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1-HYDROXY-3-AMINOPYRROLID-2-ONE (HA-966) AND KYNURENATE ANTAGONIZE *N*-METHYL-D-ASPARTATE INDUCED ENHANCEMENT OF [^{3}H]DOPAMINE RELEASE FROM RAT STRIATAL SLICES

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Abstract—Excitatory amino acid receptors, including those of conventional *N*-methyl-D-aspartate (NMDA) type, are believed to be located on terminals of the nigrostriatal dopaminergic projection in the rat. Activation of these receptors enhances depolarization-induced dopamine release. Rat striatal slices were preloaded with [^{3}H]dopamine (DA) and its subsequent release and possible modulation during excitatory amino acid receptor activation were investigated. Superfusion of slices with K^+ (20 mM) produced a robust increase in [^{3}H]DA release, which was markedly enhanced by the inclusion of *N*-methyl-D-aspartate (NMDA) or NMDLA in the buffer. No enhancement was observed following addition of glycine, suggesting that either the glycine binding site on the NMDA receptor complex was lacking, or that it was tonically fully activated. The latter appears to be the case since 1-hydroxy-3-aminopyrrolid-2-one (HA-966) (a purported antagonist at strychnine-insensitive glycine receptors) was able to antagonize the NMDA-induced enhancement of [^{3}H]dopamine release. This action of HA-966 could subsequently be reversed by inclusion of glycine in the medium. While the action of NMDA was readily prevented by the inclusion of the competitive antagonist 2-amino-7-phosphonoheptanoate (AP7), this antagonism could not be reversed by either glycine or D-serine. Kynurenate behaved in an apparently identical manner to HA-966. Strikingly, the enhancement of dopamine release by kainate was unaffected by HA-966. These data indicate that NMDA receptor-mediated enhancement of [^{3}H]DA release from striatal dopaminergic terminals can be modulated through strychnine-insensitive sites.

In addition to the well established role of glycine as a central inhibitory neurotransmitter at strychnine-sensitive receptors, it has been reported that low concentrations of glycine greatly facilitate the frequency of channel opening at the NMDA receptor complex via a strychnine-insensitive mechanism [1]. More recent observations on NMDA receptors expressed in xenopus oocytes, suggest that the presence of glycine may be an absolute requirement for receptor activation [2]. The evidence for a linkage between NMDA and glycine sites is strengthened by the autoradiographical finding of a parallel distribution of NMDA and glycine recognition sites in rat brain [3]. Of considerable interest are the observations that the excitatory amino acid antagonists HA-966 (1-hydroxy-3-aminopyrrolid-2-one) kynurenate and 7-chlorokynurenate [4–6] appear selectively to antagonize NMDA responses on cortical wedge preparations, by competitively antagonizing the tonic influence of glycine. In support of this, is the finding that HA-966 inhibits strychnine-insensitive [^{3}H]glycine binding to brain synaptic membranes [7].

Functionally, it is well established that NMDA heteroreceptors may regulate the release of other CNS inhibitory transmitters, including dopamine [8, 9], acetylcholine [10] and noradrenaline [11]. The effect of excitatory amino acids on the striatal release of dopamine is generally reported to be insensitive to tetrodotoxin and is therefore considered to be due to a direct action on receptors localized on nigrostriatal dopaminergic terminals. Since this system has been particularly well characterized, the present study set out to investigate firstly whether glycine modulation of NMDA-enhanced [^{3}H]dopamine release from rat striatal slices could be demonstrated, and secondly to examine the effects of HA-966 and kynurenate. Either NMDA or the racemic DL-form were used, since their activities were found to be completely equivalent at the concentrations employed.

MATERIALS AND METHODS

Male adult Wistar rats were killed by cervical dislocation followed by decapitation. The striata were rapidly removed and 400 μm longitudinal sections cut using a McIlwain tissue chopper. Slices were incubated at 37° for 15 min in Krebs bicarbonate medium (in mM: NaCl 112.5, KCL 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5, $CaCl_2$ 1.3), pH 7.4, containing 60 nM [^{3}H]dopamine (40 Ci/mmol; New England Nuclear Research, Boston, MA) ascorbate (1 mg/ml) and pargyline (10 μM). Magnesium was routinely omitted due to its known ability to suppress responses elicited through the NMDA receptor on somatodendritic sites. Although its omission was probably unnecessary for release enhanced by excitatory amino acids during K^+-depolarization, this paradigm permitted parallel assessment where required, of drug effects on spontaneous release.

