Cannabinoid Agonists Stimulate Both Receptor- and Non-Receptor-Mediated Signal Transduction Pathways in Cells Transfected with and Expressing Cannabinoid Receptor Clones

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SUMMARY

The physiologic activity of $(-)$-Δ^9-tetrahydrocannabinol, the most active component of marijuana, and of many synthetic cannabimimetics may be mediated either through receptor binding and functional coupling to specific signal transduction pathways or through nonspecific interaction with cell membrane components. The cloning of the human and rat cannabinoid receptors has provided the opportunity to investigate the binding properties and signal transduction pathways directly associated with these receptors. Cannabinoid receptor cDNA was transfected into and stably expressed in fibroblast cell lines that do not contain native cannabinoid receptors, thus allowing comparison with untransfected cells. Binding constants measured using [^{3}H]CP55,940 indicated that the rat and human cloned cannabinoid receptors were similar to native cannabinoid receptors measured in brain and neural cell lines. The cloned receptors coupled to the inhibition of cAMP accumulation, as previously demonstrated. CP55,940 binding and inhibition of cAMP accumulation were absent in untransfected cells. Cannabinoid agonist-stimulated release of arachidonic acid and increase in intracellular calcium were observed in both transfected and untransfected cells. Stereoselectivity of cannabinoid agonists was demonstrated for binding and functional inhibition of cAMP accumulation, but not for the release of arachidonic acid and intracellular calcium. Therefore, cannabinoid agonists can stimulate signaling pathways through both receptor- and non-receptor-mediated pathways in the same cell.

Marijuana has been used recreationally and therapeutically for centuries with little knowledge of its mechanism of action. After the elucidation of the structure of Δ^9-THC (1), the active principle in marijuana, a number of more potent synthetic cannabinoids were developed and shown to have behavioral effects in both animals and humans similar to those of Δ^9-THC (2). Using these synthetic compounds, Howlett and co-workers (3, 4) described and pharmacologically characterized membrane-associated receptors that bind to cannabinoid agonists stereospecifically and with a rank order of potency similar to their behavioral effectiveness. They were also able to demonstrate that cannabinoid receptors couple to the inhibition of cAMP accumulation (3, 4). Distribution of cannabinoid receptor binding in specific brain regions was demonstrated by Herkenham *et al.* (5) by autoradiography. This further substantiated that the known central nervous system effects of can-

nabinoids were mediated at least in part through receptor binding. Because of their high lipid solubility, cannabinoids have been reported to partition into cell membranes and alter the activity of a number of membrane-associated enzymes, providing an alternate non-receptor-mediated mechanism of action (6).

The recent cloning of the cannabinoid receptor (7) has provided an opportunity for direct analysis of receptor binding properties and coupling to signal transduction pathways through functional expression of cannabinoid receptor clones in cell lines devoid of the receptor. With untransfected cells as a control, it is now possible to compare cannabinoid receptor- or non-receptor-mediated actions of cannabinoid agonists within the same cell. In this report, comparisons have been made between the cloned rat and human cannabinoid receptors expressed in CHO and L cell fibroblasts and cannabinoid

eptors previously identified in brain preparations. CHO cells expressing the cannabinoid receptor have been used as a ntrol to evaluate non-receptor-mediated effects. Natural and nthetic cannabinoid agonists were selected based on their uctural diversity and were examined for displacement of [³H] 55,940 binding and functional inhibition of cAMP accumu- ion. Cannabinoid agonists were also tested for their ability modulate signal transduction pathways mediated through ospholipase A_2, phospholipase C, or calcium mobilization.

Experimental Procedures

terials

P55,940, CP50,556 (levonantradol), and CP55,244 were generously ided by Dr. Larry Melvin (Pfizer Inc., Groton, CT.); WIN55212-2 l WIN55212-3 by Dr. Susan Ward (Sterling-Winthrop Research titute, Rensselaer, NY); HU-210, HU-211, RM-7, and RM-8 by Dr. phael Mechoulam (Hebrew University of Jerusalem, Jerusalem, ael); 2-iodo-Δ^8-THC and 2-iodo-Δ^8-THC-dimethylheptyl by Dr. Al- ndros Makriyannis (University of Connecticut School of Pharmacy, rrs, CT); (−)-Δ^9-THC and (+)-Δ^9-THC by the National Institute of Drug Abuse; nabilone by Eli Lilly (Indianapolis, IN); O,2-propano- THC by Dr. Herbert Seltzman (Research Triangle Institute, Re- rch Triangle Park, NC) and Dr. Patricia Reggio (Kennesaw State lege, Marietta, GA); THC-7-oic acid by Dr. Sumner Burstein (Uni- sity of Massachusetts Medical School, Worcester, MA); and TMA Dr. Solomon Snyder (Johns Hopkins University, Baltimore, MD). I]CP55,940 and [5,6,8,9,11,12,14,15-³H(N)]Arachidonic acid were rchased from New England Nuclear (Boston, MA) and myo-[2- N)]inositol from American Radiolabeled Chemicals Inc. (St. Louis,). RO 20-1724, a phosphodiesterase inhibitor, was purchased from mol (Plymouth Meeting, PA). Fura-2/acetoxymethyl ester was pur- sed from Molecular Probes Inc. (Eugene, OR). All other reagents e purchased from Sigma (St. Louis, MO). Plasmids hSKR6p1 and muARS-4 were provided by Dr. Tom Bonner (National Institute of ntal Health, Bethesda, MD) and Dr. Ulrich H. Weidle (Boehringer nnheim GmbH, Penzberg, FRG).

