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Effect of Extracellular pH on the Potency of *N*-Methyl-D-aspartic Acid Receptor Competitive Antagonists

MORRIS BENVENISTE[1] and MARK L. MAYER

Laboratory of Cellular and Molecular Neurophysiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

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SUMMARY

Structure-activity analysis reveals that acidic α-amino acids containing an ω-PO_3H_2 group are more potent antagonists at *N*-methyl-D-aspartate (NMDA) receptors than are analogs with ω-COOH or ω-tetrazole groups. At physiological values of extracellular pH the ω-PO_3H_2 group is only partially deprotonated and the corresponding antagonists exist as ions with one or two negative charges. In contrast, competitive antagonists with ω-COOH and ω-tetrazole groups are fully ionized at physiological pH but carry only a single negative charge. Dose-inhibition analysis was performed with (2*R*)-AP7 and its piperidine derivative LY 257883 to determine whether ionization of the ω-PO_3H_2 group influences NMDA receptor antagonist potency; these experiments revealed a >3-fold increase in potency on raising of the extracellular pH from 7.3 to pH 8.2, consistent with the increase in the relative concentration of the ionic form of the antagonist in which the ω-PO_3H_2 group contains two negative charges. Experiments with the ω-COOH-containing analog of LY 257883 and with SDZ EAB 515, an ω-PO_3H_2-containing antagonist of novel structure, revealed only 1.5- and 1.3-fold increases in potency, respectively, over the same pH range. Analysis of the kinetics of block of NMDA-activated currents resulting from rapid application of LY 257883 suggests that the increase in potency on raising of the extracellular pH results largely from an increase in the antagonist association rate constant but also from a small decrease in the dissociation rate constant. Together, these results suggest that the fully ionized forms of the *R*-enantiomers of AP7 and LY 257883 act as the active antagonist species at NMDA receptors.

The NMDA receptor has been implicated in nerve cell death due to ischemia (1), in chronic neurodegenerative disease (2), and in the promotion of seizure activity (3); thus, drugs that effectively block activation of NMDA receptors have high therapeutic potential. Considerable effort has been spent on the synthesis of novel competitive NMDA receptor antagonists, with the goal of developing compounds that cross the blood-brain barrier and are of high potency but that lack the behavioral side effects common to MK-801, phencyclidine, and ketamine (4). By analyzing structure-activity relationships for ligands that act as NMDA receptor competitive antagonists, it has been determined that the majority are acidic α-amino acids that contain another negatively charged group, positioned four or six atoms away from the α-carbon atom (5, 6). Active ω-substituents include the COOH, PO_3H_2, and tetrazole groups (5–11). Marked increases in the potency of NMDA receptor antagonists have been achieved by incorporating the requisite acidic amino acids into a conformationally restrained framework (7–11). In every case studied, antagonists with an ω-PO_3H_2 group are more potent than their ω-COOH- or ω-tetrazole-substituted derivatives (7–10). The law of mass action requires that at equilibrium the antagonist dissociation constant (K_i) is determined by the ratio of the rate constants for the dissociation (k_{off}) and association (k_{on}) of an antagonist (i.e., $K_i = k_{off}/k_{on}$). In view of this, it is surprising that kinetic experiments reveal the apparent association rate constant for the binding to NMDA receptors of antagonists with an ω-PO_3H_2 group to be slower than that of the corresponding ω-tetrazole- and ω-COOH-substituted analogs (12).

Differences in the geometry, charge, and chemical composi-

[1] National Research Council Fellow.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartic acid; MK-801, 1-[*cis*-2-carboxypiperidine-4-yl]methyl-1-phosphonic acid; AP5, 2-amino-5-phosphonopentanoic acid; AP7, 2-amino-7-phosphonoheptanoic acid; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; *K*, antagonist microscopic dissociation constant; K_d, agonist apparent microscopic dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; LY 257883, *cis*-4-(3-phosphonoprop-1-yl)piperidine-2-carboxylic acid; LY 221501, *cis*-4-(3-carboxyprop-1-yl)piperidine-2-carboxylic acid; SDZ EAB 515, (2*S*)-2-amino-5-phosphonomethyl[1,1′-biphenyl]-3-propanoic acid; CGP 37849, (*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid; CGS 19755, *cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid; LY 233053, *cis*-4-(1(2)*H*-tetrazol-5-yl)methylpiperidine-2-carboxylic acid; LY 235959, (3*S*,4a*R*,6*S*,8a*R*)-6-phosphonomethyl-decahydroisoquinoline-3-carboxylic acid; LY 202157, (3*S*,4a*R*,6*S*,8a*R*)-6-(1(2)*H*-tetrazol-5-yl)methyl-decahydroisoquinoline-3-carboxylic acid; CsBAPTA, tetracesium salt of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tion of the acidic ω-group would be expected to account for the variation in potency of antagonists with otherwise similar structure. Tetrazole and COOH groups are planar structures that contain one negative charge when fully ionized; in contrast, PO_3H_2 groups are approximately tetrahedral and have two negative charges when fully ionized. Although the ω-tetrazole and ω-COOH groups of α-amino acid derivatives are completely ionized at physiological pH, pK_a values of approximately 2.5 and 7.8 for AP7 (13, 14) indicate that the ω-PO_3H_2 group in AP7 will exist with either one or two negative charges at physiological pH and that both forms will be present in significant proportions.

