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In vitro-evaluation of lipid emulsions as vehicles for the administration of xenon: Interaction with NMDA receptors

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The noble gas xenon is an antagonist of the NMDA (N-methyl-D-aspartate)-type glutamate receptor which may account for the ideal anesthetic profile and potent neuroprotective properties demonstrated even at subanesthetic concentrations. Because lipid emulsions also promote NMDA antagonistic effects they may serve as ideal carriers for xenon. In this *in vitro* study, we investigated the efficacy of xenon dissolved in various lipid emulsions (Intralipid®, Lipofundin®, ClinOleic® and Abbolipid®) on NMDA-evoked currents in cultured cortical neurons. The NMDA receptor blocking property at a clinically relevant concentration seen in the lipid emulsions tested may contribute to the anesthetic, analgetic and neuroprotective effects of xenon administered by way of these lipid carriers. Abbolipid® may serve as the most acceptable carrier since the NMDA antagonistic effect of xenon was enhanced in combination with this emulsion.

Key words: xenon, lipid emulsions, ligand-gated ion channels, NMDA receptor

INTRODUCTION

Most general anesthetics potentiate the action of the neurotransmitter γ-aminobutyric acid (GABA) at inhibitory synapses by a direct action of anesthetics on GABA-activated channels (Franks and Lieb 1994). In contrast xenon is an effective inhibitor of glutamatergic N-methyl-D-aspartate (NMDA) receptors with little or no effects on $GABA_A$-receptors (Franks et al. 1998, de Sousa et al. 2000). The specific inhibition of the NMDA-receptor may explain the attractive pharmacological properties of xenon in the context of anesthesia including a potent neuroprotection by xenon even at subanesthetic concentrations which could be demonstrated in *in vivo* and *in vitro* models (Ma et al. 2002).

Anesthetics act on different regions of the nervous system. Hypnosis and amnesia are supraspinal effects whereas it is remarkable that anesthetic-induced ablation of movement in response to pain is mediated primarily by the spinal cord (Eger et al. 1997, Antognini and Carstens 2002). It was hypothesized that the delivery of xenon to the target tissue may be improved by dissolving the gas in a lipid emulsion in which the xenon solubility is greater than in blood (Lavini et al. 2000). The authors demonstrated in a compartment model that in the emulsion formulation the predicted tissue concentrations of hyperpolarized xenon in the gray and white matter of the brain are comparable using respiration techniques (Lavini et al. 2000).

Lipid emulsions are well-suited to deliver lipophilic compounds to cells and whole animals and therefore may serve as a pharmaceutically acceptable carrier for xenon (Decker et al. 1995, Weigt et al. 2003). However, lipid emulsions by their own show a complex interaction with the NMDA receptor modulating the channel by different mechanisms dependent from the emulsion and the lipid concentration (Weigt et al. 2002a). Importantly, in lower clinically relevant concentrations emulsions by their own inhibit NMDA evoked currents in cortical neurons and may therefore promote the NMDA-antagonistic activity of xenon at the target tissue (Weigt et al. 2002b, 2003).

In a series of *in vitro* experiments we investigated the effects of xenon and various lipid emulsions (Intralipid® 20%, Abbolipid® 20%, Lipofundin MCT® 20% and ClinOleic® 20%) on NMDA receptors and

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tested the hypothesis that the combined application of xenon in the presence of lipid emulsions is associated with pronounced NMDA signaling.

METHODS

Animals and cell culturing

Adult Balb/c mice were kept in a 12/12 h dark/light cycle and fed a diet of standard pellets as approved by the Institutional Animal Care and Use Committee. The animals were allowed to mate during a 12 h period, and the day after mating was defined as day 0 of pregnancy. On embryonic day 15, pregnant mice were anesthetized with 25% chloralhydrate (1 ml/100 g body weight) and killed by decapitation.

The cerebral cortex of the fetal brains was dissected after removal of the striatal and hippocampal structures. Neuronal cell cultures were initiated as described (Beyer et al. 1991). Briefly, the cortical tissue was dissociated both enzymatically (0.1% trypsin) and mechanically. Cells were plated at a density of 1.8×10^5 cells/cm^2 on poly-DL-ornithine-coated (0.5 mg ml) plastic culture dishes (10 cm^2). Cultures were raised with minimum essential medium (MEM, Biochrom, Germany) supplemented with 5% fetal calf serum, and grown for at least two weeks. The medium was exchanged every second day. The cultured cells were used for experimentation after 10 to 14 days of cultivation. Before use they were rinsed twice with extracellular solution and thereafter placed in a Petri dish containing a medium with 140 mM NaCl, 2.7 mM KCl, 1.5 mM $CaCl_2$, 10 mM glucose, 2.5 µM glycine, 100 µM strychnine, 12 mM HEPES; pH 7.3.

