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Anandamide synthesis is induced by arachidonate mobilizing agonists in cells of the immune system

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Abstract

The hypothesis that the capability of agents to mobilize arachidonic acid (AA) could predict increased anandamide (ANA) synthesis in a macrophage cell line has been examined. Lipopolysaccharide (LPS), platelet-activating factor (PAF) and cannabinoids such as Δ^9-tetrahydrocannabinol (THC) and anandamide were all found to be agonists for the release of AA and led to increased ANA synthesis in RAW264.7 mouse macrophage cells. Nitric oxide, in contrast, stimulated AA release without raising ANA levels. ANA stimulation of its own synthesis indicates the existence of a positive feedback mechanism. The possible involvement of the CB2 receptor in THC-mediated AA release and ANA synthesis is addressed using the antagonist SR144528. ANA synthesis is also increased by the combination of calcium ionophore and indomethacin, suggesting that ANA is metabolized by a cyclooxygenase in this system. The data imply that ANA could play a role in the response of the immune system to cannabinoids and bacterial endotoxins and that AA mobilization is a predictor for increased ANA synthesis.

Keywords: Anandamide; Cannabinoid; Platelet-activating factor; Lipopolysaccharide; Arachidonic acid; Macrophage

1. Introduction

The existence of naturally occurring ethanolamide derivatives of fatty acids was reported as early as 1957 [1,2]; however, it was not appreciated until recently that several members of this family of substances could mimic the actions of plant derived and synthetic cannabinoids [3,4]. Of the various molecules in this group, arachidonyl ethanolamide (ANA) most closely resembles THC, the psychoactive principle of Cannabis. Much effort has been expended in characterizing the pharmacological activities of the endogenous cannabinoid, ANA, and its related fatty acid ethanolamide analogs; however, relatively little has been reported on the factors that regulate their levels under physiological conditions. ANA as such, does not appear to be stored by cells in the same way that certain transmitter molecules are. By contrast, it seems that it is rapidly synthesized in response to the appropriate stimuli much like the closely related prostaglandins and leuko-

Abbreviations: AA, arachidonic acid; ANA, anandamide; BSA, bovine serum albumin; CB, cannabinoid; DMSO, dimethyl sulfoxide; EA, ethanolamine; LPS, lipopolysaccharide; NAPE, *N*-arachidonylphosphatidylethanolamine; PEA, palmitoylethanolamide; RPMI, RPMI-1640; PAF, platelet activating factor; THC, Δ^9-tetrahydrocannabinol; TLC, thin layer chromatography

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trienes. Therefore, a full understanding of the physiological role of the anandamides requires a detailed knowledge of their biosyntheses and the factors that control it.

Thus far, two distinct pathways have been proposed for the production of fatty acid ethanolamides in biological systems. The earlier and more straightforward mechanism involves a direct condensation of EA with free fatty acid in an ATP and CoA independent process [2,5]. While the enzymic mediator for this reaction has not been fully characterized, it appears that it may be an ANA amidohydrolase operating in reverse. The supply of precursors, which could be under agonist-receptor control, would be the rate-limiting step. There are data suggesting that this pathway operates under some conditions; however, the relatively high concentrations of precursors needed to drive the reaction in vitro detract somewhat from its possible relevance in physiological circumstances [2–7]. Another, more complex pathway has been suggested involving *N*-acylphosphatidyl ethanolamine precursors that can act as storage pools for anandamide or its analogs [3]. Receptor-coupled phospholipase D activity would release the endogenous cannabinoid following activation by the appropriate ligand. Considerable evidence has been reported in support of this mechanism [3]; however, no receptor or ligand has yet been identified that is able to initiate this process, thereby showing its physiological importance.

Our working hypothesis is that substances which are able to mobilize arachidonate may also lead to increased levels of ANA. We have recently shown that THC increased the synthesis of ANA in N18 mouse neuroblastoma cells under conditions where the release of AA was also stimulated in a CB1 receptor-mediated process [8,9]. This finding was supported by earlier observations that cannabinoids generally act to promote eicosanoid synthesis [10] and that this effect involves the activation of phospholipases A_2 and D, MAP kinases and diacylglyceride lipases [11,12]. A possible rationale for a connection between AA mobilization and ANA synthesis via the PLD pathway is provided by a recent report [13] describing the incorporation of free AA into the *sn*-1 position of PC which could then lead to ANA synthesis by transacylation to give NAPE that, in turn, has been shown to result in PLD-mediated ANA synthesis [14].

