Benzimidazole-type Glycine Antagonists: The Role of the Ring Nitrogen Atoms☆

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Summary

Several derivatives of 1*H*-benzimidazole-2-carboxylic acid (BICA, **2a**) were tested *in vitro* in comparison to 1*H*-indole-2-carboxylic acid (ICA, **1e**) for their ability to displace [^{3}H]glycine from rat hippocampal membranes. Compound **2a** was 8 times more potent than **1e** (K_i 5.3 μM, as compared to 42 μM). However, introduction of a carboxymethyl group or a corresponding ester at position 3 had no positive effect on the potency of **2a**, while this type of structural modification increased the potency of **1e** significantly. Nevertheless, 1-carboxymethyl-BICA (**2b**) displaced [^{3}H]glycine with similar potency as the corresponding 3-carboxymethyl-ICA **1c**, indicating that also a nitrogen atom lacking a hydrogen atom can be engaged in glycine receptor interaction. *N*-Methylation strongly reduced the potencies of both BICA and ICA derivatives.

Introduction

Binding studies indicate that *1H*-benzimidazole-2-carboxylic acid (BICA, **2a**) is a selective antagonist of the NMDA receptor complex associated glycine binding site[10, 12]. The compound is the aza-bioisoster of *1H*-indole-2-carboxylic acid (ICA, **1e**), which has been introduced in an electrophysiological study by Huettner[7] as a selective antagonist of the NMDA receptor associated glycine site (with moderate potency, K_i = 24 μM). Subsequent attempts to use ICA (**1e**) as an inhibitor of [^{3}H]glycine binding to neuronal membranes have not been successful (IC_{50} > 100 μM[5,6,8]). Nevertheless, compound **1e** has become the lead structure of a series of potent indole type glycine antagonists, most of them – apart from halogen substituents at the phenyl nucleus – bearing a side chain at position 3 (for a recent review, see Leeson and Iversen[11]). Bearing in mind that **2a** is more potent than **1e**, it is surprising that the effect of introducing a side chain into **2a** has not been studied until now. Even with indole compounds there is no information available on the role of the ring nitrogen, with the exception of one study[5] mentioning the *N*-methylated compound **3c** as inactive (K_i > 100 μM).

In ICA (**1e**), the free electron pair of the nitrogen atom participates in the aromatic electron decet of the ring system, whereas in BICA (**2a**) the electron pair of one of the two ring nitrogens is not engaged in such an interaction. Although this electron pair, in principle, is prone to protonization, **2a** at physiological pH does not bear a positive charge[16]. To clarify the significance of these ring nitrogens for the interaction with the glycine binding site, the potencies and selectivities of a series of non-halogenated 1*H*-indole- and 1*H*-benzimidazole-2-carboxylic acids with varying substituents in the 1- and the 3 positions were compared to each other.

Neuronal membranes prepared from the CA1- and dentate gyrus part of rat hippocampi were used for [^{3}H]glycine binding assays, since the NMDA receptor complex is abundant in that region. Rigorous control for background glycine increased the sensitivity of our [^{3}H]glycine binding assay. It allowed reliable quantification of still considerable weaker inhibition constants (up to K_i = 300 μM)[1] and provided the basis for a quantitative comparison of the potencies.

Results and Discussion

Table 1 presents the K_i-values of all tested compounds for the inhibition of [^{3}H]glycine, [^{3}H]MK-801, and [^{3}H]glutamate binding. At 400 μM, none of the tested substances inhibited the specific binding of [^{3}H]kainic acid or of [^{3}H]AMPA by more than 30%.

