Cellular Actions of Urethane on Rat Visual Cortical Neurons In Vitro

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Sceniak, Michael P. and M. Bruce MacIver. Cellular actions of urethane on rat visual cortical neurons in vitro. J Neurophysiol 95: 3865–3874, 2006. - Urethane is widely used in neurophysiological experiments to anesthetize animals, yet little is known about its actions at the cellular and synaptic levels. This limits our ability to model systems-level cortical function using results from urethane-anesthetized preparations. The present study found that action potential discharge of cortical neurons in vitro, in response to depolarizing current, was strongly depressed by urethane and this was accompanied by a significant decrease in membrane resistance. Voltage-clamp experiments suggest that the mechanism of this depression involves selective activation of a Ba2+-sensitive K+ leak conductance. Urethane did not alter excitatory glutamate-mediated or inhibited GABAergic synaptic transmission. Neither the amplitude nor decay time constant of GABAAR- or GABAB-mediated synaptic currents was altered by urethane, nor was the frequency of spontaneous IPSCs. These results are consistent with observations seen in vivo during urethane anesthesia where urethane produced minimal disruption of signal transmission in the neocortex.

INTRODUCTION

In the present study we used an in vitro brain slice preparation to study urethane [ethyl carbamate (NH2COOCH2CH3)] effects on synaptic responses and neuronal excitability. Much is known about the electrophysiological properties of neurons in the neocortex, both in urethane-anesthetized preparations as well as in brain slices, particularly for layer 4 excitatory pyramidal neurons. These neurons are considered the dominant information-processing stream of the neocortex. There is a need to understand the impact of urethane on neocortical pyramidal neurons under urethane anesthesia to help interpret results from electrophysiological recordings and imaging studies of visual system function (Coffey et al. 2002; Heynen et al. 2003; Ohki et al. 2005; Petersen et al. 2003).

Urethane has been reported to produce minimal disruption of physiologically relevant measures including reflex responses (Albrecht and Davidowa 1989; Dringenberg and Vanderwolf 1995; Maggi and Meli 1986). Unlike other general anesthetics that depress neuronal responses primarily through synaptic mechanisms (Franks and Lieb 1994; Nicoll et al. 1975; Nishikawa and MacIver 2000; Pittson et al. 2004), urethane produced little effect on the time course or amplitude of synthetically evoked inhibitory responses in olfactory cortex (Scholfield 1980)—in the only previous study to address urethane effects on synaptic transmission. Basic cellular properties such as resting membrane potential, action potential amplitude, and membrane capacitance also did not vary significantly between measures in vitro and those made in vivo from urethane-anesthetized rats (Bindman et al. 1988).

Although urethane appears to preserve synaptic signal transmission, it has also been reported to produce depressive effects on neuronal excitability (Albrecht and Davidowa 1989; Dyer and Rigdon 1987; Girman et al. 1999; Ikeda and Wright 1974; Mercer Jr et al. 1978). In a recent study, using frog oocytes transfected with several recombinant neurotransmitter receptors, it was also shown that urethane nonselectively affected excitatory and inhibitory currents (Hara and Harris 2002; Koblin 2002), and thus actions on synaptic transmission would seem likely. The present study investigated the cellular and synaptic mechanism(s) of urethane’s action on neocortical pyramidal cells.

METHODS

Brain slice preparation

All procedures and protocols were approved by the Institutional Animal Care Committee at Stanford University and adhered to published guidelines of the National Institutes of Health. All experiments were performed on rat brain slices dissected from the visual cortex of young [postnatal days 21 to 28 (P21–P28)] male Long–Evans rats (Charles River Laboratories, Wilmington, MA). Rats were initially anesthetized with diethyl ether. The entire brain was removed and briefly (<20 s) placed in ice-cold (1–4°C) oxygenated (95% O2–5% CO2) artificial cerebral spinal fluid [ACSF containing the following (in mM): 124, NaCl; 2, KCl; 1.5, MgSO4; 1.25, NaH2PO4; 26, NaCH3CO3; 10, dextrose; and 2.5, CaCl2]. Brain slices were cut in oxygenated ice-cold ACSF into 350-μm-thick sections.

Electrophysiology

Brain slices were transferred from the holding chamber to a submersion-recording chamber (Warner Instruments, Hamden, CT). Slices were perfused with oxygenated room-temperature (22–24°C) gravity-fed ACSF (2–4 ml/min). Slices were visualized with a Zeiss Axioskop upright microscope (Zeiss, Jena, Germany), using a water immersion objective (40X) with near-infrared illumination and a CCD camera (COHU, San Diego, CA) with contrast enhancement.

