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Specific binding of [^{3}H]GppNHp to extracellular membrane receptors in chick cerebellum: possible involvement of kainic acid receptors

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Abstract Guanine nucleotides (GNs), including GMP, displace [^{3}H]kainic acid binding to chick cerebellar lysed and vesiculated membranes. Saturation studies of [^{3}H]GppNHp binding, under conditions that prevent the occupation of the nucleotide binding sites in G-proteins, demonstrate the existence of extracellular membrane receptors specific for guanine nucleotides. Affinity-labeling of a vesicle preparation with [α-^{32}P]GTP gives one single labeled band, upon electrophoresis, with an apparent molecular mass of 50 kDa. Additional experiments with partially purified kainate receptors suggest that the GN extracellular sites may overlap, at least partially, the kainic acid binding sites, being then responsible for the displacement of [^{3}H]kainic acid by GNs. The physiological significance of these findings remains unclear.

Key words: Kainic acid receptor; Guanine nucleotide; G-protein; Extracellular guanine nucleotide receptor; Excitotoxicity; Chick cerebellum

1. Introduction

The role of the guanine nucleotides (GNs) GTP and GDP in the activation/inactivation cycle of G-proteins and other intracellular GTPases involved in cell-signalling-related processes is well understood [1–4]. One of the constant features of G-protein-coupled receptors is the displacement of agonists (but not of antagonists) by either GTP or GDP, and their analogs. In the case of ionotropic glutamate receptors, however, the displacement of different agonists by GNs, under conditions that presumably exclude the hypothetical coupling of these receptors to G-proteins, has been reported ([5–11] but see also [12]). We have furthermore demonstrated that [^{3}H]kainic acid (KA) is displaced not only by GTP and GDP, but also by GMP (which is never active on G-protein-coupled receptors) in chick tectal and cerebellar membranes [8]. The displacement of specific [^{3}H]KA binding by GNs is not modified by pre-saturation of the nucleotide sites of G-proteins with GppNHp, and we have recently concluded that GNs displace KA acting extracellularly at, or close to, the KA receptor itself [11]. This furthermore agrees with our own results using closed membrane vesicles, where G-proteins are not accessible to the GNs [8]. In the present paper we describe the characterization of these extracellular GN binding sites by means of [^{3}H]GppNHp, a non-hydrolyzable GTP analog, and explore the relationship between the new GN sites and the KA receptor sites.

2. Materials and methods

2.1. Animals

Young white Leghorn chicks (10 ± 1 days old) were raised in our facilities. They were kept at 37°C, on a 12-h light/12-h darkness cycle.

2.2. Materials

β,γ-Imido[8-^{3}H]guanosine 5′-triphosphate (GppNHp: 19.8 Ci/mmol) and [α-^{32}P]GTP (>400 Ci/mmol) were obtained from Amersham International, UK; [^{3}H]kainic acid (58 Ci/mmol) was from New England Nuclear, Germany; protein kinase, for cAMP determinations, guanine nucleotides and ATP were from Sigma or Boehringer Mannheim. All other chemicals were obtained from standard commercial suppliers.

2.3. Membrane preparations

Lysed and vesiculated membrane preparations were carried out at 4°C as described previously [8,11], with some modifications to improve yield and reproducibility.

In the case of lysed membranes, 10-day chick cerebella were homogenized (Dounce glass/glass homogenizer, A+B pestles) in 20 volumes of 0.32 M sucrose prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$. The homogenate was centrifuged at 1000×g for 15 min, and the pellet resuspended and centrifuged again. The second pellet was discarded and the supernatants pooled and centrifuged at 100 000×g for 30 min. The resulting pellet was lysed in 20 volumes (relative to initial weight) of 1 mM Tris-HCl buffer, pH 7.4, for 30 min, and centrifuged at 100 000×g as above. This pellet was washed twice more in lysis buffer, under the same conditions, and resuspended finally in 10 mM Tris-HCl buffer, pH 7.4, at an adequate protein concentration, for use in binding (and other) experiments.

