PERTURBATION OF VOLTAGE-SENSITIVE Ca\(^{2+}\) CHANNEL FUNCTION BY VOLATILE ORGANIC SOLVENTS

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ABSTRACT

The mechanisms underlying the acute neurophysiological and behavioral effects of volatile organic compounds (VOCs) remain to be elucidated. However, the function of neuronal ion channels is perturbed by VOCs. The present study examined effects of toluene (TOL), trichloroethylene (TCE) and perchloroethylene (PERC) on whole cell calcium current (I$_{Ca}$) in nerve growth factor-differentiated pheochromocytoma (PC12) cells. All three VOCs affected I$_{Ca}$ in a reversible, concentration-dependent manner. At +10 mV test potentials, VOCs inhibited I$_{Ca}$, whereas at test potentials of -20 and -10 mV, they potentiated it. The order of potency for inhibition (IC$_{50}$) was PERC (270 μM) > TOL (720 μM) > TCE (1525 μM). VOCs also changed I$_{Ca}$ inactivation kinetics from a single to double exponential function. Voltage-ramp experiments suggested that VOCs shifted I$_{Ca}$ activation in a hyperpolarizing direction; this was confirmed by calculating the half maximal voltage of activation ($V_{1/2,act}$) in the absence and presence of VOCs using the Boltzmann equation. $V_{1/2,act}$ was shifted from ~ -2 mV in control to -11, -12 and -16 mV by TOL, TCE and PERC, respectively. Similarly, VOCs shifted the half-maximal voltage of steady-state inactivation ($V_{1/2,inact}$) from ~ -16 mV in control to -32, -35 and -20 mV in the presence of TOL, TCE and PERC, respectively. Inhibition of I$_{Ca}$ by TOL was confirmed in primary cultures of cortical neurons, where 827 μM TOL inhibited current by 61%. These data demonstrate that VOCs perturb voltage-sensitive Ca$^{2+}$ channel function in neurons, an effect that could contribute to the acute neurotoxicity of these compounds.
INTRODUCTION

Volatile organic compounds (VOCs) such as toluene (TOL), 1,1,1-trichloroethylene (TCE) and 1,1,2,2-tetrachloroethylene (perchloroethylene, PERC) are widely utilized as paint thinners, industrial degreasing agents, and dry-cleaning agents. Acute exposure to sufficient concentrations of these compounds results in behavioral and neurological deficits, characterized by biphasic changes in locomotor activity (Bushnell et al., 1985), psychomotor impairment (Moser and Balster 1986) incoordination, sedation (Tegeris and Balster 1994) and alterations in cognitive ability (Echeverria et al., 1991; Bushnell, 1997). The mechanism(s) underlying the acute effects of these and other VOCs are not well understood and generally have received little attention.

VOCs share many effects with CNS depressant compounds such as ethanol, barbiturates, benzodiazepines and volatile anesthetics (for review, see Evans and Balster 1991). Previously, the neuroactivity of volatile anesthetics and related compounds was thought to be due to their ability to perturb the fluidity of the plasma membrane, as their potency correlates strongly with their solubility in the organic phase (Meyer, 1899). However, increasing evidence suggests that ethanol (Crews et al., 1996), volatile anesthetics (Yamakura et al., 2001; Dilger 2002) and VOCs exert acute effects on neuronal function via interactions with a variety of voltage- and ligand-gated ion channels. Specifically, TOL has been shown to potentiate currents mediated by GABA_A receptors (Beckstead et al., 2000), and to inhibit currents mediated by NMDA (Cruz et al., 1998; 2000) and nicotinic acetylcholine (nAChR) receptors (Bale et al., 2002; 2005). Recently, effects of TOL were demonstrated on voltage-sensitive Ca^{2+} channels (VSCC) in pheochromocytoma (PC12) cells (Tillar et al., 2002).
Voltage-sensitive calcium channels regulate important neuronal functions including neurotransmitter release. Alcohol and volatile anesthetics have been demonstrated to disrupt function of VSCC (Study, 1994; Kameyama et al., 1999; Kamatchi et al., 1999; McMahon et al., 2000) and this action has been suggested to contribute to effects of these compounds on the nervous system. The present studies were designed to test further the hypothesis that VOCs interact with VSCC. PC12 cells are a clonal cell line that expresses both N- and L-type VSCC following differentiation with nerve growth factor (NGF). This cell line has been utilized as a model system to study effects of neurotoxicants (for review, see Shafer and Atchison 1991) and ethanol (McMahon et al., 2000) on Ca\(^{2+}\) channel function. Toluene reduced KCl-induced Ca\(^{2+}\) responses measured by fura-PE3 as well as reduced whole cell Ca\(^{2+}\) currents in PC12 cells (Tillar et al., 2002). Thus, this cell line was chosen as an appropriate model to characterize the effects of TOL on VSCC, as well as to examine the effects of TCE and PERC. Specifically, the abilities of TCE and PERC to inhibit VSCC function were examined, and the interactions of TOL, TCE and PERC with VSCC function in NGF-differentiated PC12 cells were characterized. This included examining the reversibility of VOC effects, concentration-response relationships and VOC effects on steady-state activation and inactivation. Finally, because PC12 cells are a clonal cell line, the sensitivity of neuronal VSCC to VOCs were confirmed by examining TOL inhibition of \(I_{Ca}\) in primary cultures of cortical cells.
MATERIALS AND METHODS

Chemicals. Toluene (99.5% purity), 1,1,1-trichloroethylene (99.5% purity) and 1,1,2,2-tetrachloroethylene (99.5% purity) were obtained from Sigma-Aldrich (St. Louis, MO). Toluene solutions were prepared immediately before use by addition of TOL to extracellular solution (see below), followed by vigorous shaking in a teflon-capped vial. Solutions of TCE and PERC were prepared by addition of these solvents to extracellular solution followed by sonication in a water bath for several minutes. In all cases, solutions were prepared immediately before use, the amount of headspace in the containers was minimized and the total volume of solvent added did not exceed 0.05% of the volume of extracellular solution (see below). Omega-conotoxin GVIA (GVIA) was obtained from Peninsula Laboratories (Belmont, CA) and was dissolved in extracellular buffer to prepare 100 μM stock solutions. All other chemicals were purchased from commercial vendors and were of the highest available quality.

