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Kinetic Study of the Interactions Between the Glutamate and Glycine Recognition Sites on the *N*-Methyl-D-aspartic Acid Receptor Complex

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SUMMARY

The *N*-methyl-D-aspartate (NMDA) receptor is unique among the ligand-gated ion channels, in that the gating process requires the binding of two independent coagonists, glutamate and glycine. Receptor binding experiments have suggested that the coagonist recognition sites interact with one another in an allosteric manner, and previous work in this laboratory has provided additional functional support in favor of an allosteric coupling; the affinity of glutamate for its recognition site was reduced when a partial agonist, (+)-HA-966, occupied the glycine site, compared with the affinity when glycine itself was bound to the receptor. The present experiments have taken these observations a step further and compare the effects of several glycine site ligands with different affinities and intrinsic activities (determined from equilibrium concentration-response curves) on glutamate off-rate. Thus, the dissociation rate for the decay of glutamate-activated membrane currents in voltage-clamped rat cortical neurons was fastest (160 ± 28 msec) in the presence of saturating concentrations of (+)-HA-966 and progressively slower in the presence of D-cycloserine (258 ± 27 msec), aminocyclopropanecarboxylic acid (330 ± 21 msec), L-alanine (375 ± 28 msec), and glycine (502 ± 42 msec). We have also measured the affinities and intrinsic activities of several NMDA receptor ligands and report that a reciprocal interaction exists, such that the off-rate of glycine is influenced by the properties of the agonist occupying the glutamate coagonist site. Thus, the time constant for current decay after a brief exposure to glycine was fastest in the presence of a saturating concentration of *cis*-2,3-piperidinedicarboxylic acid (449 ± 26 msec) and progressively slower in the presence of quinolinate (689 ± 73 msec), NMDA (721 ± 36 msec), and L-glutamate (1260 ± 36 msec). The data suggested that the extent of the modulation of one site by the other is related to the intrinsic activity of the agonist, rather than its affinity. Specifically, we suggest that a partial agonist occupying one of the agonist recognition sites produces a conformational change that results in an accelerated off-rate for coagonist dissociation from the receptor; the lower the intrinsic activity, the greater is the effect on coagonist off-rate.

The NMDA receptor is unique among ligand-gated ion channels in that it requires the binding of two different coagonists, glutamate and glycine, to activate the receptor. Both electrophysiological and radioligand binding studies have indicated that there is a complex allosteric interaction between the glutamate and glycine recognition sites on the NMDA receptor complex. Mayer and colleagues (1) described a model whereby the glycine-sensitive "desensitization" of NMDA responses was due to an allosteric reduction in the affinity of glycine for its recognition site consequent to the binding of NMDA to its recognition site. Our own previous studies (2) demonstrated that glutamate had a higher affinity for its recognition site when glycine was bound to the coagonist site, compared with its affinity when the low intrinsic activity, partial agonist (*R*)-(+)-HA-966 was bound. This latter finding supported radioligand binding studies that showed that HA-966 noncompetitively reduced L-[^{3}H]glutamate binding to cortical membranes and increased the binding affinity of competitive NMDA receptor antagonists (3, 4). In contrast, however, the glycine site antagonist 7-chlorokynurenic acid had no allosteric effect on the glutamate recognition site (2, 3).

A reciprocal interaction has also been observed, with some competitive NMDA receptor antagonists producing a reduction in the affinity of [^{3}H]glycine binding to the strychnine-insensitive glycine site on the NMDA receptor complex (5, 6). This situation is complicated, however, by differences between antagonists with backbones composed of five or seven carbon atoms (5, 7).

