Propofol Inhibits Phosphorylation of N-methyl-D-aspartate Receptor NR1 Subunits in Neurons

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Background: Anesthetics may interact with ionotropic glutamate receptors to produce some of their biologic actions. Cellular studies reveal that the ionotropic glutamate receptors, *N*-methyl-D-aspartate receptors (NMDARs), can be phosphorylated on their NR1 subunits at the C-terminal serine residues, which is a major mechanism for the regulation of NMDAR functions. It is currently unknown whether anesthetics have any modulatory effects on NMDAR NR1 subunit phosphorylation.

Methods: The possible effect of a general anesthetic propofol on phosphorylation of NR1 subunits at serine 897 (pNR1S897) and 896 (pNR1S896) was detected in cultured rat cortical neurons.

Results: Propofol consistently reduced basal levels of pNR1S897 and pNR1S896 in a concentration-dependent manner. This reduction was rapid as the reliable reduction of pNR1S896 developed 1 min after propofol administration. Pretreatment of cultures with the protein phosphatase 2A inhibitors okadaic acid or calyculin A blocked the effect of propofol on the NR1 phosphorylation, whereas okadaic acid or calyculin A alone did not alter basal pNR1S897 and pNR1S896 levels. In addition, propofol decreased tyrosine phosphorylation of protein phosphatase 2A at tyrosine 307, resulting in an increase in protein phosphatase 2A activity. In the presence of propofol, the NMDAR agonist–induced intracellular Ca^{2+} increase was impaired in neurons with dephosphorylated NR1 subunits.

Conclusions: Together, these data indicate an inhibitory effect of a general anesthetic propofol on NMDAR NR1 subunit phosphorylation in neurons. This inhibition was mediated through a signaling mechanism involving activation of protein phosphatase 2A.

ONE subtype of ionotropic glutamate receptors, *N*-methyl-D-aspartate receptors (NMDARs), is broadly distributed in the central nervous system.[1,2] Upon activation of ligand- and voltage-gated NMDARs, NMDAR channels mediate small cation ion influxes (Ca^{2+} and Na^+), which induce the excitatory postsynaptic current and/or modulate Ca^{2+}-sensitive intracellular signaling pathways/cascades.[3,4] As a heteromeric assembly, the NMDAR consists of obligatory NR1 subunits and modulatory NR2A–D subunits.[5,6] Cellular studies reveal that NR1 subunits can be phosphorylated, which represents a major mechanism for the regulation of NMDAR functions.[7–9] The NR1 shows phosphorylation at three distinct serine sites (897, 896, and 890) in its intracellular carboxy tail region.[10] Although the precise impact of the NR1 phosphorylation on NMDARs is poorly understood, available data show that (1) serine phosphorylation of the NR1 subunit is positively correlated to enhanced glutamatergic transmission in response to various experimental manipulations[11–14] and (2) phosphorylated NR1 prevents calmodulin from binding to the NR1 subunit and thereby inhibits the inactivation of NMDARs by calmodulin.[15] Therefore, serine phosphorylation represents a major posttranslational modification for NMDARs in their fundamental regulation of the receptor function. However, up to now, no attempt has been made to investigate a putative influence of a widely used general intravenous anesthetic propofol over the phosphorylation of NMDAR NR1 subunits at serine 897 and serine 896 in neuronal cells.

Protein phosphatase 2A (PP2A) is one of the major serine/threonine phosphatases that are highly expressed in cortical neurons.[16,17] As a negative regulator of protein phosphorylation, PP2A plays a key role in the dephosphorylation of various phosphoproteins (receptors, enzymes, structural proteins, and others) in relation to many fundamental cellular activities.[17] There is evidence showing that PP2A induces the dephosphorylation of serine 897 of the NMDAR NR1 subunit in the HEK-293 cell line.[18] Therefore, it is intriguing to investigate whether PP2A participates in the regulation of NR1 phosphorylation by propofol in neurons.

In this study, the role of propofol in regulating the phosphorylation of NMDAR NR1 subunits was investigated by examining effects of propofol on cellular levels of phospho-NR1 at 897 (pNR1S897) and phospho-NR1 at 896 (pNR1S896) in a well-characterized cortical neuronal culture model.

