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Specific [^{3}H]-guanosine binding sites in rat brain membranes

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1 Extracellular guanosine has diverse effects on many cellular components of the central nervous system, some of which may be related to its uptake into cells and others to its ability to release adenine-based purines from cells. Yet other effects of extracellular guanosine are compatible with an action on G-protein linked cell membrane receptors.

2 Specific binding sites for [^{3}H]-guanosine were detected on membrane preparations from rat brain. The kinetics of [^{3}H]-guanosine binding to membranes was described by rate constants of association and dissociation of 2.6122×10^7 M^{-1} min^{-1} and 1.69 min^{-1}, respectively.

3 A single high affinity binding site for [^{3}H]-guanosine with a K_D of 95.4 ± 11.9 nM and B_{max} of 0.57 ± 0.03 pmol mg^{-1} protein was shown.

4 This site was specific for guanosine, and the order of potency in displacing 50 nM [^{3}H]-guanosine was: guanosine = 6-thio-guanosine > inosine > 6-thio-guanine > guanine. Other naturally occurring purines, such as adenosine, hypoxanthine, xanthine caffeine, theophylline, GDP, GMP and ATP were unable to significantly displace the radiolabelled guanosine. Thus, this binding site is distinct from the well-characterized receptors for adenosine and purines.

5 The addition of GTP produced a small concentration-dependent decrease in guanosine binding, suggesting this guanosine binding site was linked to a G-protein.

6 Our results therefore are consistent with the existence of a novel cell membrane receptor site, specific for guanosine.

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Abbreviations: ATP, adenosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; HEPES, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonicacid]; NBTG, S-(4-Nitrobenzyl)-6-thio-guanosine; NBTI, S-(4-Nitrobenzyl)-6-thioinosine; PNP, purine nucleoside phosphorylase

Introduction

Guanosine is a naturally occurring compound that, like its adenine-based counterpart adenosine, shows a spectrum of biological activities. Adenine-based purines are released extracellularly from many cell types, including neurons and astrocytes, and function as important intracellular signalling molecules that mediate diverse biological effects (Rathbone *et al*., 1998a,b; 1999). Recently it has become recognized that both neurons and glia also release guanine-based purines. GTP is stored in synaptic vesicles (Wagner *et al*., 1978) and indirect evidence indicates that guanosine can be released from neurons following depolarization (Fredholm & Vernet, 1979). Moreover the amount of guanine-based purines released from astrocytes under both basal conditions and after various types of stimulation, including hypoxia/hypoglycemia, is much greater than that of their adenine-based counterparts (Ciccarelli *et al*., 1999). In cultured astrocytes inhibition of ecto-5′-nucleotidase activity by α,β-MeADP significantly reduces accumulation of extracellular guanosine indicating that it, like extracellular adenosine, is largely derived from the extracellular metabolism of guanine-derived nucleotides (Caciagli *et al*., 2000).

Both extracellular guanosine and GTP can exert trophic effects in the nervous system (Rathbone *et al*., 1998b; 1999), including stimulation of astrocyte proliferation (Kim *et al*., 1991; Ciccarelli *et al*., 2000) synthesis and release of trophic factors from astrocyte cultures (Middlemiss *et al*., 1995; Caciagli *et al*., 2000) and the enhancement of differentiation of PC12 cells and hippocampal neurons *in vitro* (Gysbers & Rathbone, 1996a; Rathbone & Juurlink, 1993). Guanosine also exerts neuroprotective effects *in vivo* (Caciagli, personal communication) and *in vitro* (Rathbone *et al*., 1998b; Caciagli *et al*., 2000).

Some of the actions of the guanosine may be mediated intracellularly after its uptake. Yet many trophic effects of guanine-based purines are not substantially affected by the nucleoside uptake inhibitors, such as NBTI or dipyridamole (Gysbers & Rathbone, 1992), indicating that they are independent of intracellular mechanisms. Guanosine also stimulates the release of adenine-based purines from astrocytes, which may, in turn, be responsible for some other effects of guanosine (Ciccarelli *et al*., 2000). But this explanation is also incomplete, since many of the effects of guanine-based purines persist in the presence of adenosine-receptor and/or P2 purine receptor antagonists (Gysbers & Rathbone, 1992).