In the experiments described here, we have tested the hypothesis that the different ionized forms of antagonists with an ω-PO_3H_2 group do not bind to NMDA receptors with equal affinity and that a change in pH that promotes ionization of the ω-PO_3H_2 group will increase both antagonist equilibrium potency and k_{on}. Previous attempts to determine whether the ionized forms of AP7 have different affinities for NMDA receptors used ligand binding experiments with a derivative of AP7 in which ionization of the ω-PO_3H_2 group was increased by introducing electron-withdrawing fluorine atoms covalently bonded to the carbon atom adjacent to the ω-PO_3H_2 group. The resulting compound, 7,7-difluoro-AP7, was 34-fold less potent than AP7 and was completely ionized at physiological pH, because the ω-PO_3H_2 group had pK_a values of 3.0 and 5.7 (14). One interpretation of the lower potency of 7,7-difluoro-AP7 as an NMDA receptor antagonist would be that 7,7-difluoro-AP7 molecules in which the ω-phosphonate group has two negative charges bind with lower affinity to NMDA receptors than does the partially ionized form with a single negative charge; however, steric hindrance due to the substitution of fluorine for hydrogen atoms could lower the potency of 7,7-difluoro-AP7 independently of any change in ionization (14). Differences in the potency of NMDA receptor antagonists containing the structurally similar ω-phosphonate (two charges, high potency) and ω-phosphinate (one charge, low potency) groups support the latter possibility and suggest that the high affinity binding of ω-phosphonates does indeed require the presence of two negative charges (6). In support of this, experiments with [^{3}H]CPP show optimal binding to NMDA receptors at pH 8.0 (15), consistent with the suggestion that high affinity binding is achieved in antagonist molecules in which the ω-PO_3H_2 group is of the doubly charged form; however, these experiments did not address an alternative explanation, that changes in ionization of functional groups within the NMDA receptor itself also contribute to the pH-sensitive binding of antagonists.

To address the issues described above, we measured the ability of two competitive antagonists containing an ω-PO_3H_2 group (Fig. 1) to inhibit NMDA receptor currents at pH 7.3, where the singly charged phosphonate group is the predominant ionic species, and at pH 8.2, where the doubly charged species is predominant. Similar experiments were performed for an antagonist containing an ω-COOH group, ionization of which does not change over this range of pH, and for a novel ω-PO_3H_2 antagonist of 2*S* instead of 2*R* stereochemical conformation (Fig. 1). Our results suggest that for (2*R*)-AP7 and for its piperidine derivative LY 257883 the fully ionized form is the active antagonist species at NMDA receptors.

Materials and Methods

Culture conditions and electrophysiology. Neurons were isolated from a dissection of gestational day 16–18 C57BL/6 mouse embryo hippocampi and were plated on a confluent glial cell feeder layer grown from a dissection of postnatal day 1 mouse hippocampi, as previously described (16, 17). Experiments were generally performed 10–14 days after neurons were plated.

The conditions for electrophysiological experiments, involving rapid application and removal of NMDA receptor competitive antagonists, have also been described in detail (17–19). Briefly, neurons were voltage clamped with an Axoclamp 2 amplifier (Axon Instruments) at a holding potential of −60 mV. Experiments were performed at 24–27°. The intracellular solution consisted of (in mM) $CsMeSO_3$, 125; CsCl, 15; HEPES, 10; $CaCl_2$, 0.5; $MgCl_2$, 3; CsBAPTA, 5; and Na_2ATP, 2; adjusted to pH 7.2 with CsOH and to 305 mOsm with sucrose. Extracellular solution consisted of (in mM) NaCl, 160; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; and HEPES, 10; with 10 μg/ml phenol red; titrated to pH 7.3 or 8.2 with NaOH, (increasing the final Na^+ concentration by 4 and 8 mM, respectively) and then adjusted to 325 mOsm with sucrose. Tetrodotoxin (400 nM) and bicuculline methochloride (5 μM) were also added to block sodium currents and inhibitory postsynaptic currents, respectively. Agonist and antagonist solutions were similar to the control extracellular solution but contained only 0.2 mM Ca^{2+} and lacked Mg^{2+}. Glycine was present at 3 μM for all experiments, and NMDA was applied at 10 μM. Solutions were applied from a 356-μm-internal diameter flowpipe (part of a nine-barrel array) positioned approximately 100 μm from the cell soma. Rapid solution changes were achieved by electronically switching the flow between adjacent flowpipe barrels containing different solutions, immediately after a stepper motor moved the appropriate barrel into position above the neuron. Previously, we showed that the time constant for solution exchange around the cell soma and surrounding dendrites was approximately 10 msec or less (18).

(2*R*)-AP7 (D-AP7) was purchased from Tocris Neuramin, LY 257883 and LY 221501 were a gift from Dr. P. Ornstein (Eli Lilly Pharmaceuticals, Indianapolis, IN), and SDZ EAB 515 was a gift from Dr. P. Herrling (Sandoz, Berne, Switzerland).

Analysis. Each antagonist studied was applied at five concentrations, and analysis of dose-response curves for equilibrium block of responses to 10 μM NMDA was accomplished with a nonlinear least squares fit of the two-binding site inhibition isotherm:

$$\%\ \text{of control} = \frac{100}{\left(1 + \frac{[\text{antagonist}]}{K_o}\right)^2} \quad (1)$$

where K_o is defined as the concentration of antagonist that displaces half of the agonist molecules occupying receptor binding sites. The antagonist microscopic dissociation constant (K_i) was then determined from the relationship (20)

$$K_i = \frac{K_o}{1 + \frac{[\text{NMDA}]}{K_d}} \quad (2)$$

where K_d is the apparent microscopic equilibrium dissociation constant for binding of NMDA at any given pH value. The K_d values for NMDA were determined from equilibrium measurements taken at the end of a 1.5-sec pulse of agonist for six concentrations of NMDA (3–1000 μM) at pH 7.3 and 8.2; the resulting dose-response curves were fit to a two-equivalent binding site model (21, 22). Although K_i values determined from eq. 2 are corrected for the 1.3-fold increase in affinity for NMDA on raising of the pH from 7.3 to 8.2, it is possible that this correction may be inappropriate if the agonist and antagonist binding sites on the NMDA receptor only partially overlap (5, 32).

