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Binding of the glutamate carboxypeptidase II (NAALADase) inhibitor 2-PMPA to rat brain membranes

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Abstract

2-Phosphonomethyl pentanedioic acid (2-PMPA) is a potent and selective inhibitor of glutamate carboxypeptidase II (NAALADase), and has shown robust neuroprotective activity in both in vitro and in vivo models of ischemia. In the brain, glutamate carboxypeptidase II (GCPII) (EC3.4.17.21) hydrolyzes the neuropeptide *N*-acetylaspartylglutamate (NAAG) to glutamate and *N*-acetylaspartate. We report the development and characterization of a [^{3}H]2-PMPA binding assay. [^{3}H]2-PMPA binding was dependent on protein concentration, saturable, and displaceable. The association (k_{on}) and dissociation (k_{off}) rate constants were 3×10^6 M^{-1} s^{-1} and 0.01 s^{-1}, respectively. The dissociation equilibrium constant (K_d) determined from the ratio of the rate constants ($K_d = k_{off}/k_{on}$) was 1 nM. Scatchard analysis revealed one binding site with $K_d = 2$ nM and $B_{max} = 0.7$ pmol/mg. Binding exhibited similar pharmacological properties to GCPII enzyme activity, including chloride dependency, cobalt stimulation and inhibition by phosphate and quisqualate. The binding of [^{3}H]2-PMPA also showed tissue specificity in that tissues previously reported to be devoid of GCPII enzymatic activity were devoid of [^{3}H]2-PMPA binding. [^{3}H]2-PMPA binding represents an additional probe for the study of GCPII activity, and may be useful as a high throughput screening assay.

Keywords: Glutamate carboxypeptidase; [^{3}H]2-(phosphonomethyl) pentanedioic acid ([^{3}H]2-PMPA); Glutamate carboxypeptidase II (NAALADase); *N*-acetylaspartylglutamate (NAAG); Prostate specific membrane antigen

1. Introduction

Glutamate carboxypeptidase II (GCPII, EC3.4.17.21) is a metallopeptidase that hydrolyzes the neuropeptide *N*-acetylaspartylglutamate (NAAG) to glutamate and *N*-acetylaspartate. The enzymatic activity of GCPII was first detected in rat brain using a radioenzymatic assay, which measured the release of [^{3}H] glutamate product from *N*-acetylaspartyl-[^{3}H]glutamate (Robinson et al., 1988). GCPII has been located in both neural and non-neural tissues, such as kidney, prostate and small intestine (Blakely et al., 1988; Slusher et al., 1990; Cassidy and Neale, 1993a; Serval et al., 1990; Halsted et al., 1998). GCPII was recently cloned, characterized and found to be homologous to the prostate cancer marker prostate specific membrane (PSM) antigen (Carter et al., 1998a,c). The pharmacological and molecular profile of the enzyme in prostate tissue was nearly identical to that of the brain-localized enzyme (Carter et al., 1998b; Tiffany et al., 1999).

In the brain, the GCPII substrate NAAG is removed from synaptic spaces by a high affinity transport system (Williamson and Neale, 1992; Cassidy and Neale, 1993b) and by enzymatic degradation (Tsai et al., 1991). NAAG is an abundant neuropeptide, found in mM concentrations (Coyle et al., 1991; Coyle, 1997). It is released from neurons by Ca^{2+}-dependent depolarization (Tsai et al., 1991) and acts as an agonist at group II metabotropic glutamate receptors (Wrobleska et al., 1997). NAAG also has partial agonist actions at the *N*-methyl-D-aspartate (NMDA) receptor (Westbrook et al., 1986; Puttfarcken et al., 1993; Vallivullah et al., 1994). Coyle et al. (1991) and Coyle (1997) have proposed that GCPII functions both to terminate the neurotransmitter activity of NAAG as well as to liberate glutamate from NAAG synaptosomal stores for action at various glutamate receptors. Potent and selective inhibitors of GCPII would be useful in probing the complexities of NAAG and glutamate synaptic transmission.

