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Effect of Propofol on the Release of [^{3}H] Acetylcholine from Rat Hippocampal Synaptosomes

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Abstract: Several studies have demonstrated that propofol interferes with acetylcholine (ACh) release in the central nervous system but the mechanism (s) involved remains unclear. The present study aimed to evaluate the mechanism (s) by which this agent interferes with the release of this neurotransmitter from rat hippocampal synaptosomes. Synaptosomes labeled with [^{3}H]ACh were superfused using an apparatus set up to collect 2 min fractions. The synaptosomes were perfused in the absence or presence of propofol, tetrodotoxin (Na^+ channel blocker), EGTA (chelator of extracellular Ca^{2+}), dantrolene (inhibitor of Ca^{2+} release through ryanodine receptors), 2-APB (antagonist of IP_3 receptors) or BAPTA-AM (chelator of intracellular Ca^{2+}) prior to the evoked-release of [^{3}H]ACh by KCl (50 mM) which is a Na^+-independent stimuli or veratridine (50 μM) which is a Na^+-dependent stimuli. Basal release of [^{3}H]ACh was significantly decreased in the presence of propofol (1-1000 μM). The anesthetic also decreased the release of [^{3}H]ACh evoked by veratridine and KCl ($p<0.05$). Tetrodotoxin (1.0 nM) reduced the veratridine-evoked release of [^{3}H]ACh ($p<0.05$). The simultaneous perfusion of the synaptosomes with tetrodotoxin (1.0 nM) and propofol (100 μM) did not enhance the effects of these agents on veratridine-induced release of [^{3}H]ACh. EGTA (0.2 mM) significantly reduced the KCl-evoked release of [^{3}H]ACh and increased the inhibitory effect of propofol (25 μM) on the KCl-induced release of [^{3}H]ACh ($p<0.05$). BAPTA-AM, dantrolene and 2-APB decreased the KCl-evoked release of the neurotransmitter ($p<0.05$) but simultaneous perfusion of the synaptosomes with propofol and BAPTA-AM, propofol and dantrolene or propofol and 2-APB did not enhance the effects of these agents on KCl-induced release of [^{3}H]ACh. In conclusion, it seems that propofol decreased the release of ACh in the rat hippocampus by interfering with Na^+ channels and the release of Ca^{2+} from internal stores.

Key words: Propofol, hippocampus, acetylcholine, synaptosomes

INTRODUCTION

Acetylcholine (ACh) is a widely distributed cerebral neurotransmitter found in the mammalian brain that is involved in the regulation of consciousness, awakening, cognitive function, sleep and memory control (Gold, 2003; Sarter and Parikh, 2005). Some in *vivo* observations have suggested that cholinergic transmission might be a target of anesthetic action (Bertorelli *et al*., 1990; Shichino *et al*., 1998).

Propofol (2,6-diisopropylphenol) is an intravenous anesthetic that has gained wide acceptance for inducing and maintaining anesthesia due to its fast onset and the rapid emergence from propofol-induced general anesthesia (Vasileiou *et al*., 2009). This agent is capable of modifying synaptic transmission by altering the release of neurotransmitters from the presynaptic region and by modulating the response of the postsynaptic region (Wei *et al*., 2002; Baars *et al*., 2006). Propofol anesthesia has effects at the levels of several neurotransmitters (choline compounds, glutamate, gamma-aminobutyric acid and aspartate) in normal human brains as evaluated by magnetic resonance spectroscopy (Zhang *et al*., 2009). Using *in vivo* microdialysis it has been shown that propofol decreases ACh release in the rat hippocampus (Kikuchi *et al*., 1998; Wang *et al*., 2000; Inagawa *et al*.,

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2004) which is an important area for memory processing that receives its main cholinergic innervations from cells in the medial septal area (Gorman *et al*., 1994). Additionally, microinjection of propofol into the nuclei of the cholinergic pathway and its related pathways decreases cortical brain ACh release in rats (Gamou *et al*., 2010).

