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Potentiation of the action of anandamide on hippocampal slices by the fatty acid amide hydrolase inhibitor, palmitylsulphonyl fluoride (AM 374)

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Abstract

The electrically-evoked release of [^{3}H]acetylcholine from hippocampal brain slices is inhibited by cannabinoid receptor agonists. The effect of palmitylsulphonyl fluoride (AM 374), a recently developed inhibitor of fatty acid amide hydrolase, in influencing the potency of exogenously added anandamide in this preparation was examined. Anandamide alone had relatively little effect on [^{3}H]acetylcholine release. By contrast, in the presence of AM 374 (0.1 μM), anandamide produced a significant inhibition of [^{3}H]acetylcholine release at all concentrations tested (0.1–10 μM). In addition to experiments with AM 374 the effects of *N*-(4-hydroxyphenyl)arachidonamide (AM 404), a putative anandamide uptake inhibitor, was also examined. However, AM 404 at concentrations up to 10 μM, was not found to significantly enhance the effect of anandamide on electrically-evoked [^{3}H]acetylcholine release. These results indicate that AM 374 potently inhibits endogenous amidase activity and thus facilitates access of exogenous anandamide to cannabinoid receptors in the hippocampal tissue.

Keywords: Anandamide; Cannabinoid receptor; Acetylcholine; Brain slice; Hippocampus; Fatty acid amide hydrolase

1. Introduction

Anandamide is an endogenous lipid, which can activate brain cannabinoid receptors and is thought to serve as the endogenous neurotransmitter for these receptors (Di Marzo et al., 1994). In brain tissue anandamide is rapidly degraded to arachidonic acid and ethanolamine by fatty acid amide hydrolase, also termed anandamide amidase (Deutsch and Chin, 1993) and this enzyme may provide a pathway for inactivation of endogenous anandamide after it has been released. Fatty acid amide hydrolase has been cloned and is highly expressed in the brain and in the liver (Thomas et al., 1997).

Phenylmethylsulfonyl fluoride (PMSF), a non-selective inhibitor of serine proteases, has been found to act as an irreversible inhibitor of fatty acid amide hydrolase (Deutsch and Chin, 1993). By preventing anandamide breakdown, inclusion of phenylmethylsulphonyl fluoride in receptor binding assays increases the apparent potency of anandamide (Childers et al., 1994; Adams et al., 1995). In in vitro tissue preparations phenylmethylsulphonyl fluoride has been found to increase the potency of anandamide in inhibiting contractions in the isolated guinea-pig myenteric plexus (Pertwee et al., 1995). However, phenylmethylsulphonyl fluoride suffers the drawbacks of both having both a low potency and selectivity against the amidase enzyme and this has severely limited its effectiveness as a pharmacological tool for investigating anandamide actions or in identifying therapeutic uses for amidase inhibitors. In a search for more potent inhibitors of this enzyme several fatty acid sulphonyl fluorides have recently been synthesized (Deutsch et al., 1997) which can inhibit the amidase enzyme with IC_{50}s in the low nanomolar range. Palmitylsulfonyl fluoride (AM 374; Fig. 1) was found to be the most selective of the compounds developed, with an affinity for the amidase enzyme of 7 nM (Deutsch et al., 1997).

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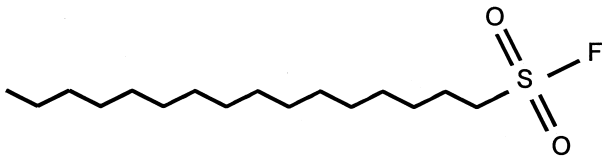

Fig. 1. Structure of AM 374.

In addition to hydrolysis by an amidase enzyme it has been proposed that termination of the action of anandamide that has been released into the synaptic cleft is also facilitated by the presence of a specific transporter for anandamide on the cell membranes. The existence of such a carrier is supported by the observation that the accumulation of [^{3}H]anandamide into neuronal cells is rapid, temperature-dependent and saturable (Hillard et al., 1997). A search for anandamide derivatives that could inhibit this uptake process led to the identification of *N*-(4-hydroxyphenyl)arachidonylamide (AM 404; Beltramo et al., 1997). This compound is both efficacious and moderately potent as an inhibitor of the cellular uptake of anandamide.

