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Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum

Murilo Monteiro Paz[a], Milagros Ramos[b], Galo Ramírez[b,*], Diogo Souza[a]

[a]*Departamento de Bioquímica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[b]*Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain*

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Abstract In G protein-coupled receptors, neurotransmitter-induced binding of GTP to G proteins triggers the activation of effector systems while simultaneously decreasing the affinity of the transmitter for its specific binding site within the receptor–G protein complex. In the present study we show that, in the chick optic tectum, guanine nucleotides inhibit the binding of the glutamate analog, kainate, and activate adenylate cyclase by different mechanisms and acting on different sites. GMP-PNP, a non-hydrolyzable analog of GTP, binds tightly to G proteins so that the binding is stable even after exhaustive washing. By use of this property, we have prepared membrane samples in which G protein GTP-binding sites are pre-saturated with GMP-PNP. Experiments carried out with these membranes show that GMP-PNP, GDP-S and GMP inhibit the binding of [^{3}H]kainate by interacting with site(s) unrelated to G proteins, whereas GMP-PNP activates adenylate cyclase activity by binding to G proteins.

Key words: Kainic acid receptor; Guanine nucleotide; G protein; Adenylate cyclase; Chick optic tectum

1. Introduction

Cells receive much of their information through membrane-coupled signalling pathways that use GTP-binding proteins (G proteins) to transduce and convey signals from cell surface receptors to cellular effector proteins [1–4]. The interaction of agonists with some receptors triggers the binding of GTP to specific G proteins which, in turn, modulate the activities of specific effectors, while simultaneously decreasing the agonist–receptor binding affinity. Intrinsic GTPase activity hydrolyzes bound GTP to bound GDP, thereby inactivating the G protein–effector system.

This G protein-mediated inhibitory effect of GTP (or GDP) on agonist binding has been well demonstrated in many neurotransmitter systems [1,2]. In contrast, there are results indicating that guanine nucleotides may inhibit the binding of glutamate analogs [5–12] by mechanisms not involving G proteins.

Glutamate is the major excitatory neurotransmitter in the CNS. Pharmacological and molecular studies have established the existence of two major classes of glutamate receptors: ionotropic receptors, which are ligand-gated ion channels, and metabotropic receptors, which are coupled to cellular effectors through G proteins. By using agonists and antagonists, glutamate receptors may be further differentiated into five subtypes: NMDA, KA, AMPA (which may overlap with the KA site), L-AP4 and *trans*-ACPD [13–15].

Well-known examples of effector proteins regulated by agonists through G proteins are adenylate cyclase (AC), cGMP phosphodiesterase, phospholipase C and ion channels [1–4]. Coupling between receptors and AC is mediated by two types of G proteins, namely G_s and G_i, which respectively activate or inhibit AC [2,4]. AC produces cAMP, a second messenger that regulates the phosphorylation of cellular proteins, thereby modulating synaptic transmission and neural function [16–17].

In the present study, a direct comparison of the effects of guanine nucleotides on KA binding and on AC activity provides further evidence to the effect that, in CNS membrane preparations, guanine nucleotides bind to KA-recognizing sites, as well as to G proteins.

2. Materials and methods

2.1. Animals

White Leghorn young chicks (6–12 days old) were used (from Aviário Jari). They were kept at 37°C, on a 12-h light/12-h dark cycle.

2.2. Materials

Imido [8-^{3}H]guanosine 5′-triphosphate (GMP-PNP, 19.1 Ci/mmol), [8-^{3}H]guanosine 5′-diphosphate (GDP, 11.6 Ci/mmol), and [^{3}H]kainic acid (KA, 58 Ci/mmol) were obtained from Amersham International, UK; protein kinase, for cAMP determinations, guanylyl-imidodiphosphate (GMP-PNP), GTP, GDP-S, GMP and ATP were from Sigma or Boehringer-Mannheim. All other chemicals of analytical grade were obtained from standard commercial suppliers.

