Biochemical Pharmacology, Vol. 35, No. 6, pp. 991–994, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

PURINE NUCLEOTIDES INHIBIT THE BINDING OF DL-[^{3}H] 2-AMINO-4-PHOSPHONOBUTYRATE (DL-[^{3}H] APB) TO L-GLUTAMATE-SENSITIVE SITES ON RAT BRAIN MEMBRANES

STEVEN P. BUTCHER,*† PETER J. ROBERTS*‡ and JAMES F. COLLINS§

* Department of Physiology and Pharmacology, University of Southampton, Southampton, U.K. and § Department of Chemistry, City of London Polytechnic, London, U.K.

(*Received* 27 *June* 1985; *accepted* 4 *October* 1985)

Abstract—The effects of purine nucleotides on the binding of DL-[^{3}H] 2-amino-4-phosphonobutyrate (DL-[^{3}H] APB) to rat brain membranes were investigated. Certain guanine nucleotides, especially cyclic GMP and GTP, were found to be potent inhibitors of binding. Kinetic studies revealed that both cyclic GMP and GTP acted to decrease receptor affinity without affecting significantly binding site density. These endogenous substances may therefore play an important role in the regulation of excitatory amino acid receptor function.

The regulation of excitatory amino acid receptor function can be probed conveniently using the radioligand binding technique. A number of endogenous substances, including sodium, calcium and chloride ions [1–5], as well as membrane associated factors such as phosphatidylserine [6] and gangliosides [7], have been reported to influence L-[^{3}H] glutamate binding. The regulatory properties of at least some of these agents may be of physiological significance.

Purine nucleotides have, in common with their effects on several other neurotransmitter binding sites [8–12], also been shown to modulate L-[^{3}H] glutamate binding [5]. Since excitatory amino acids are known to elevate neuronal levels of cyclic nucleotides [13, 14], this may represent another mechanism by which receptor function is controlled. In the present study we have used the novel, glutamate-like radioligand, DL-[^{3}H] 2-amino-4-phosphonobutyrate (DL-[^{3}H]APB),‖ which labels a discrete subpopulation of glutamate binding sites [15, 16], to examine the specificity and possible significance of this process.

METHODS AND MATERIALS

The methods used to prepare crude synaptic membranes and to determine specific DL-[^{3}H] APB binding have been described in detail previously [15]. Briefly, albino Wistar rats (250–300 g; either sex) were killed by decapitation and their brains were removed rapidly. Following homogenisation in 0.32 M sucrose/5 mM HEPES–KOH buffer (pH 7.4), a mitochondrial-synaptosomal enriched (P_2) pellet was prepared by differential centrifugation. This was lysed in hypotonic 5 mM HEPES–KOH buffer (pH 7.4) and washed several times by rapid centrifugation followed by resuspension in fresh buffer. The final synaptic membrane enriched pellet was resuspended in 50 mM HEPES–KOH buffer (pH 7.4) containing 2.5 mM calcium chloride and the appropriate nucleotide. Specific DL-[^{3}H] APB binding was determined by incubation of the membrane suspension at 37° for 10 min with 25 μl of radioligand (26.1 Ci/mmol), NEN; 30 nM final concentration except where indicated) and 25 μl of either buffer (total binding) or 1 mM L-glutamate (non-specific binding). Binding assays were terminated by rapid centrifugation and the radioactive content of each pellet was determined by liquid scintillation counting.

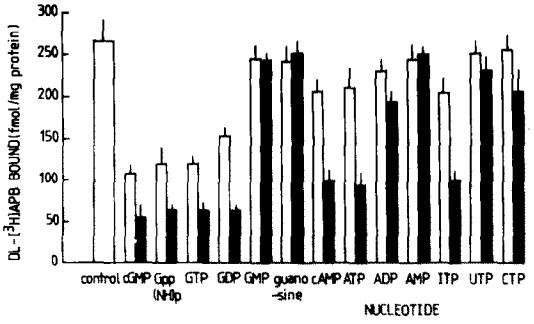

Fig. 1. The effect of nucleotide analogues on specific DL-[^{3}H] APB binding. Whole rat brain synaptic membranes were incubated in 50 mM HEPES-KOH buffer (pH 7.4) containing 30 nM DL-[^{3}H] APB, 2.5 mM calcium chloride and nucleotides as indicated. Assays were continued for 10 min at 37° in the presence, or absence of L-glutamate (1 mM). Results are the means ± S.E.M. of three experiments performed in quadruplicate. Key: filled bars, 1 mM final nucleotide concentration; open bars, 100 μM final nucleotide concentration.

† Present address: Department of Neurobiology, University of Göteborg, Göteborg, Sweden.

‡ To whom all correspondence and reprint requests should be sent.

‖ Abbreviations used: APB, 2-amino-4-phosphonobutyrate; HEPES, N-2-hydroxyethyl-N^1-2-ethanesulphonic acid.