The apparent association and dissociation rate constants for NMDA (k_{Aon} and k_{Aoff}) required for the analysis of antagonist kinetics were determined at both pH 7.3 and 8.2 by the method previously described

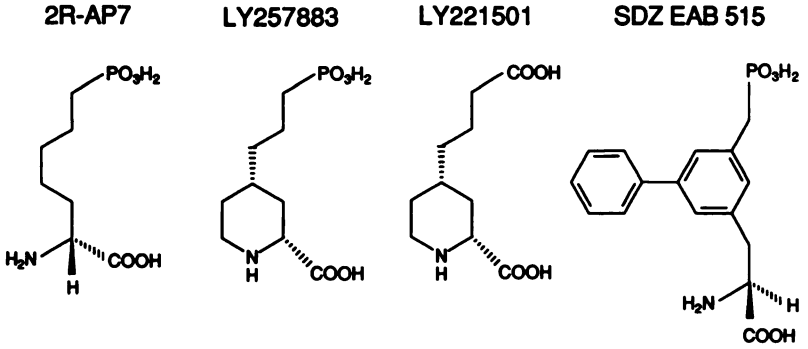
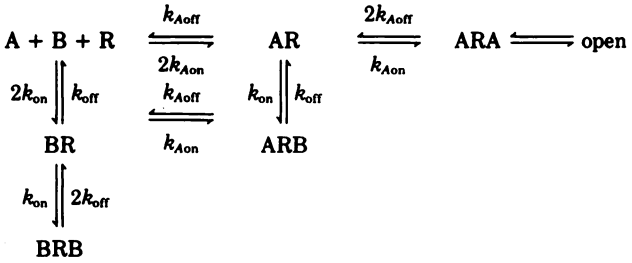


Fig. 1. Structural features of AP7 and some derivatives. Depicted are (2*R*)-AP7, LY 257883, LY 221501, and SDZ EAB 515. In our experiments AP7 and SDZ EAB 515 were applied as single enantiomers; for LY 257883 and LY 221501 the compounds tested contained both the 2*R*- and 2*S*-enantiomers; the structures drawn are of the active enantiomers.

(17). The antagonist association and dissociation rate constants (k_{on} and k_{off}, respectively) were determined at pH 7.3 and 8.2 from an iterative nonlinear least squares fit of a two-equivalent site model for competitive antagonism, according to the scheme:



where R represents the receptor, A the agonist, and B the antagonist. The analysis of antagonist association and dissociation rate constants was generally performed at two antagonist concentrations, corresponding to 80 and 95% inhibition of the response to 10 μM NMDA, and the results were pooled, as described previously (17). To increase the accuracy with which comparisons between antagonists could be determined, paired applications of antagonists were made to the same neuron at both pH 7.3 and 8.2 (e.g., Fig. 4). Only two of the four antagonists studied were optically resolved; however, from binding studies with the optical isomers of AP5, CPP, and CGP 37849 (5, 23, 24) and from physiological studies with AP5, CPP, and SDZ EAB 515 (11, 23, 25, 31), it is known that potent NMDA receptor antagonist activity is produced selectively by a single enantiomer. Thus, for the racemic compounds studied (as indicated in Tables 1 and 2), a 2-fold correction was made for K_i and k_{on}, assuming one enantiomer to be inactive.

The relative concentrations of antagonist ionic species were determined from the Henderson-Hasselbalch equation:

$$\frac{[A^-]}{[HA]} = 10^{(pH-pK_a)} \quad (3)$$

where A^- represents the proton acceptor and HA represents the proton donor. Experimental values in the text are given as mean ± standard deviation.

pH-sensitive action of NMDA receptor agonists. The activation of the NMDA receptor by agonists is sensitive to changes in extracellular pH (26–29); however, protons serve as noncompetitive inhibitors of the response to aspartate and glutamate, and there is no obvious change in the EC_{50} for aspartate over the pH range of 6.8 to 7.6 (29). Thus, block of NMDA receptor activity by protons would not be expected to interfere with the binding of agonists and antagonists. Because this is a critical assumption for our experiments, it was tested directly; before analysis of the effect of extracellular pH on antagonist equilibrium potency and antagonist binding kinetics, we determined values for K_d, k_{Aon}, and k_{Aoff} for NMDA at both pH 7.3 and pH 8.2, because these values were required for subsequent calculation of antagonist K_i, k_{on}, and k_{off} values. The K_d values for NMDA were 17.8 ± 4.4 μM and 13.8 ± 3.8 μM at extracellular pH 7.3 and 8.2, respectively, in good agreement with previous measurements (21, 29, 30).

The apparent association and dissociation rate constants for NMDA at pH 7.3 analyzed by a two-equivalent site model were previously determined to be 2.1 ± 0.9 × 10^6 M^{-1} sec^{-1} and 22.9 ± 4.3 sec^{-1}, respectively (17). At pH 8.2, the apparent association rate constant decreased slightly to 1.8 ± 0.6 × 10^6 M^{-1} sec^{-1} and the apparent dissociation rate constant increased slightly to 25.5 ± 4.6 sec^{-1} (six cells, 65 observations). The ratio k_{off}/k_{on} for NMDA should be similar to the K_d determined from equilibrium dose-response analysis. These ratios were 11.0 ± 4.0 μM and 15.7 ± 5.1 μM at pH 7.3 and 8.2, respectively, and are within 1.6-fold of the steady state values determined from dose-response analysis mentioned above. Because the apparent rate constants for binding of NMDA at pH 8.2 were slightly different from those at pH 7.3, for the analysis of antagonist concentration-jump responses the rate constants for NMDA at the appropriate pH were used for greater accuracy.

Results

The first goal of our experiments was to compare antagonist potency at two values of extracellular pH and thus provide information on the number of charges on the ω-PO_3H_2 group likely to be required for high affinity binding of antagonists to NMDA receptors. Fig. 2 shows that higher concentrations of (2*R*)-AP7 are required to inhibit NMDA-activated currents at pH 7.3 than at pH 8.2. This is exemplified by the similar degree of inhibition produced by 3 μM (2*R*)-AP7 at pH 8.2 (Fig. 2B) and 10 μM (2*R*)-AP7 at pH 7.3 (Fig. 2A). Dose-response analysis yielded K_o values for (2*R*)-AP7 of 8.7 ± 1.2 μM at pH 7.3 (five cells) and 2.6 ± 0.5 μM at pH 8.2 (seven cells) (Fig. 2C). When these values and the steady state K_d values for NMDA are substituted; into eq. 2, the calculated K_i for (2*R*)-AP7 is 5.6 ± 0.8 μM at pH 7.3 and 1.5 ± 0.3 μM at pH 8.2. Thus, (2*R*)-AP7 is 3.7-fold more potent at pH 8.2 than at pH 7.3 (Table 1).

