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Agonist-stimulated [^{35}S]GTPγS autoradiography: optimization for high sensitivity

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Abstract

The receptor-stimulated accumulation of [^{35}S]GTPγS provides a measure of functional coupling of G proteins with receptors. Sensitivity for autoradiographic visualization of [^{35}S]GTPγS binding was improved two- to threefold in rat brain sections by optimizing assay conditions. Non-specific (NSB), basal and agonist-stimulated [^{35}S]GTPγS binding were measured, using methadone, 5-carboxamidotryptamine and epinephrine for μ-opiate receptors, 5-HT$_{1A}$ receptors and α$_2$-adrenoceptors. Basal and NSB were low in glycylglycine buffer compared to Tris or HEPES buffers, and agonist-stimulated [^{35}S]GTPγS binding was more easily observed. Further optimization using glycylglycine buffer found increased signal-to-noise ratio with inclusion of dithiothrietol, increased [^{35}S]GTPγS incubation time (2–4 h) and guanosine 5′-diphosphate (GDP) preincubation (20–30 min), and use of [^{35}S]GTPγS at 0.1 nM. Improved sensitivity was due to decreased NSB and basal [^{35}S]GTPγS binding and agonist-stimulated binding were similarly affected for each receptor system. The assay conditions described should extend the use of agonist-stimulated [^{35}S]GTPγS autoradiography to receptors, which produce low levels of [^{35}S]GTPγS binding and to the measurement of changes in receptor–G protein coupling. © 2001 Published by Elsevier Science B.V.

Keywords: Signal transduction; α$_2$ adrenergic receptor; G protein-coupled receptor; μ-Opiate receptor; 5-HT$_{1A}$ receptor

1. Introduction

The seven transmembrane receptor superfamily initiates cellular responses primarily by coupling to heterotrimeric guanine nucleotide binding regulatory proteins (G proteins), which in turn regulate intracellular effector systems (Gilman, 1995). The G protein-coupled receptors mediate the majority of intracellular signaling and regulation by intercellular neurotransmitters, hormones and other signaling factors, and by photo-transduction (Birnbaumer et al., 1990). Intracellular effector systems regulated by G proteins include adenylyl cyclases, phospholipases, phosphodiesterases and ion channels. Agonist stimulation promotes interaction between G protein-coupled receptors and G proteins, leading to G protein activation as the first step in signal transduction mechanisms.

When G protein-coupled receptors are stimulated, guanosine 5′-diphosphate (GDP) is released from the G protein–receptor complex, allowing GTP to bind in its place. This leads to the dissociation of the α from the βγ subunits of the heterotrimeric G protein complex and the subsequent regulation of signal transduction systems within the cell by these subunits. The system is turned off by the intrinsic GTPase activity of the α subunit, hydrolyzing GTP to GDP. Using [^{35}S]GTPγS, a GTP analog that is not hydrolyzed, and in the presence of high GDP concentrations, receptor-stimulated activation of G protein can be studied directly (Hilf et al., 1989). Because activation of G proteins is a functional consequence of G protein-coupled receptor stimulation, this assay provides a measure of the functional activity of these receptors. To date, this assay works best for receptors coupled to the pertussis toxin-sensitive G$_{i/o}$ family.

Recently, agonist-stimulated [^{35}S]GTPγS binding has been adapted to slide mounted tissue sections for autoradiographic studies (Sim et al., 1995). The primary factor which enabled the autoradiographic detection of agonist-stimulated [^{35}S]GTPγS binding was the discovery that tissue sections required incubation in very high (2 mM) GDP concentrations compared to membrane preparations,

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which require much lower (3–10 μM) GDP concentrations (Sim et al., 1995). High concentrations of GDP are necessary to shift G proteins to an inactive state, thus reducing basal levels of [^{35}S]GTPγS binding and allowing the agonist-stimulated increase in [^{35}S]GTPγS binding to be observed.

Autoradiographic localization of agonist-stimulated [^{35}S]GTPγS binding has been demonstrated in the central nervous system (CNS) for several $G_{i/o}$-coupled neurotransmitter receptor systems (Waeber and Moskowitz, 1997; Sim et al., 1997a; Happe et al., 2000; Rodriguez-Puertas et al., 2000; Newman-Tancredi et al., 2000), each displaying anatomic localization in the same brain regions where the receptors are found with receptor-binding autoradiography. It is clear that the level of receptor-stimulated [^{35}S]GTPγS binding varies among different G protein-coupled receptors and detection depends on several factors, including the density of the receptors, the catalytic efficiency of the receptor–G protein complex (Sim et al., 1997a), the G protein subtype that is activated (Waeber and Moskowitz, 1997), and the level of basal [^{35}S]GTPγS binding, which is heterogeneously distributed in the CNS (Happe et al., 2000).

Using previously published assay conditions (Sim et al., 1995), we did not obtain significant levels of agonist-stimulated [^{35}S]GTPγS binding in tissue sections using α_2-adrenoceptor agonists, although agonists for other receptors stimulated binding as expected (Waeber and Moskowitz, 1997; Sim et al., 1997a). We found that alteration of the original protocol (Sim et al., 1995), primarily by changing buffer composition and by inclusion of dithiothreitol, as described by Waeber and Moskowitz (1997), improved the signal-to-noise ratio between α_2-adrenoceptor agonist-stimulated and basal binding so that a consistent stimulation of [^{35}S]GTPγS binding could be seen in brain regions with high levels of α_2-adrenoceptors (Happe et al., 2000). Based on this, we carried out the current studies to clearly establish the optimal conditions that allow autoradiographic demonstration of agonist-stimulated [^{35}S]GTPγS binding for neurotransmitter systems with low expression levels or weak coupling to G proteins. The increased sensitivity described here will extend use of this assay for quantitative anatomic analysis of G protein coupling for many other G protein-coupled receptors, and for measuring changes in coupling, which can vary with physiologic status, development, pathologic conditions and in response to drug treatments. Part of this work has been presented in abstract form (Happe et al., 1999a).

2. Materials and methods

2.1. Materials

[^{35}S]GTPγS (1000–1500 Ci/mmol; guanosine 5′-(γ-thio)triphosphate) was purchased from NEN Life Science Products (Boston, MA). Epinephrine bitartrate, 5-carboxamidotryptamine maleate, dithiothreitol (DTT), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), glycylglycine HCl, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), Tris–HCl and Tris base were purchased from Sigma (St. Louis, MO); HEPES was purchased from Research Organics (Cleveland, OH), methadone HCl was purchased from Research Biochemicals (Natick, MA) and guanosine 5′-diphosphate sodium (GDP) was purchased from United States Biochemical (Cleveland, OH). All other chemicals were research grade.

2.2. Animals and tissue preparation

Adult Sprague–Dawley rats (185–250 g; Sasco, Kingston, NY) were anesthetized with halothane and killed by decapitation. Brains were rapidly removed, frozen on dry ice and stored wrapped in Parafilm and foil at −70°C. The animal care and use procedures were in strict accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. Serial 16-μm sections were cut in the horizontal plane at the level of the lateral septum, thaw mounted onto subbed slides and stored with desiccant at −20°C until used, usually within 2 weeks. If necessary, tissue was stored at −80°C for longer times, as recommended (Sim et al., 1997a). Sections were brought to room temperature and air dried 30 min prior to use.