We have investigated this reciprocal relationship further and

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; ACPC, aminocyclopropanecarboxylic acid; *cis*-2,3-PDA, *cis*-2,3-piperidinedicarboxylic acid; HA-966, 3-amino-1-hydroxypyrrolid-2-one.

have found that, like that of glutamate, the affinity of glycine for its recognition site on the NMDA receptor complex is dependent upon the coagonist occupying the glutamate recognition site. In an attempt to identify which property of the agonist determines its ability to allosterically modulate coagonist binding, we have examined the effects of several glycine site ligands, with differing affinities and intrinsic activities, on the kinetics of glutamate dissociation from its recognition site and, conversely, the effects on glycine dissociation rate resulting from the binding of several different glutamate receptor agonists.

Materials and Methods

Preparation of rat cortical cell cultures. Cultures of rat cortical neurons were prepared from the cerebral hemispheres of rat fetuses (16–18 days of gestation) as described previously (8).

Whole-cell voltage-clamp recordings. Cell cultures were grown on polylysine-coated glass coverslips and were used for electrophysiological experiments after 5–10 days *in vitro*. On the day of the experiment, a single coverslip was transferred to a glass-bottomed, Perspex recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. Cultures were observed using phase-contrast optics and were continuously perfused with a salt solution of the following composition (in mM): NaCl, 149; KCl, 3.25; $CaCl_2$, 2; $MgCl_2$, 2; D-glucose, 11; tetrodotoxin, 0.0003; HEPES buffer, 10. The pH of the perfusate was adjusted to 7.35 using NaOH and the osmolarity was adjusted to 350 mOsM using sucrose. To ensure optimum efficiency of space clamp and speed of drug equilibration, we restricted our recordings to small isolated cells that possessed only one or two fine neurites.

Drugs were diluted from concentrated stock solutions into a modified salt solution that lacked $MgCl_2$ but that was supplemented with NBQX (10 μM) to block non-NMDA receptor-mediated events. Additional steps were taken in the case of experiments involving the measurement of glycine kinetics, where the drug solutions were prepared in ultrapure high performance liquid chromatography-grade water and all glassware was rinsed extensively in the same water to remove traces of glycine.

Patch pipettes with a tip diameter of ~2–3 μm (mean resistance, 2.76 ± 0.14 MΩ, n = 11) were formed from borosilicate glass (1.2-mm o.d × 0.94-mm i.d.; Clark Electromedical) using a Mechanex BBCH puller; they were not additionally fire polished or treated to reduce capacitance. The patch pipettes were filled with a solution of the following composition (in mM): CsF, 120; CsCl, 10; HEPES, 10; EGTA, 10; $CaCl_2$, 0.5; the pH was adjusted to 7.25 with CsOH and the osmolarity was adjusted to 330 mOsM with sucrose. Whole-cell currents were recorded from cultured neurons at a holding potential of −60 mV using either List EPC-7 or Axopatch-200A patch-clamp amplifiers. Pipette seal resistances were not routinely measured but were generally in excess of 100 GΩ; pipette capacitance transients were cancelled before the membrane was ruptured but no additional capacitance neutralization or series resistance compensation was applied.

L-Glutamate (high performance liquid chromatography grade; Sigma), NMDA (Tocris), quinolinic acid (Sigma), *cis*-2,3-PDA (Tocris), glycine (BDH), L-alanine (Sigma), ACPG (Tocris), and D-cycloserine (Sigma) were prepared as aqueous stock solutions. (*R*)-(+)-HA-966 was synthesized in our laboratories. All drugs were diluted in the extracellular medium detailed above but with $MgCl_2$ omitted. They were applied to localized regions of the culture by fast perfusion from a double-barreled pipette assembly. Multiple drug applications were made to individual cells and the resulting responses were averaged. The solutions in the wash barrel also lacked $MgCl_2$. The internal diameter of each of the perfusion tubes was 375 μm; the tubes were positioned close to the bottom of the dish and ~300 μm from the cell. Solution equilibration times around the cells were determined by stepping from a solution of kainate (100 μM) in 10 mM NaCl to one containing 140 mM NaCl. The mean time constant of the exponential increase in membrane current after such a step was 17.2 ± 1.3 msec (n = 10).