Standard experimental procedures

The Petri dish was placed on an inverted microscope. While the medium in the dish was continuously exchanged at a rate of 4.5 ml/min, a jet of solution containing 50 µM NMDA was directed against a cell attached to a patch pipette. Membrane currents were recorded with the membrane potential clamped to –80 mV using the whole-cell recording technique (Hamill et al. 1981). All experiments were performed at room temperature. The equipment consisted of an EPC-9 amplifier and TIDA software (HEKA, Lambrecht, Germany). The patch pipettes were drawn from borosilicate glass, with a pipette resistance of 3–6 MΩ when filled with 140 mM $CsCl_2$, 2 mM $MgCl_2$, 2 mM ATPx2Na, 2 mM EGTA, 10 mM HEPES; pH 7.2. In order to improve sealing, the electrodes were briefly dipped into 2% dimethylsilan dissolved in methylene chloride (Tabuchi et al. 1997). All chemicals were purchased from Sigma (Deisenhofen, Germany) if not otherwise stated.

Preparation of solutions

To produce the various xenon-containing solutions we equilibrated xenon either in an aqueous solution or in commercially available lipid emulsions. Intralipid® 20% and ClinOleic® 20% were purchased from Baxter (Unterschleissheim, Germany), Lipofundin MCT® 20% from Braun-Melsungen (Melsungen, Germany), and Abbolipid® 20% from Abbott (Wiesbaden, Germany). The xenon-concentration of each fluid, taken from the supply vessel (syringe) was routinely analyzed by gas chromatography followed by mass spectrometry. Saturated aqueous solutions (n=7) contained 103.8 µl ± 3.4 µl xenon/ml buffer and saturated Lipofundin MCT® 20 % contained 571 ± 11 µl/ml. With respect to the experimental conditions (25°C and 600 m above sea level), 100 µl xenon/ml buffer corresponded to a xenon-concentration of 3.94 mM. Loss of xenon from the tubings (Teflon® FEP; inner diameter 0.8 mm; wall thickness 0.4 mm) was determined from the difference of the xenon-concentrations of the solutions in the syringe and at the end of the tubing. Loss of xenon from the aqueous solution was on average 16.1% (n=7), from the solution containing 0.5% lipid it 12.1% (n=3) and from all other lipid-containing solutions between 7.6 and 8.9% (n=12). The xenon concentrations given in Results were corrected accordingly. Xenon was obtained from Messer Griesheim GmbH (Krefeld, Germany).

A chemical analysis of the lipid emulsions yielded unexpected low concentrations (in mM) for Na (12.8), K (0.53) and Ca (0.6). To abolish artifacts experiments at a dilution of 1:5 were performed following appropriate ion supplementation to achieve equimolar concentrations with the external solution [Na (140), K (2.7) and Ca (1.5)].

Application of test solutions

Test solutions were applied to the cell used for the assay via a modified L/M-SPS-8 superfusion system (List, Darmstadt, Germany). Since xenon was to be

administered in closed vessels, we used infusion pumps at a rate of 1 ml/min instead of gravity for the generation of drug flow. To restrict the presence of xenon to a small volume within the dish, a combination of two perfusion systems was installed, i.e. (1) a global bath perfusion with the inflow set at 4.5 ml/min and an outflow which removed any excess fluid and (2) a local bath perfusion that generated a continuous fluid stream containing the agent in the desired concentration. The local inlet (tip of a seven-barreled pipette) was positioned at a distance of 50–100 µm upstream and the local outlet at about 300 µm downstream of the measuring field. The selection from the 7 supply vessels connected to the seven-barreled pipette was controlled with magnetic valves.

Statistical analysis

The paired *t*-test was used for comparing the mean results from the same lipid emulsion sample and unpaired *t*-tests were used for comparing different samples. A difference between results was considered significant when $P<0.05$. Results are presented as means ± SD.