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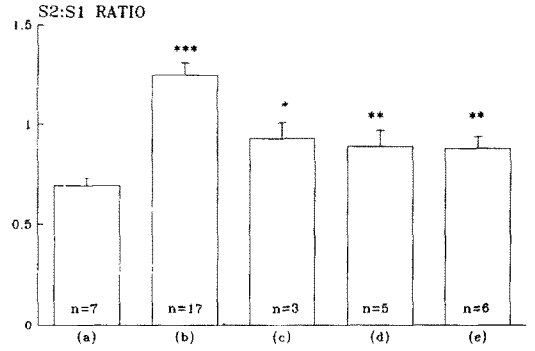

Fig. 1. Effect of HA-966 upon the enhancement by NMDLA of K^+-evoked [^{3}H]dopamine release from rat striatal slices. (a) 20 mM K^+, (b) 20 mM K^+ + 1 mM NMDLA, (c) 20 mM K^+ + 1 mM NMDLA + 1 μM HA-966, (d) 20 mM K^+ + 1 mM NMDLA + 10 μM HA-966, (e) 20 mM K^+ + 1 mM NMDLA + 100 μM HA-966. Results are means ± SE; the number of observations is shown in parentheses. Statistical analysis: (b) significantly different from (a), ***$P < 0.001$; (c), (d) and (e) each significantly different from (b), *$P < 0.05$, **$P < 0.01$ by Student's t-test.

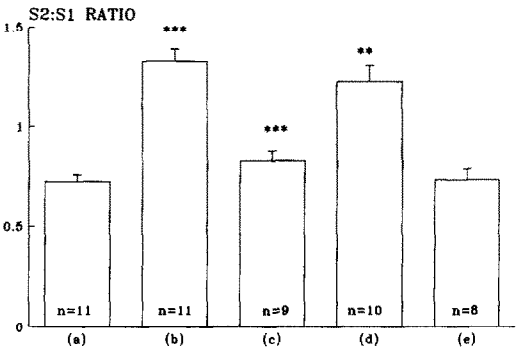

Fig. 2. Reversal by glycine of HA-966 antagonism of enhancement by NMDLA of K^+-evoked [^{3}H]dopamine release from striatal slices. (a) 20 mM K^+, (b) 20 mM K^+ + 0.6 mM NMDA, (c) 20 mM K^+ + 0.6 mM NMDA + 100 μM HA-966, (d) 20 mM K^+ + 0.6 mM NMDA + 100 μM HA-966 + 1 mM glycine, (e) 20 mM K^+ + 1 mM glycine. Results are means ± SE; the number of observations is shown in parentheses. Statistical analysis: (b) significantly different from (a), ***$P < 0.001$; (c) significantly different from (b), ***$P < 0.001$; (d) significantly different from (c), $P < 0.01$ by Student's t-test.

Following prelabelling, the slices were transferred on nylon mesh to small chambers of a perspex superfusion apparatus and then superfused with medium at a rate of 0.5 ml/min for a 50 min washout period, followed by two 4 min periods (S_1 and S_2) of depolarization, accomplished by the inclusion of 20 mM K^+ (K^+ substituted iso-osmotically for Na^+) in the medium. Test compounds were included during the S_2 period of depolarization only and superfusate collected every 2 min and assayed for released radioactivity. Thin layer chromatography confirmed that it was authentic dopamine that was primarily being released. The activity of test compounds was examined by their inclusion in the superfusion medium during the S_2 period of depolarization. Fractional release rates were calculated and stimulated release data expressed as S_2:S_1 ratios.

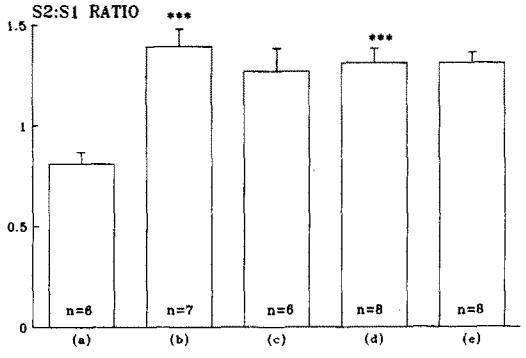

Fig. 3. Effect of HA-966 upon the enhancement by L-glutamate and kainate of K^+-evoked [^{3}H]dopamine release. (a) 20 mM K^+, (b) 20 mM K^+ + 1 mM L-glutamate, (c) 20 mM K^+ + 1 mM L-glutamate + 10 μM HA-966, (d) 20 mM K^+ + 1 mM kainate, (e) 20 mM K^+ + 1 mM kainate + 10 μM HA-966. Results are means + SE; the number of observations is shown in parentheses. Statistical analysis: (b) and (d) significantly different from (a), ***$P < 0.001$ by Student's t-test.

