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Differences in G-protein activation by μ- and δ-opioid, and cannabinoid, receptors in rat striatum

Laura J. Sim, Dana E. Selley, Ruoyu Xiao, Steven R. Childers *

Department of Physiology and Pharmacology, and Center for Investigative Neuroscience, Bowman Gray School of Medicine, Wake Forest University, Medical Center Blvd., Winston-Salem, NC 27157, USA

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Abstract

Receptor activation of G-proteins can be measured by agonist-stimulated [^{35}S]GTPγS binding in the presence of excess guanosine diphosphate (GDP). To determine whether opioid and cannabinoid receptor-mediated G-protein activation correlate with their receptor densities, this study compared opioid- and cannabinoid-stimulated [^{35}S]guanylyl-5′-*O*-(γ-thio)-triphosphate (GTPγS) binding with the corresponding B_{max} values of receptor binding in rat striatum. Scatchard analysis revealed that the B_{max} of cannabinoid receptor binding was approximately ten times higher than that of μ- or δ-opioid receptor binding. However, comparable levels of cannabinoid- and μ- and δ-opioid-stimulated [^{35}S]GTPγS binding were observed in the caudate-putamen by [^{35}S]GTPγS autoradiography in brain sections. Scatchard analysis of net agonist-stimulated [^{35}S]GTPγS binding in membranes showed that the B_{max} of cannabinoid-stimulated binding was only twice that of μ- or δ-opioid-stimulated binding. Thus, the calculated amplification factors for μ- and δ-opioid receptors are seven times that of cannabinoid receptors.

Keywords: [^{35}S]GTPγS autoradiography; G-protein-coupled receptor

1. Introduction

Opioid (including μ-, δ- and κ-) and cannabinoid receptors belong to the superfamily of GTP binding regulatory protein (G-protein)-coupled receptors (Matsuda et al., 1990; Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993), which produce a biological response by regulation of enzyme or ion channel activity through the selective activation of signal-transducing G-proteins (Gilman, 1987; Birnbaumer et al., 1990; Childers, 1991). Both opioid and cannabinoid receptors inhibit adenylyl cyclase activity (Kurose et al., 1983; Howlett, 1985; Howlett et al., 1986; Pacheco et al., 1991) and increase potassium channel conductance and decrease calcium channel conductance (Aghajanian and Wang, 1986; North et al., 1987; Gross et al., 1990; Mackie and Hille, 1992; Hampson et al., 1995) via pertussis toxin-sensitive G-proteins of the G_i/G_o family.

An assay has been developed in which agonist-stimulated [^{35}S]guanylyl-5′-*O*-(γ-thio)-triphosphate (GTPγS) binding is used to measure receptor activation of G-proteins in isolated membranes (Hilf et al., 1989; Traynor and Nahorski, 1995). We have recently developed an autoradiographic technique based upon this method, in which [^{35}S]GTPγS binding is used to identify receptor-activated G-proteins in brain sections (Sim et al., 1995). This assay is based upon the fact that the inactive state of the G-protein α subunit has a relatively high affinity for GDP over GTP, while activation of receptors by agonist shifts the α subunit into a higher affinity state for GTP versus GDP. Therefore, the [^{35}S]GTPγS assay utilizes excess GDP to shift the G-proteins into the inactive state and lower basal activity. Addition of [^{35}S]GTPγS and agonist decreases the affinity of the α subunit for GDP and increases its affinity for GTP, so that the receptor-stimulated G-protein binds [^{35}S]GTPγS. In our initial anatomical studies, we demonstrated that [^{35}S]GTPγS binding in sections was dependent upon agonist concentration and was blocked by specific antagonists (e.g. naloxone + DAMGO for μ-opioid and WIN 55212-2 + SR 141716A for cannabinoid receptors, respectively) (Sim et al., 1995). A unique anatomical distribution of agonist-stimulated [^{35}S]GTPγS binding was also identified for each receptor system. Thus, μ-opioid-stimulated [^{35}S]GTPγS binding was identified in regions including the caudate-putamen, medial thalamus, amyg-

* Corresponding author. Tel.: 910-716-3791; fax: 910-716-7282.

