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Neuroprotective effects of RPR 104632, a novel antagonist at the glycine site of the NMDA receptor, in vitro

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Abstract

The NMDA antagonist and neuroprotective effects of RPR 104632 (2*H*-1,2,4-benzothiadiazine-1-dioxide-3-carboxylic acid), a new benzothiadiazine derivative, with affinity for the glycine site of the NMDA receptor-channel complex are described. RPR 104632 antagonized the binding of [^{3}H]5,7-dichlorokynurenic acid to the rat cerebral cortex, with a K_i of 4.9 nM. This effect was stereospecific, since the (−)-isomer was 500-fold more potent than the (+)-isomer. The potent affinity of RPR 104632 for the glycine site was confirmed by the observation that RPR 104632 inhibited [^{3}H]*N*-[1-(2-thienyl)cyclohexyl]-3,4-piperidine ([^{3}H]TCP) binding in the presence of *N*-methyl-D-aspartate (NMDA) (IC_{50} = 55 nM), whereas it had no effect on the competitive NMDA site or on the dissociative anaesthetic site. RPR 104632 inhibited the NMDA-evoked increase in guanosine 3′,5′-cyclic monophosphate (cGMP) levels of neonatal rat cerebellar slices (IC_{50} = 890 nM) in a non-competitive manner and markedly reduced NMDA-induced neurotoxicity in rat hippocampal slices and in cortical primary cell cultures. These results suggest that RPR 104632 is a high-affinity specific antagonist of the glycine site coupled to the NMDA receptor channel with potent neuroprotective properties in vitro.

Keywords: RPR 104632; Glycine receptor antagonist; Glutamate; NMDA receptor-channel complex; Neurotoxicity

1. Introduction

The discovery of a strychnine-insensitive glycine modulatory site on NMDA receptors by Johnson and Ascher (1987) stimulated the search for a new generation of antagonists of glutamatergic transmission. The quinoline derivative, kynurenic acid, which represents the first generation of such compounds, had been described as an antagonist of the NMDA receptor (Perkins and Stone, 1984; Ganong and Cotman, 1986), but was found later to modulate the NMDA complex via the glycine modulatory site (Birch et al., 1989). Since then, various derivatives of kynurenic acid with better affinity and specificity for the glycine site have been reported (see Carter, 1992; Kemp and Leeson, 1993; Bigge, 1993; Ornstein et al., 1994).

In the present work, we investigated the biochemical activity of a new ligand of the glycine site, RPR 104632 (2*H*-1,2,4-benzothiadiazine-1-dioxide-3-carboxylic acid; Fig. 1). This molecule is chemically unrelated to kynurenate and therefore can be considered to represent a new generation of glycine site antagonists. We compared the effects of RPR 104632 to those of previously reported glycine site ligands, 5,7-dichlorokynurenic acid and L-689,560 (Baron et al., 1990, Baron et al., 1991; Leeson et al., 1991). To do this, we first tested whether RPR 104632 directly competes with [^{3}H]5,7-dichlorokynurenic acid binding for the glycine site.

We then tested the effect of RPR 104632 on [^{3}H]*N*-[1-(2-thienyl)cyclohexyl]-3,4-piperidine ([^{3}H]TCP) binding in the presence of *N*-methyl-D-aspartate (NMDA), glycine or the combination of both these ligands, in a functional model that can be used to determine which site is involved in the opening of the NMDA-receptor channel.

Glycine antagonists modulate the NMDA-induced increase in guanosine 3′,5′-cyclic monophosphate (cGMP) levels in neonatal rat cerebellar slices (Baron et al., 1990). Thus, the activity of RPR 104632 was also evaluated in this model. Finally, to test whether RPR 104632 exerts a neuroprotective effect, we used an in vitro model of neuro-

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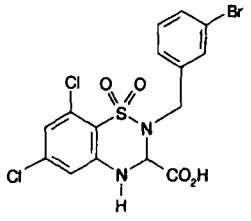

Fig. 1. Structure of RPR 104632.

toxicity, in which neurodegeneration is induced by NMDA in immature rat hippocampal slices (Malgouris et al., 1994) and in primary cultures of cortical neurones (Choi et al., 1988).

A preliminary report of some of these data has already been presented (Doble et al., 1994).

2. Materials and methods

2.1. [^{3}H]5,7-Dichlorokynurenic acid binding

2.1.1. Membrane preparation

Membranes from rat cerebral cortex were prepared according to the method published by Snell et al. (1988). Briefly, rats were decapitated and their cerebral cortices removed on ice and frozen at −80°C for at least 1 h. The tissue was rapidly thawed, homogenised with a Polytron in 10 volumes of cold (4°C) sucrose (0.32 M) and centrifuged at 1000 × *g* for 10 min. The supernatant was recovered and recentrifuged at 20000 × *g* for 20 min. The resulting pellet was resuspended in 20 volumes of ice-cold distilled water and centrifuged at 8000 × *g* for 20 min. The supernatant and buffy layer were collected and centrifuged at 48000 × *g* for 20 min. The pellet was resuspended in 20 volumes of ice-cold distilled water and recentrifuged at 48000 × *g* for 20 min. The final pellet was frozen at −20°C until use. On the day of the binding assay, the membranes were thawed and resuspended in 20 volumes of Hepes-KOH buffer (50 mM, pH 7.5), incubated at 37°C for 20 min and then recentrifuged at 48000 × *g* for 10 min. This procedure was repeated once more. The final pellet was resuspended in the appropriate buffer for use in the binding assay.

