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\(_{\text{Ca}}\)) in nerve growth factor-differentiated pheochromocytoma (PC12) cells. All three VOCs affected I\(_{\text{Ca}}\) in a reversible, concentration-dependent manner. At +10-mV test potentials, VOCs inhibited I\(_{\text{Ca}}\), 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\(_{\text{Ca}}\) inactivation kinetics from a single- to double-exponential function. Voltage-ramp experiments suggested that VOCs shifted I\(_{\text{Ca}}\) 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 Boltzman equation. V\(_{1/2}\), act was shifted from approximately −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 approximately −16 mV in control to −32, −35, and −20 mV in the presence of TOL, TCE, and PERC, respectively. Inhibition of I\(_{\text{Ca}}\) 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.

Volatile organic compounds (VOCs), such as toluene (TOL), 1,1,1-trichloroethylene (TCE), and 1,1,2,2-tetrachloroethylene (perchloroethylene; PERC), are widely used 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 is not well understood and generally has 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 attributed to their ability to perturb the fluidity of the plasma membrane, because 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

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ABBREVIATIONS: VOC, volatile organic compound; TOL, toluene; TCE, 1,1,1-trichloroethylene; PERC, perchloroethylene, 1,1,2,2-tetrachloroethylene; CNS, central nervous system; VSCC, voltage-sensitive calcium channel; VSSC, voltage-sensitive sodium channel; NMDA, N-methyl-D-aspartate; NGF, nerve growth factor; GVIA, ω-conotoxin GVIA; DMEM, Dulbecco’s modified Eagle’s medium; I\(_{\text{Ca}}\), whole-cell Ca\(^{2+}\) current; TTX, tetrodotoxin; ANOVA, analysis of variance; I/V, current-voltage.
nicotinic acetylcholine receptors (Bale et al., 2002, 2005). Recently, effects of TOL were demonstrated on voltage-sensitive Ca$^{2+}$ channels (VSCCs) in pheochromocytoma (PC12) cells (Tillar et al., 2002).

Voltage-sensitive calcium channels regulate important neuronal functions, including neurotransmitter release. Alcohol and volatile anaesthetics have been demonstrated to disrupt function of VSCCs (Study, 1994; Kamatchi et al., 1999; Kameyama 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 VSCCs. PC12 cells are a clonal cell line that expresses both N- and L-type VSCCs after differentiation with nerve growth factor (NGF). This cell line has been used as a model system to study effects of neurotoxins (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 VSCCs 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 VSCCs to VOCs was confirmed by examining TOL inhibition of calcium current (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 the addition of these solvents to extracellular solution followed by sonication in a water bath for several minutes. In all of the 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). α-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 PC12 cells were grown in polystyrene culture flasks in Dulbecco’s modified Eagle’s medium (DMEM), containing 44 mM NaHCO$_3$, 2 mM HEPES, 7.5% fetal bovine serum, and 7.5% horse serum. Cells were incubated at 37°C in a humidified incubator with a 5% CO$_2$ atmosphere, fed with fresh medium every 3 to 4 days, and passaged once weekly. In all of the experiments, cells from passage 10 (designated from the original vial received as a gift from the late Dr. G. Guroff, National Institutes of Health, Bethesda, MD) were used. For electrophysiological recordings, undifferentiated cells were plated at a density of 7.5 × 10^4 cells/ml into six-well plates (2 ml/well) containing poly-l-lysine-coated (50 μg/ml) glass coverslips. After a 2-h period in DMEM/F-12 containing 10% fetal bovine serum, the medium was replaced with 2 ml of serum-free DMEM/F-12 medium containing 50 ng/ml human recombinant NGF. NGF was replenished every 48 h by removing 1 ml of medium in each well and replacing it with 1 ml of medium containing 100 ng/ml NGF. Recordings were made using cells that had been exposed to NGF for 4 to 6 days.

Primary cultures of rat cerebrocortical cells were prepared as described previously (Inglefield et al., 2002). In brief, cells were plated (3 ml) onto poly-l-lysine-coated coverslips at a density of 2 × 10^6 cells/ml. After 3 days in vitro, 5 μM β-cytoxin 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 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; rundown 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 used as noted under Results. Currents were amplified and filtered (2 kHz) using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA), digitized at a rate of 25 kHz using a Digidata 1200 A/D converter (Molecular Devices), and stored online on a personal computer for later analysis. pCLAMP 6.0 software (Molecular Devices) 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 Instruments, Hamden, CT) and had resistances of 2 to 4 MΩ when filled with intracellular recording solution containing 125 mM CaCl$_2$, 10 mM tetraethylammonium chloride, 5 mM EGTA, 10 mM HEPES, 1 mM MgCl$_2$, 10 mM d-glucose, and 4 mM ATP, pH 7.3, adjusted to −315 mOsm with sucrose. Extracellular recording solution contained 125 mM NaCl, 20 mM HEPES, 10 mM d-glucose, 10 mM tetraethylammonium chloride, 10 mM CaCl$_2$, and 1 mM MgCl$_2$, pH 7.3, and was adjusted to −330 mOsm with sucrose. For determination of steady-state inactivation, BaCl$_2$ was substituted for CaCl$_2$ 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 shown in eq. 1.