Although several studies have previously demonstrated that propofol interferes with ACh release, the mechanism underlying this effect remains unresolved. Therefore, the aim of the present study was to investigate the effects of propofol on basal and evoked release of [^{3}H]ACh from rat hippocampal synaptosomes to unravel the mechanism (s) by which this agent alters the release of this neurotransmitter.

MATERIALS AND METHODS

Drugs: Propofol, veratridine, tetrodotoxin (TTX), EGTA, BAPTA-AM, 2-APB, dantrolene and diethyl β-nitrophenyl phosphate (paraoxon) were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

[^{3}H]ACh loading of rat brain hippocampal synaptosomes and measurement of the release of [^{3}H] ACh: The local ethical committee approved all of the experimental procedures. Isolated nerve terminals (synaptosomes) were prepared as described previously by Westphalen and Hemmings Jr. (2003). Adult Wistar rats (180-250 g) of either sex were decapitated and the brains were removed and placed on ice. The cerebral hippocampus was removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a motor-driven homogenizer for 10 strokes and the homogenate was centrifuged for 2 min at 4000 g. Crude rat synaptosomes (supernatant) were demyelinated by centrifugation through 0.8 M sucrose (10 volumes of supernatant layered onto 10 mL of 0.8 M sucrose) for 30 min at 36000 g. The pellet containing the synaptosomes was resuspended in ice-cold incubation medium containing 140 mM NaCl, 4 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.56 mM Na_2HPO_4, 10 mM glucose, 20 mM HEPES, 2 mM $CaCl_2$ and 10 μM paraoxon at pH 7.4.

The ACh stores of the synaptosomes were depleted by incubation with high-concentration KCl (50 mM) for 15 min. The depolarization medium was similar to the incubation medium with the exceptions of the NaCl and KCl concentrations which were 95 and 50 mM, respectively. The synaptosomes were then centrifuged for 10 min at 15000 g. The final pellet was incubated with [methyl-^{3}H] choline chloride (82 Ci/mmol, Amersham Searle International, England) for 30 min at 37°C. The synaptosomes were centrifuged for 10 min at 15000 g and resuspended with the incubation medium. Aliquots (200 μL) of the synaptosomal suspension were confined between Whatman GF/B filter discs and superfused using an apparatus set up to collect 2 min fractions as described by Garcia-Sanz *et al*. (2001).

Aliquots (100 μL) of the samples were assessed radioactivity by liquid scintillation spectrophotometry using a Perkin Elmer Tri-Carb® Liquid Scintillation Analyzer. In each group of experiments, [^{3}H] ACh and [^{3}H] choline were quantified using EnzyChrom™ Choline Assay Kits and EnzyChrom™ Acetylcholine Assay Kits that were obtained from BioAssay Systems (Hayward, CA, USA). [^{3}H]ACh represented approximately 60 and 50% of the total radioactivity released when the neurotransmitter release was evoked by KCl and veratridine, respectively.

The amount of radioactivity released in each fraction is expressed as the Fractional Release (FR) as described by Garcia-Sanz *et al*. (2001). The magnitudes of the release induced by KCl and veratridine were determined by subtracting the baseline release (i.e., the average of the basal release before and after the stimuli) from the cumulative fractional release values of the evoked-release (ΔΣFR) (Westphalen and Hemmings Jr., 2003).

Administration of drugs: Propofol was prepared in dimethyl sulfoxide (DMSO) and diluted with the incubation medium. The final concentration of DMSO was 0.01% and this DMSO concentration had no effects on the release of the neurotransmitter. The synaptosomes were perfused in the absence or presence of propofol, TTX, EGTA, dantrolene, 2-APB or BAPTA-AM for 12 min prior to the evoked-release of [^{3}H] ACh by KCl or veratridine. These latter two compounds were perfused for 2 min in the absence or in the presence of the antagonists. The times used for the antagonists and the stimuli in the present study were based on previous studies that have used the same perfusion system (Westphalen and Hemmings Jr., 2003, 2006). The concentrations of the antagonists used in the present study were based on those traditionally used in previous studies.

