Effect of Common Anesthetics on Dendritic Properties in Layer 5 Neocortical Pyramidal Neurons

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Potez S, Larkum ME. Effect of common anesthetics on dendritic properties in layer 5 neocortical pyramidal neurons. J Neurophysiol 99: 1394–1407, 2008. First published January 16, 2008; doi:10.1152/jn.01126.2007. Understanding the impact of active dendritic properties on network activity in vivo has so far been restricted to studies in anesthetized animals. However, to date no study has been made to determine the direct effect of the anesthetics themselves on dendritic properties. Here, we investigated the effects of three types of anesthetics commonly used for animal experiments (urethane, pentobarbital and ketamine/xylazine). We investigated the generation of calcium spikes, the propagation of action potentials (APs) along the apical dendrite and the somatic firing properties in the presence of anesthetics in vitro using dual somatodendritic whole cell recordings. Calcium spikes were evoked with dendritic current injection and high-frequency trains of APs at the soma. Surprisingly, we found that the direct actions of anesthetics on calcium spikes were very different. Two anesthetics (urethane and pentobarbital) suppressed dendritic calcium spikes in vitro, whereas a mixture of ketamine and xylazine enhanced them. Propagation of spikes along the dendrite was not significantly affected by any of the anesthetics but there were various changes in somatic firing properties that were highly dependent on the anesthetic. Last, we examined the effects of anesthetics on calcium spike initiation and duration in vivo using high-frequency trains of APs generated at the cell body. We found the same anesthetic-dependent direct effects in addition to an overall reduction in dendritic excitability in anesthetized rats with all three anesthetics compared with the slice preparation.

INTRODUCTION

Most of our knowledge of dendritic properties comes from in vitro recordings. The relatively few studies in vivo so far have required the use of urethane, barbiturate, or ketamine anesthesia (Helmchen et al. 1999; Hirsch et al. 1995; Larkum and Zhu 2002; Svoïboda et al. 1997; Waters et al. 2003). However, these anesthetics, as most other anesthetics, are known to affect ion channel activity and the firing properties of neurons (Antkowiak 2002; Arhem et al. 2003; Urban 2002; Vahle-Hinz and Detsch 2002). Dendritic data obtained in vivo can thus be interpreted only after understanding the direct effects of commonly used anesthetics on dendritic excitability.

The choice of anesthetic is always critical when examining neuronal properties in animals. General anesthesia is presumed to result in loss of consciousness but the particular actions at the cellular level are diverse. The main criteria for the choice of anesthetics in this study were their widespread use in rats and water solubility for comparison to in vitro data. The three anesthetics chosen (urethane, pentobarbital, and a mixture of ketamine and xylazine) belong to different classes. Urethane is a long-acting steady-level anesthetic with myorelaxant properties. It is advantageous for long experiments requiring few or even no supplemental doses and involves minimal disruption of physiologically relevant measures (Field and Lang 1988; Maggi and Meli 1986). On the other hand, it is highly carcinogenic and is rarely used for recovery experiments. Pentobarbital sodium (Nembutal) is a short-acting barbiturate that primarily increases the activity of γ-aminobutyric acid type A (GABA_A) receptors in the CNS (Drafts and Fisher 2006) and has a long history of use in neuroscience. Ketamine is a dissociative general anesthetic that has a short duration and fast recovery time with few adverse effects. It is reported to act as a noncompetitive antagonist of N-methyl-D-aspartate (NMDA) receptors leading to the blockade of glutamatergic neurotransmission (Liu et al. 2006). It is not recommended for use alone because of its poor analgesic effect and is therefore often used in combination with xylazine, an alpha-2 adrenergic receptor agonist causing sedation and mild analgesia (Schwartz and Clark 1998). Beside their principal actions, all of these drugs have diffuse low-affinity targets including many of the voltage-sensitive channels (Urban 2002) found in the apical dendrite of layer 5 (L5) neocortical pyramidal neurons.

The aim of this study was to show the effect of these commonly used anesthetics on firing properties, spike propagation, and calcium spike generation in the dendrites of L5 pyramidal neurons. Active spike propagation acts as an intracellular messaging mechanism (for instance, in spike-timing-dependent plasticity). Dendritic calcium spikes occur in the distal dendrites of L5 neocortical pyramidal neurons (Kim and Connors 1993; Schiller et al. 1997). They can modify the output firing properties of the neuron by generating a burst of somatic action potentials (APs) (Larkum et al. 2001; Williams and Stuart 1999; Zhu 2000) and are also important for synaptic plasticity in the dendrites (Letzkus et al. 2006; Sjöström and Häusser 2006). We examined dendritic properties both in vitro in acute brain slices and in anesthetized rats using whole cell patch-clamp recordings. We combined direct dendritic recordings with simultaneous somatic recordings in slices. We also took advantage of a reliable method for activating dendritic conductances using trains of APs elicited at the soma (the “critical frequency” test; Larkum et al. 1999a) to compare the effect of anesthetics in vitro to in vivo.
METHODS

Animal handling was in strict accordance with the guidelines given by the veterinary office of the canton Bern-Switzerland.

In vitro brain slice preparation

Acute parasagittal brain slices of the primary somatosensory neocortex were obtained from Wistar rats (P35–P56). After decapitation without previous anesthesia the brain was quickly removed into ice-cold, oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF) solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 25 NaHCO₃, 2 CaCl₂, 25 glucose (pH 7.4, 315–320 mOsmol). Slices (300 μm thick) were cut using a Microm vibratome and kept at 35 ± 0.5°C for 15 min and then kept at room temperature until use. Slices were continuously perfused with ACSF during the experiments that were performed at 32 ± 2°C.