2.3. Agonist-stimulated [^{35}S]GTPγS binding assay

Agonist-stimulated [^{35}S]GTPγS binding conditions were based on previously published methods (Sim et al., 1995, 1997a; Waeber and Moskowitz, 1997), which we modified to improve the signal-to-noise ratio (Happe et al., 1999a, 2000). The standard assay conditions established in this study are as follows. Sections were re-hydrated in assay buffer (50 mM glycylglycine, 3 mM MgCl2, 1 mM EGTA, 100 mM NaCl, pH 7.5) at room temperature for 10 min. Sections were incubated in assay buffer containing 2 mM GDP for 30 min, then incubated at room temperature for 2 to 4 h in assay buffer containing 0.1 nM [^{35}S]GTPγS, 2 mM GDP and 0.2 mM DTT. Receptor-stimulated [^{35}S]GTPγS binding was determined by inclusion of agonists at 1–100 μM, as specified. Basal [^{35}S]GTPγS binding was determined in the absence of agonist, and non-specific [^{35}S]GTPγS binding was determined in the presence of 1 μM unlabeled GTPγS. Following incubation, sections were washed twice for 3 min in ice-cold 50 mM glycylglycine, pH 7.4, containing 0.2 mM DTT, briefly dipped in ice-cold distilled water and rapidly dried under a stream of cool air. For autoradiography, sections were apposed to film (HyperFilm-βMAX, Amersham Life

Science, Arlington Heights, IL), generally for 24 h. Films were developed by standard techniques and analyzed using the MCID-M5 system (Imaging Research, St. Catherines, ON, Canada). For most assays, all samples were exposed to film for the same length of time and [^{35}S]GTPγS binding density was measured as relative optical density. When different film exposure times were required, film images were normalized using commercial ^{14}C standards (ARC146, American Radiolabeled Chemicals, St. Louis, MO) calibrated for ^{35}S (Miller, 1991). Identification of brain regions was confirmed by direct comparison to the sections used to produce autoradiograms following staining with Cresyl violet and by comparison to the atlas of Paxinos and Watson (1986).

For optimization studies, only the indicated assay conditions, individual assay components or incubation times were varied. Effects of changes in standard assay conditions were determined for non-specific, basal and agonist-stimulated [^{35}S]GTPγS binding in both the lateral septum and the striatum. Agonist-stimulated [^{35}S]GTPγS binding was determined for three different neurotransmitter receptors. Epinephrine (EPI; 100 μM) was used for α_2-adrenoceptors (Happe et al., 2000); 5-carboxamidotryptamine (5-CT; 10 μM) was used for 5-HT$_{1A}$ receptors (Waeber and Moskowitz, 1997); and methadone (1 or 10 μM), a partial agonist, was used for μ-opiate receptors (Sim et al., 1995). Data were analyzed using Prism3 (GraphPad, San Diego, CA). Statistical analyses used InStat (GraphPad). Differences were considered statistically significant when $p < 0.05$.

3. Results

Characterization of assay conditions for [^{35}S]GTPμS binding in tissue sections was carried out in detail to improve the signal-to-noise ratio between agonist-stimulated and basal binding. Changes in incubation times and assay components were examined for effects on basal, non-specific and agonist-stimulated [^{35}S]GTPμS binding. Agonist-stimulated [^{35}S]GTPμS binding was determined for three different neurotransmitter systems using methadone, 5-carboxamidotryptamine and epinephrine as agonists for μ-opiate receptors, 5-HT$_{1A}$ receptors and α_2-adrenoceptors, respectively. The first two represent receptor systems with well characterized, robust responses using this assay while the last is a system with a weak, difficult to detect response.

3.1. Effect of buffer system

The affect of different buffer systems was tested using the standard optimized assay conditions as described in Materials and methods. Significant levels of agonist-stimulated binding were most easily observed with glycylglycine buffer due to comparatively low levels of non-specific and basal [^{35}S]GTPγS binding (Fig. 1). In each buffer, non-specific and basal [^{35}S]GTPγS binding showed significant variation between brain regions ($P < 0.05$; one-way analysis of variance (ANOVA)) and the same distribution was found in each buffer. However, non-specific binding was lowest in glycylglycine buffer, low in HEPES buffer and significantly greater in Tris buffer compared to glycylglycine in almost all brain regions (Fig. 1, Table 1). Basal binding in each buffer system had the same heterogeneous regional distribution as non-specific binding. Basal binding was significantly greater than non-specific binding in each buffer for most brain regions examined (Fig. 1, Table 1) and basal [^{35}S]GTPγS binding was significantly greater in Tris and HEPES buffers than in glycylglycine buffer for most brain regions. In summary, non-specific and basal [^{35}S]GTPγS binding are both lowest in glycylglycine buffer, while Tris buffer has higher levels of both non-specific and basal binding and HEPES has low non-specific binding but the highest levels of basal binding. The effect of buffer composition on non-specific and basal [^{35}S]GTPγS binding does not show regional specificity.

Although total binding was lower in glycylglycine buffer compared to Tris or HEPES buffers, the visual identification of regions with significant levels of specific agonist-stimulated [^{35}S]GTPγS binding was most clear in glycylglycine buffer (Fig. 1, 3A–5A). The reduction in basal binding was especially important when levels of agonist-stimulated binding are low, as was the case for epinephrine stimulated-[^{35}S]GTPγS binding (compare Fig. 1, 3A–3C with 2A–2C)). The net affect of buffer composition on the signal-to-noise ratio for agonist-stimulated [^{35}S]GTPγS binding is indicated by expressing agonist-stimulated binding data as percent increase over basal binding (Table 2). The percent increase over basal binding was generally higher in glycylglycine buffer and Tris buffer than in HEPES buffer for brain regions where the agonist-stimulated binding is detectable. Similarly, the percent increase of agonist-stimulated binding over basal binding was consistently higher in glycylglycine buffer than in Tris buffer, but this was generally not statistically significant. The increased sensitivity for detection of agonist-stimulated [^{35}S]GTPγS binding was most apparent for epinephrine-stimulated binding, which was less than that produced by 5-CT and methadone. In brain regions such as the central grey and locus coeruleus, where basal binding is relatively high and epinephrine-stimulated [^{35}S]GTPγS binding is relatively low, agonist-stimulated binding was significantly greater than basal binding only in glycylglycine buffer (Table 2). Receptor stimulated [^{35}S]GTPγS binding for each agonist displayed similar anatomic distribution and relative abundance in each buffer system, indicating that buffers did not differentially affect receptor activation of G proteins for the μ-opiate receptors, 5-HT$_{1A}$ receptors or α_2-adrenoceptors.

Two other buffer systems were also examined (data not shown). In ammonium acetate buffer, [^{35}S]GTPγS binding

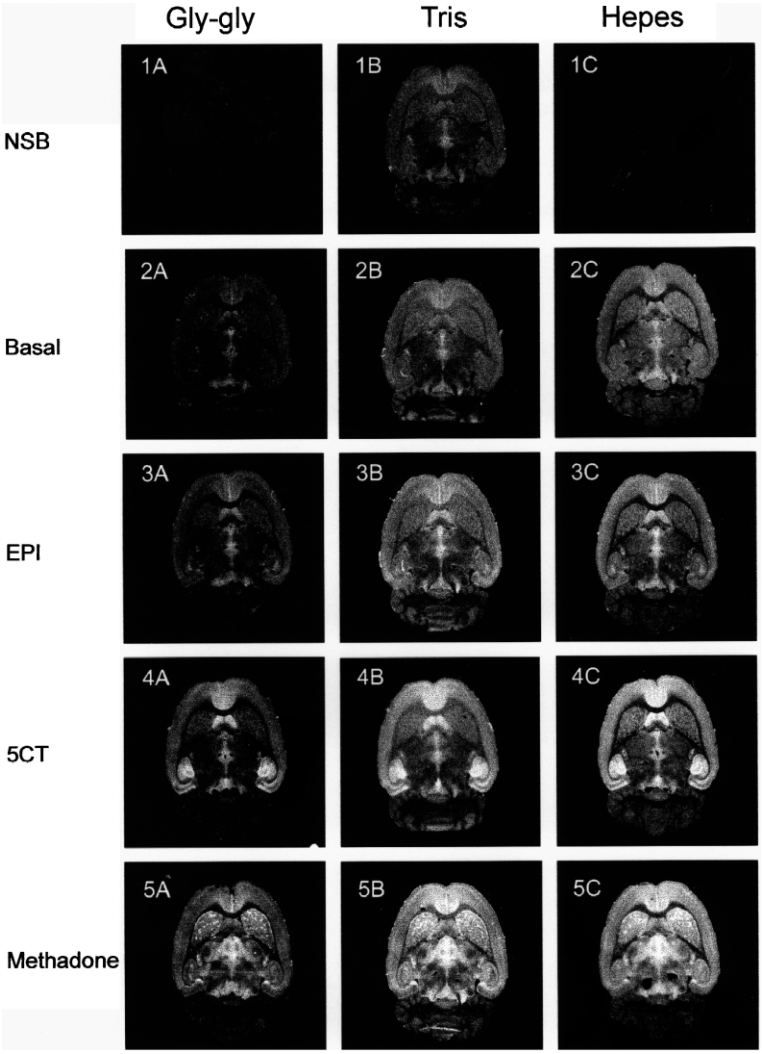

Fig. 1. The binding of [^{35}S]GTPγS was determined in different buffer systems. Shown are darkfield images of serial horizontal sections from one adult rat brain. The images are representative of results obtained with at least nine separate animals in three independent determinations. Each buffer ((A) glycylglycine (Gly–gly), (B) Tris–HCl (Tris) and (C) HEPES) was 50 mM, pH 7.5, and for each condition the same buffer was maintained in all incubation and wash steps. Other incubation buffer components were identical, each containing 3 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl and 0.2 mM DTT. Binding of [^{35}S]GTPγS is shown for (1) non-specific binding (NSB; containing 1 μM cold GTPγS), (2) basal binding in the absence of added agonist, (3) epinephrine-stimulated binding (EPI; 100 μM), (4) 5-carboxamidotryptamine-stimulated binding (5-CT; 10 μM), and (5) methadone-stimulated binding (10 μM).

