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RESEARCH PAPER

Molecular determinants of state-dependent block of voltage-gated sodium channels by pilsicainide

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Background and purpose: Pilsicainide, an anti-arrhythmic drug used in Japan, is described as a pure sodium channel blocker. We examined the mechanisms by which it is able to block open channels, because these properties may be especially useful to reduce hyperexcitability in pathologies characterized by abnormal sodium channel opening.
Experimental approach: The effects of pilsicainide on various heterologously expressed human sodium channel subtypes and mutants were investigated using the patch clamp technique.
Key results: Pilsicainide exhibited tonic and use-dependent effects comparable to those of mexiletine and flecainide on hNav1.4 channels. These use-dependent effects were abolished in the mutations F1586C and Y1593C within segment 6 of domain IV, suggesting that the interaction of pilsicainide with these residues is critical for its local anaesthetic action. Its affinity constants for closed channels (K_R) and channels inactivated from the closed state (K_I) were high, suggesting that its use-dependent block (UDB) requires the channel to be open for it to reach a high-affinity blocking site. Accordingly, basic pH, which slightly increased the proportion of neutral drug, dramatically decreased K_R and K_I values. Effects of pilsicainide were similar on skeletal muscle hNav1.4, brain hNav1.1 and heart hNav1.5 channels. The myotonic R1448C and G1306E hNav1.4 mutants were more and less sensitive to pilsicainide, respectively, due to mutation-induced gating modifications.
Conclusions and implications: Although therapeutic concentrations of pilsicainide may have little effect on resting and closed-state inactivated channels, it induces a strong UDB due to channel opening, rendering the drug ideally suited for inhibition of high-frequency action potential firing.
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Keywords: pilsicainide; anti-arrhythmics; voltage-gated sodium channels; state-dependent block; hNav1.4; local anaesthetic receptor; paramyotonia congenita; K^+-aggravated myotonia; open channel block; pKa

Abbreviations: *h* and (1 – *h*), proportions of closed and inactivated channels at a particular potential; I_{CTRL}, peak current amplitude measured in the same cell before drug application; I_{DRUG}, peak current amplitude measured in the presence of drug; I_{Na}, whole-cell sodium current; K_{APP}, apparent affinity constant at a given potential; K_I, affinity for inactivated channels; K_R, affinity for closed channels; LA, local anaesthetic; *S*, slope factor; TB, tonic block; UDB, use-dependent block; *V*, conditioning pulse potential; $V_{1/2}$, half-maximum inactivation potential

Introduction

Due to their ability to act as sodium channel blockers, local anaesthetic (LA)-like drugs can be used to treat a large spectrum of disorders including cardiac arrhythmias, epilepsy, myotonia and neuropathic pain (Conte Camerino *et al.*, 2007). Many of these drugs present a tertiary amine associated with a hydrophobic aromatic tail through an amide, ester or ether link. The two ends of the drug may interact with channel pore-lining amino acids through hydrophobic or π–cation interactions (Ragsdale *et al.*, 1994; 1996; Wright *et al.*, 1998; Nau *et al.*, 1999; Wang *et al.*, 2000; Yarov-Yarovoy *et al.*, 2001; 2002; O'Leary and Chahine, 2002; McNulty *et al.*, 2007; Ahern *et al.*, 2008; Sunami *et al.*, 2009). Because the binding site for LAs lies within the sodium channel pore, both the lipophilicity and pKa of the drugs are important chemical determinants of channel block. At physiological pH, LAs equilibrate between a liposoluble neutral form that may reach

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Table 1 Molecular weight (MW) is given for the free base

Drug	*MW*	*pKa*	*Log P*	*pH*	*Log D*	*Ionization (mol%)*	K_R (μM)	K_I (μM)
Pilsicainide	414	10.18 ± 0.01	2.12 ± 0.01	7.4	–0.66	99.8	3000	150
				8.2	0.14	98.9	1500	24
Flecainide	309	9.43 ± 0.07	2.61 ± 0.01	7.4	0.58	99.0	480	18
Mexiletine	179	9.28 ± 0.01	2.19 ± 0.01	7.4	0.30	98.7	800	6

Log *P* and pKa were determined experimentally. Log *D* was calculated from equation: log D = log P – log$(1 + 10^{(pKa-pH)})$. Ionization was calculated from Henderson–Hasselbalch equation: ionization = $1–10^{(pH-pKa)}$. Affinity of mexiletine and flecainide for closed (K_R) and inactivated (K_I) hNav1.4 channels were determined in previous studies (Desaphy *et al.,* 2001; 2004).

the receptor through a hydrophobic pathway even if the channel is closed (Ragsdale *et al*., 1994; Sunami *et al*., 2001; Bruhova *et al*., 2008; Hanck *et al*., 2009), and a protonated form that may need the channel to be open before it can enter the pore and inhibit sodium currents in a use-dependent manner (Hille, 1977a; Hondeghem and Katzung, 1977).

On the other hand, according to the modulated receptor hypothesis, conformational changes during channel activity modify the binding affinity of a drug as a function of channel state: the binding affinity of LAs is far greater for open and/or inactivated sodium channels than for closed channels (see Hanck *et al.,* 2009 for a recent description). However, there is a debate as to whether the higher affinity of a drug for a specific sodium channel isoform with respect to others depends on subtle differences in the receptor site or on differences in channel gating that secondarily affect receptor accessibility. The understanding of these fundamental interactions between LAs and sodium channels is an important issue, because this knowledge may constitute the initial step in the search for the best drug for a given sodium channel-related disease.

In previous studies using human skeletal muscle sodium (hNav1.4) channel mutants responsible for human hereditary myotonia, we showed that the effects of mexiletine on sodium currents at a given holding potential are highly dependent on the availability of the channels at this potential, because of the high affinity of mexiletine for inactivated channels compared to closed channels (Desaphy *et al*., 2001). This result predicts that mexiletine may be more effective on sodium channel mutants that have a more negative voltage dependence of inactivation. Furthermore, although the other LA examined, flecainide, has a pKa and log *D* values very similar to mexiletine (Table 1), it exhibited a smaller gap between affinities for closed and open/inactivated channels; thus, flecainide may be more efficient than mexiletine at blocking sodium channel mutants with a less negative voltage dependence of availability (Desaphy *et al*., 2004). In the present study, we examined the mechanisms involved in this hypothesis in more detail by comparing the effects of mexiletine and flecainide with those of a drug defined as a pure open-channel blocker, pilsicainide (Ono *et al*., 2000).

Pilsicainide is a class IC anti-arrhythmic drug widely used in the treatment of supraventricular and ventricular tachyarrhythmias in Japan (Hashimoto and Nakashima, 1993). Results from voltage clamp experiments have suggested that pilsicainide is a pure sodium channel blocker with little or no effect on other cardiac channels, probably with the exception of HERG channels (Hattori and Inomata, 1992; Yamashita *et al*., 1998; Wu *et al*., 2003). Pilsicainide is an *N*-2,6-(dimethylphenyl) acetamide derivative, similar to lidocaine, but its tertiary amine group is included in a pyrrolizidinyl cycle that probably confers a very high pKa and consequently a reduced lipophilicity with respect to most LAs (Table 1). We speculated that such specific properties are important for defining the behaviour of pilsicainide on sodium channels.

In the present study, we investigated the effects of pilsicainide on hNav1.4 channels to compare its mechanism of action with that of mexiletine and flecainide. We demonstrated that pilsicainide requires specific residues within the LA receptor in a manner reminiscent of other LAs. Nevertheless, because of its elevated pKa, the drug displayed very low affinities for closed channels and channels inactivated from the closed state, which limits dependence of drug block on membrane voltage. A basic pH, which slightly increases the proportion of neutral drug, allows a dramatic gain in sodium channel block. Thus, the opening of sodium channels is a critical prerequisite for the action of pilsicainide, thereby contributing to the use dependence of the sodium channel block.

Methods

Mutagenesis and expression of recombinant sodium channels

Drug and ion channel nomenclature in this study conforms to the British Journal of Pharmacology's *Guide to Receptors and Channels* (Alexander *et al*., 2008). Full-length cDNA encoding α-subunits of wild-type (WT) hNav1.4 (skeletal muscle isoform), R1448C hNav1.4 mutant (paramyotonia congenita mutant), G1306E hNav1.4 mutant (potassium-aggravated myotonia mutant), hNav1.5 (cardiac isoform) and hNav1.1 (brain isoform) channels were subcloned in the mammalian expression vector pRc-CMV, as previously described (Yang *et al*., 1994; Wang *et al*., 1996; Lossin *et al*., 2002). New hNav1.4 mutants, F1586C and Y15893C, were engineered by standard two-step PCR-based site-directed mutagenesis. All PCR reactions were performed using P*fu* DNA polymerase (Stratagene, La Jolla, CA, USA) for high-fidelity amplification. The complete coding region of channel mutant cDNAs was sequenced to exclude any polymerase errors.

