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α_2-Adrenoceptor-stimulated GTPγS binding in rat brain: an autoradiographic study

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Abstract

Agonist-stimulated [^{35}S]GTPγS binding by α_2-adrenoceptors was examined in rat brain by autoradiography. Epinephrine, norepinephrine, dexmedetomidine and brimonidine stimulated [^{35}S]GTPγS binding in a dose-dependent manner. Agonist-stimulated binding was blocked by the specific α_2-adrenoceptor antagonist (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX821002). Each α_2-adrenoceptor agonist stimulated [^{35}S]GTPγS binding in the same brain regions, corresponding to α_2-adrenoceptor distribution determined by [^{125}I]para-iodoclonidine autoradiography. The order of antagonist potency (RX821002 > idazoxan > rauwolscine > phentolamine > prazosin), and weak inhibition by propranolol and selective serotonin antagonists, indicate that epinephrine-stimulated [^{35}S]GTPγS binding is mediated primarily by α_2-adrenoceptors. Several antagonists increased [^{35}S]GTPγS binding at very high concentrations, and this effect had anatomic and pharmacologic characteristics of binding mediated by 5-HT$_{1A}$ receptors. These studies demonstrate functional linkage of α_2-adrenoceptors to G proteins in tissue sections, thus providing data on neuroanatomic localization and a means to examine drug specificity at α_2-adrenoceptors in different brain regions.

Keywords: Signal transduction; G protein; α_2-Adrenoceptor; G protein coupled receptor; Second messenger; 5-HT receptor; 5-HT$_{1A}$ receptor

1. Introduction

α_2-Adrenoceptors are members of the superfamily of heterotrimeric G protein coupled-receptors. They are one of the three major classes of receptors mediating the effects of the neurotransmitters norepinephrine and epinephrine (Bylund, 1988; Bylund et al., 1994), and their function is pertussis toxin sensitive, indicating signal transduction is mediated primarily through interaction with $G_{i/o}$ proteins (Ruffolo et al., 1993; Limbird et al., 1995). Activation of α_2-adrenoceptors inhibits adenylate cyclase activity, inhibits Ca^{2+} channels and activates inwardly rectifying K^+ channels. They have roles in a wide range of physiological and pathological processes, including regulation of blood pressure, nociception, locomotion, and processing of stressful stimuli (Ruffolo et al., 1993, 1995). α_2-Adrenoceptor agonists have been used to ameliorate withdrawal symptoms from opiates and alcohol, as anesthetic adjuvants in surgery, and may be of some benefit in treating cognitive deficits in the elderly (Ruffolo et al., 1993, 1995). The therapeutic effect of several newer antidepressant agents is associated with their α_2-adrenoceptor antagonistic properties (Potter and Manji, 1994; Sussman and Stahl, 1996). All of these actions and uses involve α_2-adrenoceptor functions in the CNS and point to the importance of understanding the role of these receptors in CNS function.

An assay of G protein coupled receptor function based on the binding of [^{35}S]GTPγS has been developed (Hilf et al., 1989). When G protein coupled receptors are stimulated, guanosine 5′-diphosphate sodium (GDP) is released from the heterotrimeric G protein complex, allowing GTP to bind in its place, leading to the dissociation of the α from the $\beta\gamma$ subunits of the complex and the subsequent regulation of signal transduction systems within the cell. The system is turned off by the intrinsic GTPase activity of the α subunit hydrolyzing GTP. Using [^{35}S]GTPγS, a GTP analog that is not hydrolyzed, receptor-stimulated activation of the G protein can be studied.

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Recently, agonist-stimulated [^{35}S]GTPγS binding has been adapted for use on slide mounted tissue sections for autoradiographic studies (Sim et al., 1995) and applied to several neurotransmitter receptor systems (Sim et al., 1997; Waeber and Moskowitz, 1997). This allows detailed anatomic analysis of agonist-stimulated [^{35}S]GTPγS binding in CNS. Thus far, only receptor systems coupled to pertussis toxin sensitive $G_{i/o}$ subtypes have been detected by [^{35}S]GTPγS binding with autoradiography, but some $G_{i/o}$ coupled receptors, including α_2-adrenoceptors, have not been amenable to this approach (Sim et al., 1997; Waeber and Moskowitz, 1997). α_2-Adrenoceptors are coupled to $G_{i/o}$ and the [^{35}S]GTPγS binding assay has been successfully used to study α_2-adrenoceptors expressed at high levels in cell lines (Tian et al., 1994; Wise et al., 1997) and recently in tissue homogenates (Happe et al., 1999a). The autoradiographic distribution of α_2-adrenoceptor-stimulated [^{35}S]GTPγS binding, however, has been difficult to detect.

We report here the characterization of agonist-stimulated [^{35}S]GTPγS binding to measure α_2-adrenoceptor coupling to G proteins by autoradiography in rat brain. Activation of G proteins is a functional consequence of α_2-adrenoceptor stimulation and, as such, provides a measure of the functional activity of these receptors. Under conditions described here, the functional activity of α_2-adrenoceptors can be examined in different brain structures with great regional resolution. This work has been presented in abstract form (Happe et al., 1999b).

2. Materials and methods

2.1. Materials

[^{35}S]GTPγS (1000–1500 Ci/mmol; guanosine 5′-(gamma-thio)triphosphate) and *p*-[^{125}I]iodoclonidine (2200 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA); epinephrine bitartrate, DL-propranolol HCl, dithiothreitol and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) were purchased from Sigma (St. Louis, MO); rauwolscine HCl, prazosin HCl, (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX821002), phentolamine mesylate, L(−)-norepinephrine bitartrate, methiothepin mesylate and *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide maleate (WAY-100635) were purchased from Research Biochemicals (Natick, MA); GDP was purchased from United States Biochemical (Cleveland, OH); and yohimbine HCl was purchased from Fisher Scientific (Pittsburgh, PA). We gratefully acknowledge the gifts of brimonidine from Allergan (Irvine, CA), methysergide maleate from Sandoz Pharmaceuticals, (E. Hanover, NJ), and dexmedetomidine from Abbott Laboratories (North Chicago, IL). All other chemicals were research grade.