Cell culture. Rat pheochromocytoma (PC12) cells were grown in polystyrene culture flasks in Dulbecco’s Modified Eagle Medium (DMEM), containing 44 mM NaHCO₃, 2 mM HEPES, 7.5% fetal bovine serum (FBS) and 7.5% horse serum. Cells were incubated at 37°C in a humidified incubator with a 5% CO₂ atmosphere, were fed with fresh medium every 3-4 days and passaged once weekly. In all experiments, cells from passage 10 (designated from the original vial received as a gift from the late Dr. G. Guroff, NIH), were utilized. For electrophysiological recordings, undifferentiated cells were plated at a density of 7.5 x 10⁴ cells/ml into 6 well plates (2 ml/well) containing poly-L-lysine-coated (50 μg/ml) glass coverslips. Following a 2 hr period
in DMEM/F-12 containing 10% FBS, the medium was replaced with 2 ml of serum-free DMEM/F-12 media containing 50 ng/ml of human recombinant nerve growth factor (NGF). NGF was replenished every 48 hr by removing 1 ml of medium in each well and replacing it with 1 ml of medium containing 100 ng/ml of NGF. Recordings were made using cells that had been exposed to NGF for 4 to 6 days.

Primary cultures of rat cerebro-cortical cells were prepared as previously described (Inglefield et al., 2002). Briefly, cells were plated (3 ml) onto poly-L-lysine coated coverslips at a density of 2 x 10^6 cells/ml. After 3 days in vitro, 5 µM β-cytosine arabinofuranoside was added to prevent overgrowth of glial cells. After 13 days in vitro, cells were used for recordings of I_{ca}.

**Electrophysiology.** Voltage-sensitive Ca^{2+} currents (I_{ca}) were recorded at room temperature (~20 °C) using the whole-cell configuration of the patch-clamp technique. Experiments were completed within 10 min of obtaining the whole-cell configuration; run-down of Ca^{2+} current is insignificant over this time period. Unless otherwise noted, the holding potential was -70 mV. Current-voltage relationships were recorded by delivering depolarizing voltage steps (200 ms duration) between -50 and +60 mV (10 mV increments) from the holding potential. Voltage ramps (140 ms duration) from -70 to +60 mV were also utilized as noted in the Results. Currents were amplified and filtered (2 kHz) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), digitized at a rate of 25 kHz using a Digidata 1200 A/D converter (Axon Instruments) and stored online on a personal computer for later analysis. pCLAMP 6.0 software (Axon Instruments) was used for data collection and analysis. Whole-cell capacitance and series...
resistance compensation were used to minimize capacitive transients; greater than 80% compensation was commonly achieved for series resistance. Leak subtraction was performed online using the P/N (N=4) protocol within the pCLAMP program. Patch electrodes were fabricated from glass blanks (Warner Instrument, Hamden, CT) and had resistances of 2-4 MΩ when filled with intracellular recording solution containing (mM): CsCl (125), tetraethylammonium (TEA) chloride (10), EGTA (5), HEPES (10), MgCl₂ (1), d-glucose (10) and ATP (4), pH 7.3, adjusted to ~315 mOsm with sucrose. Extracellular recording solution contained (mM): NaCl (125), HEPES (20), d-glucose (10), and TEACl (10), CaCl₂ (10) and MgCl₂ (1), pH 7.3 and was adjusted to ~330 mOsm with sucrose. For determination of steady-state inactivation, BaCl₂ was substituted for CaCl₂ on an equimolar basis. Currents through voltage-sensitive sodium channels were blocked by inclusion of 1 μM tetrodotoxin (TTX) in the extracellular solution. This concentration of TTX blocks completely currents mediated via voltage-sensitive sodium channels in PC12 cells (data not shown).

Inactivation of I_{Ca} during test pulses was described by either a single (control) or double (VOC exposure) exponential process, as follows:

\[ I = A_1 \exp[-(t-k)/\tau_1] + A_2 \exp[-(t-k)/\tau_2] + C \]  

(eq 1)

where \( A_1, A_2 \) and C are the slow, fast and non-inactivating components of current, and \( \tau_1 \) and \( \tau_2 \) are the time constants for the inactivating components of current. In the case of control currents, a single exponential function was sufficient to fit current decay.

To determine steady-state activation, current amplitudes at test potentials between -30 (-50 mV for TCE) and +10 mV were expressed as a fraction of current amplitude at the +10 mV
test potential and plotted vs test potential. To determine steady-state inactivation, pre-pulses (5s) to potentials ranging from -70 to +10 mV were delivered to the cell in 10 mV increments, followed by a test pulse to +10 mV. Current amplitude of the test pulse following each pre-pulse was expressed as a fraction of current amplitude following the -70 mV pre-pulse, and plotted vs pre-pulse potential. Data for both activation and steady-state inactivation were then fitted using a Boltzman function:

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V - V_{1/2}}{k}\right)} \quad \text{(eq 2)}
\]

where \(I\) is the current amplitude at a given test potential \(V\), (or pre-pulse potential, for inactivation), \(I_{\text{max}}\) is the maximum current amplitude, \(V_{1/2}\) is the voltage of half-maximal activation \((V_{1/2,\text{act}})\) or inactivation \((V_{1/2,\text{inact}})\), and \(k\) is the slope.

Finally, the inhibition of \(I_{\text{Ca}}\) by TOL was compared in the presence and absence of the N-type VSSC antagonist \(\omega\)-conotoxin GVIA. Effects of TOL on peak current amplitude were measured following a 200 msec step depolarization from -70 to +10 mV prior to and after application of GVIA (1 \(\mu\)M).