Membrane potentials and currents were monitored with a Multiclamp 700A patch clamp amplifier (Axon Instruments, Foster City, CA) and digitized with a Digidata 1322A analog to digital converter (Axon Instruments). Current and voltage signals were generated digitally in pCLAMP 9.0. Voltage and current traces were sampled at 10 kHz. Data acquisition was controlled using the pCLAMP 9.0 software package (Axon Instruments).

Recording electrodes were filled with either KCl-based or K-glutamate–based internal solutions. K-glutamate–based solution contained K-glutamate, EGTA, HEPES, MgCl2, ATP, and GTP in the following mM concentrations: 100, 10, 40, 5, 2, and 0.3 (pH 7.3 and 8.0).
osmolarity 290–295 mOsm). KCl-based internal solution contained KCl, EGTA, HEPES, MgCl₂, ATP, and GTP in the following mM concentrations: 100, 10, 40, 5, 2, and 1.5 (pH 7.3 and osmolarity 290–295 mOsm).

Synaptically evoked responses were elicited using a bipolar stimulating electrode fabricated with theta glass pipettes (Harvard Apparatus, Holliston, MA). Stimulating electrodes (10- to 30-μm tip) were positioned near the dendritic field of targeted pyramidal cells in layer 4. Stimulus amplitude was optimized for each cell to produce mono- synaptic excitatory postsynaptic potentials (EPSPs). Paired-pulse stimulation current pulses were separated by 150 ms with cells at resting membrane potential.

To test intrinsic excitability, each cell was tested for its spiking response to an injected current step input. The appropriate current amplitude for each cell was determined manually by adjusting the input current and monitoring the spiking response. Input current amplitudes were chosen to give rates within the linear range (typically eight to 15 spikes). The rates were coarsely optimized for each cell to reduce variability and prevent spike-rate saturation. Rates <5 s⁻¹ tended to produce greater variability and rates >15 s⁻¹ tended to be near saturation. However, there was cell-to-cell variability for these parameters. To account for this variability in absolute spike rates across cells, analysis was performed normalized to the control condition’s rate on a cell-by-cell basis.

Synaptic γ-aminobutyric acid type A (GABA_A)-receptor-mediated inhibitory postsynaptic currents (IPSCs) were isolated by bath application of (±)-2-amino-5-phosphonopentanoic acid (APV, 100 μM) and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX, 17.2 μM) to block N-methyl-D-aspartate (NMDA)- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor-mediated synaptic currents. During voltage-clamp experiments, action potentials were blocked by adding N-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide (QX-314, 1 mM) to the internal pipette solution. Synaptically evoked IPSC responses were elicited through extracellular current pulses to the dendritic zone of recorded neurons with the membrane potential clamped at its resting membrane potential. IPSCs were completely blocked by additional bath application of picrotoxin (100–200 μM). Spontaneous miniature IPSCs were recorded with each cell voltage clamped at its resting membrane potential using KCl-based internal solution.

GABA_A-mediated IPSPs were measured in the presence of CNQX and APV. Combined GABA_A and GABA_B IPSPs were elicited with a train of extracellular current pulses injected into the input fibers of the dendritic layers (layer 1–2 border) of neurons in layer 4. Pulse trains consisted of nine 2-ms-duration pulses separated by 50 ms (Johannes et al. 1995). GABA_B IPSPs were blocked with CGP-35348 (100 μM) and the remaining GABA_A responses were blocked with picrotoxin (100 μM).

It has been shown that acid-sensitive two-pore K⁺ channels (TASK or K₂TP3.1) are blocked with acidic pH (7.0–6.0) (Gutman et al. 2003; Talley et al. 2003). We used a HEPES-based ACSF solution containing NaCl, KCl, MgCl₂, HEPES, glucose, and CaCl₂ in the following mM concentrations: 130, 3, 2, 10, and 2 (300 ± 5 mOsm) to easily modify our ACSF. Normal HEPES-based ACSF was replaced with acidic (pH 6.0–6.5) HEPES-based ACSF to block TASK channels (K₂TP3.1).

**Histology**

To perform histological reconstructions the internal solution contained 0.5 to 1% neurobiotin (Vector Laboratories, Burlingame, CA). Slices were processed with the Elite VectaStain ABC kit (Vector Laboratories) according to the protocol described by Hamam and Kennedy (2003). Stained slices were mounted wet with Vectashield mounting medium (Vector Laboratories). Digital images were reconstructed using Adobe Photoshop (Adobe Systems, San Jose, CA) to determine cell morphology and the presence of dendritic spines.

**Data collection and analysis**

Average electrode series resistance ranged from 10 to 30 MΩ after break in. The seal impedance of whole cell recordings ranged from 1 to 3 GΩ. Resting membrane potentials ranged from −55 to −75 mV. Recordings with seals <1 GΩ or resting membrane potentials greater than −55 mV were not included in the analysis. In voltage-clamp experiments, access resistance was monitored using a 20-ms voltage step deviation from the holding potential and repeated throughout the duration of the recording.

