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Volatile organic compounds inhibit human and rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes☆

Ambuja S. Bale[a], Connie A. Meacham[a], Vernon A. Benignus[b], Philip J. Bushnell[a], Timothy J. Shafer[a,*]

[a]*Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA*

[b]*Human Studies Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA*

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Abstract

The relative sensitivity of rats and humans to volatile organic compounds (VOCs) such as toluene (TOL) and perchloroethylene (PERC) is unknown and adds to uncertainty in assessing risks for human exposures to VOCs. Recent studies have suggested that ion channels, including nicotinic acetylcholine receptors (nAChRs), are targets of TOL effects. However, studies comparing TOL effects on human and rat ligand-gated ion channels have not been conducted. To examine potential toxicodynamic differences between these species, the sensitivity of human and rat nAChRs to TOL was assessed. Since PERC has similar effects, in vivo, to TOL, effects of PERC on nAChR function were also examined. Two-electrode voltage-clamp techniques were utilized to measure acetylcholine-induced currents in neuronal nAChRs ($\alpha4\beta2$, $\alpha3\beta2$, and $\alpha7$) expressed in *Xenopus* oocytes. PERC (0.065 mM) inhibited $\alpha7$ nAChR currents by 60.1 ± 4.0% (human, $n = 7$) and 40 ± 3.5% (rat, $n = 5$), and inhibited $\alpha4\beta2$ nAChR currents by 42.0 ± 5.2% (human, $n = 6$) and 52.2 ± 5.5% (rat, $n = 8$). Likewise, $\alpha3\beta2$ nAChRs were significantly inhibited by 62.2 ± 3.8% (human, $n = 7$) and 62.4 ± 4.3% (rat, $n = 8$) in the presence of 0.065 mM PERC. TOL also inhibited both rat and human $\alpha7$, $\alpha4\beta2$, and $\alpha3\beta2$ nAChRs. Statistical analysis indicated that although there was not a species (human vs. rat) difference with PERC (0.0015–0.065 mM) or TOL (0.03–0.9 mM) inhibition of $\alpha7$, $\alpha4\beta2$, or $\alpha3\beta2$ nAChRs, all receptor types were more sensitive to PERC than TOL. These results demonstrate that human and rat nACh receptors represent a sensitive target for VOCs. This toxicodynamic information will help decrease the uncertainty associated with animal to human extrapolations in the risk assessment of VOCs. Published by Elsevier Inc.

Keywords: Neurotoxicity; Toluene; Perchloroethylene; Human; In vitro

Introduction

Toluene (TOL) and perchloroethylene (PERC) are volatile organic compounds (VOCs) widely used in industrial products and processes. Briefly, PERC has been used in dry cleaning and metal degreasing processes and TOL is commonly found in paints, paint thinners, and gasoline sources. Effects of these VOCs on function of the central nervous system (CNS) are of great interest due to their widespread use and exposure potential. For both compounds, inhalation is the typical route of human exposure, although they can be absorbed dermally and in some cases ingested (for reviews, see Arlien-Soborg, 1992;

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* Corresponding author. Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, MD B105-05 NHEERL, Research Triangle Park, NC 27711, United States. Fax: +1 919 541 4849.

E-mail address: shafer.tim@epa.gov (T.J. Shafer).

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Østergaard, 2000). Based on similarities in their adverse behavioral effects, it is hypothesized that VOCs disrupt nervous system function via common mechanisms (for a review, see Balster, 1998). Specifically, TOL and PERC cause acute neurological deficits, including cognitive and visual impairments (Baelum, 1991; Dudek et al., 1990; Echeverria et al., 1989, 1991, 1995; Muttray et al., 1999; Olson et al., 1985). Due to limitations associated with testing these compounds in humans, rats and other animal models have been used to characterize further VOC neurotoxicity. VOCs produce similar acute neurotoxic effects in rats and humans; therefore, animal studies have been used to estimate the hazard of humans exposed to VOCs (Boyes et al., 2000, 2003; Bushnell et al., 1994; Miyagawa et al., 1995; Rebert et al., 1995).

The quantitative prediction of VOC effects in humans from animal models has been examined by using meta-analyses of published studies. These studies suggested that humans were more sensitive than rats to the behavioral effects of both PERC (Guth et al., 1997) and TOL (Benignus et al., 1998). However, different behavioral endpoints were measured in the human and rat studies, and there were considerably fewer human than animal studies available for analysis. Thus, considerable uncertainty remains regarding the relative sensitivities of the two species to these VOCs. Species differences in sensitivity to VOCs can be due to differences in toxicokinetics and/or toxicodynamics. The meta-analysis of TOL (Benignus et al., 1998) has accounted for potential toxicokinetic differences by relating the effects of TOL in rats and humans to its internal dose (concentration in blood), so the difference in sensitivity to TOL is unlikely to depend on kinetics. However, toxicodynamic differences between humans and rats have not been examined to date.

Recently, TOL, 1,1,1-trichloroethane, and trichloroethylene have been demonstrated to inhibit the function of excitatory receptors [*N*-methyl-D-aspartate (NMDA), Cruz et al., 1998, 2000; nicotinic acetylcholine receptors (nAChR), Bale et al., 2002] and potentiate the function of inhibitory receptors [γ-aminobutyric acid type A ($GABA_A$), glycine receptors; Beckstead et al., 2000]. In addition, VOCs also disrupt normal function of voltage sensitive calcium channels (VSCCs; Shafer, 2003; Tillar et al., 2002). Despite the similarity of in vivo effects between PERC and other VOCs, effects of PERC on ion channel function have not been examined to date.

The ability of TOL and other solvents to disrupt ion channel function has resulted in the hypothesis that these effects contribute to VOC neurotoxicity. Therefore, potential toxicodynamic differences between humans and rats can be examined by comparing effects of VOCs on rat and human isoforms of these channels. In the present study, possible toxicodynamic differences were examined by expressing individual subtypes of cloned human and rat ion channels in *Xenopus* oocytes to compare VOC effects under the same conditions.

