J Anesth (2013) 27:98–103
DOI 10.1007/s00540-012-1466-7

ORIGINAL ARTICLE

The anesthetic urethane blocks excitatory amino acid responses but not GABA responses in isolated frog spinal cords

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Received: 26 January 2012 / Accepted: 26 July 2012 / Published online: 25 August 2012

Abstract
Purpose The anesthetic urethane is commonly used in physiological experiments. We tested urethane's actions on GABA receptors on the primary afferents in the spinal cord, which are one of the few areas in the adult central nervous system (CNS) that are depolarized by GABA, and on ligand-gated excitatory amino acid (EAA) receptors located on motoneurons. Both receptor types are critically important during anesthetic immobilization.
Methods We used the isolated hemisected spinal cord of the frog in a sucrose gap chamber to record glutamate-, *N*-methyl-D-aspartate (NMDA)-, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, kainate-, and gamma-aminobutyric acid (GABA)-induced depolarizations of the dorsal root (DR) and ventral root (VR). DR potentials (DRPs) and VR potentials (VRPs) evoked by single supramaximal afferent stimuli were also studied. Urethane (10–80 mM) was applied for 10–30 min.
Results Urethane depressed EAA responses on the motoneurons in a dose-dependent manner. At a clinical anesthetic concentration (10 mM), EAA-induced depolarizations were reduced by 8.1 ± 2.2 % ($n = 7$, $P = 0.025$), but increasing the concentration to 40 mM revealed a larger, 24.7 ± 3 % ($n = 53$, $P = 0.0001$) depressing effect of urethane on all EAA responses in the motoneurons. However, GABA and K^+ responses recorded in the DR were not altered by the presence of 10 or 40 mM urethane. Evoked DRPs and VRPs were reduced by urethane and spontaneous DR and VR potentials were suppressed by 10 or blocked by 40 mM urethane.
Conclusion Urethane appears to be selective for EAA-, sparing GABA responses at a clinical anesthetic concentration. Only a 10 % reduction of EAA activity seems to be necessary to induce anesthesia.

Keywords Urethane · Spinal cord · EAA · GABA

Introduction

Urethane (ethyl carbamate), an anesthetic that results from the combination of ethanol and urea, was introduced as an injectable agent more than a century ago [1]. It is considered to have little effect upon circulatory, respiratory, or autonomic systems at concentrations of 10–15 mM that are achieved by the application of a single dose between 1 and 1.5 g/kg [2]. Urethane is used by many investigators to induce immobilization and long-lasting anesthesia, in spite of warnings that it is a carcinogenic agent [3], and it produces a sleep-like brain rhythmic alteration very similar to sleep conditions [4].

In the past decade, it has been recognized that the spinal cord is the primary site mediating immobilization of most anesthetic agents, and the isolated spinal cord is the preparation of choice when studies of anesthetics and their actions on motoneurons, primary afferents, and interneurons are investigated [5]. The first report about the mechanism of action of urethane in isolated spinal cords was published by Evans and Smith [6], who reported that it blocked excitatory amino acid (EAA)-induced responses at a concentration of 50 mM. These results on excitatory neurotransmission were

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reproduced in the rat hippocampus, at doses of 1 g/kg [7]; in *Xenopus* oocytes expressing NR1A/NR2A *N*-methyl-D-aspartate (NMDA) and GluR1/GluR2 alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, at a concentration of 10 mM [8]; and in behavioral studies in mice, at sub-anesthetic doses (400–800 mg/kg) [9]. In contrast to these findings, it was reported that, in experiments using the whole-cell configuration of the patch-clamp technique in the nucleus of the solitary tract of the rat, 20 mM urethane neither changed the spontaneous activities nor the evoked excitatory postsynaptic currents induced by NMDA and non-NMDA receptors agonists [10]. These findings appear to be conflicting.

It has been reported that some anesthetics, particularly nitrous oxide, xenon [5, 11, 12], and ethanol [13], depress EAA receptors independently of gamma-aminobutyric acid $(GABA)_A$ and glycine receptors. Pharmacological evidence that urethane may depress EAA receptors but not GABA- or glycine-mediated receptor inhibition was demonstrated when the depressing effect of 25 mM urethane on spontaneous electrical activity was not reversed by the addition of picrotoxin, a $GABA_A$ R antagonist, or strychnine, a glycine receptor antagonist, to the isolated spinal cord [6].

