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In situ binding of a photo-affinity GTP analog to synaptic membrane G-proteins

Distribution of bound GTP analog reflects the status of adenylate cyclase

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Regulation of synaptic membrane adenylate cyclase is likely to involve interaction between neurotransmitter receptors, G-proteins and the adenylate cyclase catalytic unit as well as several other membrane proteins and lipids. Despite intensive study of this system, regulation of guanine nucleotide binding by the G-proteins which stimulate [G_s] or inhibit [G_i] adenylate cyclase has been examined only when those proteins have been purified and removed from the influence of the membrane environment. The hydrolysis-resistant photoaffinity GTP-analog, P^3-(4-azidoanilido)-P^1 5′-GTP (AAGTP) is able to bind specifically to the G-proteins in rat cerebral cortex synaptic membranes and, in this study, we have used this probe to examine the specificity and selectivity of guanine nucleotide binding to each G-protein without removing those proteins from the synaptic membrane. Marked differences were noted between guanine nucleotide binding data obtained with detergent-soluble G-proteins and data from this in situ approach. In these studies it was found that the affinity of the G-proteins binding AAGTP correlated well with the expression of adenylate cyclase activity, the affinity of both forms of G_s increasing under conditions favoring the stimulation of that enzyme.

Signal transduction; Receptor-effector coupling; Cytoskéleton; Cyclic nucleotide; Adenylyl cyclase; GTP-binding protein

1. INTRODUCTION

GTP-binding proteins in the synaptic membrane appear to mediate the stimulation (G_s) and inhibition (G_i) of adenylate cyclase [1] and have been implicated in the regulation of other elements of neuronal signal transduction as well [2]. The regulation, specificity and selectivity of guanine nucleotide binding by G-proteins has been studied, extensively, in the photoreceptor G-protein (Gr) system, which has the advantage of being largely buffer soluble [3–5]. Attempts to study guanine nucleotide binding among the adenylate cyclase G-proteins have been confined to detergent solubilized, purified, preparations in which GTP binding was studied in solution [6] or in phospholipid vesicles [7]. Despite the elegant simplicity of such experiments, components of the adenylate cyclase system are known to be influenced by lipid and protein components of the plasma membrane [8–10]. One study has attempted to account for the contribution of various membrane components, by assessing [^{3}H]GppNHp binding to brain membrane fractions [11], but individual G-protein targets of this ligand could not be determined. AAGTP has been used as a photoaffinity probe for adenylate cyclase G-proteins in both pigeon erythrocytes [12] and synaptic membranes [13,14], and this compound is a potent, hydrolysis-resistant activator or inhibitor of that enzyme [14].

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Abbreviations: AAGTP, P^3-(4-azidoanilido)-P^1 5′-GTP; G, GTP-binding protein α subunits; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GppNHp, guanylylimidodiphosphate

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A major distinction between the synaptic membrane adenylate cyclase system and that enzyme in other cell types is the ineffectiveness of hormone or neurotransmitter in promoting the stimulation (and weak effectiveness in promoting inhibition) of the enzyme. Serendipitously, significant inhibition or stimulation can be achieved by the incubation of guanine nucleotide to these membranes in the absence of neurotransmitter. Further, inhibition or stimulation of adenylate cyclase provoked by hydrolysis-resistant GTP analogs, persists subsequent to washing of the membranes [14,15]. In this study, we use AAGTP to demonstrate that the in situ binding of GTP analogs to synaptic membrane G-proteins shows kinetics which are distinct from those which might be predicted from studies with purified proteins. Further, minor changes in assay conditions which favor stimulation of adenylate cyclase, increase the binding affinity of the G-proteins associated with that process.

2. EXPERIMENTAL

2.1. *Membrane preparations*

Male Sprague-Dawley, weanling (21-day-old) rats (BioLab, Chicago) were killed by cervical dislocation. Brains were removed, and enriched synaptic membrane fractions were prepared from cerebral cortices as described previously [16] with modifications [14]. Membranes were stored under liquid nitrogen until use.

