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A comparative study of the action of tolperisone on seven different voltage dependent sodium channel isoforms

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Abstract

The specific, acute interaction of tolperisone, an agent used as a muscle relaxant and for the treatment of chronic pain conditions, with the $Na_{v1.2}$, $Na_{v1.3}$, $Na_{v1.4}$, $Na_{v1.5}$, $Na_{v1.6}$, $Na_{v1.7}$, and $Na_{v1.8}$ isoforms of voltage dependent sodium channels was investigated and compared to that of lidocaine. Voltage dependent sodium channels were expressed in the *Xenopus laevis* oocyte expression system and sodium currents were recorded with the two electrode voltage clamp technique. Cumulative dose response relations revealed marked differences in IC_{50} values between the two drugs on identical isoforms, as well as between isoforms. A detailed kinetic analysis uncovered that tolperisone as well as lidocaine exhibited their blocking action not only via state dependent association/dissociation with voltage dependent sodium channels, but a considerable fraction of inhibition is tonic, i.e. permanent and basic in nature. Voltage dependent activation was affected to a minor extent only. A shift in steady-state inactivation to more negative potentials could be observed for most drug/isoform combinations. The contribution of this shift to overall block was, however, small at drug concentrations resulting in considerable overall block. Recovery from inactivation was affected notably by both drugs. Lidocaine application led to a pronounced prolongation of the time constant of the fast recovery process for the $Na_{v1.3}$, $Na_{v1.5}$, and $Na_{v1.7}$ isoforms, indicating common structural properties in the local anesthetic receptor site of these three proteins. Interestingly, this characteristic drug action was not observed for tolperisone.

Keywords: Voltage dependent sodium channel; Tolperisone; Lidocaine; *Xenopus laevis* oocyte; Local anesthetic

1. Introduction

Tolperisone has a lengthy history as a drug used as a muscle relaxant and for the treatment of chronic pain (Bajaj et al., 2003; Meilinger et al., 2003). Since it is structurally related to local anesthetics, such as lidocaine and procaine, it is likely to be a sodium channel antagonist. Furthermore, local anesthetics are also used systemically for the treatment of cardiac arrhytmias (Chaudhry and Haffajee, 2000), neuropathic pain (Lai et al., 2004) and other nervous diseases (Czapinski et al., 2005; Yogeeswari et al., 2004). Although of prodigious therapeutic value, systemically applied sodium channel blockers are, at high doses, often associated with severe side-effects on the heart and the central nervous system (see Mao and Chen, 2000, for example). It is known that 10 distinct genes encode isoforms of the channel forming α-subunit of voltage dependent sodium channels (Goldin et al., 2000). By alternative splicing, this number is multiplied to give rise to manifold potential drug targets with different toxicological profiles (Narahashi et al., 1994; Narahashi and Herman, 1992), but potentially also distinct pharmacological properties. Expression patterns of

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sodium channel gene products are tissue specific, depend on stage of development and also on pathophysiological conditions (Waxman, 2001; Zamponi et al., 1994; Gould et al., 2004; Raymond et al., 2004). Hence, the potential application of subtype-specific sodium channel blockers could provide better management of distinctive pathophysiological conditions and in particular neuropathic pain symptoms. Despite apparent usefulness in the treatment of chronic pain conditions, little is known about the molecular targets and mechanisms underlying the therapeutic effects of tolperisone. It has been reported that tolperisone reduces sodium and potassium permeability in the node of Ranvier of frogs (Hinck and Koppenhofer, 2001), blocks calcium currents in snail neurons (Novales-Li et al., 1989) and inhibits current through heterologously expressed $Na_{v1.6}$ channels (Quasthoff et al., 2003). The current investigation aims to study the acute effect of tolperisone on seven different isoforms of the $Na_{v1.x}$ protein in order to elucidate possible isoform specific differences in its mode of action. In addition, the findings are compared to the efficacy of lidocaine as a reference substance in this respect.[2]

2. Materials and methods

2.1. Electrophysiology

Oocytes were placed in a recording chamber that allowed superfusion with ND96 solution (see below) by gravity. Two electrode voltage clamp using agarose cushion electrodes was performed at room temperature (20 °C) as described previously (Schreibmayer et al., 1994). Voltage jumps were delivered and resulting currents were simultaneously recorded by a TL-1-125 interface connected to a standard PC, using PClamp 5.0 software (Axon Instruments; Burlingame; USA). Cumulative dose response curves for the effects of both tolperisone and lidocaine on I_{pNa+} were performed: starting from a holding potential E_{h1}, sodium currents were elicited at constant frequency (f) by a suprathreshold voltage jump to the command potential (E_{com1}) of duration t_1. In order to make the resulting I_{pNa+} sensitive to eventual changes in excitation threshold, inactivation, and recovery from inactivation, these parameters varied for each isoform studied as listed here: E_{h1} (mV)/f (Hz)/E_{com1} (mV)/t_1 (ms): $Na_{v1.2}$, $Na_{v1.8}$: −70/0.1/+20/155; $Na_{v1.3}$, $Na_{v1.7}$: −100/0.1/0/155; $Na_{v1.4}$: −100/0.1/+20/155; $Na_{v1.5}$: −70/0.14/−20/62; $Na_{v1.6}$: −100/0.1/−5/155. At the end, washout of the drug was tested by superfusion with regular ND96 solution. In another set of experiments voltage dependent sodium channel kinetics were quantified, first under control conditions and then in the presence of drug at a concentration close to the empirically determined IC_{50}. These concentrations were (in μmol/L; tolperisone/lidocaine): $Na_{v1.2}$: 100/100; $Na_{v1.3}$: 700/600; $Na_{v1.4}$: 100/300; $Na_{v1.5}$: 100/300; $Na_{v1.6}$: 250/500; $Na_{v1.7}$: 350/500; $Na_{v1.8}$: 100/100. Voltage jump protocols were optimized in order to start and to finish with apparent equilibrium between channel states. Voltage dependent activation was evaluated by keeping the oocyte membrane potential constant at E_{h2} for t_2. Then, the potential was stepped to various command (E_{com3}) potentials in 5 mV increments for the duration of t_3. These parameters were as follows: (E_{h2} (mV)/t_2 (s)/E_{com3} (mV; 1st pulse to last pulse)/t_3 (ms): $Na_{v1.2}$, $Na_{v1.4}$, $Na_{v1.5}$, $Na_{v1.6}$: −120/6/−60 to +40/182; $Na_{v1.3}$, $Na_{v1.7}$: −120/6/−40 to +50/182; $Na_{v1.8}$: −100/3/−20 to + 70/182. Assessment of steady-state inactivation was performed by keeping the membrane potential constant at E_{h4} for the time t_4. Thereafter, steady-state inactivation was allowed to proceed during t_5, to reach equilibrium, at various command potentials E_{com5}, in 5 mV increments. Finally, available I_{pNa+} was elicited by a suprathreshold pulse to E_{com6}. The parameter set for steady-state inactivation of the different isoforms was as follows: (E_{h4} (mV)/t_4 (s)/t_5 (s)/E_{com5} (mV; 1st pulse–last pulse)/E_{com6} (mV)): $Na_{v1.2}$, $Na_{v1.5}$: −120/6/1/−120 to −50/−10; $Na_{v1.3}$: −120/6/1/−100 to +5/+10; $Na_{v1.4}$, $Na_{v1.6}$: −120/6/1/−115 to −45/−10; $Na_{v1.7}$: −100/6/1/−90 to −25/0; $Na_{v1.8}$: −100/6/1/−80 to −5/+20. Recovery from inactivation was measured by keeping the potential constant at E_{h7} for the time span t_7, to allow for complete recovery, then steady-state inactivation was allowed to proceed for t_8 at E_{com8} to yield channels in both the fast and the slow inactivated state. Finally, recovery was allowed at −100 mV for a time interval that was incremented at a quasi-logarithmical scale (1/2/5/10/20/50/100/200/500/1000/2000/5000/10,000/20,000/50,000 (ms)), before available I_{pNa+} was elicited by a suprathreshold pulse to E_{com9}. The following parameter values were used for the different isoforms: (E_{h7} (mV)/t_7 (s)/t_8 (ms)/E_{com8} (mV)/E_{com9} (mV)): $Na_{v1.2}$, $Na_{v1.3}$, $Na_{v1.5}$:−120/6/3000/0/−10; $Na_{v1.4}$, $Na_{v1.6}$: −120/6/50/0/−10; $Na_{v1.7}$: −120/6/3/+20/0; $Na_{v1.8}$: −90/6/100/+20/+20.

