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Accepted for publication January 26, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Activation of N-methyl-D-aspartate (NMDA) receptors is known to produce arachidonic acid release, which has been implicated in excitotoxicity. Antagonists and partial agonists at the glycine site of the NMDA receptor, despite exhibiting functional differences in electrophysiological studies, inhibit glutamate-induced neurotoxicity and ischemia-induced neurodegeneration. The objective of this study was to investigate the effects of both glycine site antagonists and partial agonists on NMDA receptor-mediated [3H]arachidonic acid (AA) release evoked by glutamate, NMDA or a competitive inhibitor of the glutamate/aspartate uptake carrier. The [3H]AA release evoked by a maximally effective concentration of glutamate (100 μM) was blocked by the glycine site antagonists 7-chlorokynurenic acid (7-CKYN) and 5,7-dichlorokynurenic acid (5,7-DCKYN) and by a low intrinsic efficacy glycine partial agonist (+)-1-hydroxy-3-aminopyrrolid-2-one ([(+)]-HA-966). 1-Aminocyclopentanecarboxylic acid (ACPC), a high intrinsic efficacy glycine partial agonist, did not modify [3H]AA release evoked by 100 μM glutamate. However, ACPC blocked (in a glycine reversible manner) the [3H]AA release induced by NMDA (100 μM) with an IC50 of 131 ± 2 μM. Furthermore, L-trans-pyrrolidine-2,4-dicarboxylate (PDC), a competitive inhibitor of the glutamate transporter, also released [3H]AA (Emax and EC50 of 127 ± 4% and 30 ± 1 μM, respectively). ACPC, 7-CKYN and (±)-2-amino-7-phosphonoheptanoic acid (AP-7), a competitive NMDA receptor antagonist, inhibited [3H]AA release evoked by PDC. These results demonstrate that both glycine site antagonists and partial agonists can inhibit NMDA receptor-mediated [3H]AA release in cerebellar granule cells, an action consistent with the neuroprotective effects of these compounds.

Activation of diverse neurotransmitter receptors as well as cell depolarization by elevation of extracellular K+ concentration can induce membrane phospholipid degradation and increase AA release in a variety of neuronal preparations (Lazarewicz et al., 1992; Volterra et al., 1994; Faroqui and Horrocks, 1991). Although AA release may be effected through diverse mechanisms, several studies indicate that the AA release evoked by excitatory amino acids in primary neuronal cell cultures is largely dependent on activation of NMDA receptor (Lazarewicz et al., 1992). Thus, NMDA has been shown to induce AA release in primary cultures of striatum (Dumuis et al., 1988), hippocampus (Sanfeliu et al., 1990) and cerebellar granule cells (Lazarewicz et al., 1988; Lazarewicz et al., 1990). These effects can be blocked by both competitive (e.g., 2-amino-5-phosphonopentanoic acid, AP-5) and noncompetitive (e.g., Mg2+) NMDA receptor antagonists (Lazarewicz et al., 1988; Sanfeliu et al., 1990). Moreover, phospholipase A2 is the primary effector enzyme responsible for NMDA receptor-evoked release of AA in neuronal cultures (Dumuis et al., 1988; Sanfeliu et al., 1990; Lazarewicz et al., 1990), and the coupling of NMDA receptors to phospholipase A2 has been shown to be directly linked to extraacellular Ca2+ entry through NMDA receptor-coupled cation channels (Lazarewicz et al., 1990).

The NMDA receptor is a ligand-gated ion channel that contains discrete but interdependent regulatory domains (reviewed in Mcbain and Mayer, 1994). NMDA receptors post-

ABBREVIATIONS: AA, arachidonic acid; ACPC, 1-aminocyclopentanecarboxylic acid; (±)-AP-5, 2-amino-5-phosphonopentanoic acid; (±)-AP-7, 2-amino-7-phosphonoheptanoic acid; ASP, aspartate; BSA, bovine serum albumin; 7-CKYN, 7-chlorokynurenic acid; 5,7-DCKYN, 5,7-dichlorokynurenic acid; GLU, glutamate; GLY, glycine; (±)-HA-966, 1-hydroxy-3-aminopyrrolid-2-one; HPLC, high-pressure liquid chromatography; LH-BSA, Locke-HEPES buffer with fatty acid-free bovine serum albumin; MK-801, 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine hydrogen, dizocilpine maleate; NMDA, N-methyl-D-aspartate; PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid.