Materials and Methods

Primary Cortical and Striatal Neuronal Cultures

Standardized procedures were used to prepare primary cortical and striatal neuronal cultures from 18-day Wistar rat embryos or neonatal 1-day rat pups (Charles River, New York, NY).[19–21] Predominant neuronal cells were obtained using the procedures as evidenced by the fact that more than 90% of total cells were immunoreactive to the specific marker for neurons (microtubule-associated protein 2a + 2b), but not for glia (glial fibrillary acidic protein). Cells were cultured for 16–18 days before use. All procedures performed were approved by the Institutional Animal Care and Use Committee (Kan-

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sas City, Missouri) and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.[22]

Western Blot Analysis

Cell lysates from cultures were sonicated in a sample buffer (RIPA) containing 50 mM Tris-HCl, pH 7.5; 1% Nonidet P-40; 4% ionic detergent sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM dithiothreitol; 1 mM phenylmethanesulfonyl fluoride; 5 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM Na_3VO_4; and 1 mM NaF. Protein concentrations were determined with a Pierce BCA assay kit (Rockford, IL). The equal amount of protein (20 μg/20 μl/lane) was separated on NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk and 0.1% Tween 20) for 1 h. The blots were incubated in primary rabbit polyclonal antibodies (Upstate, Charlottesville, VA) against pNR1S897, pNR1S896, or NR1 at 1:1,000 overnight at 4°C. For detecting tyrosine phosphorylation of PP2A catalytic subunit, PP2A catalytic subunits were immunoprecipitated with rabbit PP2A catalytic subunit antibodies (Upstate). PP2A immunoprecipitates were then blotted with rabbit antiphosphotyrosine antibodies (Upstate) at 1:1,000 overnight at 4°C. This was followed by 1 h of incubation in goat anti-rabbit horseradish peroxidase–linked secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA) at 1:5,000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and captured into Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) were used for protein size determination. The density of immunoblots was measured using Kodak 1D Image Analysis software (Eastman Kodak Company), and all bands of phospho-NR1 were normalized to total NR1 amount and then to basal values. Values are expressed as percentages of basal values.

PP1 and PP2A Phosphatase Activity Assay

The activity was assessed by dephosphorylation of the synthetic PP1/PP2A-specific phosphopeptide (K-R-pT-I-R-R) using a serine-threonine phosphatase assay kit from Upstate (cat. No. 17–127) as described previously.[23] Following the manufacturer's instructions, cultures were scraped with 0.3 ml phosphatase extraction buffer containing 20 mM imidazole-HCl; 2 mM EGTA, pH 7.0; 10 μg/ml each of aprotinin, leupeptin, antipain, and soybean trypsin inhibitor; 1 mM benzamidine; and 1 mM phenylmethanesulfonyl fluoride. Cells were sonicated (10 s) and centrifuged at 2,000g for 5 min. Supernatants were used for phosphatase activity assays. To immunoprecipitate PP1 or PP2A, rabbit antibodies against PP1δ

(4 μg; Upstate) or mouse antibodies against the catalytic subunit of PP2A (4 μg; Upstate) were added, respectively, to a total of 150 μg/200 μl lysate, followed by addition of 50% protein A agarose/Sepharose bead slurry (Amersham) and incubation for 1–2 h at 4°C. Beads were washed three times with phosphate buffer solution (PBS), followed by one wash with assay buffer, before the phosphopeptide was added to a final concentration of 0.75 mM for incubation for 10 min at 30°C. The samples were centrifuged briefly, and an amount of 25 μl of sample was transferred into a well of a 96-well microtiter plate. A detection solution containing malachite green (100 μl) was added into each well and incubated for 10–15 min at room temperature for color development. A colorimetric assay was made by reading at 650 nm in a microtiter plate reader. Absorbance values of samples were compared with negative controls containing no enzyme and a freshly prepared phosphate standard. Specific activity was determined as the amount of picomoles of phosphate released per minute per microgram of protein.