**VOC exposure and analysis.** Cells were exposed to VOCs via perfusion in the extracellular solution. Briefly, extracellular solution (~10 ml) containing TOL, TCE or PERC was added to a 50 ml glass syringe and gravity perfused into the recording chamber at a rate of 1-2 ml/min via a short length of polyethylene tubing. To determine actual concentrations of VOCs in recording solutions, Hamilton gas-tight syringes were used to take 500 \(\mu\)l samples directly from the recording chamber under conditions identical to those during recordings. These samples were
transferred directly to glass analysis vials, capped, frozen and shipped to National Medical Services (Willow Grove, PA) for analysis. For TOL, duplicate samples were collected over the nominal concentration range of 0.3 to 5 mM. Similarly, TCE and PERC duplicate samples were collected over the nominal concentration ranges of 0.5 to 5 mM and 0.25 to 0.825 mM, respectively. The concentration of volatile organic compound in each sample was determined by gas chromatography with flame ionization detection under contract with National Medical Services (Willow Grove, PA).

To measure VOC concentration in each sample, ten-point calibration curves were prepared for PERC (0-0.72 mM), TOL (0-3.3 mM) and TCE (0-6.1 mM) samples and checked against reference standards (Absolute Standards, Inc., Hamden, CT). Each 500 µl VOC sample was heated, pressurized and the vapor was injected into an Agilent 6890 (Agilent Technologies, Palo Alto, CA) gas chromatograph equipped with a flame ionization detector. The measured concentrations from this study are the values reported in the manuscript.

**Statistical Analysis.** *Effects of VOCs on peak and end current amplitude and activation rate.* VOC inhibition of current amplitude and activation rate were examined within the same set of data obtained to characterize concentration-response relationships. Thus, for each VOC two dependent variables were analyzed from the same data set and statistical analyses for each variable were not independent. To maintain an experiment-wise $p = 0.05$, a Bonferroni correction was applied so that the corrected $p$ value was $0.05/2 = 0.025$. For current amplitude, a three-factor ANOVA was conducted examining peak vs end (within 20 ms of the end of the voltage
step) current, VOC type, and VOC concentration. Similarly, a three-factor ANOVA was conducted to examining activation rate, VOC type and VOC concentration.

Because the effects of up to two VOC concentrations were usually measured on a single cell, but not all concentrations were tested in all cells, a mixed model (Proc Mixed (SAS, Carey, NC)), repeated measures analysis of variance (ANOVA) was utilized. When an ANOVA yielded a $p < 0.025$, stepdown tests were conducted as described in the results.

For all relationships that were found to be statistically significant in these tests, a concentration-effect curve was fitted using the logistic curve model (Benignus 2001). The logistic curve has a general sigmoidal shape and its equation is

$$y = \frac{1}{1+e^{(\beta_1 + \beta_2 \ln(\text{conc}))}}$$

(eq 3)

in which $y$ is the dependent variable, $e$ is the base of the natural logarithm, $\beta_1$ and $\beta_2$ are empirical parameters which jointly control the location of the curve and its rate of approach to asymptote, ln is the natural logarithm and conc is the concentration of VOC.

When effects of two solvents were significantly different, a concentration-equivalence equation was derived, including 95% confidence limits calculated by Monte-Carlo methods (Benignus, 2001). The concentration-equivalence equation expresses the relative potency of two chemicals over a range of concentrations.

**Current/voltage relationships and steady-state activation and inactivation.** Data for current/voltage (I/V) relationships and activation/inactivation were analyzed by repeated-measures ANOVAs. In each ANOVA, significance of solvent effects (treated vs. control) was determined using a repeated-measures test and significant differences between
solvents (comparison of the solvents to each other) were determined by independent-measures tests. Data for the steady-state activation were derived from the I/V relationships, resulting in two analyses being performed on the data derived from the same group of cells. Therefore, a Bonferroni correction was applied so that the experiment-wise $p$ value remained at 0.05; for each comparison $p = 0.05/2 = 0.025$.

Toluene effects in the presence and absence of $\omega$-conotoxin GVIA (GVIA). Solvent effects in the presence and absence of GVIA were analyzed by a two factor ANOVA, followed by step-down ANOVAs for comparisons between two treatment groups. Effects were considered significant when $p < 0.05$. 


RESULTS

Effects of VOCs on the amplitude and kinetics of $I_{Ca}$. TOL (857 μM), TCE (827 μM) and PERC (152 μM) rapidly and reversibly blocked $I_{Ca}$ in NGF-differentiated PC12 cells (Figures 1 and 2). Leak and capacitive currents were not altered by any of the VOCs (data not shown). To confirm that effects of VOCs were not due to solubilization of unknown compounds from the delivery system tubing (Glossmann et al., 1993), separate experiments were conducted wherein extracellular solution containing each VOC was added manually via glass pipette directly to the recording chamber (i.e. there was no contact of the VOC containing solution with polyethylene tubing). Using this exposure method, effects of TOL, TCE and PERC were identical to those observed when VOCs were delivered via tubing (data not shown). The rate of current activation was not significantly affected by any of the VOCs tested (no significant results in the three factor ANOVA). In contrast, the kinetics of current inactivation during the voltage step were clearly altered by exposure to the three VOCs (e.g. Figure 2). The inactivation kinetics of $I_{Ca}$ were described by a single exponential function prior to treatment with VOCs. However, after exposure to VOCs, current inactivation was better described by kinetics with two exponential decay components (Table 1). To test whether the altered inactivation rate was due to changes in channel inactivation or to a preference of VOCs for the open state of VSCC, PC12 cells were exposed to VOCs in the absence of any stimulation for 3 minutes. Upon resumption of test potentials, the degree of block of $I_{Ca}$ was similar to that in which cells received continued stimulation (data not shown). This indicates that VOCs do not have a preference for the open state of the channel, and that changes in the inactivation are due to alterations of channel kinetics.
Because all three VOCs have relatively high volatility and limited solubility in aqueous solution, the actual concentration of each VOC in the extracellular solution in the recording chamber was determined by gas chromatography. Thus, the concentrations reported here represent measured exposure concentrations rather than the concentrations calculated based on the amount added to the extracellular solution.

