Anesthesiology 2005; 102:1235–41

Effects of Remifentanil on N-*methyl-*D*-aspartate Receptor*

An Electrophysiologic Study in Rat Spinal Cord

Emmanuel Guntz, M.D., Hélène Dumont, M.D.,* Céline Roussel, Ph.D.,† David Gall, Ph.D.,† François Dufrasne, Ph.D.,‡ Laetitia Cuvelier,§ David Blum, Ph.D.,‖ Serge N. Schiffmann, M.D., Ph.D.,# Maurice Sosnowski, M.D., Ph.D.***

Background: **Remifentanil hydrochloride contained in Ultiva® (GlaxoSmithKline, Genval, Belgium) has been incriminated in difficult postoperative pain management, promotion of hyperalgesia, and direct *N*-methyl-D-aspartate (NMDA) receptor activation, but the involved mechanisms have remained unclear. In the current study, the authors investigated the effects of remifentanil hydrochloride, with and without its vehicle, glycine, on the activation of NMDA receptors and the modulation of NMDA-induced current on neurons inside the lamina II from the dorsal horn of rat spinal cord.**

Methods: **To test these effects, whole cell patch clamp recordings were conducted on acute rat lumbar spinal cord slices. Considering that both components of Ultiva® (remifentanil hydrochloride and glycine) could be involved in NMDA receptor activation, experiments were performed first with remifentanil hydrochloride, second with glycine, and third with the two components within Ultiva®.**

Results: **Remifentanil hydrochloride does not induce any current, whereas 3 mM glycine induced a current that was abolished by the specific NMDA glutamate site antagonist D-2-amino-5-phosphonovalerate. Ultiva® (remifentanil hydrochloride with its vehicle, glycine) also evoked an inward current that was abolished by D-2-amino-5-phosphonovalerate and not significantly different from the glycine-induced current. Application of remifentanil hydrochloride potentiated the NMDA-induced inward current, and this potentiation was abolished by the μ-opioid receptor antagonist naloxone.**

Conclusion: **These results show that remifentanil hydrochloride does not directly activate NMDA receptors. The NMDA current recorded after application of Ultiva® is related to the presence of glycine. Induced NMDA current is potentiated by application of remifentanil hydrochloride through a pathway involving the μ-opioid receptor.**

CHRONIC administration of opioids has been shown to induce development of tolerance that may vary according to the drug efficacy.[1,2] It has been recognized that tolerance can also occur after acute opioid exposure in animals[3,4] and humans.[5,6] Furthermore, animal studies evoke a decreased pain threshold after repeated heroin administration[7] and fentanyl boluses.[8,9] These new concepts indicate that acute opioid tolerance may correspond to a lasting increase in pain sensitivity and not only to a decrease of the analgesic effects. This increase in pain sensitivity is related to the activation of pronociceptive pathways that lead to the diminution of the pain threshold and is clinically expressed as hyperalgesia.[7,8]

There is strong evidence that *N*-methyl-D-aspartate (NMDA) receptors play a major role in the central sensitization processes associated with hyperalgesia.[10] Recent animal[8,11] and human studies[12–16] confirmed this hypothesis, providing evidence that blocking NMDA receptors can prevent opioid-induced hyperalgesia.

Among opioids, remifentanil presents distinguishing characteristics compared with the others. It is a potent, short-acting opioid metabolized by plasma and tissue esterases. These interesting properties allow infusion of high doses during a short time period without compromising a predictable and rapid recovery.[17] However, recent human studies have demonstrated difficult postoperative pain management[18–21]; Ultiva® (GlaxoSmithKline, Genval, Belgium) was incriminated in promoting hyperalgesia.[16,22,23]

Two mechanisms regarding opioid activity on NMDA receptors have been considered: the direct activation of NMDA receptors and their potentiation *via* an intracellular pathway.

Direct activation by remifentanil hydrochloride of human NMDA receptors expressed in *Xenopus* oocytes was recently shown by Hahnenkamp *et al.*[24] Other μ-opioid agonists are known for increasing NMDA receptor activity *via* an intracellular pathway[25–27] involving protein kinase C.[28–32]

In this study, using the whole cell patch clamp technique on acute rat spinal cord slices, we investigated NMDA receptor activation on dorsal horn neurons by remifentanil hydrochloride and by Ultiva® (remifentanil hydrochloride with glycine as vehicle). Thus, we tested the two following hypotheses: first, the direct activation of NMDA receptor by remifentanil hydrochloride, with and without its vehicle glycine, and second, the potentiation of NMDA-induced current by remifentanil hydrochloride through a pathway involving μ-opioid receptors.

Materials and Methods

Materials

Type A porcine gelatin, glycine, HEPES, EGTA, glutamate and d-gluconic acid lactone were obtained from

* Resident, Department of Anesthesiology, Hôpital Universitaire Saint-Pierre, and Research Fellow, Laboratory of Neurophysiology, † Research Fellow, # Associate Professor and Chair, Laboratory of Neurophysiology, ‡ Research Fellow, Laboratory of Pharmaceutical Organic Chemistry, § Research Technician, ‖ Research Fellow, Laboratory of Experimental Neurosurgery, Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire, ** Professor, Department of Anesthesiology, Hôpital Universitaire Saint-Pierre, Université Libre de Bruxelles, Brussels, Belgium.