was similar to binding in HEPES buffer with very low non-specific binding, high basal binding and moderate distinction between agonist-stimulated binding from basal binding. Sodium phosphate buffer, like glycylglycine buffer, had low non-specific and basal binding. The distinction between agonist-stimulated [^{35}S]GTPγS binding and basal binding was also very good. However, absolute [^{35}S]GTPγS binding was low and required exposure times seven times longer to obtain autoradiographic images equivalent to sections assayed in glycylglycine buffer. Based on the low levels of non-specific and basal binding, and the increased distinction between agonist-stimulated [^{35}S]GTPγS binding and basal binding, glycylglycine buffer was chosen as the buffer system for further optimization studies. For these studies, agonist-stimulated [^{35}S]GTPγS binding for 5-HT_{1A} receptors and α_2-adrenoceptors was measured in lateral septum and for μ-opiate receptors in striatum. These are large brain regions, providing sufficient serial sections for optimization studies, and they have high levels of the respective receptors.

3.2. Effect of dithiothreitol

Reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol are often used to prevent non-specific binding of ^{35}S-labeled compounds to tissue sections. Sections incubated in the absence of DTT had significantly higher levels of non-specific and basal [^{35}S]GTPγS bind-

Table 1
Effect of buffer composition on non-specific and basal [^{35}S]GTPγS binding
Rat brain sections were processed as described in the legend to Fig. 1 and in Materials and methods. Data are means ± S.E.M. from 15 animals and five separate experiments. Values are expressed as fmol/g tissue, as determined by image analysis using ^{14}C standards as described in Materials and methods. Data were analyzed by one-way analysis of variance. Where significant variation was found, the Tukey–Kramer Multiple Comparisons Test was used to determine differences between treatments within each brain region.

Region	[^{35}S]GTPγS binding (mean ± S.E.M., $n = 15$)					
	Gly–gly		Tris		HEPES	
	NSB	Basal	NSB	Basal	NSB	Basal
cc	69 ± 13	123 ± 16	218 ± 24[a]	273 ± 29[b]	137 ± 22	348 ± 30[b,c]
Cbm	63 ± 13	120 ± 23	111 ± 15	162 ± 21	96 ± 20	240 ± 22[1,b]
CG	121 ± 18	356 ± 33[c]	365 ± 40[a]	547 ± 38[b,c]	204 ± 39[d]	749 ± 45[b,c,e]
CgCtx	173 ± 31	338 ± 33[c]	386 ± 34[a]	540 ± 30[b]	273 ± 56[a]	731 ± 40[b,c,e]
CPu	126 ± 18	250 ± 26[c]	264 ± 26[a]	406 ± 28[b,c]	214 ± 35	543 ± 25
FrCtx	126 ± 21	236 ± 26[c]	250 ± 24[a]	377 ± 21[b,c]	214 ± 36	534 ± 27[b,c,e]
Hip	99 ± 12	215 ± 22[c]	258 ± 19[a]	346 ± 18[b,c]	172 ± 27[d]	498 ± 19[b,c,e]
LC	126 ± 22	296 ± 34[c]	282 ± 39	429 ± 42	186 ± 42	579 ± 51[b,c]
LS	155 ± 25	337 ± 23[c]	353 ± 32[a]	500 ± 13[b,c]	263 ± 53	704 ± 17[b,c,e]
PVA	169 ± 28	436 ± 28[c]	476 ± 65[a]	696 ± 55[b]	302 ± 66	996 ± 67[b,c,e]
EntCtx	99 ± 15	189 ± 22	262 ± 21	501 ± 86[b,c]	155 ± 22	407 ± 29[b,c]
VL	69 ± 9	137 ± 22	156 ± 18[a]	215 ± 20[b]	124 ± 17	322 ± 18[b,c,e]

Abbreviations: cc, corpus callosum; Cbm, cerebellum; CG, central gray; CgCtx, cingulate cortex; CPu, caudate putamen; FrCtx, frontal cortex; Hip, hippocampus; LC, locus coeruleus; LS, lateral septum; PVA, paraventricular thalamic nucleus; EntCtx, entorhinal cortex; VL, ventrolateral thalamic nucleus.
[a] Non-specific binding is significantly greater in the indicated buffer compared to glycylglycine buffer ($P < 0.05$).
[b] Basal binding is significantly greater in the indicated buffer compared to glycylglycine buffer ($P < 0.05$).
[c] Basal binding is greater than non-specific binding within the same buffer system ($P < 0.05$).
[d] Non-specific binding is significantly lower in HEPES than in Tris buffer ($P < 0.05$).
[e] Basal binding is significantly greater in HEPES than in Tris buffer ($P < 0.05$).

ing compared to those with added DTT (Fig. 2A). There was no significant change in total or specific epinephrine- or 5-CT-stimulated [^{35}S]GTPγS binding. Non-specific and basal binding were not further reduced by increasing the concentration of DTT from 0.2 to 1 mM DTT. The reduction in background was due to the presence of DTT

Table 2
Agonist-stimulated [^{35}S]GTPγS binding in different buffers
Rat brain sections were processed as described in the legend to Fig. 1 and in Materials and methods. Data are means ± S.E.M. from 8 to 12 animals and three to five separate experiments. Values for agonist-stimulated [^{35}S]GTPγS binding are expressed as percent increase over basal binding, calculated from raw data as [% basal = ((Agonist-stimulated binding − basal binding)/basal binding) × 100]. Abbreviations are listed in the legend to Table 1.
Data were analyzed by one-way analysis of variance. Where significant variation was found, the Tukey–Kramer Multiple Comparisons Test was used to determine differences between treatments.

Region	Percent increase over basal [^{35}S]GTPγS binding (mean ± S.E.M., $n = 8$–12)								
	Epinephrine			5-Carboxamidotryptamine			Methadone		
	glygly	Tris	HEPES	glygly	Tris	HEPES	glygly	Tris	HEPES
cc	3 ± 5	14 ± 9	2 ± 5	15 ± 6	1 ± 3	1 ± 3	44 ± 6[a]	27 ± 10	16 ± 5[b]
Cbm	2 ± 7	24 ± 8	3 ± 4	11 ± 9	9 ± 5	16 ± 6	7 ± 9	7 ± 11	17 ± 12
CG	36 ± 5[a]	53 ± 11	22 ± 5[c]	58 ± 10[a]	33 ± 6[b]	37 ± 4[a]	105 ± 18[a]	66 ± 13[a]	32 ± 5[a,b]
CgCtx	74 ± 11[a]	58 ± 13[a]	32 ± 4[a,b]	102 ± 8[a]	95 ± 11[a]	75 ± 9[a]	88 ± 7[a]	86 ± 12[a]	41 ± 8[a,b,c]
CPu	45 ± 9	33 ± 6	6 ± 4[b,c]	18 ± 6	14 ± 2	12 ± 2	250 ± 26[a]	202 ± 15[a]	175 ± 23[a]
FrCtx	48 ± 9[a]	41 ± 8[a]	24 ± 3[a,b]	65 ± 6[a]	45 ± 6[a]	41 ± 6[a,b]	60 ± 7[a]	33 ± 7[a]	22 ± 6[a,b]
Hip	115 ± 13[a]	92 ± 15[a]	50 ± 7[a,b,c]	270 ± 35[a]	245 ± 26[a]	169 ± 17[a,b]	95 ± 15[a]	106 ± 24[a]	42 ± 8[a,c]
LC	50 ± 7[a]	46 ± 7	27 ± 7	20 ± 5	17 ± 4	11 ± 3	85 ± 9[a]	93 ± 18[a]	45 ± 8[a,c]
LS	85 ± 6[a]	73 ± 8[a]	48 ± 4[a,b,c]	146 ± 11[a]	132 ± 9[a]	112 ± 11[a]	36 ± 4[a]	26 ± 6	11 ± 5[b]
PVA	60 ± 7[a]	59 ± 11[a]	32 ± 4[c]	26 ± 6	15 ± 5	11 ± 2	128 ± 14[a]	98 ± 18[a]	73 ± 12[a]
EntCtx	106 ± 16[a]	83 ± 10[a]	57 ± 6[a,b]	234 ± 23[a]	186 ± 23[a]	186 ± 20[a]	107 ± 15[a]	52 ± 10[b]	45 ± 7[a,b]
VL	12 ± 4	42 ± 13	6 ± 5[c]	10 ± 7	9 ± 5	1 ± 6	89 ± 14[a]	57 ± 13[a]	44 ± 7[a]

