

Remifentanil Directly Activates Human N-*methyl-*D-*aspartate Receptors Expressed in* Xenopus laevis *Oocytes*

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Background: **Clinical studies suggest that intraoperative administration of the clinical remifentanil formulation Ultiva® (GlaxoWellcome GmbH & Co, Bad Oldesloe, Germany) increases postoperative pain and postoperative analgesic requirements, but mechanisms remain unclear. *N*-methyl-D-aspartate (NMDA) receptors are thought to play a major role in development of postoperative pain and opiate tolerance. The authors hypothesized that Ultiva® directly stimulates human NMDA receptors.**

Methods: **To test this hypothesis, the authors expressed human NR1A/NR2A and NR1A/NR2B NMDA receptors in *Xenopus laevis* oocytes by injection of messenger RNA prepared *in vitro*. After protein expression, they used a two-electrode voltage clamp to measure currents induced by NMDA receptor agonists and opioids.**

Results: **Noninjected cells were unresponsive to all compounds tested. Glutamate/glycine (1 nM–1 mM each) or Ultiva® (0.01 pM–0.1 mM) stimulated NMDA receptors concentration dependently. NR1A/2A EC_{50} values were 8.0 μM/12 μM for glutamate/glycine and 3.5 nM for Ultiva®, and NR1A/2B EC_{50} values were 3.9 μM/1.9 μM for glutamate/glycine and 0.82 μM for Ultiva®. Glycine in combination with Ultiva® showed no additive effect compared with Ultiva® alone. Ultiva®-induced currents were inhibited by MK-801 (pore blocker) but not by 7-CK (glycine antagonist), D-AP5 (glutamate antagonist), or naloxone. Fentanyl (10 μM) did not stimulate NMDA receptors.**

Conclusion: **These data indicate that Ultiva® but not fentanyl stimulates NMDA receptors of different subunit combinations (NR1A/2A, NR1A/2B). The mechanism seems to be allosteric activation of the NMDA receptor.**

THE synthetic opioid remifentanil has gained wide clinical acceptance by anesthesiologists. Its pharmacologic characteristics—short context-sensitive half-time (time to a 50% decrease of effective site concentration after infusion is stopped) and organ-independent metabolism—allow predictable and rapid recovery within minutes.[1,2] Even high doses of remifentanil administered until skin closure do not affect early postoperative recovery or respiratory function.[3] However, supplemental analgesics (such as morphine or piritramide) are required routinely to treat postoperative pain adequately.[3,4] Indeed, postoperative opioid requirements after remifentanil-based intraoperative analgesia were shown to be unusually high,[4,5] and postoperative pain control seems to be more difficult than is the case with other opiates administered intraoperatively.[6,7] Clinical studies[4,5,8] as well as studies in human experimental pain models[9–11] suggest that intraoperatively administered remifentanil results in acute opioid tolerance or hyperalgesia manifested by increased postoperative pain and opioid consumption.

N-methyl–D-aspartate (NMDA) receptors play a critical role in the development of opioid tolerance and secondary hyperalgesia.[12–15] Wong *et al.*[16] showed in rats that NMDA receptor antagonists prevented the down-regulation of μ-opioid receptor high affinity sites, suggesting interactions between NMDA and opioid receptors. It is not known, however, whether opioids directly affect NMDA receptor functioning.

We hypothesized a direct stimulating effect of remifentanil on NMDA channels. This would be expected to result in changes in neuronal plasticity with consequent opioid tolerance and hyperalgesia and therefore would provide a link between clinical and experimental observations. To test this hypothesis, we compared effects of fentanyl and the clinically used remifentanil formulation (Ultiva®; GlaxoWellcome GmbH & Co, Bad Oldesloe, Germany) on NMDA receptors, expressed recombinantly in *Xenopus laevis* oocytes.

Materials and Methods

Oocyte Harvesting and Preparation

Procedures for *Xenopus laevis* oocyte isolation, messenger RNA (mRNA) synthesis and microinjection technique were published previously.[17,18] In brief, after approval of the Animal Care and Use Committee of the City of Muenster, Germany, female *Xenopus laevis* frogs were housed in a frog colony. Animals were anesthetized by immersion in cold 0.2% 3-aminobenzoic-methyl-ester until they were unresponsive to a painful stimulus (toe pinching). Approximately 200 oocytes were surgically removed. The oocytes were then defolliculated by digestion for 2 h in collagenase type 1A diluted in oocyte Ringer's solution (containing 82.5 mM NaCl, 2 mM KCl,

