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A mutation in the local anaesthetic binding site abolishes toluene effects in sodium channels

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Abstract

Toluene is a solvent of abuse that inhibits cardiac sodium channels in a manner that resembles the action of local anaesthetics. The purpose of this work was to analyze toluene effects on skeletal muscle sodium channels with and without β_1 subunit ($Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$, respectively) expressed in *Xenopus laevis* oocytes and to compare them with those produced in the F1579A mutant channel lacking a local anaesthetic binding site. Toluene inhibited $Na_v1.4$ sodium currents (IC_{50}=2.7 mM in $Na_v1.4+\beta_1$ and 2.2 mM in $Na_v1.4-\beta_1$) in a concentration dependent way. Toluene (3 mM) blocked sodium currents in $Na_v1.4$ channels proportionally throughout the entire current–voltage relationship producing inactivation at more negative potentials. Minimal inhibition was produced by 3 mM toluene in F1579A mutant channels. Recovery from inactivation was slower both in $Na_v1.4$ and F1579A channels in the presence of 3 mM toluene. The solvent blocked sodium currents in a use-dependent and frequency-dependent manner in $Na_v1.4$ channels. A single mutation in the local anaesthetic binding site of $Na_v1.4$ channels almost abolished toluene effects. These results suggest that this site is important for toluene action.

Keywords: Toluene; Sodium channel; Local anaesthetic; *Xenopus* oocyte; β_1 subunit

1. Introduction

Toluene is an organic solvent widely available in many commercial products such as thinners, paints and adhesives (Arlien-Søborg, 1992). It produces acute effects on the nervous system after exposure by inhalation (Evans and Balster, 1991) and is abused mainly by children and adolescents (Kurtzman et al., 2001). In recent years there has been an increasing interest in the study of the molecular action mechanisms of inhalants. Since the first description of toluene as an *N*-methyl-D-aspartate receptor inhibitor (Cruz et al., 1998), evidence has accumulated to show that this solvent acts on a wide variety of molecular targets. In 2000, Beckstead et al. showed that toluene, 1,1,1-trichloroethane and trichloroethylene significantly and reversibly enhanced neurotransmitter-activated ion currents in γ-aminobutyric acid ($GABA_A$) receptors expressed in *Xenopus* oocytes. More recently, Bale et al. (2002) found that toluene has inhibitory effects on different cholinergic nicotinic receptor subtypes in vitro, while Lopreato et al. (2003) reported that toluene, 1,1,1-trichloroethane and trichloroethylene increase ionic currents activated by serotonin through 5-HT_3 receptors.

The action of solvents of abuse on voltage-gated ion channels has also been a matter of investigation. According to Tillar et al. (2002), toluene inhibits voltage-sensitive calcium channels expressed in pheochromocytoma cells in a dose-dependent and reversible fashion. These effects occur at micromolar concentrations that do not compromise cell membrane integrity and are relevant to solvent abuse. Recently, we have reported that toluene inhibits cardiac sodium channels ($Na_v1.5$) with an IC_{50} of approximately 300 μM in *Xenopus* oocytes (Cruz et al., 2003). This effect could be responsible at least in part, for the well-described toluene arrhythmogenic actions (Bass, 1970; Taylor and Harris, 1970; Shepherd, 1989; Wilcosky and Simonsen, 1991; Einav et al., 1997). As far as we know, other sodium channel subtypes have not been studied.

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Toluene, like local anaesthetics, blocks sodium currents in a use-dependent and frequency-dependent manner (Hille, 2001). Interestingly, benzene, another aromatic solvent of abuse, and the local anaesthetic benzocaine, reduce sodium currents in squid giant axons (Hendry et al., 1985; Elliot et al., 1987). It is well documented that co-expression of β_1 subunit with rat brain and skeletal muscle α subunit in *Xenopus* oocytes changes both channel kinetics (Isom et al., 1992; Cannon et al., 1993) and sensitivity to local anaesthetics (Makielski et al., 1996).

Based on these findings, the purpose of this paper was to determine if toluene blocks sodium currents in skeletal muscle sodium channels ($Na_v1.4$), and if so, to investigate its molecular action site. For this purpose, we compared toluene's effects on skeletal sodium channels with and without β_1 subunit ($Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$) and on the mutant channel F1579A that lacks a local anaesthetic binding site (Wang et al., 1998), all of them expressed in *Xenopus laevis* oocytes.

2. Materials and methods

2.1. Animals

Adult *Xenopus laevis* female frogs (Xenopus I, Ann Arbor, MI, USA) were used in this study. Our local Animal Experimentation Ethics Committee approved all experimental procedures, which followed the regulations established in the Mexican official norm for the use and care of laboratory animals "NOM-062-ZOO-1999".

2.2. Drugs

Toluene (99.75%) was bought from Baker (Xalostoc, Mexico), alkamuls EL-620 (emulphor, ethoxylated castor oil) from Rhone-Poulenc (Princeton, NJ, USA), MS-222 (tricaine methanesulphonate), collagenase (type I), lidocaine, and other reagents from Sigma (St. Louis, MO, USA). Skeletal muscle ($Na_v1.4$) α-subunit cDNA (pGEM-11F, Promega; Madison, WI, USA), F1579A α-subunit cDNA (pGEM-11F), and β_1 subunit cDNA (pGW1H, British Biotechnology, London, UK) were used.