Inhibition of $I_{Ca}$ by the three VOCs was concentration-dependent (Figure 3A-C). Statistical analysis of normalized peak and end current amplitudes for all three VOCs indicated statistically significant effects of peak vs end current, VOC type and VOC concentration as well as an interaction between VOC type and VOC concentration. Nonlinear mixed model procedures (SAS Proc NLMIXED) were used to fit separate concentration-response relationships and 95% confidence limits for effects of each of the three solvents on peak (Figure 3D) and end current amplitude. Based on these determinations, the IC$_{50}$ values for inhibition of peak $I_{Ca}$ were 270, 720 and 1525 μM for PERC, TOL and TCE, respectively. End current amplitude was slightly but significantly (three factor ANOVA) more sensitive to VOC effects, with IC$_{50}$ values of 173, 306 and 1170 μM for PERC, TOL and TCE, respectively. For each VOC, the 95% confidence limits for peak and end currents overlapped. The slightly increased sensitivity of the end current is likely to reflect the ability of VOCs to increase the inactivation rate of $I_{Ca}$ (see Discussion). Because the differences in sensitivity for each VOC were less than one-half of an order of magnitude, had overlapping confidence limits, and likely reflect changes in inactivation rate, all further analyses were conducted on the peak current amplitude.

Because TOL has been most extensively studied and utilized of the three VOCs, it was
used as the "standard" for determination of concentration-equivalence equations (Figure 4). The concentration-equivalence relationships are

\[ CTOL_{\text{equiv}} = e^{0.2598 + 1.118 \times \ln(CPERC)} \]  \hspace{1cm} (eq 4)

for PERC / TOL equivalence and

\[ CTOL_{\text{equiv}} = e^{-1.572 + 1.098 \times \ln(CTCE)} \]  \hspace{1cm} (eq 5)

for TCE/TOL equivalence.

In the above equations, CTOL_{\text{equiv}} is the concentration of TOL which is equivalent in potency to the concentrations of either PERC or TCE (CPERC or CTCE) and e is the base of the natural logarithm, \ln.

Effects of VOCs on Ca^{2+} current-voltage relationships, and VSCC activation and inactivation.

All three VOCs had similar effects on the current voltage-relationship; current amplitude was decreased at test potentials depolarized from 0 mV and increased at test potentials of -10 and -20 mV (Figure 5A). A three-way ANOVA indicated that there were significant effects of treatment (presence or absence of VOC), VOC type (TOL, TCE or PERC) and voltage, as well as interactions between treatment and voltage, and voltage and VOC type. Stepdown two-way ANOVAs indicated significant interactions between TOL and voltage, with TOL effects being statistically significant at the -10, 10, 20 and 30 mV test potentials. To eliminate the possibility that increases in I_{Ca} at more hyperpolarized potentials were due to solvent-mediated activation or unblocking (by TTX) of voltage-sensitive sodium channels, extracellular solutions were prepared in which NaCl was replaced on an equimolar basis by choline chloride. Choline chloride does not
permeate voltage sensitive Na\(^+\) channels, eliminating any current mediated via voltage-sensitive Na\(^+\) channels. However, exposure of cells (n = 3) to TOL in choline containing solutions did not eliminate the enhancement of current amplitude at -10 mV, indicating that the enhanced current is not mediated via sodium (Figure 5A, inset). Similar responses were observed in two cells exposed to TCE (PERC was not tested). Currents elicited by a voltage ramp from -70 to +60 mV exhibited similar responses to VOCs. When VOCs were present, there was a clear increase in ramp-induced I\(_{\text{ca}}\) at more hyperpolarized potentials, as well as a shift in the potential at which peak current occurred (Figure 5B). As with other effects, these changes were reversible upon washout of the solvents (data not shown).

The increase in I\(_{\text{ca}}\) at hyperpolarized test potentials suggests that VOCs shift the activation of VSCC in a hyperpolarizing direction. This was tested by determining the half maximal voltage of activation (V\(_{1/2,\text{act}}\)) using the Boltzman equation. Using the Boltzman equation, V\(_{1/2,\text{act}}\) was determined in the absence and presence of each VOC. In all cases, V\(_{1/2,\text{act}}\) shifted significantly in a hyperpolarizing direction in the presence of VOCs (Table 2 and Figure 6).

Effects of VOCs on steady-state inactivation of VSCC were examined by subjecting cells to 5 sec prepulses between -70 and +10 mV prior to a 150 ms test pulse to +10 mV from a holding potential of -70 mV. The steady-state inactivation curves shifted significantly in a hyperpolarized direction in the presence of all three VOCs (Figure 7). The calculated V\(_{1/2,\text{inact}}\) values for TOL, TCE and PERC were significantly different from their respective control values (Table 3).
Effects of TOL on ω-conotoxin GVIA-insensitive current in PC12 cells. Undifferentiated PC12 cells express predominantly L-type VSCC, whereas NGF-differentiated PC12 cells express both N and L-type VSCC (for review, see Shafer and Atchison 1991). Previous work with TOL suggested that N-type VSCC might be more sensitive to VOCs than are L-type VSCC: for example KCl-induced increases in intracellular Ca\(^{2+}\) and whole cell Ca\(^{2+}\) currents are more sensitive to TOL in differentiated than in undifferentiated PC12 cells (Tillar et al., 2002). To examine further whether differences in sensitivity to TOL exist between N- and L-type VSCC, the ability of TOL to block I\(_{Ca}\) was examined in the absence and presence of the specific N-type VSCC antagonist ω-conotoxin GVIA (GVIA). As shown in Figure 8, TOL (857 μM) reduced I\(_{Ca}\) by ~45% in the absence of GVIA, and this effect was nearly completely reversible (recovery to 86% of control). Addition of 1 μM GVIA to the extracellular solution blocked 45% of the current present after washout of TOL as well as eliminated the inactivating component of current completely in 4 of 6 cells and dramatically reducing it in the remaining 2 cells (Figure 8, traces labeled CON, GVIA). In the presence of GVIA, a second exposure to 857 μM TOL blocked only 30% of the GVIA-resistant current. In addition, TOL did not alter kinetics of inactivation, even when a slight inactivating component was present (Figure 8; traces labeled GVIA and GVIA+TOL). TOL effects on GVIA insensitive current amplitude were significantly (ANOVA, \(p <0.05\)) different from its effect on control current, indicating that this current component is less sensitive to TOL than is N-type current.