Based on the effects of VOCs on ion channels, the present study tested two hypotheses related to VOC neurotoxicity: (1) that PERC will inhibit nAChRs in a manner similar to TOL and (2) that human nAChRs will be more sensitive to VOCs (TOL and PERC) than rat nAChRs. For the following studies, the effects of TOL and PERC on nAChRs were examined because of the receptors association with learning, memory, and attention (Albuquerque et al., 1997; Levin, 1992). Memory loss is an adverse effect related to acute (Meulenbelt et al., 1990; Welch et al., 1991) and chronic solvent exposure (Echeverria et al., 1991, 1995). Rodent nAChRs have been shown to be sensitive to TOL (Bale et al., 2002). Three forms of the nAChR, present in both rats and humans, were selected for this study. The α4β2 and α7 receptors were chosen due to their global receptor expression patterns in the CNS. The α3β2 nAChR was also selected because of its high expression in the autonomic ganglia, although there is also some expression in the CNS (for a review, see Leonard and Bertrand, 2001). Recombinant receptors of each type were expressed in *Xenopus* oocytes for all studies. The effects of PERC on nAChRs were compared to those of TOL to determine whether the similar qualitative behavioral effects are reflected in similar profiles of receptor inhibition in vitro. Furthermore, comparisons were made between human and rat nAChRs to determine whether differences in toxicodynamic sensitivity of the receptors could contribute to differences in behavioral sensitivity to VOCs.

Methods

Chemicals. TOL (99.0% pure, HPLC-grade), PERC (99.0% pure, HPLC-grade), acetylcholine, α-bungarotoxin, and dihydro-β-erythroidine (DHβE) were purchased from Sigma-Aldrich (St. Louis, MO). α-conotoxin MII was purchased from Tocris-Cookson, Inc (Ellisville, MO). All other chemicals were purchased from commercial vendors and were of the highest available grade.

Synthesis of messenger RNA (mRNA). cDNA clones of human nAChRs (α4, α3, α7, and β2 kindly provided by Dr. J. Lindstrom, University of Pennsylvania Medical School, Philadelphia, Pennsylvania) and rat nAChRs (α4, α3, α7, and β2 kindly provided by Dr. M. Nowak, Medical University of South Carolina, Charleston, SC) were linearized downstream from the coding sequence with restriction enzymes, and purified by phenol/chloroform extraction. The extracts were precipitated in ethanol and resuspended in diethyl pyrocarbonate-treated water. The SP6 (human nAChRs) or T7 (rat nAChRs) mMessage mMachine kits (Ambion, Austin, TX) were used to synthesize mRNA from the linearized transcripts.

Preparation of oocytes. Adult female *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI) and housed in an approved Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility in a dechlorinated water tank and fed twice weekly with frog pellets. Frogs were anesthetized in a 0.1% tricaine methanesulfonate (Sigma-Aldrich) solution buffered with 0.05% sodium bicarbonate. This procedure was approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee (IACUC). Oocytes were surgically removed and placed in OR-2 buffer (containing in mM): NaCl; 82, KCl; 2, HEPES; 5, $MgCl_2$; 1, pH 7.3). Prior to mRNA injection, oocytes were treated with collagenase (1 mg/ml) in OR-2 buffer for 1 h to remove the follicular membrane. Oocytes (stage V and VI) were injected with 10–30 ng (total volume of either 41.4 or 46.0 nl per oocyte) each of various nAChR subunit mRNAs (1:1 ratio) and maintained in 0.5× L-15 Leibovitz media (pH 7.4) supplemented with 10 mg/l penicillin, 10 mg/l streptomycin, and 15.5 mg/l gentamycin. Oocytes were incubated at 18 °C for 5–7 days before stable nAChR expression.

Preparation of drug solutions. Drug solutions were prepared using a barium-containing extracellular solution (containing in mM): NaCl; 115, KCl; 2.5, HEPES [4-2-(hydroxyethyl)-1-piperazine-ethanesulfonic acid]; 10, $BaCl_2$; 1.8, pH 7.4. Barium was used as the divalent cation to prevent activation of any endogenous calcium-dependent chloride currents (see Bale et al., 2002). Atropine (100 nM) was added to block endogenous muscarinic receptors expressed by the oocyte. TOL and PERC were added directly to the buffer and mixed vigorously until the solvent dissolved in the buffer. All solutions were prepared fresh before performing the experiments.

Two-electrode voltage-clamp recordings. Oocytes were placed in an RC-3Z oocyte chamber (Warner Instruments, Hamden, CT) and continually perfused with extracellular buffer at a flow rate of 10 ml/min. Using the Axoclamp 2B amplifier (Axon Instruments Inc., Union City, CA), oocytes were voltage clamped at a membrane potential of −80 mV with two microelectrodes filled with 3 M KCl (0.5–3 MΩ). The nAChRs were stimulated by applying a solution containing ACh (1, 5, or 150 μM for α4β2, α3β2, or α7 receptors, respectively). After the first ACh stimulation, the nAChRs were pretreated for 30 s with either PERC or TOL in buffer alone before restimulating the receptors with ACh in the presence of the solvent. After the solvent exposure, oocytes were washed extensively with the extracellular buffer for 3–10 min before a third (washout) application of ACh. Currents generated from the oocyte were filtered and digitized with a 16-bit analog-to-digital interface (Digidata 1200B, Axon Instruments) and the data was saved to a PC. pClamp 8.0 software (Axon Instruments) was utilized for all data collection and analysis.

Collection of VOC samples. Stock solutions of TOL (concentrations in mM: 0.05, 0.1, 0.3, 1, 3) and PERC (concentrations in mM: 0.005, 0.01, 0.05, 0.1, 0.5) were prepared in external buffer solution in 50-ml aliquots in the same manner as in the oocyte exposure studies. Each solution was placed in a syringe reservoir and perfused through the oocyte recording chamber system that was used for exposure to VOCs. Samples (500 μl) were taken using a gas-tight Hamilton syringe at the position where the oocyte was typically placed. The 500-μl aliquots were immediately transferred to a vial, capped, and crimped to prevent solvent loss. Immediately after sample collection, the capped vials were placed at −20 °C and frozen. The frozen samples were shipped on dry ice overnight to National Medical Services (Philadelphia, PA) for VOC concentration analysis by gas chromatograph.