[a] Significantly greater than basal binding ($P < 0.05$).
[b] Significantly lower than agonist-stimulated binding in glycylglycine buffer ($P < 0.05$).
[c] Significantly lower than agonist-stimulated binding in Tris buffer ($P < 0.05$).

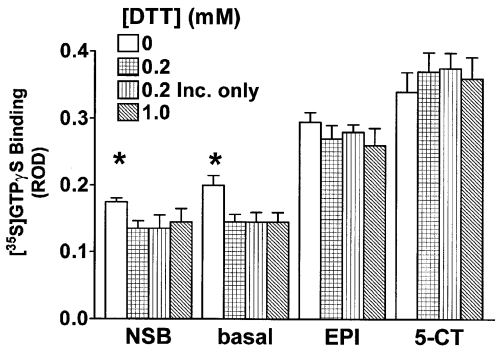

Fig. 2. The effect of dithiothreitol (DTT) on [^{35}S]GTPγS binding. These studies used 50 mM glycylglycine buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl. (A) The effect of varying concentrations of DTT on non-specific binding (NSB; with 1 μM cold GTPγS), basal, total epinephrine-(EPI; 100 μM) and 5-CT-stimulated (10 μM) [^{35}S]GTPγS binding. In some studies, 0.2 mM DTT was included in the incubation buffer but not the wash buffer. Data are means ± S.E.M., $n = 3$. The experiment was repeated two more times with the same results. In the absence of DTT non-specific and basal [^{35}S]GTPγS binding was significantly higher than in the presence of DTT ($^{*}p < 0.05$). (B) The effect of various concentrations of DTT on specific agonist-stimulated [^{35}S]GTPγS binding (basal binding subtracted). (C) The effect of various concentrations of DTT on specific agonist-stimulated [^{35}S]GTPγS binding, expressed as percent increase over basal binding. This panel indicates the increased signal-to-noise ratio obtained with DTT most clearly.

during the binding incubation and DTT is not required in the wash buffer. The net effect of reduced basal binding was to modestly increase the level of specific agonist-stimulated [^{35}S]GTPγS binding (Fig. 2B), which translates into an increase in signal-to-noise ratio of about 50% when agonist-stimulated binding is expressed as percent increase over basal binding (Fig. 2C).

3.3. Time course of agonist-stimulation of [^{35}S]GTPγS binding

Agonist-stimulated [^{35}S]GTPγS binding is a catalytic process; that is, an individual receptor can activate several G proteins during the course of the incubation period. The rate at which a given receptor stimulates [^{35}S]GTPγS binding is a measure of catalytic efficiency, which varies between different receptors (Sim et al., 1997a). Therefore, increasing the duration of the binding incubation should increase [^{35}S]GTPγS binding, which will improve the detection of agonist-stimulated binding and may be especially important for receptors expressed at low levels or with poor catalytic efficiency.

Total binding increased through 4 h for basal, non-specific and agonist stimulated [^{35}S]GTPγS binding in the lateral septum (Fig. 3A) and striatum (Fig. 3C). Agonist-stimulated binding increased more rapidly than basal or non-specific binding in each case. When basal binding was subtracted, the specific agonist-stimulated [^{35}S]GTPγS binding was found to be linear to 4 h (Fig. 3B,D).

3.4. Dependence on GDP concentration

The addition of GDP is required to detect agonist-stimulated [^{35}S]GTPγS binding. In agreement with previous studies using Tris buffer (Sim et al., 1995), 2 mM GDP provided the best distinction between agonist-stimulated [^{35}S]GTPγS binding and basal binding in glycylglycine buffer (Fig. 4).

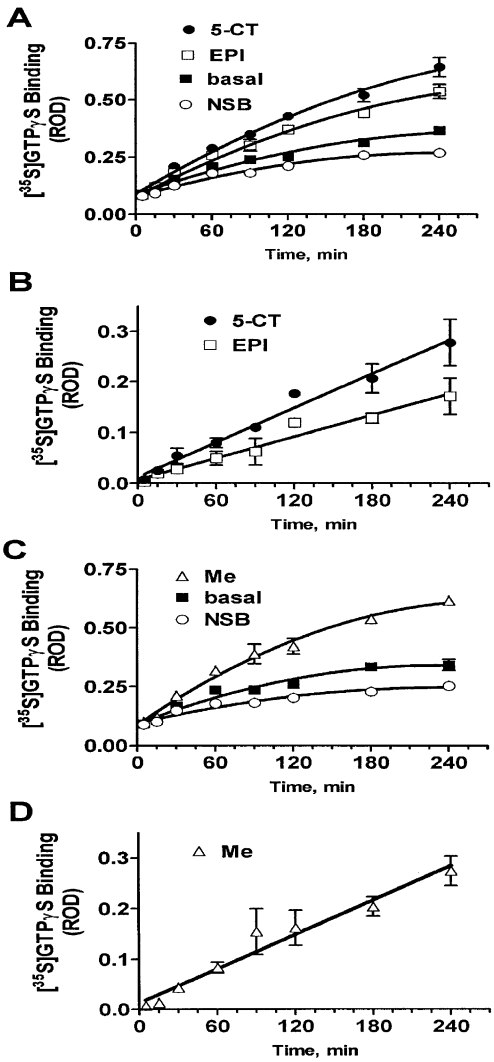

Fig. 3. The time course of [^{35}S]GTPγS binding. [^{35}S]GTPγS binding was determined for non-specific (NSB; 1 μM cold GTPγS), basal, and agonist-stimulated binding for the indicated times. Epinephrine (EPI; 100 μM), 5-carboxamidotryptamine (5-CT; 10 μM) or methadone (Me; 1 μM) were used as agonists. [^{35}S]GTPγS binding was measured in the lateral septum (A, B) and striatum (C, D) by computer-assisted densitometry. Values represent the mean relative optical density (ROD) ± S.E.M. for three animals. The experiment was repeated two more times with the same results. Total binding (A, C) and specific binding (B, D; total binding–basal binding) are shown for EPI-, 5-CT- and Me-stimulated [^{35}S]GTPγS binding. In each case, specific binding out to 4 h is best fit by linear regression analysis as compared to a second order polynomial ($r^2 = 0.96$, 0.98 and 0.96, for specific EPI-, 5-CT-, and Me-stimulated binding, respectively).