Recently 2-arachidonylglycerol has been proposed as a second endogenous ligand for the cannabinoid receptors. This compound has an approximately ten fold lower affinity for the cannabinoid CB1 receptor than anandamide but is present in the brain at much higher levels (Mechoulam et al., 1995; Stella et al., 1997). Despite possessing an ester group rather than an amide group 2-arachidonylglycerol also serves as a substrate for fatty acid amide hydrolase (Goparaju et al., 1998; Lang et al., 1999) and thus may be inactivated in the brain via the same enzymatic mechanism as anandamide.

The present experiments were conducted with the objective of determining whether inhibition of anandamide hydrolysis with AM 374 or inhibition of anandamide uptake with AM 404 would influence the potency of anandamide in producing a pharmacological response in an intact tissue preparation. For these experiments we examined the electrically-evoked release of acetylcholine from hippocampal brain slices, which we have found to be potently inhibited by the cannabinoid receptor agonists (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN 552-12-2) and (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol (CP 55,940), (Gifford and Ashby, 1996; Gifford et al., 1997, 1998).

2. Materials and methods

2.1. [^{3}H]acetylcholine release in slices

Male Sprague–Dawley rats (200–350 g, Taconic, Germantown NY) were decapitated, their brains removed, and the hippocampus dissected out. Following dissection, 300 μm tissue slices were cut with a vibratome and the slices transferred to 2 ml of Krebs buffer (119.5 mM NaCl, 3.3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 0.03 mM EDTA, pH 7.4), saturated with 95% O_2/5% CO_2, containing 10 μCi [^{3}H]choline. After incubation in the [^{3}H]choline for 15 min at 37°C the slices were transferred to 10 superfusion chambers (two slices per chamber). The slices were sandwiched between wire mesh screens positioned midway between two platinum electrodes. Slices were superfused at 37°C, at a rate of 1.6 ml/min, with oxygenated Krebs buffer containing 1 μM physostigmine to prevent hydrolysis of the released acetylcholine and 0.3 μM quinuclidinyl benzilate to prevent auto-inhibition of release via presynaptically located muscarinic receptors. Additionally, after being drawn into the tubing running to the superfusion chambers, the Krebs saline containing drugs was mixed with bovine serum albumin, added via a second pump, to act as a carrier for the drugs (final bovine serum albumin concentration was 0.13%).

To evoke neurotransmitter release the tissue slices were given either two or four periods of electrical stimulation (S1, S2, S3 and S4). Stimulation periods were of 3-min duration, beginning 120 min, 135 min, 205 and 220 min after superfusion was started and each consisted of a train of unipolar, pulses (60 mA, 2 ms) at a rate of 1 Hz. For each stimulation period the stimulation-evoked release of [^{3}H]acetylcholine was determined by subtracting the counts obtained in a 4 min fraction collected immediately prior to stimulation from the counts obtained in a 4 min fraction collected immediately after initiating stimulation.

2.2. Drug additions

AM 374 and AM 404 were dissolved at a concentration of between 1 and 10 mg/ml in dimethylsulphoxide and added to the superfusion medium 45 min prior to S1. Anandamide, supplied as an emulsion in soya oil/water (1:4) at a concentration of 10 mg/ml, was added to the superfusion medium 60 min prior to S3. WIN 55212-2 was dissolved at a concentration of 1 mg/ml in 40% 2-hydroxypropyl-β-cyclodextrin and also added to the superfusion medium 60 min prior to S3. 2-Arachidonylglycerol and AM 1191 were dissolved in dimethylsulphoxide and added 50 min prior to S2. Previous studies have indicated that the final concentrations of dimethylsulphoxide and 2-hydroxypropyl-β-cyclodextrin in the superfusion medium are not sufficient to affect neurotransmitter release (Gifford and Ashby, 1996). Slices in two of the ten chambers used in each experiment were used as controls and were either not exposed to drugs or were given drug-vehicles only. After being added drugs were kept in the superfusion medium until the termination of the experiment.