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Received from the Department of Anesthesiology and Intensive Care, University Hospital Muenster, Muenster, Germany. Submitted for publication January 5, 2003. Accepted for publication December 5, 2003. Supported by the Department of Anesthesiology and Intensive Care, University Hospital Muenster, Muenster, Germany, and by European Society of Anaesthesiology Research Award 2002. Dr. Nollet was supported by the European Academy of Anesthesiology Clinical Scholar Research Award 2000 of the European Academy of Anesthesiology. The authors thank Paul Whiting, Ph.D. (Merck Sharp & Dohme Research Laboratories, Harlow, United Kingdom), for providing the human *N*-methyl-D-aspartate receptors. Presented in part as a poster at the Sixth International Mechanisms of Anesthesia Conference, Bonn, Germany, June 28–30, 2001, and presented at the International Anesthesia Research Society 76th Clinical and Scientific Congress, San Diego, California, March 16–20, 2002.

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1 mM $MgCl_2$, and 5 mM HEPES, pH adjusted to 7.5). Microscopic observation confirmed that the follicle cells had been removed.

NMDA Receptor Expression

The NMDA receptor combinations tested consist of NR1 and NR2 subunits. The human NR1A (approximately 3,000 bp), NR2A (approximately 5,500 bp), and NR2B (approximately 5,000 bp) subunits were obtained from Paul Whiting, Ph.D. (Merck Sharp & Dohme Research Laboratories, Harlow, United Kingdom) as a complementary DNA in pcDNAI/Amp vectors. These constructs were linearized by either the nuclease *Xba*I (NR1A) or *Eco*RV (NR2A, NR2B) and transcribed in the presence of capping analog by bacteriophage RNA polymerase T7, using a commercial RNA preparation kit (mMESSAGE mMACHINE TM T7 Kit; Ambion Inc., Austin, TX). Oocytes were injected, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA), with 6 ng NR1A/NR2A or NR1A/NR2B subunits in a 1:5 weight ratio in 30 nl RNase-free sterile water. Correct injection was confirmed by noting a slight increase in cell size. Oocytes were then incubated for 48–72 h in modified Barth solution (containing 88 mM NaCl, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM Ca_2NO_3, 10 μg/ml gentamycin, 10 μg/ml penicillin, and 15 mM HEPES, pH adjusted to 7.4) at 16°C.

Electrophysiology

A single oocyte was positioned in a continuous-flow chamber with 0.5 ml volume and superfused (3 ml/min) with Mg^{2+}/Ca^{2+}-free Tyrode solution with Ba^{2+} (TyrBa; containing 150 mM NaCl, 5 mM KCl, 1.8 mM $BaCl_2$, 10 mM dextrose, and 10 mM HEPES, pH adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass on a vertical computer-controlled electrode puller (model 773; Campden Instruments Ltd., Lafayette, IN). Electrode tips were broken to a diameter of approximately 10 μm, providing a resistance of 1–3 MΩ, and filled with 3 M KCl. The oocytes were voltage clamped using a two-electrode voltage clamp amplifier (OC725C; Warner Instruments Corp., New Haven, CT) connected to an IBM-compatible personal computer for data acquisition and analysis (software by Joachim Kardeous, Research Assistant, University of Muenster, Muenster, Germany). All measurements were performed at a holding potential of −70 mV and recorded for 5 s before and 85 s after drug administration.

Because pure remifentanil is not available, the clinically used Ultiva® preparation had to be used. This contains remifentanil-hydrochloride and glycine as the sole constituents.

Glutamate, glycine, Ultiva®, fentanyl, and NMDA were diluted in TyrBa solution to the required concentrations and adjusted to a pH of 7.4. They were delivered into the continuous buffer flow over a period of 5 s. The antagonists 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), D-2-amino-5-phosphonovalerate (D-AP5), 7-chlorokynurenic acid (7-CK), and naloxone were diluted in TyrBa solution (pH adjusted to 7.4) to the required concentrations, and oocytes were incubated for 10 min in antagonists before stimulation with Ultiva® or glutamate/glycine in the continued presence of antagonists. Responses were quantified by measurement of peak currents and are reported in milliamperes.

Statistical Analysis

Unless stated otherwise, results are reported as mean ± SEM. Differences between treatment groups were analyzed using the Student *t* test. Because variability between batches of oocytes is common, at least 10 oocytes from at least 3 frogs were studied for each data point. $P < 0.05$ was considered significant. Concentration–response curves were fit to the following logistic function, derived from the Hill equation: $y = y_{min} + (y_{max} - y_{min})\,(1 - x^n/[x_{50}{}^n + x^n])$, where y_{max} and y_{min} are the maximum and minimum responses obtained, n is the Hill coefficient, and x_{50} is the half-maximal effect concentration (EC_{50}).