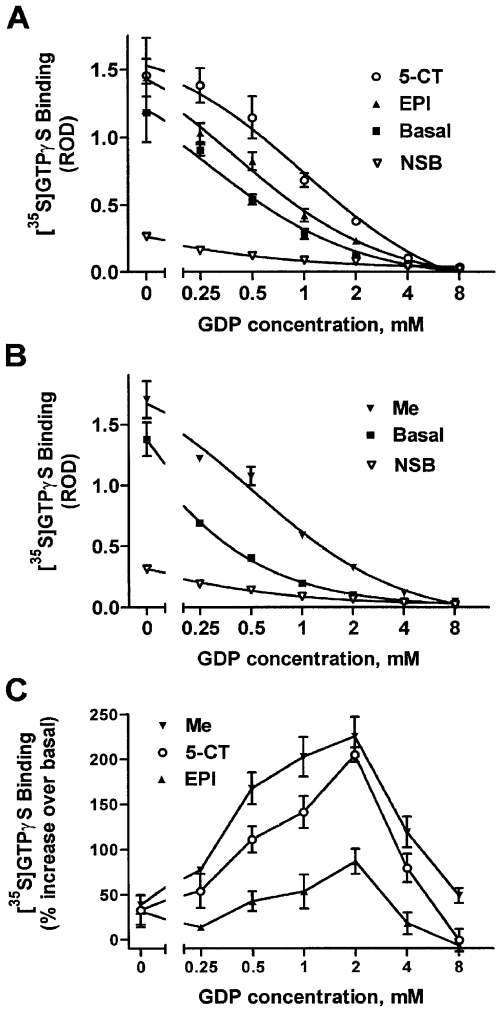

Fig. 4. Effect of GDP concentration on [^{35}S]GTPγS binding. The concentration of GDP was varied between 0 and 8 mM in both the 30-min GDP pre-incubation and the 4-h [^{35}S]GTPγS (0.1 nM) binding incubation. Total [^{35}S]GTPγS binding levels were determined for non-specific (NSB; 1 μM cold GTPγS), basal, and agonist-stimulated binding. Binding in the presence of 100 μM epinephrine (EPI) and 10 μM 5-carboxamidotryptamine (5-CT) were determined in the lateral septum (A), and in the presence of 1 μM methadone (Me) in the striatum (B). Data represent the means ± S.E.M., $n = 3$. The experiment was repeated two more times with the same results. Values in (A) and (B) are expressed as relative optical density (ROD). Specific agonist-stimulated binding, expressed as percent increase over basal binding, is shown in panel C.

As GDP concentration increased, total [^{35}S]GTPγS binding decreased for basal and agonist-stimulated conditions in both the lateral septum (Fig. 4A) and striatum (Fig. 4B). Non-specific binding, on the other hand, was much lower than basal and agonist-stimulated binding in the absence of GDP and decreased very little with increasing GDP concentration. In the absence of GDP, agonist-stimulated and basal [^{35}S]GTPγS binding could not be distinguished reliably (Fig. 4A,B) due to increased non-specific binding in all brain regions, including white matter (data not shown). At high GDP concentration (8 mM), basal and agonist-stimulated [^{35}S]GTPγS binding were low, indistinguishable from non-specific binding.

Although total [^{35}S]GTPγS binding decreased with increasing GDP concentration, the net effect was to increase the difference between basal binding and agonist-stimulated binding at concentrations up to 2 mM GDP and then reduced differences at higher GDP concentrations. Specific agonist-stimulated [^{35}S]GTPγS binding, expressed as percent increase over basal binding, was greatest at 2 mM GDP (Fig. 4C).

3.5. Time course of pre-incubation in GDP

Tissue sections were incubated in assay buffer containing 2 mM GDP for various times prior to the [^{35}S]GTPγS binding incubation. Total binding of [^{35}S]GTPγS declined as the time of pre-incubation in 2 mM GDP increased to 20 min and did not decline further with pre-incubation time to 30 min (Fig. 5A). Basal, non-specific and receptor-stimulated [^{35}S]GTPγS binding were all reduced by pre-incubation with GDP. However, as percent increase

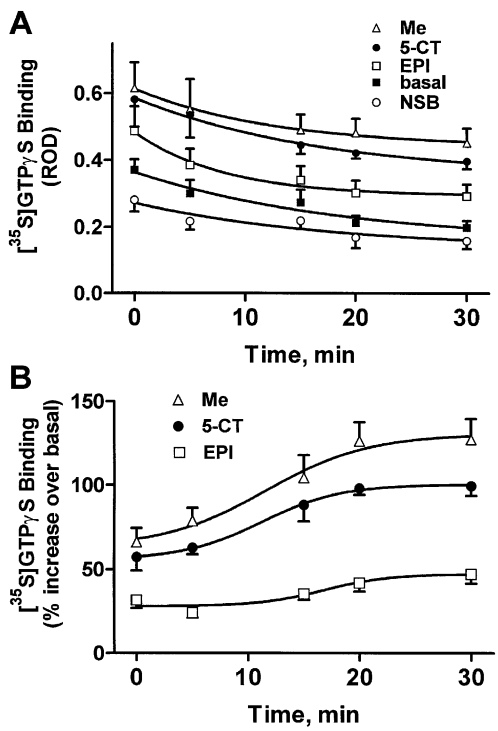

Fig. 5. Effect of pre-incubation on [^{35}S]GTPγS binding. The time of pre-incubation of sections in 2 mM GDP was varied between 0 and 30 min. Sections were then incubated for 2 h with [^{35}S]GTPγS as described in Materials and methods. (A) Total [^{35}S]GTPγS binding for non-specific (NSB; 1 μM cold GTPγS), basal, and agonist-stimulated binding. Binding in the presence of 100 μM epinephrine (EPI) and 10 μM 5-carboxamidotryptamine (5-CT) were determined in the lateral septum. Binding in the presence of 1 μM methadone (Me) was determined in the striatum. Data represent the means ± S.E.M., $n = 3$. Values are expressed as relative optical density (ROD). The experiment was repeated two more times with the same results. (B) Specific agonist-stimulated binding expressed as percent increase over basal binding.

over basal binding, specific epinephrine-, 5-CT- and methadone-stimulated [^{35}S]GTPγS binding increased to 20 min of pre-incubation in GDP (Fig. 5B). Values at 20 and 30 min were not statistically greater than at 15 min.

3.6. Time course of post-binding washes

Following the [^{35}S]GTPγS binding step, unbound nucleotide was washed from sections in ice-cold 50 mM glycylglycine buffer, pH 7.5, with 0.2 mM DTT. The length of wash steps was varied between 1 and 12 min with no effect on total, non-specific, basal or agonist-stimulated [^{35}S]GTPγS binding (data not shown). Two washes of 3-min each were routinely used, followed by a brief dip of sections in ice-cold H_2O to remove buffer and salts before the slides were dried.

3.7. Dependence on [^{35}S]GTPγS concentration

As expected, total [^{35}S]GTPγS binding was highly dependent on the concentration of [^{35}S]GTPγS. Total binding increased with concentration of [^{35}S]GTPγS, primarily due to increased non-specific and basal binding (Fig. 6A). With 0.02 nM [^{35}S]GTPγS films had to be exposed for 10 days to achieve sufficient signal for analysis, whereas with 1.0 nM [^{35}S]GTPγS film exposure was reduced to 4 h. Non-specific and basal binding at 1.0 nM [^{35}S]GTPγS were high in all brain regions, including white matter.

Specific agonist-stimulated binding, after subtraction of basal [^{35}S]GTPγS binding, increased for each agonist through 0.2 nM [^{35}S]GTPγS and leveled off at 0.5 nM (Fig. 6B). With 1.0 nM [^{35}S]GTPγS, specific agonist-stimulated binding was not detectable due to the large increase in basal binding (data not shown). The best signal-to-noise ratio for specific agonist-stimulated binding was obtained at 0.1 nM [^{35}S]GTPγS for each agonist (Fig. 6C), though this was not statistically better than at 0.05 nM [^{35}S]GTPγS. Analysis of specific agonist-stimulated binding over [^{35}S]GTPγS concentrations between 0.02 and 0.5 nM showed that the affinity of [^{35}S]GTPγS for binding to G proteins was similar for each agonist. In each case, data were best fit by single site saturation binding curve ($r^2 > 0.99$). Calculated affinities for [^{35}S]GTPγS were 0.21 ± 0.07, 0.21 ± 0.06 and 0.17 ± 0.05 nM for epinephrine-, 5-carboxamidotryptamine- and methadone-stimulated [^{35}S]GTPγS binding, respectively. The affinity of [^{35}S]GTPγS for binding under basal conditions, 1.25 ± 0.07 nM, determined after subtraction of non-specific binding (data not shown), was significantly different from the affinity of agonist-stimulated [^{35}S]GTPγS binding ($P < 0.001$, one-way ANOVA).