In vitro electrophysiology

Whole cell recordings were performed from L5 pyramidal neurons somata and apical dendrites using thick-walled (0.5 mm) borosilicate pipettes with filament (OD, 2 mm) (Hilgenberg, Malsfeld, Germany). Somatic (5–7 MΩ) and dendritic (12–25 MΩ) patch-clamp electrodes were filled with intracellular solution (in mM): 135 K-glucuronate, 4 KCl, 10 HEPES, 10 Na₂-phosphocreatine, 4 Mg-ATP, 0.3 and Na-ATP (pH 7.3, 291–293 mOsmol). For somatodendritic recordings, 10 μM Alexa 594 and 0.4% biocytin were added to facilitate neuron localization and to reconstruct the cell, respectively. For cell visualization infrared differential interference contrast optics or oblique localization and to reconstruct the cell, respectively. For cell visualization infrared differential interference contrast optics or oblique localization and to reconstruct the cell, respectively. For cell visualization infrared differential interference contrast optics or oblique localization and to reconstruct the cell, respectively.

Patch-clamp recordings were made first under control conditions, perfused with ACSF, and data acquisition was started. Anesthetics (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min (urethane, pentobarbital, and ketamine/xylazine) were then added to perfused with the extracellular ACSF solution and perfused on the slices for 10 min.

Histology

After the in vitro electrophysiology brain slices were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS) and kept at 4°C. After in vivo electrophysiology the rat was perfused with 4°C 0.1 M PBS followed by 4°C 4% paraformaldehyde in 0.1 M PBS. The brain was removed and kept in 4°C 4% paraformaldehyde in 0.1 M PBS, then sliced at a thickness of 250 μm. Slices were processed using the avidin-biotin-peroxidase method. Cells were stained with a computerized reconstruction system (Neurolucida software).

Analysis

The critical frequency protocol is an indirect method to evoke dendritic calcium activity using high-frequency trains of APs evoked at the cell body (Larkum et al. 1999a) (Supplemental Fig. S1). The amplitude for a single time point and the integral of the falling phase 5–15 ms after the initiation of the last AP in the somatic train [afterdepolarizing potential (ADP)] was measured in each neuron at frequencies between 30 and 250 Hz. Such values were reported as the ADP amplitude and the ADP integral, both similarly revealed the activation of calcium channels involved in dendritic calcium spiking. The critical frequency (CF) in vitro was defined as the turning point of a sigmoidal fit to the ADP versus frequency curve. The sigmoidal fit is a heuristic used throughout the study to provide a consistent classification of APs (BPAPs) in the initiation zone for calcium spikes (Larkum et al. 1999a) and therefore correspond to the threshold for a calcium spike. The ADP amplitude and ADP integral are dependent on the influence of the dendritic Ca²⁺ spike on somatic membrane potential (V_m) and therefore correspond to the amplitude and/or duration of the calcium spike. Under control conditions (ACSF) in vitro an average CF of 98.2 ± 22.4 Hz (SD) and an average ADP amplitude of 6.6 ± 3.0 mV (SD, n = 99, L5 pyramidal neurons) were obtained. The CF was stable for ≥30 min after patching (Supplemental Fig. S2). Because of baseline fluctuations of the signals in vivo, multiple trials (3–10) were averaged. The existence of a CF in vivo was determined through the ADP integral by comparing low and high values on either side of the turning point from a sigmoidal fit (as in vitro) and testing for statistical difference (two-tailed paired t-test).
t-test). The up states were not specifically segregated from the down states, although data are likely to be biased toward recordings in the down state because traces in which background activity obscured the ADP were not used in the analysis.

Dendritic calcium current was approximated by measuring the voltage–time integral of the calcium spikes evoked by dendritic current injection (50 ms or 1 s duration). The voltage–time integral was calculated as the area underlying the active potential component of the dendritic calcium spike close to threshold. To estimate the active component, the response to current injection was extrapolated from subtreshold traces and subtracted from the suprathreshold traces. The firing properties were assessed by examining the responses to suprathreshold depolarizing pulses (1 s) to the soma in vitro and the following parameters were measured: resting membrane potential, rheobase (current threshold), AP peak amplitude, AP half-width, input resistance, amplitude of sag, action potential frequency adaptation, and “burstiness.” Input resistance was measured from the slope of the current–voltage relationship to long current injections (1 s, −300 pA to threshold in steps of 100 pA) using the steady-state region of the response. Amplitude of sag was measured as the percentage of the steady-state response to −300 pA current injection compared with the maximum response. The action potential frequency adaptation was measured for discharges elicited by depolarizing 1 s pulses by calculating the proportional change in the frequency of the last interspike interval (ISI) compared with the frequency of the first ISI. Only discharges with an initial frequency <150 Hz were analyzed (Christophe et al. 2005). We defined a new measure, degree of “burstiness,” as the proportion of bursts (trains of APs with ISIs <20 ms) in the total number of trains following a long-step (1 s) current injection to the soma. We also noted “missing spikes”: gaps in otherwise regular spike trains that were multiples of the most frequent ISI.

**Statistical analysis**

Individual values and/or means ± SE are reported, unless stated otherwise. Statistical differences of the means were determined by using one- and two-tailed Student’s t-tests paired in vitro (which is insensitive to the cell-to-cell variability in the group, since matched data were analyzed) and a nonpaired t-test in vivo. In vitro comparisons to in vivo data were performed using two-way ANOVA with repetitions, followed by the Tukey test for multiple comparisons. The two-way ANOVA with repetitions allowed us to test interactions between the variables “in vitro/in vivo” and “anesthetics.” To compare means in the degree of “burstiness” we used the Wilcoxon signed-rank test in vitro. Comparisons of frequencies over populations in vivo were made with a chi-square test. *P < 0.05 was considered significant (*P < 0.05).

**Chemicals**

Urethane (ethyl carbamate), xylazine hydrochloride [2-(2,6-dimethylphenylamino)-5,6-dihydro-4H-thiazine hydrochloride], pentobarbital-Na [5-ethyl-5-(1-methylbutyl)-2,4,6-trioxohexahydropyrimidine], and lidocaine were acquired from Sigma–Aldrich Chemie (Buchs, Switzerland). Ketamine hydrochloride [(±)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride] was acquired from Alexis Biochemicals (Lausen, Switzerland) and Alexa fluor 568 hydrazide (Na from Molecular Probes (Eugene, OR).

