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ORIGINAL ARTICLE

Effects of propofol and pentobarbital on calcium concentration in presynaptic boutons on a rat hippocampal neuron

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Abstract
Purpose Numerous reports suggest that intravenously administered (IV) anesthetics affect postsynaptic events in the central nervous system. However, there is little evidence about how general anesthetics influence the presynaptic processes. The level of presynaptic calcium (Ca^{2+}) concentration ($[Ca^{2+}]_{pre}$) regulates neurotransmitter release. In this study, we investigated the effects of anesthetic propofol IV and the barbiturate pentobarbital on neurotransmitter release by measuring $[Ca^{2+}]_{pre}$ in the presynaptic nerve terminals (boutons) on a dissociated single hippocampal rat neuron.
Methods Sprague-Dawley rats 10–14 days old were decapitated under pentobarbital anesthesia, and brain slices were prepared. The hippocampal CA1 area was touched with a fire-polished glass pipette, which vibrated horizontally, and neurons were dissociated, along with the attached presynaptic boutons. The presynaptic boutons were visualized under a confocal laser-scanning microscope after staining with FM1-43 dye, and $[Ca^{2+}]_{pre}$ was measured with acetoxymethyl ester of fluo-3 (fluo-3 AM).
Results High potassium (K^+) (15–90 mM) increased the $[Ca^{2+}]_{pre}$ in the Ca^{2+}-containing solution in a concentration-dependent manner. Whereas propofol (10 μM) and pentobarbital (300 μM) suppressed the high K^+ (60 mM)-induced increase in $[Ca^{2+}]_{pre}$ in the boutons attached to the dendrite, they did not affect $[Ca^{2+}]_{pre}$ in the boutons attached to the soma or dendrite base. As a large majority of excitatory synapses are located on dendritic spines, these agents may affect Ca^{2+} mobilization in the excitatory presynaptic boutons.
Conclusions Propofol and pentobarbital may affect neurotransmitter release from the excitatory presynaptic nerve terminals due to inhibition of increase in $[Ca^{2+}]_{pre}$.

Keywords Propofol · Pentobarbital · Calcium signaling · Presynaptic terminals · Hippocampus

Introduction

Numerous reports suggest that general anesthetics affect postsynaptic events in the central nervous system (CNS). The intravenously administered (IV) anesthetic propofol and the barbiturate pentobarbital are used in daily clinical medicine, and the main sites of postsynaptic inhibition of these agents are γ-aminobutyric acid type A ($GABA_A$) receptors. Both agents modulate $GABA_A$-receptor-mediated inhibition during postsynaptic events through various mechanisms [1–7]. Further, propofol enhances postsynaptic $GABA_A$ receptor function at GABAergic synapses and depresses sodium-channel functions in the human brain [8] and cultured hippocampalneurons [9]. Propofol also inhibits activation of *N*-methyl-D-aspartate (NMDA) receptors in hippocampal neurons [10, 11]. General anesthetics are also known to affect presynaptic processes. Propofol affects the level of neurotransmitters [12, 13] and increases the release of GABA through presynaptic $GABA_A$ receptors [14]. However, at clinically relevant

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concentrations, propofol has fewer effects than the volatile anesthetic isoflurane on the presynaptic events [15]. There are several reports on the presynaptic effects of pentobarbital. Pentobarbital inhibits voltage-sensitive calcium (Ca^{2+}) channels [16] and reduces the amplitude of calcium transients in presynaptic terminals (boutons) [17]. As a consequence, this agent depresses excitatory synaptic transmission in the CNS [18]. However, there is not enough evidence to characterize the influence of general anesthetics on presynaptic processes. Neurotransmitter release is regulated by the level of presynaptic Ca^{2+} concentration ($[Ca^{2+}]_{pre}$) [19, 20]; therefore, to determine the influence of anesthetics on presynaptic events, it is necessary to investigate their effects on Ca^{2+} movement in the presynaptic boutons. In this study, we investigated the effects of propofol and pentobarbital on neurotransmitter release by measuring Ca^{2+} concentration in the presynaptic boutons of a dissociated rat hippocampal neuron.

