combined and used at the following final concentrations: AC4 hybridoma culture supernatant (monoclonal antibody recognizing SSEA-1), 1:1; antibody to SSEA-1, 1:200; polyclonal rabbit antibodies against TH and DBH (Eugene Tech International, Inc., Allendale, NJ), 1:60. Secondary antibodies (fluorescein- and rhodamine-conjugated affinity-purified goat antibodies to rabbit immunoglobulins and goat antibodies to mouse gamma globulins; Organon Teknika, Malvern, PA) were added together as well at a final concentration of 1:10.

28. I thank D. Noden for stimulating discussions; J. Dodd, T. Jessell, and D. Solter for gifts of AC4 and antibody to SSEA-1; J. Wright for technical assistance; C. Snyder and S. Tjepkema-Burrows for help with the illustrations; and D. Riley, F. Sieber, and M. Wong-Riley for critically reading the manuscript. Supported by USPHS grant HD21423 from the National Institute of Child Health and Human Development.

31 October 1988; accepted 31 December 1988

Indole-2-Carboxylic Acid: A Competitive Antagonist of Potentiation by Glycine at the NMDA Receptor

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The *N*-methyl-D-aspartate (NMDA) class of excitatory amino acid receptors regulates the strength and stability of excitatory synapses and appears to play a major role in excitotoxic neuronal death associated with stroke and epilepsy. The conductance increase gated by NMDA is potentiated by the amino acid glycine, which acts at an allosteric site tightly coupled to the NMDA receptor. Indole-2-carboxylic acid (I2CA) specifically and competitively inhibits the potentiation by glycine of NMDA-gated current. In solutions containing low levels of glycine, I2CA completely blocks the response to NMDA, suggesting that NMDA alone is not sufficient for channel activation. I2CA will be useful for defining the interaction of glycine with NMDA receptors and for determining the in vivo role of glycine in excitotoxicity and synapse stabilization.

VERTEBRATE CENTRAL NEURONS express two classes of excitatory amino acid receptors that mediate synaptic transmission throughout the brain and spinal cord (*1*). Fast transmission of single impulses involves mainly the kainate-quisqualate receptor class. A number of integrative phenomena, including long-term potentiation and eye-specific terminal segregation, require activation of the second receptor type (*2*), which is named for the selective agonist NMDA (*1*). Binding of transmitter to NMDA receptors activates ion channels that conduct monovalent cations and Ca^{2+} (*3*). Several additional factors regulate the conductance increase elicited by NMDA: (i) extracellular Mg^{2+} produces voltage-dependent block of the ion channel (*4*); (ii) Zn^{2+} inhibits channel opening by a different mechanism that is much less voltage-dependent (*5*); and (iii) the amino acid glycine (*6*) greatly increases the frequency of channel openings evoked by NMDA but does not open the channel when applied alone. The high affinity of the glycine potentiation site [half-maximal dose (EC_{50}) = 100 to 700 n*M*] has made it difficult to assess the role of glycine in vivo, because extracellular fluid is likely to contain a saturating concentration of glycine. Recent work (*7, 8*) on NMDA receptors expressed in *Xenopus* oocytes has raised the possibility that glycine may be absolutely required for NMDA-gated channels to open; the small current observed in the absence of added glycine could be due to nanomolar levels of contaminating glycine typically found in physiological solutions (*6, 7*). Interest in these problems has sparked a search for antagonists of the glycine potentiation site (*9*). I report that I2CA (Fig. 1) and several active derivatives competitively inhibit the action of glycine on the NMDA receptor.

Current elicited by NMDA was recorded in primary cultures of the rat visual cortex or spinal cord (*10*) with the whole-cell configuration of the patch-clamp technique (*11, 12*). In agreement with earlier work (*6–8*), glycine produced a dose-dependent increase in whole-cell current evoked by NMDA (Fig. 2). At −70 mV, NMDA activated only a few picoamperes of current in the absence of added glycine (see below); saturating concentrations of glycine potentiated the response to NMDA by 5 to 100 times. The dose-response relation for glycine can be fitted well by the equation for one-to-one binding (Fig. 2C) (*7, 8*), which suggests that the physiological response to glycine is directly proportional to receptor occupancy. I2CA and several active derivatives competitively antagonized the potentiation by glycine, causing a shift in the dose-response relation to higher glycine concentrations