Cell Viability Assay

Cell viability was measured using a double fluorescein diacetate/propidium iodide staining procedure.[24] Fluorescein diacetate is membrane permeable and freely enters intact cells, in which it is hydrolyzed by cytosolic esterase and converted to membrane-impermeable fluorescein with a green fluorescence, exhibited only by live cells. Propidium iodide is nonpermeable to live cells, but penetrating the membranes of dying/dead cells, showing red fluorescence. Cells were rinsed twice with 1× PBS and incubated at 37°C for 5 min with 1× PBS (0.5 ml/per well) containing 10 μg/ml fluorescein diacetate (Sigma, St. Louis, MO) and 5 μg/ml propidium iodide (Sigma). Cultures were washed once with PBS and examined under fluorescent light microscopy. The total numbers of viable cells stained by green fluorescein and dead cells stained by red propidium iodide were determined by counting cells in five random fields. Positive control was produced by treating cultures with kainic acid (500–1,000 μM, 24 h).

Intracellular Ca^{2+} Measurements

Intracellular Ca^{2+} ([Ca^{2+}]$_i$) measurements were performed according to our previous procedures.[21,23] Briefly, the culture was loaded with HEPES-buffered balanced salt solution aerated with 95% O_2–5% CO_2, pH 7.4, and contained 3 μM fura-2 AM (Sigma). The fluorescence of fura-2 was sequentially excited at 340 and 380 nm. Emitted lights were collected from the sample through a cooled, intensified charge-coupled device video camera (IC-110; Photon Technology International Inc., Lawrenceville, NJ). The fluorescent signal was measured at a single neuronal cell. Baseline was recorded for 3–5 min before bath application of drugs. The [Ca^{2+}]$_i$ concentra-

tion was calculated from ratios of the intensities of emitted fluorescence at two excitation wavelengths (F340/F380) with Northern Eclipse Image software (Empix Imaging, Inc., Mississauga, Ontario, Canada). When needed, fluorescence ratios (340/380) were converted to an absolute $[Ca^{2+}]_i$ concentration using the equation $[Ca^{2+}]_i = K_d(F_{min}/F_{max})[(R - R_{min})/(R_{max} - R)]$.

Drugs and Drug Treatments

Okadaic acid (OA), calyculin A, and bicuculline were purchased from Tocris Cookson Inc. (Ballwin, MO). Propofol (2,6 di-isopropylphenol) was purchased from Sigma. Cultures were washed with PBS and preincubated at 37°C in HEPES-buffered balanced salt solution consisting of 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 5.5 mM glucose, and 20 mM HEPES-KOH or HEPES-NaOH, pH 7.4, for 60 min. Cells were treated by adding drugs freshly made to the HEPES-buffered balanced salt solution. For N-methyl-D-aspartate (NMDA) treatments, $MgSO_4$ was omitted from, and 10 μM glycine was added to, the HEPES-buffered balanced salt solution. For extracellular Ca^{2+}-dependency studies, $CaCl_2$ was omitted from the HEPES-buffered balanced salt solution with substitution of a Ca^{2+}-chelator EGTA (2 mM). At the end of drug treatment, the cells were quickly washed with ice-cold PBS (pH 7.4; Ca^{2+}-free) and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. Drugs were dissolved in 1× PBS with or without dimethyl sulfoxide. Propofol was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.1%, at which dimethyl sulfoxide itself had no effect on NR1 phosphorylation at serine 897 or 896.

Statistics

The results are presented as mean ± SEM and were evaluated using one- or two-way analysis of variance, as appropriate, followed by Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of less than 0.05 were considered statistically significant.

