LY320135, a Novel Cannabinoid CB1 Receptor Antagonist, Unmasks Coupling of the CB1 Receptor to Stimulation of cAMP Accumulation[1]

CHRISTIAN C. FELDER, KELLY E. JOYCE, EILEEN M. BRILEY, MICHELLE GLASS, KENNETH P. MACKIE, KENNAN J. FAHEY, GEORGE J. CULLINAN, DAVID C. HUNDEN, DOUGLAS W. JOHNSON, MICHAEL O. CHANEY, GARY A. KOPPEL and MICHAEL BROWNSTEIN

*The Laboratory of Cellular and Molecular Regulation and **Laboratory of Cell Biology (M.B.), National Institute of Mental Health, Bethesda, Maryland, *Departments of Physiology and Anesthesiology (K.P.M.), University of Washington, Seattle, Washington, and Eli Lilly Research Labs (C.C.F., K.J.F., G.J.C., D.C.H., D.W.J., M.O.C., G.A.K.), Neuroscience and Endocrine Divisions, Indianapolis, Indiana*

ABSTRACT

LY320135 is a selective antagonist for the brain CB1 receptor, having greater than 70-fold higher affinity for the CB1 than the peripheral CB2 receptor. The K_i values for LY320135 at the CB1 and CB2 receptors, transfected and stably expressed in cell lines, were 224 nM and >10 μM, respectively. Similar K_i values were measured in binding studies performed on cerebellum and spleen membrane preparations endogenously expressing the CB1 (203 nM) and CB2 (>10 μM) receptors, respectively. LY320135 functionally reversed anandamide-mediated adenylate cyclase inhibition in Chinese hamster ovary (CHO) cells stably expressing the CB1 receptor. Pertussis toxin treatment of CHO cells expressing the CB1 receptor attenuated the anandamide-mediated inhibition of adenylate cyclase and unmasked a stimulatory effect of anandamide on adenylate cyclase. The stimulatory component was blocked with LY320135. This compound also blocked WIN 55212–2-mediated inhibition of N-type calcium channels and activation of inwardly rectifying potassium channels in N18 and AtT-20-CB2 cells, respectively. LY320135 is a promising lead compound for the further development of novel, potent and selective cannabinoid antagonists of novel structure.

Marijuana has been used for recreational and medicinal purposes for centuries. The wide appeal of this drug lies predominantly in its ability to alter mood and behavior. The therapeutic effects of marijuana are surprisingly broad and include its ability to act as an antiemetic, anti-inflammatory, antiglaucoma, analgesic and appetite-enhancing agent. These diverse physiological effects are mediated by the active principal in marijuana, THC, for which specific receptors were identified in the brain (Devane *et al.*, 1988). Three subtypes of cannabinoid receptors, CB1 (Matsuda *et al.*, 1990), CB1A (Shire *et al.*, 1995) and CB2 (Munro *et al.*, 1993), have been identified, cloned and sequenced to date and are members of the superfamily of G-protein-coupled receptors. CB1 receptors are primarily located in the central nervous system. The CB1 receptor is functionally linked to the inhibition of adenylate cyclase (Howlett *et al.* 1988; Felder *et al.*,

1992), and more recently has been shown to inhibit N- and Q-type voltage-dependent calcium channels (Mackie and Hille, 1992; Caufield and Brown, 1992; Mackie *et al.*, 1995) and to stimulate an inwardly rectifying potassium current (K_{ir} current) (Mackie *et al.*, 1995). Based on the high abundance of CB1 receptors in hippocampus, cerebellum and basal ganglia (Herkenham *et al.*, 1991), and the well known behavioral effects of cannabinoid agonists (Mechoulam, 1986), it is likely that this receptor regulates short-term memory, coordination of movement and emotions. The CB2 receptor is found predominantly in hematopoietic cells but not as yet in the brain (Munro *et al.*, 1993; Lynn and Herkenham, 1994) and has been shown to couple to the inhibition of adenylate cyclase, but not to Q-type calcium channels or K_{ir} current (Felder *et al.*, 1995). The CB2 receptor may mediate some of the peripheral effects of THC, such as immunosuppression (Martin, 1986). High concentrations of THC can also activate signal transduction pathways through cannabinoid receptor-independent mechanisms (Felder *et al.*, 1992). The discovery of brain receptors for THC led to the discovery of the endogenous cannabinoid agonist anandamide (Devane *et*

ABBREVIATIONS: THC, $(-)\Delta^9$-tetrahydrocannabinol; CHO, Chinese hamster ovary; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

al., 1992) and other potential cannabimimetic fatty acid amides (Felder *et al.*, 1993; Hanus *et al.*, 1993; Di Marzo *et al.*, 1994). These discoveries have led to a growing interest in determining the physiological role of anandamide, other fatty acid amides and cannabinoid receptors.

Cannabinoid receptor pharmacology has been hampered by the availability of selective antagonists. Three aminoalkylindoles have been shown to display cannabinoid receptor antagonist properties. WIN 56,098 was found to be a cannabinoid antagonist in *in vitro* experiments (Pacheco *et al.*, 1991; Compton *et al.*, 1992). More recently, two more potent antagonists, bromopravadoline (Casiano *et al.*, 1990) and iodopravadoline (Pertwee *et al.*, 1995), have been described. The most potent antagonist to date, SR141716A, is based on a pyrazole structure (Rinaldi-Carmona *et al.*, 1994). SR141716A was found to be selective and potent at the CB1 receptor and blocked WIN 55212–2-mediated behavioral responses. SR141716A had a weak affinity for the CB2 receptor expressed in clonal cell lines (Felder *et al.*, 1995). Here we describe a CB1 receptor antagonist of novel structure that is selective for the CB1 receptor.