In the present report, data on the synthesis of ANA in cells of peripheral origin, such as the mouse macrophage cell line, RAW264.7, is presented. In addition, we show evidence that not only THC, but also physiological ligands such as ANA, LPS and PAF can lead to increased ANA synthesis. The question of whether the effects of cannabinoids are mediated via their receptors is addressed using SR144528, an antagonist of the CB2 receptor [15]. We also show that when AA release in RAW264.7 cells is induced by calcium ionophore and, combined with indomethacin pretreatment, an elevation of ANA levels results. Our findings raise the possibility that ANA may be involved in modulation of the immune response by macrophages.

2. Materials and methods

2.1. Materials

RAW264.7 murine monocyte cells were obtained from ATCC (Rockville, MD) and maintained by the University tissue culture facility. RPMI-1640, fetal bovine serum and penicillin–streptomycin solution were obtained from Gibco-BRL (Grand Island, NY). [1-^{14}C]AA (55 Ci/mol), [1,2-^{14}C]ethanolamine (55 Ci/mol), [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) and [^{3}H]ANA (200 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). Insta-Fluor and Opti-Fluor counting fluids were purchased from Packard Instruments (Meriden, CT). BSA, LPS (*Escherichia coli*, serotype 0127:B8), DMSO, calcium ionophore A23187 and indomethacin were obtained from Sigma (St. Louis, MO). ANA and C-16 PAF were obtained from Biomol (Plymouth Meeting, PA). SR144528 was a gift from Sanofi Research (Montpellier, Cedex, France). Sep-Pak Plus C-18 cartridges were purchased from Waters (Milford, MA). Thin layer chromatography plates were obtained from EM Science (Gibbstown, NJ). THC was provided by the National Institute on Drug Abuse (Rockville, MD).

2.2. Cell culture, AA release and time course measurements

The cells were grown in RPMI with 1% penicillin–streptomycin and 10% fetal bovine serum at 37°C under 95% oxygen/5% carbon dioxide to about 85% confluence. Cells were contained in 24-well plates and labeled with 100 000 dpm/ml/well of [^{3}H]AA at 37°C for 20 h. The wells were washed four times with 0.5% BSA in RPMI followed by incubation in 1 ml 0.1% BSA–RPMI for 60 min. THC, ANA, PAF, SR144528, indomethacin, or the calcium ionophore (A23187) in 50% DMSO (final DMSO concentration, 0.5%), or LPS in water was then added to each well and the incubation continued at 37°C for the indicated time. Vehicle alone was used as control. Media were collected, centrifuged at $3000 \times g$ to remove cells, and 0.1 ml withdrawn and assayed for radioactivity using an LKB Rackbeta 1211 liquid scintillation counter.

For the time course measurements, cells were grown, labeled with AA and determinations carried out as described above. They were treated with 5 μg/ml LPS and measurements made at 1, 15, 30, 45 and 60 min. The data were plotted and the pseudo first-order rate constant obtained using Kaleidagraph 2.0 data analysis software. The values for AA are the means of four determinations ± S.E.; the ANA values are means of duplicate determinations.

2.3. Measurement of ANA

Cells were grown in either 24-well or 6-well plates and labeled with 0.05 μCi/ml [^{3}H]AA or 0.5 μCi/ml [^{14}C]EA for 20 h. They were then washed as described above and stimulated with agonist as indicated. The media were collected, acidified with 1/10 vols. of 10% acetic acid and subjected to reverse-phase, solid-matrix extraction using Sep-Pak Plus C-18 cartridges. After washing off with water all of the material not retained on the cartridge, the lipophilic products were recovered by elution with either methanol or absolute ethanol. In some experiments, the cells were covered with methanol at room temperature for 1 h and the extracts were pooled with the media. These were then extracted with chloroform. Extracts in all experiments were concentrated under reduced pressure and analyzed by TLC on 250 μm silica gel plates using chloroform/methanol/acetic acid 90:6:6 (v/v). For ANA measurements, the unlabeled standard was visualized with iodine vapor, removed from the plate and quantitated by liquid scintillation counting. The TLC zones were removed and leeched with methanol (1 ml) in the counting vial prior to the addition of counting fluid. Two-dimensional TLC was done using the above system in the first direction and the system published by Deutsch and Chin [16] in the second direction. ANA showed Rf values of 0.55 and 0.58 in the first and the second system, respectively.