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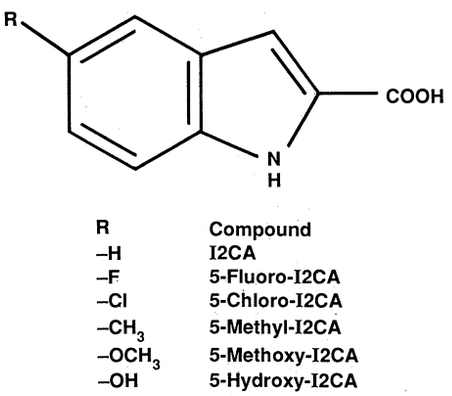

R	Compound
–H	I2CA
–F	5-Fluoro-I2CA
–Cl	5-Chloro-I2CA
$–CH_3$	5-Methyl-I2CA
$–OCH_3$	5-Methoxy-I2CA
–OH	5-Hydroxy-I2CA

Fig. 1. Chemical structures of I2CA and five substituted derivatives of I2CA.

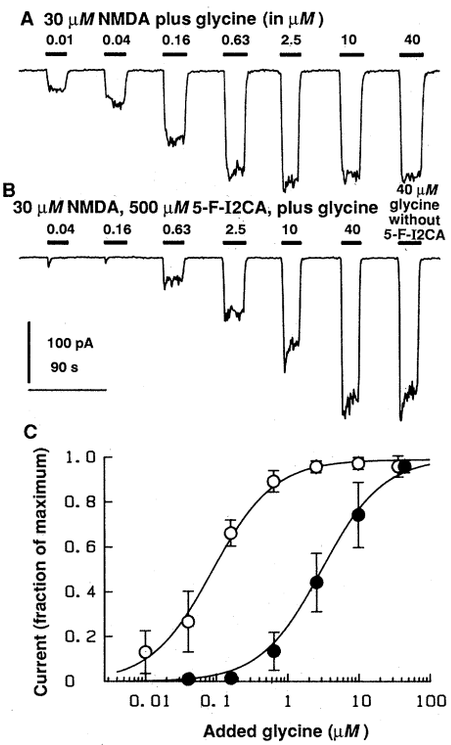

Fig. 2. The potentiation by glycine of current gated by NMDA is inhibited by 5-F-I2CA. (**A**) Whole-cell recording of current activated by 30 μ*M* NMDA plus 10 n*M* to 40 μ*M* added glycine; holding potential, −70 mV. (**B**) In the same cell, current activated by 30 μ*M* NMDA plus 40 n*M* to 40 μ*M* glycine in the presence of 500 μ*M* 5-F-I2CA and by 30 μ*M* NMDA plus 40 μ*M* glycine without 5-F-I2CA. Cortical neuron 8G19A from a P 5 donor, 6 days in vitro. (**C**) Dose-response relation for glycine in three neurons in the absence (○) or presence (●) of 500 μ*M* 5-F-I2CA; 30 μ*M* NMDA was included in each test solution. Points show mean ± SD of the current averaged over the last 10 to 20 s of each application, plotted as a fraction of the maximal current. The full dose-response relation for glycine alone and for glycine plus 5-F-I2CA was measured in three cortical neurons a total of eight times. The curves are least-squares fits of the Langmuir equation (*13*), with EC_{50} = 89 n*M* (○) or 3 μ*M* (●). The dose-response relation for glycine was also shifted to the right by I2CA, 5-Cl-I2CA, 5-methyl-I2CA, and 5-methoxy-I2CA.

(Fig. 2, B and C). The inhibition constant (K_i) of 5-fluoro-I2CA (5-F-I2CA) can be obtained from the shift in the EC_{50} for glycine (*13*); the results in Fig. 2 indicate a K_i of 15 μM.

Five derivatives of I2CA (Fig. 1) were tested for activity at the glycine site (*14*). The dose dependence of antagonism can be fitted fairly well by a one-to-one binding curve (Fig. 3). The IC_{50} values presented in Table 1 indicate significantly higher affinities for derivatives with fluorine or chlorine in the 5 position than for native I2CA or for 5-methyl-I2CA; the 5-methoxy derivative has much lower affinity and 5-hydroxy-I2CA is without effect at concentrations up to 1 mM (*15*). A number of other related compounds, pyrrole-2-carboxylic acid, indoline-2-carboxylic acid, quinaldic acid, picolinic acid, quinoxaline-2-carboxylic acid, and D,L-pipecolic acid, were tested and had no effect (at 1 mM).