Molecular pharmacology studies have contributed additional conflicts. For instance, using $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor subunits expressed in *Xenopus laevis* oocytes, it has been reported that urethane produced significant effects on GABA-induced currents at a concentration of 30 mM [14]; however, using recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor subunits, urethane at a smaller and clinically relevant concentration (10 mM) was able to enhance chloride currents by 23 % [8].

Urethane is also used by some neuroscientists to anesthetize animals in pain studies because it is thought that it has little analgesic action. In fact, urethane appears to have little effect on substance P (SP), a major neurotransmitter involved in pain sensation, because it does not affect SP responses in isolated spinal cord preparations [15] nor does it affect nociceptive behaviors induced by SP intrathecal injections in mice [9].

Considering that primary afferents in the dorsal roots are one of the few areas in the adult central nervous system (CNS) that are depolarized by GABA and in which the motoneurons are excited by EAAs, both these factors being critically important during anesthetic immobilization, we used the hemisected isolated spinal cords of frogs to further elucidate the mechanism of action of this commonly used anesthetic agent in experimental research.

Materials and methods

Adult grass frogs (*Rana pipiens*, 30–55 g) were anesthetized to the point of unresponsiveness by cooling on crushed ice according to a protocol approved by the Animal Care and Use Committee of the Veterans' Affairs Medical Center, using National Institutes of Health guidelines outlined in "The Care and Use of Laboratory Animals". The animals were decapitated, the brain destroyed by pithing, and a laminectomy was performed to remove the spinal cord. The lumbar spinal cord was hemisected sagittally and a hemicord with attached IXth and Xth dorsal root (DR) and ventral root (VR) was transferred to a sucrose gap chamber, previously described (see Fig. 1) [16].

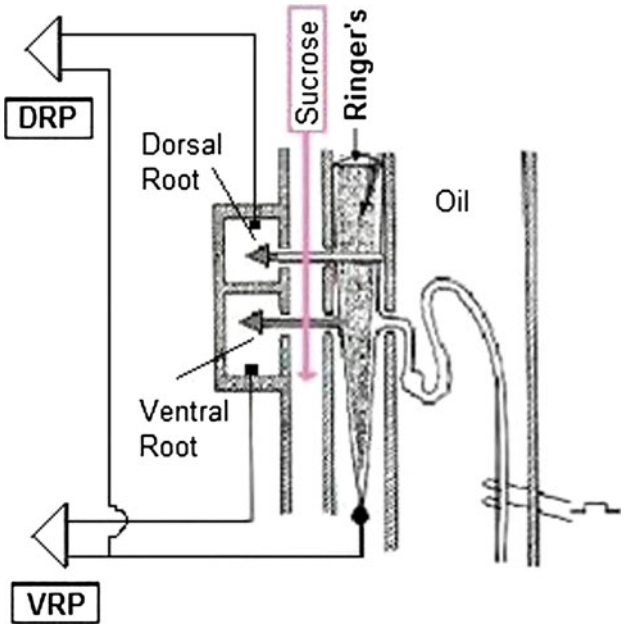

Fig. 1 Diagram of the sucrose gap recording apparatus. A hemisected spinal cord is placed in a bath that is superfused with Ringer's solution. A long dorsal root (DR) with ganglion attached placed in mineral oil is used to stimulate dorsal root potentials (DR-*DRP*s) and ventral root potentials (DR-*VRP*s). The dorsal and ventral roots to be recorded are maintained in a pool of Ringer's solution. Sucrose flows between the spinal cord and the distal roots to provide a high-resistance bridge. Direct current (DC) recordings are made with a differential amplifier

Amphibian motoneurons have been used extensively in research [17]. Amphibian tissue has been found to be less susceptible than mammalian to tissue damage from lack of oxygenation, and the whole cord preparation allows motoneurons to maintain their dendritic arborization and length—in the frog the length is approximately 2.2 mm—as well as minimizing any disruption of cell surface proteins that may be important for membrane integration of current changes. One advantage of this technique is that the extra- and intracellular environments remain intact, permitting study of the in situ signal transduction pathways affecting motoneuronal modulation. Adult amphibian motoneurons possess ionic conductances similar to those described in other amphibian and mammalian neurons, including INa, ICa. IK(v), IK(Ca), and IK(A) [18], as well

as having receptors with similar pharmacology for neurotransmitters and neuromodulators including glutamate [19].