2.3. Membrane preparations

Preparations were carried out as described in [10]. Optic tectal lobes were homogenized in 0.32 M sucrose prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the pellet resuspended and centrifuged again. The second pellet was discarded and the supernatants pooled and centrifuged at 27,000 × *g* for 20 min. The resulting pellet was lysed in 1 mM Tris-HCl buffer, pH 7.4, for 30 min, and centrifuged at 27,000 × *g* for 20 min. This pellet was washed three times in lysis buffer, at 27,000 × *g* for 20 min. To pre-saturate, and thus block, GTP-binding sites in G proteins the final pellet was preincubated in 50 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, together with 10 μM GMP-PNP (or with variable [^{3}H]GMP-PNP concentrations to monitor the saturation process – see below), at 30°C for 15 min. In all these experiments, control membrane preparations were preincubated in the same buffer, under the same experimental conditions, without GMP-PNP. Then, the membranes were kept at 4°C for 15 min, centrifuged at 27,000 × *g* for 20 min, and washed three times with preincubation buffer (without GMP-PNP) to remove unbound and labile, bound GMP-PNP. The final pellet was diluted in the same buffer and used for determination of [^{3}H]KA and [^{3}H]guanine nucleotide binding, and of AC activity.

*Corresponding author. Fax: (34) (1) 397 4799.

Abbreviations: AC, adenylate cyclase; ACPD, 1-amino-cyclopentyl-1,3-dicarboxylate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; GDP-S, guanosine-5′-*O*-(2-thio) diphosphate; GMP-PNP, 5′-guanylyl-imidodiphosphate; KA, kainic acid; L-AP4, L-2-amino-4-phosphonobutyrate; NMDA, *N*-methyl-D-aspartate.

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2.4. Binding assays

Binding assays of [^{3}H]GMP-PNP, [^{3}H]GDP or [^{3}H]KA were performed at 30°C in small polycarbonate tubes (total volume 1 ml), containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mg membrane protein, and the radioactive ligand, with or without the displacer (non-radioactive ligand). Incubation was started by addition of membrane and stopped after 5 min ([^{3}H]GDP), or after 15 min ([^{3}H]GMP-PNP and [^{3}H]KA) by centrifugation at 15,000 × *g*, for 2 min ([^{3}H]GDP and [^{3}H]GMP-PNP), or at 27,000 × *g* for 30 min ([^{3}H]KA) [10]. The supernatant was discarded and the walls of the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were processed for radioactivity and protein measurement. In binding experiments using radioactive GMP-PNP during preincubation (monitoring of pre-saturation experiments – see above), the membranes were also washed three times after preincubation before being processed for radioactivity and protein measurement. Specific binding was defined as the part of total binding displaced by a concentration of non-labeled ligand 10^4 times the radioligand concentration.