2.2. Animals and tissue preparation

Adult female Sprague–Dawley rats (185–250 g; Sasco, Kingston, NY) were anesthetized with halothane and killed by decapitation. The brains were rapidly removed and frozen on dry ice. Brains were stored wrapped in Parafilm and foil at −70°C. The animal 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. 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., 1997; Waeber and Moskowitz, 1997), modified to improve signal to noise ratio (Happe et al., 1999b). Sections were re-hydrated in assay buffer (50 mM glycylglycine, 3 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, pH 7.4) at room temperature for 10 min. Sections were incubated at room temperature in assay buffer containing 2 mM GDP for 15 min, and then for 2–4 h in assay buffer containing 0.1 nM [^{35}S]GTPγS, 2 mM GDP and 0.25 mM dithiothreitol. Receptor-stimulated [^{35}S]GTPγS binding was determined by including agonist at 100 μM (unless otherwise specified). Basal [^{35}S]GTPγS binding was determined in the absence of agonist, and nonspecific [^{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 0.25 mM dithiothreitol, 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, Ontario, Canada). Identification of brain regions was confirmed by direct comparison to the sections used to produce autoradiograms following staining with cresyl violet, using the atlas of Paxinos and Watson (1986).

To test the concentration dependence of agonist-stimulated [^{35}S]GTPγS binding, agonists were used at concentrations from 1×10^{-9} to 1×10^{-4} M. Antagonists were included at 1×10^{-11} to 1×10^{-4} M for inhibition studies. Data were analyzed using Prism3 (GraphPad, San Diego, CA). Statistical analyses used InStat (GraphPad). Differences were considered statistically significant if $P < 0.05$.

Degradation of the agonist, epinephrine, was not a factor in these assays, as indicated by several experiments.

Specific α_2-adrenoceptor-stimulated [^{35}S]GTPγS binding was linear throughout the 4-h assay, indicating no significant decrease in agonist concentration. Dose–response studies carried out at 1, 2 and 4 h assay times gave the same dose–response curves for epinephrine, indicating no significant difference in agonist concentrations at these time points (data not shown). Finally, inclusion of the monoamine oxidase inhibitor, pargyline, at 10^{-6} M gave the same level of α_2-adrenoceptor stimulated [^{35}S]GTPγS binding as found without pargyline (data not shown).

2.4. p-[^{125}I]Iodoclonidine autoradiography

α_2-Adrenoceptor localization was examined on sections adjacent to those used for agonist-stimulated [^{35}S]GTPγS binding to compare the distribution of receptors with the distribution of receptor-stimulated GTPγS binding. Sections were incubated in 50 mM Tris–HCl buffer, pH 7.4 with 50 pM *p*-[^{125}I]iodoclonidine as ligand. Nonspecific binding determined by addition of 10 μM rauwolscine was not different from film background. Sections were incubated for 3 h at room temperature, washed two times for 5 min in ice-cold 50 mM Tris–HCl, pH 7.4, briefly dipped in ice-cold distilled water and rapidly dried under a stream of cool air. Sections were apposed to HyperFilm-^{3}H (Amersham) for 48 h and developed by standard procedures.

3. Results

3.1. [^{35}S]GTPγS binding in rat brain

Agonist-stimulated [^{35}S]GTPγS binding was measured in relation to basal binding, because basal binding contributed a large portion of the total [^{35}S]GTPγS binding. Epinephrine stimulated [^{35}S]GTPγS binding in most brain regions, with exceptions in white matter, lateral thalamus, mesencephalon and cerebellum (Fig. 1A–C). α_2-Adrenoceptor agonists stimulated [^{35}S]GTPγS binding less than agonists for 5-HT (see Fig. 5), μ-opioid, muscarinic and $GABA_B$ receptors (data not shown). To increase the magnitude of autoradiographic signal, labeled sections were exposed to film for sufficient time to generate images where the most intensely labeled brain regions were at the upper end of the linear response range for the film. Under