When preparing closed membrane vesicles, the cerebella were first carefully minced and then homogenized (Dounce homogenizer, A pestle only) in a Krebs-Ringer solution comprising 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4 and 10 mM glucose, adjusting the pH to 7.4 with 100 mM Na_2HPO_4 (KRG). Differential centrifugation steps and washings were carried out as with lysed membranes, but using only the A pestle for a more gentle resuspension of vesicles. The integrity and sidedness (inside-in) of the vesicle preparation was checked before binding experiments by measuring the ability of GppNHp to stimulate adenylate cyclase (methods as in [11]), so to confirm the non-accessibility of G-proteins. Vesicle preparations with higher than 5% sensitivity to GppNHp stimulation were discarded.

2.4. Binding assays

Binding assays of [^{3}H]GppNHp or [^{3}H]KA were performed at 30°C in small polycarbonate tubes (total volume 1 ml), containing 10 mM Tris-HCl, pH 7.4 (or KRG in the case of vesicles), 0.3 mg membrane protein, and the radioactive ligand, with or without the displacer (non-radioactive ligand). Incubation was started by addition of membranes and terminated after 30 min by centrifugation at 12 000×g for 30 min (as efficient as 30 min at 100 000×g before incubation). 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 then processed for radioactivity and protein determinations. Specific binding was defined as that part of total bind-

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Abbreviations: GLU, glutamate; GNs, guanine nucleotides; GppNHp, 5′-guanylyl-imidodiphosphate; KA, kainic acid

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ing displaced by a concentration of non-labeled ligand 10^4 times the radioligand concentration.

2.5. Discrimination of intracellular (G-protein and related sites) and extracellular GN binding sites

Whereas in the case of membrane vesicles only extracellular binding was expected, lysed membranes should contain typical GTP-binding sites belonging to G-proteins and related GTPases plus the hypothetical extracellular sites that would explain the interaction between GNs and the KA receptor. To give an independent estimate of both types of receptors we took advantage of the ability of some non-hydrolyzable GTP analogs, such as GppNHp, to bind in a quasi-irreversible manner to the nucleotide site of G-proteins [11,13]. Two identical sets of samples were assayed in each experiment: in the first set, the part of [^{3}H]GppNHp binding resistant to three washes in the absence of ligand was considered to represent the G-protein (and related) binding sites. To measure the extracellular sites the second set of samples was preincubated with an excess (10 μM) of cold GppNHp, so to saturate G-protein sites [11]. After three washes as above the preparation was again incubated with [^{3}H]GppNHp, as in the general method, to label any additional GN binding sites.

2.6. Affinity labeling of the extracellular GN binding sites

Freshly prepared membrane vesicle samples were incubated, in a multiwell plate, as described in Section 2.4, in the presence of 250 nM [α-^{32}P]GTP. Some wells contained also GppNHp or KA at a $10^3\times$ concentration. After 15 min, the plate was put over ice and irradiated under a GTE Sylvania 15 W UV germicidal (254 nm) lamp (15 min/5 cm). The samples were transferred to plastic tubes and washed by centrifugation. The pellets were finally subjected to SDS-PAGE, stained and exposed for autoradiography.

2.7. Purification of the KA receptor protein

The KA receptors were partially purified, from our lysed membrane preparation, as described in [14]. Triton X-100 was used at 0.5% (v/v), this being the concentration that solubilizes a maximum of [^{3}H]KA binding sites and a minimum of [^{3}H]GppNHp binding sites (results not shown). Only the DEAE-cellulose and the concanavalin A-agarose steps were carried out. Aliquots of the solubilized receptor preparation were assayed for [^{3}H]KA and [^{3}H]GppNHp binding sites at different times during the purification. Incubations were for 1 h at 4°C, and were terminated by precipitation with polyethylene glycol 6000, in the presence of carrier γ-globulin [14]. The precipitates were carefully washed with 8% polyethylene glycol and incubated overnight in 0.1% SDS prior to scintillation counting. Protein values used in calculations were input values corrected for carrier protein recovery.