2.2. *Photoaffinity labelling*

Membranes were thawed, resuspended in 2 mM Hepes, 1 mM MgCl (pH 7.4) and washed to remove phenylmethylsulfonyl fluoride and dithiothreitol. 100 μg aliquots were then incubated with indicated concentrations of AAGTP and other nucleotides (as noted) in a final volume of 50 μl at 23°C. After 3 min, membranes were washed twice in 2 ml of the above buffer, resuspended in 50 μl and irradiated 5 min with a mineralight (8 W, 254 nM) at distance of 6 cm. The reaction was quenched with the above buffer containing 4 mM dithiothreitol and membranes were subjected to SDS-PAGE on 10% gels. No significant hydrolysis of AAGTP occurs during the assay period (as indicated by thin-layer chromatography) and no phosphorylation occurs as verified by the lack of radioactive incorporation in membranes not subjected to UV irradiation. All experiments were done under yellow light (Philips F40G0). See [14] for further details.

2.3. *AAGTP binding studies*

Saturation binding studies were performed by incubating membranes (as above) with increasing concentrations of [^{32}P]AAGTP, followed by UV irradiation and SDS-PAGE. Companion incubations (for the determination of non-specific binding) contained a 100-fold excess of cold AAGTP along with each concentration of [^{32}P]AAGTP. Radiofluorographs were used to localize and excise labelled bands, and radioactivity in those bands was determined by scintillation counting. After determination of specific activity, the Lundon 1 program [17] was used for non-linear least squares regression analysis of the binding isotherms and for the determination of K_d values for each G-protein.

Determination of K_i values was performed by incubation of 10^{-5} M [^{32}P]AAGTP with concentration of cold GTP analogs from 10^{-8} to 10^{-4} M. After determination of radioactivity in bands, K_i values were estimated by a 'symplex' curve fitting program written for the IBM PC by J.H.G. using assumptions and equations from [18].

2.4. *Materials*

P-azidoaniline was a gift from G.L. Wheeler. Purified G-proteins were the gift of J. Codina and L. Birnbaumer and islet-activating protein (a pertussis toxin) was the gift of T. Saitoh. [α-^{32}P]GTP was obtained from ICN (Irvine, CA) and all nucleotides were purchased from Boehringer Mannheim (Elkhart, IN). Purity was verified by thin-layer chromatography prior to use. All other chemicals were of the highest grade obtainable.

3. RESULTS AND DISCUSSION

A brief incubation of AAGTP with membranes followed by washing and UV photolysis causes the covalent incorporation of AAGTP to several membrane proteins. Fig.1 shows the pattern of AAGTP labelling obtained when experiments are done as indicated in the legend. Proteins have been identified, previously as G_{SH} (a protein with an apparent molecular mass of 52 kDa which is a cholera toxin substrate), G_s (apparent molecular mass 42 kDa, also a cholera toxin substrate), $G_{i/o}$ two pertussis toxin substrates of about 40 kDa and G_{32}, a novel, neural G-protein of unknown function [14]. The identity of these proteins has been verified by toxin labelling and co-electrophoresis with purified G proteins [14].

When individual bands were cut and the radioactivity quantitated, the binding was seen to be specific and saturable. Binding was rapid, and complete in less than 2 min. The non-specific bound [^{32}P]AAGTP was estimated by incubating membranes in the presence of a 100-fold excess of unlabelled AAGTP and this accounted for 34 ± 2% (SD) of the total radioactivity. The specific bound isotherms for each of the G-proteins studied were characteristic of a one site model (fig.2a) as both the linear Scatchard plot (inset fig.2a) and the statistical analysis of the non-linear regression best