RESULTS

NMDLA (1 mM), or NMDA (0.6 mM) produced a large, sub-maximal enhancement of the 20 mM K^+-evoked release of [^{3}H]dopamine; this effect was significantly attenuated by the pyrrolidone, HA-966 (1, 10 and 100 μM) (Fig. 1), although it failed to reduce the stimulated release to control (20 mM K^+) levels. There appeared to be no significant difference in efficacy between the lowest and highest concentration. Inclusion of glycine in the buffer failed to influence either the K^+- or K^+/NMDLA-evoked tritium release (Fig. 2) suggesting that in the striatal slice, glycine concentrations are sufficient to saturate its binding site on the NMDA receptor complex. None of the compounds investigated, except for the excitatory amino acids themselves, influenced the spontaneous, non-stimulated release of [^{3}H]dopamine.

The specificity of the interaction of HA-966 with the NMDA receptor complex was tested by examining its ability to influence the enhancement of [^{3}H]dopamine release observed in the presence of kainate (1 mM). In this situation, HA-966 was completely wihout effect (Fig. 3). Interestingly, 1 mM L-glutamate did not significantly inhibit NMDA-enhanced [^{3}H]DA release (Fig. 3), indicating that its dominant action in this system is not being exerted through the NMDA receptor. The NMDA block by HA-966 (100 μM) could be fully reversed by glycine (1 mM) (Fig. 2), suggesting simple competitive antagonism by HA-966 at the glycine binding site.

The NMDA-enhanced K^+-evoked release of

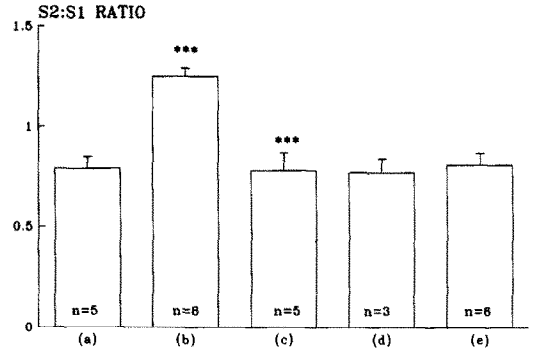

Fig. 4. Effects of 2-amino-7-phosphonoheptanoate (AP7), glycine and D-serine on the enhancement by NMDA of K^+-evoked [^{3}H]dopamine release. (a) 20 mM K^+, (b) 20 mM K^+ + 1 mM NMDA, (c) 20 mM K^+ + 1 mM NMDA + 300 μM AP7, (d) 20 mM K^+ + 1 mM NMDA + 300 μM AP7 + 1 mM glycine, (e) 20 mM K^+ + 1 mM NMDA + 300 μM AP7 + 1 mM D-serine. Results are means ± SE; the number of observations is shown in parentheses. Statistical analysis: (b) significantly different from (a), $P < 0.001$; (c) significantly different from (b), ***$P < 0.001$, by Student's *t*-test.

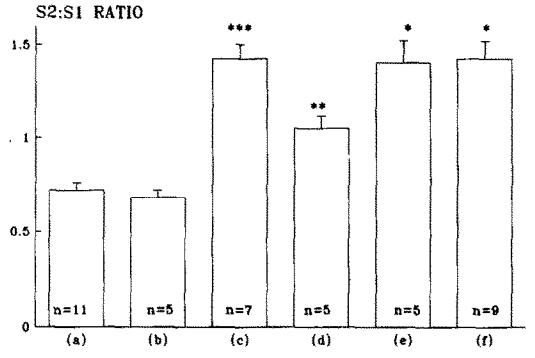

Fig. 5. Antagonism of enhancement by NMDLA of K^+-evoked [^{3}H]dopamine release by kynurenate and reversal by glycine and serine. (a) 20 mM K^+, (b) 20 mM K^+ + 200 μM kynurenate, (c) 20 mM K^+ + 1 mM NMDLA, (d) 20 mM K^+ + 1 mM NMDLA + 200 μM kynurenate, (e) 20 mM K^+ + 1 mM NMDLA + 200 μM kynurenate + 1 mM glycine, (f) 20 mM K^+ + 1 mM NMDLA + 200 μM kynurenate + 1 mM D-serine. Results are means ± SE; the number of observations is shown in parentheses. Statistical analysis: (c) significantly different from (a), ***$P < 0.001$; (d) significantly different from (c), **$P < 0.01$; (e) and (f) both significantly different from (d), *$P < 0.05$ by Student's *t*-test.

