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Subtype-selective antagonism of NMDA receptors by nylidrin

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Abstract

The 1,4-di-substituted piperidines ifenprodil, eliprodil, CP 101,606 ((1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol) and Ro 25-6981 ((*R*-(*R**,*S**))-α-(4-hydroxyphenyl)-β-methyl-4-(phenyl-methyl)-1-piperidinepropanol) are allosteric antagonists of NMDA receptors. Inhibition of diheteromeric NMDA receptors by this class of antagonist is characterized by pronounced selectivity for NR1/2B subunit combinations. In the current study, we assayed effects of nylidrin, a structurally-related non-piperidine, on recombinant and neuronal NMDA receptors. Nylidrin was a potent (IC_{50} = 0.18 μM) antagonist of NR1A/2B receptors expressed in *Xenopus* oocytes and was at least 150-fold weaker against NR1A/2A and NR1A/2C receptors. The blockade of NR1A/2B responses by nylidrin was not surmounted by increasing the concentrations of glutamate or glycine and was not voltage-dependent. Potency of inhibition increased ~3-fold upon lowering extracellular pH from 8 to 6.8. Nylidrin inhibited NMDA responses in cultured rat cortical neurons with similar potency and apparent mechanism of action as the NR1A/2B receptors. Our results suggest that nylidrin interacts with the same allosteric inhibitory site previously described for the related piperidine antagonists, and should serve as a structural lead for designing novel subtype-selective inhibitors of NMDA receptors. © 1997 Elsevier Science B.V

Keywords: Glutamate receptor; Allosteric antagonism; *Xenopus* oocyte; Ifenprodil; CP 101,606; Ro 25-6981; Haloperidol

1. Introduction

Mammalian (NMDA) receptors are ligand-gated ion channels composed of at least two types of subunit assembled into di- or tri-heterooligomeric combinations (Moriyoshi et al., 1991; Hollmann and Heinemann, 1994; Sheng et al., 1994). The rat subunits have been designated NMDA receptor (NR) 1 and NR2. NR1 subunits are transcribed from a single gene but are found in eight different isoforms (Sugihara et al., 1992; Hollmann and Heinemann, 1994). The NR2 subunits (NR2A–NR2D) are transcribed from four separate genes (Kutsuwada et al., 1992; Monyer et al., 1992, 1994). Different subunit combinations generate NMDA receptors with discrete biophysical and pharmacological properties (Williams, 1993; Hollmann and Heinemann, 1994; Priestly et al., 1995; Woodward et al., 1995). In addition, the NR1 isoforms and the NR2 subunits have distinct anatomical and developmental patterns of expression in mammalian central nervous systems, implying that different brain regions contain different NMDA receptor subtypes (Monyer et al., 1994; Sheng et al., 1994; Zukin and Bennett, 1995). In support of this, NMDA receptor properties have been shown to vary between brain regions and during development (Ben-Ari et al., 1988; Williams et al., 1993; Hollmann and Heinemann, 1994). The idea that pharmacologically distinct NMDA receptors occur in mammalian brain opens the possibility that subtype-selective antagonists will have therapeutic potential without the behavioral and neurotoxic side effects associated with some types of non-selective antagonists (Willetts et al., 1990; Small and Buchan, 1997).

The influx of Ca^{2+} that results from sustained activation of NMDA receptors is thought to play a role in neuronal death induced by ischemic stroke, head trauma and possibly chronic neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Choi and Rothman, 1990; Muir and Lees, 1995). In the search for clinically useful neuroprotectants, inhibitors for a number of the sites on the NMDA receptor complex have been identified and characterized. These include ligands for the glutamate site, the glycine site and the ion channel itself (Sucher et al., 1996; Small and Buchan, 1997). Antagonists at these sites generally demonstrate only modest levels of subtype-selectivity (Woodward et al., 1995; Sucher et al., 1996; Small and Buchan, 1997). In contrast, there is a class of NMDA antagonist, which includes ifenprodil, eliprodil, CP 101,606

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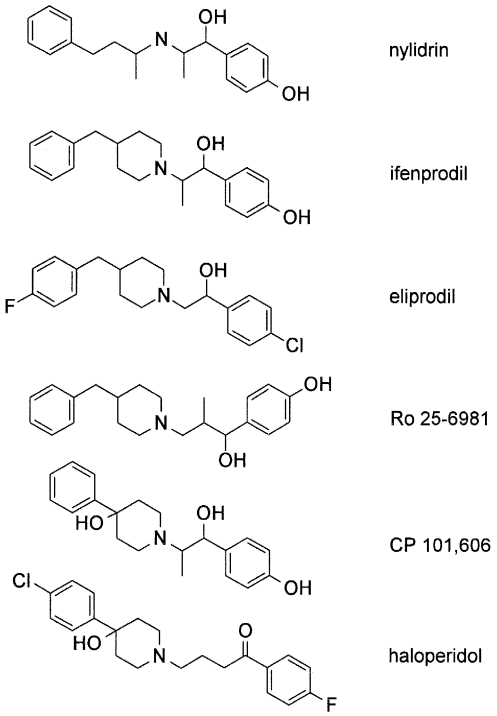

Fig. 1. Structures of nylidrin, ifenprodil, eliprodil, Ro 25-6981, CP101,606 and haloperidol.

((1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenyl-piperidino)-1-propanol), Ro 25-6981 ((*R*-(*R**,*S**))-α-(4-hydroxyphenyl)-β-methyl-4-(phenyl-methyl)-1-piperidine-propanol) and haloperidol that are highly selective for receptors composed of NR1/2B subunit combinations (Fletcher and MacDonald, 1993; Williams, 1993; Ilyin et al., 1996a; Mott et al., 1996; Trube et al., 1996; Whittemore et al., 1997). These compounds are structurally-related disubstituted piperidines (Fig. 1), which block via a non-competitive allosteric mechanism at a site that is not located deep in the channel pore (Williams, 1993; Ilyin et al., 1996a; Mott et al., 1996; Trube et al., 1996, Whittemore et al., 1997). All the compounds have demonstrated neuroprotective properties, both in vitro and in vivo, suggesting that this class of antagonists may have potential for clinical development (MacDonald and Johnston, 1990; Shalaby et al., 1992; Scatton et al., 1994; Chenard et al., 1995; Vartanian and Boxer, 1996). To begin to explore the structural requirements for activity in NR1/2B-selective ligands we have investigated the NMDA receptor antagonist properties of nylidrin; a compound initially developed in the 1960s as a β-adrenoceptor agonist (Ariëns et al., 1962; Yen and Pearson, 1979). Nylidrin is a structural analogue of compounds like ifenprodil and CP 101,606 wherein the piperidine ring has been opened to generate a secondary amine (Fig. 1). A preliminary report of this work has appeared in abstract form (Whittemore et al., 1996).

2. Materials and methods

2.1. Preparation of RNA

cDNA clones encoding the rat NMDA receptor subunits NR1A, NR2A, NR2B, NR2C were provided by Dr. P.H. Seeburg (Heidelberg University, Heidelberg, Germany) (see Monyer et al., 1992). Clones were prepared using conventional techniques and cRNA was synthesized with T3 RNA polymerase. cRNA was diluted to 400 ng/μl and stored at −80°C.