Materials and methods

Preparation

This experiment was performed under the Guidance for Animal Experiments at the Faculty of Dental Science, Kyushu University, Fukuoka, Japan. Ethical approval for this study (approval number A20-125-0) was obtained from the Animal Care and Use Committee, Kyushu University (chairperson Prof R. Takayanagi) on 8 December 2008. Sprague-Dawley rats (10–14 days old) were decapitated under pentobarbital anesthesia. The brain was quickly removed and cut into slices 400-μm thick using a microslicer (DTK-3000W; Dosaka EM, Kyoto, Japan). The slices were placed in an incubation solution for 1 h and saturated with 95% oxygen (O_2) and 5% carbon dioxide (CO_2) at room temperature (23–27°C). The slices were placed in a chamber containing standard external solution (500 μl, see description below); the hippocampal CA1 region was touched with a fire-polished glass pipette, and the pipette was vibrated horizontally for 1 min using a custom-made device. The mechanical vibration caused dissociation of pyramidal neurons, along with the attached presynaptic boutons [21, 22].

Presynaptic calcium measurement and staining with FM 1-43

The dissociated neurons were incubated in standard external solution containing acetoxymethyl ester of fluo-3 (fluo-3 AM; 10 μM) and Pluronic F-127 (0.01%) for 30 min at room temperature (23–27°C). Next, fluo-3 fluorescent images of the neurons with the presynaptic boutons were obtained using a confocal laser-scanning microscope (IX70; Olympus, Tokyo, Japan). The neurons were excited at 488 nm by passing an argon laser beam through an objective lens (UPlanApo 40X, Olympus). Next, we recorded fluo-3 fluorescent green signals through a 505– to 525-nm bandpass filter using a scan unit (FVX-SU, Olympus) at 0.42-s intervals, as previously described [23]. After measuring the changes in fluo-3 fluorescence intensity, individual presynaptic boutons were visualized by high potassium (K^+) depolarization in the presence of FM1-43. A dissociated neuron was exposed to a 60-mM K^+-containing standard external solution [prepared by replacing 55 mM sodium chloride (NaCl) with equimolar potassium chloride (KCl) for 30 s in the presence of 10 μM FM1-43 and then washed with a standard external solution for 10 min. The FM1-43 dye was excited with an argon laser, and the green fluorescence emitted was recorded using a 560-nm long-pass filter. The FM1-43-labelled boutons expressed a pseudo red color that distinguished them from the fluo-3 green signals. The morphology of the hippocampal neurons was clearly visualized in the fluo-3 images, and therefore, the regions to which the presynaptic boutons attached were distinguished in the fluorescent images during and after measurement of the fluo-3 and FM1-43 fluorescence. To evaluate $[Ca^{2+}]_{pre}$, the mean intensity (F) of the boutons was calculated, and data were expressed as the ratio of fluorescence intensity change ($\Delta F = F - F_0$) relative to the control values before stimulation (F_0), namely $\Delta F/F_0$.

Solutions

The incubation solution was saturated with 95% O_2 + 5% CO_2 and contained (in mM) NaCl (125), KCl (2.5), calcium chloride ($CaCl_2$) (2), magnesium chloride ($MgCl_2$) (2), sodium phosphate (NaH_2PO_4) (1.5), sodium bicarbonate ($NaHCO_3$) (26), and glucose (20). The standard external solution (pH 7.4) contained (in mM) NaCl (150), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), hydroxyethyl-1-piperazine ethanesulfonic acid (HEPES) (5), and glucose (10). K^+ concentration was modified by isosmotically replacing NaCl with KCl.

Drugs

Drugs used were dimethyl sulfoxide (DMSO), HEPES, Pluronic F-127 (Sigma-Aldrich, Inc., St. Louis, MO, USA), fluo-3 AM (Dojindo Laboratories, Kumamoto, Japan), FM1-43 (Molecular Probes, Inc., Eugene, OR, USA), pentobarbital sodium salt (Nacalai Tesque, Inc., Kyoto, Japan), and 2,6-diisopropylphenol (propofol; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan).

Statistics

Data are presented as mean ± standard error of the mean (SEM). Results were tested with analysis of variance (ANOVA), which was followed with Dunnett's test. Differences between control and test values were considered significant when $P < 0.05$. Analyses were carried out using KaleidaGraph software in Japanese (version 4.1; Synergy Software, Reading, PA, USA).