Statistical analyses: Statistical analyses of the data were performed using one-way ANOVAs followed by Newman-Keuls *post hoc* tests. The results are presented as the Mean±SEM. The $p<0.05$ was considered significant and these values were calculated with the GraphPad Prism 5.0 software (La Jolla, USA). The IC_{50} value was obtained using GraphPad Prism 5.0.

RESULTS

Figure 1a shows that propofol (1, 10, 30, 100 and 1000 μM) significantly decreased the basal release of [^{3}H] ACh from rat hippocampal synaptosomes ($p<0.05$; Newman-Keuls test). As the basal release of [^{3}H]ACh was very low, we used veratridine (50 μM) which is a drug that interacts directly with Na^+ channels and thus leads directly to activation (by slowing inactivation) and consequently depolarizes the plasma membrane (Barnes and Hille, 1988), as a stimuli to induce the release of the neurotransmitter to identify the mechanism (s) by which the anesthetic reduced the release of [^{3}H]ACh. Figure 1b shows that propofol reduced the release of [^{3}H]ACh evoked by veratridine with an IC_{50} of 100 μM.

Under physiological conditions, neurotransmitter release requires an influx of Na^+ and Ca^{2+} into the presynaptic terminals. Thus, propofol interferes with the release of [^{3}H] ACh either by decreasing Na^+ or Ca^{2+} influx to cause a reduction in the transmitter output. To test these possibilities, we first used TTX which is a Na^+ channel blocker (Hille *et al*., 1987), to test the influence of this agent on the effects of propofol on ACh release. It was observed that TTX (0.5 μM) abolished the release of ACh, evoked by veratridine and thus investigated the effect of a low concentration of this antagonist on the release of the neurotransmitter. Figure 1c shows that TTX (1.0 nM) decreased the release of [^{3}H]ACh from rat hippocampal synaptosomes ($p<0.05$; Newman-Keuls test). The simultaneous perfusion of the synaptosomes with TTX (1.0 nM) and propofol (100 μM) did not enhance the effects of these agents on veratridine-induced [^{3}H] ACh release which suggests that the effects of propofol on [^{3}H]ACh release at least partially involved the block of voltage-gated Na^+ channels.

To test the role of Ca^{2+} in the mechanism of action of propofol, we elevated the extracellular KCl concentrations (50 mM) which is a pharmacological method of evoking the release of neurotransmitters in a Na^+-independent manner (Nicholls, 1993). Propofol decreased the release of [^{3}H] ACh evoked by KCl with an IC_{50} of 25 μM (Fig. 2a); $p<0.05$; Newman-Keuls test). Figure 2b shows that TTX

