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Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction

(arachidonylethanolamide/adenylate cyclase/phospholipase A_2/calcium/calcium current)

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ABSTRACT **Arachidonylethanolamide (anandamide), a candidate endogenous cannabinoid ligand, has recently been isolated from porcine brain and displayed cannabinoid-like binding activity to synaptosomal membrane preparations and mimicked cannabinoid-induced inhibition of the twitch response in isolated murine vas deferens. In this study, anandamide and several congeners were evaluated as cannabinoid agonists by examining their ability to bind to the cloned cannabinoid receptor, inhibit forskolin-stimulated cAMP accumulation, inhibit N-type calcium channels, and stimulate one or more functional second messenger responses. Synthetic anandamide, and all but one congener, competed for [^{3}H]CP55,940 binding to plasma membranes prepared from L cells expressing the rat cannabinoid receptor. The ability of anandamide to activate receptor-mediated signal transduction was evaluated in Chinese hamster ovary (CHO) cells expressing the human cannabinoid receptor (HCR, termed CHO-HCR cells) and compared to control CHO cells expressing the muscarinic m5 receptor (CHOm5 cells). Anandamide inhibited forskolin-stimulated cAMP accumulation in CHO-HCR cells, but not in CHOm5 cells, and this response was blocked with pertussis toxin. N-type calcium channels were inhibited by anandamide and several active congeners in N18 neuroblastoma cells. Anandamide stimulated arachidonic acid and intracellular calcium release in both CHOm5 and CHO-HCR cells and had no effect on the release of inositol phosphates or phosphatidylethanol, generated after activation of phospholipase C and D, respectively. Anandamide appears to exhibit the essential criteria required to be classified as a cannabinoid/anandamide receptor agonist and shares similar nonreceptor effects on arachidonic acid and intracellular calcium release as other cannabinoid agonists.**

Both the psychoactive and medicinal properties of marijuana have been known for centuries, but not until the last decade has a clear mechanism of action been ascribed to Δ^9-tetrahydrocannabinol (THC), the active principle of marijuana. It is now known that THC and other more potent synthetic cannabinoid agonists bind to specific cannabinoid receptors and couple functionally to inhibit adenylate cyclase (1, 2) and inhibit N-type calcium channels via a pertussis toxin-sensitive guanine nucleotide binding protein (G protein) (3, 4). The existence of the cannabinoid receptor was corroborated with the cloning of a cannabinoid receptor gene from both rat and human (5, 6). To date only a single cannabinoid receptor gene has been identified and its nucleotide sequence indicates that it belongs to the superfamily of G-protein-coupled receptors. Expression studies indicate that the cloned receptor and the native receptor display similar binding and functional coupling to the inhibition of adenylate cyclase (5–7). The cannabinoid receptor is found primarily in brain tissue with the highest concentrations in the substantia nigra pars reticulata, globus palidus, and molecular layer of the cerebellum (8, 9). Cannabinoid receptors have also been identified in testis (6) and in spleen cells (10).

In a variety of cell types, cannabinoid agonists cause a concentration-dependent increase in arachidonic acid release presumably through a combination of phospholipase A_2 activation and acyltransferase inhibition (11–14). We have demonstrated (7) both receptor- and non-receptor-mediated activation of signal transduction pathways by cannabinoid agonists in mammalian cell lines transfected with and stably expressing the cannabinoid receptor. In these cells, cannabinoid-receptor-coupled signaling utilized adenylate cyclase as an effector enzyme, whereas noncannabinoid receptor effects included activation of phospholipase A_2 and intracellular calcium release.