Received for publication October 9, 1997.

1 This work was supported by a grant from Dirección General de Investigación Científica y Técnica, Ministry of Education of Spain, DGICYT, PB94–1119. 2 E.V. was supported by a predoctoral fellowship from Direcció General de Investigacions Cientifics, Barcelona, Spain. 3 A.Z. is a Severo Ochoa Fellow from Ferrer International Foundation. 4% and 30 ± 1 μM, respectively). ACPC, 7-CKYN and (±)-2-amino-7-phosphonoheptanoic acid (AP-7), a competitive NMDA receptor antagonist, inhibited [3H]AA release evoked by PDC. These results demonstrate that both glycine site antagonists and partial agonists can inhibit NMDA receptor-mediated [3H]AA release in cerebellar granule cells, an action consistent with the neuroprotective effects of these compounds.

Abbreviations: AA, arachidonic acid; ACPC, 1-aminocyclopentanecarboxylic acid; (±)-AP-5, 2-amino-5-phosphonopentanoic acid; (±)-AP-7, 2-amino-7-phosphonoheptanoic acid; ASP, aspartate; BSA, bovine serum albumin; 7-CKYN, 7-chlorokynurenic acid; 5,7-DCKYN, 5,7-dichlorokynurenic acid; GLU, glutamate; GLY, glycine; (±)-HA-966, 1-hydroxy-3-aminopyrrolid-2-one; HPLC, high-pressure liquid chromatography; LH-BSA, Locke-HEPES buffer with fatty acid-free bovine serum albumin; MK-801, 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine hydrogen, dizocilpine maleate; NMDA, N-methyl-D-aspartate; PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid.
sess a transmitter recognition site for acidic amino acids such as L-glutamate; a cation channel with a unique voltage-dependent regulation by Mg\(^{2+}\), a polyamine site, and a coagonist site for glycine. The binding of glycine to its recognition site on the NMDA receptor is strychnine insensitive, and occupation of this site by an agonist appears essential for channel activation (Kleckner and Dingledine, 1988; Curras and Pallotta, 1996).

There is evidence suggesting that glycine is present continuously within the extracellular space at levels that are at or near saturation (Mcbain and Mayer, 1994). Both the physiological role of glycine in the operation of NMDA receptors in vivo and the pharmacological activity of antagonists and partial agonists (Foster et al., 1992; Baron et al., 1992; Grimwood et al., 1995; Grimwood et al., 1993; Marvizon et al., 1989) at this site remain controversial (Kehne et al., 1995; Lanthorn, 1994). We have hypothesized that if occupation of the glycine site by agonists is essential for the operation of the NMDA receptor complex, then partial agonists at this glycine site, in the presence of the extracellular glycine concentrations found in situ, may function as NMDA antagonists (Skolnick et al., 1989; Trullas et al., 1989).

In the present experiments, we have further explored this hypothesis by examining the effects of both high and low intrinsic efficacy glycine partial agonists and glycine site antagonists on NMDA receptor-mediated AA release in primary granule cell cultures. Furthermore, delayed neuronal cell death in ischemic brain injury has been associated with high levels of extracellular glutamate produced by reverse operation of the high-affinity glutamate/aspartate uptake carrier (GLU/ASP). Thus, we also investigated the ability of these glycine site ligands to prevent the effects of a competitive inhibitor of the GLU/ASP carrier PDC on \(^{3}H\)AA release. We now report that glycine partial agonists and antagonists reduce glutamate-, NMDA- and PDC-evoked AA release. These results provide evidence that in the presence of glycine, both glycine partial agonists and antagonists can function as NMDA receptor antagonists.