**RESULTS**

The results are summarized as follows: first, we show the direct effect of anesthetics (urethane, pentobarbital, and ketamine/xylazine) on calcium spike threshold, amplitude and duration using dual recordings in vitro. We then examine the shift in critical frequency and the change in the afterdepolarizing potential (ADP) amplitude in vitro before and after the application of the anesthetics. We demonstrate that the anesthetics have little influence on AP propagation in the dendrite and that both the CF and change in ADP amplitude are reliable assays of dendritic Ca^{2+} spike activation. In addition, we examine the effect of the anesthetics on basic cellular firing properties using current injection at the soma. Last, we measure the critical frequency and ADP in vivo following ip injection of the anesthetics.

**Effects of anesthetics in vitro**

**DENDRITIC PROPERTIES.** Dendritic current injection. We first tested the direct effect of commonly used anesthetics on dendritic spike activity. We obtained dual-patch recordings from the somata and apical dendrites (Fig. 1) in the region of the calcium spike initiation zone [727.3 ± 117.4 μm (SD) from the soma, n = 11; Larkum and Zhu 2002]. Long depolarizing current pulses (1 s) evoked calcium spikes in the apical dendrite (Fig. 1B) and bursts of APs at the soma (Fig. 1C). We examined the dendritic calcium spikes before (black traces) and after addition of anesthetics to the extracellular medium (red traces). We measured the voltage–time integral and the current threshold (rheobase) of the first dendritic response following long (1 s) current injection to the dendritic site (Figs. 1 and 2). To calculate the regenerative component due to ion channel activity in the voltage–time integral, we subtracted the nonregenerative subthreshold component due to the current injection from the suprathreshold traces. The voltage–time integral of this regenerative component should correspond closely to the net positive influx of charge into the dendrite (Fansek and Redman 1973). Interestingly, the anesthetics had opposing effects on dendritic properties. On the one hand, 20 mM urethane and 200 μM pentobarbital significantly reduced the integral by 33.9 ± 8.6% (n = 5) and 42.5 ± 10.4% (n = 3), respectively. In one cell, pentobarbital had such a strong effect that we could no longer evoke suprathreshold activity (this cell was excluded from analysis). On the other hand, 400 μM ketamine/50 μM xylazine had the opposite effect, significantly increasing the integral by 106.5 ± 53.4% (n = 4), showing that this combination of anesthetics actually enhances calcium spike activity in the apical dendrite of L5 pyramidal neurons (Fig. 2, A and B, Table 1). The case shown in Fig. 1, A1–C3 was for a cell that, by chance, had an unusually low level of dendritic Ca^{2+} activity under control conditions. In this case, ketamine/xylazine dramatically increased the number of dendritic calcium spikes and their duration. Additionally, pentobarbital significantly increased the current threshold for evoking dendritic regenerativity, whereas ketamine/xylazine significantly decreased the current threshold (by 61.6 ± 9.8%, n = 3; 16.6 ± 4.4%, n = 4, respectively; Fig. 2C). Despite their effects on threshold and duration, none of the anesthetics significantly changed the peak amplitude of the dendritic calcium spike (Table 1). Taken together these results show that there are direct effects on the threshold and duration of dendritic calcium spikes that are anesthetic specific, which in turn have an impact on somatic firing activity.

**Reliability of the critical frequency test.** Dual dendritic/somatic patch-clamp recordings are currently not feasible in vivo and single dendritic patch-clamp recordings in vivo,
although feasible (Larkum and Zhu 2002; Waters and Helmchen 2004), tend to be much more difficult and less reliable than somatic recordings. Since our aim was to examine the direct effects of the same anesthetics in vivo, we needed a robust method to test their effect on dendritic activity using somatic patch-clamp recordings. We used the “critical frequency” test (Larkum et al. 1999a), which we show here can be used in vitro as a surrogate for dendritic recordings to determine the effects of anesthetics on dendritic activity (Fig. 3, D and E, Supplemental Fig. S1). Using this method, the effects of the anesthetics already seen on threshold would be reflected in a change in the critical frequency and the effects on duration would be reflected in a change in the amplitude of the after-depolarizing potential (ADP). This approach also had the advantage that the site of dendritic patch recording was no longer an arbitrary parameter and that stable long-term recordings could be performed regularly.

To test the reliability of the critical frequency protocol as a measure of the direct effects of anesthetics on dendritic activity we again used dual dendritic/somatic recordings in vitro.

**FIG. 1.** Effect of anesthetics on dendritic calcium spikes evoked by long current injection to the distal apical dendrite. A1–C1: 20 mM urethane. A2–C2: 200 μM pentobarbital (Pento). A3–C3: 400 μM ketamine/50 μM xylazine (Ket/Xyl). A1–A3: reconstruction morphologies of 3 biocytin-filled dual-recorded L5 pyramidal neurons with the positions of the somatic and dendritic pipettes indicated schematically. Long steps (1 s) of positive current (I_{dend; black: control; red: anesthetic}) were injected with the dendritic electrode. The recordings show the dendritic responses (V_{dend}) in B1–B3 and their related somatic discharges (V_{soma}) in C1–C3 under control conditions (black) and with the anesthetics (red).