All assays were performed in glass test tubes, which were treated by king in dichlorodimethylsilane/toluene (1:10, v/v) for 1 hr and then 100% methanol for 30 min, followed by a final rinse with 100% hanol. Test tubes were allowed to air dry overnight before use.

ethods

Cell culture and stable expression of cannabinoid receptor nes. CHO cells and murine Ltk⁻ cells were obtained from The erican Type Culture Collection (Rockville, MD). CHO cells were intained as previously described (8). Ltk⁻ cells were cultured at 37° °C CO₂ in Dulbecco's modified essential medium (0.45% glucose) taining 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml icillin, and 50 µg/ml streptomycin. The rat cannabinoid receptor NA was stably expressed in CHO cells as previously described (7). human cannabinoid cDNA was stably expressed in L cells with following construct designed to amplify receptor expression levels, reby reducing the number of cells required for radioligand binding alysis. A 2.2-kilobase $SstI$-$EcoRI$ fragment containing the complete ling region of the human cannabinoid receptor gene was subcloned o the $SstI$ and $EcoRI$ sites of the pCD-PS vector (9) to create smid hSKR6p1. From hSKR6p1, a 3.3-kilobase $SalI$-$NdeI$ fragment removed, the ends were blunted, and the fragment was inserted to the blunted $SalI$ site of the plasmid ptkmuARS-4 (10). The sulting plasmid contained the receptor gene coding sequence flanked the simian virus 40 early region promoter and polyadenylation quence originally engineered into the cloning vector pCD (11). This asmid was transfected into murine Ltk⁻ cells by calcium phosphate recipitation (12). After transfection, L cells were selected in HAT ledium. Individual HAT-resistant colonies were isolated after 3-4

weeks, grown to established cell lines, and cultured for at least 3 months to allow for expression of the receptor to stabilize (13).

Plasma membrane preparation. Cells, grown to confluency in 175-cm² culture flasks, were washed once with cold phosphate-buffered saline and scraped in assay buffer (50 mM Tris, 5 mM MgCl₂, 2.5 mM EDTA, 5 mg/ml BSA, pH 7.4) with added 200 mM sucrose. Cells were then centrifuged at 1000 × g for 10 min at 4°. The supernatant was discarded and the pellet was resuspended in ice-cold assay buffer and homogenized with a Tekmar Tissumizer (Cincinnati, OH) at 95% maximal speed for 30 sec, followed by centrifugation at 2000 × g for 15 min at 2-4°. The supernatant was centrifuged again at 43,000 × g for 30 min. The pellet was resuspended in a minimal volume of assay buffer containing 200 mM sucrose and was stored at −80° until use.

Radioligand binding assays. Competition and saturation binding assays were performed with [³H]CP55,940 as the labeled ligand. A rapid filtration binding assay was developed based on a previously published method (14), with the following modifications. All ligands were diluted in assay buffer containing 50 mg/ml fatty acid-free BSA, with the final BSA concentration not exceeding 5 mg/ml. Assay solutions were in- cubated in silicone-treated test tubes for 1 hr at 30°, with a final assay volume of 0.5 ml and a final membrane concentration of 40–400 µg of protein/ml. Membranes were rapidly filtered over GF/B filters (What- man, Maidstone, England) that had been pretreated for 3 hr with 0.1% polyethyleneimine (v/v) (pH 7.4), using an Inotech (Lansing, MI) 96- or 48-position cell harvester. Membranes were washed with 3 × 3 ml of ice-cold wash buffer (50 mM Tris, 0.5 mg/ml BSA, pH 7.4). Filters containing washed membranes were transferred to scintillation vials, 1 ml of 0.1% (v/v) Triton X-100 was added to each vial, and vials were incubated overnight before addition of scintillation cocktail (Hydro- fluor; National Diagnostics, Manville, NJ). Protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce, Rockford, IL), as described (15). Binding data were analyzed with the program LIGAND (16) or with the program GraphPad (GraphPad Software, San Diego, CA), which performs weighted nonlinear least squares curve-fitting to the general model of Feldman (17).

Assay of [³H]arachidonic acid release and uptake. Cells were labeled overnight with [³H]arachidonic acid (0.25 µCi/ml) in growth medium, and release of free arachidonic acid was analyzed as previously described (18), with the following modifications. Cannabinoid ligands were diluted in silicone-treated glass test tubes with Eagles no. 2 buffer containing 20 mM HEPES and 50 mg/ml fatty acid-free BSA. The final BSA concentration was 5 mg/ml. Assays were performed in silanized glass, with approximately 1 × 10⁶ cells/reaction volume, over a period of 15 min at 37°. The reaction was stopped by cold immersion and centrifugation to separate cells from released [³H]arachidonic acid.

[³H]Arachidonic acid uptake experiments were initiated with the addition of [³H]arachidonic acid (0.25 µCi/ml) in growth medium. Cells were maintained under an atmosphere of 5% CO₂ for the duration of the experiment. Uptake was terminated by cold immersion and cen- trifugation, followed by two washes with ice-cold Eagles no. 2 buffer containing 20 mM HEPES and 2 mg/ml fatty acid-free BSA. Pellets were resuspended in 0.1 N NaOH before addition to scintillation vials for counting.

Assay of cAMP accumulation. Measurement of cAMP accumu- lation was performed as described previously (18), with the following modifications. Cannabinoid ligands were diluted in silicone-treated glass test tubes with Eagles no. 2 buffer containing 20 mM HEPES and 50 mg/ml fatty acid-free BSA. The final BSA concentration was 5 mg/ ml. Assays were performed in silicone-treated glass, with approximately 1 × 10⁶ cells/0.25-ml final assay volume, over a period of 5 min at 37°. The reaction was stopped with the addition of an equal volume of 0.1 N HCl, after which 50 µl were removed for radioimmunoassay of cAMP as previously described, without modification (18).