Received from the Laboratory of Neurophysiology, Université libre de Bruxelles, and Hôpital Saint-Pierre, Department of Anesthesiology, Université libre de Bruxelles, Brussels, Belgium. Submitted for publication November 29, 2004. Accepted for publication March 7, 2005. Supported by Fondation Erasme, Brussels, Belgium; Fond Médical Reine Elisabeth, Brussels, Belgium; Fond National de la Recherche Scientifique, Brussels, Belgium; Fond pour la Recherche dans l' Industrie et l' Agriculture, Brussels, Belgium; and Action de Recherche Concertée, Brussels, Belgium. Presented at the International Narcotics Research Congress, Kyoto, Japan, July, 21, 2004.

Address reprint requests to Dr. Guntz: Rue haute, 322, 1000 Brussels, Belgium. Address electronic mail to: eguntz@ulb.ac.be. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Sigma (Bornem, Belgium); NMDA, bicuculline, and D-2-amino-5-phosphonovalerate (D-AP5) were obtained from Tocris (Bristol, United Kingdom); strychnine was obtained from RBI (Bornem, Belgium); and tetrodotoxin was obtained from Alexis (Zandhoven, Belgium). Ultiva®, 1 mg, was obtained from GlaxoSmithKline (Genval, Belgium); naloxone was obtained from Bristol-Myers Squibb Pharma (Braine-l'Alleud, Belgium); and [^{3}H]-D,L(E)-2-amino-4-propyl-5-phosphono-3-pentanoic acid (CGP39653), 44.0 Ci/mmol, was obtained from NEN (Zaventem, Belgium).

Electrophysiology

Whole cell patch clamp recordings were conducted on 300-μm-thick lumbar spinal cord slices obtained from 10-day-old Wistar rats (Iffa Credo, Reims, Paris, France).

After being anesthetized with halothane, rats were killed by cervical dislocation, and a lumbar laminectomy was performed. The lumbar enlargement was removed, cleared of any remaining dura membrane, embedded in a 10% gelatin block, and glued to the stage of a vibrating microslicer (Leica, Wetzlar, Germany) with cyanoacrylate glue (Roth, Karlsruhe, Germany). The spinal tissue was immersed in ice-cold Krebs solution and saturated with 95% oxygen and 5% carbon dioxide throughout all of these procedures. Slices were maintained in an incubation chamber at room temperature for at least 1 h before recordings. Krebs solution used for this procedure had the following composition: 206 mM sucrose, 25 mM $NaHCO_3$, 25 mM D-(+) glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, and 2 mM $MgCl_2$.[33]

All efforts to avoid animal suffering were made according to the standards of the Institutional Ethical Committee of the School of Medicine of the Université Libre de Bruxelles (Brussels, Belgium).

Cells currents were recorded in the whole cell configuration of the patch clamp technique[34] at room temperature inside lamina II of the dorsal horn.

Cells from lamina II (substantia gelatinosa) were identified using a Zeiss (Oberkochen, Germany) Axioskop II FS MOT microscope. Because of its relatively high neuronal density, lamina II could be readily visualized at low magnification (×100). Lamina II could also be distinguished from lamina I characterized by a striated appearance.[35] At higher magnification (×400), morphologically distinct stalk and islet cells served as markers for the boundaries of lamina II, outer and inner, respectively.[36] Thus, the substantia gelatinosa contains three morphologic types of cells that can be visually identified: projection neurons on which recordings were performed and stalk and islet interneurons.

During the patch clamp recordings, slices were perfused with standard Krebs-Henseleit solution containing 125 mM NaCl, 25 mM $NaHCO_3$, 25 mM D-(+) glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 μM bicuculline, 0.5 μM strychnine, and 0.1 μM tetrodotoxin, with a pH of 7.4 (95% oxygen and 5% carbon dioxide). Bicuculline is used as a blocker of γ-aminobutyric acid type A receptor, strychnine is used as a blocker of glycine receptors, and tetrodotoxin is used as a blocker of sodium channel.

The following components were diluted into the former solution: 10 μM NMDA; 10 or 3,000 μM glycine; Ultiva® containing 50 μM remifentanil hydrochloride and 3,000 μM glycine; 50 or 3 μM remifentanil hydrochloride, 50 μM D-AP5, the specific NMDA glutamate site antagonist; and 20 μM naloxone, the μ-opioid receptor antagonist.

Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, United Kingdom) and had a resistance of 6–10 MΩ. The intracellular solution contained 130 mM Cs^+ gluconate, 6.8 mM CsCl, 0.1 mM EGTA, 10 mM HEPES, and 2 mM Mg_2ATP, with pH adjusted to 7.3 with NaOH and osmolarity adjusted to 305–310 mOsm with sucrose.

Whole cell currents were recorded with an EPC-8 amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany) at a membrane potential of −60 mV.

Data were obtained using the acquisition software Pulse (Heka) and were analyzed with IGOR software (WaveMetrics, Lake Oswego, OR). Passive cellular parameters were extracted in voltage clamp by analyzing current relaxation induced by a 10-mV step change from a holding potential of −60 mV as described previously.[37] Visually identified neurons were selected based on a low and stable leak current. Access resistance was monitored to ensure that recording conditions remained stable during the whole experiment. Neurons showing more than 15% modification of this access resistance were excluded.

Synthesis of Remifentanil Hydrochloride

Remifentanil conditioned without glycine is not available; purification of Ultiva® is difficult and exposes the component to degradations and contaminations. Therefore, we proceeded to the synthesis of remifentanil. Synthesis has been conducted following two general published procedures. The first intermediate, *i.e.*, 4-[(1-oxopropyl) phenylamine]-1-benzylpiperidine-4-carboxylic acid methyl ester, has been obtained in five steps from *N*-benzylpiperidinone.[38] After *N*-debenzylation,[39] the piperidine derivative has been reacted with methyl acrylate.[40] Purification by column chromatography afforded remifentanil base, which was further transformed into hydrochloride salt by addition of hydrochloric acid solution in diethylether. The solid was filtered and crystallized into isopropanol–diethylether.