VOC sample analysis. A 10-point calibration curve was constructed for PERC (0–0.72 mM) and TOL (0–3.3 mM) samples and checked against reference standards (Absolute Standards, Inc., Hamden, CT) to verify VOC concentrations on a capillary gas chromatograph (Agilent 6890, Agilent Technologies, Palo Alto, CA). After calibrating the gas chromatograph for each solvent, the 500 μl samples for PERC, and then TOL were heated, pressurized, and the vapor was injected into the gas chromatograph, which used a flame ionization technique for detection. The measured concentrations from this analysis are the values reported in the manuscript.

Data and statistical analysis. Current amplitudes, rise times, and decay constants were measured and analyzed using Clampfit version 8.2 (part of the pClamp 8 software package). The current amplitude obtained from nAChRs in the presence of solvent and agonist was subtracted from the average control current amplitude and this difference was divided by the average control response to calculate the percent inhibition of the receptor response. Concentration response curves were graphed using Prism 3.0 (GraphPad Software Inc., San Diego, CA). IC50 values and were determined where applicable on Microsoft Excel spreadsheets designed by Dr F. Smith (Virginia Commonwealth University, Richmond, VA) using least squares linear regression analysis, followed by calculation of the confidence limits (Bliss, 1967).

A four-dimensional analysis of variance (ANOVA) was conducted to determine significant differences between treatment groups. There were four independent variables in the experiment; species (*S*), receptor (*R*), VOC type (*V*), and solvent concentration (*C*). Because the effects of up to two concentrations of VOC were usually measured on a single oocyte, a repeated measures design was incorporated for this variable. Further, a mixed model approach was

required because only some of the concentrations were tested per oocyte and concentrations were repeated for only some oocytes. The ANOVA was conducted using the Proc Mixed protocol (SAS, Cary, NC) with *C* as the sometimes-repeated dimension and *S*, *R*, and *V* as variables not repeated. Least-squares equations were fitted to effects of concentration (as log *C*) that were found to be statistically significant (a priori $\alpha = 0.05$) using Proc Mixed to estimate empirical parameters of the regressions.

In the event that either *S*, *R*, or *V* differed statistically, a concentration-equivalence equation was derived algebraically from the concentration–effect functions, and its 95% confidence limits were calculated by Monte-Carlo simulations (Benignus, 2001). The concentration-equivalence equation was used to express the relative potency for inhibiting current flow over a range of concentrations of the two VOCs, or of a given VOC in two situations (i.e., between species or receptors).

Results

Characterization of nAChR responses in oocytes

Prior to examining VOC effects, experiments were conducted to compare ACh concentration–response relationships between human and rat receptors, as well as to verify pharmacological sensitivity of receptor types. Application of ACh to oocytes injected with mRNA for the various nAChR subtypes resulted in reproducible inward currents between 100 and 2000 nA. ACh concentration–response relationships were not significantly different between human and rat nAChRs (data not shown). Additionally, the estimated EC50 values for ACh were consistent with previously published values using oocytes (α7, 150 μM; Papke and Porter Papke, 2002; Briggs and McKenna, 1998; α4β2, 1 μM; Buisson et al., 1996; α3β2, 5 μM; Colquhoun and Patrick, 1997). Thus, subsequent experiments with VOCs were conducted using 150, 1, and 5 μM ACh to activate α7, α4β2, and α3β2 nAChRs in oocytes, respectively.

Expression of the various nAChR types was confirmed using nAChR subtype-specific antagonists. ACh-induced currents from oocytes injected with rat and human α7 mRNA were blocked completely by α-bungarotoxin (100 nM), a specific antagonist for the α7 receptor (Albuquerque et al., 1997). Similarly, rat and human α4β2 and α3β2 receptors were completely blocked with receptor-specific antagonists DH-β-E (100 μM; Albuquerque et al., 1997) and α-conotoxin MII (10 μM; Harvey et al., 1997), respectively (Fig. 1). Thus, rat and human receptors expressed in oocytes have similar responses to ACh and exhibit the expected sensitivities to specific antagonists. As such, this model system allows for comparison of VOC effects between human and rat nAChRs. It should be noted that for all receptor types, neither TOL nor

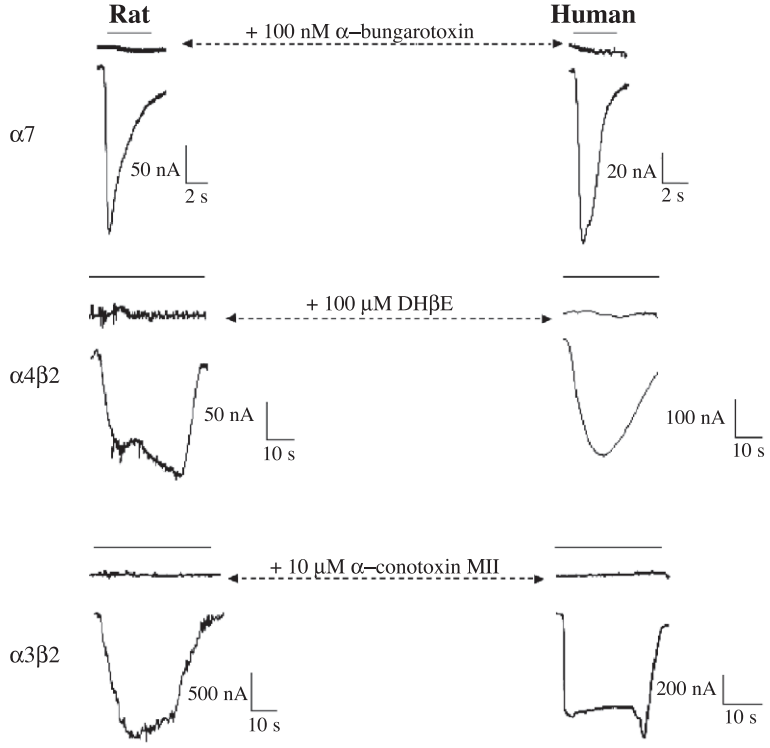

Fig. 1. Pharmacological inhibitors completely block human and rat nAChRs. Representative currents from individual *Xenopus* oocytes expressing either human or rat nAChRs are depicted and labeled above. For each set of currents, the upper current is the response obtained in the presence of ACh and the respective pharmacological inhibitor (α-bungarotoxin—α7; α-conotoxin MII—α3β2; DHβE—α4β2) as indicated by the dashed arrows. The lower current is the control ACh response obtained prior to the application of the inhibitor. ACh application times are indicated in the figure by the respective lines above the individual current. Upon application of the respective nAChRs pharmacological inhibitor, rat and human receptors were equally and completely inhibited.