**Experimental Procedures**

**Cerebellar granule cell cultures.** Primary cultures of granule cells were prepared from cerebella of 7-day-old Wistar rat pups essentially as described (Fossom et al., 1995b). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (DL no. 116, GU supplement 40, February 18, 1992) and international (EEC Council Directive 86/609,OJ L 358,1,12 December 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH publication no. 85–23, 1985) laws and policies. Dissociated cells were plated in 24-well plastic plates (2 cm\(^2\)) previously coated with poly-l-lysine hydrobromide (10 μg/ml, MW > 300,000, Sigma), and the density was adjusted to give \(\approx 4 \times 10^5\) cells/cm\(^2\). Cells were cultured in Eagle’s basal medium with the following additions of 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1 mg/ml gentamicin, and 25 mM KCl. The replication of non-neuronal cells was monitored using independent \(t\) tests.

**Materials.** Heat-inactivated fetal calf serum, culture media and gentamicin were purchased from Gibco BRL (Cergy Pontoise, France). Other cell culture reagents were from Sigma (Madrid, Spain). \(^{3}H\)AA was obtained from NEN (Brussels, Belgium). NMDA, 7-CKYNO, 5,7-DCKYN, (+)-HA-966 and (-)-AP-7 were from Research Biochemicals (Natick, MA). ACPC and PDC were from Tocris-Cookson (Bristol, UK), and glutamate and glycine were from Fluka Chemistry (Buchs, Switzerland).

**Statistical analysis.** Data from inhibition curves were fitted to a four-parameter logistic equation using nonlinear regression analyses with Prism computer program (GraphPAD, San Diego, CA). Statistical significance of differences among curve parameters was evaluated using independent \(t\) tests.

**Results**

**Effects of glutamate and NMDA on \(^{3}H\)AA release in cerebellar granule cells.** Treatment of cerebellar granule cells with glutamate or NMDA (under Mg\(^{2+}\)-free conditions) produced a concentration-dependent increase of \(^{3}H\)AA release in the absence of added glycine (fig. 1). Removal of extracellular Ca\(^{2+}\) completely blocked the effects of both glutamate (fig. 1) and NMDA (results not shown). In the presence of 1.3 mM Ca\(^{2+}\), glutamate enhanced \(^{3}H\)AA release with an EC\(_{50}\) of 13 ± 1 μM and \(E_{max}\) of 473 ± 6% (\(n = 12\)). NMDA exhibited a significantly lower potency (EC\(_{50}\) = 62 ± 1 μM) and a lower maximal effect (\(E_{max}\) = 285 ± 24%, \(n = 7\)) than glutamate in this measure (\(P < .05\), Student’s \(t\) test). Neither the EC\(_{50}\) nor maximal effect of glutamate and NMDA were significantly altered in the presence of 10 μM glycine (EC\(_{50}\) = 14 ± 1 μM and 59 ± 3 μM; \(E_{max}\) = 432 ± 18% and 290 ± 3% for glutamate, \(n = 4\), and NMDA, \(n = 4\), respectively) (fig. 1). Removal of extracellular Ca\(^{2+}\) or the incubation with NMDA or glutamate during a 15-min period did not have any significant effect on cell viability because the total radioactivity incorporated into the cells and the basal release were not significantly altered by these treatments.
Fig. 1. Concentration-dependent release of [3H]AA by glutamate and NMDA in cerebellar granule cells. Granule cells were pre-incubated with [3H]AA for 24 hr and treated for 15 min with varying concentrations of glutamate or NMDA in BSA-containing, Mg"⁺ free medium, in the presence or absence of 10 μM Gly or 1.3 mM Ca"⁺++. Results are expressed as percent mean ± S.E.M. of the total incorporated radioactivity after subtracting the basal release in the absence of agonists. Basal [3H]AA release was 368 ± 25 dpm, total [3H]AA incorporated was 37,073 ± 2,347 dpm. GLU, n = 12; GLU + 10 μM GLY, n = 4; GLU no Ca"⁺⁺, n = 2; NMDA, n = 7; NMDA + 10 μM GLY, n = 4.