Results

Selectivity of Phospho- and Site-specific Antibodies

Control experiments were conducted first to verify the selectivity of phospho- and site-specific antibodies raised against phospho-NR1 subunits on two specific serine residues. Omission of the primary antibodies against pNR1S897 or pNR1S896 in Western blot analysis produced no visible immunoreactive bands. Addition of primary antibodies produced a single band for each of two phospho- and site-specific antibodies in a molecular weight predicted for the size of NR1 subunits (120 kd) on protein extracts from the cortical cultures. When the
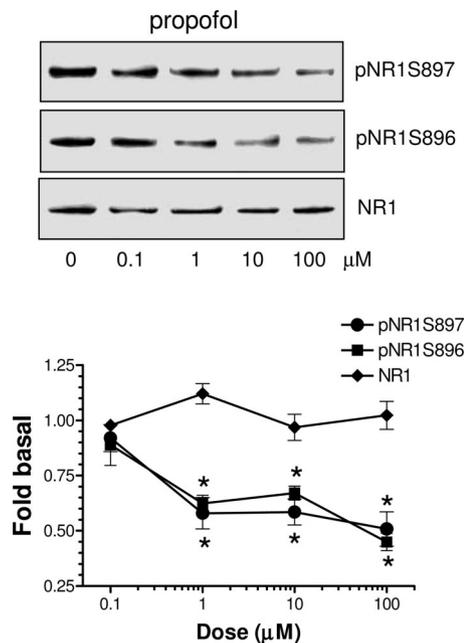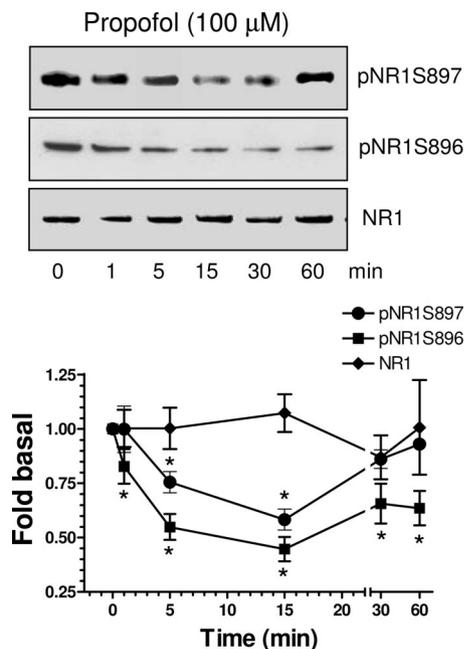

Fig. 1. Effects of propofol incubated at different concentrations on serine phosphorylation of N-methyl-D-aspartate receptor NR1 subunits in cultured rat cortical neurons. Propofol at four different concentrations (0.1, 1, 10, and 100 μM) was added to cultures for 15-min incubation. Representative immunoblots are shown above to the quantified data of three proteins analyzed from separate experiments (mean ± SEM, n = 10–11). Note that propofol incubation reduced cellular levels of pNR1S897 and pNR1S896, but not NR1 subunits, in a concentration-dependent fashion. The fold basal was determined by dividing band intensities obtained at different propofol concentrations by basal band intensity obtained at 0 μM propofol. * P < 0.05 versus basal levels.

extracts were pretreated with lambda protein phosphatase (Upstate; 400 U/ml for 4 h) for dephosphorylation of phosphorylated NR1 subunits, no immunoblot band was visualized for pNR1S897 and pNR1S896 from Western blot results with the phospho- and site-specific antibodies.