PERC altered resting membrane current at any concentration tested (data not shown). This is consistent with previous reports of TOL perfusion on oocytes (Bale et al., 2002; Cruz et al., 1998, 2000).

Measurement of VOC concentrations in the oocyte bath

Due to the high volatility of VOCs, stock solutions of PERC (0.005–0.5 mM) and TOL (0.05–3 mM) were prepared, and concentrations of the solvent in the oocyte bath were measured by gas chromatograph (National Medical Services) to account for solvent loss in the oocyte chamber, evaporation in the buffer syringe reservoir, and through the polyethylene tubing of the perfusion system. The measured PERC (Fig. 2A) and TOL (Fig. 2B) concentrations were plotted in relation to the theoretical concentrations and a linear regression analysis was performed. There was an average of 13% recovery of PERC and 30% recovery of TOL over all the concentrations tested. The subsequent experiments report PERC and TOL actual concentrations extrapolated from the linear regression analysis from the nominal concentrations.

VOC effects on human and rat nAChRs

α7 nAChRs

The homomeric α7 nAChR subtype has been reported to be sensitive to compounds such as ethanol (Aistrup et al., 1999; Yu et al., 1996) and toluene (Bale et al., 2002). Therefore, effects of PERC on this receptor were examined, and effects of VOCs on rat and human receptors were compared. PERC, 0.065 mM, reduced the ACh-induced

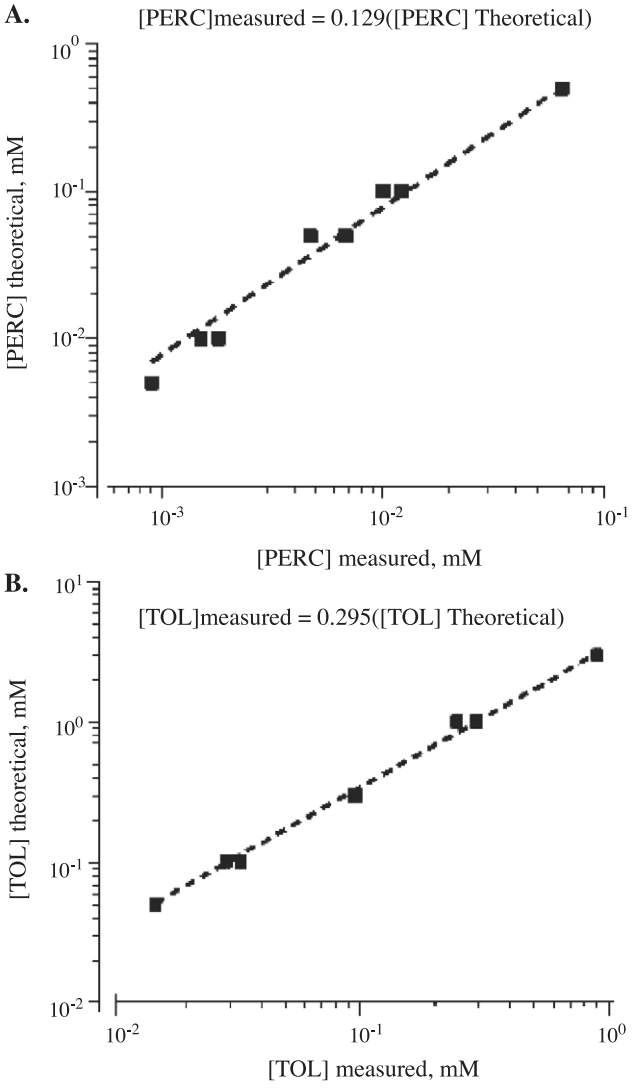

Fig. 2. PERC and TOL buffer concentrations decreased after perfusion in the oocyte bath. (A,B) Linear regression plots comparing measured PERC and theoretical PERC concentrations (A) and measured TOL and theoretical TOL concentrations (B). Each point on the graph represents an individual PERC (A) or TOL (B) sample that was collected from the oocyte bath and measured using a flame ionizing detection gas chromatograph. There was a 13% recovery of the PERC (0.005–0.5 mM, theoretical) and a 30% recovery of the TOL (0.05–3 mM, theoretical) samples over the entire concentration range.

current by 60.1 ± 4.0% (n = 7) in human α7 receptors and 40 ± 3.5% (n = 5) in rat α7 receptors (Fig. 3A); inhibition was rapidly reversed in PERC-free solution. Furthermore, human and rat α7 receptors were inhibited by PERC (0.0015–0.065 mM) in a concentration-dependent manner (Fig. 3B). The IC50 values (with confidence limits) for PERC inhibition were 0.024 mM (0.016–0.036 mM) and 0.074 mM (0.044–0.121 mM) for the human and rat receptors, respectively.

As with PERC, the ACh-induced α7 currents in human and rat receptors were also inhibited by TOL. Rat α7 receptors were inhibited by 43.5 ± 6.2% (n = 5) with 0.30 mM TOL, whereas human α7 receptor currents were inhibited by 32 ± 5.7% (n = 5) at this same concentration (Fig. 3C). Effects of TOL (0.03–0.9 mM) on human and rat α7 nAChRs were also concentration-dependent and reversible (Fig. 3D). Calculated IC50 values for TOL inhibition were 0.62 mM (0.53–0.71 mM) for human and 0.34 mM (0.27–0.42 mM) for rat α7 receptors. Neither PERC nor TOL significantly altered current rise or decay times for either human or rat α7 receptors (data not shown).