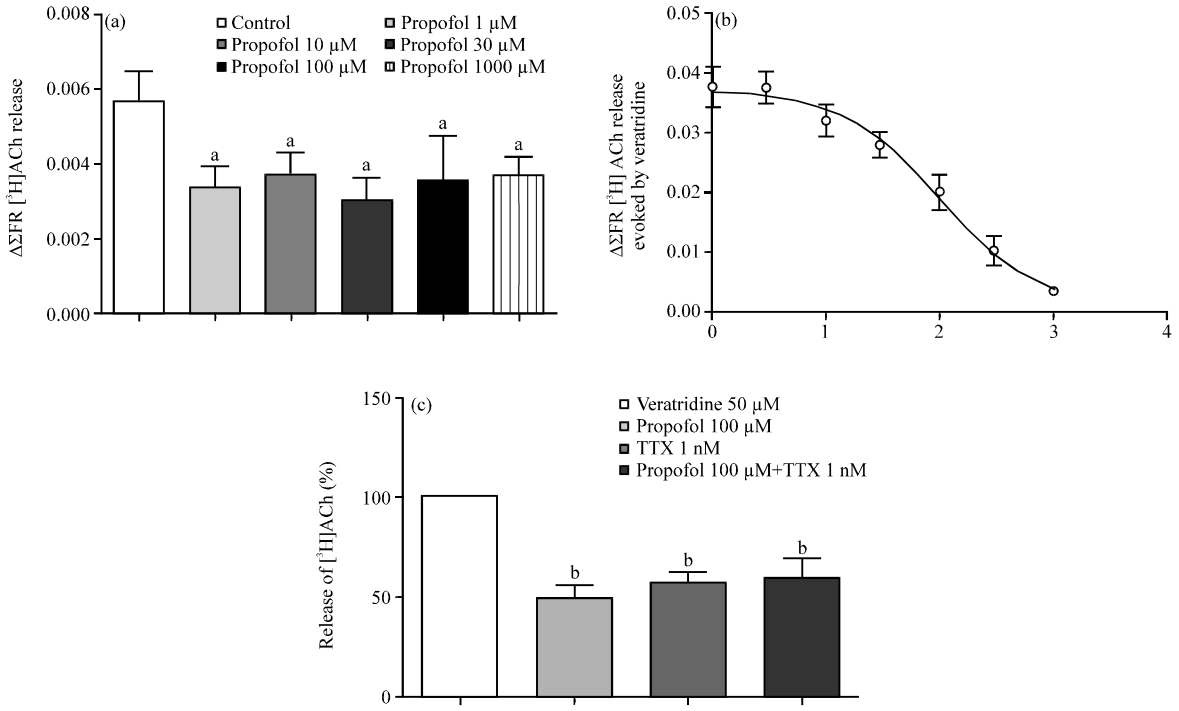

Fig. 1(a-c): Propofol decreases basal and veratridine-evoked release of [^{3}H]ACh in rat hippocampal synaptosomes by blocking Na^+ voltage channels, (a) Propofol (1, 10, 30, 100 or 1000 μM) decreases the basal release of [^{3}H] ACh in rat hippocampal synaptosomes, (b) Propofol (1, 10, 30, 100 or 1000 μM) decreases veratridine-evoked release of [^{3}H]ACh in rat hippocampal synaptosomes with an IC_{50} of 100 μM and (c) TTX (1.0 nM) decreases the release of [^{3}H]ACh from rat hippocampal synaptosomes. Simultaneous perfusion of the synaptosomes with TTX and propofol (100 μM) did not enhance the effect of propofol. [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. veratridine

did not interfere with the KCl-induced release of the neurotransmitter ($p>0.05$; Newman-Keuls test). In contrast, EGTA (0.2 mM) which is a chelator of extracellular Ca^{2+}, significantly reduced the KCl-evoked release of [^{3}H]ACh ($p<0.05$; Newman-Keuls test). Moreover, EGTA increased the inhibitory effect of propofol (25 μM) on the KCl-induced release of [^{3}H]ACh which suggests that the anesthetic action occurred via a mechanism that did not involve extracellular Ca^{2+} but might be related to an effect of propofol on intracellular Ca^{2+} (Fig. 2b). To address this possibility, we perfused the preparation with BAPTA-AM (6 μM) which is a specific chelator of intracellular Ca^{2+}. Figure 2c shows that BAPTA-AM decreased KCl-evoked release of the neurotransmitter ($p<0.05$; Newman-Keuls test) but the simultaneous perfusion of the synaptosomes with BAPTA and propofol (25 μM) did not enhance the effects of these agents on KCl-induced [^{3}H] ACh release which suggests that the of the anesthetic on KCl-evoked [^{3}H]ACh release might involve an effect on intracellular Ca^{2+} concentrations.

