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EFFECTS OF ANANDAMIDE ON CANNABINOID RECEPTORS IN RAT BRAIN MEMBRANES

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Abstract—Anandamide (arachidonylethanolamide) is a compound recently isolated from porcine brain as a putative endogenous ligand at cannabinoid receptors. The present studies examined the effects of anandamide on cannabinoid receptor binding sites and adenylyl cyclase in rat brain membranes. Receptor binding experiments, conducted at 25° for 90 min, apparently resulted in significant degradation of anandamide, since anandamide (10 μM) had little effect on [^{3}H]WIN 55212-2 binding in cerebellar membranes. Addition of the general serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) protected against this degradation, resulting in an IC_{50} value of 90 nM for anandamide versus [^{3}H]WIN 55212-2 binding. Anandamide inhibited adenylyl cyclase in cerebellar membranes in a GTP-dependent manner, exhibiting a maximal inhibition level slightly less than that of WIN 55212-2 and CP-55,940, with an IC_{50} value of 1.9 μM. The effect of anandamide on adenylyl cyclase was region-specific, with maximal inhibition occurring in cerebellum and striatum. These results suggest that anandamide acts at G-protein-coupled cannabinoid receptors in brain with properties similar to those of exogenous cannabinoids.

Key words: Δ-tetrahydrocannibinol; adenylyl cyclase; arachidonylethanolamide; phenylmethylsulfonyl fluoride

The discovery that THC† and more potent cannabinoid analogs bound to specific receptors [1–3] represented an important breakthrough in elucidating the mechanism of action of cannabinoids. The subsequent cloning of these receptors by Matsuda *et al.* [4] established the cannabinoid receptor as a typical member of the G-protein-coupled superfamily of receptors. Like many other members of this superfamily, cannabinoid agonists inhibit adenylyl cyclase [1, 2, 5–7]. Moreover, recent studies have shown that cannabinoid receptors are negatively coupled to Ca^{2+} channels in NG108-15 cells [8, 9] and positively coupled to potassium A channels in hippocampal neurons [10]. Although these studies have clearly established the specificity of these sites by demonstrating that the regional distribution in brain [11–14] and the pharmacology [15] of these sites correlate with many of the known behavioral effects of THC in animals, the major unsolved problem in this area has been the question of whether endogenous ligands exist for these receptors in mammalian brain. Preliminary studies by Evans *et al.* [16] showed that stimulation of brain slices released material that inhibited cannabinoid receptor binding in brain membranes. However, the structure of this material was not known then.

Recently, Devane *et al.* [17] reported the structure of a putative endogenous cannabinoid ligand, anandamide. This compound was the ethanolamine amide of arachidonic acid, and inhibited cannabinoid receptor binding to brain membranes with an IC_{50} value of 90 nM. It also inhibited electrically induced contractions of the mouse vas deferens like traditional cannabinoid ligands. However, pharmacological information about anandamide was relatively limited since its binding potency was examined against only one cannabinoid ligand. Moreover, no information was known about its ability to inhibit adenylyl cyclase. A recent study [18] showed that anandamide inhibited adenylyl cyclase in COS cells transfected with cannabinoid receptors. The present studies examined these issues in rat brain membranes, and demonstrated not only that anandamide inhibits adenylyl cyclase in brain with properties consistent with a cannabinoid ligand, but also showed that receptor binding assays with anandamide must be conducted in the presence of amidase inhibitors.

MATERIALS AND METHODS

[^{3}H]WIN 55212-2 binding was conducted in rat cerebellar membranes according to the method of Haycock *et al.* [19] and Kuster *et al.* [20]. Male Sprague–Dawley rats (150–200 g; Zivic-Miller, Zeleinople, PA) were decapitated and the cerebella were dissected quickly on ice. The tissue was homogenized in 10 mL of HEPES-Mg^{2+} buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.0), with a Polytron homogenizer and centrifuged at 48,000 *g* for 10 min. To inhibit anandamide breakdown, 50 μM PMSF (diluted in buffer from a 10 mM stock solution in *n*-butanol) was added to the HEPES