**FIG. 2.** Anesthetics directly affected the duration of calcium spikes evoked with current injection into the dendrite in vitro. Calcium spikes were evoked with 50 ms or 1 s current steps injected directly in the distal apical dendrite. A: representative traces of dendritic calcium spikes under control conditions with artificial cerebrospinal fluid (ACSF, black) and 20 mM urethane, 200 μM pentobarbital, or 400 μM ketamine/50 μM xylazine applications (red). The voltage–time integrals were calculated to estimate the regenerative component of underlying ion channel activity. B: average effects of urethane 20 mM, pentobarbital 200 μM, and 400 μM ketamine/50 μM xylazine, respectively, on the voltage–time integral of the calcium spike normalized to control. C: average change in calcium spikes current threshold (rheobase) normalized to the control. *P < 0.05 with one-tailed paired Student’s t-test (SE).
Trains of three to four BPAPs were generated with short-duration current steps (2 ms) at frequencies between 30 and 250 Hz at the soma (Fig. 3). At high frequencies (supra-CFs) there was a dramatic increase in the amplitude and width of the dendritic potential generated near the last BPAP in the train (Fig. 3C, black traces). We used a sigmoidal fit to this dendritic potential amplitude to estimate the critical frequency (Fig. 3, D and E). A sigmoidal fit to the ADP at the soma at different frequencies revealed a CF that was not significantly different from the CF estimated using the dendritic potential (85.8 ± 2.5 vs. 86.1 ± 2.5 Hz; P = 0.72; n = 11; Fig. 3, D and E). This showed that the CF could be measured accurately with a somatic recording under control conditions. Bath application of the anesthetics (Fig. 3, red traces) changed the CF, the dendritic depolarization, and the somatic ADP amplitude at high frequencies. In the presence of anesthetics, there was still no significant difference between the CF measured at the dendrite or at the soma in each cell (91.9 ± 6.9 vs. 92.8 ± 6.7 Hz; P = 0.58; n = 11; Fig. 1, D and E, red traces). We concluded that the CF measured at the soma in vitro accurately determines the effect of the anesthetics. As with the direct dendritic current injection (Figs. 1 and 2), the anesthetics had opposing effects on the activation of dendritic activity using the critical frequency test measure either at the soma or at the dendrite (Fig. 3). We found that urethane and pentobarbital tended to increase the CF and to decrease the somatic ADP amplitude, both indications of an inhibitory effect on Ca2+ spike generation. On the other hand, ketamine/

**TABLE 1. Summary of the effects of different anesthetics on dendritic properties in vitro**

<table>
<thead>
<tr>
<th>Dendritic Properties</th>
<th>Urethane, 20 mM</th>
<th>Pentobarbital, 200 μM</th>
<th>Ketamine, 400 μM/Xylazine, 50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Somatic recordings</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical frequency (CF)</td>
<td>+24.0 ± 2.9% (n = 16)*</td>
<td>+16.4 ± 3.2% (n = 12)*</td>
<td>-22.2 ± 3.2% (n = 14)*</td>
</tr>
<tr>
<td>ADP amplitude</td>
<td>-10.7 ± 5.7% (n = 16)*</td>
<td>-54.0 ± 7.5% (n = 12)*</td>
<td>+151.0 ± 19.3% (n = 14)*</td>
</tr>
<tr>
<td><strong>Dendritic recordings</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca2+ spike amplitude</td>
<td>-9.2 ± 5.5% (n = 4)</td>
<td>-12.8 ± 9.5% (n = 3)</td>
<td>+8.7 ± 6.5% (n = 4)</td>
</tr>
<tr>
<td>Ca2+ spike half-width</td>
<td>-0.8 ± 24.2% (n = 3)</td>
<td>-55.2 ± 21.8% (n = 2)</td>
<td>+71.1 ± 48.8% (n = 5)</td>
</tr>
<tr>
<td>Ca2+ spike integral</td>
<td>-38.5 ± 8.9% (n = 5)*</td>
<td>-52.4 ± 2.6% (n = 4)*</td>
<td>+80.5 ± 23.4% (n = 5)*</td>
</tr>
<tr>
<td>CF test</td>
<td>-33.9 ± 8.6% (n = 5)*</td>
<td>-42.5 ± 10.4% (n = 3)*</td>
<td>+106.5 ± 53.4% (n = 4)*</td>
</tr>
<tr>
<td><strong>Propagation properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back-AP half-width</td>
<td>-5.2 ± 3.4% (n = 5)</td>
<td>-8.0 ± 7.5% (n = 3)</td>
<td>+32.5 ± 16.5% (n = 3)</td>
</tr>
<tr>
<td>Back-AP amplitude</td>
<td>+1.5 ± 1.7% (n = 5)</td>
<td>+1.0 ± 3.2% (n = 3)</td>
<td>-12.1 ± 7.9% (n = 3)</td>
</tr>
</tbody>
</table>

Values are percentages ± SE (n values in parentheses) and represent the change compared with control values (no change would be 0%). Somatic recordings were pooled from dual somatic/dendritic and single somatic recordings. Action potential (AP) statistics were calculated from a single AP or the first AP in a train. *P < 0.05 with one-tailed paired Student’s t-test (SE).
xylazine decreased the CF and increased the somatic ADP amplitude, indicating an enhancing effect on Ca^{2+} spike generation (Fig. 3, D and E). The data from direct dendritic current injection (Figs. 1 and 2) suggest that the anesthetics had direct effects on the initiation and duration of the dendritic Ca^{2+} spike.

Effect of anesthetics on the propagation of action potentials along the dendrite. We also investigated the effect of the anesthetics on the amplitude and half-width of BPAPs in the apical dendrite, as well as the integral of the dendritic potential of the train of BPAPs. There was no significant effect on either the amplitude or the half-width of the dendritic BPAP by either urethane or pentobarbital relative to the somatic AP [a change by +1.5 ± 1.7% (n = 5) and +1.0 ± 3.2% (n = 3), respectively; Table 1] and a change by −5.2 ± 3.4% (n = 5) and −8.0 ± 7.5% (n = 3); Table 1]. Ketamine/xylazine also had no significant effect on either the amplitude (a change by −12.1 ± 7.9%) or the half-width of the BPAPs (a change by +32.5 ± 16.5%, n = 3; Table 1) relative to the somatic AP. However, both the amplitude and duration of the somatic AP were affected by ketamine/xylazine. (The corresponding effects on somatic APs for all anesthetics are subsequently discussed in detail and shown in Table 2.) The fidelity of propagation along the dendrite could also, in principle, affect the summation of the BPAPs in the distal tuft region. We found that there were no significant effects of the anesthetics on the integral of the BPAPs measured in the dendrite at sub-CFs (Fig. 4, light gray bars), showing that the anesthetics did not change the integration of BPAPs per se. In contrast, the integral of the BPAPs at supra-CFs changed significantly after application of each anesthetic (Fig. 4, dark gray bars) The effect of ketamine/xylazine on integration of BPAPs was complicated by the change in the shape of the somatic AP. Here, the decrease in amplitude coupled with an increase in half-width are likely to have had opposing effects on the integral measured in the dendrite. Nonetheless, even in this case, the effect of ketamine/xylazine was significant only for supra-CF BPAPs. Taken together, these data show that the anesthetics affect the generation of Ca^{2+} spikes in the dendrite (and in the case of ketamine/xylazine the generation of Na^{+} spikes at the soma/axon) but do not affect the propagation of APs along the dendrite.