Effects of TOL on \(I_{Ca}\) in cortical neurons. PC12 cells are derived from a rat pheochromocytoma, and express neuronal characteristics only upon exposure to nerve growth factor. In order to
confirm that neuronal VSSCs are sensitive to VOCs, the ability of TOL to inhibit $I_{\text{ca}}$ was examined in primary cultures of neocortical cells. Primary neocortical cultures contain both neurons and glia, but previous work has demonstrated that morphology is a reliable discriminator of neurons from glia, with pyramidal-shaped cells expressing MAP-2 but not GFAP (Inglefield et al., 2002). In recordings from cells with pyramidal morphology, TOL (415 µM) inhibited peak and end current by 12.1 ± 2.8 and 28.0 ± 6.6 %, respectively ($n = 3$). In the presence of 857 µM TOL, peak and end currents were inhibited by 61.4 ± 5.0 and 82.7 ± 7.6 %, respectively ($n = 3$). These results confirm that the sensitivity of $I_{\text{ca}}$ to TOL is not unique to PC12 cells.
DISCUSSION

The present data demonstrate that TOL, TCE and PERC disrupt VSCC function by altering steady-state activation and inactivation, current amplitude and inactivation kinetics. These effects were rapid and readily reversible, with a rank order of potency of PERC>TOL>TCE. These results confirm our previous demonstration that TOL inhibits VSCC in PC12 cells (Tillar et al., 2002) and extends the understanding of this effect by characterizing in detail the interaction of TOL with VSCC. Further, these results demonstrate that TCE and PERC, which have acute behavioral effects similar to those of TOL, also disrupt I_{ca} in a manner nearly identical to TOL. Finally, the present results confirm that VSSC in cortical neurons in primary culture are sensitive to inhibition by TOL.

The characteristic effects of VOCs on VSCC bear striking similarities to those of volatile anesthetics, such as halothane and isoflurane. These drugs decrease the amplitude of both peak and residual (similar to “end current” here) Ca^{2+} currents, shift Ca^{2+} current inactivation to hyperpolarizing potentials, and increase the inactivation rate of native VSCC in hippocampal (Study 1994) and dorsal root ganglion neurons (Kameyama et al., 1999) as well as VSSC expressed in oocytes (Kamatchi et al., 1999). One difference between the VOCs and volatile anesthetics is the ability of the former to shift the activation potential to more hyperpolarized potentials. However, the overlap in their effects at the channel level is not surprising considering the overlap in effects of these compounds at the behavioral level. Indeed, VOCs have anesthetic effects at high concentrations and have been used for this purpose in the past.

Recently, NMDA (Cruz et al., 1998; 2000), nACh (Bale et al., 2002; 2005), 5HT₃
GABA_A and glycine (Beckstead et al., 2000; 2001) receptors have been demonstrated to be sensitive targets of TOL and other VOCs. Previously TOL (Tillar et al., 2002) and 1,1,1-trichloroethane (Okuda et al., 2001) have been reported to alter VSSC function. The present data further establishes VSCC as potential targets of VOC action by demonstrating that TCE and PERC have similar effects to TOL. In addition, preliminary evidence indicates that xylene also interacts with VSCC in a manner similar to the VOCs examined in the present study (unpublished data). Thus, effects on VSCC and other ion channels, may play important roles in the acute neurotoxicity of VOCs.

Not all ion channels are sensitive to modulation by VOCs. For example, non-NMDA currents were insensitive to TOL concentrations up to 9 mM (Cruz et al., 1998) and nAChR containing the β3 subunit were less sensitive to TOL than those containing the β2 subunit (Bale et al., 2002). The present data also indicate differences in sensitivity of different VSCC types to inhibition by TOL. Because PC12 cells express both N- (GVIA-sensitive) and L- (GVIA insensitive) type VSCC, the diminished effect of TOL in the presence of GVIA suggests that L-type VSCC may be less sensitive to TOL than are N-type VSCC. This is consistent with previous data demonstrating that KCl-induced fura-3PE responses and I_{Ca} were less sensitive to TOL in undifferentiated compared to NGF-differentiated PC12 cells (Tillar et al., 2002). Alternatively, TOL and GVIA may share a common binding site. In undifferentiated PC12 cells, I_{Ca} is predominantly (although not entirely) L-type, whereas in NGF-differentiated PC12 cells, I_{Ca} is comprised of both N- and L-type currents (Shafer and Atchison 1991). The slightly greater sensitivity of the end vs peak current to VOCs may at first appear to be inconsistent with greater
sensitivity of N-type current, as end current should have a greater contribution of L-type current due to inactivation of N-type current during the voltage step. TOL increased $I_{Ca}$ inactivation rate in the absence of GVIA, but was without this effect on GVIA-insensitive (presumably L-type) current. Thus, the slight difference in peak and end current sensitivity is likely due to the altered inactivation rate. The lack of TOL effect on inactivation rate in the presence of GVIA indicates that the change of inactivation kinetics are due to effects on N-type VSCC. Additional studies will be needed to characterize further the potential differences in sensitivity among the different VSCC types; effects on several channel types not expressed readily in the PC12 cells, including P/Q-, R- and T-type have yet to be examined.

