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LACK OF EVIDENCE OF κ_2-SELECTIVE ACTIVATION OF G-PROTEINS

κ Opioid Receptor Stimulation of [^{35}S] GTPγS Binding in Guinea Pig Brain

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ABSTRACT. Although only one gene for κ opioid receptors has been cloned to date, κ_1 and κ_2 receptors have been defined pharmacologically, with drugs such as bremazocine binding to both putative κ receptor subtypes. To examine whether κ receptor subtypes can be distinguished at the level of the G-protein, the ability of the κ_1 agonist (*trans*-(dl)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) methane sulfonate (U-50488H) to stimulate [^{35}S]guanosine-5′-O-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding in guinea pig brain was compared with that of bremazocine and dynorphin. In membranes prepared from guinea pig striatum, both bremazocine and U-50488H stimulated [^{35}S]GTPγS binding with the same relative efficacy, while dynorphin produced at least two-fold greater efficacy than the other two agonists. *In vitro* autoradiography of agonist-stimulated [^{35}S]GTPγS binding revealed similar regional distributions of bremazocine- and U-50488H-activated G-proteins. In striatal membranes, the κ antagonist nor-binaltorphimine (nor-BNI) blocked both bremazocine- and U-50488H-stimulated [^{35}S]GTPγS binding with similar K_e values. In agonist additivity experiments, the stimulation of [^{35}S]GTPγS binding by the δ agonist [D-pen^{2,5}, *p*-Cl-Phe^4]enkephalin (p-Cl-DPDPE) was approximately additive with the two κ agonists. Stimulation of [^{35}S]GTPγS binding by the μ agonist [D-Ala^2, *N*-Me^4, Gly^5-ol]-enkephalin (DAMGO) was additive with U-50488H, but not with bremazocine, reflecting the μ antagonist properties of this compound. The combination of bremazocine and U-50488H together produced no greater stimulation of binding than either agonist alone, indicating that they were binding to the same site. These results demonstrate that bremazocine and U-50488H activate G-proteins in guinea pig brain through the same receptor, and suggest that κ_2 receptors are not coupled through the same signal transduction mechanisms as κ_1 receptors. BIOCHEM PHARMACOL **56**;1:113–120, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. [^{35}S]GTPγS; bremazocine; U-50488H; κ opioid receptors; guinea pig brain; dynorphin

Three genes for opioid receptors have been cloned, representing the three types of opioid receptors classically defined pharmacologically: δ [1, 2], μ [3, 4], and κ [5–7]. The pharmacology of the cloned κ receptor corresponds to that of the putative κ_1 receptor, with high affinity for agonists such as U-50488H† and dynorphin, and antagonists like nor-BNI. These pharmacological properties are found not only in cloned receptors, but also in κ receptor-containing immune cells as well [8]. However, earlier pharmacological studies have suggested the existence of several κ receptor subtypes [9–12]. One example is the putative κ_2 receptor, which is labeled with [^{3}H]bremazocine and which binds U-50488H with lower relative affinity than κ_1 sites. In receptor binding studies, κ_1 sites are often labeled directly with [^{3}H]U-69,593 (a compound closely related to U-50488H), whereas [^{3}H]bremazocine is used to label κ_2 sites. Because bremazocine is a relatively non-selective ligand, with μ antagonist as well as κ agonist properties, the binding of κ_2 receptors must be measured in the presence of more selective drugs to block the binding of [^{3}H]bremazocine to μ, δ, and κ_1 sites. In general, both subtypes of κ receptors are particularly dense in guinea pig brain, and *in vitro* receptor autoradiographic studies suggest different anatomical distributions for the two κ sites [9, 13]. In the guinea pig brain, κ_1 sites are highest in the claustrum, endopiriform nucleus, nucleus accumbens, caudate-putamen, neocortex, and cerebellum. The distribution of κ_2 sites is more widespread, with localization in these regions, as well as in the hippocampus, thalamus, and brainstem.

One of the features of the cloned opioid receptor genes is that they belong to the family of G-protein-coupled receptors. Each of these receptor types couples to $G_{i/o}$ proteins, and their signal transduction mechanisms result in the inhibition of adenylyl cyclase, a decrease in Ca^{2+} conductance, and an increase in K^+ conductance through specific

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† *Abbreviations:* DAMGO, [D-Ala^2, *N*-Me^4, Gly^5-ol]-enkephalin; GTPγS, guanosine-5′-O-(γ-thin)-triphosphate; nor-BNI, nor-binaltorphimine; *p*-Cl-DPDPE,[D-Pen^{2,5}-*p*-Cl-Phe^4]enkephalin; and U50,488H, (*trans*-(*d*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) methane sulfonate.