To determine whether the change in potency of (2*R*)-AP7 with pH was due to a change in the number of charges on the ω-PO_3H_2 group or due to a nonselective change in affinity of the NMDA receptor itself the effect of pH on dose-inhibition relationships was analyzed for a pair of piperidine-based ω-PO_3H_2- and ω-COOH-substituted antagonists that otherwise have identical structures (Figs. 1 and 3). An increase in ionization of the ω-PO_3H_2 group in the piperidine LY 257883 on raising of the extracellular pH from 7.3 to 8.2 would be expected to produce an increase in potency similar that observed for AP7. The pK_a of the ω-COOH group in the piperidine LY 221501 would be expected to be well below the physiological range, and thus should be fully ionized over the pH range 7.3 to 8.2; as a result, any change in antagonist potency would be likely to result from pH-dependent changes in functional groups on the NMDA receptor. Fig. 3 shows a leftward shift in

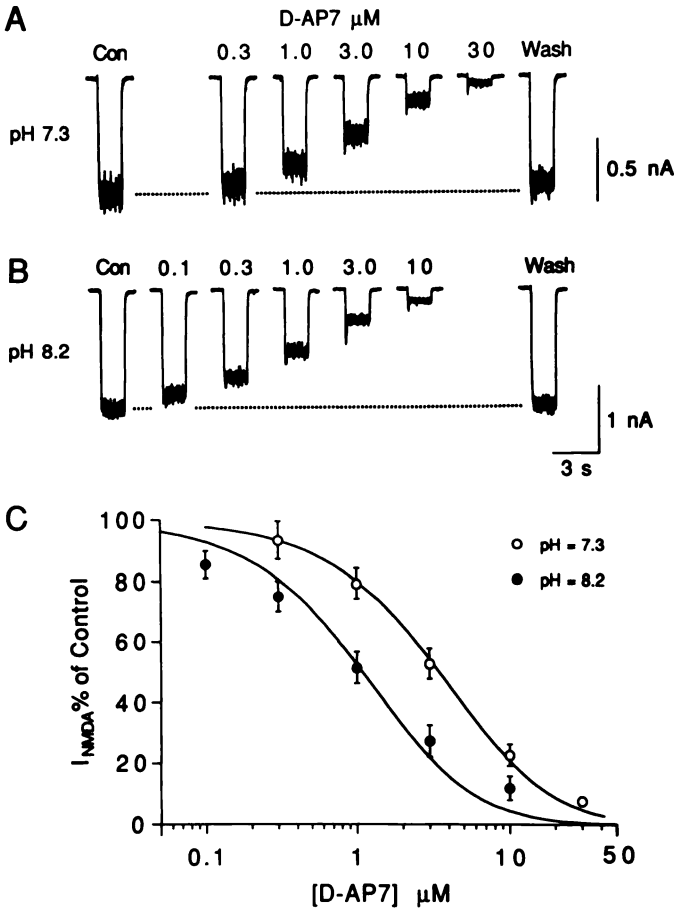


Fig. 2. pH-dependent action of the NMDA receptor competitive antagonist (2*R*)-AP7. A and B, Inward current responses to 10 μM NMDA applied simultaneously with the indicated concentrations of (2*R*)-AP7 at pH 7.3 (A) and pH 8.2 (B); 3 μM glycine was present continuously. Antagonist responses at pH 8.2 were approximately 3-fold more potent than those at pH 7.3; however, at equipotent doses the time course of responses was similar. C, Equilibrium dose-inhibition curves at pH 7.3 (○) and pH 8.2 (●). Smooth curves were fit by nonlinear regression using the equation for a two-binding site isotherm. K_o values were 8.38 μM at pH 7.3 and 2.63 μM at pH 8.2.

TABLE 1

pH dependence of equilibrium dissociation constants for NMDA receptor competitive antagonists

Measurements at each pH were made on different cells. K_i values were determined from K_o values and eq. 2, using a K_d for NMDA of 17.8 μM and 13.8 μM at pH 7.3 and 8.2, respectively.

Antagonist	K_i		
	pH 7.3	pH 8.2	pH 7.3/pH 8.2
	μM		
(2*R*)-AP7	5.56 ± 0.76	1.52 ± 0.30	3.66
LY 257883[a]	0.45 ± 0.05	0.14 ± 0.01	3.21
LY 221501[a]	1.34 ± 0.15	0.92 ± 0.10	1.46
SDZ EAB 515	0.14 ± 0.02	0.11 ± 0.01	1.27

[a] The compound was a racemic mixture of two optical isomers, only one of which is active. Values in the table have been corrected to reflect the concentration of the active optical isomer.

the dose-response curve for the piperidine ω-PO_3H_2-containing antagonist LY 257883 (9) on raising of the pH from 7.3 to 8.2, whereas over the same pH range dose-response curves for the ω-COOH-containing analog LY 221501 (9) nearly overlap. The K_i values for the ω-phosphonate LY 257883 were 0.45 ± 0.05 μM at pH 7.3 (five cells) and 0.14 ± 0.01 μM at pH 8.2 (six cells), whereas for the ω-COOH-containing analog LY 221501 the K_i values were 1.34 ± 0.15 μM at pH 7.3 (five cells) and 0.92 ± 0.10 μM at pH 8.2 (five cells). Thus, similar to AP7, LY 257883 is 3.2-fold more potent at pH 8.2 than at pH 7.3, whereas LY 221501 is <1.5-fold more potent at pH 8.2 than at pH 7.3 (Table 1). At pH 7.3 the equilibrium affinity of this pair of piperidine derivatives differs only 3-fold, whereas at pH 8.2 LY 257883 is 6.6-fold more potent than LY 221501 (Table 1).

The increase in equilibrium potency of ω-PO_3H_2-based antagonists observed on raising of the extracellular pH would be expected to reflect an increase in the apparent association rate constant for the binding of antagonists, due to an increase in the concentration of antagonist molecules with a fully ionized ω-phosphonate group. To test this hypothesis, we measured the kinetics of action of two of the aforementioned antagonists at pH 7.3 and 8.2. No analysis was performed for (2*R*)-AP7 at pH 8.2 because at high antagonist concentrations the association and dissociation rates are relatively fast, such that the predicted change in k_{on} would be difficult to measure accurately under the conditions of our experiments; in practice we did not analyze responses with time constants faster than 10 msec. In contrast, such measurements were relatively easy to obtain for conformationally restricted antagonists (e.g., Ref. 12). Fig. 4 shows examples of responses to the application of 3 μM LY 257883 and 15 μM LY 221501 at pH 7.3 and 8.2 in the presence of 10 μM NMDA; note the faster antagonist association kinetics at pH 8.2 for LY 257883 but not LY 221501. Because the amplitude of the steady state inhibition evoked by LY 257883 is smaller at pH 7.3 than at pH 8.2, for illustrative purposes the amplitude of the responses was normalized.

