Neuropharmacology 105 (2016) 124–132

Contents lists available at ScienceDirect

Neuropharmacology

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

Differential regulation of synaptic and extrasynaptic α4 GABA(A) receptor populations by protein kinase A and protein kinase C in cultured cortical neurons

John Peyton Bohnsack [a, c], Stephen L. Carlson [c], A. Leslie Morrow [a, b, c, *]

[a] Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599-7365, USA
[b] Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599-7365, USA
[c] Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill NC, 27599-7178, USA

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
7 December 2015
Accepted 4 January 2016
Available online 6 January 2016

Keywords:
PKA
PKC
α4 subunit
Serine phosphorylation
Expression
$GABA_A$ receptor

ABSTRACT

The $GABA_A$ α4 subunit exists in two distinct populations of $GABA_A$ receptors. Synaptic $GABA_A$ α4 receptors are localized at the synapse and mediate phasic inhibitory neurotransmission, while extrasynaptic $GABA_A$ receptors are located outside of the synapse and mediate tonic inhibitory transmission. These receptors have distinct pharmacological and biophysical properties that contribute to interest in how these different subtypes are regulated under physiological and pathological states. We utilized subcellular fractionation procedures to separate these populations of receptors in order to investigate their regulation by protein kinases in cortical cultured neurons. Protein kinase A (PKA) activation decreases synaptic α4 expression while protein kinase C (PKC) activation increases α4 subunit expression, and these effects are associated with increased β3 S408/409 or γ2 S327 phosphorylation respectively. In contrast, PKA activation increases extrasynaptic α4 and δ subunit expression, while PKC activation has no effect. Our findings suggest synaptic and extrasynaptic $GABA_A$ α4 subunit expression can be modulated by PKA to inform the development of more specific therapeutics for neurological diseases that involve deficits in GABAergic transmission.

1. Introduction

$GABA_A$-Receptors ($GABA_A$-Rs) are ligand-gated ion channels that mediate the majority of inhibitory neurotransmission in the CNS. $GABA_A$-Rs are normally heteropentamers that are composed of two α(1–6), two β(1–3), and either a γ(1–3) or δ subunit. The presence of either the γ or δ subunit in the assembled receptor influences receptor localization and consequentially the type of $GABA_A$ergic neurotransmission. $GABA_A$-Rs containing the γ subunit are located synaptically and mediate phasic inhibition (Nusser et al., 1995, 1998). Conversely, the δ-containing $GABA_A$-Rs are located exclusively extrasynaptically and mediate tonic inhibition (Nusser et al., 1998; Farrant and Nusser, 2005). Both synaptic and extrasynaptic $GABA_A$-Rs are crucial to maintaining overall neuronal excitability (Farrant and Nusser, 2005).

The α4 subunit is present in both synaptic and extrasynaptic $GABA_A$-Rs in the mammalian cerebral cortex. The α4γ- and α4δ-containing $GABA_A$-Rs have unique physiological and pharmacological properties. α4γ2-containing $GABA_A$-Rs have a lower affinity for GABA but faster desensitization than α4δ-containing $GABA_A$-Rs (Brown et al., 2002; Carlson et al., 2014; Bianchi et al., 2001). In addition, other endogenous modulators, such as $GABA_A$ergic neuroactive steroids, exhibit higher potency at α4δ-containing $GABA_A$-Rs than α4γ2-containing $GABA_A$-Rs (Brown et al., 2002). Both α4-containing $GABA_A$-Rs assemblies are insensitive to classic benzodiazepine agonists such as diazepam (Brown et al., 2002) although the structurally related benzodiazepine derivatives, imidazobenzodiazepines, such as Ro15-4513, display activity at both receptor subtypes (Wallner et al., 2006). α4δ $GABA_A$-Rs are also potentiated by low millimolar concentrations of ethanol while α4γ2 $GABA_A$-Rs require a higher concentration (Wallner et al.,

Abbreviations: PKA, protein kinase A; PKC, protein kinase C; Sp-cAMPS, Sp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt; Rp-cAMPS, Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt; PDBu, Phorbol 12,13-dibutyrate; CalC, Calphostin C; PSD-95, postsynaptic density protein-95.

* Corresponding author. Bowles Center for Alcohol Studies, UNC School of Medicine, 3027 Thurston-Bowles Building, CB#7178, Chapel Hill, NC 27599, USA.
E-mail address: morrow@med.unc.edu (A.L. Morrow).

http://dx.doi.org/10.1016/j.neuropharm.2016.01.009
0028-3908/

2006, 2003; Sundstrom-Poromaa et al., 2002), although this data is controversial, as not all labs have found that δ-containing $GABA_A$-Rs are sensitive to low concentrations of ethanol (Borghese et al., 2006). Thus, differences in the pharmacological and physiological characteristics of these $GABA_A$-Rs have generated considerable interest in the contributions of these receptors to both physiological and pathological disease states.

Both α4-containing $GABA_A$-R populations have been implicated in multiple disease states, such as alcohol dependence, fragile X syndrome, epilepsy, schizophrenia, and depression (Whissell et al., 2015). In some disease states, such as alcohol dependence (Liang et al., 2007), epilepsy (Zhang et al., 2007; Lund et al., 2008), and schizophrenia (Maldonado-Avilés et al., 2009), downregulation of the δ subunit is accompanied by upregulation of α4γ2-containing $GABA_A$-Rs, suggesting that this change in overall $GABA_A$-R population may be important to the pathogenesis of these diseases. In non-pathological states, genetic ablation of the δ subunit also resulted in an increase in γ2 subunit expression in the cerebellar granule cells (Tretter et al., 2001). Despite these observations, the intracellular mechanisms that regulate changes in expression of the α4δ and α4γ2 receptors are still largely unknown.