RESULTS

To analyze interactions between xenon and lipid emulsion with NMDA receptors, we determined effects of lipid incorporated xenon in a series of experiments with high lipid concentrations in a 1:5 dilution. For these experiments lipid emulsions were diluted in extracellular solution with correcting for a dilution of ion concentrations to avoid an underestimation of the actual effect (see methods section).

Figure 1A demonstrates representative traces illustrating effects on the NMDA response by Abbolipid® in a 1:5 dilution and by xenon in combination with Abbolipid®. Since lipid emulsions can solvate approximately 20 times more xenon than water the xenon content in these experiments was approximately 100 µl/ml, which is corresponding to 1 MAC a xenon-saturated aqueous solution. In further experiments effects of lipid incorporated xenon on NMDA response was investigated at a 1:80 dilution which represents a clinically relevant lipid concentration. Representative traces for effects of Abbolipid® and xenon included in Abbolipid® in a 1:80 dilution are shown in Fig. 1B. Since xenon was incorporated in the lipid emulsion, that was diluted 1:80 the xenon-concentration in the test solution is 6 µl/ml, corresponding to 0.2 MAC. Figure 1C illustrates average inhibition of NMDA-induced currents by Abbolipid® alone and in combination with xenon. In both dilutions the peak response was not modulated by Abbolipid® alone. However, there was a significant reduction in plateau current decreasing the amplitude by 26.4 ± 10.1% (dilution 1:5) and 32.5 ± 13.3% (dilution 1:80). In contrast to Abbolipid® (Fig. 1C), Lipofundin MCT® (Fig. 2B) and ClinOleic® (Fig. 2C) which did not modulate the peak NMDA currents and significantly reduced plateau currents Intralipid® in a dilution of 1:5 enhanced the peak NMDA response by 52.8 ± 14.7% and did not affect plateau current (7.9 ± 12.9%, Fig. 2A) . This may be due to the NMDA agonistic effect of Intralipid® itself which has been demonstrated for such a high lipid concentration (Weigt et al. 2002a) Accordingly, when xenon was incorporated in the lipid emulsions at a 1:5 dilution the inhibiting effects on NMDA response were more pronounced in combination with Abbolipid®, Lipofundin MCT® and ClinOleic® rather than Intralipid®. The xenon-containing Abbolipid® (Fig. 1C) and Lipofundin MCT® (Fig. 2B) in a dilution of 1:80 with a corresponding xenon content of 6 µl/ml reduced both peak and plateau current significantly by 17.9 ± 7.2% and 13.0 ± 3.1%. The incorporation of xenon in Intralipid® and ClinOleic® did not affect significantly the NMDA response at a 1:80 dilution compared to the lipid emulsion effect alone.

To investigate an interaction between lipids and xenon on NMDA channels we evaluated in further experiments the effects of xenon in the presence of the various lipid emulsions and compared these results with xenon-effects in aqueous solution. Figure 3A illustrates a representative trace for the inhibitory effects of 6 µl/ml xenon in the continued presence of Abbolipid® in a 1:80 dilution. The same xenon-content in aqueous solution did not modulate the NMDA response as demonstrated at a representative trace (Fig. 3A). The data in Figs 3C and 3D represent xenon-induced effects since the lipid emulsions were present in the same dilution during control and xenon-application. Xenon in the continued presence of Abbolipid® (dilution 1:80) decreased the amplitude of the peak/plateau response significantly by 18 ± 7% / 19 ± 5% (Fig. 3C). Xenon (6 µl/ml) in aqueous solution or in combination with ClinOleic®, Lipofundin MCT®, or Intralipid® did not significantly inhibit peak or plateau