2.2. The Xenopus oocyte expression system

Preparation and micro-injection of oocytes were performed as reported previously (Woodward et al., 1995). Individual oocytes were injected with 1–10 ng of NMDA receptor-encoding cRNAs (amounts depended on expressional potency). NR1A and NR2A were injected at a 1:4 ratio, all other binary subunit combinations were injected 1:1. Oocytes were stored in Barth's medium containing (in mM): NaCl, 88; KCl, 1; $CaCl_2$, 0.41; $Ca(NO_3)_2$, 0.33; $MgSO_4$, 0.82; $NaHCO_3$, 2.4; Hepes 5; pH 7.4, with 0.1 mg/ml gentamycin sulfate. Electrical recordings were made with a Dagan TEV-200 voltage clamp (Minneapolis, MN, USA). Preliminary experiments were done in frog Ringer containing (in mM): NaCl, 115; KCl, 2; $CaCl_2$, 1.8; Hepes, 5; pH 7.4. For the detailed pharmacological measurements, recordings were made in nominally Ca^{2+}-free Ringer in which Ca^{2+} was replaced by equimolar Ba^{2+}. Drugs were applied via a linear array system made from microcapillary tubes (Hawkinson et al., 1996), or by bath perfusion (7–10 ml/min) in a conventional flow-through chamber (volume approx. 0.2 ml).

2.3. Culture of rat cortical neurons

Primary cultures of mixed cortical neurons were prepared using a modification of procedures described previously (Ilyin et al., 1996a). Briefly, cortices were obtained from Sprague–Dawley rat embryos (Charles River, Hollister, CA, USA) at gestation day (E) 16 or 17. Neurons were dissociated by trypsinization (10 min), light triturization, and passage through a nylon mesh. Cells were then plated at a density of $(3–5)\times10^4/cm^2$ into 35 mm culture dishes containing a confluent monolayer of rat cortical astrocytes. The plating and feeding medium was Minimal Essential Medium (MEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA). Cultures were maintained at 37°C in a humidified incubator (5%CO_2/95%air), with replacement of half of the medium three times weekly.

2.4. Electrophysiology in cultured neurons

Whole-cell currents were recorded using standard patch-clamp techniques (Hamill et al., 1981), as described

previously (Ilyin et al., 1996a,b). In most experiments, drugs and intervening wash were applied from a microcapillary linear array (Microcaps, 2 μl in volume, Drummond Scientific, Broomall, PA, USA) with fine positioning governed by remote control. For experiments investigating open-channel blockade, drugs were applied using a rapid perfusion system (DAD-12, Adams & List Associates, Westbury, NY, USA) which allows well defined timing and gives better reproducibility of peak NMDA responses. The external solution for all drug applications and wash was (in mM): NaCl 155; $CaCl_2$, 2; Na-Hepes 10 (pH 7.4); tetrodotoxin, 400 nM; (−)-bicuculline methochloride, 10 μM (305 mOsm). In addition, the recording chamber was continuously perfused (approx. 5 ml/min) by the same solution supplemented with 4 mM KCl, 2 mM $MgCl_2$ and 10 mM glucose, with tetrodotoxin and (−)-bicuculline omitted. The internal pipette solution for whole-cell recordings was (in mM): KF, 134; K-Hepes, 10.6 (pH 7.4); $CaCl_2$, 1; $MgCl_2$, 2; EGTA, 10 (285 mOsm). Recordings were made with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). The holding potential was −60 mV in all experiments except for those testing voltage dependence. Pipettes (3–8 MΩ) were pulled from thick-walled, filamented, borosilicate capillaries (World Precision Instruments, New Haven, CT, USA) and were fire-polished. The series resistance (5–12 MΩ) was routinely compensated by approx. 80% when membrane currents exceeded 1 nA. Whole cell currents were filtered at 2 kHz with a 4-pole, −3 dB, lowpass Bessel filter and were stored digitally on a NIC-310 digital oscilloscope (Nicolet Instrument, Madison, WI, USA). Recordings were analyzed off-line using software provided by the laboratory of Dr. Ricardo Miledi (University of California, Irvine, CA, USA).

2.5. Data analysis – Pharmacology

Pharmacology of whole cell currents was analyzed as reported previously (Woodward et al., 1995; Ilyin et al., 1996b). Briefly, data for glycine and glutamate concentration–response relations were fit using the logistic equation

$$I/I_{max} = 1/\left(1 + \left(EC_{50}/[\text{agonist}]\right)^{n}\right), \quad (1)$$

where I is the measured current, I_{max} is the maximum steady-state current, n is the slope factor and EC_{50} is the concentration of drug that elicits a half-maximal response. Concentration–inhibition curves for nylidrin were fit with

$$I/I_{control} = \left\{(1 - \text{min})/\left\{1 + \left([\text{antagonist}]/IC_{50}\right)^{n}\right\}\right\} + \text{min}, \quad (2)$$

where $I_{control}$ is the current in the absence of antagonist, min (minimum) is the residual fractional response at saturating concentration of antagonist and IC_{50} is the concentration of drug that causes half this level of inhibition. Data in the text are mean ± standard error (S.E.).

2.6. Drugs

Nylidrin was purchased from Research Biochemicals International (Natick, MA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA), or as noted in the text. Nylidrin was initially dissolved in dimethylsulfoxide (DMSO) and diluted in a series of DMSO stocks over the range 0.01–100 mM. Ringer solutions (0.01–100 μM) were made by 300–3000-fold dilution of stocks.

3. Results

3.1. Subunit-selective inhibition of cloned NMDA receptors expressed in oocytes

Nylidrin was assayed for effects on cloned NMDA receptors by measuring inhibition of currents elicited by fixed concentrations of agonists applied at saturating, or near saturating, concentrations: 10 μM glycine plus 100 μM glutamate for NR1A/2A; 1 μM glycine plus 100 μM glutamate for NR1A/2B and NR1A/2C. Oocyte experiments were performed primarily in zero-Ca^{2+} Ringer, with Ba^{2+} in place of Ca^{2+}. Under these conditions, provided levels of expression were moderate, co-application of glutamate and glycine elicited a monophasic NMDA response largely free of Ca^{2+}-activated Cl^- currents (Williams, 1993; Arellano et al., 1995). In typical experiments oocytes were exposed to agonists until a steady-state current was obtained, and then superfused with a mixture of agonists and increasing concentrations of nylidrin (e.g., Fig. 2). Exposures to nylidrin were maintained long enough to see a steady-state level of inhibition.