Sodium channel expression was achieved by transfecting 1 $\mu g \cdot mL^{-1}$ of plasmid either transiently in tsA201 cells using the calcium phosphate precipitation method (hNav1.4, hNav1.5, R1448C, G1306E and Y1593C) or permanently in HEK293 cells using geneticin (Gibco-Invitrogen, Milan Italy)

for clone selection (hNav1.4, hNav1.1, F1586C). For transient transfection, the channels α-subunits were co-transfected in 10:1 ratio with the gene reporter CD8 (Y1593C and R1448C mutants), or with a plasmid containing both CD8 and the auxiliary human voltage-gated sodium channel β1 subunit (pCD8-IRES-hβ1) (hNav1.4 and hNav1.5). Cells identified with microbeads coated with anti-CD8 antibody (Dynal-Invitrogen, Milan, Italy) were used for patch clamp experiments 36–96 h after transfection. The HEK293 clone expressing hNav1.1 permanently was transiently transfected with both pCD8-IRES-hβ1 (0.1 $\mu g \cdot mL^{-1}$) and pGFP-IRES-hβ2 (0.1 $\mu g \cdot mL^{-1}$), a plasmid containing the human sodium channel auxiliary β2 subunit together with the gene reporter encoding the green fluorescent protein (GFP). Only cells positive for CD8 antigen and GFP fluorescence were used for electrophysiology (Lossin *et al.,* 2002).

Electrophysiology

Whole-cell sodium currents (I_{Na}) were recorded at room temperature (20–22°C) using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Voltage clamp protocols and data acquisition were performed with pCLAMP 6.0 software (Axon Instruments) through a 12-bit A-D/D-A interface (Digidata 1200, Axon Instruments). Pipettes made with Corning 7052 glass (Garner Glass, Claremont, CA, USA) had resistance that ranged from 1 to 3 MΩ. Currents were low-pass filtered at 2 kHz (–3 dB) by the four-pole Bessel filter of the amplifier and digitized at 10–20 kHz.

After the patch membrane had been ruptured, a 25-ms-long test pulse to –20 mV from a holding potential of –120 mV was applied to the cell at a low frequency until stabilization of I_{Na} amplitude and kinetics was achieved (typically 5 min). Only those data obtained from cells exhibiting peak current amplitudes of 0.6–6 nA and series resistance errors <5 mV were considered for analysis. Little (<5%) or no rundown was observed within the experiments.

The concentration–response relationships were produced by obtaining the peak current amplitude measured in the presence of drug (I_{DRUG}), normalized to the peak current amplitude measured in the same cell before drug application (I_{CTRL}), as a function of drug concentration [(DRUG)]. Each data point is the mean ± SEM from at least three cells. The relationships were fitted with a first-order binding function:

$$I_{DRUG}/I_{CTRL} = 1/\left[1 + ([DRUG]/IC_{50})^{nh}\right] \quad (1)$$

where IC_{50} is the half-maximum inhibitory concentration, and n_h is the slope factor.

The voltage dependence of the steady-state inactivation of sodium channels was determined using a standard two-pulse protocol; sodium currents were elicited by a 20 ms test pulse to –30 mV after 50 ms conditioning pulses to potentials ranging from –150 to –30 mV in 10 mV increments. Holding potential was –180 mV and interval duration between two pulses was 15 s. The normalized peak sodium current recorded during the test pulse ($I_{Na}/I_{Na,max}$) was plotted against the conditioning pulse potential (V), and the relationships were fitted using a Boltzmann equation:

$$I_{Na}/I_{Na,max} = 1/\{1 + \exp[(V - V_{1/2})/S]\} \quad (2)$$

where $V_{1/2}$ is the half-maximum inactivation potential, and S is the slope factor.

The modulated receptor hypothesis predicts that the affinity of drugs for the sodium channels changes with channel state, being greater for inactivated than for resting, closed channels. Consequently, the apparent affinity measured at a given voltage results from the combination of block of closed channels with affinity for closed channels (K_R), together with block of inactivated channels with affinity for inactivated channels (K_I), a concept described by the Bean's equation:

$$1/K_{APP} = (1 - h)/K_I - h/K_R \quad (3)$$

where K_{APP} is the apparent affinity constant at a given potential, and the terms h and $(1 - h)$ are the proportions of closed and inactivated channels at this potential (Bean *et al*. 1983; Desaphy *et al*., 2004). Using a very negative holding potential (–180 mV), all the channels are in the resting state (h = 1), allowing the calculation of K_R as $K_R = K_{APP}$. An estimation of K_I is obtained from Equation (3), using the values of K_{APP} and h measured at a less negative holding potential, at which a significant number of channels $(1 - h)$ are inactivated.

Drugs and solutions

All reagents except pilsicainide were purchased from Sigma-Aldrich (Milan, Italy). Patch clamp pipette solution contained in mM: 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA and 5 HEPES, and the pH was set to 7.2 with CsOH. Bath solution for patch clamp recordings contained (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 5 glucose. The pH was set to 7.4 with NaOH, except a series of experiments performed at pH 8.2.

Pilsicainide was synthesized in our laboratories as a hydrochloride salt, as previously described (Bruno *et al*., 2007). Pilsicainide was diluted in bath solution at desired concentration, and the pH was adjusted to 7.4 or 8.2 with NaOH. The patched cell was continuously exposed to a stream of control or drug-supplemented bath solution flowing out from a plastic capillary.

Experimental determination of pKa and log P

The pKa and log P values of pilsicainide, mexiletine and flecainide were determined with a potentiometric method using Sirius GlpKa (Sirius Analytical Instrument Ltd, Forest Row, East Sussex, UK) as described previously (Avdeef *et al*., 1999; Comer and Tam, 2001). Because the compounds showed a low water solubility, methanol was used as co-solvent (methanol–water 10–30% w/w) for the determination of pKa.

Results

Pilsicainide binds to the LA receptor

To allow direct comparison with previous results obtained with mexiletine and flecainide, the ability of pilsicainide to block hNav1.4 channels (α-subunit alone) was evaluated by