In the present study, VOCs disrupted VSCC function in a complex manner, altering current kinetics, steady-state activation and inactivation kinetics. These data demonstrate that disruption of VSCC function is not due to block of the channel pore, as effects observed with VOCs differ from those of divalent cation blockers such as Pb$^{2+}$, or Cd$^{2+}$ (for review, see Shafer, 2000). Instead, these complex VOC effects suggest that these compounds interfere with VSCC gating and voltage-sensing. In ligand-gated ion channels, transmembrane regions are important for interactions of ethanol, volatile anesthetics and to a lesser extent, VOCs (Krasowski and Harrison 2000; Ronald et al., 2001; Lopreato et al., 2003a). The fourth membrane spanning region of the $\alpha$ subunit of VSCC contains highly conserved residues responsible for sensing membrane voltage (Franciolini 1994). In addition, transmembrane regions, especially the 6th membrane spanning region, contribute to inactivation properties of high voltage-activated Ca$^{2+}$ channels (for review see, Stotz and Zamponi 2001). Based on this, it is reasonable to hypothesize
that transmembrane regions may be important in mediating VOC effects observed in the present study. However, in contrast to effects on \( I_{\text{Na}} \), TOL did not alter inactivation kinetics, steady-state inactivation nor the I/V relationships for \( \text{Na}_1.5 \) channels expressed in oocytes, but did shift activation in a depolarizing direction and exhibit use- and frequency-dependent block (Cruz et al., 2003a). Such differences in actions could be due to differences between expression in oocytes vs a clonal cell line. Regardless, elucidation of voltage-gated channels regions important to VOC effects currently lags behind that for ligand-gated channels.

It is difficult to compare the sensitivity of different types of receptor and voltage-gated channels to VOCs, as these studies have been conducted in different laboratories using different preparations. Toluene inhibited VSCC over a concentration range of 91-1500 \( \mu \text{M} \), with an approximate \( \text{IC}_{50} \) of 720 \( \mu \text{M} \) (65 \( \mu \text{g/ml} \)). This is comparable to concentrations reported to disrupt function of GABA\(_A\) (420 \( \mu \text{M} \); Beckstead et al., 2000), NMDA (100 - 1000 \( \mu \text{M} \); Cruz et al., 1998) and nACh (30 - 900 \( \mu \text{M} \); Bale et al., 2005) receptors in other preparations. The levels of inhibition in the present study were not corrected for the VOC-induced shift in activation to more hyperpolarized potentials, which may result in slight underestimations of inhibition. Blood TOL levels associated with impaired shock avoidance behavior in rodents are estimated to be between 30 and 100 \( \mu \text{g/ml} \) (Benignus et al., 1998). Based on models (Benignus 2001), brain TOL concentrations associated with impairment are approximately three times higher than blood concentrations. Thus, the sensitivity of VSCC, as well as the other channel types, is within the brain concentration range associated with impairments. Similarly, the TCE levels used in the present experiments (24-260 \( \mu \text{g/ml} \); \( \text{IC}_{50} = 196 \mu \text{g/ml} \)) are consistent with brain levels associated
with visual evoked potential impairment in rodents (20-120 μg/ml; Boyes et al., 2005). Finally, the relative potencies for VOC effects on VSCC agree with \textit{in vivo} data, where PERC and TCE are the most and least potent, respectively. Using a swimming protocol that induced “behavioral despair immobility” DeCeaurriz and co-workers (1983) demonstrated that PERC was a more potent inhibitor of immobility than was TOL following inhalation exposure. Further, PERC was the most potent and TCE the least potent of these VOCs at increasing motor activity following inhalation exposure (Kjellstrand et al., 1985). Thus, the concentrations and relative potencies for VOC effects on VSCC are consistent with those for \textit{in vivo} exposures.

Although acute exposure to TOL and other VOCs causes effects similar to ethanol and CNS depressants (Evans and Balster 1991), the roles of specific receptors mediating VOC effects \textit{in vivo} have not yet been thoroughly examined. Recently, it has been demonstrated that TOL, but not trichloroethane, can provide protection against NMDA-induced seizures in mice (Cruz et al., 2003b), suggesting that, in vivo, NMDA receptors are targets of TOL, but not trichloroethane. Transgenic mice expressing a mutant glycine receptor with reduced sensitivity to ethanol were less sensitive than wild-type mice to some behavioral impairments (Findlay et al., 2002), supporting a role for effects on ion channels in neurotoxicity observed with CNS depressants. Additional studies are needed to determine the relative contributions of different ion channel types, including VSCC, to the clinical or behavioral effects of VOCs.

In summary, the present results have demonstrated that TOL, TCE and PERC inhibit VSCC in a manner similar to volatile anesthetics and at concentrations that are consistent with brain levels of VOCs that result in behavioral alterations. The present data support the hypothesis
that effects on VSCC could contribute to the acute neurotoxicity of VOCs. By incorporating such information into models for solvent neurotoxicity (Bushnell et al., 2005), the scientific basis for predicting the risks associated with exposure to these and possibly other VOCs will be improved.
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REFERENCES


dioxide production and locomotor activity in mice. Fundam Appl Toxicol 5:971-977.

Bushnell PJ (1997) Concentration-time relationships for the effects of inhaled
trichloroethylene on detection of visual signals in rats. Fundam Appl Toxicol 36:30-38.

Bushnell PJ, Shafer TJ, Bale AS, Boyes WK, Simmons JE, Eklund C and Jackson TL
(2005) Developing an exposure-dose-response model for the acute neurotoxicity of organic
solvents: overview and progress on in vitro models and dosimetry. Env Toxicol Pharmacol

Crews FT, Morrow AL, Criswell H and Breese G (1996) Effects of ethanol on ion

Cruz SL, Mirshahi T, Thomas B, Balster RL and Woodward JJ (1998) Effects of the
abused solvent toluene on recombinant N-methyl-D-aspartate and non-N-methyl-D-aspartate


Cruz SL, Orta-Salazar G, Gauthereau MY, Millan-Perez Peña L and Salinas-Stefanón
EM (2003a) Inhibition of cardiac sodium currents by toluene exposure. Br J Pharmacol 140:653-
660.


