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Modulation of neurosteroid potentiation by protein kinases at synaptic- and extrasynaptic-type $GABA_A$ receptors

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ABSTRACT

$GABA_A$ receptors are important for inhibition in the CNS where neurosteroids and protein kinases are potent endogenous modulators. Acting individually, these can either enhance or depress receptor function, dependent upon the type of neurosteroid or kinase and the receptor subunit combination. However, *in vivo*, these modulators probably act in concert to fine-tune $GABA_A$ receptor activity and thus inhibition, although how this is achieved remains unclear. Therefore, we investigated the relationship between these modulators at synaptic-type $\alpha1\beta3\gamma2L$ and extrasynaptic-type $\alpha4\beta3\delta$ $GABA_A$ receptors using electrophysiology.

For $\alpha1\beta3\gamma2L$, potentiation of GABA responses by tetrahydro-deoxycorticosterone was reduced after inhibiting protein kinase C, and enhanced following its activation, suggesting this kinase regulates neurosteroid modulation. In comparison, neurosteroid potentiation was reduced at $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors, and unaltered by PKC inhibitors or activators, indicating that phosphorylation of β3 subunits is important for regulating neurosteroid activity. To determine whether extrasynaptic-type $GABA_A$ receptors were similarly modulated, $\alpha4\beta3\delta$ and $\alpha4\beta3^{S408A,S409A}\delta$ receptors were investigated. Neurosteroid potentiation was reduced at both receptors by the kinase inhibitor staurosporine. By contrast, neurosteroid-mediated potentiation at $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ receptors was unaffected by protein kinase inhibition, strongly suggesting that phosphorylation of α4 and β3 subunits is required for regulating neurosteroid activity at extrasynaptic receptors. Western blot analyses revealed that neurosteroids increased phosphorylation of $\beta3^{S408,S409}$ implying that a reciprocal pathway exists for neurosteroids to modulate phosphorylation of $GABA_A$ receptors.

Overall, these findings provide important insight into the regulation of $GABA_A$ receptors *in vivo*, and into the mechanisms by which GABAergic inhibitory transmission may be simultaneously tuned by two endogenous neuromodulators.

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1. Introduction

Neurosteroids and protein kinases are among the most potent modulators of the $GABA_A$ receptor, which, when acting individually, can enhance or depress receptor function depending on the nature of the neurosteroid or protein kinase present and to some extent, on the subunit combination of the receptor (Belelli and Lambert, 2005; Moss and Smart, 1996). However, *in vivo*, these agents are unlikely to be temporally discrete modulators at $GABA_A$ receptors, and are far more likely to act in concert to achieve high-precision fine-tuning of inhibitory neurotransmission.

A number of previous studies have indicated that the activity of protein kinases, and presumably phosphorylation, can modulate the potentiating effects of selected neurosteroids on both recombinant and native $GABA_A$ receptors. For example, in *Xenopus* oocytes expressing recombinant $\alpha1\beta2\gamma2L$ $GABA_A$ receptors, the potentiation of $GABA_A$ receptor-mediated currents by the naturally-occurring neurosteroid, tetrahydro-deoxycorticosterone (THDOC) is enhanced by the activation of protein kinase C (PKC) (Leidenheimer and Chapell, 1997). These results are supported by other studies showing that inhibition of PKC and/or protein kinase A (PKA) resulted in a reduction of neurosteroid sensitivity in neurons from both the hippocampus (Harney et al., 2003) and hypothalamus (Fáncsik et al., 2000). However, whilst many studies

Abbreviations: THDOC, tetrahydro-deoxycorticosterone; BIM-I, bisindolylmaleimide I; PMA, phorbol-12-myristate-13-acetate; THIP, 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol.

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conducted to date support a role for protein kinases modulating neurosteroid activity at $GABA_A$ receptors, other results are apparently conflicting. Indeed, whilst the aforementioned studies collectively show positive regulation of neurosteroid potentiation by the activity of protein kinases, in both the lamina II neurons of the spinal cord (Vergnano et al., 2007) and the hypothalamic neurons of pregnant rats (Koksma et al., 2003), enhancement of protein kinase activity causes a reduction in the neurosteroid sensitivity of $GABA_A$ receptors. This is supported by a more recent study demonstrating that, in the pyramidal neurons of the hippocampus, kindling causes an increase in $GABA_A$ receptor phosphorylation which is accompanied by a concomitant decrease in receptor sensitivity to THDOC (Kia et al., 2011).