2.4. Identification of anandamide by double isotope analysis

RAW cells were grown in T-75 flasks, labeled with [^{14}C]AA and treated with or without LPS as described above and the AA release was measured. Tritiated ANA standard (6700–6800 cpm) was added to the media and the samples were prepared for TLC using the conditions described above. The ANA region from the TLC plate was scraped and eluted thrice with chloroform/methanol 2:1 (v/v). The eluates were pooled and concentrated under nitrogen. Aliquots were assayed for tritium and carbon-14 content by liquid scintillation counting. The remainder of the eluates were subjected to two further consecutive TLC analyses. Solvent systems with differing compositions were chosen so as to achieve maximum separation of ANA from any comigrating metabolites. The solvent systems used were: A, ethyl acetate/acetone/hexane 1:1:1 (v/v); B, chloroform/hexane/methanol 50:50:8 (v/v); and C, chloroform/methanol/acetic acid 90:6:6 (v/v). The Rf values for ANA in these systems were 0.43, 0.22 and 0.55, respectively. Samples were counted using settings giving ^{3}H and ^{14}C counts in the first and the second windows respectively with minimal spillover. The blank value was measured using scintillation fluid alone. Spillovers were calculated for each sample and these values were added to the blank values and subtracted from the total counts to arrive at the values used to calculate the isotope ratios.