An alternative possibility is that there are distinct receptors for guanine-based purines. Gysbers *et al*. (2000) found that

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PC12 cells expressed binding sites for GTP with features consistent with a cell membranes receptor. Moreover, several of the effects of guanosine may be mediated through G-protein dependent signalling pathways involving cyclic nucleotides or MAP kinase pathway (Rathbone *et al*., 1991; Gysbers & Rathbone, 1996b; Caciagli *et al*., 2000) raising the possibility that some of the effects of guanosine involve activation of cell-surface receptors. If the brain does indeed contain specific receptors for guanosine, it should be possible to identify a high-affinity binding site for guanosine in brain membranes. This study reports the characterization of such a guanosine binding site.

Methods

Preparation of membranes

Crude rat brain membranes were prepared under conditions similar to those previously used to identify specific binding of [^{3}H]-adenosine (Schwabe *et al*., 1979; Traversa *et al*., 1984). Male Wistar rats (200–250 g) were used. The brains without cerebellum were quickly removed and homogenized in 9 volume of ice-cold buffer (0.25 M sucrose, 5 mM HEPES pH 7.4) by motor driven teflon-glass homogenizer rotating at 350 r.p.m., 7 up and down strokes. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The pellet, resuspended in 5–10 ml buffer, was centrifuged as above and the supernatants were pooled. The supernatant was centrifuged at $11{,}000 \times g$ for 20 min at 4°C. The pellet was resuspended in cold HEPES 20 mM pH 7.4, and was centrifuged at $100{,}000 \times g$ for 30 min at 4°C. The final pellet was resuspended in the same buffer to a final protein concentration of 3–5 mg per 1 ml. Aliquots (1 ml) were stored at −80°C. Before using in binding experiments, the membranes were washed twice with PBS buffer, 8.8 mM, and pH 7.4. The protein concentration was determined by the method of Lowry *et al*. (1951).

Binding assay

The measurement of [^{3}H]-guanosine binding in rat brain membranes was performed by the filtration technique, using GF/B glass fibre filters pre-soaked in 0.5% polyethylenimine for 2 h. Aliquots of 200–300 μg protein were incubated for 10 min at 25°C with 50 nM [^{3}H]-guanosine in a total volume of 0.5 ml PBS buffer. Unlabelled 1 mM guanosine was used to detect non-specific binding. The reaction was stopped by adding 2 ml cold PBS and the samples rapidly filtered by vacuum filtration. The filters were washed four times with 2 ml cold PBS. The procedure required less than 6 s to be completed. The dried filters were transferred in polyethylene vials to be counted by a liquid scintillation counter (Tri-Carb 2100TR, Packard, Meridien, CT, U.S.A.). Since guanosine is metabolized by the enzyme purine nucleoside phosphorylase (PNP), preliminary experiments were performed in the presence of several concentrations (5–200 μM) of acyclovir, an inhibitor of this enzyme (Tuttle & Krenitsky, 1984). No significant changes in total and non-specific binding were observed (data not shown). For saturation experiments a concentration range 6.25–300 nM [^{3}H]-guanosine was used. In competition experiments, displacing agents and 50 nM [^{3}H]-guanosine were added and the reaction was started by adding the membranes. The tubes were incubated for 10 min under standard assay conditions.

Identification of radioactive compounds in the incubation medium

Rat brain membranes (200 μg protein) were incubated with 50 nM [^{3}H]-guanosine in a final volume of 0.5 ml PBS buffer, pH 7.4, at 25°C. After 10 min incubation, the Eppendorf tubes were centrifuged and the supernatants collected. Aliquots of 50 μl of the radioactive supernatant or 50 μl of a standard mixture containing [^{3}H]-guanosine (2.5 pmoles), guanosine and guanine (250 pmoles) were injected.

HPLC analysis of guanosine and its metabolites was performed according to Ciccarelli *et al*. (1999) by using a reverse-phase ion pair technique. Separation was carried out with a reverse phase analytical column (LiChrospher 100 RP-18 5 μm, 250×4.6 mm I.D.-Merck) in a Kontron Instruments liquid chromatograph (50 μl loop valve injection). Elution was carried out applying a 15-min linear gradient from 100% 60 mM KH_2PO_4 plus 5 mM tetrabutylammonium phosphate, pH 6.0 (Solvent A) to 100% methanol/solvent A (30 : 70) (solvent B). The flow rate was kept constant at 1.5 ml min^{-1}. The amount of GBPs was measured on the basis of the absorption at 254 and 283 nm, by comparison with appropriate standards.

For the estimation of the radioactivity associated at each compound identified by HPLC an on-line radiochemical detector (Flo-one 500 TR Packard Instrument) was used. The liquid scintillation cocktail (Ultima-Flo M, Canberra Packard) was pumped at 3 ml min^{-1} and mixed continuously with the HPLC eluate. The mixture was passed through a 500-μl detector flow cell. Background radioactivity was subtracted from all radio-chromatograms. Radio-chromatograms were integrated and the d.p.m. under each peak was obtained. The retention times were: guanine 1.81 ± 0.20 min; guanosine 3.18 ± 0.15 min.