The resulting component was controlled with nuclear magnetic resonance spectra recorded on a Bruker Avance 300-MHz spectrometer (Illkirch, France) with tetramethylsilane as internal standard, at 293 K. Infrared analysis was performed with a Shimadzu IR-470 spectrophotometer (Antwerp, Belgium). In dimethyl sulfoxide

(DMSO)-d_6, a mixture of conformers has been observed (equilibrium between a minor isomer and a major isomer, ratio 15:85.

^{1}H-nuclear magnetic resonance (DMSO-d_6): 11.36*– 11.02** (bs, 1H, NH^+), 7.68*–7.52 (m, 3H, aromatics), 7.34 (m, 2H, aromatics), 3.72 (s, 3H, OCH_3), 3.62 (s, 3H, OCH_3), 3.40 (m, 2H, $MeOOCCH_2C\underline{H}_2$), 3.25– 3.07 (m, 4H, H2–H6), 2.85 (t, 2H, J = 7.8 Hz, CH_2COO), 2.26–1.97 (m, 4H, H3–H5), 1.75 (q, 2H, J = 7.4 Hz, $C\underline{H}_2CH_3$), 0.8 (q, 3H, J = 7.3 Hz, $CH_2C\underline{H}_3$) ppm.

^{13}C-nuclear magnetic resonance (DMSO-d_6): 174*–172.9** (piperidine $\underline{C}OO$), 173.5*– 172.5** ($CH_2\underline{C}OO$), 170 ($\underline{C}ON$), 138.6*–137.8** (C1'), 130.5*–130** (C2'C6'), 129.4*–129.2** (C3'C5'), 128.7 (C4'), 59.9*–59.3** (C4), 52.3*–52** (piperidine $COO\underline{C}H_3$), 51.6 ($CH_2COO\underline{C}H_3$), 50.5**–49.8* (C2C6), 48.5**–46.7* ($N\underline{C}H_2CH_2$), 29.7 ($\underline{C}H_2CON$), 28 ($NCH_2\underline{C}H_2$), 27.9**–27.7* (C3C5), 8.7 ($\underline{C}H_3CH_2$) ppm.

IR (KBr disk): ν = 2920 (C-H), 2465 (N-H), 1724 (C = O ester), 1645 (C = O amide), 1438, 1367, 1247, 1206, 699 (aromatic) cm^{-1}. * Minor isomer; ** major isomer; no asterisk, both isomers.

In Vitro *Receptor Autoradiography*

Radioligand bindings were performed on 20-μm frozen spinal cord sections obtained from adult Wistar rats and mounted on gelatin-coated slides with increasing concentrations of remifentanil hydrochloride (0.1–100 μM). The sections were preincubated three times for 15 min each at room temperature in 50 mM Tris-HCl buffer, with a pH of 7.5, and incubated for 60 min at 4°C in buffer containing 10 nM [^{3}H]CGP39653. Nonspecific binding of the ^{3}H-labeled ligand was assessed by the addition of 100 μM L-glutamate. Slides were washed four times for 15 s in ice-cold buffer, dipped in ice-cold distilled water, and dried under a stream of cold air.

After completion of the procedures, slides were dried under a stream of cold air, exposed to tritium-sensitive screens, and revealed by a Cyclone storage phosphore system (PerkinElmer, Boston, MA). Analyses were performed using the public domain NIH image 1.61 program (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data are reported as mean ± SEM, and statistical comparisons were performed using paired or unpaired Student *t* tests for electrophysiologic results and analysis of variance for binding assay results ($P > 0.05$, not significant).

Results

Location of lamina II is facilitated, at low magnification (×100), by the entrance of the dorsal root and the macroscopic aspect of the spinal slices. At higher magnification (×400), morphologically distinct stalk and islet cells serves as markers for the location of the cells that are recorded.[36] Membrane capacitances (23.52 ± 1.96 GΩ) and resistances (2.07 ± 0.27 GΩ) of the 22 selected cells exhibit little cell-to-cell variation. This result underlines the ability of the method to select a homogenous cells population.

Experiments were designed to determine whether remifentanil hydrochloride is able to activate NMDA receptors directly. Ultiva® contains remifentanil hydrochloride and glycine as vehicle, and NMDA receptors require both glutamate and its coagonist, glycine, to be activated. These specific characteristics mean that both components of Ultiva® can be involved in NMDA receptor activation. Therefore, we planned to perform experiments first with remifentanil hydrochloride, second with glycine, and third with the two components within Ultiva®.

Experiments were performed with 50 μM remifentanil hydrochloride, considering the saturating effect obtained with Ultiva® in a previous study.[24] Given that a 1-mg Ultiva® vial contains 15 mg glycine as adjunct, a solution of Ultiva® containing 50 μM remifentanil also contains 3 mM glycine. Therefore, we performed a first group of experiments with 50 μM remifentanil hydrochloride, a second with 3 mM glycine, and a third with Ultiva® containing 50 μM remifentanil and 3 mM glycine.

