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Different Modes of Action of 3-Amino-1-hydroxy-2-pyrrolidone (HA-966) and 7-Chlorokynurenic Acid in the Modulation of *N*-Methyl-D-aspartate-Sensitive Glutamate Receptors

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SUMMARY

The *N*-methyl-D-aspartate (NMDA)-sensitive glutamate receptors are known to be inhibited by 3-amino-1-hydroxy-2-pyrrolidone (HA-966) and 7-chlorokynurenic acid (Cl-KYN), which act at the glycine-regulated allosteric modulatory center. In this work we show that, in synaptic membranes prepared from rat brain, Cl-KYN and HA-966 inhibit the binding of [^{3}H]glycine. Moreover, Cl-KYN can also completely inhibit the binding of [^{3}H]glutamate to the primary transmitter recognition site for the NMDA receptor, whereas HA-966 only partially reduces this binding. Cl-KYN also abolishes the binding of the NMDA receptor antagonist [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP). In contrast, HA-966 increases [^{3}H]CPP binding, affecting the affinity but not the maximal number of binding sites. This increase is inhibited by glycine and Cl-KYN. The binding of [^{3}H] (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate (MK-801), used as an index of NMDA receptor activation, is completely inhibited by Cl-KYN but only partially by HA-966. In addition, HA-966, but not Cl-KYN, increases the potency of CPP in inhibiting [^{3}H]MK-801 binding. Our results demonstrate that Cl-KYN and HA-966 differ in their ability to modulate the NMDA receptor, perhaps acting at distinct but overlapping recognition sites. Furthermore, our results suggest that agonist and antagonist recognition sites of the NMDA receptor may be independently regulated by glycine and HA-966, which would result, respectively, in a positive and negative allosteric modulation of the NMDA receptor complex.

The function of NMDA-sensitive glutamate receptors is inhibited by PCP and Mg^{2+}, acting at specific recognition sites inside the receptor-operated cationic channel (for review see Ref. 1). However, NMDA receptors can be modulated in a positive manner by glycine and D-serine, which bind to a strychnine-insensitive recognition site (Gly_2) outside the channel, increase ligand binding to the primary transmitter recognition site (2, 3), and enhance neuronal responses induced by NMDA receptor agonists (4–7). However, the lack of selective antagonists for these Gly_2 sites limits the possibility of studying the modulatory role of glycine in NMDA receptor function in experimental systems where high levels of endogenous glycine are present (5, 8, 9). KYN at low doses antagonizes glycine (8–11) but at higher concentrations inhibits binding to NMDA recognition sites (8, 11). A KYN derivative, Cl-KYN, appears to be a more selective glycine antagonist (12). Recently, HA-966, originally described as an anxiolytic compound (13) and a noncompetitive NMDA receptor antagonist (14), has been shown to antagonize glycine-induced facilitation of NMDA receptor activation (15). However, differences in the intrinsic activity of Cl-KYN and HA-966 were observed in electrophysiological experiments (16).

The aim of this study was to investigate the actions of Cl-KYN, KYN, and HA-966 at various recognition sites in the NMDA receptor domain using radioligand binding assays. The binding of [^{3}H]MK-801 to the PCP recognition site was used to monitor activation of NMDA receptors.

Materials and Methods

Membrane preparation. Crude synaptic membranes were prepared as described previously (6). Cerebral hemispheres from rats (Sprague Dawley; Zivic Miller; 200–250 g) were homogenized in 20 volumes of ice-cold medium containing 0.32 M sucrose and 10 mM Tris·HCl, pH 7.2, in a glass-Teflon homogenizer (10 strokes). The homogenate was centrifuged at 1,000 × *g* for 10 min and the resulting supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in 20 volumes of ice-cold deionized water, homogenized for 30 sec (Polytron), and centrifuged at 8,000 × *g* for 20 min. The supernatant and the soft upper layer of the pellet were collected and centrifuged at 48,000 × *g* for 20 min. The crude synaptic membrane pellet was resuspended in deionized water and centrifuged at 48,000 × *g* for 20 min. The final pellets were frozen for at least 12 hr at −60°. On the day of the experiment, frozen membranes were thawed at room temperature and suspended in 30 volumes of 5 mM Tris·HCl, pH 7.4,