Equilibrium concentration-response curves. Equilibrium concentration-response curves for glutamate, NMDA, quinolinic acid, and *cis*-2,3-PDA were constructed by measuring the peak membrane current response to increasing concentrations in the presence of a constant concentration of glycine (10 μM). Similar experiments were conducted with glycine, L-alanine, and D-cycloserine in the presence of a constant concentration of glutamate (10 μM). However, the latter experiments with the glycine site ligands were compromised by the presence of residual glycine contamination in salt solutions, despite our efforts to remove all traces of glycine. The level of contamination was routinely assessed by measuring the current amplitude in response to an application of glutamate (10 μM) in nominally glycine-free solution. The amplitude of this 'basal' current response was subtracted from all subsequent agonist-evoked currents to correct concentration-response curves for the residual glycine contamination. We were careful to construct concentration-response curves only after having first established a stable base-line response to repeated agonist (glutamate or glycine) exposures at nearly maximal concentrations, to minimize problems arising from response rundown. NMDA receptor desensitization was unlikely to have a significant influence, because experiments involved fast drug application and were performed in the presence of saturating glycine concentrations and peak current was always measured.

Best-fit lines were computed for all concentration-response data using a two-equivalent site model (eq. 1). Values for a given agonist in a given cell were then normalized to the maximal current (I_{max}) predicted by the fitted line and pooled to generate a mean curve.

$$I = \frac{I_{max}}{\left(1 + \frac{mK_d}{[A]}\right)^2} \quad (1)$$

Affinity estimates are quoted as the microscopic dissociation constants (mK_d). Intrinsic activity was estimated for each NMDA receptor agonist by comparing the maximal response amplitude predicted from the fitted line and that produced by a nearly saturating concentration of glutamate (30 μM) in the same cell. Intrinsic activity comparisons were corrected for the fact that this concentration produced a 90% maximal response. Likewise, intrinsic activities of glycine site agonists were estimated by comparison with responses evoked by 10 μM glycine in the same cell. The affinity of HA-966 was derived from inhibition curves describing the antagonism of responses to combined applications of glutamate (10 μM) and glycine (300 nM) by increasing concentrations of HA-966. Inhibition curves for HA-966 were also analyzed using the two-equivalent site model, and eq. 1 was rearranged to yield the concentration (K_o) of HA-966 at which the number of binding sites occupied by the agonist is reduced by 50% at a given agonist concentration (9). The K_o value was then converted to a microscopic K_i value using the Cheng-Prusoff equation (10).

Exponential curve fitting and measurement of drug off-rates. Whole-cell currents were filtered (cut-off frequency, 10 kHz), digitized (22 kHz) using a CED1401 laboratory interface, and captured on-line to the hard disk of a Compaq Deskpro 486 computer using SCAN software (purchased from J. Dempster, University of Strathclyde). Agonist off-rates (τ_{off}) were obtained from monoexponential functions fitted to the decline of averaged current relaxations (using SCAN software).

Results

The affinities and intrinsic activities of four NMDA receptor ligands were compared by constructing equilibrium concentration-response curves (Fig. 1A). Of the compounds tested, glutamate had the highest affinity and consistently produced larger responses at saturating concentrations than did either NMDA, quinolinic acid, or *cis*-2,3-PDA. Glutamate was, there-