Drugs and chemicals

Guanosine [8-^{3}H], specific activity 7.4 Ci mmol^{-1}, was supplied by ICN, Irvine, California, U.S.A. Guanosine, inosine, guanine, GMP, GDP, GTP, ATP, adenosine, hypoxanthine, xanthine, nitrobenzylthioguanosine, nitrobenzylthioinosine, propentophylline, polyethylenimine, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonicacid]) were supplied by Sigma-Aldrich, Milano, Italy. All other chemicals were of analytical grade or the best commercially available.

Statistical analysis

The binding experiments were analysed by non linear regression analysis using the program Prism 3.02 (GraphPad Software, San Diego, CA, U.S.A.).

Results

Binding of [^{3}H]-guanosine to rat brain membranes

Both total and non-specific binding of 50 nM [^{3}H]-guanosine to rat brain membranes increased as a linear function of the

membrane concentration over a range of 50–400 μg protein (Figure 1a). Non-specific binding was measured in the presence of 1 mM guanosine; higher concentrations of guanosine, co-addition of other compounds, or washing the filter more than four times failed to further reduce non-specific binding. Specific binding amounted to about 50–70% of total membrane dependent binding. The optimal specific binding was displayed at pH 7.4 with a decline at more alkaline or acid pH values (data not shown). The binding was temperature-dependent over a range of 4–56°C (Figure 1b). Whereas non-specific binding did not change with increasing temperature, specific binding, and hence total binding, peaked between 25 and 37°C. At 56°C specific binding was completely abolished.

Association and dissociation kinetics

The time course of [^{3}H]-guanosine binding to rat brain membranes showed that the association kinetic was rapid (Figure 2a). The steady state was reached within 10 min and was maintained for up to 40 min. The observed association constant (K_{obs}) was 0.38 (0.31–0.45) min^{-1}. The dissociation of 50 nM [^{3}H]-guanosine from its binding site was measured by adding an excess of unlabeled guanosine after an equilibration time of 10 min. The addition of 1 mM guanosine reduced [^{3}H]-guanosine binding by about 80% at 1 min (Figure 2b), and reached a plateau within 2.5 min. No further reduction of binding was observed over longer periods of incubation. The dissociation constant (K_{off}) was 1.69 (1.29–2.08) min^{-1}. The calculated association rate constant (K_{on}) was 2.6122×10^7 M^{-1} min^{-1}. An independent estimate of dissociation constant at equilibrium (K_D) of 64.5 nM was obtained by the relationship $K_D = K_{off}/K_{on}$. This value was in reasonable agreement with the apparent high affinity constant calculated from the results obtained from the saturation studies described below.

Saturation isotherm studies

The saturation isotherm studies showed that the binding became saturable at 150–200 nM [^{3}H]-guanosine concentrations (Figure 3). The pooled data were fitted by a computerized non-linear regression analysis and resolved for the presence of a single high affinity binding site with an apparent $K_D = 95.4 \pm 11.9$ nM and $B_{max} = 0.57 \pm 0.03$ pmol mg^{-1} protein.

Displacement studies

To determine the specificity of the binding of guanosine for its receptor site we tested the ability of other purines to displace specifically-bound [^{3}H]-guanosine in rat brain membranes. None of the substances tested were potent displacers of [^{3}H]-guanosine. The potency order of the substances best able to displace 50 nM [^{3}H]-guanosine was of 6-thio-guanine $\geqslant$ guanosine > inosine > 6-thio-guanine > guanine. The displacement curves for guanosine, inosine and guanine are shown in Figure 4. The curves were resolved by non-linear regression analysis with a one-site model. The results of all molecules are shown in Table 1. The addition of GTP caused a weak concentration-dependent reduction of the specific binding of [^{3}H]-guanosine in a concentration range of 0.1–100 μM, and reached about 30% reduction at highest concentration. In contrast, GDP, GMP, hypoxanthine, xanthine, ATP adenosine and the guanosine derivative acyclovir, were unable to significantly displace [^{3}H]-guanosine from its binding sites even at very high concentrations. Xanthosine displayed a flat displacement curve, reaching about 50% at 1 mM concentration. To exclude the possibility that the [^{3}H]-guanosine bound to the purine transporter systems, we tested the ability of nitrobenzylthioguanosine (NBTG), hitrobenzylthioinosine (NTBI) and propentophylline, drugs which inhibit the uptake of nucleosides, to alter [^{3}H]-guanosine binding. Whereas