Nylidrin potently inhibited responses in oocytes expressing NR1A/2B subunit combinations. Inhibition curves for NR1A/2B responses were biphasic, suggesting high and low affinity components of antagonism (Fig. 2 lower panel; Table 1). The high affinity component of inhibition had an IC_{50} of 0.18 μM and saturated with about 80% of the current blocked. As seen with haloperidol (Ilyin et al., 1996a) potency of antagonism by nylidrin could vary somewhat between different batches of oocytes. Inhibition typically took 1–2 min to equilibrate and washed out over 2–15 min, depending on drug concentration. The remaining 20% of the NR1A/2B response was inhibited at low potency. The IC_{50} for the second component of inhibition was approximately 37 μM, though the accuracy of this estimate was compromised by the limited number of data points over this range. The potency of nylidrin for inhibition of NR1A/2B responses was similar in zero-Ca^{2+} Ringer and in Ca^{2+}-containing Ringer (not shown). Nylidrin also inhibited responses in oocytes expressing NR1A/2A and NR1A/2C subunit combinations, but with greatly reduced potency compared to the high affinity component at NR1A/2B. Inhibition of NR1A/2A and NR1A/2C responses was adequately fit by single compo-

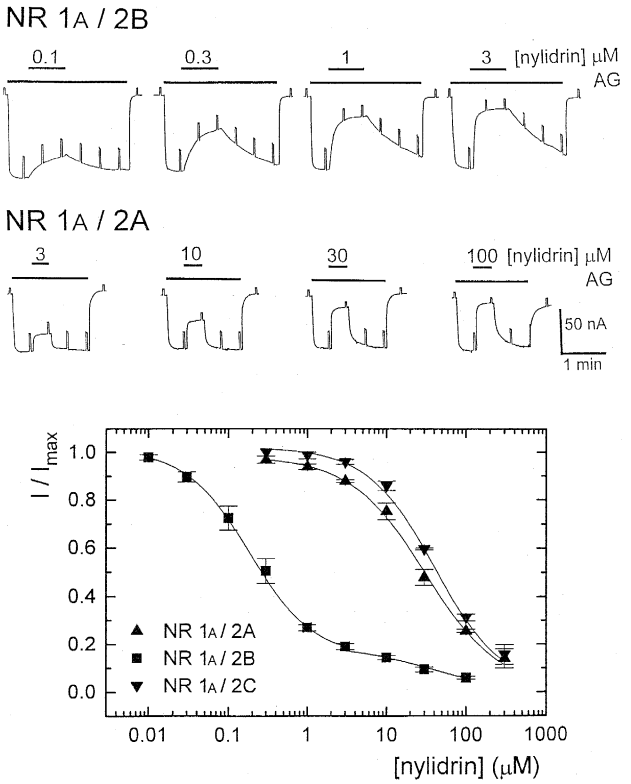

Fig. 2. Inhibition of cloned NMDA receptors expressed in *Xenopus* oocytes. Upper panel: Sample records illustrating inhibition of NMDA responses by nylidrin in oocytes expressing the NR1A/2B (top record) and NR1A/2A (bottom record) subunit combinations. Drugs were applied as indicated by bars. AG signifies a mixture of co-agonists: 1 μM glycine and 100 μM glutamate for NR1A/2B; 10 μM glycine and 100 μM glutamate for NR1A/2A. The holding potential was −70 mV, with regular steps to −60 mV to time drug applications and to assess the membrane conductance. Inward current is denoted by downward deflection. Lower panel: Concentration–inhibition curves for nylidrin; effect of varying the NR2 subunit on nylidrin sensitivity. Response amplitudes are expressed as a fraction of currents elicited by 10 μM glycine plus 100 μM glutamate for NR1A/2A, and 1 μM glycine plus 100 μM glutamate for NR1A/2B and NR1A/2C. Smooth curves are best fits of Eq. (2) to the data. For the NR1A/2B curve, curve-fitting was performed using data points from 0.01–3 μM, representing the high affinity component. The low affinity component was fitted between 3–100 μM. Curve parameters are given in Table 1, including *n* values for each subunit combination. Current ranges and mean responses were: NR1A/2A, 92 to 146 nA (117±16 nA); NR1A/2B, 79 to 190 nA (111±27 nA); NR1A/2C, 25 to 90 nA (56±19 nA).

nent curves with IC_{50} values of 32 μM and 42 μM, respectively (Fig. 1; Table 1). At the holding potential of −70 mV, nylidrin (1 nM to 100 μM) did not activate currents when applied alone to oocytes expressing any of the three subunit combinations.

3.2. Effects of nylidrin on agonist concentration–response curves

The mechanism of inhibition on NR1A/2B and NR1A/2A receptors was investigated by measuring the effects of a fixed concentration of nylidrin on concentration–response curves for glycine and glutamate (Figs. 3 and 4). Antagonism of NR1A/2B by 0.3 μM nylidrin was not surmounted by increasing the glycine concentration (Fig. 3 lower panel). A similar result was seen with NR1A/2A receptors using 30 μM nylidrin (Fig. 3 upper panel). For both subunit combinations, levels of inhibition,

Table 1
Inhibition of NMDA responses by nylidrin

Subunit combination	IC_{50} (μM)	Slope	*n*
Oocyte recordings			
NR1A/2A	32±2.0	−0.91±0.04	3
NR1A/2B (high affinity)	0.18±0.03	−1.06±0.09	4
NR1A/2B (low affinity)	37±5	−0.87±0.11	4
NR1A/2C	42±1.1	−1.0±0.06	3
Neuron recordings			
NMDA	0.22±0.02	−1.2±0.1	3

Inhibition of responses was measured at pH 7.4 using saturating, or near saturating concentrations of agonists. For NR1A/2B and NR1A/2C: glutamate (100 μM) and glycine (1 μM). For NR1A/2A: glutamate (100 μM) and glycine (10 μM). '*n*' indicates the number of independent experiments (oocytes or neurons examined). IC_{50} and slope values are from best fit of data to Eq. (2). Curve fitting for NR1A/2A, NR1A/2C and the low affinity component of NR1A/2B was performed assuming inhibition was complete. Data are presented as mean±S.E. Levels of expression are listed in Figs. 2 and 6.

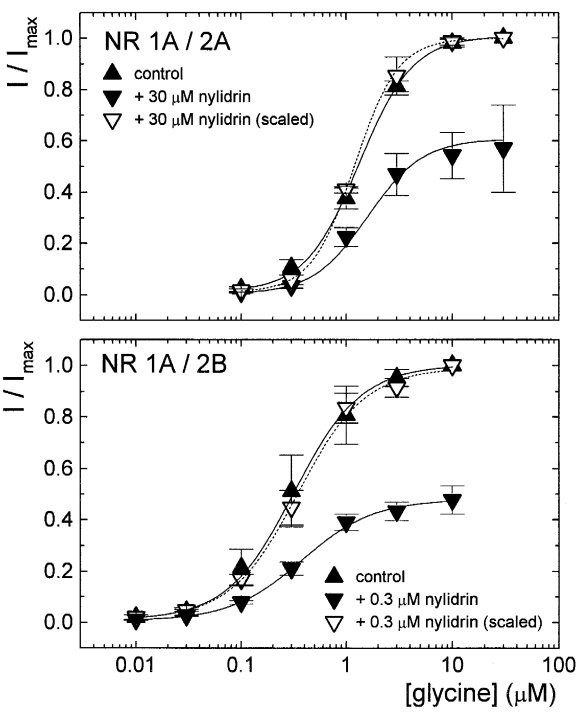

Fig. 3. Effect of nylidrin on steady-state concentration–response curves for glycine in oocytes expressing NR1A/2A (upper panel) and NR1A/2B (lower panel) subunit combinations. For both panels, glutamate was fixed at 100 μM. Response amplitudes are expressed as a fraction of maximum currents (I/I_{max}). Smooth curves are best fits of Eq. (1) to the data. Dotted lines represent data scaled to 1.0, for direct comparisons of affinity. Current ranges and mean steady-state responses were: NR1A/2A, 27 to 172 nA (110±43 nA); NR1A/2B, 25 to 138 nA (57±27 nA).