Effects of glycinergic site ligands on [3H]AA release evoked by glutamate. 7-CKYN, 5,7-DCKYN and (+)-HA-966 inhibited glutamate (100 μM) induced [3H]AA release in a concentration dependent manner. Nonlinear regression analyses of inhibition curves showed that the glycinergic antagonists 7-CKYN and 5,7-DCKYN did not significantly differ in their potencies to inhibit [3H]AA release evoked by 100 μM glutamate. The IC₅₀ values for 7-CKYN and 5,7-DCKYN were 9 ± 1 μM (n = 4) and 10 ± 2 μM (n = 3), respectively. In contrast, the low efficacy, partial agonist (+)-HA-966 inhibited 100 μM glutamate induced [3H]AA release with an IC₅₀ of 263 ± 2 μM (n = 4), a much lower potency than observed for glycine antagonists (P < .05, Student’s t test) (fig. 2).

Effects of ACPC on [3H]AA release evoked by glutamate and NMDA. ACPC, a partial agonist at the glycine site with an intrinsic activity higher than (+)-HA-966 in electrophysiological and neurochemical studies (Watson and Lanthorn, 1990; Marvizon et al., 1989; Priestley and Kemp, 1994), did not alter the [3H]AA release induced by 100 μM glutamate (results not shown). However, ACPC inhibited [3H]AA release evoked by 20 μM glutamate (in the presence of 10 μM added glycine) to an I₅₀ of 60 ± 4% with an IC₅₀ of 506 ± 18 μM (fig. 3A). ACPC also produced a complete, concentration dependent, inhibition of the [3H]AA release evoked by 100 μM NMDA (in the presence of 10 μM added glycine) with an IC₅₀ of 131 ± 2, n = 7 (fig. 3B). The inhibitory effect of ACPC was reversed by increasing the concentration of glycine from 10 μM to 1 mM (fig. 3B, inset).

Effects of glycine site ligands on [3H]AA release evoked by PDC. PDC, a competitive inhibitor of the glutamate transporter, produced a concentration dependent release of [3H]AA in cerebellar granule cells. This effect was dependent on extracellular Ca"⁺⁺. Glutamate was ~4-fold more potent and 2-fold more efficacious than NMDA (fig. 1). The magnitude of these effects was similar to that observed in previous studies using cerebellar granule cells (Rodriguez et al., 1993; Lazarewicz et al., 1988, 1990). The 4-fold lower potency of NMDA in this measure is also consistent with previous studies demonstrating that glutamate is ~10-fold more potent than NMDA as an inhibitor of radioligand binding to wild-type glutamate receptors in rodent forebrain (Monahan and Michel, 1987; Olverman et al., 1988).

The observation that NMDA is only 50% as efficacious as glutamate in stimulating [3H]AA release is consistent with previous studies (Dumuis et al., 1988; Lazarewicz et al., 1990) and substantiates radioligand binding studies demonstrating that NMDA is less efficacious than glutamate (Foster and Wong, 1987; Loo et al., 1987). The ability of glycine agonists to block glutamate-stimulated [3H]AA release (fig. 2), at concentrations that are inactive at non-NMDA receptors, indicates that in cerebellar granule neurons, this effect of glutamate is mediated solely via activation of NMDA receptors. In contrast, both NMDA and AMPA/kainate receptors are involved in the glutamate evoked release of [3H]AA in cortical and striatal cell cultures (Dumuis et al., 1988; Stella et al., 1995).