Materials and Methods

CP 55,940 was generously provided by Dr. Larry Melvin (Pfizer Inc., Groton, CT.); WIN 55212–2 by Dr. Susan Ward (Sterling-Winthrop Research Institute, Malvern, PA); HU-210 by Dr. Raphael Mechoulam (Hebrew University, Jerusalem, Israel) and (−)Δ-9-THC by the National Institute on Drug Abuse. Anandamide was obtained from Biomol (Plymouth Meeting, PA). All other reagents were purchased from Sigma Chemical Co. (Saint Louis, MO). All assays were performed in glass test tubes which were treated by exposure to dimethyldichlorosilane vapor under vacuum overnight.

Expression of CB1 and CB2 receptors, plasma membrane preparation and radioligand binding assays. CB1 and CB2 cDNA were stably transfected and expressed in either CHO cells or L cells as described previously (Felder *et al.*, 1995). Plasma membranes were prepared from murine fibroblast L cells, CHO-CB2 cells, rat cerebellum and rat spleen as described previously (Felder *et al.*, 1995). Competition binding assays were performed with 0.5 nM ^{3}H-CP 55,940 as the labeled ligand. Radioligand binding of cannabinoid agonists was measured by a previously described rapid filtration assay (Felder *et al.*, 1993). Phenylmethylsulfonyl fluoride was included where indicated to inhibit the degradation of anandamide during radioligand binding experiments (Deutsch and Chin, 1993; Childers *et al.*, 1994). Binding assays for data shown in table 1 were performed as described previously (Foreman *et al.*, 1992). The K_i values for competition binding experiments and EC_{50} values for the inhibition of forskolin-stimulated cAMP accumulation were calculated by nonlinear regression analysis of the primary data with the computer software Graph Pad (San Diego, CA). Levels of cAMP accumulation were measured by radioimmunoassay as described previously (Felder *et al.*, 1992).

Electrophysiological recordings. N18 cells were cultured as described previously (Mackie *et al.*, 1993) and were used for measurement of calcium currents. AtT-20-CB1 cells were cultured as described previously and were used to measure of potassium currents (Mackie *et al.*, 1995). Currents were recorded by the whole-cell voltage-clamp technique (Hamill *et al.*, 1981) with pipettes pulled from microhematocrit glass (VWR Scientific, Plainfield, NJ) 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 a series of solenoid valves which allowed solution changes in less than 1 min. Voltage protocols were generated, and data were digitized, recorded and analyzed with Basic-Fastlab (Indec Systems, Capitola, CA). Junction potentials were uncorrected.

Potassium currents were measured with a pipette solution containing (mM): 130,, KCl; 20, HEPES; 10, EGTA; 5, MgCl$_2$; 3, Na$_2$ATP; 0.6, GTP; 0.08, leupeptin, pH 7.25 with KOH. The external solution contained (mM): 40, KCl; 110, NaCl; 1, CaCl$_2$; 25, HEPES; 10, glucose, pH 7.35 with NaOH. Fatty acid-free bovine serum albumin (3 mM) was added to decrease the adsorption of cannabinoids. The K_{ir} current was defined as the component of the current sensitive to 1 mM Ba^{++} elicited during the final 150 ms of a 250-ms hyperpolarizing pulse to −100 mV from a holding potential of −45 mV. Currents were sampled at 1 kHz. The magnitude of the K_{ir} current depended on cell size, therefore aggregate current data are presented as current densities normalized to cell capacitance.

Calcium currents (I_{Ca}) were measured with a pipette solution containing (mM): 100, CsCl; 40, HEPES; 10, EGTA; 5 MgCl$_2$; 3, Na$_2$ATP; 0.2, GTP; 0.08, leupeptin, pH 7.30 with CsOH. The external solution contained (mM): 160, NaCl; 10, BaCl$_2$; 4, KCl; 1, MgCl$_2$; 10, HEPES; 8, glucose, pH 7.35 with NaOH. Tetrodotoxin (200 nM) was added to block sodium currents and fatty acid-free bovine serum albumin was added to decrease the adsorption of cannabinoids. I_{Ca} was measured near the end of a 25-ms depolarizing pulse to 0 mV from a holding potential of −90 mV and was defined as that component of the current sensitive to 100 mM CdCl$_2$. Currents were sampled at 4 kHz. To control for possible response variations with passage number and to avoid sources of systematic bias, experimental and control measurements were alternated whenever possible and concurrent controls were always performed. Data are expressed as the mean ± standard error of the mean.

Results

LY320135 is a substituted benzofuran which is structurally distinct from the aminoalkylindole and pyrazole type cannabinoid antagonists, AM630 and SR141716A, respectively (fig. 1). LY320135 was selected from a series of related compounds as having the highest affinity for the CB1 receptor. The ability of LY320135 to compete for ^{3}H-CP55,940 binding was evaluated at the cannabinoid CB1 and CB2 receptors (fig. 2). The K_i values for LY320135 are listed in table 1 and are compared with the K_i values for the cannabinoid antagonist, SR141716A, and selected cannabinoid agonists. LY320135 was essentially equipotent for binding to the CB1 receptor ectopically expressed in L cells (L-CB1) and to rat cerebellum (an endogenous source of CB1 receptors) (fig. 2) and completely displaced ^{3}H-CP55,940 binding at saturating

TABLE 1

Comparison of K values for competition of LY320135 and selected cannabinoid ligands for 0.5 nM ^{3}H-CP 55,940 binding to the CB1 and CB2 receptors

The K_i values for LY320135 and SR141716A are presented in the top of the table. Cannabinoid agonists are ranked according to their K_i value at the CB1 receptor. K_i values for the agonists are provided for comparison purposes and were evaluated under the identical conditions used for the antagonists [reprinted with permission from Felder *et al.* (1995)]. Data are the mean ± S.E.M. of at least three experiments, each performed in triplicate. (Radioligand binding data: L-CB1: K_d = 3.3 ± 0.7 nM, B_{max} = 7.0 ± 0.5 pmol/mg; CHO-CB2: K_d = 7.5 ± 0.9 nM, B_{max} = 1.21 ± 0.1 pmol/mg; CP55,940 was used as the labeled ligand.)