Expression of cannabinoid receptors in the brain suggested the presence of an endogenous ligand and a normal physiological role for the receptor in brain function. Arachidonylethanolamide (anandamide), a candidate endogenous cannabinoid ligand, has recently been isolated from porcine brain and inhibited cannabinoid-agonist-specific binding to synaptosomal membrane preparations (15). Anandamide mimicked cannabinoid-induced inhibition of the twitch response in isolated murine vas deferens, suggesting it may be functionally similar to THC. However, functional activity of anandamide at the cannabinoid receptor and stimulation of one or more second messenger responses have not been definitively demonstrated. In this study, mammalian cells stably expressing the transfected human cannabinoid receptor were used to determine whether anandamide bound specifically to the cannabinoid receptor and coupled to the inhibition of cAMP accumulation. In addition, chemical modifications were made to both the arachidonyl and ethanolamide moieties of anandamide to establish the structurally important elements of anandamide required for activity. The efficacy of these analogs in inhibiting high-voltage-activated calcium currents (N-type calcium channels) in N18 neuroblastoma cells was also assessed. Finally, selectivity of the cannabinoid receptor for coupling to various signal transduction pathways was evaluated and comparison was made to the action of anandamide on muscarinic m5 receptor-transfected control cells.

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Abbreviations: HCR, human cannabinoid receptor; THC, Δ^9-tetrahydrocannabinol; G protein, guanine nucleotide binding protein.

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MATERIALS AND METHODS

[*Side chain*-2,3,4-^{3}H(N)]CP55,940 and [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid were purchased from New England Nuclear and *myo*-[2-^{3}H(N)]inositol and [9,10-^{3}H(N)]palmitic acid were from American Radiolabeled Chemicals (St. Louis). RO-20,1724, a phosphodiesterase inhibitor, was purchased from Biomol (Plymouth Meeting, PA). Fura-2 tetrakis(acetoxymethyl) ester was purchased from Molecular Probes. Fatty acids were purchased from Cayman Chemicals (Ann Arbor, MI). Arachidonamide and all other reagents were purchased from Sigma. (*R*)-(+)-{2,3-Dihydro-5-methyl-3-[(4-morphonolinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl}(1-naphthalenyl)methanone monomethanesulfonate (WIN 55,212-2) was a gift from Sterling. All assays were performed in glass test tubes, which were treated by exposure to dichlorodimethylsilane vapor under vacuum overnight.

Cell Culture and Stable Expression of Cannabinoid Receptor Clones. CHO and murine Ltk$^-$ (L) cells were obtained from the American Type Culture Collection and maintained as described (5, 16). The human cannabinoid receptor (HCR) cDNA (7) was stably expressed in CHO cells and L cells. N18 neuroblastoma cells (passage 32–44) were grown on coverglass fragments in Dulbecco's modified Eagle's medium/5% (vol/vol) fetal bovine serum and "differentiated" in DMEM/0.5% fetal bovine serum/2% (vol/vol) dimethyl sulfoxide for 1–2 weeks prior to recording.

Plasma Membrane Preparation and Radioligand Binding Assays. Plasma membranes were prepared from L cells expressing the human cannabinoid receptor as described (7). Competition binding assays were performed with [^{3}H]-CP55,940 as the labeled ligand. Radioligand binding of hydrophobic cannabinoid agonists was measured using a rapid filtration assay (7).

Synthesis of Anandamide and Congeners. Anandamide and various congeners were synthesized and purified by TLC as described (15). Briefly, the acid chloride of arachidonic acid and various other fatty acids was prepared by combination with oxalyl chloride (17), dissolved in methylene chloride, and added to a 10-fold molar excess of ethanolamine or other amino alcohols at 0°C for 15 min. The reaction mixture was washed five times with water, reduced in volume by evaporation under nitrogen gas, and purified by TLC (silica gel 60A, Whatman). Chemical structures were verified by GC/mass spectrometry and eluted as predominantly one peak.

Electrophysiological Recording. Whole-cell calcium currents, defined as the cadmium-sensitive fraction of the current elicited by a 25-ms step depolarization to 0 mV from a holding potential of −65 mV, were recorded as described (3). Anandamide and its congeners were first prepared as a 10 mM stock solution in ethanol. Working dilutions were prepared by successive dilution of the compounds in external recording solution containing 3 μM bovine serum albumin (fatty acid free) to the desired final concentration. All compounds were applied by bath perfusion from freely flowing reservoirs and solution changes were accomplished in <1 min.

