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Dihydropyridine inhibition of the glycine receptor: Subunit selectivity and a molecular determinant of inhibition

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ABSTRACT

The dihydropyridines (DHPs), nifedipine and nicardipine, modulate native glycine receptors (GlyRs) at micromolar concentrations. Nicardipine has a biphasic potentiating and inhibitory effect, whereas nifedipine causes inhibition only. The present study sought to investigate (1) the molecular mechanism by which these compounds inhibit recombinant GlyRs, and (2) their potential utility as subunit-selective inhibitors of α1, α1β, α3 and α3β GlyRs. The rate of onset of inhibition in the open state was accelerated by pre-application of DHP in the closed state, with the degree of acceleration proportional to the concentration of pre-applied DHP. This implies a non-inhibitory binding site close to the DHP inhibitory site. DHP inhibition was use-dependent and independent of glycine concentration, consistent with a pore-blocking mode of action. DHP sensitivity was abolished by the G2′A mutation, providing a strong case for a DHP binding site in the pore. Nifedipine exhibited an approximately 10-fold higher inhibitory potency at α1-containing relative to α3-containing receptors, whereas nicardipine was only weakly selective for α1-containing GlyRs. The differential sensitivities of nifedipine and nicardipine for different GlyR isoforms suggest that DHPs may be a useful resource to screen as pharmacological tools for selectively inhibiting different synaptic GlyR isoforms.

1. Introduction

Glycine receptors (GlyRs) mediate inhibitory neurotransmission in the adult rat spinal cord and brainstem (Lynch, 2004). They incorporate an anion-selective pore and are members of the pentameric Cys-loop family of ligand-gated ion channels. Individual subunits of the Cys-loop receptor family contain a large N-terminal ligand-binding domain followed by a bundle of four transmembrane α-helical domains (Unwin, 2005). Each of the five subunits contributes its second transmembrane domain (M2) to the lining of the central water-filled pore. To date, five GlyR subunits have been identified (α1–α4, β). Embryonic receptors generally comprise α2 homomers or α2β heteromers, whereas the dominant adult subtype is the α1β heteromer (Lynch, 2004). Heteromeric GlyRs are thought to exist in a 2α:3β stoichiometry (Grudzinska et al., 2005). The β subunit, which is widely distributed throughout the adult nervous system, mediates the anchoring of GlyRs to the cytoskeleton via a direct binding interaction with the cytoplasmic protein, gephyrin (Kim et al., 2006). The α1–α4 subunits exhibit differential central nervous system distribution patterns that are particularly evident in the superficial dorsal horn of the spinal cord (Harvey et al., 2004) and the retina (Haverkamp et al., 2003, 2004; Heinze et al., 2007). The physiological consequences of the differential distribution patterns are difficult to establish as there are currently few pharmacological probes that can selectively inhibit different β subunit-containing GlyR isoforms (Betz and Laube, 2006; Webb and Lynch, 2007). The identification of compounds that can pharmacologically discriminate between α1- and α3-containing GlyRs may help in understanding the role of α3 GlyRs in inflammatory pain processing in spinal nociceptive neurons (Ahmadi et al., 2002; Harvey et al., 2004; Reinold et al., 2005; Zeilhofer, 2005) and in cone signal processing in the retina (Haverkamp et al., 2003).

Dihydropyridines (DHPs) such as nifedipine (NF) and nicardipine (NC) are important for the treatment of hypertension, angina, atrial arrhythmia and myocardial ischaemia (Struyker-Boudier et al., 1990). These drugs act therapeutically by inhibiting calcium flux through L-type Ca^{2+} channels, thereby inducing the relaxation

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of vascular smooth muscle and the suppression of cardiac contractility. They inhibit L-type Ca^{2+} channels at nanomolar concentrations but inhibit other Ca^{2+} channels at micromolar concentrations (Struyker-Boudier et al., 1990; Dunlap et al., 1995). Low micromolar concentrations of NF and NC have also been shown to modulate native GlyRs in cultured ventral spinal cord neurons (Chesnoy-Marchais and Cathala, 2001). These effects were selective for GlyRs over $GABA_A$ receptors in the same cells.

In the present study, we examined whether NF and NC may be useful as subunit-selective inhibitors of the α1, α1β, α3 and α3β GlyRs. The results suggest that NF may have a reasonable degree of selectivity for α1-containing GlyRs over α3-containing GlyRs. In addition, we have identified a molecular determinant of the inhibitory effect of these compounds.

2. Materials and methods

2.1. Mutagenesis and expression of GlyR cDNAs

The human GlyR α1, rat α3L and human β subunit cDNAs were subcloned into the pCIS, 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. A chimera of the α3 subunit was created whereby the α3 M4 domain residues, A401–D431, were replaced by the corresponding residues (I393–Q421) of the α1 M4 domain. This chimera was generated according to the "seamless" protocol (Padgett and Sorge, 1996). Briefly, long PCR of the vector encoding α3L was performed with primers incorporating EarI sites at the termini and this fragment was ligated to a PCR fragment of α1 M4 incorporating compatible EarI sites at each termini. The final construct was confirmed by DNA sequencing.

HEK293 cells, cultured in Dulbecco's modified Eagles medium, were transfected 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.