2.5. Statistical analysis

Values are the means ± S.E. for three determina-

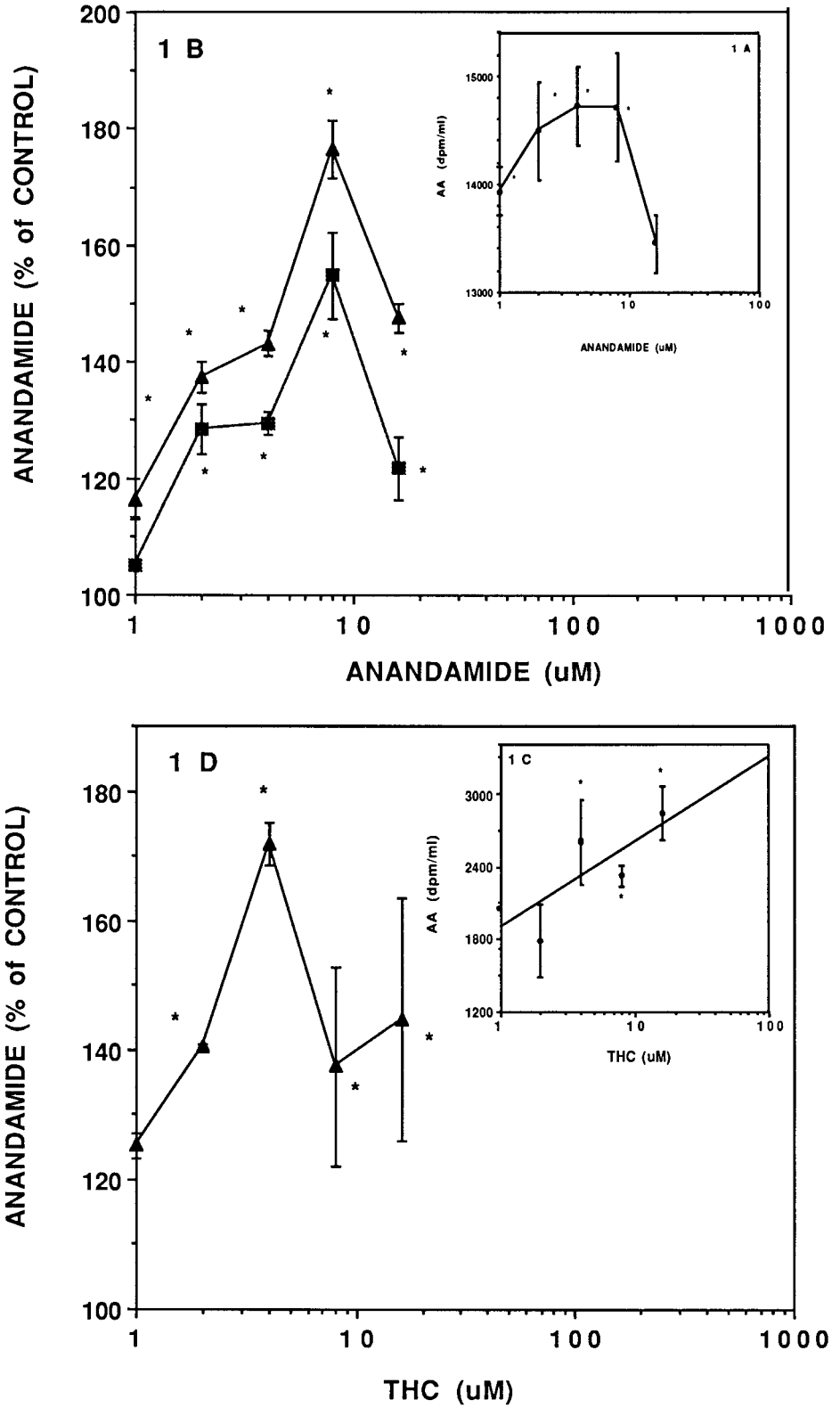

Fig. 1 (*continued on next page*).

