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Original Article

Effect of intravenous anesthetic propofol on synaptic vesicle exocytosis at the frog neuromuscular junction

Luciana Ferreira LEITE[1], Renato Santiago GOMEZ[2, *], Matheus de Castro FONSECA[1], Marcus Vinicius GOMEZ[3], Cristina GUATIMOSIM[1, §]

[1]Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; [2]Department of Surgery, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; [3]Graduate Program of Santa Casa, Belo Horizonte, Minas Gerais, Brazil

Aim: To investigate the presynaptic effects of propofol, a short-acting intravenous anesthetic, in the frog neuromuscular junction.
Methods: Frog cutaneous pectoris nerve muscle preparations were prepared. A fluorescent tool (FM1-43) was used to visualize the effect of propofol on synaptic vesicle exocytosos in the frog neuromuscular junction.
Results: Low concentrations of propofol, ranging from 10 to 25 µmol/L, enhanced spontaneous vesicle exocytosis monitored by FM1-43 in a Ca^{2+}-dependent and Na^+-independent fashion. Higher concentrations of propofol (50, 100, and 200 µmol/L) had no effect on spontaneous exocytosis. By contrast, higher concentrations of propofol inhibited the Na^+-dependent exocytosis evoked by 4-aminopyridine but did not affect the Na^+-independent exocytosis evoked by KCl. This action was similar and non-additive with that observed by tetrodotoxin, a Na^+ channel blocker.
Conclusion: Our data suggest that propofol has a dose-dependent presynaptic effect at the neuromuscular transmission which may help to understand some of the clinical effects of this agent on neuromuscular function.

Keywords: propofol; neuromuscular junction; frog; FM1-43; synaptic vesicle; general anesthetic

Introduction

During the last decade, there was a significant progress related to the knowledge of the mechanism of action of general anesthetic. Cellular and molecular mechanisms underlying general anesthesia are not yet fully elucidated but general anesthetics seems to act in both presynaptic and postsynaptic molecular targets[1-4]. There is now a great body of evidences that clinical concentrations of most general anesthetics act on specific ligand-gated ion channels like GABA and glutamate receptors and/or other important ion channels, such as voltage gated sodium channels, potassium channels and HCN-pacemaker channels[3]. Nevertheless, characterizing the molecular targets of general anesthetics has challenged many research groups over the years.

Propofol is one of the most widely used general anesthetic agent for induction and maintenance of anesthesia. Previous works performed in central nervous system (CNS) synapses have shown an inhibition of calcium channels by propofol[5-7]. Other works showed that propofol inhibited calcium-dependent glutamate release evoked by veratridine and 4-AP with greater potency than Na^+ channel-independent release evoked by KCl[8, 9]. In addition, there were evidences in the literature suggesting that high doses of propofol might have a direct effect on Na^+ channels[9-13].

It is well described the sedative and hypnotic effects of propofol on CNS. Nevertheless, there are few studies investigating its effect on neuromuscular transmission. Indeed, it has been proposed that propofol has a dual effect on the neuromuscular junction. Low concentrations of propofol stimulate skeletal muscle contractions elicited direct and indirectly[14] but high concentrations of this agent inhibit skeletal muscle contractions evoked direct and indirectly[15, 16]. The mechanisms underlying the neuromuscular effects of propofol include reduction of muscular blood flow, a direct effect on the post-junctional membrane receptors, and reduction on acetylcholine (ACh) release on the neuromuscular junction[17-23].

FM1-43 is a fluorescent tool that has been used to study synaptic vesicle recycling at the neuromuscular junction[24–26]. The molecular characteristics of this fluorescent marker allow its internalization during synaptic vesicle endocytosis as well as its release during exocytosis[25]. In the present work, we investigated the effect of propofol on synaptic vesicle exocytosis, a crucial step for neurotransmitter release. We used FM1-43 to visualize the effect of several concentrations of propofol on spontaneous and evoked exocytosis at frog neuromuscular junction.