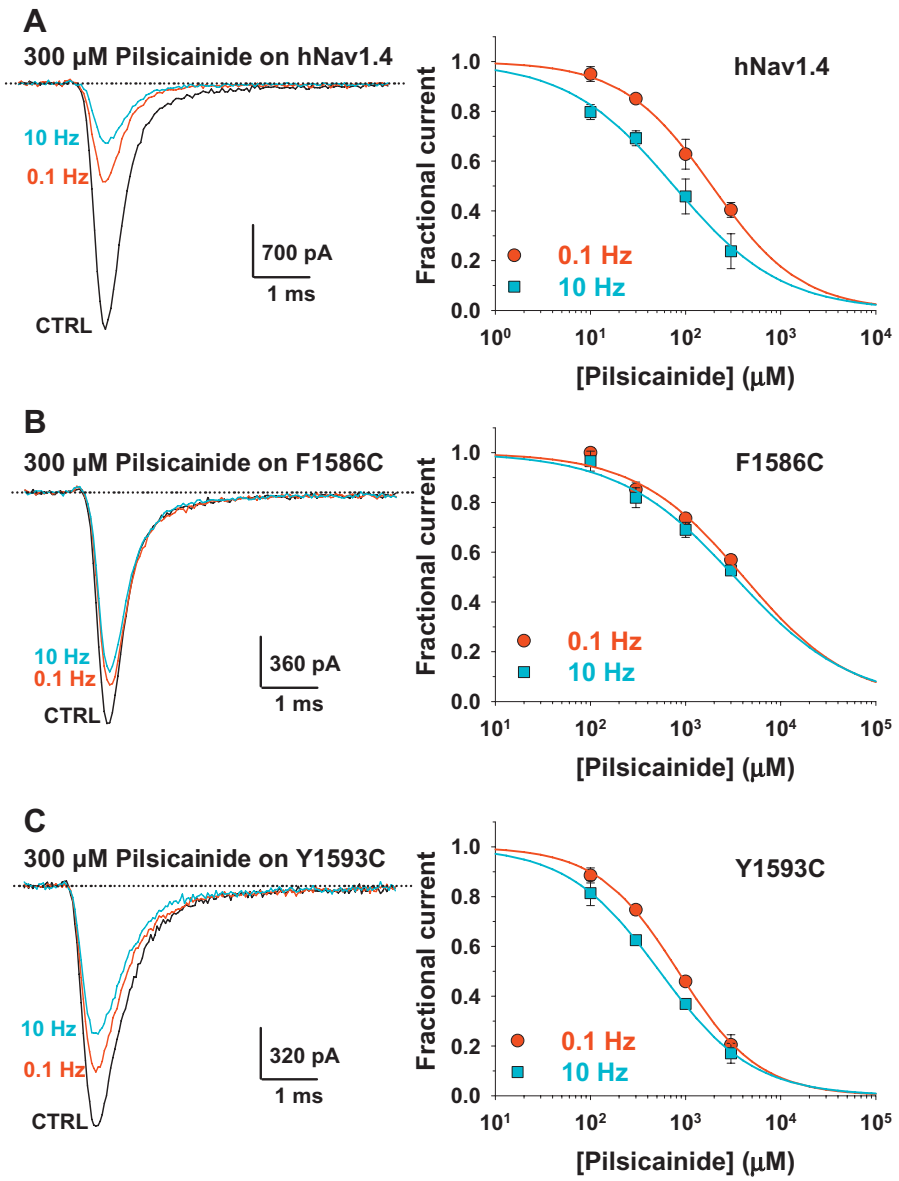

Figure 1 Effects of pilsicainide on WT hNav1.4 channels (A) and mutants, F1586C (B) and Y1593C (C). Left column, representative sodium current traces elicited from a holding potential of –120 mV to a test potential of –30 mV in the absence of drug (CTRL) and in the presence of 300 µM pilsicainide at 0.1 or 10 Hz frequency stimulation. Right column, concentration–response relationships were constructed at 0.1 and 10 Hz frequency stimulation, and fitted with Equation (1). Each point is the mean ± SEM from at least three cells. The calculated IC_{50} values ± SE of the fit were for WT hNav1.4: 189 ± 10 µM at 0.1 Hz, and 76 ± 7 µM at 10 Hz; for F1586C: 4084 ± 1086 µM at 0.1 Hz and 3297 ± 666 µM at 10 Hz; for Y1593C: 835 ± 27 µM at 0.1 Hz and 533 ± 9 µM at 10 Hz. The slope factors ranged between 0.71 and 1.03.

measuring the reduction of I_{Na} elicited from a holding potential of –120 to –30 mV at stimulation frequencies of 0.1 and 10 Hz. In the absence of drug, this protocol did not produce any significant change in I_{Na} amplitude (not shown). Examples of current traces recorded before (control) and during drug application at 0.1 Hz, and then between the 100th and 110th pulse at 10 Hz are illustrated in Figure 1A. With 300 µM pilsicainide, the amplitude of peak I_{Na} was reduced by 59.6 ± 3.0% at 0.1 Hz, and 76.2 ± 7.0% at 10 Hz (n = 3). The concentration–response curves were fitted with Equation (1) (see Methods), and the half-maximum inhibitory concentrations (IC_{50}) were 189 ± 10 µM at 0.1 Hz, and 76 ± 7 µM at 10 Hz. The slope factors (n_h) were close to 1. Thus, under these conditions, the IC_{50} value of pilsicainide at 0.1 Hz is intermediate between those of mexiletine (~236 µM) and flecainide (~84 µM), while the IC_{50} at 10 Hz is about twofold greater than that of these two drugs (Desaphy *et al.*, 2001; 2004). The characteristics of the sodium channel block and the chemical similarity with lidocaine suggest that pilsicainide binds to the LA receptor. In particular, the aromatic moiety and the charged amine of LAs have been proposed to interact with two aromatic residues of segment 6 in domain IV (Phe1764 and Tyr1771 in the Nav1.2 channel) through hydrophobic and π–cation interactions (Ragsdale *et al.*, 1994). To test this hypothesis, we engineered the corresponding F1586C and Y1593C

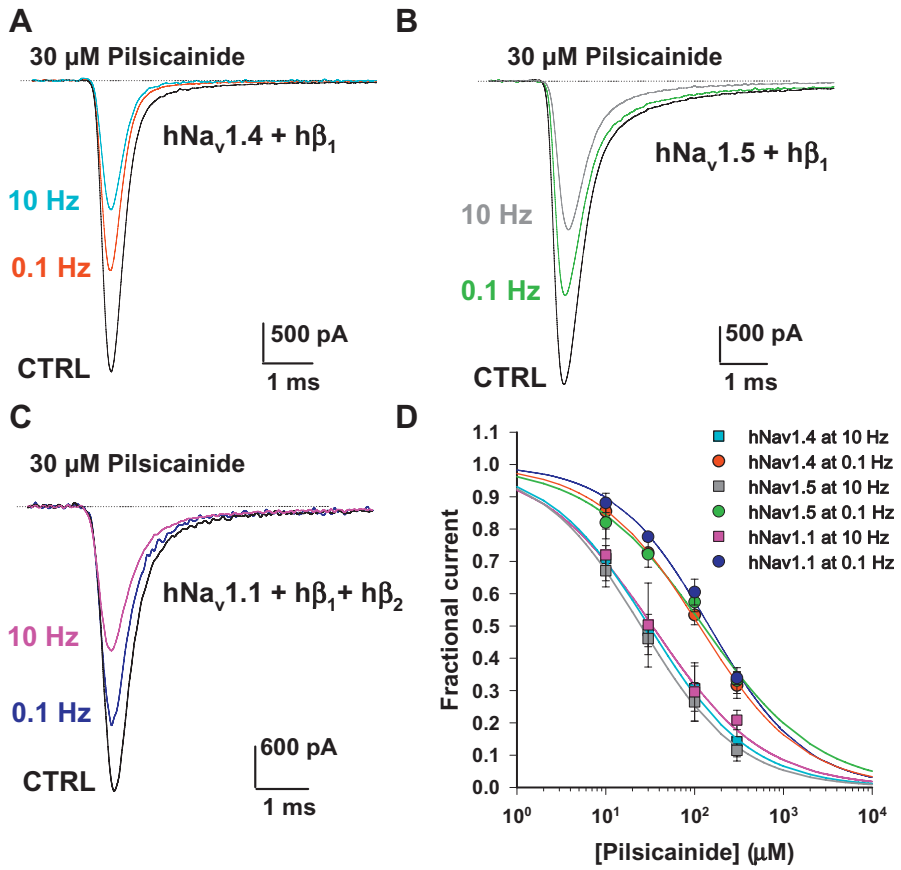

Figure 2 Effects of pilsicainide on hNav1.4 (A), hNav1.5 (B) and hNav1.1 (C) sodium channel subtypes, co-expressed with hβ1 alone (hNav1.4, hNav1.5) or hβ1 and hβ2 (hNav1.1) auxiliary subunits. (A–C) Representative sodium current traces are shown. Currents were elicited from a holding potential of −120 mV to a test potential of −30 mV in the absence of drug (CTRL) and in the presence of 30 μM pilsicainide at 0.1 or 10 Hz frequency stimulation. (D) Concentration–response relationships were constructed at 0.1 and 10 Hz frequency stimulation and fitted with Equation (1). Each point is the mean ± SEM from at least three cells. The calculated IC_{50} values ± SE of the fit were for hNav1.4: 113 ± 4 μM at 0.1 Hz and 31 ± 2 μM at 10 Hz; for hNav1.5: 125 ± 18 μM at 0.1 Hz and 25 ± 1 μM at 10 Hz; for hNav1.1: 146 ± 14 μM at 0.1 Hz and 34 ± 4 μM at 10 Hz. The slope factors ranged between 0.67 and 0.81.

mutations into hNav1.4. Both mutants were expressed well in HEK cells, and clones with uniform stable expression were obtained. The F1586C mutation in hNav1.4 induced a positive 5–10 mV shift in the voltage dependence of channel availability similar to F1764A in Nav1.2, and prevents the block of inactivated channels by mexiletine (Desaphy *et al.*, 2009). The effects of mexiletine were also drastically reduced in the Y1593C mutation, without any shift in channel availability voltage dependence (not shown). Representative current traces and the concentration–effect relationships of pilsicainide on these mutant sodium currents are shown in Figure 1B,C. Pilsicainide at 300 μM had little effect on the F1586C mutant I_{Na}, as the affinity of pilsicainide at 0.1 Hz in this mutation was dramatically decreased by 36-fold. The IC_{50} value obtained on F1586C channels (~4 mM) was comparable to the affinity constant for closed WT channels (K_R = ~3 mM, calculated below), and use-dependent block (UDB) was greatly attenuated, thereby suggesting that the preferential binding of pilsicainide to inactivated and/or open channels is abolished in this mutation. Although less impressive, the Y1593C mutation also greatly reduced (~4-fold) the affinity of pilsicainide at 0.1 Hz, as well as the use dependence.