Results

Identification of the presynaptic boutons attached to a dissociated rat neuron

FM dyes are generally used for labelling synaptic vesicles that actively release neurotransmitters; these dyes brightly stain presynaptic boutons [22, 24, 25]. Single presynaptic boutons on a hippocampal CA1 pyramidal neuron were stained with FM1-43 after Ca^{2+} measurement so that the green fluorescence emitted by FM1-43 would not affect the measurement of fluo-3 fluorescence intensity changes. The spots, which were pseudo red in color and could be distinguished from the fluo-3 green signals, appeared to represent single presynaptic boutons (Fig. 1). To confirm that these FM1-43-labelling spots represented single boutons, a 60-mM K^+-containing external solution was applied to the neurons. A red spot that disappeared after high K^+ stimulation was considered a presynaptic bouton (data not shown) [22]. The mean intensity of the fluo-3 red spot that was accepted as a presynaptic bouton was calculated to estimate $[Ca^{2+}]_{pre}$.

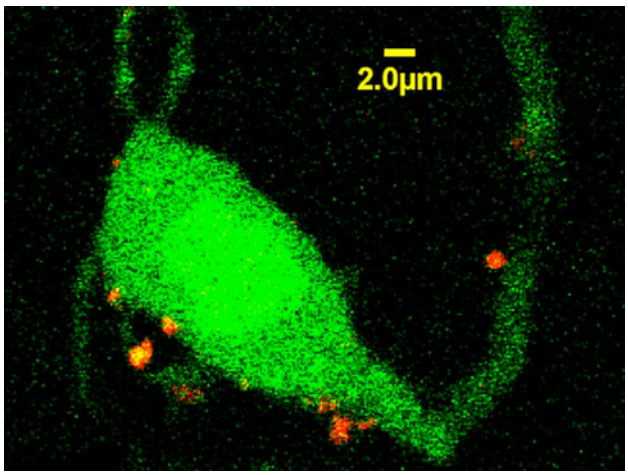

Fig. 1 Fluorescence image of a hippocampal CA1 pyramidal neuron stained with acetoxymethyl ester of fluo-3 and FM1-43. After changes in fluo-3 fluorescence intensity were measured, a dissociated hippocampal neuron was exposed to 10 μM FM1-43 in a 60-mM potassium (K^+)-containing standard external solution for 30 s; subsequently, the neuron was washed with the standard solution. FM1-43-labelled presynaptic boutons expressed a pseudo red color that distinguished them from the fluo-3 green fluorescence

High K^+-induced increase in $[Ca^{2+}]_{pre}$ in the presence of external Ca^{2+}

Figure 2a shows a representative trace of 90 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ in the mechanically dissociated rat hippocampal CA1 pyramidal neuron. High K^+ (90 mM) applied for 10 s produced a rapid increase in $[Ca^{2+}]_{pre}$ in the Ca^{2+}-containing external solution. Figure 2b shows the average peak value of the changes in