2.1.2. [^{3}H]5,7-Dichlorokynurenic acid binding assay

The extent of [^{3}H]5,7-dichlorokynurenic acid binding to the glycine recognition site on the NMDA receptor was determined by the method described by Canton et al. (1992). Briefly, membranes were suspended in Hepes-KOH buffer (0.1 mg protein/ml) and incubated for 10 min at 4°C with [^{3}H]5,7-dichlorokynurenic acid (20 nM), the studied compound or 1 mM glycine for determination of the non-specific binding. The binding interaction was terminated by filtration through Whatman GF/B glass fibre filters, and filters were immediately rinsed three times with 4 ml of cold Hepes-KOH buffer (pH 7.5, containing 10 mM magnesium sulfate). Each determination was performed in duplicate. The radioactivity remaining on the filters was measured by liquid scintillometry in Ready-Gel scintillant.

2.2. [^{3}H]TCP binding

2.2.1. Membrane preparation

Rats were decapitated and their cerebral cortices removed on ice and frozen at −80°C for at least 24 h. The tissue was rapidly thawed, homogenised with a Polytron in 20 volumes of cold (4°C) sucrose (0.32 M) and centrifuged at 1000 × *g* for 20 min. The supernatant was recovered and recentrifuged at 17500 × *g* for 20 min. The resulting pellet was resuspended in 50 volumes of ice-cold distilled water, incubated for 30 min at 37°C and centrifuged at 32000 × *g* for 20 min. This procedure was repeated once more. The pellet was resuspended in 50 volumes of Hepes-NaOH 10 mM, pH 7.5 and centrifuged at 32000 × *g* for 20 min. The resulting pellet was resuspended in 30 volumes of Hepes buffer and frozen at −80°C until use. On the day of the binding assay, the membranes were thawed and centrifuged at 32000 × *g* for 20 min. This procedure was repeated once more. The final pellet was resuspended in the appropriate buffer for use in the binding assay.

2.2.2. [^{3}H]TCP binding assay

The extent of [^{3}H]TCP binding to the dissociative anaesthetic binding site was determined by the method described by Hori et al. (1991). Membranes (0.2 mg protein/ml) suspended in 10 mM Hepes buffer (pH 7.5) were incubated for 45 min at 25°C with 2.5 nM [^{3}H]TCP and the studied compound, plus NMDA 100 μM, glycine 1 mM or both. MK-801 (10 μM) was added to some of the aliquots for determination of the non-specific binding. The binding interaction was terminated by filtration through Whatman GF/B glass fibre filters with a Skatron cell harvester, and filters were immediately rinsed with 3 × 4 ml of cold buffer. Each determination was performed in duplicate. The radioactivity remaining on the filters was measured by liquid scintillometry in Ready Solv HP scintillant.

2.3. cGMP accumulation

The accumulation of cGMP in cerebellar slices was determined by the method of Ferrendelli et al. (1973) with modifications. Cerebella from immature rats were sliced in two dimensions (0.5 × 0.5 mm) with a McIlwain tissue chopper. The slices were preincubated for 1.5 h at 37°C in a Krebs-Ringer bicarbonate medium (pH 7.4), which contained, in mM, NaCl: 120; KCl: 4.7; $CaCl_2$: 2.5:, $MgSO_4$: 2.4; KH_2PO_4: 1.2; $NaHCO_3$: 25; glucose 10, and which was continuously gassed with 95% O_2/5% CO_2. Aliquots

of suspensions of slices were transferred to microtubes containing test compounds. Slices were preincubated in the presence of RPR 104632 or studied compounds in a final volume of 1 ml for 5 min and then incubated with NMDA for 5 min. The accumulation of cGMP was stopped by aspiration of the supernatant, addition of 500 μl of boiling 50 mM Tris, 4 mM EDTA, buffer pH 7.5 and maintenance of the sample at 100°C for 10 min. Each sample was sonicated, and after centrifugation, the amounts of cGMP in the supernatants were determined by radioimmunoassay with a commercially available cGMP assay kit (Amersham TRK 500). Results are expressed as the means of cGMP levels per milligram of protein ± S.E.M.

2.4. NMDA-induced neurotoxicity in hippocampal slices

2.4.1. Slice preparation and induction of neurotoxicity

The in vitro model of neurotoxicity we used was that described by Ellrén and Lehmann (1989), with a slight modification. Briefly, 8-day-old Sprague-Dawley rats of both sexes were decapitated, and transverse hippocampal slices (400 μm) were prepared with a McIlwain tissue chopper, then preincubated for 60 min in gassed (95% O_2/5% CO_2) Krebs-Ringer bicarbonate buffer (KRB) containing, in mM, NaCl: 122, KCl: 3, $MgSO_4$: 1.2, KH_2PO_4: 0.4, $NaHCO_3$: 25, $CaCl_2$: 1.2 and D-glucose: 10. Slices were then rinsed twice with 5 ml of fresh KRB, and aliquots (300 μl) containing approximately 10 slices were transferred to test tubes containing 5 ml KRB and assigned in duplicate to the following groups: control, NMDA, NMDA + glycine, in the presence or in the absence of tested drugs. The tubes were placed in a slowly shaking water-bath (37°C), then sealed. A mixture of 95% O_2 and 5% CO_2 was blown over the surface of the incubation medium through a cannula in the lid of the tube. The slices were incubated for 45 min with RPR 104632 (1, 10 μM). Fifteen minutes after the beginning of the incubation, NMDA (100 μM) or NMDA (100 μM) + glycine (1 mM) was added to the incubation medium. Slices were then transferred to fresh agonist/antagonist-free medium (5 ml) for the recovery period (3 h).

2.4.2. Histology and image analysis

For histological examination, slices were then fixed with glutaraldehyde (5%) in 0.1 M phosphate buffer. After embedding and freezing, serial sections (10 μm) were cut at −20°C using a Leica 1720 cryostat microtome and stained with Cresyl violet for light microscopy and neurone counts.

Living neurones in the pyramidal cell layer of the CA1 and CA3 areas of the hippocampus were counted in 100 × 450 μm fields at a magnification of 400 ×. The living neurones were detected with a specific image analysis software using a computerised image analysis system (Quantimet 970, Leica). At least five fields from two slices per group were examined.