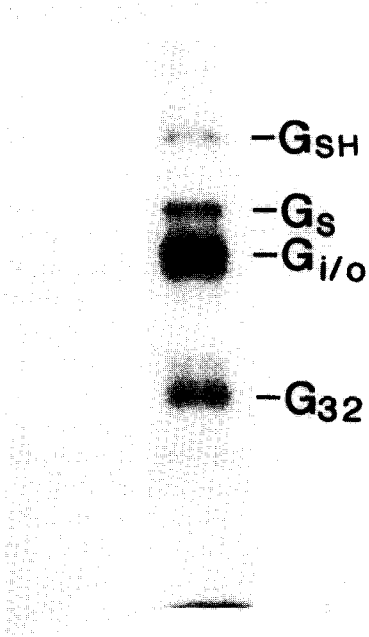

Fig.1. AAGTP labelled synaptic membrane proteins. Synaptic membranes (100 μg), prepared as described were incubated with 1.2×10^{-5} M [^{32}P]AAGTP (final volume 50 μl). After washing, resuspension UV irradiation and SDS-PAGE were carried out as indicated and a representative autoradiograph is shown. Radioautography shows incorporation of [^{32}P]AAGTP into 4 major bands. Exposure of membranes to 10^{-3} M AAGTP (or GppNHp or GTPγS) and washing prior to incubation of membranes with [^{32}P]AAGTP blocked incorporation of that compound. Likewise, if membranes were not UV irradiated, no ^{32}P was incorporated. The G protein bands are: G_{SH}, SDS-PAGE M_r = 52000 (sequence M_r = 46000). This is a cholera toxin substrate which stimulates adenylate cyclase [26,27]; G_s, SDS-PAGE M_r = 42000 (sequence M_r = 45000). This is a cholera toxin substrate which stimulates adenylate cyclase [26,27]; G_i, SDS-PAGE M_r = 40000 (about the same as sequence M_r). This is a pertussis toxin substrate which inhibits adenylate cyclase and may also regulate K^+ channels [28]; G_o, slightly smaller than G_i. This is a pertussis toxin substrate without clear function in second messenger systems. It has been reported to regulate some Ca^{2+} channels [29]; G_{32}, SDS-PAGE M_r = 32000. This is a major AAGTP binding protein in rat cerebral cortex synaptic membranes [14]. It is not clear whether this species functions in signal transduction.