Materials

N-methyl-D-aspartate, glutamate, glycine, MK-801, D-AP5, and 7-CK were obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany), remifentanil-hydrochloride (Ultiva®) was obtained from GlaxoWellcome GmbH & Co. (Bad Oldesloe, Germany), naloxone was obtained from CuraMed Pharma GmbH (Karlsruhe, Germany), and fentanyl (Fentanyl®-Janssen) was obtained from Janssen-Cilag GmbH (Neuss, Germany).

Results

Functional Expression of NMDA Receptors in Xenopus *Oocytes*

Uninjected oocytes were unresponsive to either glutamate/glycine or to NMDA (data not shown). In contrast, oocytes injected with either NR1A/2A or NR1A/2B receptor mRNA 48–72 h previously responded to glutamate (1 nM–1 mM) in the presence of 10 μM glycine with inward currents. The currents consisted of a rapid initial peak current,[19] followed by a gradual return to baseline (fig. 1A). Peak current values were used for further analysis. Glutamate activation of either receptor subunit combination in the presence of glycine 10 μM was concentration-dependent. No statistical difference was observed between the EC_{50} values for the subunit combinations (table 1), but Emax obtained on NR1A/2B was significantly greater than that obtained on NR1A/2A (3.1 ± 0.23 *vs.* 2.0 ± 0.16 μA, respectively; $P < 0.05$; fig. 1B and table 1). The selective agonist NMDA (1 mM) in combination with glycine (10 μM) was applied to both

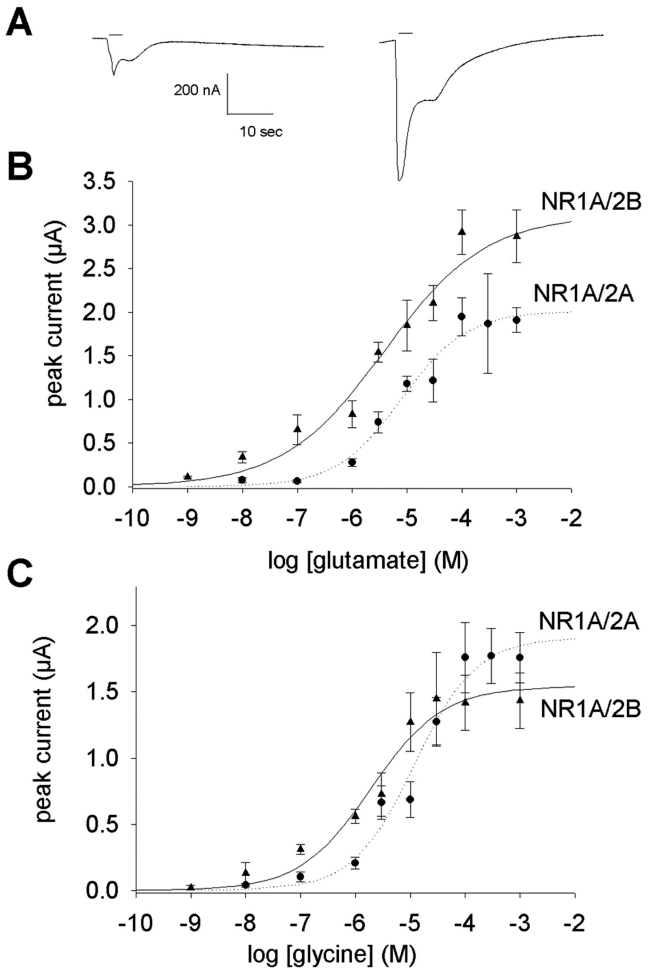

Fig. 1. NR1A/NR2A and NR1A/NR2B *N*-methyl-D-aspartate receptors expressed recombinantly in *Xenopus laevis* oocytes. (*A*) Representative traces of *N*-methyl-D-aspartate receptor responses induced by different concentrations of glutamate (*left trace*, 0.1 μM; *right trace*, 0.1 mM) in the presence of 10 μM glycine in oocytes expressing NR1A/NR2B receptors; *black line* shows duration of agonist application. (*B*) Glutamate, in the presence of 0.1 μM glycine, evokes inward currents in a concentration-dependent manner in oocytes expressing NR1A/2A (EC_{50} = 8.0 μM) (*circles*) or NR1A/2B (EC_{50} = 3.9 μM) (*triangles*) receptors (n = 10 for each data point; means ± SEMs). (*C*) Glycine, in the presence of EC_{50} glutamate, evokes inward currents in a concentration-dependent manner in oocytes expressing NR1A/2A (EC_{50} = 12 μM) (*circles*) or NR1A/2B (EC_{50} = 1.9 μM) (*triangles*) receptors (n = 10 for each data point; means ± SEMs).

receptor subunit combinations. Responses were indistinguishable from those stimulated by glutamate/glycine (data not shown). Therefore, for further experiments, the physiologic agonist glutamate in combination with glycine at EC_{50} concentration was used.