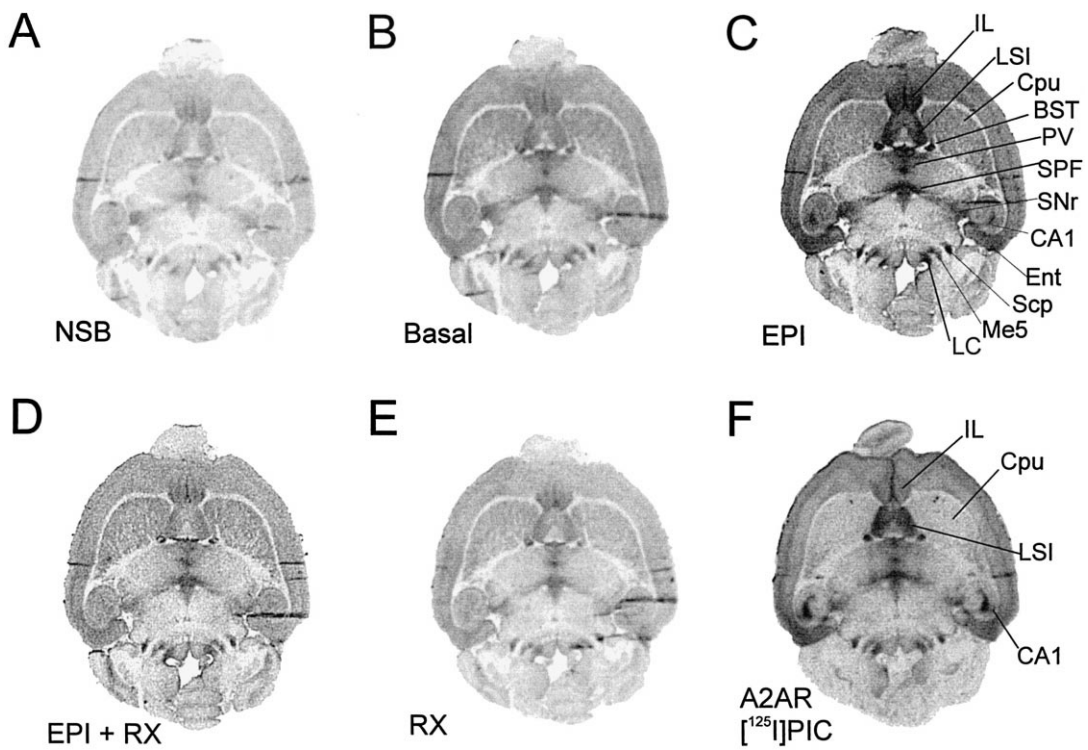

Fig. 1. Distribution of [^{35}S]GTPγS and p-[^{125}I]iodoclonidine binding sites in rat brain horizontal sections at the level of the lateral septum and locus coeruleus. (A) Non-specific [^{35}S]GTPγS binding (1 μM cold GTPγS) is low and mostly homogeneous. (B) Basal [^{35}S]GTPγS binding is low and heterogeneous, but higher than non-specific binding throughout grey matter brain regions. (C) Epinephrine (EPI; 100 μM) markedly stimulates [^{35}S]GTPγS binding above basal binding levels in several brain regions, including infralimbic cortex (IL), lateral septum (LSI), striatum (CPu), bed nucleus of the stria terminalis (BST), paraventricular thalamic nuclei (PV), subparafasicular thalamic nuclei (SPF), substantia nigra pars reticulata (SNr), the caudal CA1 region of the hippocampus (CA1), entorhinal cortex (Ent), superior cerebellar peduncle (Scp), mesencephalic trigeminal nucleus (Me5) and locus coeruleus (LC). (D) Epinephrine-stimulated (100 μM) [^{35}S]GTPγS binding is blocked by the addition of 10 μM RX821002, a specific α_2-adrenoceptor antagonist. (E) RX821002 (10 μM) does not by itself reduce basal binding. (F) The distribution of α_2-adrenoceptor, determined with *p*-[^{125}I]iodoclonidine in an adjacent section, shows comparably high levels of binding sites in the same brain regions that show epinephrine-stimulated [^{35}S]GTPγS binding.

these conditions, images for both basal and non-specific [^{35}S]GTPγS binding were well above film background and a heterogeneous pattern of labeling is apparent. Basal binding in all brain regions was reduced by 1 μM unlabeled GTPγS (non-specific binding; Fig. 1A,B). Both basal and non-specific [^{35}S]GTPγS binding were relatively higher in most of the brain regions where [^{35}S]GTPγS binding was stimulated by epinephrine (Fig. 1A–C), suggesting greater endogenous G protein levels.

Epinephrine-stimulated [^{35}S]GTPγS binding was reduced to basal levels by the specific α_2-adrenoceptor antagonist RX821002 (10 μM; Fig. 1C,D). RX821002 reduced epinephrine-stimulated binding in all brain regions showing specific agonist induced increases, but had no effect by itself (Fig. 1E).

The pattern of specific epinephrine-stimulated [^{35}S]GTPγS binding corresponds well with the distribution of α_2-adrenoceptor binding sites determined by receptor autoradiography using *p*-[^{125}I]iodoclonidine (Fig. 1C,F). High levels of α_2-adrenoceptors and epinephrine-stimulated [^{35}S]GTPγS binding are found in the infralimbic cortex, lateral septum, bed nucleus of the stria terminalis, some midline thalamic nuclei, locus coeruleus, mesencephalic trigeminal nucleus, and on the lateral edge of the superior cerebellar peduncle. Moderate binding densities for both markers are found in the entorhinal cortex, a portion of the caudal hippocampus CA1 lacunosum moleculare layer, substantia nigra pars reticulata, and the full extent of the cerebral cortex. Binding for both markers is very low in the olfactory bulb, ventral thalamus, mesencephalon, cerebellum and white matter tracts. Epinephrine-stimulated [^{35}S]GTPγS binding is higher than would be expected based on α_2-adrenoceptor density in the infralimbic cortex and the striatum, and lower than expected in the lateral septum, layer I of the cortex, including the midline layer of the infralimbic and cingulate cortex, and hippocampus CA1 field (Fig. 1C,F).

The binding of [^{35}S]GTPγS stimulated by α_2-adrenoceptor activation was characterized in more detail using the lateral septum for quantitative data because this brain region has high levels of alpha-2 adrenergic agonist-stimulated [^{35}S]GTPγS binding and binding levels are consistent through a large number of serial sections.

3.2. Time course

Total, basal and non-specific [^{35}S]GTPγS binding each increased throughout the time course of the assay out to 4 h, being fit better by a second order polynomial non-linear regression than by a straight line. This was due primarily to the non-linearity of non-specific binding (Fig. 2). Specific agonist-induced [^{35}S]GTPγS binding, however, was linear for at least 4 h (Fig. 2). Because epinephrine-stimulated binding increased more rapidly than basal binding, the magnitude of specific agonist-induced [^{35}S]GTPγS binding was greater at longer incubation times and incubation times of 2–4 h were used for all subsequent studies.

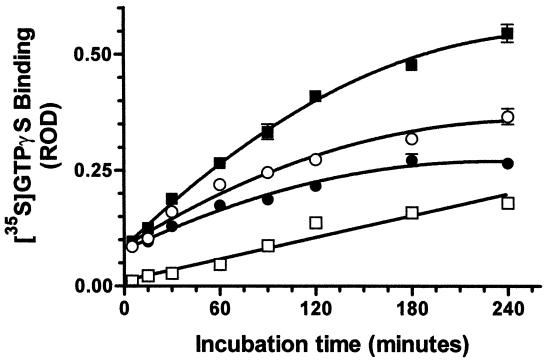

Fig. 2. Time course for [^{35}S]GTPγS binding. Sections were incubated for the indicated times. Binding levels for [^{35}S]GTPγS were measured in the lateral septum by computer assisted densitometry. Values represent the mean relative optical density (ROD) ± S.E.M. for 3 animals. Binding increased with time up to 4 h. Basal binding (○; no added drugs), total binding (■; 100 μM epinephrine) and non-specific binding (●; 1 μM unlabeled GTPγS) were best fit by non-linear regression. Specific α_2-adrenoceptor-stimulated [^{35}S]GTPγS binding (□; total minus basal) was best fit by linear regression ($r^2 = 0.983$).