The failure of exogenous glycine to augment glutamate- and NMDA-stimulated AA release (fig. 1) indicates that the concentration of glycine in the medium is sufficient to permit the action of glutamate and NMDA. In the presence of saturating concentrations of glycine, the glycine site antagonists 7-CKYN and 5,7-DCKYN, as well as a low intrinsic efficacy glycine partial agonist, (+)-HA-966, all inhibited [3H]AA release evoked by 100 μM glutamate. These results provide further evidence that activation of the glycine site is necessary for the operation of NMDA receptors (Kleckner and Dingledine, 1988; Lerma et al., 1990; Curras and Pallotta, 1996), demonstrating that this principle also applies to NMDA-evoked arachidonic acid release. The potentials of...
7-CKYN (IC$_{50}$ = 9 \text{ mM}) and (+)-HA-966 (IC$_{50}$ = 263 \text{ mM}) to inhibit glutamate (100 \text{ mM})-evoked $[^3H]$AA release are similar to values previously reported for these compounds to act as NMDA antagonists in brain slices (Foster and Kemp, 1989; Kemp et al., 1988) and in preventing hypoxia-induced neurodegeneration in rat cortical cell cultures (Priestley et al., 1990). Moreover, the potency of 5,7-DCKYN (IC$_{50}$ = 10 \text{ mM}) to inhibit glutamate-evoked $[^3H]$AA release is similar to the value reported (IC$_{50}$ = 4 \pm 1 \text{ \text{ mM}}) for inhibition of NMDA-stimulated cGMP accumulation in cerebellar slices (Baron et al., 1990). Nonetheless, both the absolute values and rank order potencies of these glycine site compounds differ from data obtained in radioligand binding and electrophysiological studies. For example, in forebrain membranes, 5,7-DCKYN exhibits higher affinity ($K_i$ 80 \text{ nM}) for the glycine site than 7-CKYN ($K_i$ 360 \text{ nM}) (Baron et al., 1992). The higher potencies of these compounds in radioligand binding assays can be explained by incubation conditions (e.g., temperature, ionic milieu, elimination of endogenous glycine, and a homogeneous tissue preparation) intended to optimize ligand affinities that cannot be duplicated using intact cell or tissue preparations. The differences in rank order potency among glycine site ligands may be attributed, at least in part, to preparatory tissue preparation (intended to optimize ligand affinities) and subunit composition. Thus, studies in recombinant receptors indicate that subunit composition is the primary determinant of ligand affinity for a wide variety of structurally diverse compounds (Laurie and Seeburg, 1994; Wafford et al., 1993). Consistent with this interpretation, the potencies of a series of glycine site ligands to inhibit $[^3H]$glycine binding can vary by >20-fold between hippocampus and cerebellum (Yoneda and Ogita, 1991).

The ability of (+)-HA-966, a partial agonist at the glycine site, to inhibit glutamate evoked $[^3H]$AA release with an efficacy similar to the observed with competitive glycine antagonists is in agreement with previous studies showing that HA-966 and 7-CKYN display equivalent neuroprotective effects against glutamate induced neurotoxicity in vitro (Boje...
et al., 1993). The similar inhibition obtained with a partial agonist and an antagonist can be explained by the very low intrinsic activity of HA-966 obtained in most preparations (Priestley and Kemp, 1994; Henderson et al., 1990). However, ACPC, a partial agonist with a higher intrinsic activity than HA-966 (Watson and Lanthorn, 1990; Priestley and Kemp, 1994), did not alter the [3H]AA release evoked by a maximally effective concentration of glutamate (100 μM) but inhibited [3H]AA release induced by 20 μM glutamate by a maximum of 60% (fig. 3A). Moreover, ACPC completely blocked (in a glycine reversible manner) the [3H]AA release induced by 100 μM NMDA (fig. 3B).