Measurement of intracellular calcium concentration in single fura-2-loaded cells. Cells grown on glass coverslips coated with Vitrogen (300 µg/ml; Collagen Corp., Palo Alto, CA) were loaded with 5 µM fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR) for

30 min at 37° in growth medium. washed once. and stored in Eagles no. 2 medium containing 1 mg/ml BSA and 20 mM HEPES buffer. pH 7.4, at 25° for no more than 45 min before the experiment. Fura-2 fluorescence was measured photometrically at an emission wavelength of 510 nm. in a single cell mounted on a Nikon Diaphot microscope illuminated alternately with 340-nm and 380-nm light (bandpass. 4 nm). using a SLM-Aminco DMX-1000 spectrofluorometer (SLM-Aminco. Urbana. IL). Ratios of 340 nm/380 nm were converted to calcium concentrations based on calibration that was performed as previously described (19).

Measurement of inositol phosphate release. Cells were grown to approximately 80% confluence and labeled overnight with 1 μCi/ml [³H]inositol. Released [³H]inositol phosphates were measured as previously described (18), with the following modifications. Cannabinoid ligands were diluted in silicone-treated glass test tubes with Eagles no. 2 buffer containing 20 mM HEPES and 50 mg/ml fatty acid-free BSA. The final BSA concentration was 5 mg/ml. Assays were performed in silicone-treated glass with approximately 1×10^6 cells/assay, over a period of 15 min at 37°.

Results

Characterization of rat and human cannabinoid receptors. L cells and CHO cells were transfected with the human and rat cannabinoid receptors, respectively, and expression level and receptor affinity were determined in radioligand binding experiments. The human cannabinoid receptor was placed into a high expression plasmid construct and transfected into L cells in order to facilitate receptor characterization. Saturation binding experiments were performed in which [³H] CP55,940 was used to define total binding and unlabeled CP55,940 was added to saturate specific binding. Greater than 90% specific binding was observed in L cell plasma membranes, with binding constants of $K_d = 3.3$ nM and $B_{max} = 7.0$ pmol/ mg of protein (Fig. 1A). [³H]CP55,940 failed to display any significant binding to membranes prepared from untransfected L cells (data not shown), indicating that specific binding occurred only in L cells expressing the cannabinoid receptor. Similar binding experiments were conducted with CHO cells expressing the rat cannabinoid receptor and with plasma membranes prepared from rat cerebellum (Fig. 1, B and C). Binding constants were similar for all membranes tested and are presented in Fig. 1.

Competition experiments were conducted using various cannabinoid agonists that were tested for their ability to displace [³H]CP55,940 binding to cannabinoid receptor-containing L cell plasma membranes. The K_i values for both natural and synthetic agonists are listed in Table 1. (−)-Δ^9-THC is the natural active compound found in marijuana and (+)-Δ^9-THC is its less active stereoisomer. HU-210, an 11-OH-Δ^8-THC metabolite with a dimethylheptyl side chain modification, is the most potent synthetic analog yet synthesized (20). Competition curves for (−)-Δ^9-THC and for HU-210 are shown in Fig. 2A. Also included in this figure are their less active enantiomers, (+)-Δ^9-THC and HU-211. 2-Iodo-Δ^8-THC-dimethylheptyl, synthesized by Makriyannis and co-workers, was tested and found to have a lower affinity than the unmodified structure but retained high enough affinity to be used as a potential ¹²⁵I-labeled ligand (Table 1). A novel benzofuran structure, RM-8, and its less active stereoisomer, RM-7, displayed appropriate cannabinomimetic properties as previously described for these compounds (21). Four other analogs of Δ^9-THC (nabilone, 0,2-propano-Δ^8-THC, TMA, and THC-7-oic acid) were tested and displayed similar rank orders of potency as shown in previous

studies (2, 44, 45). Nonclassical cannabinoids unlike THC in structure (CP55,940, CP50,556, CP55,244, WIN55212-2, and WIN55212-3) were tested and compared with the natural cannabinoids.