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G-protein-coupled mechanisms [14]. Traynor and Nahorski [15] applied methodology previously described for other receptor systems [16, 17] to examine agonist-stimulated [^{35}S]GTPγS binding, thus defining the ability of opioid agonists to activate G-proteins. When assayed in the presence of excess GDP, agonist-stimulated [^{35}S]GTPγS binding provides an *in vitro* estimate of agonist efficacy at the level of G-protein activation. Our laboratory has adapted this technique to *in vitro* autoradiography in brain sections [18, 19], which allows the neuroanatomical localization of receptor-activated G-proteins in brain. Experiments using agonist-stimulated [^{35}S]GTPγS binding assays in both brain sections and membranes have been used to examine the amplification of receptor response through G-proteins [20, 21], the effect of chronic agonist treatment on receptor activation of G-proteins [22–24], and mechanisms of agonist efficacy at the G-protein level [25].

Although most of the earlier work on opioid-stimulated [^{35}S]GTPγS binding has focused on μ and δ receptors, more recent evidence has established the utility of this method for studying κ receptors as well. Liu-Chen and colleagues [26] have determined the relative efficacy of κ receptors in stimulating [^{35}S]GTPγS binding in transfected CHO cells, and our laboratory has described the distribution of U-50488H-stimulated [^{35}S]GTPγS binding in guinea pig brain [27]. However, none of these studies have explored the question of whether κ_2 sites can be identified using the [^{35}S]GTPγS binding assay. Because κ receptors exist in high density in the guinea pig brain, we chose this tissue to compare the ability of the κ_1 agonist U-50488H and the non-selective agonist bremazocine to stimulate [^{35}S]GTPγS binding. If κ_2 receptors were coupled to G-proteins in the same way as κ_1 sites, we predicted that bremazocine-stimulated [^{35}S]GTPγS binding would display a different pharmacological profile and neuroanatomical distribution than that of U-50488H. In the present study, we provide evidence that the pharmacology and localization of G-protein activation in guinea pig brain are the same for both drugs, and that dynorphin activates G-proteins through a combination of μ, δ, and κ opioid receptors.

MATERIALS AND METHODS

Materials

Male guinea pigs were purchased from Sasco, and male Sprague–Dawley rats were purchased from Zivic-Miller. [^{35}S]GTPγS (1250 Ci/mmol) was obtained from DuPont/NEN. Dynorphin(1-17), DAMGO,(*p*-Cl-DPDPE, and U50,488H were obtained from the Sigma Chemical Co. Bremazocine and nor-BNI were obtained from Research Biochemicals International. GDP and GTPγS were purchased from Boehringer Mannheim. Reflections® autoradiography film was purchased from DuPont/NEN. Ecolite scintillation fluid was obtained from ICN. All other reagent grade chemicals were obtained from Sigma or Fisher.

[^{35}S]GTPγS Autoradiography

Guinea pigs were killed by decapitation. The brains were removed and immersed in isopentane at −35°. Coronal sections (20 μm) were cut on a cryostat at −20°, and thaw-mounted onto gelatin-coated slides. Slides were rinsed in assay buffer (50 mM of Tris–HCl, 3 mM of $MgCl_2$, 0.2 mM of EGTA, 100 mM of NaCl, pH 7.4) at 25°, and then were pretreated with 2 mM of GDP and protease inhibitors (10 μL/mL of a solution containing 0.2 mg/mL each of bestatin, leupeptin, pepstatin A, and aprotinin) in assay buffer. Sections were incubated in 0.04 nM of [^{35}S]GTPγS and 2 mM of GDP, with 1 μM of U50,488H, 0.1 μM of bremazocine, or 1 μM of dynorphin in assay buffer at 25° for 2 hr. Basal activity was assessed in the absence of agonist. Slides were rinsed twice in cold Tris buffer (50 mM of Tris–HCl buffer, pH 7.4) and once in deionized water, thoroughly dried, and exposed to film for 5 days. Films were digitized with a Sony XC-77 video camera and analyzed using the National Institutes of Health 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 [22]. Agonist-stimulated activity was calculated by subtracting the optical density in basal sections (GDP only) from that of agonist-stimulated sections. For each agonist, triplicate sections of brains from two different animals were used.

