0026-895X/99/030611-08$3.00/0

MOLECULAR PHARMACOLOGY, **56:**611–618 (1999).

A Mutation in the Second Transmembrane Region of the CB1 Receptor Selectively Disrupts G Protein Signaling and Prevents Receptor Internalization

JOHN P. ROCHE, SID BOUNDS, SEAN BROWN, and KEN MACKIE

Departments of Anesthesiology (Se.B., K.M.), and Physiology and Biophysics (J.P.R., K.M.), University of Washington School of Medicine, Seattle, Washington; and MDS Panlabs, Bothell, Washington (Si.B.)

Received March 1, 1999; accepted June 14, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

We mutated a conserved aspartate in the second transmembrane domain of the cannabinoid CB_1 receptor to asparagine (D164N), stably transfected it into AtT20 cells, and examined the coupling of this mutant receptor to several intracellular effectors that are targets of wild-type CB_1 receptor activation. We found that the D164N receptor binds the CB_1 agonist WIN 55,212-2 with an affinity matching that of the wild-type CB_1 receptor and inhibits Ca^{2+} currents and cAMP production with an equivalent potency and efficacy. This mutation, however, blocks coupling of the receptor to the potentiation of inwardly rectifying potassium channel (KIR) currents and prevents internalization of the receptor after exposure to agonist. Although the mutant receptor did not internalize, we found it was still capable of activating p42/44 MAP kinase. In addition, we made a reciprocal mutation that exchanged the aspartate with an asparagine in the seventh transmembrane region (D164N/N394D). In other seven-membrane-spanning receptors, this reciprocal mutation is known to restore functions disrupted by the mutation of the single conserved aspartate. However, activation of D164N/N394D did not potentiate KIR current, nor did it internalize. We conclude that D164 is necessary for potentiation of KIR current and internalization of receptor but not necessary for agonist binding, inhibition of cAMP production, inhibition of Ca^{2+} currents, or activation of p42/44 MAP kinase. Furthermore, CB_1 receptor internalization is not necessary for MAP kinase activation.

The cannabinoid CB_1 receptor is a member of the seven-membrane-spanning receptor family (Matsuda et al., 1990) that mediates the actions of cannabinoids in the central nervous system (Pertwee, 1997). The CB_1 receptor activates heterotrimeric pertussis toxin (PTX)-sensitive G proteins (G_i/G_o) in many tissues, which results in a variety of actions within the cell. These actions include activation of an inwardly rectifying potassium channel (KIR; Mackie et al., 1995), inhibition of voltage-gated Ca^{2+} channels (Mackie et al., 1995), inhibition of cAMP production (Howlett, 1985; Howlett et al., 1986), and activation of the MAP kinase cascade (Bouaboula et al., 1995). The seven-membrane-spanning receptor family has numerous highly conserved amino acids thought to be responsible for universally critical actions of the receptor family (Savarese and Fraser, 1992). An aspartate residue in the second membrane-spanning helix (D164 in the rat CB_1 receptor) is among these conserved amino acids. Mutation of this residue in various receptors has a variety of effects, including decreasing agonist affinity (Strader et al., 1987; Chung et al., 1988; Ho et al., 1992; Chanda et al., 1993; Brodbeck et al., 1995; Parent et al., 1996; Chakrabarti et al., 1997), blocking coupling to KIR current (Surprenant et al., 1992), decreasing phosphoinositide hydrolysis (Brodbeck et al., 1995; Jagerschmidt et al., 1995; Sealfon et al., 1995), blocking inhibition of cAMP production (Chakrabarti et al., 1997), and eliminating allosteric receptor modulation by sodium ions (Horstman et al., 1990; Kong et al., 1993; Parent et al., 1996). This conserved aspartate in the second transmembrane region is thought to interact with an asparagine in the seventh transmembrane helix (Zhou et al., 1994), and many of the actions resulting from mutation of the aspartate are thought to result from disruption of this interaction. Consistent with this idea, a double point mutation that exchanges these conserved aspartate and asparagine residues in the $5\text{-}HT_{2A}$ receptor restores coupling of this

[1] This work was supported by National Institutes of Health Grants DA08934, NS 07332, NS 08174, and DA 00286; and by the WM Keck Foundation.

ABBREVIATIONS: PTX, pertussis toxin; KIR, inwardly rectifying potassium channel; PD98095, [2-(2′-amino-3′-methoxyphenyl)-oxanphthalen-4-one]; CP 55,940, {1α-2-(*R*)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol}; SR141716A, [*N*-9-piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride]; WIN 55,212-2, {*R*-(+)-(2,3-dihydro-5-methyl-3-[{4-morpholinyl}methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphtalenyl)methanone monomethanesulfonate}; GPCR, G protein-coupled receptor; PMA, phorbol-12-myristate-13-acetate; MAP kinase, mitogen-activated protein kinase; G$\beta\gamma$, G protein $\beta\gamma$ subunit.

receptor to phosphoinositide hydrolysis, which is lost by mutation of the aspartate residue (Sealfon et al., 1995).

To determine the role of this highly conserved aspartate residue in cannabinoid CB_1 receptors, we mutated this amino acid and tested the ability of the mutant CB_1 receptor to couple to several intracellular signaling cascades which normally couple to the wild-type CB_1 receptors in AtT20 cells. We also examined whether any disruptive effects of D164N were a consequence of loss of an interaction between D164 and N394 in the seventh transmembrane region. For this purpose, we compared the coupling of D164N to that of D164N/N394D. The results reveal that D164 has a role, independent of an interaction with N394, in receptor internalization and G protein-mediated modulation of KIR.

Materials and Methods

Radioligand Binding and Adenylyl Cyclase Activity. For binding studies, AtT20 cells transfected with pcDNA3 (control), CB_1, or CB_1-D164N were grown to confluency in 500-cm^2 plates. The monolayers were washed twice with TEM (25 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.4) and homogenized in TEM (1 ml/plate). The homogenate was centrifuged at 800*g* for 10 min; the resulting pellet was homogenized in TEM and spun at 800*g* for 10 min. The supernatants were combined and centrifuged at 100,000*g* for 1 h; then, the pellets were homogenized in TEM at a final protein concentration of 1 to 5 mg/ml. Membranes (50 μg) were incubated in 20 mM HEPES and 1 mg/ml bovine serum albumin (BSA), pH 7.5 (final volume 200 μl) in 0.325 to 6 nM [^{3}H]WIN 55,212-2 [{*R*-(+)-(2,3-dihydro-5-methyl-3-[{4-morpholinyl}methyl]pyrol [1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphtalenyl)methanone monomethanesulfonate}]. After 90 min at 30°C, the membranes were filtered on a Tomtec Harvester 96 (Tomtec, Orange, CT) programmed to collect and wash captured membranes rapidly three times with 5 ml HEPES/BSA (Kuster et al., 1993). Specific binding was defined as the fraction of binding displaced by 1 μM unlabeled WIN 55,212-2. Saturation studies were analyzed by computer with the INPLOT program (Graphpad, San Diego, CA). For adenylyl cyclase studies, CB_1-expressing cells grown on 24-well plates were rinsed twice with PBS and preincubated first with Dulbecco's modified Eagle's medium for 60 min, then with Dulbecco's modified Eagle's medium, 1 mM isobutylmethylxanthine, and 3 μM BSA for 10 min. When appropriate, 100 nM WIN 55,212-2 or WIN 55,212-3 was added, and the sample was incubated for 5 min. The samples were next incubated for 10 min at 37°C with or without 10 μM forskolin and lysed with 0.1 N HCl; cAMP was measured by a scintillation proximity assay kit (Amersham Corp., Arlington Heights, IL).

