Modulation of AMPA receptor GluR1 subunit phosphorylation in neurons by the intravenous anaesthetic propofol

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Background. The ionotrophic glutamate receptor is a potential molecular site in the central nervous system that general anaesthetics may interact with to produce some of their biological actions. Protein phosphorylation has been well documented to occur in the intracellular C-terminal domain of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of glutamate receptors, which represents a pivotal mechanism for the post-translational modulation of AMPA receptor functions. In this study, we investigated a possible influence of an i.v. anaesthetic agent propofol on the phosphorylation of AMPA receptor GluR1 subunits in cultured neurons.

Methods. The effect of propofol on phosphorylation of GluR1 subunits at serine 831 and 845 was assayed in cultured rat striatal and cortical neurons by western blot with phospho- and site-specific antibodies.

Results. Propofol consistently elevated phosphorylation of GluR1 subunits at the C-terminal serine 845 site in both striatal and cortical neurons. The elevation in phosphorylation was concentration-dependent and started at a low concentration (3 μM). This increase in serine 845 phosphorylation was rapid and sustained during the entire course of propofol exposure. In contrast to serine 845, phosphorylation of GluR1 at serine 831 was not altered by propofol in striatal and cortical neurons. Total GluR1 abundance remained unchanged in response to propofol incubation.

Conclusions. These data indicate that propofol possesses the ability to upregulate AMPA receptor GluR1 subunit phosphorylation at a specific serine 845 site in neurons and provide evidence supporting the AMPA receptor as a molecular target for general anaesthetics.

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at two distinct serine sites (831 and 845) in its intracellular carboxy tail region. The phosphorylation of these two sites is specifically driven by different kinases: protein kinase C (PKC) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) on serine 831 and protein kinase A (PKA) on serine 845. Since phosphorylation of serine 831 by CaMKII or serine 845 by PKA was found to augment AMPA receptor-mediated currents in transfected HEK cells, the phosphorylation status at either site is believed to have a significant influence over AMPA receptor function.

The widely used i.v. general anaesthetic propofol affects a broad variety of molecular targets in the central nervous system. Among these targets is the AMPA receptor. Available data show that propofol, although at a relatively high concentration range (0.1 or 0.2 to 1 mM), alters current responses of homomeric GluR1 receptors or heteromeric GluR1/GluR2 receptors to AMPA in transfected Xenopus oocytes or HEK-293 cells. However, no attempt to date has been made to investigate a possible effect of this anaesthetic on phosphorylation status of AMPA glutamate receptors in neuronal cells. Given a recent finding that propofol inhibited phosphorylation of the N-methyl-D-aspartate (NMDA) receptor NR1 subunit in brain cells and the NMDA receptor-mediated activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinases, it is important to examine whether propofol has any effect on phosphorylation status of the AMPA receptor.

In this study, we therefore evaluated the role of propofol in regulating phosphorylation of AMPA receptor GluR1 subunits. Changes in cellular levels of phospho-GluR1 at serine 831 (pGluR1-Ser831) and phospho-GluR1 at serine 845 (pGluR1-Ser845) after propofol administration were monitored in a well-characterized striatal and cortical neuronal culture model with phospho-site-specific antibodies.

**Methods**

**Primary striatal and cortical neuronal cultures**

Primary striatal and cortical neuronal cultures from 18 day Wistar rat embryos or neonatal 1 day rat pups (Charles River, New York, NY, USA) were prepared according to standardized procedures in this laboratory. Predominant neuronal cells were obtained using the procedures as evidenced by the fact that >90% of total cells were immunoreactive to the specific marker for neurons (microtubule-associated protein-2a/2b), but not 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 (Kansas City, MO, USA) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Western blot analysis**

Cell lysates from cultures were sonicated in sample buffer (RIPA) containing 50 mM Tris–HCl, pH 7.5, 1% Non-ident P-40, 4% ionic detergent sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 μg ml−1 each of aprotinin, leupeptin, and pepstatin, 1 mM Na2VO4, and 1 mM NaF. Concentrations of proteins were determined with a Pierce BCA assay kit (Rockstate, IL, USA). An equal amount of protein (usually 20 μg 20 μL−1 lane−1) was separated on NuPAGE Novex 4–12% gels (Invitrogen, Carsbad, CA, USA). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (5% non-fat dry milk and 0.1% Tween 20) for 1 h. The blots were incubated in primary rabbit polyclonal antibodies against pGluR1-Ser831 (Upstate, Charlotteville, VA, USA), pGluR1-Ser845 (Chemicon, Temecula, CA, USA), GluR1 (Upstate), or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500–1000 overnight at 4°C. This was followed by 1 h incubation in goat anti-rabbit horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and captured using a Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA, USA) and MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software (Eastman Kodak Company), and all bands of phospho-GluR1 were normalized to total GluR1 and then to basal values. Data are expressed as percentages of basal values. Actin levels in each sample were detected simultaneously as equal protein loading controls.