Values are expressed as means ± S.E.M. of neurones remaining alive. The protective effect of tested drugs was calculated from the number of living neurones.

2.5. NMDA-induced neurotoxicity in primary cortical neurones

2.5.1. Preparation of cultured cerebral cortical cells

Cerebral cortical cells from 17-day-old rat foetuses (Sprague-Dawley strain) were isolated and cultured according to a previously reported procedure (Dichter, 1978). Briefly, the dissected cerebral cortices were minced with scissors in phosphate buffer saline (PBS) buffer without Ca^{2+} and Mg^{2+}, supplemented with 33 mM glucose and digested with 0.25 mg/ml trypsin in the same buffer for 10 min incubation at 37°C. The reaction was stopped by addition of 7 μg/ml DNAse and 4 μg/ml trypsin inhibitor in the same buffer. The complete dissociation was achieved by repeated trituration using a serum-coated Pasteur pipette with a fire-polished tip in the buffer containing 27 μg/ml DNAse and 160 μg/ml trypsin inhibitor. Finally, the isolated cells were suspended in the following culture medium: Dulbecco modified Eagle medium with Glutamax and 4.5 g/l glucose supplemented with 3% foetal calf serum and hormones (100 μg/ml transferrin, 25 μg/ml insulin, 60 μM putrescine, 20 nM progesterone and 30 nM sodium selenite). They were plated at an initial density of around 8×10^5 cells per well onto 96-well Costar plates previously coated with 10 μg/ml poly-L-lysine. The plates were then kept for 8 days before use without medium change.

2.5.2. Neurotoxic effect and its measurement

The primary cell cultures were exposed to NMDA 1 mM with or without test compounds in buffer (Tris-HCl 25 mM with 2.3 mM $CaCl_2$, 5.6 mM KCl and 155 mM NaCl at pH 8) substituted for culture medium by triple exchange. After a 30 min incubation at room temperature, the buffer was replaced by the culture medium.

The day after the start of the exposure, the neurotoxicity was visualized by trypan blue exclusion and measured by a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (1983). Briefly, MTT was incubated directly in the wells for 2 h at 37°C. The optical density was then measured at 540 nm using an SLT Rainbow spectrophotometer.

The optical density measured on NMDA-treated cultures was considered to be 100% of toxicity. Each point was determined in sextuplicate, and the data are presented as the mean ± S.E.M. of two or three independent experiments.

2.6. Data analysis

For binding and cGMP accumulation studies, the data points within each experiment were averaged. The binding

Table 1
Affinity for the glycine site ([^{3}H]5,7-dichlorokynurenic acid binding)

Ligand	K_i (nM)
(±)-RPR 104632	4.9 ± 0.2
(−)-RPR 104632	2.2 ± 0.5
(+)-RPR 104632	1115 ± 276
DCKA	27 ± 2
L-689560	15 ± 1
7-CKA	117 ± 12
(±)-HA-966	19950 ± 530
(−)-HA-966	> 100000
Glycine	441 ± 21

Comparison of the potency of RPR 104632 and other glycine site ligands at inhibiting [^{3}H]5,7-dichlorokynurenic acid binding. Specific binding of 20 nM [^{3}H]5,7-dichlorokynurenic acid was determined in the presence or in the absence of at least 4 concentrations of studied compounds. 5,7-Dichlorokynurenic acid: DCKA; 7-chlorokynurenic acid: 7-CKA. The data shown are means ± S.E.M. of 3–6 independent experiments.

observed in the presence of appropriate cold ligand was defined as the non-specific binding. This value was subtracted from the other data points. The binding remaining in the presence of RPR 104632 or reference compound was calculated as a percentage of the specific binding observed with the radioligand alone. Where appropriate, the concentration of compound inhibiting 50% (IC_{50}) of the specific binding (or 50% of the biochemical response in the case of cGMP) was calculated by computer-assisted iterative non-linear regression analysis, for which the ENZFITTER software package was used. The K_i value was calculated from the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + L/K_D)$. The dissociation constant (K_D) was obtained from Scatchard analysis of saturation isotherms. Protein levels were measured by the method of Bradford (Bio-Rad Protein Assay).

2.7. Sources of materials

L-689,560 and 5,7-dichlorokynurenic acid were synthesized by the Chemistry Department at Rhône Poulenc Rorer, CRVA, Vitry-sur-Seine, France. 2-APV (DL-2-amino-5-phosphonovaleric acid) was purchased from Sigma (La Verpillière, France) and MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine), CPP (3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid) and CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline) from Research Biochemicals (Natick, MA, USA). [^{3}H]TCP (specific activity: 1.83–1.86 TBq/mmol) and [^{3}H]5,7-dichlorokynurenic acid (specific activity: 0.58–0.68 TBq/mmol) were obtained from NEN (Les Ulis, France).

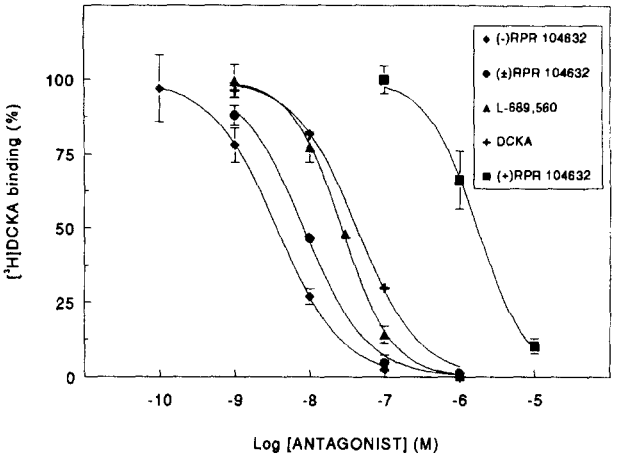

Fig. 2. Inhibition of [^{3}H]5,7-dichlorokynurenic acid ([^{3}H]DCKA) binding to rat cerebral cortex membranes. Specific binding of 20 nM [^{3}H]5,7-dichlorokynurenic acid was measured as described in Materials and methods. Competition curves were determined for (±)-RPR 104632, its (−)-isomer, its (+)-isomer, 5,7-dichlorokynurenic acid (DCKA) and L-689,560. The data shown are the mean ± S.E.M. of data obtained from at least three independent experiments.