[^{35}S]GTPγS Membrane Assay

Brain regions (striatum and cerebellum) were dissected and homogenized in 20 vol. buffer (50 mM of Tris–HCl, 3 mM of $MgCl_2$, 1 mM of EGTA, pH 7.4). The homogenate was centrifuged twice at 48,000 *g* at 4° for 10 min and resuspended in assay buffer (50 mM of Tris–HC, 3 mM of $MgCl_2$, 0.2 mM of EGTA, 100 mM of NaCl, pH 7.4). Protein was assayed by the method of Bradford [28]. For concentration–effect curves, membranes (10 μg protein) were incubated at 30° for 1 hr in assay buffer with various concentrations of agonist, 20 μM of GDP, and 0.05 nM of [^{35}S]GTPγS in a total volume of 1 mL. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM of unlabeled GTPγS. 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 ± SE values of at least three experiments, which were each performed in triplicate. Data are expressed as percent stimulation of [^{35}S]GTPγS binding, which was calculated by dividing the net agonist-stimulated binding (i.e. agonist-stimulated minus basal binding) by the basal [^{35}S]GTPγS determined in the absence of agonist. Nonlinear iterative regression analy-

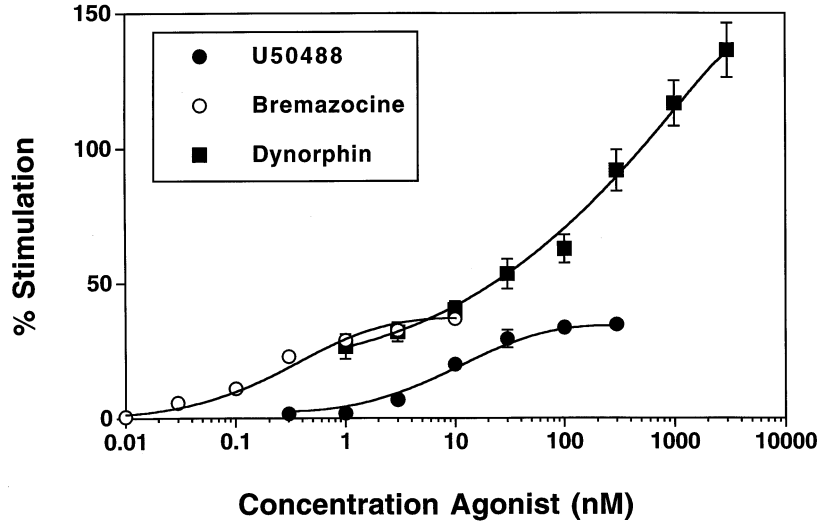

FIG. 1. Stimulation of [^{35}S]GTPγS binding in membranes from guinea pig striatum by U-50488H, bremazocine, and dynorphin(1-17). Data are expressed as percent stimulation of [^{35}S]GTPγS binding, and represent mean values ± SEM from at least three separate experiments. Basal [^{35}S]GTPγS binding was 159 ± 25 fmol/mg.

ses of agonist and antagonist concentration–effect curves were performed with JMP (SAS Institute).

RESULTS

κ Opioid Stimulation of [^{35}S]GTPγS Binding in Guinea Pig Brain Membranes

Previous autoradiographic studies showed that guinea pig striatum contains a high density of both κ_1 and κ_2 sites [13]. Therefore, to determine whether the stimulation of [^{35}S]GTPγS binding by bremazocine contained appreciable κ_2 activity, membranes were prepared from guinea pig striatum and assayed for agonist-stimulated [^{35}S]GTPγS binding with either bremazocine, U-50488H, or dynorphin (1-17). The results (Fig. 1) showed that the maximal effects of both bremazocine and U-50488H in stimulating [^{35}S]GTPγS binding were virtually the same (E_{max} = 37 ± 2% for bremazocine, and 35 ± 2% for U-50488H), whereas the EC_{50} of bremazocine (0.20 ± 0.12 nM) was significantly ($P < 0.001$) lower than that of U-50488H (9.5 ± 1.6 nM). In contrast, dynorphin(1-17) produced a higher maximal level of stimulation: the E_{max} could not be defined because stimulation continued to increase at 1–10 μM of dynorphin, but the maximal stimulation observed at 10 μM of dynorphin, 150%, was over three-fold greater than that observed for either bremazocine or U-50488H.