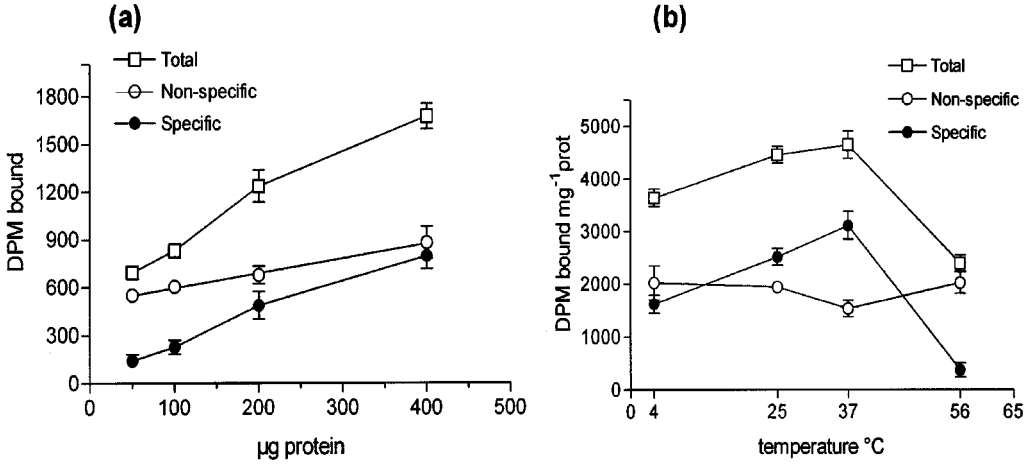

Figure 1 (a) Binding of [^{3}H]-guanosine to rat brain membranes as a function of protein concentration. Membranes were incubated with [^{3}H]-guanosine 50 nM for 20 min under standard assay conditions in the absence or the presence of 1 mM guanosine. The values are the mean ± s.e. of three experiments, each point in duplicate. (b) Temperature dependence of [^{3}H]-guanosine binding to rat brain membranes. Membranes were incubated with [^{3}H]-guanosine 50 nM for 20 min under standard assay conditions. One mM guanosine was used to detect non-specific binding. The values are the mean ± s.e. of two experiments, each point in triplicate.

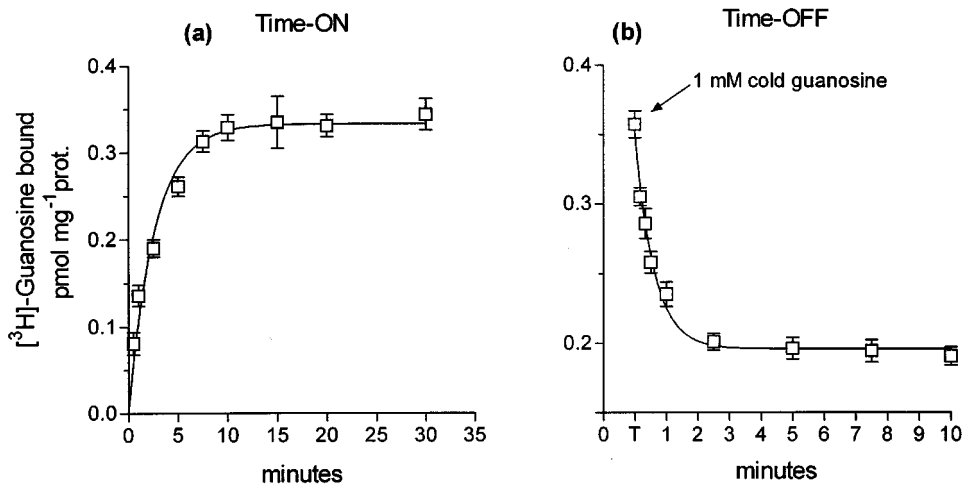

Figure 2 Kinetic of association and dissociation of [^{3}H]-guanosine binding to rat brain membranes. (a) Membranes were incubated with [^{3}H]-guanosine 50 nM under standard assay conditions for the various time intervals indicated. One mM guanosine was used to detect non-specific binding. The values are the mean ± s.e. of six experiments, each point in triplicate. $K_{obs} = 0.38$ (0.31 – 0.45) min^{-1}; 95% C.L. are given in parentheses. (b) Membranes were incubated with [^{3}H]-guanosine 50 nM for 10 min under standard assay conditions. At this time point, 1 mM guanosine was added to the mixture to prevent re-association, i.e. to initiate dissociation. The binding was measured at the time intervals indicated. The values are the mean ± s.e. of five experiments, each point in triplicate. $K_{off} = 1.69$ (1.29 – 2.08) min^{-1}; 95% C.L. are given in parentheses.