Electrophysiological studies indicate that ACPC is a partial agonist with a very high intrinsic activity (80–95%) (Watson and Lanthorn, 1990; Priestley and Kemp, 1994). However ACPC is neuroprotective in vivo (Long and Skolnick, 1994; Fossom et al., 1995b) and also mimics several other pharmacological actions, such as anticonflict (Trullas et al., 1989; Przegalinski et al., 1996), antidepressant (Trullas and Skolnick, 1990), and blockade of opiate tolerance (Kolesnikov et al., 1994) of NMDA antagonists. The apparent divergence between the NMDA receptor antagonist profile of ACPC in vivo and the high efficacy that this compound shows in electrophysiological experiments has led to the suggestion that the effects of ACPC in vivo cannot be attributed to an action of this compound at the NMDA receptor (Kemp and Leeson, 1993; Wood, 1995). However, the inhibition by ACPC, in a glycine reversible manner, of NMDA evoked [3H]AA release observed in the present study in cerebellar granule cells provides strong evidence to suggest that ACPC reduces NMDA receptor function at the glycine site. Moreover, consistent with a high intrinsic efficacy partial agonist action, ACPC partially inhibited glutamate evoked [3H]AA release (fig. 3A). As the concentration of glutamate (or NMDA) increases, the ability of exogenous glycine to augment the actions of glutamate (or NMDA), such as generation of cGMP or neurotoxicity, is lost in cerebellar granule cell cultures (Boje et al., 1993; Fossom et al., 1995b). Both electrophysiological and neurochemical evidence indicates that this augmentation by glycine may in part be attributed to a “left shift” in the concentration effect curve of glutamate (or NMDA) (Johnson and Ascher, 1987; Monaghan et al., 1988; Hood et al., 1990). At low to moderate concentrations of NMDA or glutamate, ACPC would result in a smaller increase in the affinity of glutamate relative to that observed with glycine, resulting in an apparent “functional NMDA antagonist” action. However, at higher glutamate (or NMDA) concentrations, this dampening effect would be negligible. This hypothesis is consistent with the ability of ACPC to produce a concentration dependent but partial reduction in granule cell neurotoxicity at glutamate concentrations (<25 μM) that induce low to moderate damage (Boje et al., 1993; Fossom et al., 1995a), while lacking this neuroprotective action against a maximally effective glutamate insult. Similarly, ACPC reduced cGMP formation in these cultures at low to moderate but not high concentrations of NMDA (Fossom et al., 1995b; Fossom et al., 1995a).

Pathological conditions of hypoglycemia or ischemic brain injury are associated with both AA and excitatory amino acid release (Lazarewicz et al., 1992). It has been suggested that under conditions of energy deprivation, like anoxia or ischemia, there is an excessive increase of extracellular excitatory amino acids produced by reverse operation of the high-affinity glutamate/aspartate (GLU/ASP) uptake carrier (Sztatkowski and Attwell, 1994). In the present study, we mimicked the reversal of this carrier by treating cells with PDC, a competitive substrate of the high affinity GLU/ASP transporter (Bridges et al., 1991; Griffiths et al., 1994) that does not show NMDA receptor agonist activity at concentrations in the low micromolar range (Balcar et al., 1995). In the present studies, PDC increased [3H]AA release in a concentration-dependent manner and this increase was NMDA receptor mediated since it was blocked by AP-7. In agreement with the results obtained with glycine antagonists and partial agonists on glutamate and NMDA mediated [3H]AA release, both 7-CKY and ACPC inhibited the [3H]AA release evoked by a maximally effective concentration of PDC. These results suggest that glycine site antagonists and partial agonists may reduce the effects of excessive excitatory amino acid release induced by alterations of the GLU/ASP uptake system and are consistent with previous studies showing that glycine antagonists and partial agonists reduce neuronal damage after ischemia in animal models (Wood et al., 1993; Von Lubita et al., 1992; Pellegrini-Giampietro et al., 1994; Fossom et al., 1995b).

AA release and its metabolism has been hypothesized to play a significant role in the cell damage produced by the excitotoxic cascade (Bigge and Boxer, 1994; Volterra et al., 1994; Katsuki and Okuda, 1995; Bazan et al., 1995). AA oxidation produces free radicals that may contribute to neuronal damage via inflammatory reactions or may act as downstream mediators of excitotoxicity and further enhance glutamate release (Bigge and Boxer, 1994). The ability of glycine partial agonists and antagonists to attenuate NMDA receptor mediated AA release and protect against cell damage induced by a variety of excitotoxic treatments (Boje et al., 1993; Von Lubita et al., 1992; Patel et al., 1990; Long and Skolnick, 1994; Foster et al., 1990; Priestley et al., 1990; Fossom et al., 1995b; Zapata et al., 1996) is consistent with this hypothesis.

In summary, the present results show that both glycine antagonists and partial agonists can inhibit glutamate and NMDA stimulated [3H]AA release. These findings are consistent with both in vivo and in vitro studies demonstrating the neuroprotective properties of these compounds. Furthermore, these results provide evidence that in the presence of saturating concentrations of glycine, both glycine partial agonists and antagonists may function as NMDA receptor antagonists.

References
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line-2,3-dione (CNQX) and [3H]-kainate studied by autoradiography in rat forebrain. Neurochem Int 26:135–144.