2.2. Molecular biology

Plasmid vectors were grown in bacteria, isolated, and linearized using standard procedures (Sambrook and Russel, 2001). cRNA was synthesized as described (Dascal and Lotan, 1992). Plasmid vectors containing full-length inserts encoding the appropriate voltage-dependent sodium channel isoform were used as described previously: α-subunits: $Na_{v1.2}$ (Auld et al., 1990), $Na_{v1.3}$ (Patton et al., 1994), $Na_{v1.4}$ (Trimmer et al., 1989), $Na_{v1.5}$ (Gellens et al., 1992), $Na_{v1.6}$ (Smith et al., 1998), $Na_{v1.7}$ (Klugbauer et al., 1995) and $Na_{v1.8}$ (Akopian et al., 1996). β_1-subunit (Wallner et al., 1993). Alignments of the primary structure of the different *vdsc* isoforms were produced using the Wisconsin Package, version 10.3 (Accelrys Inc., San Diego CA, USA).

2.3. Solutions (in mmol/L)

ND96: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, buffered with NaOH to pH 7.4; NDE: same as ND96, but contained 2.5 mmol/L pyruvate, 0.1% antibiotics (G-1937, 1000× stock; Sigma Chemical Co.) and 1.8 mmol/L $CaCl_2$.

[2] Part of this study has been communicated at the American Biophysical Society Meeting 2004 (Hofer et al., 2004).

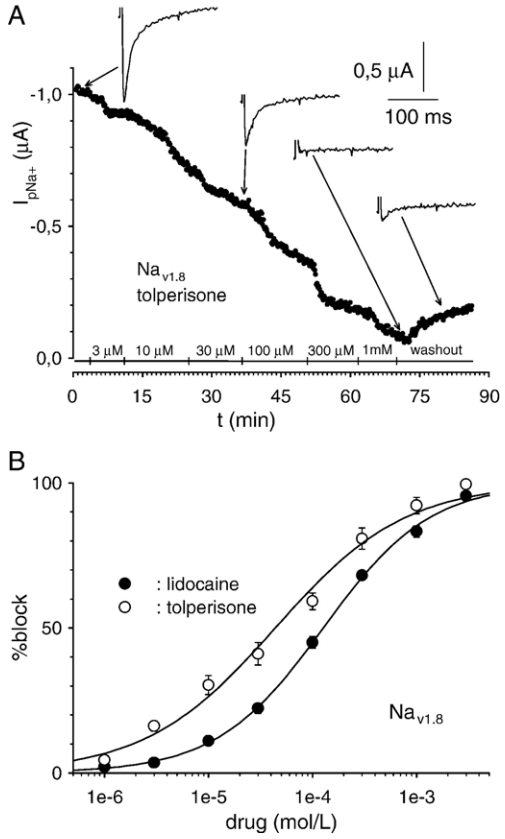

Fig. 1. Comparison of cumulative dose/response relations of tolperisone vs. lidocaine on the $Na_{v1.8}$ isoform. A: I_{pNa+} was measured following a suprathreshold voltage jump from −70 to +20 mV at a frequency of 0.1 Hz while increasing concentrations of tolperisone were applied. Note that washout of the drug was only partial. Representative original current recordings are shown. B: Cumulative dose/response relation for tolperisone (○) and lidocaine (●; $N=6$). Bars represent S.E.M. The difference in IC_{50} between the two drugs was significant at the $P<0.001$ level.

2.4. Chemicals

All chemicals used were reagent grade. Reagents for molecular biology were purchased from Promega or MBI Fermentas. Collagenase was Type IA from Sigma.

2.5. Cell culture

Prior to surgery frogs were placed for 10–15 min in MS 222 for anesthesia. When the frog did not respond any more to mechanical stimuli it was placed on its back and a ~5 mm long incision was made into the skin and the underlying abdominal fascia with sharp, small scissors. Small pieces of ovary, containing approx. 200–500 oocytes, were removed, put in CS and shaken at a low frequency (0.2–0.5 Hz) at room temperature (<22 °C). The CS solution was replaced approx. every 30 min, in order to avoid contamination by cell debris. When about 50% of the oocytes had lost their follicular layer the enzymatic disaggregation process was abandoned and the oocytes were washed several times with ND96Ca0 and then put in NDE. From oocytes still surrounded by the follicular layer, this layer was removed mechanically by sucking them into fire-polished, sterilized Pasteur pipettes. The following amounts of cRNA were injected one day after disaggregation (ng/oocyte): $Na_{v1.2}$: 0.05–0.25; $Na_{v1.3}$: 10–15; $Na_{v1.4}$: 0.25–1.5 (+5 ng cRNA encoding the β_1 subunit); $Na_{v1.5}$: 0.25–1.5; $Na_{v1.6}$: 2.5; $Na_{v1.7}$: 25–50; $Na_{v1.8}$: 50 (+5 ng cRNA encoding the β_1 subunit). Incubation of oocytes was performed in NDE solution at 19 °C for 3–5 days, prior to the electrophysiological recording.

2.6. Data evaluation and statistics

Original current traces were analysed using the Clampfit 9.0 software (Axon Instruments; Burlingame; USA). The following parameters were evaluated from the original traces:

2.6.1. Cumulative dose–response relations

I_{pNa+} as a function of drug concentration was fitted to the original data according to the following equation:

$$I_{pNa+} = I_{pNa+}^{max} \cdot \frac{1}{1 + 10^{(\log IC_{50} - \log[d]) \cdot n}} \quad (1)$$

where I_{pNa+}^{max} represents the full I_{pNa+}, in the absence of drug, IC_{50} the drug concentration required to achieve 50% block, [d] the drug concentration (in mol/L) and n the Hill slope of the dose–response curve.