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† Abbreviations: THC, Δ^9-tetrahydrocannabinol; PMSF, phenylmethylsulfonyl fluoride; and BSA, bovine serum albumin.

buffer before homogenization of tissue. The pellet was then resuspended in HEPES-Mg^{2+} buffer and centrifuged at 48,000 *g* for 10 min; the resulting pellet was resuspended in HEPES-Mg^{2+} buffer to provide a final concentration of 2.5 mg protein/mL in the assay tubes. [^{3}H]WIN 55212-2 (60 Ci/mmol; New England Nuclear, Boston, MA) was diluted in HEPES-Mg^{2+} buffer containing 5 mg/mL BSA. Membranes were added to assay tubes containing 80,000 dpm (0.67 nM) of [^{3}H]WIN 55212-2 and various drugs in HEPES-Mg^{2+} buffer containing 0.1% BSA, with a total volume of 1 mL. Non-specific binding was determined in the presence of 1 μM unlabeled WIN 55212-2.Tubes (in triplicate) were incubated at 25° for 90 min and filtered through Whatman GF/B filters presoaked in HEPES buffer containing 5 mg/mL BSA. The filters were washed three times with 5 mL each of HEPES buffer containing 0.05% BSA, and radioactivity was determined by liquid scintillation spectrophotometry (50% efficiency) after overnight extraction of the filters in 5 mL of Ecolite (ICN) scintillation fluid. The typical binding experiment resulted in 4400 dpm total binding, with 360 dpm non-specific binding, thus providing 0.13 pmol [^{3}H]WIN 55212-2 binding/mg protein.

Adenylyl cyclase was determined in rat brain membranes as previously described [6]. Briefly, male Sprague–Dawley rats (150–200 g) were decapitated and cerebella or selected brain regions were dissected quickly on ice. Because PMSF had little effect on anandamide inhibition of adenylyl cyclase (see Results), these membranes were not routinely prepared with PMSF. The tissue samples were homogenized in a Polytron homogenizer in assay buffer (50 mM Tris–HCl, 2 mM $MgSO_4$, pH 7.4) and centrifuged at 20,000 *g* for 10 min. Membranes were pretreated at low pH as previously described [21, 22] by incubating membranes in pH 4.5 buffer (50 mM sodium acetate, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 4.5) on ice for 10 min. The low pH treatment was terminated by the addition of a 10-fold excess volume of cold assay buffer (pH 7.4), and centrifugation at 48,000 *g* for 10 min. The resulting membranes were resuspended in assay buffer containing 10 mM theophylline, 50 μM GTP, 30 μM cAMP, 100 μM ATP (with 1 μCi [^{3}H]ATP), 20 mM creatine phosphate and 10 U of creatine phosphokinase in a total volume of 100 μL. Adenylyl cyclase activity was assayed as previously described [23]. Protein values were determined by the method of Bradford [24]. Results were expressed as percent of basal adenylyl cyclase activity. In both binding and adenylyl cyclase experiments, IC_{50} values represent mean values ± SEM of at least three separate experiments.

Anandamide was prepared as a stock solution of 10 mg/mL (29 mM) in 95% ethanol; the working solution was 1 mM in 25% ethanol. In the adenylyl cyclase assays, the highest concentration of anandamide was 40 μM, containing 1% ethanol. Control experiments (not shown) revealed no effect of ethanol on basal adenylyl cyclase at this concentration. Maximal levels of ethanol were much lower in the receptor binding assays (0.025% ethanol), which had no effect on [^{3}H]WIN 55212-2 binding (not shown).

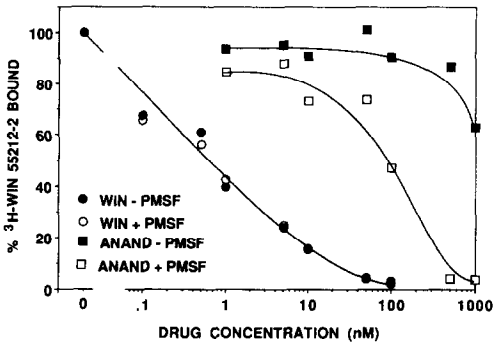

Fig. 1. Effect of PMSF on displacement of [^{3}H]WIN 55212-2 binding to rat cerebellar membranes by unlabeled WIN 55212-2 (WIN) and anandamide (ANAND). Membranes were pretreated with PMSF or buffer as described in Materials and Methods, and then incubated with [^{3}H]WIN 55212-2 and drugs to 90 min at 25°. Results are from a typical experiment that was replicated three times.