No Direct Activation of NMDA Receptors by Remifentanil Hydrochloride

Whole cell current is first recorded in control conditions (standard Krebs-Henseleit solution). When the current is stable during 100 s, 50 μM remifentanil hydrochloride and 10 μM glycine are added to the solution perfusing bath, and the current is recorded during 400 s. Remifentanil hydrochloride does not induce any current variation (n = 5); the current recorded during the perfusion of remifentanil hydrochloride is similar to the one recorded during the control conditions (−14.69 ± 3.04 *vs.* −12.09 ± 2.61 pA; $P > 0.05$, n = 5; figs. 1A and B).

In accord with these results, the binding study conducted on spinal cord sections from adult rats shows that increasing concentrations (0.1–100 μM) of remifentanil hydrochloride do not modify the binding of [^{3}H]CGP39653 from the glutamate site (fig. 2). These results indicate that remifentanil hydrochloride seems to be devoid of NMDA receptor activator properties.

Direct Activation of NMDA Receptors by Glycine

Whole cell current is recorded using a similar protocol. When the current is stable, 3 mM glycine is added to the perfusing bath. Glycine induces an inward current (−94.53 ± 16.29 pA). When this current remains stable, 50 μM of the specific NMDA glutamate site antagonist D-AP5 is added to the bath. The glycine-induced current is abolished by application of D-AP5, revealing an NMDA current. There is a significant difference between the current induced by glycine and the one recorded during

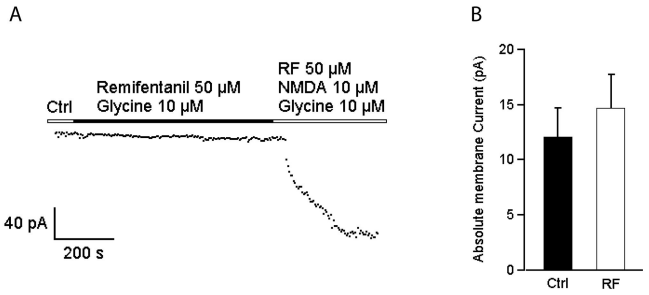

Fig. 1. (*A*) No direct activation of *N*-methyl-D-aspartate (NMDA) receptor by remifentanil hydrochloride. Representative trace of the recording of a single neuron during application of remifentanil. Whole cell current is first recorded in control conditions (Ctrl). Remifentanil hydrochloride (RF), 50 µM, and 10 µM glycine are added to the perfusing bath during 400 s. After this period, the presence of NMDA receptor is checked by addition of 10 µM NMDA. RF, 50 µM, does not induce any current, whereas application 10 µM NMDA and 10 µM glycine induce an NMDA current in the same cell. (*B*) There is no significant difference between the current recorded during control conditions and the one recorded during application of 50 µM RF (n = 5, $P > 0.05$).

the control condition (−94.53 ± 16.29 *vs.* −20.90 ± 5.56 pA; $P < 0.05$, n = 5). No significant difference between the current induced by glycine plus D-AP5 and the current recorded during control condition is observed (−45.74 ± 10.54 *vs.* −20.90 ± 5.56 pA; $P > 0.05$, n = 5; figs. 3A and B). These results show that 3 mM glycine alone is able to activate NMDA receptors in rat spinal cord. Ultiva® contains remifentanil and glycine. When the dilution of Ultiva® provides a 50 µM remifentanil hydrochloride concentration, the concentration of glycine is 3 mM. This concentration of remifentanil does not induce an NMDA current, whereas 3 mM glycine does induce this current.

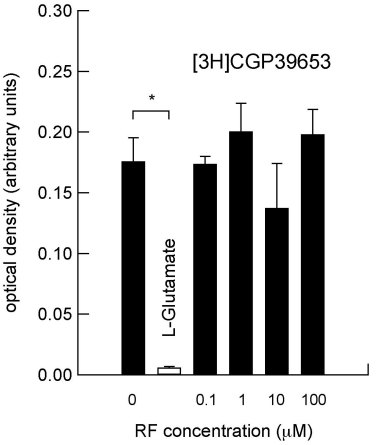

Fig. 2. No displacement of [^{3}H]CGP39653 by remifentanil hydrochloride. Binding assays do not show any displacement of [^{3}H]CGP39653 from its site after application of increasing concentrations of remifentanil hydrochloride (RF). There is no significant difference between the remifentanil hydrochloride concentrations (0–100 µM) regarding the optical density (analysis of variance, $P > 0.05$). Optical density values in the presence of the cold competitor L-glutamate are significantly different from the optical density measured with 0 µM RF (analysis of variance, * $P < 0.05$).

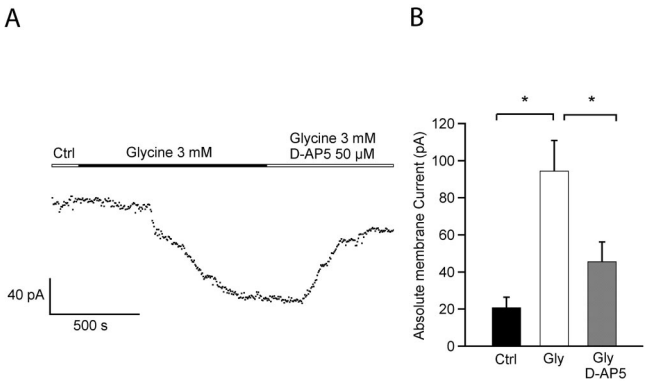

Fig. 3. (*A*) Direct activation of *N*-methyl-D-aspartate receptor by glycine (Gly). Representative trace of *N*-methyl-D-aspartate current induced by 3 mM glycine. Whole cell current is first recorded in control conditions (Ctrl). Glycine, 3 mM, added to the perfusing bath induces an inward current. When this current remains stable, 50 µM D-2-amino-5-phosphonovalerate (D-AP5) is added to the perfusing bath. The glycine-induced current is abolished by application of 50 µM D-AP5. (*B*) There is a significant difference between the current recorded during control conditions and the one recorded during application of 3 mM glycine (n = 5, * $P < 0.05$). There is no significant difference between the current recorded during application of D-AP5 and the one recorded during control conditions (n = 5, $P > 0.05$).