As a first result, BICA (**2a**) turned out to be 8 times more potent than the isosteric compound **1e** (ICA) in displacing [^{3}H]glycine and [^{3}H]MK-801. The higher potency of the benzimidazole compound in comparison to the indole compound may have the following reasons:

1. The N-H bond at position 1 is more polarized in **2a** than in **1e**, which is indicated by a lower pK_a-value for the equilibrium $R_2NH = R_2N^- + H^+$ (**1e**: 17.0[18]; **2a**: 13.1[16]).
2. The second, hydrogen-free nitrogen in **2a** is weakly basic (pK_a for the equilibrium $R_2NH^+ = R_2N + H^+$ is 0.4[16]) and might be more appropriate for receptor interaction than the hydrogen-bearing, neutral nitrogen at position 1.
3. The second nitrogen in **2a** might provide an additional site for receptor interaction.

Our data allow the second possibility to be ruled out, since BICA **2a** was practically inactivated by *N*-methylation (compound **2d**). This inactivation was not due to steric hindrance

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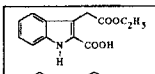
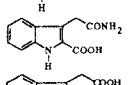
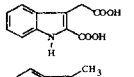
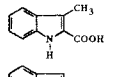
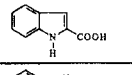
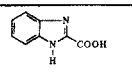
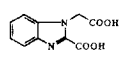
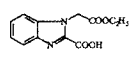
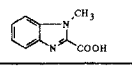
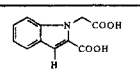
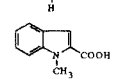
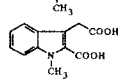
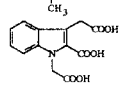
Table 1. Structures of indole and benzimidazole derivatives and their inhibitory effect on the specific binding of [^{3}H]gly (10 nM), [^{3}H]MK-801 (5 nM), and [^{3}H]glu (10 nM, NMDA sensitive); K_i (μM; mean ± S.D.), or % change in presence of 400 μM compound, number of experiments in parentheses.

	Structure	[^{3}H]gly	[^{3}H]MK-801[a]	[^{3}H]glu (NMDA)
1a		2.62 ± 0.62 (8)	3.03 ± 0.74 (3)	+ 2.0 % (2)
1b		6.53 ± 1.40 (6)	4.76 ± 1.39 (4)	- 46.7 % (3)
1c		7.43 ± 1.76 (9)	7.0 ± 3.0 (3)	20.4 ± 7.5 (4)
1d		31.9 ± 3.80 (3)	n.d.	- 3.3 % (3)
1e		42.0 ± 5.80 (5)	46 ± 13 (3)	- 22.4 % (3)
2a		5.30 ± 1.75 (5)	6.92 ± 1.90 (6)	- 53.4 % (1)
2b		10.0 ± 1.13 (5)	9.8 ± 7.7 (5)	22.5 ± 8.4 (3)
2c		274 ± 48 (3)	n.d.	- 36.0 % (3)
2d		- 33.3 % (2)	n.d.	- 4.3 % (2)
3a		- 27.6 % (2)	n.d.	- 49.3 % (3)
3b		- 18.0 % (2)	n.d.	- 6.0 % (2)
3c		- 3.9 % (2)	n.d.	- 30.3 % (3)
3d		- 28.0 % (1)	n.d.	- 50.0 % (3)

[a] 10 μM glutamate, background glycine 12–180 nM; n.d., not determined.

by the introduced methyl group, since the methylated reference compound **1d**, which bears a hydrogen atom at the indole nitrogen atom, was even slightly more potent than ICA (**1e**) itself ($P < 0.05$). [*N*-Methylation of ICA (**1e**) also resulted in a practically inactive compound (**3b**)].