RESULTS

In previous studies [17], the ability of anandamide to displace radioligand binding to cannabinoid receptors utilized only one cannabinoid radioligand, [^{3}H]HU-243. To help determine its cannabinoid specificity, the present study examined its ability to displace binding of a structurally unrelated cannabinoid ligand, [^{3}H]WIN 55212-2 [13, 19, 20]. In preliminary assays, (data not shown), anandamide had little effect on [^{3}H]WIN 55212-2 binding in rat cerebellar membranes up to concentrations of 10 μM. Because it is a derivative of arachidonic acid, and likely to be metabolized quickly by amidase activities in brain membranes, binding experiments were performed in membranes treated with the general serine esterase/protease inhibitor, PMSF. The results (Fig. 1) showed a significant effect of PMSF on the binding potency of anandamide. In the absence of PMSF, anandamide had no effect on [^{3}H]WIN 55212-2 binding until 1 μM, where it inhibited binding by 35%. In membranes pretreated with 50 μM PMSF, anandamide displaced [^{3}H]WIN 55212-2 binding in a concentration-dependent manner, with an IC_{50} value of 90 ± 11 nM. The ability of unlabeled WIN 55212-2 to displace [^{3}H]WIN 55212-2 binding was not affected by PMSF, since it displayed an IC_{50} value of 0.45 ± 0.09 nM in both the presence and absence of PMSF.

Since cannabinoid receptors are present in relatively high density in the cerebellum [11, 12], cannabinoid agonists produce maximum inhibition of adenylyl cyclase in membranes from this region [6]. Figure 2 shows concentration–effect curves of two cannabinoid agonists, CP-55,940 and WIN 55212-2, compared with anandamide, in inhibiting adenylyl cyclase in cerebellar membranes. These data showed that anandamide inhibited adenylyl cyclase activity in a concentration-dependent

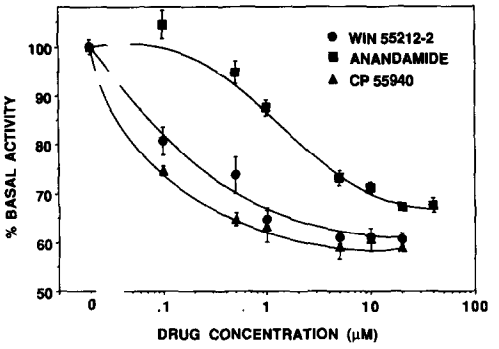

Fig. 2. Inhibition of adenylyl cyclase activity in rat cerebellar membranes by WIN 55212-2 CP-55,940 and anandamide. Adenylyl cyclase was assayed in the presence of the indicated concentrations of drugs in cerebellar membranes pretreated at pH 4.5 as described in Materials and Methods. Data are expressed as percent of basal activity (360 pmol/min/mg), and represent mean values ± SEM of three separate experiments.

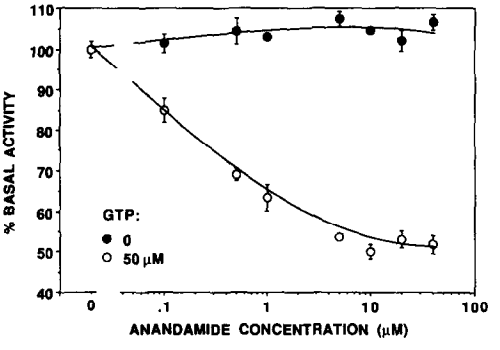

Fig. 3. Effect of GTP on inhibition of adenylyl cyclase activity in rat cerebellar membranes by anandamide. Adenylyl cyclase was determined in cerebellar membranes with various concentrations of anandamide, in the presence and absence of 50 μM GTP. Data are expressed as percent of basal activity (385 pmol/min/mg), and represent mean values ± SEM of three separate experiments.