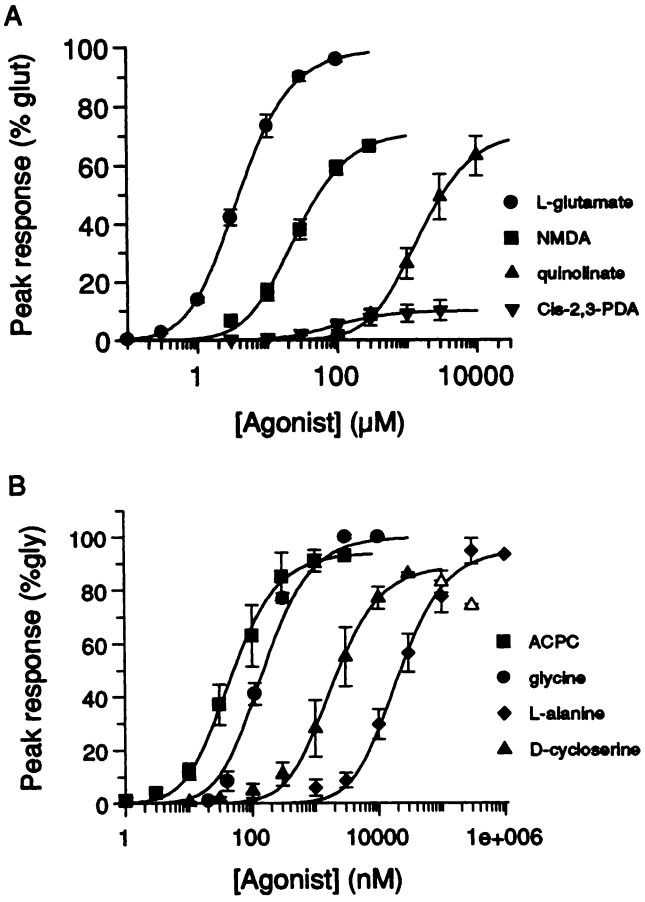

Fig. 1. Equilibrium concentration-response curves for whole-cell currents evoked by fast application of NMDA recognition site ligands and NMDA receptor glycine site ligands to voltage-clamped rat cultured cerebrocortical neurons. A, Responses to increasing concentrations of L-glutamate, NMDA, quinolinate, and *cis*-2,3-PDA were measured in the continuous presence of a saturating concentration of glycine (10 μM). Responses evoked by each of the agonists were expressed as a function of the current evoked by a nearly maximally effective concentration (30 μM) of L-glutamate applied to the same neuron. The *fitted lines* represent best-fit computations through mean data points using a two-equivalent binding site model, as described in Materials and Methods. B, Responses to increasing concentrations of ACPC, glycine, D-cycloserine (Δ, points excluded from curve fit), and L-alanine were measured in the continuous presence of a saturating concentration of glutamate (10 μM). Responses evoked by each of the agonists were expressed as a function of the current evoked by a maximally effective concentration (10 μM) of glycine applied to the same neuron. Curve fitting was performed using the two-equivalent site model, after subtraction of basal current responses obtained in nominally glycine-free solutions.

TABLE 1

Affinity and intrinsic activity values for glycine site and NMDA site ligands

Recognition site	Ligand	Microscopic K_d	Intrinsic activity[a]	n[b]
		μM	%	
Glycine	Glycine	0.063 ± 0.003	100	3
	L-Alanine	9.6 ± 2.3	96 ± 4	4
	ACPC	0.009 ± 0.001	95 ± 6	4
	D-Cycloserine	1.0 ± 0.3	86 ± 5	4
	(*R*)-(+)-HA-966	5.6 ± 0.6[c]	10 ± 1	5
NMDA	Glutamate	0.68 ± 0.07	100	5
	NMDA	11.4 ± 1.04	71 ± 3	8
	Quinolinate	728 ± 121	72 ± 6	5
	cis-2,3-PDA	46.9 ± 4.9	10 ± 3	4

[a] Expressed as percentage of response to glycine (10 μM) or glutamate (30 μM).
[b] n, number of neurons tested.
[c] Microscopic K_i value.

fore, defined as a full agonist (Table 1) and, by comparison, NMDA, quinolinate, and *cis*-2,3-PDA each produced smaller maximal responses, having approximately 71, 72, and 10%, respectively, of the intrinsic activity of glutamate (Table 1). Of equal importance, with respect to the present study, was the fact that these compounds showed marked differences in their affinities for the NMDA receptor (Table 1).