Direct Activation of NMDA Receptors by Ultiva®

Using a similar protocol, application of Ultiva® containing 50 µM remifentanil hydrochloride and 3 mM glycine induces an inward current (−96.42 ± 10.99 pA) that is abolished by application of 50 µM D-AP5, revealing an NMDA current.

There is a significant difference between the current induced by Ultiva® and the one recorded during the control condition (−96.42 ± 10.99 *vs.* −23.92 ± 3.16 pA; $P < 0.05$, n = 7). No significant difference between the current induced by Ultiva® plus D-AP5 and the current recorded during the control condition is observed (−39.43 ± 5.47 *vs.* −23.92 ± 3.16 pA; $P > 0.05$, n = 7; figs. 4A and B).

There is also no significant difference between the NMDA current induced by Ultiva® containing 50 µM remifentanil hydrochloride and glycine and the NMDA current induced by 3 mM glycine (−96.42 ± 10.99 pA, n = 7 *vs.* −94.53 ± 16.29 pA, n = 5; $P > 0.05$).

These results indicate that the NMDA current induced by Ultiva® preparation is related to the presence of glycine and that remifentanil hydrochloride by itself seems to be devoid of NMDA receptor activation properties.

Modulation of NMDA Receptor Activity by Remifentanil Hydrochloride through µ-Opioid Receptors

Modulation of NMDA receptors activity by remifentanil hydrochloride through the activation of µ-opioid recep-

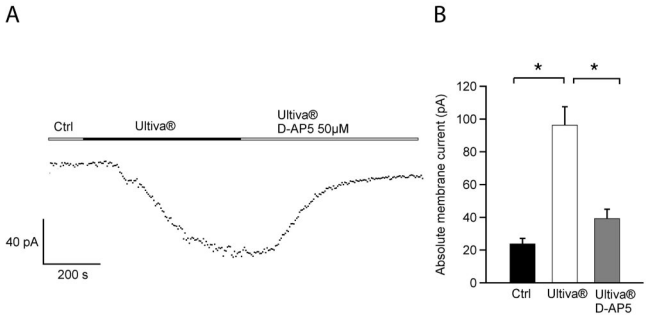

Fig. 4. (*A*) Direct activation of *N*-methyl-D-aspartate receptors by Ultiva®. Representative trace of *N*-methyl-D-aspartate current induced by application of Ultiva® containing 50 μM remifentanil hydrochloride and 3 mM glycine. Whole cell current is first recorded in control conditions (Ctrl). Ultiva® added to the perfusing bath induces an inward current. When this current remains stable, 50 μM D-2-amino-5-phosphonovalerate (D-AP5) is added to the perfusing bath. Ultiva®-induced current is abolished by application of 50 μM D-AP5. (*B*) There is a significant difference between the current recorded during control conditions and the one recorded during application of Ultiva® containing 50 μM remifentanil hydrochloride and 3 mM glycine (n = 5, * *P* < 0.05). There is no significant difference between the current recorded during application of D-AP5 and the one recorded during control conditions (n = 5, *P* > 0.05).

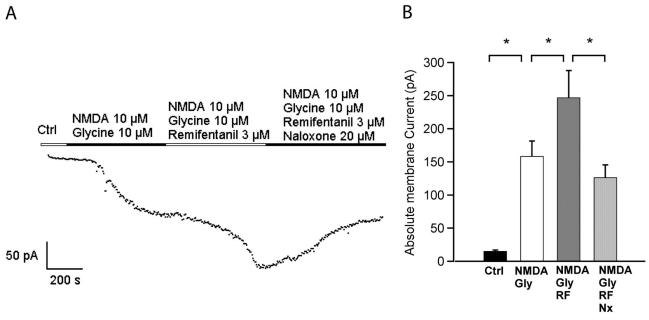

Fig. 5. (*A*) Modulation of *N*-methyl-D-aspartate (NMDA) receptors activation by remifentanil. Representative trace of the increase of NMDA-induced current by application of 3 μM remifentanil hydrochloride (RF). Whole cell current is first recorded in control conditions (Ctrl). NMDA, 10 μM, and 10 μM glycine (Gly) added to the perfusing bath induce an NMDA current. When this current remains stable, 3 μM remifentanil hydrochloride is added to the perfusing bath. When the resulting current remains stable, 20 μM naloxone (Nx) is added to the bath. Application of remifentanil increases NMDA current, and this increase is abolished by application of naloxone. (*B*) There is a significant difference between the NMDA-induced current and the NMDA-induced current in the presence of remifentanil hydrochloride (n = 5, * *P* < 0.05). There is no significant difference between the NMDA-induced current and the NMDA-induced current in the presence of remifentanil hydrochloride and naloxone (n = 5, *P* > 0.05).

tors, as described for other opioids,[27,28] was our second hypothesis. Therefore, we applied remifentanil hydrochloride on cells with preactivated NMDA receptors. The opioid agonist DAMGO has been described to increase NMDA receptors activity when applied within the micromolar range.[28] In accord with this observation, we started our experiments with 3 μM remifentanil hydrochloride.