Table 1
IC_{50} values for tolperisone and lidocaine on different sodium channel isoforms

	Tolperisone			*Lidocaine*		
	IC_{50} (µmol/L)	n	*N*	IC_{50} (µmol/L)	n	*N*
$Na_{v1.2}$	116 ± 45	0,89 ± 0,05	5	68 ± 12	0,77 ± 0,04	5
$Na_{v1.3}$	802 ± 162	1,11 ± 0,18	5	687 ± 178	1,19 ± 0,39	5
$Na_{v1.4}$	96 ± 18	0,93 ± 0,05	7	289 ± 90	1,03 ± 0,35	5
$Na_{v1.5}$	131 ± 363	0,77 ± 0,05	7	109 ± 52	0,80 ± 0,09	6
$Na_{v1.6}$	326 ± 88[a]	0,85 ± 0,11[a]	5	612 ± 103	1,30 ± 0,10	5
$Na_{v1.7}$	394 ± 66[a]	1,10 ± 0,02[a]	5	614 ± 42	1,30 ± 0,06	5
$Na_{v1.8}$	49 ± 15[c]	0,70 ± 0,04[a]	6	128 ± 7	0,86 ± 0,06	6

Tolperisone

	$Na_{v1.2}$	$Na_{v1.3}$	$Na_{v1.4}$	$Na_{v1.5}$	$Na_{v1.6}$	$Na_{v1.7}$
$Na_{v1.3}$	b					
$Na_{v1.4}$	n.s.	b				
$Na_{v1.5}$	n.s.	b	n.s.			
$Na_{v1.6}$	n.s.	a	a	n.s.		
$Na_{v1.7}$	b	a	b	b	n.s.	
$Na_{v1.8}$	n.s.	b	a	n.s.	a	b

Lidocaine

	$Na_{v1.2}$	$Na_{v1.3}$	$Na_{v1.4}$	$Na_{v1.5}$	$Na_{v1.6}$	$Na_{v1.7}$
$Na_{v1.3}$	a					
$Na_{v1.4}$	a	n.s.				
$Na_{v1.5}$	n.s.	a	n.s.			
$Na_{v1.6}$	b	n.s.	a	b		
$Na_{v1.7}$	c	n.s.	a	c	n.s.	
$Na_{v1.8}$	b	a	n.s.	n.s.	b	c

Mean value of IC_{50} and slope of dose–response curves (n) are given +/− standard error of mean value (S.E.M.). *N* signifies the number of experiments. a, c: the mean values between tolperisone and lidocaine differed significantly at the $P<0.05$ and 0.001 levels, respectively, between identical isoforms.
Insets: IC_{50} values for tolperisone (*left*) or lidocaine (*right*) were tested for statistically significant differences between the different isoforms tested. a, b, c: The IC_{50} values differed significantly at the $P<0.05$, 0.01, and 0.001 levels, respectively, between the isoforms. *n.s.*: difference was not significant.

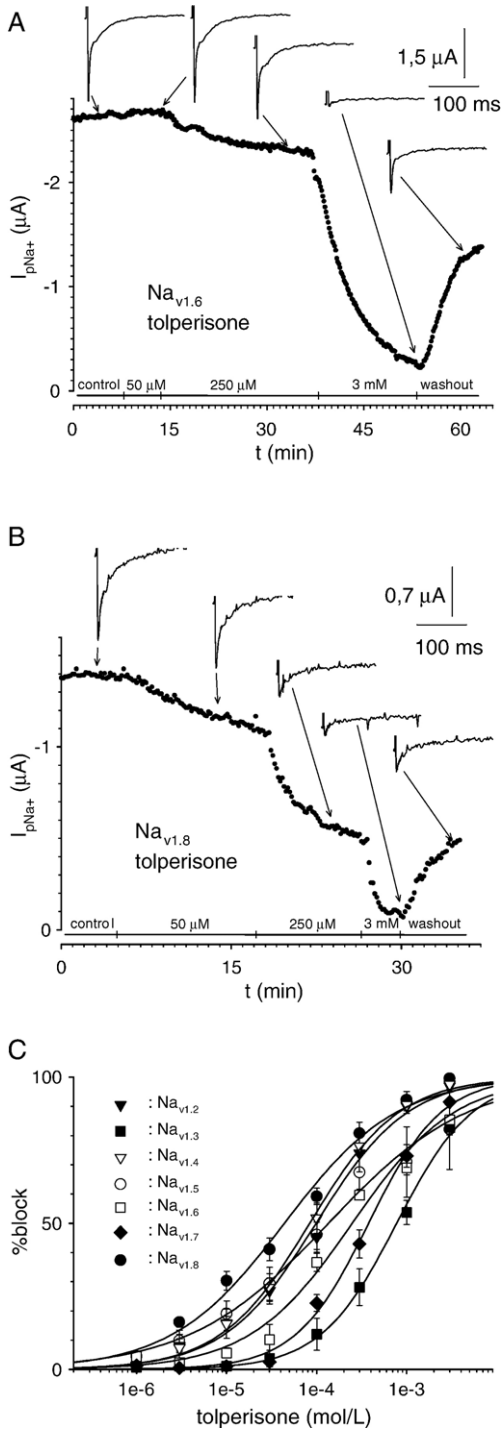

Fig. 2. Comparison of cumulative dose/response relations of tolperisone on the different voltage dependent sodium channels isoforms studied. A: I_{pNa+} was measured following a suprathreshold voltage jump from −100 to −5 mV at a frequency of 0.1 Hz while three concentrations of tolperisone (50 µmol/L, 250 µmol/L and 3 mmol/L) were applied successively to block $Na_{v1.6}$. Representative original current recordings are shown. B: I_{pNa+} was measured following a suprathreshold voltage jump from −70 to +20 mV at a frequency of 0.1 Hz while three concentrations of tolperisone, identical to the ones used in panel A (50 µmol/L, 250 µmol/L and 3 mmol/L), were applied to $Na_{v1.8}$. C: Cumulative dose/response relation for tolperisone on the different voltage dependent sodium channel isoforms studied. Bars represent S.E.M. See Table 1 for number of experiments and statistically significant differences.