All data analysis was performed using custom-written functions in Matlab Release 12 (The MathWorks, Natick, MA). All statistics are expressed as the mean ± SD unless otherwise stated. Statistical significance of data from control and urethane groups was determined using the Student’s t-test. One-way ANOVA was used to compare differences from three or more groups. Time-locked current and voltage traces were averaged in Matlab to produce mean evoked IPSCs and EPSPs. Spontaneous IPSCs were fitted with an exponential equation to determine the decay time constant estimated from the time of the IPSC peak.

**Animals/chemicals**

All rats were obtained from Charles Rivers Laboratories (Wilmington, MA). Chemicals for the ACSF were reagent grade or better and obtained from J. T. Baker (Philadelphia, PA) or Sigma–Aldrich (St. Louis, MO).

**RESULTS**

The effects of urethane were investigated on neurons in input layer 4 of rat visual cortex. Each neuron was initially characterized according to its spiking response (Connors and Gutnick 1990; Connors et al. 1982; Kawaguchi 1995; McCormick et al. 1985). Those cells that were determined to be regular-spiking (RS) excitatory cells were characterized for intrinsic cellular excitability and synaptic transmission. Many neurons were filled with neurobiotin and histologically processed to identify them anatomically as pyramidal neurons (see Fig. 11A).

**Urethane depressed neuron excitability**

Initial observations revealed a consistent depressive effect of urethane on spike responses but not on synaptic responses of pyramidal neurons in the rat neocortex. Spike rates were reduced when urethane (10 mM) was bath applied to whole cell patch-clamped neurons recorded in current-clamp mode (Fig. 1A). Spike-rate depression was reversible after a 30-min wash (Fig. 1A). We tested intrinsic excitability by applying constant-amplitude 1-s current steps with amplitudes optimized to produce moderate spike rates (7–12 spikes) and repeated 35 times. Blank periods with a minimum duration of 15 s were interdigitated between current steps.

An increased shunting conductance turned on by urethane was evident in the current–response functions (Fig. 1B). The absolute response was depressed in the presence of urethane and the response gain or slope of the f-I curve was reduced. Urethane decreased membrane-induced resistance, observed by measuring the slope of the voltage–current relationships (Fig. 1D). Effects on input resistance were reversible after washout.

The dose–response curve for spike-rate depression showed that urethane acts in a concentration-dependent manner (Fig.

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Urethane compared with control (Gtatively). F (n averaged over all action potentials elicited by a 1-s depolarizing current step not appreciably affected by urethane. Representative action potentials shown in the presence of urethane (10 mM), the action potential discharge rates were not appreciably altered in the presence of urethane (3–10 mM; Fig. 2A). The remaining population of cells (n = 34) showed no change in firing rate in the presence of urethane [mean % difference (control – treated) = 4 ± 26%]. For those cells with a ≥20% spike-rate depression in the presence of urethane (3–10 mM), there was a 74 ± 20% significant decrease in the mean firing rate (Fig. 2B; P < 0.01, t-test). There was also a significant change in the firing rate across the entire population [n = 77; mean % difference (control – treated) = 43 ± 38%; P < 0.01, t-test].

Effects on membrane electrical properties

Intrinsic membrane properties were analyzed for those cells that showed a ≥20% spike depression in the presence of urethane (n = 43, Fig. 2, C–E). Slope resistance for each cell was determined from steady-state current–voltage responses (500–900 ms after the onset of the 1-s-long current step, 0–20 mV hyperpolarized from resting membrane potential). Slope resistance was significantly decreased in the presence of urethane [mean % difference (control – treated) = 18 ± 14%; P < 0.01, t-test; Fig. 2C]. When the entire population of cells (n = 77) was considered there was no change in the mean slope resistance [mean % difference (control – treated) = 9 ± 18%]. Cells that showed <20% spike-rate depression (n = 34) also showed no change in slope resistance [mean % difference (control – treated) = −3 ± 16%]. There was a significant decrease in slope resistance only for cells that exhibited a decrease in firing rate in the presence of urethane. We also found no consistent effect of urethane on initial sag currents (data not shown).

There was even greater decrease in input resistance in the presence of urethane when estimated from chord resistance measures for responses near spiking threshold (Fig. 2D). Steady-state responses were measured 500–900 ms after onset of the 1-s-long current step. Chord resistance was significantly decreased (29 ± 20%; P < 0.01, t-test) in the presence of urethane. When the entire population was considered (n = 56), there was no significant change in the chord resistance under urethane [mean % difference (control – treated) = 11 ± 30%; P > 0.05, t-test]. For cells that showed <20% spike-rate depression (n = 19) there was also no significant change in chord resistance [mean % differ-
On average (mean difference across the entire population of cells tested (urethane compared with control for the cells in the population [mean % difference (control − treated) = −2 ± 5%; P > 0.05, Wilcoxon ranked-sum test; Fig. 2E] for cells that showed >20% spike-rate depression (n = 43) or for the entire population [mean % difference (control − treated) = −0.4 ± 5%; P > 0.05, t-test, n = 77].