	L-CB1	CHO-CB2	K_i Ratio (CB2/CB1)
	nM	*nM*	
Antagonists			
LY320135	141 ± 4	14,900 ± 403	106
SR141716A	11.8 ± 2.0	13,200 ± 700	1119
Agonists			
HU210	0.0608 ± 0.0070	0.524 ± 0.045	8.6
CP55940	3.72 ± 0.090	2.55 ± 0.19	0.7
(−) Δ°-THC	53.3 ± 8.3	75.3 ± 8.4	1.4
WIN55212-2	62.3 ± 31	3.3 ± 0.40	0.1
Anandamide	543 ± 83	1940 ± 240	3.6

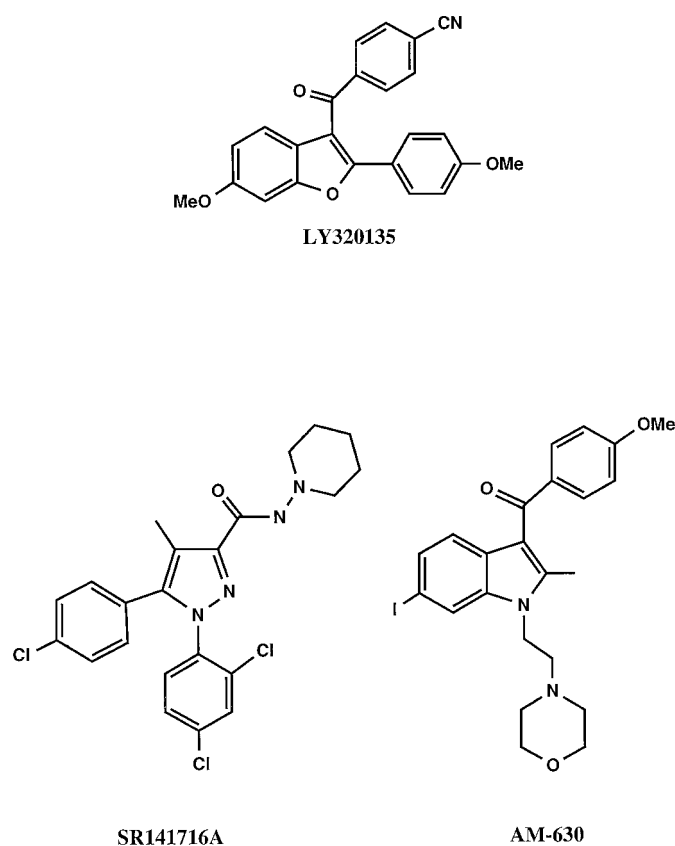**LY320135**

SR141716A   **AM-630**

Fig. 1. Structure of LY320135, SR141716A and AM-630.
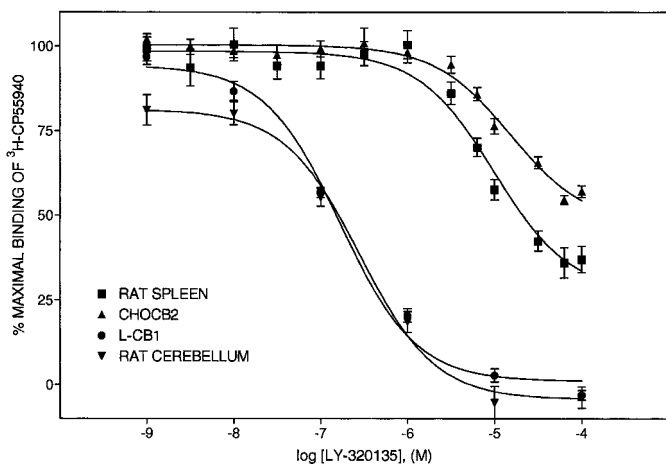

Fig. 2. LY320135-mediated inhibition of ^{3}H-CP 55,940 binding to membranes prepared from rat spleen, CHO-CB2 cells, rat cerebellum and L-CB1 cells, and rat spleen cell membranes. Plasma membranes were prepared from CHO and L cells stably transfected with the CB2 and CB1 receptor, respectively (CHO-CB2 and L-CB1, respectively) as well as rat spleen and cerebellum as described under "Materials and Methods." Competition binding curves were analyzed by nonlinear regression analysis, and the derived binding constants are the mean ± S.D. of at least three experiments, each performed with triplicate determinations. K_i values are listed in table 1. The curves shown are from representative experiments.

concentrations. LY320135 had a greater than 70-fold higher affinity for the CB1 receptor than the CB2 receptor expressed in CHO cells (CHO-CB2) or rat spleen membranes (an endogenous source of CB2 receptors), respectively. LY320135 failed to completely displace ^{3}H-CP55,940 binding to the CB2 receptor in

either spleen or CHO-CB2 membranes because of solubility limitations above 100 μM (fig. 2). Previously reported K_i values for cannabinoid receptor agonists, HU210, CP55940, THC and the endogenous cannabinoid receptor agonist anandamide, were included in table 1 to provide comparisons with known compounds by the same methodology (Felder *et al.*, 1995). Ki ratios of CB1/CB2 receptors are listed in the right-hand column of table 1.

