdong Chen, Douglas A. Bayliss, Jiu Liu and Zhong-yuan Xia.

Conducted experiments: Qing-tao Meng and Xiangdong Chen.

Contributed new reagents or analytic tools: Xiangdong Chen and Douglas A. Bayliss.

Performed data analysis: Qing-tao Meng and Xiangdong Chen.

Wrote or contributed to the writing of the manuscript: Xiangdong Chen and Douglas A. Bayliss.

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FIGURE LEGENDS

Figure 1. ***Local anesthetic lidocaine differentially inhibits HCN channel currents expressed in Xenopus oocytes.***

A. Sample currents from Xenopus oocytes expressing mHCN1, mHCN2 and mHCN1-mHCN2 channel constructs evoked by hyperpolarizing voltage steps from -40 mV to -130 mV, before and during exposure to lidocaine (100 μM); conditioning voltage steps were of different duration for the three constructs (3 s, 4s and 3s) followed by a step to -90 mV for tail current analysis. **B**. Summary data showing averaged (± SEM) current inhibition (% from control; *left*) and shift in half activation potential (V½; *right*) evoked by lidocaine for each of the indicated HCN channel constructs. *, $P<0.05$ by ANOVA for lidocaine vs. control (n=6, 5 & 8 for mHCN1, mHCN2 and mHCN1-mHCN2).

Figure 2. ***Local anesthetic inhibits HCN channel currents in HEK293 cells.***

A. Sample currents from HEK293 cells expressing mHCN1, mHCN2 or a linked heteromeric mHCN1-mHCN2 construct evoked by hyperpolarizing voltage steps (Δ-10 mV) from -40 mV to -120 mV before and during exposure to lidocaine (100 μM); voltage steps were followed by a step to -90 mV for tail current analysis. A sample current trace from untransfected HEK293 cell is also shown at the bottom. **B.** Activation curves were determined from tail currents (*lower*) and steady-state *I*-V curves from currents at the end of the voltage steps (*upper*) under control conditions (*filled squares*), during exposure to lidocaine (*filled triangles*) for mHCN1, mHCN2 or a linked mHCN1-HCN2 constructs. *, $P<0.05$ vs. control (n=5, 5 & 6 for mHCN1, mHCN2 and mHCN1-HCN2).

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Figure 3. ***Lidocaine inhibits HCN4 channel currents.***

A. Sample currents from HEK293 cells expressing mHCN4 channel construct evoked by hyperpolarizing voltage steps from -40 mV to -130 mV, before and during exposure to lidocaine (100 μM); conditioning voltage steps (6s-14s) were followed by a step to -90 mV for tail current analysis. **B**. Steady-state *I*-V curves from currents at the end of the voltage steps (*upper*) and activation curves were determined from tail currents (*lower*) under control conditions (*filled squares*) and during exposure to lidocaine (*open triangles*). **C**. Summary data showing averaged (± SEM) current inhibition (% from control; *left*) and shift in half activation potential (V½; *right*) evoked by lidocaine metabolite monoethylglycinexylidide (MEGX) for each of the indicated HCN channel constructs.*, $P<0.05$ vs. control.

Figure 4. ***Lidocaine inhibits tonic HCN channel currents.***

A. Instantaneous I-V relationships were obtained from a holding potential of -40 mV in HEK293 cells expressing mHCN1, mHCN2, mHCN1-HCN2 and mHCN4 under control conditions (*filled spuares*), in the presence of 100 μM lidocaine (*filled triangles*) and 3 mM CsCl (*open spuares*). Solid lines represent linear fits through averaged data (± SEM; n=5, n=5, n=6 and n=5 for HCN1, HCN2, HCN1-HCN2 and HCN4), representing the input conductance at -40 mV. **B.** Input conductance was determined from slopes of instantaneous *I*-V curves in individual cells expressing the HCN channel constructs, and averaged for each condition as indicated (C=control, L=lidocaine, Cs=CsCl). Lidocaine decreased input conductance in all HCN expressing cells (*, $P<0.05$ vs. control).