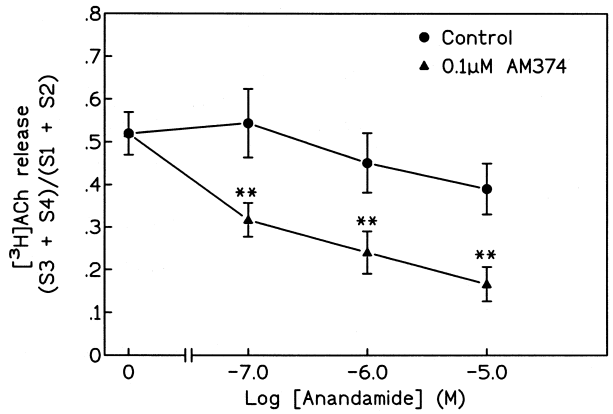

Fig. 2. Effect of AM 374 on the inhibition of electrically-evoked [^{3}H]acetylcholine release by anandamide. AM 374 was present in all stimulation periods whereas anandamide was present in (S3 + S4) only. Data points are means (± S.E.M.) of 8–18 determinations (ANOVA, $F(6,82) = 6.0$, $P < 0.01$). $^*P < 0.05$, $^{**}P < 0.01$ vs. control (Dunnett's test).

2.3. Drug sources

AM 374, AM 404 and AM 1181 were synthesized in the laboratory of Dr. Makriyannis. *R*(+)WIN 55212-2 mesylate, (±)-quinuclidinyl benzilate and 2-hydroxypropyl-β-cyclodextrin were purchased from Research Biochemicals (Natick, MA). Anandamide was purchased from Tocris (Ballwin, MO). Physostigmine (eserine) was obtained from Sigma (St. Louis, MO). 2-Arachidonylglycerol was both synthesized in the laboratory of Dr. Makriyannis and purchased from Research Biochemicals.

2.4. Data analysis

To determine the effect of anandamide and WIN 55212-2 on stimulation-evoked release, data was expressed as the ratio of the total amount of evoked release of radioactivity before adding drug (S1 + S2) relative to the total amount of evoked release after adding drug (S3 + S4). Calculating the release as a ratio rather than an absolute amount (viz. C.P.M./slice) can compensate for substantial variations in the amount of electrically-evoked release from individual slices resulting from differences in their size and orientation in the electrical field. Similarly, any effects on the electrically-evoked release of drugs or drug-vehicles which have been added to the superfusion medium *prior* to the stimulation periods (i.e., BSA, AM 374 and AM 404) is also compensated for by this method of analysis.

Median electrically-evoked release (C.P.M./chamber) in S1 + S2 for Figs. 2–5 was as follows: Fig. 2: 1116 (control), 728 (AM 374); Fig. 3: 1520 (control), 1572 (AM 404); Fig. 4: 506 (control), 510 (AM 404 + AM 374); Fig. 5: 644 (control), 578 (AM 374). Median electrically-evoked release in Fig. 6 (S1 only) was 366 (control) and 294 (AM 374). To avoid inaccurate release ratios from those slices showing relatively little stimulation-evoked overflow, chambers having a stimulation-evoked release in S1 + S2 (or S1 only for Fig. 6) of < 100 C.P.M. were excluded from the analysis. Chambers not showing a stable level of release (> 50% variation in the amount of release between S1 and S2) were also excluded from the analysis.