2.3. Oocyte preparation and microinjections

Frogs were anesthetized by immersion in 0.2% MS-222. Stage V and VI oocytes were surgically removed and placed in OR-2 buffer containing (in mM): 82.5 NaCl, 2.5 KCl, 2 $MgCl_2$, and 5 HEPES at pH 7.6, after which they were treated with collagenase (1.3 mg/ml) to remove the follicular membrane. Oocytes were coinjected into the nucleus with 2–10 ng of cDNA by a nanolitre automatic injector (model A203XVY; WPI, Sarasota, FL, USA). Three series of experiments were done: In the first, $Na_v1.4$ α subunit cDNA was co-injected with β_1 subunit cDNA (Cannon et al., 1993) at a ratio of 1:5 to

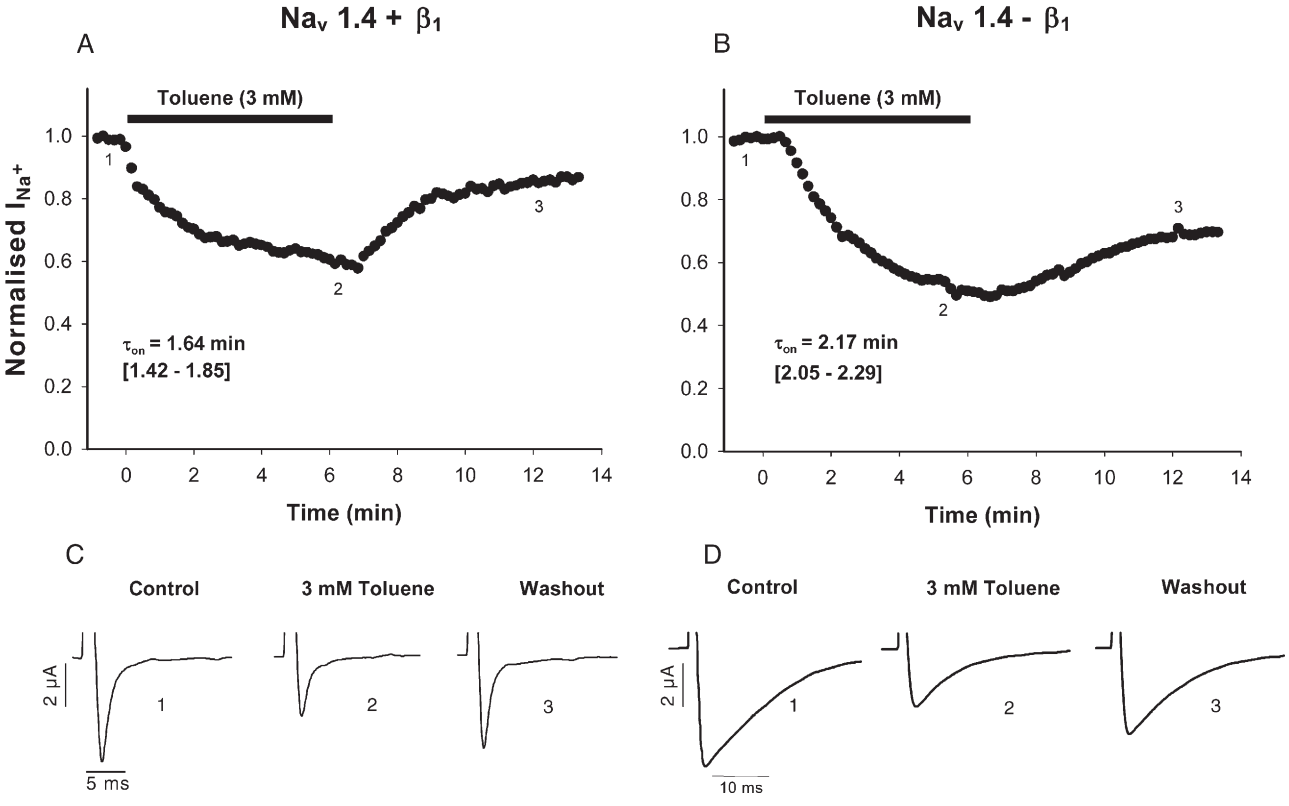

Fig. 1. Time course of 3 mM toluene inhibition of sodium current (I_{Na^+}) on $Na_v1.4+\beta_1$ (A) and $Na_v1.4-\beta_1$ channels (B). Each point represents the maximum current elicited by a 30-ms pulse to −20 mV from a holding potential of −100 mV. The stimulation frequency was 0.1 Hz. The bars indicate the time of toluene perfusion. (C and D) Sample currents of $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$ channels under three different conditions: control, with 3 mM toluene, and after washout.

reconstitute native channel behaviour. In the second, only the α subunit was used. In the third, the β_1 subunit was co-injected with the F1579A α subunit, which is a mutant isoform insensitive to local anaesthetics having alanine instead of phenylalanine at the 1579 amino acid position of domain IV's transmembrane segment S6 (Wang et al., 1998). Eggs were then maintained at 16 °C in ND-96 solution (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, and 1.8 $CaCl_2$, pH 7.6, supplemented with 0.5 mM theophylline, 2 mM pyruvate, and 50 μg/ml gentamycin for up to 3 days before recording.

2.4. Electrophysiological recordings in oocytes

Oocytes were placed in a 1.6-ml recording chamber and continuously superfused with a barium-containing solution at a flow rate of approximately 1 ml/min. Two-electrode voltage-clamp recordings were performed at room temperature (20–22 °C), using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA, USA). Electrodes pulled on a vertical pipette puller (model 700C, David Kopf Instruments, Tujunga, CA, USA) filled with agarose 0.8% and 3 M KCl (Schreibmayer et al., 1994) were used with a final resistance of 0.4–1.0 MΩ. Sodium current signals were digitised at a sampling rate of 10 kHz by an analog-to-digital converter (Digidata 1322A, Axon Instruments, Foster City, CA, USA) and stored in a computer for analysis with pClamp software (Version 8.1, Axon Instruments, Foster City, USA). Sodium currents were elicited by step depolarisations from a holding potential of −100 mV at an electrical stimulation frequency of 0.1 Hz, unless otherwise stated. Only oocytes with peak sodium currents lower than 7 μA were used in the present study to minimise voltage-clamp errors (Sah et al., 1998; Li et al., 1999). Several experimental protocols were used with the three Na^+ channel variants. Concentration–response curves were plotted based on peak sodium current values observed in current–voltage curves. These current–voltage curves were obtained measuring sodium currents elicited by 30 ms, 10 mV steps from a holding potential of −100 up to +50 mV. The voltage dependence of Na^+ channel steady-state inactivation was determined by a two-pulse protocol. A first variable voltage-conditioning pulse lasting 1000 ms was applied to inactivate different fractions of sodium channels. After 2 ms, a second 10-ms-long test pulse was applied to −20 mV. Data were normalised to the maximum sodium current recorded at this test pulse. Recovery from inactivation was examined by applying a 500-ms conditioning pulse to −20 mV from a