Equilibrium concentration-response curves for several glycine site ligands were also constructed, to determine their affinities at the glycine site on the NMDA receptor complex (Fig. 1B). The cyclopropyl analogue ACPC was the highest affinity glycine site ligand of those tested, with an ~7-fold higher affinity for the glycine site, compared with glycine. However, ACPC consistently produced slightly smaller maximal responses, having approximately 95% intrinsic activity, compared with glycine (Table 1). L-Alanine had a level of intrinsic activity similar to that of ACPC but had an approximately 150-fold lower affinity than did glycine (Table 1). D-Cycloserine had the profile of a lower affinity partial agonist, having ~86% of the intrinsic activity of glycine and ~15-fold lower affinity (Table 1). At high concentrations (>30 μM) of D-cycloserine the response to NMDA was consistently reduced, such that concentration-response curves were bell-shaped (Fig. 1B). In applying curve-fitting routines to such data we did not include data points generated by D-cycloserine concentrations above 30 μM; the intrinsic activity was, therefore, estimated from the maximal response predicted from the fitted line. HA-

966, as we reported previously (2), had ~10% of the intrinsic activity of glycine [based on the residual current response to 30 μM NMDA in nominally glycine-free solution containing a saturating concentration of (+)-HA-966 (100 μM) and expressed as a percentage of the response obtained with a nearly maximal concentration of glycine (3 μM)] and, hence, antagonized responses evoked by glycine. The inhibition curve for the antagonism of responses to glycine by (*R*)-(+)-HA-966 (Fig. 2) yielded an estimated microscopic K_i of 5.6 μM (Table 1).

Glycine off-rate time constants for the exponential current decay after a change to glycine-free solution were measured in the presence of saturating concentrations of either glutamate, NMDA, quinolinate, or *cis*-2,3-PDA. These experiments revealed that the decay in current amplitude varied depending upon the agonist occupying the NMDA recognition site (e.g., Fig. 3). Thus, the decay in the membrane current was slowest when glutamate was used as the coagonist and was progressively accelerated in the presence of NMDA, quinolinate, and *cis*-2,3-PDA (Table 2). Similar experiments in which the glutamate off-rate time constant was measured in the presence of various glycine site ligands revealed an analogous relationship. Thus, the decay in membrane current after a change to glutamate-free solution was slowest in the presence of a saturating concentration of glycine and was progressively faster when L-alanine, ACPC, D-cycloserine, and (*R*)-(+)-HA-966 (Fig. 3) occupied the glycine site (Table 3).

Discussion

These results provide additional evidence that the affinities of both glutamate and glycine for their respective recognition sites are influenced allosterically by the compound occupying the corresponding coagonist site. The relationships between the rate of decay of the agonist responses and the estimates of intrinsic activity and affinity have been plotted in Fig. 4. As is apparent from these plots, there is a clear and statistically

