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Subunit-specific potentiation of recombinant glycine receptors by NV-31, a bilobalide-derived compound

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Abstract

Bilobalide, a major bioactive component of *Ginkgo biloba* herbal extracts, exhibits neuroprotective and anti-ischaemic activity. However, its therapeutic potential is limited because of its instability. Attempts to synthesise a more stable analogue culminated in the development of NV-31. This compound recapitulates some aspects of bilobalide pharmacology. However, although bilobalide inhibits recombinant glycine receptor Cl channels (GlyRs), NV-31 potentiates hippocampal neuron GlyRs. Because of the possible therapeutic relevance of this effect, the present study investigated the molecular mechanism and subunit specificity of NV-31 actions at recombinantly expressed α1, α1β, α2 and α3 GlyRs. NV-31 potentiated α1 GlyRs by approximately 135 % with an EC_{50} near 170 nM. Its potentiating effect was observed only at low (EC_{10}) glycine concentrations. The magnitude of its potentiating effect was reduced at α1β GlyRs and it had no effect at all at α2 and α3 GlyRs. NV-31 was unlikely to bind at the bilobalide pore-binding site as its efficacy was not affected by the α1 subunit G2′A and T6′S mutations. However, the S15′C mutation to the alcohol-binding site abolished its effects, suggesting that NV-31 modulates the GlyR via a specific (steric or allosteric) interaction with S15′. GlyRs are potential therapeutic targets for chronic anti-inflammatory pain and movement disorders. NV-31, as a positive modulator of these receptors, thus remains viable as a therapeutic candidate for these disorders.

Keywords: Chloride channel; Glycinergic; Inhibitory neurotransmission; *Ginkgo biloba*; Molecular pharmacology

Ginkgolides A, B, C and J and bilobalide are the main bioactive components of EGb 761, a standardised herbal extract derived from leaves of the *Ginkgo biloba* tree [26]. These compounds are multi-cyclic terpene lactones with closely related structures [29]. Bilobalide, the single most abundant component of EGb 761, exhibits potent neuroprotective and anti-ischaemic actions [2,28]. However, its therapeutic potential is limited because of its instability. Attempts to synthesise a more stable analogue lead to the development of NV-31 (see structure in Fig. 1). This molecule incorporates a bilobalide moiety comprising a *tert*-butyl group in close apposition to a hydroxyl, but otherwise the two molecules are chemically different.

NV-31 also exhibits neuroprotective and anti-ischaemic properties [1]. However, as expected from its different chemistry, NV-31 does not completely recapitulate bilobalide pharmacology. Although ginkgolides and bilobalide potently inhibit the inhibitory neurotransmitter GABA type-A receptor ($GABA_AR$) and glycine receptor (GlyR) chloride channels [12,14–16,19], NV-31 potentiates hippocampal neuronal $GABA_ARs$ and GlyRs [4]. Despite the therapeutic potential of substances that enhance current flow through these receptors, the subunit specificity and molecular mechanism of NV-31 have not been investigated at either receptor.

$GABA_ARs$ and GlyRs are member of the pentameric Cys-loop ion channel receptor family. Individual subunits of this family comprise a large extracellular N-terminal agonist-binding domain followed by 4 α-helical transmembrane bundle. The second transmembrane (M2) domains line the channel pore and recent structure–function analyses suggest that ginkgolides and bilobalide effect inhibition by binding directly to GlyR M2 domain pore-lining residues [11,13,17].

Four GlyR subunits have been identified in humans (α1–α3, β). Embryonic receptors generally comprise α2 homomers or α2β heteromers, whereas the dominant adult subtype is α1β heteromer [3,21]. Although the β subunit is widely distributed throughout the adult central nervous system, α subunits exhibit differential distribution patterns that are especially dramatic in the superficial laminae of the spinal cord dorsal horn [8] and

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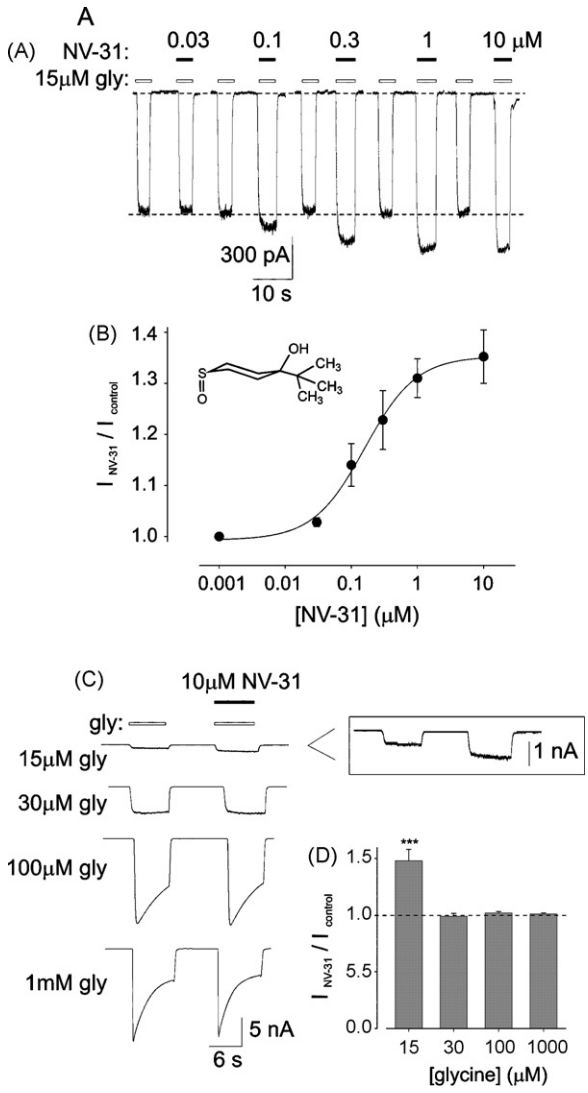