Analysis of Second Messenger Pathways. Measurement of arachidonic acid release, cAMP accumulation, and inositol phosphate release was performed as described (7) and was used as an index of phospholipase A_2, adenylate cyclase, and phospholipase C activation, respectively. Changes in intracellular free calcium concentration were measured by loading cells with the calcium-sensitive fluorescent dye fura-2 as described (7). Phospholipase D activation was determined in suspended cells by measuring both phosphatidylethanol and phosphatidic acid release by modifications of a published method (18). Briefly, cells prelabeled for 18 h with [^{3}H]palmitic acid were washed twice with assay buffer (Eagle's 2 medium). Cells were incubated with or without anandamide for 15 min, in the presence of 1% ethanol, and the reaction was stopped by rapid centrifugation at 4°C. Phosphatidylethanol was extracted with methanol/chloroform/H_2O, 1:2:1 (vol/vol), and analyzed by TLC on silica gel 60A TLC plates developed in chloroform/pyridine/formic acid, 50:30:7 (vol/vol). Lipids that corresponded to authentic standards were detected by autoradiography, the spots were scraped, and radioactivity was measured in a scintillation spectrophotometer.

RESULTS

Binding and Functional Activity of Anandamide. Anandamide competed, in a concentration-dependent manner, for cannabinoid agonist [^{3}H]CP55,940 binding to plasma membranes isolated from L cells transfected with and expressing the human cannabinoid receptor (Fig. 1*A*). Only nonspecific [^{3}H]CP55,940 binding was observed in nontransfected control L cells; this binding was not displaced with up to 1 mM anandamide (data not shown). The ability of anandamide to elicit a functional response through the cannabinoid receptor was evaluated by its ability to inhibit forskolin-stimulated cAMP accumulation in CHO-HCR cells. Anandamide inhibited forskolin-stimulated cAMP accumulation in CHO-HCR cells (Fig. 1*B*) but not in control CHOm5 cells (data not

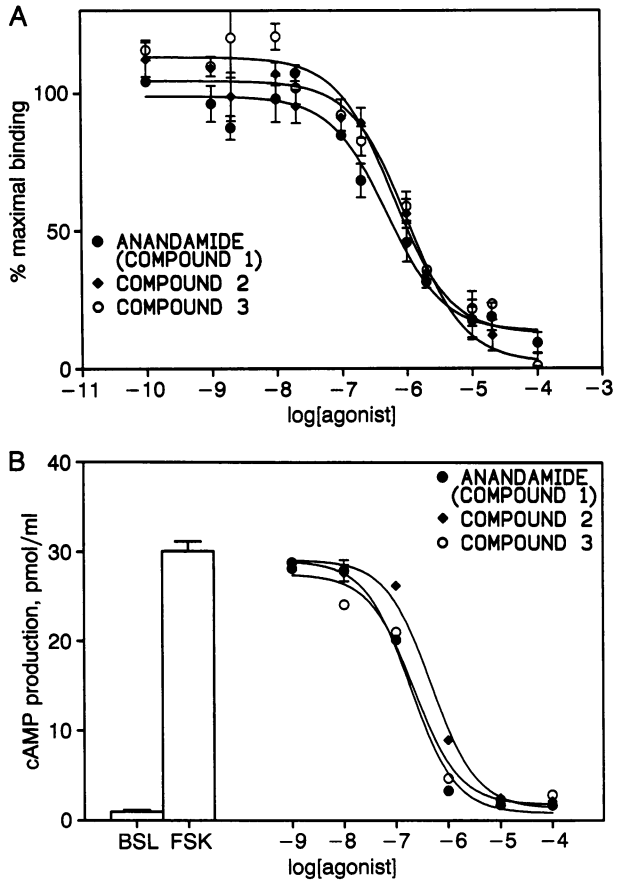

FIG. 1. (*A*) Competition for [^{3}H]CP55,940 binding by anandamide, compound 2, and compound 3 (Fig. 2) in L-cell membranes expressing the human cannabinoid receptor. [^{3}H]CP55,940 (50 pM) was incubated with competing compounds at 30°C for 1 h and bound ligand was separated from free by rapid filtration. Data are the mean ± SEM of triplicate determinations from a representative of three experiments. (*B*) Inhibition of forskolin (500 nM)-stimulated cAMP accumulation in CHO-HCR cells by anandamide, compound 2 and compound 3. cAMP accumulation was measured over 5 min in whole cells in suspension at 37°C and cAMP was quantitated by RIA. The results are the mean ± SEM of triplicate determinations from a single representative experiment. BSL, basal; FSK, forskolin.

shown). The CHOm5 cell was chosen as a control cell since it and the CHO-HCR cells had been subjected to similar transfection and selection procedures. Both the K_i value for binding and the IC_{50} value for inhibition of cAMP accumulation are shown for anandamide (compound 1) in Fig. 2.