UDB of hNav1.4, hNav1.5 and hNav1.1 channels by pilsicainide

The effects of pilsicainide on three sodium channel isoforms encoded by three different genes were examined. While the hNav1.4 channel (*SCN4A* gene) is expressed exclusively in skeletal muscle, the hNav1.5 channel (*SCN5A* gene) is the main cardiac isoform and is also expressed in some areas of the nervous system, as well as in immature or denervated skeletal muscle, and the hNav1.1 channel (*SCN1A* gene) is expressed in central and peripheral neurones, and cardiac myocytes (Catterall *et al.*, 2005). The hNav1.4 and hNav1.1 channels have <20% difference in amino acid sequence and are both sensitive to the pufferfish toxin tetrodotoxin. Human Nav1.5 is phylogenetically more distant from the two others, with >20% difference in amino acid sequence, and is less sensitive to tetrodotoxin. To mimic physiological conditions, hNav1.4 and hNav1.5 channels were co-expressed with the auxiliary hβ$_1$ subunit, and hNav1.1 channels were co-expressed with both hβ$_1$ and hβ$_2$ subunits. Figure 2 illustrates representative current traces of the three channel subtypes, elicited from a holding potential of −120 mV to a test pulse at −30 mV. In all three channel isoforms, 30 μM pilsicainide reduced the peak I_{Na} by ~25% at 0.1 Hz, and ~45% at

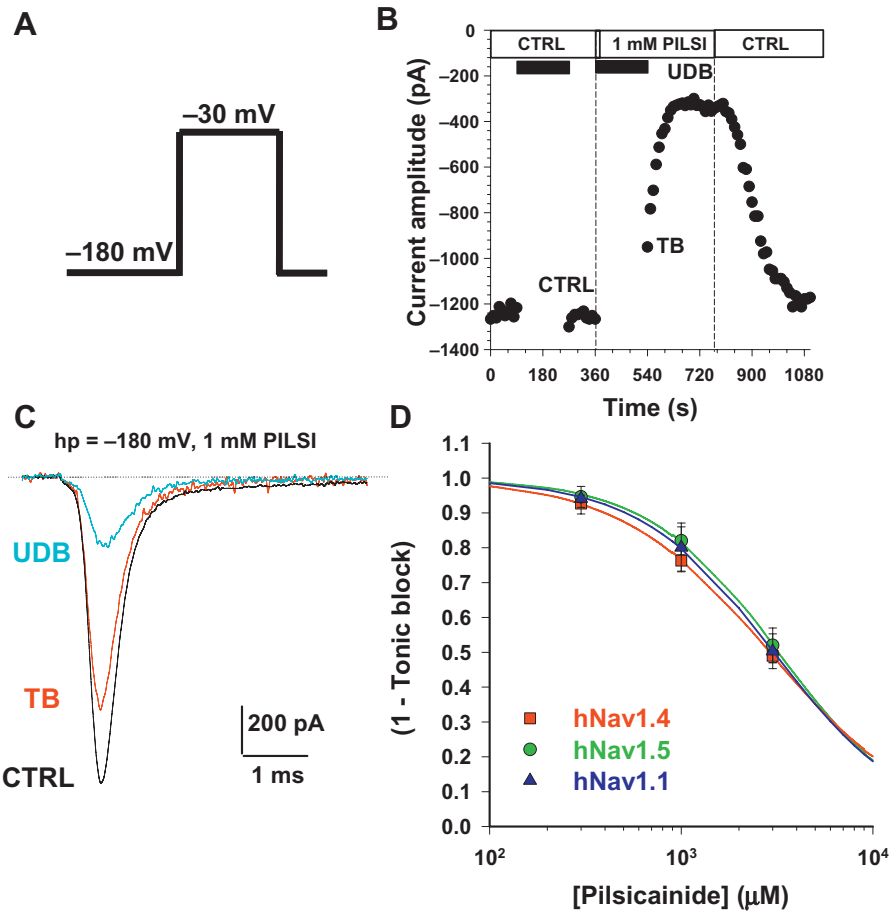

Figure 3 Determination of the affinity constant of pilsicainide for resting, closed channels (K_R). (A) Description of the voltage clamp protocol. (B) Time-course of hNav1.4 current amplitude in a representative cell exposed to 1 mM pilsicainide at a holding potential of –180 mV. For TB, the drug was applied while the cells were maintained at the holding potential for 180 s without depolarization (solid bars). TB was measured on the first test pulse at –30 mV, while UDB developed at 0.1 Hz frequency of stimulation. No current variation was observed with the same protocol in the absence of drug (CTRL). Drug effects were fully reversed upon washout. (C) Current traces were recorded in the same cell as in (B) at the time-points CTRL, TB and UDB shown in (B). (D) TB of hNav1.4 (α + β1), hNav1.5 (α + β1) and hNav1.1 (α + β1 + β2) channel subtypes was determined at hp = –180 mV using various pilsicainide concentrations. Concentration–TB relationships were fitted with Equation (1), where IC_{50} corresponds to K_R. Each data point is the mean ± SEM of at least three cells. The calculated IC_{50} values ± SE of the fit were 2888 ± 24 μM (hNav1.4), 3209 ± 93 μM (hNav1.5) and 3040 ± 57 μM (hNav1.1). The slope factors ranged between 1.11 and 1.27.

10 Hz. The concentration–response curves overlapped extensively, and the IC_{50} values determined at both frequencies were not different among the three channels.

State-dependent affinity of pilsicainide for sodium channel subtypes

According to the modulated receptor hypothesis, the use-dependent effect of pilsicainide on sodium channel subtypes suggests that it has different binding affinities for the various channel states (Hille, 1977a; Desaphy *et al.*, 2004). To obtain an estimation of pilsicainide affinities for resting channels (K_R), we measured tonic block (TB) of hNav1.4 (α + β1 subunits), hNav1.5 (α + β1) and hNav1.1 (α + β1 + β2) on the first test pulse applied at –30 mV, after the cells have been maintained at a holding potential of –180 mV for 180 s (Figure 3A,B). UDB was then observed during subsequent test pulses applied at a frequency of 0.1 Hz. The observed effects were fully reversed upon return to control solution. Representative current traces in Figure 3C illustrate TB and UDB of hNav1.4 channels by 1 mM pilsicainide. On average, 1 mM pilsicainide induced 24 ± 3% of TB and 77 ± 4% of UDB (n = 4). The concentration–TB relationships obtained for the three sodium channel subtypes were superimposed (Figure 3D). Because TB measured at a holding potential of –180 mV results exclusively from the block of closed channels, the IC_{50} values calculated from Equation (1) provide a good estimate of K_R. The calculated K_R values were 2888 μM for hNav1.4, 3209 μM for hNav1.5 and 3040 μM for hNav1.1. Thus, no difference was found among the three channel isoforms. For comparison, the K_R values for mexiletine and flecainide on hNav1.4 channels were ~800 and ~480 μM, respectively (Desaphy *et al.*, 2001; 2004). We concluded that pilsicainide is a very weak blocker of closed sodium channels.