Effect of anesthetics on dendritic activity evoked by BPAPs shown with single somatic recordings. Since the somatic ADP was strictly correlated to the dendritic Ca^{2+} activity under control conditions and in the presence of anesthetics, we could readily increase the number of recordings using single somatic patch-clamp recordings. This also facilitated the testing of the same anesthetics with various doses on the same neuron. Similar to the experiments with dendritic recordings shown in Fig. 3, experiments with single somatic recordings revealed that 20 mM urethane increased the CF by 24.0 ± 2.9% (n = 16; Fig. 5, A and G, Table 1), 200 µM pentobarbital increased the CF by 16.4 ± 3.2% (n = 12; Fig. 5, B and G) and 400 µM ketamine/50 µM xylazine decreased the CF by 22.2 ± 3.2% (n = 14; Fig. 5, C and G), showing that ketamine/xylazine had an effect opposite to that of urethane and pentobarbital. Similarly, the somatic ADP amplitude was decreased by 10.7 ± 5.7% in urethane (Fig. 5, D and H), by 54.0 ± 7.5% (n = 12) in pentobarbital (Fig. 5, E and H), and increased by 151.0 ± 19.3% (n = 14) in ketamine/xylazine (Fig. 5, F and H). Thus our conclusions remained the same: that urethane and pentobarbital inhibited the generation of dendritic calcium spike, whereas ketamine/xylazine enhanced it.

Lower concentrations of anesthetics had partially similar effects. Urethane (10 mM) significantly increased the CF by 12.5 ± 1.8% of control, n = 11, P < 0.001) but did not affect the somatic ADP amplitude (a change by −10.4 ± 5.5% of control, n = 11, P = 0.18). On the other hand, 100 µM pentobarbital did not significantly alter the CF (a change by −2.8 ± 1.9% of control, n = 10) but decreased the somatic ADP amplitude (by 15.9 ± 5.8% of control, n = 10, P < 0.05). In a few experiments we used a very high concentration of pentobarbital (1 mM) that had such large depressive effects on somatic spike generation and propagation that the effects on CF could not be tested. We tested ketamine and xylazine on their own and the mixture of ketamine/xylazine was tested at various doses (Table 1) relative to the somatic AP. Values are means ± SE (n values in parentheses) and correspond to parameters measured at the soma for long (1 s) somatic current injections. Current pulses started at negative values and increased by steps of 100 pA after each sweep. Measurements relative to the AP were done for the first action potential of the sweep. *P < 0.05 (SE).
n = 5, P < 0.05) without significantly altering the somatic ADP amplitude (a change by 30.8 ± 12.8% of control, n = 5). A fourfold decrease (to 100 μM ketamine/12.5 μM xylazine) significantly decreased both the CF (by 12.4 ± 3.4% of control, n = 6, P < 0.05) and significantly increased the somatic ADP amplitude (by 43.7 ± 13.8% of control, n = 6, P < 0.05). Taken together, these results indicate that the facilitatory versus depressive effects of the anesthetics were not dependent on the concentrations used.

SOMATIC FIRING PROPERTIES. Anesthetics have been reported to affect the basic firing properties of neurons (Antkowiak 1999; Christophe et al. 2005; Sceniak and MacIver 2006). We tested the effect of urethane, pentobarbital, and ketamine/xylazine on AP firing properties using long-step (1 s) current injection at the soma (Fig. 6). We analyzed a number of parameters and particularly those that might be related to dendritic properties (Fig. 7, Table 2). For instance, burst firing in pyramidal neurons can be an indication of dendritic activity (Helmchen et al. 1999; Larkum et al. 1999b; Schwindt and Crill 1999; Williams and Stuart 1999; Wong and Prince 1978). We determined the change in burstiness with 10 and 20 mM urethane in vitro. Under control conditions, the average burstiness of cells was 37.8 ± 6.8% (n = 16), which indicates that normally about one third of the spike trains were in the form of bursts. Although there was a slight decrease from control (37.8 ± 6.8%, n = 16) to 20 mM urethane (29.2 ± 6.7%, n = 16), this was not statistically significant. There was also no significant difference in the number of “missing spikes” in a train that gave another measure of the “irregularity” of a train (see METHODS). We found a small difference in the threshold of the APs with 20 mM urethane (41.32 ± 1.6 mV, n = 16 vs. 43.97 ± 1.2 mV, n = 16 under control conditions, P < 0.05) (Fig. 7D). In general urethane appeared to have minor effects and preserved the firing properties quite well compared with the other anesthetics tested.

Pentobarbital (100 or 200 μM) did not alter the degree of burstiness. However, pentobarbital (unlike urethane and ketamine/xylazine) led to a specific firing behavior in which the spike trains appeared to be interrupted (i.e., single spikes appeared to be missing in otherwise regular trains of APs). This “missing spikes” effect happened in 60.0% (n = 10) of the neurons treated with 100 μM pentobarbital and in 91.7% (n = 12) of the neurons treated with 200 μM pentobarbital.
compared with 0.0% \( (n = 13) \) under control conditions. With 100 and 200 \( \mu M \) pentobarbital the current threshold or rheobase \( (475.0 \pm 38.9 \text{ pA}, n = 10, P < 0.05; 658.3 \pm 72.5 \text{ pA}, n = 12, P < 0.001, \text{ respectively}) \) was increased compared with control conditions \( (363.9 \pm 40.6 \text{ pA}, n = 12) \) (Table 2). To reach threshold with 200 \( \mu M \) pentobarbital we needed to increase the amplitude of current injected by 80.9%. With 100 and 200 \( \mu M \) pentobarbital the input resistance \( (30.2 \pm 2.6 \text{ nS/m}) \) was increased compared with control conditions. The effect on input resistance is shown in Table 2.