Fig. 1. Effects of NV-31 on α1 GlyRs. (A) In this and subsequent figures, unfilled and filled bars indicate glycine and NV-31 applications, respectively. The upper dashed line represents the zero current level. EC_{10} (15 μM) glycine applications activated an inward current to a magnitude as indicated by the lower dashed line. Co-application of 0.03, 0.1, 0.3, 1 and 10 μM NV-31 facilitated the glycine current in a dose-dependent manner. (B) Averaged NV-31 dose response. Data points represent mean fractional increase in glycine current mediated by the indicated NV-31 concentrations in the same five cells. In this and all subsequent figures, error bars represent ± S.E.M. The curve represents a Hill equation fit to the averaged data. Averaged parameters of best fit to individual Dose–response relationships are given in the text. The structure of NV-31 is displayed in the inset. (C) Example of the effects of 10 μM NV-31 on currents activated by EC_{10} (15 μM), EC_{40} (30 μM), EC_{80} (100 μM) and EC_{100} (1 mM) glycine in a single cell. The uppermost trace is expanded in the inset. (D) Mean fractional change in GlyR current plotted as a function of glycine concentration. All results were averaged from three cells. ***$P < 0.001$ relative to control current magnitude using paired Student's *t*-test.

the retina [9,10]. Of particular interest, α1 and α3 subunits represented equally at glycinergic synapses on dorsal horn nociceptive neurons, although α1 subunits predominate at other spinal cord glycinergic synapses [8]. Thus, compounds that selectively increase GlyR current flux through α3-containing GlyRs may specifically inhibit the firing of nociceptive projection neurons and could thus be useful lead compounds for the development of novel analgesics [22,34]. Substances that potentiate α1-containing GlyRs may have therapeutic application for movement disorders, including spasticity, but could also have analgesic potential.

The aim of this study was to characterise the GlyR subunit specificity of NV-31 in an attempt to assess its therapeutic potential and to gain some insight into its molecular mechanism of action.

The human α1, human α2, rat α3 and human β subunit GlyR cDNAs were subcloned into the pCIS, pcDNA3.1, pcDNA3.1 and pIRES2-EGFP plasmid vectors, respectively. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) and the successful incorporation of mutations was confirmed by DNA sequencing. HEK293 cells, cultured in Dulbecco's Modified Eagles Medium, were transfected with the cDNAs using a calcium phosphate precipitation protocol. When co-transfecting GlyR α and β subunit constructs, the respective plasmid DNAs were combined in a ratio of 1:10. After exposure to transfection solution for 24 h, cells were washed twice using calcium-free phosphate buffered saline, then returned to standard culture medium and used for recording over the following 24–72 h.

The glycine concentration–response profiles for all wild type and mutant receptors used in this study have recently been quantitated in our laboratory [31,32].

During experiments cells were perfused by a control solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had tip resistances of 1–2 MΩ when filled with the pipette solution which contained (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. Whole-cell currents were recorded by patch-clamp recording. Cells were voltage-clamped at −40 mV and membrane currents were recorded using an Axopatch 1D amplifier and pClamp9.1 software (Molecular Devices, Sunnyvale, CA, USA). The cells were perfused by a parallel array of microtubular barrels through which solutions were gravity-induced. Experiments were conducted at room temperature (19–22 °C).

Because α homomers form functional GlyRs, the successful incorporation of β subunits into functional receptors was inferred by the characteristic reduction in picrotoxin (PTX) sensitivity [27]. PTX (from Sigma, St Louis, MO, USA) and NV-31 (kindly provided by Dr Shyam Chatterjee, Retired pharmacologist; Stettiner Str.1; D 76139 Karlsruhe, Germany) were both stored frozen as 50 mM stocks in dimethylsulfoxide.

Results are expressed as mean ± S.E.M. of three or more independent experiments. The Hill equation was used to calculate the half-maximal concentration (EC_{50}) and Hill coefficient (n_H) values for NV-31 potentiation. Dose–response curves were fitted using a non-linear least squares algorithm (Sigmaplot 9.0, Jandel Scientific, San Rafael, CA, USA). Statistical significance was determined by paired or unpaired Student's *t*-test, as appropriate, with $P < 0.05$ representing significance.