3.3. Dose–response of [^{35}S]GTPγS binding for α_2-adrenoceptor agonists

The agonist concentration dependence of specific [^{35}S]GTPγS binding in the lateral septum was compared for epinephrine, norepinephrine, and the α_2-adrenoceptor selective agonists dexmedetomidine and brimonidine (Fig. 3). Significant agonist stimulation of [^{35}S]GTPγS binding required high agonist concentrations ($> 10^{-5}$ M). The low potency of agonists was likely due to the presence of very high concentrations of GDP (2 mM) required to distinguish agonist-stimulated from basal [^{35}S]GTPγS binding in autoradiographic studies and is similar to findings with other receptor systems (Sim et al., 1995). Epinephrine-stimulated [^{35}S]GTPγS binding does not saturate at concentrations up to 10^{-2} M in the presence of 2 mM GDP (data not shown). At 0.5 mM GDP, which increases agonist affinity for the receptors compared to 2 mM (Wurster et al., 1998), epinephrine stimulation is saturable ($EC_{50} = 2$ μM; data not shown), as would be expected with such a receptor system. However, lower GDP concentrations also reduce the signal to noise ratio significantly. Due to the low level of α_2-adrenoceptors in brain tissue, we chose to maximize signal to noise ratio in the current studies by using higher GDP concentrations.

In the present studies, the highest stimulation was achieved at 100 μM for each agonist. However, because higher concentrations were not used, saturating agonist concentrations were not determined and EC_{50} values could not be calculated. At 100 μM epinephrine and norepinephrine stimulated [^{35}S]GTPγS binding to about the same extent in the lateral septum, and dexmedetomidine and brimonidine stimulated binding to a lesser extent. At lower

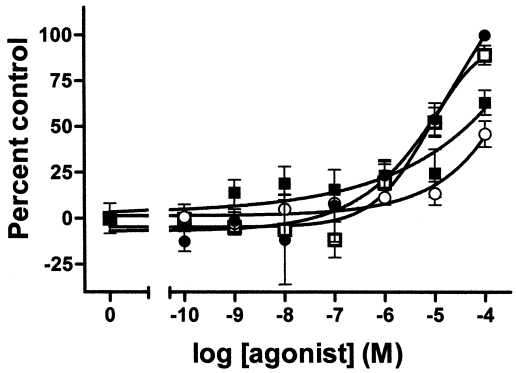

Fig. 3. Agonist dose–response curves for epinephrine (●), norepinephrine (□), dexmedetomidine (■) and brimonidine (○) stimulating [^{35}S]GTPγS binding in lateral septum. Data are mean ± S.E.M., $n = 3$ animals. Values are expressed as percent control calculated from the density of epinephrine-stimulated [^{35}S]GTPγS binding at 100 μM. Binding for each agonist at 100 μM is blocked by inclusion of the specific α_2-adrenoceptor antagonist RX821002 at 100 μM (data not shown).

agonist concentrations, both dexmedetomidine and brimonidine increased [^{35}S]GTPγS binding somewhat, but this was not statistically significant (Fig. 3).

Regional differences between the different agonists were examined at 100 μM (Table 1). Epinephrine and norepinephrine stimulated [^{35}S]GTPγS binding to a similar extent in all regions examined, with the exceptions of the hippocampus and locus coeruleus where the binding stimulated by norepinephrine was 30–40% lower compared to epinephrine. The binding stimulated by dexmedetomidine and brimonidine was similar to epinephrine and norepinephrine only in the frontal cortex; otherwise the binding stimulated by both specific α_2-adrenoceptor agonists was lower in all other brain regions examined. The binding induced by dexmedetomidine was 27–42% lower than epinephrine in these other brain regions except locus coeruleus, where it was nearly 70% lower. Of the four agonists, brimonidine stimulated [^{35}S]GTPγS binding the least. In the cingulate cortex, entorhinal cortex, hippocampus CA1 field and lateral septum, brimonidine-stimulated binding was 40–50% of that obtained with epinephrine. In locus coeruleus and striatum, brimonidine-stimulated [^{35}S]GTPγS binding was only 15–20% of levels obtained with epinephrine. Specific [^{35}S]GTPγS binding stimulated by each agonist at 100 μM was blocked by 100 μM RX821002, a specific α_2-adrenoceptor antagonist (data not shown).

3.4. Antagonist specificity and potency for inhibiting epinephrine-stimulated [^{35}S]GTPγS binding

The specific α_2-adrenoceptor antagonist RX821002 was the most potent inhibitor of epinephrine-stimulated [^{35}S]GTPγS binding in the lateral septum with an IC_{50} of about 4 nM (Fig. 4, Table 2). Other α_2-adrenoceptor antagonists, idazoxan, rauwolscine and yohimbine, were also potent inhibitors of epinephrine-stimulated [^{35}S]GTPγS binding with IC_{50} values of about 27, 524 and 660 nM, respectively. The non-selective α_1-adrenoceptor and α_2-adrenoceptor antagonist, phentolamine, was a relatively potent inhibitor ($IC_{50} = 760$ nM). The α_1-adrenoceptor selective antagonist, prazosin ($IC_{50} = 2$ μM), was much less potent than RX821002 or idazoxan, indicating that epinephrine-stimulated [^{35}S]GTPγS binding was not mediated by α_1-adrenoceptors. The low potency of prazosin may also mean that the α_2-adrenoceptor stimulated binding is due primarily to the α_{2A} adrenoceptor subtype. The selective β-adrenoceptor antagonist propranolol inhibited binding by less than 50% at 100 μM (data not shown). The 5-HT receptor selective antagonists methio-

Table 1
Brain region distribution and agonist relative activity for α_2-adrenoceptor-stimulated [^{35}S]GTPγS binding

Brain region	Agonist			
	Epinephrine	Norepinephrine	Dexmedetomidine	Brimonidine
frontal cortex	0.14 ± 0.03[a] (100)	0.15 ± 0.01[a] (107)	0.12 ± 0.03[a] (86)	0.12 ± 0.02[a] (86)
cingulate cortex	0.33 ± 0.02[a] (100)	0.33 ± 0.04[a] (100)	0.19 ± 0.01[a,b] (58)	0.17 ± 0.01[a,b] (52)
entorhinal cortex	0.30 ± 0.01[a] (100)	0.26 ± 0.02[a] (87)	0.22 ± 0.02[a] (73)	0.12 ± 0.01[a,b,c] (40)
hippocampus caudal CA1	0.40 ± 0.03[a] (100)	0.28 ± 0.01[a,b] (70)	0.27 ± 0.02[a,b] (68)	0.18 ± 0.01[a,b,c] (45)
locus coeruleus	0.21 ± 0.05[d] (100)	0.13 ± 0.08 (62)	0.07 ± 0.05 (33)	0.04 ± 0.06 (19)
lateral septum	0.37 ± 0.05[a] (100)	0.32 ± 0.03[a] (86)	0.22 ± 0.04[a] (59)	0.16 ± 0.03[a,b] (43)
ventrolateral thalamus	0.01 ± 0.02	0.06 ± 0.01	0.02 ± 0.02	0.01 ± 0.02
striatum	0.13 ± 0.02[a] (100)	0.12 ± 0.01[a] (92)	0.08 ± 0.02 (62)	0.02 ± 0.03[b] (15)

Data are given as means ± S.E.M., $n = 3$ animals. Specific binding is expressed as relative optical density (ROD). Agonist relative activity values (in parentheses) are in relation to epinephrine-stimulated [^{35}S]GTPγS binding, defined as 100. Each agonist was used at 100 μM.

