Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Direct posttranslational modification of astrocytic connexin 43 proteins by the general anesthetic propofol in the cerebral cortex

Mutsuo Nuriya [a, b, *], Daisuke Yasui [a], Tomoko Yamada [a], Takuma Aoki [a], Masato Yasui [a]

[a] Department of Pharmacology, School of Medicine, Keio University, Japan
[b] Graduate School of Environment and Information Sciences, Yokohama National University, Japan

ARTICLE INFO

Article history:
Received 6 February 2018
Accepted 16 February 2018
Available online xxx

Keywords:
Astrocyte
Connexin 43
Gap junction
General anesthetic
Phosphorylation
Propofol

ABSTRACT

Propofol is widely used as a general anesthetic and is generally considered to exert its action by regulating neuronal firing via facilitation of $GABA_A$ receptors. However, accumulating evidence suggests that propofol also acts on astrocytes, including inhibitory effects on gap junctional coupling, but the underlying molecular mechanisms remain largely unknown. Here, using acute cortical brain slices prepared from mice, we characterize propofol-induced molecular changes in astrocytic gap junction protein connexin 43 (Cx43). Propofol does not change the protein expression level of Cx43 or its incorporation into gap junctional plaques, according to biochemical and immunohistochemical analyses. However, propofol alters migration pattern of Cx43 on western blot, suggesting changes in its posttranslational modifications. Indeed, this change is accompanied by an increase in the phosphorylation of Cx43 at serine 368, which is known to reduce permeability of Cx43 gap junctions. Finally, we show that this change occurs in the absence of neuronal firing or glutamatergic transmissions. Overall, these results show that propofol induces posttranslational modification of Cx43 directly on astrocytes at the site of gap junctional plaques, exerting direct pharmacological action on astrocytes in parallel with its action on neurons.

1. Introduction

Propofol (2,6-diisopropylphenol) is widely used for induction and maintenance of general anesthesia and is considered to exert its function by facilitating GABAergic neuronal transmission by binding to $GABA_A$ channels [1]. Recently, however, evidence is accumulating to suggest that astrocytes play key roles in many aspects of brain physiology, pathology and pharmacology [2–4]. This raises the possibility that general anesthetics may modulate astrocytes in addition to neurons, which in turn may contribute to the pharmacological effects of the drug on the brain. In fact, a pioneering study showed that propofol can inhibit gap junction function in dissociated cortical astrocytes [5]. This observation was further extended by a recent study suggesting that propofol inhibits gap junctions and hemichannels differentially in brain slices [6]. However, the molecular mechanisms contributing to the functional changes in gap junctions are largely unknown. A reduction in gap junctional communication may arise from downregulation of connexin proteins, undocking of connexins from gap junctional plaques, changes in channel properties, or by other mechanisms. Understanding these molecular mechanisms is the first and most crucial step in pharmacological characterization of the anesthetic and other effects of propofol and in the future development of new general anesthetics.

One of the major reasons for the lack of detailed knowledge of the molecular mechanisms underlying the pharmacological effect of psychoactive drugs on astrocytes is the difficulty associated with the choice of assay system. Despite the ease in preparation and accessibility, the elaborate morphologies of astrocytes are lost in cell culture, leading to marked changes in their physiological properties that are supported by their structure. Of particular importance is the change in the endfeet that serve as a specialized intracellular compartment [7–9], which is the first place of action of systemically administered drugs, including propofol, in astrocytes and in the brain parenchyma as a whole. In contrast, while physiological in principle, pharmacological manipulations and subsequent biochemical analyses are difficult to achieve *in vivo*. This is particularly true for research related to anesthesia, because the actions of anesthetic agents on the whole body changes various

* Corresponding author. 35 Shinanomachi, Shinjuku, Tokyo, 160-8582, Japan.
E-mail address: mnuriya@z2.keio.jp (M. Nuriya).

https://doi.org/10.1016/j.bbrc.2018.02.142
0006-291X/

physiological aspects, such as blood pressure, oxygen concentration and body temperature, to name only a few, all of which significantly affect the physiological properties of astrocytes. Further complications arise from changes in neuromodulators induced by anesthesia, which significantly affects the physiology of astrocytes in addition to that of neurons [10]. Finally, despite its importance, distinguishing the direct action of a drug of interest on astrocytes from its indirect action, mediated by the changes in neuronal activity is difficult to achieve *in vivo*, as animals do not tolerate general manipulations of neuronal activity well. Acute cortical brain slices can circumvent these technical difficulties and offer a unique opportunity to investigate molecular changes in endogenous proteins that are localized in the astrocytes of native structures and functions, under strict pharmacological controls [9].

Therefore, in this study, we attempted to characterize the molecular mechanisms underlying the action of the general anesthetic propofol on astrocytic gap junctions by focusing on the major gap junction channel, Cx43, by applying biochemical approaches to acute cortical brain slices.

2. Materials and methods

2.1. Slice preparation

All procedures related to the care and treatment of animals were approved by the Keio University Institutional Animal Care and Use Committee. Acute cortical slices for biochemical and immunohistochemical analyses were prepared from P14-21 C57BL/6J mice (Japan SLC, Shizuoka, Japan) of either sex anesthetized with isoflurane as described previously [9].