Materials and methods
Reagents
FM1-43 was purchased from Molecular Probes (Eugene, OR, USA), *d*-tubocurarine, 4-aminopyridine (4AP), tetrodotoxin (TTX), 2APB, dantrolene and omega-conotoxin GVIA were purchased from Sigma-Aldrich (St Louis, MO, USA). Propofol was obtained from Fresenius (Uppsala, Sweden) and azumolene was obtained from Proctor & Gamble (Norwich, NY, USA). All other chemicals and reagents were of analytical grade. All procedures were approved by the local animal care committee (CETEA-UFMG).

Staining and destaining with FM1-43
The frog neuromuscular junction has been an invaluable experimental model for elucidating many aspects of neurotransmission which is the basis of neuronal communication. Two decades ago, Betz and colleagues have introduced the use of the fluorescent dye FM1-43 to visualize synaptic vesicles recycling in motor nerve terminals of the frog neuromuscular junction[24, 25, 27]. Using this powerful tool, it was possible to elucidate the mechanisms that governed synaptic vesicle recycling and consequently neurotransmitter release in several neuronal cell types[28].

In the present work, frog cutaneous pectoris nerve muscle preparations were dissected from Rana catesbiana (about 60 g) and mounted in a sylgard-lined chamber containing frog Ringer solution (115 mmol/L NaCl, 2.5 mmol/L KCl, 1.8 mmol/L CaCl$_2$, 5 mmol/L HEPES, pH 7.2). FM1-43 (4 µmol/L) was used to stain the recycling pool of synaptic vesicles[25]. This dye presents a hydrophobic tail that reversibly binds to membranes and a polar head that impairs it to fully permeate the plasma membrane[24–28]. Therefore, FM1-43 binds to synaptic membrane and when the nerve terminal is submitted to a stimulus that causes exocytosis of synaptic vesicles and, consequently, a compensatory endocytosis, the fluorescent dye is incorporated, resulting in a typical pattern of staining[25]. After a new round of stimulation, in the absence of FM1-43 in the external medium, the dye is released to the hydrophilic medium, resulting in a decrease of fluorescence intensity, reflecting the exocytosis of synaptic vesicle[24–26]. In our experiments, the muscles were incubated with *d*-tubocurarine (16 µmol/L) to prevent contractions during stimulation. The muscles were stimulated for 10 min with modified Ringer solution (57.5 mmol/L NaCl, 60 mmol/L KCl, 1.8 mmol/L CaCl$_2$, 5 mmol/L HEPES, pH 7.2) in the presence of FM1-43

(4 µmol/L). Thereafter, the preparation was kept resting for 15 min to guarantee FM1-43 uptake. The excess of FM1-43 adhered to the muscle membranes was removed during an one hour washing period in frog Ringer solution. Images were acquired in intervals of 5 min until the end of the experiments. The destaining at the absence of stimulus (photobleaching) was used as a control.

After labeling with FM1-43, neuromuscular preparations were exposed to different concentrations of propofol during 30 min to evaluate its effect on spontaneous exocytosis. Experiments were also performed to investigate the effect of propofol on Na$^+$-dependent exocytosis evoked by 4AP. After labeling neuromuscular preparations with FM1-43, muscles were exposed to 4AP (1 mmol/L) during 30 min. To test the role of extracellular Na$^+$ on 4AP-evoked exocytosis, the neuromuscular preparation was initially incubated for 30 min in frog Ringer containing 1.0 µmol/L TTX and, thereafter, it was exposed to 4AP for 30 min. The propofol effect on 4AP-induced exocytosis was investigated by pre-incubation in propofol (100 µmol/L) solution during 10 min before 4AP exposure. Similar protocols were applied to investigate the effects of propofol and TTX on Na$^+$-independent exocytosis evoked by 60 mmol/L KCl.