The hemisected cord was superfused with a Ringer's solution containing (in mM): NaCl 114, KCl 2.0, $CaCl_2$ 1.9, $NaHCO_3$ 10, and glucose 5.5. The pH was adjusted to 7.4 by bubbling with 95 % O_2/5 % CO_2. All experiments were cooled by a Peltier unit to a temperature of 18 ± 1 °C.

Direct current (DC) recordings of electrotonically conducted changes in the membrane potential of primary afferent terminals and motoneurons were made by placing the Xth DR and IXth VR, respectively, across a 3-mm sucrose gap. The distal ends of the DR and VR were maintained in a pool of Ringer's solution; 2 % agar-Ringer's bridges connected the distal pool of Ringer solution to a pool of KCl. Calomel electrodes, placed in the KCl pool, measured the difference in potential between the spinal cord bath and the distal DR or VR, via a differential amplifier. The preparation was left ungrounded. After amplification100×, the signals were recorded using a rectilinear pen writer. Rapid (1–2 s) solution changes of the Ringer's bath to apply drugs or agonists to the hemisected cord were made using a solenoid valve assembly.

The peak amplitudes of responses to EAAs on the VR of the isolated spinal cord, as well as the responses to GABA on the DR, known as primary afferent depolarization (PAD), were measured. Dorsal root-stimulated dorsal root potentials (DR-DRPs) and dorsal root-stimulated ventral root potentials (DR-VRPs) evoked by single supramaximal afferent stimuli were also studied. Urethane (10–80 mM) was applied for 10–30 min; amino acids and other agonists were applied for 10 s.

Data are expressed as means ± SEM. The statistical significance of differences was assessed using Student's *t*-test for paired samples.

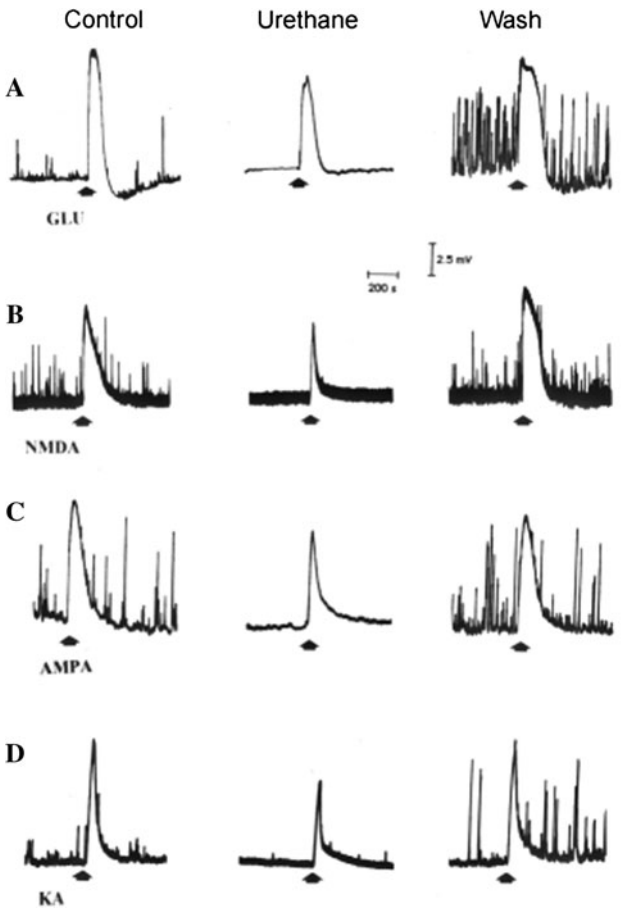

Fig. 2 Urethane inhibits excitatory amino acid (EAA)-induced depolarization of the VR. **a** Glutamate (*Glu* 1.0 mM), **b** *N*-methyl-D-aspartate (*NMDA*) (100 μM), **c** alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (*AMPA*) (10 μM), and **d** kainate (*KA* 30 μM). All agonists were applied for 10 s, and urethane (40 mM) was applied for 10–30 min before application of the agonists. Displacements of the pen recorder upwards indicate negative potential or depolarization. *Vertical* (2.5 mV) and *horizontal* (200 S) *bars* apply for all recordings. For all EAAs $P < 0.0001$