[a] Statistical analysis used one-way ANOVA with Tukey–Kramer multiple comparisons post-test, comparing different agonists and basal binding within the same brain region; significantly greater than basal binding, $P < 0.05$.

[b] Statistical analysis used one-way ANOVA with Tukey–Kramer multiple comparisons post-test, comparing different agonists and basal binding within the same brain region; significantly less than epinephrine-stimulated binding, $P < 0.05$.

[c] Statistical analysis used one-way ANOVA with Tukey–Kramer multiple comparisons post-test, comparing different agonists and basal binding within the same brain region; significantly less than dexmedetomidine-stimulated binding, $P < 0.05$.

[d] Statistical analysis used a two-tailed Student's *t*-test; significantly greater than basal binding, $P < 0.05$.

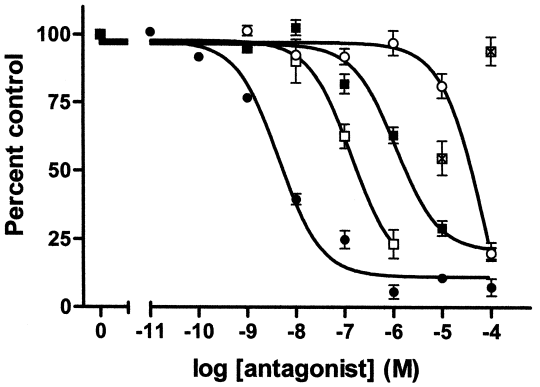

Fig. 4. Inhibition of epinephrine-stimulated [^{35}S]GTPγS binding in lateral septum by RX821002 (●), rauwolscine (□), prazosin (■) and WAY-100635 (○). Data represent the mean ± S.E.M. of 4–9 animals. Values are expressed as percent specific [^{35}S]GTPγS binding stimulated by epinephrine (100 μM) in the absence of antagonists. The estimated pIC_{50} values are listed in Table 2. The effect of rauwolscine at 10 and 100 μM on epinephrine-stimulated [^{35}S]GTPγS binding (⊠) demonstrates the apparent agonist activity of rauwolscine at high concentrations, presumably acting at 5-HT_{1A} receptors.

thepin and WAY-100635 were also weak inhibitors with IC_{50} values of about 10 and 32 μM, respectively.

By itself RX821002 (10 μM) had no effect on [^{35}S]GTPγS binding (Fig. 1E). However, idazoxan, rauwolscine, yohimbine and propranolol each exhibited agonist activity at 10 μM, increasing [^{35}S]GTPγS binding over basal levels (data not shown). Several α_2-adrenoceptor antagonists have previously been shown to be weak agonists at 5-HT_{1A} receptors (Newman-Tancredi et al., 1998).

Epinephrine-stimulated [^{35}S]GTPγS binding in the presence of 100 μM phentolamine, prazosin, methiothepin or WAY-100635 was less than or equal to binding levels in the presence of each antagonist at 10 μM, as would be expected. On the other hand, apparent epinephrine-stimulated [^{35}S]GTPγS binding in the presence of 100 μM idazoxan, rauwolscine, yohimbine or propranolol, was greater than epinephrine-stimulated binding in the presence of these drugs at 10 μM (e.g. rauwolscine in Fig. 4), indicating that at very high concentrations, these drugs produce agonist effects independent of epinephrine, apparently at 5-HT_{1A} receptors. Due to the non-specific effect of these drugs at very high concentrations, 30 μM was the highest concentration used in the analysis of inhibition curves. To examine this issue further, inhibition of epinephrine-stimulated [^{35}S]GTPγS binding by rauwolscine was tested in the presence of 1 μM WAY-100635, a specific 5-HT_{1A} receptor antagonist. At this concentration, WAY-100635 alone inhibited epinephrine-stimulated [^{35}S]GTPγS binding less than 20%. Addition of WAY-100635 did not alter the dose–response curve of rauwolscine for inhibition of epinephrine stimulation of [^{35}S]GTPγS binding; but it did block the 100 μM rauwolscine induced increase in [^{35}S]GTPγS binding (data not shown). This further supports the involvement of 5-HT_{1A} receptors in the stimulatory effects of rauwolscine.

3.5. Comparison of α_2-adrenoceptor- and 5-HT receptor-stimulated [^{35}S]GTPγS binding

The [^{35}S]GTPγS binding in several brain regions is increased over basal levels by the addition of epinephrine, rauwolscine and 5-carboxamidotryptamine (5-CT), a selective 5-HT_{1A} receptor agonist (Fig. 5A–D). Epinephrine-stimulated [^{35}S]GTPγS binding is not as robust as 5-HT receptor stimulated binding, as noted earlier. There is a great deal of overlap in the brain regions where both epinephrine and 5-CT stimulate [^{35}S]GTPγS binding, including the lateral septum, infralimbic cortex, frontal cortex, striatum, and some central thalamic nuclei. The distribution of [^{35}S]GTPγS binding sites stimulated by 100 μM rauwolscine is similar to 5-HT receptor stimulated binding, but is much less dense. In only a few brain regions is the distribution of epinephrine-stimulated [^{35}S]GTPγS binding clearly distinct from 5-CT- and rauwolscine-stimulated binding. In the hippocampus, epinephrine-stimulated [^{35}S]GTPγS binding is more restricted, being most dense in the caudal CA1 field within the lacunosum moleculare layer. For 5-CT- and rauwolscine-stimulated binding, there is more diffuse binding in CA1–CA3 and a distinct density of binding in the dentate gyrus. In the entorhinal cortex, 5-CT- and rauwolscine-stimulated [^{35}S]GTPγS binding is laminar, being most dense in the outermost and innermost layers, whereas epinephrine-stimulated binding is diffuse (Fig. 5C). The locus coeruleus has higher epinephrine-stimulated binding than 5-CT- or rauwolscine-stimulated binding.