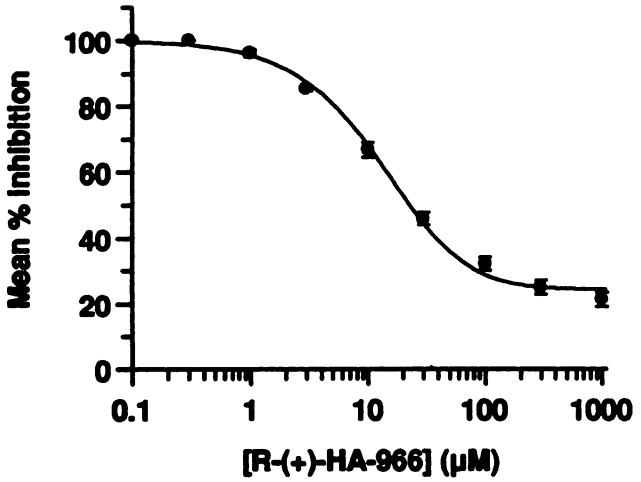

Fig. 2. Inhibition curve for the antagonism by (*R*)-(+)-HA-966 of responses to NMDA plus glycine. The antagonism of combined applications of NMDA (30 μM) and glycine (300 nM) by increasing concentrations of HA-966 was expressed as a function of the control, i.e., pre-HA-966 response amplitude. The figure shows the *fitted curve* (two-equivalent site model) through the mean data obtained from five neurons and reveals HA-966 to be a partial agonist at the glycine site. The curve falls short of complete antgonism, indicating a level of intrinsic activity that is approximately 20% that of glycine, a value that is, in fact, an overestimate (see Table 1) due to the use of a submaximal (~70%) concentration (300 nM) of glycine in these experiments.

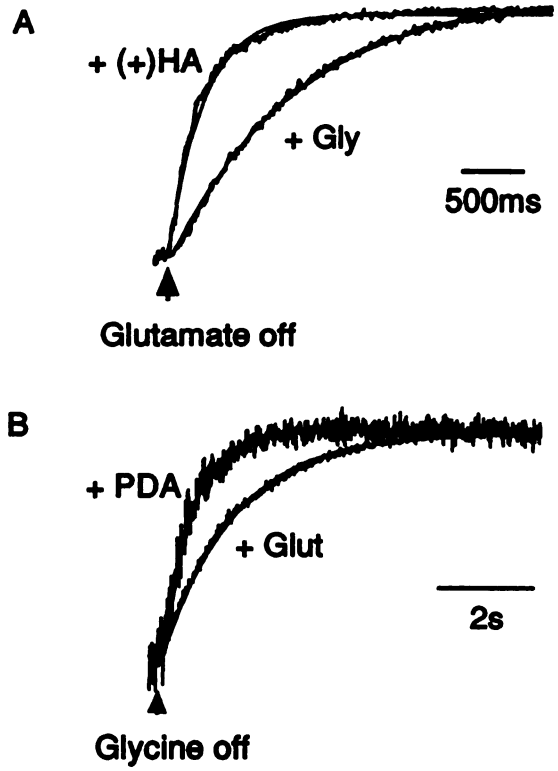

Fig. 3. Relaxation of inward membrane currents recorded from voltage-clamped rat cortical neurons, after the removal of either glutamate or glycine, with different coagonist combinations. A, Averaged current relaxations after a rapid step from glutamate (3 μM) to glutamate-free solution (*arrowhead*) in the continuous presence of either (*R*)-(+)-HA-966 (300 μM) [*+(+)HA*] or glycine (300 nM) (*+Gly*). The responses have been scaled to the same amplitude to facilitate direct visual comparison (actual amplitudes: HA-966, 400 pA; glycine, 2600 pA) and are shown with superimposed exponential curves fitted to the digitized data. Best-fit exponential decays revealed glutamate off-rates of 200 msec and 740 msec for currents obtained in the presence of HA-966 and glycine, respectively. B, Averaged current relaxations after a rapid step from glycine (300 nM) to glycine-free solution (*arrowhead*) in the continuous presence of either *cis*-2,3-PDA (1 mM) (*+PDA*) or glutamate (3 μM) (*+Glut*). Best-fit monoexponential curves are shown superimposed onto the digitized scaled (actual amplitudes: PDA, 135 pA; glutamate, 2000 pA) responses and revealed glycine off-rates of 580 msec and 1500 msec in the presence of PDA and glutamate, respectively. The figure illustrates the fact that agonist decay rates are dependent upon the compound occupying the coagonist binding site and that decay rates are faster when that compound is a partial agonist, rather than a full agonist.

TABLE 2

Glycine off-rate time constants (τ_{off}) in the presence of different glutamate site ligands

Ligand	τ_{off}	n^a
	msec	
Glutamate	1260 ± 36	6
NMDA	721 ± 36	13
Quinolinate	689 ± 6	6
cis-2,3-PDA	449 ± 26	11

[a] *n*, number of neurons tested.

significant ($p < 0.05$) correlation between the increase in response decay rate and reduction in coagonist intrinsic activity, whereas there is no relationship ($p > 0.05$) between coagonist affinity and response decay rate.