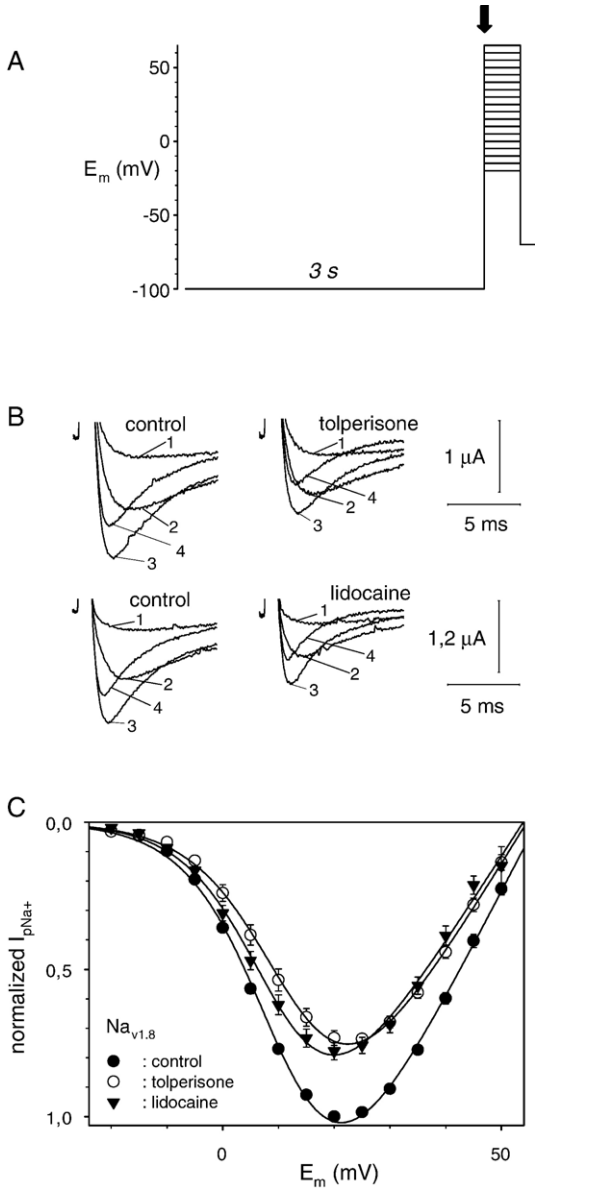

Fig. 3. Effect of tolperisone and lidocaine on the voltage dependent activation of $Na_{v1.8}$. A: Voltage jump protocol: The oocyte membrane was held at −100 mV for 3 s and then stepped to various test potentials in 5 mV increments to elicit I_{pNa+} (indicated by arrow). B: Representative original current recordings under control conditions (*left*) and under the influence of 100 µmol/L tolperisone (*right*, *upper*) and 100 µmol/L lidocaine (*right*, *lower*). Test potential was, 1: −5 mV; 2: +5 mV; 3: +25 mV and 4: +35 mV, respectively. C: Normalized and averaged I_{pNa+}/E_m relations for control conditions (●), 100 µmol/L tolperisone (○), and 100 µmol/L lidocaine (▼). Bars represent S.E.M. Solid lines represent a least square fit to the data according to a Boltzmann isotherm.

2.6.2. Voltage dependent activation

I_{pNa+} as a function of command potential (E_{com3}) was fitted to the original data according to the following equation:

$$I_{pNa+} = G_{max} \cdot (E_{com3} - E_{rev}) \cdot \left(1 - \frac{1}{1 + e^{\frac{E_{com3} - Ea_{50}}{ka}}}\right) \quad (2)$$

where, G_{max} represents the maximum sodium conductance of the oocyte, E_{rev} the reversal potential of the sodium current,

Table 2
Effect of tolperisone and lidocaine on voltage dependent activation

		$Ea_{0.5}$ (mV)	ka (mV^{-1})	N
$Na_{v1.2}$	Control	−18.4±1.3	4.85±0.34	16
	Tolperisone	−16.5±1.8	5.36±0.47	7
	Lidocaine	−17.0±2.2	4.84±0.40	9
$Na_{v1.3}$	Control	−10.6±1.4	3.80±0.17	10
	Tolperisone	−6.1±0.4[b/α]	4.64±0.28[a]	5
	Lidocaine	−9.4±1.3	4.10±0.23	5
$Na_{v1.4}$	Control	−23.0±1.3	4.28±0.36	12
	Tolperisone	−20.2±2.0	4.25±0.30	11
	Lidocaine	−20.8±1.1	5.02±0.38	8
$Na_{v1.5}$	Control	−24.5±0.5	6.24±0.16	15
	Tolperisone	−22.3±1.0	6.41±0.28	12
	Lidocaine	−23.7±0.6	6.49±0.33	8
$Na_{v1.6}$	Control	−17.9±1.7	5.92±0.34	14
	Tolperisone	−13.1±2.1	6.67±0.30[α]	6
	Lidocaine	−17.3±2.1	5.49±0.41	8
$Na_{v1.7}$	Control	−10.6±0.9	5.63±0.13	16
	Tolperisone	−9.2±0.7	6.07±0.14[a]	9
	Lidocaine	−9.7±1.1	5.89±0.17	7
$Na_{v1.8}$	Control	11.3±0.5	7.19±0.10	15
	Tolperisone	13.3±1.0[a]	7.07±0.31	11
	Lidocaine	11.6±0.7	7.32±0.07	8

Mean values of potential of half maximum activation ($Ea_{0.5}$) and slope of Boltzmann isotherm (ka) are given±S.E.M. N signifies the number of experiments. [a, b] The mean value differed significantly at the $P<0.05$ and 0.01 levels, respectively, from control conditions. [α] The mean values between tolperisone and lidocaine differed significantly at the $P<0.05$ level.

$Ea_{0.5}$ the potential where the steady-state activation achieves 50% and ka the slope of the Boltzmann isotherm describing steady-state activation.

2.6.3. Steady-state inactivation

For the pulse sequence used to measure steady-state inactivation, I_{pNa+} as a function of prepulse test potential (E_{com5}) was fitted to the original data according to the following equation:

$$I_{pNa+} = I_{pNa+}^{max} \cdot \frac{1}{1 + e^{\frac{E_{com5} - Ei_{0.5}}{ki}}} \quad (3)$$

where I_{pNa+}^{max} represents, in this case, the maximum sodium peak current when all sodium channels are available, $Ei_{0.5}$ the potential where the steady-state inactivation achieves 50% and ki the slope of the Boltzmann isotherm describing steady-state inactivation.

2.6.4. Recovery from inactivation

I_{pNa+} as a function of duration of recovery time interval (Δt) was fitted to the original data as the sum of two exponentials according to the following equation:

$$I_{pNa+} = I_1^{max} \cdot e^{-\frac{\Delta t}{\tau_1}} + I_2^{max} \cdot e^{-\frac{\Delta t}{\tau_2}} \quad (4)$$

where I_1^{max} and I_2^{max} are the maximal sodium currents of the fast and the slow component, respectively, when recovery is complete. τ_1 and τ_2 are the time constants for the fast and the slow recovery processes. The fraction of channels exhibiting fast recovery (f_1) was calculated accordingly:

$$f_1 = \frac{I_1^{max}}{I_1^{max} + I_2^{max}} \quad (5)$$

The unpaired Student's t-test (Sigmastat 9.0; Jandel Scientific) was used for the comparison of experimental parameters obtained under different conditions.