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dala, periaqueductal gray, locus coeruleus, parabrachial nucleus and nucleus tractus solitarius, and cannabinoid-stimulated [^{35}S]GTPγS binding was high in the globus pallidus, caudate-putamen, hippocampus, cerebellum, cortex and substantia nigra (Sim et al., 1995). These distributions of agonist-stimulated [^{35}S]GTPγS binding corresponded to known receptor distributions, as measured by receptor autoradiography (Goodman et al., 1980; Herkenham and Pert, 1982; Herkenham et al., 1991; Jansen et al., 1992). However, the apparent levels of [^{35}S]GTPγS binding stimulated by opioid and cannabinoid agonists were comparable, despite the fact that cannabinoid receptor density in the brain is approximately ten-fold greater than that of opioid receptors (Herkenham and Pert, 1982; Devane et al., 1988; Herkenham et al., 1991; Kuster et al., 1993). We attributed this observation to differences in the efficiency of G-protein coupling between these receptor systems. To understand the relationship between the number of receptors and the number of G-proteins they activate, a quantitative assessment must be obtained of both parameters in the same preparation. In the present study, we report on the comparison between μ- and δ-opioid, and cannabinoid, receptor densities in striatum measured by radioligand binding, versus the number of G-proteins activated by each receptor as measured by agonist-stimulated [^{35}S]GTPγS binding.

2. Materials and methods

2.1. Materials

Male Sprague-Dawley rats were purchased from Zivic-Miller (Zelienople, PA). [^{35}S]GTPγS (1150–1395 Ci/mmol), [^{3}H][D-Ala^{2},*N*-Me-Phe^{4},Gly^{5}-ol]enkephalin (DAMGO) (41.55 Ci/mmol), [^{3}H][D-Pen^{2,5}, Phe^{4}]enkephalin (DPDPE) (34.3 Ci/mmol) and [^{3}H]WIN 55212-2 (37.0 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). [D-Ala^{2}]methionine enkephalinamide (DAME), DAMGO, DPDPE, [D-Pen^{2,5}, *p*-Cl-Phe^{4}]enkephalin (pCl-DPDPE) and trans-3,4-dichloro-*N*-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide (U-50,488H) were obtained from Sigma Chemical Co. (St. Louis, MO). WIN 55212-2 was purchased from Research Biochemicals International (Natick, MA). GDP and GTPγS were purchased from Boehringer Mannheim (New York, NY). Reflections autoradiography film was purchased from New England Nuclear Corp. (Boston, MA). Ecolite scintillation fluid was obtained from ICN (Irvine, CA). All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher.

2.2. [^{35}S]GTPγS autoradiography

Male Sprague-Dawley rats (150–200 g) were killed by decapitation. The brains were removed and immersed in isopentane at −35°C. 20 μm coronal sections of the caudate-putamen at the level of the anterior commissure were cut on a cryostat at −20°C, and thaw-mounted onto gelatin coated slides. Slides were rinsed in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) at 25°C, then pretreated with 2 mM GDP in assay buffer. Sections were incubated in [^{35}S]GTPγS (0.04 nM) and 2 mM GDP, with 3 μM WIN 55212-2, 10 μM pCl-DPDPE, 1 μM U50,488H or 3 μM DAMGO in assay buffer at 25°C for 2 h. The incubation media for WIN 55212-2 also contained 0.5% bovine serum albumin. Basal activity was assessed in the absence of agonist, and non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Slides were rinsed twice in cold Tris buffer (50 mM Tris-HCl buffer, pH 7.4) and once in deionized water, thoroughly dried and exposed to film for 48 h. Films were digitized with a Sony XC-77 video camera and analyzed using the NIH IMAGE program for Macintosh computers. Quantification of images was obtained from densitometric analysis using ^{14}C standards and corrected for ^{35}S using brain paste standards as previously described (Christie, 1991). Agonist-stimulated activity was calculated by subtracting the optical density in basal sections (GDP only) from that of agonist-stimulated sections and results are expressed as percent stimulation over basal activity. For each agonist, triplicate sections of brains from five animals were used.

2.3. [^{35}S]GTPγS membrane assay

Striata were dissected and homogenized in 20 vol buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4). The homogenate was centrifuged twice at 48 000 × *g* at 4°C for 10 min and resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Protein was assayed by the method of Bradford (1976). For concentration-effect curves, membranes (4 μg protein) were incubated at 30°C for 1 h in assay buffer with various concentrations of agonist, 20 μM GDP and 0.05 nM [^{35}S]GTPγS in a 1 ml total volume. Basal binding was assessed in the absence of agonist, and non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. The assay buffer for WIN 55212-2 also contained 0.1 mg/ml bovine serum albumin. For [^{35}S]GTPγS assays for Scatchard analysis of agonist-stimulated [^{35}S]GTPγS binding, membranes (10 μg) were incubated with 20 μM GDP, 0.05 nM [^{35}S]GTPγS and 0 to 20 nM unlabeled GTPγS with and without 3 μM DAMGO, 10 μM pCl-DPDPE or 3 μM WIN 55212-2 in a 1 ml total volume. The reaction was terminated by filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after extraction overnight in Ecolite scintillation fluid. Data are reported as mean ± standard error values of at least three

experiments, which were each performed in triplicate. Nonlinear iterative regression analyses of agonist concentration-effect curves were performed with JMP (SAS Institute, Cary, NC). Scatchard analysis was conducted using the computer curve fitting program EBDA.