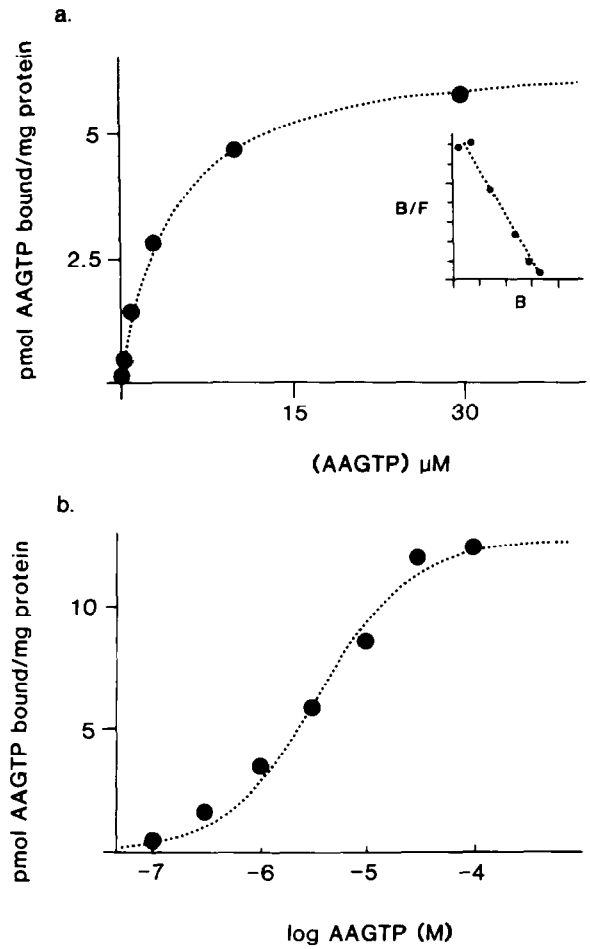

Fig.2. Binding isotherms for [^{32}P]AAGTP binding to synaptic membranes from rat cortex. (a) Bound vs free graph (with Scatchard inset) [30] of [^{32}P]AAGTP binding isotherm to the G_s protein in synaptic membranes. (b) Bound vs log free graph [31] of [^{32}P]AAGTP binding isotherm to the $G_{i/o}$ protein in synaptic membranes. The binding of [^{32}P]AAGTP was conducted as described in section 2 and the legend to fig.1. Nonspecific binding was estimated with a 100-fold excess of cold AAGTP. The final protein concentration was kept between 2.8 and 3.4 mg/ml. After counts were collected the specific activity at the time of counting was determined. Curve fitting was performed with the Lundon 1 program and data from one of four similar experiments are displayed for G_s and $G_{i/o}$. Similar curve fitting was performed in each experiment for each of the proteins, and in two additional experiments for separate G_i and G_o. Hill coefficients calculated from the above data are: 0.963 ± 0.097, G_{SH}; 0.858 ± 0.092, G_s; 0.915 ± 0.142, $G_{i/o}$ and 0.962 ± 0.155, G_{32}.

fit a single site [17]. [^{32}P]AAGTP binding to the various G-proteins appeared saturable, and the plateau indicative of saturation is apparent in the isotherm for the $G_{i/o}$ protein band when graphed in the bound vs log free coordinate system (fig.2b). Although a single plot for G_s (fig.2a) and for $G_{i/o}$ (fig.2b) is displayed, similar fits were obtained for each G-protein in four separate experiments. The ability of various guanine nucleotide analogues to displace the [^{32}P]AAGTP for the different G-

protein bands is also indicative of specificity of the binding (table 1).

The apparent K_d for [^{32}P]AAGTP ranged from 2.12 to 5.70 μM, with the lowest K_d (highest affinity) displayed by the $G_{i/o}$ band. The $G_{i/o}$ band consists of two distinct proteins (G_i and G_o), however when these bands were separated on a 26 cm gel, no significant difference was noted in the maximum AAGTP bound (G_i = 2.78 ± 0.09, G_o = 2.94 ± 0.26 pmol/mg membrane protein) or the K_d (G_i = 2.73 ± 0.42, G_o = 2.88 ± 1.15 μM) of the individual protein components.

The K_i value for the various GTP analogues was internally consistent, as the same rank order was noted for each protein band, i.e. $G_{i/o} > G_{32} > G_s > G_{SH}$ (table 1). The rank order of potency for AAGTP displacement by each of the GTP analogues studied was the same for each of the G-proteins, i.e. GPP(NH)P > GDPβS > GTPγS > AAGTP > GTP. Adenosine analogues (ATP and AppNHp) did not displace [^{32}P]AAGTP binding at concentrations to 100 μM.

When the [^{32}P]AAGTP binding isotherms were determined under conditions in which AAGTP stimulates, rather than inhibits, synaptic membrane adenylate cyclase (i.e. 30°C with 5 mM Mg^{2+}), the amount of AAGTP bound/mg membrane protein for each of the G-proteins was unchanged. Nonetheless, apparent affinity of [^{32}P]AAGTP for the two stimulatory G proteins (i.e. G_{SH} and G_s) was increased while the affinity of AAGTP for the other proteins was not changed significantly by these assay conditions (fig.3). Thus it would appear that one significant result of a change in adenylate cyclase assay conditions from 23°C to 30°C and Mg^{2+} concentrations from 1 to 5 mM, is a shift in the affinity of G_s and G_{SH} for GTP analogues.

We report an average K_d for AAGTP of 3.12 μM for the four proteins studied. This is tenfold higher than the 0.33 μM K_d reported for AAGTP binding to pigeon erythrocyte membranes [12] but the different conditions and membranes employed render moot, attempts at a direct comparison. Furthermore, we are unable to determine a valid B_{max} for AAGTP at each of these proteins. The primary reason for this is that we cannot calculate a precise efficiency for AAGTP photoincorporation without knowing the quantities of each G-protein on the synaptic membrane. The efficiency of AAGTP photoincorporation into tubulin has been calculated as 2.4% [19] and AAGTP incorporation for G-proteins released from the synaptic membrane ranges from 1.5 to 2.2% [13]. However, G-proteins constitute too small a fraction of the total membrane protein to measure by protein staining and densitometry, and neither toxin labelling nor immunoblotting can provide accurate quantitation of synaptic membrane G-proteins.