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

where $A_1$, $A_2$, and $C$ are the slow, fast, and noninactivating 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 versus test potential. To determine steady-state inactivation, pre pulses (5 s) 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 after each prepulse was expressed as a fraction of current amplitude after the −70 mV prepulse and plotted versus prepulse potential. Data for...
both activation and steady-state inactivation were then fitted using a Boltzman function as shown in eq. 2,

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp((V - V_{1/2})/k)}^{-1}$$

(2)

where $I$ is the current amplitude at a given test potential $V$ (or prepulse 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 α-conotoxin GVIA. Effects of TOL on peak current amplitude were measured following a 200-ms step depolarization from $-70$ to $+10$ mV before and after application of GVIA (1 µM).

### VOC Exposure and Analysis

Cells were exposed to VOCs via perfusion in the extracellular solution. In brief, extracellular solution ($\sim 10$ 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 to 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-µl 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, 10-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 and 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 this article.

### 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 analysis of variance (ANOVA) was conducted examining peak versus 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 examine 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 Institute, Cary, NC), repeated measures ANOVA was used. When an ANOVA yielded a $p < 0.025$, step-down tests were conducted as described under 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 (eq. 3) is shown as follows,

$$y = \frac{1}{1 + e^{(β1 + β2/\text{conc}(β3))}}$$

(3)

in which $y$ is the dependent variable, $e$ is the base of the natural logarithm, $β1$ and $β2$ are empirical parameters that 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 (I/V) Relationships and Steady-State Activation and Inactivation.

Data for I/V relationships and activation/inactivation were analyzed by repeated measures ANOVAs. In each ANOVA, significance of solvent effects (treated versus 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 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 $I_{\text{Ca}}$.** TOL (857 µM), TCE (827 µM), and PERC (152 µM) rapidly and reversibly blocked $I_{\text{Ca}}$ in NGF-differentiated PC12 cells (Figs. 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 cur-
VOCs do not have a preference for the open state of the channel and that changes in the inactivation were 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 (Fig. 3, A–C). Statistical analysis of normalized peak and end current amplitudes for all three VOCs indicated statistically significant effects of peak versus 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 (Fig. 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 probably reflected changes in inactivation rate, all further analyses were conducted on the peak current amplitude.

Because TOL has been most extensively studied and the most used of the three VOCs, it was used as the “standard” for determination of concentration-equivalence equations (Fig. 4). The concentration-equivalence relationships are shown in eqs. 4 for PERC/TOL equivalence and 5 for TCE/TOL equivalence,

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

$$CTOL_{equiv} = e^{1.572 + 0.998 \times \ln(CTCE)}$$  \hspace{1cm} (5)

where $CTOL_{equiv}$ is the concentration of TOL that is equivalent in potency to the concentrations of either PERC or TCE (PERC 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 (Fig. 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. Step-down 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. 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 (Fig. 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_{Ca} at more hyperpolarized potentials as well as a shift in the potential at which
peak current occurred (Fig. 5B). As with other effects, these changes were reversible upon washout of the solvents (data not shown).

The increase in ICa at hyperpolarized test potentials suggests that VOCs shift the activation of VSCCs in a hyperpolarizing direction. This was tested by determining the half-maximal voltage of activation (V1/2, act) using the Boltzman equation. Using the Boltzman equation, V1/2, act was determined in the absence and presence of each VOC. In all cases, V1/2, act shifted significantly in a hyperpolarizing direction in the presence of VOCs (Table 2; Fig. 6).

Effects of VOCs on steady-state inactivation of VSCCs were examined by subjecting cells to 5-s prepulses between −70 and +10 mV before a 150-ms test pulse to +10 mV from a holding potential of −70 mV. The steady-state inactivation curves shifted significantly in a hyperpolarizing direction in the presence of all three VOCs (Fig. 7). The calculated V1/2, 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 VSCCs, whereas NGF-differentiated PC12 cells express both N- and L-type VSCCs (for review, see Shafer and Atchison, 1991). Previous work with TOL suggested that N-type VSCCs might be more sensitive to VOCs than are L-type VSCCs: for example KCl-induced increases in intracellular Ca²⁺ and whole-cell Ca²⁺ currents are more sensitive to TOL in differentiated than in undiffer-

![Fig. 5. I/V relationships in the presence and absence of VOCs. A, 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 μM TOL (top; n = 6), 2.1 mM TCE (middle; n = 7), and 152 μM PERC (bottom; n = 5). The arrows indicate current amplitudes that 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 μM TOL in extracellular solution containing NaCl (top pair of traces; scale bars, 200 pA and 50 ms) or choline chloride (bottom pair of traces; scale bars, 100 pA and 50 ms). B, current responses to a voltage ramp from −70 to +60 mV (140 ms) in three separate cells before and after exposure to TOL (top), TCE (middle), and PERC (bottom) at the indicated concentrations. Similar responses were demonstrated in five additional cells for TOL, four additional cells for TCE, and two additional cells for PERC.