Concerning the third possibility, detailed structure activity relationship studies have revealed a substantial increase in potency of indole-type glycine antagonists bearing electron pair donating substituents in position 3[5,6,14], thus pointing to a discrete receptor domain particularly amenable for interaction with hydrogen acceptors (for a review: see Kemp and Leeson[9]); similar observations have been made with quinoline-type antagonists[11]. The two main interaction sites reflect the geometry of glycine itself, with site one interacting with the carboxyl group and site two with the amino group. Our data could be interpreted in such a way that the electron donating element, which leads to the increase in potency by interaction with domain three, might not only be introduced into 1*H*-indole-2-carboxylic acid (**1e**) via suitable substituents in position 3 (yielding **1a**, **1b**, **1c**), but also by isosteric replacement of the carbon atom in position 3 of the five membered ring by an electron pair donating nitrogen atom (as in **2a**). Additionally or alternatively, a more pronounced polarization of the N–H bond might also play a role in the higher potency of BICA (**2a**) versus its homologous indole derivative ICA (**1e**).

As already mentioned above, the potency of BICA (**2a**) as a glycine antagonist was eliminated by *N*-methylation (compound **2d**). In principle, this *N*-methylated benzimidazole could present its geometrically equivalent second nitrogen atom to binding domain two, thereby circumventing steric hindrance by the methyl group. (Steric requirements at this site are demonstrated by inactivity of *N*-methylated compounds **3b** and **3c**.) From the inactivity of compound **2d** we concluded that receptor domain two did not accept a nitrogen atom with a free electron pair as a substitute for the hydrogen-bearing nitrogen atom. Thus, receptor domain two could be regarded as hydrogen acceptor. However, to our surprise, compound **2b** inhibited [^{3}H]glycine and [^{3}H]MK-801 binding almost as potently as BICA (**2a**) and **1c**. (Concerning inhibition of [^{3}H]glycine binding, the small difference in potency between **2b** on one side and **2a** and **1c** on the other side was statistically significant: $p < 0.05$.) In addition, both dicarboxylic acids turned out as moderately potent inhibitors of NMDA-sensitive [^{3}H]glutamate binding, with similar potencies (see Table 1), although the nature of their nitrogen atoms interacting with site two of the receptor differed greatly. This result may be explained assuming that the potency of compounds **1c** and **2b** was caused mainly by the two carboxy groups. One might even be tempted to assume that the free nitrogen atom in compound **2b** was not involved in any receptor interaction at all. However, the importance of the presence of a nitrogen atom is illustrated by the inactivity of compound **3a**, where the hydrogen-free nitrogen atom of **2b** has been replaced by an sp^2-hybridized carbon atom; (**3a** is an isomer of **1c** and an isoster of **2b**). The fact that in a benzimidazole type ligand a nitrogen atom lacking a hydrogen does in one case (**2b**) contribute crucially to its potency, but in another case (**2d**) does not (or only very weakly), suggests that the hydrogen-free nitrogen atom can only contribute as an additional attachment point, once two robust contacts have been formed, but is unable to serve as a primary recognition site, a role, which only a hydrogen-bearing nitrogen atom can fulfil.

The interpretation of relating the potency of compounds **1c** and **2b** mainly to the two carboxy groups serving as primary interaction sites is dramatically illustrated by the consequence of esterification of one of the carboxy groups of 2-carboxy-1*H*-benzimidazole-1-acetic acid **2b**: Compound **2c** turned out to be a very weak glycine antagonist, much to our disappointment, since corresponding esterification of the indole type analogue **1c** yielded the most potent compound in this series (**1a**). We interpret this discrepancy as a further indication for the subordinate role of the hydrogen-free nitrogen atom as suggested above.

In conclusion, careful control for background glycine allowed, for the first time, a detailed and quantitative study of structure activity relationships of non-halogenated indole and benzimidazole type inhibitors of [^{3}H]glycine binding. The simplest molecule with high potency and selectivity was BICA (**2a**). In contrast to indole compounds, its potency was not increased by side chain introduction. Nevertheless, the carboxymethyl derivative of **2a** represents a novel type of glycine antagonist, with a hydrogen-free nitrogen atom engaged in receptor interaction.