3. Results

Dose dependent action of tolperisone and lidocaine on the different sodium channel isoforms expressed in *Xenopus laevis* oocytes was investigated. Cumulatively increasing concentrations of the drug were added to the bath, while I_{pNa+} was monitored. In Fig. 1, original recordings taken from the $Na_{v1.8}$ isoform, under the influence of augmented doses of tolperisone are shown. Onset of action of both drugs was within a few minutes for all the isoforms under investigation. In general, washout of the substance was possible, but never complete. Under otherwise identical experimental paradigms, marked differences between the effects of the two drugs on identical isoforms were found (see Fig. 1B, for example). IC_{50}s were in the μmol/L range (exact values are given in Table 1). The differences in IC_{50}s between tolperisone and lidocaine was statistically significant for the neuronal $Na_{v1.6}$, $Na_{v1.7}$ and $Na_{v1.8}$ isoforms. Tolperison exerted 50% block at lower doses, when

Table 3
IC_{50} values for the tonic component of block, exerted via tolperisone and lidocaine, on voltage dependent sodium channel isoforms

	Tolperisone		*Lidocaine*	
	IC_{50} (mmol/L)	N	IC_{50} (mmol/L)	N
$Na_{v1.2}$	1,22 ± 0,57	7	2,64 ± 0,73	8
$Na_{v1.3}$	1,44 ± 0,15[c]	8	2,73 ± 0,28	6
$Na_{v1.4}$	2,64 ± 1,19	8	2,60 ± 0,54	6
$Na_{v1.5}$	0,61 ± 0,08[a]	9	2,48 ± 0,81	8
$Na_{v1.6}$	1,07 ± 0,60	6	9,54 ± 4,78	8
$Na_{v1.7}$	0,78 ± 0,13[c]	9	2,40 ± 0,28	7
$Na_{v1.8}$	0,40 ± 0,10	10	0,72 ± 0,15	4

Tolperisone

	$Na_{v1.2}$	$Na_{v1.3}$	$Na_{v1.4}$	$Na_{v1.5}$	$Na_{v1.6}$	$Na_{v1.7}$
$Na_{v1.3}$	n.s.					
$Na_{v1.4}$	n.s.	n.s.				
$Na_{v1.5}$	n.s.	c	n.s.			
$Na_{v1.6}$	n.s.	n.s.	n.s.	n.s.		
$Na_{v1.7}$	n.s.	b	n.s.	n.s.	n.s.	
$Na_{v1.8}$	n.s.	c	n.s.	n.s.	n.s.	a

Lidocaine

	$Na_{v1.2}$	$Na_{v1.3}$	$Na_{v1.4}$	$Na_{v1.5}$	$Na_{v1.6}$	$Na_{v1.7}$
$Na_{v1.3}$	n.s.					
$Na_{v1.4}$	n.s.	n.s.				
$Na_{v1.5}$	n.s.	n.s.	n.s.			
$Na_{v1.6}$	n.s.	n.s.	n.s.	n.s.		
$Na_{v1.7}$	n.s.	n.s.	n.s.	n.s.	n.s.	
$Na_{v1.8}$	n.s.	c	a	n.s.	n.s.	b

Mean values for IC_{50}s of the tonic component of block, calculated from the fractional change in G_{max} (±S.E.M.). N signifies the number of experiments. [a, c] The mean values between tolperisone and lidocaine differed significantly at the $P<0.05$ and 0.001 levels respectively.
Insets: IC_{50} values of the tonic component of block for tolperisone (*left*) or lidocaine (*right*) were tested for statistically significant differences between the different isoforms tested. [a, b, c] The IC_{50} values differed significantly at the $P<0.05$, 0.01, and 0.001 levels, respectively, between the corresponding isoforms. *n.s.*: difference was not significant.

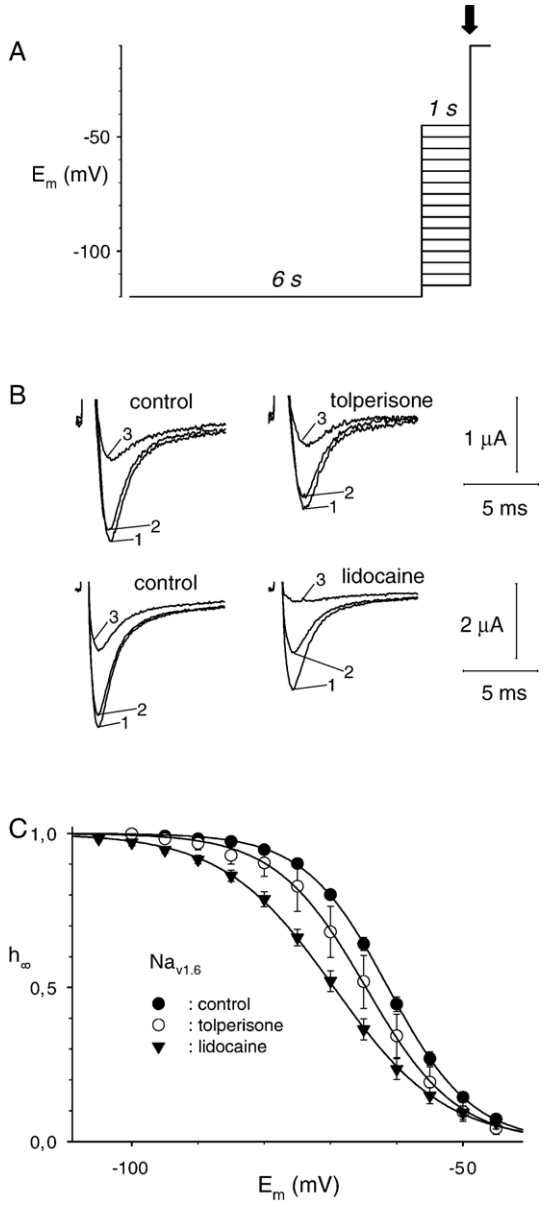

Fig. 4. Steady-state inactivation of $Na_{v1.6}$. A: Voltage jump protocol: The oocyte membrane was held at −120 mV for 6 s to allow for recovery, then steady-state inactivation was allowed to proceed for 1 s at various test potentials (in 5 mV increments) and subsequently available I_{pNa+} was elicited by a suprathreshold pulse to −10 mV. B: Representative original current recordings under control conditions (*left*) and under the influence of 250 µmol/L tolperisone (*right*, *upper*) and 500 µmol/L lidocaine (*right*, *lower*). Test potential was, 1: −100 mV; 2: −75 mV and 3: −60 mV, respectively. C: Average voltage dependence of h_8 for control conditions (●), 250 µmol/L tolperisone (○), and 500 µmol/L lidocaine (▼). Bars represent S.E.M. Solid lines represent a least square fit according to a Boltzmann isotherm.

compared to lidocaine. Not only was there a difference between the two drugs on identical isoforms, but the IC_{50} for the same drug differed between different isoforms. When, for example, three selected concentrations of tolperisone were applied sequentially to oocytes expressing either the $Na_{v1.6}$ or the $Na_{v1.8}$ proteins, clear differences in their blocking abilities could be observed (Fig. 2A and B). Dose response relations for tolperisone on all seven isoforms studied are shown in Fig. 2C (see insets in Table 1 for statistical significant differences between the different isoforms).

In order to study the interaction of both tolperisone and lidocaine with voltage dependent sodium channel isoforms, concentrations leading to partial inhibition were applied and the change in kinetic parameters monitored in detail. In order to obtain a molecularly valid measurement for comparison of drug action, optimized experimental paradigms were used in order to start with fully resting channels for each individual isoform (see Materials and methods section for details of paradigms). A typical experiment for the assessment of voltage dependent activation is shown in Fig. 3. Under the influence of both drugs peculiar effects were observed. Voltage dependent activation itself was hardly affected by both drugs, other than small, but significant effects of tolperisone on the threshold of $Na_{v1.3}$ and $Na_{v1.8}$ channels (see Table 2 for comparison of half maximal activation potential, slope of Boltzmann isotherm and statistics). On the other hand, G_{max} was significantly reduced under the influence of both drugs (with the exception of lidocaine on $Na_{v1.2}$; see Table 3 for exact values and statistics). This fraction of the block is independent of the voltage jump protocol and, hence, represents the fraction of channels that are permanently blocked at a given drug concentration. Therefore this fraction of block is termed "*tonic block*." Compared to lidocaine, the extent of this tonic block on the same isoform was significantly stronger for tolperisone ($Na_{v1.2}$, $Na_{v1.3}$, $Na_{v1.7}$ and $Na_{v1.8}$).