α4β2 nAChRs

Like the α7 receptor, the α4β2 heteromeric receptor is also sensitive to general anesthetics (Flood et al., 1997) and TOL (Bale et al., 2002). Therefore, the sensitivity of this channel to PERC was examined, as were potential differences in sensitivity of rat and human channels to PERC or TOL. Effects of PERC and TOL on this receptor type were concentration-dependent and reversible (Fig. 4). PERC inhibited the ACh current for both receptor types up to a maximum of 42.1 ± 5.2% (n = 6, human) and 48.8 ± 3.4% (n = 7, rat) at 0.065 mM (Fig. 4A). However, inhibition never surpassed 50% for either the human or rat α4β2 receptors (Fig. 4B) and IC50 values (the concentration at which 50% inhibition is produced) for PERC could not be calculated. Half-maximal effect values (EC50) of 0.016 mM (0.011–0.026 mM) for human and 0.021 mM (0.013–0.034 mM) for rat α4β2 receptors were calculated based on a maximal effect) at 0.065 mM PERC.

In contrast to PERC, 0.30 mM TOL inhibited both human and rat α4β2 receptors by greater than 70% (Fig. 4C). Furthermore, TOL (0.90 mM) almost completely inhibited α4β2 receptor currents by 83.3 ± 2.7% (n = 5, human) and 86.5 ± 4.6% (n = 5, rat) (Fig. 4D). IC50 values for TOL for human and rat α4β2 receptors were 0.15 mM (0.11–0.19 mM) and 0.09 mM (0.08–0.42 mM), respectively. For comparison to PERC, EC50 values were also calculated for TOL and were 0.11 and 0.085 mM for human and rat α4β2 receptors, respectively.

α3β2 nAChRs

Previous studies found that α3β2 receptors are sensitive to ethanol (Cardoso et al., 1999; Covernton and Connolly,

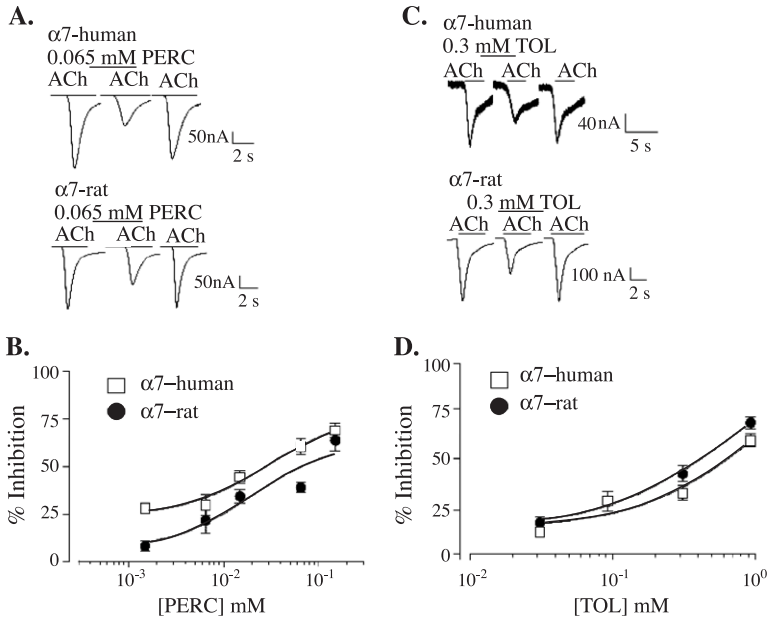

Fig. 3. PERC and TOL inhibit human and rat α7 receptors in a concentration-dependent manner. (A,C) Representative ACh-induced currents from individual *Xenopus* oocytes expressing either human (top) or rat (bottom) α7 nAChRs. ACh (150 μM), PERC (0.065 mM), and TOL (0.3 mM) application times are indicated in the figure by the respective lines above the individual currents. PERC and TOL were pre-applied for 30 s before activating the receptors with ACh. (A) 0.065 mM PERC inhibited human α7 nAChR function by 60% (A, top) and rat α7 nAChR by 45% (A, bottom). (B) PERC (0.0015–0.13 mM) concentration–response curves were generated for both rat and human α7 receptors. Each point (●, rat; □, human) on the graph represents mean ± SEM from 5–9 oocytes. (C) 0.3 mM TOL application inhibited human (top) and rat (bottom) α7 nAChRs by 45%. (D) TOL (0.03–0.9 mM) was applied to rat and human α7 receptors and inhibition was concentration-dependent. Each point (●, rat; □, human) on the graph represents mean ± SEM from 4–8 oocytes. In all cases, receptor function returned to pre-exposure levels following a 5-min perfusion of extracellular buffer.

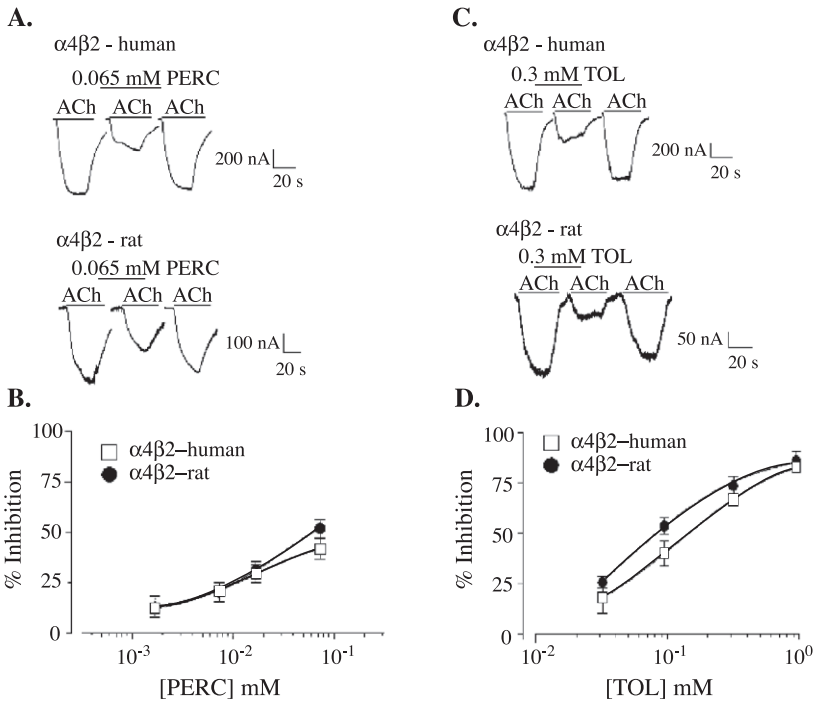

Fig. 4. Rat and human α4β2 nAChRs are inhibited by PERC and toluene in a similar manner. (A,C) Representative ACh-induced currents from individual *Xenopus* oocytes expressing either human (top) or rat (bottom) α4β2 nAChRs. ACh (1 μM), PERC (0.065 mM), and TOL (0.3 mM) application times are indicated in the figure by the respective lines above the individual currents. PERC and TOL were pre-applied for 30 s before activating the receptors with ACh. (A) 0.065 mM PERC decreased human and rat α4β2 nAChR function by 45%. (B) Rat and human α4β2 receptors were exposed to PERC concentrations ranging from 0.0015 to 0.065 mM. Each point (●, rat; □, human) on the graph represents mean ± SEM from 5–10 oocytes. (C) 1 mM TOL inhibited human (top) and rat (bottom) α4β2 nAChRs by 75%. (D) TOL (0.03–0.9 mM) concentration curves were generated for rat and human α4β2 receptors. Each point (●, rat; □, human) on the graph represents mean ± SEM from 5–8 oocytes.