**Cell viability assay**

Cell viability was measured using a double fluorescein diacetate–propidium iodide staining procedure. Fluorescein diacetate is membrane permeable and freely enters intact cells, in which it is hydrolysed by cytosolic esterase and converted to membrane-impermeable fluorescein with a green fluorescence, exhibited only by live cells. Propidium iodide is non-permeable to live cells, but able to penetrate 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−1 of fluorescein diacetate (Sigma, St Louis, MO, USA) and 5 μg ml−1 of 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. A positive control was produced by treating cultures with kainic acid (500–1000 μM, 24 h).

**Drugs and drug treatments**

Propofol (2,6-di-isopropylphenol) was purchased from Sigma. Cultures were washed with PBS and pre-incubated at 37°C in HEPES-buffered balanced salt solution consisting of (mM) 154 NaCl, 5.6 KCl, 2 CaCl₂, 2 MgSO₄, 5.5 glucose, and 20 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. At the end of drug treatment, the cells were quickly washed with ice-cold PBS (pH 7.4; Ca²⁺-free) and immediately placed on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. Propofol was dissolved in dimethyl sulphoxide as described previously.¹¹ ¹² The final concentration of dimethyl sulphoxide was ≤0.1% at which dimethyl sulphoxide itself had no effect on GluR1 phosphorylation at serine 831 or 845.

**Statistics**

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

**Results**

**Selectivity of phospho- and site-specific antibodies**

We first carried out a series of control experiments to verify the selectivity of phospho- and site-specific antibodies raised against phospho-GluR1 subunits on two specific serine residues. Omission of the primary antibodies against pGluR1-Ser831 or pGluR1-Ser845 in western blot analysis produced no visible immunoreactive bands. The addition of primary antibodies produced a single band for each of two phospho- and site-specific antibodies at a molecular weight predicted for the size of GluR1 subunits (106 kDa) on protein extracts from striatal cultures (Fig. 1). When the extracts were pretreated with lambda protein phosphatase (400 units ml⁻¹, 4 h) which induced dephosphorylation of phosphorylated GluR1 subunits (L3 and L4).

**Effects of propofol on serine phosphorylation of GluR1: a concentration–response study**

We first conducted experiments in cultured striatal neurons to test whether propofol at different concentrations alters GluR1 phosphorylation at serine residues 831 and 845.

Propofol at five different concentrations (0.1, 1, 3, 10, or 30 μM) was added to cultures and incubated for 5 min before culture/protein collection. We found that propofol did not affect GluR1 phosphorylation at serine 831, but elevated GluR1 phosphorylation at serine 845 in a concentration-dependent fashion (Fig. 2). At the two lower concentrations (0.1 and 1 μM), propofol caused no significant change in basal levels of pGluR1-Ser845. At 3 μM, propofol induced a reliable increase in pGluR1-Ser845 levels. Greater increases in this phosphoprotein were seen at the two higher concentrations of propofol (10 and 30 μM). In contrast to pGluR1-Ser845, cellular levels of GluR1-Ser831 were not significantly affected by propofol application at all concentrations used (Fig. 2). Similarly, total levels of GluR1 and actin did not show any changes in response to propofol administration (Fig. 2). There was no significant difference in cell viability between control and propofol-treated cultures as detected by the double fluorescein diacetate–propidium iodide staining.

We next conducted the same concentration–response experiments in cultured cortical neurons. Like the response of GluR1 phosphorylation at serine 831 in cultured striatal neurons, phosphorylation at this site was not responsive to propofol exposure in cortical neurons (Fig. 3). A concentration-dependent increase in GluR1 serine phosphorylation was only observed at serine 845 in these neurons (Fig. 3). Total levels of GluR1 subunits and actin protein remained unchanged after propofol application (Fig. 3).