2.4. Membrane receptor binding assays

Rats were decapitated and brains (minus cerebellum) were homogenized in 20 vol cold Tris buffer with a Polytron homogenizer. Membranes were centrifuged at 48 000 × *g* for 10 min and resuspended in fresh buffer. Membranes were centrifuged again at 48 000 × *g* for 10 min, then resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, pH 7.4 for opioid receptor binding assay or 20 mM Hepes + 1 mM $MgCl_2$ buffer for WIN 55212-2). Protein was assayed by the method of Bradford (1976). For opioid binding assays, membranes (0.2 mg protein) were incubated in assay buffer with the appropriate concentrations of unlabeled agonist with either 1 nM [^{3}H]DAMGO or 3 nM [^{3}H]DPDPE in a final volume of 1 ml. Nonspecific binding was measured in the presence of 5 μM DAME. For the cannabinoid assay, membranes (0.06 mg protein) were incubated in 20 mM Hepes + 1 mM $MgCl_2$, pH 7.0 with various concentrations of unlabeled WIN 55212-2 and 1 nM [^{3}H]WIN 55212-2 in 1 ml total volume. Nonspecific binding was measured in the presence of 1 μM WIN 55212-2 for cannabinoid receptors. The assay was incubated for 60 min ([^{3}H]DAMGO), 90 min ([^{3}H]WIN 55212-2) or 180 min ([^{3}H]DPDPE) at 25°C. The reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters followed by three washes with Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction overnight in 5 ml Ecolite scintillation fluid. Data are reported as mean ± standard error values of at least three experiments performed in triplicate. Scatchard analysis was conducted using the computer curve fitting program EBDA.

3. Results

3.1. Autoradiographic localization of opioid- and cannabinoid-activated G-proteins in caudate-putamen

In vitro autoradiography of agonist-stimulated [^{35}S]-GTPγS binding provides a high-resolution quantitative localization of receptor-mediated G-protein activation in the brain. To directly compare the activation of G-proteins by μ-, δ- and κ-opioid receptors with that of cannabinoid receptors, adjacent brain sections were incubated with maximally effective concentrations of DAMGO, pCl-DPDPE, U50,488H and WIN 55212-2, respectively, in the presence of [^{35}S]GTPγS (0.04 nM) and excess GDP (2 mM). Results (Fig. 1) showed significant stimulation of [^{35}S]GTPγS binding by the μ, δ and cannabinoid agonists. The distribution of agonist-stimulated [^{35}S]GTPγS binding in the caudate-putamen was unique for each receptor, as seen in representative sections shown in Fig. 1. High levels of μ-opioid-stimulated [^{35}S]GTPγS binding were identified in the patches and subcallosal region of the caudate-putamen in sections incubated with 3 μM DAMGO. The highest level of cannabinoid-stimulated

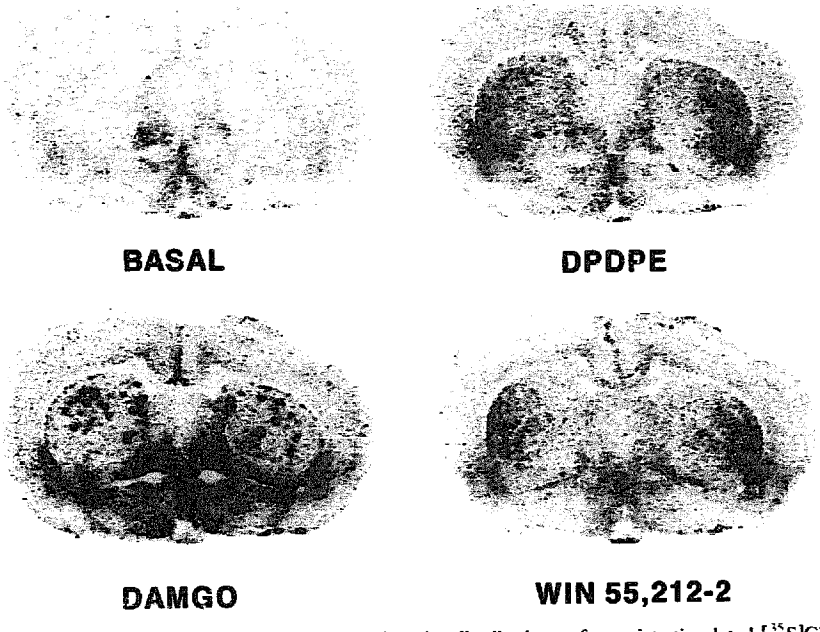

Fig. 1. Representative sections at the level of the caudate-putamen showing the distributions of agonist-stimulated [^{35}S]GTPγS binding. Sections were incubated with 2 mM GDP, then [^{35}S]GTPγS (0.04 nM) with 2 mM GDP and 3 μM DAMGO, 3 μM WIN 55212-2 or 10 μM pCl-DPDPE. Basal binding was assessed in the absence of agonist.