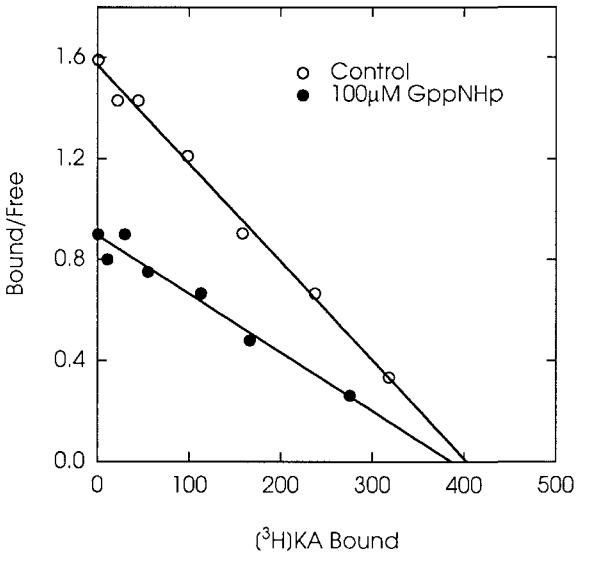

Fig. 1. Competitive displacement of [^{3}H]KA by GppNHp in 10-day chick cerebellar membranes. Saturation studies were carried out using the vesiculated membrane preparation (Section 2.3), varying the ligand concentration between 1 and 1000 nM. The graph shows the result of a typical experiment which was replicated three times. Control (○): K_d, 256 nM; B_{max}, 403 pmol/mg. GppNHp (●): K_d, 431 nM; B_{max}, 387 pmol/mg.

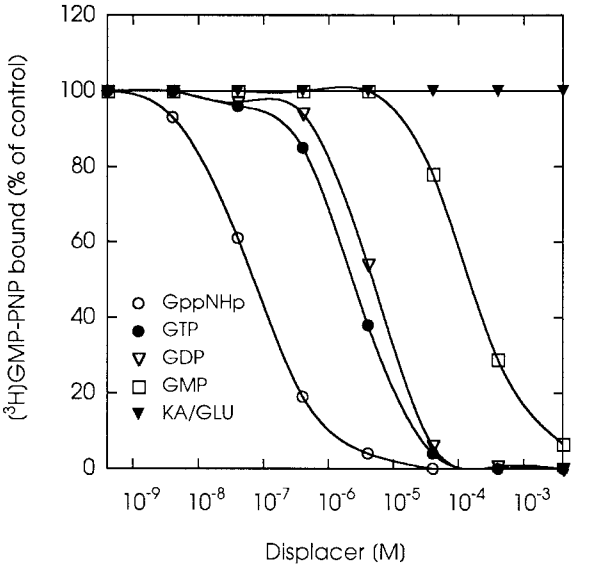

Fig. 2. Displacement of 40 nM [^{3}H]GppNHp from chick cerebellar receptor sites by different GNs and glutamate agonists. The experiments were carried out in a 10-day chick cerebellum lysed membrane preparation. Neither KA nor GLU had any effect at the concentrations shown. The figure shows a typical experiment which was replicated three times. IC_{50}s: GppNHp, 7×10^{-8} M; GTP, 2×10^{-6} M; GDP, 5×10^{-6} M; GMP, 1.5×10^{-4} M.

2.8. Protein measurement

Protein was measured according to the method of Lowry [15].

3. Results

3.1. Competitive displacement of [^{3}H]KA by GppNHp in membrane vesicles

We have previously characterized KA binding sites in chick tectal and cerebellar membranes from pharmacological and developmental standpoints [16,17]. We have also shown that GNs, including GMP, are efficient displacers of [^{3}H]KA [8,11]. In the present paper we just give some additional data on the [^{3}H]KA binding to the closed vesicle preparation that has proved especially useful to distinguish between intra- and extracellular GN binding sites.

Fig. 1 shows again that the non-hydrolyzable GTP analog GppNHp, used in this work, is an efficient displacer of [^{3}H]KA in chick vesiculated cerebellar membranes. The kinetic parameters measured in the presence of the nucleotide suggest that the displacement is purely competitive, a result compatible with the notion of a direct interference of the GNs at the KA receptor site.

3.2. Characterization of [^{3}H]GppNHp binding sites in chick cerebellar membranes

[^{3}H]GppNHp binds to specific, saturable sites in chick cerebellar membranes. This binding is displaced by all three native GNs but, surprisingly, is not affected by either KA or glutamate (GLU) (Fig. 2).

