J. Pharm. Pharmacol. 1992, 44: 812–816
Received November 20, 1991



# A Rapid Filtration Assay for the Glycine Binding Site on the NMDA Receptor in Rat Cortical Membranes using [$^3$H]Dichlorokynurenic Acid

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**Abstract**—A filtration binding assay using [$^3$H]dichlorokynurenic acid to label the glycine binding site on the *N*-methyl-D-aspartic acid receptor has been evaluated on rat cortical membranes. This ligand binds to a single population of binding sites following mass action kinetics with a $K_D$ of 29 nM and a capacity of 5·73 pmol (mg protein)$^{-1}$. The pharmacological specificity of the binding site is identical to that previously reported for this binding site using [$^3$H]glycine as a radioligand. Agonists showed lower affinity and antagonists higher affinity when [$^3$H]dichlorokynurenic acid was used compared with [$^3$H]glycine. The higher affinity of [$^3$H]dichlorokynurenic acid compared with [$^3$H]glycine make it the more suitable compound with which to label the glycine site.

The *N*-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors is unusual in that it possesses a secondary agonist site for the amino acid glycine, whose occupation is necessary for normal receptor activation (Thomson 1989). Antagonists at this site are allosteric blockers of NMDA receptors, and may be useful in the treatment of disorders in which these receptors are implicated; such compounds have been shown to be active in certain animal models of epilepsy (Singh et al 1990) and of brain damage following cerebral ischaemia (Germano et al 1988). Amongst the most potent of these antagonists is the quinoleic acid derivative 5,7-dichlorokynurenic acid (DCKA (Baron et al 1990)).

The glycine site has been labelled in radioligand binding studies using [$^3$H]glycine (Marvizon et al 1989). Binding to the glycine site on the NMDA receptor can be distinguished from binding to the inhibitory glycine receptor by its insensitivity to strychnine and by the activity of a number of D-amino acids. However, the low affinity of [$^3$H]glycine (around 300 nM) limits its usefulness as a radioligand, the assay usually having to be performed by centrifugation. A recent publication (Baron et al 1991) has described the use of [$^3$H]DCKA to label the glycine site, again using a centrifugation assay. We now report that, by virtue of its higher affinity, it is possible to measure [$^3$H]DCKA binding to the glycine site using a simple filtration assay.

## Materials and Methods

[$^3$H]DCKA was purchased from New England Nuclear: 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dichloro-3-hydroxy-2-quinoxaline carboxylic acid (DHQX) and 7-chlorokynurenic acid were obtained from Tocris Neuramin, Buckhurst Hill, UK. 5,7-Dichlorokynurenic acid (DCKA) and 3-(4,6-dichloro-2-carboxyindol-3-yl)-propionic acid (DCCPA) were synthesized according to published methods (Harrison et al 1990; Salituro et al 1990). Other compounds and reagents were from Sigma Chemical Co., St Louis, USA.

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Buffy-coat membranes from rat cerebral cortex were prepared using the method published by Snell et al (1988). Male Sprague-Dawley rats (180–220 g, IFFA-CREDO, France) were killed by decapitation and their cerebral cortices removed on ice and frozen at −80°C for at least 24 h. The tissue was rapidly thawed and homogenized with a Polytron in 10 vol of cold (4°C) sucrose (0·32 M) and centrifuged for 10 min at 1000 *g*. The supernatant was recovered and recentrifuged for 20 min at 20 000 *g*. The resulting pellet was resuspended in 20 vol of ice-cold distilled water and centrifuged at 8000 *g* for 20 min. The supernatant and buffy layer were collected and centrifuged at 50 000 *g* for 20 min. The pellet was resuspended in 20 vol of ice-cold water and recentrifuged at 50 000 *g* for 20 min. The final pellet was frozen at −20°C until use. On the day of the binding assay, the membranes were thawed and resuspended in 20 vol of HEPES-KOH (50 mM, pH 7·5), incubated at 37°C for 20 min and then recentrifuged at 50 000 *g* for 10 min. The pellet was washed twice by resuspension in HEPES-KOH buffer and recentrifugation in order to remove endogenous glycine and glutamic acid. The final pellet was resuspended in HEPES-KOH buffer for use in the binding assay. Protein concentrations were determined using the bicinchoninic acid method (Smith et al 1985).