LY320135 was evaluated as a functional antagonist by its ability to reverse the inhibition by anandamide of forskolin-stimulated cAMP accumulation in CHO-CB1 cells (fig. 3A). Anandamide (10 μM) completely attenuated the forskolin-stimulated accumulation of cAMP (fig. 3A). LY320135 reversed the inhibitory effect of anandamide, but also caused an increase in cAMP accumulation above levels observed with forskolin alone. LY320135 (10 μM) alone had no effect
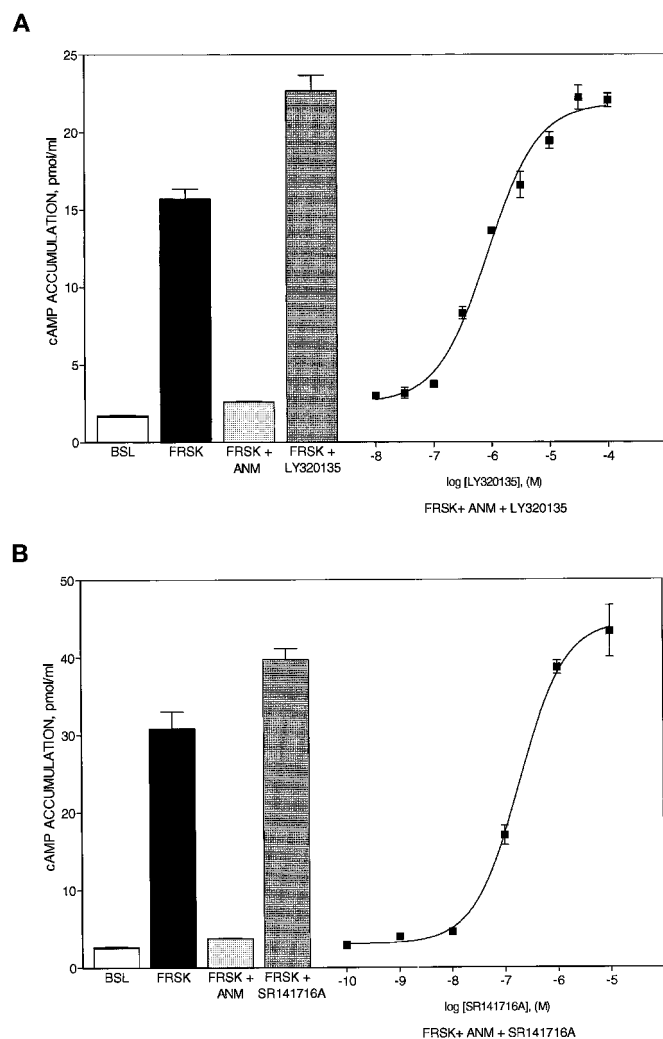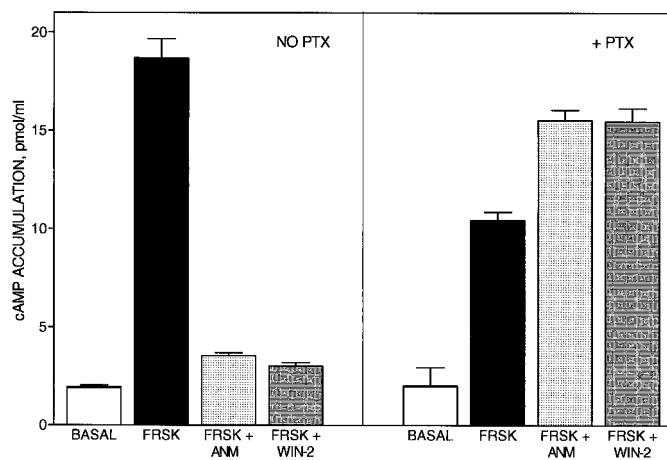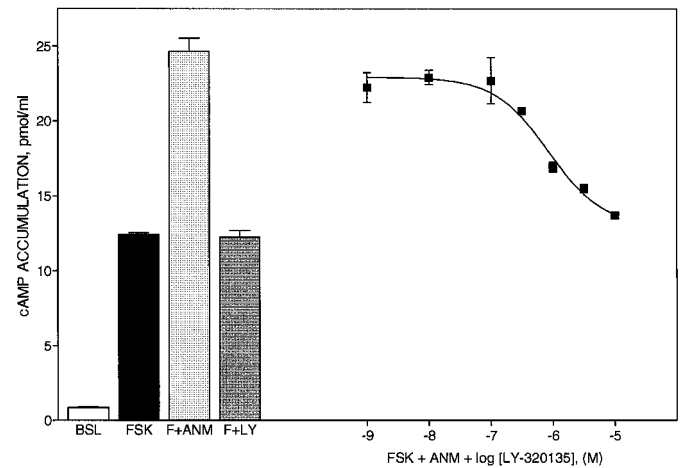
A