In the lateral septum, epinephrine-stimulated [^{35}S]GTPγS binding was potently inhibited by the α_2-adrenoceptor specific antagonist RX821002, and weakly inhibited by the 5-HT_{1A} receptor specific antagonist WAY-100635 (Fig. 4, Table 2). As shown in Fig. 5E and F, rauwolscine- and 5-CT-stimulated [^{35}S]GTPγS binding is pharmacologically distinct from epinephrine-stimulated

Table 2
Antagonist inhibition of epinephrine-stimulated [^{35}S]GTPγS binding

Compound	pIC_{50} (M)
RX821002	8.4 ± 0.2 (9)
Idazoxan	7.6 ± 0.2 (4)
Rauwolscine	6.3 ± 0.2 (6)
Yohimbine	6.2 ± 0.1 (6)
Phentolamine	6.1 ± 0.1 (5)
Prazosin	5.7 ± 0.2 (6)
Methiothepin	5.0 ± 0.1 (5)
WAY-100635	4.5 ± 0.1 (6)

Data are mean ± S.E.M. of *n* values (in parentheses). pIC_{50} values are for inhibition of [^{35}S]GTPγS binding stimulated by 100 μM epinephrine (EPI).

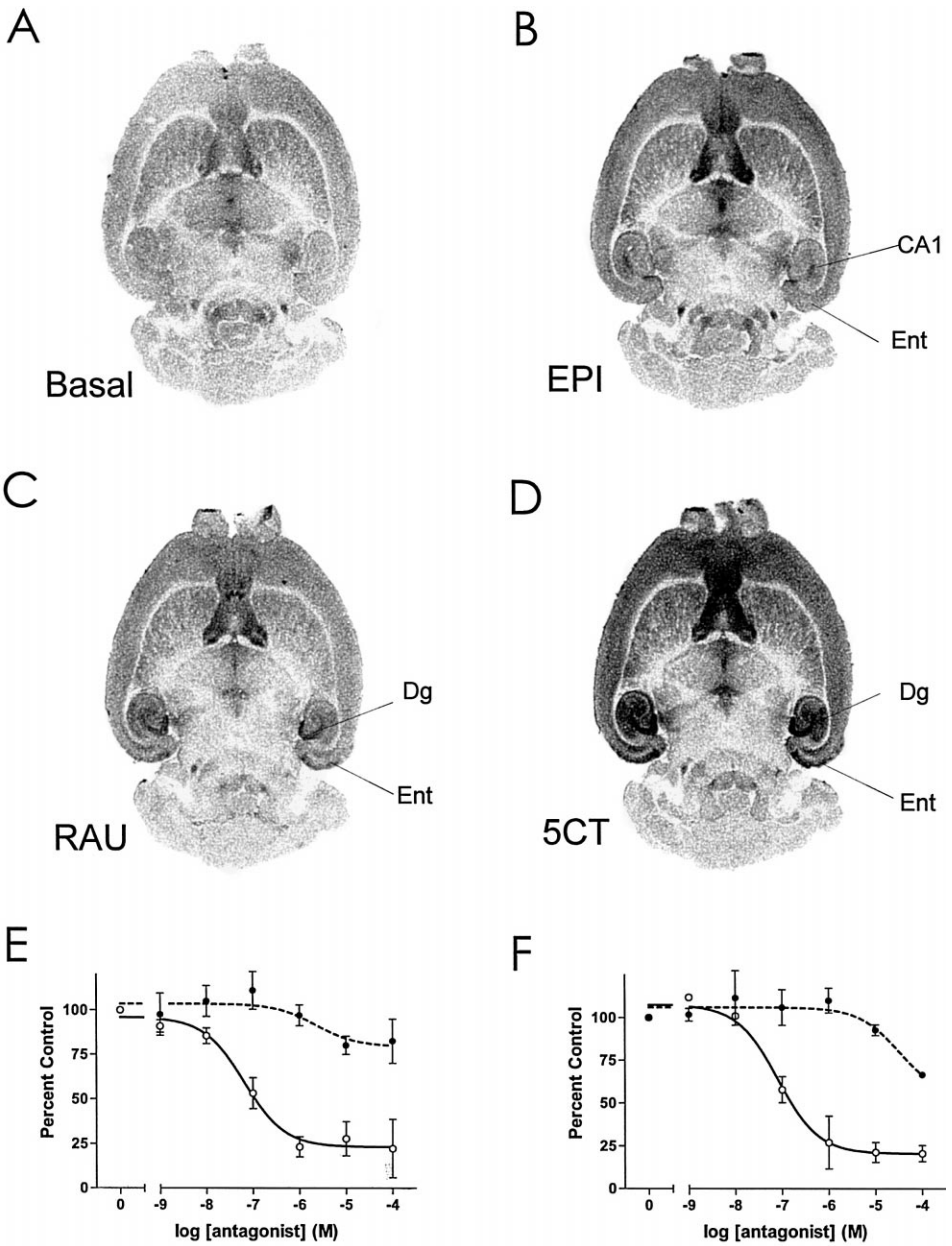

Fig. 5. [^{35}S]GTPγS binding in rat brain. (A) Basal binding. Binding stimulated by addition of (B) 10 μM epinephrine (EPI), (C) 100 μM rauwolscine (RAU) or (D) 10 μM 5-carboxamidotryptamine (5-CT). Although epinephrine-stimulated and 5-CT-stimulated [^{35}S]GTPγS binding appear in many of the same regions, 5-CT-stimulated binding is distinguished by the laminar pattern in the entorhinal cortex (Ent) and by the larger field of stimulation in the hippocampus, including the dentate gyrus (Dg). Rauwolscine-stimulated [^{35}S]GTPγS binding shares the regional characteristics of 5-HT receptor-stimulated binding. In the lateral septum (E) 100 μM rauwolscine-stimulated and (F) 10 μM 5-CT-stimulated [^{35}S]GTPγS binding were inhibited by the α_2-adrenoceptor specific antagonist RX821002 (■) and the 5-HT$_{1A}$ receptor specific antagonist WAY-100635 (●). Data represent the mean ± S.E.M. of 3 animals. Values are expressed as percent of agonist-stimulated binding in the absence of competing drug.