Autoradiographic Localization of κ Opioid-Stimulated [^{35}S]GTPγS Binding in Guinea Pig Brain Sections

To compare the neuroanatomical localization of these three κ agonists, *in vitro* [^{35}S]GTPγS autoradiography was performed on sections of guinea pig brain using 0.1 μM of bremazocine, 1 μM of U-50488H, and 1 μM of dynorphin. U-50488H and bremazocine stimulated [^{35}S]GTPγS binding in the same brain regions, including the caudate-putamen and cerebellum (Fig. 2), as well as the neocortex, nucleus accumbens, endopiriform nucleus, claustrum, hippocampus, and substantia nigra. Densitometric analysis of these regions showed no significant differences in the levels of [^{35}S]GTPγS stimulation produced by U-50488H and bremazocine (data not shown). Dynorphin-stimulated [^{35}S]GTPγS binding was found in the same brain regions as κ-stimulated [^{35}S]GTPγS binding. However, the level of dynorphin-stimulated [^{35}S]GTPγS binding was generally higher than that produced by the other agonists. Dynorphin-stimulated [^{35}S]GTPγS binding was also higher in some regions with relatively low levels of bremazocine- or U-50488H-stimulated [^{35}S]GTPγS binding, such as hypothalamus and nucleus accumbens, consistent with the lower receptor selectivity of dynorphin.

Pharmacological Characterization of κ Opioid-Stimulated [^{35}S]GTPγS Binding in Guinea Pig Striatal Membranes

To confirm the pharmacological specificity of the three κ agonists in stimulation of [^{35}S]GTPγS binding in guinea pig striatal membranes, agonist-stimulated [^{35}S]GTPγS binding was measured in the presence of the κ antagonist nor-BNI. The results for bremazocine and U-50488H are shown in Fig. 3. In both cases, 0.5 nM nor-BNI significantly increased the agonist EC_{50} values with no apparent change in the maximal efficacy. The calculated K_e values for nor-BNI, as obtained in these experiments, were 0.012 ± 0.004 nM vs bremazocine, and 0.056 ± 0.019 nM vs U-50488H. The effect of nor-BNI on dynorphin-stimulated [^{35}S]GTPγS binding was more complex, with a greater effect of the antagonist evident at lower concentrations (<1 μM) of dynorphin (Fig. 3).

Another test for the specificity of these drugs at κ

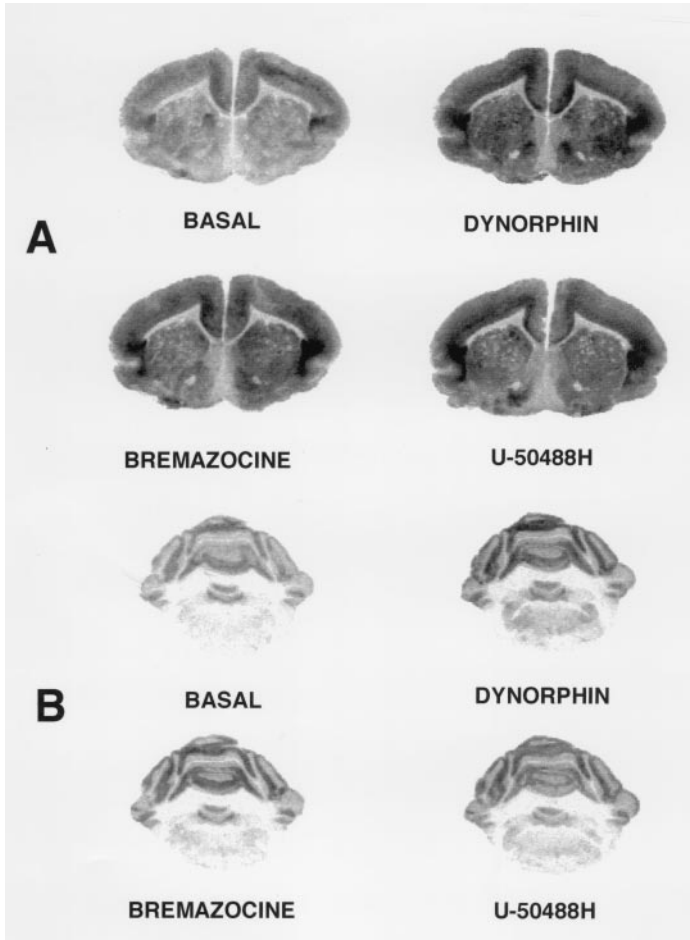

FIG. 2. Autoradiographic localization of κ opioid-stimulated [^{35}S]GTPγS binding in sections of guinea pig brain. Sections were incubated with 0.04 nM of [^{35}S]GTPγS and 2 mM of GDP, in the absence of agonist (BASAL), or with 1 μM of U-50488H, 0.1 μM of bremazocine, or 1 μM of dynorphin. (A) Coronal sections cut at the level of striatum; and (B) coronal sections cut at the level of brainstem and cerebellum.