Structure–Activity Studies. Anandamide is a combination of arachidonic acid and ethanolamine coupled through an amide linkage. Several congeners were chemically synthesized with substitutions for either the arachidonic acid moiety or for the ethanolamine moiety. The substitutions contained a carbon chain of various lengths and various numbers of double bonds. These chemical structures were tested for their ability to displace [^{3}H]CP55,940 binding to L-cell membranes expressing the human cannabinoid receptor and to inhibit forskolin-stimulated cAMP accumulation in CHO-HCR cells. Fig. 2 summarizes the K_i values for binding and the IC_{50} values for cAMP accumulation for each of these compounds. The upper half of Fig. 2 lists the data for modifications to the arachidonyl moiety of anandamide. Compounds are listed in descending rank order of potency. Anandamide and two compounds, in particular, in which dihomo-γ-linolenic acid (compound 2) and adrenic acid (compound 3) were substituted for arachidonic acid, displayed similar effectiveness in both binding and functional assays (Figs. 1 and 2). Compound 2 was also active in inhibiting the calcium current in N18 cells (Fig. 3). Compound 6 was synthesized with palmitic acid instead of arachidonic acid and was found to be inactive up to 1 mM. The lower half of Fig. 2 lists the data for modifications to the ethanolamide moiety of anandamide. Compound 7 was synthesized with 3-amino-1-propanol instead of ethanolamine, resulting in the addition of one carbon atom, and was found to be less active than anandamide (Figs. 2 and 3). Compounds 8 and 9 were synthesized with the racemic mixtures of DL-2-amino-1-propanol and DL-1-amino-2-propanol, respectively. Though less active than compound 7, it is possible that one of the enantiomers could display higher activity. Compound 10, arachidonamide, was considerably less active than anandamide.