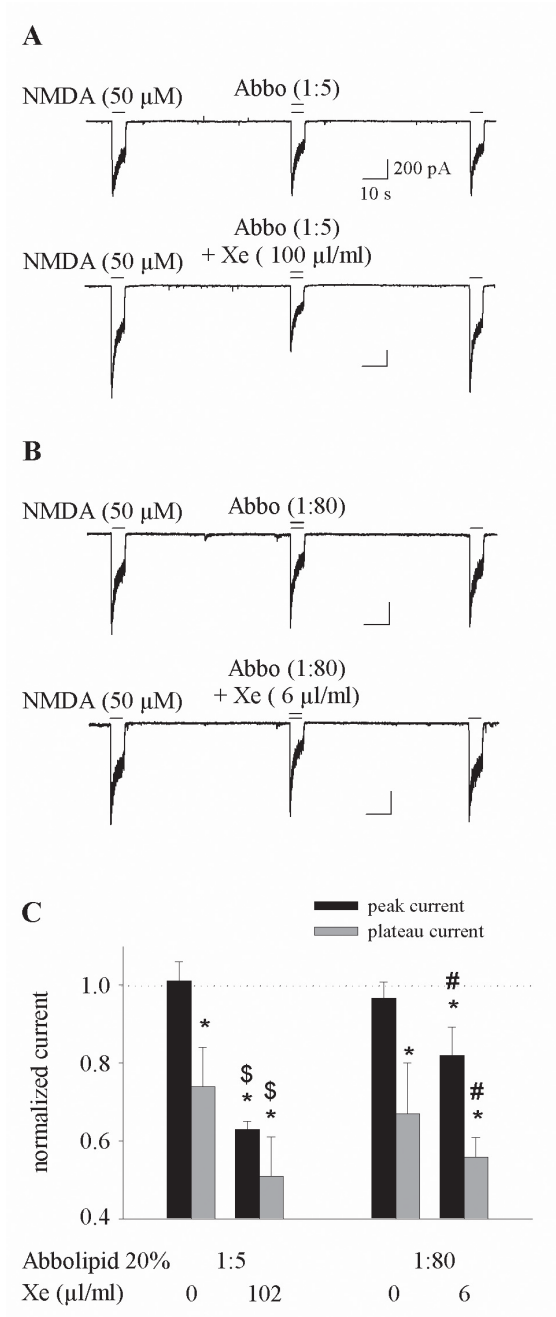

Fig. 1. Inhibition of NMDA-response by Abbolipid® and when xenon was incorporated in the lipid carrier. Representative traces of current induced by 50 µM NMDA are shown for control, respectively (A, B). The period of perfusion is indicated by the horizontal bar at the top of the traces. The effect on NMDA-response is shown for a dilution of 1:5 (A) and a dilution of 1:80 (B) after application of Abbolipid® 20% alone and when xenon was included in Abbolipid®, respectively. (C) The average depressant effects of peak and steady-state currents, recorded in the presence of Abbolipid® with and without xenon are illustrated for the two tested lipid concentrations. Averaged data of eight to 18 cells per experimental group are shown. Error bars denote SD; * Significantly different *versus* control (NMDA 50 µM). $ Significantly different *versus* correspondent peak/plateau current after application of Abbolipid® 20%, dilution 1:5. # Significantly different *versus* correspondent peak/plateau current after application of Abbolipid® 20%, dilution 1:80. The effects on the NMDA response were completely reversible. Neurons were voltage-clamped at a holding potential of –80 mV.