3. Results

3.1. Effect of RPR 104632 on [^{3}H]5,7-dichlorokynurenic acid binding

Fig. 2 illustrates the dose-response curves obtained for RPR 104632, its two isomers, L-689,560 and 5,7-dichlorokynurenic acid itself. From previous experiments in which K_D values for [^{3}H]5,7-dichlorokynurenic acid binding were 29 ± 4 (n = 4), the data are expressed as K_i values in

Table 2
Affinity for glycine, NMDA and dissociative site (IC_{50} for [^{3}H]TCP binding in nM)

Competing ligand	NMDA site (in the presence of glycine)	Glycine site (in the presence of NMDA)	TCP site (in the presence of NMDA and glycine)
(±)-RPR 104632	> 100000	55 ± 15	> 100000
(−)-RPR 104632	67400 ± 28000	21 ± 7	> 100000
(+)-RPR 104632	> 100000	5750 ± 2750	> 100000
2-APV	6595 ± 2335	> 100000	> 100000
DCKA	> 100000	685 ± 105	> 100000
PCP	67 ± 8	54 ± 12	56 ± 13

Effects of RPR 104632 on the specific binding of [^{3}H]TCP in various experimental conditions. Specific binding of 2.5 nM [^{3}H]TCP was determined in the presence of glycine, NMDA or both co-agonists of the NMDA-receptor channel complex. At least 4 concentrations of each compound were used. 5,7-Dichlorokynurenic acid: DCKA. The data shown means ± S.E.M. of 3–6 independent experiments.

Table 1. RPR 104632 (K_i = 4.9 nM) was more potent than L-689,560 and 5,7-dichlorokynurenic acid (K_i = 15 and 27 nM, respectively) in inhibiting binding. The inhibition was stereospecific: the K_i values for (−)-RPR 104632 and (+)-RPR 104632 were 2.2 and 1115 nM, respectively. Table 1 also indicate K_i values for other ligands of the glycine site.

3.2. Effect of RPR 104632 on [^{3}H]TCP binding

NMDA (100 μM), glycine (1 mM) or the combination of both agents enhanced basal [^{3}H]TCP binding (fmol/mg protein: basal = 30 ± 7.6; NMDA present = 174 ± 33; glycine present = 157 ± 22; NMDA + glycine present = 232 ± 28 (mean of 9 experiments in duplicate).

As shown in Table 2, RPR 104632 potently inhibited (IC_{50} = 55 nM) the binding of [^{3}H]TCP in the presence of NMDA, suggesting it to be an antagonist at the glycine site. This effect was specific, since RPR 104632 did not inhibit [^{3}H]TCP binding in the presence of either glycine alone (which leaves the NMDA site exposed) or glycine plus NMDA (which leaves exposed only the dissociative anaesthetic site). The stereospecificity of RPR 104632 observed with [^{3}H]5,7-dichlorokynurenic acid as a ligand was confirmed by the [^{3}H]TCP binding experiments: the (−)-isomer (IC_{50} = 21 nM) was 273 times more potent than the (+)-isomer (IC_{50} = 5750 nM). In the presence of glycine, but not in the presence of both glycine and NMDA, 2-APV antagonized the binding of [^{3}H]TCP, a result expected from the specific affinity of this compound for the competitive NMDA site. Phencyclidine antagonized the binding of [^{3}H]TCP whatever the ligand present (i.e. glycine, NMDA or both), a result which fits well with its reported affinity for the dissociative anaesthetic site.

3.3. cGMP accumulation

Basal cGMP levels in slices from cerebellum from rats aged 8–11 days averaged 1.48 ± 0.2 pmol/mg protein; a sub-maximal concentration (80 μM) of NMDA increased cGMP levels in cerebellar slices by approximately 100 times (157 ± 7 pmol/mg protein; mean of 8 experiments, at least in triplicate). As shown in Fig. 3, RPR 104632 dose-dependently antagonized the increase of cerebellar cGMP levels induced by NMDA (IC_{50} = 890 ± 110 nM). This effect was not observed in the presence of glycine (1 mM). The effect of RPR 104632 was stereospecific: the (−)-isomer (IC_{50} = 400 ± 200 nM) was more potent than the (+)-isomer (IC_{50} = 12 ± 2 μM). As shown in Fig. 4, the response to NMDA in the presence of various RPR 104632 concentrations was reduced in a noncompetitive manner.

3.4. Neuroprotective effects of RPR 104632

3.4.1. Rat hippocampal slices

The CA1 and CA3 pyramidal neurones in the hippocampal slices exposed to 100 μM NMDA underwent a complete necrosis (Fig. 5, Table 3). When hippocampal slices were incubated with NMDA and 5,7-dichlorokynurenic acid (100 μM), the CA1 and CA3 pyramidal neurones were totally protected; this protective effect was reversed in a dose-dependent manner by glycine (Fig. 5).