receptors was agonist additivity experiments, where maximally effective concentrations of agonists were assayed for stimulation of [^{35}S]GTPγS binding either alone or in combination. Agonists acting at different receptors should produce an additive response, whereas no additivity would be seen if maximally effective concentrations of agonists were binding to the same receptor. Table 1 shows the results of additivity experiments in guinea pig striatal membranes, using combinations of the μ agonist DAMGO and the δ agonist *p*-Cl-DPDPE, as well as bremazocine, U-50488H, and dynorphin(1-17). These results demonstrate several points: 1) The combination of bremazocine and U-50488H was completely nonadditive (58% stimulation), because the two agonists together produced no stimulation beyond that produced by either agonist alone (50 and 57% stimulation). 2) Both bremazocine and U-50488H were additive with the δ agonist *p*-Cl-DPDPE. For example, the combination of U-50488H and *p*-Cl-DPDPE (96%) was similar to the addition of *p*-Cl-DPDPE (50%) and U-50488H (57%) alone. The combination of bremazocine and *p*-Cl-DPDPE (76%) was not quite additive, but was still significantly different from the stimulation of either agonist alone (50% for either *p*Cl-DPDPE or bremazocine). 3) The combination of U-50488H and DAMGO (87%) was also approximately additive compared with the effects of either agonist alone (57 and 40%). However, the combination of bremazocine and DAMGO (45%) was nonadditive compared with DAMGO (40%) and bremazocine (50%) alone. This anomalous result is best explained by the fact that bremazocine is also a μ antagonist [29], as demonstrated below. 4) Most agonists were nonadditive with dynorphin: combinations of other agonists together with dynorphin did not produce significantly greater stimulation than dynorphin alone. The one exceptional result was the combination of dynorphin and bremazocine, which produced significantly less stimulation (51%) than dynorphin alone (92%). This result can be explained if bremazocine is a μ antagonist and if a portion of the dynorphin response is mediated by μ receptors.

Testing the question of whether bremazocine was a μ antagonist in these assays required determination of agonist-stimulated [^{35}S]GTPγS binding in rat striatal membranes, since the activity of bremazocine as a κ agonist complicated the interpretation of the same experiments in