Table 1
Effect of opioid and cannabinoid agonists on [^{35}S]GTPγS binding: autoradiographic analysis in the caudate-putamen

Agonist	nCi/g	% Stimulation
Basal	330±15	N.A.
DAMGO	580±18	59±2%
pCl-DPDPE	634±18	72±5%
WIN 55212-2	606±9	66±5%
U50,488H	362±10	8±3%

Sections were incubated with 2 mM GDP, then [^{35}S]GTPγS (0.04 nM) with 2 mM GDP and 3 μM DAMGO, 3 μM WIN 55212-2 or 10 μM pCl-DPDPE. Basal binding was assessed in the presence of 2 mM GDP alone. Data represent mean values ± S.E.M. of triplicate sections from the brains of five animals. N.A., not applicable.

[^{35}S]GTPγS binding was identified in the dorsal lateral caudate-putamen after incubation with 3 μM WIN 55212-2. δ-opioid-stimulated [^{35}S]GTPγS binding in sections incubated with 10 μM pCl-DPDPE was distributed throughout the caudate-putamen, particularly in the lateral region. Image analysis was performed by measuring the optical density of the caudate-putamen for each receptor agonist (Table 1). Since excess GDP was present, very low levels of basal binding were observed. The μ, δ and cannabinoid agonists stimulated [^{35}S]GTPγS binding to approximately the same degree: 59% by DAMGO, 72% by pCl-DPDPE and 66% by WIN 55212-2, with no statistically significant differences in stimulation among these three agonists. Sections were also incubated with U50,488H to measure [^{35}S]GTPγS binding stimulated by κ-opioid receptors (not shown). The κ agonist U50,488H did not appreciably stimulate [^{35}S]GTPγS binding above basal (Table 1).

3.2. Agonist-stimulated [^{35}S]GTPγS binding in striatal membranes

Concentration-effect curves of agonist-stimulated [^{35}S]GTPγS binding were also examined in isolated striatal membranes to further quantitate maximal stimulation of [^{35}S]GTPγS binding by opioid and cannabinoid agonists (Fig. 2). In each case, agonist-stimulated [^{35}S]GTPγS binding was concentration-dependent and saturable. Maximal stimulation (E_{max}) of [^{35}S]GTPγS binding by WIN 55212-2 was 125 ± 9%, with an ED_{50} value of 0.02 ± 0.01 μM. The E_{max} values for DAMGO and pCl-DPDPE were 68 ± 5% and 57 ± 4%, respectively, and were significantly different from that of WIN 55212-2 ($P < 0.001$). The ED_{50} values for DAMGO and pCl-DPDPE were 0.56 ± 0.12 μM and 0.21 ± 0.02 μM, respectively. Thus, in isolated striatal membranes the cannabinoid agonist stimulated approximately twice the level of [^{35}S]GTPγS binding compared to the μ- and δ-opioid agonists.

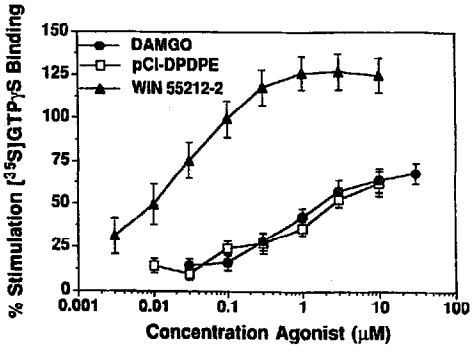

Fig. 2. Concentration-effect curves of agonist-stimulated [^{35}S]GTPγS binding in isolated striatal membranes. Membranes were incubated with 20 μM GDP with and without various concentrations of DAMGO, pCl-DPDPE, or WIN 55212-2. Data are expressed as mean percent basal [^{35}S]GTPγS binding ± SEM.

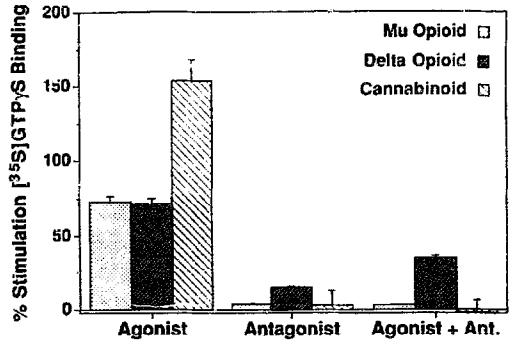

Fig. 3. Effects of antagonists on basal and agonist-stimulated [^{35}S]GTPγS binding. Membranes were incubated with 0.05 nM [^{35}S]GTPγS and 20 μM GDP with 5 μM naloxone, 10 μM ICI 184,764 or 1 μM SR 141716A in the presence and absence of 5 μM DAMGO, 5 μM pCl-DPDPE or 0.5 μM WIN 55212-2, respectively. Data are expressed as mean percent basal [^{35}S]GTPγS binding ± SEM.