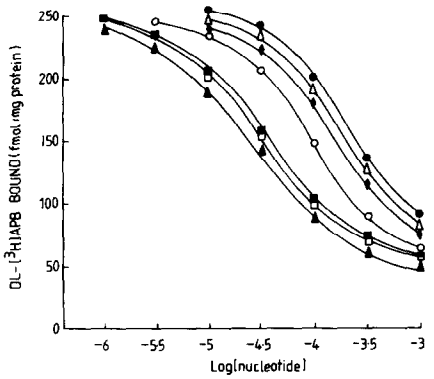

Fig. 2. Concentration dependence of the effects of nucleotide analogues on specific DL-[^{3}H] APB binding. The inhibitory effects of active nucleotide derivatives were examined by incubation of synaptic membranes with 50 mM HEPES–KOH buffer (pH 7.4) containing 2.5 mM calcium chloride and a wide range of nucleotide concentrations (1 μM–1 mM). Assays were performed at 37° for 10 min with the inclusion of 30 mM DL-[^{3}H] APB. Non-specific binding was defined by addition of L-glutamate (1 mM). Results are the means of quadruplicate determinations from three independent experiments and errors were less than 15% in each case. Key: ▲, cyclic GMP; □, Gpp(NH)p; ■, GTP; ○, GDP; △, cyclic AMP; ◆, ITP; ●, ATP.

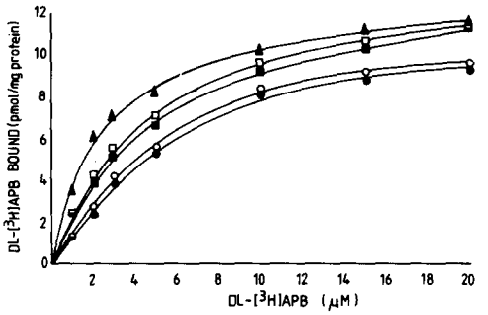

Fig. 3. Saturation analysis of specific DL-[^{3}H] APB binding in the presence, and absence, of added nucleotides. Specific DL-[^{3}H] APB binding was determined over a wide ligand concentration range (0.5–20 μM) in 50 mM HEPES–KOH buffer (pH 7.4) containing 2.5 mM calcium chloride and either cyclic GMP or GTP (final concentration 100 μM or 1 mM). Binding assays were performed at 37° for 10 min and non-specific binding was defined by addition of L-glutamate (1 mM). Results are the means of two experiments performed in quadruplicate. Key: ▲, control; □, 100 μM cyclic GMP; ■, 100 μM GTP; ○, 1 mM cyclic GMP; ●, 1 mM GTP.

RESULTS

Preliminary results revealed that several nucleotides (added as their monosodium salts) decreased specific DL-[^{3}H]APB binding to rat brain membranes (Fig. 1). When added at a final concentration of 1 mM, the most effective inhibitors were found to be cyclic GMP, GTP, Gpp(NH)p and GDP. Although certain other nucleotides, notably cyclic AMP, ATP and ITP, also decreased binding, little or no inhibition was observed in the presence of GMP, guanosine, AMP, CTP or UTP. At a lower concentration (100 μM) only cyclic GMP, GTP and Gpp(NH)p were found to reduce binding to less than 50% of the control value. With the exception of GDP, which inhibited binding to a lesser extent, all other nucleotides possessed only weak or negligible activity. In order to quantify these effects, concentration-response curves were constructed using a wide range of nucleotide concentrations (Fig. 2). IC_{50} values (the concentration of nucleotide required to inhibit binding by 50%) were calculated directly from these plots (Table 1). Data obtained using this approach revealed that cyclic GMP was the most potent inhibitor of binding. GTP and GPP(NH)p were approximately equipotent and were both only marginally less active than cyclic GMP. GDP exhibited an intermediate potency whereas cyclic AMP, ATP and ITP were 7–8 times less effective inhibitors.

Table 1. Inhibition of DL-[^{3}H] APB binding to rat brain membranes by nucleotide analogues

Nucleotide	IC_{50} (μM)
cGMP	42
Gpp(NH)p	63
GTP	69
GDP	125
ITP	251
cAMP	300
ATP	346
Guanosine, GMP, ADP, AMP, UTP and CTP	>1000

Synaptic membranes were incubated at 37° for 10 min with 30 nM DL-[^{3}H] APB in the presence, or absence, of a wide range of concentrations (1 μM–1 mM) of the compound under test. IC_{50} values (the nucleotide concentration required to produce 50% inhibition of binding) were read directly from log concentration–% inhibition curves (see Fig. 2). Results are the means of three independent experiments.