Steady-state inactivation was assessed using another voltage pulse protocol. Interestingly, the potential of half maximal steady-state inactivation ($Ei_{0.5}$) was shifted to more negative potentials for some drug/$Na_{v1.x}$ combinations, but not in all

Table 4
Tolperisone vs. lidocaine and steady-state inactivation

		$Ei_{0.5}$ (mV)	ki (mV^{-1})	N
$Na_{v1.2}$	Control	−46.8±0.9	7.24±0.26	16
	Tolperisone	−51.7±1.0[b]	7.60±0.45	7
	Lidocaine	−49.5±2.6	7.80±0.48	9
$Na_{v1.3}$	Control	−26.1±1.0	7.75±0.24	10
	Tolperisone	−30.8±1.9[a]	10.38±0.33[c]	5
	Lidocaine	−26.7±1.1	9.28±0.51[a]	5
$Na_{v1.4}$	Control	−41.1±2.15	4.38±0.21	12
	Tolperisone	−52.6±3.4[b/α]	4.60±0.11[α]	11
	Lidocaine	−43.4±1.3	4.06±0.18	8
$Na_{v1.5}$	Control	−71.7±0.8	5.72±0.14	15
	Tolperisone	−72.6±1.4	6.11±0.30	12
	Lidocaine	−79.6±1.3[c]	6.16±0.27	8
$Na_{v1.6}$	Control	−60.8±0.7	6.28±0.26	14
	Tolperisone	−65.2±2.0	6.71±0.59	6
	Lidocaine	−69.4±1.0[c]	8.36±0.77[a]	8
$Na_{v1.7}$	Control	−62.6±0.6	7.47±0.20	16
	Tolperisone	−68.0±1.3[b]	8.97±0.22[c]	9
	Lidocaine	−69.4±1.5[b]	9.50±0.36[c]	7
$Na_{v1.8}$	Control	−37.5±1.5	12.45±0.51	15
	Tolperisone	−40.0±1.7	12.90±1.13	11
	Lidocaine	−42.0±0.9[a]	12.11±1.00	8

Mean values of potential of half maximum steady-state inactivation ($Ei_{0.5}$) and slope of Boltzmann isotherm (ki) are given±S.E.M. N signifies the number of experiments. [a, b, c] The mean values differed significantly at the $P<0.05$, 0.01, and 0.001 levels, respectively, from control conditions. [α] The mean values between tolperisone and lidocaine differed significantly at the $P<0.05$ level.

cases (see Fig. 4 and Table 4 respectively). For tolperisone, this shift ranged from −11.5 mV ($Na_{v1.4}$) to −4.7 mV ($Na_{v1.3}$), whereas for lidocaine it ranged between −8.6 mV ($Na_{v1.6}$) and −4.5 mV ($Na_{v1.8}$). This shift of $Ei_{0.5}$ to more negative potentials was significantly less pronounced for lidocaine, when compared to tolperisone with respect to the $Na_{v1.4}$ isoform. Moderate, but significant, changes in the steepness of the Boltzmann isotherm also were observed in some instances (see Table 4).

A major principle of action of voltage dependent sodium channel specific drugs is their effect on the recovery process at negative potentials, leading to an increase in refractoriness in most cases. Therefore, both the recovery from fast and slow inactivation was measured under the influence of the two drugs. The analysis resulted in two time constants, one for the fast and another one for the slow recovery process (with the exception of $Na_{v1.4}$, where the experimental paradigm resulted only in the fast recovery process). In addition, the contribution of the fast process to total recovery was also measured. Most notably, both substances exerted profound effects on the recovery process, with the exception of $Na_{v1.4}$, where the applied doses proved to be ineffective with respect to the recovery process (although the dose produced significant overall block in $Na_{v1.4}$). Regarding the fast process of recovery, tolperisone was unable to induce significant changes in this particular time constant, whereas lidocaine produced a statistically significant increase in the time constant for the $Na_{v1.3}$, $Na_{v1.5}$, $Na_{v1.7}$ and to some extent also of the $Na_{v1.8}$ proteins (see Table 5). This increase in the fast time constant amounted for up to an approximately *35-fold* deceleration in the case of the $Na_{v1.5}$ channel, when compared to control conditions (see also Fig. 5D). For the other voltage dependent sodium channel isoform subunits, lidocaine, like tolperisone, did not produce statistically significant effects on the fast time constant of recovery. With respect to the fast recovery time constant, the differences between lidocaine and tolperisone were statistically significant for the $Na_{v1.3}$, $Na_{v1.5}$ and $Na_{v1.7}$ isoform. The contribution of the fast time course to the fast recovery process (f_1) was significantly reduced by tolperisone for the $Na_{v1.3}$ and $Na_{v1.5}$ isoforms. Lidocaine, on the other hand, reduced f_1 significantly for $Na_{v1.5}$, $Na_{v1.6}$ and $Na_{v1.8}$. The difference in action on f_1 was significant for $Na_{v1.3}$, $Na_{v1.6}$ and $Na_{v1.8}$. Tolperisone, at the given concentration, resulted in a significant prolongation of the slow time course of recovery from inactivation (τ_2) for the $Na_{v1.2}$, $Na_{v1.5}$ and $Na_{v1.8}$ isoform. Similarly, lidocaine increased τ_2 for $Na_{v1.5}$ and $Na_{v1.8}$.