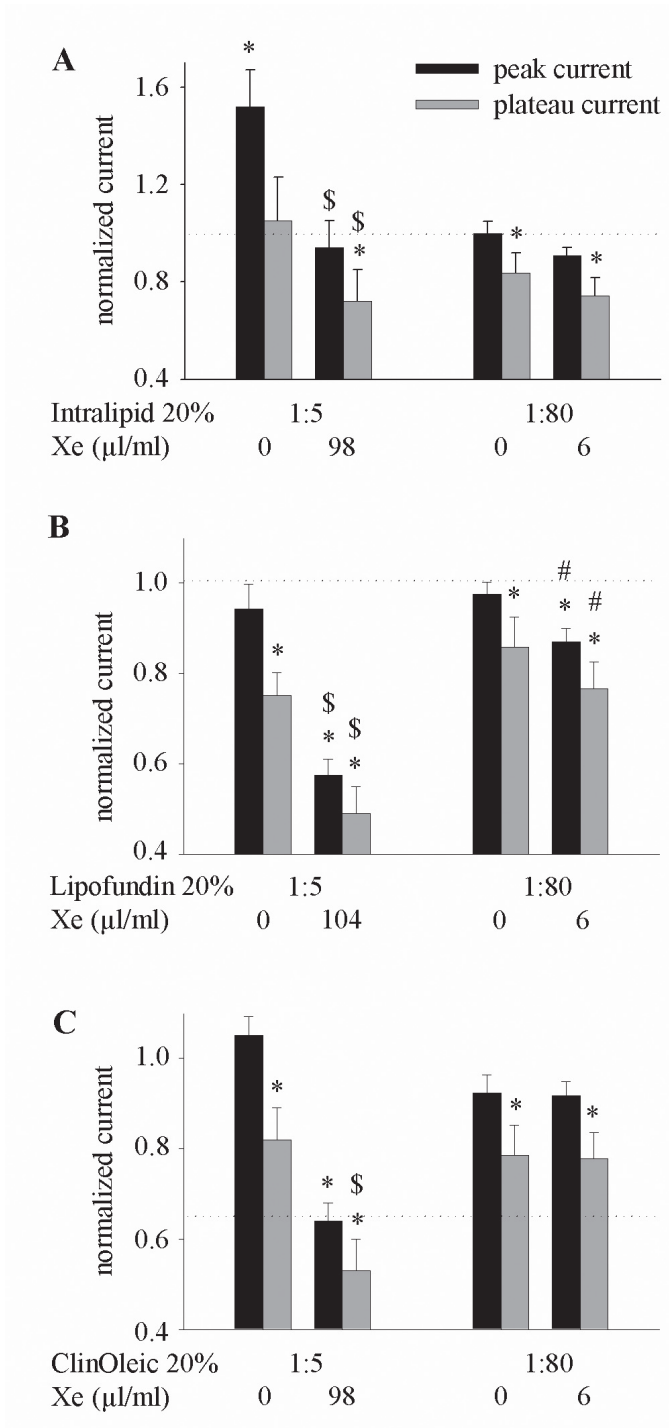

Fig. 2. Average depressant effects on NMDA-activated currents by Intralipid® 20%, Lipofundin MCT® 20% and ClinOleic® 20% alone and when xenon was incorporated in the lipid carrier, respectively. The average depressant effects of peak and steady-state currents on NMDA-response is shown for a dilution of 1:5 and 1:80 after application of Intralipid® 20% (A), Lipofundin MCT® 20% (B) or ClinOleic® 20% (C) alone and when xenon was included in the lipid emulsions, respectively. Averaged data of eight to 20 cells per experimental group are shown. Error bars denote SD; * Significantly different *versus* control (NMDA 50 µM). $ Significantly different *versus* correspondent peak/plateau current after application of the lipid emulsion at a dilution of 1:5. # Significantly different *versus* correspondent peak/plateau current after application of the lipid emulsion at a dilution of 1:80.

NMDA-response (Fig. 3C). Thus, the inhibitory effect of xenon was enhanced in combination with Abbolipid® suggesting an interaction between xenon and component(s) of the lipid emulsion. Unexpectedly, at a dilution of 1:5 the effects of xenon (xenon content between 98 and 104 µl/ml) on the NMDA response in the continued presence of the tested lipid emulsions were significantly diminished compared to the xenon-effect in aqueous solution (Fig. 3D).

In vivo pharmaceutically active agents like lipid emulsions and xenon have access to the NMDA receptor for a longer period of time and therefore may desensitize the receptor. To determine the effects of xenon in combination with Abbolipid® at that situation at a clinically relevant concentration we pre-applied xenon in combination with Abbolipid® at a dilution of 1:80 for 40 seconds. An example of this protocol is illustrated in Fig. 4A. Pre-application of xenon and Abbolipid® in a dilution of 1:80 inhibited both peak and plateau NMDA response significantly by 34.7 ± 5.7% and 40.5 ± 8.1% (Fig. 4B).

DISCUSSION

One of the aims of the present study was to use various lipid emulsions as carriers for xenon to examine combined effects on NMDA evoked currents, since lipid emulsions may have NMDA antagonistic effects by itself (Weigt et al. 2002a,b). Our *in vitro* results show that xenon dissolved in all tested lipid emulsions inhibits NMDA evoked currents in neuronal cells. Furthermore, we could demonstrate that there are markedly differences in the NMDA antagonistic potency between the tested lipid emulsions which were loaded with xenon. At a dilution of 1:80 that is easily attainable in clinical settings we found the strongest block of receptor channels by xenon dissolved in Abbolipid®.