As shown in Table 3, RPR 104632 potently protected CA1 neurones against NMDA-induced toxicity: 100% and 50% of the pyramidal neurones remained alive in the presence of 10 and 1 μM RPR 104632, respectively. This neuroprotection, like that conferred by 5,7-dichlorokynurenic acid, was inhibited by 1 mM glycine, as illus-

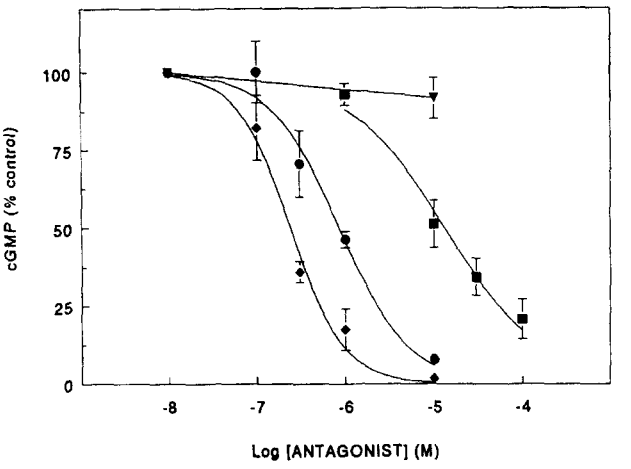

Fig. 3. Inhibition by RPR 104632 (●), (−)-isomer (♦), and (+)-isomer (■) of the increase in cGMP levels induced by 80 μM NMDA in immature rat cerebellar slices. The NMDA-induced increase in cGMP was also measured in the presence of a combination of 10 μM RPR 104632 and 1 mM glycine (▼). The data shown are the mean ± S.E.M. of data obtained from at least three independent experiments.

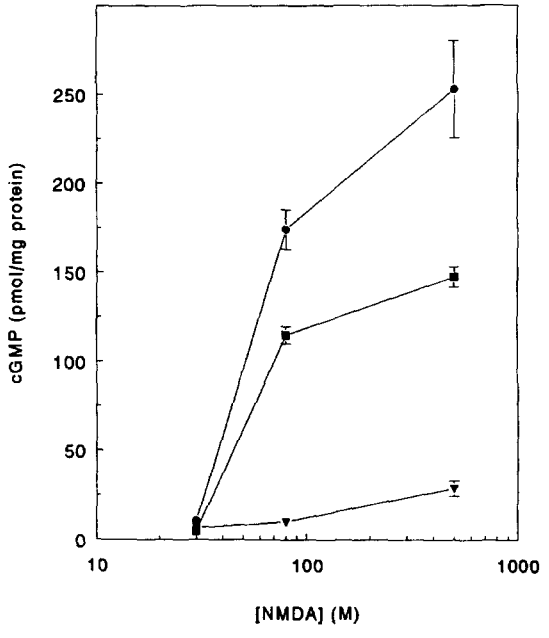

Fig. 4. Dose-response curve for NMDA in the absence (●) or in the presence of 1 μM (■) and 10 μM RPR 104632 (▼). The accumulation of cGMP was measured in cerebellar slices as reported in Fig. 3. The data shown are the mean ± S.E.M. of data obtained from three experiments.

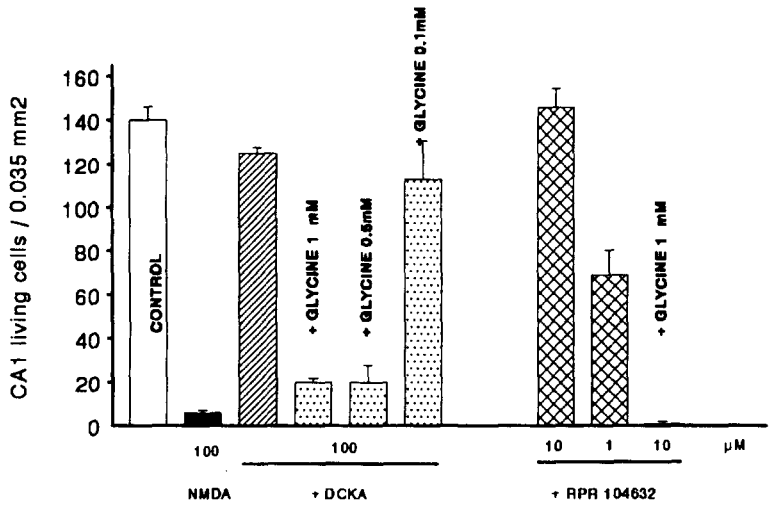

Fig. 5. Effect of 5,7-dichlorokynurenic acid and RPR 104632 and glycine on CA1 pyramidal cells viability. Hippocampal slices were incubated with 5,7-dichlorokynurenic acid (100 μM) or RPR 104632 (1–10 μM) 15 min before and during the incubation (30 min) with NMDA (100 μM) in the presence or absence of glycine (0.1–1 mM), followed by 180 min recovery as described in Materials and methods. Living pyramidal cells were detected using a computerised image analysis system (Quantimet 970, Leica) as described in Materials and methods. The data shown are the mean ± S.E.M of data from two independent slice preparations and neurone counts were performed on five sections for each preparation ($n = 10$).

trated in Fig. 6. Similar results were found for the CA3 pyramidal cells. The protective effect of RPR 104632 was stereospecific, since the (−)-enantiomer was about 100 times more potent than the (+)-enantiomer. As shown in Table 3, 50% of the CA1 pyramidal neurones were protected against NMDA toxicity by 1 μM of the (−)-enantiomer, whereas a concentration of 100 μM of the (+)-enantiomer was necessary to obtain an equivalent protective effect. This stereospecificity was also observed for CA3 pyramidal neurones.