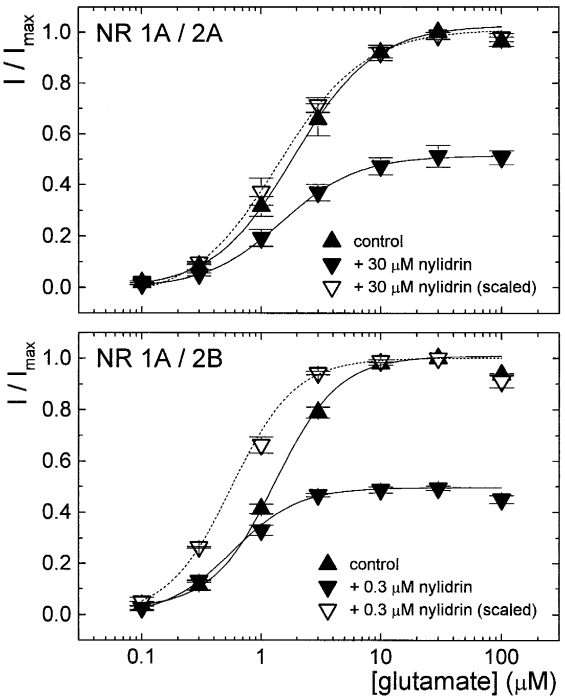

Fig. 4. Effect of nylidrin on steady-state concentration–response curves for glutamate in oocytes expressing NR1A/2A (upper panel) and NR1A/2B (lower panel) subunit combinations. For both panels glycine was fixed at 10 μM. Data plotted as in Fig. 3. Curve parameters are given in Table 2. Current ranges and mean steady-state responses were: NR1A/2A, 29 to 218 nA (132±55 nA); NR1A/2B, 24 to 144 nA (65±40 nA).

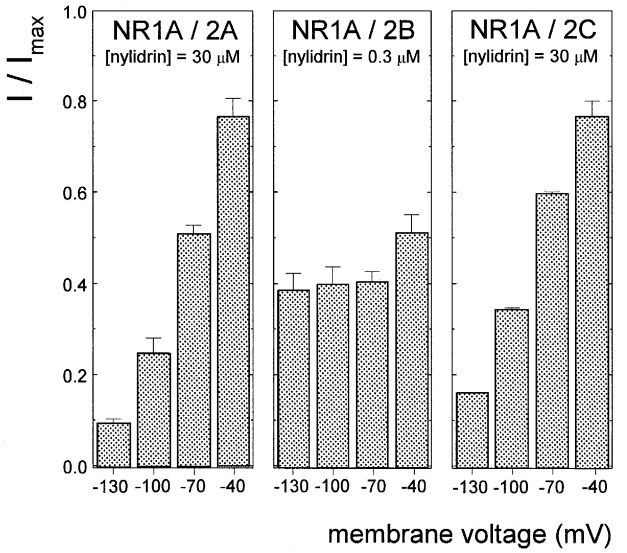

Fig. 5. Effect of membrane voltage on inhibition by nylidrin of NR1A/2A, NR1A/2B and NR1A/2C responses in oocytes. Response amplitudes are expressed as a fraction of currents elicited by 10 μM glycine plus 100 μM glutamate for NR1A/2A, and 1 μM glycine plus 100 μM glutamate for NR1A/2B andNR1A/2C. Concentrations of nylidrin used were 0.3 μM at NR1A/2B, and 30 μM for NR1A/2A and NR1A/2C. Each point is data from 3 different oocytes. Current ranges and mean steady-state responses measured at −70 mV were: NR1A/2A, 57 to 142 nA (104±25 nA); NR1A/2B, 30 to 99 nA (71±21 nA); NR1A/2C, 22 to 77 (49±16).

assessed in terms of EC_{50} values, were independent of glycine concentration ($P = 0.55$ for NR1A/2A and $P = 0.32$ for NR1A/2B). Antagonism of NR1A/2B induced by 0.3 μM nylidrin was also not surmountable by raising concentrations of glutamate (Fig. 4 lower panel). In this case, however, inhibition was associated with a significant increase in apparent affinity for the agonist ($P = 0.004$) (Table 2). For NR1A/2A receptors, inhibition produced by 30 μM nylidrin was not associated with appreciable changes in glutamate affinity ($P = 0.10$) (Fig. 4 upper panel).

3.3. Voltage-dependence of inhibition on oocytes

To test whether antagonism of NR1A/2A, NR1A/2B and NR1A/2C responses by nylidrin was dependent upon membrane voltage levels of inhibition were measured in oocytes at holding potentials over the range of −130 to −40 mV. Antagonism NR1A/2B receptors by 0.3 μM nylidrin was independent of voltage (Fig. 5). There was a

Table 2
Effect of nylidrin on glycine and glutamate concentration–response curves in oocytes

Subunit combination	Condition	EC_{50} (μM)	Slope	*n*
NR1A/2A	glycine (control)	1.3 ± 0.1	1.7 ± 0.2	4
	glycine + 30 μM nylidrin	1.2 ± 0.04	2.1 ± 0.3	4
	glutamate (control)	1.9 ± 0.3	1.3 ± 0.2	3
	glutamate + 30 μM nylidrin	1.4 ± 0.4	1.2 ± 0.3	3
NR1A/2B	glycine (control)	0.32 ± 0.09	1.4 ± 0.3	3
	glycine + 0.3 μM nylidrin	0.34 ± 0.05	1.3 ± 0.2	3
	glutamate (control)	1.3 ± 0.07	1.6 ± 0.1	3
	glutamate + 0.3 μM nylidrin	0.56 ± 0.04	1.6 ± 0.1	3

EC_{50} and slope values are the best fits of pooled data to Eq. (1). Data are presented as mean ± S.E. For NR1A/2B receptors, glutamate curves at NR1A/2B and NR1A/2C were measured in the presence of 10 μM glycine, and at NR1A/2A in the presence of 1 μM glycine. All glycine curves were measured in the presence of 100 μM glutamate. '*n*' indicates the number of independent experiments (oocytes examined). Levels of expression are given in Figs. 3 and 4.

Table 3
Effect of pH on inhibition of NR1A/2B responses by nylidrin

Subunit combination	pH	IC_{50} (μM)	Slope	*n*
NR1A/2B	6.8	0.12 ± 0.05	−1.1 ± 0.5	3
	7.4	0.23 ± 0.04	−0.94 ± 0.2	3
	8.0	0.37 ± 0.06	−1.0 ± 0.2	3

IC_{50} and slope values are the best fits of pooled data to Eq. (2). Data are presented as mean ± S.E. Inhibition was measured using 1 μM glycine plus 100 μM glutamate. '*n*' indicates the number of independent experiments (oocytes examined). Current ranges and mean responses were: pH 6.8, 50 to 101 nA (84 ± 17 nA); pH 7.4, 308 to 403 nA (362 ± 28 nA); pH 8.0, 275 to 330 nA (226 ± 78 nA).

slight decrease in inhibition at −40 mV, but this was not significantly different from that at −70 mV ($P = 0.25$). In contrast, antagonism at NR1A/2A and NR1A/2C by 30 μM nylidrin showed statistically significant voltage-dependence over this range ($P < 0.01$ and $P < 0.001$, respectively). Testing nylidrin at more positive voltages in oocytes was complicated by activation of slowly developing endogenous currents (Arellano et al., 1995).