Experiments that investigated the role of extracellular Ca^{2+} on the vesicular release induced by propofol were performed in modified Ringer solution without Ca^{2+} but containing EGTA (2.0 mmol/L), an extracellular Ca^{2+} chelator. The preparations were incubated in modified Ringer during 30 min before application of propofol. In experiments performed with the calcium channel blocker omega-conotoxin GVIA, the preparations were pre-incubated in Ringer containing toxin for 30 min before propofol. The participation of intracellular Ca^{2+} stores on the exocytosis evoked by propofol was also investigated. For this purpose, preparations were incubated for 30 min in Ringer containing 2APB (100 µmol/L), an IP$_3$ receptor blocker, or azumolene (100 µmol/L), a ryanodine receptors blocker before the addition of propofol.

Fluorescence microscopy and imaging analyses
Images were acquired using a fluorescence microscope (Zeiss Axioskop) coupled to a CCD camera (Micromax) and visualized in a computer. The microscope was equipped with water immersion objectives (63×, 0.95 NA and 40×, 0.75 NA). Excitation light came from a 100 W Hg lamp and passed through filters (505/530 nm) to select the fluorescence spectrum. The experimental parameters for collection of images were always identical in control and test contralateral muscles in a given trial.

Statistical analysis
Image analysis was performed using the softwares Image J and Microsoft Excel. The mean fluorescence intensity was determined for each group of spots and plotted against the time as percentage of its mean initial fluorescence using the software Sigma Plot 9.0. Statistical analysis was performed using paired Student's *t*-Test or ANOVA. *P*<0.05 values were

considered statistically significant.

Results

FM1-43 staining and destaining of nerve terminals

Frog cutaneous-pectoris neuromuscular junctions were stained with FM1-43 as previously described[25]. The nerve terminal that was stained with FM1-43 shows fluorescent spots corresponding to clusters of synaptic vesicles that were able to pick up the dye (Figure 1A, upper panel). When this previously labeled terminal was submitted to a new depolarizing stimulus by modified Ringer containing 60 mmol/L KCl, in the absence of FM1-43 in external medium, we observed a significant reduction in fluorescence (Figure 1A, lower panel). The loss of fluorescence was due to dye release to external medium, that correspond to synaptic vesicle exocytosis[25]. Exposure of nerve terminals to illumination without any depolarizing stimulus resulted in a small decrease on fluorescence (maximum 10%) attributable to dye photobleaching[28]. A representative image of the terminal before (upper panel) and after illumination (lower panel), in the absence of stimulus, is shown in Figure 1B. To quantify the decrease in fluorescence that occurs after stimulation with 60 mmol/L KCl and photobleaching, the mean fluorescence intensity for each synaptic vesicle cluster was measured and plotted as gray levels against time (Figure 1C). The small reduction in fluorescence observed corresponds to photobleaching whereas the larger destaining curve obtained during depolarization stimulus corresponds to synaptic vesicle exocytosis.
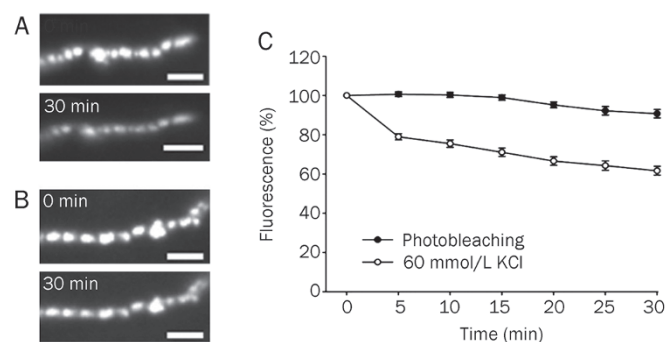