3.3. Antagonist effects on basal and agonist-stimulated [^{35}S]GTPγS binding

[^{35}S]GTPγS binding experiments were conducted with agonists and antagonists alone and in combination to confirm receptor specificity and examine the effects of antagonists on basal [^{35}S]GTPγS binding (Fig. 3). As observed previously, the stimulation by the cannabinoid agonist was approximately two-fold greater than that of either the μ- or δ-opioid agonists; stimulation in all three cases was significantly attenuated by the addition of the selective μ, δ and cannabinoid antagonists, naloxone, ICI 174,864 and SR 141716A, respectively. The smaller effect of the δ antagonist ICI 174,864 in reducing pCl-DPDPE-stimulated [^{35}S]GTPγS binding is consistent with its relatively low potency (McKnight et al., 1985; Selley and Bidlack, 1992). None of these antagonists had any significant effect on basal [^{35}S]GTPγS binding (in the absence of agonist). The lack of antagonist effects on basal [^{35}S]GTPγS binding suggests that the potential presence of endogenous agonists does not appreciably affect binding. Moreover, these antagonists do not have appreciable negative intrinsic activity on their own in brain under the conditions used in this

study, as has been reported for ICI 174,864 and naloxone in cell culture systems (Costa et al., 1990; Wang et al., 1994).

3.4. Scatchard analyses of receptor binding sites and agonist-stimulated [^{35}S]GTPγS binding

Previous reports on cannabinoid receptor binding (Devane et al., 1988; Herkenham et al., 1991; Kuster et al., 1993) have suggested that this receptor is present in brain at a density at least ten times greater than that of most other G-protein-coupled receptors. However, the results from Fig. 2 showed that cannabinoid-stimulated [^{35}S]-GTPγS binding was only two-fold greater than that of μ and δ agonist-stimulated activity. In order to quantitatively explore the basis of this discrepancy, receptor binding and [^{35}S]GTPγS binding were conducted in the same membrane preparations for these three receptor types. These assays were conducted so that B_{max} values could be obtained from both assays for direct comparison. For receptor binding, Scatchard analysis was performed using [^{3}H]DAMGO for μ sites, [^{3}H]DPDPE for δ sites, and [^{3}H]WIN 55212-2 for cannabinoid sites. Fig. 4 shows a typical Scatchard plot for all three receptor types. The cannabinoid receptor B_{max} value is 3.6 ± 0.3 pmol/mg, compared to 0.30 ± 0.04 and 0.29 ± 0.05 pmol/mg for μ and δ receptors, respectively. The calculated B_{max} values from three separate experiments for each receptor type (Table 2) confirmed this finding, with the cannabinoid receptor B_{max} value over ten times higher than that of μ- or δ-opioid receptors.

To quantitatively assess the relative number of G-proteins activated by these receptor systems, Scatchard analysis was performed on agonist-stimulated [^{35}S]GTPγS binding, using methods previously described (Gierschik et al., 1991; Traynor and Nahorski, 1995). In this method, [^{35}S]GTPγS binding was assayed in the presence of excess GDP and increasing concentrations of GTPγS, in the presence and absence of agonists. Basal [^{35}S]GTPγS binding was subtracted from agonist-stimulated binding to obtain net agonist-stimulated binding. This net stimulated binding was used to construct Scatchard plots to provide estimated B_{max} values of agonist-activated G-proteins in striatal membranes. Fig. 5 shows the effect of DAMGO, pCl-DPDPE, and WIN 55212-2 on [^{35}S]GTPγS binding as a function of increasing concentrations of unlabeled GTPγS. These data show that the binding of [^{35}S]GTPγS is increased by all three agonists at low concentrations of GTPγS, indicating an agonist-induced increase in high affinity GTPγS binding. Direct Scatchard analysis of basal and agonist-stimulated binding (not shown) indicated that the effect of each agonist was to decrease the K_D for

Table 2
Catalytic amplification factors for opioid- and cannabinoid-stimulated G-protein activation

Receptor	Receptor B_{max} pmol/mg	G-protein B_{max} pmol/mg	Amplification factor
μ-Opioid	0.30	5.15	17
δ-Opioid	0.29	6.27	22
Cannabinoid	3.56	10.05	3