The effect of the two most potent endogenous nucleotides, cyclic GMP and GTP, on the kinetic characteristics of DL-[^{3}H]APB binding was also examined. Synaptic membranes were incubated with a wide range of DL-[^{3}H] APB concentrations (0.5–20 μM) in the presence, or absence, of added nucleotides. The appropriate saturation curves were constructed (Fig. 3), and using a computer assisted curve fitting program [17], binding site affinity (K_d) and density (B_{max}) were determined (Table 2). A single, saturable site appeared to participate in the binding process under all conditions. Addition of cyclic GMP and GTP (100 μM final concentration) decreased the affinity of the binding site but did not affect binding site density. When the final concentration of these nucleotides was raised to 1 mM, a further reduction in binding site affinity and, in addition, a small decrease in the number of binding sites was noted.

DISCUSSION

These data demonstrate that the binding of the novel radioligand, DL-[^{3}H] APB, to L-glutamate sensitive sites on rat brain membranes is inhibited by purine nucleotides. This effect exhibited a high degree of specificity and was most obvious in the presence of certain guanosine containing nucleo-

Table 2. Effect of cyclic GMP and GTP on the kinetic characteristics of DL-[3H] APB binding to rat brain membranes

Nucleotide	Dissociation constant (K_D) (μM)	Binding site density (B_{max}) (pmol/mg protein)
No addition	1.32 ± 0.36	12.77 ± 0.88
100 μM cyclic GMP	3.43 ± 0.59	12.06 ± 0.88
100 μM GTP	3.11 ± 0.70	12.35 ± 0.68
1 mM cyclic GMP	4.58 ± 0.61	10.26 ± 1.32
1 mM GTP	4.56 ± 0.52	10.15 ± 1.43

Whole rat brain synaptic membranes were incubated at 37° for 10 min with DL[3H] APB (0.5–20 μM) in the presence, or absence, of nucleotide derivatives. Non-specific binding was defined by addition of 1 mM L-glutamate. Results are means ± S.E.M. of quadruplicate determinations from two independent experiments, and were determined from untransformed binding data by the method of Wilkinson [19].

tides. The most potent inhibitors were found to be cyclic GMP, GTP and Gpp(NH)p. These derivatives produced half-maximal inhibition of binding in the low- to mid-micromolar range. Other guanine nucleotides, with the exception of GDP, were essentially inactive. A number of non-guanine nucleotides, notably cyclic AMP, ATP and ITP, also possessed inhibitory activity. However, when compared with the IC_{50} value of the corresponding guanine nucleotide, a 7–8 times higher concentration of these adenosine and inosine derivatives was required to obtain a similar effect. Although this inhibitory profile resembles that reported for other neurotransmitter binding sites [5, 8–12], a number of anomalies are apparent. Non-guanine nucleotides are certainly more effective as inhibitors of the binding of DL-[3H] APB than of the other radioligands tested, and a potent effect of cyclic GMP has only been reported in one previous investigation of L-[3H] glutamate binding [5] and in the present study. The physiological significance of this pattern of specificity is difficult to assess because the precise concentration of individual nucleotides at the site of action is unknown. A regulatory role for cyclic GMP and GTP might be anticipated on the basis of their increased inhibitory activity within a concentration range likely to be of relevance physiologically. Kinetic studies demonstrated that low concentrations of these endogenous nucleotides act primarily to decrease the affinity of DL-[3H] APB binding sites. When nucleotide concentrations were raised, a reduction in binding site density was also noted and this may be related to the detergent properties of nucleotides [18].

A further complication is introduced by the use of monosodium nucleotide salts in the present study. We have shown previously that sodium ions can themselves inhibit DL-[3H]APB binding in the millimolar concentration range [19], and this effect may become relevant when high concentrations of nucleotides are used (sodium inhibits DL-[3H]APB binding by 20% at 1 mM final concentration [19]). In contrast, cyclic GMP and GTP reduce DL-[3H] APB binding in the micromolar concentration range and sodium ions are unlikely to influence binding under these conditions.

Previous studies on other neurotransmitter binding sites also indicate that a decrease in binding site affinity underlies the inhibitory properties of purine nucleotides [5, 8–12]. A common molecular mechanism, perhaps an alteration in the configuration of binding site proteins may therefore be responsible for the effects of these compounds. Nucleotide modulated receptors are generally coupled to the adenylate cyclase enzyme complex and a role for the regulatory protein component of this unit, the "N" protein, in the actions of purine nucleotides has been suggested previously. However, available data suggest that glutamate and other excitatory amino acids elevate neuronal levels of cyclic GMP rather than cyclic AMP [13, 14]. This hypothesis may therefore not be valid in the case of excitatory amino acid receptors. Obviously, this does not preclude the possibility that cyclic GMP acts either directly at a site on the binding site protein or indirectly at another site to inhibit the interaction between excitatory amino acid receptors and their endogenous ligand following receptor activation and stimulation of its own formation. This may represent a mechanism for the prevention of neuronal hyperexcitation. Unfortunately, the radioligand binding technique does not permit further evaluation of this possibility.