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Figure 5. ***Lidocaine causes a slowing of HCN subunit currents.***

Activation data were obtained from biexponential fits at -120 mV and the time constant (τ) describing the fastest (and largest) current component were determined under control and during lidocaine application for mHCN1, mHCN1-mHCN2, mHCN2 and mHCN4 subunit currents under control conditions and in the presence of 100 μM lidocaine. Lidocaine caused a slowing of all HCN subunits current activation examined. *, $P<0.05$ vs. control (n=5, 5, 6 & 5 for HCN1, HCN2, HCN1-HCN2 and HCN4, respectively).

Figure 6. ***Lidocaine modulates HCN channel currents at -70 mV and at clinically relevant concentrations.***

A. Sample HCN1 current at -70 mV under control conditions and during administration of lidocaine (100 μM) and CsCl (3 mM), an HCN channel blocker. HCN1 currents include two components: a voltage and time-dependent component that was almost strongly inhibited by lidocaine and totally blocked by Cs^+; and a Cs^+-sensitive instantaneous current component that was partly reduced by lidocaine. **B**. Summary data showing effects of lidocaine on voltage and time-dependent (*left*) and tonic (*right*) HCN currents measured at -70 mV in cells expressing HCN1, HCN2, HCN1-HCN2 and HCN4 channel constructs. Calculation of % inhibition of tonic current is relative to the Cs^+-sensitive instantaneous current component (i.e., the HCN current). Lidocaine inhibited instantaneous and voltage-dependent currents for HCN1, HCN1-HCN2 and HCN4 channels; for HCN2, the instantaneous current is predominant at -70 mV, and was also reduced by lidocaine. *, $P<0.05$ vs. control (n=5, 5, 6 & 5 for HCN1, HCN2,

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HCN1-HCN2 and HCN4, respectively). **C**. Averaged values for shift in amplitude inhibition (*left*) and V½ (*right*) of mHCN1 (*squares*), mHCN2 (*triangles*) and heteromeric mHCN1-mHCN2 (*circles*) currents expressed in oocytes at different concentrations of lidocaine. The effects of lidocaine (0, 20, 50,100 and 200 μM) on current amplitude and V½ were averaged (± SEM) and fitted with logistic equations.