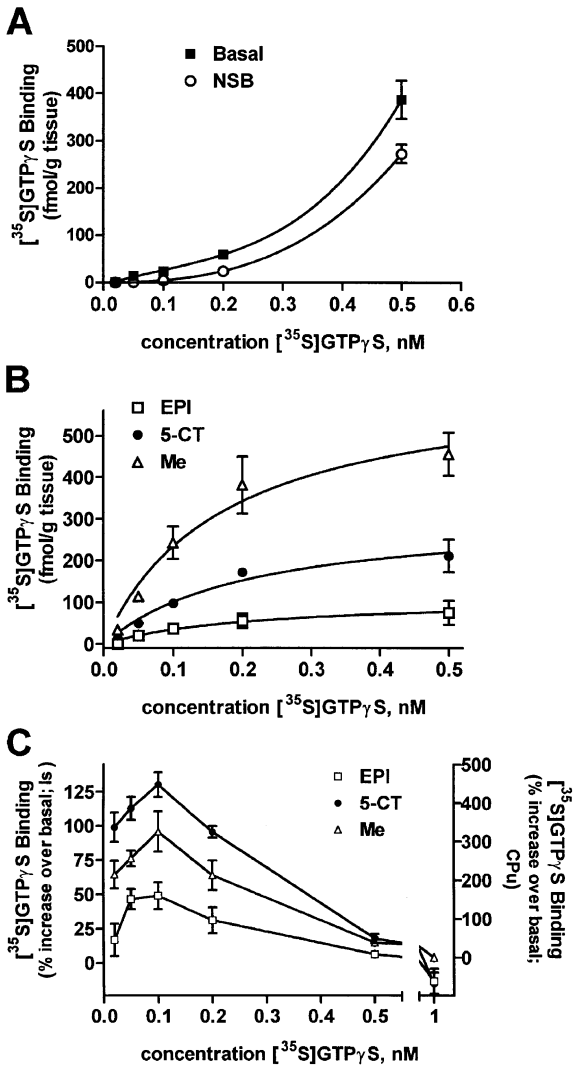

Fig. 6. Effect of [^{35}S]GTPγS concentration on [^{35}S]GTPγS binding. The concentration of [^{35}S]GTPγS was varied between 0.02 and 1.0 nM and each condition included 2 mM GDP. Incubation times were 2 h. Data represent the means ± S.E.M., $n = 3$. The experiment was repeated two more times with the same results. Films were exposed for different times ranging from 4 h to 10 days, and all films were analyzed using commercial standards calibrated for [^{35}S] at each exposure time. Values are expressed as fmol/g tissue and were measured in the lateral septum and striatum. (A) Basal and non-specific binding (NSB; 1 μM unlabeled GTPγS) are shown. At high [^{35}S]GTPγS concentrations, both basal and non-specific binding increased rapidly in all brain regions. At 1 nM [^{35}S]GTPγS, both basal and non-specific binding were greater than 900 fmol/g tissue (data not shown). (B) Specific [^{35}S]GTPγS binding stimulated by 100 μM epinephrine (EPI), 10 μM 5-carboxytryptamine (5-CT), and 10 μM methadone (Me). (C) Data for agonist-stimulated [^{35}S]GTPγS binding expressed as percent increase over basal binding. EPI- and 5-CT-stimulated binding in lateral septum (ls) are plotted on the left axis. Methadone-stimulated binding in the striatum (CPu) is plotted on the right axis.

3.8. Dependence on Mg^{2+} concentration

Agonist-stimulated [^{35}S]GTPγS binding is known to be Mg^{2+}-dependent (Hilf et al., 1989; Sim et al., 1995). Using the standard assay conditions, non-specific [^{35}S]GTPγS binding was not affected by added Mg^{2+} (Fig. 7A). On the other hand, whereas basal and agonist-

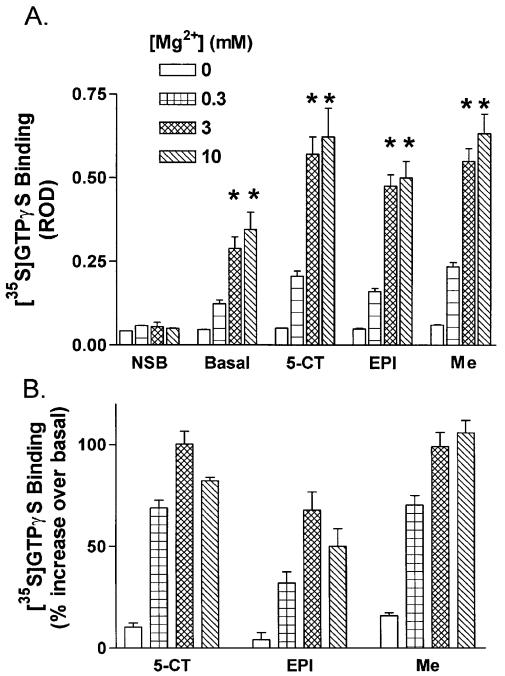

Fig. 7. Effect of Mg^{2+} concentration on [^{35}S]GTPγS binding. Binding of [^{35}S]GTPγS was measured in standard incubation buffer with either 0, 0.3, 3 or 10 mM $MgCl_2$. Data represent the means ± S.E.M., $n = 3$. The experiment was repeated two more times with the same result. (A) Total binding expressed as relative optical density (ROD) for non-specific (NSB; 1 μM cold GTPγS), basal, and agonist-stimulated binding. Binding in the presence of 100 μM epinephrine (EPI) and 10 μM 5-carboxamidotryptamine (5-CT) were determined in the lateral septum. Binding in the presence of 1 μM methadone (Me) was determined in the striatum. [^{35}S]GTPγS binding was significantly higher than in binding at 0 or 0.3 mM $MgCl_2$ ($^{*}p < 0.05$). (B) Agonist-stimulated binding expressed as percent increase over basal binding is shown.

stimulated binding were at the same level as non-specific binding in the absence of Mg^{2+}, both basal and agonist-stimulated [^{35}S]GTPγS binding increased with added Mg^{2+} (Fig. 7A). Basal and agonist-stimulated [^{35}S]GTPγS binding were significantly increased at 3 and 10 mM Mg^{2+}, but not at 0.3 mM Mg^{2+}, compared to binding in the absence of added Mg^{2+}. When specific agonist-stimulated [^{35}S]GTPγS binding data were expressed as percent increase over basal binding, the signal-to-noise ratio was optimal at 3 mM Mg^{2+} for each receptor system (Fig. 7B).

3.9. Dependence on NaCl concentration

Agonist-stimulated [^{35}S]GTPγS binding is dependent on the concentration of NaCl in the incubation buffer (Hilf et al., 1989; Sim et al., 1995). With the standard incubation conditions used in this study, non-specific, basal and total agonist-stimulated [^{35}S]GTPγS binding were all reduced by the addition of NaCl (Fig. 8A). Significant levels of specific agonist-stimulated [^{35}S]GTPγS binding were present in the absence of NaCl, indicating that NaCl is not an absolute requirement (Fig. 8B). However, the net effect of increasing NaCl concentration, which reduces total [^{35}S]GTPγS binding for all conditions, was to improve the detection of specific agonist-stimulated binding. The signal-to-noise ratio for agonist-stimulated binding was best at 100 to 200 mM NaCl (Fig. 8B).