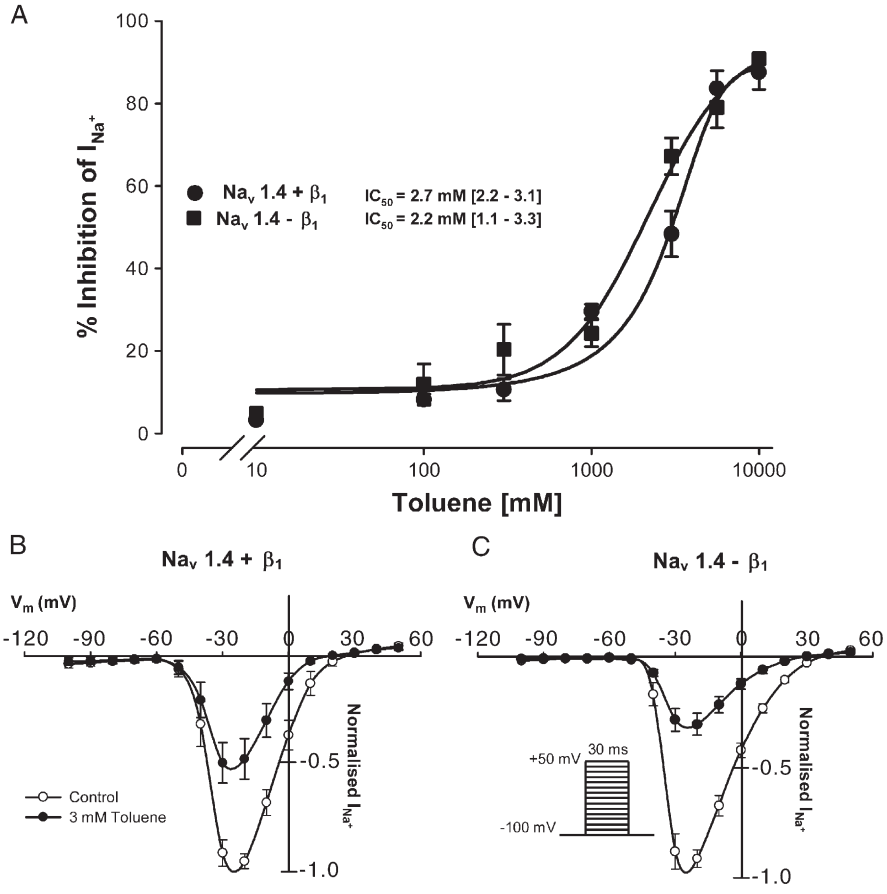

Fig. 2. (A) Concentration–response curves showing the blocking effects of toluene on sodium currents (I_{Na^+}) through $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$ channels. Sodium currents were elicited by 30-ms pulses to −20 mV from a holding potential of −100 mV. The stimulation frequency was 0.1 Hz. Lines through the points correspond to the best fit using the Hill equation. (B and C) Effects of 3 mM toluene on the current–voltage curves (I–V) of $Na_v1.4+\beta_1$ (B) and $Na_v1.4-\beta_1$ channels (C). Each point represents the mean±S.E.M. of six oocytes from at least two different frogs. Current–voltage relationships were determined from peak currents elicited by 30 ms, 10 mV steps from a holding potential of −100 up to +50 mV.

holding potential of −100 mV (to completely inactivate sodium channels), followed by a variable recovery interval (Δt) of 1 to 1000 ms and a test pulse to −20 mV. A train of 20 pulses of 30 ms each to −20 mV at 1, 2 or 4 Hz from a holding potential of −100 mV was used to evaluate Na^+ channel use-dependent and frequency-dependent block. Sodium current amplitudes were normalised to peak sodium currents from the first pulse of each train.

2.5. Toluene and recording solutions

Toluene was mixed with alkamuls EL-620 (emulphor, ethoxylated castor oil) at a 1:1 ratio (v/v) as previously described (Cruz et al., 1998). This mixture was then diluted as needed with an extracellular recording solution of the following composition (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, and 1 $BaCl_2$; pH 7.6. Barium was used as the divalent cation to prevent activation of any endogenous calcium-dependent chloride currents (Lupu-Meiri et al., 1988). The final toluene concentrations tested varied from 10 μM to 10 mM. This range of concentrations was selected because it has proven to be effective in modulating various physiologically relevant channels without producing a non-specific disruption of the oocyte membrane (Cruz et al., 1998; Beckstead et al., 2000; Tillar et al., 2002; Bale et al., 2002; Lopreato et al., 2003). Sodium current was recorded before, during and after toluene perfusion.

2.6. Statistics and data analysis

Results are expressed as the mean±S.E.M. Differences between mean data were analysed with a paired or unpaired Student's *t*-test as appropriate. The dose–response curves for toluene's effects were adjusted using the Win-nonlin program (Pharsight Co., version 2.1) to calculate IC_{50} values and their corresponding 95% confidence limits according to the model: Sodium current $(I_{Na^+}) = 1/(1+([\text{toluene}]/IC_{50})^n)$. Normalised inactivation–voltage curves were fitted to Boltzmann relationships of the form $y = 1/\{1+\exp[V-V_{m1/2}]/k\}+A$, where y is normalised sodium current, A the baseline, V the