Cannabinoid receptor activation has been shown to be linked to the inhibition of cAMP accumulation; therefore, functional
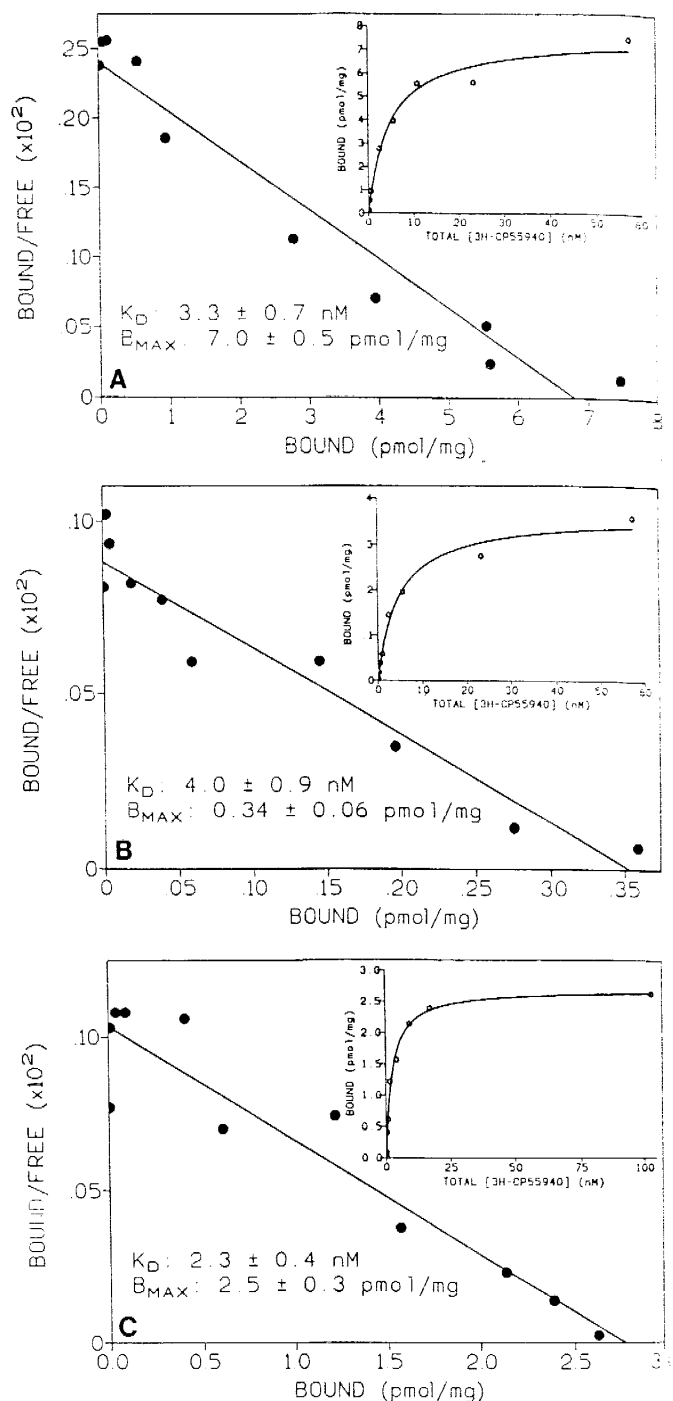
Fig. 1. Saturation binding analysis was performed as described in Experimental Procedures. using [³H]CP55,940 as the labeled ligand and 100 nM unlabeled CP55,940 to define nonspecific binding. *Inset.* Saturation binding isotherms of specific binding. Plasma membranes were prepared from L cells expressing the human cannabinoid receptor (A) CHO cells expressing the rat cannabinoid receptor (B), or rat cerebellum (C). The data presented are representative of at least three experiments each performed in triplicate.

BLE 1

mparison of K_i and IC_{50} data for cannabinoid agonists

Agonists	K_i^a	IC_{50}^b
	nM	nM
IC analogs		
(−)-δ^9-THC	53 ± 0.1	55 ± 1.2
(+)-δ^9-THC	>900	>900
HU-210	0.06 ± 0.0002	0.02 ± 0.001
HU-211	1364 ± 14	191 ± 2.6
2-Iodo-δ^8-THC-dimethyl heptyl	164 ± 21	36.8 ± 1.4
RM-8	32.7 ± 2.5	8.5 ± 0.2
RM-7	3994 ± 53	2060 ± 24
Nabilone	79.1 ± 2.5	3.1 ± 0.1
0.2-Propano-δ^8-THC	262 ± 2.7	78.3 ± 4.2
TMA	>2000	>2000
THC-7-oic acid	>2000	>2000
onclassical cannabinoid agonists		
CP55,940	3.7 ± 0.1	1.8 ± 0.2
CP50,556	81.4 ± 0.9	2.4 ± 0.4
CP55,244	0.51 ± 0.02	0.2 ± 0.01
WIN55212-2	564 ± 12.5	24 ± 3.7
WIN55212-3	>3500	>2000

" Competition with [^{3}H]CP55,940.
Inhibition of cAMP accumulation.

pling of the cloned rat cannabinoid receptor expressed in HO cells was investigated using the same agonists used in the nding studies. Cannabinoid receptor-mediated inhibition of .MP accumulation was significantly reduced in L cells over-pressing the human receptor and, therefore, CHO cells were d for these studies. IC_{50} values for the cannabinoid receptor-diated inhibition of cAMP accumulation are shown in Table and selected curves are shown in Fig. 2B. Enantiomeric activity was also displayed in functional experiments. The antiomeric pair displaying the widest separation in affinity re the 11-OH-Δ^9-THC-dimethylheptyl derivatives HU-210 a HU-211. HU-210 also displayed the highest affinity for the nabinoid receptor both in competition experiments and in ctional assays, compared with all agonists tested. RM-7 and M-8, (−)-Δ^9-THC and (+)-Δ^9-THC, and the aminoalkylin-es WIN55212-2 and WIN55212-3 also displayed enantiom-c selectivity. 2-Iodo-Δ^9-THC-dimethylheptyl was less effec-e both in binding and in functional coupling to the canna-oid receptor than was HU-210. At the concentrations tested IC-7-oic acid, TMA, and WIN55212-3 were essentially in-ve at the cloned cannabinoid receptors (Table 1).

Cannabinoid agonists increase free arachidonic acid ease independently of receptor activation. Previous dies have suggested a role for cannabinoid agonists in unes-tified arachidonic acid release and membrane phospholipid nover. CHO cells transfected with the cannabinoid receptor re compared with untransfected control CHO cells to deter-ne whether the cannabinoid receptor was involved in the ase of arachidonic acid. Release of [^{3}H]arachidonic acid was mulated by CP55,940 in a concentration-dependent manner inning at 100 μM (Fig. 3A) in both transfected and untrans-ted CHO cells, suggesting a receptor-independent mecha-m. Concentrations above 1 mM exceed the solubility limit of '55,940, and 1 mM was, therefore, the highest concentration ted. Similar concentration-dependent release of arachidonic d was observed when cells were stimulated with the enan-meric agonists HU-210 and HU-211 (Fig. 3, B and C). These