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HA-966, 3-amino-1-hydroxy-2-pyrrolidone; Cl-KYN, 7-chlorokynurenic acid; KYN, kynurenic acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine maleate; PCP, phencyclidine; CPP, 3-(2-carboxy piperazin-4-yl)propyl-1-phosphonic acid; APV, 2-amino-5-phosphonovalerate.

for the [^{3}H]MK-801 binding assay or 50 mM Tris-acetate, pH 7.4, for all other binding assays. The membrane suspensions were incubated for 20 min at 37° and centrifuged at 48,000 × *g* for 20 min. The resulting pellets were resuspended in the appropriate buffers and centrifuged at 48,000 × *g* for 20 min. The washing procedure was repeated five times.

Binding assays. The binding assays for [^{3}H]glutamate, [^{3}H]CPP, and [^{3}H]glycine were performed in 50 mM Tris-acetate, pH 7.4, containing 100–300 μg of the membrane protein, 20 nM radioactive ligand, and the indicated additions, in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 1 mM NMDA, 100 μM CPP, or 1 mM glycine, respectively. Incubations were carried out for 30 min at 4°. In the case of [^{3}H]glutamate and [^{3}H]glycine binding, the incubations were terminated by centrifugation at 20,000 × *g* for 10 min, followed by three washes of the pellet with 5 ml of ice-cold buffer. In [^{3}H]CPP binding experiments, incubations were terminated by filtration through Whatman GF/C filters, followed by three washes with 5 ml of ice-cold buffer. The binding assay for [^{3}H]MK-801 was performed in 5 mM Tris·HCl, pH 7.4, containing 100 μg of membrane protein, 5 nM [^{3}H]MK-801, 100 nM glutamate, and the indicated additions, in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM MK-801. The incubations were carried out for 60 min at room temperature and were terminated by filtration through Whatman GF/C filters, followed by three washes with 5 ml of ice-cold buffer.

Data analysis. Competition experiments were analyzed by nonlinear regression, from which the IC_{50} values were derived. The values of K_i were calculated according to the equation of Cheng and Prusoff (17). The K_d and B_{max} values were obtained from the Eddie-Hofstee analysis of saturation experiments. All results are expressed as means from three to five experiments (unless indicated otherwise) with the standard error used as a measure of variation. Student's *t* test was used for pairwise comparisons.

Chemicals. [^{3}H]Glutamate (69.7 Ci/mmol), [^{3}H]CPP (30.7 Ci/mmol), [^{3}H]MK-801 (17.8 Ci/mmol), and [^{3}H]glycine (43.7 Ci/mmol) were purchased from Dupont/NEN (Boston, MA); CPP and MK-801 from Research Biochemicals Inc. (Natick, MA); HA-966 and Cl-KYN from Tocris Neuramin (Buckhurst Hill, England); and all other chemicals from Sigma Chemical Co. (St. Louis, MO).

Results

The specific binding of [^{3}H]glutamate to the primary transmitter recognition site and that of [^{3}H]glycine to the modulatory site of the NMDA receptor was inhibited by KYN, Cl-KYN, and HA-966 (Fig. 1). The potencies of HA-966 and KYN for inhibiting [^{3}H]glycine binding were similar (K_i = 17 ± 2.5 and 29 ± 4.4 μM, respectively), whereas Cl-KYN was 30- to 50-fold more potent (K_i = 0.55 ± 0.027 μM) (Fig. 1A). The specific binding of [^{3}H]glutamate was inhibited completely and with similar potencies by Cl-KYN and KYN (K_i = 67 ± 8.0 and 110 ± 9.7 μM, respectively). However, 1 mM HA-966 inhibited this binding by only 40% (Fig. 1B). The addition of 10 μM glycine failed to change the inhibition of [^{3}H]glutamate binding by the above mentioned compounds (data not shown).