Glycine (1 nM–10 mM), as expected, evoked no inward currents in the absence of glutamate. In combination with glutamate, responses to glycine (1 nM–1 mM) were concentration dependent for both subunit combinations (fig. 1C and table 1). EC_{50} concentrations for glutamate and glycine were comparable with those obtained by others.[20,21]

Ultiva® Induces Inward Currents in Xenopus *Oocytes Expressing NMDA Receptors*

Application of Ultiva® to uninjected cells induced no inward currents (data not shown). In contrast, oocytes expressing NR1A/2A or NR1A/2B receptors showed concentration-dependent responses to Ultiva® (fig. 2). EC_{50} measured in NR1A/2A-expressing oocytes (3.5 ± 0.2 nM) was significantly less than that obtained in NR1A/2B-expressing cells (0.82 ± 0.01 μM). There was no statistically significant difference in Emax between subtypes (fig. 2 and table 1). The ratios for the Emax of Ultiva® compared with the Emax effect of glutamate/glycine on NMDA receptors were 56% for NR1A/2A and 29% for NR1A/2B, respectively (fig. 2 and table 1).

Fentanyl Does Not Activate NMDA Receptors

To test the hypothesis that the agonist effect of Ultiva® is specific to this opioid, we studied the effects of fentanyl on both combinations of NMDA receptors. Cells expressing NMDA receptors were unresponsive to fentanyl, even when applied in high concentrations (10 μM), but responded appropriately to glutamate/glycine applied subsequently (fig. 3A).

Although endogenous opioid receptors in *Xenopus* oocytes have not been reported, we investigated a potential indirect effect through μ-opioid receptors by determining the effects of Ultiva® in the presence of naloxone (0.1 mM). No effect on Ultiva®-induced currents was observed (fig. 3B).

Table 1. Fitting Values for NMDA (NR1A/2A and NR1A/2B) Receptor Stimulation in the Presence of Different Agonists

NMDA NR1A/	Agonists	Hill Coefficient, Mean ± SEM	EC_{50}, Mean ± SEM, M	Emax, Mean ± SEM, μA	r^2
2A*	Glu/gly	0.7 ± 0.1	$8.0 \pm 2.9 \times 10^{-6}$	2.0 ± 0.16	0.97
2B*	Glu/gly	0.5 ± 0.08	$3.9 \pm 2.8 \times 10^{-6}$	3.1 ± 0.23	0.96
2A	Gly/glu	0.8 ± 0.1	$1.2 \pm 0.4 \times 10^{-5}$	1.9 ± 0.2	0.96
2B	Gly/glu	0.7 ± 0.1	$1.9 \pm 0.7 \times 10^{-6}$	1.5 ± 0.1	0.97
2A†	Ultiva®	0.3 ± 0.04	$3.5 \pm 0.2 \times 10^{-9}$	1.1 ± 0.006	0.98
2B†	Ultiva®	0.3 ± 0.07	$8.2 \pm 0.1 \times 10^{-7}$	0.9 ± 0.3	0.94

* $P < 0.05$ Emax NR1A/2A *vs.* NR1A/2B. † $P < 0.05$ EC_{50} NR1A/2A *vs.* NR1A/2B.

NMDA = *N*-methyl-D-aspartate; glu/gly = glutamate in combination with 10^{-5} M glycine; gly/glu = glycine in combination with EC_{50} glutamate (n = 10).