The effects of anandamide on the (S1 + S2)/(S3 + S4) ratio was analysed using a single factor analysis of vari-

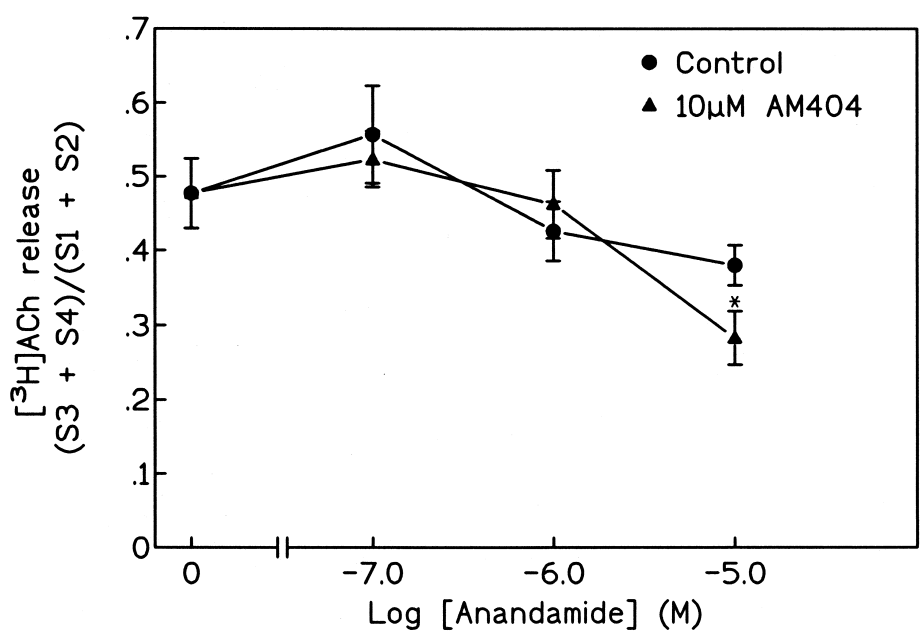

Fig. 3. Effect of AM 404 on the inhibition of electrically-evoked [^{3}H]acetylcholine release by anandamide. AM 404 was present in all stimulation periods whereas anandamide was present in (S3 + S4) only. Data are means (± S.E.M.) of 6–10 determinations for the anandamide data points and 12 determinations for the control data point (ANOVA, $F(6,50) = 3.14$, $P < 0.05$). $^*P < 0.05$ versus control (Dunnett's test).

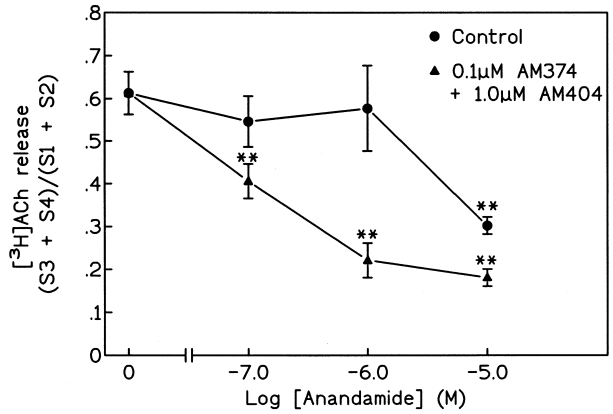

Fig. 4. Effect of the combined presence of both AM 374 and AM 404 on the inhibition of electrically-evoked [^{3}H]acetylcholine release by anandamide. AM 374 and AM 404 were present in all stimulation periods whereas anandamide was present in (S3 + S4) only. Data are means (± S.E.M.) of 9–17 determinations for the anandamide data points and 18 determinations for the control data point (ANOVA, $F(6,80) = 11.8$, $P < 0.01$). $^*P < 0.05$, $^{**}P < 0.01$ versus control (Dunnett's test).

ance, followed by a Dunnett's test for comparing drug-treatment means to a single control mean. Sample sizes were usually greater for the control mean than for drug treatment means so as to provide an optimal statistical comparison.

3. Results

Inclusion of 0.1 μM AM 374 in the superfusion medium to inhibit anandamide hydrolysis resulted in a significant potentiation of the inhibition of [^{3}H]acetylcholine release by anandamide over a range of anandamide concentrations (Fig. 2). In contrast, the putative anandamide uptake inhibitor, AM 404, at a concentration of 10 μM, did not appear to significantly enhance the effect of anandamide on electrically-evoked [^{3}H]acetylcholine release (Fig. 3). 1 μM AM 404 was also without effect (data not shown). Combining both 0.1 μM AM 374 and 1 μM AM 404 in the superfusion medium produced a potentiation of the effect of anandamide that was similar to that of AM 374 alone (Fig. 4).