Figure 1. Synaptic vesicles recycling visualized by the fluorescent dye FM1-43. (A) Upper panel: Representative nerve terminal that was incubated in modified Ringer containing 60 mmol/L KCl for 10 min in the presence of FM1-43. Note the formation of fluorescent spots, corresponding to clusters of synaptic vesicles that were able to pick up dye. Lower panel: The same terminal after a second round of depolarization with modified Ringer containing 60 mmol/L KCl, now in the absence of extracellular dye. Note a pronounced destaining of fluorescent spots, corresponding to exocytosis of synaptic vesicles and dye release to the external medium. (B) Representative image of fluorescence loss due to photobleaching during illumination for 30 min. Upper panel: before illumination. Lower panel: after 30 min of illumination. (C) Quantification of FM1-43 fluorescence loss due to photobleaching and KCl fluorescence loss due to exocytosis of synaptic vesicles. The results are mean±SEM of 65 spots from 8 nerve terminals of 4 animals. Scale Bar=10 μm.

Effect of low concentrations of propofol on spontaneous synaptic vesicle exocytosis

Nerve terminals labeled with FM1-43 were bathed in different concentrations of propofol (10 to 200 μmol/L) for 30 min (Figure 2). Fluorescence of representative terminals before and after photobleaching (Figure 2A); propofol 10 μmol/L (Figure 2B), and propofol 200 μmol/L (Figure 2C) were obtained. Low concentrations of propofol ranging from 10 to 25 μmol/L significantly reduced FM1-43 fluorescence, corresponding to exocytosis of previously labeled vesicular clusters (*P<0.01 compared to photobleaching) (Figure 2D). On the other hand, high doses of propofol (50–200 μmol/L) had no effect on FM1-43 destaining from motor nerve terminals (Figure 2D), suggesting a dose-dependent effect of the anesthetic agent.
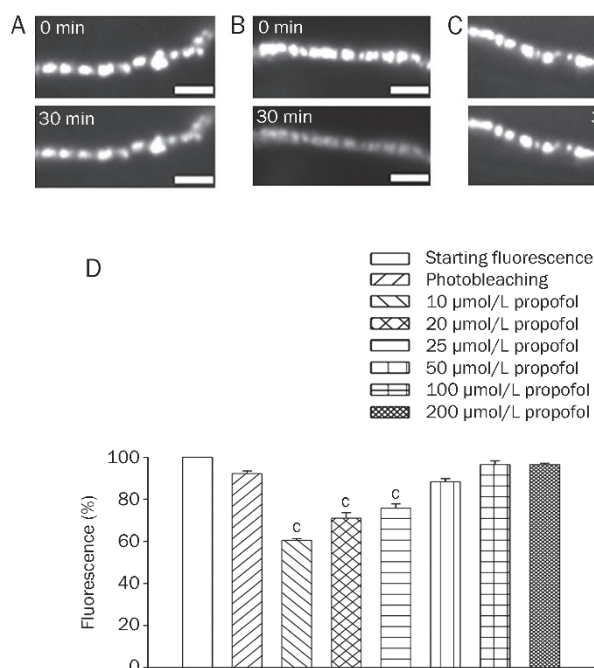

Figure 2. Dose-response curve of exocytosis induced by propofol. (A) Representative image of fluorescence loss due to photobleaching during illumination for 30 min. Upper panel: before illumination. Lower panel: after 30 min of illumination. (B) Representative image of fluorescence loss before (upper panel) and after (lower panel) 30 min in the presence of propofol 10 μmol/L. (C) Representative image of fluorescence loss before (upper panel) and after (lower panel) 30 min in the presence of propofol 200 μmol/L. (D) Quantification of exocytosis evoked by different concentrations of propofol. The results are mean±SEM of 131 fluorescent spots from 15 nerve terminals of 8 animals. $^{c}P<0.01$ compared to the second bar (photobleaching). Scale Bar=10 μm.