Acknowledgement

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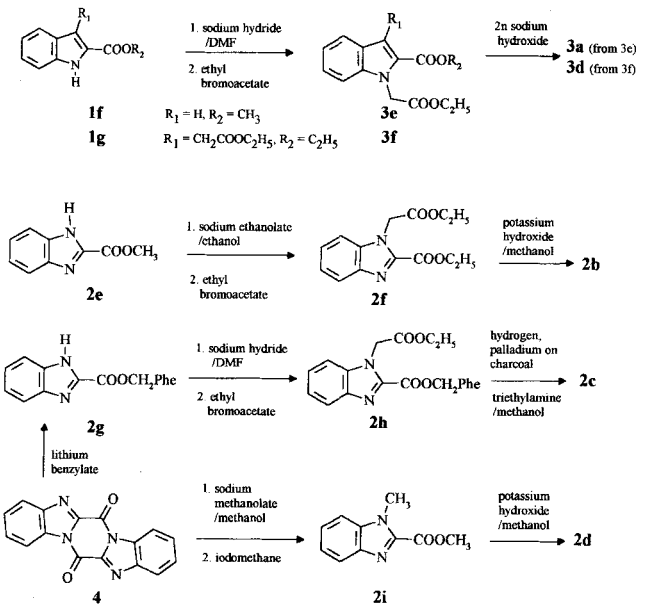
Experimental

Equipment and Chemicals

Vacuum flash chromatography was performed on 0.04–0.06 mm silica gel (Merck). Petroleum ether, ether, dichloromethane, and ethyl acetate (over sodium hydrogen carbonate) were distilled before use. DMF was dried over a molecular sieve (3Å), THF and dioxane were refluxed over sodium and distilled. Melting points were measured on a Kofler apparatus and are uncorrected. 200 MHz ^{1}H NMR- and 50 MHz ^{13}C NMR-spectra were recorded on a Bruker 200 FS FT-NMR spectrometer; chemical shifts were reported in parts per million relative to tetramethylsilane or sodium trimethylsilylpropanesulfonate. (*RS*)-α-Amino-3-hydroxy-5-methylisoxazole-4-propanoic acid (AMPA), quisqualic acid, 5,7-dichlorokynurenic acid (DCKA), and 2-D-amino-5-phosphonovaleric acid (D-APV) were obtained from Tocris & Cookson, kainic acid and NMDA from Sigma, D-serine from Merck, and compound **1e** (1*H*-indole-2-carboxylic acid, ICA) from Fluka. All other tested compounds were synthesized. Chemicals for syntheses were used in standard commercial quality.

Syntheses

Compounds **1a**[13], **1c**[13], **1d**[17], and **2a**[16] were prepared as described in the literature. The syntheses of compounds **1b**, **2d**, **3a**, **3b**, **3c**, and **3d** have also been previously described, but alternative, more convenient routes were used. Compound **1b** was synthesized by refluxing compound **1c** in acetic anhydride. The resulting cyclic anhydride was opened with ammonia in dioxane (for experimental details see[15]). Products **2b** and **3a–3d** were synthesized by an *N*-alkylation sequence starting from BICA methyl ester (**2e**), ICA methyl ester (**1f**), and 2-carboxy-1*H*-indole-3-acetic acid diethyl ester (**1g**), respectively. The educt esters were deprotonated by sodium hydride/DMF or by sodium ethanolate/ethanol. Addition of ethyl bromoacetate and subsequent hydrolysis by 2N sodium hydroxide in 50 % aqueous methanol resulted in the *N*-carboxylated compounds **3a** and **3d** (see Scheme 1). Replacing ethyl bromoacetate by iodomethane yielded the *N*-methylated compounds **3b** and **3c** (not illustrated). Due to the instability of the free 2-carboxy groups of *N*-substituted 1*H*-benzimidazole derivatives, compounds **2b**, **2c**, and **2d** were isolated as their salts (see Scheme 1). It was verified that the counter ions at the concentrations used did not influence