Effects on excitatory synapses

To determine the effects of urethane on excitatory synaptic transmission, we evoked monosynaptic EPSPs through extracellular current pulse stimulation of input fibers near the dendrites of pyramidal cells in layers 1/2. Monosynaptic responses were estimated as the initial EPSP slope fitted for responses from baseline to 80% of peak response (Fig. 1F). There was no significant change in EPSP slope in the presence of urethane [n = 67; mean % difference (control − treated) = −6 ± 40%; P > 0.05, t-test; Fig. 2, G and H]. The EPSP slope ratio (pulse 2/pulse 1) from paired-pulse stimulation was also unaffected in the presence of urethane [mean % difference (control − treated) = −5 ± 23%; Fig. 2, F and H].

Voltage-clamp analysis

Current responses were measured in the presence of urethane (10 mM) for a subpopulation of the neurons that showed spike-rate depression (Fig. 3). Membrane potentials were voltage clamped at resting level and current responses were collected in response to 2-s-long voltage steps (Fig. 3A). Resulting currents (urethane − control; 1 to 1.5 s after the beginning of the test step; Figs. 3A) were tonic and positive, indicating that urethane produced a steady-state conductance increase. The current–voltage relationship indicated that urethane increased the input conductance in a linear fashion (Fig. 3, B and C).

GABA receptor

Urethane produced a spike-rate depression in pyramidal neurons through a tonic leak conductance increase, or shunting inhibition, with no effect on properties of individual action potentials. To determine the mechanism of shunting inhibition, we attempted to reverse the spike-rate depression by bath-applying a GABA_A receptor antagonist or Cl⁻ channel blocker in the presence of urethane (Bieda and MacIver 2004). Adding the GABA_A-specific antagonist gabazine (20 μM) to urethane-depressed neurons (n = 6) did not reverse the spike-rate depression (Figs. 4A and 8B). Gabazine’s blocking effect on tonic GABA_A currents could be seen as an increase in the cell’s input resistance (n = 5, Figs. 4A and 8B). However, there was no significant reversal of urethane-dependent spike-rate depression (mean % recovery = −6 ± 3%). The Cl⁻ channel blocker picrotoxin (100 μM) also did not reverse the spike-rate depression produced in the presence of urethane (mean % recovery = −5 ± 8%; n = 5; Figs. 4B and 8B).

GABA synapses

Urethane’s action on synaptic inhibition was investigated for electrically evoked IPSCs as well as spontaneous IPSCs (Figs. 5 and 6). IPSCs were isolated using a KCl-based internal
FIG. 3. Urethane produced a tonic conductance increase in voltage-clamp recordings. A: underlying currents resulting from application of urethane for a representative cell that exhibited reduction in spike rate in the presence of urethane. Each current trace shown was averaged from 10 repeats of voltage steps either above or below resting membrane potential. Currents resulting from 2-s-long voltage steps from resting potential are shown for control and urethane conditions. Bottom: currents produced by urethane are shown with control currents subtracted. B: current–voltage (I–V) relationships are shown for urethane and control conditions (open and closed circles, respectively). C: I–V relationship for currents produced by urethane with control currents subtracted (gray circles).

solution containing QX-314 and by including APV and CNQX in bath ACSF (see METHODS). Averaged IPSC responses ($n = 10$) showed no difference in shape or amplitude in the presence of urethane (6–10 mM urethane, Fig. 5A). Picrotoxin (200 µM) added to the bath completely eliminated IPSCs, verifying that responses were Cl⁻-dependent IPSCs (Fig. 5A).

GABA$_B$-mediated IPSPs were not appreciably affected by the presence of urethane (10 mM) in amplitude or decay time constant (Fig. 5B). GABA$_B$-mediated IPSPs ($n = 6$) were collected in response to a train of extracellular current pulses delivered to input fibers of the distal dendritic region (border of layers 1/2) of neurons in layer 4, to selectively activate GABA$_B$ synapses (Johannes et al. 1995) (see METHODS). GABA$_B$-mediated IPSPs were blocked with CGP (100 µM) and the remaining GABA$_B$ responses were blocked with picrotoxin (150 µM).