The non-competitive and competitive NMDA receptor-channel antagonists, MK-801 (1 μM) and CPP (100 μM) completely protected the CA1 and CA3 pyramidal neurones against NMDA-induced toxicity, but these effects

Table 3
NMDA-induced neurotoxicity in rat hippocampal slices – protective effect of compounds on pyramidal cell death

Compounds		CA1 pyramidal cells				CA3 pyramidal cells			
				+ glycine 1 mM				+ glycine 1 mM	
		Living cells/ 0.045 mm^2	% of protection	Living cells/ 0.045 mm^2	% of protection	Living cells/ 0.045 mm^2	% of protection	Living cells/ 0.045 mm^2	% of protection
Control		140 ± 6		ND		90 ± 4		ND	
NMDA 100 μM		6 ± 1		ND		10 ± 1		ND	
+ DCKA	10 μM	34 ± 8	21						
	100 μM	125 ± 3	89	18 ± 2	9	91 ± 4	100	7 ± 2	0
+ MK 801	1 μM	157 ± 13	100	161 ± 14	100	77 ± 7	84	116 ± 10	100
+ CPP	100 μM	145 ± 10	100	136 ± 10	97	77 ± 5	84	80 ± 11	88
+ CNQX	10 μM	3 ± 2	0	0	0	20 ± 3	13	0	0
+ (±)-RPR 104632	1 μM	72 ± 12	49			34 ± 4	30		
	10 μM	152 ± 9	100	0	0	90 ± 3	100	0	0
+ (−)-RPR 104632	1 μM	78 ± 10	54			33 ± 6	29		
	10 μM	145 ± 5	100	0	0	79 ± 3	86	0	0
+ (+)-RPR104632	10 μM	0 ± 0	0			1 ± 1	0		
	100 μM	81 ± 10	56	56 ± 3	37	23 ± 8	16	0	0

Hippocampal slices were incubated with 100 μM NMDA for 30 min in the presence or absence of 1 mM glycine and tested compounds, followed by 180 min recovery, as described in Materials and methods. Slices were then fixed and stained for light microscopic examination and neurone counts. The quantification of living neurones was assessed using a computerised image analysis system (Quantimet 970, Leica). Values are the means ± S.E.M. of 2 independent slice preparations and neurone counts are performed on 5 sections for each slice preparation. 5,7-Dichlorokynurenic acid: DCKA. Percent of protection was calculated from hippocampal slices co-exposed to NMDA and tested compounds compared to that from hippocampal slices exposed to NMDA alone.

were not blocked by glycine. The non-NMDA receptor antagonist CNQX (10 μM) failed to protect the slices against NMDA-induced toxicity (Table 3).

3.4.2. Rat cortical primary cell cultures

Exposure of rat cortical cell cultures to a high dose of NMDA (1 mM) resulted in about 30% of reproducible

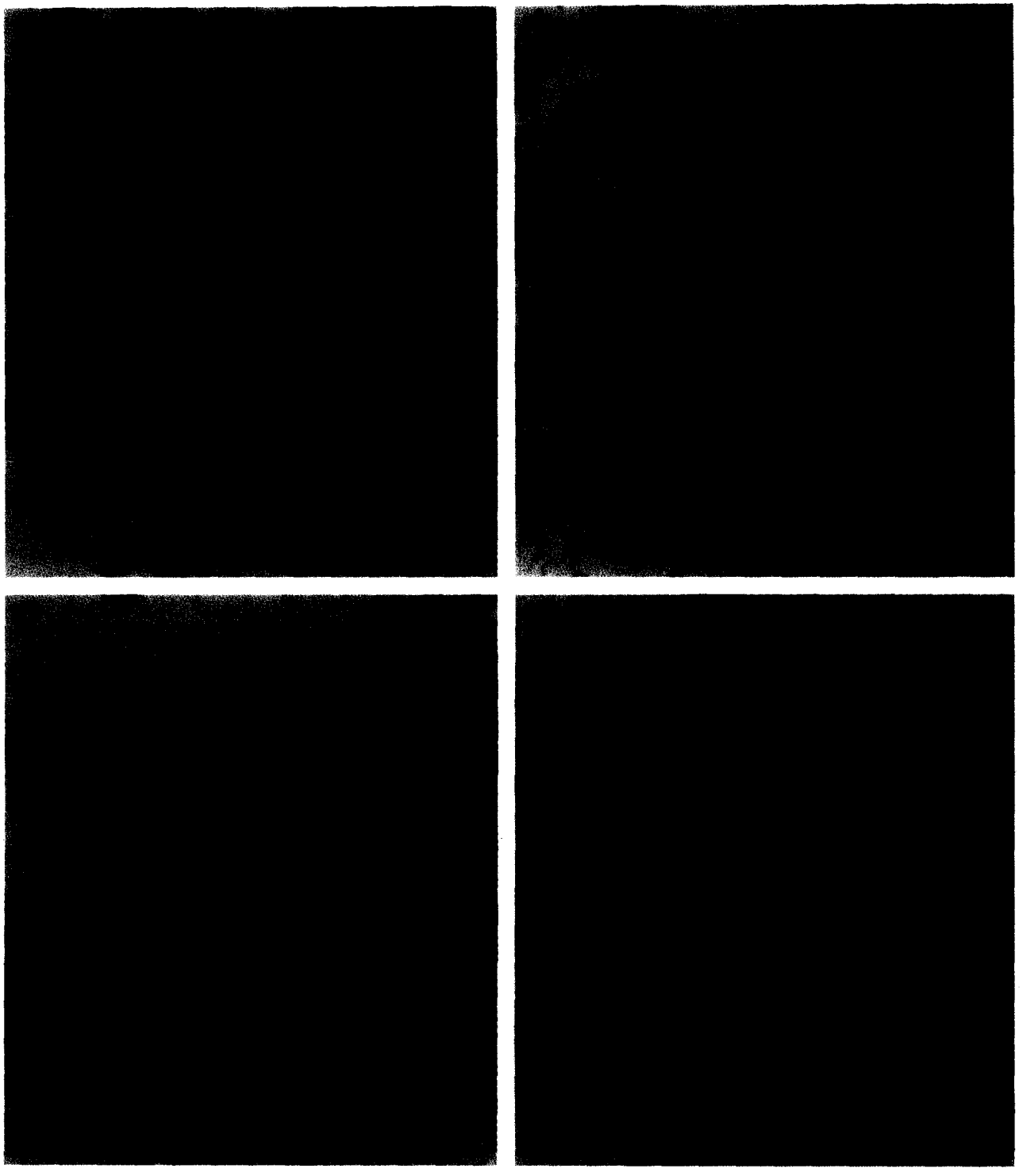

Fig. 6. Neuroprotective effect of RPR 104632 against NMDA toxicity in immature rat hippocampal slices. Photomicrographs of cresyl violet-stained sections showing CA1 pyramidal cells from hippocampal slices exposed to 100 μM NMDA for 30 min in the absence (A) or in the presence of 1 μM (B), 10 μM (C) RPR 104632, or in the presence of 10 μM RPR 104632 + 1 mM glycine (D), followed by recovery in normal medium for 180 min. In (A), neurones injured by NMDA exhibited pronounced clumping of chromatin. Note the complete protection by RPR 104632 against NMDA injury in (C).