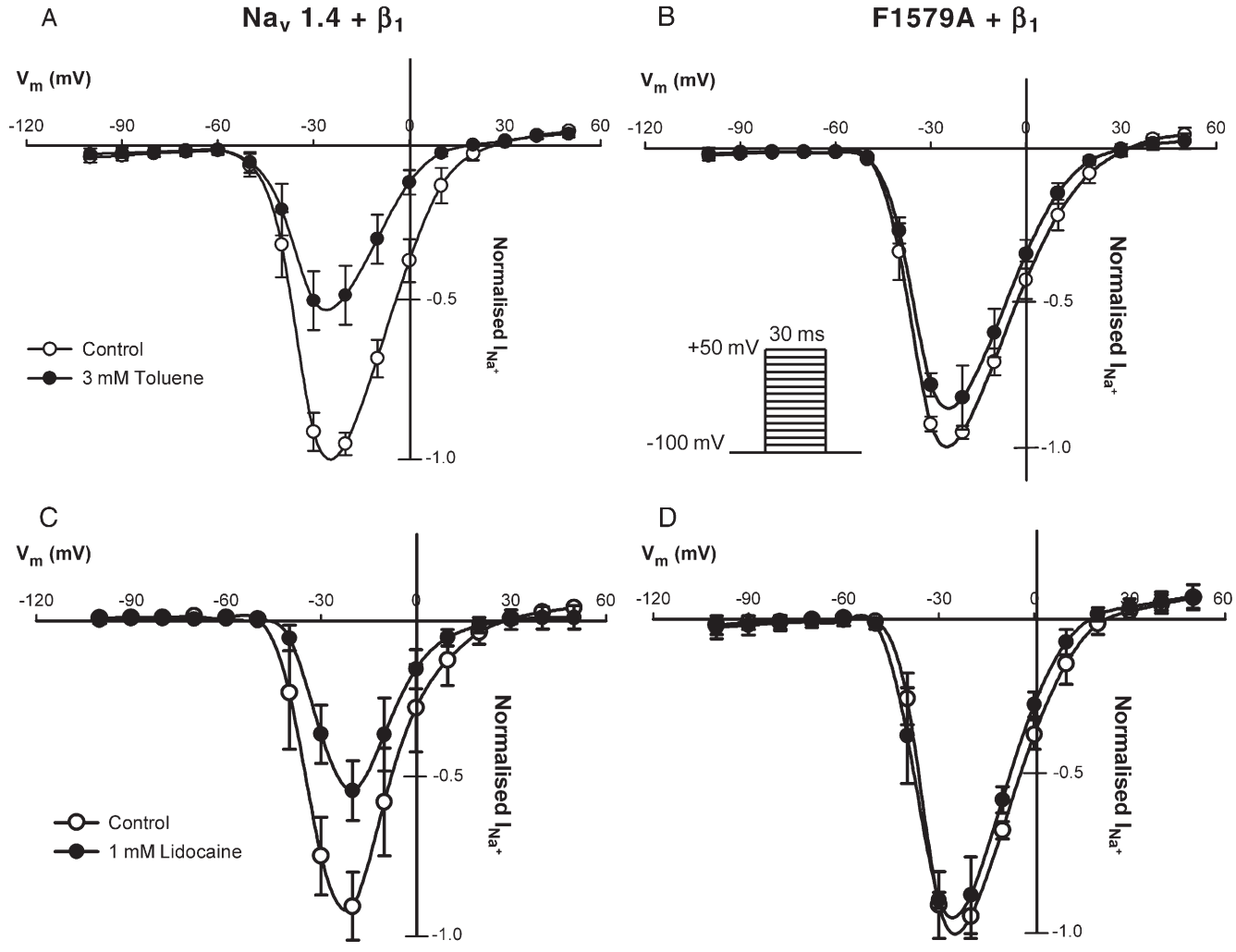

Fig. 3. Effects of 3 mM toluene (A and B) and lidocaine (C and D) on current–voltage curves (*I–V*) of $Na_v1.4+\beta_1$ channels (A and C) and F1579A mutant channels (B and D). Each point represents the mean±S.E.M. of six oocytes from different frogs in control conditions (○) and perfused with 3 mM toluene or 1 mM lidocaine (●). Current–voltage relationships were determined from peak currents elicited by 30 ms, 10 mV steps from a holding potential of −100 up to +50 mV.

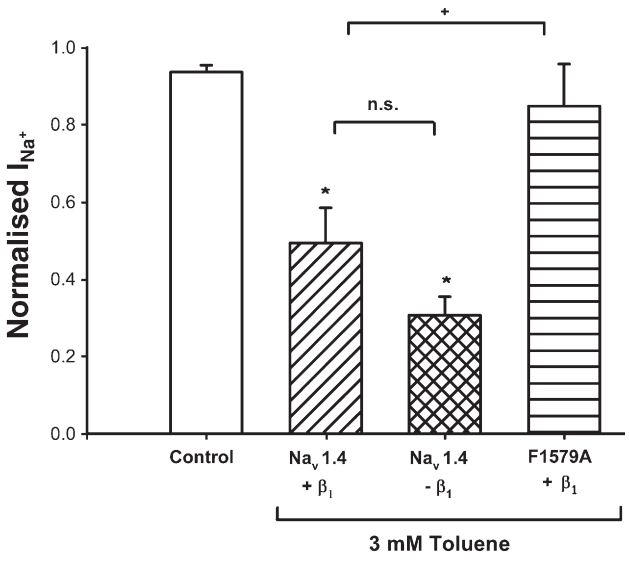

Fig. 4. Effects of 3 mM toluene on sodium current (I_{Na^+}) through Nav1.4+β1, $Na_v1.4-\beta_1$, and F1579A mutant channels. Each bar represents the mean ±S.E.M. of maximal current of six oocytes from different frogs. The data were taken from maximal inhibition in current–voltage curves. *$P<0.05$ vs. control, $^+P<0.05$ vs. $Na_v1.4+\beta_1$; n.s., non significant, Student's *t*-test.

membrane potential, $V_{m1/2}$ the voltage of half-maximal inactivation, and k a slope factor. A P value of <0.05 was used to denote statistically significant differences between groups.

3. Results

Fig. 1 shows the time course of sodium current inhibition by 3 mM toluene on $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$ (panels a and b). Each point represents the normalised current recorded at each depolarising pulse at 0.1 Hz. After an initial recording of several control pulses, oocytes were superfused with toluene for 6 min, time after which an apparent steady state inhibition was reached (40% and 50%, respectively). In both cases, the inhibition was slow with calculated block time constants (τ_{on}) of 1.64 [1.42–1.85] and 2.17 min [2.05–2.29] after adjustment with a mono-exponential function. Based on these and on previous findings (Cruz et al., 2003), toluene effects were measured after reaching the steady-state inhibition in subsequent experiments. Once completing the exposure time (6 min), superfusion was changed to normal barium-recording solution to determine the recovery time course after washout. Under our experimental conditions, sodium currents recovered approximately 90% in $Na_v1.4+\beta_1$ and 70% in $Na_v1.4-\beta_1$, which is consistent with the typical slow inactivation kinetics in oocytes lacking β_1 subunit (Cannon et al., 1993) (Fig. 1).