Effects of urethane on spontaneous IPSCs were measured in a subpopulation of cells ($n = 12$, Fig. 6A). Spontaneous events recorded in control ($n = 165$ events) and urethane-exposed conditions ($n = 172$ events) (10 mM) were averaged over 30 s of recording (Fig. 6B). The average IPSCs were indistinguishable between the control and urethane conditions and well within the response variability (Fig. 6B, dotted lines represent ± SD). Estimates from population averages indicated that there were no significant differences between control and urethane-exposed IPSCs as quantified by mean rate (control = 6.0 ± 3.3 Hz, urethane = 5.9 ± 2.9 Hz, $P > 0.05$, t-test), mean amplitude (control = 34 ± 28 pA, urethane = 36 ± 25 pA, $P > 0.05$, t-test), and decay time constant $\tau$ (control = 10.0 ± 3.5 ms, urethane = 11.0 ± 3.9 ms, $P > 0.05$, t-test; Fig. 6C).

$K^+$ channels

We studied $K^+$ channels as possible mechanisms for action potential depression and tonic background conductance increases produced by urethane. The broad-spectrum $K^+$ channel blocker tetraethylammonium (TEA) was used to test for $K^+$ channel involvement in spike-rate depression elicited by urethane ($n = 12$). Relatively high concentrations (>20 mM) of TEA were successful at reversing spike-rate depression (Fig. 7A). TEA significantly ($P < 0.01$, t-test) reversed spike-rate depression in 11 of 12 cells by 35 ± 10% (see also Fig. 11B). Individual action potential half-widths were increased in the presence of TEA, consistent with a block of delayed rectifying $K^+$ currents, among others.

To more specifically identify the mechanism of urethane’s spike-rate adaptation, we tested increasingly selective $K^+$ channel blockers (Gutman et al. 2003). Ba$_2^+$ at low concentrations (<2 mM) is known to selectively block $K^+$ channels that contribute to the resting membrane potential such as the inward rectifier family and the two-pore family (K$_{2P}$) (Goldstein et al. 2001; Gutman et al. 2003; Patel and Honore 2001).
urethane did not appear to alter GABAB-mediated inhibitory postsynaptic currents (IPSCs) in the presence of urethane (gray line) and in the control condition (bottom black line). IPSCs were completely eliminated by the application of picrotoxin (top black line). B: urethane did not appear to alter GABA_A-mediated inhibitory postsynaptic potentials (IPSPs). IPSPs are shown for control and urethane-treated conditions (dashed line and thick gray line, respectively). GABA_A-mediated IPSPs were blocked with CGP (100 μM, thin gray line). Remaining GABA_A-mediated IPSPs were blocked by picrotoxin (150 μM, thin black line).

When low concentrations of Ba^{2+} (50–150 μM) were added to the perfusate of neurons that demonstrated urethane-dependent spike-rate depression (>20%), there was a significant reversal of the spike-rate depression (81 ± 7%; P < 0.01, t-test; Figs. 7B, 8, and 11B). Individual action potentials were not affected in shape or amplitude, indicating that urethane-induced depression does not involve effects on delayed rectifier K^+ currents (Fig. 7B).

To test for the specificity of Ba^{2+} reversal of spike-rate depression, Ba^{2+} was added before urethane (Fig. 8A). Adding Ba^{2+} at low concentrations (150 μM) did not significantly increase the firing rate within our sample of cortical pyramidal neurons (n = 8, P > 0.05, t-test; Fig. 8, B and C). When urethane was added to the perfusate in the presence of Ba^{2+}, there was no significant difference in the firing rate compared with control (P > 0.05, t-test; Fig. 8, B and C). However, after switching to urethane without Ba^{2+} there was a significant spike-rate depression (>50% of control, P < 0.01, t-test) in five of eight cells (Fig. 8, B and C).

We examined underlying current responses for cells that exhibited urethane induced spike-rate depression. For cells that showed spike-rate depression in the presence of urethane (10 mM), there was a significant increase in the slope conductance (150 ± 25%; P < 0.01, t-test, see Fig. 9, inset indicated by **). Ba^{2+} (150 μM) reversed the urethane-induced conductance increase and the resulting slope was not different from control slope (88 ± 14%; Fig. 9, inset).

Several other K^+ channel blockers failed to reverse urethane-induced spike-rate depression. The broad-spectrum K^+ channel blocker 4-AP (1 mM) did not reverse firing-rate depression (n = 5, Fig. 10A). The reduction in firing rate after adding 4-AP in Fig. 10A likely resulted from continued increase in urethane’s action over time.

Cs^+ has been shown to block inward-rectifier K^+ channels with low concentrations (100 μM) (Gutman et al. 2003; Soh and Park 2001). However, Cs^+ (100 μM) was also ineffective at reversing urethane-dependent spike-rate depression (n = 5, Fig. 10B). When Cs^+ was added to neurons as a control, it caused a significant increase in firing rate (15 ± 9%, P < 0.01, t-test) in the absence of urethane despite not reversing urethane-induced spike-rate depression.