Propofol Reduces Serine Phosphorylation of NR1

We first set out to test whether propofol at different concentrations affects NR1 phosphorylation at serine residues 897 and 896. In cultured cortical neurons, propofol at four different concentrations was added to cultures and incubated for 15 min. We found that propofol reduced phosphorylation of NR1 subunits at two serine sites in a concentration-dependent fashion (fig. 1). At a concentration of 0.1 μM, no significant change in basal levels of pNR1S897 or pNR1S896 seemed to be induced by propofol. Starting at a low concentration (1 μM), propofol induced reliable decreases in both pNR1S897 and pNR1S896 levels. Greater decreases in the two phosphoproteins were induced by two higher concentrations of propofol (10 and 100 μM). In contrast to phosphoproteins, no significant changes were seen in cellular levels of NR1 after propofol application at all concentrations surveyed (fig. 1). Similar concentration-dependent inhibition of NR1 serine phosphorylation was
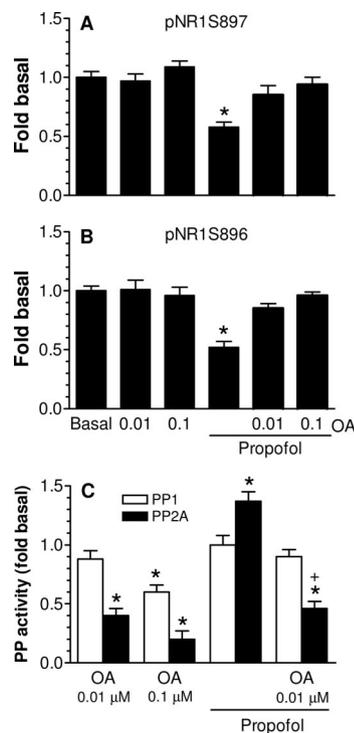
Fig. 2. Effects of propofol incubation at different durations on serine phosphorylation of *N*-methyl-D-aspartate receptor NR1 subunits in cultured rat cortical neurons. Propofol at 100 μM was added to cultures for different durations of incubation. Representative immunoblots are shown above to the quantified data of three proteins analyzed from separate experiments (mean ± SEM, n = 8–10). Note that propofol induced a time-dependent reduction of cellular levels of pNR1S897 and pNR1S896 without causing any change in levels of NR1 subunits. * *P* < 0.05 *versus* basal levels.

also observed in cultured striatal neurons (data not shown). There was no significant difference in cell viability between control and propofol-treated cultures as detected by the double fluorescein diacetate-propidium iodide staining.

Upon above demonstration of the inhibitory effect of propofol on NR1 phosphorylation, a complete time course evaluation was conducted to characterize the kinetics of propofol action. In cultured cortical neurons, propofol was applied at 100 μM for different durations. It was found that a time-dependent decrease in NR1 phosphorylation at either serine site occurred with no changes in basal NR1 levels (fig. 2). Although a reliable decrease in pNR1S897 was not shown 1 min after addition of propofol, a significant reduction of pNR1S896 levels was seen at this time point. Incubation of propofol for 5 or 15 min induced consistent decreases in both pNR1S897 and pNR1S896 levels. At 30 min, the reduced pNR1S897 returned to the normal level, whereas the significant reduction of pNR1S896 remained at this time as well as at 60 min.

Given the well-known actions of propofol on GABA$_A$ receptors, it is possible that propofol may affect NR1 phosphorylation indirectly *via* a GABAergic mechanism. To test this possibility, the effect of propofol on NR1 phosphorylation was examined in the presence of a GABA$_A$ receptor antagonist bicuculline. We found that

Fig. 3. Effects of the protein phosphatase 1/2A inhibitor okadaic acid (OA) on the propofol-induced inhibition of serine phosphorylation of *N*-methyl-D-aspartate receptor NR1 subunits in cultured rat cortical neurons. Note that propofol did not reduce basal levels of pNR1S897 (*A*) and pNR1S896 (*B*) in the presence of OA. OA (0.01 or 0.1 μM) was added 1 h before and during 15-min incubation of propofol (100 μM). Values are expressed as mean fold changes of basal levels (n = 8–10). (*C*) Effects of OA and propofol on protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) activity in cultured cortical neurons measured by the protein phosphatase activity assays (n = 5–6). OA (0.01 or 0.1 μM) or propofol (100 μM) was incubated for 1 h or 15 min before the activity assay, respectively. When coincubated, OA (0.01 μM) was added 1 h before and during 15-min incubation of propofol (100 μM). OA at 0.01 μM reduced PP2A but not PP1 activity. In addition, propofol increased PP2A but not PP1 activity, which was blocked by 0.01 μM of OA. * *P* < 0.05 *versus* basal levels. + *P* < 0.05 *versus* propofol alone.

the propofol (100 μM, 15 min)–induced reduction of pNR1S896 and pNR1S897 levels was not altered by 20 μM of bicuculline (data not shown). Therefore, the GABAergic transmission plays an insignificant role in the propofol effect on NR1 phosphorylation.