In contrast, the binding of [^{3}H]CPP to NMDA receptors was affected in a different manner by HA-966, Cl-KYN, and KYN. The specific binding of [^{3}H]CPP was inhibited by Cl-KYN and KYN with potencies similar to those for inhibiting [^{3}H]glutamate binding (Fig. 2). Furthermore, their inhibitory potencies were not reduced by the addition of 10 μM glycine (data not shown). On the other hand, HA-966 increased [^{3}H]CPP binding with EC_{50} = 8.3 ± 0.5 μM. The addition of glycine decreased the potency of HA-966 for enhancing [^{3}H]CPP binding (Fig. 2). The addition of 30 μM HA-966 shifted the K_d value for [^{3}H]CPP binding from 106 ± 8.4 to 66.3 ± 1.8 nM ($p < 0.05$), while

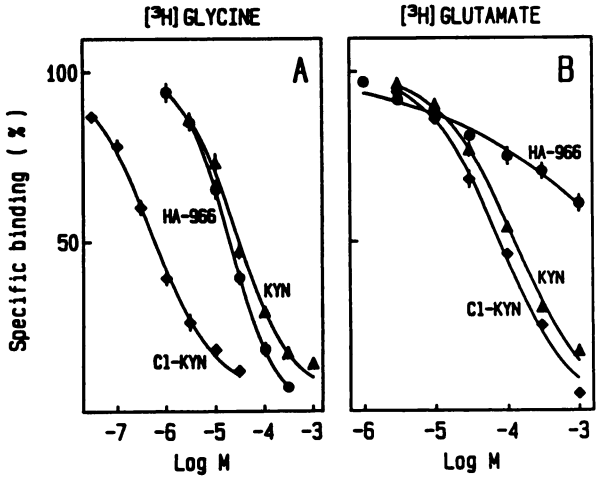

Fig. 1. Inhibition of [^{3}H]glycine binding (A) and NMDA-sensitive [^{3}H]glutamate binding (B) by HA-966 (●), Cl-KYN (♦), and KYN (▲). Specific binding is expressed as percentage of control, which amounted to 0.6 pmol/mg of protein for [^{3}H]glycine and 0.25 pmol/mg of protein for [^{3}H]glutamate.

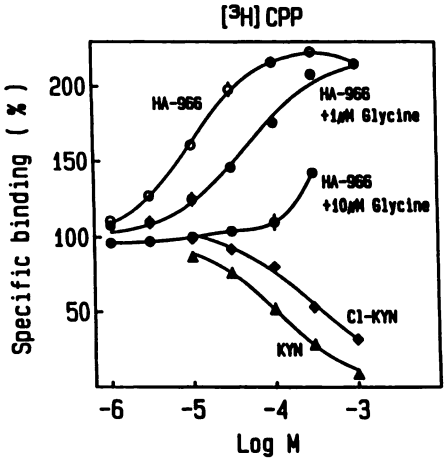

Fig. 2. Stimulation of [^{3}H]CPP binding by HA-966 in control conditions (○) (12 experiments) and in presence of 1 or 10 μM glycine (●) (six experiments). Inhibition produced by Cl-KYN (♦) and KYN (▲) is shown for comparison. Specific [^{3}H]CPP binding is expressed as percentage of control, which amounted to 0.4 pmol/mg of protein.

the B_{max} was not affected (4.3 ± 0.5 pmol/mg of protein). Additional experiments (not shown) confirmed that the increase in [^{3}H]CPP binding induced by HA-966 was inhibited by glutamate and APV, eliminating the possibility that, in the presence of HA-966, [^{3}H]CPP might bind to a site unrelated to the NMDA receptor. The increase of [^{3}H]CPP binding elicited by HA-966 was not mimicked by D-cycloserine and cycloleucine (not shown).

