

# Characterization of CB<sub>1</sub> Cannabinoid Receptors Using Receptor Peptide Fragments and Site-Directed Antibodies

ALLYN C. HOWLETT, CHAO SONG,<sup>1</sup> BARBARA A. BERGLUND, GERALD H. WILKEN, and J. JILL PIGG

Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri 63104

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## ABSTRACT

The mechanism by which CB<sub>1</sub> cannabinoid receptors are coupled to the G<sub>i</sub>/G<sub>o</sub> class of G proteins was studied. A peptide representing the juxtamembrane carboxyl terminus robustly stimulated guanosine-5'-O-(3-thio)triphosphate binding. Peptides simulating subdomains of the third intracellular loop (IL3) activated minimally when present alone but produced additive effects when present in combination. Peptides representing the amino-side IL3 and the juxtamembrane carboxyl terminus autonomously inhibited adenylate cyclase, and this response was not significantly augmented or inhibited by peptides representing other intracellular domains. Site-directed antipeptide anti-

bodies developed against the domains of the amino terminus, first extracellular loop, amino-side IL3, and juxtamembrane carboxyl terminus of CB<sub>1</sub> receptors failed to influence binding of [<sup>3</sup>H]CP-55940. However, IgG raised against the amino-side IL3 diminished the agonist-dependent inhibition of adenylate cyclase. These experiments suggest that the juxtamembrane carboxyl terminus is critical for G protein activation by CB<sub>1</sub> cannabinoid receptors and that the amino-side IL3 also may interact with G<sub>i</sub> proteins leading to inhibition of adenylate cyclase.

Δ<sup>9</sup>-Tetrahydrocannabinol, the active compound in *Cannabis sativa*, and synthetic cannabimimetic compounds of cannabinoid, aminoalkylindole, and eicosanoid classes interact with cannabinoid receptors in the nervous system to produce their effects (for a review, see Howlett, 1995a). Stimulation of CB<sub>1</sub> cannabinoid receptors results in the pertussis toxin-sensitive inhibition of forskolin- or receptor-stimulated adenylate cyclase activity and regulation of ion channels, indicating that the cannabinoid receptor is coupled to G<sub>i</sub> and G<sub>o</sub> proteins (for a review, see Howlett, 1995b). The sequence of CB<sub>1</sub> cannabinoid receptors has been deduced for both rat and human species (Matsuda *et al.*, 1990; Gerard *et al.*, 1991).

In the current study, we sought to determine topographic regions of the CB<sub>1</sub> cannabinoid receptor that would be critical regions for agonist-induced activity. Peptides representing regions of the IL3 or the juxtamembrane carboxyl terminus were tested for their ability to compete for receptor/G protein interactions or to act autonomously to stimulate G proteins. Site-directed antibodies were generated against synthetic peptides representing the amino terminus, EL1, amino-side segment of the IL3, and juxtamembrane carboxyl-terminal

regions of the rat brain CB<sub>1</sub> receptors. These antisera were tested for their ability to occlude ligand binding to the receptor or coupling of the receptor to G proteins in membrane preparations. Based on the findings of the peptide and antibody interactions, it was concluded that the juxtamembrane carboxyl terminus is critical for G protein activation by CB<sub>1</sub> cannabinoid receptors and that the amino-side IL3 also may interact with G<sub>i</sub> proteins leading to inhibition of adenylate cyclase.

## Materials and Methods

**Peptides.** Peptides (Table 1) CB1–14, CB174–188, CB301–317, and CB401–417 were synthesized using *t*-butyloxycarbonyl chemistry and purified by C-18 reverse-phase high performance liquid chromatography eluted with a gradient extending over 35 min from 0.1% trifluoroacetic acid to 80% acetonitrile plus 0.085% trifluoroacetic acid. Peptides CB316–327, CB329–344, and CB401–417 were synthesized by 9-fluorenylmethoxycarbonyl chemistry and purified by C-18 reverse-phase high performance liquid chromatography (Princeton Biomolecules, Columbus, OH). The amino acid sequence of peptide CB401–417 was verified by amino acid sequence analysis using automated Edman degradation and gas chromatography/mass spectrometry. There is 100% identity in the amino acid sequences of these six peptides between rat and human CB<sub>1</sub> receptors (Matsuda *et al.*, 1990; Gerard *et al.*, 1991). CB1–14 is 64 amino acids proximal to the first of three potential amino-linked glycosylation sites on the

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<sup>1</sup> Current affiliation: Department of Surgery, Beth Israel Deaconess Hospital, Harvard Medical School, Boston, MA 02115.

**ABBREVIATIONS:** IL3, third intracellular loop of the G protein-coupled receptor; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; DALN, desacetyllevonantradol; EL1, first extracellular loop of the G protein-coupled receptor; ELISA, enzyme-linked immunosorbent assay; GTPγS, guanosine-5'-O-(3-thio)triphosphate; KLH, keyhole limpet hemocyanin.

TABLE 1

Rat brain cannabinoid receptor peptide fragments and corresponding antisera

The peptide numbers correspond to inclusive amino acid residues of each sequence of the CB<sub>1</sub> cannabinoid receptor as reported by Matsuda *et al.* (1990). Amino acids in parentheses were added to peptide sequences to facilitate coupling peptides to protein carriers. For peptide CB401–417, the amino-terminal lysine added for coupling was not present in the peptide used for biochemical studies. The underlined amino acid replaced a cysteine in the sequence.

Peptide	Amino acid sequence	Location	Antisera
CB1–14	MKSILDGLADTTFR(C)	Amino terminus	Anti-CB1–14
CB174–188	(K)SFVDFHVFHRKDSPN	EL1	Anti-CB174–188
CB301–317	KAHSHAVRMIQRGTQKS	N-side IL3	Anti-CB301–317
CB316–327	KSIIHTSEDGK	Central IL3	
CB329–344	QVTRPDQARMDIRLAK	C-side IL3	
CB401–417	(K)RSKDLR <sup>H</sup> AFRSMF <u>PP</u> SS <u>E</u>	Carboxyl terminus	Anti-CB401–417

amino terminus, and therefore the epitope on the receptor is not likely to be obscured by the bulky oligosaccharides. The peptides have poor homology (0–30%) with related domains of the CB<sub>2</sub> receptor, with the exception of CB174–188 (EL1), which exhibits 64.3% identity and 5 consecutive amino acids.