**FIG. 6.** Effect of anesthetics on firing properties in in vitro dual recordings. A1–A3: morphological reconstructions of the L5 pyramidal neurons and the positions of the somatic and dendritic electrodes. B1–B3: somatic and corresponding dendritic discharges of the neurons for a long step \( (1 \text{ s}) \) of positive current injection at the soma near threshold, in the control conditions (black traces) and with the anesthetics (red traces). C1–C3: expanded view of B1–B3. Concentrations: urethane, 20 mM; pentobarbital, 200 \( \mu M \); and ketamine and xylazine mixture, 400 \( \mu M \) and 50 \( \mu M \), respectively.

**FIG. 7.** Summary of effects of anesthetics on firing properties in vitro. A: normalized input resistance. B: normalized sag voltage amplitude. C: relative burstiness of the neuron for somatic long current injection. D: normalized membrane potential threshold needed to generate action potentials. E and F: normalized half-width and height of the action potential, respectively. * \( P < 0.05 \) (SE).
pyramidal neurons in vivo at depths of 825–1500 μM, n = 12, P < 0.01, respectively) was decreased compared with control conditions (35.3 ± 3.6 μM, n = 12) (Fig. 7A). The AP frequency adaptation increased from the control conditions (10.8 ± 6.5%, n = 8) to 200 μM pentobarbital (41.8 ± 11.4%, n = 9, P < 0.01). With 1 mM pentobarbital firing was either abolished completely or only one or two APs occurred at very high thresholds (n = 8; data not shown). Under such conditions we could not properly use the critical frequency protocol. We concluded that pentobarbital depressed generation of sodium APs, likely through the increasing of ion channel conductances.

With 400 μM ketamine/50 μM xylazine (but not for lower concentrations), burstiness increased from 16.1 ± 8.9% under control conditions to 51.2 ± 7.5%, n = 14, P < 0.005 (Figs. 6B and 7C), a relative increase of 35.1%. This is consistent with the enhancement by ketamine/xylazine of dendritic calcium spike regenerativity. Another specific feature of 400 μM ketamine/50 μM xylazine was a progressive decrease in the amplitude of the APs in a train and this decrease became stronger with increased current injection. This AP amplitude adaptation was observed in 92.9% (n = 14) of the neurons and did not exist either under control conditions or for lower concentrations tested. Ketamine/xylazine also increased the AP frequency adaptation (from 30.1 ± 7.7%, n = 11 under control conditions to 86.5 ± 2.3%, n = 10, P < 0.001) as well as the AP half-width (from 0.757 ± 0.037 to 0.836 ± 0.048 ms, n = 14, P < 0.01) (Fig. 7E). Ketamine/xylazine also decreased the peak amplitude of the first AP in a train (from 89.7 ± 4.4 mV under control conditions to 65.3 ± 3.0 mV, n = 14, P < 0.001) (Fig. 7F). The AP threshold was depolarized from −42.6 ± 1.2 mV under control conditions to −37.2 ± 1.5 mV (n = 14, P < 0.001) with ketamine/xylazine (Fig. 7D) and the resting membrane potential was hyperpolarized from −67.0 ± 0.7 to −68.9 ± 1.1 mV (n = 14, P < 0.05). In other words, ketamine/xylazine had a double impact on neurons. On the one hand, ketamine/xylazine enhanced dendritic properties, resulting in an increase in burstiness at the soma. On the other hand, ketamine/xylazine inhibited the generation of somatic APs.

Among the three anesthetics tested in vitro, urethane affected the firing properties the least and ketamine/xylazine the most. Such information might be important in the context of functional studies at the network level. For this study the salient point was that there was a general correspondence between the effects of the anesthetics on dendritic properties and on somatic firing properties: pentobarbital depressed both dendritic calcium spikes and generation of sodium APs, whereas ketamine/xylazine enhanced dendritic calcium spikes and increased burstiness.

Effects of anesthetics in vivo

We wanted to test the direct effects of anesthetics on the generation of Ca2+ spikes in vivo and to compare dendritic excitability with the in vitro data. We recorded from L5 pyramidal neurons in vivo at depths of 825–1500 μm from the pial surface [average 1141 ± 154 μm (SD), n = 53] using the blind patch-clamp technique (Larkum and Zhu 2002; Pei et al. 1991; Zhu and Connors 1999). The ip doses of anesthetic injected to the rats were 1.4 g/kg for urethane, 35 mg/kg for pentobarbital and 75 mg/kg of ketamine mixed with 10 mg/kg of xylazine. As in vitro, we evoked trains of three to four BPAPs and recorded the critical frequency, the ADP amplitude, and calculated the ADP integral (Fig. 8). Resting membrane potentials tended to fluctuate more in vivo due to greater background activity. In many cases, the CF could still be determined from a single trial (i.e., one measurement of the ADP for each frequency across a given range); however, in cases of high activity, we took the average of several (3–10) trials. To calculate the CF, we measured the ADP at frequencies between approximately 30 and 250 Hz. This is referred to herein as a “trial.” Occasionally in vivo we recorded more than once at each frequency (3–10 times) and this is still referred to as one trial.

In vitro, nearly all L5 pyramidal neurons showed a CF for ADP enhancement and the CF was robustly reproducible from trial to trial. In contrast, we found that the CF was not always detectable in vivo (Fig. 9A). On the other hand, none of the anesthetics abolished the CF in all cells. Our criterion in vivo for attributing a CF to a cell was a P < 0.05 significance value when performing a t-test on the points either side of the midpoint of a sigmoidal fit. Under urethane anesthesia, in 14 of 19 neurons (73.7%) the ADP amplitude varied sharply around a critical frequency. Under pentobarbital and ketamine/xylazine this proportion decreased slightly to 62.5% (n = 16) and 66.7% (n = 21). Even in cells that showed a CF there were trials (where the ADP was measured across a range of frequencies) in which no CF could be detected (Fig. 9B). The rate of occurrence for the CF from trial to trial in vivo was 64.7% (n = 34 trials) for urethane, 56.1% (n = 57 trials) for pentobarbital, and 40.7% (n = 54 trials) for ketamine/xylazine (Fig. 9B). This variability suggested that some additional factor inhibits the dendritic Ca2+ spikes in vivo, which would be consistent with fluctuations in inhibition impinging on the dendrite since inhibition is known to strongly suppress dendritic Ca2+ spikes (Pérez-García et al. 2006). Note that the anesthetics that had the least effect on somatic firing properties in vitro had the highest occurrence of the CF in vivo and vice versa.