The reasons underling these discrepancies are currently unclear, but one factor which may affect the relationship between neurosteroids and protein kinase activity is the subunit combination of the $GABA_A$ receptor. Although the neurosteroids appear to display only modest changes in potency across most $GABA_A$ receptor subtypes (Belelli et al., 2002; Herd et al., 2007), phosphorylation by protein kinases has been shown to differentially alter $GABA_A$ receptor function, depending on the receptor isoform (Moss and Smart, 1996), which can even distinguish between different receptor β subunits (McDonald et al., 1998). Therefore, when acting together, it could be envisaged that protein kinases may modulate the activity of neurosteroids at the $GABA_A$ receptor in a manner that is dependent upon the receptor isoform. This may explain the variation in previous studies which have utilized different neuronal populations likely to reflect the presence of a mixture of different subsets of $GABA_A$ receptors.

In order to examine how protein kinases modulate the activity of neurosteroids in more detail, we investigated the relationship between neurosteroids and protein kinases at $GABA_A$ receptors with defined subunit compositions, replicating typical synaptic- and extrasynaptic-type receptor isoforms, by controlling expression in a secondary cell line. In addition, by mutating specific target residues for protein kinases on $GABA_A$ receptor subunits, we unveil a mechanism by which protein kinases can reciprocally act to modulate the actions of neurosteroids at these receptors.

2. Methods

2.1. Molecular biology

cDNAs encoding murine α1, $\alpha1^{Q241W}$, α4, β3, $\beta3^{S408A}$, $\beta3^{S409A}$, $\beta3^{S408A,S409A}$, γ2L, $\gamma2L^{S327A,S343A}$ and δ $GABA_A$ receptor subunits have all been described previously (Moss et al., 1991; Connolly et al., 1996; McDonald et al., 1998; Hosie et al., 2006, 2009). These cDNA constructs were cloned into the plasmid vector pRK5. The cDNA construct encoding murine $\alpha4^{S443A}$ was generated by site-directed mutagenesis of the wild-type α4 subunit gene using standard PCR methods and the following oligonucleotides: Forward, gccactcgccctgcatttggatctag and reverse, agctgaccccaaagaagctggc, obtained from Eurofins Genomic.

2.2. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% v/v foetal calf serum, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and incubated at 37 °C in 95% air/5% CO_2. Cells were transfected using the calcium phosphate precipitation method (using 1 μg of each subunit cDNA and enhanced green fluorescent protein (eGFP) to a total of 4 μg): 20 μl $CaCl_2$ (340 mM) plus 24 μl of 2× HBS (280 mM NaCl, 2.8 mM Na_2HPO_4, 50 mM HEPES, pH 7.2) per 22 mm coverslip. Cells were used for electrophysiology 24–48 h later. For biochemistry (60 mm culture dishes) cells were transfected with a total of 9 μg of the appropriate cDNA mix.

2.3. Electrophysiology

GABA-activated currents were recorded from transfected HEK293 cells continuously perfused with Krebs solution containing: 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.52 mM Glucose, 11 mM HEPES and 5 mM $CaCl_2$ (pH 7.4). Whole-cell recordings were performed using patch pipettes (4–5 MΩ) filled with an internal solution (120 mM KCl, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$ and 4 mM ATP, pH 7.11) in conjunction with an Axopatch 200B amplifier (Axon Instruments). Cells were voltage clamped at −10 mV and currents filtered at 3 kHz (8 pole Bessel filter), digitized (Digidata 1322A, Molecular Devices) and viewed and analysed using Clampex and Clampfit ver 9.2, respectively (Molecular Devices). Cells were continually monitored for access resistance and discarded if this changed by >20%. Drugs were rapidly-applied using a modified U-tube system with 10–90 % response time of 100–150 msec. THDOC was prepared as a 10 mM stock solution in DMSO, and diluted to the appropriate final concentration in Krebs. The effect of the DMSO vehicle alone was negligible. The EC_{20} GABA concentration was pre-determined by constructing GABA concentration–response curves and new curves were generated regularly to adjust for any drift in EC_{20}. The EC_{20} GABA currents were recorded at 2 min intervals. The neurosteroid-mediated potentiation was measured by co-application of EC_{20} GABA with 50 nM THDOC, followed by a recovery EC_{20} GABA response. Recording ceased if the responses did not return to baseline GABA-activated levels. Subsequently, cells were perfused with a protein kinase inhibitor or activator diluted in Krebs solution, and applied continuously via the bath. The exceptions to this were the protein kinase G (PKG) inhibitor (KT5823) and protein kinase A (PKA) activator (cAMP), which were applied via the patch electrode. Stocks of staurosporine (1 mM), BIM-I (1 mM), KT5823 (3 mM) and phorbol-12-myristate-13-acetate (PMA; 1 mM) were prepared in DMSO. cAMP (10 mM) was dissolved in distilled water.