**Antibodies and immunoblots.** Peptide CB1–14, modified by a cysteine at the carboxyl terminus (CB1–14), was conjugated at a 40-fold molar excess to sulfo-SMCC-KLH (Pierce Chemical, Rockford, IL). CB301–317 and lysine-modified CB174–188 and CB401–417 were conjugated to KLH in an 8–40-fold molar excess of peptides using glutaraldehyde (Song and Howlett, 1995). In CB401–417, Cys416 was replaced with serine to avoid potential artifactual disulfide bond formation. New Zealand White rabbits were injected intradermally with peptide-KLH conjugates (50 µg/rabbit), and antisera were screened by ELISA using peptide-BSA conjugates on Immulon 2 plates (Dynatech, Chantilly, VA). Plates were incubated and washed sequentially with antisera, goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce) (1:6,000), and 2,2-azino-di-(3-ethylbenzthiazoline)-6 sulfonate (Pierce) in 2.5 mM H<sub>2</sub>O<sub>2</sub>, 0.1% Tween 20, and 100 mM sodium acetate, pH 4.2, and the absorbance at 405 nm was determined with a Molecular Devices (Menlo Park, CA) plate reader. By ELISA, all antisera recognized their respective immunization peptides in the peptide-BSA conjugates but not unconjugated BSA or alternative peptide conjugates; the corresponding preimmune sera showed no significant reaction. Titers achieved were anti-CB1–14, 1:60,000; anti-CB174–188, 1:40,000; anti-CB301–317, 1:8,000; and anti-CB401–417, 1:2,300. Low-titer antisera may have been the result of a low molar ratio of peptides to KLH, which was limited by significant precipitation of conjugates having high molar ratios. IgG fractions for anti-CB301–317 and anti-CB401–417, and their preimmune sera were obtained by incubation with Protein A agarose (1 hr at 23°) according to the Pierce kit instructions. Protein concentrations were measured by absorbance at 280 nm.

**Membrane preparation and signal transduction assays.** Rat brain membranes were prepared from entire rat brain minus the brainstem as described previously (Devane *et al.*, 1988) with the addition of a protease inhibitor cocktail to the buffers (15 µg/ml benzamide, 5 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.7 µg/ml pepstatin). N18TG2 cell membranes were prepared as described previously (Howlett, 1985).

Radioligand binding assay of the CB<sub>1</sub> cannabinoid receptor was performed and analyzed using [<sup>3</sup>H]CP-55940 as described previously (Pinto *et al.*, 1994), with specific binding defined as [<sup>3</sup>H]CP-55940 binding that was displaced by 100 nM DALN.

Adenylate cyclase assays were performed as described previously using N18TG2 neuroblastoma cell membranes (Howlett, 1985). Secretin (600 nM) was present as the hormonal stimulator, and Ro20–1724 (100 µM) was used as the phosphodiesterase inhibitor. Final concentrations of fatty acid-free BSA present in these assay mixtures were 0.16–0.21 mg/ml. The hormone-stimulated enzyme activities from this set of studies were 66.4 ± 3.67 (mean ± standard error, 13 experiments) pmol/min/mg of protein.

[<sup>35</sup>S]GTPγS binding to rat brain membrane G proteins was performed according to a modification of the procedure of Lorenzen *et al.*

(1993). Rat brain P2 membranes (5 µg of protein) were added to a reaction mixture containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM dithiothreitol, 10 µM GDP, 0.375 nM [<sup>35</sup>S]GTPγS, and the indicated concentrations of peptides or cannabinoid ligands (100 µl final volume). Binding of [<sup>35</sup>S]GTPγS (30° for 60 min) was stopped by rapid filtration over GF/B (Whatman, Clifton, NJ) filters using a TOMTEC (Orange, CT) Harvester, and radioactivity was quantified using a 1205 Betaplate counter (Wallac, Gaithersburg, MD). Nonspecific binding was calculated as binding that could not be displaced by 100 µM GTPγS. Data were calculated as pmol of [<sup>35</sup>S]GTPγS bound/mg of protein from an average of four replicates. Background values averaged 623 ± 68 fmol/mg (mean ± standard error, six experiments), and values stimulated by 1 µM DALN averaged 1150 ± 100 fmol/mg (six experiments).

## Results

### Determination of Functional Domains on Rat Brain Cannabinoid Receptors

**Agonist binding sites.** All study antisera specifically recognized a band with an apparent molecular mass of 64 ± 1.0 kDa (13 experiments), whereas preimmune sera did not (Song and Howlett, 1995; data not shown). The 64-kDa band is believed to be CB<sub>1</sub> cannabinoid receptors with carbohydrates at two glycosylation sites (Song and Howlett, 1995). To determine whether the domains of the receptor occluded by the antibodies overlap regions important for agonist ligand binding, rat brain membrane preparations were preincubated with each of the four antisera, and specific binding of [<sup>3</sup>H]CP-55940 was determined. Compared with the effects of cognate preimmune sera, none of the antisera interfered with ligand binding to rat brain cannabinoid receptors (Table 2). The more concentrated antisera and preimmune sera (1:20 dilution) both attenuated the specific binding of [<sup>3</sup>H]CP-55940, probably as the result of an interaction of serum lipoproteins and albumin with radioligand. Serum lipoprotein and albumin interactions with cannabinoid ligands have been reported previously (Wahlqvist *et al.*, 1970; Widman *et al.*, 1974; Poddar *et al.*, 1988) at concentrations equal to or greater than present in the assay mixture (1.2 mg of protein/ml for 1:20, 4.8 mg of protein/ml for 1:5 in Table 3).