3.4. pH dependence of inhibition at NR1A / 2B in oocytes

To test the pH dependence of NR1A/2B receptor inhibition by nylidrin concentration–inhibition curves were measured separately at pH 6.8, 7.4 and 8.0. As reported previously (Tang et al., 1990), reducing pH itself produced a substantial reduction in NMDA response. For example, the mean membrane current responses elicited by 1 μM glycine and 100 μM glutamate at pH 7.4 and 6.8 were 362 ± 28 and 84 ± 17 nA, respectively ($n = 3$). The high

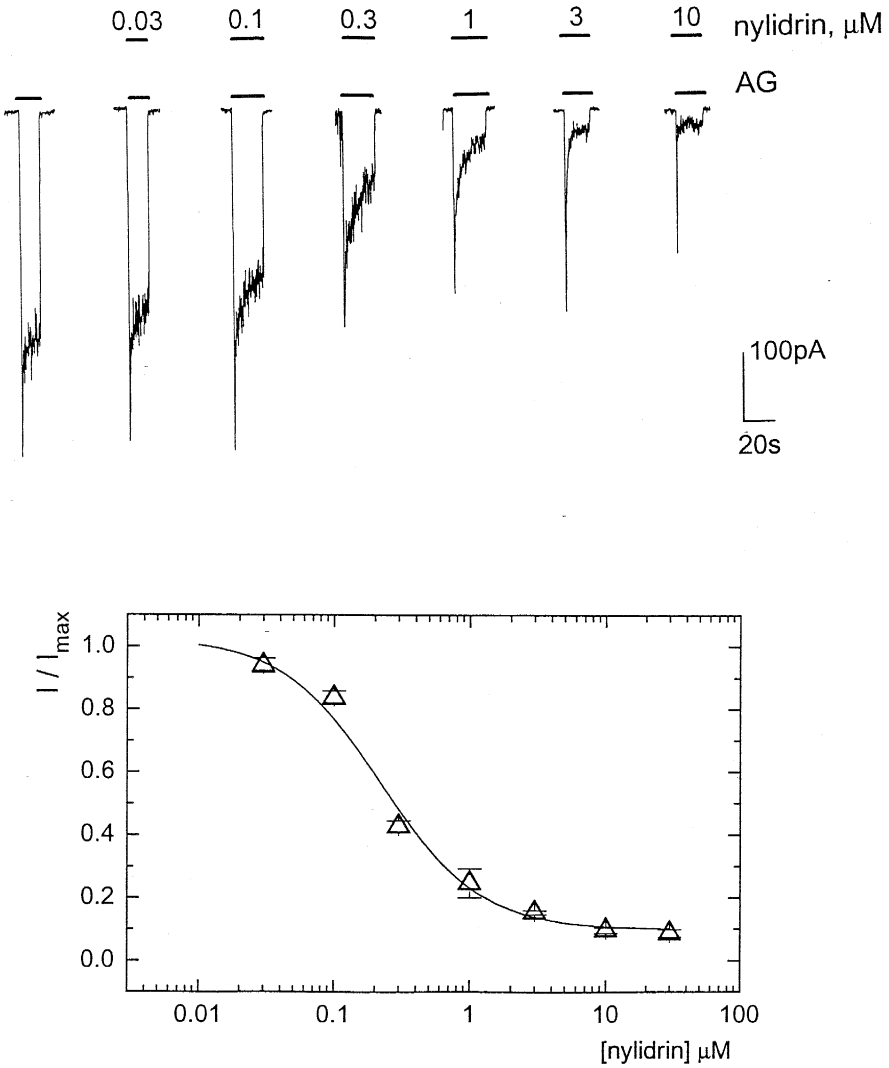

Fig. 6. Inhibition of NMDA receptors in cultured rat cortical neurons. Upper panel: Sample records illustrating inhibition of NMDA-induced currents by nylidrin in a single neuron. Co-agonists and nylidrin were applied simultaneously as indicated by bars. AG (agonists) = 1 μM glycine plus 100 μM NMDA. The holding potential was −60 mV. Inward current is denoted by downward deflection. Lower panel: Concentration–inhibition curve for nylidrin. Data from three neurons are presented. Response amplitudes are expressed as a fraction of control values. The smooth curve is the best fit of Eq. (2) to the data. Curve parameters are given in Table 1. Current ranges and mean responses were: 110 to 407 pA (254 ± 98 pA, $n = 11$).

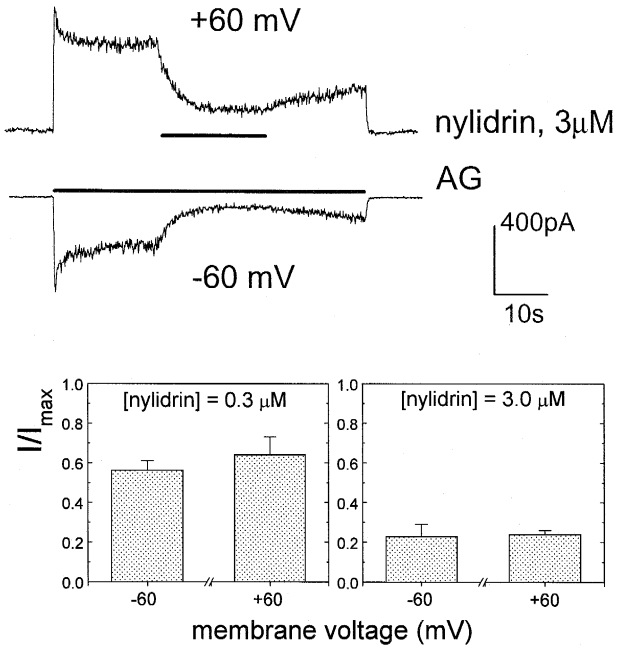

Fig. 7. Effect of membrane voltage on inhibition of NMDA responses by nylidrin in cortical neurons. Upper panel: Sample currents evoked by co-agonists at two membrane voltages, −60 mV and +60 mV. AG = 1 μM glycine plus 100 μM NMDA. Nylidrin, 3 μM was applied when the NMDA response reached its steady-state level. Lower panel: Response amplitudes in the presence of 0.3 μM and 3 μM nylidrin expressed as a fraction of control values at the two membrane voltages ($n = 5$).

affinity IC_{50} value for nylidrin progressively decreased as the pH was lowered, such that potency measured at pH 6.8 was approx. 3-fold higher than that measured at pH 8.0 (Table 3). Differences in IC_{50} were statistically significant measured between pH 6.8 and 8.0, but were insignificant between pH 6.8 and 7.4, or between pH 7.4 and 8.0.