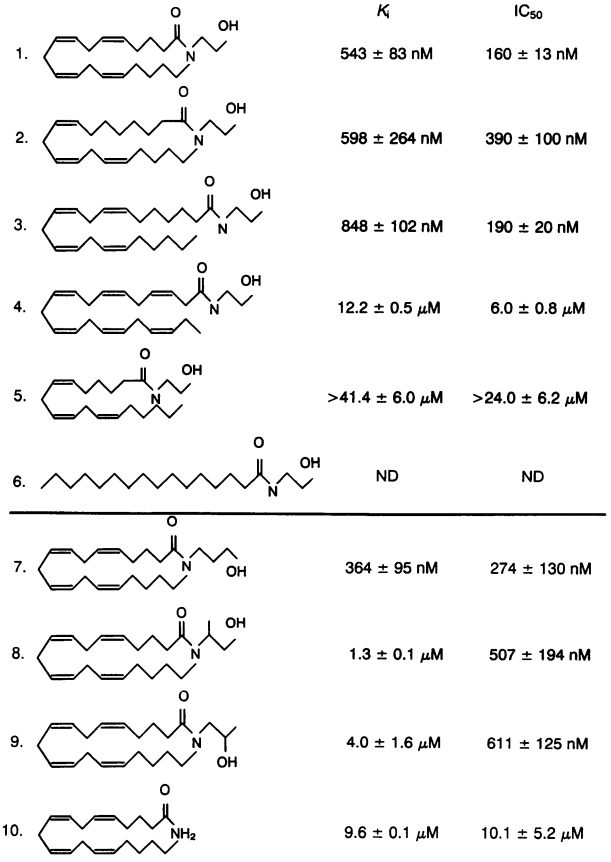

FIG. 2. Anandamide (compound 1) and congeners were examined for their ability to inhibit binding of [^{3}H]CP55,940 (50 pM) to L-cell membranes expressing the human cannabinoid receptor (K_i) and to inhibit forskolin-stimulated cAMP accumulation in CHO-HCR cells (IC_{50}). Compounds 2–6 contain fatty acid substitutions for arachidonic acid. Compounds 7–10 contain substitutions for ethanolamine. Substitutions are as follows: compound 2, dihomo-γ-linolenic acid; compound 3, adrenic acid; compound 4, docosahexaenoic acid; compound 5, γ-linolenic acid; compound 6, palmitic acid; compound 7, 3-amino-1-propanol; compound 8, DL-2-amino-1-propanol; compound 9, DL-1-amino-2-propanol; compound 10, arachadonamide. Data are the mean ± SEM of at least three experiments, each performed in triplicate except for compound 5, which was performed in duplicate. ND, no inhibition detected.

Anandamide and Congeners Inhibit N-Type Calcium Currents in N18 Cells. The ability of selected congeners to inhibit the high-voltage-activated calcium current in N18 neuroblastoma cells was assessed and compared to the inhibition by WIN 55,212-2 and anandamide. Compounds 2 and 7 were both slightly less potent than anandamide, but both inhibited a similar fraction of the calcium current (≈30%; Fig. 3). In contrast, WIN 55,212-2 was both more potent and more efficacious in inhibiting the calcium current than either compound 2 or 7. Compound 2 also behaved as a partial agonist in calcium current inhibition. During inhibition of the calcium current by 100 nM WIN 55,212-2, application of 1 μM compound 2 resulted in a partial recovery of the calcium current (38 ± 2%, n = 4; data not shown). Compound 5 was inactive in inhibiting the calcium current at concentrations up to 10 μM (Fig. 3). Thus, for the compounds examined, the ability of the anandamide congeners to inhibit calcium currents closely parallels their activity in binding to the cannabinoid receptor and inhibiting adenylate cyclase, suggesting a common receptor interaction.

Pertussis Toxin and cAMP Accumulation. Pertussis toxin blocks receptor-mediated inhibition of adenylate cyclase by ADP-ribosylating G_i type G proteins preventing the dissociation of their α from β/γ subunits (19). Pertussis toxin not only blocked anandamide-mediated inhibition of forskolin-stimulated cAMP accumulation but also unmasked a stimulation of cAMP accumulation above the forskolin-stimulated levels in CHO-HCR cells (Fig. 4). In the absence of forskolin, anandamide stimulated a more modest cAMP accumulation in pertussis toxin-treated CHO-HCR cells. Anandamide alone slightly stimulated cAMP accumulation at 100 μM, the

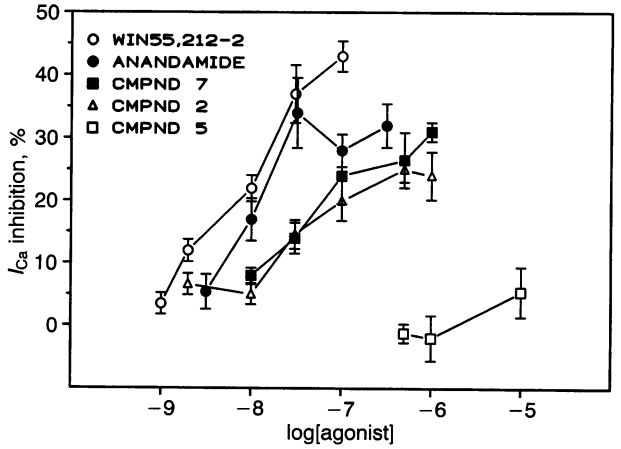

FIG. 3. Inhibition of calcium currents (I_{Ca}) in N18 neuroblastoma cells by cannabinoid agonists and anandamide analogues. Inhibition is expressed as a percentage of the total calcium current at 0 mV. Data are expressed as the mean ± SEM. Each point represents the results from 3 to 12 cells, with most determinations from 5 or 6 cells. CMPND, compound.