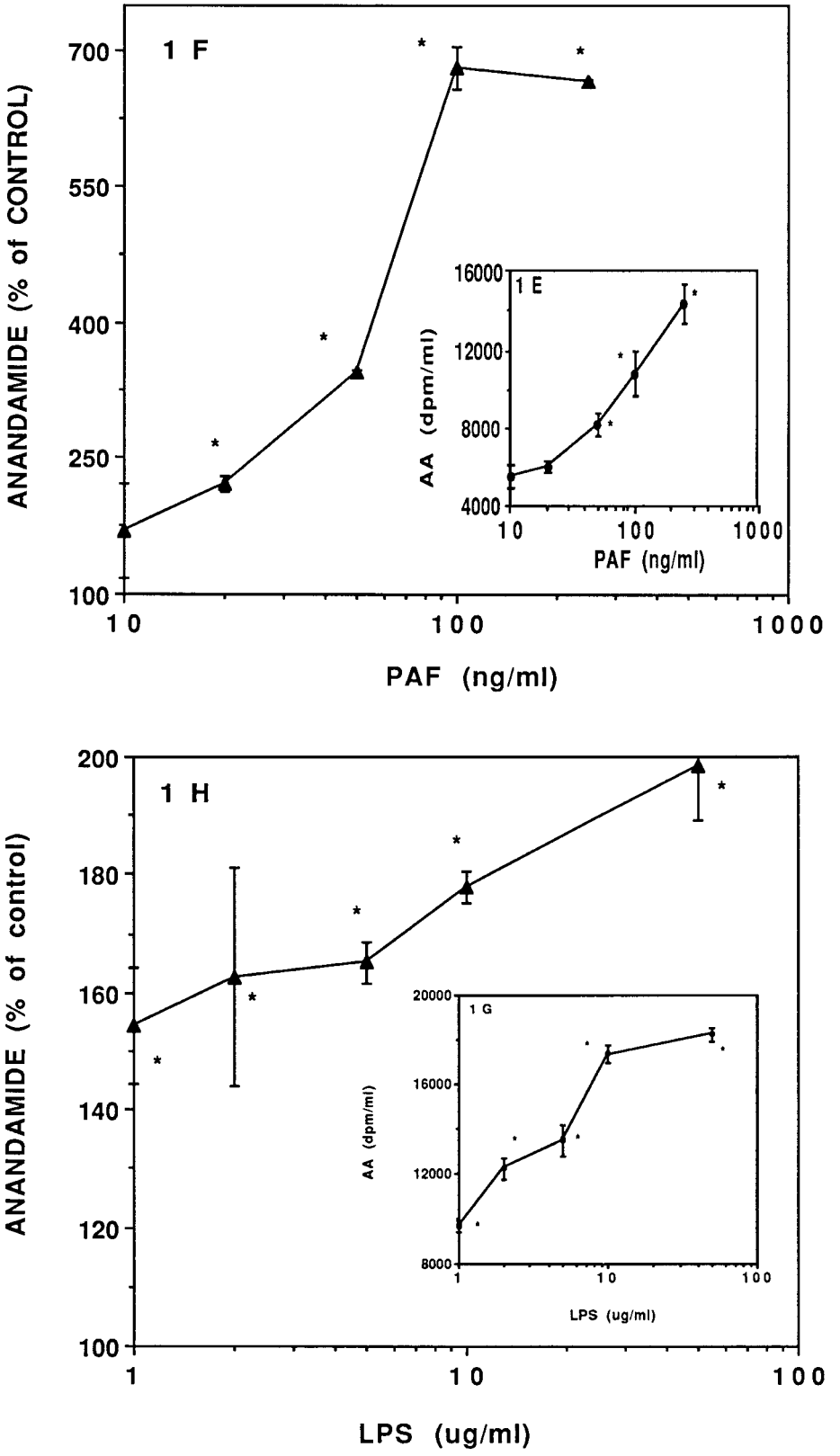

Fig. 1 (*for legend see next page*).

Fig. 1. Agonist induced AA release and ANA synthesis. Cells ($\sim 0.5 \times 10^6$/well) were grown, labeled with [^{3}H]AA or [^{14}C]EA and washed as described in Section 2. ANA, THC or PAF in 50% DMSO (A–F) or LPS in water (G,H) was added to the cells at the concentrations indicated. Incubations were carried out for 30 min (A–D), 10 min (E,F) or 60 min (G,H). Media were sampled and radioactive content was determined by liquid scintillation counting (A,C,E,G). ANA was determined by TLC as described in Section 2 (B,D,F,H). The values shown are the means of three determinations ± S.E. Mean values for the controls are: (A) 12 550 ± 485 dpm/ml; (B) for AA (triangles), 37.7 ± 3 dpm and for EA (squares), 10.3 ± 3 dpm; (C) 1483 ± 25 dpm/ml; (D) 15.3 ± 2 dpm; (E) 5548 ± 400 dpm/ml; (F) 5.4 ± 1 dpm; (G) 6758 ± 196 dpm/ml; and (H) 44.0 ± 7 dpm. *95% confidence limit vs. control.
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tions unless otherwise indicated. A one-factor ANOVA evaluation was used followed by a Fisher PLSD post-hoc test to compare the different sets of experimental data for statistical significance. The sets were considered to be significantly different at $P < 0.05$.

3. Results

3.1. ANA, THC, PAF and LPS stimulation of AA release and ANA synthesis

3.1.1. Effects of ANA

ANA was able to stimulate the mobilization of AA in RAW264.7 cells over the concentration range of 1–8 μM (Fig. 1A). A previous report also showed a similar concentration relationship for ANA-induced release of arachidonate in rat cortical astrocytes [17]. Comparable agonist activity for ANA was reported in WI-38 human lung fibroblasts [12] and, more recently, Di Marzo et al. [18] reported that ANA stimulates AA release in the J774 macrophage cell line. ANA synthesis was also stimulated by ANA itself as demonstrated using either radiolabeled EA or AA (Fig. 1B, Table 1). Both procedures, utilizing different precursors, exhibited similar dose– response curves with maximum at 8 μM and a 3.1–5.7-fold increase in ANA counts over vehicle-treated cells.