B

Fig. 3. The effect of LY320135 (A) and SR141716A (B) on anandamide-mediated inhibition of cAMP accumulation in CHO-CB1 cells. Forskolin (500 nM)-stimulated cAMP accumulation was measured in CHO-CB1 cells, as described under "Materials and Methods," in combination with 1 μM anandamide and increasing concentrations of either LY320135 (A) or SR141716A (B). Both LY320135 and SR141716A reversed anandamide-mediated inhibition of forskolin-stimulated cAMP accumulation in a concentration-dependent manner to levels above that observed with forskolin alone. EC_{50} values are provided under "Results." Data are the mean ± S.E.M. of at least three experiments, each performed in triplicate. (FRSK, forskolin; ANM, anandamide)

on cAMP levels (data not shown), but in combination with forskolin, enhanced the accumulation of cAMP (fig. 3A). The antagonist, SR141716A, also reversed the inhibitory effects of anandamide to levels above those observed with forskolin alone (fig. 3B). SR141716A alone had no effect on cAMP accumulation (data not shown), but also displayed similar enhancement of forskolin-stimulated cAMP accumulation. CHO-CB1 cells were pretreated with pertussis toxin to determine whether G_i or G_o mediated the overshoot of cAMP accumulation to levels above those achieved with forskolin alone (fig. 4). Both anandamide and WIN 55,212–2 attenuated forskolin-stimulated cAMP accumulation in CHO-CB1 cells (fig. 4). In the presence of pertussis toxin, anandamide alone had no effect on cAMP accumulation (data not shown), but augmented the forskolin-stimulated response. Similar results were observed when the aminoalkylindole agonist, WIN55212–2, was substituted for anandamide (fig. 4). Anandamide and WIN 55212–2, when applied alone, with or without pertussis toxin, did not increase cAMP accumulation above basal levels (data not shown). LY320135 blocked the anandamide-mediated augmentation of cAMP accumulation in the presence of pertussis toxin (fig. 5), which suggests that it was mediated through cannabinoid receptor stimulation (IC_{50} = 734 ± 122 nM). Indomethacin (10 μM) had no effect on the accumulation of cAMP in the CHO cell after anandamide and LY320135 addition (data not shown), which suggests that increases in cAMP were not mediated through prostaglandin release. Moreover, concentrations of anandamide and WIN 55212–2 used were below the those shown previously to stimulate arachidonic acid release in CHO cells (>10 μM) (Felder *et al.*, 1995). These results suggest that the CB1 receptor couples to the stimulation of cAMP accumulation and that the stimulation is unmasked when the inhibitory response is blocked with pertussis toxin.

The selectivity of LY320135 for other G protein-coupled receptors was evaluated in a radioligand binding screening

Fig. 4. The effect of pertussis toxin on anandamide- and WIN 55212–2-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO-CB1 cells. Forskolin (500 nM)-stimulated cAMP accumulation was measured in CHO-CB1 cells, in the presence and absence of anandamide or WIN 55212–2, as described under "Materials and Methods." Pertussis toxin (10 ng/ml) was added 18 hr before the measurement of cAMP accumulation. Data are the mean ± S.E.M. of at least three experiments, each performed in triplicate. (FRSK, forskolin; ANM, 1 μM anandamide; WIN-2, 50 nM WIN 55212–2)

Fig. 5. Effect of LY320135 on anandamide-mediated inhibition of cAMP accumulation in pertussis toxin-treated CHO-CB1 cells. Forskolin (500 nM)-stimulated cAMP accumulation was measured in CHO-CB1 cells, as described under "Materials and Methods." Pertussis toxin (10 ng/ml) was added 18 hr before the measurement of cAMP accumulation. In the presence of pertussis toxin, LY320135 blocked the anandamide + forskolin-mediated stimulation of cAMP accumulation (IC_{50} = 734 ± 122 nM). Data are the mean ± S.D. of two experiments, each performed in triplicate. (FRSK, forskolin; ANM, 1 μM anandamide; LY, LY320135)