2.2. Pharmacology

2,6-Diisopropylphenol (Propofol, Wako Pure Chemical Industries, Osaka, Japan), tetrodotoxin (TTX, Abcam, Cambridge, UK), (R,S)-α-Methyl-4-carboxyphenylglycine (MCPG, Abcam), DL-2-Amino-5-phosphonopentanoic acid (APV, Abcam) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma Aldrich, MO, USA) were purchased from the suppliers indicated.

Propofol was diluted to 150 mM in dimethyl sulfoxide (DMSO) and applied in a bath at a final concentration of 150 μM. Control slices were incubated in the same final concentration (0.1%) of DMSO. Pharmacological manipulations were performed in a custom-made chamber in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 26 mM $NaHCO_3$, 1.1 mM NaH_2PO_4, 10 mM dextrose, 3.0 mM KCl, 1.0 mM $MgCl_2$, and 3.0 mM $CaCl_2$ (pH 7.3) bubbled with 95% O_2/5% CO_2 at 37 °C. In experiments examining the involvement of neuronal activity and glutamatergic transmissions, TTX or glutamate receptor antagonist cocktail was pre-administered to brain slices for 15 min at 37 °C before propofol treatment and continued during propofol treatment.

2.3. Biochemistry

After pharmacological manipulations, slices were immediately cooled in ice-cold HEPES ACSF (150 mM NaCl, 3 mM KCl, 10 mM dextrose with 10 mM HEPES, pH 7.4) and lysed in Triton lysis buffer (1% Triton X-100 in phosphate-buffered saline, PBS) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) as well as phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) at 4 °C for 30 min with gentle rotation. The lysate was then centrifuged at 20,000 × *g* for 20 min at 4 °C and the resulting supernatant was collected as the Triton-soluble non-detergent resistant membrane (non-DRM) fraction. The pellet was then re-suspended in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, pH 7.4) supplemented with protease and phosphatase inhibitor cocktails. After solubilization for 30 min at 4 °C, the lysate was again centrifuged at 20,000 × *g* for 20 min at 4 °C and the resulting supernatant was collected as the Triton-insoluble detergent resistant membrane (DRM) fraction. These samples were then processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by adding half the volume of 3 × SDS sample buffer and incubated for 30 min at 37 °C. SDS-PAGE was performed using the custom-made gel and the proteins were transferred to polyvinylidene fluoride (PVDF) membrane, which was then used for western blotting.

Anti-phospho-connexin 43 rabbit polyclonal antibody (Cell Signaling Technologies, MA, USA, RRID: AB_2110169) and anti-connexin 43 rabbit polyclonal antibody (Thermo Fisher Scientific, NH, USA, RRID: AB_2533973) were used as primary antibodies. Chemiluminescence detections were performed using horseradish peroxide (HRP)-conjugated secondary antibodies (Jackson Laboratories, ME, USA) and Super Signal Substrate (Thermo Fisher Scientific) and the resulting signals were acquired with Image Quant LAS4000 (GE Healthcare, Little Chalfont, UK) and band intensities were quantified using ImageJ software (National Institute of Health). Reprobing of the membrane was performed after stripping it with stripping buffer (Nacalai Tesque).

2.4. Immunohistochemistry

Immunohistochemical analyses of the acute brain slices were performed essentially as described previously [9] with some modifications. Briefly, brain slices were fixed in 4% paraformaldehyde in PBS (Wako Pure Chemical Industries) for 2 h and then incubated in permeabilizing and blocking solution containing 10% normal goat serum, 0.5% Triton X-100 with 0.05% sodium azide in PBS overnight at 4 °C after washing in PBS. Anti-laminin rabbit polyclonal antibody (Sigma Aldrich, RRID: AB_477163) and anti-GLT1 rabbit polyclonal antibody (Frontier Institute, WA, USA, RRID: AB_2571718) were labeled with Alexa Fluor 555, while anti-connexin 43 rabbit polyclonal antibody (Thermo Fisher Scientific, RRID: AB_2533973) was labeled with Alexa Fluor 488 using Zenon antibody labeling kits (Thermo Fisher Scientific) according to the manufacturer's instructions before use. Slices were then incubated with the Alexa Fluor-labeled antibodies diluted in antibody dilution solution containing 2% normal goat serum, 0.1% Triton X-100 with 0.05% sodium azide in PBS overnight at 4 °C. Slices were washed in PBS and examined using FV1000 confocal microscopy (Olympus, Tokyo, Japan). Confocal images taken at different depths at 2-μm intervals were stacked for analyses.

2.5. Data analysis

Quantitative analyses of immunoblot data were performed using ImageJ software and plot profiles of the western blotting were generated essentially as previously described [11]. For quantifications of both integrated band intensity (protein amount) and band width (migration pattern), values of individual bands were normalized to those of control samples in the same membrane. Statistical analyses were performed using OriginPro (OriginLab) and significance was determined using the Mann→Whitney test. All data are shownin scatter plots and statistically significant values are represented by $^{*}\ p < 5 \times 10^{-2}$, $^{**}p < 1 \times 10^{-2}$, $^{***}p < 1 \times 10^{-3}$.

Please cite this article in press as: M. Nuriya, et al., Direct posttranslational modification of astrocytic connexin 43 proteins by the general anesthetic propofol in the cerebral cortex, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.02.142