There are several possible reasons for the different effects on NMDA-evoked currents by the various lipid emulsions alone and in combination with xenon. First, lipid emulsions act heterogeneously, dependent on its concentration on the NMDA receptor channels. At high, clinically not relevant concentrations (dilution 1:5) some lipid emulsions like Intralipid® show NMDA agonistic effects, they activate NMDA membrane currents by itself (Weigt et al. 2002a). This may explain the significant enhancement of the NMDA induced peak current by Intralipid® and the smallest relative effect of xenon in combination with Intralipid® at a 1:5 dilution. In contrast, at low concentrations which are clinically relevant (dilution 1:80) most lipid emulsions do not activate NMDA-currents by itself (Weigt et al. 2002a).

In addition they show NMDA antagonistic effects and inhibit NMDA-induced plateau currents. Lipid emulsions are oil-in-water formulations containing a mixture of oils and egg phosphatides as emulsifier, and glycerol for the adjustment of tonicity. Of course, one cannot discount the possibility that the reason for the different effects on the NMDA-response may be differences in the emulsifier of the lipid emulsions. However, a direct regulation of ion channels by a variety of fatty acids was described (Ordway et al. 1991). and it is known that fatty acids may alter the function of integral membrane proteins (Traul et al. 2000, Czyzewski et al. 2001). Lipid mediators, such as lysophosphatidic acid but not oleic acid modulate NMDA receptor activity (Tabuchi et al. 1997). Since fatty acids are highly enriched in lipid emulsions, they may, dependent from the lipid emulsion, alter the lipid environment in the vicinity of ion channels or even directly interact with ion channel proteins.

An explanation for the apparent difference in inhibition of the peak and late currents by xenon could be caused by a use-dependent block of the NMDA-receptor. Such use dependence has been observed with uncompetitive antagonists, such as ketamine or MK-801 (MacDonald et al. 1987, Zieminska and Lazarewicz 2006). On the other hand, uncompetitive blockers have much higher affinity to the open than to the closed state of the NMDA receptor channel. As a consequence the onset of, and the recovery from the blockade by ketamine is enhanced by prolonged or repeated activation of the receptor (MacDonald et al. 1987). However, xenon in combination with Abbolipid® reduced the peak and plateau responses to NMDA similar when it was pre-applied in the absence of the agonist suggesting that it can gain access to its binding site regardless of the conformation of the receptor. So, use dependence does not seem to play a role here.

A fully saturated aqueous xenon solution (98 µl/ml) depressed NMDA-activated currents recorded from cortical neurons by approximately 53%. These results referring to xenon dissolved in aqueous solution are in agreement with previous reports that show a significant inhibition of NR1a/NR2a NMDA receptors expressed in Xenopus oocytes (Yamakura and Harris