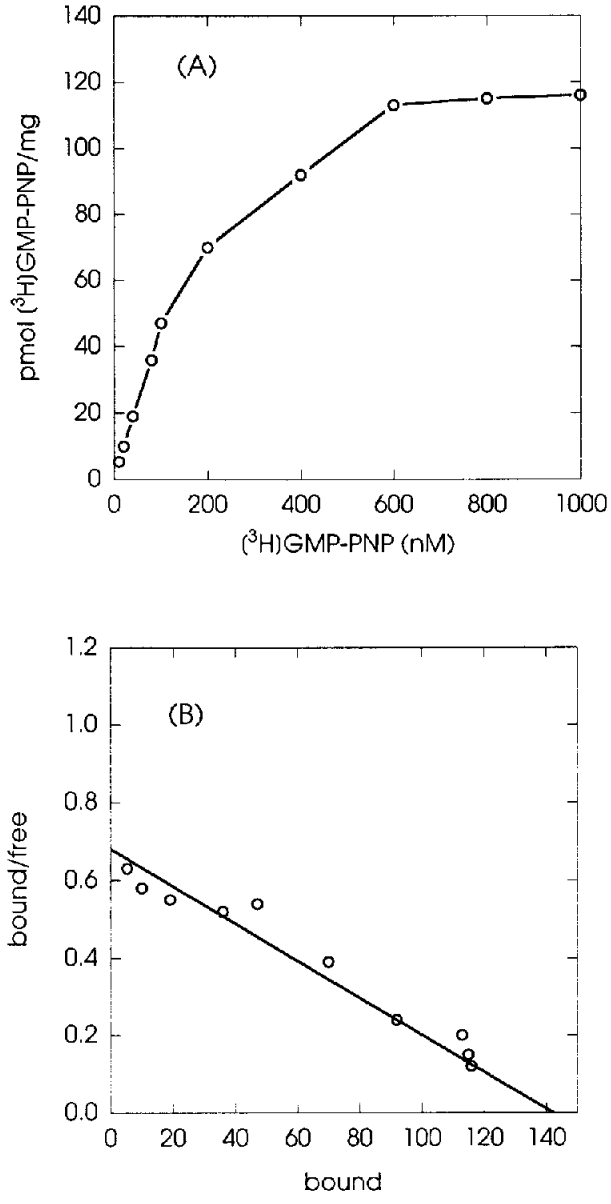

Fig. 1. Saturation curve of [^{3}H]GMP-PNP binding to chick optic tectum membranes under the conditions used in preincubation experiments with tritiated or non-radioactive GMP-PNP. Preincubation was carried out at 30°C. After that, membranes were washed 3 times, as indicated in section 2, and processed to measure bound [^{3}H]GMP-PNP. (A) Saturation curve of specific binding. (B) Scatchard analysis of saturation curve. The curve is the average of 4 experiments (standard deviation of each point was less than 15% of the mean).

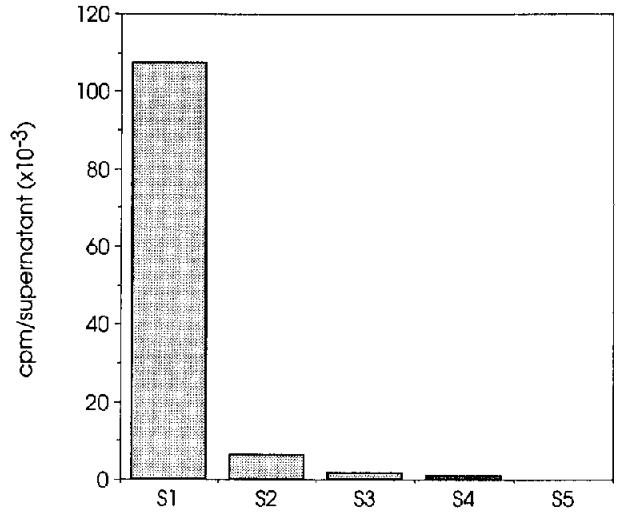

Fig. 2. Determination of free [^{3}H]GMP-PNP released by washing cycles after preincubation. After preincubation with 1 μM [^{3}H]GMP- PNP, as described in section 2 and in the legend to Fig. 1, membranes were centrifuged at 27,000 × *g* and the radioactivity in the 1-ml supernatant (S1) was measured. This procedure was repeated 4 times with additional 1-ml washings (S2–S5) The figure shows a typical experiment, which was replicated three times.

2.5. Adenylate cyclase activity

The conditions used for AC determination were the same as for binding assays, except for the inclusion of 0.1% bovine serum albumin in the incubation medium. After 15 min of incubation of the membrane samples with all other components, cAMP synthesis was started by addition of ATP (final concentration 1 mM), and stopped after 1 min by boiling the tubes for 3 min. The tubes were centrifuged at 12,000 × *g* for 2 min, and the supernatants were used for measuring cAMP content by the protein-binding method [18].