The saturation profile of [^{3}H]GppNHp binding sites in chick lysed membranes is illustrated in Fig. 3. The curved shape of the overall Eadie-Scatchard plot bears out the ex-

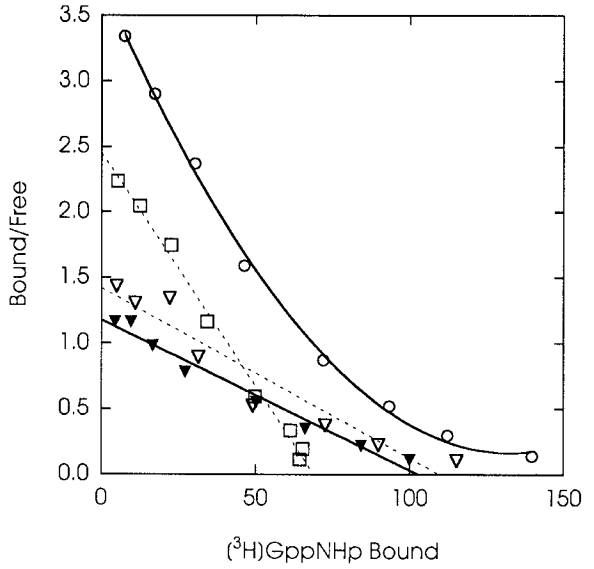

Fig. 3. Saturation analysis of [^{3}H]GppNHp binding sites in 10-day chick cerebellar lysed and vesiculated membranes. (○) Eadie-Scatchard plot of binding sites in lysed membranes. (□) G-protein and (▽) non-G-protein binding sites calculated as described in Section 2.5. (▼) Eadie-Scatchard plot of binding sites measured directly on vesiculated membranes (extracellular GN sites). The figure shows a typical experiment which was replicated three times. The kinetic parameters are given in Table 1.

pected complexity of the total population of GN binding sites (comprising intra- and extracellular, G-protein- and non-G-protein-related sites). Trying to characterize the independent populations of binding sites that make up the curved plot in Fig. 3, we have put aside the usual regression algorithms and we have actually tried to ascertain the contributions of the G-protein-related and non-related sites, as explained in Section 2.5. In this way, we have been able to distinguish one type of binding sites of somewhat higher affinity, which can be quasi-irreversibly blocked by cold GppNHp, and another lower affinity site, which cannot and is therefore a potential candidate for the extracellular site. The probable identity of this component of the overall binding with the extracellular site (which we are looking for) is supported by its near coincidence with the independently calculated Eadie-Scatchard plot of the [^{3}H]GppNHp binding sites in our closed vesicle preparation, as seen in Fig. 3. For comparative purposes, the kinetic constants for the different plots in the figure are summarized in Table 1.

On the other hand, the accessibility to GTP of the GN binding sites in the vesicle preparation is confirmed by the electrophoretic profiles in Fig. 4. The molecular weight of the only labeled band has been calculated as 50 (±1) kDa, very close to the published value for the chick cerebellum KA receptor [14]. Whereas the presence of an excess of GppNHp totally blocks the labeling of the 50 kDa band, KA is unable to displace the radioactive GTP, in agreement with the curves in Fig. 2.

3.3. Partial purification of [^{3}H]KA binding sites

Table 2 summarizes the yield and properties of the solubilized chick cerebellar KA receptors along the different steps of the purification process. As seen in Table 2, we have achieved a nine-fold purification of the KA binding sites with a simultaneous four-fold increase in [^{3}H]GppNHp binding sites, with KA binding still more than 80% displaceable by the guanine nucleotide, suggesting that the purified KA receptor still retains a GN binding site the occupation of which blocks the access of KA to its own membrane receptor [8].