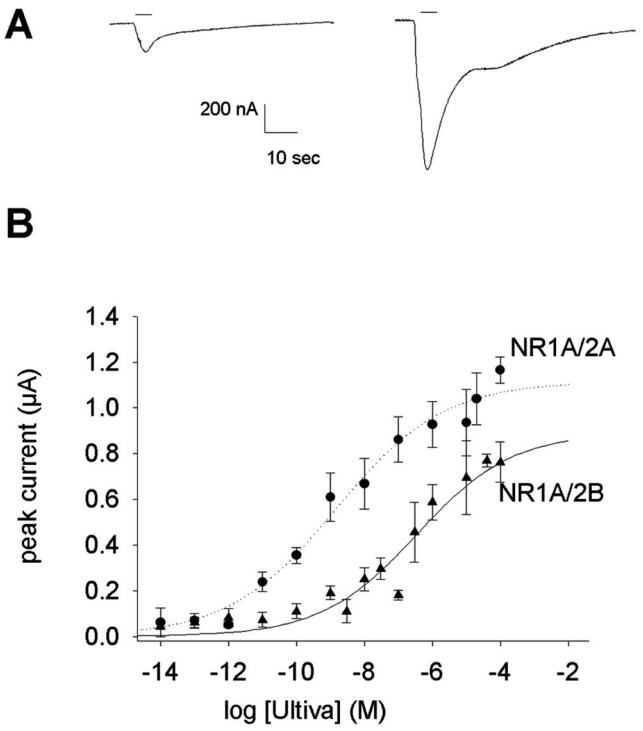

Fig. 2. (*A*) Representative traces of *N*-methyl-D-aspartate receptor responses induced by different concentrations of Ultiva® (*left trace*, 0.25 nM; *right trace*, 0.24 mM) in oocytes expressing NR1A/NR2A receptors; *black line* shows duration of Ultiva® application. (*B*) Ultiva® evokes inward currents in a concentration-dependent manner in oocytes expressing NR1A/2A (EC_{50} = 3.5 nM) (*circles*) or NR1A/2B (*triangles*) (EC_{50} = 0.8 μM) receptors (n = 10 for each data point; means ± SEMs).

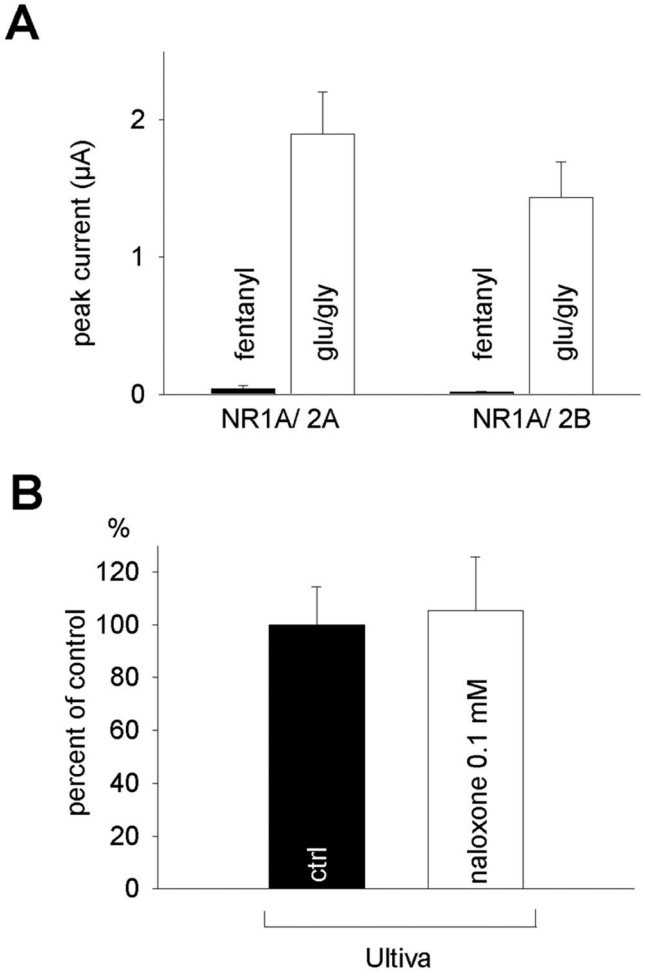

Fig. 3. (*A*) Fentanyl, 10 μM (*black bars*), does not evoke inward currents in NR1A/2A or NR1A/2B *N*-methyl-D-aspartate receptors, in contrast to glutamate 1 mM/glycine EC_{50} (glu/gly) (*white bars*) (n = 10; vertical bars ± SEMs). (*B*) Application of Ultiva® (0.1 mM remifentanil plus 3.1 mM glycine) after 10 min incubation in naloxone (0.1 mM; *white bar*) does not abolish Ultiva®-evoked *N*-methyl-D-aspartate receptor currents (NR1A/2A) (n = 10; vertical bars ± SEMs).

Activation of NMDA Receptors by Ultiva® Is Not Glycine Dependent

Each 5-mg Ultiva® (remifentanil) vial contains 15 mg glycine as adjunct. As mentioned above, both NMDA receptor subtype combinations were unresponsive to glycine alone (data not shown). It is, however, conceivable that glycine might act as an obligatory coagonist with remifentanil or a glutamate-like contaminant in our experimental system. This hypothesis could not be tested directly because glycine-free Ultiva® is not available. However, if glycine acts as coagonist, one would expect its effect to be glycine concentration dependent, as shown above for the combination with glutamate. Several experiments were performed to determine any effect of glycine. We tested the effect of additional glycine on Ultiva®-induced responses. Effects of Ultiva® (24 nM remifentanil containing 0.4 μM glycine; fig. 4A) were compared with those induced by Ultiva® (24 nM remifentanil containing 0.4 μM glycine) plus an approximately 25-fold greater concentration of glycine (10 μM). Both solutions induced similar currents (fig. 4A), indicating a non–glycine-dependent effect. In case of contamination of Ultiva® with a glutamate-like compound, the glycine (0.4 μM) contained in 24 nM Ultiva® could already represent a saturating concentration (in combination with the assumed high glutamate concentration). To exclude this possibility, Ultiva® in a low concentration (0.24 nM remifentanil, containing 4 nM glycine) was applied in a saturated glycine (10 mM) perfusate. Ultiva® in the presence of a saturating glycine concentration evoked currents similar to those without a saturating glycine concentration (fig. 4B).