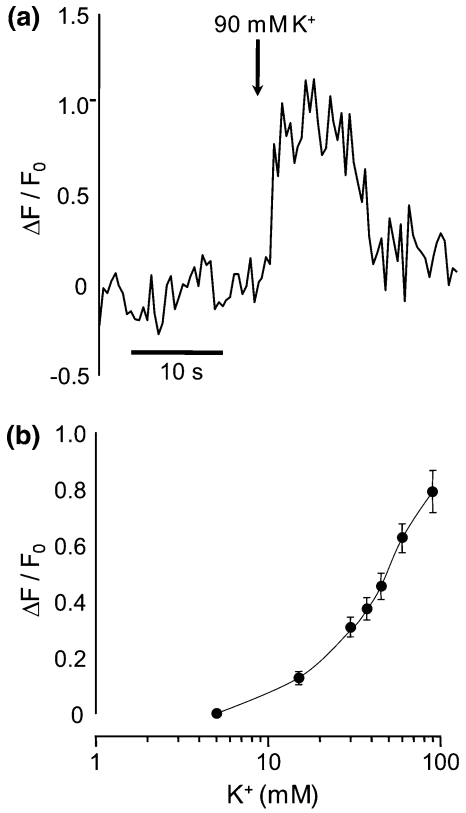

Fig. 2 High potassium (K^+) stimulation induced increase in presynaptic calcium concentration $[Ca^{2+}]_{pre}$ in the presence of external Ca^{2+}. The acetoxymethyl ester of fluo-3-loaded presynaptic boutons attached to the rat hippocampal CA1 pyramidal neurons were exposed to the standard external solution containing various concentrations of K^+ for 8–20 s at room temperature (23–27°C). **a** In the external solution containing 2 mM Ca^{2+}, 90 mM K^+ produced an increase in $[Ca^{2+}]_{pre}$ in the neuron. The trace shown is representative of 12 experiments. **b** Concentration-response relationship of K^+-induced $[Ca^{2+}]_{pre}$ increase in Ca^{2+}-containing solution is shown. The *ordinate* shows the peak $[Ca^{2+}]_{pre}$ of the response after subtracting the baseline value. To smooth the noise, we calculated the peak value as the average of 6–12 points in the maximum response in each bouton. Data points indicate mean [standard error of mean (SEM)] of 7–21 experiments. High K^+ (15–90 mM) induced a concentration-dependent increase in $[Ca^{2+}]_{pre}$ concentration in the Ca^{2+}-containing solution in the rat hippocampal neurons

$[Ca^{2+}]_{pre}$ induced by the application of K^+ (5–90 mM) for 8–20 s at room temperature (23–27°C). In order to smooth the noise, the peak value was calculated as the average of 6–12 points of the maximum response in each bouton. In the presence of external Ca^{2+}, $[Ca^{2+}]_{pre}$ was increased in a concentration-dependent manner in the hippocampal neuron.

Effects of propofol and pentobarbital on high K^+-induced increase in $[Ca^{2+}]_{pre}$

We investigated the effects of propofol and pentobarbital on high K^+ (60 mM)-induced presynaptic Ca^{2+} mobilization in the rat hippocampal CA1 pyramidal neuron. The 60 mM K^+-induced changes in $[Ca^{2+}]_{pre}$ in the Ca^{2+}-containing external solution were used as controls. Figures 3a and 4a show the representative traces of controls in the bouton attached to dendrite and soma, respectively. Single application of propofol (10 μM) or pentobarbital (300 μM) did not cause changes in $[Ca^{2+}]_{pre}$ (data not shown). In the presynaptic boutons attached to dendrites, pretreatment with propofol (10 μM) and pentobarbital (300 μM) for 3 min markedly inhibited the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ (Fig. 3b, 10 μM propofol; Fig. 3c, 300 μM pentobarbital). However, application of the same concentrations of propofol (10 μM) and pentobarbital (300 μM) to the boutons attached to the soma did not inhibit the increase in high K^+ (60 mM)-induced $[Ca^{2+}]_{pre}$ (Fig. 4b, c).

In the boutons attached to the dendrite, inhibition of the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ was 31.0% in the presence of propofol (10 μM) and 33.3% in the presence of pentobarbital (300 μM). In the boutons attached to the soma or the base of the dendrite, however, there was no significant difference between control and pretreatment with either propofol or pentobarbital (Fig. 5).

Discussion

In this study, we investigated the effects of propofol and pentobarbital on the high K^+-induced changes in $[Ca^{2+}]_{pre}$ in the presynaptic boutons on a rat hippocampal CA1 pyramidal neuron. Neurotransmitter release is mediated by Ca^{2+} entry through N- and P/Q-type Ca^{2+} channels localized exclusively at the active zones in presynaptic nerve terminals [19, 20, 26–28]. Ca^{2+} acts on the Ca^{2+} sensor to induce the exocytosis of synaptic vesicles. The Ca^{2+} sensors are not saturated during the local $[Ca^{2+}]_{pre}$ elevation induced by a presynaptic action potential; therefore, it is probable that small changes in Ca^{2+} influx during a presynaptic action potential are highly effective in modulating neurotransmitter release [19]. It has been suggested that chemical depolarization by elevated K^+ levels increases $[Ca^{2+}]_{pre}$ and induces the neurotransmitter release [29]. In our experiments, high K^+ at 15–90 mM increased $[Ca^{2+}]_{pre}$ in Ca^{2+}-containing solution in a dose-dependent manner (Fig. 2). Therefore, the influence of an agent on neurotransmitter release may be inferred from measuring changes in $[Ca^{2+}]_{pre}$.