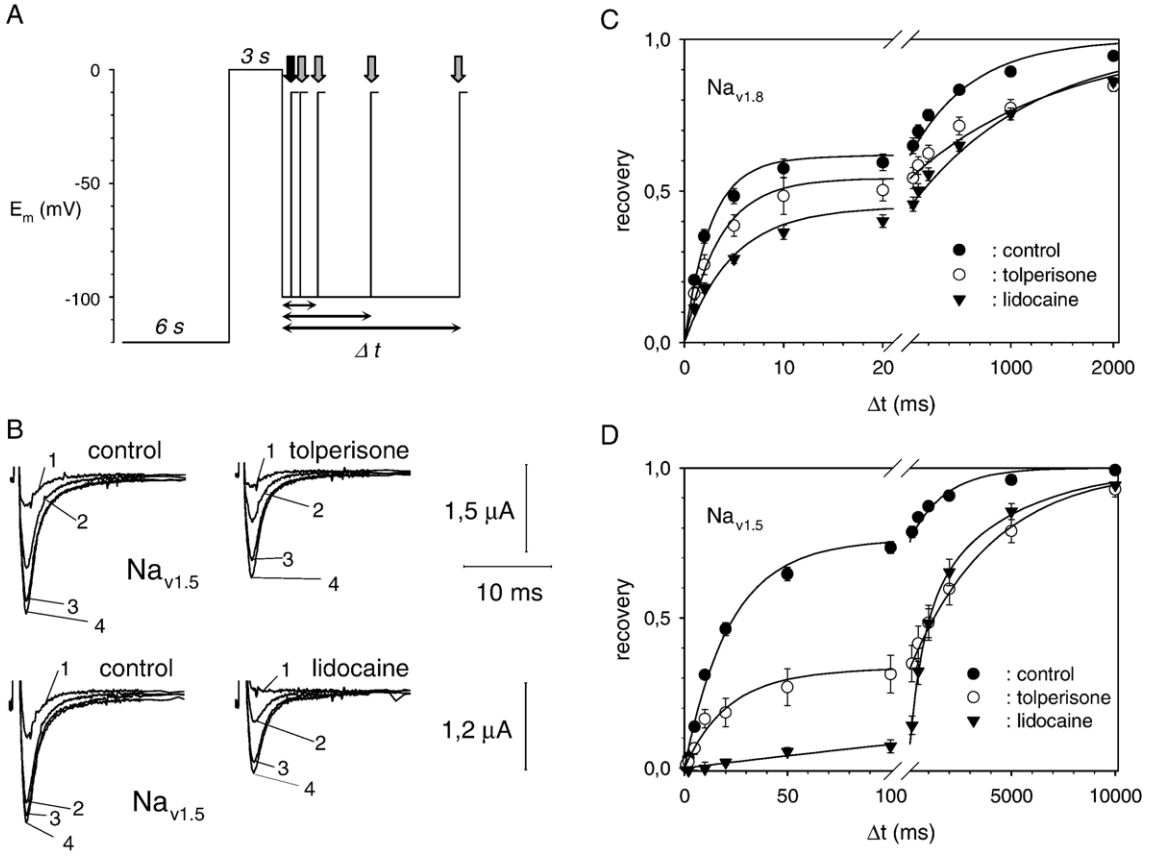

Fig. 5. Influence of tolperisone vs. lidocaine on the recovery from inactivation of $Na_{v1.5}$ and $Na_{v1.8}$. A: Voltage jump protocol: The oocyte membrane was held at −120 mV for 6 s to allow for recovery, then steady-state inactivation was allowed to proceed for 3 s at 0 mV to yield channels in both the fast and the slow inactivated states, then recovery was allowed at −100 mV for various time intervals before available I_{pNa+} was elicited by a suprathreshold pulse to −10 mV (arrows). B: Representative original current recordings from $Na_{v1.5}$ under control conditions (*left*) and under the influence of 100 µmol/L tolperisone (*right*, *upper*) and 300 µmol/L lidocaine (*right*, *lower*). Recovery time interval was, 1: 10 ms; 2: 500 ms; 3: 5 s and 4: 50 s, respectively. C: Average recovery of $Na_{v1.8}$ for control conditions (●), 100 µmol/L tolperisone (○), and 100 µmol/L lidocaine (▼). Bars represent S.E.M. Solid lines represent a biexponential fit. Time axis was split in two regions of different scaling to better visualize the domains of the fast and the slow recovery process. D: As in C, but for $Na_{v1.5}$.

Interestingly, both substances significantly decreased τ_2 for the $Na_{v1.7}$ isoform.

4. Discussion

Here we report that tolperisone acts, at micromolar concentrations, as an acute blocker of seven different isoforms of voltage dependent sodium channel, expressed in *Xenopus laevis* oocytes. The systematic comparison of the effects on different isoforms in the same cellular environment allows exact assessment of differences between drug action on divergent voltage dependent sodium channels. The drug concentrations needed to exert 50% block often differed markedly between different isoforms and also between tolperisone and lidocaine for the same isoform. The differences between voltage dependent sodium channel isoforms, however, may have been due to the different experimental paradigms, required to account for the distinct biophysical characteristics of the different sodium channels. Therefore, and in order to evaluate the contribution of distinct conformational transitions to overall block, a detailed kinetic analysis was carried out. Use-dependence of voltage dependent sodium channel blocking drugs is often a primary concern, regarding their therapeutic efficacy. On the other hand, use-dependence can lead to unwanted side-effects, such as proarrhythmogenicity (see for review: Errington et al., 2005; Yogeeswari et al., 2004; Vaughan Williams, 1989). Therefore drugs exerting a high degree of use independent, tonic block, may be useful for specific therapeutic applications, such as pain treatment. Interestingly, both tolperisone and lidocaine revealed considerable use-independent block of voltage dependent sodium channels, contributing significantly to overall voltage dependent sodium channel inhibition (accounted for by both use-dependent and use-independent block). The concentration required to achieve 50% tonic block was lower for tolperisone than for lidocaine, with the exception of at the skeletal muscle $Na_{v1.4}$ subunit, where both substances were approximately equipotent in this respect. Use-dependent drug binding generally results in a shift in the voltage dependence of steady-state inactivation to negative potentials (Hondeghem and Katzung, 1977). Both tolperisone and lidocaine exerted this negative shift in steady-state inactivation. In many cases this shift turned out to be statistically significant. The drug concentrations we used resulted in substantial partial block under our experimental conditions. They are likely to be in the range of acute therapeutic concentrations. It must be stated, however, that under these conditions, this shift in the steady-state inactivation curve will contribute only to a minor extent to overall inhibition.

Besides the use-independent, tonic block, the effect of both drugs on the recovery from inactivation was pronounced in our study. Normally, voltage dependent sodium channels exhibit recovery from inactivation in at least two different time domains: the first one occurs within milliseconds and is important for the availability of voltage dependent sodium channels from one action potential to the next. The second one occurs at much longer time intervals and is most likely responsible for the adjustment of voltage dependent sodium channel availability during prolonged trains of activity. When characterizing the mode of action of voltage dependent sodium channel blocking drugs, it is not only important to quantitate the influence of a drug on the time constants of both fast and slow recovery processes, but also whether the proportions of voltage dependent sodium channels displaying fast and slow recovery are affected by the drug. Generally, both tolperisone and lidocaine either reduced the fraction of voltage dependent sodium channels exerting fast recovery, or did not change it significantly in other voltage dependent sodium channel isoforms. Never did the fraction of voltage dependent sodium channels showing fast recovery increase in the presence of the two drugs. However, in three isoforms different behaviour between both drugs with respect to the fast recovery component was observed, lidocaine always being more effective in reducing the fraction of voltage dependent sodium channels displaying fast recovery. Most interestingly, the time constant of the fast recovery process was markedly prolonged for three voltage dependent sodium channel isoforms, exclusively by lidocaine, while tolperisone did not produce a significant prolongation of the fast recovery process. Bean and coworkers (Bean et al., 1983) reported for the first time that the fast recovery from inactivation of cardiac sodium currents is greatly prolonged by lidocaine. Here we report that the fast recovery process of two other neuronal voltage dependent sodium channel isoforms also is greatly prolonged by this drug. Moreover, this substantial influence on voltage dependent sodium channel gating is peculiar for lidocaine, since tolperisone was unable to produce such an effect. The time