Figure 1

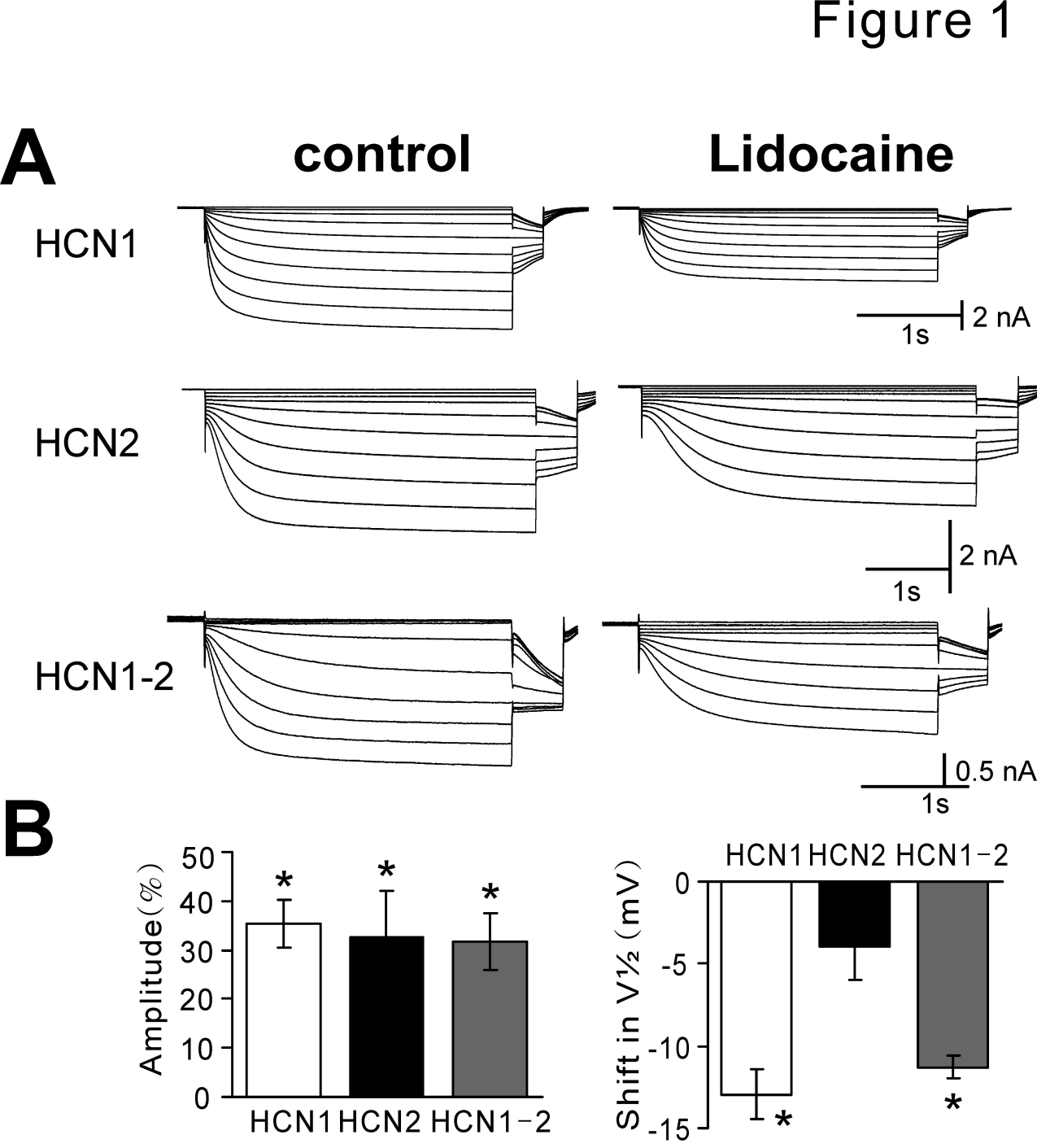

A
control
Lidocaine
HCN1
HCN2
HCN1-2
2 nA
1s
2 nA
1s
0.5 nA
1s
B
Amplitude(%)
50
40
30
20
10
0
HCN1 HCN2 HCN1-2
Shift in V½ (mV)
0
-5
-10
-15
HCN1 HCN2 HCN1-2
*
*
*
*
*