Fig. 2 shows complete dose–response curves for sodium current block by toluene (panel a) and current–voltage curves (panels b and c). The solvent was slightly more potent to inhibit Na^+ currents in $Na_v1.4-\beta_1$ channels than in those co-injected with β_1 subunit. In spite of this apparent differential sensitivity, IC_{50} confidence limits at 95% overlapped, indicating that the differences observed, although consistent, were not statistically significant: (IC_{50} for $Na_v1.4+\beta_1=2.7$ mM; confidence limits: [2.2–3.1] vs. IC_{50} for $Na_v1.4-\beta_1=2.2$ mM; confidence limits: [1.1–3.3]). The current–voltage curves shown in panels b and c correspond to $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$ channels in the presence and in the absence of 3 mM toluene. Regardless of the sodium channel variant studied or toluene absence or presence, sodium current activated at about −50 mV, reached a maximum value at −20 mV, and attained its reversal potential at approximately +30 mV. In both cases, toluene inhibited sodium currents throughout the entire current–voltage curve.

Toluene effects were studied in wild-type $Na_v1.4$ and in F1579A mutant channels co-expressing β_1 subunit (Fig. 3a and b). As a control, 1 mM lidocaine was also tested in both channels (panels c and d). Note that Fig. 3a is the same as Fig.

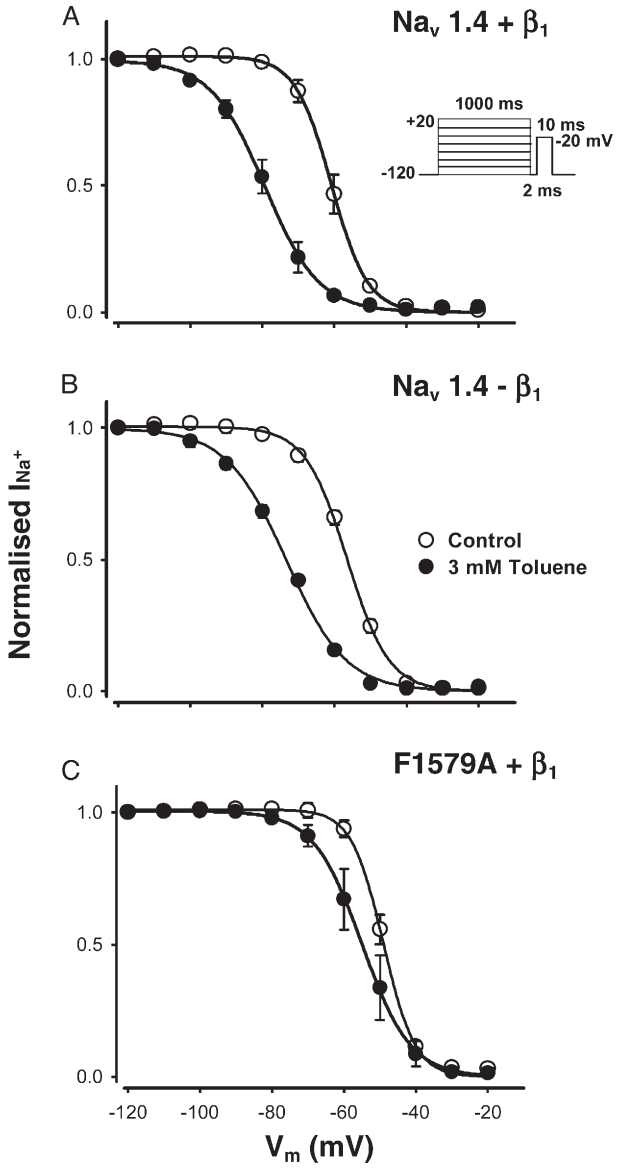

Fig. 5. Effects of toluene on the voltage dependence of steady-state inactivation of $Na_v1.4+\beta_1$ (A), $Na_v1.4-\beta_1$ (B) and F1579A mutant channels (C). Normalised data under control conditions and following toluene exposure are plotted against membrane potential. Each point represents the mean±S.E.M. of six oocytes from different frogs. Lines through the points correspond to the best fit using the Boltzmann equation.

2b and it is included here only for comparative purposes. We selected 1 mM lidocaine because this concentration approximates its IC_{50} value in *Xenopus* oocytes (Wagner et al., 1999). As shown, toluene and lidocaine produced a similar inhibition in wild-type Na^+ channels throughout the entire current–voltage curve, but none of them was able to significantly block sodium currents in F1579A mutant channels.

Fig. 4 summarizes toluene inhibitory efficacy in the three different channels studied. The most sensitive was $Na_v1.4-\beta_1$ followed by $Na_v1.4+\beta_1$. Mutant channels were practically insensitive to toluene.

The effects of toluene on voltage dependent Na^+ channel steady-state inactivation was studied using a conventional double-pulse protocol in $Na_v1.4+\beta_1$, $Na_v1.4-\beta_1$ and F1579A channels (Fig. 5). In all three cases, toluene produced a leftward shift of the steady-state inactivation curve, which was less evident in the mutant channel than in wild-type sodium channels (Table 1). Activation curves before and after toluene perfusion were similar in all three cases (data not shown).

Fig. 6 illustrates the course of sodium current inactivation recovery time in $Na_v1.4+\beta_1$, $Na_v1.4-\beta_1$, and F1579A mutant channels under control conditions and 3 mM toluene exposure. Toluene delayed recovery in all three channel variants, being the F1579A mutant channel the least sensitive of all. Maximal recovery from inactivation, i.e., that achieved at 1000 ms, was 40%, 20% and 80%, respectively. It should be taken into account that recovery in $Na_v1.4-\beta_1$ channels was also slow and incomplete (60%) in control conditions (without toluene). This kinetics is characteristic of channels lacking β_1 subunit (Nuss et al., 1995a).