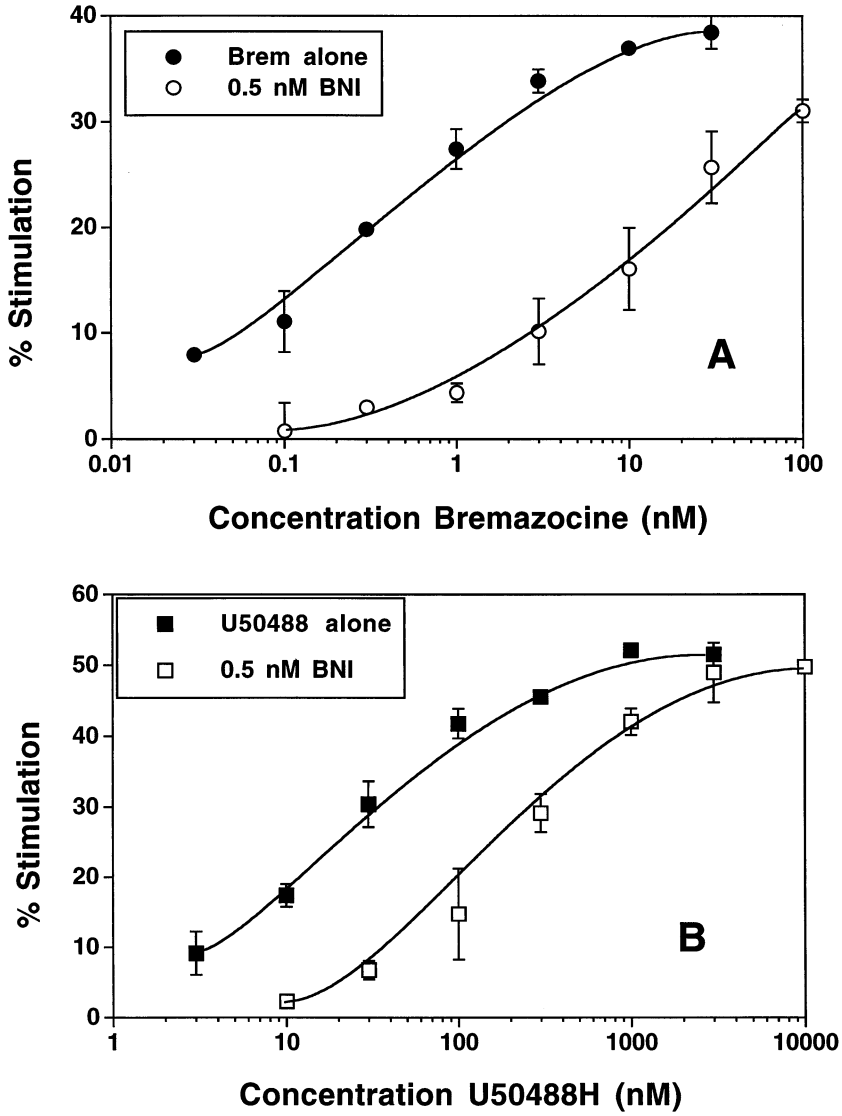

FIG. 3. Effect of nor-BNI on the stimulation of [^{35}S]GTPγS binding in guinea pig striatal membranes by bremazocine and U-50488H. Data are expressed as percent stimulation of [^{35}S]GTPγS binding, and represent mean values ± SEM from at least three separate experiments. Basal [^{35}S]GTPγS binding was 172 ± 20 fmol/mg.

guinea pig brain. Rat striatum is ideal, since it contains a high level of μ-stimulated [^{35}S]GTPγS binding, but no detectable κ-stimulated activity [20]. Figure 4 shows the effect of bremazocine on DAMGO-stimulated [^{35}S]GTPγS binding in rat striatal membranes. These results showed that bremazocine had little effect on [^{35}S]GTPγS binding alone, with maximal stimulation of [^{35}S]GTPγS binding approximately 20% of the values obtained by DAMGO alone. Addition of 10 and 100 nM of bremazocine increased the EC_{50} of DAMGO in a concentration-dependent manner, consistent with a competitive antagonist. From these data, a K_e value of 1.2 ± 0.2 nM was calculated for bremazocine antagonism of DAMGO stimulation.

DISCUSSION

The cloning of the three major classes of opioid receptors provided an important confirmation of many years of pharmacological data that suggested the existence of multiple opioid receptor types. However, the confirmation of multiple subtypes of these receptors has eluded molecular cloning techniques, and alternative hypotheses have been used to explain the pharmacological evidence for these receptor subtypes. An example is the putative κ_2 receptor. The cloned κ receptor exhibits the pharmacology of the κ_1 site [6, 7]; however, the existence of κ_2 sites has been suggested by a variety of pharmacological evidence. For example, [^{3}H]bremazocine and [^{3}H]ethylketocylazocine both bind to a sub-class of receptor that is reportedly not μ, δ, or κ_1 [30]. The interpretation of κ binding has been complex. For example, κ_2 sites have been defined as low affinity [^{3}H]ethylketocyclazocine sites [9], as β-endorphin (epsilon) sites [11], and as part of a more complex scheme involving multiple κ receptor subtypes [31]. Although the pharmacological distinction between κ_1 and κ_2 sites is not clear, most reports suggest that drugs such as U-50488H are

TABLE 1. Additivity of opioid agonists in stimulating [^{35}S]GTPγS binding in guinea pig striatal membranes

First addition	Stimulation [^{35}S]GTPγS binding (%)			
	Second addition			
	None	Bremazocine	U-50488H	Dynorphin
Bremazocine	49.9 ± 3.5			
U-50488H	56.8 ± 7.8	58.1 ± 7.3		
Dynorphin	92.1 ± 3.9	50.9 ± 3.6	110 ± 8.4	
DAMGO	39.6 ± 4.9	45.0 ± 5.4	86.9 ± 6.0*	95.4 ± 2.2
p-Cl-DPDPE	50.2 ± 4.6	76.3 ± 1.4*	95.5 ± 9.3*	114 ± 3.0*

Guinea pig striatal membranes were incubated with 0.05 nM of [^{35}S]GTPγS and 20 μM of GDP as described in Materials and Methods. Agonists were added at maximally effective concentrations either alone or in the indicated combinations; these included 0.1 μM of bremazocine, 1 μM of U-50488H, 1 μM of dynorphin, 3 μM of DAMGO, and 3 μM of p-Cl-DPDPE. Data are presented as percent stimulation of [^{35}S]GTPγS binding and represent mean values ± SEM of three experiments, each conducted in triplicate. Basal [^{35}S]GTPγS binding as 184 ± 22 fmol/mg.

*Combination effect of two agonist was significantly ($P < 0.01$) higher than the effect of either agonist alone by Student's *t*-test.

relatively selective for κ_1, whereas bremazocine binds to both κ_1 and κ_2 sites [30]. Moreover, *in vitro* autoradiography of receptor binding sites has shown that the neuroanatomical distribution of κ_2 sites in guinea pig brain is also quite distinct from that of κ_1 sites: κ_1 sites predominate in the claustrum, endopiriform nucleus, nucleus accumbens, caudate-putamen, neocortex, and cerebellum, whereas κ_2 sites are more widespread, with localization in these regions, as well as in the hippocampus, thalamus, and brainstem [9, 13].