3.5. Inhibition of neuronal NMDA responses

Whole cell recordings were made from rat cortical neurons maintained for 6 to 14 days in culture. Neurons with large cell bodies and pyramidal morphology were chosen for electrophysiology. Previous studies have shown that neurons at this age in vitro express primarily NR1/2B subunits (Williams et al., 1993; Zhong et al., 1994) and have high sensitivity to the NR1/2B-selective antagonist haloperidol (Ilyin et al., 1996a). Responses to saturating concentrations of agonists (100 μM NMDA plus 1 μM glycine) were inward currents consisting of a rapid rising phase followed by desensitization to a steady-state level (Fig. 6, upper panel). Using the linear array for drug application, peak amplitudes had poor reproducibility in successive trials due to inconsistencies in the speed of delivery. All inhibition curves were therefore measured under steady-state desensitizing conditions on the plateau phase of the response. Similar to NR1A/2B receptors in

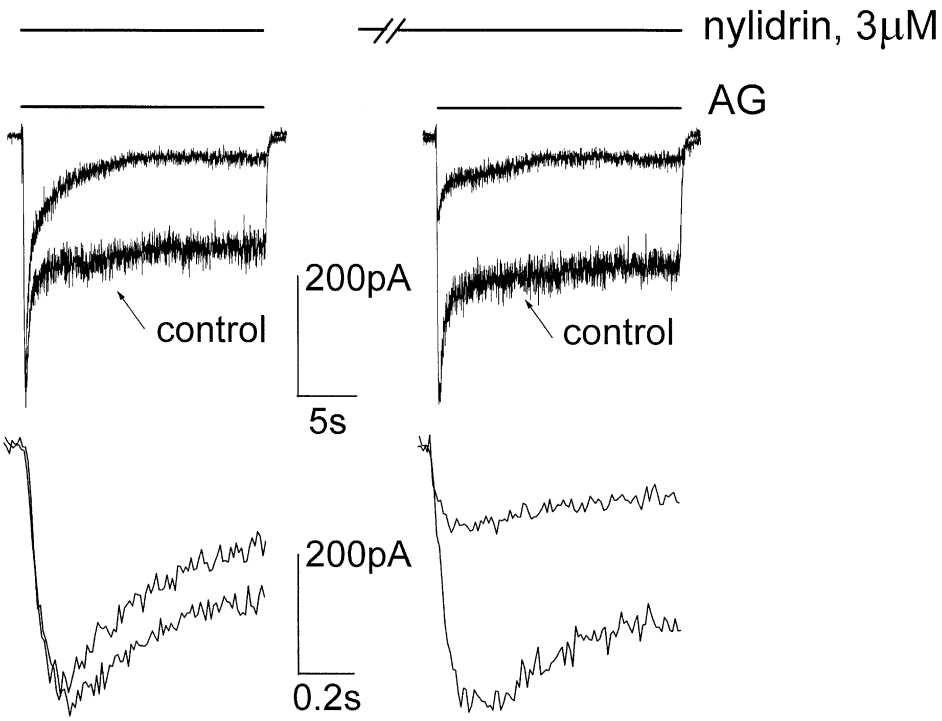

Fig. 8. Use dependence of NMDA receptor inhibition by nylidrin. Inhibition induced by 3 μM nylidrin showing the comparison between simultaneous application of antagonist (left column) and pretreatment with antagonist (right column). Time of pretreatment was 24 s. In each column lower traces are the control and drug treated responses superimposed at a higher sweep. Control = 1 μM glycine plus 100 μM NMDA.

oocytes, NMDA responses in neurons were inhibited with an IC_{50} value of 0.22 μM (Table 1). Inhibition of neuronal NMDA responses by nylidrin was also incomplete: approx. 10% of the response remained at what appeared to be saturating antagonist concentrations. In neurons, nylidrin was not assayed at concentrations >30 μM to see if a second, low affinity, component of inhibition was present. Exposure to nylidrin alone at concentrations up to 30 μM did not elicit measurable currents in neurons.

3.6. Mechanism of neuronal NMDA receptor antagonism

The mechanism of inhibition was investigated by testing for voltage-dependence and by testing whether nylidrin behaved as a classic open channel blocker. Voltage-dependence of inhibition was assessed by comparing the levels of block induced by two concentrations of nylidrin, 0.3 and 3 μM, at two different holding voltages, −60 and +60 mV (Fig. 7). For 0.3 μM nylidrin, the fractional response was 0.56 ± 0.05 at −60 mV and 0.64 ± 0.09 at +60 mV ($n = 5$). For 3 μM nylidrin the fractional response was 0.23 ± 0.06 at −60 mV and 0.24 ± 0.02 at +60 mV ($n = 5$). There was no significant difference in level of block for either concentration ($P = 0.07$ for 0.3 μM nylidrin; $P = 0.24$ for 3 μM nylidrin).

Evidence for channel trap blockade or other 'activity-dependent' mechanisms of inhibition were assessed in experiments comparing the effects of 3 μM nylidrin applied with pre-incubation or no pre-incubation. These experiments were done using a DAD-12 drug perfusion system which allowed rapid and more reproducible delivery of agonists. Simultaneous co-application of agonists with 3 μM nylidrin resulted in a spike of current which decayed to a steady-state level that was approx. 15% of the control current. The amplitude of the spike was similar to that of the control responses (Fig. 8, left traces). In contrast, after pre-incubation with 3 μM nylidrin the spike of current induced by agonists was substantially reduced (Fig. 8, right traces). In these experiments it was also possible to compare the degree to which nylidrin inhibited the peak versus the steady-state component of the NMDA response. For example, after a 24 s pre-incubation with 3 μM nylidrin the fractional peak response was significantly greater than the fractional steady-state current: 0.33 ± 0.004 and 0.15 ± 0.002, respectively ($p < 0.001$) ($n = 6$). Lastly, washout of 3 μM nylidrin inhibition was investigated by comparing rates of wash either with, or without, activation of the receptor. Washout of inhibition was largely independent of channel activity (Fig. 9).