Figure 2

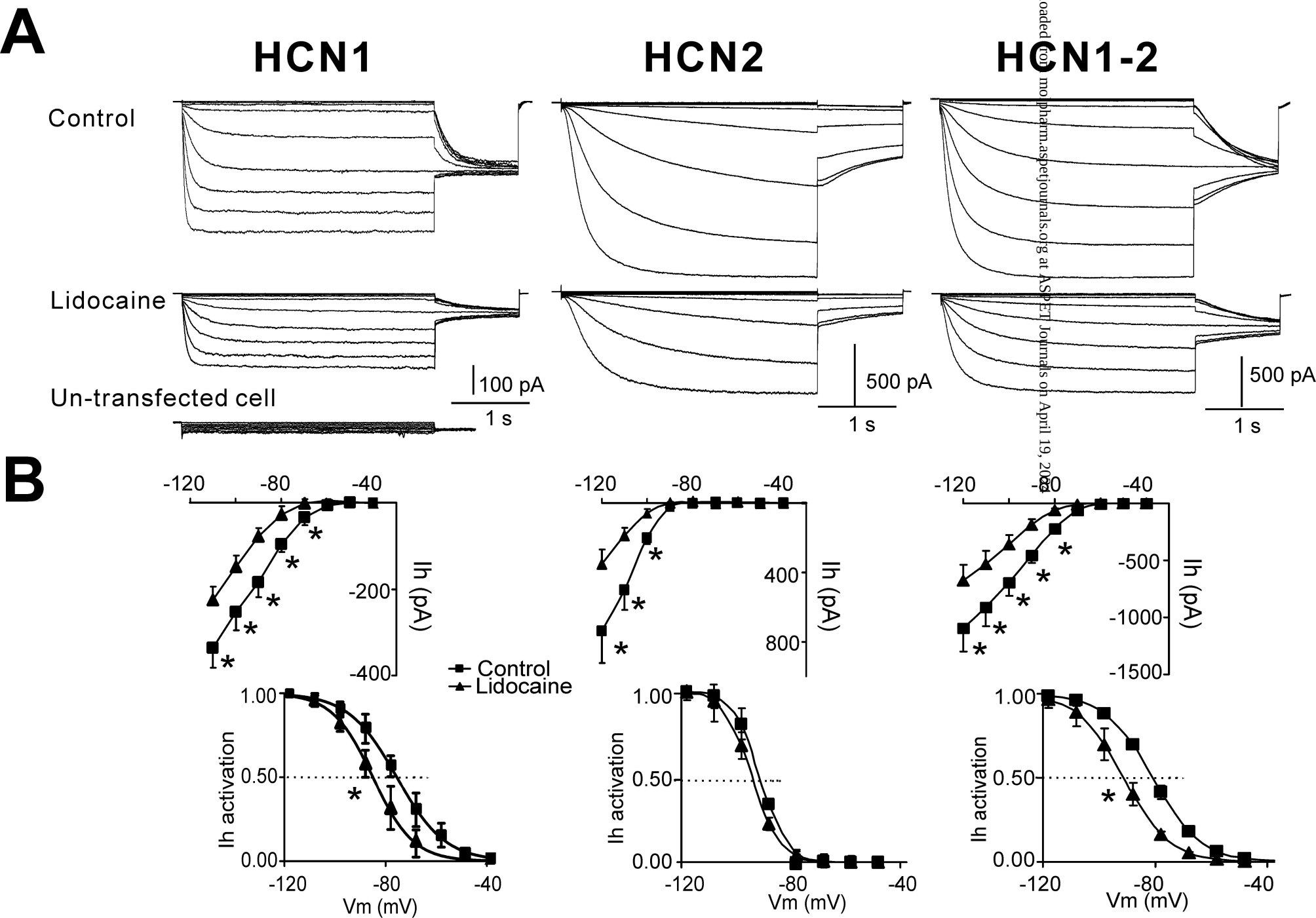


Figure 3

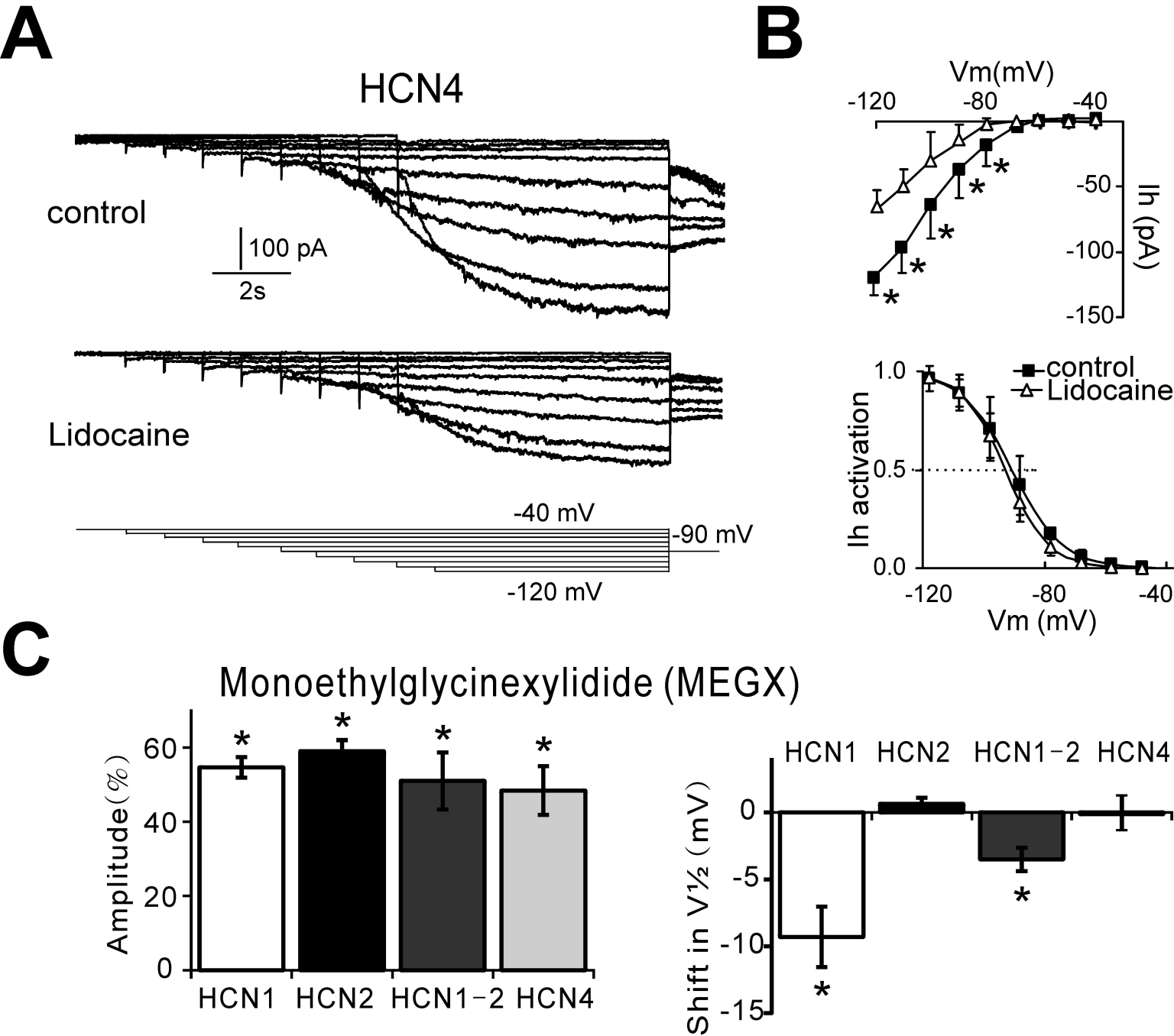

A
HCN4
control
100 pA
2s
Lidocaine
-40 mV
-90 mV
-120 mV
B
Vm(mV)
-120
-80
-40
-50
-100
-150
Ih (pA)
control
Lidocaine
Ih activation
1.0
0.5
0.0
-120
-80
-40
Vm (mV)
C
Monoethylglycinexylidide (MEGX)
Amplitude(%)
60
40
20
0
HCN1
HCN2
HCN1-2
HCN4
Shift in V½ (mV)
0
-5
-10
-15