Glossmann H, Hering S, Savchenko A, Berger W, Friedrich K, Garcia ML, Goetz MA,


Krasowski MD and Harrison NL (2000) The actions of ether, alcohol and alkane general anaesthetics on GABA\textsubscript{x} and glycine receptors and the effects of TM2 and TM3 mutations. *Br J Pharmacol* 129:731-743.


FOOTNOTES:

Preliminary results were presented at the 32nd Annual meeting of the Society for Neuroscience, November 2-7, 2002, and at the 42nd Annual Meeting of the Society of Toxicology, March 9-13, 2003, and have been published in abstract form (Program No. 437.28. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM, and Tox. Sci. 72(S1): 266, 2003). The information in this document has been funded wholly by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.
LEGENDS FOR FIGURES

Figure 1. Time-course of block of $I_{\text{Ca}}$ by TOL. Peak current amplitude vs time is plotted for a test pulse to +10 mV from a holding potential of -70 mV (frequency = 0.1 Hz). The black bar indicates the presence of 857 μM TOL in the bath solution. Current amplitude decreases in the presence of TOL and recovers rapidly following return to TOL-free extracellular solution.

Figure 2. VOCs rapidly and reversibly inhibit $I_{\text{Ca}}$ in PC12 cells. In three separate cells given a test potential from +10 mV (holding potential = -70 mV), VOCs blocked $I_{\text{Ca}}$. A) 857 μM TOL. B) 827 μM TCE and C) 152 μM PERC. Note that in the presence of VOCs, the rate of current inactivation was increased (see Table 1).

Figure 3. Concentration-response relationships for VOC effects on peak and end $I_{\text{Ca}}$. Inhibition of $I_{\text{Ca}}$ by A) TOL (n = 4-10 cells/concentration), B) TCE (n = 5-11 cells/concentration) and C) PERC (n = 4-7 cells/concentration) is shown for a test pulse from -70 to +10 mV. Effects on the peak (solid symbols) and end (open symbols) current is plotted as a function of VOC concentration. Current amplitudes were normalized to the pre-treatment value for each cell, and the “CON” represents the normalized control amplitudes for all cells treated with each respective solvent. Control peak amplitudes were: -768 ± 107 pA for TOL (n=28); -811 ± 53 pA for TCE (n=33); and -644 ± 86 pA for PERC (n=17). Three factor ANOVA indicated a significant main
effect \((p < 0.025)\) of peak vs end current, concentration of VOC, and VOC type, and a significant interaction \((p < 0.025)\) between concentration and VOC type. Step-down two-way ANOVAs indicated that all solvent pairs were significantly different from each other. Only TOL and TCE did not have a significant two-way interaction, implying that their concentration-response curves had different locations (e.g. potencies) but were not significantly different from parallel (e.g. similar shapes). For all comparisons, results were considered significant if \(p<0.025\). D) Fitted logistic concentration-effect curves for TOL, TCE and PERC (solid lines) inhibition of peak current amplitude and their 95% confidence intervals (dashed lines.)

**Figure 4. Concentration-equivalence functions for VOC effects on VSCC.** The calculated concentration equivalence curve for concentrations of PERC and TCE (solid lines) that are equivalent (produce the same degree of inhibition) as a range of TOL concentrations. The 95% confidence limits (broken lines) were computed by a Monte-Carlo method.

**Figure 5. Current-voltage (I/V) relationships in the presence and absence of VOCs.** A) The peak current amplitude obtained from a step depolarization to the indicated test potentials from a holding potential of -70 mV is plotted in the presence and absence of 857 \(\mu\)M TOL (top, \(n=6\)). 2.1 mM TCE (middle, \(n=7\)) and 152 \(\mu\)M PERC (bottom, \(n=5\)). The arrows indicate current amplitudes which are greater in the presence of VOC. A three-way ANOVA indicated significant
effects of treatment, solvent and voltage, and significant interactions between treatment and voltage and voltage and solvent. Step-down two-way ANOVAs indicated significant differences in effects of TOL at -10, 10, 20, and 30 mV test potentials (as indicated by the asterisk (*)). For all comparisons, results were considered significant if \( p < 0.025 \). Inset: effects of 857 \( \mu \text{M} \) TOL in extracellular solution containing NaCl (top pair of traces; scale bars 200\( \text{pA} \) and 50 msec) or choline chloride (bottom pair of traces, scale bars 100 \( \text{pA} \) and 50 msec). B) Current responses to a voltage ramp from -70 mV to +60 mV (140 ms) in 3 separate cells prior to and after exposure to TOL (top), TCE (middle) and PERC (bottom) at the indicated concentrations. Similar responses were demonstrated in 5 additional cells for TOL, 4 additional cells for TCE and 2 additional cells for PERC.

**Figure 6. VOCs shift the activation of \( I_{\text{ca}} \) in a hyperpolarizing direction.** Using the Boltzman equation (eq 2), the voltage of half-maximal activation (\( V_{1/2, \text{act}} \)) of \( I_{\text{ca}} \) was determined for PC12 cells in control and VOC-containing extracellular solutions. Peak current amplitudes at each test potential were normalized to the peak current amplitude at +10 mV. The data demonstrate that 857 \( \mu \text{M} \) TOL (top; \( n=6 \)), 2.1 mM TCE (middle; \( n=7 \)) and 152 \( \mu \text{M} \) PERC (bottom; \( n=5 \)) shift the activation of \( I_{\text{ca}} \) to hyperpolarized potentials, as statistically-significant changes in \( V_{1/2, \text{act}} \) are observed in each case.
Figure 7. VOCs shift steady-state inactivation in a hyperpolarizing direction. The half maximal voltage of inactivation, $V_{1/2,\text{inact}}$, was determined by delivering a 5 second pre-pulse to PC12 cells prior to a test pulse to +10 mV (from a holding potential of -70 mV). The peak current amplitude of the test pulse was normalized to current amplitude in the absence of any prepulse, plotted against the pre-pulse amplitude, and fitted by the Boltzman function (eq 2) for inactivation. TOL (n = 5 cells), TCE (9 cells) and PERC (3 cells) shifted the $V_{1/2,\text{inact}}$ to hyperpolarized potentials. A three-way, mixed model ANOVA indicated significant main effects of treatment (presence or absence of VOCs) and voltage, and a significant interaction between these variables ($p<0.025$). Stepdown ANOVAs indicated that control and treated values were significantly different at all voltages.