2.4. Western blotting

Transfected HEK293 cells were treated with 50 nM THDOC or 100 nM PMA as appropriate and were lysed to isolate total protein. Lysis buffer was supplemented with a combination of protease inhibitors (phenylmethyl sulfonyl fluoride and benzamidine) and phosphatase inhibitors (20 mM NaF, 10 mM sodium pyrophosphate and 20 nM calyculin A). Equal amounts of total protein were subjected to Western blotting to assess the expression of $GABA_A$ receptor β3 and phosphorylated β3 subunits (on S408 and S409 residues). Total protein was isolated from each transfected cell culture by homogenisation in ice-cold RIPA buffer, followed by cell disruption with repeated freeze–thaw cycles. Equal amounts of total protein were subjected to sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blotting was performed using 5% w/v milk, or 0.2% BSA, in 0.1% v/v TWEEN-supplemented TRIS-buffered saline (TBS), with blocking for 1 h at room temperature (RT), exposure to primary antibody overnight at 4 °C, and secondary horseradish peroxidase (HRP)-conjugated antibody for 1 h at RT. Following incubation with each antibody, membranes were washed 3× (10 min, RT) with phosphate buffered saline (PBS) and imaged using the ECL detection system (GE Healthcare). Images were quantified using the Western blot plug-in on ImageJ software (Version 1.44p, National Institutes of Health, USA).

2.5. Antibodies

The following primary antibodies were used for Western blotting: Rabbit anti-β3-$GABA_A$ receptor (1:1000 dilution, Millipore), Rabbit anti-phospho-β3-$GABA_A$ receptor (1:1000, phosphorylated S408/409 epitope, gift from Dr J Jovanovic, UCL, UK). A donkey anti-rabbit (1:2500, GE Healthcare) secondary antibody (IgG (H&L)) conjugated to HRP was subsequently used for detection.

2.6. Data analysis and statistics

Peak whole-cell GABA-activated currents were analysed using Clampfit ver 9.2. For protein kinase inhibitor or activator experiments, current responses were normalized to the first response elicited by EC_{20} GABA. The potentiation caused by a neurosteroid was measured relative to the preceding response to EC_{20} GABA and expressed as a percentage. All concentration response curves were constructed by plotting mean peak response amplitude against GABA concentration and the data subsequently fitted with the Hill equation:

$$I = 1/(1 + (EC_{50}/[GABA])^n),$$

where I = GABA-activated current, EC_{50} = concentration of GABA inducing 50% of the maximal current response and n is the Hill coefficient. Data were graphically represented and analysed using Origin version 6.0 (Microcal). Statistical analyses were undertaken using GraphPad Instat (v.3) employing either a student's t-test (two value comparisons) or an ANOVA (three or more value comparisons) followed by an appropriate post-hoc test (as stated in the text) to compare selected data sets.

3. Results

To examine the effects of phosphorylation by protein kinases on the potentiation of typical synaptic $GABA_A$ receptors by neurosteroids, HEK293 cells were transfected to express α1β3γ2L subunit-containing $GABA_A$ receptors. Whole-cell recording was used to assess the magnitude of neurosteroid-mediated potentiation before and after cells were treated with modulators to inhibit or activate protein kinase activity. Peak currents were recorded in response to brief (3 s) applications of either EC_{20} (the concentration eliciting 20% of the maximal GABA response) GABA alone, (baseline control responses), or EC_{20} GABA co-applied with 50 nM of the neurosteroid, THDOC (the potentiated GABA responses). At this