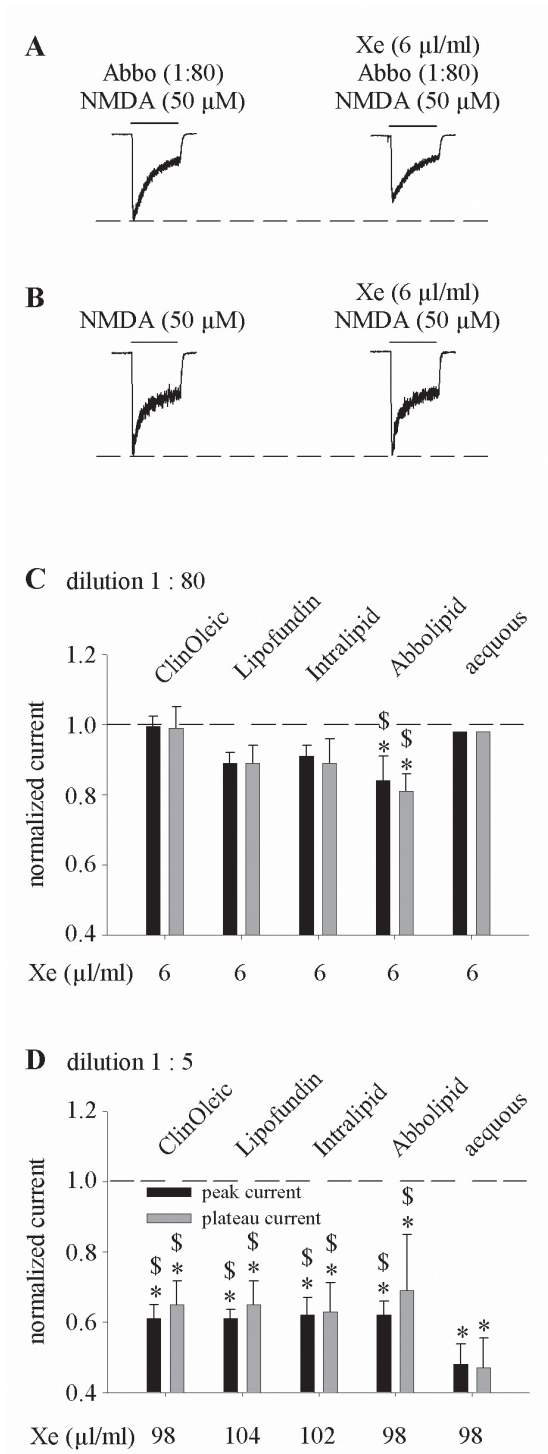

Fig. 3. Xenon-effects on NMDA-induced currents in the continued presence of lipid emulsions. The effects of lipid incorporated xenon on peak- and steady-state currents were analyzed from control, representing the base line current recorded in the presence of lipid emulsion alone. Representative traces are shown for Abbolipid® (control) and xenon (6 µl/ml) incorporated in Abbolipid® (A). (B) illustrates the corresponding effect of xenon (6 µl/ml) dissolved in aqueous solution. The effects of xenon at a dilution of 1:80 (C) and 1:5 (D) in combination with different lipid emulsions are demonstrated. Averaged data of eight to twenty cells per experimental group are shown. Error bars denote SD; * Significantly different *versus* control (NMDA 50 µM), $ Significantly different *versus* corresponding xenon effects in aqueous solution.

2000) and demonstrate that xenon inhibits NMDA-evoked responses in cultured hippocampal neurons (Franks et al. 1998). Franks and colleagues (1998) observed a reduction by approximately 60% for the peak and plateau NMDA-response when applying a solution equilibrated with a mixture of 80% xenon and 20% oxygen. It is notable that a significant NMDA antagonistic effect by xenon dissolved in Abbolipid® at a dilution of 1:80 was evident at a xenon-content of only 6 µl/ml, reducing currents by approximately 19%. However, the same xenon content (6 µl/ml) in an aqueous solution in absence of the lipid emulsion or in the presence of the other tested lipid emulsions did not affect the NMDA-response significantly. This observation suggests an interaction between xenon and Abbolipid® on the NMDA receptor channel.

However, there are several limitations to our study. In our study design, we incorporated xenon in different lipid concentrations and used two concentrations (dilution 1:5 and 1:80) for our experiments. Our main interest focuses on the effect at a lipid emulsion concentration occurring in the physiological setting. On the basis of the assumption of an injection of 20 to 40 ml of lipid emulsion in 2 500 ml of circulating blood plasma, which is realistic under clinical conditions, lipid emulsion concentrations between of 1:125 and 1:62.5 are achieved. Therefore, we used a dilution of 1:80, the overall xenon content was 6 µl/ml. However, the total dilution effect of an injected bolus in the circulatory system is dependent from the cardiac output and the injection speed (Lavini et al. 2000). Therefore, it is very difficult to predict corresponding concentrations at the site of action in the brain *in vivo*.

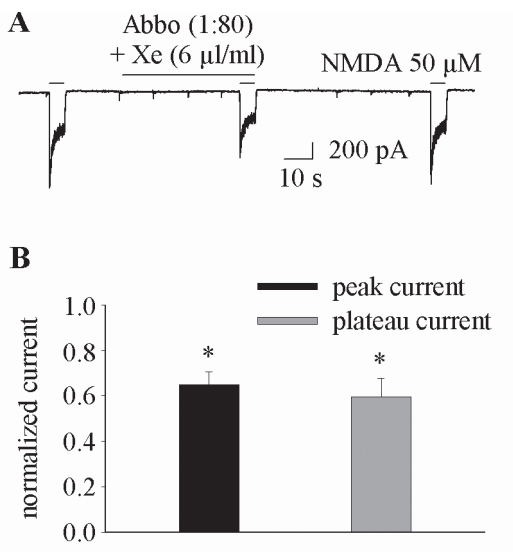

Fig. 4. Pre-application of xenon incorporated in Abbolipid ® 20%. (A) A representative trace is shown for control (NMDA 50 µM), application of xenon and Abbolipid® 20% (lipid concentration: 0.25%, dilution 1:80) for 40 s and following co-application of NMDA (50 µM) and washout. (B) Pre-application of Abbolipid® reduced the both the peak and the plateau current similar (no significant difference). Data were recorded from holding potential of –80 mV, *n*=11 cells. * Significantly different *versus* control (NMDA 50 µM).