It is noteworthy that G_o is reported to exist in great excess over G_i in homogenates of bovine brain [20,21], yet the AAGTP bound in these proteins appears equal. Further, the total G_s represents at least 100-fold less protein than $G_{i/o}$ and AAGTP binding does not reflect this. Studies for the determination of G-protein amounts relied upon protein purification and assays for G-

Table 1

Dissociation constants (K_d) for ^{32}P-AAGTP and K_i values for GTP analog binding to specific G-proteins in rat cerebral cortex synaptic membranes

Protein band	G_{SH}	G_S	$G_{i/o}$	G_{32}
K_d (μM)	5.70 ± 1.25	4.87 ± 0.64	2.12 ± 0.50	3.59 ± 0.59
K_i (μM)				
AAGTP	8.51 ± 0.89	7.47 ± 1.29	3.59 ± 0.23	5.29 ± 2.17
GTPγS	6.48 ± 1.76	2.56 ± 0.91	2.13 ± 0.86	2.26 ± 0.64
Gpp(NH)p	1.91 ± 0.48	1.56 ± 0.16	0.46 ± 0.12	1.11 ± 0.46
GDPβS	4.72 ± 0.78	1.78 ± 0.23	1.46 ± 0.49	2.01 ± 0.63
GTP	28.7 ± 3.3	33.9 ± 4.2	24.6 ± 6.2	32.0 ± 3.8

Binding isotherms for [^{32}P]AAGTP were constructed and analyzed for K_d as described in section 2 and the legend to fig.1. Values listed are means ± SE of individual determinations from four separate experiments

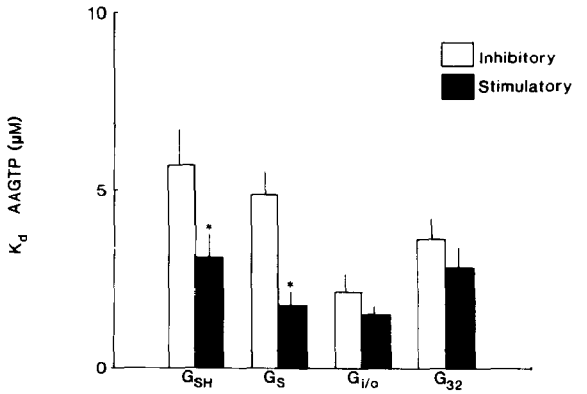

Fig.3. Effects of assay conditions on K_d values for [^{32}P]AAGTP binding to G-proteins in synaptic membranes. Binding isotherms were constructed and analyzed as described. Under inhibitory (open bars, see fig.1) and stimulatory conditions (closed bars). Stimulatory conditions included 5 mM Mg^{2+} in the Hepes buffer and incubation of binding reaction mixture at 30°C for 3 min. Data represent means ± SE of data obtained in 4 (inhibitory) or 3 (stimulatory) separate experiments. * Significant decrease in apparent K_d value relative to inhibitory conditions, analysis of variance, Duncan's test ($p < 0.05$).

proteins involved either GTPγS binding in solution or reconstitution of adenylate cyclase activity. The present studies were designed specifically to determine K_d values for the binding of GTP analogs to G-proteins in their native milieu. No attempt was made to remove previously bound GTP or GDP. Thus, it is possible that some of the G-proteins were not available for AAGTP binding under the membrane preparation and/or binding assay conditions used in this study.

Synaptic membrane adenylate cyclase functions independently of neurotransmitter receptors in broken cell preparations and we have used this independence to study G-protein binding related to adenylate cyclase activity. The EC_{50} of AAGTP for the activation of synaptic membrane adenylate cyclase is 1.1 μM and this correlates well with the K_d of 1.6 μM displayed by G_s for this compound (the K_d of G_{SH} for AAGTP is 3.11 μM). Likewise, the EC_{50} of AAGTP for the inhibition of synaptic membrane adenylate cyclase is 0.97 μM and the K_d for AAGTP displayed by $G_{i/o}$ was 2.12 μM.