Results

Urethane inhibited EAA-induced depolarization in VRs

All EAAs tested depolarized the motoneurons and interneurons and potential changes were electrotonically conducted and recorded in the distal end of the VRs as was previously described [20]. When urethane was tested at its clinically anesthetic effective concentration (10 mM) only a small inhibition of EAA responses (8.1 ± 2.2 %, $n = 7$, $P = 0.025$; glutamate, 1 mM, $n = 1$; NMDA, 100 μM, $n = 2$; AMPA, 10 μM, $n = 2$; kainate, 30 μM, $n = 2$) was recorded in the VRs. Increasing the concentration to 20 mM also produced a modest inhibition, of 16.4 ± 3.7 % ($n = 7$, $P = 0.001$, glutamate 1 mM, $n = 1$; NMDA, 100 μM, $n = 2$; AMPA, 10 μM, $n = 2$; kainate, 30 μM, $n = 2$) of EAA responses. Therefore, the concentration of the anesthetic was further increased. At 40 mM, urethane clearly inhibited the depolarizing effect of EAAs (24.7 ± 3.0 %), in comparison to control, in the following magnitudes: glutamate (1.0 mM) 24.5 ± 2 %, $P = 0.0001$, $n = 9$; NMDA (100 μM) 22.3 ± 1.3 %, $P = 0.0001$, $n = 14$; AMPA (10 μM) 25.1 ± 4.4 %, $P = 0.0001$, $n = 15$; and kainate (30 μM) 26.3 ± 4.2 %, $P = 0.0001$, $n = 15$ for all EAAs tested (Fig. 2).

GABA-induced depolarization of primary afferents was not affected by urethane at clinical anesthetic concentrations

Urethane tested at concentrations of 10 and 20 mM did not have a noticeable effect on 1 mM GABA-induced

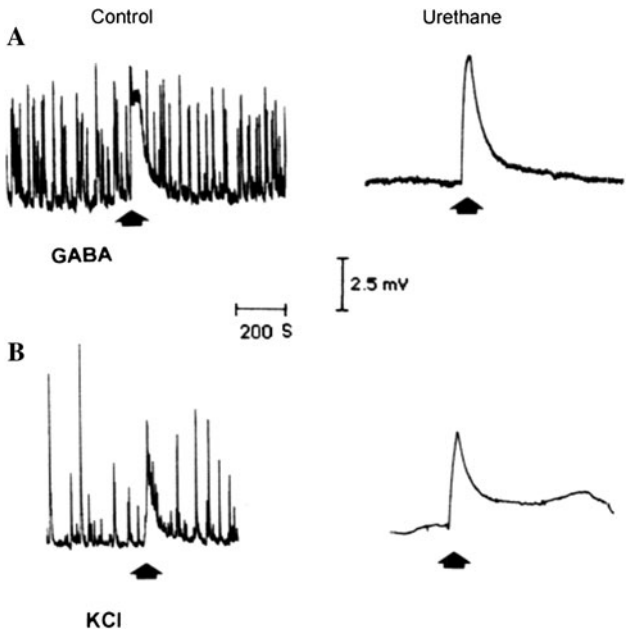

Fig. 3 Urethane did not affect primary afferent depolarization (PAD) induced by gamma-aminobutyric acid (*GABA*) and KCl but suppressed spontaneous firing in the dorsal roots. **a** GABA (1 mM) and **b** KCl (10 mM) were applied to the cord for 10 s, and urethane (40 mM) was applied for 10–30 min before application of the agonists (*right side*)

PAD, and even at 40 mM, GABA responses were not inhibited significantly (9 ± 6.1 % in comparison to control, $P = 0.239$, $n = 10$). When the concentration of urethane was increased to a non-clinical concentration of 80 mM, the inhibition of GABA-induced PAD was 53 ± 13.3 %, in comparison to control ($P = 0.03$, $n = 6$).

The direct depolarizing effect of KCl (10 mM) on PAD, which was used as the control, was little depressed or remained unchanged (2.8 ± 6.3 %, $P = 0.54$, $n = 5$) (Fig. 3).

Evoked DR and VR potentials were depressed by urethane

Urethane at an anesthetic concentration (10 mM) inhibited moderately evoked DR-DRPs (10.2 ± 1.3 %, $P = 0.0002$, $n = 8$) and DR-VRPs (15.2 ± 7.0 %, $P = 0.045$, $n = 8$), but at a concentration of 40 mM, urethane depressed evoked DR-DRPs (28.1 ± 3.6 %, $P = 0.0006$, $n = 13$) and DR-VRPs (34.5 ± 2.8 %, $P = 0.0001$, $n = 11$) compared to control. At a higher concentration (80 mM), urethane further reduced the evoked potentials (DR-DRP 60.5 ± 6.5 %, $P = 0.0095$, $n = 4$ and DR-VRP 62.5 ± 6.7 %, $P = 0.0001$, $n = 8$). These effects were completely reversed by washing for 15–30 min in normal Ringer's solution (Fig. 3). Both the mono-synaptic (fast responses) and poly-synaptic (slow decay responses) components of the DR-DRPs and DR-VRPs were equally depressed, as shown in Fig. 4.