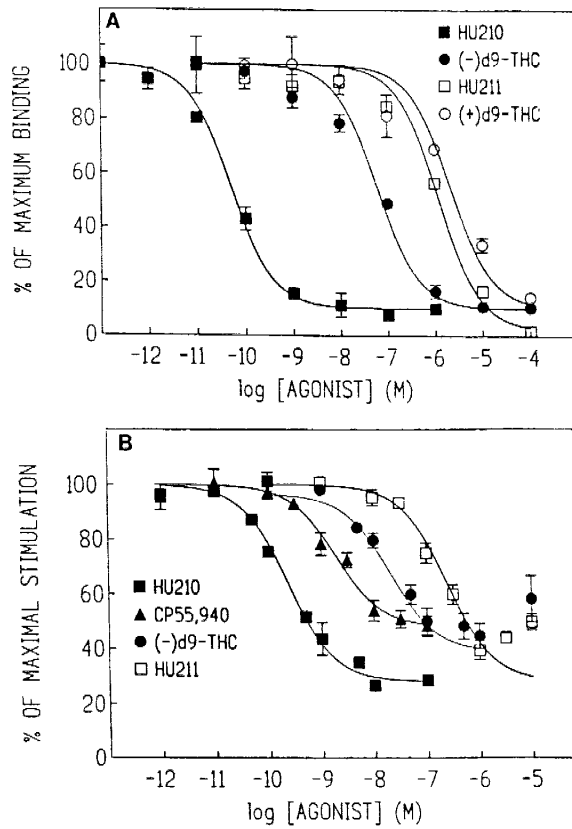**Fig. 2.** A, Competition experiments were performed in L cell plasma membranes expressing the human cannabinoid receptor, by displacing 500 pM [^{3}H]CP55,940 binding with HU-210 and (−)-Δ^9-THC and their less active enantiomers, HU-211 and (+)-Δ^9-THC, respectively. K_i values are provided in Table 1. Data are the mean and standard error of at least three experiments, each performed in triplicate. B, Inhibition of forskolin-stimulated cAMP accumulation was examined in CHO cells expressing the rat cannabinoid receptor. Data are the mean and standard error of at least three experiments, each performed in triplicate. IC_{50} data are presented in Table 1.

compounds demonstrated stereoselectivity in binding assays but not in the release of arachidonic acid. CHO cells transfected with the muscarinic m5 receptor were used as a positive control of receptor-mediated release of arachidonic acid (8). When cells were stimulated with the muscarinic agonist carbachol under identical assay conditions as those used for the cannabinoid receptor (Fig. 3D), there was a concentration-dependent release of arachidonic acid in m5 receptor-containing CHO cells but not in untransfected CHO cells. The carbachol-stimulated release of arachidonic acid was blocked by the antagonist atro-pine, further demonstrating a receptor-mediated process (Fig. 3D).