The enhancement of [^{3}H]CPP binding by HA-966 was sensitive to inhibition by Cl-KYN (Fig. 3). The addition of 1 and 10 μM Cl-KYN shifted the HA-966 dose-response curve to the right (K_i = 19.0 ± 1.2 and 470 ± 50 μM, respectively) and also decreased the maximal effect, suggesting that Cl-KYN and HA-966 may not interact at the same site. The inhibition of HA-966-stimulated [^{3}H]CPP binding by varying concentrations of Cl-KYN produced a biphasic dose dependency curve with two K_i values, 0.58 ± 0.07 and 270 ± 14 μM (Fig. 4A). These values were comparable to the potency of Cl-KYN for inhibiting binding of [^{3}H]glycine (K_i = 0.55 ± 0.027 μM; Fig. 1A) and

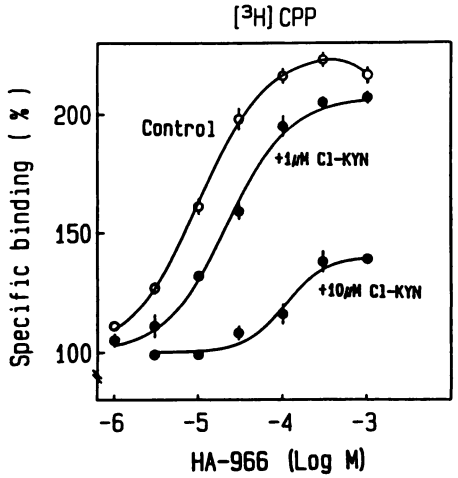

Fig. 3. Stimulation of [^{3}H]CPP binding by HA-966 in the absence (○) and in the presence of 1 or 10 μM Cl-KYN (●). Specific [^{3}H]CPP binding is expressed as percentage of control, which amounted to 0.4 pmol/mg of protein.

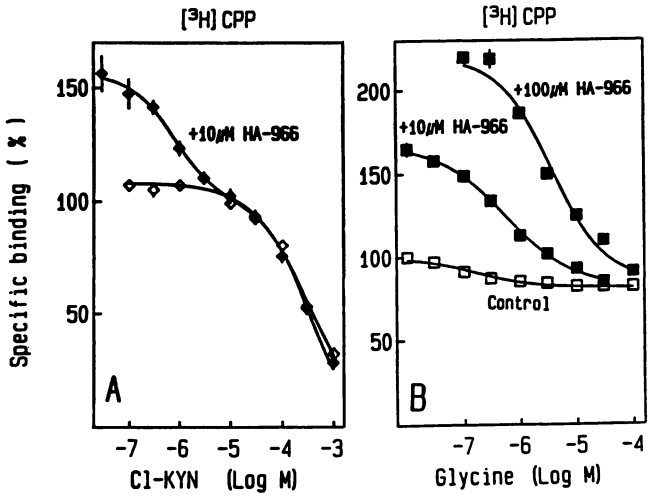

Fig. 4. A, Inhibition of [^{3}H]CPP binding by Cl-KYN in the absence (◇) and in the presence of 10 μM HA-966 (◆). B, Effect of glycine on [^{3}H]CPP binding in the absence (□) and in the presence of 10 or 100 μM HA-966 (■). Specific [^{3}H]CPP binding is expressed as percentage of control, which amounted to 0.4 pmol/mg of protein.

[^{3}H]CPP (K_i = 270 ± 12 μM; Fig. 3). When KYN was used to inhibit HA-966-stimulated [^{3}H]CPP binding (data not shown), the curve was monophasic, possibly due to a lower selectivity of KYN for the Gly_2 site. Glycine alone had a weak inhibitory effect on [^{3}H]CPP binding (less than 20%; Fig. 4B); however, in a dose-dependent manner, it inhibited the stimulation of [^{3}H]CPP binding by HA-966 (Fig. 4B). The potency of glycine for producing this inhibition depended on the concentration of HA-966; the K_i value was shifted from 0.41 ± 0.063 μM, in the presence of 10 μM HA-966, to 2.6 ± 0.008 μM in the presence of 100 μM HA-966 ($p < 0.05$).