assay (table 2). LY320135 had a 15-fold greater selectivity for the CB1 receptor than muscarinic receptors, and at least a 45-fold greater selectivity than benzodiazepine and 5-HT2 receptors. All other receptors tested in table 2 had affinities for LY320135 at least 70-fold higher than for the CB1 receptor.
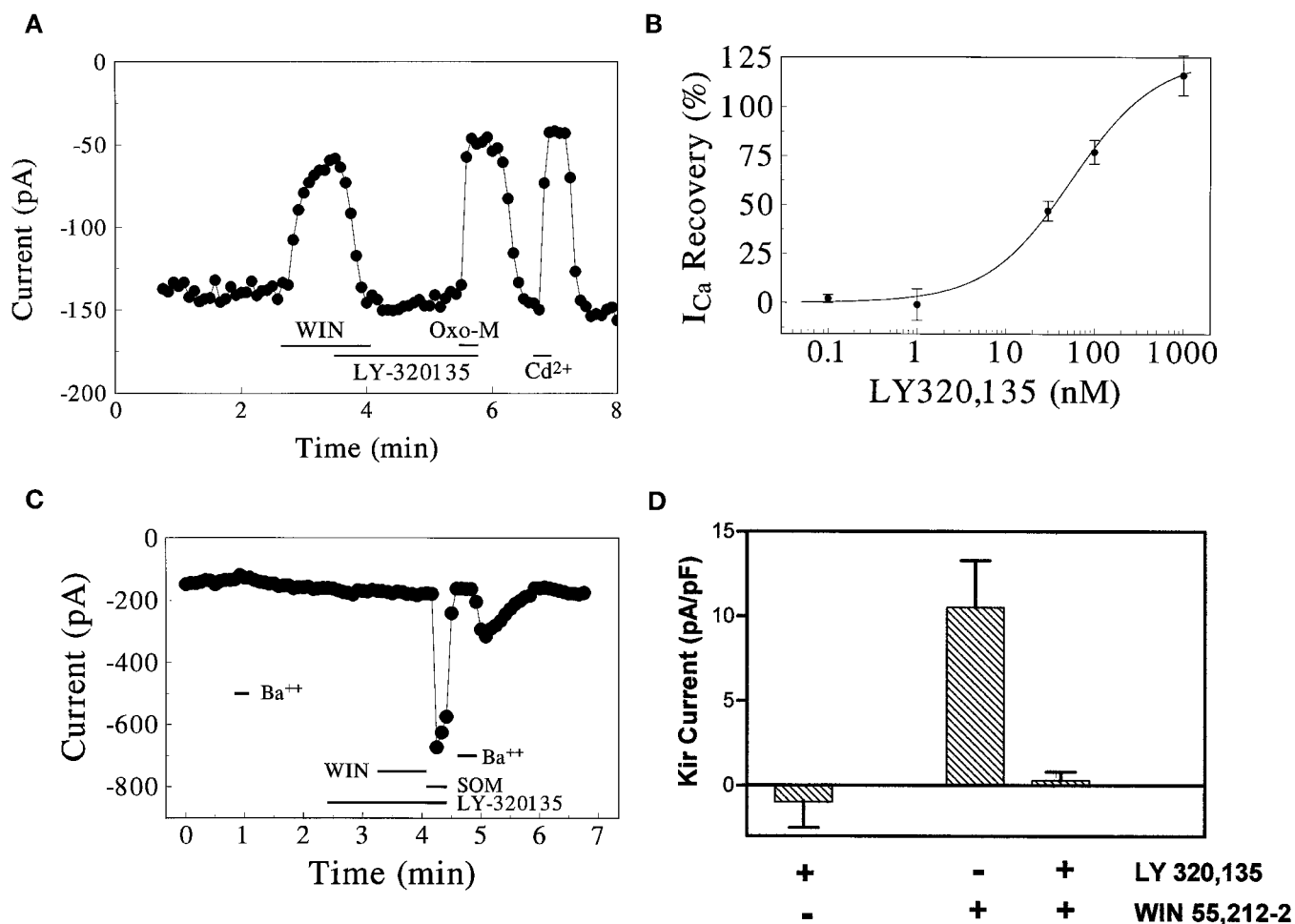
The ability of LY320135 to reverse the WIN 55212–2-mediated inhibition of N-type calcium currents (I_{Ca}) and activation of K_{ir} currents was evaluated in N18 cells and AtT-20 cells. Application of 100 nM WIN 55212–2 to N18 cells inhibited a significant fraction of the high voltage-activated I_{Ca} (fig. 6A). The inhibition was quickly reversed by coapplication of 1 μM LY320135. Despite the continued application of LY320135, the muscarinic agonist, oxotremorine-M, still inhibited I_{ca}, which suggests that LY320135, at 1 μM, doesn't interfere with muscarinic receptors or signal transduction events distal to the cannabinoid receptor. By itself, 1 μM LY320135 had little effect on the calcium current (−2.4 ± 2.1% inhibition, n = 5). LY320135 was able to potently reverse the inhibition of I_{Ca} by 100 nM WIN

TABLE 2
Comparison of K_i values for competition of LY320135 for binding to several selected neurotransmitter receptors

LY320135 was competed against ligands selective for the listed receptor. Plasma membranes were prepared from tissues expressing the receptor of interest as described under "Materials and Methods." Data are the mean ± S.E.M. of at least three experiments, each performed in triplicate.

Receptor	K_i
	μM
Alpha-1 adrenergic	45.3 ± 8.1
Alpha-2 adrenergic	17.5 ± 0.4
Alpha adrenergic	>100
D_1	897 ± 9.7
D_2	441 ± 33.7
Benzodiazepine	>10
H_1	12.9 ± 4.8
γ-Aminobutyric acid	>10
5-HT$_2$	6.4 ± 1.9
Muscarinic	2.1 ± 0.8

A

B

C

D

Fig. 6. LY320135 reverses and blocks the modulation of ion channels by cannabinoid receptor agonists. (A) LY320135 can reverse calcium current (I_{Ca}) inhibition by WIN 55212–2 in N18 cells. Application of 100 nM WIN 55212–2 inhibits a significant fraction of the I_{Ca}. Coapplication of LY320135 reverses this inhibition. In the continued presence of LY320135, inhibition by 10 μM oxotremorine-M is maintained. (B) Concentration-response curve of LY320135 reversal of I_{Ca} inhibition by 100 nM WIN 55212–2 in N-18 cells. Reversal is potent with an IC$_{50}$ of 55 $\pm$ 10 nM and noncooperative with a Hill coefficient of 0.91 $\pm$ 0.16. The solid line indicates the least squares fit for the Hill equation; n = 4 to 9 for each data point. (C) LY320135 prevents activation of K$_{ir}$ current by WIN 55212–2 in AtT-20-CB1 cells. Prior application of 1 μM LY320135 prevents activation of K$_{ir}$ current by WIN 55212–2. However, 200 nM somatostatin still activates the current. (D) Summary of LY320135 effects of K$_{ir}$ current activation by 100 nM WIN 55212–2 in AtT-20-CB1 cells. By itself, LY320135 has little effect on K$_{ir}$ current activation and in control cells 100 nM WIN 55212–2 activates a substantial current. However, in cells treated with 1 μM LY320135, 100 nM WIN 55212–2 no longer activates a K$_{ir}$ current. For each condition, n = 4 to 6. Data are the mean $\pm$ S.E.M. (WIN, WIN 55212–2; Oxo-M; oxotremorine-M; SOM, somatostatin; LY, LY320135).

55212–2 with an IC$_{50}$ of 55 $\pm$ 10 nM (fig. 6B). There was a tendency for over-recovery of I_{Ca} after applications of the higher concentrations of LY320135. After the addition of 1 μM LY320135, the current returned to 116 $\pm$ 10% of its original level (n = 9, data not shown). The effects of LY320135 could not be reversed by washing for up to 5 min (data not shown).