The first potent GCPII inhibitor, 2-(phosphonomethyl) pentanedioic acid (2-PMPA), was described in 1996; it has a K_i of 0.3 nM (Jackson et al., 1996), and it was subsequently shown to be selective for GCPII. 2-PMPA, at 10 μM, did not inhibit over 100 different receptors, trans-

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porters, ion channels, or enzymes. This selectivity screen included several glutamatergic sites such as NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), metabotropic glutamate receptors and the glutamate transporter (Slusher et al., 1999).

The purpose of this study was to develop and characterize a binding assay for GCPII using rat brain membranes and [^{3}H]2-PMPA.

2. Materials and methods

2.1. Rat brain membrane tissue preparation

Rat brain membrane P2 fraction was used as GCPII source as previously described (Robinson et al., 1988). Briefly, whole rat brains were homogenized in 10 volumes (w/v) of 0.32 M sucrose and centrifuged at $800 \times g$ for 10 min. The supernatant was removed and centrifuged at $20{,}000 \times g$ for 20 min. The resulting pellet was lysed by suspension in 10 volumes (w/v) of ice-cold Baker Ultrapure water (VWR, Bridgeport, NJ) and centrifuged at $8000 \times g$ for 10 min. The supernatant and buffy coat were removed and centrifuged at $35{,}000 \times g$ for 10 min. The resulting pellet was resuspended in 20 volumes of Tris–Cl buffer (50 mM, pH 7.4 at 37 °C), incubated for 30 min at 37 °C, and then centrifuged at $35{,}000 \times g$ for 10 min. After washing the pellet in 20-ml buffer and centrifuging twice more, the membranes were suspended in Tris–Cl buffer to a final concentration of approximately 5 mg/ml of protein, aliquoted and stored frozen at −80 °C until use. Protein measurements for rat brain and other tissue preparations were carried out with the Bio-Rad DC protein assay kit (BioRad, Hercules, CA).

2.2. Kidney, liver, prostate and small intestine tissue preparation

Tissues other than brain were prepared as membrane and soluble fractions as previously described (Tiffany et al., 1999). Briefly, tissues were homogenized with a Polytron (Brinkman, Westbury, NY) in 10 volumes (w/v) of ice-cold Ultra pure water, and then sonicated twice on ice for 30 s. Homogenates were then centrifuged at $50{,}000 \times g$ for 20 min at 4 °C. The supernatant was aliquoted to new tubes, adjusted to 50 mM Tris–Cl with 200 mM stock, and stored at −80 °C until use. The pellet was homogenized again in 5 volumes (original wet weight) of ice-cold Tris–Cl buffer, aliquoted and stored frozen at −80 °C until use. Rat tissues were obtained and processed in house. Analytical Biological Services (Wilmington, DE) provided the human tissue preparations.

2.3. [^{3}H]2-PMPA binding assay

[^{3}H]2-Phosphonomethyl-pentane-1,5-dioic acid ([^{3}H]2-PMPA) was synthesized at Guilford Pharmaceuticals as previously described (Jackson et al., 1996) and subsequently radiolabelled by Amersham Pharmacia Biotech (Arlington Heights, IL). Radiochemical purity was determined to be greater than 95%; specific activity was 35 Ci/mmol. Unless otherwise noted, the binding assays were carried out in triplicate in 96-well Packard Optiplates (Meriden, CT); the wells contained tissue membranes (200–300 μg) and [^{3}H]2-PMPA in Tris–Cl buffer (pH 7.4, 50 mM) in a total volume of 250 μl. Plates were incubated for 30 min at room temperature. Non-specific binding was determined in the presence of 0.1 mM unlabelled 2-PMPA. The incubation mixture was subsequently centrifuged at $2000 \times g$ for 10 min in a Beckman GS-6R tabletop refrigerated centrifuge equipped with a microplate capacity rotor. After centrifugation, the supernatant was removed and the membrane pellet resuspended in 250 μl ice-cold Tris–Cl buffer and centrifuged as above. Twenty microliters Solvable (Packard) and 250 μl MicroScint-20 (Packard) were added to each well, and radioactivity was counted using a Packard Topcount. The ratio of [^{3}H]2-PMPA specific binding to non-specific was 75–90%. When the effect of $CoCl_2$ was tested, the tissue was extracted and the reactions performed in 50 mM Tris–acetate.