Using two-way ANOVA we found that there was a statistically significant increase in the CF in vivo compared with in vitro (P < 0.001, df = 1, n = 78; Fig. 10A) and no interaction of this factor with the variable anesthetic; i.e., the trend was the same in vivo compared with in vitro. This provided evidence that the direct effects of the anesthetics on dendritic properties observed in vitro were also present in vivo. Interestingly, ketamine/xylazine did not lower the CF proportionately as much in vivo as in vitro, presumably because the enhancement of Ca2+ spikes by ketamine/xylazine competed with the general suppression of Ca2+ spikes in vivo.

In vivo, we found that both the ADP amplitude and the ADP integral over 10 ms (starting 5 ms after the initiation of the last AP) could be used to determine the influence of the dendritic Ca2+ spike on the soma (Fig. 8, D–F). However, because of the random fluctuations in membrane potential in vivo it was difficult to find a single time point at which to compare all the data in vivo and in vitro to establish the relative amplitude of the ADP. We therefore chose the ADP integral for comparisons made across cells in vivo and in vitro (Fig. 10B). Using this measure we found that the average ADP integral for each anesthetic was lower in vivo compared with that in vitro but, due to the huge difference in the case of ketamine/xylazine, the trend was not found to
be similar using two-way ANOVA. As with the CF, we assume that the suppressive effects on dendritic Ca\(^{2+}\) activity in vivo counteracted the direct action of ketamine/xylazine. This disproportionately large counteractive effect is consistent with the lower occurrence of detectable CFs in vivo with ketamine/xylazine. Another possible explanation for the discrepancy observed for ketamine/xylazine in vivo versus in vitro could be that the final concentration obtained in vivo is small. This possibility is unlikely, given the fact that even with an order-of-magnitude reduction in vitro, the facilitatory effect of ketamine/xylazine could still be detected. Nonetheless, to further test this possibility, we made additional experiments with local cortical application of 400 \(\mu\)M ketamine/50 \(\mu\)M xylazine at the craniotomy site (i.e., the highest concentration used in the slice). There was no further increase with 400 \(\mu\)M ketamine/50 \(\mu\)M xylazine added to the surface of the cortex compared with the control condition in which the rat was anesthetized only with ip injection (\(n = 6\); \(P = 0.89\) and \(P = 0.86\) for the CF and ADP, respectively). Thus the discrepancy observed for ketamine/xylazine in vivo versus in vitro was unlikely related to the concentration of ketamine/xylazine present in vivo. In summary, we conclude that the direct effects of the anesthetics present in vitro are also present in vivo competing...
with (or adding to) a general tendency for the suppression of dendritic Ca\textsuperscript{2+} activity in vivo.

**DISCUSSION**

We investigated the effect of the anesthetics urethane, pentobarbital and a mixture of ketamine/xylazine on dendritic properties in L5 pyramidal neurons in vitro and in vivo. There were five main conclusions.

1. All three anesthetics tested altered the initiation and duration of dendritic calcium spikes: urethane and pentobarbital suppressed dendritic activity, whereas ketamine/xylazine enhanced it.
2. All three anesthetics had negligible effects on the amplitude and shape of BPAPs.
3. Each anesthetic had particular effects on the somatic firing properties.
4. The direct effects of the anesthetics were similar in vivo to in vitro.
5. There was some additional factor or factors in vivo that further suppressed dendritic Ca\textsuperscript{2+} activity.

Although all anesthetics alter the level of consciousness, their specific actions on channel conductances and receptors both pre- and postsynaptically can be quite different (Dilger 2002; Franks and Lieb 1994; Richards 2002; Rojas et al. 2006). Even the choice of anesthetic concentration in vitro can be problematic (Franks 2006) and there is a lack of specificity at higher concentrations with most anesthetics. On the other hand, our principal aim was to investigate their effects specifically on intrinsic dendritic excitability with a view to interpreting studies of dendritic properties in vivo rather than to understand the mechanisms underlying the ability of anesthetics to occlude consciousness. In principle we cannot know the precise concentrations of the drugs in the cortex in vivo, although we chose concentrations of anesthetics that have been used in other studies in vitro (Accorsi-Mendonca et al. 2007). In addition, using several concentrations, we found no evidence that the inhibitory versus facilitatory effect on Ca\textsuperscript{2+} spikes was concentration dependent.

Our study further confirmed that urethane, pentobarbital and ketamine/xylazine had quite different specific actions in their effects both on somatic and on dendritic firing properties. By using DC injection, effects of the anesthetics on intrinsic dendritic excitability were investigated. In the case of synaptic stimulation, however, anesthetics may affect the calcium spike differently. The specific action of anesthetics on dendritic voltage-sensitive channels will require further study and is in general very difficult because most pharmacological interventions (i.e., blocking Na\textsuperscript{+} and K\textsuperscript{+} channels) influence AP shape and propagation. This will likely require focusing on each anesthetic in turn in separate studies in vitro. Ketamine/xylazine had the opposite effect on dendritic firing properties compared with that of urethane and pentobarbital. Although urethane and pentobarbital had similar effects on dendritic properties, their effects on somatic firing properties were different and even the underlying dendritic mechanisms of action may have been different. We now speculate on the mechanisms likely to be involved based on effects determined in our study and by others using somatic recordings.
Urethane