**Sites coupling to G proteins.** Receptor peptide fragments and site-directed antibodies were used to investigate domains of the CB<sub>1</sub> receptor important for receptor/G protein interaction. None of the receptor peptide fragments (at 100 µM) was able to compete for DALN-induced receptor/G protein interactions as determined in either [<sup>35</sup>S]GTPγS binding or adenylate cyclase assays (see Figs. 3 and 6). To determine whether receptor peptide fragments could autonomously activate G<sub>i</sub> and G<sub>o</sub>, these peptides were added with membranes directly into the incubation mixture in the absence of a stim-

TABLE 2

The effect of site-directed antisera on agonist ligand binding to rat brain cannabinoid receptors

Rat brain P2 membrane preparations were incubated with no sera, antisera, or preimmune sera at 4° for 4 hr before the addition (without washing) to the ligand binding reaction mixtures at 0.4 times the final assay volume. Under the conditions of the assay, the specific binding activity of 110 pM [<sup>3</sup>H]CP-55940 in the absence of serum was 56.2 ± 4.3 and 61.2 ± 0.6 fmol/mg of protein for experiments A and B, respectively. These values represented ~70% of the total binding at this concentration of radioligand and membrane fraction. The data shown are the mean ± standard error from two experiments, each with triplicate determinations. There was no significant difference (*p* < 0.05) when the effect of antisera was compared with that of preimmune sera using a paired Student's *t* test.

Antibody	Serum dilution	Specific binding	
		Percent of total binding	Normalized to control
%			
Experiment A			
None		69.9 ± 0.6	100.0
Anti-CB1-14	1:100	65.4 ± 0.5	93.5
	1:20	60.5 ± 0.3	86.5
Preimmune CB1-14	1:100	74.7 ± 2.1	106.8
	1:20	62.3 ± 1.5	89.1
Anti-CB174-188	1:100	72.8 ± 3.2	104.1
	1:20	64.2 ± 4.1	91.8
Preimmune CB174-188	1:100	72.0 ± 4.2	103.0
	1:20	60.7 ± 0.6	86.8
Experiment B			
None		68.2 ± 2.5	100.0
Anti-CB301-317	1:100	62.8 ± 4.2	92.1
	1:20	44.1 ± 6.7	64.6
Preimmune-CB301-317	1:100	67.8 ± 6.5	99.4
	1:20	45.1 ± 4.9	66.1
Anti-CB401-417	1:100	62.8 ± 4.2	92.1
	1:20	44.3 ± 3.3	64.9
Preimmune-CB401-417	1:100	70.4 ± 0.8	103.2
	1:20	51.9 ± 3.2	76.1

ulatory cannabimimetic agonist, and signal transduction activity was determined. Fig. 1 shows peptides tested for their ability to stimulate [<sup>35</sup>S]GTPγS binding to G proteins in rat brain membrane preparations. The peptides representing the extracellular amino terminus and EL1 produced a background response in the assays. This was expected because these peptides represent domains of the receptor that would interact with neither the ligand nor the intracellular effector proteins. CB401-417 promoted a robust stimulation of [<sup>35</sup>S]GTPγS binding to G proteins. When tested individually, CB301-317, CB316-327, and CB329-344, components of IL3, produced only background stimulation of [<sup>35</sup>S]GTPγS binding. However, when present in combination, peptides representing the three segments of IL3 seemed to produce an additive stimulation (Fig. 2). This response was not simply due to the presence of a 3-fold greater concentration of peptide because a 300 μM concentration of either of the peptides alone produced responses that were not different from responses to the 100 μM concentrations (data not shown). These findings could indicate the necessity for some conformational interaction of all three subdomains to select for and activate G proteins. Alternatively, each subdomain might have the ability to select and activate one of multiple G protein subtypes, and the additivity represents multiple populations of G proteins. Combinations of IL3 component peptides with CB401-417 failed to significantly augment or inhibit the [<sup>35</sup>S]GTPγS binding promoted by CB401-417 alone. Thus, activation by the juxtamembrane carboxyl-terminal domain seems to be the dominant influence on G protein activation, but the regions comprising IL3 also seem to exert a regulatory influence.

TABLE 3

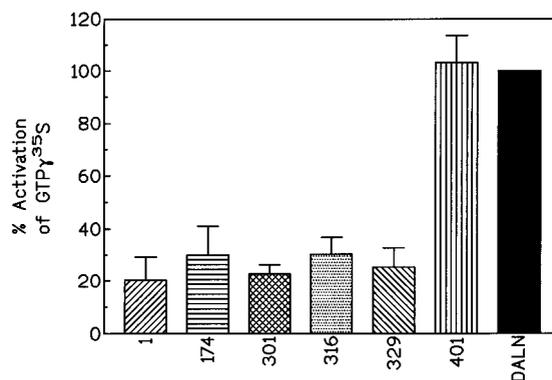
The effects of site-directed antisera on DALN-induced inhibition of adenylate cyclase

N18TG2 cell membrane preparations were preincubated with diluted preimmune sera or antisera at 4° for 4 hr before the addition to the adenylate cyclase assay mixture. Data were normalized for each experiment so the inhibition by 100 nM DALN in the absence of either antiserum or preimmune serum was 100% inhibition. Data are expressed as the mean ± standard error of two to four experiments, each with three determinations.

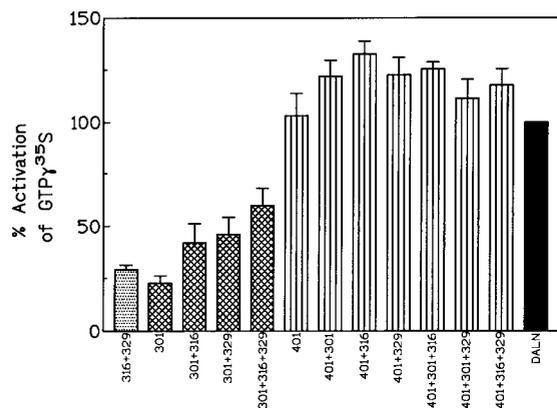
	Antisera dilution	Adenylate cyclase inhibition	Adenylate cyclase inhibition normalized to control
			%
Experiment A			
None		32.2 ± 4.1	100.0
Anti-CB1-14 ( <i>n</i> = 2)	1:100	29.0 ± 7.7	90.1
	1:20	29.7 ± 4.5	92.3
	1:5	19.4 ± 5.6	60.3
Preimmune CB1-14	1:100	30.3 ± 4.0	94.1
	1:20	28.6 ± 6.9	88.8
	1:5	16.9 ± 5.0	52.5
Anti-CB174-188 ( <i>n</i> = 2)	1:100	33.7 ± 2.6	104.6
	1:20	27.7 ± 9.6	86.0
	1:5	7.5 ± 4.9	23.3
Preimmune CB174-188	1:100	35.9 ± 5.4	111.5
	1:20	25.0 ± 3.4	77.6
	1:5	6.1 ± 2.3	18.9
Experiment B			
None		33.2 ± 1.2	100.0
Anti-CB301-317 ( <i>n</i> = 4)	1:100	32.1 ± 5.2	96.7
	1:20	14.3 ± 10.7	43.1
	1:5	7.1 ± 3.7 <sup>a</sup>	21.2
Preimmune CB301-317	1:100	32.9 ± 5.9	99.1
	1:20	23.7 ± 3.1	71.4
	1:5	21.1 ± 9.3	63.5
Anti-CB401-417 ( <i>n</i> = 3)	1:100	34.8 ± 1.4	107.4
	1:20	29.4 ± 1.5	90.7
	1:5	10.8 ± 4.5	33.3
Preimmune CB401-417	1:100	32.4 ± 2.6	100.6
	1:20	26.9 ± 2.1	83.0
	1:5	12.2 ± 4.5	37.6