Figure 8. Current insensitive to GVIA is also less sensitive to TOL. Top: Peak current amplitude resulting from a voltage step to +10 mV (h.p.=-70) was determined in cells sequentially exposed to 857 μM TOL, WASH, 1 μM GVIA and 1μM GVIA plus 857 μM TOL (n = 6 cells). Following block of N-type VSCC with GVIA, inhibition of $I_{Ca}$ by 857 μM TOL was significantly less than in the absence of GVIA (30 vs 45% inhibition in GVIA + TOL vs TOL only, respectively). The lines with asterisks indicate treatments that are significantly different ($p < 0.05$) from each other (significant repeated measures ANOVA with step-down repeated measures ANOVAs for each comparison). Bottom: Example whole cell calcium currents in each treatment (not including WASH). Note that GVIA treatment dramatically reduced the
inactivation of current compared to control (CON) and that in the presence of GVIA, TOL does not induce changes in kinetics. Scale bars are 50 pA and 25 msec.
Table 1. Inactivation kinetics of $I_{\text{Ca}}$ in PC12 cells in the absence and presence of VOCs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$A_1$ (pA)</th>
<th>$A_2$ (pA)</th>
<th>$C$ (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>146 ± 34</td>
<td>N.A.</td>
<td>-459 ± 108</td>
<td>N.A.</td>
<td>-349 ± 82</td>
</tr>
<tr>
<td>TOL (857 μM)</td>
<td>76 ± 9</td>
<td>7.8 ± 1.1</td>
<td>-140 ± 13</td>
<td>-141 ± 24</td>
<td>-43 ± 17</td>
</tr>
<tr>
<td>TCE (827 μM)</td>
<td>115 ± 21</td>
<td>14 ± 4</td>
<td>-197 ± 36</td>
<td>-147 ± 36</td>
<td>-167 ± 18</td>
</tr>
<tr>
<td>PERC (152 μM)</td>
<td>131 ± 46</td>
<td>14 ± 4</td>
<td>-300 ± 60</td>
<td>-139 ± 18</td>
<td>-124 ± 35</td>
</tr>
</tbody>
</table>

Values were determined by fitting current decay to a single (CON) or double (VOC-treated) exponential decay function (eq 1), and are the means ± SEM of 18 (CON) and 6 (TOL, TCE, PERC) cells. N.A. = not applicable.
Table 2. Effects of VOCs on the half-maximal voltage of activation ($V_{1/2,\text{act}}$) of $I_{\text{Ca}}$ in PC12 cells.

<table>
<thead>
<tr>
<th>VOC</th>
<th>CON</th>
<th>TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOL (857 μM)</td>
<td>-3.5 ± 0.9 mV</td>
<td>-12.4 ± 1.1 mV*</td>
</tr>
<tr>
<td>TCE (2.1 mM)</td>
<td>-4.2 ± 0.9 mV</td>
<td>-12.4 ± 1.1 mV*</td>
</tr>
<tr>
<td>PERC (152 μM)</td>
<td>-2.8 ± 0.6 mV</td>
<td>-16.1 ± 0.9 mV*</td>
</tr>
</tbody>
</table>

Values were determined using the Boltzmann equation (eq 2), and are the mean ± SEM of 6 (TOL), 7 (TCE) and 5 (PERC) separate cells in the absence and presence of solvent. The asterisk (*) indicates that the VOCs had a significant effect on $V_{1/2,\text{act}}$ ($p<0.025$; two-way, repeated measures ANOVA indicated a significant main effect of treatment (presence or absence of solvent) but not of solvent type and no significant interactions).
Table 3. Effects of VOCs on the half-maximal voltage of steady-state inactivation ($V_{1/2, \text{inact}}$) of $I_{\text{Ca}}$ in PC12 cells.

<table>
<thead>
<tr>
<th>VOC</th>
<th>CONTROL</th>
<th>TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOL (857 μM)</td>
<td>-16.1 ± 1.8 mV</td>
<td>-32.0 ± 2.9 mV*</td>
</tr>
<tr>
<td>TCE (2.1 mM)</td>
<td>-15.9 ± 1.6 mV</td>
<td>-35.0 ± 6.4 mV*</td>
</tr>
<tr>
<td>PERC (152 μM)</td>
<td>-13.0 ± 2.7 mV</td>
<td>-20.7 ± 3.7 mV*</td>
</tr>
</tbody>
</table>

Values were determined using the Boltzmann equation (eq 2), and are the mean ± SEM of 5 (TOL), 9 (TCE) and 3 (PERC) separate cells in the absence and presence of solvent. The asterisk (*) indicates that the VOCs had a significant effect on $V_{1/2, \text{inact}}$ (p<0.025; two-way, repeated measures ANOVA indicated a significant main effect of treatment (presence or absence of solvent) but not of solvent type and no significant interactions).
Figure 1

![Graph showing current amplitude over time with an annotation of "827 µM TOL"]
Figure 2

A

200 pA

25 ms

TOL

WASH

CON

B

TCE

WASH

CON

C

PERC

WASH

CON
Figure 6

Normalized Current

857 µM TOL

2.1 mM TCE

152 µM PERC

CON

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Figure 7

Normalized Current

- 857 μM TOL
- 2.1 mM TCE
- 152 μM PERC