Analysis of such experiments, together with the response to removal of antagonists (12, 17), revealed that for LY 257883 the value for k_{on} increased significantly with a rise in extracellular pH ($p < 0.001$, paired t test), from 3.5 ± 0.99 × 10^6 M^{-1} sec^{-1} at pH 7.3 to 6.0 ± 2.65 × 10^6 M^{-1} sec^{-1} at pH 8.2 (Table 2). The concentration-independent value for k_{off} also showed a small but significant change with pH ($p < 0.001$, paired t test), decreasing from 0.83 ± 0.24 sec^{-1} at pH 7.3 (four cells, 27 observations) to 0.71 ± 0.2 sec^{-1} at pH 8.2 (five cells, 63 observations). The experimentally measured change in antagonist equilibrium potency (Table 1) is in reasonable agreement with that estimated from the ratio k_{off}/k_{on} (Table 2). Similar analysis was performed for the ω-COOH-piperidine antagonist LY 221501 (Table 2); however, despite a small increase in equilibrium potency on raising of the pH from 7.3 to 8.2, values for k_{on} and k_{off} showed no significant difference at pH 7.3 (five cells, 49 observations) and 8.2 (five cells, 53 observations; $p > 0.2$, paired t test).

SDZ EAB 515, the structure of which is depicted in Fig. 1, is a novel, potent, ω-PO_3H_2-containing NMDA receptor antagonist, the 2*S*-enantiomer of which is approximately 150 times more potent than the 2*R*-enantiomer (31). The biphenyl moiety in SDZ EAB 515 would be expected to restrict rotation of the ω-PO_3H_2 group relative to the α-NH_2 and α-COOH groups; the effect of such conformational restriction is likely to contribute to the high potency of SDZ EAB 515, compared with (2*R*)-AP7 (see Ref. 12). Possible additional effects of this substituent include an electron-withdrawing effect, which increases ionization of the neighboring ω-PO_3H_2 group, and hydrophobic interactions of the biphenyl moiety with NMDA receptors. Fig.

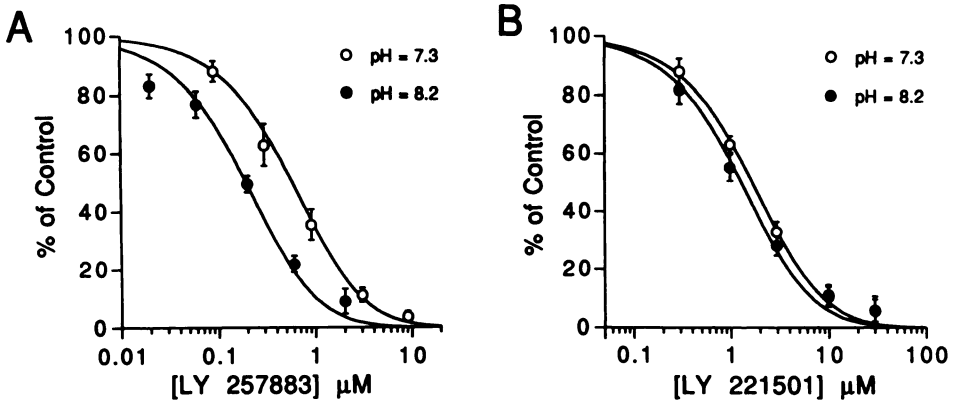


Fig. 3. The piperidine-ω-phosphonate antagonist LY 257883 is more sensitive to changes in extracellular pH than is its ω-COOH-containing analog LY 221501. A, Equilibrium dose-inhibition curves for block of responses to 10 μM NMDA in the presence of varying concentrations of LY 257883 at pH 7.3 (○) and pH 8.2 (●). A 3-fold increase in potency was observed on raising of the extracellular pH. Smooth curves were fit by nonlinear regression using the equation for a two-binding site isotherm. K_o values were 1.41 μM at pH 7.3 and 0.47 μM at pH 8.2. B, Equilibrium dose-inhibition curves for block of responses to 10 μM NMDA in the presence of varying concentrations of LY 221501 at pH 7.3 (○) and pH 8.2 (●). A 1.3-fold increase in potency was observed on raising of the extracellular pH. Smooth curves were fit by nonlinear regression using the equation for a two-binding site isotherm. K_o values were 4.11 μM at pH 7.3 and 3.16 μM at pH 8.2.

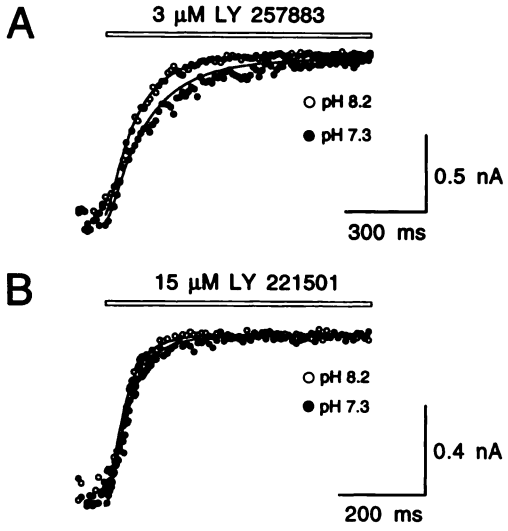


Fig. 4. Effects of extracellular pH on the kinetics of NMDA receptor block by LY 257883 and LY 221501. A, Kinetics of the decrease in inward current when 3 μM LY 257883 was applied by concentration jump in the presence of 10 μM NMDA. For illustrative purposes responses recorded at pH 7.3 (●) were normalized to those at pH 8.2 (○) by multiplying by 1.31. From analysis of these responses, together with the kinetics of the increase in current recorded after removal of LY 257883, the antagonist apparent association rate constants at pH 7.3 and pH 8.2 were calculated to be 4.70×10^6 M^{-1} sec^{-1} and 5.80×10^6 M^{-1} sec^{-1}, respectively, as estimated by least squares fits to a two-binding site model for competitive antagonism (*smooth line*). The antagonist apparent dissociation rate constants at pH 7.3 and pH 8.2 were calculated to be 0.94 sec^{-1} and 0.77 sec^{-1}, respectively. B, Similar responses recorded from the same cell during application of 15 μM LY 221501; the antagonist apparent association rate constants at pH 7.3 and pH 8.2 were calculated to be 2.70×10^6 M^{-1} sec^{-1} and 2.77×10^6 M^{-1} sec^{-1}, respectively, with apparent dissociation rate constants at pH 7.3 and pH 8.2 of 3.73 sec^{-1} and 3.57 sec^{-1}, respectively.