To determine whether pilsicainide blocks inactivated channels, we first evaluated the voltage dependence of the TB by varying the holding potential. A 100 ms prepulse at –180 mV was introduced before the test pulse at –30 mV to allow channels to recover from inactivation, thereby guaranteeing that

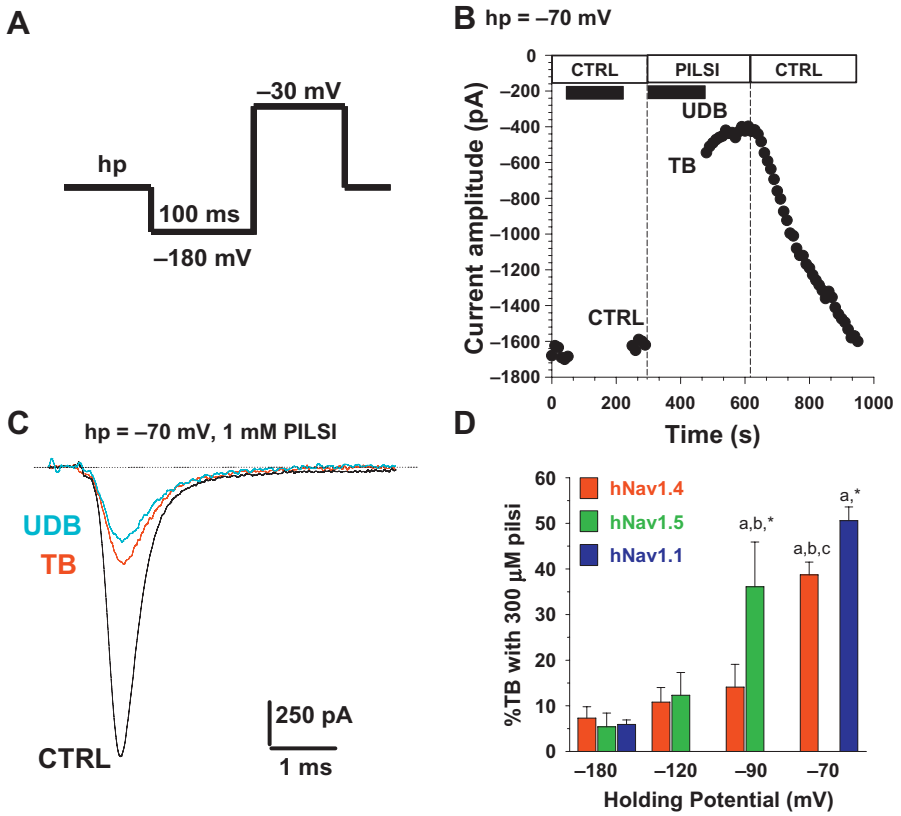

Figure 4 Voltage dependence of sodium channel TB induced by pilsicainide. (A) Description of the voltage clamp protocol. Various holding potentials were used, and a 100 ms prepulse at –180 mV was applied before the test pulses to allow the channels to recover from inactivation occurring at the less negative holding potentials. (B) Time-course of hNav1.4 current amplitude in a representative cell exposed to 1 mM pilsicainide at a holding potential of –70 mV. To determine the TB, the drug was applied while the cells were maintained at the holding potential for 180 s without depolarization (solid bars). TB was measured on the first test pulse at –30 mV, while UDB developed at 0.1 Hz frequency of stimulation. No current variation was observed with the same protocol in the absence of drug (CTRL). Drug effects were fully reversed upon washout. (C) Current traces were recorded in the same cell as in (B) at the time-points CTRL, TB and UDB shown in (B). (D) The TB of hNav1.4 (α + β1), hNav1.5 (α + β1) and hNav1.1 (α + β1 + β2) channel subtypes by 300 µM pilsicainide was determined at various holding potentials. Each column is the mean ± SEM of three to seven cells. Statistical analysis with Student's *t*-test indicated $P < 0.05$ versus TB of the same channel subtype at –180 mV (a), –120 mV (b) and –90 mV (c), or versus TB of hNav1.4 at the same holding potential (*).

the reduction of I_{Na} was attributable only to drug block (Figure 4A). The time-course of the effects of 1 mM pilsicainide on the hNav1.4 current amplitude at a holding potential of –70 mV is shown in Figure 4B, and representative current traces are shown in Figure 4C (for comparison to Figure 3B,C obtained at a holding potential of –180 mV). On average, 1 mM pilsicainide induced 65 ± 7% of TB and 74 ± 2% of UDB ($n = 4$) at –70 mV. Figure 4D shows that the TB induced by 300 µM pilsicainide is voltage dependent for the three channel isoforms. Nevertheless, the voltage dependence of the TB was negatively shifted for hNav1.5 compared to hNav1.4 and hNav1.1 subtypes. Figure 5A shows the voltage dependence of the steady-state inactivation for the three channel subtypes. The relationships were constructed using a standard two-pulse protocol, and the experimental data were fitted using Equation (2) (see Methods). Under our conditions, the hNav1.4 and hNav1.1 displayed a similar voltage dependence, whereas the hNav1.5 channel inactivated at voltages ~15 mV more negative (Figure 5A). Although we cannot definitely exclude the possibility that the voltage dependence of the inhibition of closed channels may result from the block of channels in the various non-inactivated, closed states with specific drug affinities, the parallelism between the voltage dependence of the TB for each channel subtype and the voltage dependence of the steady-state inactivation strongly suggests that pilsicainide blocks channels inactivated from the closed state. Thereafter, we assumed that pilsicainide has similar low affinities for all the non-inactivated, closed states, the mean of which is K_R, and a similar high affinity for all the fast inactivated states, the mean of which is K_I. Thus, the TB at the holding potential of –70 mV (or –90 mV for hNav1.5) results from the block of a mixed population of closed and inactivated channels with affinities K_R and K_I respectively. Values for K_I were estimated from Equation (3) (see Methods). The apparent affinity constant (K_{APP}) was calculated as the IC_{50} for the TB at a holding potential of –70 or –90 mV (Figure 5B). For hNav1.4 and hNav1.1 at –70 mV, the K_{APP} was 500 ± 53 and 307 ± 19 µM (Figure 5B), and h was 0.624 and 0.529, respectively (Figure 5A). For hNav1.5 at –90 mV, K_{APP} was 547 ± 38 µM and h was 0.691. Thus the K_I values of pilsicainide were 211 µM for hNav1.4, 153 µM for hNav1.1 and 192 µM for hNav1.5. These values are far higher than those for mexiletine ($K_I = 6$ µM) and flecainide ($K_I = 18$ µM) (Desaphy *et al.*, 2001; 2004). This sug-

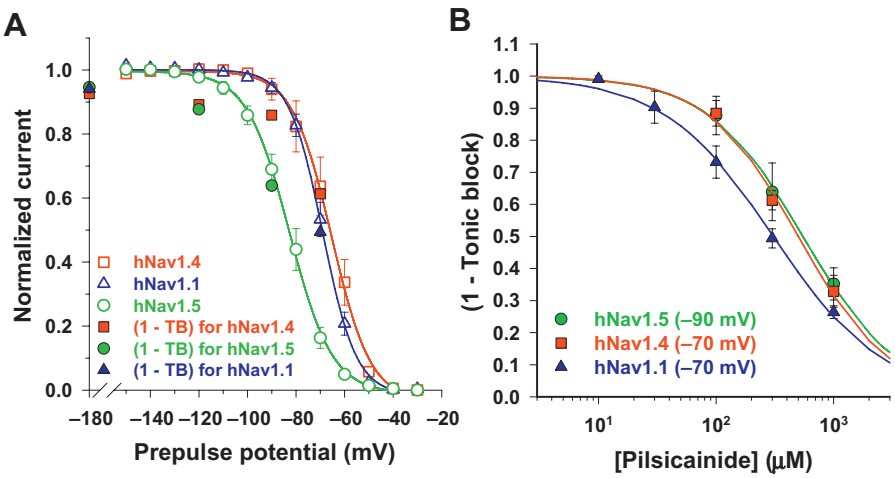

Figure 5 Determination of the affinity constant of pilsicainide for channels inactivated from the closed state (K_I). (A) The voltage dependence of steady-state availability of hNav1.4 (α + β1), hNav1.5 (α + β1) and hNav1.1 (α + β1 + β2) channel subtypes was determined by measuring sodium currents elicited by a 20-ms-long test pulse to –30 mV after 50-ms-long conditioning pulses to potential ranging from –150 to –30 mV in 10 mV increments. Holding potential was –180 mV, and interval duration between two pulses was 15 s. The normalized peak sodium current recorded during the test pulse was plotted against the conditioning pulse potential. The relationships were fitted with Equation (2), giving a half-maximum inactivation potential ($V_{1/2}$ ± SE of the fit) of –65.4 ± 0.4 mV for hNav1.4 (n = 11), –82.7 ± 0.4 mV for hNav1.5 (n = 14) and –69.0 ± 0.3 mV for hNav1.1 (n = 16). The slope factor ± SE of the fit was 8.1 ± 0.5 mV for hNav1.4, 8.8 ± 0.3 mV for hNav1.5 and 6.9 ± 0.3 mV for hNav1.1. (B) The TB was determined at hp = –70 mV for hNav1.4 and hNav1.1, and at hp = –90 mV for hNav1.5, using various pilsicainide concentrations. Concentration–TB relationships were fitted with Equation (1), where IC_{50} corresponds to K_{APP} (see text). Each data point is the mean ± SEM of at least three cells. The calculated IC_{50} values ± SE of the fit were 500 ± 53 μM (hNav1.4), 547 ± 38 μM (hNav1.5) and 307 ± 19 μM (hNav1.1). The slope factors ranged between 0.93 and 1.12.

gests that pilsicainide is a weak blocker of sodium channels inactivated from the closed state.