The ability to quantify agonist efficacy *in vitro* by assaying agonist activation of G-proteins with [^{35}S]GTPγS binding provides an opportunity to test how putative receptor binding sites may be coupled to G-proteins. If κ_2 receptors couple to G-proteins in the same way as κ_1 receptors, several predictions could be made with [^{35}S]GTPγS binding in tissue that contains both κ receptor subtypes. First, bremazocine, which binds to both κ_1 and κ_2 sites, should exhibit a higher efficacy than the κ_1-selective agonist U-50488H. As shown in Fig. 1, this was not seen in guinea pig striatal membranes, where the E_{max} values of bremazocine- and U-50488H-stimulated [^{35}S]GTPγS binding were very similar. In fact, the E_{max} of bremazocine was slightly lower than that of U-50488H (see Fig. 3), consistent with reports that bremazocine may be a partial agonist at κ_1 receptors [26, 29]. A second prediction would be that the distribution of bremazocine- and U-50488H-stimulated [^{35}S]GTPγS binding should be different in guinea pig brain, and that U-50488H-stimulated activity should parallel the distribution of κ_1 ([^{3}H]U-69,593) sites, whereas bremazocine-stimulated activity would parallel a combination of κ_1 and κ_2 sites. Results of [^{35}S]GTPγS autoradiography (Fig. 2) showed that the distributions and actual levels of stimulation by both bremazocine and U-50488H were indistinguishable. More importantly, the overall distribution of G-proteins activated by either bremazocine or U-50488H closely paralleled the distribution of [^{3}H]U-69,593 binding sites in guinea pig brain, with high levels in regions including the claustrum, endopiriform nucleus, neocortex, caudate-putamen, substantia nigra, and cerebel-

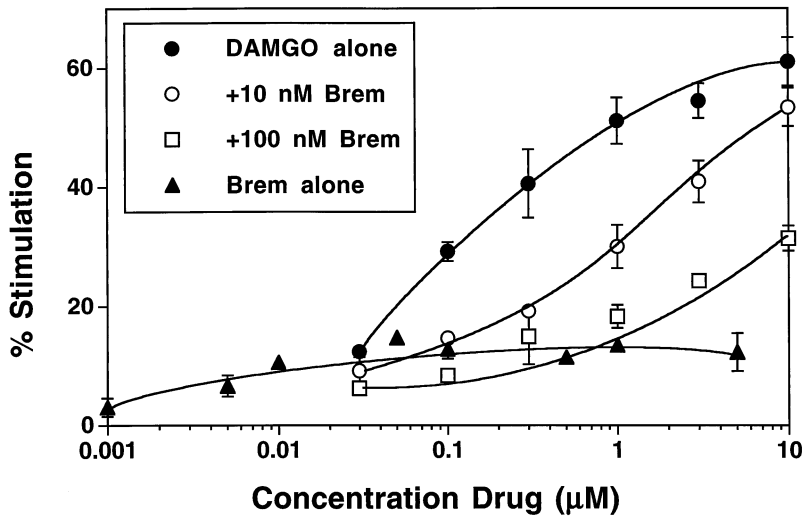

FIG. 4. Effect of bremazocine as a μ antagonist in blocking DAMGO-stimulated [^{35}S]GTPγS binding in rat striatal membranes. Membranes were incubated with the indicated concentrations of DAMGO or bremazocine alone, or with DAMGO in the presence of 10 and 100 nM of bremazocine. Data are expressed as percent stimulation of [^{35}S]GTPγS binding, and represent mean values ± SEM from at least three separate experiments. Basal [^{35}S]GTPγS binding was 160 ± 13 fmol/mg.

lum. These findings strongly suggest that both agonists are activating G-proteins through κ_1 receptors. One important caveat, however, involves the potential efficacy of bremazocine at κ_2 receptors. If bremazocine is either a low efficacy agonist or an antagonist at κ_2 receptors, then any specific stimulation of [^{35}S]GTPγS binding through κ_2 receptors may not be observed.