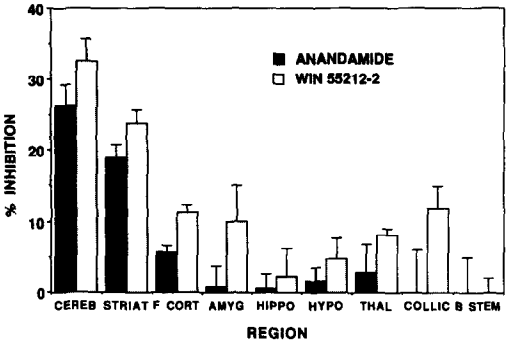

Fig. 4. Regional distribution of anandamide and WIN 55212-2 inhibition of adenylyl cyclase in rat brain. Various rat brain regions were dissected, treated at pH 4.5, and assayed with 5 μM WIN 55212-2 or 20 μM anandamide, as described in Materials and Methods. Abbreviations (with basal activity indicated in parentheses) for regions: CEREB, cerebellum (302 pmol/min/mg); STRIAT, striatum (110 pmol/min/mg); F CORT, frontal cortex (212 pmol/min/mg); AMYG, amygdala (243 pmol/min/mg); HIPPO, hippocampus (259 pmol/min/mg); HYPO, hypothalamus (285 mol/min/mg); THAL, thalamus (272 pmol/min/mg); COLLIC, superior colliculus (262 pmol/min/mg); and B STEM, brain stem (92 pmol/min/mg). Data are expressed as percent of basal activity, and represent mean values ± SEM of three separate experiments.

manner. The maximal effects of both CP-55,940 and WIN 55212-2 on adenylyl cyclase activity were both the same (40 ± 3% of basal activity for CP-55,940, and 41 ± 3% for WIN 55212-2), whereas the maximal effect of anandamide was slightly less (33 ± 4% of basal activity). The potency of anandamide was significantly lower than that of either WIN 55212-2 or CP-55,940: whereas the IC_{50} values of CP-55,940 and WIN 55212-2 were 0.06 ± 0.02 and 0.1 ± 0.07 μM, respectively, the IC_{50} value for anandamide was 1.9 ± 0.8 μM ($P < 0.05$ vs either CP-55,940 or WIN 55212-2 by Student's unpaired *t*-test).

To determine whether inhibition of adenylyl cyclase by anandamide occurred through G-proteins, the effect of anandamide on adenylyl cyclase was determined in the presence and absence of GTP (Fig. 3). In the presence of 50 μM GTP, anandamide inhibited adenylyl cyclase activity by approximately 40%, with a potency similar to that observed in Fig. 2. When GTP was omitted from the adenylyl cyclase assay, anandamide had no significant effect on basal adenylyl cyclase activity. These results confirmed that anandamide inhibited adenylyl cyclase in a GTP-dependent manner, and suggested that this inhibition occurred through inhibitory G-proteins. Other evidence of G-protein involvement came from low pH pretreatment of cerebellar membranes. Previous studies [21, 22] have shown that inhibition of adenylyl cyclase through several G_i-coupled receptors is increased by pretreatment of membranes at pH 4.5. When anandamide inhibition of adenylyl cyclase was compared in low pH pretreated versus untreated membranes, a similar increase in efficacy was observed (data not shown): maximal inhibition of adenylyl cyclase by anandamide in untreated membranes was 25%, compared with 45% maximal inhibition in low pH pretreated membranes. No effect of low pH pretreatment was observed on the potencies of these agonists in inhibiting adenylyl cyclase. In all subsequent experiments, all assays were performed in low pH pretreated membranes.

Previous studies comparing cannabinoid inhibition of adenylyl cyclase in different brain regions revealed that significant cannabinoid inhibition of activity was observed in cerebellum and striatum, with a smaller amount in frontal cortex and no significant inhibition of adenylyl cyclase in other brain regions [6]. To determine whether anandamide inhibited adenylyl cyclase with the same regional specificity as other cannabinoids, adenhylyl cyclase activity was assayed in nine different rat brain regions in the presence of either 20 μM anandamide or 5 μM WIN 55212-2 (Fig. 4). These results showed that anandamide

inhibited adenylyl cyclase with the same regional specificity as WIN 55212-2, with the largest inhibition occurring in cerebellum, followed by striatum and a small inhibition in frontal cortex. No significant inhibition for either cannabinoid agonist was observed in any other region. However, the maximal inhibition by anandamide was always slightly less than that of WIN 55212-2 in every region where there was significant inhibition by WIN 55212-2.