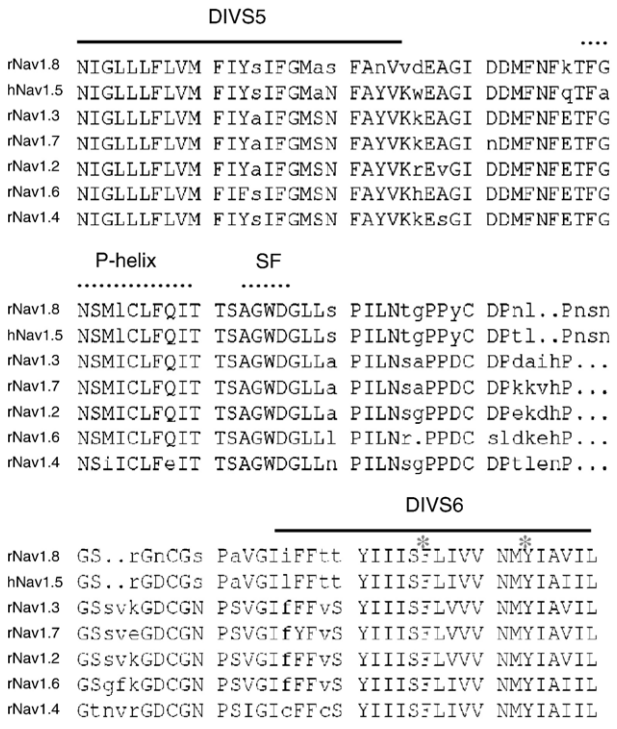

Fig. 6. Alignment of selected regions of the different voltage dependent sodium channel isoforms studied, comprising the local anesthetic receptor in the IVth transmembrane domain. *DIVS5:* S5 helix of transmembrane domain IV. *DIVS6*: S5 helix of transmembrane domain IV. *P-helix:* location of putative P-helix. *SF:* region of amino acids essentially entangled in the formation of the selectivity filter. Conserved amino acids are set in uppercase letters.

Table 5
Tolperisone vs. lidocaine and the recovery from inactivation

	τ_1 (ms)			τ_2 (s)			f_1			N
	Control	Tolperisone	Lidocaine	Control	Tolperisone	Lidocaine	Control	Tolperisone	Lidocaine	
$Na_{v1.2}$	14.2±3.3	35.5±15.0	17.3±7.0	0.75±0.86	3.31±0.48$^{b/\beta}$	1.23±0.17	0.42±0.03	0.37±0.03	0.35±0.04	14/5/9
$Na_{v1.3}$	196.3±20.0	187.4±21.1	366.8±55.5$^{a/\alpha}$	5.94±0.56	9.33±2.28	7.25±2.48	0.67±0.02	0.50±0.02$^{c/\beta}$	0.62±0.02	10/5/5
$Na_{v1.4}$	4.0±0.3	3.7±0.3	4.8±0.4	*n.d.*	*n.d.*	*n.d.*	–	–	–	15/8/7
$Na_{v1.5}$	23.1±1.4	24.4±4.1	802.2±160.8$^{b/\beta}$	1.85±0.26	4.24±0.36^{c}	4.76±0.62^{b}	0.74±0.02	0.25±0.03^{c}	0.39±0.08^{b}	20/13/7
$Na_{v1.6}$	7.9±0.8	6.1±0.8	9.0±1.8	1.89±0.26	2.41±0.34$^{\beta}$	1.27±0.19	0.77±0.03	0.57±0.09$^{\alpha}$	0.40±0.04^{c}	14/6/8
$Na_{v1.7}$	28.9±2.3	31.1±4.3	131.0±24.9 $^{b/\beta}$	8.86±0.24	5.85±0.33 $^{c/\alpha}$	6.94±0.33^{c}	0.34±0.02	0.27±0.03	0.26±0.03	15/9/6
$Na_{v1.8}$	2.8±0.2	4.6±1.1	4.8±0.4^{a}	0.62±0.07	1.60±0.12$^{c/\alpha}$	1.22±0.03^{c}	0.60±0.05	0.54±0.04$^{\beta}$	0.44±0.02^{a}	18/10/8

Mean values of time constants for fast (τ_1) and slow (τ_2) phases of recovery from inactivation are given±S.E.M. f_1 denotes the contribution of fast recovery from inactivation to the entire recovery process (±S.E.M.). *N* signifies the number of experiments for each experimental condition (control/tolperisone/lidocaine). $^{a, b, c}$ The mean values differed significantly from control at the $P<0.05$, 0.01, and 0.001 levels, respectively. $^{\alpha, \beta}$ The mean values differed significantly between tolperisone and lidocaine at the $P<0.05$ and 0.01 levels, respectively. *n.d.:* slow recovery process could not be detected under the given experimental conditions.

constant of recovery from the slow inactivated state was differentially affected by the drugs. Shortening, prolongation and no influence have been observed in different isoforms using the two agents.

Regarding this basic difference of lidocaine, affecting fast recovery from inactivation on different isoforms tested and also the differences between lidocaine and tolperisone, a closer look on the receptor site for local anesthetics, antiarrhytmics and anticonvulsants is of interest. A phenylalanine and a tyrosine in the S6 helix of the fourth transmembrane domain have been identified to contribute crucially to drug binding (Ragsdale et al., 1994, 1996). These two amino acids are conserved throughout the voltage dependent sodium channel isoforms tested and, hence, cannot account directly for the differences observed between the isoforms. More likely the surroundings of the receptor site may contribute to the differential effect of lidocaine on recovery from inactivation. It is clearly evident by looking at Fig. 6, that the S6 helix of DIV is stringently conserved. On the other hand, the voltage dependent sodium channel isoforms exert the greatest degree of sequence diversity in the region between the selectivity filter and DIVS6. Interestingly in this context, a "foot in the door" mechanism for local anesthetic binding to voltage dependent sodium channels recently has been suggested, where drug binding impairs closure of the inner vestibule of voltage dependent sodium channels (Sandtner et al., 2004). Therefore at least one additional surface of interaction between these drug and voltage dependent sodium channel isoforms is likely. Based on our findings, we consider that this non-conserved region may provide the structural basis for differential lidocaine effects on fast inactivation. The considerable difference between the two drugs, with respect to the prolongation of recovery from inactivation for the $Na_{v1.3}$, $Na_{v1.5}$ and $Na_{v1.7}$ isoforms, may be attributed to their different structures, allowing or not allowing interaction with this surface.

Both lidocaine and tolperisone are drugs routinely used for therapy, but with different indications. From the physician's point of view, both substances could exert desirable effects and undesirable side effects by altering voltage dependent sodium channel gating. Desirable effects could be the block of ectopic firing of nociceptors and the antagonism of wind up in the central nervous system leading to increased pain perception. Both substances have blocking effects (use dependent and tonic) on voltage dependent sodium channels while their efficacies on pain relevant isoforms are different. Interestingly, especially $Na_{v1.8}$ is affected which is known to be up-regulated in an animal model of neuropathic pain (Chung and Chung, 2004). The strong effect of lidocaine on the fast recovery process of $Na_{v1.5}$ may implicate a more pronounced tendency to cardiac side effects under lidocaine treatment. This has to be elucidated in clinical studies comparing both substances in regard to their effectiveness and undesirable side effects when applied in chronic pain conditions. The comparison of interaction with voltage dependent sodium channel isoforms may provide a solid basis for the refinement of such therapeutic concepts.

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