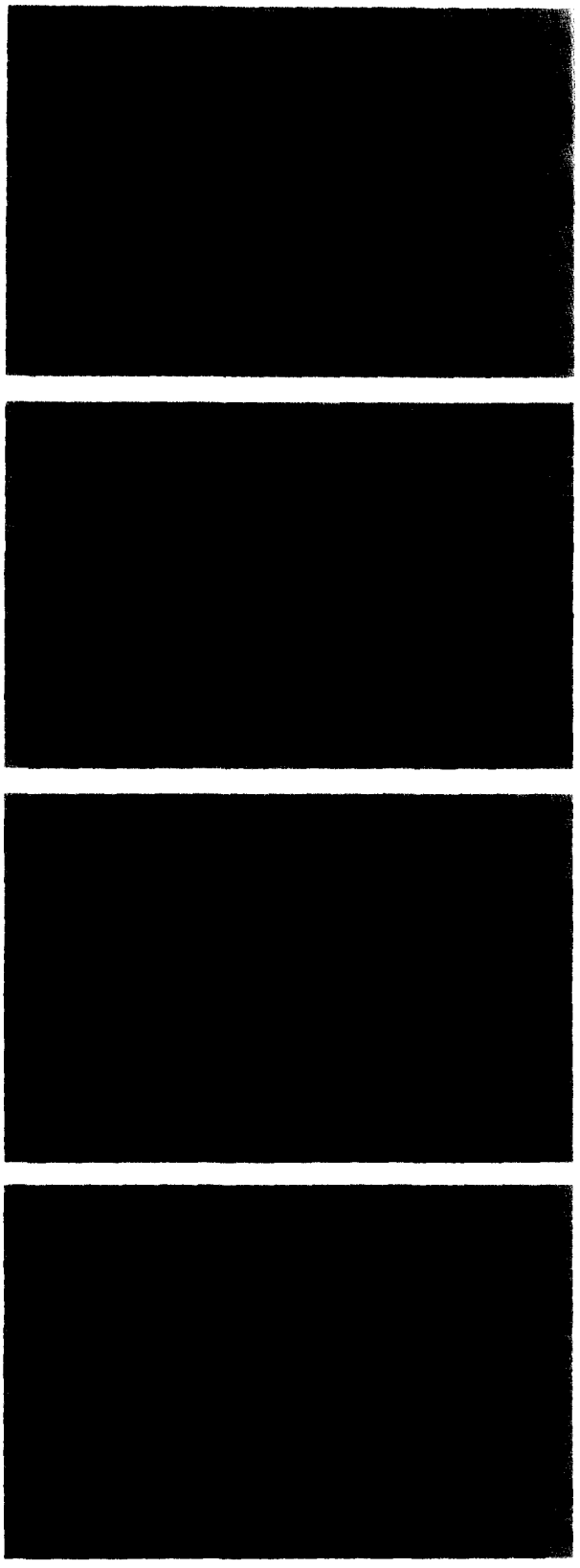

Fig. 7. Neuroprotective effect of RPR 104632 against NMDA toxicity in rat cortical primary cell cultures. The phase-contrast photomicrographs of trypan blue-stained cultures (magnification × 100) show the control cells in (A) and (C), the NMDA-treated ones in (B) and (D), with (C and D) or without (A and B) 10 μM RPR 104632.

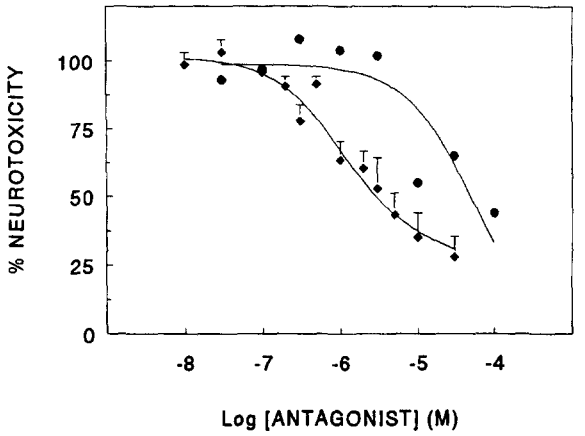

Fig. 8. Dose-response curves for the protective effects of RPR 104632 (♦) and 5,7-dichlorokynurenic acid (●) against the NMDA-induced neurotoxicity in the rat cortical primary cell cultures (about 30% toxicity). They were obtained from seven (RPR 104632) or two (5,7-dichlorokynurenic acid: DCKA) independent experiments.

toxicity (Fig. 7B) compared to that observed in control sister cultures (Fig. 7A). RPR 104632 produced a significant and consistent neuroprotective effect towards all the NMDA-induced toxicity and had no effect when it was added alone at concentrations up to 10 μM. An example of the protective effect of RPR 104632 is illustrated in Fig. 7D.