It has been reported that propofol affects neurotransmitter release [12–14]; however, another study contradicts this finding [15]. Several studies have shown that pentobarbital inhibits $[Ca^{2+}]_{pre}$ increase in the presynaptic boutons [16, 17] and depresses excitatory synaptic transmission in the CNS [18]. In these reports, the inhibitory

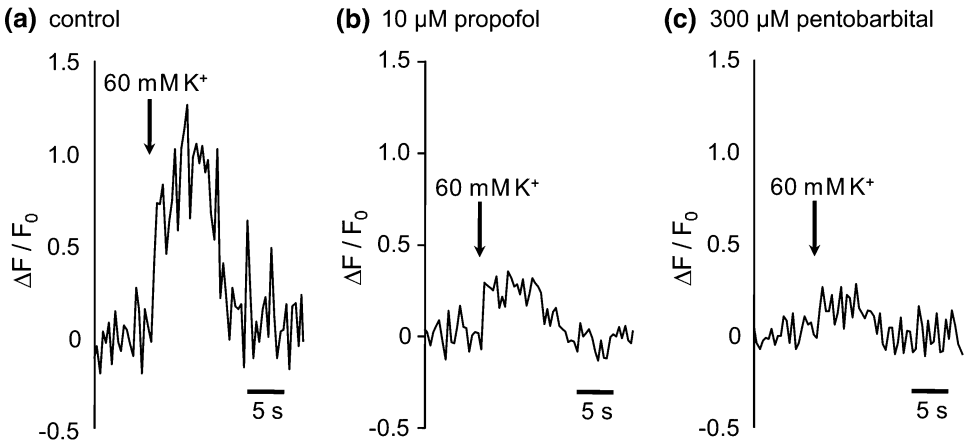

Fig. 3 Effects of propofol and pentobarbital on increase in 60 mM potassium (K^+)-induced presynaptic calcium concentration $[Ca^{2+}]_{pre}$ in rat boutons attached to the dendrite. High K^+ (60 mM) was applied for 8–20 s in the external solution containing 2 mM Ca^{2+}. **a** Control; **b** pretreatment for 3 min with 10 μM propofol; **c** pretreatment for 3 min with 300 μM pentobarbital. Results were obtained from different boutons. Propofol (10 μM) and pentobarbital (300 μM) inhibited the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ in the presynaptic boutons attached to the dendrite

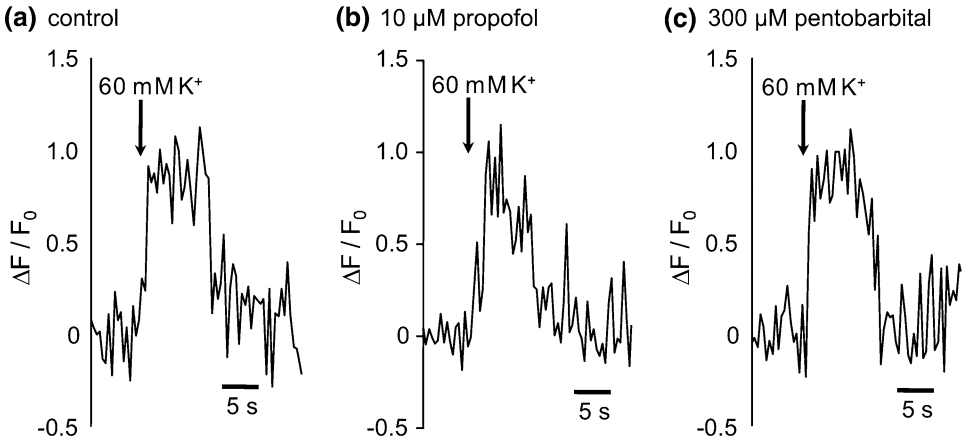

Fig. 4 Effects of propofol and pentobarbital on the 60 mM potassium (K^+)-induced increase in presynaptic calcium concentration $[Ca^{2+}]_{pre}$ in the rat boutons attached to the soma. High K^+ (60 mM) was applied for 8–20 s in the external solution containing 2 mM Ca^{2+}. **a** Control; **b** pretreatment for 3 min with 10 μM propofol; **c** pretreatment for 3 min with 300 μM pentobarbital. Results were obtained from different boutons. Propofol (10 μM) and pentobarbital (300 μM) did not inhibit the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ in the presynaptic boutons attached to the soma