The [$^3$H]glycine binding assay was performed according to the method described by Snell et al (1988). A similar method was used for the determination of [$^3$H]DCKA binding. Briefly, membranes (0·1 mg protein mL$^{-1}$ in HEPES-KOH buffer; final volume: 1 mL) were incubated for 30 min at 4°C with [$^3$H]DCKA (final concentration 20 nM) and, where necessary, glycine (1 mM) to determine the non-specific binding. All assays were performed in triplicate. The binding interaction was terminated by filtration on Whatman GF/B filters, which were rinsed immediately with 3 × 4 mL of cold HEPES-KOH buffer. Magnesium sulphate (10 mM) was included in the washing buffer since this has been claimed by

Kessler et al (1989) to slow dissociation of [$^3$H]glycine; the validity of this observation was not, however, evaluated in the present experiments. It was important that the filtration and rinsing process were finished in 5 s in order to prevent dissociation of the ligand. Radioactivity trapped on the filters was determined by liquid scintillation counting in Readi-Gel scintillant (Beckman).

## Results

Under the conditions routinely used in the assay, the binding of [$^3$H]DCKA to rat cortical membranes corresponded to around 10000 d min$^{-1}$, of which 90% could be displaced by unlabelled glycine. This compared with a specific binding component of 70% using a centrifugation assay. Binding increased linearly with protein concentration up to 0·5 mg protein mL$^{-1}$ (data not shown). Saturation analysis showed that [$^3$H]DCKA bound to a single population of binding sites according to mass action kinetics (Fig. 1). Nonlinear regression analysis of the data yielded kinetic parameters of 29 ± 4 nM for $K_D$ and 5·73 ± 0·24 pmol (mg protein)$^{-1}$ for $B_{max}$, and a Hill coefficient of 0·98 ± 0·02, (mean ± s.e.m., n = 3). These values compared favourably with those of 278 nM and 5·89 pmol (mg protein)$^{-1}$ obtained for [$^3$H]glycine binding to the same membrane preparations (data from a single experiment—not shown). Using a centrifugation assay, values of $K_D = 61 \pm 10$ nM, $B_{max} = 12{\cdot}8 \pm 1{\cdot}4$ pmol (mg protein)$^{-1}$, $n_H = 1{\cdot}02 \pm 0{\cdot}05$ were obtained.

Association of [$^3$H]DCKA to the membranes was extremely rapid, equilibrium being already virtually reached after 2 min (Fig. 2). The time-course of association followed a single exponential with a $k_{+1}$ value of 0·029 ± 0·002 min$^{-1}$ nM$^{-1}$. Similarly, dissociation of [$^3$H]DCKA initiated by the addition of glycine (1 mM), was monoexponential ($k_{-1} = 0{\cdot}978 \pm 0{\cdot}128$ min$^{-1}$) and was essentially complete by 5 min. The $K_D$ value obtained from these kinetic constants was 33·7 nM, in good agreement with that obtained from saturation experiments. It was not possible to perform these measurements with a centrifugation assay.

Binding of [$^3$H]DCKA to cortical membranes could be displaced by a variety of agonists and antagonists for the glycine site, with Hill coefficients close to unity (Table 1). The rank order of potency was comparable with that observed using [$^3$H]glycine as a ligand, although it was noticed that antagonists tended to be more potent against [$^3$H]DCKA, whereas agonists were more potent against [$^3$H]glycine (Fig. 3). The most potent inhibitors of binding were unlabelled DCKA ($K_i = 27$ nM) and DCCPA ($K_i = 28$ nM). Phencyclidine, MK 801, 2-aminophosphonovaleric acid and NMDA, compounds acting at other sites on the NMDA receptor, were unable to displace specific [$^3$H]DCKA binding, as were strychnine and quisqualic and glutamic acids. It was noticed, however, that high concentrations of NMDA and 2-aminophosphonovaleric acid produced respectively a slight enhancement and a slight decrease in [$^3$H]DCKA binding.