3.1.2. Effects of THC

The exogenous cannabinoid, THC, promoted the release of AA into the cell culture media at concentrations up to 16 μM (Fig. 1C). At these levels, THC caused an 80–90% elevation of radioactivity over the vehicle control values. Similar findings were recently reported by Shivachar et al. [17] for cannabinoid induced arachidonate release in rat cortical astrocytes. THC also stimulated ANA synthesis in RAW264.7 cells over the concentration range of 1–16 μM (Fig. 1D). These results, which were obtained using EA as the labeled precursor, were confirmed when labeled AA was employed (Table 1) supporting the identity of ANA as a product.

3.1.3. Effects of PAF

The phosphoacylglycerol derivative PAF has been extensively reported to mobilize AA in a variety of models [19]. Leukocytes, in particular, appear to be an important site for both the synthesis and actions of PAF. Thus RAW264.7 cells are an appropriate model for testing its effects on ANA synthesis. As predicted, when precursor labeled cells were exposed to C-16 PAF, a clear dose-related stimulation of

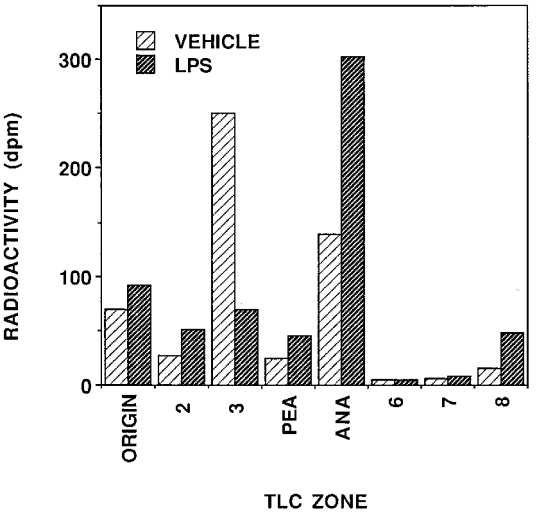

Fig. 2. TLC analysis of products following LPS treatment. RAW cells were grown to 90% confluency in T-75 flasks, labeled with [^{14}C]EA and treated with vehicle or LPS (50 μg/ml) for 60 min as described in Section 2; the vehicle was water. The media were extracted and chromatographed using silica gel TLC (system C, Table 2) as described in Section 2. Unlabeled standards of anandamide (ANA) and palmitoylethanolamide (PEA) were added prior to the extractions; the standards were visualized by exposure to iodine vapor and phosphomolybdic acid. Individual zones were removed, leeched with methanol, and assayed by liquid scintillation counting.

Table 1
Comparison of precursors in agonist-induced ANA synthesis

Agonist	Precursor	
	Arachidonic acid	Ethanolamine
ANA (8 μM)	178	155
Vehicle	37.7 ± 3	10.3 ± 3
THC (4 μM)	173	234
Vehicle	15.3 ± 2	29.0 ± 3
PAF (100 ng/ml)	670	485
Vehicle	5.4 ± 1	10.3 ± 3
LPS (50 μg/ml)	198	175
Vehicle	44.0 ± 7	8.0 ± 3

RAW cells (~0.5×106/well) were grown, labeled with AA or EA and washed as described in Section 2. ANA, THC or PAF in 50% DMSO or LPS in water was added to the cells at the concentrations indicated. Incubations were carried out for 30 min (ANA, THC); 10 min (PAF) or 60 min (LPS). ANA was determined by TLC as described in Section 2 using system C. Values shown are the radioactive content ± S.E. (dpm) of the ANA zones from TLC for the vehicle controls and percent of the control for the agonists.

both arachidonate release and ANA production was observed (Fig. 1E,F). Similar results were obtained when EA was the precursor (Table 1).

3.1.4. Increased AA mobilization and ANA synthesis following LPS exposure

LPS is known to mobilize AA in macrophages at a concentration of 50 μg/ml [20]. Lower concentrations (0.1–0.5 μg/ml) of LPS do not cause the release of AA; however, they have been shown to sensitize cells to the actions of other agonists [21]. We treated AA labeled RAW264.7 cells with LPS (1 50 μg/ml) and observed a robust stimulation of arachidonate release (Fig. 1G) with an ED_{50} (concentration of LPS at which 50% of the maximum AA release was seen) of 2.73 μg/ml. The effect of LPS on ANA synthesis in these cells was measured in a parallel manner and a similar dose-related stimulation was observed (Fig. 1H). A comparable response was obtained when the other component of ANA, EA, was the precursor (Table 1). The time course for both AA release and ANA synthesis in LPS-treated cells was also determined. A close relationship between the two re-