Internalization. AtT20 cells stably expressing either wild-type CB_1 or D164N were treated as indicated in the figure legends. At the end of the experiment, cells were washed with phosphate buffer, fixed with 4% paraformaldehyde, permeabilized in 10% nonfat dry milk/0.1% saponin in PBS, and incubated with rabbit polyclonal antibody directed against the amino terminus of the CB_1 receptor (Twitchell et al., 1997). The cells were then washed extensively with PBS, and bound primary antibody was detected by FITC-conjugated anti-rabbit IgG secondary antibody (Zymed, San Francisco, CA). After washes in PBS, phosphate buffer, and water, coverslips were dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and viewed with a Bio-Rad MRC600 confocal microscope (Bio-Rad Laboratories, Hercules, CA). Images were processed with Adobe Photoshop and Canvas. Additional experiments were performed with cells that were incubated for 24 h in serum-free medium containing 10 or 100 nM SR141716A (SR; [*N*-9-piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride]) plus 1 mg/ml BSA, which was exchanged with fresh serum-free medium containing SR and BSA 2 h before the experiment to maintain treatment consistent with that used for the MAP kinase assay with identical results.

MAP Kinase Assay. Stably transfected AtT20 cells were plated on six-well plates. Twenty-four hours before the experiment, the cells were incubated in serum-free medium containing either 10 or 100 nM SR plus 1 mg/ml BSA to reduce basal levels of MAP kinase activity. The medium was exchanged with fresh serum-free medium containing SR and BSA 2 h before the experiment. Cells were then incubated with medium appropriate for the desired experiment for 15 min. The experiment was stopped with ice-cold PBS, and the cells were lysed with buffer containing SDS, bromophenol blue, and β-mercaptoethanol. The plates were then scraped, and the resulting samples were then normalized for cell protein by using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The samples were sonicated for ~15 s, heated for 5 min, and centrifuged for 5 min; then, the supernatants were resolved by SDS-10% polyacrylamide gel electrophoresis. The gels were transferred to nitrocellulose filters, blocked with 5% milk Tris-buffered saline, probed with a primary antibody specific for the dually phosphorylated form of p44/42 MAP kinase (New England Biolabs, Beverly, MA), probed with an anti-rabbit peroxidase-labeled secondary antibody, and detected by enhanced chemiluminescence (Amersham).

Electrophysiological Recording. AtT20 cells stably transfected with rat CB_1 (Mackie et al., 1995) or D164N were plated on polylysine-coated coverslips and grown in Dulbecco's modified Eagle's medium + 10% heat-inactivated horse serum + 1:200 penicillin/streptomycin + 400 μg/ml G418 in a humidified environment with 5% CO_2 at 37°C. Cells were passaged with 0.05 mg/ml trypsin in PBS and used within 15 passages after the initial clones were isolated. Currents were recorded by using the whole-cell voltage clamp technique (Hamill et al., 1980). Pipettes were pulled from microhematocrit glass (VWR Scientific, Seattle, WA) and fire polished. For recording, a coverslip containing cells was transferred to a 200-μl chamber that was constantly perfused (1–2 ml/min) with the appropriate external solution. Solution reservoirs were selected by means of a series of solenoid valves, and solution changes were accomplished in <30 s. Voltage protocols were generated and currents were digitized, recorded, and analyzed with the Pulse program (HEKA Elektronik, Lambrecht, Germany). Liquid junction potentials are uncorrected.

For measuring KIR currents, the pipette solution contained 120 mM KCl, 10 mM HEPES, 5 mM EGTA, 3 mM $MgCl_2$, 3 mM Na_2ATP, 0.3 mM GTP, 0.1 mM leupeptin, pH 7.2, with KOH, and the external solution contained 40 mM KCl, 110 mM *N*-methylglucamine, 1 mM $CaCl_2$, 25 mM HEPES, 10 mM glucose, pH 7.35, with NaOH. Fatty acid-free BSA (3 μM) was added to decrease adsorption of cannabinoids to surfaces. The KIR current was defined as that component of the current sensitive to 1 mM Ba^{2+} elicited during the final 35 ms of a 50-ms hyperpolarizing pulse to −100 mV from a holding potential of −45 mV. Currents were sampled at 2 kHz. Because the magnitude of the KIR current was dependent on cell size, aggregate current data are presented as current densities normalized to cell capacitance.

For measuring Ba^{2+} currents in calcium channels, the pipette solution contained 100 mM CsCl, 40 mM HEPES, 10 mM EGTA, 5 mM $MgCl_2$, 3 mM Na_2ATP, 0.3 mM GTP, pH 7.2, with CsOH, and the external solution contained 140 mM NaCl, 10 mM $BaCl_2$, 5 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.3, with NaOH. Tetrodotoxin (200 nM) was added to block voltage-dependent sodium currents, 2 μM nifedipine was added to block L-type calcium channels, and BSA was added to decrease adsorption of the cannabinoids. I_{Ba} was measured at 25 ms during depolarizing voltage steps from −80 to +10 mV and was defined as the component of current sensitive to 100 μM $CdCl_2$. Currents were sampled at 2 kHz. To control for possible variations of response with passage number and to avoid one source of systematic bias, experimental and control measurements were alternated whenever possible, and concurrent controls

were always performed. Where appropriate, data are expressed as mean ± S.E.

Results

A D164N Point Mutation of the CB_1 Receptor Does Not Affect Agonist Binding to the Receptor or Receptor-Mediated Inhibition of Forskolin-Induced cAMP Production. In other members of the seven-membrane-spanning receptor family, mutation of aspartate residues homologous to D164 disrupts agonist binding to the receptor (Strader et al., 1987; Chung et al., 1988; Ho et al., 1992; Chanda et al., 1993; Brodbeck et al., 1995; Parent et al., 1996; Chakrabarti et al., 1997), leading to the hypothesis that this conserved aspartate is part of the ligand binding pocket. To test whether D164 contributed to agonist binding to the CB_1 receptor, we measured specific binding of [^{3}H]WIN 55,212-2 to membranes containing D164N and compared the values to those for wt CB_1. The specific binding of the synthetic CB_1 agonist [^{3}H]WIN 55,212-2 to AtT20 cell membranes that expressed D164N receptor (K_d = 5.5 ± 1.3 nM, Fig. 1, A and B) was comparable to binding to membranes expressing wt CB_1 receptors (K_d = 4.3 ± 2.8 nM; Fig. 1B). The B_{max} for [^{3}H]WIN 55,212-2 binding was also similar for D164N (390 ± 40 fmol/mg, Fig. 1C) when compared with wt CB_1 (450 ± 220 fmol/mg; Fig. 1C). These data indicate that expression levels as well as affinity for the synthetic agonist [^{3}H]WIN 55,212-2 are similar for D164N and the wt CB_1 receptor for the cell lines studied.