Receptor B_{max} values were determined by receptor binding in striatal membranes incubated with 1 nM [^{3}H]DAMGO, 3 nM [^{3}H]DPDPE, or 1 nM [^{3}H]WIN 55212-2 in the presence of various concentrations of unlabeled agonist. G-protein B_{max} values were determined by [^{35}S]GTPγS binding assays in which membranes were incubated with 20 μM GDP, 0.05 nM [^{35}S]GTPγS and 0–20 nM unlabeled GTPγS with and without 3 μM DAMGO, 10 μM pCl-DPDPE or 3 μM WIN 55212-2, and net-stimulated binding was determined as described in the legend of Fig. 4. B_{max} values were calculated from experiments performed in triplicate that were replicated at least four times. Amplification factors were calculated by dividing the G-protein B_{max} by receptor B_{max}.

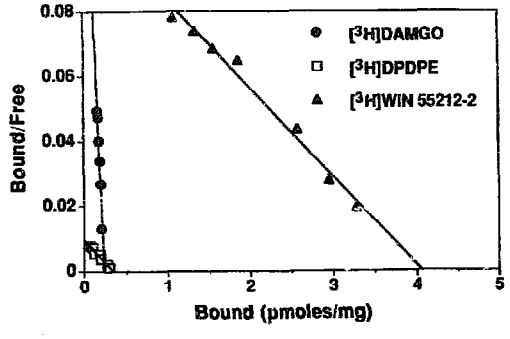

Fig. 4. Representative Scatchard plot of receptor binding in striatal membranes. Membranes were incubated with various concentrations of unlabeled DAMGO, DPDPE or WIN 55212-2 in the presence of 1 nM [^{3}H]DAMGO, 3 nM [^{3}H]DPDPE, or 1 nM [^{3}H]WIN 55212-2, respectively. Data shown are from one experiment that was performed in triplicate and replicated four times with similar results.

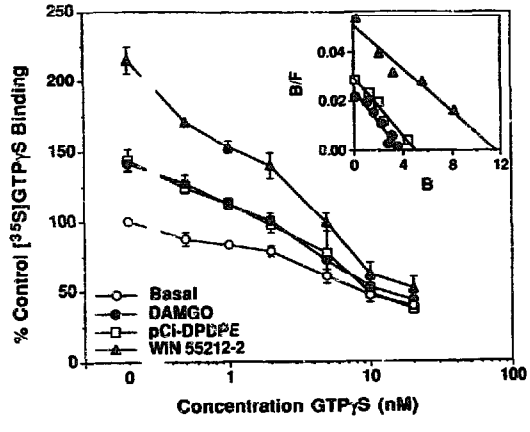

Fig. 5. Displacement of [^{35}S]GTPγS binding by GTPγS. Membranes were incubated with 0.05 nM [^{35}S]GTPγS in the presence of 0–20 nM GTPγS with and without 3 μM DAMGO, 10 μM pCl-DPDPE or 3 μM WIN 55212-2. Data represent mean percent control [^{35}S]GTPγS binding (binding in the absence of unlabeled GTPγS or agonist). Control [^{35}S]GTPγS binding was 0.25 ± 0.02 pmol/mg membrane protein. Inset, Scatchard analysis of the net agonist-stimulated [^{35}S]GTPγS binding (agonist-stimulated binding minus basal binding, measured at each concentration of GTPγS).

GTPγS. The high affinity K_D value of basal GTPγS binding (determined in the absence of agonist) was 14.0 ± 1.4 nM, whereas the K_D values determined in the presence of agonists were 4.9 ± 0.7 nM, 6.0 ± 1.2 nM, and 4.5 ± 0.5 nM for DAMGO-, pCl-DPDPE-, and WIN 55212-2-stimulated binding, respectively. Although the K_D values obtained in the presence of all three agonists were significantly different from basal [^{35}S]GTPγS binding ($P < 0.005$), there were no significant differences in the magnitude of the increase in the K_D values of [^{35}S]GTPγS binding produced by any of the three agonists. In order to specifically determine B_{max} values of [^{35}S]GTPγS binding stimulated by each agonist, Scatchard plots were constructed from net agonist-stimulated [^{35}S]GTPγS binding (Fig. 5, inset). The B_{max} values of net agonist-stimulated [^{35}S]GTPγS binding were 10 ± 2 pmol/mg and 5 ± 2 and 6 ± 2 pmol/mg for cannabinoid and μ- and δ-opioid agonists, respectively. In agreement with the data presented in Fig. 2, these B_{max} values (Table 2) showed that WIN 55212-2 stimulated approximately twice as much [^{35}S]GTPγS binding as either DAMGO or pCl-DPDPE.

