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Regulation of cerebellar L-[^{3}H]glutamate binding: influence of guanine nucleotides and Na^+ ions

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Purine nucleotides serve important functions in many biological processes. For example, the cyclic hydrolytic products of ATP and GTP (cylcic AMP and cyclic GMP, respectively) regulate cell growth and development [1], mediate hormonal [2], and certain neurotransmitter [3] responses, and, in addition purines themselves may have transmitter roles in the periphery [4] and central nervous system [5].

The receptor binding of many hormones or transmitters which effect changes in adenylate cyclase activity, is sensitive to guanine nucleotides: GTP and GDP have been demonstrated to produce selective decreases in the affinities of epinephrine [6], dopamine [7], serotonin [8] and opiates [9] for their receptors by an allosteric regulatory mechanism. It has been proposed that sensitivity to guanine nucleotides is a property of only these receptors that are linked to adenylate cyclase [10]. However, recent evidence indicates that ligand binding to the D2 dopamine receptor may also be regulated by these nucleotides [11].

We have reported previously that the binding of L-glutamate to its postsynaptic receptor on rat cerebellar membranes is influenced by an as yet unidentified endogenous inhibitor [12] and that guanine nucleotides, in contrast to adenine derivatives, produce substantial inhibition of specific binding [13].

This study was carried out in order to investigate the mechanisms underlying the inhibitory actions of guanine nucleotides and sodium ions on glutamate binding.

Materials and methods

Preparation of synaptic membranes. Female Wistar rats (250–300 g) were killed by decapitation and cerebella were homogenised in 20 vol. (w/v) 0.32 M sucrose in a Teflon–glass homogeniser (0.25 mm clearance). Synaptic membranes were then prepared as described previously [14]. The membrane pellet was suspended in glass-distilled water and subjected to a 30 min preincubation at 37°, followed by two or three washing stages with 50 vol. water, and recentrifugation of the membranes (50,000 *g* for 10 min).

The final pellet was dispersed in 50 mM Tris–citrate buffer (pH 7.1) or in buffer with added nucleotide (as the sodium salts) or sodium chloride, and used immediately for the binding assay.

During some of these experiments, aliquots of homogenates and supernatants were taken during various stages of membrane preparation, and were assayed for the presence of L-glutamate by a microenzymic fluorometric method [15]. No free glutamate was detectable in the final membrane preparation as compared with 250 nmole/g tis-

sue for the P_2 preparation (sensitivity limit of assay = 0.1 nmole).

The L-[^{3}H]glutamate binding assay was carried out in 1.5 ml polypropylene microcentrifuge tubes (Elkay Products Inc.). 0.5 ml of membrane suspension (0.1–0.5 mg protein) with or without the substance under test, and 10 μl of either unlabelled L-glutamate (1 mM final concentration) or buffer were prepared on ice and preincubated at 37° in a shaking water bath. Addition of 25 μl of L-[^{3}H]glutamate (29 Ci/mmole) at a final concentration of 1–1000 nM initiated the incubation proper, which was carried out for a further 10 min, after which the tubes were centrifuged at 10,000 g for 30 sec. The pellets remaining following aspiration of the supernatant were dissolved in 0.5 ml 12% NaOH in methanol (w/v) and bound radioactivity determined by liquid scintillation counting. Specific L-glutamate binding was calculated as described previously [12].

Results and discussion

Low concentrations of sodium ions (1–10 mM) produced 23–43% inhibition of specific [^{3}H] glutamate binding. However, salt concentrations between 25–100 mM markedly enhanced the binding (215% increase at 100 mM). In the subsequent kinetic study, the binding of various concentrations of [^{3}H] glutamate was measured at a sodium concentration of 10 mM. A significant reduction in the apparent affinity of glutamate for its receptors was observed, without any significant change in the number of binding sites as compared with control (Figs. 1 and 2).

A number of guanine derivatives were found to influence glutamate binding to cerebellar membranes. The rank order of potency was cGMP > GTP > GDP > guanosine as determined from their IC_{50} values (Table 1).

As reported previously [12, 16] [^{3}H] glutamate binding involved a single population of sites and was saturable with an apparent $Kd = 0.19 \pm 0.05$ μM and capacity, $B_{max} = 33.4 \pm 0.35$ pmole/mg protein. In the presence of guanine nucleotides (1 mM final concentration), however, the affinity was reduced at least tenfold with the density of the receptors being unaffected (Figs. 1 and 2).

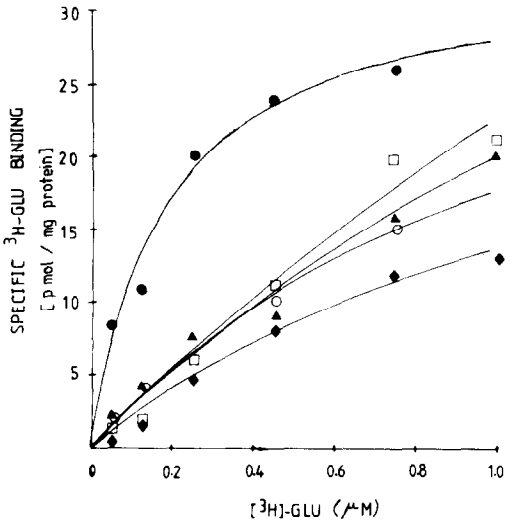

Fig. 1. The effect of guanine nucleotides and sodium on kinetics of [^{3}H[glutamate binding investigated over a range of glutamate concentrations (0.001–1.0 μM). Results are means of triplicate determinations for each point. ●, control; ○, 1 mM cGMP; □, 1 mM GDP; ▲, 1 mM guanosine; ♦, 10 mM Na^+.