The active derivatives of I2CA are specific for the glycine potentiation site on the NMDA receptor. At 1 mM, there is no effect on Cl^- current elicited in spinal cord neurons by activation of strychnine-sensitive glycine receptors (*16*) and only minimal antagonism at non-NMDA receptors (*17*). Furthermore, I2CA does not interact with the NMDA binding site. The dose-response relation for NMDA with a high level of glycine (50 μM) was not affected by the addition of 1 mM 5-F-I2CA (Fig. 4A). Decreasing the amount of added glycine to 300 nM reduced the maximum response to NMDA but did not change the level of NMDA required for half-maximal activation (Fig. 4A). This result confirms previous reports (*6–8*) and strongly suggests that glycine acts by directly increasing the probability of channel opening and not by changing the receptor's affinity for NMDA.

Kleckner and Dingledine (*7*) have proposed that glycine is absolutely required for channel activation by NMDA, on the basis of studies in *Xenopus* oocytes injected with rat brain mRNA. My results indicate that glycine is also absolutely required when the channels are expressed by central nervous system neurons. In the presence of either zero or 300 nM added glycine, 5-F-I2CA (1 mM) completely blocked the current evoked by all concentrations of NMDA (Fig. 4, A

Table 1. Inhibition of whole-cell current evoked by 30 μM NMDA plus 300 nM added glycine. Dose-response experiments were performed as in Fig. 3. IC_{50} values, presented as mean ± SEM of determinations, were obtained from the least-squares fit of $1/(1 + [drug]/IC_{50})$ to each set of six drug concentrations (see Fig. 3, inset). K_i values were estimated from the mean IC_{50} values on the basis of the Cheng-Prusoff equation (*19*); N.D., not determined.

Compound	IC_{50} (μM)	Estimated K_i (μM)	n
I2CA	105 ± 12	24	7
5-F-I2CA	61 ± 10*	14	8
5-Cl-I2CA	43 ± 5*	10	8
5-Methyl-I2CA	114 ± 19	26	7
5-Methoxy-I2CA	>1000	N.D.	6

*Differences among the means (excluding 5-methoxy-I2CA) are significant at $P < 0.01$ (analysis of variance, $F_{statistic}$). Confidence intervals for the difference between means indicate that means for 5-F-I2CA and 5-Cl-I2CA are different from that of I2CA at $P < 0.05$ and $P < 0.01$, respectively.

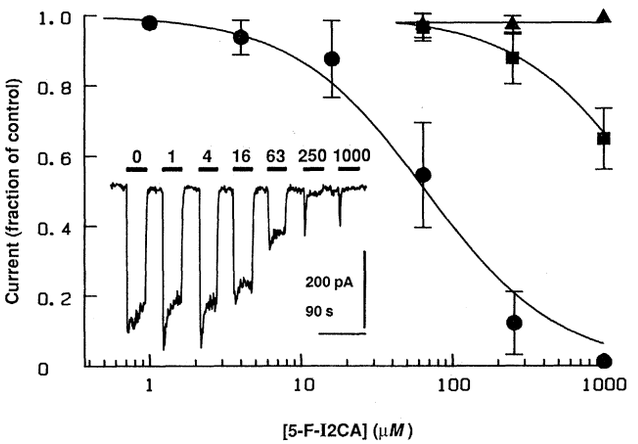

Fig. 3. Dose-response relations for 5-F-I2CA (●), 5-methoxy-I2CA (■), and 5-hydroxy-I2CA (▲). Whole-cell current evoked by 30 μM NMDA plus 300 nM glycine and various concentrations of each I2CA derivative is plotted as a fraction of the current obtained without antagonist. Inset shows a sample record for 5-F-I2CA (concentrations in micromolar units). Cortical neuron 8G21B, P 3 donor, 6 days in vitro; holding potential, −70 mV. Points show mean ± SD of seven applications in two cells (●), six applications in two cells (■), or five applications in two cells (▲). Smooth curves are least squares fits to the mean points of $1/(1 + [drug]/IC_{50})$, with IC_{50} = 67 μM (●), 2 mM (■), or >0.1M (▲).