DISCUSSION

The finding that the potency of anandamide was increased significantly when brain membranes were treated with PMSF suggests that this derivative of arachidonic acid is metabolized rapidly under conditions of standard receptor binding assays (90 min at 25°). While the identities of the enzymes that degrade anandamide are not known at this point, these results would suggest that a significant amount of anandamide degradation is produced by an amidase containing a serine residue in the active site of the enzyme. The fact that Devane *et al.* [17] did not appear to detect significant anandamide degradation in their experiments probably arises from technical differences in binding assays. Devane *et al.* [17] used relatively low concentrations of brain membrane protein in their binding experiments. Such quantities of protein provide relatively low binding levels, and may have prevented some of the degradation of anandamide. Most groups use larger levels of membrane protein in their binding assays, and under those conditions should utilize inhibitors such as PMSF to obtain relevant binding data.

Interestingly, addition of PMSF had little effect on the potency of anandamide in inhibiting adenylyl cyclase in cerebellar membranes. In fact, the potency of anandamide is similar to that of THC in inhibiting adenylyl cyclase in cerebellum [7]. This lack of effect by PMSF in adenylyl cyclase assays may be due to the shorter incubation times for adenylyl cyclase compared with binding assays (10 vs 90 min), or perhaps the low pH pretreatment of brain membranes for adenylyl cyclase assay also inhibited anandamide breakdown. In comparing the two assays in relative terms, WIN 55212-2 was approximately 15 times more potent than anandamide in adenylyl cyclase assays, compared with the 180 times difference between anandamide and WIN 55212-2 observed in binding assays. It is possible that PMSF had not completely protected anandamide degradation during the longer incubation period required for binding compared with adenylyl cyclase assays.

This study also showed that anandamide inhibited [^{3}H]WIN 55212-2 receptor binding (as long as measures are taken to protect it from degradation) with a potency that was similar to its IC_{50} value versus [^{3}H]HU-243 binding [17]. Moreover, by inhibiting adenyl cyclase in brain membranes analogous to other cannabinoid agonists, these data suggest that anandamide binds to G-protein-coupled receptors in brain in a manner that is consistent with a cannabinoid agonist. Therefore, anandamide is a good candidate for an endogenous cannabinoid ligand. However, several questions have yet to be answered about this compound. For example, the selectivity of anandamide has not yet been determined against G-protein-coupled receptors other than the cannabinoid receptor [17]. It would be particularly interesting to determine its affinity at various eicosanoid receptors. Moreover, data both from our laboratory (M. Pacheco and S. Childers, unpublished observations) and from Howlett and colleagues [+6] suggest that other cannabinoid ligands with properties significantly different from those of anandamide may be present in brain extracts. In this regard, it is interesting to note that the maximal efficacy of anandamide in adenylyl cyclase assays was slightly, but significantly, lower than that of exogenous cannabinoids like WIN 55212-2 and CP-55,940. It is possible that the vehicle used to dissolve anandamide in these experiments (ethanol) interfered with the adenylyl cyclase assay to prevent full agonist activity. Although such a problem cannot be totally ruled out, it is unlikely for several reasons. First, the vehicle had no effect on basal adenylyl cyclase at the highest concentration of ethanol added (see Materials and Methods). Second, the same concentration of ethanol had no effect on the maximal inhibition of adenylyl cyclase by CP-55,940 and WIN 55212-2 (data not shown). Finally, the lower efficacy of anandamide compared with other cannabinoids has been reported by two other groups in two different systems, including adenylyl cyclase assays in transfected cells [18], and effects on calcium conductance in neuroblastoma cells [25]. These results suggest that, in these systems, anandamide may act as a partial agonist. If so, the existence of other cannabinoid ligands is a distinct possibility. Isolation and characterization of these other ligands should answer some of these questions.

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