When expressed in AtT20 cells, the wt CB_1 receptor is capable of inhibiting the forskolin-induced production of cAMP (Mackie et al., 1995). We tested for the ability of the mutant receptor to inhibit forskolin-induced cAMP production and found that after application of 200 nM WIN to cells stably expressing D164N, forskolin-induced cAMP production was inhibited by 33%, compared with 25% for cells expressing wt CB_1 (Fig. 1D). The IC_{50} for inhibition of adenylyl cyclase was similar for wt CB_1 and D164N (data not shown). Hence, the D164N receptor remains capable of inhibiting adenylyl cyclase when the receptor is expressed in AtT20 cells.

D164 Is Not Necessary for CB_1 Receptor-Mediated Inhibition of Ca^{2+} Channels. The wild-type CB_1 receptor has been shown previously to inhibit high voltage-activated N- and P/Q-type Ca^{2+} channels in AtT20 cells (Mackie et al., 1995). The inhibition is voltage dependent, a widely observed type of G protein-mediated inhibition characterized by: 1) slowed channel activation kinetics, and 2) reversal of inhibition by large depolarizing prepulses (Marchetti et al., 1986; Bean, 1989). We found that the CB_1 agonist WIN caused an inhibition of I_{Ba} in cells expressing D164N (Fig. 2A). The inhibition was dose-dependent, with an IC_{50} of 6.8 ± 1.3 nM (Fig. 2B). A nearly identical IC_{50} was found for cells express-

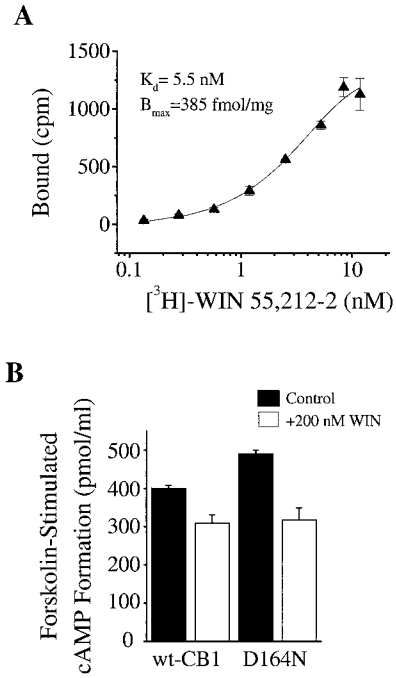

Fig. 1. A D164N point mutation of the CB_1 receptor does not affect agonist binding to the receptor, nor does it affect receptor-mediated inhibition of forskolin-induced cAMP production. A, specific binding of [^{3}H]WIN 55212-2 to membranes prepared from AtT20 cells stably expressing D164N. Specific binding was 50% of total binding at the K_d. B, forskolin induced cAMP production in the absence (■) and presence (□) of CB_1 agonist WIN (200 nM) for AtT20 cells stably expressing either wt CB_1 (left) or D164N (right) (n = 4–6).

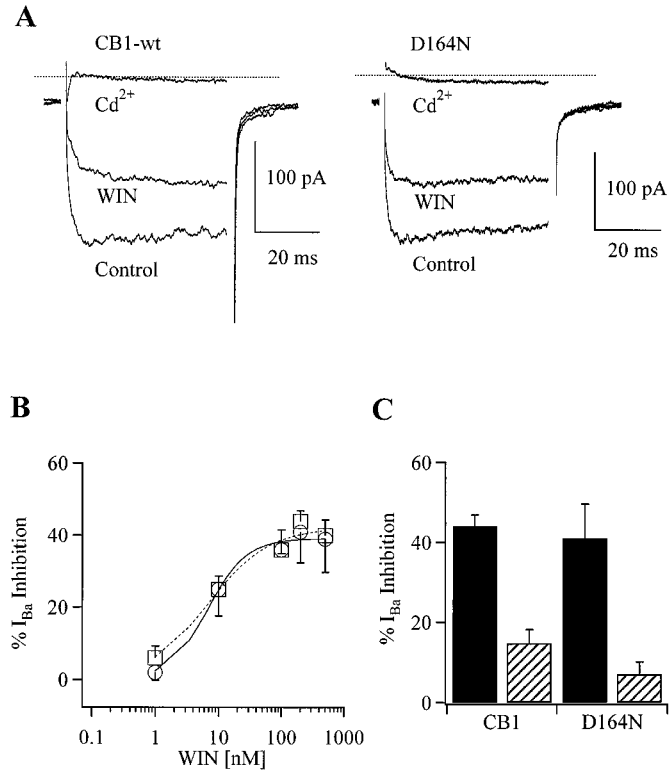

Fig. 2. D164 is not necessary for CB_1 receptor-mediated inhibition of Ca^{2+} channels. A, whole cell current recordings from AtT20 cells stably expressing the wt CB_1 (left) or the CB_1 receptor with the D164N point mutation (right). Shown are the currents elicited with a voltage step to +10 mV from a holding potential of −80 mV with no prior treatment (Control), after application of 100 nM WIN, and after application of 100 μM Cd^{2+}. A 10 mM concentration of Ba^{2+} was the charge carrier. B, dose-response curves showing inhibition of barium current as a function of increasing WIN concentration. Shown are the results obtained for wild-type CB_1 receptor (□; n = 3–9) and D164N (○; n = 2–4). C, mean inhibition (± S.E.M.) of barium current after application of 200 nM WIN (■) in AtT20 cells stably expressing either wt CB_1 (n = 9) or D164N (n = 4). Also shown is the mean inhibition of barium current after overnight incubation with pertussis toxin (▨) for cells expressing wt CB_1 (n = 3) or D164N (n = 3).

ing the wt CB_1 receptor (8.0 ± 1.6 nM; Fig. 2B). The maximal inhibition of peak current amplitude by 200 nM WIN was also similar for cells expressing either wt CB_1 or D164N-CB_1 receptors (Fig. 2C). The WIN-induced inhibition of I_{Ba} was blocked by overnight incubation with PTX in cells expressing D164N as well as in cells expressing wt CB_1 (Fig. 2C), implicating a G protein from the G_i/G_o family in the inhibition, via D164N, as well as wt CB_1. Thus, the D164N mutation does not alter the ability of the CB_1 receptor to inhibit Ca^{2+} channels or the nature of the inhibition.