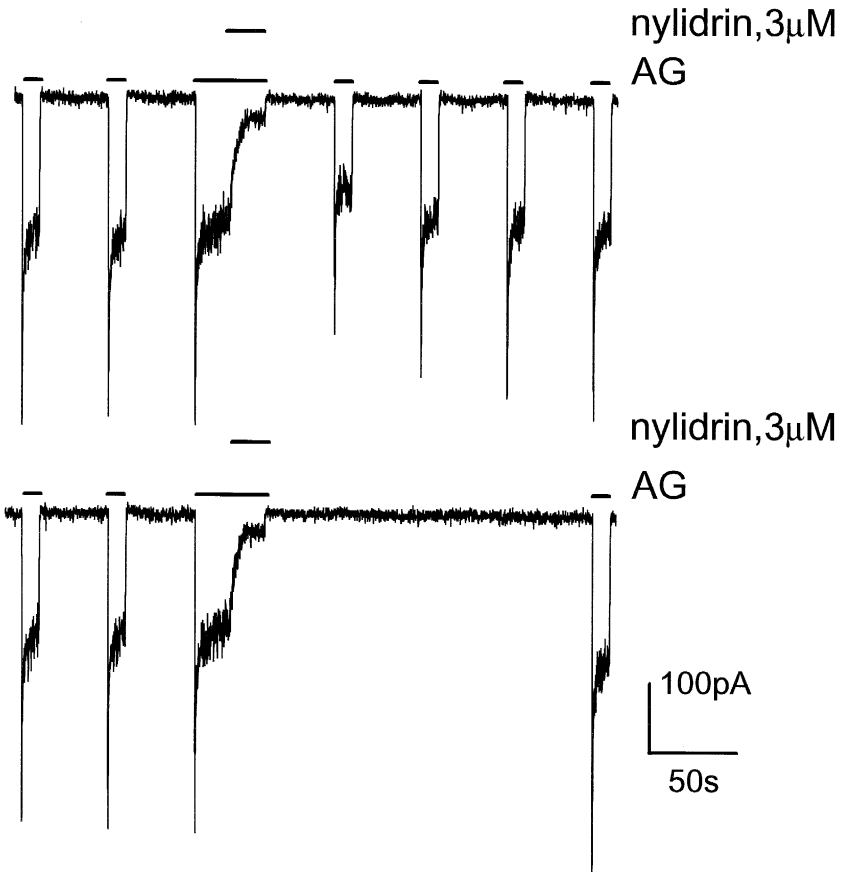

Fig. 9. Effect of channel activation on rate of washout of inhibition by 3 μM nylidrin. Co-agonists and nylidrin were applied as indicated by bars. AG = 1 μM glycine plus 100 μM NMDA. Records were taken from the same cortical neuron.

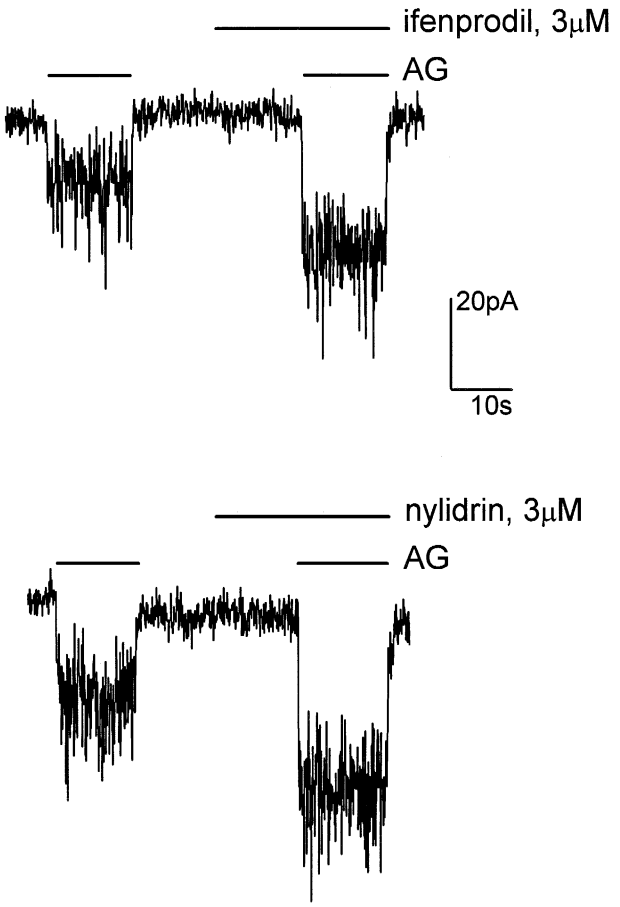

Fig. 10. Potentiation of NMDA responses in cortical neurons by nylidrin and ifenprodil at low concentrations of NMDA. Co-agonists, nylidrin and ifenprodil were applied as indicated by bars. AG = 30 μM glycine plus 1 μM NMDA (note: other neuronal recordings demonstrating inhibition by nylidrin (Figs. 6–9) were done using 100 μM NMDA). Records were taken from the same cortical neuron.

3.7. Potentiation of NMDA responses at low glutamate-site agonist concentrations

To assess whether the increase in apparent affinity of glutamate at NR 1A/2B in the presence of nylidrin (see Fig. 4, lower panel) may result in potentiation of NMDA receptor responses at low concentrations of glutamate or NMDA, we tested the effects of 3 μM nylidrin on currents evoked by 1 μM NMDA plus 30 μM glycine in 2 neurons (Fig. 10). As a positive control, effects of 3 μM ifenprodil on these responses were assayed in the same cells. Both drugs increased the control currents by approx. 100%.

4. Discussion

4.1. Subunit-selective inhibition of binary NMDA subunit combinations

The principle finding in this study is that nylidrin is a subunit-selective inhibitor of NMDA receptors. Nylidrin inhibits recombinant rat NR1A/2B receptors expressed in *Xenopus* oocytes with an IC_{50} of approx. 0.2 μM and has > 175-fold selectivity for NR1A/2B receptors as compared to NR1A/2A and NR1A/2C. High potency inhibition of NR1A/2B receptors by nylidrin is insurmountable with respect to glycine and glutamate, indicating that the drug is not a conventional competitive antagonist at the glycine or glutamate binding sites. Inhibition is, however, associated with an increase in apparent affinity for glutamate, suggesting positive allosteric coupling between glutamate and inhibitor binding sites.

Inhibition of NR1A/2B receptors by nylidrin shows pH-dependence, with increased potency at lower pH values (also see Pahk and Williams, 1997). Since the amine in nylidrin has a pK_a between 9 and 10, inhibition measured between pH 6.8 and 8 is probably not due to changes in the degree of ionization of this moiety. The most likely explanation for the effect is positive allosteric coupling between the nylidrin and proton regulatory sites (Pahk and Williams, 1997). Antagonism of NR1A/2B receptors by nylidrin is independent of membrane voltage, which argues that nylidrin is not acting at a site deep in the channel pore (Huettner and Bean, 1988). Taken together, inhibition of NMDA receptor function by nylidrin would appear to be mediated by an allosteric modulatory site located on the external surface of the receptor, which is dependent on NR2B subunits for functional integrity (see Williams, 1993; Gallagher et al., 1996).

The low affinity inhibition of NR1A/2A and NR1A/2C receptors is, in contrast, voltage dependent. The mechanism is therefore consistent with a site of inhibition located in the channel pore. Though we did not test voltage-dependence for the low affinity component of block of NR1A/2B, it is interesting to note that the apparent affinity (37 μM) is strictly comparable to the potency of inhibition at NR1A/2A (32 μM) and NR1A/2C (42 μM). The simplest explanation for this correspondence is that all three subunit combinations contain a second low affinity site for nylidrin located in the channel pore. This site would appear to be well conserved across the different subunit combinations and thus may be associated with the NR1 subunit. What relation the site has to the phencyclidine binding site, or to other sites in the pore such as those for polyamines, polyamine toxins, or Zn^{2+} (Sucher et al., 1996), remains uncertain.