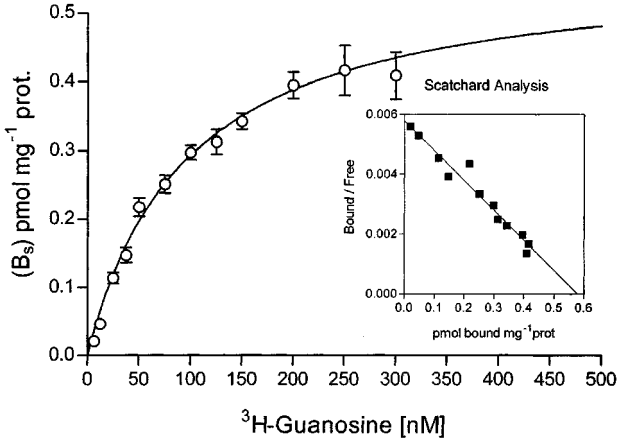

Figure 3 Saturation binding of [^{3}H]-guanosine at 25°C using membranes of rat brain. Membranes (200 μg protein) were incubated with increasing concentrations (6.25 – 300 nM) of [^{3}H]-guanosine under standard assay conditions. Non-specific binding was defined in the presence of 1 mM guanosine. Values are the means ± s.e. of five experiments, each point performed in triplicate. The data were fitted by a computerized nonlinear regression analysis and resolved with a one site model. $K_D = 95.4 \pm 11.9$ nM; $B_{max} = 0.57 \pm 0.03$ pmol mg^{-1} protein. Inset: Scatchard analysis of the data.

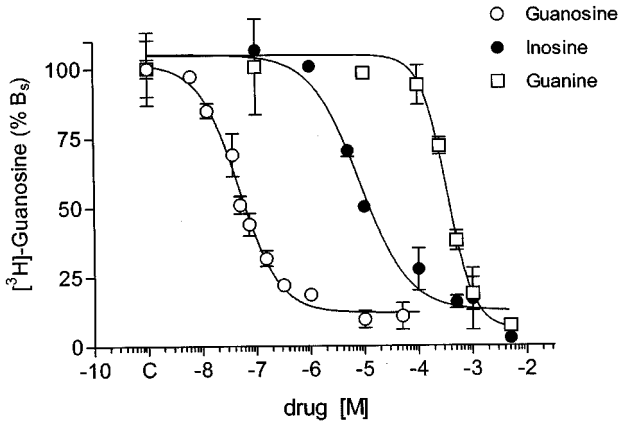

Figure 4 Displacement of 50 nM [^{3}H]-guanosine binding from membranes of rat brain by purines. The values are the mean ± s.e. of three experiments, each point in triplicate. The data were fitted by a computerized non-linear regression analysis and resolved with a one site model.

NBTI showed a small inhibition at 10 μM, neither NBTG or propentophylline had any effect on [^{3}H]-guanosine binding. Neither of the non-specific adenosine receptor antagonists, caffeine or theophylline in a concentration range 1 μM – 25 mM displaced [^{3}H]-guanosine from its binding site.

Identification of radioactive compounds in the incubation medium

Guanosine is a naturally occurring compound, subject to metabolism by ecto-enzymes bound to brain membranes. To ensure that under the conditions of our experiments [^{3}H]-guanosine was not substantially metabolized, we tested its fate using HPLC analysis. These experiments also confirmed the identity of the [^{3}H]-labelled compound bound to the membranes. As shown in Figure 5a,b in 8.8 mM PBS buffer pH 7.4, 15% of 50 nM [^{3}H]-guanosine was degraded to guanine after 10 min incubation at 25°C. At time zero, all of the radioactivity was identified as [^{3}H]-guanosine. When the reaction was performed in the presence of 1 mM inosine, no metabolism of [^{3}H]-guanosine was observed (data not shown). Since inosine inhibits purine nucleoside phosphorylase (PNP) activity (Ropp & Traut, 1991), this implies that PNP was principally responsible for the degradation of [^{3}H]-guanosine in this experiment.