5A shows typical responses to the simultaneous application of 10 μM NMDA and varying concentrations of SDZ EAB 515. When NMDA was applied simultaneously with high concentrations of this antagonist, there was a transient inward current overshoot, consistent with slow equilibration kinetics for competitive antagonists with restricted conformational flexibility

TABLE 2

pH dependence of association and dissociation rate constants for NMDA receptor competitive antagonists

Paired measurements at each pH were made on single neurons. Antagonist k_{on} and k_{off} values were estimated by a two-equivalent site model for competitive antagonism, using NMDA association and dissociation rate constants of 2.08×10^6 M^{-1} sec^{-1} and 22.87 sec^{-1}, respectively, at pH 7.3 and 1.78×10^6 M^{-1} sec^{-1} and 25.48 sec^{-1}, respectively, at pH 8.2.

Compound	pH	k_{on}	k_{off}	k_{off}/k_{on}
		μM^{-1} sec^{-1}	sec^{-1}	μM
LY 257883	7.3	3.36 ± 0.99	0.83 ± 0.24	0.28 ± 0.17
(ω-PO_3H_2)[a]	8.2	5.99 ± 2.64	0.71 ± 0.20	0.13 ± 0.05
Ratio	*7.3/8.2*	*0.56*	*1.17*	*2.15*
LY 221501	7.3	7.30 ± 2.58	3.45 ± 0.26	0.53 ± 0.17
(ω-COOH)[a]	8.2	7.06 ± 1.94	3.45 ± 0.27	0.52 ± 0.14
Ratio	*7.3/8.2*	*1.03*	*1.00*	*1.02*
SDZ EAB 515	7.3	4.79 ± 1.45	0.34 ± 0.02	0.08 ± 0.02
	8.2	3.68 ± 0.87	0.27 ± 0.03	0.08 ± 0.02
Ratio	*7.3/8.2*	*1.30*	*1.26*	*1.00*

[a] The compound was a racemic mixture of two optical isomers, only one of which is active. Values in the table have been corrected to reflect the concentration of the active optical isomer.

(see Ref. 12). Equilibrium dose-response curves for SDZ EAB 515 at pH 7.3 and 8.2 overlap (Fig. 5B), and their analysis yielded K_i values of 0.14 ± 0.02 μM at pH 7.3 (six cells) and 0.11 ± 0.01 μM at pH 8.2 (seven cells). The biphenyl phosphonate compound SDZ EAB 515 showed a significant 1.3-fold decrease in k_{on} ($p < 0.05$, paired t test) on raising of the extracellular pH from 7.3 (four cells, 25 observations) to 8.2 (six cells, 47 observations) and a significant 1.3-fold decrease in k_{off} ($p < 0.001$, paired t test) over the same pH range. These values yield k_{off}/k_{on} ratios of 80 nM at both pH values, again in reasonable agreement with equilibrium estimates (Table 1).

Discussion

The results of this study suggest that the pH sensitivity of the binding of NMDA receptor competitive antagonists reflects changes in both antagonist and NMDA receptor structure. The pK_a for ionization of the ω-acidic group in individual antagonists and the stereochemical configuration of the α-COOH and α-NH_2 groups act as major determinants of whether antagonist potency varies with changes in pH close to the physiological

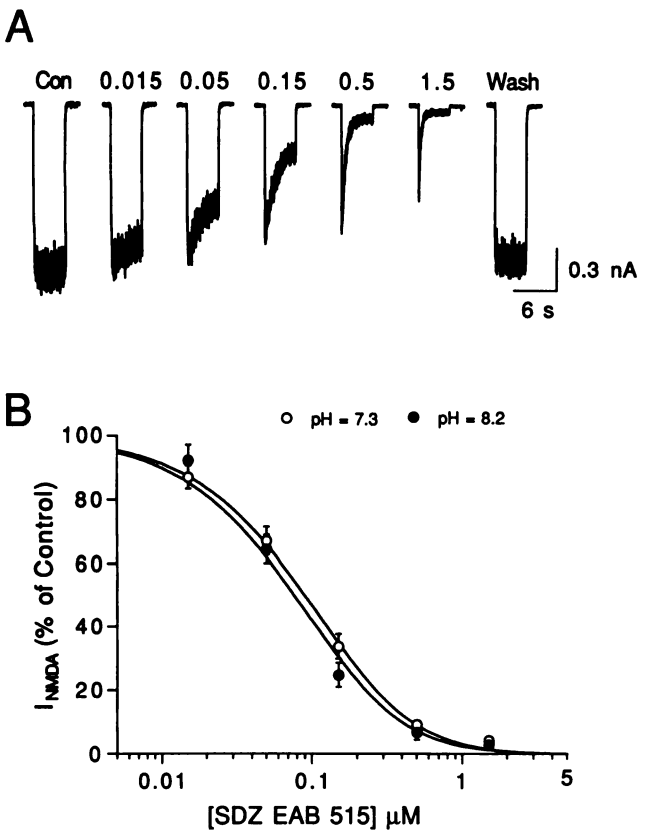


Fig. 5. The biphenyl-ω-phosphonate antagonist SDZ EAB 515 has similar potency at pH 7.3 and 8.2. A, Inward current responses to 10 μM NMDA applied simultaneously with the indicated concentrations of SDZ EAB 515 at pH 7.3. Similar responses were recorded at pH 8.2. B, Equilibrium dose-inhibition curves measured at pH 7.3 (○) and pH 8.2 (●). *Smooth curves* were fit by nonlinear regression using the equation for a two-binding site isotherm. K_o values were 0.22 μM at pH 7.3 and 0.18 μM at pH 8.2.

range. In addition, there appears to be a smaller component of pH sensitivity due to changes in ionization of functional groups within the NMDA receptor itself. Our results are consistent with the observations of Fagg and Baud (6) concerning the higher potency of ω-phosphonates (two negative charges) versus ω-phosphinates (one negative charge) as NMDA receptor antagonists and suggest that the fully ionized forms of the 2*R*-enantiomers of ω-phosphonate antagonists bind with much higher affinity to NMDA receptors than do the corresponding ionic species in which the ω-phosphonate group contains only a single negative charge.