CB-1 receptors also activate an inwardly rectifying potassium current (K$_{ir}$) (Mackie *et al.*, 1995). Pretreatment of AtT-20-CB1 cells with 1 μM LY320135 blocked WIN 55212–2-mediated stimulation of K$_{ir}$; however, the current was still activated by 200 nM somatostatin (fig. 6C). LY320135 by itself had little effect on K$_{ir}$ (0.78 $\pm$ 0.82 pA/pF, n = 4) (fig. 6D). In control cells, 100 nM WIN 55212–2 activated K$_{ir}$ by an average of 10.9 $\pm$ 3.2 pA/pF (n = 6). In cells exposed to 1 μM LY320135, activation of K$_{ir}$ by WIN 55212–2 was suppressed completely (0.08 $\pm$ 0.32 pA/pF, n = 5). However, somatostatin still activated the K$_{ir}$ in the presence of LY320135 (17.8 $\pm$ 2.0 pA/pF, n = 4) (data not shown).

Discussion

LY320135 was selected as a lead compound from a series of potential cannabinoid receptor antagonists because it displayed the highest affinity for the CB1 subtype of the cannabinoid receptor. This compound is a benzofuran analog structurally distinct from the previously described aminoalkylindole or pyrazole cannabinoid receptor antagonists and may provide a novel approach to cannabinoid receptor antagonist development. LY320135 displayed a K_i of 141 nM for the CB1 receptor ectopically expressed in a mammalian cell line and an essentially equal affinity for CB1 receptors endogenously expressed in rat cerebellum. LY320135 had a relatively low affinity for the CB2 receptor ($K_i \geq 10$ μM) and ten other unrelated receptors (table 2), and therefore offers good selectivity for the CB1 receptor. Further modifications in the structure of LY320135 are currently being evaluated for cannabinoid receptor antagonist activity. The structure of LY320135 offers considerably more acces-

sibility for structural modification than SR141716A and may be less toxic when administered *in vivo*.

LY320135 was a functional antagonist for anandamide-mediated inhibition of forskolin-stimulated cAMP accumulation. During the course of these experiments, an overshoot of cAMP accumulation elicited by LY320135 was discovered which suggested that cannabinoid receptors couple to the stimulation of cAMP accumulation which is masked by the inhibitory component coupled through G_i. In the presence of pertussis toxin, LY320135 blocked the anandamide-mediated stimulation of cAMP accumulation, which demonstrates for the first time that the stimulatory response is regulated through the CB1 receptor. The LY320135-mediated reversal of the stimulatory effect had an IC_{50} of 734 nM which is less potent than the 141 nM K_i observed for LY320135 in competition experiments, which suggests that the functional response is complex and possibly mediated by multiple effectors. Indomethacin had no effect on the stimulatory phase of cAMP accumulation, which suggests that it was not mediated through prostaglandin production as previously demonstrated in fibroblasts (Hillard and Bloom, 1983). Additional studies have observed cannabinoid agonist-induced increases in cAMP in fibroblasts (Kelly and Butcher, 1979) and mouse brain (Dolby and Kleinsmith, 1974), but the response was not directly linked to cannabinoid receptor stimulation. Other G_i-linked receptors have been shown to stimulate cAMP accumulation after pertussis toxin pretreatment, such as *alpha*-2 adrenergic (Federman *et al.*, 1992) and muscarinic m4 receptors (Jones *et al.*, 1991). However, in CHO cells, increases in cAMP were observed only after adenylate cyclase was first stimulated with forskolin. These findings are similar to those observed in liver (Hillard *et al.*, 1986) and cardiac cell membranes (Hillard *et al.*, 1990) in which THC stimulated increases in cAMP, only in combination with glucagon or isoproterenol, respectively. These results are consistent with the regulation of type II or IV adenylate cyclase by $\beta\gamma$ subunits of G proteins (Tang and Gilman, 1991). The $\beta\gamma$ effect also requires an initial priming of the adenylate cyclase. It is not know which subtypes of adenylate cyclase are expressed in the CHO cell.

The mismatch in K_i and IC_{50} for LY320135 and SR141716A may be caused by the coupling of the CB1 receptor to both G_i, mediating inhibition of adenylate cyclase, and to an unknown stimulatory component resulting in increases in cAMP accumulation. The inhibitory effect appears to dominate over the stimulatory component because the stimulatory component can only be observed when the inhibitory component is reduced by either antagonist or pertussis toxin treatment. A Schild analysis would not be practical under these circumstances because of the complex nature of these second messenger responses.

LY320135 was a functional antagonist for WIN 55212–2-mediated inhibition of N-type voltage-dependent calcium currents and stimulation of K_{ir} current. By itself, LY320135 had no effect on either current or on modulation of the currents by other agonists. Under the conditions used for evaluating N-type currents, LY320135 was potent and irreversible during the 5 min of wash perfusion. In addition, application of LY320135 resulted in an overshoot (>100% recovery in fig. 6B) in the WIN 55212–2-mediated inhibition of N-current. This result is similar to the overshoot observed in the cAMP response. The mechanism of the overshoot of

calcium channel recovery may be related to activation of the cannabinoid receptor and is currently being investigated.

Development of cannabinoid receptor selective antagonists will provide the tools necessary to more fully understand the cannabinoid receptors both in the central nervous system and in the peripheral immune system. Although the CB1 and CB2 receptors have very similar pharmacological selectivity for cannabinoid agonists, it is apparent that there are significant differences in the binding pocket of these two receptors because of their differential selectivity for the currently available antagonists. Use of antagonists in studies of cannabinoid receptor biology will be useful in separating receptor-dependent from receptor-independent effects of cannabinoid agonists. A larger arsenal of cannabinoid receptor antagonists will be helpful in characterizing known as well as new cannabinoid receptor subtypes, should they be discovered.