D164 Is Necessary for CB_1 Receptor-Mediated Potentiation of KIR Currents. Activation of the wt CB_1 receptor also increases the KIR current in AtT20 cells (Fig. 3, A and B) by coupling to a PTX-sensitive G protein (Mackie et al., 1995). This potassium current also can be activated by somatostatin receptor agonists via receptors endogenous to the AtT20 cell line (Fig. 3, A and B) (Pennefather et al., 1988). A mutation of an aspartate residue of the α_2-adrenergic receptor that is homologous to the D164N mutation of the CB_1 receptor has previously been shown to block coupling of the α_2-receptor to activation of the KIR current in these cells (Surprenant et al., 1992). We tested whether cells expressing the D164N receptor were still capable of activating KIR current in response to a CB_1 agonist. We found that, unlike wt CB_1, the D164N receptor could not activate KIR (Fig. 3). However, the KIR current could still be activated in the D164N-expressing cells by activation of the endogenous somatostatin receptor. The mean potentiation of KIR current by 200 nM WIN was 4.0 ± 0.6 pA/pF for wt CB_1-expressing cells but only 0.3 ± 0.2 pA/pF for cells expressing D164N. In contrast, the mean potentiation of KIR current produced by somatostatin application was not significantly different for these two cell lines (Fig. 3C). At all WIN concentrations tested, we found very little potentiation of KIR current in D164N cells (Fig. 3D). Thus, the D164N mutation largely abolishes the ability of the CB_1 receptor to activate KIR.

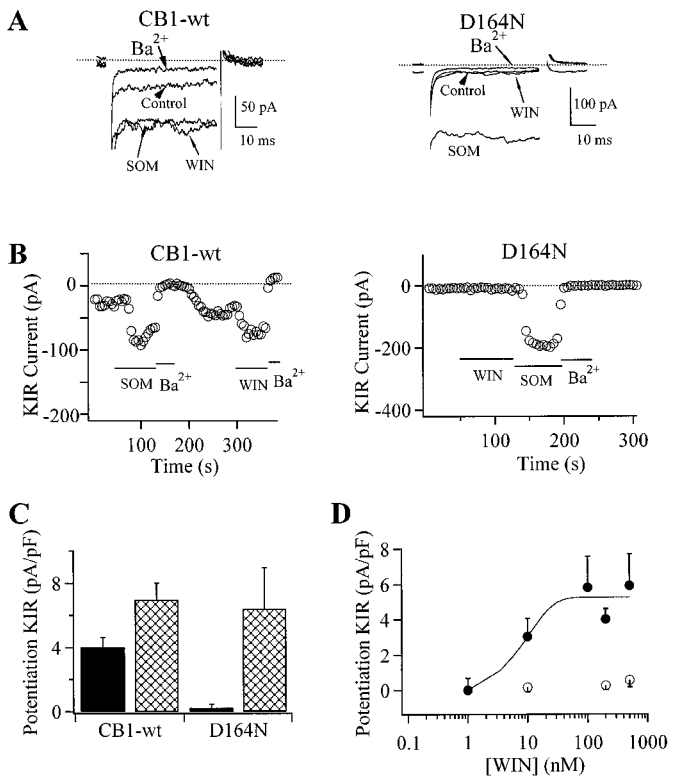

Fig. 3. D164 is necessary for CB_1 receptor-mediated potentiation KIR currents. A, whole cell current recordings from AtT20 cells stably expressing the wt CB_1 (left) or the CB_1 receptor with the D164N point mutation (right). Shown are the currents elicited with a voltage step to −100 mV from a holding potential of −45 mV with no prior treatment (Control), after application of 100 nM WIN, after application of somatostatin (SOM), and after application of 2 mM Ba^{2+}. B, the time course of KIR current potentiation for both wt CB_1 and D164N. KIR current amplitude is plotted versus time. Each circle represents the average current amplitude attained at a voltage step to −100 mV, stepped at 5-s intervals. Bars represent the period of time in which the various agents were applied to the bath. C, the mean potentiation (± S.E.M.) of KIR current after application of 200 nM WIN (■) in cells expressing either wt CB_1 (left; $n = 8$) or the D164N mutant (right; $n = 7$). Also shown is the potentiation of KIR via an endogenous somatostatin receptor (▨) in the cells expressing wt CB_1 (left; $n = 6$) or D164N (right; $n = 14$). D, dose-response curve showing potentiation of KIR current as a function of increasing concentrations of the CB_1 agonist WIN. Shown are the results obtained for both wt CB_1 (●; $n = 5–8$) and D164N (○; $n = 2–7$). The EC_{50} for the WIN-induced KIR activation of wt CB_1 was 8.0 ± 4.9 nM. The D164N dose-response curve could not be fitted.

Inability of the Mutant Receptor to Potentiate KIR Currents Is Not Agonist Specific. Previous mutations in other seven-transmembrane receptors of aspartate residues homologous to D164N in the CB_1 receptor have shown effects on agonist binding. Therefore, we wanted to test a structurally dissimilar CB_1 agonist, CP-55,940 (CP; {1α-2-(R)-5-(1,1dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol}), to preclude the possibility that the failure of WIN to activate the KIR current in D164N cells was specific to this agonist. Application of CP to cells expressing D164N did not activate the KIR current, whereas application of somatostatin caused a robust activation of KIR current in the same cells (Fig. 4, A–C). However, CP inhibited Ca^{2+} channels when applied to D164N- expressing cells (Fig. 4, D and E), similar to the results seen with application of WIN (Fig. 3). Thus, the loss of activation of the KIR current after the D164N mutation is not specific to the agonist WIN.

The Mutated Receptor Does Not Internalize on Agonist Exposure But Does Activate p42/44 MAP Kinase. Agonist-induced internalization of seven-membrane-spanning receptors on agonist application is a widely studied phenomenon and is thought to be involved in receptor down-regulation and/or resensitization (Zhang et al., 1997; Lefkowitz, 1998). When the wt CB_1 receptor is exposed to the agonist WIN, internalization occurs rapidly and is nearly complete after 15 min (Hsieh et al., 1999). Internalization is thought to result from agonist binding the receptor, activation of heterotrimeric G proteins, and subsequent activation of a number of proteins, including G protein receptor kinases and β-arrestins (Zhang et al., 1997; Lefkowitz, 1998). These proteins, particularly β-arrestin, are thought to form a complex with the receptor that aids in directing the receptor to clathrin-coated pits. These clathrin-coated pits are then endocytosed, and the receptor is either targeted to lysosomes or returned to the cell surface. Because we have documented above a selective disruption in signaling via the D164N mutant, we wanted to know whether this mutation affected receptor internalization. We exposed cells expressing mutant or wt receptors to 100 nM WIN for 15 min and compared the amount of receptor internalization. We found that the mutant showed no evidence of internalization after a 15-min WIN exposure ($n = 6$), an exposure that caused almost complete internalization of the wt receptor (Fig. 5A). Longer

exposure (up to 1 h) also failed to cause any internalization of the mutant receptor (data not shown).