Spike frequency adaptation–related K^+ currents (I_{Na}) did not appear to contribute to urethane-dependent spike-rate depression. In a subpopulation of the sampled cells (n = 5), we tested reversibility of urethane-dependent spike-rate depression with the I_{Na} channel blockers XE991 and linopirdine (Aiken et al. 1995; Schnee and Brown 1998; Wang et al. 1998) (15 and 20 μM, respectively; Fig. 11B). Neither reversed urethane-dependent spike-rate depression when added to the perfusate.

The role of acid-sensitive two-pore K^+ channels (TASK or K_{2P3.1}) in urethane-induced spike-rate depression was tested by decreasing the ACSF pH (pH 6.0–6.5; see METHODS) (Talley et al. 2003). Acidic ACSF did not reverse urethane-dependent spike-rate depression.
spike-rate depression \((n = 4, \text{Fig. } 11B)\). An additional test of TASK \((K_{2p3.1})\) channel involvement in urethane’s actions was made by determining the reversibility produced by low concentrations of bupivacaine \((20 \mu M, \text{Fig. } 11B)\) (Meuthw et al. 2003). Bupivacaine did not reverse the urethane-dependent spike-rate depression.

Pyramidal neurons of rat visual cortex provided a homogeneous and relevant population of neurons for studying the effects of urethane. Cell type was verified both physiologically and anatomically in a subset of neurons \((n = 30)\) through neurobiotin loading and histological reconstruction (Fig. 11A). There was agreement between physiological (Connors and Gutnick 1990; Connors et al. 1982; Kawaguchi 1995; McCormick et al. 1985) (based on spike responses) and anatomical classification of these cells (Azouz et al. 1997; Nowak et al. 2003). Bupivacaine did not reverse the urethane-dependent spike-rate depression.

**DISCUSSION**

The present study found that urethane depressed the intrinsic excitability of layer 4 cortical neurons and increased input spike-rate depression \((n = 4, \text{Fig. } 11B)\). An additional test of TASK \((K_{2p3.1})\) channel involvement in urethane’s actions was made by determining the reversibility produced by low concentrations of bupivacaine \((20 \mu M, \text{Fig. } 11B)\) (Meuthw et al. 2003). Bupivacaine did not reverse the urethane-dependent spike-rate depression.

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**FIG. 7.** Increased K⁺ conductance appears to account for urethane-dependent spike-rate depression. Membrane potential response of a representative neuron to a fixed-amplitude 1-s current injection. Resting membrane potentials are indicated by the values to the left of each voltage trace. Spike raster plots are shown below each membrane potential recording for 35 repeats. A: adding the broad-spectrum K⁺ blocker tetraethylammonium (TEA, 20 mM) reversed urethane-dependent spike-rate depression. B: spike-rate depression was reversed when Ba²⁺ \((100 \mu M)\) was added to the perfusion in the presence of urethane.

**FIG. 8.** Specificity of Ba²⁺ in blocking urethane-induced spike-rate depression. Effects of urethane were blocked by adding Ba²⁺ before combining with urethane. A: representative example neuron that showed a block of the urethane induced spike-rate depression by preapplication with Ba²⁺. Urethane alone \((10 \text{ mM})\) produced spike-rate depression. B: population summary \((n = 8)\) of the effects of preapplication with Ba²⁺ in preventing urethane-induced spike-rate depression and recovery of the effect with urethane alone \((10 \text{ mM})\).

**FIG. 9.** Ba²⁺ blocked the increased leak conductance produced by urethane. \(I-V\) relationships are shown for a representative neuron in control and urethane-exposed conditions (open circles and gray circles, respectively). Urethane \((10 \text{ mM})\) produced a linear conductance increase. Ba²⁺ \((150 \mu M)\) reversed the conductance increase induced by urethane (black squares with dotted line). Inset: population averages \((n = 6)\) of input slope resistance \((R_{\text{in, slope}})\) relative to control for urethane-exposed conditions \((149 ± 24\%, P < 0.01, t\)-test) and reversal by Ba²⁺.
conductance. Neurons showed a consistent decrease in their action potential firing rate in the presence of urethane combined with an increase in tonic background conductance. Action potential amplitudes, half-widths, or afterhyperpolarization characteristics were not affected. Urethane (3–15 mM) produced spike-rate depression of 20% of control rate in over 50% of the cells studied (n = 77) with surgically relevant concentrations (Coffey et al. 2002; Hara and Harris 2002; Heynen et al. 2003; Koblin 2002; Ohki et al. 2005; Petersen et al. 2003) (0.26 and 1.5 g/kg ip corresponding to 3–15 mM, respectively).