In subsequent experiments, the neuroprotective potencies of RPR 104632 and 5,7-dichlorokynurenic acid were compared (Fig. 8). From the concentration-effect curves obtained for these molecules, an EC_{50} value was calculated, defined as the concentration of antagonist that reduced to 50% the NMDA-induced toxicity (as 100%). The EC_{50} obtained clearly demonstrated that RPR 104632 ($EC_{50} = 4 \pm 2$ μM) was significantly more potent than 5,7-dichlorokynurenic acid ($EC_{50} = 50$ μM) under our experimental conditions. Moreover, as shown in Fig. 9, the

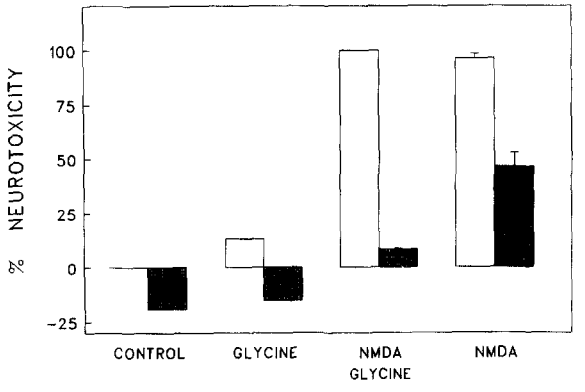

Fig. 9. Effect of RPR 104632 (10 μM) and its reversal by glycine (1 mM) on the neurotoxicity induced by NMDA (1 mM) in the rat cortical primary cell cultures. The white bars represent the toxicity obtained in the presence of buffer, glycine, NMDA and NMDA + glycine; the black bars represent the toxicity obtained with the same molecules in the presence of RPR 104632. The data were obtained from two independent experiments.

protection of RPR 104632 against the NMDA-induced neurotoxicity was abolished partially in the presence of 1 mM glycine. This result confirmed the interaction of RPR 104632 at the competitive glycine site of the NMDA channel receptor.

4. Discussion

This study reports that RPR 104632, a new member of the benzothiadiazide chemical family, binds to the glycine site coupled to the NMDA receptor channel complex. RPR 104632 exhibits a potent affinity ($K_i = 4.9$ nM) for the glycine site labelled with [^{3}H]5,7-dichlorokynurenic acid and is therefore one of the most potent compounds yet described in this pharmacological class. The binding of RPR 104632 is stereospecific, as the (−)-enantiomer is 500 times more potent than the (+)-enantiomer.

As discussed by Kloog et al. (1990), the binding of [^{3}H]TCP provides a means to assess the specificity of a given ligand for the glycine site, the NMDA site or the dissociative anaesthetic site located inside the channel. A consequence of the increase in the time the channel is open is greater accessibility to the dissociative anesthetic sites, hence, more binding of [^{3}H]TCP. In the presence of NMDA and the absence of added glycine, glycine antagonists potently inhibit the binding of [^{3}H]TCP. Conversely, in the absence of NMDA and the presence of glycine, antagonists acting at the NMDA site antagonize [^{3}H]TCP binding (see Kloog et al., 1990). In the presence of both NMDA and glycine, only ligands for the dissociative anaesthetic site are active. Effectively, under our experimental conditions [^{3}H]TCP binding was increased by NMDA, glycine or the combination of these two agonists. The increase in [^{3}H]TCP binding brought about by NMDA stimulation was antagonized by RPR 104632. This effect was stereospecific, as predicted from the results obtained with [^{3}H]5,7-dichlorokynurenic acid. RPR 104632, unlike 2-APV, did not modify the increase in [^{3}H]TCP binding induced by an excess of glycine. Moreover, unlike PCP, RPR 104632 was inactive when [^{3}H]TCP binding was increased by a combination of NMDA and glycine. From these results it can be concluded that RPR 104632 interferes specifically with the glycine site in an in vitro model of the NMDA receptor-channel complex, which responds functionally to stimulation with both co-agonists.

The effects of RPR 104632 were also studied in another functional model: the accumulation of cGMP in cerebellar slices that occurs after stimulation with NMDA. RPR 104632 antagonized potently the NMDA-induced increase in cGMP levels, an effect which can also be prevented by adding an excess of glycine to the incubation medium. The inhibition observed with RPR 104632 was noncompetitive with respect to the NMDA site. These results fit well with those obtained in binding experiments with [^{3}H]5,7-dichlorokynurenic acid and [^{3}H]TCP. Thus, it is highly probable that RPR 104632 interacts specifically with NMDA receptors at the glycine site.

To complete this study, we used in vitro models of toxicity to determine whether RPR 104632 exerts a neuroprotective effect in hippocampal slices and in cortical cell culture. As previously reported (Malgouris et al., 1994), stimulation of NMDA receptors can cause necrosis of the pyramidal cells in the CA1 and CA3 areas of the hippocampus. At a concentration as low as 1 μM, RPR 104632 antagonized the NMDA-induced injury, whereas 5,7-dichlorokynurenic acid exhibited a significant activity only at a much higher (100 μM) concentration. Unlike the protective effect of MK-801 or CPP, that of RPR 104632 could be reversed by glycine, providing further evidence that RPR 104632 protects against NMDA-induced neurotoxicity through a glycine site interaction. Finally, the stereospecificity of RPR 104642 was confirmed by the use of this model, as the (−)-isomer was 100 times more potent than the (+)-isomer.

A dose-dependent protective effect of RPR 104632 against NMDA-induced toxicity in cultured cortical neurones has also been found. Similarly, in this model RPR 104632 antagonizes the toxicity of NMDA at lower concentrations than 5,7-dichlorokynurenic acid, and this effect is also reversed by glycine.

In conclusion, RPR 104632 represents a new potent glycine antagonist which modulates functionally the activity of the NMDA receptor-channel complex. Excessive NMDA stimulation has been suggested to play a role in pathological situations such as cerebral ischaemia, stroke, trauma, epilepsy and transmission of pain. Thus, blockade of NMDA transmission with glycine antagonists may be useful for the therapy of these disorders.

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