N-methyl-D-aspartate receptors are first activated by 10 μM NMDA and 10 μM glycine. When this current remains stable, 3 μM remifentanil hydrochloride, added to the bath, increases the NMDA-induced inward current by 61.3 ± 13% (−158.76 ± 22.66 *vs.* −247.31 ± 40.45 pA; $P < 0.05$, n = 5). This increase is abolished by 20 μM of the μ-opioid receptor antagonist naloxone (−158.76 ± 22.66 *vs.* −126.63 ± 18.81 pA; $P > 0.05$, n = 5; figs. 5A and B).

These results indicate that remifentanil hydrochloride potentiates the NMDA current through a μ-opioid receptor pathway.

Discussion

The current study provides strong evidence that remifentanil hydrochloride does not directly activate NMDA receptors in rat dorsal horn neurons. The NMDA current recorded after application of Ultiva® is related to the drug vehicle glycine. In these neurons, the NMDA current is majored by application of remifentanil hydrochloride in the micromolar range, and this potentiation is abolished by the μ-opioid receptor antagonist naloxone. Therefore, remifentanil hydrochloride increases NMDA current, and this potentiation is mediated by μ-opioid receptor activation, probably through an intracellular pathway.

Application of remifentanil hydrochloride on rat dorsal horn neurons does not induce any current. The binding assay technique does not provide any evidence that remifentanil hydrochloride is able to bind to the glutamate sites of the NMDA receptor. Considering these latter results together with the patch clamp recordings, it is unlikely that remifentanil hydrochloride directly stimulates the NMDA receptor in rat spinal cord.

N-methyl-D-aspartate receptor is also activated in the presence of Ultiva® containing remifentanil hydrochloride and glycine. Considering the results of the experiments performed first with remifentanil hydrochloride and second with glycine, it seems that this current is related to the presence of glycine. NMDA receptors require both glutamate and its coagonist, glycine, to be activated, and the potentiation of NMDA receptor by glycine is specific for this receptor.[41] Previous studies in the dorsal horn of spinal cord showed that, in the presence of tetrodotoxin, bicuculline, and strychnine, nerves terminals spontaneously release quanta of glutamate. These small amounts of glutamate do not induce NMDA currents.[42] Nevertheless, when the concentration of glutamate remains unchanged, increasing concentrations of glycine induce increasing NMDA currents.[43] Therefore, a possible explanation would be that the small amount of endogenous glutamate present inside the spinal cord slices is sufficient to activate NMDA receptors with a 3 mM saturating concentration of glycine.

Moreover, the Ultiva®-induced current is abolished by the specific glutamate site antagonist D-AP5, suggesting

that remifentanil hydrochloride contained in Ultiva® cannot activate NMDA receptors through another site or mechanism; otherwise, the induced current would not be totally reversed. This result confirmed the first experiment performed with remifentanil hydrochloride alone.

We also show that application of remifentanil hydrochloride on dorsal horn neurons in the micromolar range potentiates the NMDA-induced current. These results are in accord with recent reports showing similar results with the μ-opioid agonist DAMGO.[27,28] This potentiation is reversed by the μ-opioid antagonist naloxone, indicating the role of μ-opioid receptor as a trigger of an intracellular pathway, able to positively regulate the activity of NMDA receptors. Therefore, during application of Ultiva® on dorsal horn neurons, glycine appears as a primer of NMDA receptor activation, whereas remifentanil by itself enhances this activation.

Glycine was recently described as an important NMDA receptor facilitator.[44] During acute pain, spillover of glycine from spinal cord interneurons causes NMDA receptor facilitation and contributes to the development of chronic pain. Intravenous glycine injection dose-dependently increases the concentration of glycine contained inside the cerebrospinal fluid,[45] and extracellular concentrations are below the levels required for maximal NMDA receptor activation.[46] In contrast, behavioral studies with rats showed that the infusion of glycine alone did not modify the pain threshold while it induced a transitory motor impairment and that infusion of remifentanil hydrochloride with its vehicle glycine did not modify the pain threshold compared with infusion of remifentanil hydrochloride.[47,48] It should not also be forgotten that glycine receptors are the major inhibitory receptors in spinal cord.[49]

Glycine contained in Ultiva® has not been incriminated as an NMDA receptor activator in a recent study conducted by Hahnenkamp *et al.*[24] on NMDA receptors expressed in *Xenopus* oocytes. Despite the absence of experiments using remifentanil hydrochloride out of the presence of glycine, these results suggest a direct allosteric activation of NMDA receptor by remifentanil hydrochloride. Differences with our results could be explained by the differences between the two models. Considering that the functional properties of NMDA receptors are modulated by the composition of the subunits,[50] relating data obtained with recombinant NMDA receptors to those obtained with native NMDA receptors is challenging. Moreover, the composition of the multiproteic complexes known to anchor and regulate NMDA receptors at the postsynaptic membrane plays also a critical role.[51] We must note here that the current study was conducted under physiologic conditions. Currents were recorded from neurons inside their environment. The opportunity to perform experiments with a pure and controlled preparation of remifentanil hydrochloride in the absence of glycine must also be noted.

In conclusion, remifentanil hydrochloride does not directly stimulate NMDA receptors in spinal cord. Ultiva® activates NMDA receptors, but this activation is related to the presence of glycine. Application of remifentanil hydrochloride increases the activity of NMDA receptors, and this potentiation is mediated through a μ-opioid receptor–initiated cascade.