2.4. Data analysis

2.4.1. Determination of the association rate constant (k_{on})

Association data was plotted according to the integrated form of the second-order rate equation (Siegel et al., 1989):

$$\ln Y = k_{on} t\left[\left(LB_{max}/B_e\right) - B_e\right] \quad (1)$$

This is the equation of a straight line where $Y = [B_e(L - BB_e/B_{max})/L(B_e - B)]$ and k_{on} can be written as a function of the slope:

$$k_{on} = \text{Slope}/\left[\left(LB_{max}/B_e\right) - B_e\right] \quad (2)$$

where B_e is the concentration of radioligand at equilibrium, L is the concentration of radioligand, and B is the concentration of receptor–radioligand complex at various times. B was obtained from each time point in association experiments; dpm values were converted to concentration values using the specific activity of the radioligand (35 Ci/mmol). B_{max} is the concentration of GCPII: 1 nM. It is the B_{max} from Scatchard analysis of equilibrium experiments (0.7 pmol/mg protein) converted to molarity by using the concentration of synaptosomal protein (1.48 mg protein/ml).

2.4.2. Determination of the dissociation rate constant (k_{off})

Dissociation data was plotted according to the integrated form of a first-order rate equation:

$$\ln\left[B/B_o\right] = k_{off} t \quad (3)$$

where B/B_o is the ratio of radioligand bound at a given time (B) to the amount of radioligand bound at the begin-

ning of dissociation (B_o). The slope of this plot is the negative of the dissociation rate constant k_{off} (Siegel et al., 1989).

Scatchard analysis was performed using the Radlig Program (BIOSOFT, Cambridge, UK). Other graphics and statistics were generated using the Microcal Origin program (Northampton, MA).

3. Results

3.1. Dependence of binding of [^{3}H]2-PMPA on protein concentration

[^{3}H]2-PMPA binding increased as a function of protein concentration up to 0.6 mg/0.25 ml (Fig. 1) and then attained saturation (data not shown).

3.2. Binding of [^{3}H]2-PMPA to GCPII

The specific binding of 2-PMPA at 0.3 nM to rat brain membrane was time-dependent, and it reached plateau levels at about 10 min at room temperature (Fig. 2A); when higher ligand concentrations were used, the rate of association was faster and plateau levels were reached earlier (data not shown). The association rate constant, k_{on}, was determined from the results in Fig. 2A and the integrated form of a second-order rate equation (Materials and methods). B_e, the concentration of radioligand at equilibrium was estimated from least square analysis of the specific binding as 965 dpm = 0.050 nM. The concentration of radioligand, L, was 0.3 nM. The concentration of