For these experiments, we used the whole-cell recording method and single neuronal cells as a model system. With this technique, interactions between lipid emulsions and xenon with the NMDA receptor channels can be examined under controlled conditions. Effects of the blood-brain barrier on the intravenously administered agents are not considered in this model.

The fundamental difference between conventional solution phase drug delivery and delivery using emulsions is the presence of two distinct phases in the latter system: an aqueous phase and a lipid phase, separated by the amphiphilic interface. The delivery of drugs from emulsions to cells is dominated by the concentration of the compound in the lipid phase of the emulsion which is directly proportional to the concentration of the compound in cells at equilibrium (Decker et al. 1995). Unlike many other NMDA antagonists, xenon when inhaled and solved in blood is able to rapidly equilibrate with the brain by diffusing across the blood brain barrier (Peled et al. 1996). In addition, a theoretical study with hyperpolarized ^{129}xenon has demonstrated that inhalation of gaseous xenon and injection of xenon mixed with a lipid emulsion can lead to comparable concentrations of ^{129}xenon in the grey or white matter of the brain, depending on the xenon solubility in the carrier agent and injection rate (Lavini et al. 2000). For this reasons it is certainly possible that xenon applied in an emulsion formulation could block NMDA receptors in neuronal cells in the brain and spinal cord *in vivo*.

Because lipid emulsions on their own do not cause anesthesia or analgesia it is difficult to discern whether *in vivo* the effects of the lipid emulsions are different as seen in the present *in vitro* study. However, intravenous administration of medium-chain triglycerides in dogs with intact blood brain barrier showed significant dose related EEG changes and central nervous system toxicity suggesting a diffusion of medium chain fatty acids across the blood brain barrier (Miles et al. 1991).

But we do not know whether the compound(s) of the lipid emulsions inhibiting the NMDA receptor described here have access to its molecular targets in neurons, and if so, whether its action of xenon is additionally potentiated by the lipid emulsion. However, the observation that more than twice the dose of propofol was required to achieve EEG burst suppression in rats with the lipid-free formulation compared to emulsion formulation (Dutta et al. 1997) and intravenous administration of a lipid emulsion loaded with halothane revealed a lower MAC in swine than that for inhaled halothane (Musser et al. 1999) may suggest a potential mechanisms for the emulsion effect by blockade of NMDA receptors. However, other mechanisms like a decrease of the volume of distribution by emulsions may also contribute (Dutta et al. 1997).

NMDA-receptor antagonists, e.g. ketamine and xenon have been shown to be neuroprotective during many clinically relevant pathological circumstances, e.g. ischemia and brain trauma (Harada et al. 1999). Interestingly, a potent neuroprotection by xenon even at subanesthetic concentrations could be demonstrated in *in vivo* and *in vitro* models (Ma et al. 2002). Because lipid emulsions also promote NMDA antagonistic effects they may serve as ideal carriers for xenon and might enhance the efficacy of the individual compounds through a synergistic or additive mechanism. Our *in vitro* results show that xenon in combination with Abbolipid® as a carrier potently inhibits NMDA receptor channels in a clinically relevant concentration. Remarkably, the NMDA antagonistic effect of xenon was enhanced in the presence of Abbolipid®. Therefore, the intravenous administration of xenon included in Abbolipid® may represent a potent neuroprotectant for preemptive use in clinical settings. Moreover, intracerebroventricular Intralipid® application reduced the extracellular glutamate accumulation during the peri-ischemic period indicating neuroprotective effects through a different mechanism than NMDA receptor blockade (Yano et al. 2000). However, further research will be required to ascertain potential neuroprotective properties of lipid emulsions alone or in combination with xenon *in vivo* since in present no data are available

CONCLUSION

In summary, xenon included in lipid emulsions promotes the NMDA-antagonistic activity of xenon in a clinically relevant concentration. Abbolipid® may serve as a pharmaceutically acceptable carrier since the NMDA antagonistic effect of xenon was enhanced in combination with this emulsion. The present *in vitro* results suggest a significant inhibition of NMDA evoked currents by xenon loaded lipid emulsions in neuronal cells, however, the *in vivo* relevance has to be assessed.

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