3.10. Adenosine A_1 receptor effects on basal binding

We investigated the effects of adenosine deaminase (100 mU/ml) and the adenosine A_1 receptor antagonist, DPCPX (10 μM), in our system. Previous reports (Laitinen, 1999; Moore et al., 2000) found that this reduced basal binding, improving signal-to-noise ratio, by blocking the effects of endogenous adenosine at A_1 receptors. These studies used Tris buffer and we found similar results in Tris or HEPES buffer, i.e., basal binding was reduced 30% to 50% ($P < 0.05$, one way ANOVA, $n = 6$). However, addition of adenosine deaminase or DPCPX to the glycylglycine buffer system had much smaller effects that were not statistically significant (data not shown). Basal binding was not reduced significantly. Specific [^{35}S]GTPγS binding and specific to non-specific binding ratios were not

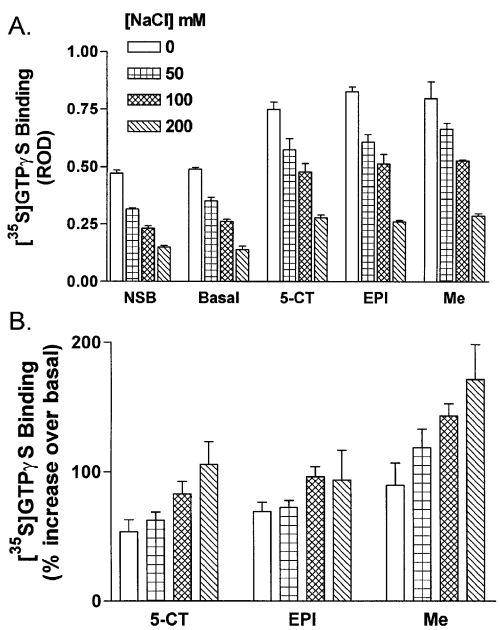

Fig. 8. Effect of NaCl on [^{35}S]GTPγS binding. Binding of [^{35}S]GTPγS was measured in standard incubation buffer with either 0, 50, 100 or 200 mM NaCl. Data represent the means ± S.E.M., $n = 3$. The experiment was repeated two more times with the same results. (A) Total binding expressed as relative optical density (ROD) for non-specific (NSB;1 μM cold GTPγS), basal, and agonist-stimulated binding. Binding in the presence of 100 μM epinephrine (EPI) and 1 μM 5-carboxamidotryptamine (5-CT) were determined in the lateral septum. Binding in the presence of 1 μM methadone (Me) was determined in the striatum. (B) Agonist-stimulated binding expressed as percent increase over basal binding is shown.

increased significantly. For this reason, we did not routinely use these reagents.

4. Discussion

Agonist-stimulated [^{35}S]GTPγS binding measures the functional state of G protein-coupled receptors, monitoring the initial step in G protein activation, and represents an important addition to the methods for studying G protein-coupled receptor regulation of intracellular effector systems. Agonist-stimulated [^{35}S]GTPγS binding can be used in tissue sections (Sim et al., 1995), so the functional activity of G protein-coupled receptors can be determined by autoradiography, maintaining a high level of anatomic resolution. In this study, we analyze conditions for agonist-stimulated [^{35}S]GTPγS binding autoradiography and provide an approach for improved resolution between agonist-stimulated and basal [^{35}S]GTPγS binding. The increase in signal-to-noise ratio should prove valuable in extending the use of this technique to receptors, which have low sensitivity in this assay, and improve studies in which dynamic changes in receptor activation of G proteins are monitored, such as during development, under pathologic conditions or due to drug effects.

To date, agonist-stimulated [^{35}S]GTPγS binding autoradiography has been demonstrated for several G protein-coupled receptors, including μ-, δ-, and K_1-opiate, cannabinoid, γ-aminobutyric acid type B, opioid receptor-like peptide, 5-HT_{1A} and 5-HT_{1B}, muscarinic M_2, histamine H_3, α_2-adrenoceptors, and adenosine A_1 receptors (Sim et al., 1995, 1996a,b, 1997a,b; Sim and Childers, 1997; Waeber and Moskowitz, 1997; Laitinen, 1999; Happe et al., 2000; Platzer et al., 2000; Moore et al., 2000). This method appears to be limited to receptors coupled to the pertussis toxin-sensitive $G_{i/o}$ class of G proteins. The inability to detect receptor stimulation through G_s is probably due to the slower dissociation of GDP from G_s (Carty and Iyengar, 1994; Wieland and Jakobs, 1994) and to the relative abundance of G protein classes, $G_o > G_i > G_s$, in the CNS (Sternweis and Robishaw, 1984). Although the particular receptor examined is controlled by the specificity of the agonist being used, the G protein subtypes that are activated are not clearly defined. Agonist-stimulated [^{35}S]GTPγS binding autoradiography probably demonstrates receptor activation of a heterogeneous population of G proteins (Prather et al., 2000).

It has been difficult to detect G protein-coupled receptors for which agonist-stimulated [^{35}S]GTPγS binding is low due either to low receptor density or to low catalytic efficiency of the receptor–G protein complex (Sim et al., 1997a). These are intrinsic properties that cannot be readily changed through alterations in the assay methods. The improved signal-to-noise ratio demonstrated in this study is primarily due to decreasing basal [^{35}S]GTPγS binding to a greater extent than agonist-stimulated binding. Whereas this allows detection of more G protein-coupled receptors, there may be others that will remain undetectable by these methods.

In this study, we sought to optimize the signal-to-noise ratio for agonist-stimulated [^{35}S]GTPγS binding. This could be accomplished through several mechanisms, such as increased binding of agonist to the receptor, increased interaction of the receptor with G proteins or a decrease in basal binding. We found no evidence that changes in assay conditions differentially affected any of the three G protein-coupled receptors studied, indicating that effects on agonist binding to receptors are not important. Although assay components such as NaCl concentration, buffer composition and the inclusion of GDP and GTP are known to affect the affinity of agonists and antagonists for G protein-coupled receptors in ligand binding studies, when these were varied in the [^{35}S]GTPγS binding assay we found no differential effects on agonist-stimulated binding.

Alterations to the original protocol (Sim et al., 1995, 1997a) that we found effective include the use of glycylglycine buffer, the inclusion of 0.2 mM dithiothreitol during the [^{35}S]GTPγS incubation (cf. Waeber and Moskowitz, 1997), increasing the length of the [^{35}S]GTPγS binding incubation and the length of preincubation with GDP, and increasing the concentration of [^{35}S]GTPγS in the binding incubation. In this autoradiographic study using glycylglycine buffer, the concentration dependencies of Na^+ and Mg^{2+} were the same as earlier studies in membrane preparations (Cerione et al., 1986; Hilf et al., 1989) and tissue sections (Sim et al., 1995, 1997a)using Tris buffer. Tissue sections require much higher concentrations of GDP than used in membrane preparations and our studies found the same optimal concentration of GDP for autoradiographic studies in glycylglycine buffer as previously shown in Tris buffer (Sim et al., 1995).

All conditions which increased the signal-to-noise ratio between agonist-stimulated and basal [^{35}S]GTPγS binding affected basal binding to a greater extent than agonist-stimulated binding. Our data are consistent with predictions of the ternary complex model of receptor-activated G proteins (De Lean et al., 1980; Costa et al., 2000), in that formation of the receptor–G protein complex can occur in the absence of agonist. However, agonists induce a conformational change in uncoupled receptors that greatly favors the formation of or stabilizes the receptor–G protein complex. Furthermore, the exchange of GTP or [^{35}S]GTPγS for GDP bound to the inactive G protein is enhanced by agonist-bound receptors compared to unoccupied receptors (Gilman, 1987). The increased activity in the presence of agonist and the catalytic nature of the receptor–G protein interaction probably accounts for the differential effects of changes in assay conditions on basal and agonist-stimulated [^{35}S]GTPγS binding.

Both basal and non-specific binding were decreased in glycylglycine buffer compared to Tris buffer. In HEPES buffer, non-specific binding was very low, but basal bind-

ing was high. Buffer effects on non-specific binding may not be related to effects on receptor–G protein mechanisms and it is the high non-specific binding present in Tris buffer that makes it difficult to discern agonist-stimulated [^{35}S]GTPγS binding for some receptor systems, such as the α_2-adrenoceptor. For α_2-adrenoceptors, Tris buffer acts as an allosteric modulator of agonist and antagonist binding, whereas glycylglycine buffer does not (Deupree et al., 2000), indicating that glycylglycine buffer does not produce conformational changes in the receptor. Tris buffer has also been shown to alter the conformation of the α_1-adrenoceptor, destabilizing a salt bridge important for producing agonist-stimulated changes in the receptor (Porter et al., 2000). The differential effects of glycylglycine and Tris buffers on [^{35}S]GTPγS binding support the idea that buffers can affect receptor conformation. The low non-specific [^{35}S]GTPγS binding in HEPES buffer differs from Tris buffer. However, basal binding is high in both HEPES and Tris buffers, indicating that buffer effects on basal and non-specific binding are independent. Furthermore, the increase in basal [^{35}S]GTPγS binding suggests that Tris and HEPES buffers may modulate the interaction of receptors with G proteins in the absence of agonist to increase the level of precoupled receptors and/or increase the affinity of the unoccupied receptor–G protein complex for [^{35}S]GTPγS. Consistent with an increase in precoupled receptors due to buffer effects, drugs known to act as inverse agonists at the α_2-adrenoceptor (Murrin et al., 2000) reduce apparent basal binding (Tian et al., 1994) in a region specific manner and the reduction of basal binding is larger in Tris and HEPES buffers than in glycylglycine buffer (Happe et. al, unpublished data).