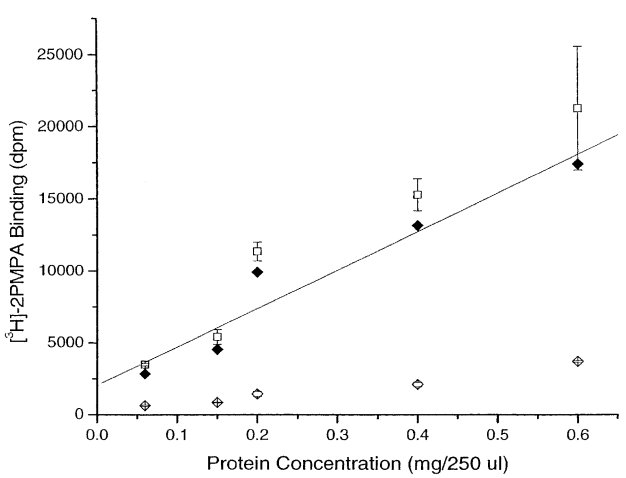

Fig. 1. Dependence of binding on protein concentration. Different concentrations of protein in brain membrane P2 preparations were incubated with [^{3}H]2-PMPA (5 nM) in a total volume of 250 μl for 30 min. After incubation, the assay mixture was centrifuged at 2000× g for 10 min, the supernatant removed, the pellet resuspended in Tris buffer and radioactivity was measured in a scintillation counter. The results illustrate the mean of four separate experiments; each point was assayed in octuplicate. (□ total, ◆ non-specific, ● specific). SEM values are shown on total binding only for clarity.

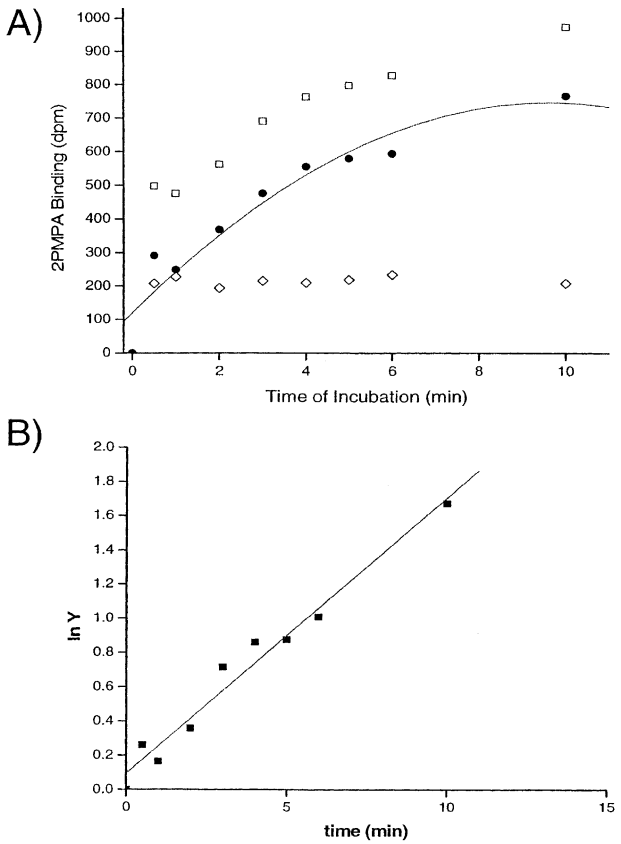

Fig. 2. Dependence of 2-PMPA binding on time of incubation. [^{3}H]2-PMPA (0.3 nM, 3 Ci/mmol) was added to a brain membrane P2 preparation (total protein = 1 mg/ml). Binding reaction was carried out at 4 °C in Tris buffer (pH 7.7, 50 mM) in a total volume of 5 ml. At the times indicated, 0.25-ml aliquots were taken out, diluted fivefold with ice-cold buffer and centrifuged to precipitate the protein. The supernatant was discarded and the radioactivity in the pellet was determined in a scintillation counter. Data points correspond to the average of two parallel determinations in a representative experiment. (□ total, ◇ non-specific, ● specific) (panel A). Plot of association data according to the integrated form of a second-order rate equation as outlined in Materials and methods (panel B).

enzyme–radioligand complex at various times, B, was obtained from each time point. B_{max}, the total concentration of GCPII, was 1 nM (Materials and methods). A value of Y was determined for each time point and a plot of ln$Y$ vs. time gave a straight line (Fig. 2B). Substitution of the values for the different parameters into Eq. (2) gave $k_{on} = 3 \times 10^7\ M^{-1}\ s^{-1}$.