Urethane did not appear to have an influence on excitatory or inhibitory synaptic transmission at physiologically relevant concentrations (<15 mM). EPSPs and IPSCs showed no difference in amplitude or decay time course in the presence of urethane. There was also no observable difference in the frequency, amplitude, or time course of spontaneous GABA$_A$ IPSCs or GABA$_B$-mediated IPSPs. Unlike the effects observed for recombinant neurotransmitter receptors in frog oocytes (Hara and Harris 2002; Koblin 2002), neocortical pyramidal cell synaptic responses were not altered by urethane. This discrepancy most likely results from differences in the density and distribution of ion channels between transfected cells and pyramidal neurons.

Spike-rate depression observed in the presence of urethane appeared to be mediated by a background K$^+$ leak conductance. In the presence of urethane, pyramidal neurons that showed spike-rate depression also showed significant decreases in their input resistance. Overall, urethane produced a change in the slope of the $f$–$I$ curve as well as a rightward shift (Fig. 1B). The opening of background leak ion channels changed the input resistance and caused a divisive change in firing rate that suppressed the maximum firing rate. There was not a consistent hyperpolarization of the resting membrane potential nor a pure rightward shift in the $f$–$I$ curve as would result from a hyperpolarization without a change in the input resistance. This could have resulted from the opening of nonselective K$^+$ channels or from multiple types of K$^+$ channels.

Changes in input resistance were not strongly correlated with changes in spiking when compared directly ($r^2 = 0.42$). This is not surprising given the nonlinear relationship between membrane conductance change and spike encoding. For example, if the channels contributing to the effect were located at the axon hillock they could have a much greater effect on spiking than if they were located on the distal dendrites.

**FIG. 10.** Representative examples of K$^+$ channel blockers that were ineffective at reversing urethane-induced spike-rate depression. A: broad-spectrum K$^+$ channel blocker 4-AP (1 mM) did not reverse the effects of urethane on intrinsic spike rate. B: low concentrations of Cs$^+$ (100–200 μM) were also not successful at reversing urethane-induced spike-rate depression.

**FIG. 11.** Effects of urethane on physiologically and anatomically identified excitatory pyramidal neurons. A: camera lucida reconstructions of neurobiotin-filled example neurons from a sample of 30 reconstructed neurons in the study. Representative examples of excitatory pyramidal neurons and interneurons recorded. Pyramidal neurons were easily identifiable and homogeneous compared with the interneuron population. B: population summary of pharmacological reversal effects on urethane-induced spike-rate depression. Reversal of urethane-dependent spike-rate depression is shown here as the percentage recovery of firing rate from urethane-depressed levels. Vertical error bars represent SE (n = 5). Asterisks indicate those conditions (Ba$^{2+}$ and TEA) where channel blockers significantly reversed the spike-rate depression induced by urethane ($P < 0.01$, ANOVA).
In voltage-clamp recordings this conductance increase was tonic and linear over the voltage range tested. Blocking neither GABA\textsubscript{A}-mediated currents nor Cl\textsuperscript{−} channels reversed the effects of this urethane-induced leak conductance (Figs. 4 and 11B). However, K\textsuperscript{+} channel blockers TEA (>20 mM) and Ba\textsuperscript{2+} (50–150 \textmu M) reversed urethane-dependent spike-rate depression (Figs. 7A and 11B).

Through the use of pharmacological blockers, urethane effects on several K\textsuperscript{+} channel families could be ruled out. Voltage-gated K\textsuperscript{+} channels showed no obvious involvement in the actions of urethane because the effects were insensitive to 4-AP and relatively insensitive to TEA (effects observed only at concentrations >20 mM). Urethane-induced currents showed no obvious inward rectification (Fig. 3) and were not blocked by Cs\textsuperscript{+} (Figs. 10 and 11B). Therefore inward rectifier K\textsuperscript{+} channels (Kir) could be ruled out. Urethane-dependent spike depression was also independent of delayed rectifier K\textsuperscript{+} channels (KCNQ/M) because neither XE991 nor linopirdine (Aiken et al. 1995; Schnee and Brown 1998; Wang et al. 1998) reversed the urethane-dependent spike-rate depression (Fig. 11B).

Neither acidic ACSF (pH 6.0–6.5) nor low concentrations of bupivacaine (Meuth et al. 2003) (20 \textmu M) reversed the urethane-dependent spike-rate depression (Fig. 11B). Therefore the two-pore acid-sensitive K\textsuperscript{+} channels (TASK or K\textsubscript{2P3.1}) did not appear to be the dominant mechanism of urethane-induced spike-rate depression.