The possible existence of an endogenous inhibitor, or inhibitors of the glutamate receptor system, was suggested by the earlier finding of a 67% reduction in [^{3}H]glutamate binding following the re-addition of the supernatant from incubated, to control membranes [12]. This inhibitory effect may be due, at least in part, to the presence of endogenous glutamate, since extensive washing procedures are required

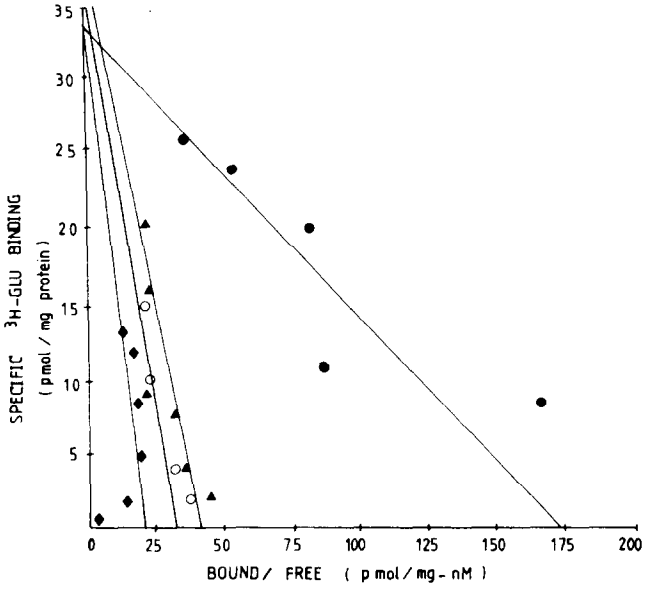

Fig. 2. Eadie–Hofstee plot of [^{3}H]glutamate binding in presence or absence of added nucleotide or sodium chloride. Data replotted from Fig. 1. ●, control; ○, cGMP; ▲, guanosine; ♦, Na^+.

Table 1. Effects of guanine derivatives and Na^+ on kinetic parameters of [^{3}H]glutamate binding

Compound	IC_{50} (μM)	Kd (μM)	Bmax (pmol/mg protein)
- (control)	-	0.19 ± 0.05	33.40 ± 0.35
cGMP	30.1	1.98 ± 0.47**	38.50 ± 10.00
GTP	63.0	N.D.	N.D.
Guanosine	457.0	2.42 ± 1.71*	68.00 ± 36.80
GDP	257.0	3.00 ± 2.63*	87.35 ± 62.15
Na^+ (10mM)	-	1.60 ± 0.79*	35.30 ± 12.00

IC_{50} Values were determined from log dose/percentage inhibition plots over a wide range of inhibitor concentrations (quadruplicate determinations) at an L-[^{3}H]glutamate concentration = 450 nM. Kinetic analyses were carried out in the presence (1 mM) or absence of added guanosine derivatives. Values for *Kd* and B_{max} were calculated by computer, using the method of Wilkinson [24] as applied to the data depicted in Figs. 1 and 2. Significance of difference of kinetic parameters from control values by unpaired *t*-test; * $P < 0.05$, ** $P < 0.01$.

to remove this substance. It is, however, probable that other inhibitory agents such as endogenous purines and membrane components, for example, free fatty acids, may be liberated during preparation and assay. Indeed, membrane gangliosides have been reported to inhibit glutamate receptor activity in synaptic membranes [17].

The inhibitory action of Na^+ on glutamate binding is in accord with other reports of monovalent cations reducing ligand–receptor interactions [14, 18, 19]. However, the results contrast with those of Baudry and Lynch [20] for the binding of [^{3}H]glutamate to hippocampal membranes, where no change in affinity was apparent in the presence of Na^+, although there was a reduction in the number of sites. This discrepancy may be due to dissimilar preparative methods and to the different brain regions studied.

In hormonal and neurotransmitter systems where adenylate cyclase-linked receptors exist, guanine-based purine nucleotides facilitate receptor–cyclase coupling, whilst attenuating the ligand–receptor interaction. In this study, where there is no evidence for any coupling between the glutamate receptor and adenylate cyclase, it is particularly interesting that a very marked effect of the guanine derivatives was observed. The kinetic studies performed in the presence of GTP, GDP, cGMP or guanosine indicated that the reduction in glutamate binding induced by these compounds was essentially attributable to a 12-fold decrease in receptor affinity (*Kd*'s increase from 0.19 to 2.4 μM) with the apparent B_{max} remaining unchanged. An explanation of these findings may be that the guanyl compounds accelerate the rates of association and dissociation of [^{3}H]glutamate from the receptors (with a resultant increase in *Kd*), as may be the case for the effects of GTP on [^{3}H]serotonin [21] and opiate binding [9]. It would seem likely that the modulation of [^{3}H]glutamate binding is through an allosteric mechanism which alters the conformation of the binding site. However, the large variance of the data seen with the guanine derivatives (Table 1) precluded any convincing assessment of possible co-operative effects by construction of Hill slopes.

In summary, the major finding of this study was that guanine derivatives markedly influence the binding of glutamate, by reducing the apparent affinity for the receptor. This therefore adds further doubt to the concept that only adenylate cyclase-coupled receptor systems can so be regulated. It is, however, worth noting that in the cerebellum, glutamate and structural analogues are powerful activators of guanylate cyclase [22]. It is possible that here also there is a purine binding site located on the nucleotide regulatory protein which is thought to be associated with the receptor, as is the case for adenylate cyclase-coupled systems in membranes derived from various tissues [23].

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