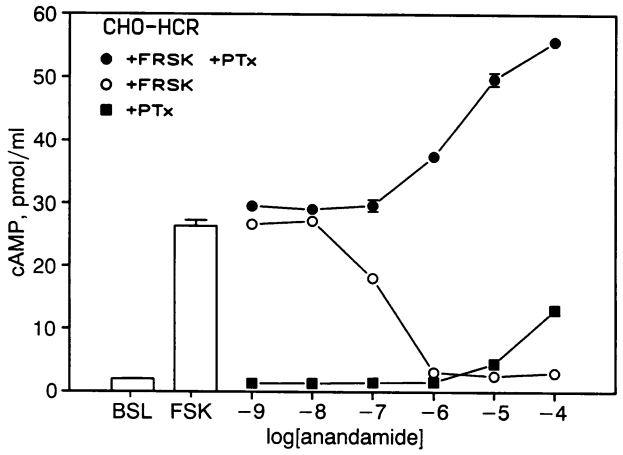

FIG. 4. Effect of pertussis toxin on anandamide plus forskolin and anandamide-stimulated cAMP accumulation in CHO-HCR cells. CHO-HCR cells were treated with or without pertussis toxin (PTx; 5 ng/ml) for 18 h and cAMP accumulation was measured over 5 min at 37°C. The results are the mean ± SEM of triplicate determinations from a single representative experiment. BSL, basal; FSK, forskolin.

highest concentration tested, but not at lower concentrations (basal = 1.1; 100 μM anandamide = 5.9 pmol/ml). Anandamide stimulated cAMP accumulation to a similar extent in CHOm5 cells and CHO-HCR cells in both the presence and absence of pertussis toxin, suggesting anandamide stimulation of adenylate cyclase was not mediated by the cannabinoid receptor (data not shown).

Anandamide and Receptor- vs. Non-Receptor-Mediated Signal Transduction. Cannabinoid agonists mediate cannabinoid-receptor-dependent inhibition of adenylate cyclase (7) and N-type calcium current (3, 4) and cannabinoid-receptor-independent activation of phospholipase A_2 and intracellular calcium mobilization (7). Similar investigations were conducted with anandamide, including signaling mediated by phospholipase D, in CHO-HCR cells and in CHOm5 cells. CHOm5 cells were selected as a control for signal transduction studies because they display muscarinic-receptor-mediated activation of phospholipase A_2, C, and D, adenylate cyclase, calcium influx, and intracellular release of calcium (20). Anandamide stimulated the release of arachidonic acid in both CHO-HCR and CHOm5 cells beginning at 1 μM and saturating at 100 μM, suggesting a non-cannabinoid-receptor-mediated stimulation of phospholipase A_2 (data not shown). Anandamide stimulated the release of intracellular calcium at 10 μM, but not at lower concentrations, in both CHO-HCR and CHOm5 cells, suggesting a non-cannabinoid-receptor-mediated effect (data not shown). No effect of anandamide was observed for the release of inositol phosphates, phosphatidic acid, or phosphatidylethanol in either CHO-HCR or CHOm5 cells (data not shown). A transduction pathway was recently demonstrated for G_i-coupled receptors that augments a previously stimulated arachidonic acid release (21). Anandamide was examined as a possible modulator of ATP-stimulated arachidonic acid release acting through the cannabinoid receptor. Anandamide did not augment ATP-stimulated arachidonic acid release in either CHO-HCR or CHO control cells expressing the m2 muscarinic receptor (data not shown). Carbachol, a muscarinic receptor agonist, did augment ATP-stimulated arachidonic acid release in CHOm2 cells under identical assay conditions (7).

DISCUSSION

Anandamide, the first candidate endogenous ligand reported for the cannabinoid receptor, was isolated from porcine brain, its structure was determined by mass spectrometry and NMR and verified by chemical synthesis, and its functional identity was demonstrated with receptor binding and inhibition of vas deferens twitch response (15). In this study, anandamide was evaluated as a possible cannabinoid agonist through radioligand binding analysis and functional coupling to adenylate cyclase inhibition in clonal mammalian cell lines stably expressing the human cannabinoid receptor cDNA. Mammalian cell lines stably expressing the cannabinoid receptor have been proven to be suitable model systems in which to study cannabinoid receptor binding and functional responses because they are free of receptor subtype or cell-type heterogeneity typical of native tissues, and the same cell line transfected with a noncannabinoid receptor serves as an excellent control (7). The affinity of anandamide for the cloned human receptor was 10-fold less than that reported (15) in synaptosomal preparations, and anandamide was 2-fold less potent for the inhibition of cAMP accumulation in CHO-HCR cells compared to inhibition of the twitch response in the vas deferens. These discrepancies may reflect methodological differences between the two binding assays or possible metabolism of anandamide within cell membrane preparations reducing its availability for receptor interaction. The relatively low affinity of anandamide for this receptor in comparison to classical neurotransmitters, which typically exhibit low nanomolar affinities, may reflect some difference in the physiological role for this compound in the brain.