Elevated extracellular arachidonic acid levels might occur as a result of receptor-mediated release or by the inhibition of acyl-transferases responsible for incorporating arachidonic acid into phospholipids. The effect of cannabinoid agonists on ara-chidonic acid uptake was evaluated as an index of acyl-trans-ferase activity. HU-210 decreased [^{3}H]arachidonic acid uptake both in CHO cells expressing the cannabinoid receptor and in untransfected cells (Fig. 4). L cells expressing the human cannabinoid receptor demonstrated similar receptor-independ-ent arachidonic acid release and reduction in labeling with [^{3}H] arachidonic acid as seen in the CHO cells (data not shown), suggesting that the response was not cell specific. The uptake
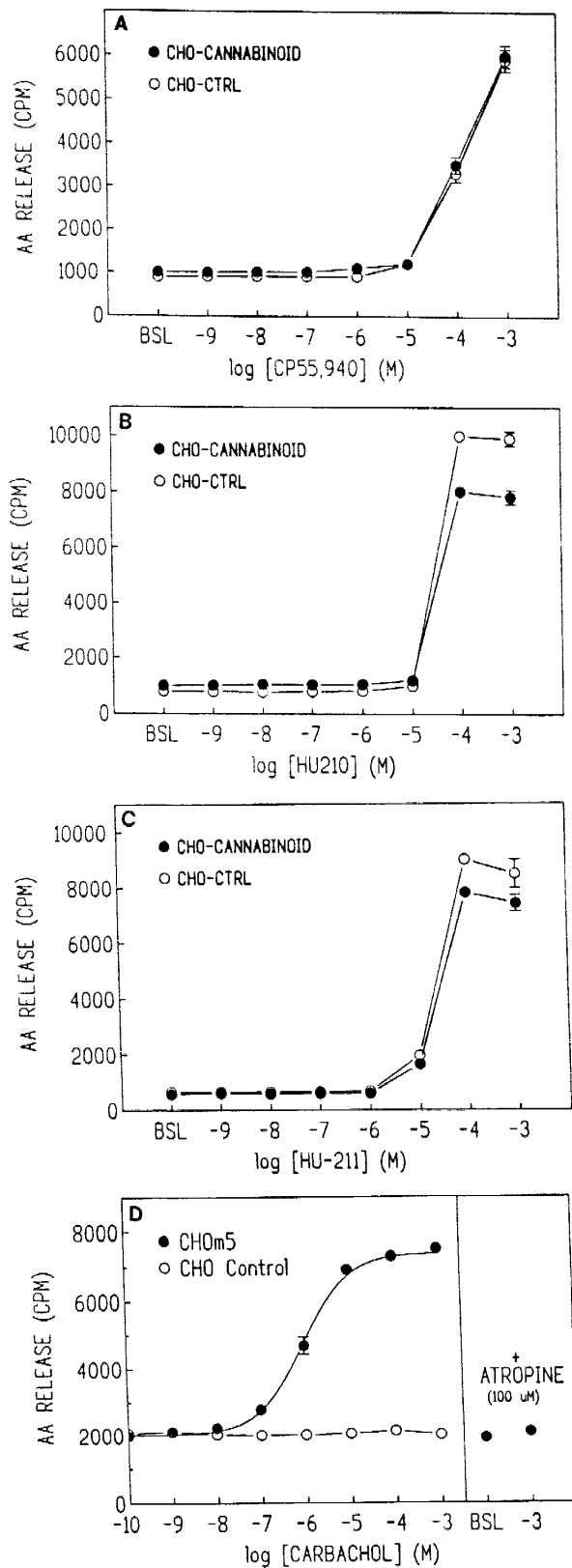
Fig. 3. A, B, and C, CHO cells expressing the rat cannabinoid receptor (*CHO-CANNABINOID*) or untransfected CHO cells (*CHO-CTRL*) were prelabeled overnight with [^{3}H]arachidonic acid (*AA*), and release was monitored over 15 min with increasing concentrations of the indicated cannabinoid agonist. D, CHO cells expressing the muscarinic m5 receptor (*CHOm5*) or untransfected CHO cells (*CHO-CTRL*) were prelabeled overnight with [^{3}H]arachidonic acid, and release was monitored over 15
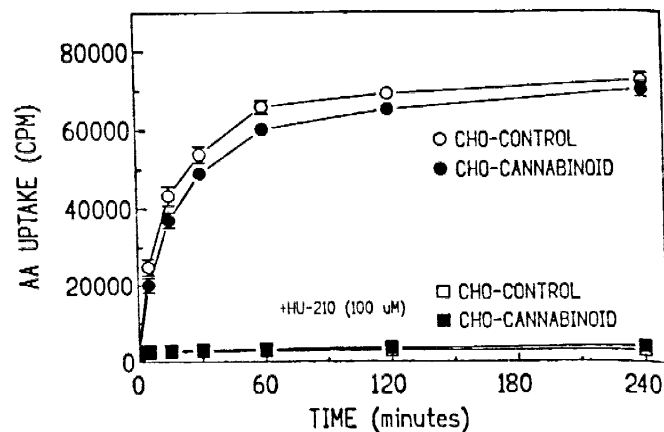
Fig. 4. [^{3}H]Arachidonic acid (*AA*) uptake was examined in CHO cells expressing the rat cannabinoid receptor and in untransfected control cells after the addition of HU-210. Data are the mean and standard error of at least three experiments, each performed in triplicate.
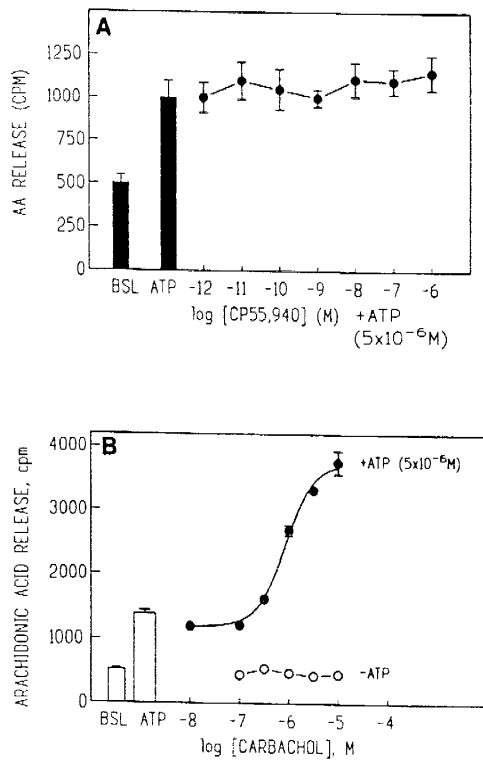
of [^{3}H]arachidonic acid in CHO cells expressing the muscarinic m5 receptor was identical with or without added carbachol and was examined under experimental conditions identical to those used for CHO cells expressing the cannabinoid receptor or untransfected cells. Therefore, arachidonic acid uptake inhibition by cannabinoid agonists was not mimicked by carbachol.

Recently, a novel transduction pathway has been described for receptors that normally couple to the inhibition of adenylate cyclase through the guanine nucleotide-binding protein G_i. The stimulation of G_i-coupled receptors results in the potentiation of ATP-stimulated arachidonic acid release (22, 23). In contrast to these findings, however, the cannabinoid receptor failed to augment ATP-stimulated release of arachidonic acid when stimulated with up to 10 μM CP55,940 (Fig. 5A). As a positive control, m2 muscarinic receptor-transfected CHO cells did augment ATP-stimulated arachidonic acid release when assayed under assay conditions identical to those used for the cannabinoid receptor-containing CHO cells. As previously shown, no augmentation of arachidonic acid release was seen after m2 receptor stimulation in the absence of ATP (Fig. 5B). HU-210 and WIN55212-2 also failed to augment arachidonic acid release in cannabinoid receptor-containing CHO cells at up to 100 μM (data not shown). These results indicate a functional difference between cannabinoid receptor signaling and signaling of other guanine nucleotide-binding protein-coupled receptors linked to the inhibition of adenylate cyclase and augmentation of arachidonic acid release [dopamine D2, muscarinic m2 and m4, α_2-adrenergic (23), and adenosine A1[1]].

Cannabinoid agonists do not increase inositol phosphate production. HU-210, at up to 100 μM, did not increase IP, IP_2, or IP_3 production over basal levels in CHO cells or L cells expressing the cannabinoid receptor. No increase was seen in untransfected cells. For comparison, carbachol, a muscarinic receptor agonist, increased IP, IP_2, and IP_3 levels in CHO cells expressing the m5 muscarinic acetylcholine receptor under

min with increasing concentrations of the muscarinic agonist carbachol. These cells were used as a positive control for receptor-activated release of arachidonic acid. Atropine, a muscarinic receptor antagonist, was added to block the m5 receptor-mediated arachidonic acid release. Data are the mean and standard error of at least three experiments, each performed in triplicate. *BSL*, basal.