The next set of experiments was performed to identify the mechanism(s) by which low doses of propofol induced exocytosis of synaptic vesicles. Pre-incubation of nerve terminals with TTX (1.0 μmol/L), a voltage-gated Na+-channel blocker, did not affect the vesicle exocytosis evoked by propofol (data not shown). By contrast, the effect of low doses of propofol

on spontaneous exocytosis monitored by FM1-43 destaining was Ca^{2+}-dependent (Figure 3A). Indeed, we observed a statistically significant inhibition of exocytosis evoked by low doses of propofol in the presence of the external Ca^{2+} chelator EGTA (2.0 mmol/L) or in the presence of omega-toxin GVIA (5 μmol/L), that blocks N-type calcium channels (Figure 3A). The FM1-43 destaining in the presence of GVIA was not significantly different from that due to photobleaching ($P>0.05$). In addition, we observed that the effect of low doses of propofol on synaptic vesicles exocytosis was independent on intracellular Ca^{2+} stores (Figure 3B). Our data suggest that FM1-43 destaining induced by propofol (10 μmol/L) was Ca^{2+} dependent and Na$^+$-independent.

Effects of high concentrations of propofol on exocytosis evoked by depolarizing stimuli

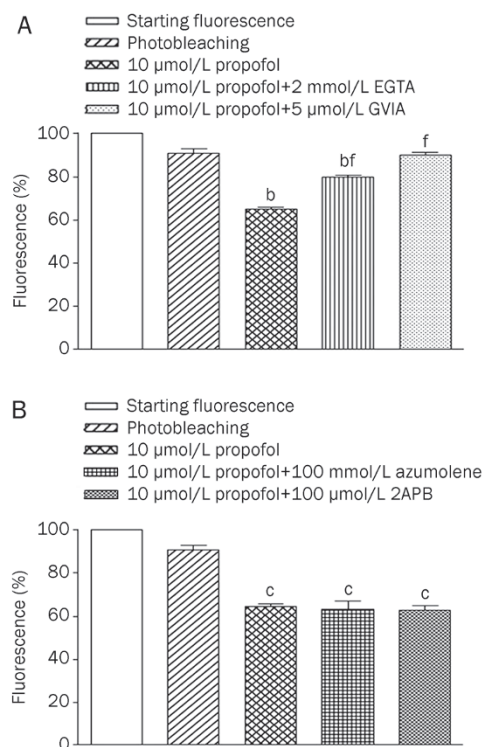It has been shown that propofol at concentrations around 100 μmol/L inhibits skeletal muscle evoked contractions[15, 16]. We therefore tested in our system if this concentration of propofol could alter with 4AP-evoked FM1-43 destaining, which is dependent on extracellular Na$^+$. The incubation of nerve terminals with 4AP (1.0 mmol/L) during 30 min induced a significant FM1-43 destaining (Figure 4A). The destaining evoked by 4AP was reduced by TTX ($P<0.01$ compared to 4AP alone). Pre-incubation of FM1-43 stained nerve terminals with propofol (100 μmol/L) also reduced the 4AP-evoked exocytosis (Figure 4A; $P<0.01$ compared to 4AP alone). Moreover, the simultaneous pre-incubation with propofol (100 μmol/L) and TTX (1.0 μmol/L) produced a significant inhibition of 4AP evoked FM1-43 destaining without any additive effect (Figure 4A), suggesting that propofol reduces the 4AP-induced vesicle exocytosis by blocking Na$^+$ channels sensitive to TTX.