If κ_2 receptors are coupled to G-proteins in the same way as κ_1 receptors, a third prediction is that the κ_1 antagonist nor-BNI should exhibit differential potencies in blocking activation of G-proteins by κ_1 compared with κ_2 receptors. However, as shown in Fig. 3, the K_e values of nor-BNI against bremazocine and U-50488H were similar. These K_e values (0.012 to 0.056 nM) were similar to the reported K_e value (0.05 nM) of nor-BNI at κ receptors in guinea pig ileum [32]. There was a small difference between the K_e values for nor-BNI against bremazocine (0.012 nM) compared with U-50488H (0.056 nM), but it is not clear that this 4.5-fold difference in potency is significant. A final prediction is that maximally effective concentrations of agonists binding to different receptors should produce additive responses. The data in Table 1 strongly support the hypothesis that U-50488H and bremazocine are stimulating [^{35}S]GTPγS binding through the same receptor, since the combination of these two agonists produced no further stimulation of [^{35}S]GTPγS binding than either agonist alone. In contrast (with one exception), the addition of both drugs with μ or δ agonists produced at least some degree of additivity. The one exception was the combination of DAMGO and bremazocine, which produced the same degree of stimulation as bremazocine alone, in contrast to DAMGO and U-50488H which produced a fully additive response. This paradoxical result was explained by the fact that bremazocine is also a μ antagonist, as confirmed in rat striatum (Fig. 4), where bremazocine blocked DAMGO-stimulated [^{35}S]GTPγS binding with a K_e value of 1.2 nM. This is similar to the 1.6 nM value previously reported for bremazocine as a μ antagonist in guinea pig ileum [29]. The small amount of stimulation in [^{35}S]GTPγS binding produced in rat striatal membranes by bremazocine alone (less than 20% of the stimulation produced by DAMGO) may suggest that bremazocine may be a low efficacy partial μ agonist under these conditions, or that a small amount of κ activity may be present in these membranes. It is unlikely to be produced by bremazocine actions at δ receptors, since addition of 1 nM of naltrindole had no effect on bremazocine-stimulated [^{35}S]GTPγS binding in rat striatal membranes (data not shown).

The interpretation of the actions of dynorphin was more complex, since dynorphin was clearly acting at multiple sites. These studies suggested that the high concentrations of dynorphin used in these experiments produced stimulation of [^{35}S]GTPγS binding not only through κ receptors but also through μ and δ receptors as well. First, the efficacy of dynorphin was considerably higher than any of the specific opioid agonists alone (μ, δ, or κ). This additional component of [^{35}S]GTPγS stimulation by dynorphin is probably a combination of activity produced at μ and δ receptors, because the combination of dynorphin with DAMGO or *p*-Cl-DPDPE (Table 1) produced no greater response than that of dynorphin alone. In addition, although U-50488H had no effect on dynorphin-stimulated [^{35}S]GTPγS binding, bremazocine actually inhibited dynorphin stimulation to a level close to that produced by either κ agonist alone. This result is explained by the actions of bremazocine as a μ antagonist: in this manner, bremazocine should block the μ component of dynorphin-stimulated [^{35}S]GTPγS binding and leave only the κ and δ components. The fact that the residual dynorphin-stimulated activity remaining after the addition of bremazocine was similar to the activity of the κ agonists alone suggests that the δ component at this concentration of dynorphin was relatively small, an idea supported by the fact that dynorphin has a higher affinity at μ receptors than δ receptors [33]. These results suggest that although low concentrations of dynorphin may preferably act at κ receptors under the conditions where agonist-stimulated [^{35}S]GTPγS binding can be observed (i.e. in the presence of NaCl and GDP), the κ selectivity of this endogenous peptide is not large under these conditions.

The results of these studies strongly suggest that bremazocine and U-50488H activate G-proteins in guinea pig brain through the same κ_1 receptor type. It is clear, therefore, that these studies provide no evidence for selective κ_2-mediated G-protein activation in tissues that should have a significant level of κ_2 receptors. These findings have important implications for the proposed structure of the putative κ_2 receptor. One possibility is that the κ_2 receptor is not coupled to G-proteins, but is instead a member of a different receptor superfamily. If that is the case, it explains why no κ_2 subtype has emerged from cloning studies using homologies with μ, δ, and κ_1 receptors. Another possibility is that κ_2 receptors may be coupled to different types of G-proteins compared with κ_1 receptors. In brain, $G_{i/o}$ proteins predominate, and therefore the signal for agonist-stimulated [^{35}S]GTPγS binding is greater for receptors coupled to these types of G-proteins. The κ receptors have been reported to couple to other G-protein α subunits [34, 35]. Therefore, if κ_2 receptors were coupled to G_q, G_z, or other G-protein classes, the agonist-induced stimulation of [^{35}S]GTPγS binding might be too small to observe in brain membranes. If this explanation were correct, the κ_2 receptor would still have the same seven-transmembrane structure as other G-protein-coupled receptors, but the homology with the other three opioid receptor types might be lower than originally expected. In either case, these experiments suggest that the structure and function of putative κ_2 sites are likely to be quite different from the opioid receptor types cloned thus far.

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