The binding of [^{3}H]MK-801 to NMDA receptor channels, which is enhanced by glutamate and glycine, reflects the level of NMDA receptor activation (18–21). Cl-KYN completely inhibited [^{3}H]MK-801 binding with IC_{50} = 2.9 ± 1.1 μM (Fig. 5A) and, in the presence of 1 μM glycine, its potency was decreased (IC_{50} = 9.0 ± 1.2 μM; $p < 0.05$). In contrast, HA-966, up to 1 mM, produced only a partial decrease (60%) of [^{3}H]MK-801 binding in the absence of added glycine (Fig. 5B). The

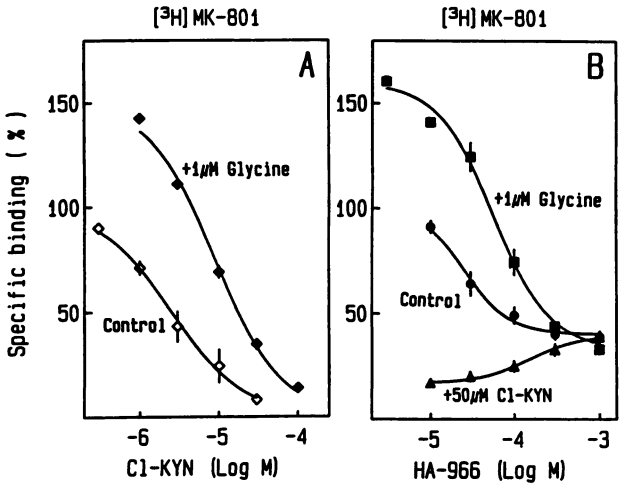

Fig. 5. Inhibition of [^{3}H]MK-801 binding by Cl-KYN in the absence (◇) and in the presence of 1 μM glycine (◆) (A) and by HA-966 without (●) or with 1 μM glycine (■) or 50 μM Cl-KYN (▲) (B). Specific [^{3}H]MK-801 binding is expressed as percentage of control, which amounted to 0.8 pmol/mg of protein.

addition of 1 μM glycine decreased the potency of HA-966 (IC_{50} changed from 26 ± 3.6 μM to 60 ± 4.1 μM). Moreover, HA-966 reduced the 85% inhibition of [^{3}H]MK-801 binding produced by 50 μM Cl-KYN to a 60% level, the maximal inhibition obtained with HA-966 alone. The ability of HA-966 to increase the affinity of NMDA receptor antagonists was reflected also in the inhibition of [^{3}H]MK-801 binding. The addition of 30 μM HA-966 increased the potency of CPP for inhibiting [^{3}H]MK-801 binding; the IC_{50} value decreased from 2.5 ± 0.22 μM (eight experiments) to 1.2 ± 0.14 μM (four experiments) ($p <$ 0.05). Both glycine and Cl-KYN failed to change the potency of CPP for inhibiting [^{3}H]MK-801 binding (data not shown).

Discussion

The activity of NMDA-sensitive glutamate receptors is allosterically modulated in a positive manner by glycine acting at strychnine-insensitive sites (Gly_2) within the domain of the NMDA receptor (1). Several studies have shown that KYN, Cl-KYN, and HA-966 inhibit the positive allosteric modulation of NMDA responses produced by glycine (8–11, 15, 16). Our present results demonstrate that, although Cl-KYN, KYN, and HA-966 all inhibit [^{3}H]glycine binding, they show striking differences in modulation of other sites located in the NMDA receptor domain.

The difference between KYN and Cl-KYN appears to be mainly in their potencies for inhibiting the binding of [^{3}H]glycine, Cl-KYN being 50-fold more potent than KYN. However, at higher concentrations, both compounds inhibit with similar potencies the binding of [^{3}H]glutamate and [^{3}H]CPP, the respective agonist and antagonist of the NMDA recognition site. Because the inhibition of either [^{3}H]glutamate or [^{3}H]CPP binding by KYN and Cl-KYN is not counteracted by glycine, one may infer that both compounds, at high concentrations, interact isosterically with the primary transmitter recognition site of the NMDA receptor. The poor (4-fold) selectivity of KYN for the glycine recognition site has been previously reported (8, 9, 11). However, the selectivity of Cl-KYN for the allosteric modulatory center is 100-fold greater than that for the primary transmitter recognition site and,

therefore, Cl-KYN can be used successfully to antagonize the effects of glycine without directly affecting the NMDA recognition site.