<sup>a</sup> Significantly lower than preimmune serum control at *p* < 0.05 using paired Student's one-tailed *t* test.

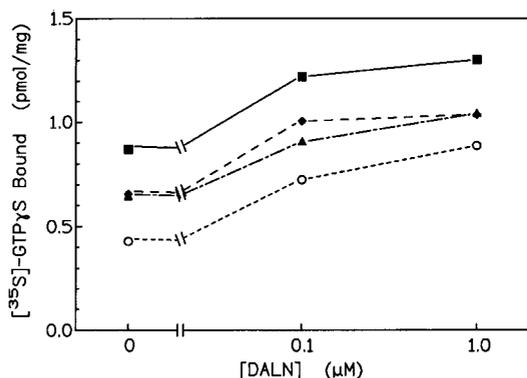
The response to peptides was additive with agonist-dependent stimulation by DALN (Fig. 3). This might suggest that the agonist/CB<sub>1</sub> receptor complex is a limiting factor in G protein stimulation in rat brain homogenates. An explana-



**Fig. 1.** Effect of CB<sub>1</sub> receptor peptide fragments on [<sup>35</sup>S]GTPγS binding to rat brain membrane G proteins. Peptides (100 μM) were included in the incubation mixture without pretreatment, and accumulation of [<sup>35</sup>S]GTPγS was determined. Data are presented as activation promoted by the peptide expressed as a percentage of the response to 1 μM DALN (i.e., stimulated by agonist/receptor complex), where 0% is the [<sup>35</sup>S]GTPγS binding in the absence of hormonal or peptide stimulators. Error bars, standard error from three to five experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test, and no significant differences (*p* < 0.05) were noted between peptides except for CB401-417, which differed from all others.



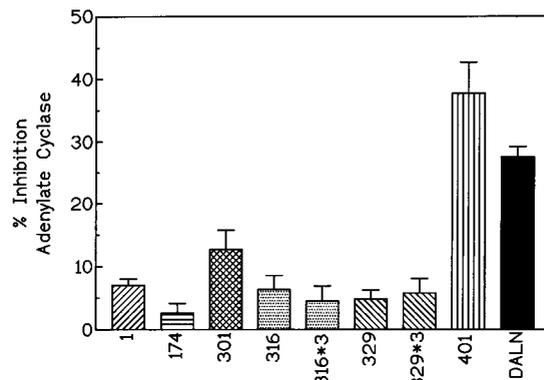
**Fig. 2.** Effects of combinations of CB<sub>1</sub> receptor peptide fragments on [<sup>35</sup>S]GTP $\gamma$ S binding to rat brain membrane G proteins. Peptides (100  $\mu$ M) were included in the incubation mixture in the indicated combinations and are presented as percentage of the response to 1  $\mu$ M DALN (mean  $\pm$  standard error) from three to five experiments for each peptide combination. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. Peptide combinations containing CB301–317 (▨) were not different from each other except for the combination of CB301–317 plus CB316–327 plus CB329–344, which differed from CB301–317 and from CB316–327 plus CB329–344 ( $p < 0.05$ ). Peptide combinations containing CB401–417 (▩) were not different from each other at the ( $p < 0.05$ ) level of significance.



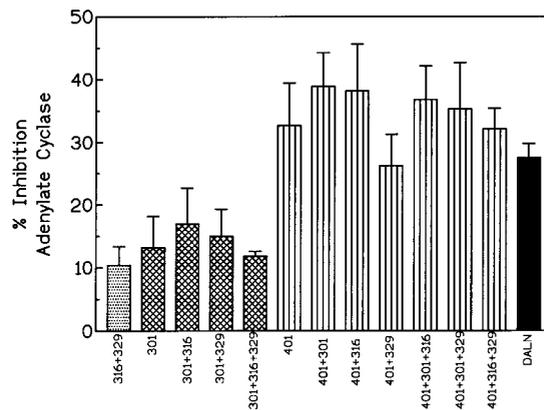
**Fig. 3.** Additivity of responses to peptides with that of the cannabinoid agonist-stimulated receptor. DALN was absent or present at the indicated concentrations in the presence of vehicle (○) or 100  $\mu$ M CB316–327 (▲), CB329–344 (◆), or CB401–417 (■). Data are expressed as pmol of [<sup>35</sup>S]GTP $\gamma$ S/mg of protein specifically bound. This is a single representative experiment.

tion for the additivity could be that the peptide fragments were activating G proteins that were not associated with agonist/receptor complexes. One could hypothesize that these G proteins would be of the G<sub>i/o</sub> class; however, the activation of other G protein classes cannot be dismissed.