effects of propofol and pentobarbital have been established with concentrations of 1–10 μM [13, 14] and 150–300 μM [17, 18], respectively. Pentobarbital concentration of 200 μM was sufficient to inhibit depolarization-induced secretion by 30–50% [18]. Buggy et al. [13] observed that propofol inhibited synaptic release of glutamate in the cerebral cortex at a single concentration (10 μM), which is similar to the clinical serum concentrations of anesthetic agents. However, Westphalen et al. [15] reported that propofol (15 μM) did not affect K^+-evoked neurotransmitter release from isolated rat cerebrocortical nerve terminals. From another point of view, propofol concentration of 10 μM is equal to 1.78 μg/ml, and hence, it was considered reasonable for a neurological study comparing the target-controlled concentrations of propofol in clinical anesthesia. Taking these lines of evidence into consideration, propofol and pentobarbital concentrations used in this study were regarded as acceptable for examining the effects of these anesthetics on Ca^{2+} mobilization in presynaptic boutons.

More than 90% of all excitatory synapses in the CNS are located in the dendritic spines [30, 31]. In addition, most GABAergic boutons are located on the soma or the base of the apical dendrite in the hippocampus [22]. Almost all excitatory presynaptic boutons attach to dendritic spines, and the inhibitory boutons are largely located on the soma or at the base of the dendrite. Therefore, it seemed reasonable to examine the effects of anesthetic agents on Ca^{2+} movement by examining the attachment sites of the presynaptic boutons. In our experiments, propofol (10 μM) and pentobarbital (300 μM) suppressed the high K^+ (60 mM)-induced changes in $[Ca^{2+}]_{pre}$ in the presynaptic boutons attached to the dendrite (Figs. 3, 5) but not in the boutons attached to the soma or the base of the dendrite (Figs. 4, 5). These results suggest that propofol and pentobarbital may inhibit Ca^{2+} mobilization in the excitatory presynaptic boutons that are attached to dendrites but not in the inhibitory boutons that are attached to the soma or to the base of the dendrite. To confirm the validity of these effects of the anesthetic agents, it is desirable to verify the exact locations of the boutons. Therefore, it is necessary to observe the presynaptic boutons under high magnification using upgraded experimental apparatus.

Many reports demonstrate that the increase in $[Ca^{2+}]_{pre}$ that triggers neurotransmitter release is caused by Ca^{2+} entry through voltage-gated Ca^{2+} channels in the presynaptic boutons [16, 18–20, 26–28]. In our experiments, high K^+ (15–90 mM) increased the $[Ca^{2+}]_{pre}$ in Ca^{2+}-containing solution (Fig. 2); 90 mM K^+, however, did not increase $[Ca^{2+}]_{pre}$ in Ca^{2+}-free solution (data not shown). These findings suggest that the increase in $[Ca^{2+}]_{pre}$ induced by high K^+ in the Ca^{2+}-containing solution was largely due to the influx of Ca^{2+} through voltage-gated Ca^{2+} channels. Although the presence of the endoplasmic reticulum that acts as an intracellular Ca^{2+} store in the presynaptic boutons may have been involved, Ca^{2+} release from intracellular stores is probably not the main mechanism in fast synaptic transmission [18]. It has been reported that anesthetic agents, including propofol and pentobarbital, inhibit glutamate release from rat cerebrocortical slices and that these inhibitory effects appear to be due mainly to the direct inhibition of P/Q-type voltage-gated Ca^{2+} channels [16]. Moreover, Richards [18] demonstrated that many general anesthetics inhibited the action potential-evoked secretion of neurotransmitter in the CNS and that their inhibitory actions could be fully accounted for by their