Prediction of changes in NMDA receptor antagonist potency based on ionization of the ω-acidic group. Because the pK_a values for AP7 are known (12, 13), the Henderson-Hasselbalch equation can be used to calculate the relative concentration of individual ionic species present at a particular pH. At pH 7.3, 72% of AP7 molecules will have one negative charge on the ω-phosphonate group (denoted HPO_3^{1-} ionic species) and 28% will have two negative charges (denoted PO_3^{2-} ionic species). At pH 8.2, 25% of AP7 molecules will be of the HPO_3^{1-} ionic species and 75% will be of the PO_3^{2-} ionic species. Experiments were not performed above pH 8.2 because of the increasing contribution of the ionic species of AP7 in which the amino group is deprotonated (pK_a = 9.8) (13); at pH 8.2, the contribution of this form is only 1.7%. To determine which charged species produces high affinity antagonism at NMDA receptors, the change in potency over the range between pH 7.3 and 8.2 for (2*R*)-AP7 was compared with the change in concentration of the HPO_3^{1-} and PO_3^{2-} ionic species as calculated above.

If the HPO_3^{1-} ionic species is the only form of the antagonist that binds to the receptor with high affinity, then the potency of (2*R*)-AP7 should decrease by two thirds on raising of the extracellular pH from 7.3 to 8.2. In the second case, if the HPO_3^{1-} and PO_3^{2-} ionic species bind with similar relative affinities, then the potency of (2*R*)-AP7 should be approximately equivalent at pH 7.3 and 8.2. In the third case, if only the PO_3^{2-} ionic species binds with high affinity, then the potency of (2*R*)-AP7 should increase 2.7-fold on going from pH 7.3 to 8.2. Results summarized in Table 1 reveal a 3.7-fold increase in the potency of (2*R*)-AP7 on raising of the extracellular pH from 7.3 to 8.2, suggesting that the PO_3^{2-} ionic species of (2*R*)-AP7 binds with high affinity to NMDA receptors and that the HPO_3^{1-} ionic species binds with much lower affinity. The 3.2-fold increase in potency for the piperidine ω-phosphonate antagonist LY 257883 on raising of the extracellular pH from 7.3 to 8.2 also supports the hypothesis that the PO_3^{2-} form is the active ionic species.

Assuming the HPO_3^{1-} ionic species to be inactive, the maximum increase in potency for (2*R*)-AP7 over the range between pH 7.3 and 8.2, calculated from the Henderson-Hasselbalch equation using published pK_a values, is only 2.7-fold; this is less than the experimentally observed increase (Table 1). Furthermore, the ω-COOH-containing derivative LY 221501 shows a 1.5-fold increase in potency on raising of the extracellular pH from 7.3 to 8.2, even though its ω-COOH group has a pK_a value of <6 and should be completely ionized at either pH (Table 1). This suggests an additional effect of pH on the potency of NMDA receptor antagonists that does not result from a shift in the relative concentration of different ionized forms of antagonist molecules and may reflect titration of charged residues within the NMDA receptor itself. Although this is a small effect, the pH-sensitive 1.5-fold increase in potency observed for LY 221501 is very likely to be real; comparison of results obtained at pH 7.3 for AP7, LY 257883, and LY 221501 in the present series of experiments with those obtained previously (12) reveal K_i values that differ on average <1.2-fold, indicating a high degree of reliability for equilibrium measurements of antagonist potency.

To separate the effect of extracellular pH on antagonist ionization from the effect of pH on the NMDA receptor itself, the increase in potency observed for (2*R*)-AP7 on raising of the pH from 7.3 to 8.2 was corrected for the pH-sensitive increase in potency observed for the completely ionized ω-COOH-containing antagonist LY 221501. With this correction the potency increase for (2*R*)-AP7 is 2.5-fold. This value approximates the 2.7-fold increase in potency predicted by the Henderson-Hasselbalch equation, providing further evidence that the PO_3^{2-} ionic species of (2*R*)-AP7 is a considerably more potent NMDA receptor antagonist than the HPO_3^{1-} ionic species, although we cannot exclude the possibility that the HPO_3^{1-} ionic species could make a small contribution to overall potency of this antagonist.

Our hypothesis that the increase in equilibrium potency of AP7 and LY 257883 that occurs on raising of the pH from 7.3 to 8.2 is due to an increase in concentration of the active ionic

species predicts that the antagonist apparent association rate constant should also increase as the pH is raised. Although it is more difficult to make accurate measurements of rate constants because of their sensitivity to the model used for analysis, the results of our experiments utilizing a two-equivalent site model are in good agreement with values predicted from the equilibrium analysis of antagonist action. At physiological pH, the apparent association rate constant for the piperidine ω-COOH-containing antagonist LY 221501 is 2.2-fold higher than that of the ω-PO_3H_2-containing analog LY 257883 (Table 2). This difference decreases to 1.2-fold at pH 8.2 (Table 2) and, if we correct for the still incomplete ionization of the ω-PO_3H_2 group at pH 8.2, the k_{on} value for the fully ionized form of LY 257883 increases to $7.8 \pm 3.4 \times 10^6$ M^{-1} sec^{-1}, slightly greater than that for LY 221501. Previously, we have shown that estimates of k_{on} for other ω-PO_3H_2-containing antagonists, such as the piperidine AP5 derivative CGS 19755 and the bicyclic antagonist LY 235959, were also 3-fold slower than those for the corresponding ω-tetrazole-substituted analogs (12). Correction for incomplete ionization of the ω-phosphonate group (assuming a 2-fold increase in k_{on} on raising of the pH from 7.3 to 8.2 and only 70% ionization of the ω-phosphonate group at pH 8.2) increases the k_{on} values to 2.0×10^7 M^{-1} sec^{-1} for CGS 19755 and 3.1×10^6 M^{-1} sec^{-1} for LY 235959. These values are nearly identical to those obtained for the corresponding ω-tetrazoles, i.e., 2.0×10^7 M^{-1} sec^{-1} for LY 233053 and 3.4×10^6 M^{-1} sec^{-1} for LY 202157 (12). This analysis suggests that, once corrected for partial ionization, k_{on} values for these competitive antagonists are not strongly dependent on the chemical properties of the ω-substituted acidic moiety but do vary with the degree of conformational flexibility of the α- and ω-acidic groups, as shown previously (12).