Cannabinoid receptor-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in rat brain homogenates would be likely to represent predominantly G<sub>o</sub> (Garibay *et al.*, 1991). To determine whether peptide fragments of the CB<sub>1</sub> receptor could autonomously activate G<sub>i</sub>, these peptides were added directly to the adenylate cyclase incubation mixture, and enzyme activity was measured in the absence of an agonist (Fig. 4). The peptides representing the extracellular amino terminus and EL1 produced a minimal response in the adenylate cyclase assays. The juxtamembrane carboxyl terminus robustly promoted inhibition of adenylate cyclase, with an efficacy that generally exceeded that of cannabinoid agonist/receptor-stimulated activity. The amino-side IL3 also inhibited adenylate



**Fig. 4.** Effect of CB<sub>1</sub> receptor peptide fragments on inhibition of adenylate cyclase activity. Peptides (100  $\mu$ M, except \*3 denotes 300  $\mu$ M) are denoted by the number corresponding to the first amino acid of the peptide in the rat CB<sub>1</sub> receptor sequence (see Table 1). Data are presented as the percentage inhibition promoted by the presence of peptide (mean  $\pm$  standard error) from three to seven experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. CB401–417 was significantly different from all other peptides ( $p < 0.05$ ).



**Fig. 5.** Effects of combinations of CB<sub>1</sub> receptor peptide fragments on inhibition of adenylate cyclase activity. Peptides (100  $\mu$ M) were included in the incubation mixture in the indicated combinations and are presented as the percentage inhibition promoted by the presence of peptides (mean  $\pm$  standard error) from three to six experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. Peptide combinations within the groups containing CB301–317 (▨) or CB401–417 (▩) were not different from each other ( $p < 0.05$ ).

cyclase, but the extent was limited to only ~40% of that promoted by the agonist/receptor complex. The magnitude of the response was not increased when 300  $\mu$ M peptide was used (data not shown). The peptide fragments representing the central IL3 and carboxyl-side IL3, even at 300  $\mu$ M, failed to evoke a response greater than those of the two extracellular domain fragments.

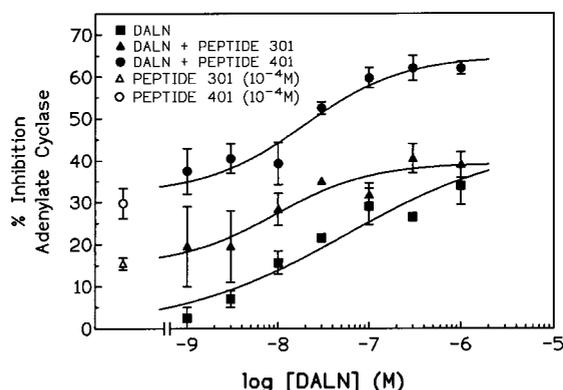
Combinations of peptides were tested to determine whether discrete CB<sub>1</sub> receptor domains could interact with each other to promote G<sub>i</sub> activation. No synergism was observed on the addition of peptides representing the central and/or carboxyl-side regions of IL3 to the amino-side IL3 peptide (Fig. 5). Peptide combinations comprising IL3 failed to significantly alter the response to the juxtamembrane carboxyl-terminal domain peptide. Thus, the dominant activating effect of the amino-side carboxyl-terminal domain was neither attenuated nor augmented by the presence of any of the subdomains of IL3.

The cannabinoid agonist DALN inhibited adenylate cy-

class activity with an  $EC_{50}$  value of 31 nM (confidence interval, 16–64 nM) and an asymptotic approach to a maximal inhibition of 40% of the hormone-stimulated activity in these experiments. Peptide CB301–317 alone inhibited adenylate cyclase activity by 15% and produced a less-than-additive response with increasing concentrations of DALN (Fig. 6). In the presence of peptide CB301–317, the  $EC_{50}$  value for DALN was 9.5 nM (confidence interval, 2–47 nM) and approached a maximal inhibition of 40% of the hormone-stimulated activity. Thus, there seemed to be a limitation in the amount of  $G_i$  activated in the presence of this peptide, and the response to agonist/receptor complex seemed to supersede that of the receptor fragment.

Peptide CB401–417 alone produced a robust inhibition of adenylate cyclase (32%) at 100  $\mu$ M, a concentration that produced the maximum response (Mukhopadhyah S and Howlett AC, unpublished observations). This concentration of peptide CB401–417 produced additive effects with the agonist/receptor complex throughout the entire dose-response range for the agonist. In the presence of CB401–417, the  $EC_{50}$  value for DALN was 19 nM (confidence interval, 4–90 nM) and approached a maximal inhibition of 65% of the hormone-stimulated activity. Analysis of the curves indicates that the small decreases in  $EC_{50}$  values in the presence of peptides are not statistically significant. The increase in efficacy produced by peptide CB401–417 suggests a greater number of  $G_i$  proteins are activated in the presence of the receptor peptide fragment than are activated by the agonist/receptor complex alone. This suggests that the population of  $G_i$  proteins available for interaction with the peptide is distinct from the population of  $G_i$  proteins coupled to  $CB_1$  receptors. The observations that the actions of hormone-stimulated receptor and peptide CB401–417 are neither synergistic nor competitive, and that the peptide did not alter the affinity of the hormone for the receptor suggests the peptide does not affect receptor/G protein coupling.

Agonist-induced inhibition of adenylate cyclase was assayed in N18TG2 membranes that had been preincubated with each of the four site-directed antisera or preimmune sera. Anti-CB1–14 and anti-CB174–188, antibodies that would occlude the amino terminus and EL1 of  $CB_1$  receptors, each failed to alter the DALN-induced inhibition compared

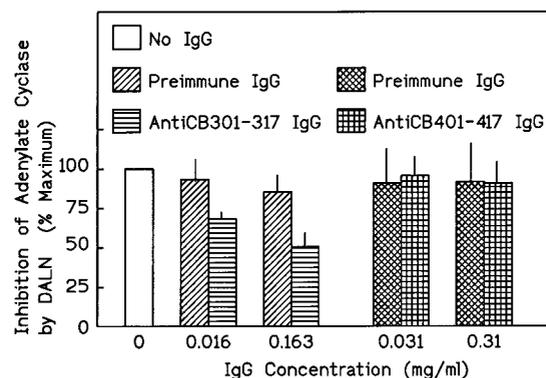


**Fig. 6.** Additivity between activating peptides and agonist/receptor complex for  $G_i$  activation. Peptides CB301–317 and CB401–417 (100  $\mu$ M) were included in the incubation mixture in the presence or absence of increasing concentrations of the cannabinoid agonist DALN. Data are mean  $\pm$  standard error from three experiments, and the curves were analyzed using GraphPAD InPlot.