Additional experiments (results not shown) were conducted with this partially purified receptor. For instance, we still could not displace the [^{3}H]GppNHp with 10^4 times KA (still higher concentrations of KA gave erratic results). Finally, to better compare the actual number of binding sites for GN and KA in the purified receptor preparation we also performed binding experiments at high concentrations of tritiated ligand (1000 nM, instead of the 40 nM ligand concentration in Table 2). In four such experiments we measured 1853 ± 167 pmol/mg (mean ± S.D.) of KA bound vs. 1594 ± 98 for GppNHp. Taking into account the 40 nM binding values, and assuming a linear Scatchard plot, this would allow a rough estimate of K_d/B_{max} values of 420 nM/2640 pmol/mg and 600 nM/2560 pmol/mg, for KA and GppNHp, respectively. This is a much closer match than the 4 to 1 ratio of the respective B_{max} values obtained in the membrane preparations (Fig. 1 and Table 1).

4. Discussion

Ionotropic glutamate receptor agonists, including KA, have been shown to be displaced by guanine nucleotides in different species and membrane preparations [5–11]. Results with chick KA receptors are specially illustrative in this respect [8,10,11]. Chick tectal or cerebellar membranes contain abundant medium/low affinity binding sites (K_d in the range of 100–300 nM) that have been analyzed in different laboratories [14,16,17] and, in more recent years, purified and cloned [14,18].

Our previous studies on the displacement of [^{3}H]KA in chick optic tectum and cerebellum by guanine nucleotides, including GMP [8,11], have led us to suggest the existence of extracellular GN receptors overlapping the KA receptors in a way to block the free access of the latter agonist. To characterize these hypothetical receptors we analyzed the binding of [^{3}H]GppNHp to preparations of lysed or vesiculated chick cerebellar membranes, using specially devised procedures to discriminate G-protein- and internal GTPase-related binding sites from the alleged extracellular sites. Although more relevant than purely mathematical methods, these procedures may possibly overestimate the number of G-protein sites (due to incomplete washing) so that the graphical

Table 1
Kinetic parameters of binding modes in Fig. 3

	Lysed membranes		Vesiculated membranes
	G-protein sites (□) (estimated)	Non-G-protein sites (▽) (estimated)	Extracellular sites (▼) (measured)
K_d (nM)	28	77	87
B_{max} (pmol/mg)	69	109	102

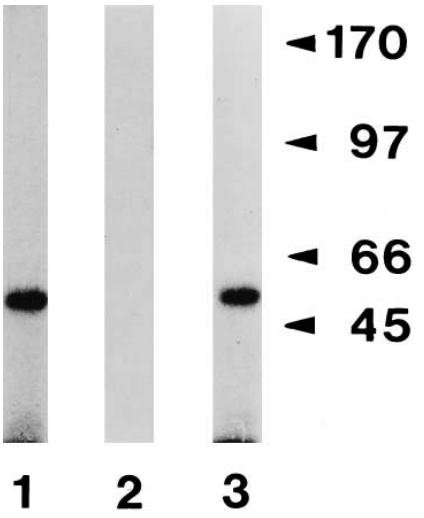

Fig. 4. Photoaffinity labeling of chick cerebellar proteins that bind [α-^{32}P]GTP in a vesiculated membrane preparation. Lane 1: Membrane preparation incubated with 250 nM [α-^{32}P]GTP. Lanes 2 and 3: Preincubation with 250 μM GppNHp and 250 μM KA, respectively, prior to exposure to [α-^{32}P]GTP. The molecular weight of the labeled band is 50 ± 1 kDa (3 runs). Molecular weight markers are, from top to bottom, reduced α_2-macroglobulin, phosphorylase b, bovine serum albumin and ovalbumin.

addition of the two discontinuous lines in Fig. 3 would result in an composite curve shifted to the right. However, it is reassuring to note the excellent agreement between the non-G-protein component of the overall binding and the independent plot of the vesicle preparation, where only the extracellular sites are accessible to the ligand. The overall binding curve would therefore reflect the existence of two types of GN binding sites: the already known G-protein and related sites and the extracellular sites that remain accessible in the vesiculated preparation.

Recent developments in the study of the spatial configuration of the glutamate recognition site in the different ionotropic receptors (NMDA or non-NMDA) suggest the involvement of three different regions in the transmitter/receptor interaction [19]. One of them includes the glycine-rich sequence (G)XGXXG known to be similar to the GN recognition site in the α subunit of G-proteins [10,20,21]. Occupation of this subsite by GNs would explain the displacement of glutamate and related agonists, as it has been finally demonstrated by use of directed mutagenesis and specific antibodies [21].