The present study has also provided information about the specificity of the effects of nucleotides on acidic amino acid binding sites. In the one previous report concerning the effects of purine nucleotides on excitatory amino acid receptors L-[3H] glutamate was used as the radiolabelled probe [5], and an inhibitory action of guanine nucleotides on the binding of this radioligand to cerebellar membranes were clearly demonstrated. However, it is clear that L-[3H] glutamate binds to more than one site on synaptic membranes [3, 20]. The use of DL-[3H]APB in the present study has permitted an investigation of the effects of nucleotides on one discrete subpopulation of glutamate binding sites. This site, defined as the A4 binding site by Foster and Fagg [20], is labelled only by L-[3H] glutamate (and DL-[3H] APB) in the presence of chloride/calcium ions and is exquisitely sensitive to quisqualate [3, 20]. A non-ionic buffer (Tris–citrate) was used in the previous study [5], and it may therefore be assumed that the A4 site did not participate to any great extent in the binding process. Since the inhibitory effects of purine nucleotides were observed both in ion-free and calcium chloride

containing buffers, a selective action of these compounds on any one population of glutamate binding sites would appear unlikely.

Finally, several recent reports have questioned whether the A4 binding site represents the APB-sensitive population of acidic amino acid receptors. The possibility that the binding process might be an uptake related phenomenon has been suggested [21, 22], even though there is no direct evidence to support this hypothesis (APB does not interfere with either synaptosomal D-[^{3}H] aspartate (Sharpe and Roberts, unpublished data) or vesicular L-[^{3}H] glutamate uptake [23]). Since neither possibility can at present be considered as proven, the physiological significance of the effects of purine nucleotides on DL-[^{3}H] APB binding remains uncertain.

Acknowledgements—This work was supported by an SERC project grant to PJR. We thank Dr Dick Young (NEN) for his continued interest in this work.

REFERENCES

1. M. Baudry and G. Lynch, *Nature, Lond.* **282**, 749 (1979).
2. M. Baudry, M. C. Bundman, E. K. Smith and G. Lynch, *Eur. J. Pharmac.* **90**, 161 (1983).
3. G. E. Fagg, A. C. Foster, E. E. Mena and C. W. Cotman, in *CNS Receptors: From Molecular Pharmacology to Behaviour* (Eds. P. Mandel, and F. V. De Feudis), pp. 199–209. Raven Press, New York (1983).
4. E. E. Mena, G. E. Fagg and C. W. Cotman, *Brain Res.* **243**, 378 (1982).
5. N. A. Sharif and P. J. Roberts, *Biochem. Pharmac.* **30**, 3019 (1981).
6. A. C. Foster, G. E. Fagg, E. W. Harris and C. W. Cotman, *Brain Res.* **242**, 374 (1982).
7. E. K. Michaelis, M. L. Michaelis, M. M. Chang, R. D. Grubbs and D. Kuonen, *Molec. cell. Biochem.* **38**, 163 (1981).
8. S. R. Childers and S. H. Snyder, *J. Neurochem.* **35**, 153 (1980).
9. M. E. Maguire, P. M. Van Arsdale and A. G. Gilman, *Molec. Pharmac.* **12**, 335 (1976).
10. S. J. Peroutka, R. T. Leboritz and S. H. Snyder, *Molec. Pharmac.* **16**, 700 (1979).
11. B. M. Rouot, D. C. Prichard and S. H. Snyder, *J. Neurochem.* **34**, 374 (1980).
12. N. R. Zahniser and P. B. Molinoff, *Nature, Lond.* **275**, 453 (1978).
13. G. A. Foster and P. J. Roberts, *Br. J. Pharmac.* **74**, 723 (1981).
14. J. Garthwaite, *Neurosci.* **7**, 2491 (1982).
15. S. P. Butcher, J. F. Collins and P. J. Roberts, *Br. J. Pharmac.* **80**, 355 (1983).
16. D. T. Monaghan, M. C. McMills, A. R. Chamberlint and C. W. Cotman, *Brain Res.* **278**, 137 (1983).
17. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1981).
18. P. M. Laduron, *Biochem. Pharmac.* **33**, 833 (1984).
19. S. P. Butcher, P. J. Roberts and J. F. Collins, *J. Neurochem.* **43**, 1034 (1984).
20. A. C. Foster and G. E. Fagg, *Brain Res. Rev.* **7**, 103 (1984).
21. G. E. Fagg, *Trends Neurosci.* **8**, 207 (1985).
22. J.-P. Pin, J. Bockaert and M. Recasers, *Febs. Lett.* **175**, 31 (1984).
23. S. Naito and T. Ueda, *J. Neurochem.* **44**, 99 (1985).