Use-dependent and frequency-dependent block of sodium channels was addressed using trains of depolarising pulses at three different frequencies, with and without toluene (Fig. 7). In wild-type $Na_v1.4+\beta_1$ and F1579A mutant channels no significant change was seen in control oocytes (panels a and c). In $Na_v1.4-\beta_1$ channels, due to its characteristic slow kinetics (Cannon et al., 1993), the decreased sodium current amplitude was more evident at higher frequencies. Toluene (3 mM) blocked Na^+ channels in a use-dependent and frequency-dependent fashion, i.e., the higher the frequency of depolarising pulses the greater the inhibition. Consistent with what was previously mentioned this effect was significantly less pronounced in mutant channels than in wild-type channels. It is important to keep in mind that the inhibition observed at the 20th pulse with a 1 Hz stimulation frequency corresponds to that achieved after 20 s of toluene exposure, while that recorded at the 20th pulse at 4 Hz corresponds to the inhibition seen after only 5 s.

Table 1
Toluene effects on voltage dependence of steady state inactivation in $Na_v1.4+\beta_1$, $Na_v1.4-\beta_1$, and F1579A mutant channels expressed in *Xenopus laevis* oocytes ($V_{m1/2}$: voltage of half-maximal inactivation; k: slope factors for the Boltzmann curves)

	Control		Toluene (3 mM)	
	$V_{m1/2}$ (mV)	k	$V_{m1/2}$ (mV)	k
$Na_v1.4+\beta_1$	−60.8±0.5	−5.0±0.4	−79.2±0.8[a]	−7.5±0.6[a]
$Na_v1.4-\beta_1$	−56.5±0.4	−5.8±0.3	−73.4±0.4[a]	−8.3±0.4[a]
F1579A+β_1	−49.0±0.4	−4.4±0.3	−55.0±1.1[a]	−6.7±0.9

[a] $P<0.05$ vs. control.

4. Discussion

The purpose of this work was to test if toluene could inhibit skeletal sodium channels expressed in *Xenopus* oocytes and if so, to determine its possible site of action. Our results show that skeletal muscle sodium channels can be inhibited by toluene, but they are ten times less sensitive than cardiac sodium channels. Thus, while toluene IC_{50} for $Na_v1.5$ is approximately 300 μM (Cruz et al., 2003), it is almost 3 mM for $Na_v1.4$ channels. Differences in potency have been described for lidocaine block in $Na_v1.5$ and $Na_v1.4$. For example, Nuss et al. (1995b) reported that $Na_v1.5$ channels were threefold more sensitive to lidocaine's rested-state block than $Na_v1.4$ channels (IC_{50}=0.4 mM vs. 1.2 mM). Even greater differences were observed by Bennett et al. (1995) and Wagner et al. (1999) who reported an IC_{50} value=0.04 mM vs. 1.9 mM for $Na_v1.5$ and $Na_v1.4$ channels, respectively. The homology between $Na_v1.5$ and $Na_v1.4$ is high, around 87% (Goldin et al., 2000), but the differences between these channel subtypes clearly affect toluene's potency. Although any extrapolation to humans must be done with caution, it is interesting to mention that, in agreement with these findings, the occurrence of cardiac arrhythmias and sudden sniffing death due to toluene inhalation is well recognized (Bass, 1970; Shepherd, 1989), while there are few reports on muscle problems among inhalant abusers (Kamijo et al., 1998; Kao et al., 2000).

It is well documented that co-expression of β_1 subunit with rat brain and skeletal muscle α subunit in *Xenopus* oocytes accelerates sodium current inactivation, shifts voltage dependence of inactivation to more negative membrane potentials and accelerates recovery from inactivation when compared to sodium channels composed only by α subunits (Isom et al., 1992; Cannon et al., 1993). All these functional properties were reproduced in the present study. One issue that we wanted to address was if the presence of β_1 subunit could play a role in sodium channel block by toluene, since this is the case for other drugs such as lidocaine and etidocaine (Makielski et al., 1996, 1999). Our results show that toluene blocks skeletal muscle sodium channels without β_1 subunit with an IC_{50} value of 2.2 mM, while this value is a little higher for oocytes co-injected with β_1 and α subunits (2.7 mM). Sodium current inhibition by toluene is consistently more pronounced in $Na_v1.4-\beta_1$ than in $Na_v1.4+\beta_1$. However, when the IC_{50} confidence limits were taken into account, these differences did not reach statistical significance, suggesting that, although β_1 might play a role, it is not crucial for toluene inhibitory effects. Our findings are in agreement with the observations of Makielski et al. (1996), who reported that coexpression of β_1 subunit with cardiac sodium channel α subunit in oocytes slightly decreases lidocaine block. In conclusion, the main difference between $Na_v1.4-\beta_1$ and $Na_v1.4+\beta_1$ in control and toluene-treated oocytes was the well described slow kinetics of