Table 2
Identification of anandamide using double isotope thin layer chromatographic analysis

	Tritium (cpm ± S.D.)	Carbon-14 (cpm ± S.D.)	Isotope ratio[a]
Vehicle-treated cells			
Media	6801	11 580	0.59
Media extract	6658	5 210	1.28
TLC system A	4481 ± 34.2	232.0 ± 6.45	19.30
TLC system B	1789 ± 12.8	79.1 ± 10.6	22.64
TLC system C	477 ± 43.4	23.3 ± 15.3	20.43
LPS-treated cells			
Media	6710	21 000	0.32
Media extract	6414	13 860	0.47
TLC system A	3881 ± 24.9	437.0 ± 8.39	8.91
TLC system B	718 ± 32.4	123.0 ± 8.00	5.86
TLC system C	429 ± 38.3	76.7 ± 21.8	5.61

RAW cells were grown to 90% confluency in T-75 flasks, labeled with [^{14}C]AA and treated with vehicle LPS in water (50 μg/ml) as described in Section 2. Tritiated ANA standard (6700–6800 cpm) was added to each of the collected media and the samples were prepared for TLC using the conditions described in Section 2.4. The ANA region from the TLC plate was analyzed for tritium and carbon-14 content in three successive chromatograms. See Section 2 for counting conditions. The solvent systems used were: A, ethyl acetate/acetone/hexane 1:1:1 (v/v); B, chloroform/hexane/methanol 50:50:8 (v/v); and C, chloroform/methanol/acetic acid 90:6:6 (v/v). The Rf values for ANA in these systems were 0.43, 0.22 and 0.55, respectively. The blank value was measured using scintillation fluid alone. The values shown are the means of three measurements ± S.D. and samples were counted until a confidence level of 95% was attained (media and media extracts were single measurements).
[a]Tritium (cpm) divided by carbon-14 (cpm).

sponses was found with pseudo first-order rate constants of 0.028 per min for the release of tritium, i.e. free AA and 0.020 per min for ANA synthesis.

Not all agonists led to ANA synthesis since treatment with sodium nitroprusside, a source of nitric oxide, resulted in increased AA release with little if any change in the level of anandamide (data not shown).

3.2. Identification of ANA

Fig. 2 shows the composition of a media extract from RAW cells that had been labeled with EA and challenged with 50 μg/ml of LPS for 60 min. ANA is the most abundant product present and its level is more than doubled when compared with vehicle-treated cells. The observed control value may be due to traces of endotoxins present in either the culture media or the water vehicle. The identity of the major zone of radioactivity in the control extract (Fig. 2, zone 3) is not known; however, it is noteworthy that it decreases following LPS treatment suggesting a role in ANA synthesis. As reported in Section 3.1, on silica gel TLC, both precursors, EA and AA, gave radioactivity in the region defined by the addition of unlabeled, authentic ANA (Table 1). This observation minimizes the possible involvement of other fatty acid ethanolamides [3,4] in our results, since they would not have the unique combination of EA and AA seen in our data. In addition, the data shown in Fig. 2 clearly indicate that PEA is not an interfering substance under our conditions. The identity of ANA was further established by two-dimensional TLC employing the conditions described in Section 2.3. Each agonist at optimal concentration for ANA synthesis was tested under the conditions shown in Table 1. Radioactivity above background level was found in the ANA TLC zone when either AA or EA were used as precursors (data not shown).