References

Casiano FM, Arnold R, Haychock D, Kuster J and Ward SJ (1990) Putative aminoindolealkyl antagonists. *NIDA Res Monogr Ser* **105:**295–296.

Caufield MP and Brown DA (1992) Cannabinoid receptor agonists inhibit Ca^{++} current in NG108–15 neuroblastoma cells via a pertussis toxin-sensitive mechanism. *Br J Pharmacol* **106:**231–232.

Childers SR, Sexton T and Roy MB (1994) Effects of anandamide on cannabinoid receptors in rat brain membranes. *Biochem Pharmacol* **47:**711–715.

Compton DR, Gold LH, Ward SJ, Balster RL and Martin BR (1992) Aminoalkylindole analogs: cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydrocannabinol. *J Pharmacol Exp Ther* **263:**1118–1126.

Deutsch DG and Chin SA (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **46:**791–796.

Devane WA, Dysarz FA, Johnson MR, Melvin LS and Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **36:**605–613.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258:**1946–1949.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz J-C and Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372:**686–691.

Dolby TW and Kleinsmith LJ (1974) Effects of Δ^9-tetrahydrocannabinol on the levels of cyclic adenosine 3′,5′-monophosphate in mouse brain. *Biochem Pharmacol* **23:**1817–1825.

Federman AD, Conklin BR, Schrader KA, Reed RR and Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. *Nature* **356:**159–161.

Felder CC, Veluz JS, Williams HL, Briley EM and Matsuda LA (1992) Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol Pharmacol* **42:**838–845.

Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K and Devane WA (1993) Anandamide, an endogenous cannabinoid agonist, couples to receptor mediated signal transduction. *Proc Natl Acad Sci USA* **90:**7656–7660.

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48:**443–450.

Foreman MM, Fuller RW, Nelson DL, Calligaro DO, Kurz KD, Misner JW, Garbrecht WL and Parli CJ (1992) Preclinical studies on LY327733, a Potent and Selective Serotonergic Antagonist. *J Pharmacol Exp Ther* **260:**51–57.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high resolution current recording from cell and cell-free membrane patches. *Pflugers Arch* **391:**85–100.

Hanus L, Gopher A, Almog S and Mechoulam R (1993) Two new unsaturated fatty acid ethanolamides in brain that bind to the cannabinoid receptor. *J Med Chem* **36:**3032–3034.

Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Coste BR and Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: A quantitative *in vitro* autoradiographic study. *J Neurosci* **11:**563–583.

Hillard CJ and Bloom AS (1983) Possible role of prostaglandins in the effects of cannabinoids on adenylate cyclase activity. *Eur J Pharmacol* **91:**21–27.

Hillard CJ, Bloom AS and Houslay MD (1986) Effects of delta-9-tetrahydrocannabinol on glucagon receptor-coupling to adenylate cyclase in rat liver plasma membranes. *Biochem Pharmacol* **35:**2797–2803.

Hillard CJ, Pounds JJ, Boyer DR and Bloom DS (1990) Studies of the effects of membrane lipid order in the effects of Δ^9-tetrahydrocannabinol on adenylate cyclase activation in heart. *J Pharmacol Exp Ther* **252:**1075–1082.

Howlett AC, Johnson MR, Melvin LS and Milne GM (1988) Nonclassical cannabinoid analgetics inhibit adenylate cyclase: Development of a cannabinoid receptor model. *Mol Pharmacol* **33:**297–302.

Jones SVP, Heilman CJ and Brann MR (1991) Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* **40:**242–247.

Kelly LA and Butcher RA (1979) Effects of Δ^1-tetrahydrocannabinol on cyclic AMP in cultured human diploid fibroblasts. *J Cyclic Nucleotide Res* **5:**303–313.

Lynn AB and Herkenham M (1994) Localization of cannabinoid receptors and non-saturable high-density cannabinoid binding sites in peripheral tissues of the rat: Implications for receptor-mediated immune modulation by cannabinoids. *J Pharmacol Exp Ther* **268:**1612–1623.

Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* **89:**3825–3829.

Mackie K, Devane WA and Hille B (1993) Anandamide, an endogenous cannabinoid, inhibits N-type calcium currents in N18 cells as a partial agonist. *Mol Pharmacol* **44:**498–503.

Mackie K, Lai Y, Westenbroek R and Mitchell R (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci* **15:**6552–6561.

Martin BR (1986) Cellular effects of cannabinoids. *Pharmacol Rev* **38:**45–74.

Matsuda LA, Lolait SJ, Brownstein MJ, Young A and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346:**561–564.

Mechoulam R (1986) *Cannabinoids as Therapeutic Agents*. CRC Press, Boca Raton, FL.

Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365:**61–65.

Pacheco M, Childers SR, Arnold R, Casiano F and Ward SJ (1991) Aminoalkylindoles: Actions on specific G-protein-linked receptors. *J Pharmacol Exp Ther* **257:**170–183.

Pertwee R, Griffen G, Fernando S, Li X, Hill A and Makriyannis A (1995) AM630, a competitive cannabinoid receptor antagonist. *Life Sci* **56:**1949–1955.

Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, Ferrara P, Soubrie P, Breliere JC and Le Fur G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350:**240–244.

Shire D, Carillon C, Kaghad M, Calandra B, Rinaldi-Carmona M, Le Fur G, Caput D and Ferrara P (1995) An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. *J Biol Chem* **270:**3726–3731.

Tang W-J. and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* **254:**1500–1503.