We next tested the effect of high doses of propofol on Na$^+$-independent exocytosis evoked by modified Ringer containing 60 mmol/L KCl. Nerve terminals were stained with FM1-43
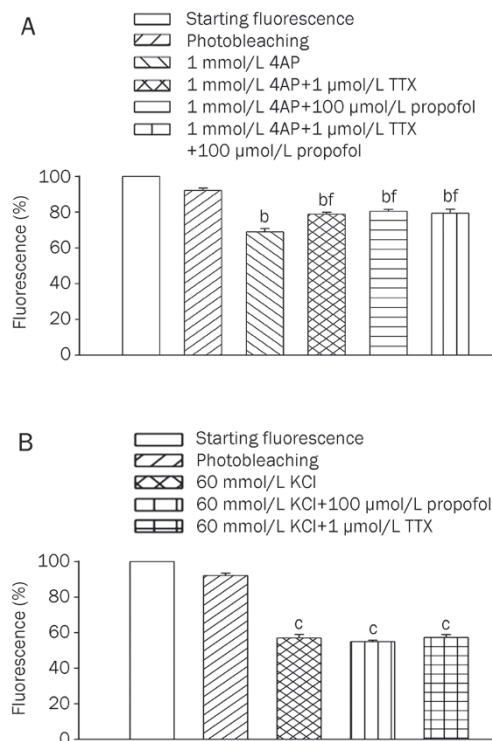

Figure 3. Low doses of propofol evoke synaptic vesicles exocytosis that is dependent on external calcium. (A) FM1-43 destaining evoked by photobleaching (second bar), propofol (10 μmol/L) (third bar), propofol (10 μmol/L)+EGTA (2 mmol/L) (fourth bar), propofol (10 μmol/L)+GVIA (5 μmol/L) (fifth bar). Note that even though EGTA was apparently slightly less effective than GVIA, both agents inhibited the propofol-evoked destaining. The results are mean±SEM of 192 fluorescent spots from 20 nerve terminals of 10 animals. $^bP<0.05$ compared to the second bar (photobleaching). $^fP<0.01$ compared to propofol (10 μmol/L). (B) FM1-43 destaining evoked by photobleaching (second bar), propofol (10 μmol/L) (third bar), propofol (10 μmol/L)+azumolene (100 μmol/L) (fourth bar), propofol (10 μmol/L)+2APB (100 μmol/L) (fifth bar). The results are mean±SEM of 237 fluorescent spots from 18 nerve terminals of 9 animals. $^cP<0.01$ compared to the second bar (photobleaching).

Figure 4. High doses of propofol inhibit synaptic vesicles exocytosis by a Na$^+$-dependent manner. (A) Graphic comparing FM1-43 destaining evoked by photobleaching (second bar), 4AP (1 mmol/L, third bar), 4AP (1 mmol/L)+TTX (1 μmol/L) (fourth bar), 4AP (1 mmol/L)+propofol (100 μmol/L) (fifth bar) and 4AP (1 mmol/L)+propofol (100 μmol/L)+TTX (1 μmol/L) (sixth bar). The results are mean±SEM of 114 fluorescent spots from 10 nerve terminals of 5 animals. $^bP<0.05$ compared to the second bar (photobleaching). $^fP<0.01$ compared to 4AP (1 mmol/L). (B) Quantification of exocytosis evoked by photobleaching (second bar), KCl (60 mmol/L) (third bar); KCl (60 mmol/L)+propofol (100 μmol/L) (fourth bar); KCl (60 mmol/L)+TTX (1 μmol/L) (fifth bar). The results are mean±SEM of 120 fluorescent spots from 10 nerve terminals of 5 animals. $^cP<0.01$ compared to the second bar (photobleaching).

and we observed a significant reduction on fluorescence after incubation with KCl for 30 min (Figure 4B). Pre-incubation of terminals with TTX had no effect on KCl-evoked reduction in fluorescence, confirming the data that depolarization induced by high concentration of K^+ is independent of extracellular Na^+ [29] (Figure 4B). In addition, pre-incubation with high doses of propofol did not inhibit KCl-evoked Na^+-independent exocytosis (Figure 4B). Taken together, these results suggest that high doses of propofol inhibit synaptic vesicles exocytosis on the neuromuscular junction by a Na^+-dependent manner.