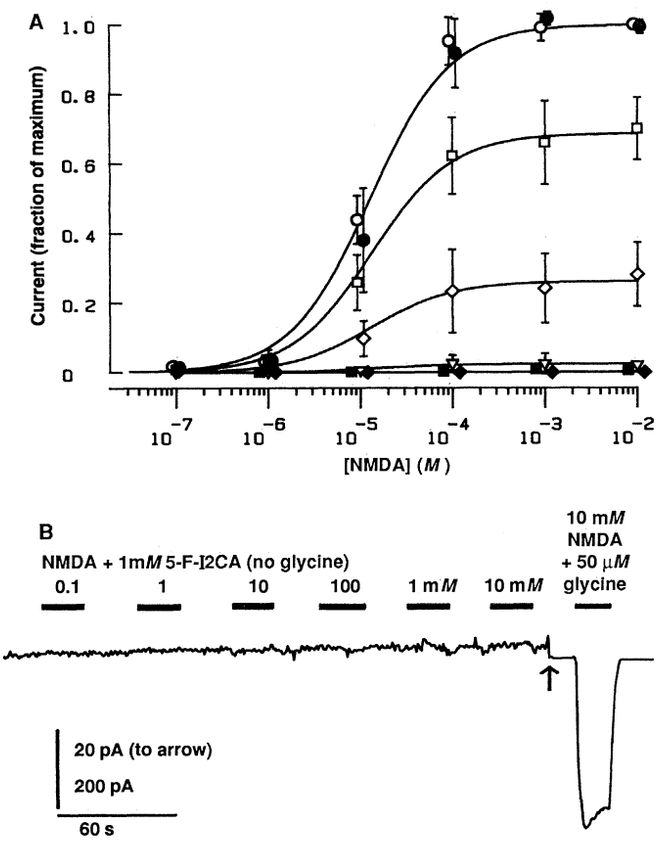

Fig. 4. Dose-response relations for NMDA. (**A**) The current (mean ± SD) obtained for each test solution as a fraction of the current evoked in the same cell by 10 mM NMDA plus 50 μM glycine (without 5-F-I2CA). Circles represent neurons in culture for 4 days tested with NMDA plus 50 μM glycine without (○, eight applications in two cells) or with (●, eight applications in two cells) 1 mM 5-F-I2CA. Squares represent neurons in culture for 5 days tested with NMDA plus 300 nM glycine without (□, eight applications in two cells) or with (■, nine applications in two cells) 1 mM 5-F-I2CA. Diamonds represent neurons in culture for 12 days tested with NMDA lacking added glycine without (◇, nine applications in three cells) or with (◆, eight applications in four cells) 1 mM 5-F-I2CA. Inverted triangles (▽) show data for two neurons in culture for 5 days tested with NMDA lacking added glycine (without 5-F-I2CA, five applications). Smooth curves are the best fit to the Langmuir equation (*13*), with EC_{50} = 13 μM (○) or with EC_{50} = 13 μM and maximum current (I_{max}) = 69% (□), 26% (◇), 2.5% (▽), or 0.6% (■, ◆) of I_{max} for 50 μM glycine. (**B**) 5-F-I2CA (1 mM) completely blocked the response to NMDA (concentration in micromolar units) without added glycine. Note the change in gain made at the arrow. Neuron 8S08K from a P 3 donor after 12 days in vitro.

and B), which suggests that NMDA alone is not sufficient to open the channel. Indeed, at early times in culture, NMDA elicited very little current unless glycine was added (Fig. 4A). By 6 to 10 days in vitro, however, the response to rapid perfusion of NMDA in solutions lacking glycine was often a substantial fraction of the maximal current obtained with glycine added (Fig. 4A). This change over time in culture probably reflects the continual release of glycine by glial cells (*6*) that proliferate to confluence during the first week in vitro. Johnson and Ascher (*6*) have shown that medium conditioned by cultures contains significant levels of glycine; rapid perfusion apparently can reduce but not eliminate this contamination.

These experiments demonstrate that I2CA selectively and competitively inhibits the potentiating action of glycine on current elicited by NMDA. Together with recent results on another antagonist, 7-chlorokynurenic acid (*9*), they strengthen the proposal (*7*) that glycine is essential for channel activation (although it cannot be ruled out that I2CA may act as an inverse agonist at the glycine site). A number of studies have implicated NMDA receptors in the control of synaptic plasticity (*2*) and in excitotoxic cell death (*18*). Investigations of antagonists of the glycine potentiation site, such as I2CA, should lead to a better understanding of glycine's role in these processes; these antagonists may have therapeutic value for the treatment of stroke, epilepsy, and other neurodegenerative disorders (*18*).