1997) and toluene (Bale et al., 2002). Human and rat α3β2 receptors were rapidly and reversibly inhibited by PERC (Fig. 5A). Sensitivity of human and rat α3β2 nAChRs to PERC (0.065 mM) was similar as this concentration reduced the ACh-induced current via receptors by 62.2 ± 3.8% (n = 7, human) and 62.4 ± 4.3% (n = 8, rat). PERC (0.0015–0.065 mM) concentration–response relationships (Fig. 5B) for both species were also nearly identical and IC50 values were 0.025 (0.018–0.034) mM for human and 0.029 (0.018–0.044) mM for rat receptors. TOL (0.3 mM) inhibited the ACh-induced current by 60.1 ± 1.7% (n = 5) in human (Fig. 5C; top) and 59.4 ± 2.8% (n = 7) in rat (Fig. 5C, bottom) α3β2 receptors, respectively. As with PERC, ACh current inhibition between the species was similar with TOL (0.03–0.9 mM; Fig. 5D). Calculated IC50 values for TOL inhibition were 0.13 mM (0.10–0.16 mM) for human and 0.18 mM (0.16–0.21 mM) for rat α3β2 receptors.

Statistical comparison of nAChR inhibition by PERC and TOL between species

A four-way mixed-model ANOVA was conducted since there were four independent variables in the experiment: species, receptor, solvent type, and concentration. This analysis allows comparison of effects of VOCs on these different variables using a single statistical analysis, thus decreasing the possibility of a Type I error. This four-way ANOVA indicated statistically significant main effects of concentration and solvent type and a statistically significant interaction between these variables ($P < 0.0001$ for all three effects). This result implies (1) that the concentration–effect curves for TOL and PERC were not parallel; (2) that human and rat receptors did not differ in sensitivity to either TOL or PERC; and (3) that the three receptor types did not differ in sensitivity to either TOL or PERC.

To analyze the interaction between concentration and solvent type, stepdown procedures (Proc Mixed) were conducted to fit separate concentration–effect curves for TOL and PERC. The resulting concentration–effect equations were:

$$IN_{\mathrm{TOL}} = 17.24\log_e(C_{\mathrm{TOL}}) - 40.11$$

and

$$IN_{\mathrm{PERC}} = 10.53\log_e(C_{\mathrm{PERC}} + 10.0)$$

in which IN is percent inhibition, C is concentration in μM, and the subscripts indicate the VOC. These

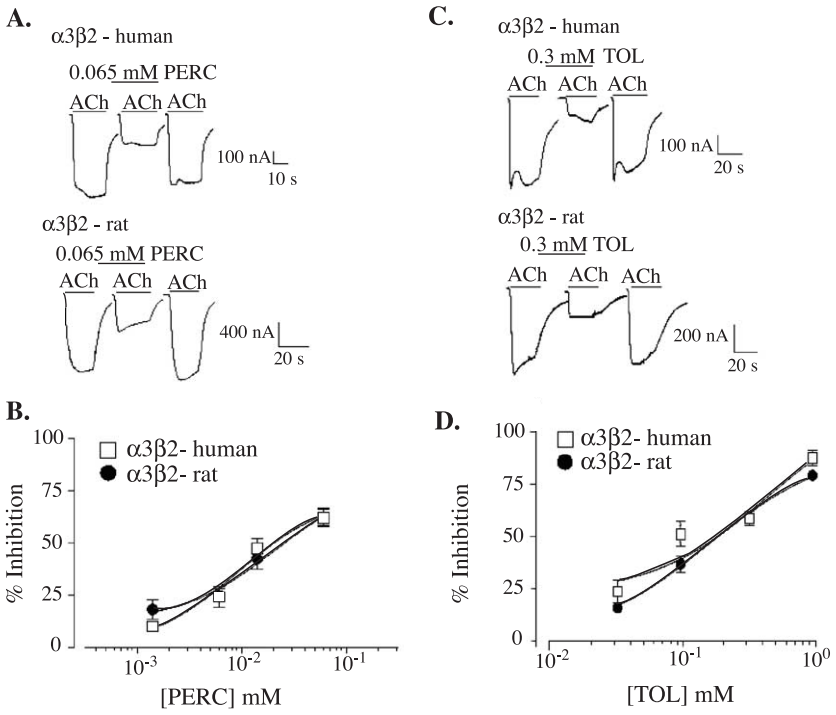

Fig. 5. Rat and human α3β2 nAChRs show a similar inhibition profile to TOL and PERC. (A,C) Representative ACh-induced currents from individual *Xenopus* oocytes expressing either human (top) or rat (bottom) α3β2 nAChRs. ACh (5 μM), PERC (0.065 mM), and TOL (0.3 mM) application times are indicated in the figure by the respective lines above the individual currents. PERC and TOL were pre-applied for 30 s before activating the receptors with ACh. (A) 0.065 mM PERC decreased human and rat α3β2 nAChR function by 62%. (B) Rat and human α3β2 receptors were exposed to PERC concentrations ranging from 0.0015 to 0.065 mM. Each point (●, rat; □, human) on the concentration curve represents mean ± SEM from 5–7 oocytes. (C) 0.3 mM TOL inhibited human (C, top) and rat (C, bottom) α3β2 nAChRs by 60%. (D) TOL (0.03–0.9 mM) was applied to rat and human α3β2 receptors. Each point (●, rat; □, human) on the graph represents mean ± SEM from 5–8 oocytes.

equations describe VOC effects for either human or rat receptors.