with the effect of the cognate preimmune sera (Table 3). Preincubation of membranes with antiserum against IL3 (anti-CB301–317) resulted in a dose-dependent attenuation of the DALN-induced inhibition of adenylate cyclase compared with the effect of cognate preimmune serum. The antiserum against the amino-side carboxyl terminus (anti-CB401–417) did not inhibit the effect of DALN on adenylate cyclase. The influence of the preimmune sera at low dilutions suggests interference that might be eliminated by isolating the IgG fraction by Protein A chromatography. The normalized DALN-induced inhibition of adenylate cyclase in the presence of IgG fractions of anti-CB301–317 versus cognate preimmune serum was 68.4% versus 93.2% at a low concentration of IgG (0.016 mg/ml) and 50.5% versus 85.5% at a high concentration (0.163 mg/ml) (Fig. 7). The IgG of anti-CB401–417, against the amino-side carboxyl terminus, failed to block the DALN response (Fig. 7). These data suggest that the  $CB_1$  receptor IL3 may be involved in  $G_i$ /receptor coupling. However, the lack of response to the IgG of anti-CB401–417 cannot necessarily be interpreted to mean that this region fails to be involved in  $G_i$ /receptor coupling. The lack of response may simply be due to a poor affinity that the antibody may have for the receptor, resulting in the inability of the antibody to compete with a receptor/G protein interaction with relatively greater affinity.

## Discussion

In the current report, we describe peptide fragments of the  $CB_1$  cannabinoid receptor and four site-directed anti-peptide antisera against  $CB_1$  receptors and the use of these tools to study functional domains of brain cannabinoid receptors. An important finding from these studies is that the juxtamembrane carboxyl-terminal domain peptide of the  $CB_1$  cannabinoid receptor is a highly effective activator of  $G_{i/o}$  proteins. Interaction of this receptor domain with G proteins has been reported for several other G protein-coupled receptors, including rhodopsin coupling to transducin as indicated by studies using synthetic peptides (Takemoto *et al.*, 1985; Phillips and Cerione, 1994) and alanine scanning mutagenesis (Osawa and Weiss, 1994). The amino-side carboxyl-terminal



**Fig. 7.** DALN-induced inhibition of adenylate cyclase in the presence of IgG fractions of anti-CB301–317 and anti-CB401–417. The IgG fractions derived from the two antisera or their respective preimmune sera were preincubated with the N18TG2 cell membrane at 4° for 4 hr before direct addition to the adenylate cyclase assay mixture at 0.4 times the final assay volume. The inhibition of cAMP synthesis by DALN in the absence of any serum was 44%, and this value was normalized to be 100%. Error bars, mean  $\pm$  standard error from triplicate determinations. Data shown are representative of four experiments.

region seemed to be important for  $\beta_2$ -adrenergic receptor coupling to G proteins in mutation studies (O'Dowd *et al.*, 1988). A juxtamembrane carboxyl-terminal peptide from the N-formyl peptide receptor interacted with a carboxyl-terminal region of  $\alpha_i$  in ELISA studies, and that peptide also disrupted the receptor/G protein complex in detergent solution (Schreiber *et al.*, 1994). A major difference between the results reported here for the CB<sub>1</sub> receptor compared with other G protein-coupled receptors is that activation of a G protein by the carboxyl terminus has not yet been reported for other receptor types.

The CB<sub>1</sub> receptor proximal amino-side IL3 peptide is able to stimulate G<sub>i</sub> activation, leading to inhibition of adenylate cyclase; however, this peptide does not exhibit as great an efficacy as the juxtamembrane carboxyl-terminal peptide. Another difference between the actions of these two peptide segments is that the amino-side IL3 peptide alone does not promote the activation of G proteins in brain membranes (predominantly G<sub>o</sub>). Perhaps the IL3 receptor region facilitates G protein activation secondarily to or only in combination with a more effective stimulus.

For a number of G protein-coupled receptors, the carboxyl-side IL3 domain has been proposed to serve as a G protein recognition or coupling region (Dixon *et al.*, 1987; O'Dowd *et al.*, 1988; Wade *et al.*, 1994; Yamada *et al.*, 1994; Liu *et al.*, 1995; Shi *et al.*, 1995), although the data presented here do not support this role for the carboxyl-side IL3 of the CB<sub>1</sub> receptor. Evidence exists for a role for the carboxyl-side IL3 domain of G protein-coupled receptors in suppressing or restricting the G protein interaction in that point mutations in the carboxyl-side IL3 domain of the  $\alpha_{1B}$ -adrenergic receptor (Cotecchia *et al.*, 1990; Ren *et al.*, 1993),  $\alpha_{2A}$ -adrenergic receptor (Ren *et al.*, 1993), and muscarinic acetylcholine (Hogger *et al.*, 1995) receptors result in constitutively active receptors. When the carboxyl-side IL3 peptide of the CB<sub>1</sub> receptor was included in combination with the peptides that activate G<sub>i</sub> and G<sub>o</sub>, it failed to suppress activation. This evidence argues against a model in which this domain of the CB<sub>1</sub> receptor would suppress activity by associating with and thereby occluding a domain that activates G proteins.

For the CB<sub>1</sub> receptor, the combination of subdomains of IL3 seems to be necessary to observe activation of G proteins in rat brain membranes. This could result from a summation of the activation of multiple G protein subtypes, each activated by a different receptor domain. Alternatively, a G protein may possess multiple sites of interaction with the receptor, and the combination of peptides may trigger recognition and activation at these distinct sites. The finding that multiple patches on the receptor facilitate interaction with the G protein has been reported for other G protein-coupled receptors, including the IL2 and IL3 regions of the muscarinic acetylcholine receptor in chimeric mutant studies (Wong *et al.*, 1990); IL2, IL3, and a region of the carboxyl terminus distal to the cysteine-palmitate anchor of rhodopsin in studies of peptides and peptide-directed antisera (Weiss *et al.*, 1988; Konig *et al.*, 1989); IL2, amino-side IL3, and amino-side IL4 of the  $\beta$ -adrenergic receptor in peptide studies (Munch *et al.*, 1991); and amino- and carboxyl-side IL3 of the  $\alpha_2$ -adrenergic receptor in studies of homodimers and heterodimers of peptides (Dalman and Neubig, 1991; Wade *et al.*, 1994). Recognition sites on  $\alpha$ - and  $\beta_2$ -adrenergic and muscarinic