At pH 8.2 the ω-PO_3H_2-containing antagonist LY 257883 is considerably more potent than its ω-COOH-containing analog LY 221501, primarily resulting from the 4.9-fold faster rate of dissociation of the ω-COOH-containing analog (Table 2). A pharmacophore recently developed for the NMDA receptor contains sites modeled as forming hydrogen bonds with each of the hydroxyl groups of ω-PO_3H_2-containing antagonists (32). We suggest that the higher affinity, more stable binding of LY 257883 results from interaction of both negative charges in the PO_3^{2-} ionic species with this pair of sites. In contrast, the ω-carboxylate has only a single negative charge, and the ketonic oxygen in the ω-COOH group would be expected to show weaker interaction with the binding site in the NMDA receptor for the ω-acidic group. In a similar manner, k_{off} values are an average of 3.8-fold faster for ω-tetrazole analogs (charge of −1) than for their ω-PO_3H_2-containing analogs (charge of −2), in agreement with the lower equilibrium potency of ω-tetrazole-containing analogs (12). Furthermore, if the rate of dissociation of ω-PO_3H_2-containing antagonists reflects a weighted average of the dissociation of low affinity HPO_3^{1-} ionic species and higher affinity PO_3^{2-} ionic species, then the decrease in k_{off} for LY 257883 at pH 8.2 in comparison with pH 7.3 might also reflect a higher proportion of PO_3^{2-} ionic species at pH 8.2.

Antagonist action of SDZ EAB 515. SDZ EAB 515, a potent derivative of AP7 containing a biphenyl substituent midway between the α- and ω-groups (31), shows unexpectedly weak pH sensitivity for an ω-PO_3H_2-containing antagonist. The biphenyl group in SDZ EAB 515 is electron withdrawing, such that pK_{a2} values for ionization of the ω-phosphonate group are reduced from 7.85 in AP7 (13, 14) to 7.4 in SDZ EAB 515.[2] The Henderson-Hasselbalch equation predicts that at pH 7.3 the HPO_3^{1-} and PO_3^{2-} ionic species of SDZ EAB 515 would represent 55 and 45% of the total population, respectively. At pH 8.2, the relative concentrations of the HPO_3^{1-} ionic species would be 12%, whereas that of the PO_3^{2-} ionic species would be 80%; it is reasonable to assume that at pH 8.2, based on a pK_a of 9.2 for the α-amino group in phenylalanine, there is an 8% contribution of a population of SDZ EAB 515 anions of valence −3, due to an additional deprotonation of the α-amino group. If the PO_3^{2-} ionic species is the only form of the antagonist that binds to the NMDA receptor with high affinity, then the potency of SDZ EAB 515 should increase by 78% on raising of the extracellular pH from 7.3 to 8.2. If only the HPO_3^{1-} ionic species binds with high affinity, then the potency of SDZ EAB 515 should decrease by 78% on going from pH 7.3 to 8.2. If the HPO_3^{1-} and PO_3^{2-} ionic species bind with similar affinities, then the potency of SDZ EAB 515 should decrease only slightly (8%) on going from pH 7.3 and 8.2, due to deprotonation of the α-amino group. Table 2 indicates that SDZ EAB 515 increases in potency by 21% on raising of the pH from 7.3 to 8.2. Recalling that a 1.5-fold increase in potency occurs over this pH range for the ω-COOH-containing derivative LY 221501, at least part of the pH-sensitive increase in potency for SDZ EAB 515 is likely to reflect titration of groups within the NMDA receptor.

Analysis of the action of SDZ EAB 515 highlights the difficulties encountered when comparing results from structurally unrelated compounds. The lower than expected pH sensitivity of SDZ EAB 515 might arise if stereochemical restrictions interfered with the correct orientation of the pair of negatively charged ω-phosphonate oxygen atoms that is required for the high affinity interaction of antagonists with binding sites in the NMDA receptor (32). The paradoxical enantiomeric selectivity of SDZ EAB 515, with the *S*-enantiomer being 150 times more potent than the *R*-enantiomer (31), is in contrast to results obtained with other antagonists, with the exception of decahydroisoquinoline derivatives (12, 33). Molecular modeling provides an explanation for unexpected enantiomeric selectivity of SDZ EAB 515, because in a low energy conformation that allows overlap of the αNH_2, α-COOH, and ω-PO_3H_2 hydroxyl groups with binding sites on the NMDA receptor pharmacophore (32) there is a collision of the biphenyl group with the receptor excluded volume in the 2*R*- but not 2*S*-enantiomer, consistent with the experimentally observed potency differences (31). However, results from molecular modeling are less informative concerning pH sensitivity of the action of the *S*-enantiomer of SDZ EAB 515, because there is good overlap of the α-NH_2, α-COOH, and both ω-PO_3H_2 hydroxyl groups with their corresponding receptor sites on a pharmacophore of the NMDA receptor (32). One explanation for the low pH sensitivity of the action of SDZ EAB 515 would be hydrophobic bonding to the receptor of the biphenyl substituent, which compensates for the difference in binding energy of the HPO_3^{1-} and PO_3^{2-} ionic species. The role of the biphenyl substituent in conferring high potency in SDZ EAB 515 through hydrophobic interactions is further supported by the observation of a 43-fold lower potency for the phenyl derivative of (2*S*)-AP7, (2*S*)-2-amino-3-(phosphonomethyl)benzenepropanoic acid (31). Further exploration of the mechanisms involved in the binding of antag-

[2] W. Müller and P. Herrling, personal communication.

onists to NMDA receptors will be required to provide a detailed molecular explanation for drug action at this site.

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Send reprint requests to: Mark L. Mayer, Building 36, Room 2A21, National Institutes of Health, Bethesda, MD 20892.