Urethane is one of the most commonly used anesthetics in research on rodents, partly because of its long-lasting action (Maggi and Meli 1986). Although most anesthetics are thought to enhance inhibition in the cortex, urethane has one of the lowest effects on GABAergic transmission (Scholfield 1980), although the underlying mechanisms are not completely understood. Urethane has been reported to inhibit NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in a concentration-dependent manner, to potentiate the functions of neuronal nicotinic acetylcholine and glycine receptors, but also to have variable effects on GABAergic neurotransmission (Accorsi-Mendonca et al. 2007; Garrett and Gan 1998; Harata and Harris 2002). Our results suggest that 10–20 mM urethane inhibited dendritic excitability, which is in agreement with Adelsberger et al. (2005) who showed that urethane suppressed calcium waves in brain slices and in vivo. We saw very little change in most cellular firing properties (e.g., input resistance, AP amplitude and width, spike adaptation), which is in agreement with Sceniak and Maclver (2006) and Accorsi-Mendonca et al. (2007); however, we did observe a significant shift of the AP threshold to more depolarized values accompanied with a slight increase in the rheobase, which suggests that urethane depressed action potential discharge. Sceniak and Maclver (2006) also described a strong depression of potential discharge accompanied with a decrease in membrane resistance, which they attributed to selective activation of a Ba2+-sensitive K+ leak conductance. Moreover, urethane has also been shown to suppress the Na,1,2 channel function (Shiraishi and Harris 2004).

Pentobarbital

The principal action of pentobarbital is its enhancement of GABAergic transmission (Drafts and Fisher 2006; Macdonald et al. 1989; Parker et al. 1986). However, pentobarbital has also been reported to act on many other targets including inhibition of voltage-activated Ca2+ channel currents (ffrench-Mullen et al. 1993; Kitayama et al. 2002; Todorovic and Lingle 1998; Todorovic et al. 2000), depression of glutamate-mediated excitatory synapses (Pittson et al. 2004), and inhibition of sodium channels (Wartenberg et al. 1999). All these targets seem consistent with the inhibition pentobarbital exerts on calcium spikes, but it is not clear yet which effects prevail. We found that pentobarbital had an action similar to that of urethane in suppressing dendritic activity. However, the effects of pentobarbital on general firing properties were stronger than those of urethane, including an increase in the rheobase, decrease in input resistance, and a general hyperpolarization of the resting membrane potential. This would be consistent with a tonic activation of GABAA receptors by pentobarbital (Farrant and Nusser 2005). It would also explain the discontinuities occurring in the spike trains of APs and the powerful blockade of firing at higher concentrations. Consistent with previous reports (Antkowiak 1999), we found that pentobarbital inhibited spontaneous AP firing in a concentration-dependent manner. However, Christophe et al. (2005) showed that a proportion of L5 pyramidal neurons switched from regular firing to intrinsic bursting mode due to prolonged pentobarbital anesthesia in vivo prior to recordings in vitro. Such differences could have resulted from homeostatic effects of the anesthetic on intrinsic membrane properties.

Ketamine/xylazine

The most unexpected result of our study was that a mixture of ketamine and xylazine in vitro enhanced dendritic excitability with a broadening of the Ca2+ spike, a lowering of the critical frequency, and a corresponding increase in ADP amplitude. Ketamine is a dissociative anesthetic whose main action is as a noncompetitive antagonist of NMDA receptors (Hirota and Lambert 1996; Kapur and Seeman 2002). At low doses and during emergence from anesthesia, ketamine is known to have various psychotomimetic effects that account for its use as a recreational drug (Krystal et al. 1994; Rowland 2005). Used in combination with xylazine, it is a very common alternative to urethane and pentobarbital in animal experiments (Green et al. 1981). Apart from its main anesthetic action on NMDA channels there is evidence that ketamine enhances GABAA receptors (Gage and Robertson 1985; Irifune et al. 2000; Scholfield 1980), although others have reported that it does not interact with these receptors (Hirota and Lambert 1996; Kitayama et al. 2002; Lingamaneni and Hemmings Jr 2003; Yamakura et al. 2000). In addition, ketamine can inhibit various voltage-sensitive channels including sodium, calcium, and potassium channels (Friederich et al. 2001; Harata and Harris 1998; Hatakeyama et al. 2001; Kitayama et al. 2002; Schnoebel et al. 2005; Zhou and Zhao 2000). This makes it very difficult to predict its action on dendritic activity.

Adding to the complexity are the effects of xylazine, which principally activates α2-adrenergic receptors (Cabral et al. 1998). The effects we observed on broadening dendritic Ca2+ spikes are consistent with studies showing that ketamine prolongs the total duration of plateau-like cardiac APs (Hatakeyama et al. 2001) and that xylazine enhances myometrial contractility mediated by α2-adrenergic receptors and calcium channels (Ko et al. 1990). A recent study showed that α2-receptors down-regulate Ih channels colocalized on the dendrites of cortical pyramidal neurons (Wang et al. 2007). Inhibition of Ih channels has been shown to enhance dendritic Ca2+ channel activity and decrease the critical frequency (Berger et al. 2003) and xylazine might act on Ca2+ channel activity through this pathway. Ketamine/xylazine had the greatest effect on firing properties of all three anesthetics. Christophe et al. (2005) measured a switch from regular firing to intrinsic bursting mode in L5 pyramidal neurons with prolonged ketamine/xylazine anesthesia previous to decapitation, which was similar to the effects we observed. Interestingly, however, they did not observe similar effects in vitro with bath application of ketamine (10–200 μM).

Critical frequencies in vivo were systematically higher than those in vitro for all anesthetics. This strongly suggests that some additional factor in vivo affects the threshold for dendritic Ca2+ activity. One possibility is due to inhibition arising from network activity not present in vitro (Rudolph et al. 2007). GABAergic transmission has a strong veto-like action on dendritic activity (Pérez-Garcia et al. 2006). Moreover, most anesthetics recruit additional inhibition (Franks and Lieb 1994). On the other hand, we did observe evidence of dendritic Ca2+ activity with all anesthetics, which is consistent with previous reports in vivo using urethane, pentobarbital, thiopen-

In conclusion, we have presented the first study identifying dendritic calcium spikes as a direct target of anesthetics. All the drugs tested here affecting consciousness also had direct effects on dendritic Ca$^{2+}$ spikes and firing properties, although the specific effects were quite different. Active propagation of dendritic spikes was not altered. Since none of the three anesthetics tested abolished dendritic Ca$^{2+}$ spikes they can all be used for investigations of dendritic function in vivo; however, it is vital in each case to take the specific effects of these anesthetics into account.

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