Furthermore, glutamate/glycine responses were inhibited in the presence of the competitive glycine antagonist 7-CK (5 μM) in a glycine concentration–dependent manner, but 7-CK did not inhibit the responses to high (containing 3.1 mM glycine) and low (containing 4 nM glycine) Ultiva® concentrations (fig. 4C).

Therefore, glycine does not seem to be responsible for the NMDA receptor activation by Ultiva®.

To study a potential contamination with glutamate, glutamate/glycine or Ultiva® was applied in the presence

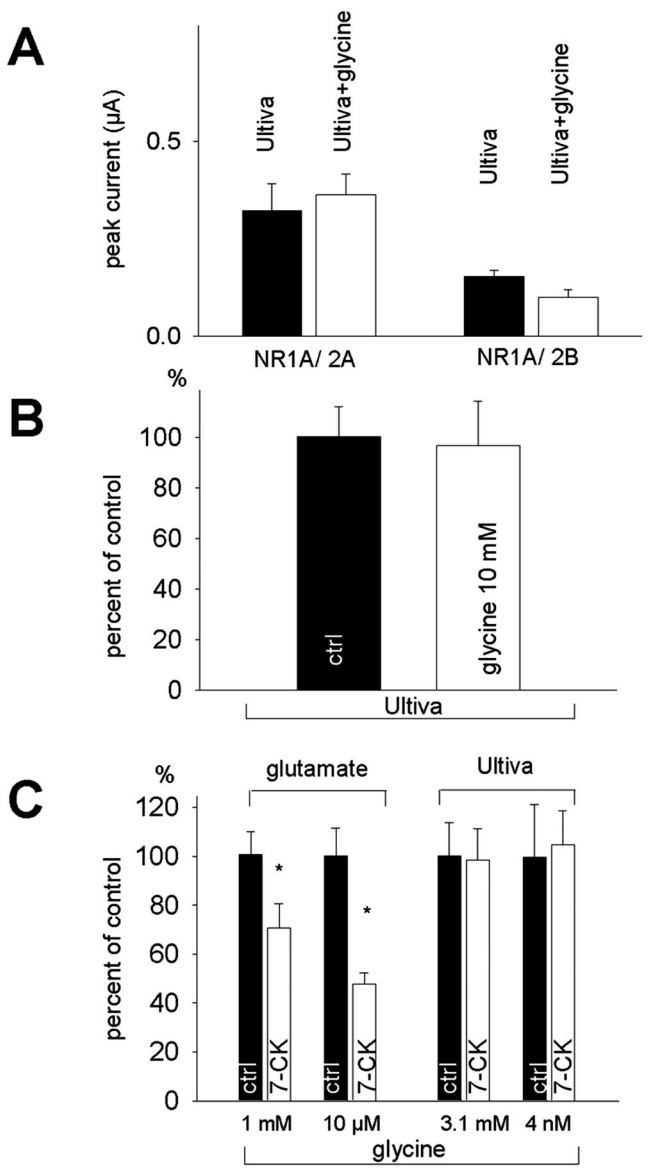

Fig. 4. (*A*) Addition of glycine (10 μM) to Ultiva® (*white bars*; 24 nM remifentanil plus 0.4 μM glycine) does not induce an additive effect on either receptor subtype combination compared with Ultiva® (*black bars*; 24 nM remifentanil plus 0.4 μM glycine alone) ($P > 0.05$; n = 10; vertical bars ± SEMs). (*B*) Preapplication of a saturating glycine concentration (10 mM; *white bar*) before application of Ultiva® (remifentanil 0.24 nM plus glycine 4 nM) does not enhance Ultiva®-induced currents in *N*-methyl-D-aspartate receptors (NR1A/2A) (n = 10; vertical bars ± SEMs). (*C*) Glutamate/glycine-evoked *N*-methyl-D-aspartate receptor responses (NR1A/2A) are inhibited by the glycine site antagonist 7-CK (5 μM; *white bars*) in a glycine concentration–dependent manner. Responses to both Ultiva® concentrations (remifentanil 0.1 mM plus glycine 3.1 mM and remifentanil 0.25 nM plus glycine 4 nM) are not affected by 7-CK (* $P < 0.05$; n = 10; vertical bars ± SEMs).