Recent reports indicate that prevention of β_2-adrenergic receptor internalization by overexpression of dominant negative mutants of β-arrestin or dynamin also block activation of the MAP kinase signaling cascade (Daaka et al., 1998). This observation suggested that receptor internalization is necessary to activate the p42/44 MAP kinase. With this hypothesis in mind, and the knowledge that activation of CB_1 receptors increases MAP kinase activity (Bouaboula et al., 1995), we asked whether the expressed D164N receptors also coupled to the MAP kinase phosphorylation cascade. Contrary to our expectations based on the β_2-adrenergic receptor results, we observed a dose-dependent increase in the amount of phosphorylated p42/44 MAP kinase in cells expressing the D164N receptor after a 10-min exposure to WIN (Fig. 5B). Similar results were obtained in five separate experiments. The increase in phospho-p42/44 was blocked by PTX, the CB_1 antagonist SR 141716A, and the MAP kinase-kinase inhibitor PD98095 (PD; [2-(2′-amino-3′-methoxyphenyl)-oxanphthalen-4-one]; Fig. 5B). We also repeated the internalization experiments under pretreatment conditions similar to those used for the MAP kinase experiments to better compare the results from these separate experiments and found no difference in either the internalization of wt CB1 or lack of internalization of D164N.

We found that WIN 55,212-2 caused only a weak increase in the amount of phosphorylated p42/44 MAP kinase in cells expressing the wt CB_1 receptor (data not shown). In other cell

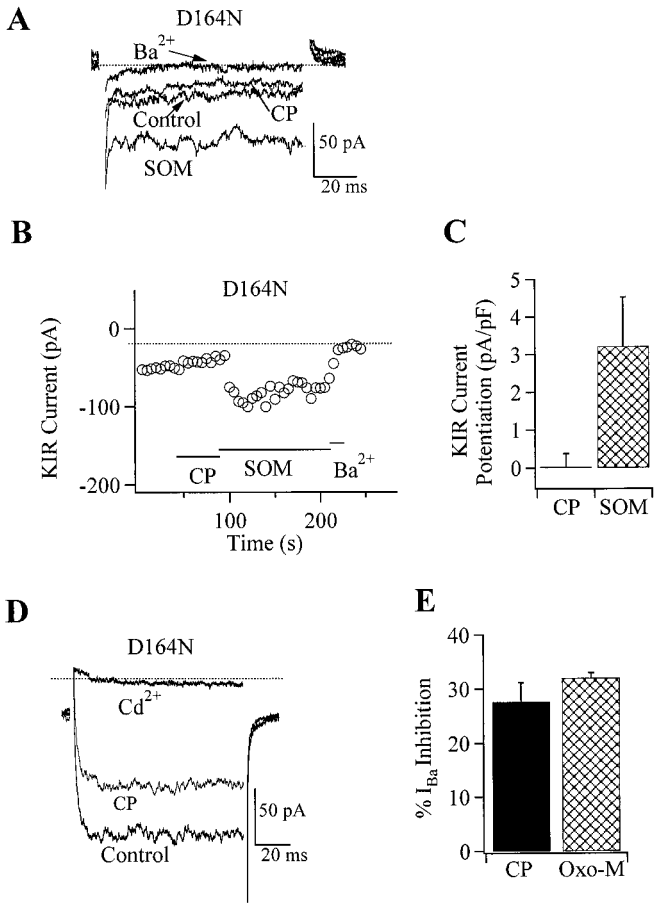

Fig. 4. Inability of the D164N mutant receptor to potentiate KIR currents is not agonist specific. A, whole cell KIR current recordings from AtT20 cells stably expressing the CB_1 receptor with the D164N point mutation. Shown are the currents elicited with a voltage step to −100 mV from a holding potential of −45 mV with no prior treatment (Control), after application of 100 nM CP-55,940 (CP), after application of somatostatin (SOM), and after application of 2 mM Ba^{2+}. B, the time course of KIR current potentiation for cells expressing D164N. KIR current amplitude is plotted versus time, each circle representing the average current amplitude attained at a voltage step to −100 mV from a holding potential of −45 mV at 5-s intervals. Bars represent the period of time during which the various agents were applied to the bath. C, the mean potentiation of KIR current via the agonists CP (solid; $n = 5$) and SOM (hatched; $n = 3$) in AtT20 cells stably expressing D164N. D, whole cell barium current recordings from AtT20 cells stably expressing D164N. Shown are the currents elicited with a voltage step to +10 mV from a holding potential of −80 mV with no prior treatment (Control), after application of 100 nM CP, and after application of 100 μM Cd^{2+}. E, the mean inhibition (± S.E.M.) of barium current after application of 200 nM CP (■; $n = 6$) or the muscarinic agonist oxotremorine (▨; $n = 2$) in AtT20 cells stably expressing D164N.

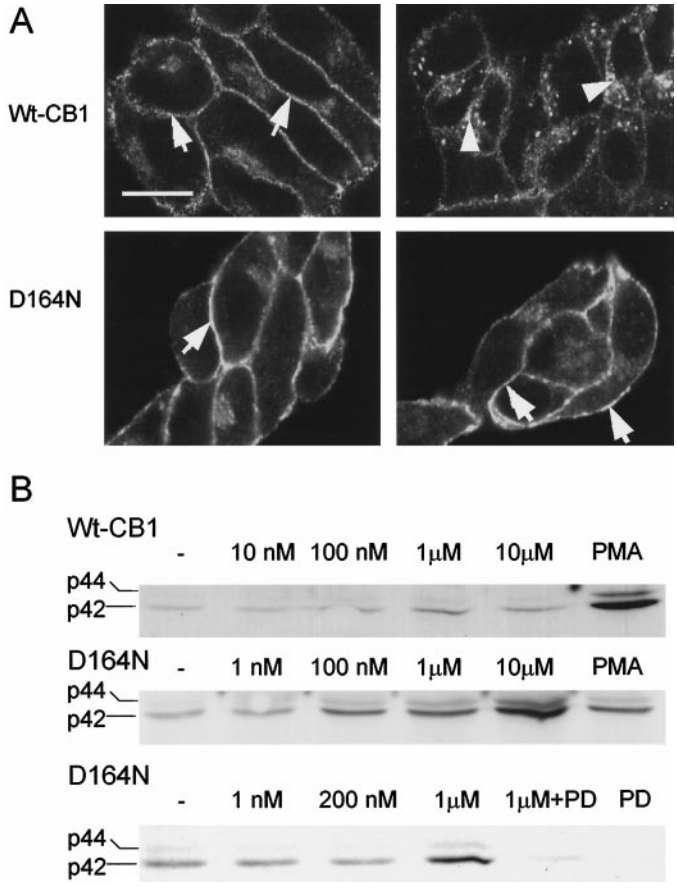

Fig. 5. The D164N mutation blocks the receptor's ability to internalize on agonist exposure but does not block MAP kinase activation. A, a confocal image of AtT20 cells stably expressing either wt CB_1 (top) or D164N (bottom) stained with antibody specific for the N terminus of the CB_1 receptor. Cells were incubated for 15 min either untreated (0 WIN) or in the presence of 100 nM WIN (100 nM WIN). Arrows highlight regions that clearly demonstrate the location of the cell surface antibody-stained receptor, whereas arrowheads highlight antibody-stained internalized receptor. The scale bar indicates a length of 10 μm. B, Western blots probed with antibody specific for the dually phosphorylated form of p42/44 MAP kinase. AtT20 cells stably expressing either wt CB_1 or D164N were incubated in serum-free media for 15 min at 37°C with the CB_1 antagonist SR (10 nM) in addition to the various concentrations of WIN indicated above each lane as well as the PKC activator PMA (1 μM) and the MAP kinase-kinase inhibitor PD (100 μM). The dash indicates that no additional drugs were added. SR was applied to all cells in order to prevent an increase in phospho-p42/44 resulting from increased receptor activity on removal of antagonist.