4.2. Inhibition of NMDA receptors in neurons

Cortical neurons prepared as described and cultured for < 14 days express primarily NR1A/2B receptors (Williams et al., 1993; Zhong et al., 1994). NMDA-evoked currents in such neurons are sensitive to inhibition by haloperidol (Ilyin et al., 1996a), ifenprodil (Kew et al., 1996) and eliprodil (Whittemore et al., 1997) and potencies and degrees of inhibition in neurons are consistent with blockade of NR1/2B receptors. The inhibition of

NMDA responses in neurons by nylidrin shares many of the properties described for NR1A/2B receptors expressed in oocytes. Specifically, the neuronal NMDA receptors are blocked at the same potency as the cloned receptors (high affinity component), the inhibition is incomplete, with 10–15% of the current remaining unblocked, and is not dependent on voltage, even at strongly positive holding potentials. Our neuronal recordings with nylidrin also demonstrate positive coupling between the glutamate site and the inhibitor site at low concentrations of glutamate-site agonists, as reported for ifenprodil (Kew et al., 1996).

The experiments comparing inhibition after simultaneous application of agonists and nylidrin with inhibition after pre-incubation with nylidrin suggest two further points: (1) The reduction in peak current after pre-incubation suggests that nylidrin is able to interact with the receptor in resting state prior to channel activation. (2) The increased fractional inhibition of the steady-state response as compared to the peak argues that, though nylidrin binds the receptor in the resting state, the potency of the interaction is increased by channel activation or desensitization. The comparisons of washout suggest that channel activation does not facilitate unbinding of nylidrin from the channel. Collectively, these experiments confirm that nylidrin is not behaving as an open-channel, or 'channel-trap', blocker where inhibition and wash would be dependent upon channel activation (Huettner and Bean, 1988).

4.3. Similarities with other NR2B-selective NMDA receptor antagonists

The present study shows that inhibition of NMDA receptors by nylidrin shares many features with NMDA receptor inhibition by ifenprodil (Legendre and Westbrook, 1991; Williams, 1993; Kew et al., 1996), CP 101,606 (Mott et al., 1996), Ro 25-6981 (Trube et al., 1996) and haloperidol (Ilyin et al., 1996a). The similarities include: (1) pronounced selectivity for NR1/2B receptors, (2) incomplete, or two component, block of current, (3) insurmountable inhibition with respect to glutamate and glycine, (4) absence of voltage-dependence or channel-trap properties and (5) pH-dependence. Additional similarities include evidence for positive allosteric coupling between the nylidrin and glutamate sites, and the indication of increased potency on the activated/desensitized receptor: both features that are consistent with the detailed model proposed for ifenprodil (Kew et al., 1996). These numerous similarities suggest that the mechanism of inhibition for nylidrin is similar or identical to ifenprodil, CP 101,606 and Ro 25-6981, and that all these drugs interact at the same, or at least overlapping, binding sites. We did not test nylidrin on other NR1 splice variants expressed in combination with NR2B, but experiments with ifenprodil and haloperidol imply that inhibition will be independent of isoform (Williams, 1993; Ilyin et al., 1996a).

In support of this idea, binding experiments indicate that nylidrin displaces [^{125}I]ifenprodil from rat cortical membranes (Beart et al., 1991; Mercer et al., 1993). However, the K_i values reported in these studies were approx. 100-fold higher (30 μM) than we observe at NR1A/2B in oocytes and neurons. It is unclear why nylidrin was so weak in these binding experiments, since in the same system ifenprodil and eliprodil were reported to have K_i values very similar to those we observe electrophysiologically at NR1A/2B (Whittemore et al., 1997). In a second binding study focussing on σ (sigma) site pharmacology, nylidrin was shown to displace [^{3}H]ifenprodil from rat cortical membranes with a K_i of 49 nM (Schoemaker et al., 1994), more in line with the IC_{50} value at NR1A/2B. In this case, however, the authors considered the principle binding site for [^{3}H]ifenprodil to be a σ site, rather than the NMDA receptor.

Ifenprodil, eliprodil, CP 101,606 and Ro 25-6981 are all related structurally (Fig. 1). Nylidrin can be considered an analog of these molecules with a secondary amine in the place of the piperidine nitrogen. The potency of NR1A/2B receptor inhibition for nylidrin is comparable to that of ifenprodil and CP 101,606 (Williams, 1993; Kew et al., 1996; Mott et al., 1996): in our hands the IC_{50} values are 0.18, 0.31 and 0.11 μM, respectively (Whittemore et al., 1997). This indicates that conformational and geometrical constraints conferred on the molecules by the piperidine ring do not result in any particular advantage in terms of affinity for the NR1/2B site. Also, potency is largely unaffected by changing the amine from tertiary in the piperidine compounds to secondary in nylidrin. The differences in pK_a between the piperidine nitrogen (approx. 8.5–9) and the secondary amine in nylidrin (approx. 9–10) are not sufficiently different to support a difference in electrical properties between the molecules. It follows that exploring the SAR of other related non-piperidine compounds should yield additional potent and selective NR1A/2B antagonists.

4.4. Other sites of action and relevance to therapeutic potential

Nylidrin was described initially as a vasodilator, with β-sympathomimetic activity (Ariëns et al., 1962). Later studies demonstrated anti-hypertensive effects at β_2 receptors (Yen and Pearson, 1979) and β-adrenergic binding with an IC_{50} of 0.37 μM (Bilezikian et al., 1978). Other reports of cardiovascular effects for nylidrin suggested actions at α-adrenergic receptors centrally (Fichtl and Felix, 1980) and binding studies have shown that nylidrin is a potent α-adrenergic ligand, with a K_d of 66 nM for α-receptors from hepatic membranes (Guellaen et al., 1978). In addition, it seems likely that nylidrin will share other sites of actions common to some, or all, of the piperidines mentioned above; e.g. dopamine D_2, and possibly D_3 and D_4 receptors, σ_1 and σ_2 sites, and blocking effects at voltage-gated Na^+, K^+ and Ca^{2+}-channels

(Guellaen et al., 1978; Beart et al., 1994; Bath et al., 1996, Whittemore et al., 1997). Ifenprodil, eliprodil, CP 101,606, Ro 25-6981 and haloperidol all have neuroprotective properties in vitro and in animal models of stroke and traumatic brain injury (Shalaby et al., 1992; Williams, 1993; Scatton et al., 1994; Chenard et al., 1995; Vartanian and Boxer, 1996). This supports the idea that selective blockade of NR1A/2B receptors is sufficient for neuroprotection, and that these types of drugs may have potential for clinical development. Though nylidrin may have central activity (Fichtl and Felix, 1980), its effects on the cardiovascular system, particularly when administered i.v., compromise the drug in terms of clinical utility as a neuroprotectant. As was found with piperidine based molecules like ifenprodil, for non-piperidine NR1/2B-selective antagonists it will be important to design molecules with significantly improved side effect profiles.

4.5. Conclusion

Nylidrin is a potent NMDA receptor antagonist which shows selectivity for NR1/2B subunit combinations. Inhibition appears to be mediated allosterically in a manner similar to that observed for ifenprodil, eliprodil, CP 101,606, Ro 25-6981 and haloperidol. Nylidrin differs from these compounds structurally being a non-piperidine, and thus should serve as a structural lead for developing novel subtype-selective NMDA receptor ligands.

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