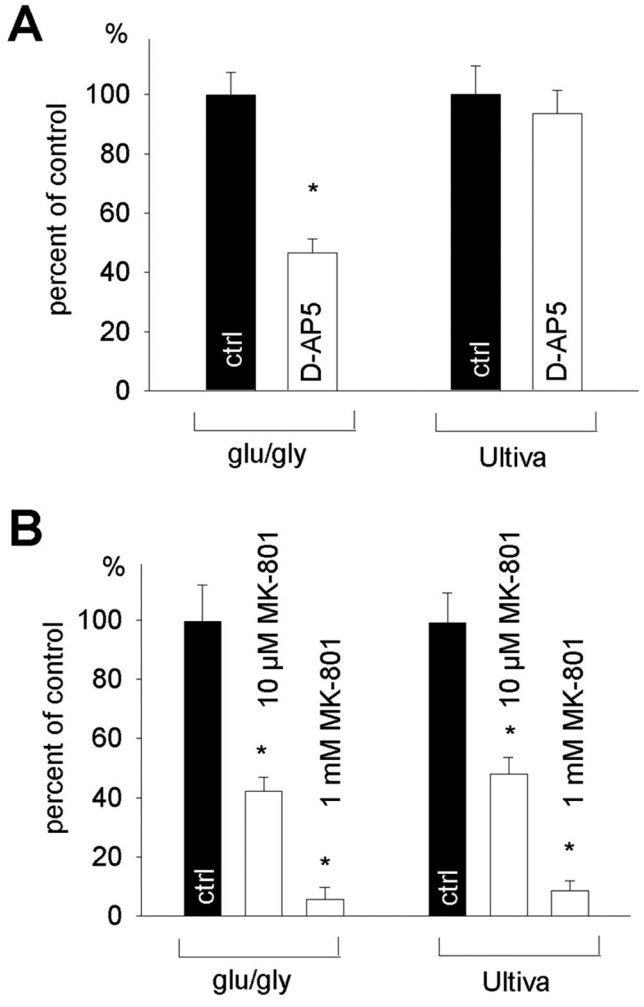

Fig. 5. (*A*) The competitive glutamate binding site antagonist D-AP5 (1 mM; *white bars*) inhibits glutamate/glycine-induced currents (EC_{50}) (* $P < 0.05$) but does not affect Ultiva®-induced (remifentanil 0.1 mM plus glycine 3.1 mM) responses (n > 10; vertical bars ± SEMs). (*B*) The noncompetitive *N*-methyl-D-aspartate receptor channel pore blocker MK-801 inhibits *N*-methyl-D-aspartate receptor responses (NR1A/2A) to Ultiva® (remifentanil 0.1 mM plus glycine 3.1 mM) and glutamate/glycine (EC_{50}) in a concentration-dependent manner (* $P < 0.05$; n = 10; vertical bars ± SEMs).

of the competitive glutamate binding site antagonist D-2-amino-5-phosphonovalerate (D-AP5, 1 mM). D-AP5 inhibited the responses to glutamate/glycine but had no effect on Ultiva®-mediated currents (fig. 5A). We also applied glutamate/glycine or Ultiva® in the presence of MK-801, a highly potent and selective noncompetitive NMDA receptor antagonist, which acts at the NMDA receptor–operated ion channel as an open channel blocker. In contrast to the findings with D-AP5, MK-801 (1 mM and 10 μM) abolished the responses to both glutamate/glycine and Ultiva® in a concentration-dependent manner (fig. 5B).

Discussion

Ultiva® stimulates NR1A/2A or NR1A/2B NMDA receptor combinations expressed in *Xenopus* oocytes. This effect is not observed after the application of fentanyl. In addition, the adjunct glycine seems not responsible for the NMDA receptor activation. These results may ex-

plain in part the clinical observation of increased postoperative opioid requirements after intraoperative Ultiva® administration.[8]

The NMDA receptor is a protein complex composed of two classes of subunits, the essential subunit NR1 and one or more of four different NR2 subunits (A–D). These subunits coassemble in various combinations to form functionally distinct NMDA receptors.[22] NR2 subunits alone cannot form functional channels, but they potentiate NR1 activity and induce functional variability of the NMDA receptor.[23,24] In this study, the NR1 subunit was coexpressed with either the NR2A or the NR2B subunit. Both combinations are widely distributed in the brain and the dorsal horn of the spinal cord[25] and are believed to play a relevant physiologic role in the development of acute opioid tolerance, hyperalgesia, and the "windup" phenomenon.[26,27] As charge carrier, we used Ba^{2+} to exclude a contribution to observed currents by activation of endogenous Ca^{2+}-dependent ion channels. Mg^{2+}-free solutions were used to exclude any inhibitory effects of magnesium on NMDA receptors.