2.6. Protein measurement

Protein was measured according to the method of Lowry [19].

3. Results

Fig. 1A demonstrates how GTP-binding sites in chick tectal G proteins become saturated by preincubation with [^{3}H]GMP-PNP; the specifically bound radioactivity was measured after three washings to take advantage of the fact that the binding of GMP-PNP is practically irreversible under the experimental conditions used in this study (it can be removed, however, by further incubation at 37°C). Scatchard analysis of the resulting saturation curve (Fig. 1B) revealed a single component, with a dissociation constant (K_d) of 210 nM, and a maximal density of binding sites (B_{max}) of 142 pmol/mg protein. Other readily washable GTP-binding sites would not be detected by this procedure. To monitor the efficiency of the washing procedure, free [^{3}H]GMP-PNP was measured in the successive supernatants. After three washings, free [^{3}H]GMP-PNP released is barely detectable (Fig. 2). The effects of preincubation with GMP-PNP described below should therefore be interpreted as due to presaturation of G protein GTP-binding sites in the

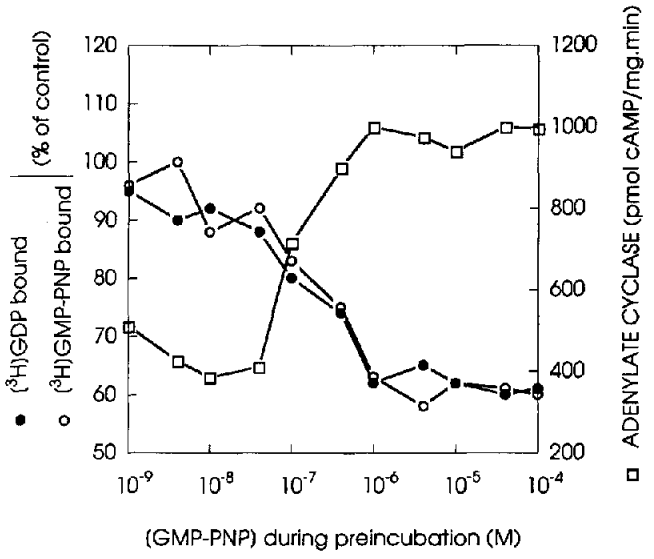

Fig. 3. Effects of preincubation with increasing concentrations of non-radioactive GMP-PNP on further binding of [^{3}H]GMP-PNP or [^{3}H]GDP, and on AC activity. Membranes were preincubated with non-radioactive GMP-PNP, at the concentrations indicated, and washed 3 times as indicated in section 2. After that, membranes were incubated with [^{3}H]GMP-PNP or [^{3}H]GDP-S (600nM), or incubated for measuring AC activity. Binding values are given as % of the control (preincubated without GMP-PNP). Basal AC activity (no GMP-PNP added) was 495 pmol cAMP/mg·min. The figure shows a typical experiment, which was replicated three times.

tectal membranes, rather than to the presence of any free GMP-PNP in the medium.

That preincubation with non-radioactive GMP-PNP actually blocks G protein GTP-binding sites is shown in Fig. 3, where the progressive occupation of this class of GTP-binding sites by increasing concentrations of non-radioactive GMP-PNP gradually decreases the further binding of either [^{3}H]GMP-PNP or [^{3}H]GDP to tectal membranes and stimulates AC activity in a strictly parallel fashion. The guanine nucleotide binding curves in Fig. 3 furthermore show that some 60% of the guanine nucleotide binding sites in the membranes remain available under these conditions: these binding sites, not blocked by preincubation with non-radioactive GMP-PNP, must therefore be unrelated to G proteins. This preparation of tectal membranes, with G protein GTP-binding sites specifically blocked, is therefore an excellent experimental system to characterize other possible GTP-binding sites in tectal membranes and to check whether the displacement of [^{3}H]KA binding by guanine nucleotides takes place at the G protein level or elsewhere. Indeed, as seen in Fig. 4, GMP-PNP, GDP-S or GMP effectively displace [^{3}H]KA binding, whether or not G proteins are presaturated with GMP-PNP. In a similar experiment (Fig. 5), the saturation of GTP-binding sites in G proteins by preincubation with GMP-PNP is clearly shown by the lack of stimulatory effect on AC of further addition of GMP-PNP to preincubated membranes. It should be noted that only GMP-PNP, and not GDP-S and GMP, stimulates AC activity in the control (preincubated without GMP-PNP) membrane preparations. Therefore, all the guanine nucleotides tested displace [^{3}H]KA binding by acting at sites unrelated to G proteins, whereas only GTP-like guanine nucleotides stimulate AC activity by interaction with G protein GTP-binding sites.