[1] C. C. Felder and H. L. Williams, unpublished observations.

Fig. 5. Augmentation of ATP-stimulated release of [^{3}H]arachidonic acid AA) was examined in CHO cells expressing the rat cannabinoid receptor A) and cells expressing the muscarinic m2 receptor (B), which were ncluded as a positive control. Data are the mean and standard error of t least three experiments, each performed in triplicate.

onditions identical to those used for cannabinoid receptor timulation (data not shown).

Cannabinoid agonists induce a nonspecific release of ntracellular calcium. Cannabinoid-mediated changes in in-racellular free calcium concentrations were examined in CHO lls loaded with the calcium-sensitive fluorescent dye fura-2. iU-210 stimulated a concentration-dependent increase in in-acellular free calcium in cells transfected with the rat can-abinoid receptor (Fig. 6A). The increase in intracellular cal-ium originated from intracellular stores, because a similar annabinoid-mediated increase was observed in the absence of xtracellular calcium (Fig. 6B). Similar intracellular calcium elease was observed in control CHO cells stimulated with HU-10. with or without added calcium (Fig. 6, C and D). HU-211, he less active stereoisomer of HU-210, also stimulated an ncrease in intracellular calcium, further suggesting a receptor-ndependent process (Fig. 6, E and F). Similar effects were seen ith the addition of >100 nM Δ^9-THC and CP55,940 (data not hown).

Discussion

Pharmacological characterization of cloned human and rat annabinoid receptors expressed in mammalian cells indicates strong similarity of these receptors to those studied in rat rain and neural cell lines (24). The cloned receptors meet the ame criteria of saturability, stereoselectivity, and rank order f potency for the cannabinoid agonists tested. The pharma-cological profiles of classical and nonclassical cannabinoids were compared for their rank order of potency both in binding to receptors expressed in mammalian cell lines and in their

functional inhibition of cAMP accumulation. Rank orders of potency were similar to those of cannabinoid agonists evaluated by *in vivo* behavioral models of cannabinoid efficacy. For example, the stereoisomers of Δ^9-THC were shown to differ 10-100-fold in causing static ataxia in dogs, depressing schedule-controlled responding in monkeys, and inducing hypothermia in mice (25). HU-210 and HU-211 contain modifications to two important sites of the THC structure, i.e., hydroxylation at carbon-11 (26) and alkyl side chain conversion to dimethylhep-tyl (27). Our results with HU-210 compare with previous reports for [^{3}H]CP55,940 displacement and for the inhibition of cAMP accumulation in rat membranes and N18TG2 neuro-blastoma cells, respectively (20). Good enantiomeric selectivity between HU-210 and HU-211 was demonstrated by the cloned receptors in both binding and functional assays. A novel ben-zofuran structure, RM-8, and its less active stereoisomer, RM-7, displayed appropriate cannabimimetic properties, as previ-ously described for these compounds (21). Nabilone, an effec-tive antiemetic agent (28), contains the same dimethylheptyl side chain modification as does HU-210 but without the carbon-11 hydroxylation. Nabilone had activity similar to that of (−)-Δ^9-THC. TMA has been shown to display potent binding in brain preparations but lacks correlation with cannabinomi-metic activity in behavioral tests (29). TMA was inactive in our studies for both binding and function, suggesting a possible second cannabinoid binding site. THC-7-oic acid displays po-tent antiinflammatory, bronchodilatory, and analgesic proper-ties without the associated psychoactive side effects (30). This compound also inhibited cylooxygenase, 5-lipoxygenase (31), and the actions of platelet-activating factor (32), which may account for its antiinflammatory properties. THC-7-oic acid was inactive for both binding and function at the cloned can-nabinoid receptor. Its activity, like that of TMA, is unlikely to be due to interaction at the cannabinoid receptor. The binding and inhibition of cAMP accumulation for three nonclassical cannabinoids were tested, and their relative potencies (CP55,244 > CP55,940 > CP50,556) were consistent with those established previously in behavioral models (4, 33).

Cannabinoid receptor activation leads to the inhibition of adenylate cyclase, which was the first signal transduction path-way described as being associated with this receptor (34). Due to their hydrophobic nature, cannabinoid agonists may also exert their effects by interacting directly with cell membranes and membrane proteins (6). Transduction pathways associated with phospholipase A$_2$, phospholipase C, calcium mobilization, and adenylate cyclase were tested for activation by cannabinoid agonists simultaneously in cells expressing the rat cannabinoid receptor and in cells devoid of the receptor. Under these con-ditions, receptor-activated transduction would appear only in cells expressing the cannabinoid receptor and nonspecific ef-fects not related to receptor activation would appear in both transfected and untransfected cells.