Scheme 1

[^{3}H]glycine binding (not shown). The two latter compounds (**2c** and **2d**) were not prepared from a BICA ester, but from the diamide dimer of BICA[4] (6*H*,13*H*-dibenzimidazo[1,2-*a*,1′,2′-*d*]pyrazine-6,13-dione, compound **4**, see Scheme 1). Since the preparation of compounds **2b** and **2c** has not been described before, the detailed description of their synthesis is given as follows:

2-(Ethoxycarbonyl)-1H-benzimidazole-1-acetic Acid Ethyl Ester (**2f**)

After dissolution of 141 mg (6.1 mmol) sodium in 25 ml dry ethanol 1.08 g (6.1 mmol) 1*H*-benzimidazole-2-carboxylic acid methyl ester **2e**[4] was added at 5 °C. After refluxing for 20 min a solution of 926 mg (6.1 mmol) ethyl bromoacetate in 2 ml dry ethanol was added and refluxed for 15 min. The reaction mixture was concentrated, diluted with water, and extracted with ethyl acetate. The organic phase was washed with water, dried over sodium sulphate, filtered, and evaporated. The residue was purified by vacuum flash chromatography (30 g silica gel, impregnated with diisopropylamine; eluent: dichloromethane:petroleum ether = 3:2) to yield 1.2 g (75%) colourless crystals, mp 83 °C.– ^{1}H NMR ($CDCl_3$): δ = 7.92 (d, 1H, H-7*), 7.43–7.29 (m, 3H, H-4*, H-5, H-6), 5.33 (s, 2H, NCH_2), 4.49 (q, 2H, $-NCH_2COOCH_2CH_3$), 4.21 (q, 2H, $-COOCH_2CH_3$), 1.46 (t, 3H, $-NCH_2COOCH_2CH_3$), 1.25 (t, 3H, $-COOCH_2CH_3$).– ^{13}C NMR ($CDCl_3$): 167.3 (s, $-NCH_2COOCH_2CH_3$), 159.9 (s, benzim.-$COOCH_2CH_3$), 141.4 (s, C-3a*), 140.6 (s, C-2*), 135.9 (s, C-7a), 125.7 (d, C-6), 123.8 (d, C-5), 122.0 (d, C-4), 109.6 (d, C-7), 62.2/61.7 (2t, -O-CH_2-), 46.4 (t, -NCH_2CO-), 14.0/13.9 (2q, $-CH_2CH_3$).– Anal. ($C_{14}H_{16}N_2O_4$).

2-Carboxy-1H-benzimidazole-1-acetic Acid (**2b**)*, Dipotassium Salt*

To a solution of 430 mg (1.65 mmol) of this diester (**2f**) in 13 ml of 50% aqueous methanol 185 mg (3.3 mmol) potassium hydroxide was added. The reaction mixture was refluxed for 2 h, evaporated and the residue treated with methanol and ether to yield 274 mg (60%) of the dipotassium salt as a colourless solid (mp > 325 °C). ^{1}H NMR (D_2O): δ = 7.75 (d, 1H, H-7*), 7.50–7.28 (m, 3H, H-4*, H-5, H-6), 5.13 (s, 2H, -NCH_2-).– ^{13}C NMR (D_2O): δ = 178.2 (s, -NCH_2COO-), 168.1 (s, benzim.-COO-), 151.0 (s, C-2), 142.7 (s, C-3a), 138.1 (s, C-7a), 127.0 (d, C-6), 125.9 (d, C-5), 122.1 (d, C-4), 113.6 (d, C-7), 51.0 (t, -CH_2-).– Anal. [$C_{10}H_6K_2N_2O_4 \times 0.2(C_2H_5)_2O$].