**TABLE 2**

Effects of VOCs on the half-maximal voltage of activation (V1/2, act) of ICa in PC12 cells.

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

* Indicates that the VOCs had a significant effect on V1/2, 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].
entiated PC12 cells (Tillar et al., 2002). To examine further whether differences in sensitivity to TOL exist between N- and L-type VSCCs, the ability of TOL to block ICa was examined in the absence and presence of the specific N-type VSCCs antagonist GVIA. As shown in Fig. 8, TOL (857 μM) reduced ICa by ~45% in the absence of GVIA, and this effect was nearly completely reversible (recovery to 86% of control). The 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 four of six cells and dramatically reduced it in the remaining two cells (Fig. 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 (Fig. 8; traces labeled GVIA and GVIA + TOL). TOL effects on GVIA-insensitive
neurons from glia, with pyramidal-shaped cells expressing microtubule-associated protein-2 but not glial fibrillary acidic protein (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 $I_{Ca}$ 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 VSCCs in PC12 cells (Tillar et al., 2002) and extends the understanding of this effect by characterizing in detail the interaction of TOL with VSCCs. Furthermore, 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 VSSCs 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 VSCCs 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), nicotinic acetylcholine (Bale et al., 2002, 2005), 5-hydroxytryptamine$_3$ (Lorenzo et al., 2003b), 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 VSCCs 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 VSCCs in a manner similar to the VOCs examined in the present study (T. J. Shafer, unpublished data). Thus, effects on VSSC 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 nicotinic acetylcholine receptors 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

### Table 3

<table>
<thead>
<tr>
<th>VOC</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>TOL (857 μM)</td>
<td>−16.1 ± 1.8</td>
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</tr>
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<td>TCE (2.1 mM)</td>
<td>−15.9 ± 1.6</td>
<td>−35.0 ± 6.4*</td>
</tr>
<tr>
<td>PERC (152 μM)</td>
<td>−13.0 ± 2.7</td>
<td>−29.7 ± 3.7*</td>
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* Indicates that the VOCs had a significant effect on $V_{1/2}$, 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).
sensitivity of different VSCC types to inhibition by TOL. Because PC12 cells express both N-type (GVIA-sensitive) and L-type (GVIA-insensitive) VSCCs, 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 VSCCs. This is consistent with previous data demonstrating that KCl-induced fura-3PE responses and $I_{Ca}$ were less sensitive to TOL in undifferentiated compared with 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, $I_{Ca}$ comprises both N- and L-type currents (Shafer and Atchison, 1991). The slightly greater sensitivity of the end versus peak current to VOCs may at first seem to be inconsistent with greater sensitivity of N-type current, because 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 it was without this effect on GVIA-insensitive (presumably L-type) current. Thus, the slight difference in peak and end current sensitivity is likely because of 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 VSCCs. 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 voltage-gated selectivity filter of the channel pore, because 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 VSCCs contains highly conserved residues responsible for sensitive membrane voltage (Franciolini, 1994). In addition, transmembrane regions, especially the sixth 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 $I_{Ca}$, TOL did not alter inactivation kinetics, steady-state inactivation, or the IV relationships for Na$_{1.5}$ channels expressed in oocytes, but it did shift activation in a depolarizing direction and exhibit use- and frequency-dependent block (Cruz et al., 2003b). Such differences in actions could be because of differences between expression in oocytes versus 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, because these studies have been conducted in different laboratories using different preparations. Toluene inhibited VSCCs over a concentration range of 91 to 1500 $\mu$M, with an approximate IC$_{50}$ of 720 $\mu$M (65 $\mu$g/ml). This is comparable with concentrations reported to disrupt function of GABA$_A$ (420 $\mu$M; Beckstead et al., 2000), NMDA (100–1000 $\mu$M; Cruz et al., 1998), and nicotinic acetylcholine (30–900 $\mu$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 $\mu$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 VSCCs 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 $\mu$g/ml; IC$_{50}$ = 196 $\mu$g/ml) are consistent with brain levels associated with visual evoked potential impairment in rodents (20–120 $\mu$g/ml; Boyes et al., 2005). Finally, the relative potencies for VOC effects on VSCCs agree with in vivo data, where PERC and TCE are the most and least potent, respectively. Using a swimming protocol that induced “behavioral despair immobility”, De Ceaurriz et al. (1983) demonstrated that PERC was a more potent inhibitor of immobility than was TOL after inhalation exposure. Furthermore, PERC was the most potent and TCE was the least potent of these VOCs at increasing motor activity after inhalation exposure (Kjellstrand et al., 1985). Thus, the concentrations and relative potencies for VOC effects on VSCCs are consistent with those for 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 in vivo have not yet been thoroughly examined. Recently, it has been demonstrated that TOL, but not trichlorehane, can provide protection against NMDA-induced seizures in mice (Cruz et al., 2003a), 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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