[^{3}H]dopamine was completely abolished by the competitive NMDA receptor antagonist, 2-amino-7-phosphonoheptanoate (AP7) (300 μM). This antagonism could not be reversed by the inclusion of either 1 mM glycine or D-serine (Fig. 4).

Kynurenate (200 μM) was found to behave in an analogous manner to HA-966. Whilst having no intrinsic effect on the K^+-stimulated release of [^{3}H]dopamine, it partially antagonized the NMDA receptor mediated enhancement (Fig. 5), an effect that was reversed by the inclusion of either glycine or D-serine in the superfusion medium.

DISCUSSION

It is now well established that the release of dopamine from striatal terminals may be facilitated by activation of excitatory amino acid receptors both *in vitro* and *in vivo*. NMDA, kainate and quisqualate are each able to enhance the spontaneous release of [^{3}H]dopamine from striatal slices (e.g. see Ref. 8). Rather more dramatic is the ability of these excitatory amino acids to enhance DA release under depolarizing conditions and in this study we focussed upon the enhancement of K^+-evoked release, particularly by NMDA. The results demonstrate that the presynaptic NMDA receptors involved, in common with conventional postsynaptic receptors, are subject to modulation by glycine, which exerts this action through strychnine-insensitive receptors. It is presently unclear whether glycine influences NMDA receptor activity under physiological conditions, or whether glycine concentrations are normally adequate to tonically facilitate NMDA responses fully. For example, in a cortical wedge preparation, there was no observable effect of glycine on NMDA depolarizations [12]. This observation is consistent with our own findings, where glycine failed to influence the enhancement of [^{3}H]DA release following NMDA receptor activation, even though slices were continuously superfused for an extended period with medium that contained no glycine. Glycine also failed to enhance the neurodegenerative actions of glutamate on cultural hippocampal neurones, while 7-chlorokynurenate antagonized glutamate-induced excitotoxicity in a glycine-reversible manner [13]. Other studies in cortical slices, however, have shown augmentations of NMDA responses by glycine [14], suggesting that perhaps the level of structural complexity is a key determinant. Another possibility is that NMDA receptor activation may result in the release of glycine from specific cellular compartments, which then interacts with its receptors. Indeed, we have found that excitatory amino acid receptor activation may influence both uptake and release of exogenous glycine in striatum (Crawford *et al.*, submitted).

Despite the lack of a demonstrable direct inhibitory action of glycine on NMDA-enhanced DA release, it proved possible to unmask its potential modulatory activity in the presence of HA-966 (1–100 μM) or kynurenate (200 μM), two agents that have been reported to antagonize glycine actions at non strychnine-sensitive receptors. HA-966 does have significant advantages over kynurenate however, in that it does not interact with the NMDA receptor agonist-recognition site [5]. When striatal slices were superfused with HA-966 or kynurenate, in conjunction with K^+/NMDA, there was a marked antagonism of the evoked release of [^{3}H]dopamine, an effect that could be reversed by inclusion of glycine or D-serine, an agonist at the strychnine-insensitive glycine recognition site. It is notable that inclusion of HA-966 did not fully antagonize the NMDA-enhancement of K^+-stimulated release of dopamine. This may indicate that the requirement

for glycine is not absolute, or that some sites occupied by glycine are not accessed by HA-966 under these experimental conditions. The ability of kainate to enhance the K^+-evoked release of [^{3}H]DA was not affected by the inclusion of HA-966, clearly demonstrating that this agonist site is not regulated by glycine. The finding that the action of the mixed agonist L-glutamate was only marginally affected by HA-966, implies that under these experimental conditions, glutamate is acting primarily through non-NMDA receptors. Experiments are in progress to investigate both the sensitivity of the glutamate response to the non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and the activity of the selective quisqualate agonist, α-amino-3-hydroxy-5-methylisoxazole proprionate (AMPA).

It is to be anticipated that glycine modulation of NMDA receptor-mediated events will be found to be extremely widespread in the CNS. This is one of the first reports of the actions of antagonists of strychnine-insensitive glycine receptors on a functional neurochemical response. While this paper was in preparation, a supporting paper by Ransom and Deschenes was published [15], where the non-selective antagonist kynurenate was reported to antagonize the NMDA-evoked release of both [^{3}H]DA and [^{3}H]acetylcholine from striatal slices.

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