2.2. Electrophysiology

The cells were viewed via an inverted fluorescent microscope and currents were recorded in the whole-cell patch-clamp configuration. Cells were superfused 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 fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had a tip resistance of 1–2 MΩ when filled with the standard pipette solution containing (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole-cell configuration, cells were voltage-clamped at −40 mV (unless otherwise indicated) and membrane currents were recorded using an Axopatch 1D amplifier and pCLAMP9 software (Axon Instruments, Union City, CA, USA). The cells were perfused by a parallel array of microtubular barrels through which solutions were gravity-induced. This system permitted complete solution exchange within 100 ms. Experiments were conducted at room temperature (19–22 °C).

Because α homomers can form functional GlyRs, it is necessary to confirm the incorporation of β subunits into functional αβ heteromers. As the GlyR β subunit cDNA was cloned into the pIRES2-EGFP plasmid vector, we used GFP fluorescence to identify cells expressing the GlyR β subunit. The successful incorporation of β subunits into functional receptors was also inferred by their characteristic reduction in picrotoxin (PTX) sensitivity (Pribilla et al., 1992; Handford et al., 1996) as demonstrated in Fig. 1.

PTX, picrotoxinin (PTXININ), NF and NC were all obtained from Sigma (St Louis, MO, USA) and stored frozen as 50 mM stocks in dimethylsulfoxide.

2.3. Data analysis

Results are expressed as mean ± standard error of the mean 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 glycine activation. A similar equation was also used to calculate the half maximal concentrations for inhibition (IC_{50}) and n_H values of the antagonists tested in this study. All 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. One-way ANOVA (GraphPad Prism, Version 4 for Windows, GraphPad Software, Inc., San Diego, CA, USA) was used for the comparison of means of more than three groups.

3. Results

3.1. Expression and functional properties of GlyR isoforms

Examples of the response of cells expressing α3 and α3β GlyRs to increasing concentrations of glycine are shown in Fig. 1A. Glycine concentration–response relationships, averaged from at least three cells expressing the α1, α1β, α3 and α3β GlyRs, are presented in Fig. 1B with the mean EC_{50} and n_H values of best fit presented in Table 1. This table also includes the averaged EC_{50} and n_H values for all mutant GlyRs examined in this study. The EC_{50} values for the α3 and α3β GlyRs are several fold higher than previously reported by others (Meier et al., 2005; Miller et al., 2005). The reasons for this are unknown. However, similar results are consistently observed in our laboratory (Yang et al., 2007). The incorporation of β subunits into functional GlyRs results in a characteristic reduction in receptor sensitivity to PTX (Pribilla et al., 1992; Handford et al., 1996). To confirm the successful incorporation of β subunits into functional GlyRs, the effects of 10 μM PTX on currents activated by an EC_{50} (30 μM) glycine concentration were compared at the α1 and α1β GlyRs. The averaged results confirm the expected reduction in PTX sensitivity in the α1β GlyR (Fig. 1D). We have recently demonstrated that PTXININ is better than PTX at pharmacologically discriminating between α3 homomeric and α3β heteromeric GlyRs (Yang et al., 2007). We therefore compared the inhibitory potency of 1 μM PTXININ on currents activated by EC_{50} (300 μM) glycine at the α3 and α3β GlyRs (e.g., Fig. 1C). The percentage of original current remaining in 1 μM PTXININ, averaged from four cells expressing each receptor, is presented in Fig. 1D. These results confirm that co-expression of α1 or α3 together with the β subunit results in the formation of heteromeric receptors.

3.2. Mechanism of DHP action

As described below (Fig. 5), NF and NC exhibited no direct effect in the absence of glycine. In addition to its inhibitory effect, NC has also been reported to potentiate currents activated by sub-saturating glycine concentrations (Chesnoy-Marchais and Cathala, 2001). We also found that NC exerts a dual potentiating and inhibitory effect on α1 and α1β GlyRs. For homomeric α1 GlyRs, potentiation was observed only at glycine concentrations less than EC_{30}. However, in many cells the NC potentiating response was labile, either disappearing completely or progressively declining in magnitude over time (data not shown). NC produced only inhibition of α3 and α3β GlyRs, regardless of the glycine concentration used (e.g., Fig. 6A, below). The onset of potentiation of α1 GlyRs by NC was faster than that of inhibition, as shown by the biphasic response to increasing concentrations of NC in the presence of EC_{20} (20 μM) glycine (Fig. 2A, upper panel). The potentiating concentration–response, averaged from 11 cells (Fig. 2B), was bell-shaped with a peak at around 30 μM, possibly due to truncation of the potentiation peak by a faster onset of inhibition at higher NC concentrations. Examples of inhibition by NC of currents activated by EC_{50} (30 μM) glycine in homomeric α1 GlyRs are shown in Fig. 2A (lower panel), with averaged concentration–responses presented in Fig. 2B. The averaged IC_{50} and n_H values for NC inhibition are given in Table 2. NF produced no potentiation at any glycine concentration. Examples of the inhibitory effects of NF on currents activated by EC_{50} glycine at the α1 GlyR are shown in Fig. 2C and the averaged inhibitory concentration–response is plotted in Fig. 2D. The averaged parameters of best fit to the inhibitory concentration–response are given in Table 2.

We next investigated whether DHP inhibition was dependent of glycine concentration. In this experiment, the inhibitory potencies of NF (10 μM) and NC (30 μM) were compared at EC_{20}, EC_{50} and saturating glycine concentrations on homomeric α1 and α3 GlyRs,