References

1. Coombs DW, Saunders RL, Lachance D, Savage S, Ragnarsson TS, Jensen LE: Intrathecal morphine tolerance: Use of intrathecal clonidine, DADLE, and intraventricular morphine. ANESTHESIOLOGY 1985; 62:358–63
2. Sosnowski M, Yaksh TL: Differential cross-tolerance between intrathecal morphine and sufentanil in the rat. ANESTHESIOLOGY 1990; 73:1141–7
3. Kissin I, Brown PT, Robinson CA, Bradley EL Jr: Acute tolerance in morphine analgesia: Continuous infusion and single injection in rats. ANESTHESIOLOGY 1991; 74:166–71
4. Larcher A, Laulin JP, Celerier E, Le Moal M, Simonnet G: Acute tolerance associated with a single opiate administration: Involvement of N-methyl-D-aspartate-dependent pain facilitatory systems. Neuroscience 1998; 84:583–9
5. Guignard B, Bossard AE, Coste C, Sessler DI, Lebrault C, Alfonsi P, Fletcher D, Chauvin M: Acute opioid tolerance: Intraoperative remifentanil increases postoperative pain and morphine requirement. ANESTHESIOLOGY 2000; 93:409–17
6. Vinik HR, Kissin I: Rapid development of tolerance to analgesia during remifentanil infusion in humans. Anesth Analg 1998; 86:1307–11
7. Celerier E, Laulin JP, Corcuff JB, Le Moal M, Simonnet G: Progressive enhancement of delayed hyperalgesia induced by repeated heroin administration: A sensitization process. J Neurosci 2001; 1:4074–80
8. Celerier E, Rivat C, Jun Y, Laulin JP, Larcher A, Reynier P, Simonnet G: Long-lasting hyperalgesia induced by fentanyl in rats: Preventive effect of ketamine. ANESTHESIOLOGY 2000; 92:465–72
9. Laulin JP, Maurette P, Corcuff JB, Rivat C, Chauvin M, Simonnet G: The role of ketamine in preventing fentanyl-induced hyperalgesia and subsequent acute morphine tolerance. Anesth Analg 2002; 94:1263–9
10. Dickenson AH, Sullivan AF: Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology 1987; 26:1235–8
11. Celerier E, Laulin J, Larcher A, Le Moal M, Simonnet G: Evidence for opiate-activated NMDA processes masking opiate analgesia in rats. Brain Res 1999; 13:18–25
12. Guignard B, Coste C, Costes H, Sessler DI, Lebrault C, Morris W, Simonnet G, Chauvin M: Supplementing desflurane-remifentanil anesthesia with small-dose ketamine reduces perioperative opioid analgesic requirements. Anesth Analg 2002; 95:103–8
13. Kissin I, Bright CA, Bradley EL Jr: The effect of ketamine on opioid-induced acute tolerance: Can it explain reduction of opioid consumption with ketamine-opioid analgesic combinations? Anesth Analg 2000; 91:1483–8
14. Koppert W, Sittl R, Scheuber K, Alsheimer M, Schmelz M, Schuttler J: Differential modulation of remifentanil-induced analgesia and postinfusion hyperalgesia by *S*-ketamine and clonidine in humans. ANESTHESIOLOGY 2003; 99:152–9
15. Buvanendran A, McCarthy RJ, Kroin JS, Leong W, Perry P, Tuman KJ: Intrathecal magnesium prolongs fentanyl analgesia: A prospective, randomized, controlled trial. Anesth Analg 2002; 95:661–6
16. Angst MS, Koppert W, Pahl I, Clark DJ, Schmelz M: Short-term infusion of the mu-opioid agonist remifentanil in humans causes hyperalgesia during withdrawal. Pain 2003; 106:49–57
17. Egan TD, Lemmens HJ, Fiset P, Hermann DJ, Muir KT, Stanski DR, Shafer SL: The pharmacokinetics of the new short-acting opioid remifentanil (GI87084B) in healthy adult male volunteers. ANESTHESIOLOGY 1993; 79:881–92
18. Fletcher D, Pinaud M, Scherpereel P, Clyti N, Chauvin M: The efficacy of intravenous 0.15 versus 0.25 mg/kg intraoperative morphine for immediate postoperative analgesia after remifentanil-based anesthesia for major surgery. Anesth Analg 2000; 90:666–71
19. Yarmush J, D'Angelo R, Kirkhart B, O'Leary C, Pitts MC II, Graf G, Sebel P, Watkins WD, Miguel R, Streisand J, Maysick LK, Vujic D: A comparison of remifentanil and morphine sulfate for acute postoperative analgesia after total intravenous anesthesia with remifentanil and propofol. ANESTHESIOLOGY 1997; 87:235–43
20. Albrecht S, Fechner J, Geisslinger G, Maass AB, Upadhyaya B, Moecke H, Haigh C, Schuttler J: Postoperative pain control following remifentanil-based anaesthesia for major abdominal surgery. Anaesthesia 2000; 55:315–22
21. Bürkle H, Dunbar S, Van Aken H: Remifentanil: A novel, short-acting, mu-opioid. Anesth Analg 1996; 83:646–51
22. Hood DD, Curry R, Eisenach JC: Intravenous remifentanil produces withdrawal hyperalgesia in volunteers with capsaicin-induced hyperalgesia. Anesth Analg 2003; 97:810–5
23. Luginbuhl M, Gerber A, Schnider TW, Petersen-Felix S, Arendt-Nielsen L,