binding in the lateral septum. Binding stimulated by 100 μM rauwolscine or 10 μM 5-CT is inhibited less than 50% at 100 μM RX821002. On the other hand, rauwolscine- and 5-CT-stimulated [^{35}S]GTPγS binding is potently inhibited by WAY-100635 with IC_{50} values of 19 ± 6 and 89 ± 18 nM, respectively. Therefore, although 5-HT$_{1A}$ receptor and α_2-adrenoceptor mediated [^{35}S]GTPγS binding overlap to a great extent in regional distribution, the pharmacologic difference indicates that different receptor systems mediate the effects of serotonin and epinephrine and they can be differentiated.

4. Discussion

The evidence presented here demonstrates the use of [^{35}S]GTPγS binding to visualize functional α_2-adrenoceptor coupling to G proteins in the rat brain using autoradiography. Epinephrine stimulation increases [^{35}S]GTPγS bind-

ing to slide-mounted tissue sections, demonstrating anatomic and pharmacologic characteristics indicative of specific α_2-adrenoceptor mediated signal transduction.

The advantages of the autoradiographic [^{35}S]GTPγS binding assay can now be applied to studies of the α_2-adrenoceptor system, as has been done for other neurotransmitter systems (Sim et al., 1997; Waeber and Moskowitz, 1997). Agonist-stimulated [^{35}S]GTPγS binding autoradiography provides information on receptor interactions with G proteins in specific brain regions, including small and difficult to dissect regions. The method is rapid and does not require the specific radioligands used for receptor binding. α_2-Adrenoceptor-stimulated [^{35}S]GTPγS binding is useful to estimate agonist activity, evaluate the specificity of agonists and antagonists, and as a method to examine the functional state of α_2-adrenoceptors receptors (especially in conjunction with receptor radioligand binding studies) in a variety of experimental conditions, including development and aging, and in pathologic conditions.

The adrenergic neurotransmitters epinephrine and norepinephrine act through three related but distinct adrenergic receptor classes, β-adrenoceptor, α_1-adrenoceptor and α_2-adrenoceptor, classified by distinct pharmacological characteristics and molecular cloning (Bylund, 1988; Bylund et al., 1994). The pharmacology and anatomy of epinephrine-stimulated [^{35}S]GTPγS binding presented in this paper is most consistent with G protein coupling mediated through the α_2-adrenoceptor family, with little or no contribution from β- or α_1-adrenoceptors.

4.1. Agonists

The endogenous neurotransmitters epinephrine and norepinephrine, and α_2-adrenoceptor selective agonists dexmedetomidine and brimonidine all stimulate [^{35}S]GTPγS binding. Although there are differences in relative agonist activity, all four agonists stimulated [^{35}S]GTPγS binding in the same brain regions. The only exception was in the striatum where brimonidine did not produce a discernable signal above basal. In transfected cells, brimonidine was shown to be a full agonist for $\alpha_{2A/D}$-adrenoceptor, but a partial agonist for α_{2C}-adrenoceptor (Jasper et al., 1998), the subtype with high levels of expression in the striatum. The pattern of [^{35}S]GTPγS binding stimulated by each agonist corresponds to brain regions known to have high levels of α_2-adrenoceptor expression (Boyajian et al., 1987; Wamsley et al., 1992; Nicholas et al., 1993; Rosin et al., 1996; Talley et al., 1996; Winzer-Serhan and Leslie, 1997) and correlates well with the regional distribution of *p*-[^{125}I]iodoclonidine binding sites determined in adjacent sections. α_2-Adrenoceptor agonist-stimulated binding was blocked in all regions by the specific α_2-adrenoceptor antagonist, RX821002 (Stillings et al., 1985). In most brain regions, epinephrine and norepinephrine appeared to be full agonists, whereas dexmedetomidine and brimonidine were partial agonists. The apparent partial agonist activity of dexmedetomidine and brimonidine is consistent with similar studies using membrane assays of recombinant receptors (Wise et al., 1997; Jasper et al., 1998). We did not try to determine the relative contribution of different α_2-adrenoceptor subtypes. However, regional differences in agonist relative activity reported here may be due to differences in α_2-adrenoceptor subtype distribution and/or regional differences in expression levels and subtypes of the $G_{i/o}$ proteins to which α_2-adrenoceptors are coupled. The low potency of the agonists in this study is similar to other studies (Sim et al., 1995; Waeber and Moskowitz, 1997) and is likely due to effects on receptor affinity resulting from the high concentration of GDP required to reduce basal binding (Wieland and Jakobs, 1994).

4.2. Antagonists

Inhibition of epinephrine-stimulated [^{35}S]GTPγS binding by α_2-adrenoceptor antagonists was examined in detail in the lateral septum. The rank order of antagonists (RX821002 > idazoxan > rauwolscine ≥ yohimbine > phentolamine ≫ prazosin ≫ propranolol) is consistent with involvement of α_2-adrenoceptors (Bylund et al., 1994). The low potencies of prazosin and of propranolol relative to RX821002 are evidence that agonist stimulation of α_1-adrenoceptors or β-adrenoceptors, respectively, are not mediating epinephrine-stimulated [^{35}S]GTPγS binding under our conditions. The potency of antagonists in this assay system was considerably lower than expected based on membrane receptor binding assays (Boyajian and Leslie, 1987; Blaxall et al., 1991; Harrison et al., 1991), but a similar shift in antagonist potency in a membrane epinephrine-stimulated [^{35}S]GTPγS binding assay has been reported previously (Jasper et al., 1998).