Anandamide appears to display binding and functional properties similar to previously studied cannabinoid agonists, including the ability to induce both receptor-mediated and non-receptor-mediated signal transduction. Anandamide inhibited a pertussis toxin-sensitive cAMP accumulation in CHO-HCR cells but not in CHOm5 cells, demonstrating that this functional response is mediated through cannabinoid receptor activation and that this response is coupled through a G_i type G protein. Anandamide has also been shown to inhibit a pertussis-toxin-sensitive N-type calcium current in N18 neuroblastoma cells (22). After pertussis toxin treatment, anandamide augmented forskolin-stimulated cAMP accumulation and stimulated basal levels of cAMP accumulation in both CHO-HCR and control CHOm5 cells, suggesting a receptor-independent mechanism. Cannabinoid agonists were shown (23) to stimulate adenylate cyclase activity over a concentration range similar to that shown for anandamide. Additional studies have shown a receptor-dependent stimulation of adenylate cyclase, after pertussis toxin treatment, by inhibitory receptors such as muscarinic m4 and α_2 adrenergic receptors, although a mechanism for this response has not been clearly identified (24–26). Stimulation of type II or IV adenylate cyclase can be mediated by the G_s α subunit alone or in combination with G-protein β/γ subunits (27, 28). Calcium- and calmodulin-dependent regulatory mechanisms have been described for both type I and type III adenylate cyclases (29). Anandamide-stimulated calcium release may modulate this class of adenylate cyclases. It is not known whether any of the G-protein-dependent mechanisms mediate anandamide-dependent adenylate cyclase stimulation in the CHO cell.

Extensive structure–activity studies have been performed on classical cannabinoid agonists such as THC (30, 31). A number of structurally important functional groups were identified, including hydrophilic hydroxyls when situated at positions 1 and 11 and a long carbon side chain situated at position 3 of the dihydrobenzopyran ring structure of THC. Anandamide, though structurally dissimilar to THC, may contain similar functional groups that play a role in binding activity. A limited structure–activity relationship study was undertaken to provide preliminary information about elements of the molecule important for binding and function. Modifications were made to both the arachidonyl and the ethanolamide moieties of the molecule through the synthetic

combination of compounds with various carbon chain lengths or degrees of saturation. Small modifications to the arachidonic acid moiety, as in compound **2** (dihomo-γ-linolenylethanolamide) having only three double bonds or compound **3** (adrenylethanolamide) having two more methylene groups, did not greatly alter activity. Compounds **2** and **3** were synthesized because both dihomo-γ-linolenic acid and adrenic acid have been detected in mammalian brain (32), although it is not known whether either compound naturally forms amides with ethanolamine *in vivo*. The large difference in activity between compound **3** and compound **4** suggests that the end pentyl chain may play a role similar to the pentyl side chain of THC. More extensive studies will be required to fully evaluate the structurally important elements of anandamide in binding and function. Such knowledge may be helpful in designing cannabinoid antagonists; to our knowledge, no cannabinoid antagonists are known.

Anandamide fits essential criteria to establish it as an endogenous agonist for the cannabinoid receptor. Anandamide has been shown to display saturable and specific binding, to stimulate functional second messenger responses, and to mimic the action of cannabinoid agonists in several behavioral paradigms (33). G-protein-coupled receptors can be generalized to belong to either the inhibitory group of receptors, which inhibit adenylate cyclase, or to the calcium mobilizing group of receptors, which can activate membrane-associated phospholipases leading to changes in intracellular calcium. The cannabinoid/anandamide receptor appears to exhibit the properties of an inhibitory receptor except for its inability to modulate the release of arachidonic acid in the CHO cell model as shown for other inhibitory receptors (21).

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