It has previously been shown that propofol inhibits thapsigargin-induced Ca^{2+} transients in cultured mouse pituitary cells by inhibiting the release of Ca^{2+} from intracellular stores (Ya Deau *et al.*, 2003). Therefore, to test the possibility that propofol interferes with the release of Ca^{2+} from intracellular stores, we next investigated the effect of dantrolene (100 μM) which inhibits the release of Ca^{2+} through ryanodine receptors (Ohta *et al*., 1990) and 2-APB (100 μM) which is an antagonist of IP_3 receptors (Peppiatt *et al*., 2003), on the KCl-induced release of [^{3}H]ACh. Figure 2d shows that both agents decreased the KCl-induced release of [^{3}H]ACh ($p<0.05$; Newman-Keuls test) but they did not increase the inhibitory effect of propofol on KCl-induced neurotransmitter release. Therefore, our data suggest that propofol might interfere with the release of Ca^{2+} from internal stores through ryanodine and IP_3 receptors.

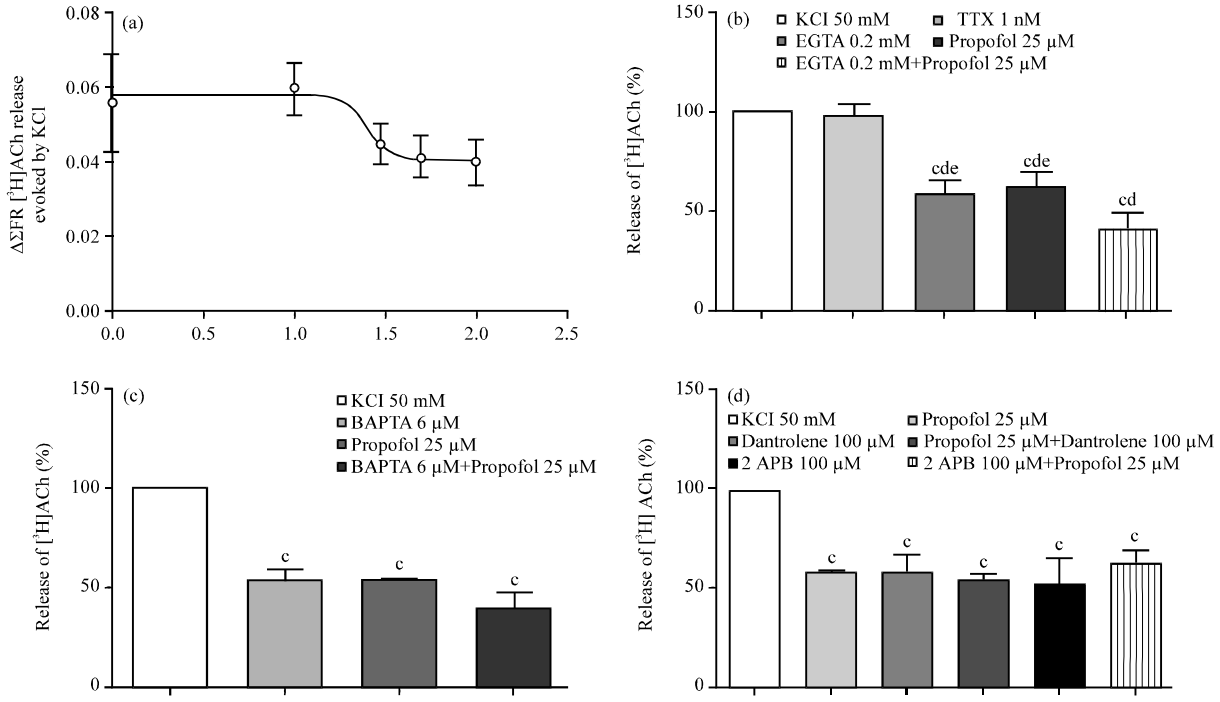

Fig. 2(a-d): Propofol inhibits the release of [^{3}H]ACh evoked by KCl in rat hippocampal synaptosomes by interfering with the release of Ca^{2+} from intracellular stores. (a) Propofol (1, 10, 30, 100 or 1000 μM) decreases KCl-evoked release of [^{3}H]-ACh in rat hippocampal synaptosomes with an IC_{50} of 25 μM. (b) TTX (1.0 nM) does not decrease the release of [^{3}H]ACh that was induced by KCl. EGTA (0.2 mM) reduces the KCl-evoked release of [^{3}H]ACh. EGTA increases the inhibitory effect of propofol (25 μM) on the KCl induced release of [^{3}H]-ACh. (c) BAPTA-AM (6 μM) decreases the KCl-evoked release of [^{3}H]ACh. Simultaneous perfusion of the synaptosomes with BAPTA and propofol (25 μM) does not enhance the effect propofol on the KCl-induced release of [^{3}H]ACh and (d) Dantrolene (100 μM) and 2-APB (100 μM) decrease the release of [^{3}H] ACh evoked by KCl. These agents did not increase the inhibitory effect of propofol on KCl-induced neurotransmitter release. $^c p<0.05$ vs. KC, $^d p<0.05$ vs. TTX, $^e p<0.05$ vs. EGTA + Propofol

DISCUSSION

Several studies have shown that ACh release is one of the targets of general anesthetics and these findings reinforce the importance of investigating the relationship between anesthetic action and the cholinergic system. There are several lines of data showing that inhibition of the cholinergic system by propofol might be responsible for the sedative/anesthetic state induced by this agent. In this study, the effect of propofol on the release of [^{3}H]ACh from rat hippocampal synaptosomes was investigated to determine the mechanism by which propofol interferes with the cholinergic system.