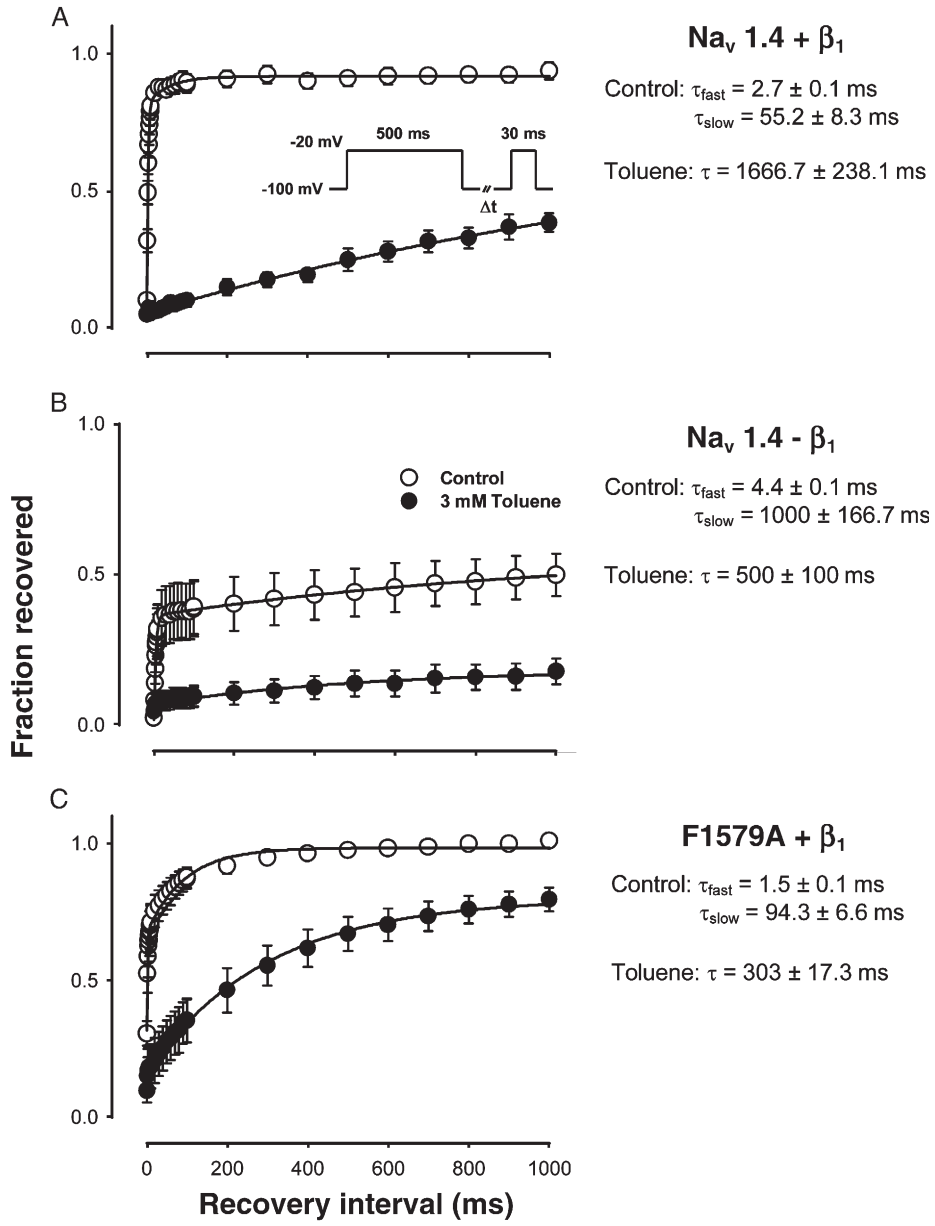

Fig. 6. Effects of 3 mM toluene on the time course of recovery of sodium current (I_{Na}) inactivation in $Na_v1.4+\beta_1$ (A), $Na_v1.4-\beta_1$ (B) and F1579A mutant channels (C). Recovery from inactivation was measured using a conventional double-pulse protocol (a first conditioning pulse followed by a test pulse) as shown in the inset. The recovery interval (Δt) varied from 1 to 1000 ms. Peak current levels during the test pulse were normalised to peak current levels during conditioning pulse, and plotted against the recovery interval. Each point represents the mean±S.E.M. of six oocytes. Lines through the points correspond to the best fit using exponential equations.

channels lacking β_1 subunit and not the amount of inhibition reached (Nuss et al., 1995a).

Alkylbenzenes (toluene, benzene, xylene) share a structural feature with local anaesthetics: an aromatic ring, as well as the characteristic use-dependent and frequency-dependent sodium channel block (Cruz et al., 2003; Hille, 2001). As early as 1987, Elliot and his colleagues reported that benzene and benzocaine inhibited sodium currents with similar efficacy, but different potency at low millimolar concentrations in squid giant axons. Both benzene and benzocaine increased the inactivation rate, reflected as a leftward shift of steady-state inactivation curves. This effect was also produced by toluene in the present work. In agreement with this, Hendry et al. (1985) observed that the aromatic hydrocarbons toluene, benzene, ethyl benzene and *n*-propyl benzene all produced a reversible inhibition of sodium and potassium currents in the squid giant axon. According to these authors, a concentration of approximately 1 mM toluene produced a 50% sodium current inhibition. Based on these findings, we considered of interest to study if a point mutation known to drastically reduce local anaesthetics' blocking efficacy also reduced toluene inhibitory effects.

Our results show that toluene inhibition is almost abolished in F1579A mutant channels lacking the local anaesthetic