Urethane inhibited spontaneous firing

Urethane produced a remarkable depression of spontaneous firing in the DRs and VRs at a concentration of 10 mM and it blocked spontaneous firing maximally at 40 mM (Figs. 2, 3). However, urethane at either of these concentrations produced no changes in the resting potentials of the DRs or VRs.

Discussion

Our results show that urethane (10–20 mM) produced an inhibition of 8.1–16.4 % of the depolarizing responses induced by EAAs in VRs. However, this change appears to be sufficient to induce immobilization in anesthetized animals and may account for the small variations in various physiological variables, including pain mechanisms, where

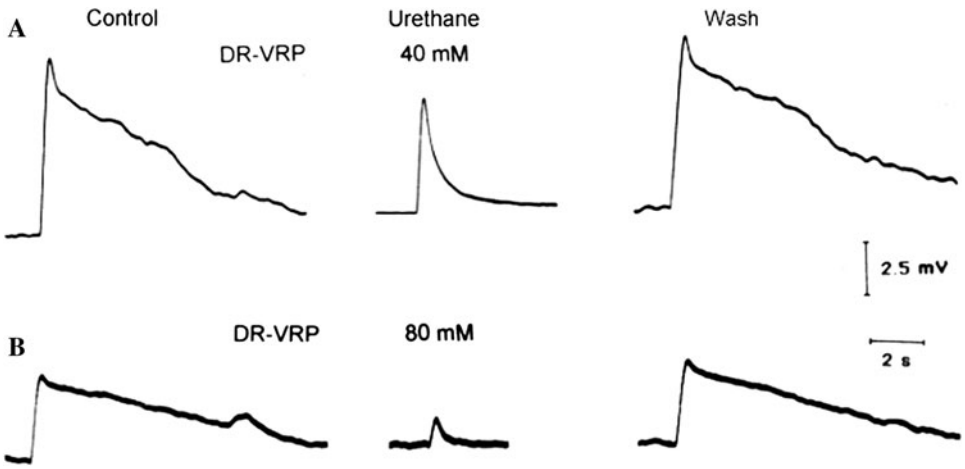

Fig. 4 Urethane markedly reduced neurotransmission in the spinal cord. **a** Urethane at 40 mM reduced DR-VRP by more than 30 %. **b** Urethane at 80 mM abolished DR-VRP. The affect of urethane was rapidly reversible after washing with normal Ringer's solution (*right side*). DR-VRPs were recorded at a greater speed than EAA or GABA applications, as shown in the *horizontal bar* (2 S)

EEAs are involved. When the urethane concentration was raised to 40 mM an inhibition of 25 % for all tested EAAs resulted. Evans and Smith [6] reported that 50 mM urethane depressed the amplitudes of sub-maximal depolarizing responses to EAAs by 50 %. Our present findings confirm those of other experimental studies that have reported that 10 mM urethane suppresses 10 % of the cation currents in *Xenopus laevis* oocytes expressing NR1A/NR2A NMDA receptors (half maximal effective concentration [EC_{50}] = 70 mM) and 18 % of the cation currents in GluR1/GluR2 AMPA receptors (EC_{50} = 34 mM) [8].

Effect of urethane on GABA responses

It is well established that $GABA_A$ receptors have an important role in anesthetic-induced loss of consciousness, but this appears not to be the case for all anesthetics. For example, nitrous oxide and xenon have little or no effect on $GABA_A$ receptors [12]. Our results indicate that urethane at a clinically relevant anesthetic concentration had no effect on GABA receptors. In order to observe a clear inhibition of GABA responses it was necessary to increase the urethane concentration to 80 mM, which is considered very high.

Similar results have been reported using other techniques. For instance, 10 mM urethane tested using recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ and α_1 glycine receptors expressed in *Xenopus laevis* oocytes enhanced the inward chloride currents induced by an agonist by 23 % for $GABA_A$ receptors and 33 % for α_1 glycine receptors [8]. The urethane EC_{50} values were estimated to be 64 and 46 mM for $GABA_A$ and α_1 glycine receptors, respectively [8], with these values being much higher than the clinical effective concentration [2].