acetylcholine receptors have been demonstrated that promote coupling but fail to activate G proteins autonomously (Hausdorff *et al.*, 1990; Luttrell *et al.*, 1993; Hawes *et al.*, 1994). For the  $\beta_2$ -adrenergic receptor, deletion mutation studies implicate a seven-amino acid segment in the carboxyl-side IL3 in transmitting the agonist-induced stimulatory signal to G<sub>s</sub> and that this region is distinct from domains that function to promote the ternary complex (Hausdorff *et al.*, 1990). Peptides from the amino-side IL3 and carboxyl-side IL3 of the  $\beta_2$ -adrenergic receptor activate G<sub>s</sub> in phospholipid vesicle GTPase and GTP $\gamma$ S binding assays (Cheung *et al.*, 1991). For the  $\alpha_2$ -adrenergic receptor, IL2 or the carboxyl-side IL3 peptides reduced high affinity agonist binding, but only the latter could stimulate GTPase activity (Dalman and Neubig, 1991). These findings favor a mode of cooperativity between different domains of G protein-coupled receptors to activate G proteins.

The CB<sub>1</sub> carboxyl-terminal peptide activates G proteins in a manner that is additive with that of cannabinoid receptor-stimulated G protein activation. The CB<sub>1</sub> receptor/G protein complex seems to be stable in the absence of agonist in CHAPS-solubilized preparations (Houston and Howlett, 1993). The peptides seem to be activating a population of G proteins that are not coupled to the cannabinoid receptor. This differs from observations made for several other G protein-coupled receptors for which peptides compete for the receptor/G protein interaction (Konig *et al.*, 1989; Phillips and Cerione, 1994; Schreiber *et al.*, 1994; Wade *et al.*, 1994).

The CB<sub>1</sub> receptor/G protein interaction could be disrupted by the antiserum or IgG fraction against the proximal segment of IL3. This finding is consistent with antibody competition for a G protein association site on the receptor. A similar disruption was reported for the D<sub>2</sub> receptor, in which an antibody against an IL3 peptide was able to disrupt the ternary complex (Boundy *et al.*, 1993). The incomplete block of the cannabinoid inhibition of adenylate cyclase may suggest that the G<sub>i</sub> protein interacts with multiple intracellular domains of the receptor that also may stabilize the receptor/G protein association and that the antibody inefficiently dislodges the G protein.

No effect of the antibody to the juxtamembrane carboxyl-terminal of the CB<sub>1</sub> receptor was observed when tested on cannabinoid-mediated inhibition of adenylate cyclase. If the juxtamembrane region of the CB<sub>1</sub> receptor association with the G protein is of high affinity, then an antibody may not be able to disrupt that preformed interaction. Alternatively, the CB<sub>1</sub> receptor/G protein interaction may occlude the juxtamembrane carboxyl-terminal domain of the receptor. This was the explanation for the failure of a carboxyl-terminal  $\alpha_{i/o}$  antibody to interact with the  $\alpha_2$ -adrenergic receptor/G protein ternary complex in the presence of agonist (Okuma and Reisine, 1992).

The current results have provided some insight into the nature of the CB<sub>1</sub> receptor/G protein interaction leading to G protein activation and signal transduction. Future studies will investigate the selectivity for G protein subtypes and the role of the  $\alpha$  and  $\beta\gamma$  subunits in the interaction with subdomains of the CB<sub>1</sub> receptor. Other studies will investigate the properties of the CB<sub>1</sub> receptor carboxyl-terminal juxtamembrane domain that regulate activation of G proteins.