If the GN receptor site is just part of the KA receptor (or, generally speaking, of the GLU receptor) the measured numbers (B_{max}) of extracellular GN receptors (Fig. 3, Table 1) and of KA receptors (Fig. 1) should be reasonably similar. However, in our experiments with membranes, we observe a 1:4 ratio, favorable to the KA site (compare the KA B_{max} in Fig. 1 and the GppNHp B_{max} in vesicles, Table 1), whereas in the case of the purified receptor preparation the number of sites labeled by either ligand seems to be much the same (although binding assays with soluble receptor have a higher degree of inaccuracy). We cannot find an easy explanation for this discrepancy since [^{3}H]KA is displaced equally well by an excess of cold GppNHp in the membrane and the purified preparations. It may be conceived that the experimental conditions prevailing in binding experiments in the case of membranes would result in a limited accessibility of the GN to the membrane site in the native conformation, thus discriminating against [^{3}H]GppNHp binding at the relatively low ligand concentrations used in the saturation experiments (only a few pseudo-high affinity sites would be labeled: compare the K_d of the membrane receptor in Table 1 and the estimated K_d of the soluble receptor in Section 3.3), this effect being scarcely noticeable when a higher (0.4 mM) concentration of cold GppNHp is used in the KA displacement experiments. The lack of reciprocity in the displacement of KA by GNs in our membrane, and even in our partially purified preparations (only a partial displacement at the highest KA concentrations, although both KA and GLU have been reported to displace radioactive GTP in more purified receptor samples [14]) is equally puzzling. Perhaps, if the tertiary structure of the receptor, with the three-site interaction model mentioned above, is maintained under our experimental conditions – as would be expected to happen in the membrane, and perhaps (but less likely) in a partially purified receptor preparation – the lack of symmetry in the displacement could be explained if the KA and GN binding sites within the glycine-rich sequence do not totally coincide and provided that the very different size and shape of the two ligands leads to unidirectional steric hindrance at the level of one of the other two (preferentially) agonist recognizing sites. The above explanations may seem too labored but if we accept the alternative possibility that the GN receptor that we have characterized is not related to the KA receptor (also, in spite of the coincidence of the molecular mass, see Fig. 4), we would have to additionally explain our failure to detect and measure the binding of GppNHp truly responsible for the displacement of the excitatory agonist.

Whatever the explanation, any sustained increase of the concentration of GNs in the vicinity of the KA receptor should effectively limit the access of the agonist, which brings us to the possible extracellular role of GNs in the CNS, if any. We lack precise data on the physiological presence of GMP or other guanine nucleotides in the extracellular space in the CNS and their hypothetical role as regulators of receptor activity. However, extracellularly acting guanine nucleotides (and nucleosides) have been, in the recent years, implicated in a number of trophic activities [22]. In any case, if they are at all present, or if they are at least released by cellular injury,

Table 2
Purification of KA receptors from 10-day chick cerebellar membranes

Purification step	Binding of 40 nM [^{3}H]KA (pmol/mg)	Purification factor	Recovery (%)	Binding of 40 nM [^{3}H]GppNHp (pmol/mg)	Displacement of 40 nM [^{3}H]KA by 400 μM GppNHp (%)
Membranes	25	1	100	44	80
Supernatant	50[a]	2	100	68[a]	84
DEAE-cellulose	160[a]	6	70	64[a]	90
ConA-agarose	230[a]	9	10	160[a]	83

[a]Binding data from membrane and soluble receptor sites were obtained by different procedures (see Section 2).

in the same way as the amino acids themselves are released, they could help slow down the propagation of the excitotoxic wave to the neighboring cells [23]. Actually, we have recently shown that locally injected GMP can prevent quinolinic acid-induced damage in the rat striatum [24]. Additionally, guanine nucleotides could serve as structural models to design a new type of glutamate antagonists potentially effective in the management of the excitotoxic component of CNS diseases.

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