In order to directly compare the efficiency of each of these three receptor types in striatal membranes, a catalytic amplification factor was obtained by dividing the B_{max} of agonist-stimulated [^{35}S]GTPγS binding by the B_{max} of receptor binding (Table 2). These results showed that the amplification factors of both μ and δ receptors were approximately equal to each other, with twenty times higher B_{max} values for G-protein activation than for receptor binding. However, the catalytic amplification factor for cannabinoid receptors was considerably less, with only a three-fold higher B_{max} value for G-protein activation than for cannabinoid receptor binding. Thus, the relative amplification factors for receptor-mediated G-protein activation were approximately 6.5-fold greater for μ- and δ-opioid receptors than for cannabinoid receptors.

4. Discussion

[^{35}S]GTPγS autoradiography and [^{35}S]GTPγS binding assays in isolated membranes are techniques that complement each other in the study of receptor-mediated G-protein activity. As seen in the present study, the results obtained by the two methods are similar in terms of the level of agonist-stimulated [^{35}S]GTPγS binding measured. One difference between the two techniques is the level of GDP which is necessary to detect agonist-stimulated [^{35}S]GTPγS binding. The concentration of GDP required for [^{35}S]GTPγS autoradiography is 1–2 mM, whereas in membranes the concentration is in the 10–30 μM range (Sim et al., 1995). Perhaps the most likely explanation for this difference is the greater level of protein in tissue sections (approximately 175 μg) compared to membranes (4–15 μg), since [^{35}S]GTPγS binding is very sensitive to protein concentration (Sim et al., unpublished observations). The results of previous [^{35}S]GTPγS autoradiography studies have shown that although the distributions of opioid- and cannabinoid-stimulated [^{35}S]GTPγS binding correlate with known receptor distributions, the relative levels of agonist-stimulated [^{35}S]GTPγS binding do not necessarily reflect known receptor densities (Sim et al., 1995). In the present study, we have confirmed this finding quantitatively in sections at the level of the caudate-putamen and in isolated striatal membranes. Although a ten-fold excess of cannabinoid over μ- and δ-opioid receptors was measured, this receptor excess did not translate into a ten-fold excess of G-protein activation. In tissue sections, opioid and cannabinoid agonists stimulated approximately the same level of [^{35}S]GTPγS binding. In isolated striatal membranes, cannabinoid-stimulated [^{35}S]GTPγS binding was twice that of opioid-stimulated binding. For the opioid agonists, the levels of stimulated [^{35}S]GTPγS binding were approximately the same between tissue sections and isolated membranes; however, cannabinoid-stimulated [^{35}S]GTPγS binding in membranes was twice that of tissue sections. This difference resulted from the anatomical resolution of the autoradiographic technique versus membrane assays, since membranes were prepared from grossly dissected striata, which include a portion of the globus pallidus. The globus pallidus contains a high density of cannabinoid receptors (Herkenham et al., 1991; Jansen et al., 1992) and a very high level of cannabinoid-stimulated [^{35}S]GTPγS binding (Sim et al., 1995), which clearly increases the levels of stimulated [^{35}S]GTPγS binding seen in striatal membrane preparations in the present study.

We have considered two possible mechanisms by which the observed differences in receptor efficiency may occur. It is possible that receptors with greater efficiency, such as opioid receptors, produce a greater change in the affinity of the G-protein for GTP versus GDP. This does not appear to be the case for these receptors in the striatum, since the calculated K_D of agonist-stimulated [^{35}S]GTPγS binding were similar for opioid and cannabinoid agonists. A second possibility is that opioid receptors catalytically activate more G-proteins than cannabinoid receptors. The B_{max} values derived from [^{35}S]GTPγS Scatchard analysis of receptor-stimulated binding indicate that this is in fact the case. Despite a ten-fold excess of cannabinoid over opioid receptors, the number of G-proteins activated by cannabinoid receptors is only twice that of opioid receptors. These values correlate with the levels of G-protein activation measured in the agonist concentration-effect curves in membranes.

In order to quantitate the discrepancy between receptor binding and receptor-mediated G-protein activation, we directly compared B_{max} values of these two parameters to obtain a catalytic amplification factor (Table 2). This amplification factor was approximately 20 for both μ- and δ-opioid receptors, but only 3 for cannabinoid receptors. This factor might be interpreted as the catalytic amplification for each receptor type. By this interpretation, each μ