types, constitutively active CB_1 receptors lead to a high basal activation of p42/44 MAP kinase. This high basal activation can be reduced by preincubation with the CB_1 antagonist SR (Bouaboula et al., 1995), suggesting, perhaps, that the lack of WIN-induced increase in phospho-p42/p44 seen in the wt receptors was the result of a high basal activity of the receptor. Increasing the amount of the CB_1 antagonist SR to 100 nM in the preincubation stage, however, did not improve the ability of WIN to increase phospho-p42/p44 activity via wt CB_1 receptors (data not shown). Differences in basal activity of the wt CB_1 and D164N receptors are also unlikely because phorbol-12-myristate-13-acetate (PMA), which activates the MAP kinase pathway in a receptor-independent manner, increased the phosphorylated MAP kinase signal equivalently in both cell lines (Fig. 5B). This result indicates that not only is internalization of the CB_1 receptor not necessary for MAP kinase activation, but that the altered G protein coupling or the lack of internalization caused by the D164N mutation actually enhances coupling of activation of the CB_1 receptor to phosphorylation of p42/44 MAP kinase.

A Reciprocal Mutation (D164N,N394D) Does Not Restore the Ability to Potentiate KIR Currents or Internalize on Agonist Exposure. The aspartate residue in the second transmembrane region is highly conserved through most members of the seven-membrane-spanning receptor family. In the GnRH receptor, however, an asparagine replaces the aspartate at this position and a highly conserved asparagine in the seventh transmembrane region is replaced by an aspartate. Molecular modeling indicates that the second and seventh transmembrane regions come into close proximity, suggesting that the highly conserved aspartate and asparagine residues in the second and seventh transmembrane regions interact with each other (Zhou et al., 1994). Indeed, when the residues at these two sites were exchanged in the 5-hydroxytryptamine (serotonin) 5-HT_{2A} receptor, the receptor functioned normally (Sealfon et al., 1995). However, mutating the second-transmembrane aspartate alone disrupted coupling of this receptor to phosphoinositide turnover. Here we tested whether a similar double mutation could restore functional coupling of the D164N receptor.

We stably expressed a double mutant D164N/N394D construct in AtT20 cells and observed that 200 nM WIN produced no activation of the KIR current (Fig. 6, A and B). Application of 200 nM WIN to these same cells did, however, inhibit Ca^{2+} channels (Fig. 6, C and D). Application of 100 nM WIN also failed to cause internalization of the D164N/N394D receptor (Fig. 6E). The same application of 100 nM WIN again caused almost complete internalization of the wt CB_1 receptor (Fig. 6E). Thus, the double mutant did not restore the functional defects of the single mutant.

Discussion

We have shown that mutation of a highly conserved aspartate residue in the second transmembrane region of the CB_1 cannabinoid receptor disrupts certain G protein signaling cascades without affecting others. The mutation did not affect binding of the CB_1 agonist WIN 55,212-2. These results suggest that D164 is required for the correct coupling of agonist-activated receptor to the stimulation of certain G proteins. The mechanism underlying the selectivity is unclear. Possibilities include: 1) selectively altering coupling of specific families of G proteins to the activated receptor; 2) reducing the activation efficiency of all coupled G proteins with effectors differentially affected by this change; and 3) altering the localization of the receptor in relation to the various effectors.

Selectively altering the coupling of specific G protein families is possible but seems unlikely. Both voltage-dependent inhibition of Ca^{2+} channels and activation of KIR are

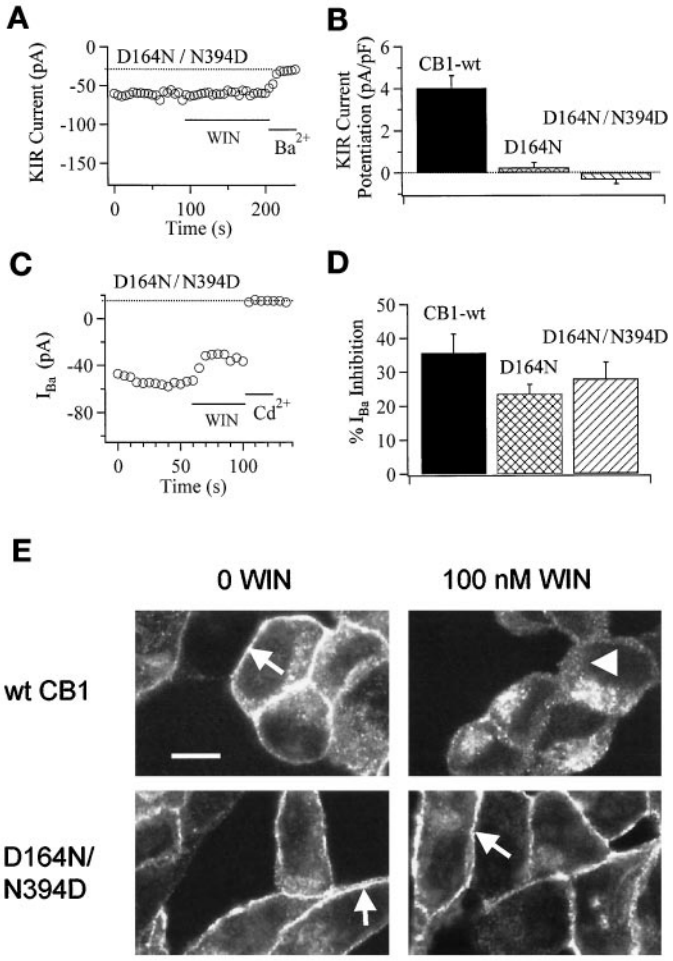

Fig. 6. A reciprocal mutation (D164N/N394D) does not restore the receptor's ability to potentiate KIR currents or internalize on agonist exposure. A, the time course of KIR current potentiation for D164N/N394D. KIR current amplitude is plotted versus time. Each circle represents the average current amplitude attained at a voltage step to −100 mV. Bars represent the period of time during which the various agents were applied to the bath. B, the mean potentiation (± S.E.M.) of KIR current after application of 200 nM WIN in cells expressing either wt CB_1 (left; ■; $n = 4$), D164N (middle; ▨; $n = 7$), or D164N/N394D (right; ▧; $n = 6$). C, the time course of I_{Ba} current inhibition for D164N/N394D. I_{Ba} current amplitude is plotted versus time. Each circle represents the average current amplitude attained at a voltage step to +10 mV from a holding potential of −80 mV. Bars represent the period of time in which the various agents were applied to the bath. D, the mean inhibition (± S.E.M.) of I_{Ba} current after application of 200 nM WIN in cells expressing either wt CB_1 (left; $n = 9$), D164N (middle; $n = 4$), or D164N/N394D (right; $n = 8$). E, confocal images of AtT20 cells stably expressing either wt CB_1 (top) or the double point mutant D164N/N394D (bottom) stained with antibody specific for the N terminus of the CB_1 receptor. Cells were incubated for 1 h either untreated (control) or in the presence of 100 nM WIN. Arrows highlight regions that clearly demonstrate the location of the cell surface antibody-stained receptor; arrowheads highlight antibody-stained internalized receptor. Scale bar indicates a length of 10 μm.