Discussion

Propofol is widely used during general anesthesia, as well as for sedation in the intensive care unit. However, fatigue of the respiratory muscles, especially the diaphragm, may cause respiratory failure. Indeed, it has been demonstrated that volatile (halothane, enflurane, isoflurane, and sevoflurane) and intravenous (propofol and midazolam) anesthetics cause diaphragmatic contractile dysfunction which may probably contributes to acute respiratory failure[19–21, 30–32]. Studies *in vivo* suggested that several mechanisms such as reduction of blood flow, failure of neuromuscular transmission, and impairment of membrane excitation and excitation-contraction (E–C) coupling may be responsible for the neuromuscular effects of propofol. However, *in vivo* studies preclude any specification regarding the mechanism involved on the anesthetic effect and the use of the fluorescent probe FM1-43 enabled us to assess specifically a presynaptic effect of this agent on the neuromuscular junction. Hemmings *et al*[30] had already performed a pioneer study on cultured hippocampal neurons using this fluorescent dye to probe synaptic vesicles exocytosis in the presence of isoflurane and they showed that isoflurane depresses exocytosis evoked by multiple presynaptic targets.

In the present work, we examined the effect of propofol on spontaneous and evoked synaptic vesicle exocytosis at the neuromuscular junction, an experimental model that provides a direct way to investigate neurotransmitter release in an isolated synapse. We found that propofol, at low concentrations ranging from 10 to 25 µmol/L, induced synaptic vesicles exocytosis monitored by FM1-43 destaining on a Ca^{2+}-dependent and Na^+-independent manner. By contrast, high concentrations of propofol ranging from 50 to 200 µmol/L were ineffective to induce synaptic vesicle exocytosis.

It is well known that exocytosis depends on external Ca^{2+}[23] and we observed that in the absence of this ion, there was a significant reduction on FM1-43 destaining evoked by low doses of propofol, suggesting that synaptic vesicles exocytosis evoked by propofol is Ca^{2+}-dependent. In addition, we investigated which calcium channel subtype could be the target of low doses of propofol at the frog neuromuscular junction and we observed an inhibition of propofol evoked exocytosis in the presence of the *N*-type inhibitor omega-conotoxin GVIA. Because the FM1-43 destaining evoked by propofol in the presence of GVIA was similar to that due to photobleaching ($P>0.05$), we suggest that propofol might act on N-type calcium channels promoting calcium influx that is coupled to

synaptic vesicles exocytosis. Different from our data, previous works performed in CNS synapses have shown a predominant inhibition of calcium channels by propofol[5–7]. However, none of them have shown evidences that *N*-type calcium channels could be a target for propofol action. This discrepancy might be due to the fact that these studies were performed in brain slices that maintain intact neuronal circuitry whereas in the present work we were looking at nerve terminals without connection with motor neuron cell bodies. Therefore, we cannot rule out the possibility that propofol may exert an inhibitory effect on calcium channels located at motor neurons' cell bodies that are located at the spinal cord. Because we are looking at events that take place exclusively at the synaptic nerve terminal, such inhibition would not be detected in our experimental model. Nonetheless, our data provide direct evidences that low doses of propofol might act through *N*-type calcium channel that are directly coupled to synaptic vesicle exocytosis at motor nerve terminals. Moreover, we showed that propofol and KCl-evoked exocytosis were not affected by TTX suggesting that both conditions did not increase the synaptic vesicle exocytosis by interfering with Na^+ channels. Therefore, propofol and KCl can induce exocytosis in a Ca^{2+}-dependent and Na^+-independent fashion suggesting that this anesthetic may act directly on Ca^{2+} entry through *N*-type voltage-gated Ca^{2+} channels.

In agreement with our data, it has been observed that low concentrations of propofol increased the amplitude of the indirectly-elicited twitch and tetanic contractions in chick biventer cervices skeletal muscle, indicating that, in low concentrations, this anesthetic may stimulate skeletal muscle[14].