receptor in striatum activates 17 G-proteins, while each δ receptor activates 22 G-proteins and each cannabinoid receptor activates only 3 G-proteins. However, this interpretation is only true in a relative sense. Agonist stimulation of [^{35}S]GTPγS binding is not an absolute constant, but instead depends upon a large number of technical factors including sodium and GDP concentrations, as well as the temperature and kinetics of GTPγS binding. Moreover, the increase in [^{35}S]GTPγS binding observed in the presence of agonist is not an increase in the absolute number of G-proteins, but instead represents an allosteric change in the G-protein structure to increase its relative affinity for GTP and GTPγS over its affinity for GDP. Since GTPγS is hydrolysis resistant, those G-protein α subunits which have bound [^{35}S]GTPγS are virtually irreversibly activated and are thus not available for re-activation by the receptor. This may slightly reduce the available pool of G-proteins for activation by receptors and underestimate actual catalytic amplification values. Another potential inaccuracy in the calculation of amplification factors is the determination of the appropriate receptor B_{max} to use in the calculation. Since GDP and NaCl in the [^{35}S]GTPγS binding assay would shift agonist binding into low affinity states (Childers and Snyder, 1980; Devane et al., 1988; Werling et al., 1988), receptor binding assay conditions were chosen in the present study which promote high affinity agonist binding sites. Thus, receptor B_{max} values could be determined with the same agonists used in the [^{35}S]GTPγS binding assay. The use of radiolabeled antagonists may be most accurate for determination of total receptor number, but it may overestimate the actual number of binding sites available to agonists. For all these reasons, the amplification factors calculated in Table 2 are true only under the particular assay conditions, and are most useful when comparing relative amplifications between receptors assayed under identical conditions. By this interpretation, each μ- and δ-opioid receptor in striatum appears to activate approximately the same number of G-proteins, while each cannabinoid receptor appears to activate a several fold lower number of G-proteins than either μ- or δ-opioid receptors.

The biochemical basis of this disparity is not clear. The simplest explanation is that the different receptors have different intrinsic abilities to catalytically activate G-proteins (i.e. the observed differences are a property of the receptors themselves). In addition, different receptors may couple to different types of G-protein subunits. There are currently more than 20 types of α subunits, of which G_s, G_i, G_o, G_z and G_q/G_{11} are found in the striatum (Worley et al., 1986; Asano et al., 1990; Hinton et al., 1990; Aronin and DiFiglia, 1992; Mailleux et al., 1992). Opioid and cannabinoid receptors may couple with one or more of three subtypes of G_i ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$) and/or two of G_o ($G_{o\alpha1}$ and $G_{o\alpha2}$) (Jones and Reed, 1987; Hsu et al., 1990). The relative affinities of different α subtypes for GDP and GTPγS may vary, which may affect efficiencies of receptor-G-protein activation in [^{35}S]GTPγS binding assays. However, this seems an unlikely explanation for the results obtained in this study for two reasons. First, the same optimal GDP concentrations were required for stimulation by either cannabinoid or μ- and δ-opioid receptors (not shown). Secondly, all three receptor agonists produced the same high affinity K_D for [^{35}S]GTPγS binding, under conditions where the range of GDP:GTPγS concentration ratios were consistent among the different receptor types studied. A more likely explanation for differences in efficiency of coupling is that the rate of catalytic activation of G-proteins may depend upon the receptor-G-protein combination. For example, in purified systems the catalytic rate of dopamine D_2 activation of $G_{i\alpha2}$ was twice that of other G-protein subtypes (Senogles et al., 1990). Similarly, in the striatum, the relative levels of G_i/G_o subtypes and their anatomical distribution may dictate receptor efficiency. Moreover, individual subtypes of G-protein α subunits have different guanine nucleotide binding and release kinetics (Carty et al., 1990; Offermanns and Schultz, 1994), which may affect the catalytic rate at which they can be activated by receptors.

Investigation of receptor efficiency has also been conducted using β-adrenoceptors. Previous studies have shown that β_2-adrenoceptors stimulate adenylyl cyclase activity to a higher degree than β_1-adrenoceptors in cardiac membranes, despite a greater than 2:1 ratio of β_1- to β_2-adrenoceptors (Kaumann and Lemoine, 1987). More recent investigations in transfected cells indicate that this difference in efficiency may be inherent to the receptors, since the β_1-adrenoceptor was always less 'efficacious' than the β_2-adrenoceptor for stimulation of adenylyl cyclase under a variety of conditions (Levy et al., 1993; Birnbaumer et al., 1994). Thus, different receptor efficiencies can be observed within the same cell.

The physiological significance of these differences in amplification between different receptor systems is not known. Differences in drug effects have often been attributed to the anatomical distribution of the receptors. This is clearly the case for some effects, such as cardiovascular and respiratory risk of opioid versus cannabinoid administration, since opioid receptors are localized in many brain stem autonomic centers, whereas there are virtually no cannabinoid receptors in these regions. However, the powerful psychological and analgesic, as well as addictive, effects of opiates could also be a result of a higher level of catalytic amplification of the receptor-mediated response, resulting in more profound behavioral and physiological effects of opioids than cannabinoids.

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