Why, then, might a partial agonist occupying one recognition site affect the current decay rate in response to removal of the coagonist for the other recogniton site? In attempting to answer

TABLE 3

Glutamate off-rate time constants (τ_{off}) in the presence of different glycine site ligands

Compound	τ_{off}	n^a
	msec	
Glycine	502 ± 42	16
L-Alanine	375 ± 28	10
ACPC	330 ± 21	5
D-Cycloserine	258 ± 27	4
(+)-HA-966	160 ± 28	9

[a] *n*, number of neurons tested.

this question we have found it useful to consider the scheme outlined below for the kinetics of glutamate responses induced in the presence of saturating concentrations of a glycine site ligand. This was based on the kinetic schemes proposed for two independent, equivalent, binding sites for both glutamate and glycine on the NMDA receptor (9, 11, 12). The same would apply for glycine responses in the presence of saturating concentrations of a glutamate site ligand.

$$2\text{Glu} + \text{R}^{2\text{Gly agonist}} \underset{K_{off}}{\overset{2k_{on}}{\rightleftarrows}} \text{Glu} + \text{R}^{2\text{Gly agonist}}_{\text{Glu}} \underset{2k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{R}^{2\text{Gly agonist}}_{2\text{Glu}}$$

$$\alpha \Updownarrow \beta$$

$$^{*}\text{R}^{2\text{Gly agonist}}_{2\text{Glu}}$$

*R is the fully bound and open (conducting) state of the receptor, k_{on} and k_{off} are rate constants for ligand binding and unbinding, and β and α are rate constants for channel opening and closing, respectively.

According to such a scheme, a glycine site full agonist would shift the equilibrium to the right, i.e., the open channel state (*R). In the case of a partial agonist, however, the receptor would spend more time in the bound but closed conformation. Consequently, when a partial agonist occupies either the glutamate or glycine recognition site, the receptor complex is more likely to exist in the state from which the coagonist could dissociate (13). This would be predicted to lead to an increased current decay rate, because both coagonist sites are required to be fully bound for channel activity.

Although this appears to be a plausible explanation, published estimates of the respective rate constants suggest that the channel closing rate, α, is ~50–200-fold faster than either $k_{-1(Gly)}$ or $k_{-1(Glu)}$ (14, 15). Consequently, α would be expected to have little influence on the overall current decay rate, which depends largely on k^1_{off}. In view of this, it must be assumed that a partial agonist at either the glutamate or glycine binding site elicits a conformational change in the receptor that increases k_{-1} for the corresponding coagonist. It is possible, of course, that the published estimates of k_{+1}, k_{-1}, α, and β are incorrect and that α and β play a much more influential role in deter-