thought to result from direct binding of G protein $\beta\gamma$ subunits. Because these two actions are differentially affected by the D164N mutation, after the D164N mutation, the CB_1 receptor would have to specifically couple to a PTX-sensitive G protein with a $\beta\gamma$ subunit that is capable of modulating I_{Ba} but not KIR. Although a G protein $\beta\gamma$ subunit ($G\beta\gamma$) with such specificity may exist, it appears unlikely, as at this time no $G\beta\gamma$ is known to have such a profile (Yan and Gautam, 1997; Garcia et al., 1998).

A reduced coupling efficiency of several G protein families has been documented previously after a similar mutation to the α_2-adrenergic receptor (Chabre et al., 1994), suggesting that this mutation may affect coupling of the receptor to all G proteins but the decreased efficiency only blocks coupling to some effectors. Differing effector sensitivity may reflect whether these effectors are modulated directly by G protein subunits or, instead, by second messenger cascades that could potentially amplify even a very small signal. Because voltage-dependent inhibition of Ca^{2+} channels and activation of KIR are both thought to result from direct binding of $G\beta\gamma$s, this possibility is unlikely. Alternatively, differing effector sensitivity may reflect the number of G protein subunits required to modulate the different effectors. Inhibition of Ca^{2+} channels and activation of KIR may have different stoichiometric requirements for $G\beta\gamma$s and thus would be differentially affected by a reduction in $G\beta\gamma$ concentrations. However, a comparison of the dose-response curves for WIN-induced modulation of Ba^{2+} current and KIR current via wt CB_1 receptors reveals almost identical sensitivity (Figs. 2B and 3D), making the possibility of different $G\beta\gamma$ requirements less likely.

Both activation of inwardly rectifying potassium currents and inhibition of Ca^{2+} channels are membrane-delimited forms of G protein inhibition, and as such they are dependent on close localization of the activated receptor to the channel. Our results could be explained if the D164N mutation interfered with colocalization of the CB_1 receptor with KIR channels but not with Ca^{2+} channels. Such an effect would selectively alter signaling to these channel types in agreement with our data. Localization of receptor is also important for internalization, because the receptors are first clustered in clathrin-coated pits on the cell membrane and subsequently internalized. The D164N mutation likely prevents internalization at a step before clustering of the receptor in clathrin-coated pits, inasmuch as the antibody staining remains evenly distributed over the entire membrane. We cannot differentiate from these data, however, whether the lack of clustered receptor is a failure in activation of the G protein signaling cascade or a failure in a localization signal requiring D164 occurring subsequent to G protein activation.

Mutations of this conserved aspartate residue in many different G protein-coupled receptors affect binding of agonist to the receptor (Strader et al., 1987; Chung et al., 1988; Ho et al., 1992; Chanda et al., 1993; Brodbeck et al., 1995; Parent et al., 1996; Chakrabarti et al., 1997; Tao and Abood, 1998). We have shown here, however, that mutation of D164 had no effect on agonist (WIN 55,212-2) binding to the CB_1 receptor, indicating that this residue was not a necessary component of the WIN binding pocket, and that any conformational change caused by the D164N mutation does not impact binding of agonist to this pocket.

The conserved aspartate in the second transmembrane region is thought to interact with a conserved asparagine in the seventh transmembrane region (Zhou et al., 1994), and several studies show that exchanging these two residues results in a receptor that functions identically to the wild type (Zhou et al., 1994; Sealfon et al., 1995). However, when we made this double mutation in the CB_1 receptor, the mutated receptor behaved similarly to the D164N mutant rather than the wild-type CB_1 receptor. These data indicate that, contrary to the results seen for other receptors, an aspartate in the 164th position of the CB_1 receptor seems to be critical for functional coupling rather than for interaction with the asparagine in the seventh transmembrane region. This aspartate could interact with another region of the protein.

Our results indicate that the D164N mutation had no effect on inhibition of cAMP production or binding of the CB_1 agonist WIN 55,212-2. These results are in contrast to a previous report that found that a similar point mutation in the human CB_1 receptor (D163N) blocked coupling of the receptor to inhibition of cAMP production and significantly reduced the receptor's affinity for WIN (Tao and Abood, 1998). Interestingly, a similar disparity was seen for the analogous mutation in the α_2-adrenergic receptor (D79N). In AtT20 cells, the D79N mutation had no effect on the coupling of this receptor to the inhibition of adenylyl cyclase (Surprenant et al., 1992), whereas in Chinese hamster ovary cells, the D79N mutation disrupted coupling of the receptor to inhibition of adenylyl cyclase (Wang et al., 1991). Thus, the disparity between our results and those of Tao and Abood (1998) is likely a product of the cell type used for expression of the receptor. The cell types used may provide different ratios of G protein families that couple to the CB_1 receptor, provide different forms of adenylyl cyclase, or localize the receptors in different manners. Comparison of receptor localization and specific G protein families activated by the CB_1 receptor in these two cell types may offer clues to the mechanism underlying the selective disruption of G protein signaling after mutation of D164.

We have found that the CB_1-D164N mutant, a GPCR that does not internalize, is capable of activating p42/44 MAP kinase, indicating that receptor internalization is not necessary for activation of this cascade. Previous experiments demonstrating that receptor internalization is necessary for p42/44 MAP kinase activation were carried out with dominant negative dynamin mutants to block internalization of β-adrenergic receptors (Daaka et al., 1998). These results, taken in light of our data, suggest either that internalization is necessary only for certain receptor types to activate MAP kinase or that the dynamin mutants block activation of p42/44 MAP kinase via a mechanism other than blockade of receptor internalization. A less likely explanation is that D164N may have a low level of internalization, undetectable through the use of specific antibodies, that is sufficient to fully activate the MAP kinase cascade. Even more surprising was the finding that the D164N mutant activated p42/44 MAP kinase better than wt receptor, suggesting that the mutated receptor coupling to G proteins involved in activation of the p42/44 MAP kinase cascade may be enhanced. Alternatively, the enhanced activation of P42/44 MAP kinase may simply reflect the lack of internalization of this mutant receptor as the receptor would be exposed to agonist at the cell surface for longer periods of time. This result makes a general decrease in receptor/G protein coupling unlikely and

favors either altering the specific G protein families coupled to the receptor or altered localization of the receptor.