Calcium-activated K\textsuperscript{+} channels (K\textsubscript{Ca}) were also ruled out as the mechanism of urethane action because these currents show inward rectified current–voltage responses (Kohler et al. 1996; Lancaster et al. 1991; Soh and Park 2001; Vogalis et al. 2003; Xia et al. 1998). Also unlike the classical anesthetics-related K\textsuperscript{+} current, \(I_{K_{Na}}\) (Franks and Honore 2004; Patel and Honore 2003; Patel et al. 1999), the urethane-induced K\textsuperscript{+} leak conductance did not show typical outward rectification; instead it had a linear current–voltage relationship.

K\textsuperscript{+} leak conductances have been attributed to three possible K\textsuperscript{+} channel families including: inward rectifier, delayed rectifier, and two-pore K\textsuperscript{+} channels (Franks and Lieb 1999; Goldstein et al. 2001; Gutman et al. 2003; Lesage 2003; Yost 1999, 2000, 2003). Of the three possible K\textsuperscript{+} channel families associated with K\textsuperscript{+} leak currents found in the neocortex, we have ruled out inward rectifiers (Kir) and delayed rectifiers (KCNQ/M). The remaining possibility is the K\textsuperscript{+} leak currents associated with the two-pore family. Within the two-pore family of channels, we have also ruled out acid-sensitive channels (TASK) because neither acidic ACSF nor bupivacaine reversed urethane-dependent spike-rate depression. The Ba\textsuperscript{2+} sensitivity (reversal at 50–150 \textmu M) and linear current–voltage relationship of the urethane current is consistent with other two-pore K\textsuperscript{+} channels such as TWIK (Goldstein et al. 2001; Gutman et al. 2003; Patel and Honore 2001) (K\textsubscript{2P1.1}); however, more specific pharmacological blockers of these channels are needed to definitively determine whether one or more two-pore K\textsuperscript{+} channels are activated by urethane.

The action of urethane on excitatory neocortical pyramidal neurons appears to be specifically through the opening of Ba\textsuperscript{2+}-sensitive K\textsuperscript{+} leak currents. Urethane’s action was independent of Cl\textsuperscript{−} channels with no effect on excitatory or inhibitory synaptic transmission. Although volatile general anesthetics have been shown to activate background two-pore K\textsuperscript{+} channels, TREK-1 (K\textsubscript{2P2.1}) and TASK (Patel et al. 1999) (K\textsubscript{2P3.1}), these classical \(I_{K_{Na}}\) currents do not appear to contribute to urethane’s action. Urethane-induced spike-rate depression was not reversed by blocking TASK channels and the Ba\textsuperscript{2+} sensitivity of the urethane current is inconsistent with that of TREK-1 channels (Patel and Honore 2001). However, the urethane-activated current is similar to a K\textsuperscript{+} leak conductance induced by isoflurane in thalamocortical neurons (Ries and Puil 1999).

The effectiveness of urethane as an anesthetic for in vivo electrophysiological recordings comes from the observation that it produces minimal depression of sensory evoked responses or signal transmission in EEG recordings while maintaining a surgical level of anesthesia (Albrecht and Davidowa 1989; Dringenberg and Vanderwolf 1995; Maggi and Meli 1986; Moore and Appenteng 1990). It has also been reported that urethane depresses cortical single-cell responsiveness (Girman et al. 1999). The actions of urethane to increase a shunting K\textsuperscript{+} background leak conductance combined with the lack of effect on either excitatory or inhibitory synaptic transmission are consistent with both observations.

Therefore unlike other anesthetics like isoflurane and barbiturates, which alter the time course of synaptic transmission and functional visual receptive field properties (Ries and Puil 1999; Villeneuve and Casanova 2003), urethane’s action on neocortical pyramidal cells can be thought of as a tonic reduction of intrinsic excitability through a specific K\textsuperscript{+} background leak conductance. Because urethane does not affect synaptic responses, the K\textsuperscript{+} channel action can be thought of as a scaling of the unaffected inputs. However, it is not a simple linear scaling because urethane does not just hyperpolarize the cell—it causes a gain change in the \(f-I\) curve (see Fig. 1). Therefore urethane produces a nonlinear divisive scaling of synaptic inputs.

It is known that background synaptic activity and voltage-gated channels may contribute to firing rate input/output response characteristics (Chance et al. 2002; Mitchell and Silver 2003). Dynamic-clamp techniques that inject conductance rather than current and allow for simulated in vivo-like background activity could be used to address these issues. Additional experiments are also needed to address urethane’s action at subcortical levels and on inhibitory cell subtypes within the cortex to understand urethanes’ effects on early visual processing. However, the findings presented here—that urethane’s action is specifically on intrinsic membrane properties rather than synaptic responses—is a significant advance toward a more complete understanding of in vivo recordings made in urethane-anesthetized preparations. Our results provide a starting point to model the influence of urethane on systems level processing.

REFERENCES