4. Discussion

It has been demonstrated that both GTP and GDP inhibit the binding of neurotransmitters to a wide variety of receptors, such as dopaminergic [20], adrenergic [21], serotoninergic [22], purinergic [23], GABAergic [24], muscarinic [25] and opioid [26] receptors. However, in these cases, GMP, cGMP and adenine derivatives had no inhibitory effects, nor did guanine nucleotides modify the binding of the respective antagonists. In contrast, evidence has accumulated that suggests that the inhibitory effects of guanine nucleotides on the binding of glutamate and analogs, including antagonists, and on the the responses of intact cells to glutamate analogs, have unusual properties [5–12]. Studies on the interactions between glutamatergic systems and guanine nucleotides have shown that GMP, cGMP and guanosine may also inhibit the binding of glutamate or related agonists to membrane preparations [5,7,10]; furthermore, guanine nucleotides inhibit the binding of kainate by acting on the external surface in closed vesicle preparations [10]. If we now take into account that GMP, cGMP and guanosine do not bind to G proteins, that GTP and GDP do not cross the cell membrane, and that activation of G proteins does not interfere with binding of antagonists, it seems reasonable to postulate that the inhibitory effects of guanine nucleotides on the binding of glutamate and analogs are exerted by acting on sites unrelated to G proteins.

The binding of guanine nucleotides to G proteins has been used as a tool to study the role of these proteins in mediating interactions between receptors and cellular effectors [27–30]. Pre-incubation with GMP-PNP maintains AC activity maximally stimulated, even after extensive washing, suggesting that stable GMP-PNP–G protein complexes are formed [31–34]. In agreement with this fact, our experiments clearly show that preincubation of tectal membrane preparations with GMP-PNP, followed by washing, is effective in saturating GTP binding sites in G proteins (Fig. 1). Pre-saturation of G protein

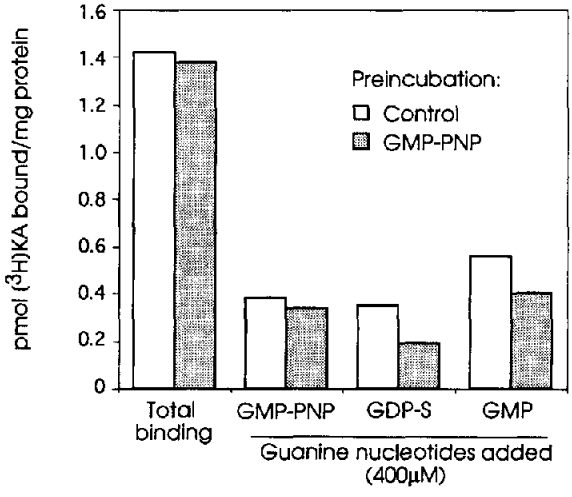

Fig. 4. Effects of preincubation of tectal membranes with non-radioactive GMP-PNP on the displacement of [^{3}H]KA binding by guanine nucleotides. Membranes were prepared, preincubated with or without (blank controls, open bars) 10 μM GMP-PNP, washed, and incubated with 40 nM [^{3}H]KA, with or without ('Total binding') the displacing guanine nucleotides (400 μM), as described in section 2. Data represent 11 experiments (standard deviation of each point was less than 15% of the mean).