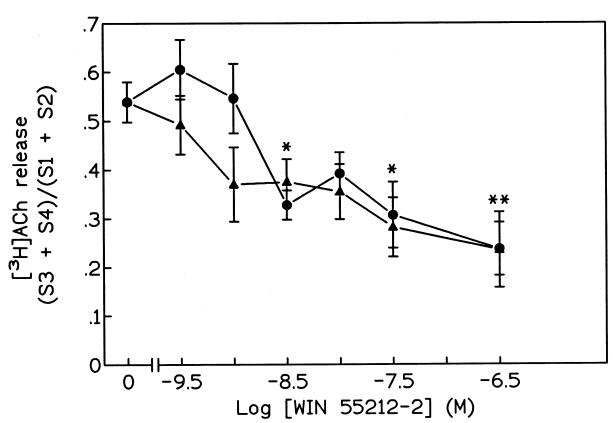

Fig. 5. Effect of AM 374 on the inhibition of electrically-evoked [^{3}H]acetylcholine release by WIN 55212-2. AM 374 was present in all stimulation periods whereas WIN 55212-2 was present in (S3 + S4) only. Data are means (± S.E.M.) of 10–19 determinations for the WIN 55212-2 data points and 40 determinations for the control data point (ANOVA, $F(12,168) = 4.4$, $P < 0.01$). $^*P < 0.05$ or $^{**}P < 0.01$ (vs. control) for data points in both dose–response curves at the 3, 30 and 300 nM WIN 55212-2 concentrations (Dunnett's test).

Table 1
Action of 2-arachidonylglycerol and AM 1181 on electrically-evoked [^{3}H]acetylcholine release in hippocampal slices
Data are means ± S.E.M. (n). WIN 55212-2, 2-arachidonylglycerol and AM 1181 were present in S2 only whereas AM 374 was present in both S1 and S2. (ANOVA, $F(4,54) = 4.8$, $P < 0.01$).

	[^{3}H]acetylcholine release (S2/S1)
Control	0.72 ± 0.12 (14)
0.3 μM WIN 55212-2	0.17 ± 0.03 (7)[a]
10 μM 2-arachidonylglycerol	0.54 ± 0.12 (16)
10 μM 2-arachidonylglycerol (+ 0.1 μM AM 374)	0.65 ± 0.10 (14)
10 μM AM 1181	0.14 ± 0.03 (8)[a]

[a] $P < 0.01$ vs. control (Dunnett's test).

In addition to examining the effect of AM 374 on the anandamide-induced inhibition of [^{3}H]acetylcholine release we also determined if AM 374 had an effect on the potency of WIN 55212-2-induced inhibition of [^{3}H]-acetylcholine release (Fig. 5), which would not be expected to be influenced by fatty acid amide hydrolase activity. In these experiments WIN 55212-2 produced a shallow dose–response curve, as we have also observed in previous studies (Gifford and Ashby, 1996). The dose–response curve for WIN 55212-2 was not affected to a significant extent by inclusion of 0.1 μM AM 374 in the superfusion medium.

The putative endogenous neurotransmitter, 2-arachidonylglycerol (10 μM), did not significantly reduce electrically-evoked [^{3}H]acetylcholine release either in the presence or absence of AM 374 (0.1 μM) to inhibit amidase activity (Table 1). By contrast an ether analogue of 2-arachidonylglycerol, AM 1181, which is resistant to hydrolysis by fatty acid amide hydrolase and has a higher affinity for cannabinoid CB1 receptors than 2-arachidonylglycerol, produced a level of inhibition of [^{3}H]-acetylcholine release equivalent to that of WIN 55212-2.

4. Discussion

We have previously reported that the electrically-evoked release of acetylcholine from hippocampal slices is inhibited by the cannabinoid receptor agonists WIN 55212-2 and CP 55,940 (Gifford and Ashby, 1996; Gifford et al., 1997). The present results indicate that acetylcholine release can also be inhibited by anandamide, the putative endogenous ligand for cannabinoid receptors.