In contrast to KYN and Cl-KYN, high concentrations of HA-966 produce only a partial inhibition of [^{3}H]glutamate binding. Moreover, HA-966 increases, instead of decreases, the affinity of [^{3}H]CPP binding. The potency of HA-966 for increasing [^{3}H]CPP binding is comparable to its potency for inhibiting [^{3}H]glycine binding. The increase of [^{3}H]CPP binding caused by HA-966 is antagonized by both glycine and Cl-KYN, suggesting that they may act in the allosteric modulatory center with different intrinsic activities. Differences in the intrinsic activity of Cl-KYN and HA-966 have been previously observed in electrophysiological experiments (16). However, at least in the case of [^{3}H]CPP binding, competition experiments (Fig. 3) do not support the interaction of HA-966 and Cl-KYN at the same site. Hence, the possibility that HA-966 and Cl-KYN act at distinct but overlapping sites in the NMDA receptor complex cannot be excluded.

The results of [^{3}H]MK-801 binding studies indicate that the glycine-induced increase of this binding is antagonized completely by Cl-KYN but only partially by HA-966. Furthermore, HA-966, but not Cl-KYN, increases the potency of CPP to inhibit [^{3}H]MK-801 binding. As proposed recently (22), the binding of [^{3}H]MK-801 may occur through a fast hydrophilic path, which is related to the opening of NMDA receptor channels, and through a slow hydrophobic path, which is functional in the closed channel conformation of the NMDA receptor complex. The combined actions of glutamate and glycine, by opening the channel, allow the fast kinetics of association of [^{3}H]MK-801 (22). Thus, in order to consider the binding of [^{3}H]MK-801 as a functional parameter describing the effects of glycine on the opening of NMDA receptor channels, short incubation times were used. This approach minimized the contribution of the glutamate- and glycine-insensitive hydrophobic path in [^{3}H]MK-801 binding.

Based on the above findings, it may be proposed that the NMDA receptor domain contains an allosteric center where the actions of glycine and HA-966 induce different conformations of the receptor complex, producing, respectively, a positive and negative modulation of receptor activation. Cl-KYN prevents the actions of both glycine and HA-966. Such an arrangement bears certain similarities to the modulatory center of the γ-aminobutyric acid$_A$ receptor, where benzodiazepines and β-carbolines act, respectively, as positive and negative modulators whereas flumazenil antagonizes both effects (23).

It has been suggested that NMDA receptors may exist in agonist- or antagonist-preferring affinity states (2). Differences in brain distribution and binding characteristics have been reported when the radiolabeled agonist ([^{3}H]glutamate) or antagonist ([^{3}H]CPP) was used to characterize NMDA recognition sites (2, 24). Glycine has been proposed to regulate the equilibrium between agonist- and antagonist-preferring states of NMDA receptors, although the observed shift does not seem to involve more than 15–20% of either [^{3}H]glutamate or [^{3}H]CPP specific binding (2). In line with these findings we have seen that glycine slightly decreases [^{3}H]CPP binding whereas Cl-KYN, at low concentrations, produces an equivalent increase, possibly due to antagonism of the endogenous glycine in the membrane preparation. However, the enhancement of [^{3}H]CPP binding by HA-966 does not seem to represent a shift into the antagonist-preferring receptor conformation, because this is not accompanied by an equivalent decrease in affinity of [^{3}H]glutamate binding. Rather, it seems that the binding of agonists and antagonist may be regulated independently. Negative modulation by HA-966 would increase the binding to antagonist-preferring site whereas positive modulation by glycine would increase binding to agonist-preferring sites of the NMDA receptor.

The functional significance of the negative modulatory role of HA-966 is obscure, because endogenous compounds with similar actions have not been yet identified. It should be stressed that the increase in affinity of the antagonist (CPP) caused by HA-966 may not have functional significance per se. However, it may reflect the ability of HA-966, but not Cl-KYN, to induce a different conformational and functional state of the NMDA receptor complex.

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