Although NMDA receptor expression in *Xenopus* oocytes is an established model,[19,28,29] some limitations of the model should be noted. First, our experiments were performed at room temperature, whereas the expressed receptor is human and normally functions at 37°C. This might theoretically influence its behavior. However, we found it more important to maintain the cell membrane in its normal state of fluidity. Second, NR2 subunits were coexpressed with NR1 subunits to enhance currents through expressed receptors and to provide a more physiologic receptor configuration.[24] To achieve heteromeric expression, the mRNA was injected into the oocytes in a weight ratio of 1:5 between NR1A and NR2A or NR2B subunits.[21] We could not determine expression levels or stoichiometry for the subunit combinations. Therefore, although we used a defined mRNA weight ratio between NR1 and NR2 subunits, it is conceivable that the ratio of NR1 to NR2 varied during experimentation. Despite these restrictions, we believe that our expression system provides an appropriate model of NMDA receptor expression in a neuron, as confirmed by the glutamate and glycine concentration–response curves observed, responsiveness of the receptors to the specific agonist NMDA, and appropriate responses to the various antagonists.

The concentrations of Ultiva® tested in this study are well within the clinical range. According to the product information of Ultiva®, every 0.1-$\mu g \cdot kg^{-1} \cdot min^{-1}$ change in the intravenous infusion rate leads to a corresponding 2.5-ng/ml change in the blood concentration of Ultiva®.[30] Given a continuous infusion of 0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ and a plasma protein binding capacity of 70%,[30] the calculated free plasma concentration of remifentanil is 8.8 ng/ml (21 nM). In healthy adults, the EC_{50} of remifentanil was 19.9 ng/ml (48 nM), resulting in a comparable free plasma concentration.[1,31] These clinical concentrations would certainly be sufficient to stimulate NMDA receptors in our model. For the NR1A/2A combination, the EC_{50} of Ultiva® was 1.4 ng/ml (3.5 nM), well within the clinically found free plasma concentration.

The clinical preparation of Ultiva® contains glycine as an adjunct.[30] Glycine is an obligatory coagonist for NMDA receptors. Therefore, it seemed conceivable that glycine itself contributes to the stimulation by the Ultiva® preparation. However, our data imply that the effect of Ultiva® is independent of the glycine concentration present, suggesting that glycine itself does not contribute to the stimulating effect of Ultiva® on the NMDA receptor subunit combinations. *In vivo* glycine concentrations are significant and not appreciably affected by administered Ultiva®. The calculated plasma concentration of glycine expected after a 0.5-$\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion of Ultiva® is approximately 0.3 μM. Physiologic plasma concentrations (0.1 mM) in healthy adults are 350-fold greater; cerebrospinal concentrations (15 μM) are 50-fold greater.[32] Therefore, even if Ultiva® activation of NMDA signaling requires the presence of glycine, it would still be expected to do so *in vivo*.

To elucidate whether other synthetic opioids may have similar effects, we studied the structurally related opioid fentanyl in our model. Clinical studies as well as studies in rat pain models have shown that fentanyl also might be associated with the development of hyperalgesia.[33] However, in our model, fentanyl, even at high concentrations, did not stimulate NMDA receptors. This finding indicates that clinically observed acute opioid tolerance cannot be attributed solely to direct NMDA receptor activation by opioids and that the mechanism of action of Ultiva® on NMDA receptors seems to be selective. Because remifentanil and fentanyl have similar actions on opioid receptors, the lack of effect of fentanyl essentially rules out an indirect effect of remifentanil through endogenous opioid receptors in the oocyte and supports our findings with naloxone.

The different Ultiva® EC_{50} for NR1A/2A and NR1A/2B receptors suggests that the binding site for the compound is neither the glutamate nor the glycine binding site (because EC_{50} values for these agonists were not different between the receptor types). In agreement, we find that Ultiva® responses are inhibited neither by the glycine-site antagonist 7-CK nor by the glutamate-site antagonist D-AP5. In combination with the finding that the pore blocker MK-801 inhibits Ultiva® responses, this suggest that the compound directly activates the receptor, but through an allosteric mechanism. Although the difference in EC_{50} between NR1/2A and NR1/2B suggests the NR2 subunit as target, it is conceivable that the NR2 subunit might modulate allosteric sites on the NR1 subunit. Hence, the exact site of action on the receptor cannot be determined in detail from this study.

In summary, Ultiva®, in clinically relevant concentrations, stimulates recombinantly expressed human NMDA receptors. This effect is not shared by fentanyl and is not caused by glycine in our model.

The authors thank Carl Lynch III, M.D. (Chair, Department of Anesthesiology, University of Virginia, Charlottesville, Virginia), for his insightful comments on the results.

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