**Effects of propofol on serine phosphorylation of GluR1: a time-course study**

After demonstration of the facilitatory effect of propofol on GluR1 phosphorylation at serine 845, a complete
time-course was constructed to characterize the kinetics of the propofol effect. In cultured striatal neurons, propofol was applied at 3 mM for different durations (1, 2, 5, 15, or 30 min). Representative immunoblots are shown above the quantified data of four proteins analysed from separate experiments [mean (SEM), n = 8–10]. Note that propofol incubation enhanced cellular pGluR1-Ser845, but not pGluR1-Ser831 and GluR1 levels, in a concentration-dependent fashion. Fold basal was determined by dividing band intensities obtained at different propofol concentrations by basal band intensity obtained at 0 µM of propofol. *P < 0.05 vs basal levels.

Fig 2 Effects of propofol incubation on serine phosphorylation of GluR1 subunits in cultured rat striatal neurons. Propofol at five different concentrations (0.1, 1, 3, 10, and 30 mM) was added to cultures for 5 min. Representative immunoblots are shown above the quantified data of four proteins analysed from separate experiments [mean (SEM), n = 8–10]. Note that propofol incubation enhanced cellular pGluR1-Ser845, but not pGluR1-Ser831 and GluR1 levels, in a concentration-dependent fashion. Fold basal was determined by dividing band intensities obtained at different propofol concentrations by basal band intensity obtained at 0 µM of propofol. *P < 0.05 vs basal levels.

Discussion

This study investigated the effect of an i.v. anaesthetic propofol on AMPA receptor GluR1 subunit phosphorylation in cultured rat neurons. It was found that propofol increased phosphorylation of GluR1 at serine 845 whereas it has no such effect on serine 831. Since propofol did not alter total cellular GluR1 levels, the increased portion of phosphorylated GluR1 is believed to be a result from an increase in the phosphorylation process at serine 845 and not from an increase in total protein abundance. Propofol can induce a sustained upregulation of serine 845 phosphorylation when incubated for up to 30 min. These results provide evidence for a notion that i.v. anaesthetic drugs have the ability to modulate central glutamatergic transmission through adjusting the phosphorylation status of AMPA receptors.

There are substantial and growing reports about the actions of general anaesthetics on a variety of neurotransmitter-gated ion channels, with the greatest attention being focused on inhibitory GABA<sub>A</sub> receptors. On the other hand, when compared with GABA receptors, glutamate receptors, the most important class of excitatory neurotransmitter-gated receptor channels, have received relatively less attention as to whether they are a molecular target for anaesthetics in the central nervous system. In this study, we discovered that propofol is capable of regulating the phosphorylation status of AMPA receptors in neurons. This regulation was characterized in several aspects. First, it was concentration-dependent, and noticeably propofol was effective at a relatively low concentration (3 µM). Secondly, the effect of propofol on GluR1 phosphorylation was time-dependent. This effect was relatively rapid and maintained in the presence of propofol.
These kinetics are in accordance with the actions of propofol in producing clinical effects in vivo. Thirdly, propofol produced a parallel increase in serine 845 phosphorylation in both striatal and cortical neurons indicating that propofol can affect GluR1 phosphorylation in broad brain areas. Finally, the effect of propofol was selective between serine 845 and serine 831 since propofol increased serine 845 phosphorylation while leaving serine 831 unaffected. The serine 845 site is a PKA phosphorylation site whereas serine 831 is a PKC or CaMKII phosphorylation site. The selective effect of propofol over serine 845 may imply a PKA-sensitive signalling mechanism involved in the propofol effect.

Snyder and colleagues recently reported the effect of propofol on phosphorylation of glutamate receptors in mice in vivo. They found that an intraperitoneal injection of propofol at an anesthetic dose, as manifested by loss of righting reflex, reduced phosphorylation of NMDA receptor NR1 subunits at serine 897 in the cerebral cortex, similar to our previous results observed in cultured rat cortical neurons. However, they also found that propofol reduced serine 831 without affecting serine 845 phosphorylation in the striatum in vivo whereas, in the cortex, propofol reduced serine 845 without affecting serine 831 phosphorylation. These results differ from those observed in vitro in this study. The difference may mainly result from the two different models (in vivo vs in vitro) used. Additionally, the differences in species and other experimental conditions may partially explain why propofol differentially altered AMPA receptor phosphorylation in the two studies. The results obtained in vivo cannot discriminate between direct and indirect effects of propofol, although they indeed have the advantage of revealing the integral effect of an agent in a complex in vivo model. The results obtained in vitro in this study more likely reflect the direct effect of propofol.