Curatolo M: Modulation of remifentanil-induced analgesia, hyperalgesia, and tolerance by small-dose ketamine in humans. Anesth Analg 2003; 96:726–32

24. Hahnenkamp K, Nollet J, Van Aken HK, Buerkle H, Halene T, Schauerte S, Hahnenkamp A, Hollmann MW, Strumper D, Durieux ME, Hoenemann CW: Remifentanil directly activates human *N*-methyl-D-aspartate receptors expressed in *Xenopus laevis* oocytes. ANESTHESIOLOGY 2004; 100:1531–7

25. Mao J, Price DD, Lu J, Mayer DJ: Antinociceptive tolerance to the mu-opioid agonist DAMGO is dose-dependently reduced by MK-801 in rats. Neurosci Lett 1998; 10:193–6

26. Martin G, Nie Z, Siggins GR: μ-Opioid receptors modulate NMDA receptor-mediated responses in nucleus accumbens neurons. J Neurosci 1997; 1:11–22

27. Kow LM, Commons KG, Ogawa S, Pfaff DW: Potentiation of the excitatory action of NMDA in ventrolateral periaqueductal gray by the mu-opioid receptor agonist, DAMGO. Brain Res 2002; 10:87–102

28. Chen L, Huang LY: Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. Nature 1992; 9:521–3

29. Chen L, Huang LY: Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a mu opioid. Neuron 1991; 7:319–26

30. Mayer DJ, Mao J, Price DD: The development of morphine tolerance and dependence is associated with translocation of protein kinase C. Pain 1995; 61:365–74

31. Smith FL, Javed RR, Elzey MJ, Dewey WL: The expression of a high level of morphine antinociceptive tolerance in mice involves both PKC and PKA. Brain Res 2003; 19:78–88

32. Celerier E, Simonnet G, Maldonado R: Prevention of fentanyl-induced delayed pronociceptive effects in mice lacking the protein kinase Cγ gene. Neuropharmacology 2004; 46:264–72

33. Green GM, Gibb AJ: Characterization of the single-channel properties of NMDA receptors in lamina I and II of the dorsal horn of neonatal rat spinal cord. Eur J Neurosci 2001; 14:1590–602

34. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth F: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981; 85–100

35. Chery N, de Koninck Y: Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. J Neurosci 1999; 19:7342–55

36. Bennett GJ, Abdelmoumene M, Hayashi H, Dubner R: Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. J Comp Neurol 1980; 15:809–27

37. D'Angelo E, Filippi GD, Rossi P, Taglietti V: Synaptic excitation of individual rat cerebellar granule cells *in situ*: Evidence for the role of NMDA receptors. J Physiol (Lond) 1995; 484:397–413

38. Van Daele PG, De Bruyn MF, Boey JM, Sanczuk S, Agten JT, Janssen PA: Synthetic analgesics: N-(1-[2-arylethyl]-4-substituted 4-piperidinyl) N-arylalkanamides. Arzneimittelforschung 1976; 26:1521–31

39. Olofson RA, Marz JT, Sene JP, Piteau M, Malfroot T: A new reagent for the selective, high-yield N-dealkylation of tertiary amines: Improved syntheses of naltrexone and nalbuphine. J Org Chem 1984; 49:2081–2

40. Feldman PL, James MK, Brackeen MF, Bilotta JM, Schuster SV, Lahey AP, Lutz MW, Johnson MR, Leighton HJ: Design, synthesis, and pharmacological evaluation of ultrashort- to long-acting opioid analgetics. J Med Chem 1991; 34:2202–8

41. Johnson JW, Ascher P: Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 1987; 325:529–31

42. Hori Y, Endo K, Takahashi T: Presynaptic inhibitory action of enkephalin on excitatory transmission in superficial dorsal horn of rat spinal cord. J Physiol 1992; 450:673–85

43. Kleckner NW, Dingledine R: Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. Science 1988; 241:835–7

44. Ahmadi S, Muth-Selbach U, Lauterbach A, Lipfert P, Neuhuber WL, Zeilhofer HU: Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. Science 2003; 27:2094–7

45. D'Souza DC, Gil R, Cassello K, Morrissey K, Abi-Saab D, White J, Sturwold R, Bennett A, Karper LP, Zuzarte E, Charney DS, Krystal JH: IV glycine and oral D-cycloserine effects on plasma and CSF amino acids in healthy humans. Biol Psychiatry 2000; 47:450–62

46. Globus MY, Busto R, Martinez E, Valdes I, Dietrich WD, Ginsberg MD: Comparative effect of transient global ischemia on extracellular levels of glutamate, glycine, and gamma-aminobutyric acid in vulnerable and nonvulnerable brain regions in the rat. J Neurochem 1991; 57:470–8

47. Buerkle H, Yaksh TL: Continuous intrathecal administration of shortlasting μ opioids remifentanil and alfentanil in the rat. ANESTHESIOLOGY 1996; 84:926–35

48. Buerkle H, Yaksh TL: Comparison of the spinal actions of the μ-opioid remifentanil with alfentanil and morphine in the rat. ANESTHESIOLOGY 1996; 84: 94–102

49. Bechade C, Sur C, Triller A: The inhibitory neuronal glycine receptor. Bioessays 1994; 16:735–44

50. Urch CE, Rahman W, Dickenson AH: Electrophysiological studies on the role of the NMDA receptor in nociception in the developing rat spinal cord. Brain Res Dev Brain Res 2001; 31:81–9

51. Rosenmund C, Westbrook GL: Calcium-induced actin depolymerization reduces NMDA channel activity. Neuron 1993; 10:805–14