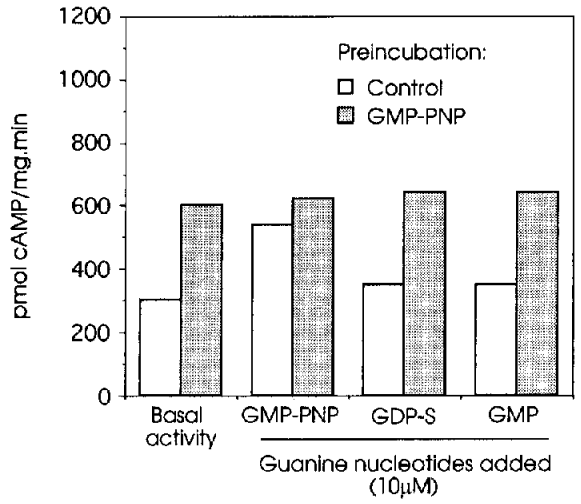

Fig. 5. Effects of preincubation with non-radioactive GMP-PNP on the modulation of AC activity by guanine nucleotides. Membranes were prepared, preincubated with or without (blank controls, open bars) 10 μM GMP-PNP, and washed, as described in section 2. AC activity was measured in the absence ('Basal activity') or in the presence of 10 μM guanine nucleotides. Data represent 9 experiments (standard deviation of each point was less than 10% of the mean). All AC activity values in samples preincubated with GMP-PNP are nearly identical. Of the blank controls only GMP-PNP produced a statistically significant increase in AC activity ($P < 0.05$).

GTP-binding sites by increasing concentrations of non-radioactive GMP-PNP effectively decreased the binding of [^{3}H]GMP-PNP or [^{3}H]GDP while simultaneously stimulating AC activity (Fig. 3). These results indicate that the decrease in GMP-PNP and GDP binding is due to the stable GMP-PNP–G protein complexes formed during preincubation. The formation of these complexes between GMP-PNP and G proteins is further supported by the concomitant stimulation of AC activity, which reaches a maximum of stimulation when all G protein GTP-binding sites are effectively occupied by GMP-PNP. The binding of [^{3}H]GMP-PNP and [^{3}H]GDP measured after preincubation with saturating concentrations of non-radioactive GMP-PNP must therefore occur at site(s) not related to G proteins.

The effects of preincubation with non-radioactive GMP-PNP on the displacement of [^{3}H]KA, and on the modulation of AC activity by guanine nucleotides, are examined in Figs. 4 and 5. Preincubation with GMP-PNP maintains AC activity maximally stimulated even after exhaustive washings (Fig. 5). However, this preincubation with GMP-PNP does not modify the ability of the different guanine nucleotides to displace [^{3}H]KA binding (Fig. 4). Besides, as previously shown by us [10], the effects of guanine nucleotides on AC activity and on [^{3}H]KA binding are markedly different. AC activity in control membranes (preincubated in the absence of GMP-PNP) was stimulated only by GMP-PNP (a GTP-like nucleotide), and AC activity in membranes preincubated with GMP-PNP remained stimulated even in the absence of guanine nucleotides; in both membrane preparations, however, the binding of [^{3}H]KA was inhibited by all guanine nucleotides. These results suggest that the effects of GTP-like guanine nucleotides on AC activity, and of all GTP-, GDP-, and GMP-like nucleotides on [^{3}H]KA binding, are mediated by distinct mechanism(s), the AC activity being stimulated by GMP-PNP (GTP-like) through G proteins and the [^{3}H]KA binding being displaced by GMP-PNP, GDP-S and GMP by interaction with a second guanine nucleotide binding site, quite possibly the kainate receptor itself.

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