Figure 4

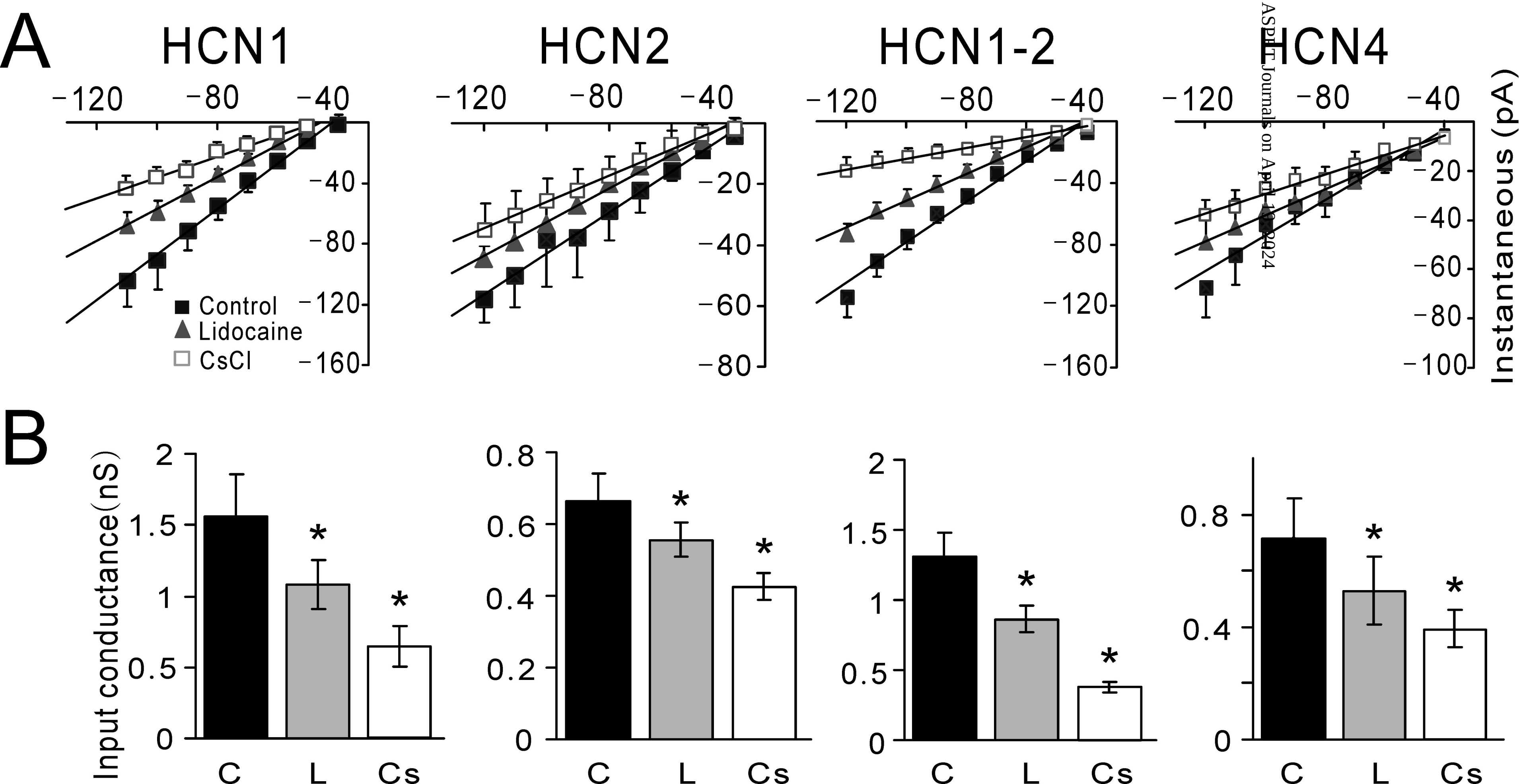
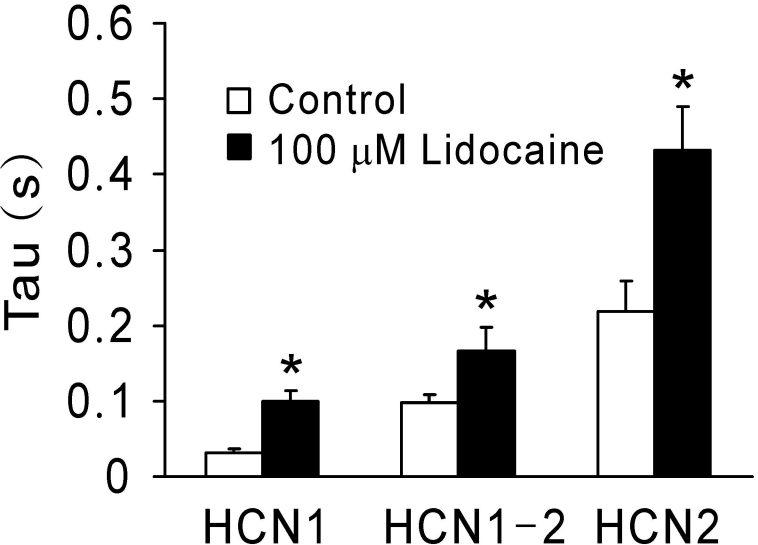


Figure 5


0.6
0.5
0.4
0.3
0.2
0.1
0
Tau (s)
Control
100 μM Lidocaine
*
*
*
HCN1
HCN1-2
HCN2

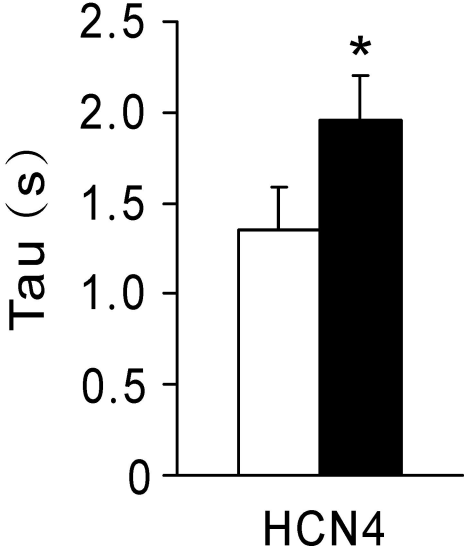

2.5
2.0
1.5
1.0
0.5
0
Tau (s)
*
HCN4

Figure 6