## Discussion

The data presented in this report confirm previous data (Baron et al 1991) suggesting that [$^3$H]DCKA can be used to label the glycine binding site on the NMDA receptor, and

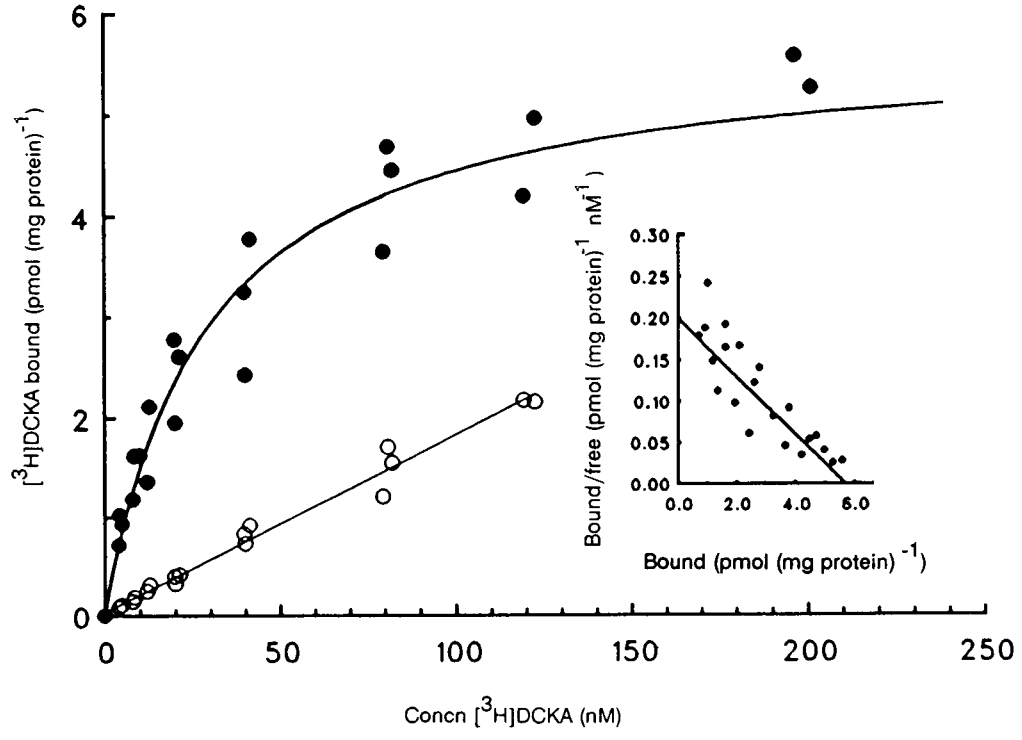


FIG. 1. Saturation analysis of the binding of [$^3$H]DCKA to rat cortical membranes. The data are pooled from three independent experiments each performed in triplicate. Non-specific (○) and specific (●) binding are shown. The line through the points (●) represents the best fit to a simple Langmuir isotherm determined by iterative nonlinear regression analysis which yielded $K_D$ and $B_{max}$ values of 29 ± 4 nM and 5·73 ± 0·24 pmol (mg protein)$^{-1}$, (mean ± s.e.m.). The inset shows a Scatchard transformation of the same data.