3.3. Dissociation of [^{3}H]2-PMPA from GCPII

Dissociation was time-dependent and it was much slower than association. Specific binding was displaced to 80% 2 h after unlabelled 2-PMPA (0.1 mM) was added to the binding mixture (Fig. 3A). The dissociation rate constant was calculated from substitution of the data in Fig. 3A into Eq. (3) (Materials and methods). The plot of ln$[B/B_o]$ vs. time (Fig. 3B) gave a straight line with a

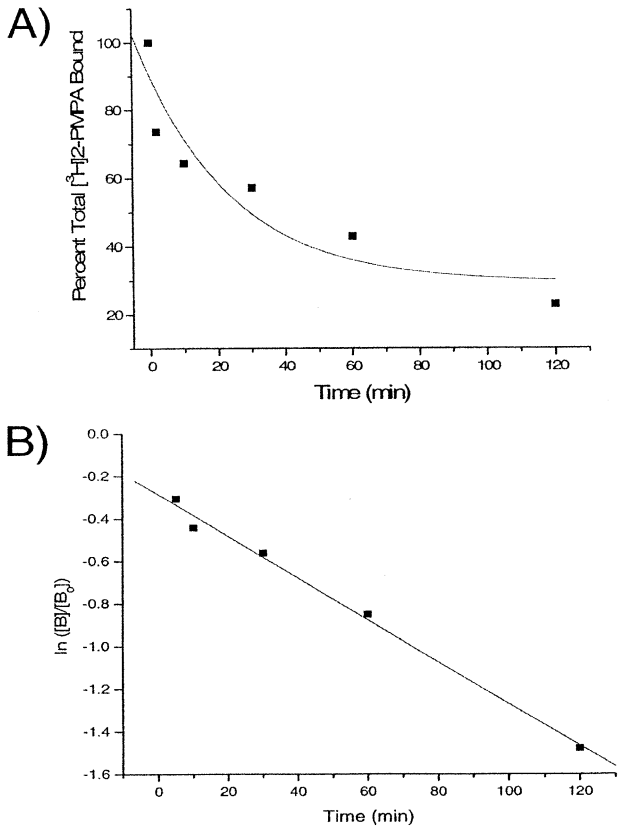

Fig. 3. Reversibility of 2-PMPA specific binding. Brain membrane P2 preparations were incubated with [^{3}H]2-PMPA (5 nM) in a total volume of 250 μl for 30 min. Several binding assays were carried out concomitantly in a 96-well format. After 30 min, 2-PMPA (100 μM) was added and the binding mixtures were incubated for various times as shown. After incubation, the assay mixture was centrifuged at 2000× *g* for 10 min, the supernatant removed, the pellet resuspended in Tris buffer and radioactivity was measured in a scintillation counter. Each point was assayed in octuplicate. The results illustrate a representative experiment ($n = 3$) (panel A). Plot of dissociation data according to the integrated form of a first order rate equation as outlined in Materials and methods (panel B).

slope equal to the negative of the dissociation rate constant; $k_{off} = 0.01\ s^{-1}$.

3.4. [^{3}H]2-PMPA Scatchard analysis

[^{3}H]2-PMPA binding was saturable at or above 10 nM (Fig. 4). Scatchard analysis showed one binding site with $K_d = 2 \pm 0.4$ nM and $B_{max} = 0.7 \pm 0.06$ pmol/mg protein.

3.5. [^{3}H]2-PMPA tissue binding specificity

To examine the tissue binding specificity of [^{3}H]2-PMPA, binding experiments were performed using rat and human tissues. Specific [^{3}H]2-PMPA binding could be detected in rat brain, spinal cord, and kidney that have previously been shown to exhibit GCPII enzyme activity (Blakely et al., 1988; Slusher et al., 1990) (Fig. 5A).