Table 1 Effects of purines and various agents in displacing 50 nM [^{3}H]-guanosine binding to rat brain membranes

Agent	IC_{50} (μM with 95% C.L.)
Guanosine	0.048 (0.038–0.060)
Inosine	8.44 (4.55–15.65)
Guanine	334.44 (204.1–548.0)
Thio-guanosine	0.046 (0.024–0.088)
Thio-guanine	317.1 (222.6–452.7)
GMP	>1000 (0%)
GDP	>1000 (0%)
GTP	>100 (34%)
Adenosine	>1000 (14%)
ATP	>5000 (20%)
Hypoxanthine	>100 (12%)
Xanthine	>1000 (15%)
Xanthosine	>1000 (50%)
Theophylline	>1000 (6%)
Caffeine	>1000 (0%)
Acyclovir	>1000 (0%)
NBTG	>100 (0%)
NBTI	>100 (30%)
Propentophylline	>100 (0%)

Values were determined at least with five concentrations of each agent in triplicate incubations, in three separate experiments. Values given in parentheses indicate the per cent inhibition obtained by the concentration indicated when the inhibition is less than 50%.

Discussion

Several lines of evidence have raised the possibility that specific cell surface receptors for guanosine may exist (Rathbone *et al*., 1999). A key prediction of this hypothesis is that the plasma membranes contain specific high affinity binding sites for guanosine. The results of the present study are the first direct evidence for the presence of a high-affinity binding site for [^{3}H]-guanosine in rat brain membrane preparations. The membranes represent principally plasma and synaptic membranes and were prepared by techniques commonly accepted to produce such fractions (Hulme & Buckley, 1992) and that have been used earlier to identify receptors for [^{3}H]-adenosine (Schwabe *et al*., 1979; Traversa *et al*., 1984). Moreover, [^{3}H]-guanosine did not bind to cytosolic fractions (data not shown). Specific binding of guanosine was rapid, reversible and saturable, and thus fulfils important criteria for the characterization of hormone and drug receptors.

The kinetics of association and dissociation of [^{3}H]-guanosine were similar to those observed for the binding of [^{3}H]-adenosine to adenosine receptors in membranes from rat brain (Schwabe *et al*., 1979; Traversa *et al*., 1984). Both association and dissociation were very rapid, which is characteristic of the binding of several natural compounds to their receptors.

The saturation curve indicates the presence of a single high affinity binding site since it is resolved by non-linear regression analysis with a one-site model with a K_D of 95.4 ± 11.9 nM and an apparent maximal number of binding sites of 0.57 ± 0.03 pmol mg^{-1} protein. This was confirmed by homologous competitive binding experiments; in which unlabelled guanosine displaced 50 nM [^{3}H]-guanosine in a monophasic manner, with an IC_{50} of 48.3 nM.

The competition curves of guanosine and related compounds, at concentrations ranging over several orders of magnitude, demonstrate the specificity of the [^{3}H]-guanosine binding site. The data confirm that this site is distinct from the well characterized adenosine and purine nucleotide binding sites. Neither adenosine, ATP, nor the non-specific adenosine receptor antagonists caffeine and theophylline, reduced [^{3}H]-guanosine binding.

The relative abilities of analogues to displace [^{3}H]-guanosine provide insight into the characteristics of the binding site. 6-Thio-guanosine and guanosine were equally effective in displacing [^{3}H]-guanosine, whereas adenosine was ineffective. These data indicate that a 6-keto or 6-thio group is important, and that a 6-amino group greatly reduces the binding affinity. Similarly, the 2-amino group appears to be important, since xanthosine, which has a 2-keto rather than a 2-amino group, could displace [^{3}H]-guanosine, although the displacement curve was flat. The ribose moiety also appears important. The bases, 6-thio-guanine and guanine were three orders of magnitude less effective than guanosine in displacing [^{3}H]-guanosine. Moreover, the guanosine analogue acyclovir (9-(2-hydroxyethoxymethyl) guanine), in which the ribose is replaced, was also ineffective.

Although our data are compatible with the [^{3}H]-guanosine binding site being a cell membrane guanosine receptor, other possible membrane binding sites also exist. These include the nucleoside transporter systems and the enzyme purine nucleoside phosphorylase. We consider these possibilities unlikely for the following reasons. First, the binding of [^{3}H]-guanosine to the membranes was unaffected by co-incubation with the inhibitors of the nucleoside transporter. Second, the inability of other purine nucleosides to displace guanosine also makes it unlikely that the binding could be to a non-specific purine nucleoside transporter. Recently a mammalian guanosine-specific transporter has been described (Flanagan & Meckling-Gill, 1997), but the concentrations of guanosine tested were four orders of magnitude higher than those used in the binding studies we describe in this study. Moreover inosine was inhibitory only at a concentration of 1 mM, whereas in our experiments inosine displaced guanosine with an IC_{50} of 8.8 μM. Finally, in analogous binding experiments using [^{3}H]-adenosine to define adenosine receptors, no significant binding was reported to the nucleoside transporters (Schwabe *et al*., 1979).