2-[[(Phenylmethoxy)carbonyl]methyl]-1H-benzimidazole-1-acetic Acid, Ethyl Ester (**2h**)

After addition of 1 g (3.96 mmol) 1*H*-benzimidazole-2-carboxylic acid benzyl ester (**2g**, prepared from lithium benzylate and 6*H*,13*H*-dibenzimidazo[1,2-*a*,1′,2′-*d*]tetrahydropyrazine-6,13-dione[4], **4**) to a stirred suspension of 95 mg (3.96 mmol) sodium hydride (applied as 60% dispersion in oil) in 25 ml dry DMF at 0 °C under N_2 atmosphere the reaction mixture was allowed to reach room temperature. After evolution of gas had ceased it was warmed to 40 °C and 598 mg (3.96 mmol) ethyl bromoacetate were added slowly. The reaction mixture was stirred at 35–45 °C for 40 min, cooled, poured onto ice, acidified, and the precipitate separated by suction filtration. The coloured solid was washed with water, dried and recrystallized from acetone:petroleum ether = 1:2 to yield 1.2 g (89%) of colourless shiny crystals, mp 139 °C.– ^{1}H NMR ($CDCl_3$): δ = 7.95 (d, 1H, 7-H*), 7.60–7.30 (m, 8H, 4-H*, 5-H, 6-H, $5H_{phe}$), 5.35 (s, 2H, -NCH_2-), 4.19 (q, 2H, -CH_2CH_3), 1.20 (t, 3H, -CH_2CH_3).– ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ = 166.2 (s, -$COOCH_2CH_3$), 158.1 (s, -$COOCH_2$-phe), 139.9 (s, C-2*), 139.2 (s, $C\text{-}1_{phe}$), 134.8/133.6 (2s, C-3a*/C-7a*), 127.2/127.1 (3d, $C\text{-}2,6_{phe}$, $C\text{-}3,5_{phe}$, $C\text{-}4_{phe}$), 124.4 (d, C-6), 122.4 (d, C-5), 120.1 (d, C-4), 109.3 (d, C-7), 66.1 (t, -OCH_2-phe), 60.2 (t, -OCH_2CH_3), 45.2 (t, -NCH_2-), 12.6 (q, -CH_3).– Anal. ($C_{19}H_{18}N_2O_4$).

1-[(Ethoxycarbonyl)methyl]-1H-benzimidazole-2-carboxylic Acid (**2c**)*, Triethylammonium Salt*

A solution of 180 mg (0.53 mmol) **2h** and 215 mg triethylamine in 35 ml methanol was hydrogenated at room temperature in a Parr apparatus at 50 psi using 108 mg 10 % palladium on charcoal as a catalyst. After 2 h the solution was filtered over Celite and evaporated. The residue was taken up in dichloromethane and extracted with water. The aqueous phase was washed with dichloromethane and evaporated to dryness to yield 50 mg (27%) of the triethylammonium salt of **2c** as a colourless oil.– ^{1}H NMR (D_2O): δ = 7.70

(d, 1H, H-7*), 7.45–7.20 (m, 3H, H-4*, H-5, H-6), 5.20 (s, 2H, -NCH_2-), 4.15 (q, 2H, -OCH_2CH$_3$), 3.05 (q, 6H, -N$^+$CH_2CH$_3$), 1.90–1.05 (m, 12H, -OCH$_2$CH_3 and -N$^+$CH$_2$CH_3).– ^{13}C NMR (D_2O): δ = 173.1 (s, -COOCH$_2$CH$_3$), 167.0 (s, -COO-), 150.4 (s, C-2), 142.2 (s, C-3a), 137.6 (s, C-7a), 127.2 (d, C-6), 126.0 (d, C-5), 122.0 (d, C-4), 112.9 (d, C-7), 65.1 (t, -OCH$_2$CH$_3$), 49.0 (t, -N$^+$CH$_2$CH$_3$), 15.6 (q, -OCH$_2$CH$_3$), 10.5 (q, -N$^+$CH$_2$CH$_3$).