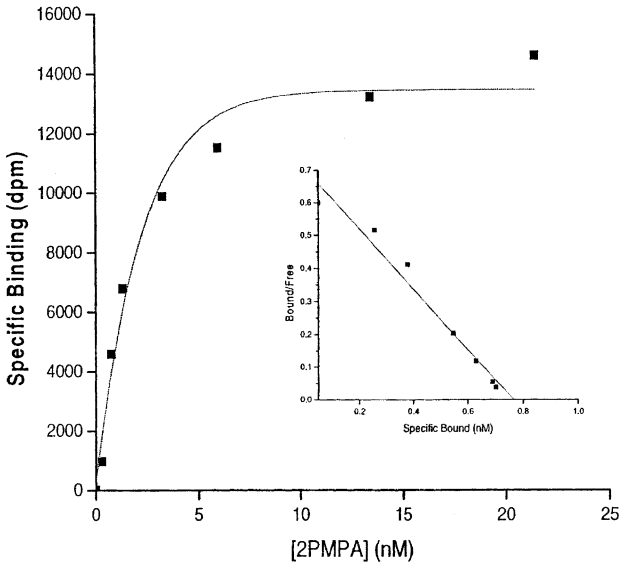

Fig. 4. Saturation isotherm and Scatchard analysis of 2-PMPA binding. Brain membrane P2 preparations were incubated with different concentrations of [^{3}H]2-PMPA in a total volume of 250 μl for 30 min. After incubation, the assay mixture was centrifuged at 2000× *g* for 10 min, the supernatant removed, the pellet resuspended in Tris buffer and radioactivity was measured in a scintillation counter. The graph is a representative of four separate experiments; each point was assayed in octuplicate (panel A). Scatchard analysis of the data (panel B).

Human kidney, brain and prostate exhibited robust specific binding of [^{3}H]2-PMPA, also consistent with GCPII enzyme activity in those tissues (Carter et al., 1998a; Tiffany

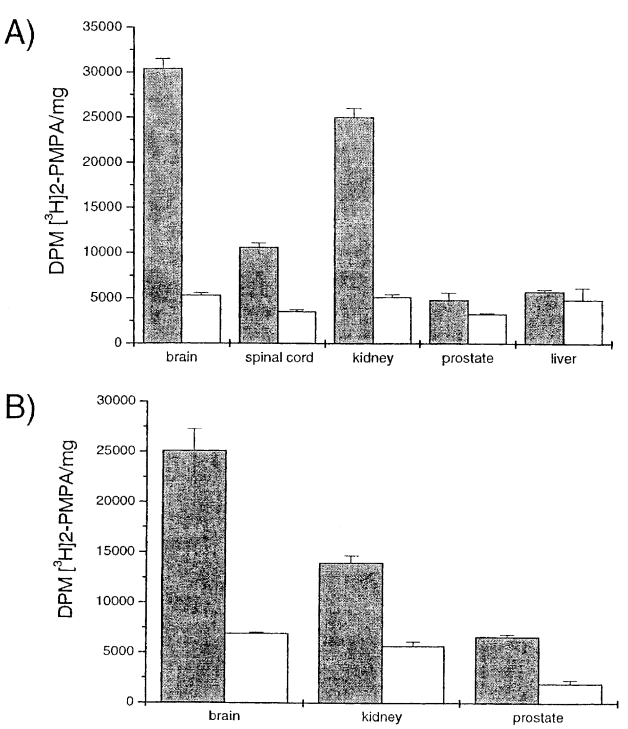

Fig. 5. Specificity of 2-PMPA binding to tissue. Tissue preparation was carried out as outlined in Materials and methods. [^{3}H]2-PMPA was incubated with rat tissues (panel A) and human tissues (panel B) (■ total, □ non-specific).