Higher affinities have been reported for [^{35}S]GTPγS binding to purified G-proteins [6,7] but, as membrane elements clearly influence adenylate cyclase activity, it is possible that these elements influence GTP-binding as well. Yamazaki et al. [3] have found this to be the case for the photoreceptor system. Curiously, calculated EC_{50} values for the stimulation of synaptic membrane adenylate cyclase are 1.61 μM for GppNHp and 0.04 μM for GTPγS while the EC_{50} values of these compounds for the inhibition of synaptic membrane adenylate cyclase are 0.05 and 0.003 μM, respectively (Marcus and Rasenick, unpublished). Consistent with the above, $G_{i/o}$ shows the highest affinity for AAGTP, however, the K_i for GppNHp and GTPγS at $G_{i/o}$ is 2–3 orders of magnitude greater than the EC_{50} of those compounds for adenylate cyclase inhibition. The simplest explanation is that only a percentage of the total G_s or G_i is required to activate or inhibit adenylate cyclase [13].

The change of incubation temperature and [Mg^{2+}] which promote stimulation rather than inhibition of adenylate cyclase cause a dramatic shift in the K_d of AAGTP for G_{SH} and G_s without affecting nucleotide affinity at $G_{i/o}$ or G_{32}. Preliminary data (Rasenick and Marcus, unpublished) indicate that changes in both [Mg^{2+}] and temperature are required for this change in AAGTP affinity. Although temperature effects have not been evaluated, the Mg^{2+} requirement for nucleotide binding to G-proteins appears to be quite low [3,7] and this is true for AAGTP as well (Hatta and Rasenick, unpublished). Mg^{2+} increased the rate of GTPγS binding to G_s and was required for activation of the GTPase, but the requirements were 20 nM and 10 μM, respectively [7]. Mg^{2+} can also provoke dissociation of the α from the βγ subunits of G-proteins, but [Mg^{2+}] concentrations in excess of 25 mM are required [22]. The subtle changes in [Mg^{2+}] and temperature which cause guanine nucleotides to stimulate rather than inhibit synaptic membrane adenylate cyclase [23] do not fit with the above schemes. Two possible explanations are (i) that G-protein affinities for and interactions with Mg^{2+} are different when those proteins are in the membrane and (ii) that observed Mg^{2+} effects involve additional synaptic membrane proteins under these circumstances.

We have proposed that G-proteins might associate and exchange nucleotide with one another on the membrane [14,18,24], however, in those experiments, increases in nucleotide binding (and affinity) at G_s were paralleled by decreases on

AAGTP binding to $G_{i/o}$. Such compensatory changes would not occur under the conditions of these experiments and, as such, G-protein nucleotide exchange is an unlikely explanation for this phenomenon. The possibility of G-proteins forming complexes which alter binding affinity cannot be ruled out, indeed, evidence exists for such cooperative interaction in the photoreceptor system [25]. However, Hill coefficients for the G-proteins average 0.925 (fig.2) and none are significantly different than 1. Thus, no 'classic' cooperativity has been observed in these studies. Nonetheless, the participation of other synaptic membrane constituents (particularly cytoskeletal elements [10,13,19,24]) to alter affinities at certain G-proteins remains a possibility.

Clearly, under most conditions, an agonist occupied receptor is required to activate G-protein mediated systems and the receptor has been shown to change the kinetics of GTP-binding to G_s and the photoreceptor G-protein. The differences between the parameters of GTP binding observed in these studies and those noted in studies with purified proteins may be due to the participation of other components in the receptor-G protein-effector syncytium. Whether these other components are soluble and lost during purification or whether the mechanical integrity of the membrane contributes to regulation of GTP binding remain open questions, but AAGTP may serve as a useful probe to address them.

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