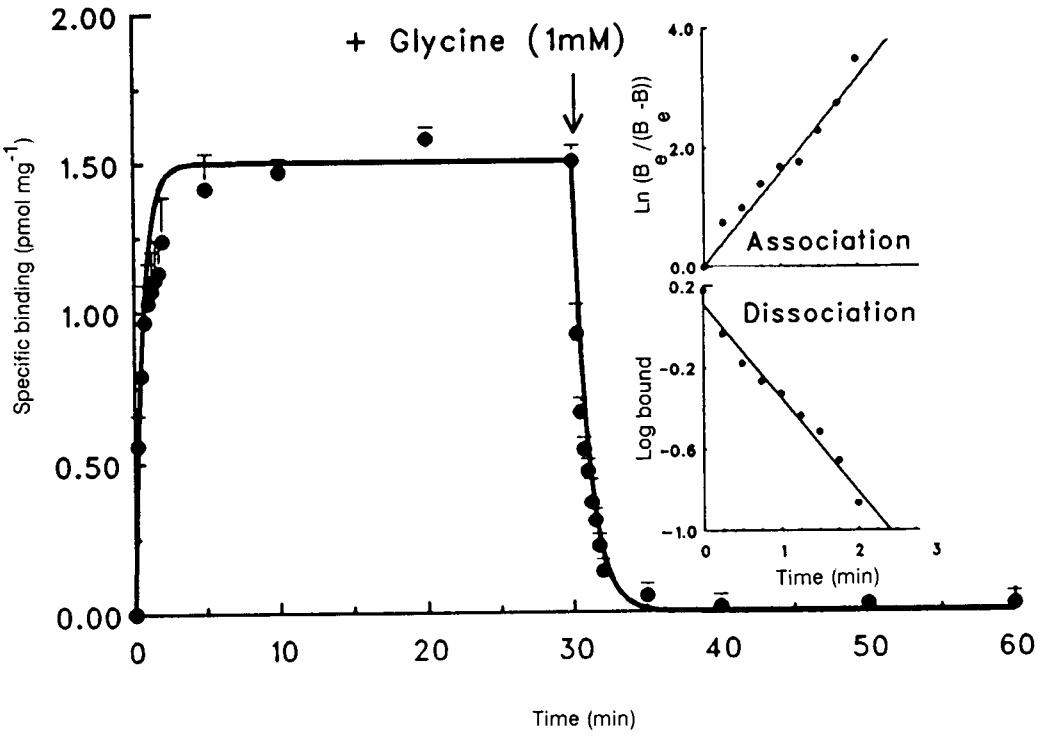


FIG. 2. Kinetic analysis of the binding of [$^3$H]DCKA to rat cortical membranes. The data represent the mean ± s.e.m. of values obtained from three independent experiments each performed in triplicate. Dissociation was initiated by addition of glycine (1 mM) as indicated by the arrow. The insets show semi-logarithmic transformations of the same data over the first two min of association and dissociation; the lines represent those obtained by linear regression analysis. The line through the points on the main graph represents the theoretical curve generated from the kinetic constants derived from the linear regressions.

Table 1. Pharmacological specificity of [$^3$H]DCKA binding sites on rat cortical membranes.

| | Compound | $K_i$ ($\mu$M) | $n_H$ |
|---|---|---|---|
| 1 | DCCPA | 0·028 ± 0·001 | 0·92 ± 0·03 |
| 2 | Indole-2-carboxylic acid | 40·5 ± 2·7 | 0·91 ± 0·09 |
| 3 | DCKA | 0·027 ± 0·002 | 0·93 ± 0·06 |
| 4 | 7-Chlorokynurenic acid | 0·122 ± 0·010 | 0·97 ± 0·05 |
| 5 | Kynurenic acid | 5·85 ± 0·53 | 0·96 ± 0·05 |
| 6 | CNQX | 1·55 ± 0·21 | 1·03 ± 0·06 |
| 7 | DHQX | 0·194 ± 0·051 | 0·82 ± 0·09 |
| 8 | DNQX | 0·334 ± 0·022 | 0·77 ± 0·12 |
| 9 | HA 966 | 22·3 ± 0·90 | 0·83 ± 0·06 |
| 10 | 1-Aminocyclopropane carboxylic acid | 0·413 ± 0·004 | 0·86 ± 0·05 |
| 11 | Glycine | 0·424 ± 0·026 | 0·84 ± 0·03 |
| 12 | L-Alanine | 84·7 ± 4·0 | 0·81 ± 0·03 |
| 13 | D-Alanine | 1·39 ± 0·10 | 0·92 ± 0·09 |
| 14 | L-Serine | 60·8 ± 2·7 | 0·83 ± 0·19 |
| 14 | D-Serine | 0·526 ± 0·049 | 0·94 ± 0·13 |
| | Phencyclidine | Inactive | |
| | MK 801 | Inactive | |
| | 2-Aminophosphonovaleric acid | Decrease (−40% at 1 mM) | |
| | Glutamic acid | Inactive | |
| | NMDA | Increase (+35% at 0·1 mM) | |
| | Quisqualic acid | Inactive | |
| | Strychnine | Inactive | |

Inhibition constants are presented as $K_i$ values, and the Hill coefficients of the displacement curves are given ($n_H$). Data represent the mean ± s.e.m. of at least three experiments each performed in triplicate.