Cannabinoid agonists inhibited cAMP accumulation in cells expressing the cloned receptors but not in untransfected cells. In contrast, cannabinoid agonists activated arachidonic acid release and an increase in intracellular calcium, as well as an inhibition of arachidonic acid uptake, in both transfected and untransfected cells, suggesting a receptor-independent mode of action. This was further substantiated when stereoisomers of potent cannabinoid agonists were equipotent at generating these responses. Cannabinoid agonists had no effect on phos-
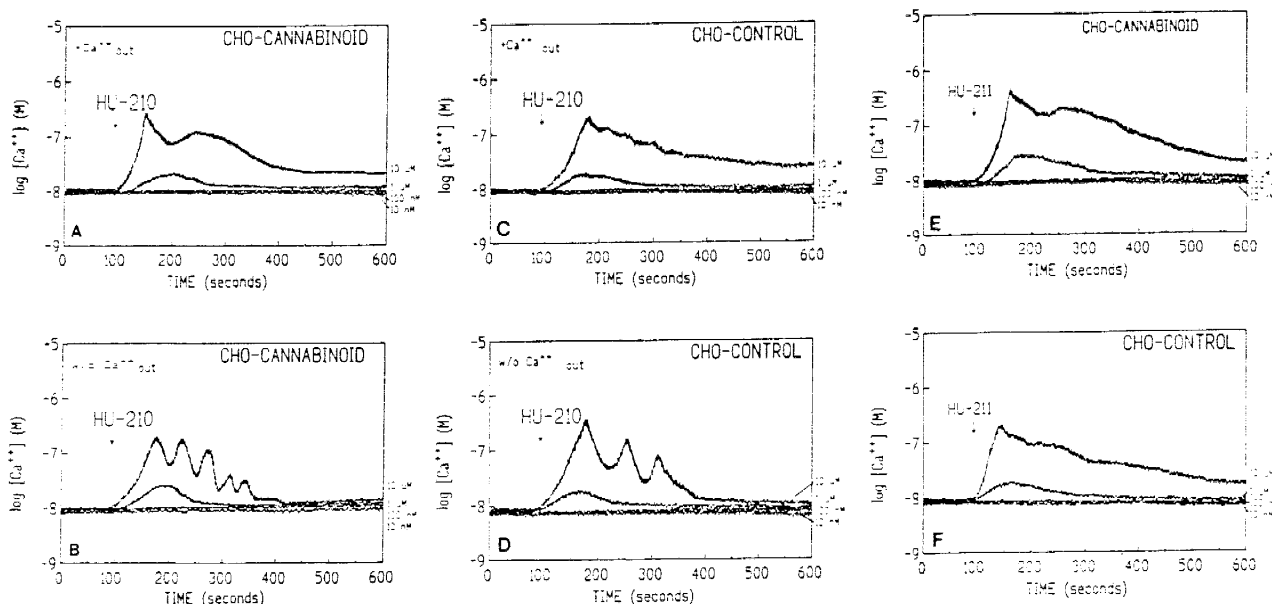
Fig. 6. Untransfected CHO cells (*CHO-CONTROL*) or CHO cells expressing the rat cannabinoid receptor (*CHO-CANNABINOID*) were loaded with the calcium-sensitive dye fura-2, and single-cell intracellular calcium concentration was measured over time. Four concentrations of the cannabinoid agonist HU-210 were added in the presence (A) or absence (B) of extracellular calcium. Similar experiments were performed on untransfected CHO cells in the presence (C) or absence (D) of extracellular calcium. Four concentrations of the minimally active cannabinoid agonist HU-211 were added to cannabinoid receptor-expression (E) or control (F) CHO cells. Data are representative of at least six experiments, and each *trace* is from a single cell. In all panels, the four concentrations of added agonist (starting from the *bottom trace*) are 10 nM, 100 nM, 1 μM, and 10 μM.

pholipase C activation, as measured by the generation of inositol phosphates. However, it is possible that small amounts of IP$_3$ may be generated that are not detectable with the currently available methodology. Cannabinoid-mediated release of arachidonic acid and subsequent conversion to eicosanoids have been described in both neural and non-neural cells (35–37). Our results confirm recent findings suggesting that cannabinoid agonist-stimulated release of arachidonic acid is not receptor mediated and may be due in part to the inhibition of free fatty acid reacylation (38). Because in our studies all [^{3}H]arachidonic acid was removed by multiple washes before the addition of agonists, and fatty acid-free BSA was included in the incubation medium to trap free arachidonic acid as it was released from the cell, it is possible that phospholipase A$_2$ was being activated concurrently with acyl-transferase inhibition.

Cannabinoids can initiate both receptor-dependent and receptor-independent signaling in the CHO cell. Receptor-dependent signaling requires nanomolar concentrations of agonist and leads primarily to the inhibition of cAMP accumulation. Receptor-independent effects require high concentrations of agonist and include the stimulation of arachidonic acid release and calcium mobilization. Phospholipase A$_2$ activation and arachidonic acid release have been shown to depend on an increase in intracellular calcium in the CHO cell (8), suggesting that cannabinoid agonist-mediated increases in intracellular calcium may play a role in the release of arachidonic acid. Although arachidonic acid has been shown to stimulate an increase in intracellular calcium in pancreatic islets (42), it is unlikely to be a factor in CHO cells, because almost all of the released arachidonic acid is either metabolized to eicosanoids or bound to extracellular BSA.[2] The increase in intracellular calcium was independent of extracellular calcium, suggesting

that its source is intracellular. The actions of cannabinoids may mimic those of thapsigargin, which penetrates the cell membrane to release intracellular calcium pools normally mobilized by IP$_3$ (39). The cannabinoid agonist-induced calcium oscillations observed in the absence of extracellular calcium are reminiscent of agonist-induced oscillations observed in many cell types. Several hypotheses have been introduced to explain oscillatory intracellular calcium fluxes, all of which require an initial increase in IP$_3$ (40, 41). Our data do not support a role for IP$_3$, because it was not detectable after cannabinoid agonist addition. Furthermore, at intracellular calcium concentrations of 300 nM achieved after muscarinic receptor stimulation in the CHO cells, significant increases in IP$_3$ are detectable.[2] It is not certain whether the micromolar concentrations of cannabinoids required to stimulate the non-receptor-mediated transduction pathways occur after ingestion or inhalation of marijuana. These levels of cannabinoids may be achieved, however, due to their hydrophobicity and tendency to accumulate in areas of high lipid density (43).

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[2] C. C. Felder, unpublished observation.

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