In summary, a highly conserved aspartate residue in the CB_1 receptor is responsible for correctly coupling receptor activation to some, but not all, intracellular signaling cascades. Future studies will determine whether the loss of coupling to specific effectors results from: 1) the loss of activation of specific subtypes of G proteins; 2) a general reduction in G protein activation with different effector sensitivity to the reduced G protein activation; or 3) altered localization of the receptor with respect to certain effectors.

Acknowledgments

We thank D. Babcock, H. Cruzablanca-Hernandez, B. Hille, E. Kaftan, and M. Shapiro for careful reading of the manuscript.

References

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium channels by changes in channel voltage dependence. *Nature (Lond)* **340:**153–156.

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312:**637–641.

Brodbeck RM, Sachais BS and Krause JE (1995) Residue 78 in the second transmembrane domain of the neurokinin-1 receptor is important in coupling high affinity agonist binding to multiple second messenger responses. *Mol Pharmacol* **47:**1065–1071.

Chabre O, Conklin BR, Brandon S, Bourne HR and Limbird LE (1994) Coupling of the alpha 2A-adrenergic receptor to multiple G proteins. A simple approach for estimating receptor-G protein coupling efficiency in a transient expression system. *J Biol Chem* **269:**5730–5734.

Chakrabarti S, Yang W, Law PY and Loh HH (1997) The mu-opioid receptor down-regulates differently from the delta-opioid receptor: Requirement of a high affinity receptor/G protein complex formation. *Mol Pharmacol* **52:**105–113.

Chanda PK, Minchin MC, Davis AR, Greenberg L, Reilly Y, McGregor WH, Bhat R, Lubeck MD, Mizutani S and Hung PP (1993) Identification of residues important for ligand binding to the human 5- hydroxytryptamine1A serotonin receptor. *Mol Pharmacol* **43:**516–520.

Chung FZ, Wang CD, Potter PC, Venter JC and Fraser CM (1988) Site-directed mutagenesis and continuous expression of human beta-adrenergic receptors. Identification of a conserved aspartate residue involved in agonist binding and receptor activation. *J Biol Chem* **263:**4052–4055.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG and Lefkowitz RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* **273:**685–688.

Garcia DE, Li B, Garcia-Ferreiro RE, Hernandez-Ochoa EO, Yan K, Gautam N, Catterall WA, Mackie K and Hille B (1998) G Protein beta-subunit specificity in the fast membrane-delimited inhibition of Ca2+ channels [In Process Citation]. *J Neurosci* **18:**9163–9170.

Ho BY, Karschin A, Branchek T, Davidson N and Lester HA (1992) The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT1A receptor: A site-directed mutation study. *FEBS Lett* **312:**259–262.

Horstman DA, Brandon S, Wilson AL, Guyer CA, Cragoe EJ Jr and Limbird LE (1990) An aspartate conserved among G protein receptors confers allosteric regulation of alpha 2-adrenergic receptors by sodium. *J Biol Chem* **265:**21590–21595.

Howlett AC (1985) Cannabinoid inhibition of adenylate cyclase: Biochemistry of the response in neuroblastoma cell membranes. *Mol Pharmacol* **27:**429–436.

Howlett AC, Qualy JM and Khachatrian LL (1986) Involvement of G_i in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* **29:**307–313.

Hsieh C, Brown S, Derleth C and Mackie (1999) Internalization and recycling of the CB1 cannabinoid receptor. *J Neurochem* **73:**493–501.

Jagerschmidt A, Guillaume N, Goudreau N, Maigret B and Roques BP (1995) Mutation of Asp100 in the second transmembrane domain of the cholecystokinin B receptor increases antagonist binding and reduces signal transduction. *Mol Pharmacol* **48:**783–789.

Kong H, Raynor K, Yasuda K, Bell GI and Reisine T (1993) Mutation of an aspartate at residue 89 in somatostatin receptor subtype 2 prevents Na+ regulation of agonist binding but does not alter receptor-G protein association. *Mol Pharmacol* **44:**380–384.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* **273:** 18677–18680.

Mackie K, Lai Y, Westenbroek R and Mitchell R (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type voltage-dependent calcium currents in AtT-20 cells transfected with rat brain cannabinoid receptor. *J Neurosci* **15:**6552–6561.

Marchetti C, Carbonne E and Lux HD (1986) Effects of dopamine and noradrenaline on Ca2+ channels of cultured sensory and sympathetic neurons of chick. *Pfluegers Arch* **406:**104–111.

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.

Parent JL, Le Gouill C, Rola-Pleszczynski M and Stankova J (1996) Mutation of an aspartate at position 63 in the human platelet-activating factor receptor augments binding affinity but abolishes G protein-coupling and inositol phosphate production. *Biochem Biophys Res Commun* **219:**968–975.

Pennefather PS, Heisler S and MacDonald JF (1988) A potassium conductance contributes to the action of somatostatin-14 to suppress ACTH secretion. *Brain Res* **444:**436–450.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74:**129–180.

Savarese TM and Fraser CM (1992) In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem J* **283:**1–19.

Sealfon SC, Chi L, Ebersole BJ, Rodic V, Zhang D, Ballesteros JA and Weinstein H (1995) Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT2A receptor. *J Biol Chem* **270:**16683–16688.

Strader CD, Sigal IS, Register RB, Candelore MR, Rands E and Dixon RA (1987) Identification of residues required for ligand binding to the beta-adrenergic receptor. *Proc Natl Acad Sci USA* **84:**4384–4388.

Surprenant A, Horstman DA, Akbarali H and Limbird LE (1992) A point mutation on the α_2-adrenoreceptor that blocks coupling to potassium but not calcium channels. *Science (Wash DC)* **257:**977–980.

Tao Q and Abood ME (1998) Mutation of a highly conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB1 and CB2, disrupts G protein coupling. *J Pharmacol Exp Ther* **285:**651–658.

Twitchell W, Brown S and Mackie K (1997) Cannabinoids inhibit N- and P/Q-type calcium currents in cultured rat hippocampal neurons. *J. Neurophysiol* **78:**43–50.

Wang CD, Buck MA and Fraser CM (1991) Site-directed mutagenesis of alpha 2A-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* **40:**168–179.

Yan K and Gautam N (1997) Structural determinants for interaction with three different effectors on the G protein beta subunit. *J Biol Chem* **272:**2056–2059.

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ and Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: Role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptors Channels* **5:**193–199.

Zhou W, Flanagan C, Ballesteros JA, Konvicka K, Davidson JS, Weinstein H, Millar RP and Sealfon SC (1994) A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. *Mol Pharmacol* **45:** 165–170.

Send reprint requests to: Dr. Ken Mackie, Departments of Anesthesiology and Physiology and Biophysics, University of Washington School of Medicine, Box 357290, Seattle, WA 98195-7290. E-mail: kmackie@u.washington.edu