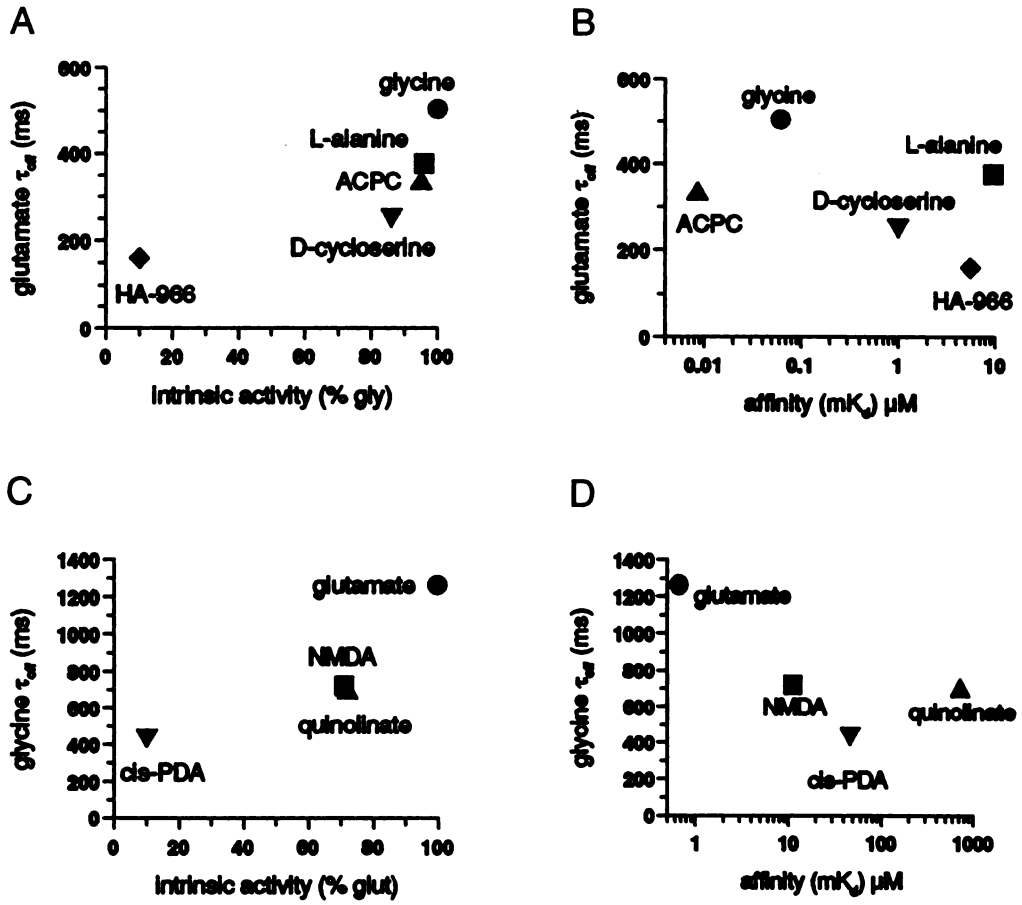

Fig. 4. Relationship between agonist intrinsic activity, affinity, and coagonist response off-rate. The dependence of glutamate off-rate on glycine receptor agonist intrinsic activity (A) and the apparent lack of a correlation with agonist affinity (B) are shown for the glycine receptor agonists glycine, L-alanine, ACPC, D-cycloserine, and (*R*)-(+)-HA-966. Similarly, glycine off-rate is influenced by NMDA receptor agonist intrinsic activity (C) but not by agonist affinity (D), as illustrated for the NMDA receptor agonists L-glutamate, NMDA, quinolinate, and *cis*-2,3-PDA. Intrinsic activities for all of the agonists are represented as percentages of those for the full agonists glycine and glutamate, respectively, and are presented with the estimated affinities in Table 1. mK_d, microscopic K_d.

mining agonist affinity. However, there are two reasons why this is unlikely to be the case. Firstly, a dominant role for α and β in determining agonist affinity would make it impossible for a full agonist to have low affinity, whereas there are examples of low affinity, high intrinsic activity compounds for both of the coagonist sites, as illustrated by the present data, e.g., quinolinate and L-alanine. Secondly, when Benveniste and Mayer (9) simulated the effect of independently changing α and β while keeping experimentally determined binding and unbinding rates constant, they found surprisingly little effect of α on either agonist or antagonist affinity, provided that its value was fast (>200 sec^{-1}) relative to the binding constants. A considerable range of values for β had even less influence on measured affinity.

In conclusion, these experiments have provided additional functional data to support the concept of coupling between the NMDA and glycine recognition sites on the NMDA receptor complex. Agonist affinity at either site appears to be dependent upon the properties of the ligand occupying the other site. Specifically, the present data suggest that the extent of the modulation of one site by the other is a property of the intrinsic activity of the agonist, rather than its affinity.

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