Recently, it was shown that extracellular guanine nucleotides, and to lesser extent the nucleoside guanosine, exerted *in vitro* effects on glutamatergic transmission (Baron *et al*., 1989; Migani *et al*., 1997), inhibiting the binding of several glutamatergic ligands as well as excitotoxic cell response to glutamate and analogues (Baron *et al*., 1989; Schmidt *et al*., 2000). To exclude that the observed [^{3}H]-guanosine binding site could somehow be related to glutamate receptors, competition experiments with glutamate were performed. Up to 1 mM concentration of glutamate no displacement was observed (data not shown).

The enzyme purine nucleoside phosphorylase (PNP) is principally cytosolic, but recent studies have shown the existence of an ecto-PNP activity in cultured astrocytes (Giuliani *et al*., 1999; Caciagli *et al*., 2000). In our experiments we observed a small amount, 15%, of conversion of labelled guanosine to guanine. This conversion was inhibited by 1 mM inosine, high concentrations of which are

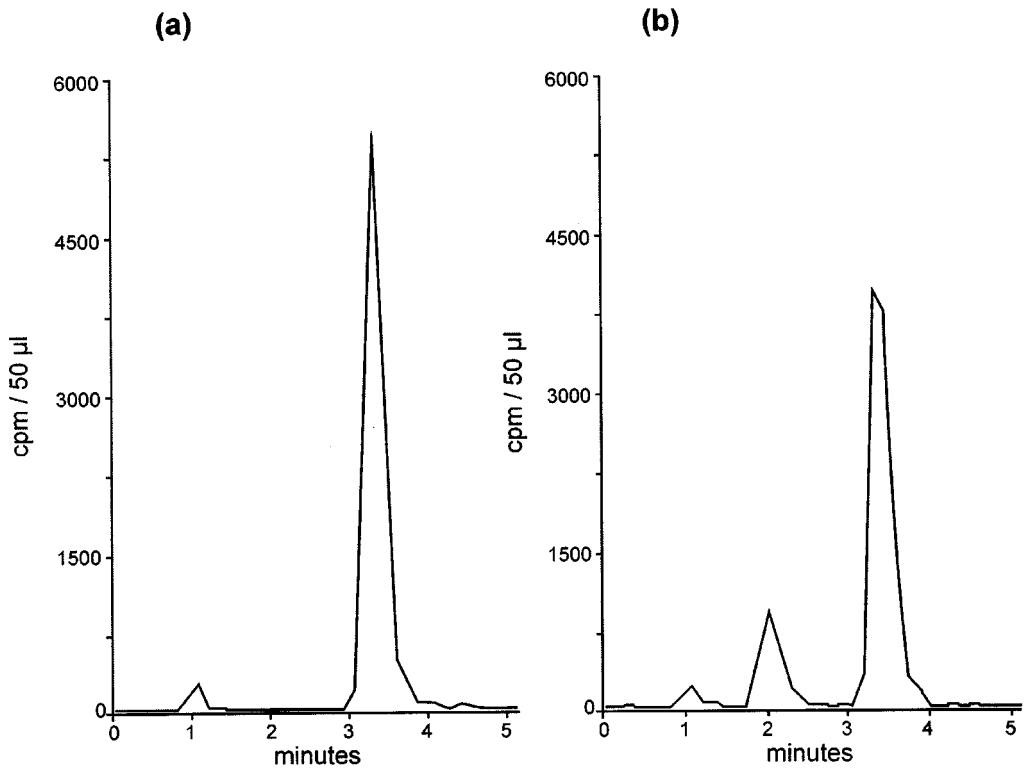

Figure 5 (a) HPLC analysis of [^{3}H]-guanosine added to rat brain membranes, at time 0. The chromatogram is from a representative experiment that was repeated three times. [^{3}H]-guanosine (25 pmol) was added to rat brain membranes in a final volume of 500 μl under the same conditions as the binding assay. The tube was immediately centrifuged. Fifty μl of the supernatant was injected into an HPLC apparatus and guanosine was detected as a single peak at 3.2 min elution time. The peak of [^{3}H]-guanosine eluted from the experimental samples was at the same position as that obtained by analysing a standard solution of radioactive guanosine by HPLC. The amount recovered by integrating the peaks of 3 experiments was 2.456 ± 0.029 pmol. (b) HPLC analysis of [^{3}H]-guanosine added to rat brain membranes, at 10 min. The chromatogram is from a representative experiment that was repeated three times. The experiment was performed as described above, but the tube was centrifuged after 10 min of incubation. Fifty μl of the supernatant was injected into an HPLC apparatus. Guanosine was detected as a large peak at 3.2 min elution time, and the small peak at 2.00 min elution time was identified as guanine, based on a comparison with a reference solution of guanine analysed by HPLC with u.v. detection. The amounts recovered by integrating the peaks of three experiments were 2.141 ± 0.02 pmol and 0.383 ± 0.02 pmol for guanosine and guanine, respectively.

known to inhibit the activity of PNP (Ropp & Traut, 1991). These data suggest the presence of ecto-PNP in rat brain membrane preparations. The ecto-PNP could be an isoform of the well known cytosolic PNP. Certainly that would be compatible with the inability of acyclovir, an inhibitor of cytosolic PNP (Tuttle & Krenitsky, 1984), to increase [^{3}H]-guanosine binding in our experiments. Moreover, it is unlikely that the [^{3}H]-guanosine binding sites we detected are localized on PNP, since the kinetics of ecto-PNP activity, with a K_M of 18 μM for guanosine (Caciagli *et al*., 2000), are incompatible with the binding data obtained in the experiments reported here. The small amount of degradation of [^{3}H]-guanosine under the conditions of our binding experiments does not materially affect our conclusions regarding the characteristics of the site, other than a potential over estimation of the binding affinity (K_D) of guanosine at this site.