In rat brain homogenates AM 374 has been found to be a potent inhibitor of fatty acid amide hydrolase activity (Deutsch et al., 1997). In the present experiments addition of AM 374 to the superfusion medium resulted in a substantial potentiation in the ability of anandamide to inhibit electrically-evoked [^{3}H]acetylcholine release in the brain slices, suggesting that endogenous fatty acid amide hydrolase activity is similarly present in the intact hippocampal slices and can be effectively inhibited by this compound. Fatty acid amide hydrolase activity in the hippocampus may be relatively high compared to other brain regions, as suggested by the presence of moderate to high levels of mRNA for fatty acid amide hydrolase in this region (Thomas et al., 1997).

AM 374 has a relatively high potency in inhibiting anandamide breakdown when compared with phenylmethylsulphonyl fluoride, which has previously been widely used to inhibit anandamide breakdown. Thus the 0.1 μM concentration of AM 374 found to be effective in the present study contrasts with a concentration of between 2 and 20 μM phenylmethylsulphonyl fluoride found to be effective in potentiating anandamide actions in the isolated guinea-pig myenteric plexus preparation (Pertwee et al., 1995). In cultured cells AM 374 has also been found to have a high potency, showing over a thousand fold greater potency in increasing anadamide levels than phenylmethylsulphonyl fluoride (Deutsch et al., 1997). However, although the relatively high potency of AM 374 should make this compound a useful research tool this compound does suffer a drawback of having a slight degree of affinity for cannabinoid CB1 receptors (IC_{50} of 520 nM, Deutsch et al., 1997). Hence direct effects of AM 374 on cannabinoid receptors may be produced if too high a concentration is employed in biochemical or physiological experiments.

WIN 55212-2 produced a dose-dependent inhibition of [^{3}H]acetylcholine release similar to that we have reported previously (Gifford and Ashby, 1996; Gifford et al., 1998). Inclusion of AM 374 in the superfusion medium did not appear to augment the WIN 55212-2 induced-inhibition of acetylcholine release to a significant extent, suggesting that the effect of AM 374 is selective for anandamide.

In contrast to the results obtained with AM 374 on acetylcholine release, inclusion of the putative uptake transport inhibitor, AM 404, in the perfusion medium at concentrations of up to 10 μM did not appear to produce a significant enhancement of the anandamide-induced inhibition of acetylcholine release. In previous studies performed on cultured cells AM 404 has been found to produce an up to 70% decrease in [^{3}H]anandamide accumulation, having an EC_{50} of approximately 1 μM in neurons and 5 μM in astrocytes (Beltramo et al., 1997). Some evidence has also been obtained that AM 404 may be active in potentiating anandamide actions in vivo (Calignano et al., 1997). However, our results indicate that in the hippocampal slices either AM 404 was not able to effectively inhibit cellular uptake anandamide at the concentrations examined, or else that transport mechanisms may not be of importance in terminating anandamide action in this preparation.

2-Arachidonylglycerol is an endogenous brain lipid, which like anandamide is capable of activating cannabinoid receptors. Its affinity for the cannabinoid CB1 receptor it's lower than that of anandamide, with a K_i of 470 nM (Mechoulam et al., 1995). This compares with a K_i of 89 nM for anandamide (Showalter et al., 1996). In the present study 2-arachidonylglycerol failed to significantly inhibit [^{3}H]acetylcholine release even in the present of AM 374 to inhibit hydrolysis by fatty acid amide hydrolase. One possible explanation for the lack of effect of 2-arachidonylglycerol in the hippocampal slices may be that other enzymatic mechanisms operate in the tissue to inactivate this compound. The low affinity of 2-arachidonylglycerol for cannabinoid CB1 receptors may also have contributed to its lack of effect since AM 1181 an ether analogue with an approximately 5-fold greater affinity for the cannabinoid CB1 receptor (unpublished observations) did produce an inhibition of [^{3}H]acetylcholine release in the brain slices.

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