Effect of urethane on DR and VR potentials

Urethane depressed the evoked potentials to a greater magnitude than responses to application of EAAs. The average inhibition of DR-DRPs and DR-VRPs was 38 %. The hydrosolubility and the feasibility of including this agent in physiological solutions have permitted its use in medium at a concentration of 22 mM [21], in order to protect mature spinal cord preparations in rats during the dissection and manipulation of the tissue.

The remarkable effect of urethane in inhibiting DR and VR potentials by more than 30 % and to a greater extent than the depression of EAA-induced depolarization could be attributable to the inhibitory effects of other neurotransmitter systems or to Na^+ channel blocking activity, as previously reported [22].

To understand the effect of urethane on the CNS, its affect on neurotransmitter systems in the spinal cord must be considered. In the spinal cord the first neurotransmitter system that was studied was the cholinergic system. In the spinal cord acetylcholine (Ach) receptors can affect neuronal transmission, but the blockade of nicotinic or muscarinic receptors does not modify the potency of anesthetics; thus, Ach receptors are not implicated in anesthetic-induced immobility [5]. Many volatile anesthetics inhibit neuronal Ach receptors, often at sub-anesthetic concentrations, and these receptors might be involved in the nociceptive effects of the volatile agents [23]. However, urethane appears to have an opposite effect, because, in recombinant $\alpha4\beta2$ Ach receptors expressed in *Xenopus laevis* oocytes, 10 mM urethane enhanced currents by 15 % [8]. In addition, 50 mM urethane antagonizes the depolarization evoked by carbachol [6].

The second neurotransmitter system in the spinal cord is the serotoninergic system, with 5-hydroxytryptamine (5-HT) being the most investigated neurotransmitter. Several inhaled anesthetics can block the *in vitro* effect of 5HT on 5HT2A receptor subtypes [5]. In the spinal cord, activation of 5HT2A receptors mediates antinociception in rats [24]. In the frog spinal cord, a low concentration of 5-HT directly hyperpolarized, while a high concentration indirectly depolarized motoneurons, effects that are mediated by 5HT1 and 5HT2 receptor subtypes [25]. The effect of urethane on 5HT-induced depolarization has not been investigated yet; but in rat thoracic aortic rings, 11 mM urethane reduced maximal contraction to 5-HT by 30.8 % and enhanced the action of ketaserin, a 5-HT2 receptor antagonist [26].

A third possible explanation of the effect of urethane is via the interaction of this agent with ions such as K^+ as a result of DR stimulation. It is clear that K^+ ions are released as a result of DR electric stimulation [27]; however, in the present work we present evidence that the direct depolarization produced by the application of KCl was not altered by 40 mM urethane. In agreement with our results, in thoracic aortic rings the maximal contraction to KCl was not affected by 11 mM urethane [26]. The lack of direct action of urethane on the resting potentials of DRs and VRs suggests that K^+ currents are not directly activated by urethane.

Effect of urethane on neuron spontaneous activities

The depressing effect of urethane on spontaneous firing has been the focus of early investigations because this property is shared by many volatile and intravenous anesthetics. However, urethane, in contrast to many intravenous anesthetics, has a significant effect on the Na^+ channel that is responsible for spontaneous activity [22]. It has been shown that urethane (10 and 20 mM) inhibited the current in recombinant voltage-gated Na^+ channels (Na(v)1.2

channel), which are predominantly expressed in the brain and it are sensitive to tetrodotoxin [22].

Conclusions

Urethane appears to have a very modest depressing effect on EAA receptors at a clinical anesthetic concentration, but increasing its concentration by four times revealed a unique depressing effect on all EAA responses, the two components of evoked potentials and neuronal spontaneous activity, but not on GABA responses, which required much higher doses in order to be altered significantly. Therefore, care should be exercised when a high dose of this agent is used during data collection in urethane-anesthetized animals.

Acknowledgments This work was supported by VA Medical Research Funds and USPHS #37946 and by The Research Council (CDCHT) of The Universidad Centroccidental Lisandro Alvarado to N.L.D. We express our gratitude to Dr. Robert Davidoff for his encouragement and great support and to Dr. Alice Holohean for her valuable suggestions.

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