PP2A Is Involved in Mediating the Effect of Propofol

To explore the mechanism underlying the propofol effect, we tested the role of PP2A in this event. We found that pretreatment of cortical cultures with the PP2A selective inhibitor OA at either 0.01 or 0.1 μM blocked the propofol (100 μM)–induced reduction of pNR1S897 levels (fig. 3A). Similarly, OA prevented the propofol (100 μM)–induced reduction of pNR1S896 levels (fig. 3B). OA itself did not affect basal levels of pNR1S897, pNR1S896, and NR1. Another commonly used PP2A inhibitor, calyculin A, was also tested for its effects on

the two phosphoproteins in the same protocol for OA. In the presence of calyculin A (0.1 μM), 100 μM propofol no longer induced a significant change in either pNR1S897 or pNR1S896 levels (data not shown). These results indicate a significant link of PP2A in processing propofol-induced dephosphorylation of NR1 serine phosphorylation.

To test the efficacy and selectivity of OA in this culture system, the PP1 and PP2A phosphatase activity was assayed in cultures treated with OA at different concentrations for 15 min. From figure 3C, OA at 0.01 μM, a dose that blocked the propofol effect, reduced PP2A but not PP1 activity. At 0.1 μM, OA reduced PP1 and, to a greater extent, PP2A activity. Interestingly, propofol (100 μM) increased PP2A activity without significantly affecting PP1 activity. In the presence of OA (0.01 μM), PP2A activity obtained after propofol treatment was not significantly different from that observed after OA treatment alone at 0.01 μM. These data suggest a stimulatory effect of propofol on PP2A, which results in dephosphorylation of NR1 subunits in response to exposure to propofol.

Propofol Reduces Tyrosine Phosphorylation of PP2A

The catalytic subunit of PP2A undergoes phosphorylation at Tyr[307] of its conserved C-terminus *in vitro* and in living cells, which controls phosphatase activity of PP2A in an inhibitory manner.[24–30] To determine whether the effect of propofol on PP2A activity is related to PP2A tyrosine phosphorylation, we tested the effect of propofol on tyrosine phosphorylation of PP2A in cultured cortical neurons. Tyrosine phosphorylation of the PP2A catalytic subunit was tested in PP2A immunoprecipitates using immunoblots with antiphosphotyrosine antibodies. Propofol (100 μM, 15 min) decreased the basal level of tyrosine phosphorylated PP2A by more than 50%, without affecting basal levels of PP2A (fig. 4). Moreover, propofol (100 μM incubated for 1 or 5 min) induced a rapid decrease in tyrosine phosphorylation of PP2A (data not shown) similar to the kinetics of propofol-induced dephosphorylation of pNR1S897/pNR1S896 (fig. 2). These results indicate that propofol reduces the tyrosine phosphorylation of the PP2A catalytic subunit without changing cellular levels of PP2A, which in turn results in potentiation of phosphatase activity of PP2A.

Propofol Attenuates NMDAR-mediated Ca²⁺ Influx

To determine whether the dephosphorylated NMDAR NR1 subunits by propofol have any impact on NMDAR-mediated functions, we tested the effect of propofol on NMDAR-mediated intracellular Ca^{2+} increases in cultured cortical neurons. A typical rapid increase in $[Ca^{2+}]_i$ was induced after addition of NMDA (50–100 μM) into the culture as detected by somatic fura-2 ratio fluorescent measurements (fig. 5), which was blocked by the open channel blocker MK801 (0.1 μM) or removing
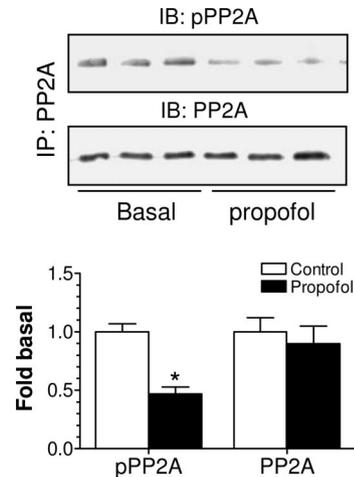