Protein phosphorylation has been documented to control AMPA receptor function. Electrophysiologically, serine 845 phosphorylation increased channel open probability and the peak amplitude of the current. Biochemically, PKA phosphorylation of serine 845 facilitated synaptic incorporation of AMPA receptors. In contrast, dephosphorylation of serine 845 via a mechanism involving recruitment and activation of protein phosphatase 2B resulted in the internalization of AMPA receptors, leading to a weakened synaptic strength and the expression of long-term depression. Collectively, available data seem to favour a model that serine 845 phosphorylation promotes surface expression of AMPA receptors and thereby enhances the strength of synapses containing AMPA receptors. The current study has not directly evaluated changes in AMPA receptor function as a result of changed GluR1 phosphorylation at serine 845. However, Yamakura and colleagues have observed an augmented AMPA receptor current through GluR1-containing AMPA receptors expressed in Xenopus oocytes after propofol application at 0.2–1 mM as opposed to an inhibited NMDA receptor-mediated current. Similarly, the relative steady-state current through GluR1-containing AMPA receptors expressed in HEK-293 cells was increased in the presence of propofol (0.1–1 mM) probably due to decelerated desensitization of AMPA receptors. Thus, propofol unlike xenon seems to possess the ability to potentiate GluR1/AMPA receptor currents, which is in line with the finding in this study that propofol elevates serine 845 phosphorylation which may lead to an enhancement of AMPA receptor function. It should be pointed out that propofol started to modify transfected AMPA receptors in oocytes/HEK-293 cells at approximately 100–200 μM. This concentration range is higher than the concentration (3 μM) effective to alter serine 845 phosphorylation in cultured neurons (this study). Perhaps, native AMPA receptors in neurons are more sensitive to propofol than transfected recombinant AMPA receptors in heterologous expression systems in terms of their responses to propofol.

It is unclear whether the enhancement of AMPA receptor GluR1 phosphorylation contributes to any specific biological action of propofol. Propofol is noted to increase GluR1 serine 845 phosphorylation at 3 μM which is above the EC50 value (0.4–2.2 μM) for free (non-protein-bound) propofol for producing general anaesthesia. Thus, this event is less likely to contribute to the anaesthetic effect of propofol. In support of this notion, propofol has been well documented to induce anaesthesia through the enhancement of GABAergic transmission. Moreover, enhanced excitatory synaptic transmission through AMPA receptors in broad brain areas

### Table 1 Time-course for propofol effects on the phosphorylation of GluR1 in rat cultured striatal and cortical neurons. Data are expressed as per cent changes in protein levels of controls [mean (SEM), n=5–9 per group]. *P<0.05 vs controls at corresponding time points

<table>
<thead>
<tr>
<th>Protein</th>
<th>Neurons</th>
<th>Propofol incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pGluR1-Ser831</td>
<td>Striatum</td>
<td>124.6 (21.3)</td>
</tr>
<tr>
<td></td>
<td>Cortex</td>
<td>89.2 (7.3)</td>
</tr>
<tr>
<td>pGluR1-Ser845</td>
<td>Striatum</td>
<td>127.4 (21.8)</td>
</tr>
<tr>
<td></td>
<td>Cortex</td>
<td>133.2 (16.7)</td>
</tr>
<tr>
<td>GluR1</td>
<td>Striatum</td>
<td>104.1 (22.6)</td>
</tr>
<tr>
<td></td>
<td>Cortex</td>
<td>88.5 (13.6)</td>
</tr>
</tbody>
</table>
is less likely to contribute to the production of anaesthesia that typically reflects a general suppression of synaptic transmission. Propofol has been reported to facilitate the convulsive potency of quisqualic acid, a glutamate receptor agonist acting on AMPA receptors, in vivo. Future studies will need to be carried out to elucidate the possible contribution of upregulated GluR1 phosphorylation in the propofol effect on AMPA receptor-mediated convulsions or other biological activity.

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