Fig. 6 is a plot of the concentration–effect functions for TOL and PERC with their 95% confidence intervals. The plotted points are the means calculated by summing data over both species and all receptors. The derived concentration-equivalence equation was

$$\mathrm{EQUIV}(C_{\mathrm{PERC}}) = \exp\{1.64\log_e(C_{\mathrm{TOL}}) - 2.86\}$$

in which EQUIV(C_{PERC}) is the concentration of PERC that produces the same inhibition as any given concentration of TOL (C_{TOL}). Table 1 gives the variance–covariance matrix from fitting the equations to TOL and PERC, respectively, and which was used in the Monte-Carlo computation of the confidence interval for the concentration–equivalence curve (Fig. 7).

Discussion

This is the first study to examine PERC effects on ion channel function as well as to compare directly VOC effects on human and rat nAChRs. Results of the present study demonstrate that PERC inhibited nAChRs in a concentration-dependent and reversible manner. Additionally, the present results confirm TOL inhibition of rat nAChRs (Bale et al., 2002) and extend this effect to human nAChRs. Overall, there were no significant species differences in sensitivity of the α7, α4β2, and α3β2 nAChRs to VOCs. However, PERC effects on nAChR occurred at significantly lower concentrations than TOL.

The VOC concentrations applied to the nAChR-expressing oocytes in these studies were comparable to measured brain concentrations associated with functional changes such as alterations in attention tasks and visual evoked potentials (Bushnell et al., 1994; Rebert et al., 1989). PERC (0.0015–0.065 mM) concentrations were at or lower than the predicted brain PERC concentration of ~167 μM (174 μg/g) after a 2-h inhalation exposure to 500 ppm PERC in rats (Dallas et al., 1994). Likewise, the TOL (0.03–0.9 mM) concentration range was at or below arterial TOL blood concentrations in both humans and rats (3–13 mM; Benignus et al., 1998) and measured brain concentrations in rats (0.5–3 mM; Kishi et al., 1988; van Asperen et al., 2003). Concentrations above 0.13 mM (1 mM) PERC or 0.9 mM (3 mM) TOL (theoretical values in parentheses) were not used because higher concentrations could not be solubilized adequately.

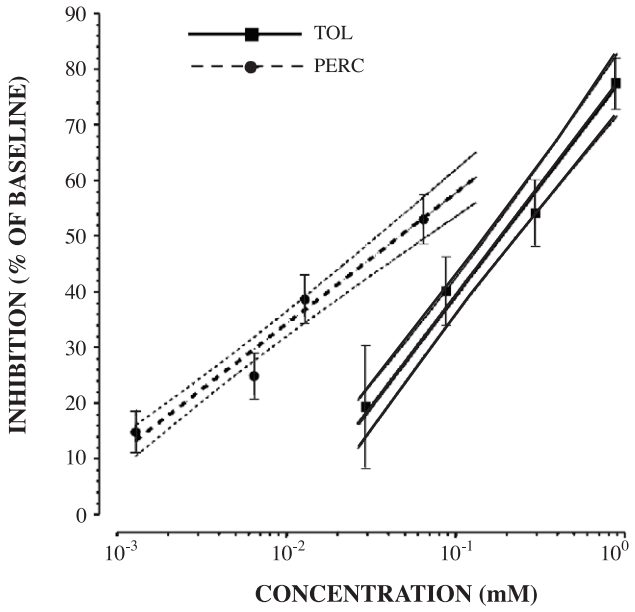

Fig. 6. Concentration–effect curves for TOL and PERC plotted as concentration on a natural log scale. The plotted points are means of data summed over both species and three receptors (these were not significantly different). The error bars are 95% confidence limits computed as univariate limits for each mean.

It was originally believed that lipophilic compounds, such as TOL and PERC, produced their adverse behavioral effects in humans and animals by perturbing the cellular membrane. Recent studies have demonstrated that the CNS depressive effects of these compounds, in vivo, may be due to interactions with specific ion channels and membrane receptors (Franks and Lieb, 1984, 2004). In our study, neither TOL (0.03–0.9 mM) nor PERC (0.0015–0.065 mM) altered resting membrane potentials. This finding is in agreement with previous studies (Bale et al., 2002; Cruz et al., 1998, 2000) that demonstrated lack of membrane activity during perfusion of VOC alone. These results suggest that both TOL and PERC are not perturbing the oocyte membrane to produce changes in nAChR activity.

In the present study, there was a 13% recovery of PERC (0.005–0.05 mM, theoretical) and a 30% recovery of TOL (0.05–3 mM, theoretical) from prepared stock solutions. These findings are consistent with the results presented in Beckstead et al. (2000) where they reported a 42% recovery of TOL in their oocyte perfusion system and a 32% recovery of TOL in their hippocampal slice system (PERC was not examined in this study). Therefore, VOC concentrations in buffer appear to be dependent on the experimental setup and the buffer perfusion system that is used for VOC exposure in addition to the volatility associated with these compounds.

In a similar experiment, Cruz et al. (2000) examined solvent evaporation over a period of 30 min with toluene and observed a recovery in solvent concentration of nearly 80%. The properties varied from solvent to solvent; 1,1,1-trichloroethane almost completely evaporated after 30 min whereas nearly 75% of ethylbenzene was retained. Similarly, TOL has minimal evaporation after 30 min (Cruz et al., 1998, 2000). Although the solvent loss for TOL was less than what was observed in the present study, solvent concentrations in the Cruz et al. (2000) study were measured without flowing through a buffer perfusion system. PERC evaporation rates have not been examined in this manner although there is most likely a loss of PERC, as has been reported with TOL.

There was a conservation in relative VOC sensitivity with the three nAChRs tested (human and rat types). As evidenced by the concentration–equivalence function that quantified the relative potency of two substances (TOL and PERC; Fig. 6), it is clear that the equivalent concentrations of PERC and TOL depend on their concentration. At low concentrations, PERC is more potent than TOL by nearly an order of magnitude. However, as the concentrations increase, the potency differences diminish between the solvents (at theoretical ranges). Both human and rat nAChRs were more sensitive to PERC than TOL at the tested concentrations in this study. At the lower VOC concentrations, this effect was not surprising because in comparative studies (PERC and TOL), PERC causes adverse behavioral effects at lower exposure concentrations (Echeverria et al., 1991, 1995; Kjellstrand et al., 1985).