Fig. 4. Effects of propofol on the tyrosine phosphorylation of protein phosphatase 2A (PP2A) catalytic subunit in cultured rat cortical neurons. Representative immunoblots (IBs) are shown above to the quantified data of phospho-PP2A (pPP2A) and PP2A immunoreactivity analyzed from separate experiments (mean ± SEM, n = 5). Propofol decreased the level of pPP2A but not PP2A. PP2A in samples were precipitated through immuno-precipitation (IP) with rabbit antibodies against the PP2A catalytic subunit, and pPP2A levels in PP2A precipitates were then detected through immunoblots with antiphosphotyrosine antibodies. * P < 0.05 *versus* basal levels.

extracellular Ca^{2+} ions (data not shown), confirming an NMDAR-mediated Ca^{2+} increase. In the presence of propofol (100 μM, 15 min), NMDA (50 μM) was still able to induce a significant increase in $[Ca^{2+}]_i$ as compared with basal levels (fig. 5). However, the amplitude of Ca^{2+} increase was significantly smaller than that observed in the absence of propofol (fig. 5). Propofol itself had no effect on basal levels of intracellular Ca^{2+} (fig. 5). Furthermore, in the presence of both OA (0.01 μM) and propofol (100 μM), NMDA induced a Ca^{2+} increase which was insignificantly different from that seen after application of NMDA alone (fig. 5). These results indicate that reduced phosphorylation of NR1 by propofol may impair the NMDAR-mediated intracellular Ca^{2+} increase.
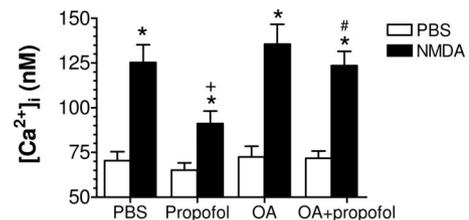

Fig. 5. Changes in intracellular calcium levels in cultured cortical neurons after bath application of *N*-methyl-D-aspartate (NMDA) in the absence or presence of propofol or okadaic acid (OA). The $[Ca^{2+}]_i$ levels were measured by somatic fura-2 ratio fluorescence. In the presence of propofol, NMDA induced a significantly less increase in $[Ca^{2+}]_i$ levels. Propofol (100 μM) was incubated 15 min before addition of NMDA (50 μM). The values are expressed as mean fold changes of basal levels in terms of the peak amplitude of Ca^{2+} responses measured within 30 s after the start of NMDA treatment from 16–24 neurons. * P < 0.05 *versus* basal levels. + P < 0.05 *versus* NMDA given in the absence of propofol. # P < 0.05 *versus* NMDA given in the presence of propofol.

Discussion

The current study investigated effects of a general anesthetic propofol on NMDAR NR1 subunit phosphorylation in cultured neurons. We found that propofol reduced NR1 phosphorylation at serine 897 and 896. The reduction seems to be mediated by activation of PP2A because (1) propofol reduced tyrosine phosphorylation of PP2A, which resulted in enhancement of phosphatase activity of PP2A, and (2) the PP2A inhibitors blocked the propofol-induced reduction of NR1 phosphorylation. Finally, the dephosphorylation of NR1 subunits seen after propofol may lead to a reduced intracellular Ca^{2+} rise induced by activation of NMDARs with NMDA. These results reveal that propofol possesses the ability to negatively modulate NMDAR NR1 phosphorylation *via* a mechanism involving PP2A activation.

An interesting finding in this study is that a PP2A-dependent mechanism is involved in mediating the propofol effect on NR1 subunit phosphorylation. Several lines of evidence support the role of PP2A. First, the PP2A inhibitors blocked the reduction of NR1 phosphorylation induced by propofol. Second, phosphatase activity of PP2A was enhanced in response to propofol exposure. And last, in an effort to unravel how propofol enhances PP2A activity, we found that propofol reduced tyrosine phosphorylation of PP2A. Because tyrosine phosphorylation suppresses phosphatase activity of PP2A, the reduction of tyrosine phosphorylation by propofol could increase PP2A activity. Together, the results here favor a signaling model that propofol initiates its effects by reducing tyrosine phosphorylation of PP2A, which activates PP2A to dephosphorylate NR1 subunits. Although the detailed interaction between PP2A and NR1 subunits is unclear, one study has revealed that PP2A can bind to the NMDAR NR3 subunit to dephosphorylate NR1 at serine 897.[18] In addition to PP2A, protein kinase A and protein kinase C phosphorylates serine 897 and 896, respectively.[10] Therefore, propofol may alter NR1 phosphorylation *via* a signaling mechanism involving these protein kinases.