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## References

- Boundy VA, Luedtke RR, Artymyshyn RP, Filtz TM, and Molinoff PB (1993) Development of polyclonal anti-D<sub>2</sub> dopamine receptor antibodies using sequence-specific peptides. *Mol Pharmacol* **43**:666–676.
- Cheung AH, Huang RR, Graziano MP, and Strader CD (1991) Specific activation of G<sub>s</sub> by synthetic peptides corresponding to an intracellular loop of the beta<sub>2</sub>-adrenergic receptor. *FEBS Lett* **279**:277–280.
- Cotecchia S, Exum S, Caron MG, and Lefkowitz RJ (1990) Regions of the  $\alpha_1$ -adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc Natl Acad Sci USA* **87**:2896–2900.
- Dalman HM and Neubig RR (1991) Two peptides from the  $\alpha_{2A}$ -adrenergic receptor alter receptor G protein coupling by distinct mechanisms. *J Biol Chem* **266**:11025–11029.
- Devane WA, Dysarz FA 3d, Johnson MR, Melvin LS, and Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34**:605–613.
- Dixon RA, Sigal IS, Candelore MR, Register RB, Scattergood W, Rands E, and Strader CD (1987) Structural features required for ligand binding to the  $\beta$ -adrenergic receptor. *EMBO J* **6**:3269–3275.
- Garibay JLR, Kozasa T, Itoh H, Tsukamoto T, Matsuoka M, and Kaziro Y (1991) Analysis by mRNA levels of the expression of six G protein  $\alpha$ -subunit genes in mammalian cells and tissues. *Biochim Biophys Acta* **1094**:193–199.
- Gerard CM, Mollereau C, Vassart G, and Parmentier M (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279**:129–134.
- Hausdorff WP, Hnatowich M, O'Dowd BF, Caron MG, and Lefkowitz RJ (1990) A mutation of the  $\beta_2$ -adrenergic receptor impairs agonist activation of adenylyl cyclase without affecting high affinity agonist binding: distinct molecular determinants of the receptor are involved in physical coupling to and functional activation of G<sub>s</sub>. *J Biol Chem* **265**:1388–1393.
- Hawes BE, Luttrell LM, Exum ST, and Lefkowitz RJ (1994) Inhibition of G protein-coupled receptor signaling by expression of cytoplasmic domains of the receptor. *J Biol Chem* **269**:15776–15785.
- Hogger P, Shockley MS, Lameh J, and Sadee W (1995) Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. *J Biol Chem* **270**:7405–7410.
- Houston DB and Howlett AC (1993) Solubilization of the cannabinoid receptor from rat brain and its functional interaction with guanine nucleotide-binding proteins. *Mol Pharmacol* **43**:17–22.
- Howlett AC (1985) Cannabinoid inhibition of adenylyl cyclase: biochemistry of the response in neuroblastoma cell membranes. *Mol Pharmacol* **27**:429–436.
- Howlett AC (1995a) Cannabinoid compounds and signal transduction mechanisms, in *Cannabinoid Receptors: Molecular Biology and Pharmacology* (Pertwee RG, ed) pp 167–204, Academic Press, London.
- Howlett AC (1995b) Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35**:607–634.
- Konig B, Arendt A, McDowell J, Kahlert M, Hargrave P, and Hofmann K (1989) Three cytoplasmic loops of rhodopsin interact with transducin. *Proc Natl Acad Sci USA* **86**:6878–6882.
- Liu J, Conklin B, Blin N, Yun J, and Wess J (1995) Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. *Proc Natl Acad Sci USA* **92**:11642–11646.
- Lorenzen A, Fuss M, Vogt H, and Schwabe U (1993) Measurement of guanine nucleotide-binding protein activation by A<sub>1</sub> adenosine receptor agonists in bovine brain membranes: stimulation of guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate binding. *Mol Pharmacol* **44**:115–133.
- Luttrell LM, Ostrowski J, Cotecchia S, Kendall H, and Lefkowitz RJ (1993) Antagonism of catecholamine receptor signaling by expression of cytoplasmic domains of the receptors. *Science (Washington DC)* **259**:1453–1457.
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346**:561–564.
- Munch G, Dees C, Hekman M, and Palm D (1991) Multisite contacts involved in coupling of the  $\beta$ -adrenergic receptor with the stimulatory guanine-nucleotide-binding regulatory protein: structural and functional studies by  $\beta$ -receptor-site-specific synthetic peptides. *Eur J Biochem* **198**:357–364.
- O'Dowd BF, Hnatowich M, Regan JW, Leader WM, Caron MG, and Lefkowitz RJ (1988) Site-directed mutagenesis of the cytoplasmic domains of the human  $\beta_2$ -adrenergic receptor: localization of regions involved in G protein-receptor coupling. *J Biol Chem* **263**:15985–15992.
- Okuma Y and Reisine T (1992) Immunoprecipitation of  $\alpha_{2A}$ -adrenergic receptor-GTP-binding protein complexes using GTP-binding protein selective antisera: changes in receptor/GTP-binding protein interaction following agonist binding. *J Biol Chem* **267**:14826–14831.
- Osawa S and Weiss E (1994) The carboxyl terminus of bovine rhodopsin is not required for G protein activation. *Mol Pharmacol* **46**:1036–1040.
- Phillips WJ and Cerione RA (1994) A C-terminal peptide of bovine rhodopsin binds to transducin  $\alpha$ -subunit and facilitates its activation. *Biochem J* **299**:351–357.
- Pinto JC, Potie F, Rice KC, Boring D, Johnson MR, Evans DM, Wilken GH, Cantrell CH, and Howlett AC (1994) Cannabinoid receptor binding and agonist activity of amides and esters of arachidonic acid. *Mol Pharmacol* **46**:516–522.
- Poddar MK, Ghosh SK, and Haque SJ (1988) Cannabinoids and fatty acids: interaction with bovine serum albumin, in *Marijuana: An International Research Report* (Chesher G, Consroe P, and Musty R, eds) pp 309–314, Australian Government Public Service, Canberra.
- Ren Q, Kurose H, Lefkowitz RJ, and Cotecchia S (1993) Constitutively active mutants of the  $\alpha_2$ -adrenergic receptor. *J Biol Chem* **268**:16483–16487.
- Schreiber R, Prossnitz E, Ye R, Cochrane C, and Bokoch G (1994) Domains of the human neutrophil N-formyl peptide receptor involved in G protein coupling: mapping with receptor-derived peptides. *J Biol Chem* **269**:326–331.
- Shi W, Osawa S, Dickerson C, and Weiss E (1995) Rhodopsin mutants discriminate sites important for the activation of rhodopsin kinase and G<sub>s</sub>. *J Biol Chem* **270**:2112–2119.
- Song C and Howlett AC (1995) Rat brain cannabinoid receptors are N-linked glycosylated proteins. *Life Sci* **56**:1983–1989.
- Takemoto D, Takemoto L, Hansen J, and Morrison D (1985) Regulation of retinal transducin by C-terminal peptides of rhodopsin. *Biochem J* **232**:669–672.
- Wade SM, Dalman HM, Yang S-Z, and Neubig RR (1994) Multisite interactions of receptors and G proteins: enhanced potency of dimeric receptor peptides in modifying G protein function. *Mol Pharmacol* **45**:1191–1197.
- Wahlqvist M, Nilsson IM, Sandberg F, and Agurell S (1970) Binding of delta-1-tetrahydrocannabinol to human plasma proteins. *Biochem Pharmacol* **19**:2579–2584.
- Weiss ER, Kelleher DJ, and Johnson GL (1988) Mapping sites of interaction between rhodopsin and transducin using rhodopsin antipeptide antibodies. *J Biol Chem* **263**:6150–6154.
- Widman M, Agurell S, Ehrnebo M, and Jones G (1974) Binding of (+)- and (–)-delta-1-tetrahydrocannabinols and (–)-7-hydroxy-delta-1-tetrahydrocannabinol to blood cells and plasma proteins in man. *J Pharm Pharmacol* **26**:914–916.
- Wong SK, Parker EM, and Ross EM (1990) Chimeric muscarinic cholinergic:  $\beta$ -adrenergic receptors that activate G<sub>s</sub> in response to muscarinic agonists. *J Biol Chem* **265**:6219–6224.
- Yamada M, Yamada M, Watson MA, and Richelson E (1994) Deletion mutation in the putative third intracellular loop of the rat neurotensin receptor abolishes polyphosphoinositide hydrolysis but not cyclic AMP formation in CHO-K1 cells. *Mol Pharmacol* **46**:470–475.

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**Send reprint requests to:** Dr. Allyn C. Howlett, Department of Pharmacological and Physiological Science, 1402 South Grand Boulevard, St. Louis, MO 63104. E-mail: howletta@slu.edu

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