PKA and PKC have long been known to regulate $GABA_A$-R expression either through direct phosphorylation of $GABA_A$-R subunits or through proteins associated with $GABA_A$-Rs (McDonald et al., 1998; Brandon et al., 2000; Abramian et al., 2010). PKA is known to modulate expression and function of $GABA_A$-Rs through direct phosphorylation on the β3 subunit serine sites S408/409 (McDonald and Moss, 1997). PKC has been shown to phosphorylate sites on the $GABA_A$ subunits at α4 S443 (Abramian et al., 2010), β2 S410, β3 S408/409 (McDonald and Moss, 1997), and γ2 S327 (Krishek et al., 1994). Phosphorylation on these sites contributes to different trafficking (Connolly et al., 1999), stabilization (Abramian et al., 2014), internalization (Bright and Smart, 2013), or expression (Werner et al., 2011), depending on both the phosphorylation site and the composition of the $GABA_A$-R (Nakamura et al., 2015). In addition to direct regulation by protein kinases, indirect regulation of signal transduction by G-coupled protein receptors has also been shown to effect $GABA_A$-R expression and function (Connelly et al., 2013; Janssen et al., 2009; Brunig et al., 1999).

However, it is still unknown whether PKA and PKC regulate both synaptic and extrasynaptic populations of α4-containing $GABA_A$-Rs. Therefore, we were interested in the role of these two kinases in $GABA_A$-R subunit expression. Previous work in our lab has suggested that PKA and PKC have opposing effects on $GABA_A$ α4 subunit expression in the presence of ethanol in cortical neurons (Werner et al., 2011; Carlson et al., 2013). Thus, the present study sought to determine if PKC and PKA were involved in regulation of both the synaptic and extrasynaptic populations of α4 $GABA_A$-Rs in the absence of ethanol.

2. Materials and methods

2.1. Primary cortical neuron cell culture and treatments

All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee at the University of North Carolina. Postnatal day 0–1 Sprague Dawley rat pups of both sexes were decapitated and cortices were isolated and cultured as previously described (Kumar et al., 2010). Neurons were maintained *in vitro* for 18 days in DMEM, B27 (2%, Invitrogen), and penicillin/streptomycin (15 days, 50 U, Invitrogen). On day 18, drugs were diluted in ddH_2O or DMSO. PKA was activated with Sp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Sp-cAMPs, 50 μM, Sigma Aldrich) and inhibited with Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS, 50 μM, Sigma Aldrich). PKC was activated with Phorbol 12,13-dibutyrate (PDBu, 100 nM, Sigma Aldrich) and inhibited with Calphostin C (CalC, 300 nM, Sigma Aldrich) All control experiments were exposed to equal volume ddH_2O. All drug exposures were for 1 h since previous experiments showed PKA and PKC both alter $GABA_A$ receptors at this time point (Werner et al., 2011; Carlson et al., 2013).

2.2. Quantitative PCR

Following treatment, cells were homogenized in Trizol according to manufacturers instructions. RNA was purified using Direct-zol RNA miniprep kits (Zymo) and 260/280 and 260/230 ratios >1.8 were determined using a Nanodrop 1000 (ThermoScientific). RNA was reversed transcribed into cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). qPCR was performed using 10 ng of cDNA per reaction, TaqMan Gene Expression Assays (Life Technologies), and TaqMan Gene Expression Master Mix (Life Technologies). Each reaction was run in duplicate and analyzed with the $\Delta\Delta C_t$ method with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a loading control.

2.3. Membrane, synaptic and extrasynaptic fractionation

Membrane, synaptic, and extrasynaptic fractions were produced as described previously (Carlson et al., 2014; Goebel-Goody et al., 2009; Carlson et al., 2016; Huttner et al., 1983; Devaud et al., 1997). Following drug exposures, cells were lysed by brief sonication in PBS containing 0.32 M sucrose. The nuclear fraction and cell debris were removed by centrifugation at 1000 × g for 10 min at 4 °C. The membrane fraction was produced by centrifugation of the supernatant at 12,000 × g for 30 min at 4 °C. The resulting pellet was resuspended in 0.32 M sucrose PBS buffer containing 0.5% (v/v) Triton-X100 and incubated at 4 °C under rotation for 20 min. The synaptic fraction was produced by centrifugation at 32,000 × g for 20 min at 4 °C. The resulting pellet containing the synaptic fraction was resuspended in PBS containing protease and phosphatase inhibitors. The supernatant containing the extrasynaptic fraction was incubated in acetone (1:8 v/v) overnight at −20 °C to insolubilize and concentrate the protein. This solution was pelleted by centrifugation at 3000 × g for 15 min at 4 °C. The resulting pellet containing the extrasynaptic fraction was resuspended in PBS containing protease and phosphatase inhibitors (Halt™, ThermoScientific).

2.4. Biotinylation for isolation of surface proteins

Isolation of surface proteins with biotinylation was performed using the Cell Surface Protein Isolation Kit (Pierce) according to manufacturers instructions. An aliquot was taken before avidin pulldown in order to analyze expression in the total fraction. The eluted biotinylated fraction was then subjected to western blot analysis. Surface expression was analyzed as the ratio of α4 in the biotinylated fraction over α4 in the total fraction. β-actin was probed in the biotinylated fraction as a control to insure that there were no intracellular proteins in the biotinylated fraction. Results were then normalized to the control for each fraction.

2.5. Western blot analysis

Protein concentrations were determined using the BCA assay (Pierce). 30–50 μg of protein was electrophoresed on 4–16% Tris-Glycine polyacrylamide gels (Biorad) and transferred to iBlot PVDF membranes (Invitrogen), blocked for 1 h in 1–5% BSA and