Effects of external pH on the effects of pilsicainide

The weak block of sodium channels, either closed or inactivated from the closed state, may be due to a weak affinity of the drug for these channel states, according to the modulated receptor model (Hille, 1977a; Hondeghem and Katzung, 1977), and/or to reduced access of the drug to the closed or inactivated receptor through the closed pore (Starmer *et al*., 1984). The second hypothesis is supported by the high pKa of pilsicainide (Table 1). At pH 7.4, more than 99.8% of the drug is charged compared to 99.0% for flecainide and 98.7% for mexiletine. Under these conditions, it may be very difficult for pilsicainide to reach the receptor within the closed pore. To test this hypothesis, we measured the effects of pilsicainide on hNav1.4 channels using an external bath solution buffered at pH 8.2, which predicts pilsicainide ionization to be 98.9%, a value similar to that of the two other drugs at pH 7.4. Currents measured at pH 8.2 were very similar to those measured at pH 7.4, although the steady-state inactivation occurred at potentials slightly more negative (not shown). The half-maximum inactivation potential was –72.8 ± 0.2 mV (n = 12) at pH 8.2, compared to –65.4 ± 0.4 mV (n = 11) at pH 7.4. The TB of hNav1.4 channels by pilsicainide was determined at pH 8.2 at the holding potentials of –180 and –70 mV (Figure 6). The K_R, determined at –180 mV, was 1191 ± 187 μM, which is ~2.5 times smaller than that at pH 7.4. At –70 mV, the K_{APP} value was 39.7 ± 6.9 μM, that is a 13-fold reduction with respect to that at pH 7.4. Using Equation (3) with h = 0.392, we calculated a K_I value of 24 μM for pilsicainide at pH 8.2. Thus, by increasing the proportion of neutral pilsicainide, we obtained channel state-specific affinity constants more similar to those of flecainide and mexiletine.

Pilsicainide effects on the myotonic R1448C and G1306E $hNa_v1.4$ mutants

The R1448C and G1306E mutations in hNav1.4 are responsible for paramyotonia congenita and K^+-aggravated myotonia, respectively, which are two skeletal muscle disorders characterized by overexcitability of the sarcolemma (George, 2005). Both mutations had a slower decay rate of I_{Na}, resulting in an increased inward current that increases membrane excitability (Desaphy *et al*., 2001). Moreover, the voltage dependence of inactivation of G1306E channels was shifted by ~15 mV to less negative potentials, as shown in Figure 4A, thereby increasing the propensity for episodes of overexcitation. In contrast, the voltage dependence of inactivation of R1448C channels was shifted by ~15 mV to more negative potentials, which in theory may limit the severity of the other gating defects in producing myotonia.

In previous studies, we demonstrated that mexiletine and flecainide block R1448C more potently than the WT channels at a holding potential of –120 mV (Desaphy *et al*., 2001; 2004). According to the modulated receptor hypothesis, the apparent block of R1448C channels at –120 mV is greater than WT channels because a substantial number of R1448C channels are inactivated at this potential. Based on the same principle, the block of G1306E channels by mexiletine was reduced with respect to WT channels, because fewer mutant channels are inactivated at –120 mV. In contrast, the block of WT and G1306E channels was similar for flecainide, owing to a lower K_R/K_I ratio of this drug as compared to mexiletine.

We tested the effects of pilsicainide on the two mutants using similar voltage clamp protocols. At a holding potential

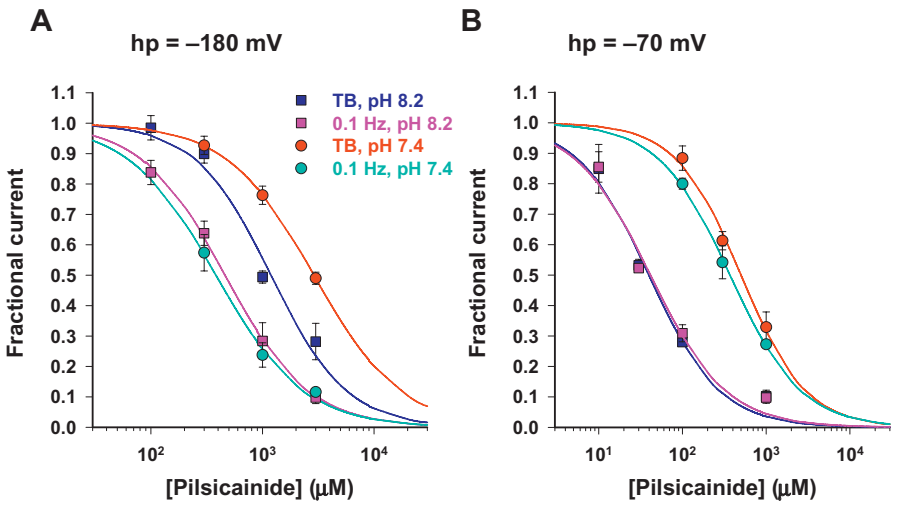

Figure 6 Effects of external pH on tonic and UDB of hNav1.4 channels by pilsicainide. TB and UDB at 0.1 Hz stimulation frequency of hNav1.4 were determined at pH 8.2 and compared to those obtained at pH 7.4, using a holding potential of –180 mV to calculate K_R (A, as in Figure 3) and a holding potential of –70 mV to calculate K_{APP} (B, as in Figure 4). Each data point is the mean ± SEM of at least three cells. Concentration–effect relationships were fitted with Equation (1). (A) For the TB at –180 mV, the calculated IC_{50} values ± SE of the fit was 1191 ± 187 μM at pH 8.2, compared to 2888 ± 24 μM at pH 7.4. For the UDB at –180 mV, the IC_{50} values were 462 ± 20 μM at pH 8.2, and 383 ± 36 μM at pH 7.4. (B) For the TB at –70 mV, the IC_{50} value was 39 ± 7 μM at pH 8.2, compared to 500 ± 53 μM at pH 7.4. For the UDB at –70 mV, the IC_{50} values were 42 ± 8 μM at pH 8.2, and 370 ± 16 μM at pH 7.4. In (A) and (B), the slope factors ranged between 0.96 and 1.30.

–120 mV, the reduction in the I_{Na} amplitude of R1448C channels with 30 μM pilsicainide was 41.4 ± 3.0% at 0.1 Hz, and 72.9 ± 2.0% at 10 Hz (n = 3), which is comparable to the reduction of WT channels with 100–300 μM of the drug (Figure 7A). The IC_{50} values of pilsicainide in the R1448C mutant were 40 ± 3 μM at 0.1 Hz, and 11 ± 1 μM at 10 Hz, values that are ~5 times smaller than for WT channels (Figure 7C). On the other hand, a twofold higher concentration of pilsicainide was needed at 0.1 Hz to block GE mutants to the same extent as WT channels (Figure 7B), while the use dependence was greatly reduced. The IC_{50} values of pilsicainide in the G1306E mutant were 572 ± 94 μM at 0.1 Hz, and 331 ± 53 μM at 10 Hz (Figure 7C).

Because the UDB in the G1306E mutant was greatly attenuated, we hypothesized that the mutation may modify the affinity constant of pilsicainide for the inactivated channels. To test this, we measured the K_R using a holding potential of –180 mV and K_{APP} at a holding potential of –70 mV for the G130E channels, and found values of 3005 ± 301 and 723 ± 31 μM, respectively (Figure 7D). With h = 0.8414 in Equation (3), the K_I calculated for G1306E was 144 μM. Thus, no differences were found for the K_R and K_I values between the WT hNav1.4 and G1306E mutant.

Discussion

Pilsicainide is an anti-arrhythmic drug in use in Japan. Based on current clamp and a few voltage clamp studies, it has been described as a pure sodium channel blocker with preferential binding to open channels.