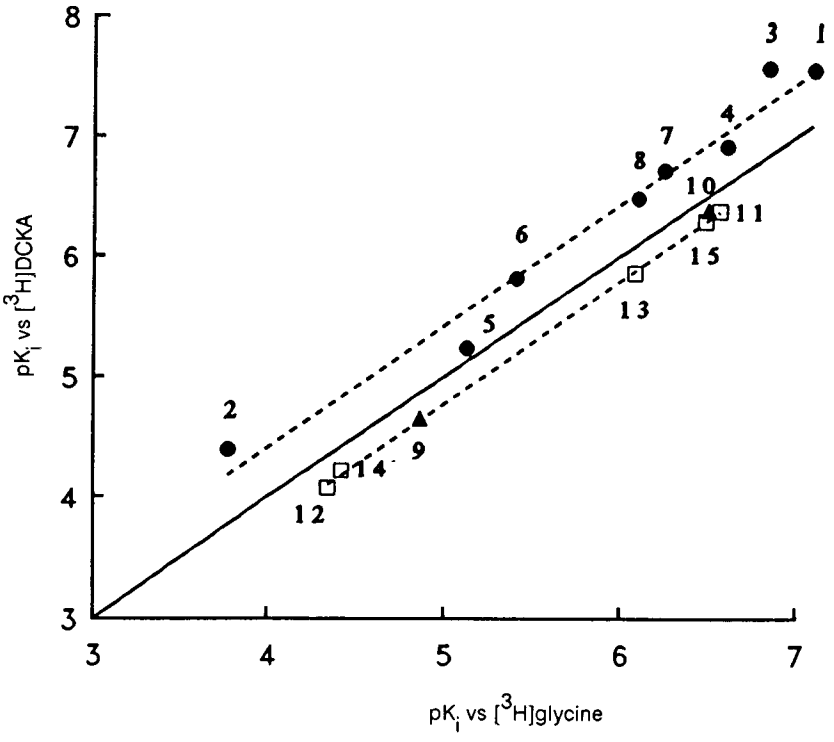


FIG. 3. Correlation between the affinities of a range of compounds for binding sites for [$^3$H]glycine and [$^3$H]DCKA. Data are presented as $pK_i$ values; the compounds are referred to by the numbers given in Table 1. Antagonists are indicated by ●, partial agonists by ▲ and agonists by □. The solid line indicates theoretical equivalence (x = y), and the dotted lines the best fit to the points obtained by linear regression for agonists (r = 0·99) and antagonists (r = 0·99). The slope of both regression lines was unity.

demonstrate for the first time that a rapid filtration technique can be used. This ligand labelled an apparently identical population of sites to that labelled by [$^3$H]glycine itself. Kinetic analysis of the binding process is consistent with a simple bimolecular interaction between [$^3$H]DCKA and the NMDA receptor according to a classical Langmuir isotherm. The affinities of a number of agonists and antagonists for this site were comparable with those previously obtained using [$^3$H]glycine (Snell et al 1988; Marvizon et al 1989). Unlike the data obtained by Baron et al (1991), we noticed that antagonists were slightly more active, and agonists somewhat less active at displacing [$^3$H]DCKA binding, than at displacing [$^3$H]glycine. Such a distinction between the binding of an agonist and that of an antagonist has been noted previously for a number of other receptors (e.g. the $GABA_A$ receptor (Möhler & Okada 1977)). The finding that NMDA slightly enhanced [$^3$H]DCKA binding may reflect an allosteric interaction between the NMDA and glycine sites, similar, for example, to the increase in benzodiazepine binding to $GABA_A$ receptors induced by GABA (Martin & Candy 1978). The $K_D$ and $B_{max}$ values obtained by filtration were lower than those measured by centrifugation, suggesting that in the latter method a further population of lower affinity, rapidly dissociating sites, may be recruited at high ligand concentrations. The $K_D$ we obtained by centrifugation is similar to that observed by Baron et al (1991), who noticed that the binding capacity for [$^3$H]DCKA measured by centrifugation was higher than that for [$^3$H]glycine.

The use of [$^3$H]DCKA to label the glycine site has several advantages compared to [$^3$H]glycine itself, stemming largely from its tenfold higher affinity. This is reflected in a higher signal-to-noise ratio (90% specific binding compared with 60%), enabling the assay to be performed at lower protein concentrations (0·1 vs 0·3 (mg protein) $mL^{-1}$), and thus on smaller samples of brain tissue. Likewise, smaller quantities of ligand need to be handled.

More importantly, unlike [$^3$H]glycine binding, which is generally carried out using a centrifugation assay (apart from one report (Snell et al 1988) using filtration), [$^3$H]DCKA binding can readily be performed using a rapid filtration technique. This has obvious advantages in terms of the rapidity and simplicity of the assay and in the improved signal-to-noise ratio. Furthermore, the swiftness of the separation procedure allows detailed kinetic analysis of the binding interaction to be performed.

In conclusion, [$^3$H]DCKA seems to be the most suitable available radioligand with which to label the glycine site on the NMDA receptor.

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