A previous study showed that ACh release in the rat cerebral cortex *in vivo* is strongly suppressed immediately following the intravenous infusion of propofol but returns to control levels once the infusion is stopped (Nemoto *et al*., 2013). A clinical study with 10 healthy volunteers ranging from 20-40 years old showed that intravenous administration of propofol decreases the level of choline compounds in the brain as measured by magnetic resonance spectroscopy (Zhang *et al*., 2009). As choline is a precursor of ACh, a decrease in choline levels might affect the synthesis of ACh which reinforces the notion that the cholinergic system might be one of the important targets of propofol in the central nervous system.

In the present study, it was shown that propofol decreased the basal release of [^{3}H]ACh from rat hippocampal synaptosomes (Fig. 1a). We next investigated the effects of propofol on the release of ACh evoked by pharmacological tools to gain insight on the mechanism (s) by which the anesthetic reduced the release of this neurotransmitter. It was observed that propofol decreased the release of [^{3}H]ACh induced by veratridine which is an agent that increases the release of ACh in a Na^+-dependent manner (Fig. 1b). Indeed, TTX which is a voltage-dependent Na^+ channel blocker, decreased the effect of veratridine but did not increase the effect of propofol on veratridine-evoked ACh release. This result suggests that the anesthetic effect on ACh release might, at least partially, involve the blocking of Na^+ channels which agrees with an early observation that Na^+ channels are one of the molecular targets of the action of propofol (Frenkel *et al*., 1993).

The present results are also consistent with the observation that the effect of propofol on voltage-dependent Na^+ channels seems to be due to a preferential interaction with the inactivated state of the channel (Ratnakumari and Hemmings Jr., 1996). Additionally, it has been shown that propofol inhibits veratridine-evoked $22Na^+$ influx and alters intracellular Na^+ concentrations in cerebrocortical synaptosomes in a dose-dependent manner (Ratnakumari and Hemmings Jr., 1997). It has also been demonstrated that propofol decreases Na^+ currents in mammalian cell lines expressing rat $Na_v1.2$ (Rehberg and Duch, 1999) and voltage-gated Na^+ currents in isolated neurohypophysial nerve terminals in a concentration and voltage dependent manner (Ouyang *et al*., 2003).