Our results presented here show that the block induced by pilsicainide depends upon the same critical residues required by LAs and other class I anti-arrhythmic drugs. Specifically, we found that non-conserved mutations at phenylalanine 1586 and, to a lesser extent, tyrosine 1593 residues in D4S6 of hNav1.4 dramatically reduced the UDB, indicating that these amino acids are involved in the binding of pilsicainide to inactivated channels, as originally proposed for LA drugs by Ragsdale *et al*. (1994). In addition, the Brugada syndrome-responsible mutation N406S within D1S6 of hNav1.5 has been shown to completely abolish the UDB mediated by pilsicainide without affecting its TB (Itoh *et al*., 2005). This asparagine residue has been proposed to either interact directly with LAs in inactivated rNav1.4 (Nau *et al*., 1999) or to modify the binding of the LAs through gating defects in rNav1.2 (Yarov-Yarovoy *et al*., 2002). In addition, we found that the mutation G1306E, associated with potassium-aggravated myotonia, greatly reduced the UDB of pilsicainide. Although we previously found no evidence for a direct interaction between G1306E and mexiletine or flecainide (Desaphy *et al*., 2001; 2004), a previous study suggested that lidocaine has a reduced affinity for inactivated hNav1.4 channels carrying the paramyotonia congenita-associated T1313M mutation (Fan *et al*., 1996). Both G1306E and T1313M are within the intracellular D3–D4 linker, very close to the IFM tripeptide motif, which is thought to constitute the inactivation gate. Mutating IFM residues to glutamine disrupts fast inactivation and impairs high-affinity binding of lidocaine, suggesting that, at least for some LAs, the fast inactivation gate may be critical for their action (Bennett *et al*., 1995a). Nevertheless, a direct interaction between the inactivation gate and lidocaine can be excluded, based on observations that the fast gate recovers from inactivation at the same rate whether lidocaine is present or not (Vedantham and Cannon, 1999). Importantly, we found that the affinity of pilsicainide for closed and inactivated channels was not modified in the G1306E mutation, thereby indicating that the impairment of

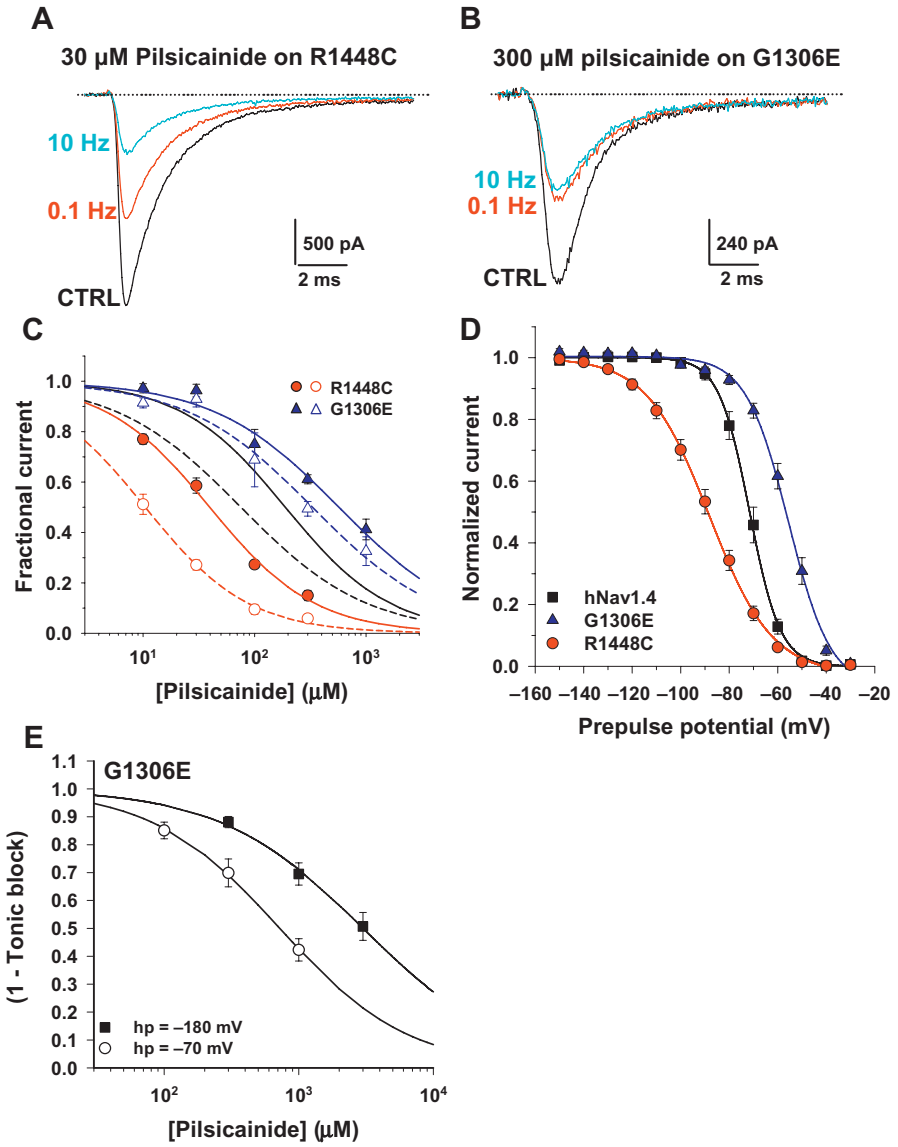

Figure 7 Effects of pilsicainide on the myotonic hNav1.4 mutants, R1448C and G1306E. (A,B) Representative R1448C and G1306E sodium current traces are shown. Currents were elicited from a holding potential of –120 mV to a test potential of –30 mV in the absence of drug (CTRL) and in the presence of 30 (R1448C) or 300 (G1306E) µM pilsicainide at 0.1 or 10 Hz frequency stimulation. (C) The concentration–response relationships were constructed at 0.1 Hz (filled circles and solid line) and 10 Hz (open circles and dashed line), and fitted with Equation (1). Each point is the mean ± SEM from at least three cells. For R1448C, the calculated IC_{50} values ± SE of the fit were 40 ± 3 µM at 0.1 Hz, and 11 ± 1 µM at 10 Hz. For G130E, the IC_{50} values were 572 ± 94 µM at 0.1 Hz, and 331 ± 53 µM at 10 Hz. The slope factors ranged between 0.77 and 0.94. For comparison, solid and dashed lines without symbols show concentration–responses curves for pilsicainide on WT hNav1.4. (D) The voltage dependence of steady-state availability of WT hNav1.4 channels (α subunit alone), and R1448C and G1306E mutants were determined by measuring sodium currents elicited by a 20-ms-long test pulse to –30 mV after 50 ms conditioning pulses to potentials ranging from –150 to –30 mV in 10 mV increments. Holding potential was –180 mV, and interval duration between two pulses was 15 s. The normalized peak sodium current recorded during the test pulse was plotted against the conditioning pulse potential. The fit of relationships with Equation (2) gave a half-maximum inactivation potential ($V_{1/2}$ ± SE of the fit) of –71.3 ± 0.2 mV for WT hNav1.4 (n = 12), –88.2 ± 0.5 mV for R1448C (n = 12) and –55.6 ± 0.8 mV for G1306E (n = 16). The slope factor was 6.3 ± 0.2 mV for WT hNav1.4, 12.9 ± 0.5 mV for R1448C and 8.7 ± 0.7 mV for G1306E. (F) The TB of G1306E by pilsicainide was determined at a holding potential of –180 or –70 mV, as in Figures 3 and 4. Each data point is the mean ± SEM of at least three cells. Concentration–TB relationships were fitted with Equation (1) to calculate K_R at hp = –180 mV and K_{APP} at hp = –70 mV. The calculated K_R ± SE of the fit was 3005 ± 301 µM and K_{APP} was 723 ± 31 µM. The slope factors were 0.82 ± 0.08 at –180 mV and 0.91 ± 0.04 at –70 mV.

pilsicainide use dependence probably results from a gating effect of the mutation rather than from a modification of the binding site.