4.3. Anatomy

In addition to the pharmacologic evidence, anatomic evidence also supports the conclusion that epinephrine-stimulated [^{35}S]GTPγS binding is mediated via α_2-adrenoceptors. There is a close correspondence between the regional distribution and relative levels of *p*-[^{125}I]iodoclonidine binding to α_2-adrenoceptors and epinephrine-stimulated [^{35}S]GTPγS binding, indicating this is due to α_2-adrenoceptor stimulation. Although there is overlap between the regional expression of α_2-adrenoceptors, α_1-adrenoceptor and β-adrenoceptor in the CNS (Nicholas et al., 1996), regions expressing high levels of the latter two adrenergic receptors (Rainbow et al., 1984; Jones et al., 1985) do not have high levels of epinephrine-stimulated [^{35}S]GTPγS binding, such as cerebellum and lateral thalamus. On the other hand, high levels of epinephrine-stimulated [^{35}S]GTPγS binding in the cingulate cortex, lateral

septum and bed nucleus of the stria terminalis correspond to high levels of α_2-adrenoceptors (Boyajian et al., 1987; Wamsley et al., 1992), whereas α_1-adrenoceptor or β-adrenoceptor are at low levels in these regions (Rainbow et al., 1984; Jones et al., 1985).

Whereas epinephrine and norepinephrine are the endogenous neurotransmitters for all three adrenergic receptor subtypes, epinephrine-stimulated [^{35}S]GTPγS binding appears to be mediated either completely or predominantly by α_2-adrenoceptors. This is not surprising because CNS α_2-adrenoceptor-mediated effects are primarily pertussis toxin-sensitive, indicating coupling of α_2-adrenoceptors with $G_{i/o}$, whereas α_1-adrenoceptor and β-adrenoceptor are coupled to pertussis toxin-insensitive G_q and G_s, respectively (Bylund et al., 1994). For brain membrane preparations and slide mounted tissue sections, all receptors reported to stimulate [^{35}S]GTPγS binding are coupled to $G_{i/o}$, whereas receptors coupled to other G proteins have not been detected (Carty and Iyengar, 1994; Sim et al., 1997; Waeber and Moskowitz, 1997). Differences that may account for this include the relative abundance of $G_{i/o}$ in the brain (Sternweis and Robishaw, 1984) and the different rates of GDP dissociation from the G protein α subunit ($G_{i/o} > G_s$) (Carty and Iyengar, 1994; Wieland and Jakobs, 1994).

Most epinephrine-stimulated [^{35}S]GTPγS binding is likely due to the $\alpha_{2A/D}$-adrenoceptor receptor subtype because this is the predominant α_2-adrenoceptor expressed in the CNS (Uhlen et al., 1992; Ordway et al., 1993). The regional distribution of epinephrine-stimulated [^{35}S]GTPγS binding is generally consistent with distribution of the $\alpha_{2A/D}$-adrenoceptor subtype, however the relatively high epinephrine-stimulated [^{35}S]GTPγS binding in striatum and substantia nigra indicate involvement of α_{2C}-adrenoceptor receptors (Wamsley et al., 1992; Uhlen et al., 1997). Given the predominance of $\alpha_{2A/D}$-adrenoceptor over α_{2C}-adrenoceptor, this may indicate the α_{2C}-adrenoceptor subtype has a higher amplification factor for stimulation of [^{35}S]GTPγS binding. The α_{2B}-adrenoceptor subtype mRNA is expressed in very low levels in the CNS (Nicholas et al., 1993; Winzer-Serhan and Leslie, 1997), and the lack of [^{35}S]GTPγS binding in the lateral thalamus, where α_{2B}-adrenoceptor mRNA is at its highest level, indicates that this receptor subtype is not detected. There is evidence that receptors coupled to some $G_{i/o}$ subtypes may not stimulate [^{35}S]GTPγS binding (Waeber and Moskowitz, 1997). Therefore α_2-adrenoceptor coupled to different G protein subtypes may exhibit differences between brain regions or between subcellular localizations (Wozniak and Limbird, 1996) which could limit the detection of some α_2-adrenoceptors.

4.4. 5-HT receptor agonists

Several α_2-adrenoceptor antagonists inhibited epinephrine-stimulated [^{35}S]GTPγS binding in a concentration dependent manner, but at higher concentrations produced an increase in [^{35}S]GTPγS binding. Our results support previous descriptions of 5-HT receptor agonist properties of the α_2-adrenoceptor antagonists idazoxan, rauwolscine and yohimbine, and the β-adrenoceptor antagonist propranolol (Schoeffter and Hoyer, 1989; Kawai et al., 1992; Newman-Tancredi et al., 1998). In view of the 5-HT receptor agonist properties of some α_2-adrenoceptor antagonists and the considerable overlap in brain regions expressing 5-HT receptor- and epinephrine-stimulated [^{35}S]GTPγS binding, we considered the possibility that at high concentrations epinephrine stimulation of 5-HT receptors may occur. WAY-100635, a selective inhibitor of 5-HT_{1A} receptors, blocked the 5-HT_{1A} receptor and the rauwolscine-stimulated increases in [^{35}S]GTPγS binding with high affinity (81 and 64 nM), but it was a weak inhibitor of epinephrine-stimulated [^{35}S]GTPγS binding in all regions examined. This supports our conclusion that the epinephrine-mediated effects are through α_2-adrenoceptor, and that stimulation of [^{35}S]GTPγS binding at high concentrations of some α_2-adrenoceptor antagonists is through 5-HT_{1A} receptors. In addition, rauwolscine- and 5-CT-stimulated [^{35}S]GTPγS binding are both found in hippocampus and entorhinal cortex in a pattern distinct from α_2-adrenoceptor mediated binding.

These findings indicate autoradiographic screening of drugs using our method provides a relatively simple way to initially examine drugs for agonist and antagonist specificity by visual inspection, allowing selection of promising drugs for more detailed pharmacologic studies.

4.5. Conclusion

In conclusion, we here demonstrate and characterize α_2-adrenoceptor receptor-mediated increases in [^{35}S]GTPγS binding in rat brain using autoradiographic methods. Epinephrine and norepinephrine had higher efficacy than other agonists examined. The anatomic distribution and pharmacologic properties indicate that epinephrine-stimulated [^{35}S]GTPγS binding is measuring specific α_2-adrenoceptor interaction with G proteins, a measure of the functional activity of these receptors in tissue.

While this paper was in review, two reports appeared which demonstrated, but did not characterize, α_2-adrenoceptor stimulation of [^{35}S]GTPγS binding in tissue sections (Newman-Tancredi et al., 2000; Rodriguez-Puertas et al., 2000).

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