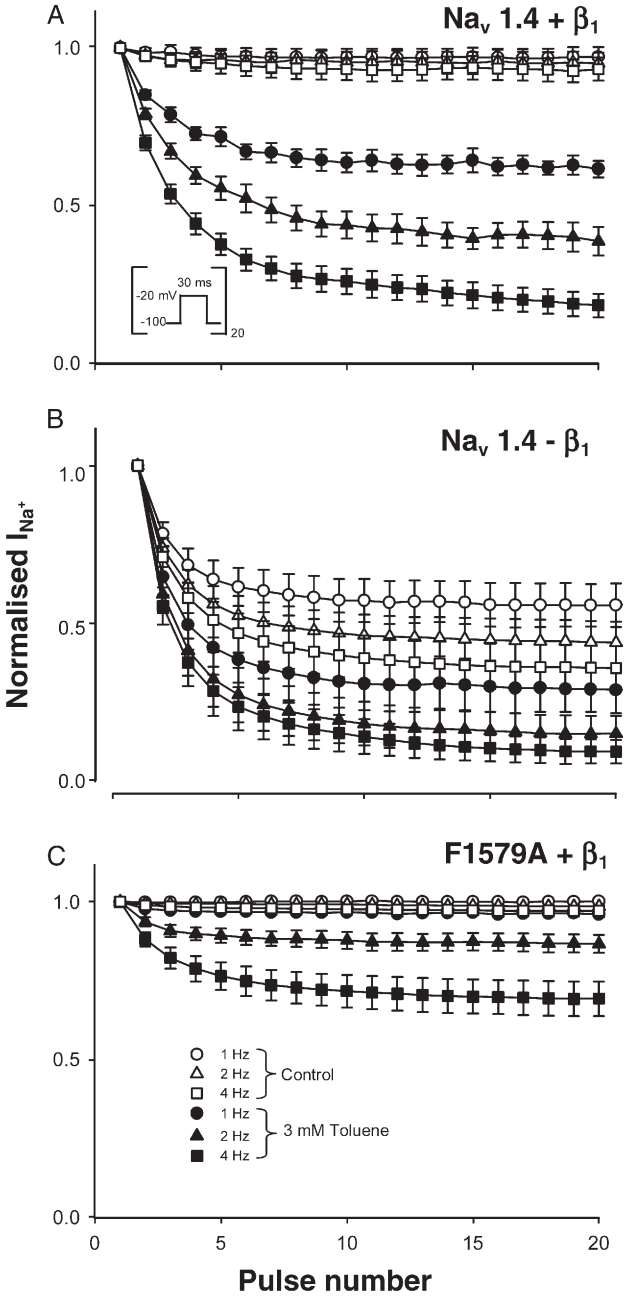

Fig. 7. Use- and frequency-dependent block of sodium current (I_{Na^+}) by toluene. (A and B) Wild-type $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$ channels and (C) F1579A mutant channels. A train of 20, 30-ms long pulses to −20 mV from a holding potential of −100 mV was applied at three different frequencies (1, 2 and 4 Hz) under control conditions (empty symbols) and in the presence of 3 mM toluene (filled symbols). The means±S.E.M. of maximal current values during the 20 pulses at the three different frequencies are shown.

binding site. A very similar result was obtained when testing lidocaine as a control under the same experimental conditions. Since 1994, Ragsdale et al. studied the effects of etidocaine, another local anaesthetic with a benzene ring, on channels with a F1764A mutation. This mutation in neuronal sodium channels is functionally equivalent to F1579A mutation in skeletal muscle sodium channels (Wang et al., 1998). Both mutations are located in the first third of S6 transmembrane segment in the α subunit domain IV and their occurrence results in almost complete abolition of both use-dependent and frequency-dependent local anaesthetic block (Ragsdale et al., 1994). It is important to mention that we found a residual inhibitory toluene effect in F1579A channels. In the same direction, Wang et al. (1998) using a different experimental preparation found that 1 mM benzocaine and 30 μM etidocaine had an average 15% blocking effect in F1579A mutant sodium channels instead of the 50% seen in wild-type channels expressed in HEK-293t cultured cells. The residual effect observed in our experiments could be attributed to the use of a single-mutated α subunit when it is known that the local anaesthetic binding site is composed of at least three amino acids. Similar findings were reported by Wagner et al. (1999), who found that 2 mM lidocaine inhibited sodium current amplitude nearly three times less in F1579A mutant channels than in wild-type $Na_v1.4$ channels expressed in *Xenopus* oocytes.

The most consistent finding of this work is that toluene delayed recovery from inactivation in all three channel variants studied, being the wild-type $Na_v1.4+\beta_1$ the most sensitive. According to Nuss et al. (1995b) and Wagner et al. (1999), although lidocaine slows the rate of sodium channel recovery, complete recovery is eventually achieved. In the present study, we observed maximal recoveries after 1 s of only 40% and 20% in $Na_v1.4+\beta_1$ and $Na_v1.4-\beta_1$, respectively, in oocytes treated with toluene. If sodium channels eventually recover from toluene inhibition, it is a very slow process. As previously mentioned, the sodium channels least sensitive to toluene inhibition were F1579A mutant channels. In spite of this, recovery from inactivation was also delayed by toluene with respect to what happened in mutant channels not exposed to the solvent. In this case, sodium channels recovered approximately 80% in 1s. Interestingly, a similar differential sensitivity was seen for recovery from inactivation in F1764A mutant channels exposed to 200 μM etidocaine, in which recovery was 20 times faster than in wild-type brain channels. Based on these data it has been suggested that F1764A mutation reduces the affinity of open and fast inactivated channels to etidocaine (Ragsdale et al., 1994). If this is similar to what happens with toluene effects on F1579A mutant channels is still the matter of investigation.

Our results show that use-dependent and frequency-dependent block in F1579A mutant channels is significantly less pronounced than in wild-type channels, but nevertheless occurs at least at the highest stimulation frequency. One could speculate that the mutation changes channel configuration, and thus toluene can reach the action site more easily than local anaesthetics because of its smaller size, therefore exerting a mild effect.

Since this is the first report addressing toluene's effects on F1579A mutant sodium channels, we do not know the mechanism by which this solvent of abuse interacts either with the local anaesthetic binding site or nearby. It has been described that the local anaesthetic binding site is located in the Na^+ channel pore (Ragsdale et al., 1994), but the exact way by which local anaesthetics interact with it is unknown. Bokesch et al. (1986) reported that lidocaine and a series of lidocaine homologs varying in alkyl substituents on the tertiary amine nitrogen have a potency for tonic block that uniformly increases

with increasing partition coefficients, but does not correlate well with the degree of ionization in solution (p*K*a) or the molecular weight of the anaesthetic in experiments done in sciatic nerves of frogs. Based on this, it is reasonable to suppose that something similar could happen with toluene and other related compounds. Contrary to this idea is the finding that several alkylbenzenes inhibit NR1/2B *N*-methyl-D-aspartate receptors in a concentration-dependent manner (Cruz et al., 1998, 2000), but their inhibitory potency correlates poorly with hydrophobicity. Instead, *N*-methyl-D-aspartate inhibitory potency strongly correlates with drug ability to engage in cation–π interactions (Raines et al., 2004). Benzene and toluene possess regions of highly negative electrostatic potential over their aromatic rings, reflecting the high electron density or their ð systems. We suggest that toluene could interact through cation–π interactions with a partial cationic charge in the local anaesthetic binding site. As before, confirmation or denial of this hypothesis could come from structure–activity studies currently on their way.

Taken together, our results show that toluene blocks skeletal muscle sodium channels as a function of its concentration in a use-dependent and frequency-dependent manner, and that the local anaesthetic binding site is important not only for local anaesthetics but also for abused alkylbenzene solvents like toluene.

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