Although the block of sodium currents by pilsicainide at hp –120 mV and a stimulation frequency of 0.1 Hz was of comparable magnitude to that developed by mexiletine and flecainide, the calculated affinity constants of pilsicainide for channels either closed or inactivated from the closed state were much higher than that of the two other drugs. This indicates that the block at 0.1 Hz was mainly use dependent

and depended essentially on channel opening. UDB was also observed at 0.1 Hz using the very negative hp of –180 mV (see Figure 3B), whereas mexiletine induces little or no UDB under these conditions (Desaphy *et al.*, 2001). The use dependence of an LA-like drug may arise from either the incomplete recovery of block between two successive depolarizations or the need for the channel to open for the drug to reach its high-affinity receptor within the open and/or inactivated pore. Neutral pilsicainide is very lipid soluble (as indicated by log *P* value), but, because of the very high pKa, the drug is almost exclusively charged at pH 7.4, resulting in a negative log *D* value. Thus, the hydrophobic pathway, which is involved in the effect of LAs, is less important for pilsicainide, which may need the hydrophilic pathway, which is channel opening, to reach its receptor (Hille, 1977a,b). Importantly, increasing the proportion of neutral pilsicainide slightly by increasing the pH led to the attainment of affinity constants for closed or closed-state inactivated channels more similar to those of mexiletine and flecainide, thereby supporting the latter hypothesis. One might also hypothesize that the differences in affinity between the two pH conditions may arise from pH-dependent specific interactions at the binding site. However, because the steady-state block at hp –180 mV and 0.1 Hz stimulation was the same at both pHs, it is likely that the difference in the K_R and K_I values is due more to a change in the drug access rate to the binding site than to a different drug interaction at the binding site. Thus, our data strongly support the hypothesis that, at physiological pH, drug transit through the open pore is a prerequisite for high-affinity block of sodium channels by pilsicainide. This is also consistent with the observation that pilsicainide is a slow acting drug (Hattori and Inomata, 1992).

Although pilsicainide may be almost exclusively charged at pH 7.4, the drug does not act in exactly the way as permanently charged quaternary amines, like QX314. The latter is completely impermeant to cell membranes and needs to be applied intracellularly to reach its receptor and inhibit sodium currents. The substitution of an isoleucine residue in the extracellular third of D4S6 (I1760 in rat Nav1.2, I1575 in rat Nav1.4; I1756 in human Nav1.5) with alanine, cysteine or glutamate was shown to create an external access pathway, thereby allowing QX314 to block sodium channels from the outside and facilitate LA drug escape from the closed channel (Ragsdale *et al.*, 1994; Wang *et al.*, 1998; Sunami *et al.*, 2001; O'Leary *et al.*, 2003). In contrast to other isoforms, the cardiac Nav1.5 channel is blocked by external QX314, and replacement of threonine 1765 in rNav1.5 by valine, which is a native residue in rNav1.2 reduced this block (Qu *et al.*, 1995); conversely, a cysteine mutation to threonine at this site in rNav1.4 creates an external access for QX314 (Sunami *et al.*, 2000). These two amino acids are probably involved in the hydrophobic pathway for neutral drug, which is probably located at the III/IV domain interface (Bruhova *et al.*, 2008). The effect of mutations at these sites on the action of pilsicainide is not known, but it is probable that mutations may favour resting channel block by pilsicainide and/or may reduce the UDB by accelerating its escape from the closed channel. Nevertheless, in contrast to QX314, external pilsicainide conserves the possibility to cross membranes and block closed sodium channels via the hydrophobic pathway, although with a reduced efficiency compared to other tertiary amines showing a positive logD.

Whether the high-affinity receptor of an LA-like drug is the open or the inactivated channel is not an easy question to answer, in part because the channel transits very quickly from the open to the inactivated state and because the inactivated state is non-conducting. Indeed, there are numerous examples in the literature of studies with opposite conclusions about this matter. For instance, the mechanism of action of flecainide was recently revisited, and the drug was proposed to be either an open channel blocker (Grant *et al.*, 2000; Nagamoto *et al.*, 2000; Wang *et al.*, 2003) or an inactivated channel blocker (Viswanathan *et al.*, 2001; Liu *et al.*, 2003; Desaphy *et al.*, 2004). With regard to pilsicainide, our data are more consistent with it having an interaction with the inactivated channel. Indeed, significant open channel blockade should have produced UDB at a holding potential of –70 mV in a manner similar to that observed at –180 mV. On the contrary, very little UDB was observed at –70 mV (compare Figure 4B with Figure 3B), indicating that during the 3 min channels were held at –70 mV, the drug had enough time to enter the pore through the hydrophobic pathway and to reach equilibrium in binding to the inactivated channel.

This hypothesis appears to conflict with data derived from two previous studies that supported an open-channel blocking mechanism of pilsicainide, based on the effects of the drug on channels with impaired inactivation (Inomata *et al.*, 1989; Ono *et al.*, 2000). In the first study (Inomata *et al.*, 1989), the effects of pilsicainide (formerly SUN 1165) were tested on whole-cell sodium currents with delayed inactivation, induced by a scorpion toxin. In this experiment, the drug applied during the depolarizing pulse showed little or no effect on the current, while UDB developed on the successive pulses. This use-dependent inhibition was interpreted by the authors to be a result of open channel blockade, because the inactivation of the channel is impaired by the toxin. However, the toxin did not delete the inactivation completely, and an equally plausible interpretation is that the use-dependent inhibition resulted from the block of inactivated channels by the drug that had entered the pore during the preceding depolarizing pulse. The latter may also explain why the drug had little effect on the first test pulse. In the second study, using single-channel analysis of ΔKPQ cardiac sodium channel mutant in cell-attached macropatches, Ono *et al.* (2000) showed that pilsicainide decreased a persistent current more than the peak current, owing essentially to the shortening of burst duration, and attributed this effect to open channel blockade. In the ΔKPQ channel mutant, burst activity is the result of an increased propensity towards a modal gating, characterized by a very rapid flickering of the channel between the open and a non-conducting state (Bennett *et al.*, 1995b). The repeated opening of the channel during the burst may increase the likelihood for the drug to enter the pore and bind to its receptor, thereby resulting in a reduction of burst duration. Nevertheless, the analysis of this effect of pilsicainide did not allow these investigators to discriminate between the open and the inactivated channel for the high-affinity receptor, because channel inactivation can occur within a burst, appearing as a gap corresponding to the slowest of the two closed time constants (Undrovinas *et al.*,

2002; Maltsev and Undrovinas, 2006). Hence, the effects of pilsicainide on toxin-modified sodium channels and on ΔKPQ channel mutants may be consistent with our hypothesis that pilsicainide binds to the inactivated state of the channel, and this is mediated through the open pore.

Nevertheless, it is possible that both the open and the inactivated states of the sodium channels contribute similarly to the high-affinity binding of LA drugs. Indeed, mutation of the tyrosine in the D4S6 segment (Y1767 in human Nav1.5) was shown to weaken both the inactivation-dependent block in fast-inactivating channels and the open-channel block in inactivation-deficient channels by cocaine (O'Leary and Chahine, 2002). Under these conditions, the access of LA drugs to these states appears to be the actual determinant of sodium channel inhibition. Pilsicainide cannot easily use the hydrophobic pathway, in contrast to other LA drugs, such as mexiletine, which can exploit the hydrophobic pathway to bind inactivated channels without channel opening. Hence, pilsicainide needs the channel to be open for it to be able to bind to open/inactivated channels with high affinity.

In conclusion, pilsicainide shows an unusual behaviour compared to other LA drugs, such as mexiletine and flecainide. Because the latter can reach the high-affinity receptor via the hydrophobic pathway, their block of sodium channels shows a strong voltage dependence, and the specific affinities of these drugs for the low-affinity receptor (i.e. the resting state) and high-affinity receptors (i.e. the open/inactivated state) define their specificity of action on the various sodium channel subtypes or mutants (Desaphy *et al*., 2001; 2004). In contrast, with pilsicainide it is the access to the receptor that is the main determinant of its effect. Because of its unusual negative logD, a clinical concentration of pilsicainide may not be able to block pre-open channels, either closed or closed inactivated, as it needs the channel to be open in order to reach its receptor. Such a difference between pilsicainide and other LA drugs, such as mexiletine, may be of interest in the clinical situation. The limited voltage dependence of the pilsicainide block may favour its use in the myotonic syndromes: where the skeletal muscle fibres are more hyperpolarized than other excitable tissues rendering this tissue less sensitive than heart and neurones to LA drugs. At present, the antimyotonic drug of choice is mexiletine, which needs to be used in a high-concentration range with the significant risk of developing adverse CNS and cardiac effects. In the case of pilsicainide, the most important factor determining selectivity will be the frequency of channel opening, which is increased in myotonic muscle or other overexcited tissues. Importantly, the low lipophilicity of pilsicainide may also limit its access to the central nervous system, which is consistent with the relative rarity of CNS adverse effects (Hashimoto and Nakashima, 1993). These observations reveal the possible benefits of pilsicainide in patients suffering from peripheral disorders characterized by overexcitability, especially myotonic syndromes.

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Conflict of interest

The authors state no conflict of interest.

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