Several laboratories have demonstrated that single amino acid changes in the transmembrane domains (TMD) of a receptor subunit, particularly in the second or third TMD, can change the sensitivity of the entire receptor to ethanol or general anesthetics ($GABA_A$/glycine receptors: Beckstead et al., 2001; Mihic et al., 1997; NMDA receptor: Ronald et al., 2001; nicotinic receptor: Yamakura et al., 2000). Yamakura et al. (2000) found that single amino acid changes in the second TMD of nAChRs β subunits conferred changes in general anesthetic sensitivity. It is possible that sensitivities of the heteromeric receptors to VOCs were not significantly different between the species because potential VOC sites on α4β2 and α3β2 receptors were unchanged between rat and human receptors. These nAChRs share over an 85% homology in amino acid sequence (Chavez-Noriega et al., 1997). A change of valine to a phenylalanine at position 253 in the second TMD in the β2 subunit altered general anesthetic (Yamakura et al., 2000) and toluene (Bale et al.,

Table 1
Variance–covariance matrix for the solution of the confidence intervals for the concentration–equivalence equation relating the potency of PERC and TOL for percent inhibition

	TOL intercept	TOL slope	PERC intercept	PERC slope
TOL intercept	25.59	−5.070	0.0	0.0
TOL slope	−5.070	1.117	0.0	0.0
PERC intercept	0.0	0.0	2.275	−0.729
PERC slope	0.0	0.0	−0.729	0.412

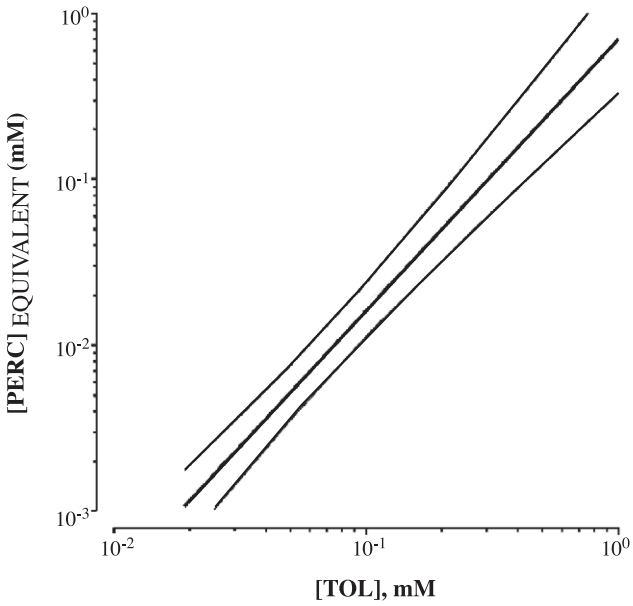

Fig. 7. The concentration equivalence curve for PERC as a range of TOL concentrations. The curve was computed by a Monte-Carlo method from the means and variance–covariance matrix (Table 1).

2002) sensitivity in β-containing nAChRs. However, this amino acid (valine 253) as well as the residues around this valine are highly conserved between the human and rat β2 subunits. Thus, it is not entirely surprising that there were no significant differences in VOC sensitivity between the human and rat variants of the α4β2 or α3β2 nAChR. To date, no known studies have examined potential amino acid sites with the α7 nAChR subunit. However, amino acid sequences in human and rat α7 subunits are 93% conserved [Peng et al., 1994; Seguela et al., 1993; PubMed sequences NM_012832 (rat), NM_000746 (human)]. The high homology of these nAChR subunits between rats and humans support the absence of species differences in VOC-induced receptor inhibition that were observed in this study.

It can be difficult to characterize potential hazards of the VOC compounds using only rat models when there may be different VOC sensitivities between rats and humans. The method utilized in this study can compare effects between species using the same VOC concentrations and exposures. Furthermore, differences can be easily characterized at the receptor–ligand level. In this study, the human and rat nACh receptor types did not display a significant difference in VOC (PERC and TOL) sensitivity. These findings are not consistent with either the PERC meta-analysis (Guth et al., 1997) where results predicted that humans were two times more sensitive to this compound in comparison to rats, or with the TOL meta-analysis (Benignus et al., 1998) which found that humans were five times more sensitive to TOL than rats. Thus, the species differences observed in the meta-analyses are unlikely to arise from species differences in nAChR susceptibility at the toxicodynamic level.

Many factors could contribute to possible species differences to VOC effects. For example, there are differences in human and rat sensitivity and toxicokinetics to both TOL (Benignus et al., 1998) and PERC (Guth et al., 1997). Although no differences in VOC sensitivity and current responses were present between the human and rat nACh receptors, perhaps other receptors or ion channels may be also associated with changes in VOC sensitivity between species.

VOCs act at many receptors in the central nervous system. For example, in addition to the effects on the nAChRs, VOCs inhibit NMDA receptors (Cruz et al., 1998, 2000), potentiate $GABA_A$ and glycine receptors (Beckstead et al., 2000, 2001), increase in serotonin receptor activity (Lopreato et al., 2003), and alter calcium channel activity (Tillar et al., 2002). Species differences at these receptors have yet to be examined. A combination of species differences at these sites as well as pharmacokinetic differences in the rat and human may contribute to differences in resultant VOC effect.

This study is part of an ongoing effort to develop an exposure–dose response model for VOC effects on CNS function. When conducting risk assessments for VOCs, uncertainty factors have to be utilized to extrapolate from rodents to humans. The present study provides a non-invasive, in vitro method for directly comparing VOC effects between humans and rats. By examining cellular sites of action, the risk assessment of volatile organic compounds will be strengthened by decreasing uncertainty factors between animal to human extrapolations. The human and rat comparisons made at the receptor level for the nAChR are similar between the two species, although there are instances where VOC sensitivity is different. VOCs such as TOL and PERC also have several other possible targets in the central nervous system, which also need to be examined before completely evaluating differences between rodents

and humans. Once these potential targets are investigated, a clearer comparison of VOC effects should be possible between the two species.

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