Membrane Preparation and Binding Assays

The preparation of Triton-treated membranes from CA1 and dentate gyrus parts of rat hippocampi, [^{3}H]kainic acid and [^{3}H]MK-801 binding assays have been performed as described[2,3]. [^{3}H]MK-801 (5 nM, NEN, 21–30 Ci/mmol) was incubated for 2 h in presence of 10 µM glutamic acid and the nominal absence of glycine. Nonspecific binding was defined by replacing glutamate with 10 µM D-aminophosphovaleric acid and 1 µM 5,7-dichlorokynurenic acid. [^{3}H]Glutamic acid (10 nM, NEN, 17.3 Ci/mmol) was incubated for 20 min at 2 °C in 50 mM Tris-acetate (pH 7.0), with rat hippocampal membranes which, during membrane preparation, had been treated for 15 min with 0.08% Triton-X-100 at 2 °C; 10 µM glycine, 2 µM kainic acid and 1 µM quisqualic acid have been present. Nonspecific binding was determined in presence of 400 µM NMDA. The same procedure was adapted for the [^{3}H]glycine binding assay, described in detail elsewhere[1]. The concentration of [^{3}H]glycine (NEN, 45 Ci/mmol) was 10 nM, and 10 µM glutamate was present. Nonspecific binding was determined in presence of 100 µM D-serine. [^{3}H]AMPA (3 nM, NEN, 56.6 Ci/mmol) was incubated for 20 min at 2 °C with rat striatal membranes treated during membrane preparation for 10 min with 0.03% Triton-X-100 at 37 °C. Assays were conducted in presence of 100 mM NH_4SCN. Nonspecific binding was determined in the presence of 10 µM AMPA. In all binding assays, bound and free radioligand were separated from each other by centrifugation at 35,000–40,000 × g. Only for [^{3}H]MK-801, the filtration method was used.

Data Analysis

For compounds reducing specific binding of a radioligand by more than 50% at a concentration of 400 µM, IC_{50} values were determined using five different inhibitor concentrations up to 800 µM and computerized curve fitting of the displacement curves to the function

$$B(I) = B_0 \times IC_{50}^{n_H}/(IC_{50}^{n_H} + I^{n_H}) + NB$$

where I is the inhibitor's concentration, B_0 the amount of radioligand bound in the absence of inhibitor, n_H is the Hill coefficient, and NB the nonspecific binding. For the transformation of IC_{50} to K_i values, the true affinity constant of the radioligand for its binding site has to be known. For [^{3}H]glycine, this value is not accessible directly, since the concentration of environmental background glycine cannot be reduced to a value which is negligible in comparison to the affinity constant. We have indirect evidence that, in the presence of 10 µM glutamic acid, the true affinity constant of [^{3}H]glycine (K_D) to rat hippocampal membranes is close to 40 nM[1] and that the observed values (K_{obs}) contain, in addition, the concentration of environmental glycine. Therefore, K_i values were calculated using a modified Chen-Prusoff correction,

$$K_i = IC_{50}/[1 + (L + K_{obs} - 40)/40],$$

where L is the concentration of [^{3}H]glycine, and K_{obs} – 40 the concentration of background glycine (in nM). K_i values for the inhibition of [^{3}H]glutamic acid binding were calculated from the conventional equation $K_i = IC_{50}/(1 + L/K_D)$. Glycine sensitive inhibition of [^{3}H]MK-801 binding was evaluated by establishing concomitantly glycine stimulation curves and fitting them to the equation $B(L) = B_M \cdot (L + L_E)/(EC_{50} + L + L_E)$, where B_M is the amount of [^{3}H]MK-801 bound at saturating glycine concentrations, and L_E is the concentration of environmental glycine. The values obtained by computerized curve fitting for EC_{50} and for L_E were used to convert IC_{50} to K_i values via the Chen-Prusoff correction. Differences in mean K_i values were tested for significance by analysis of variance with Newman-Keuls test.

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