To test the role of Ca^{2+} on the effect of propofol on ACh release, the effects of the anesthetic on the synaptosomal neurotransmitter release evoked by KCl which is a Na^+ channel-independent tool that induces the release of ACh. We showed that propofol and EGTA, a chelator of extracellular Ca^{2+}, decreased the release of ACh evoked by KCl (Fig. 2a, b). We also observed an increase in the effect of propofol during the simultaneous perfusion of the hippocampus with EGTA which suggests that the anesthetic action might have resulted from a mechanism that does not involve extracellular Ca^{2+} but might be related to an effect of propofol on intracellular Ca^{2+}. In contrast to our result, it has been shown that propofol reversibly inhibits the voltage-gated Ca^{2+} currents of hypothalamic paraventricular nucleus neurons in the rat (Shirasaka *et al*., 2004) and inhibits the norepinephrine and dopamine release evoked by KCl which are predominantly mediated through P/Q-type voltage sensitive calcium channels, in rat striatal slices (Hirota *et al*., 2000).

In this present study, BAPTA-AM, a specific chelator of intracellular Ca^{2+}, decreased KCl-evoked release of ACh but did not increase the effect of propofol on KCl-induced [^{3}H]ACh release which suggests that the effect of the anesthetic on [^{3}H]ACh release might involve an action on intracellular Ca^{2+} concentrations (Fig. 2c; $p<0.05$). The intracellular Ca^{2+} concentration has an important role in triggering the release of neurotransmitters (Berridge *et al*., 2000). To test the hypothesis that propofol interferes with the release of Ca^{2+} from intracellular stores, the effects of dantrolene and 2-APB on the release of ACh induced by KCl were investigated. It was observed that both agents decreased the release of [^{3}H]ACh evoked by KCl but did not increase the inhibitory effect of propofol; these findings are suggestive of a putative effect of the anesthetic on the intracellular Ca^{2+} concentration that is mediated by blocking the release of Ca^{2+} from internal stores via ryanodine and IP_3 receptors (Fig. 2). In agreement with these data, it has been demonstrated that propofol inhibits thapsigargin-induced Ca^{2+} transients in cultured mouse pituitary cells independently of the blockade of L-type Ca^{2+} channels which indicates that the anesthetic might inhibit the release of Ca^{2+} from intracellular stores (Ya Deau *et al*., 2003). However, further studies are

necessary to confirm that propofol interferes with the intracellular Ca^{2+} concentrations of other types of neuronal cells.

In the present study, we observed that propofol inhibited the ACh release evoked by KCl (IC_{50} = 25 μM) with greater potency than it inhibited the release evoked by veratridine (IC_{50} = 100 μM). In contrast to our data, it has been demonstrated that propofol decreases glutamate release evoked by veratridine to a greater extent than the release evoked by KCl in synaptosomes prepared from rat hippocampi (Ratnakumari *et al*., 2001). This discrepancy might be due to differences of the sensitivities of different neurotransmitters to propofol and further investigations are necessary to address this point. It is not easy to correlate the concentrations of anesthetics used in *in vitro* experiments to those used during clinical anesthesia and one limitation of the present study might be the concentrations of propofol (25-100 μM) used in the present experiments. However, is it notably that the doses of intravenous anesthetics required for experimental animals are 10-100 times higher than those used for humans (Wakasugi *et al*., 1999). Moreover, plasma concentrations of propofol during anesthesia in humans are estimated to range between 70 and 106 μM (Smith *et al*., 1994; Ratnakumari and Hemmings Jr., 1997; Kazama *et al*., 1998; Haeseler *et al*., 2001) and the brain concentrations of propofol are approximately eight-fold higher than the free plasma concentrations (Shyr *et al*., 1995). Finally, the clinically relevant concentrations of anesthetic are important for examinations of the integrated responses in the intact animal but their relevance to *in vitro* studies should be assessed with care due to our lack of understanding of how to integrate *in vitro* systems into the anesthesia model (Eckenhoff and Johansson, 1999).

In conclusion, it was demonstrated that propofol decreased the release of [^{3}H]ACh from rat hippocampal synaptosomes and that this effect seems to be related to the blockage of voltage-gated Na^+ channels and the inhibition of Ca^{2+} release from intracellular stores.

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