Okadaic acid and calyculin A are the selective PP2A inhibitors, but they inhibit PP1 as well at the relatively high doses. To differentiate the relative importance of PP2A and PP1 in regulating NR1 phosphorylation, effects of the two inhibitors were tested at a dose (0.01 μM) that inhibited PP2A but not PP1 activity. We found that the inhibitors at this dose blocked propofol-induced reduction of NR1 phosphorylation. Therefore, the two inhibitors are believed to inhibit PP2A rather than PP1 to antagonize the effect of propofol. In support of this, propofol was found to selectively enhance PP2A but not PP1 activity in this study.

The catalytic subunit of PP2A can be phosphorylated on Tyr[307] *in vivo* and *in vitro* by many receptor and nonreceptor tyrosine kinases.[25-31] As an important neg-

ative regulatory mechanism, tyrosine phosphorylation inactivates the phosphatase. In this study, we found that propofol reduced constitutive tyrosine phosphorylation of PP2A. Therefore, propofol basically acts as an activator of PP2A to up-regulate phosphatase activity of PP2A. How propofol activates PP2A is unknown because of the current lack of studies in this area. Future studies are needed to investigate whether propofol activates PP2A *via* inhibiting tyrosine kinases that tonically drives tyrosine phosphorylation of PP2A or stimulating PP2A dephosphorylation on the tyrosine site.

Although PP2A is activated to dephosphorylate NR1 in response to propofol administration, this phosphatase is not seemingly involved in the regulation of basal NR1 phosphorylation. This is evidenced by the finding that the PP2A inhibitor OA did not alter basal levels of phosphorylation of NR1 at serine 897 and 896. Basal serine 897 and 896 phosphorylation is catalyzed by protein kinase A and protein kinase C, respectively.[10] Therefore, under normal conditions, protein kinases predominantly control serine phosphorylation of NR1 with a minimal influence from the phosphatase. Propofol may therefore be able to use-dependently counteract the driving force of protein kinases by augmenting the influence of phosphatase, leading to declined phosphorylation (dephosphorylation) of NR1.

To investigate whether dephosphorylation of NR1 subunits has any impact on NMDAR functions, we tested an NMDA-triggered Ca^{2+} rise in the presence of propofol. Noticeably, the NMDA-induced Ca^{2+} rise was reduced in neurons exposed to propofol. This seems to support that propofol exerts an inhibitory effect on an NMDAR-mediated event, although a question as to whether this inhibition is directly related to the dephosphorylation of the NR1 subunit remains to be elucidated experimentally.

It is unclear whether the inhibition of NMDAR NR1 phosphorylation contributes to any specific biologic action of propofol. Although the reduction of NR1 phosphorylation was induced by propofol at the low concentrations (1–10 μM), which are comparable to the clinically relevant concentrations (approximately 5–28 μM) for maintaining general anesthesia,[32] propofol is less likely to produce anesthesia through an NMDAR-dependent mechanism. This is because a large number of reports have documented that propofol produces anesthesia *via* enhancing GABAergic transmission. For example, the GABA$_A$ receptor antagonist bicuculline or picrotoxin completely reversed propofol anesthesia.[33-35] A site-directed mutation in the GABA$_A$ receptor β3 subunit also abolished the anesthetic effect of propofol.[36] In contrast to the well-defined role of GABA receptors, little evidence has shown a reliable role of NMDARs in propofol anesthesia. Two studies show an inhibitory effect of propofol on the NMDAR-mediated current *in vitro*.[37,38] However, in these studies, propofol had to be

applied at supra–clinically relevant concentrations to observe the inhibition of the NMDAR current. Future studies will have to be performed to elucidate the possible contribution of the inhibition of NR1 phosphorylation to other biologic actions of propofol.

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