Increasing incubation time takes advantage of the catalytic nature of receptor-stimulated activation of G proteins. Each receptor can interact with multiple G proteins during the course of the binding incubation, thus amplifying the receptor stimulation of [^{35}S]GTPγS binding (Hilf et al., 1989; Gierschik et al., 1991). Different receptor types are known to have different rates of G protein activation in the presence of agonist (Sim et al., 1996b). Like agonist-stimulated [^{35}S]GTPγS binding, basal binding also increases with length of incubation. To the extent that basal binding is determined by G protein–receptor interactions in the absence of agonist, its slower rate of [^{35}S]GTPγS binding indicates a lower catalytic efficiency for basal binding compared to binding stimulated by agonists.

NaCl is not an absolute requirement for detection of agonist-stimulated [^{35}S]GTPγS binding. The addition of sodium reduces total [^{35}S]GTPγS binding for agonist-stimulated, basal and non-specific binding. The net effect of Na^+ addition is to increase the signal-to-noise ratio between agonist-stimulated and basal binding, as discussed previously (Hilf et al., 1989; Tian et al., 1994; Sim et al., 1997a). Basal binding is reduced at high Na^+ concentrations, but the ability of agonist-stimulated receptors to promote G protein activation is not affected.

In addition to effects on basal binding, our data indicate that Na^+ also affects the level of non-specific [^{35}S]GTPγS binding, perhaps by reducing [^{35}S]GTPγS binding at higher ionic strength. In the absence of Na^+, greater non-specific binding is observed in all brain regions, including white matter, indicating this effect may not be related to effects on receptors or G proteins. Similar homogeneous increases in non-specific binding are found in the absence of dithiothreitol.

Detection of agonist-stimulated [^{35}S]GTPγS binding requires the addition of GDP, and the concentration required for tissue sections is considerably higher than in membranes (Hilf et al., 1989; Sim et al., 1995). In the absence of GDP, agonist-stimulated binding disappeared and high levels of basal [^{35}S]GTPγS binding were found in all brain regions, including white matter. Similar results were found with a very high concentration of [^{35}S]GTPγS (1 nM) even in the presence of 2 mM GDP. This indicates that the ratio of GDP to [^{35}S]GTPγS, not simply the presence of GDP, is an important determinant of basal binding. The requirement for higher GDP concentrations in tissue sections may be due, in part, to slow penetration of GDP into tissue sections, since it requires more than a 15-min preincubation with GDP to produce the maximal increase in signal-to-noise ratio. The effect of tissue section thickness was not examined in this study. Decreasing section thickness has been reported to increase the signal-to-noise ratio for agonist-stimulated [^{35}S]GTPγS binding (Waeber and Moskowitz, 1997), perhaps due to changes in GDP requirements.

The optimal signal-to-noise ratio was obtained at 0.1 nM [^{35}S]GTPγS. This was determined using a single concentration of GDP. Since GDP and GTP (or [^{35}S]GTPγS) compete for the same site, the apparent affinities of these nucleotides are interdependent and therefore the optimal concentration of [^{35}S]GTPγS is likely to change when other GDP concentrations are used. At 2 mM GDP, the K_d for agonist-stimulated [^{35}S]GTPγS binding was 0.2 nM and the K_d for basal binding (after subtraction of non-specific binding) was 1.7 nM, a difference that has been demonstrated previously (Hilf et al., 1989; Tian et al., 1994; Sim et al., 1996b; Happe et al., 1999b). In the current study, the affinities for [^{35}S]GTPγS in agonist-stimulated and basal binding are higher than in previous reports. In membrane studies, [^{35}S]GTPγS affinity ranged from 1 to 10 nM for agonist-stimulated binding and 14 to 100 nM for basal binding. These differences may be due to the use of tissue sections instead of membrane preparations, to the change in buffer composition, or to differences in the concentrations of GDP and [^{35}S]GTPγS.

High levels of basal binding may also be due to stimulation of receptors by endogenous agonist(s) in tissue sections. While this report was in preparation, two papers (Laitinen, 1999; Moore et al., 2000) demonstrated that the release of adenosine from tissue sections stimulates adenosine A_1 receptors to significantly increase apparent basal

[^{35}S]GTPγS binding, an effect blocked by using either adenosine deaminase to remove endogenous adenosine or by the addition of adenosine receptor antagonists. We included adenosine deaminase and the adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine in our standard assay conditions using glycylglycine, Tris, and HEPES buffers. In agreement with previous studies (Laitinen, 1999; Moore et al., 2000), we found a 30% to 50% reduction in basal binding in the Tris buffer system, and also in the HEPES buffer system. However, in glycylglycine buffer adenosine deaminase and DPCPX reduced basal binding only slightly. Non-specific binding was not reduced by adenosine deaminase or DPCPX treatment in any buffer system. Basal binding in Tris and HEPES buffer was not reduced to the level found in glycylglycine buffer. Since basal binding in glycylglycine buffer is already low, the small reduction by adenosine deaminase or DPCPX treatment does not significantly improve the signal-to-noise ratio of agonist-stimulated [^{35}S]GTPγS binding. Comparison of percent increase over basal binding values in this study and for μ-opiate receptor stimulated [^{35}S]GTPγS binding in rat striatum (Moore et al., 2000) and 5-HT$_{1A}$ receptor stimulated binding in rat hippocampus (Waeber and Moskowitz, 1997), indicate our optimized conditions are two to three times more sensitive than previously described assay conditions.

Residual endogenous neurotransmitters for other receptor systems have not been shown to stimulate [^{35}S]GTPγS binding to date. Attempts to dissociate endogenous neurotransmitters by pre-incubation in high GTP (Waeber and Moskowitz, 1997) and by preincubation steps with high salt washes or changes in pH (Happe et al., unpublished data) did not decrease basal [^{35}S]GTPγS binding. In the current study, basal binding is very low when using glycylglycine buffer and the small difference between basal and non-specific binding indicates that endogenous neurotransmitters do not greatly affect the sensitivity of the assay.

In summary, we demonstrate conditions which improve the sensitivity and anatomic resolution for agonist-stimulated [^{35}S]GTPγS autoradiography. The use of glycylglycine rather than Tris or HEPES buffers reduces non-specific and basal [^{35}S]GTPγS binding so that the signal from agonist-stimulated [^{35}S]GTPγS binding is more readily discerned. This is particularly important for receptors with a low level of agonist-stimulated [^{35}S]GTPγS binding, such as α$_2$-adrenoceptor. When using glycylglycine buffer, the NaCl, Mg^{2+} and GDP concentration dependencies were the same as in previous studies using Tris buffer (Sim et al., 1995). Factors other than buffer that increased the difference between agonist-stimulated and basal [^{35}S]GTPγS binding were inclusion of DTT in the incubation, increasing the concentration of [^{35}S]GTPγS to 0.1 nM in the presence of 2 mM GDP, increasing the length of the preincubation to 20–30 min and increasing the length of the [^{35}S]GTPγS binding incubation to 2–4 h. Agonist-stimulated [^{35}S]GTPγS binding in tissue sections is a powerful method to quantitatively measure receptor activation of G proteins, a measure of functional activity, while maintaining high anatomic resolution. The conditions described in this study extend the use of agonist-stimulated [^{35}S]GTPγS autoradiography to receptors which have low levels of [^{35}S]GTPγS binding, as shown here for α$_2$-adrenoceptor. This will be valuable for anatomic studies of many G protein-coupled receptors and for studies measuring changes in receptor activation of G proteins in different pathologic conditions, during development and due to drug effects.

Acknowledgements

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