Previous work showed that propofol, at concentrations of 42 and 112 µmol/L, inhibited contraction of isolated rat diaphragm by decreasing ACh release on neuromuscular junction[16]. These authors also observed that this agent inhibited muscle contraction evoked by electrical field stimulation, suggesting that propofol, at this concentration range, may act by inhibiting Ca^{2+} entry through presynaptic voltage-gated Ca^{2+} channels. In the present work high doses of propofol inhibited Na^+-dependent exocytosis evoked by 4AP but did not have any significant effect on Na^+-independent exocytosis evoked by KCl. The decrease on vesicle exocytosis induced by 4AP in the presence of high doses of propofol is in correspondence with data showing a decreasing on the contractility of fatigued canine diaphragm with propofol in a dose-related fashion[21]. Previous studies in rat brain synaptosomes showed that propofol inhibits Ca^{2+}-dependent glutamate release evoked by veratridine and 4-AP with greater potency than Na^+ channel-independent release evoked by KCl[33–36]. In addition, it has been observed that high concentrations of propofol selectively inhibited 4AP-evoked but not KCl-evoked [³H]norepinephrine release[37]. Considering that high doses of propofol inhibited 4AP-evoked synaptic vesicles exocytosis in a similar manner to TTX and did not interfere with KCl-evoked exocytosis, we could suggest that the effect of propofol on neuromuscular junction is thus caused primarily by inhibition of action potential-evoked synaptic vesicle exocytosis at a step upstream of

Ca^{2+} entry through voltage-gated Ca^{2+} channels, possibly as a result of Na^+ channel blockade. In addition, there are evidences in the literature suggesting that high doses of propofol might have a direct effect on Na^+ channels[10, 12, 13]. Finally, experiments in dogs show that administration of propofol decreases diaphragm contractility in a dose-dependent manner and that high dose of this anesthetic produces a progressive decrease in contractility[18]. Based on the data presented here, we could speculate that such diaphragmatic dysfunction might be due to a presynaptic effect of high doses of propofol. In addition, propofol and midazolam seems to cause diaphragm dysfunction through mechanisms other than E–C coupling failure[23]. However, the clinical importance of the present data requires further investigations and we are unable to extrapolate our *in vitro* findings to the clinical practice.

It is difficult to correlate the concentrations of anesthetics used *in vitro* experiments with those used during clinical anesthesia and it has been shown that the doses of intravenous anesthetics required in the case of experimental animals are 10-100 times higher than those used for humans[38]. Plasma concentrations of propofol during anesthesia in humans are estimated to range between 70 and 106 μmol/L[35, 39–41]. Because of the high protein binding (98%), the half-maximal effect free concentration of propofol is around 2.0 μmol/L[35, 42, 43]. It has been suggested that brain concentrations of propofol are about eight-fold higher than plasma free concentrations[44] but there is no estimative of the concentrations on neuromuscular junction. Moreover, the propofol concentration producing loss of righting reflex in Rana pipiens tadpoles ranged between 1 and 10 μmol/L[45] an end point that usually occurs at a lower concentration than surgical anesthesia. Finally, it has been argued that clinically relevant concentrations are difficult to estimate[1-4] and it is important to mention that clinically relevant concentrations of anesthetic are important to examine the integrated responses in the intact animal but their relevance to *in vitro* studies should be taken with care due to our lack of understanding of how to integrate *in vitro* systems into the anesthesia model[46].

In conclusion, we showed that propofol has a dual effect on the exocytosis of cholinergic synaptic vesicles from the neuromuscular transmission and the present results may contribute to understanding some of the clinical effects observed with this agent on neuromuscular function.

Author contribution
Luciana Ferreira LEITE and Matheus de Castro FONSECA performed research and analysed data; Renato Santiago GOMEZ designed research and wrote the paper; Marcus Vinicius GOMEZ wrote the paper; Cristina GUATIMOSIM design research and analysed data.

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