Table 1
Pharmacology of [^{3}H]2-PMPA binding vs. GCPII radioenzymatic assay

	Binding assay K_i (nM)	Radioenzymatic assay K_i (nM)
2-PMPA	1.9 ± 0.4	1.1 ± 0.2
2-PMSA	480 ± 160	2800 ± 550
Quisqualate	6000 ± 2800	5500 ± 1700
Phosphate	$750{,}000 \pm 27{,}000$	$170{,}000 \pm 12{,}000$
AMPA	$> 10{,}000{,}000$	$> 10{,}000{,}000$
NMDA	$> 10{,}000{,}000$	$> 10{,}000{,}000$
Kainic acid	$> 10{,}000{,}000$	$> 10{,}000{,}000$
$CoCl_2$, 1 mM	2-fold increase	2-fold increase

et al., 1999) (Fig. 5B). Specific binding was observed in rat prostate or rat liver. These two tissues have been shown to display little or no GCPII enzyme activity (Slusher et al., 1990; Tiffany et al., 1999) (Fig. 5A and B).

3.6. Pharmacological characteristics of [^{3}H]2-PMPA binding

2-PMPA, 2-PMSA, quisqualate, and phosphate displayed the same rank order of potency in inhibiting binding of [^{3}H]2-PMPA as they did for inhibiting GCPII enzymatic activity (Table 1). In addition, $CoCl_2$ stimulated binding and enzymatic activity to the same extent: twofold (Table 1).

4. Discussion

This is the first description and characterization of the highly selective [^{3}H]2-PMPA binding to rat brain GCPII. 2-PMPA is a newly described GCPII inhibitor (Jackson et al., 1996) which is potent ($K_d \sim 0.3$–1 nM) and selective in that it has no effect at 10 μM concentrations in more than 100 different receptors, ion channel and enzyme systems (Slusher et al., 1999). In animal studies, 2-PMPA has been shown to decrease extracellular glutamate during ischemia and provide neuroprotection (Slusher et al., 1999), to enhance myelination (Shah et al., 1998) and to ameliorate hyperalgesia (Brown et al., 1999). These effects could be mediated by 2-PMPA's ability to decrease the amount of glutamate and/or increase NAAG (Slusher et al., 1999). Decreased glutamate would be an 'upstream' mechanism that could reduce transmission at all glutamatergic receptors, while increased NAAG could indirectly decrease glutamate release by its activation of the mGluR3 receptor and partial agonist action on the NMDA receptor. Thus, GCPII represents a novel target for regulating glutamate transmission, and therefore could be a novel therapeutic intervention.

The current radioactive enzyme assay for GCPII is cumbersome and time consuming. In order to facilitate high throughput screening of chemical libraries for potential GCPII inhibitors, we developed and characterized a [^{3}H]2-PMPA binding assay using synaptosomal preparations. This binding has high affinity, is protein- and time-dependent, saturable, and reversible. The association and dissociation rate constants had values of 3×10^7 M^{-1} s^{-1} and 0.01 s^{-1}, respectively. The dissociation rate constant, K_d, determined by the ratio k_{off}/k_{on} was 1 nM, in close agreement with the K_d value determined independently by equilibrium experiments (2 nM). The association rate constant (k_{on}) for 2-PMPA and GCPII (3×10^7 M^{-1} s^{-1}) is similar to the apparent association rate constant for NAAG and GCPII ($k_{cat}/K_m = 2 \times 10^7$ M^{-1} s^{-1}) determined from steady state kinetic experiments where the enzymatic assay rather than the binding assay was used (Frazier et al., in press). 2-PMPA meets the criteria of a slow binding inhibitor exhibiting a fast rate of association and a slow rate of dissociation (Schloss, 1988).

Binding of 2-PMPA in tissue correlates with GCPII enzyme activity; rat spinal cord, rat and human kidney and brain, human prostate tissue (Blakely et al., 1988; Slusher et al., 1990; Tiffany et al., 1999) exhibit both GCPII activity and 2-PMPA specific binding. On the other hand, there was no specific binding of [^{3}H]2-PMPA in rat prostate or liver, tissues that do not exhibit appreciable GCPII activity (Slusher et al., 1990; Tiffany et al., 1999). The 96-well format of this [^{3}H]2-PMPA binding assay is amenable to high throughput screening, and it could provide a valuable tool in the search for novel GCPII inhibitors.

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