The high affinity [^{3}H]-guanosine binding site may well be a guanosine receptor. Certainly, there is extensive biological and biochemical evidence that extracellular guanosine exerts a plethora of biological effects, some of which may be mediated by a specific guanosine receptor. For example, guanosine stimulates cell proliferation (Kim *et al*., 1991; Ciccarelli *et al*., 2000), enhances outgrowth of neurites from cultured cells (Gysbers & Rathbone, 1996a; Rathbone & Juurlink, 1993), stimulates the synthesis and release of a variety of trophic factors from astrocytes and other cells (Middlemiss *et al*., 1995; Caciagli *et al*., 2000) and enhances the release of adenosine and adenine nucleotides from astrocytes (Ciccarelli *et al*., 1999; 2000). Some actions of guanosine may be mediated intracellularly, but many effects of extracellular guanosine are unaffected by nucleoside uptake inhibitors, such as NBTI or propentofylline (Rathbone *et al*., 1998b), indicating that they are independent of intracellular mechanisms. Other effects of extracellular guanosine may be mediated *via* the release of adenine-based purines, which have an autocrine and paracrine effect (Ciccarelli *et al*., 2000). However, many of the effects of extracellular guanosine persist in the presence of adenosine and/or P2 purine receptor antagonists (Gysbers & Rathbone, 1992). This indicates that many of the effects of exogenous guanosine do not involve activation of adenosine receptors. These observations, together with the binding data presented here, lead us to suggest the presence of a putative cell surface receptor for guanosine. The signal transduction mechanisms linked to these receptors are unknown. However, they may be

linked *via* G-proteins to the MAPK cascade since, for example, the ability of guanosine to enhance synthesis of trophic factors in astrocytes is associated with an increase in phosphorylation of ERK1 and ERK2 and is blocked by pre-treatment with pertussis toxin (Caciagli *et al*., 2000).

Both neurons and glia release guanine-based purines under physiological and pathological conditions (Fredholm & Vernet, 1979; Di Iorio *et al*., 1998; Ciccarelli *et al*., 1999). Extracellular guanosine, like extracellular adenosine, is largely derived from the corresponding nucleotides, through the activity of ecto 5′-ectonucleotidases (Giuliani *et al*., 1999; Caciagli *et al*., 2000). At least in astrocytes, the amount of guanine-based purines released under basal conditions and under various types of stimulation is much greater than that of adenine-based purines (Ciccarelli *et al*., 1999). The extracellular concentration of guanosine in brain under basal conditions is 0.47 μM (Zetterstrom *et al*., 1982) and increases several-fold under pathological conditions (Hagberg *et al*., 1987; Ciccarelli *et al*., 1999). Thus under physiological and pathological conditions, at least some of the effects of extracellular guanosine could be exerted by binding to the specific cell membrane sites described.

Currently our laboratories are elucidating the structure of the guanosine binding site and investigating the signal transduction pathways mediated by the putative guanosine receptor in normal and pathological neuronal function.

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