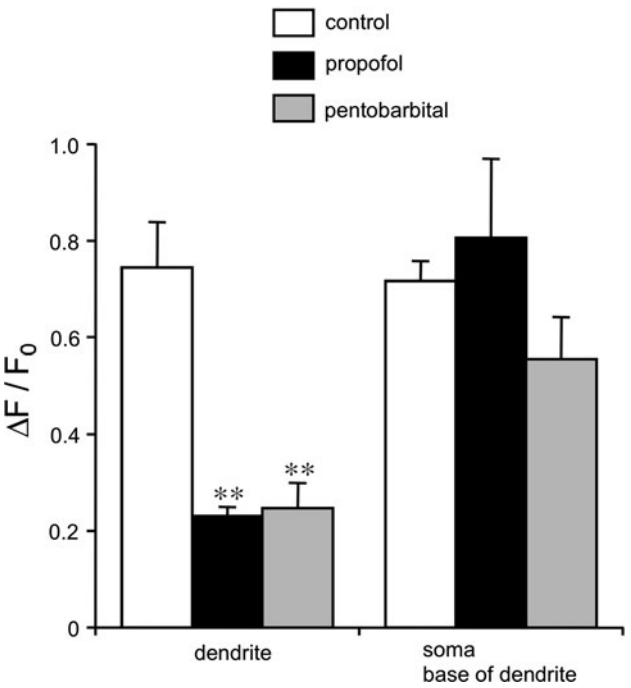

Fig. 5 Effects of propofol and pentobarbital on the 60 mM potassium (K^+)-induced increase in presynaptic calcium concentration $[Ca^{2+}]_{pre}$. Each *column* indicates the average of the peak value of the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ in the bouton on the dendrite and on the soma or the base of the dendrite. To smooth the noise, the peak value was estimated as the average of 6–12 points in the maximum response in each bouton. *Open columns* control; *closed columns* in the presence of 10 μM propofol; *shaded columns* in the presence of 300 μM pentobarbital. Results are expressed as mean ± standard error of the mean. $^{**}P < 0.01$ represents significant difference from control. In the boutons attached to the dendrite, pretreatment with propofol (10 μM; six boutons from four neurons) or pentobarbital (300 μM; six boutons from four neurons) markedly inhibited the 60 mM K^+-induced increase in $[Ca^{2+}]_{pre}$ (control; eight boutons, six neurons). In the boutons attached to the soma or the base of the dendrite, there was no significant difference between control (18 boutons, 11 neurons) and pretreatment with either propofol (10 μM; six boutons, four neurons) or pentobarbital (300 μM; six boutons, four neurons) ($P < 0.05$)

effects on the calcium channels that regulate transmitter release. Thus, it may be inferred that in the presynaptic boutons of a rat hippocampal pyramidal neuron, propofol and pentobarbital inhibit the changes in $[Ca^{2+}]_{pre}$ caused by Ca^{2+} influx through voltage-gated Ca^{2+} channels.

Propofol and pentobarbital modulate $GABA_A$-receptor-mediated inhibition in postsynaptic events [1–7]. $GABA_A$ receptors are also located on presynaptic boutons [13, 14, 32–35]. These receptors reduce Ca^{2+} entry by affecting (either directly or indirectly) voltage-gated Ca^{2+} channels, thereby reducing neurotransmitter release [34]. Therefore, propofol and pentobarbital may also exert inhibitory effects on Ca^{2+} mobilization by way of presynaptic $GABA_A$ receptors. Actually, it has been reported that propofol inhibits K^+-evoked glutamate release from rat cerebrocortical slices by mediating activation of $GABA_A$ receptors [13]. However, it has also been suggested that presynaptic $GABA_A$ receptors are not implicated in depressant actions of IV anesthetic agents in the presynaptic boutons [16, 17]. Furthermore, it has been reported that $GABA_A$ receptors also reduce neurotransmitter release by another mechanism that does not affect Ca^{2+} entry [35]. Hence, at present, mechanisms of the inhibitory effects of propofol and pentobarbital on the changes in $[Ca^{2+}]_{pre}$ are not clear. In conclusion, propofol and pentobarbital possibly have some inhibitory effects on the increase in $[Ca^{2+}]_{pre}$ via unknown mechanisms, and these anesthetics may mediate neurotransmitter release from the presynaptic nerve terminals.

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