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12. Neurons were bathed in a continuous stream of control or agonist-containing external solution flowing from one of a linear array of microcapillary tubes. A complete change of solution could be achieved within a few hundred milliseconds by moving the array of tubes relative to the cell. The external solution contained 160 m*M* NaCl, 10 m*M* Hepes (*p*H 7.4), 1 m*M* EDTA, 3 m*M* $CaCl_2$, and 300 n*M* tetrodotoxin. Pipettes were filled with 120 m*M* cesium methanesulfonate, 5 m*M* CsCl, 10 m*M* Cs_2EGTA, 10 m*M* Hepes (*p*H adjusted to 7.4 with CsOH), 5 m*M* MgATP, and 1 m*M* Na_2GTP (ATP, adenosine triphosphate; GTP, guanosine triphosphate).
13. Dose-response relations were fitted with the Langmuir equation [H. P. Rang, *Nature* **231**, 91 (1971)]:

$$I/I_{max} = 1/(1 + EC_{50}/[glycine])$$

A competitive inhibitor shifts the EC_{50} for agonist by a factor of

$$(1 + [inhibitor]/K_i)$$

14. I2CA and its derivatives were obtained from Aldrich.
15. The differences in potency among the six compounds may be due to direct interaction of the substituent group with the receptor or could result from the tendency of the substituent to release or withdraw electrons, which would affect the stability of structures in which nitrogen forms four bonds and bears a positive charge. Further experiments with other derivatives of I2CA should resolve these two possibilities.
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17. Dose-response relations for kainate, quisqualate, and glutamate [plus 30 μM D(−)2-amino-5-phosphonovaleric acid and 2 m*M* $MgCl_2$] shifted to the right in the presence of 5-F-I2CA. The shifts in EC_{50} values indicate K_i values for 5-F-I2CA of 609 μM versus kainate, 700 μM versus quisqualate, and 926 μM versus glutamate.
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19. The Cheng-Prusoff equation [Y. C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.* **22**, 3099 (1973)] relates the K_i to the concentration of inhibitor producing 50% inhibition (IC_{50}) in the presence of agonist (with dissociation constant K_d): $K_i = IC_{50}/(1 + [glycine]/K_d)$. In Table 1, K_i values were calculated with 300 n*M* for the glycine concentration and the EC_{50} from Fig. 2 (89 n*M*) used as an estimate for the glycine K_d. Although both of these values are somewhat uncertain because of the possibility of contamination by nanomolar levels of glycine, the K_i of 14 μM obtained from the IC_{50} for 5-F-I2CA agrees very well with the value of 15 μM calculated from the shift in the dose-response relation for glycine.

20. I am grateful to B. Bean for advice, encouragement, and support and to L. Regan and D. Sah for helpful comments on the manuscript. Supported by the Harvard University Society of Fellows and the Esther A. and Joseph Klingenstein Fund and by grants to B. P. Bean from the National Institutes of Health (HL-35034) and the Rita Allen Foundation.

3 October 1988; accepted 19 December 1988

Technical Comments

Asymmetries of Clade Shape and the Direction of Evolutionary Time

We argued recently (*1*) that an "arrow of time" could be demonstrated by characteristic differences between early and late arising clades in the fossil record. Early arising clades tend to be more bottom heavy than late arising clades, in the sense that they tend to diversify more rapidly after their origin. Jennifer A. Kitchell and Norman MacLeod (*2*) criticize our thesis that asymmetries of clade shape are useful for inferring temporal directionality. Their challenge is based principally on their statement that "neither the early arising (Cambro-Ordovician) nor the later arising clades have an asymmetry that is distinguishable from clades produced by a random branching process." Their objection is invalid for several reasons.

First, as Kitchell and MacLeod acknowledge, our criterion for establishing directionality hinges on the following question: "If you were handed a chart of clade diversity diagrams with unlabeled axes, would you know whether you were holding the chart upside down or right side up?" (*1, 2*). We held that an affirmative answer would be a sufficient demonstration of temporal directionality. As our question was clearly meant to be answered using real *empirical* data, it is inappropriate (and also wrong in this case) to criticize our thesis by comparing our results to those of a *theoretical* model. The relevant comparisons are between early and late arising fossil clades. The regression analyses we presented, which were not mentioned by Kitchell and MacLeod, clearly show that early arising clades are significantly more bottom heavy than those arising later (*3*). New comparisons of mean centers of gravity (*CG*) of Cambro-Ordovician and later arising clades in which standard statistical tests were used also show early arising clades to be significantly more bottom heavy ($P = 0.013$, *t* test; $P = 0.017$, Mann-Whitney *U* test) (*4*).

However, even if a comparison of our empirical data to the results of their theoretical model were relevant, Kitchell and MacLeod's own analysis would have proved their contention wrong if they had performed the correct statistical test. They found that the mean *CG* determined using their model was 0.503 and that the 95%