Additional confirmation of the identity of ANA was obtained using a double isotope technique as described in Section 2.4 (Table 2). This method has been described in the literature [22] and is particularly suited where both sensitivity and specificity are needed. LPS being a potent stimulator of AA release and ANA synthesis (Fig. 1G,H), was chosen as the agonist for these studies. The media from LPS-stimulated cells showed a significant increase in the ^{14}C content as compared to the extract from vehicle-treated cells. We did not detect radioactivity due to [^{3}H]ANA in the material not retained showing that ANA is efficiently removed by this procedure. Subsequent purification of the ANA region was evidenced by a higher $^{3}H/^{14}C$ ratio for each of the control and LPS samples. In comparison with the control, this ratio was significantly lower in LPS-

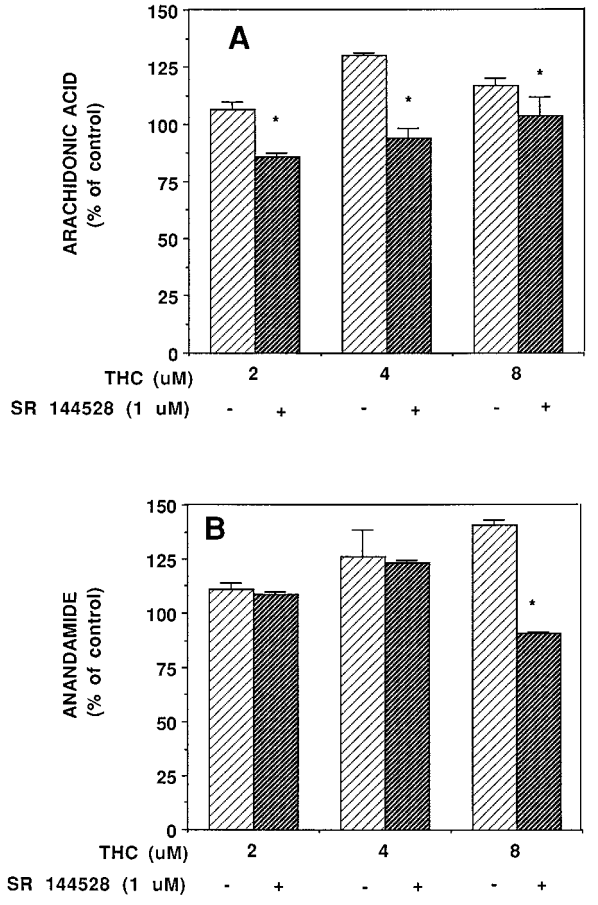

Fig. 3. SR144528 inhibits THC induced AA release and ANA synthesis. Cells ($\sim 1.5 \times 10^6$/well) were labeled with [^{3}H]AA and washed as described in Section 2. They were incubated with 1 μM SR144528 at 37°C for 30 min, after which, THC in 50% DMSO was added at the concentrations indicated. (A) After 30 min, media were sampled and radioactive content was determined by liquid scintillation counting (THC alone, light bars; SR144528 and THC, dark bars). (B) ANA was determined from combined extracts of cells and media as described in Section 2 (THC alone, light bars; SR144528 and THC, dark bars). The values shown are the means of three determinations ± S.E. Mean values for the controls are: (A) vehicle control, 2360 ± 74 dpm/ml; SR144528 control, 2873 ± 58 dpm/ml; (B) vehicle control, 477 ± 22 dpm; SR144528 control, 612 ± 12 dpm. *95% confidence limit vs. control.

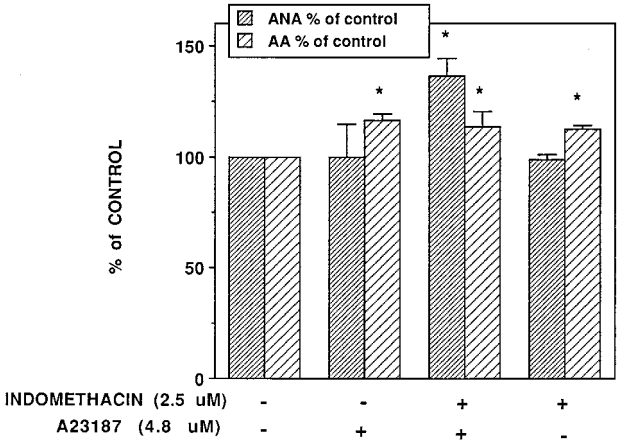

Fig. 4. Modulation of ANA levels in RAW264.7 cells by calcium ionophore requires indomethacin. Cells ($\sim 1.5 \times 10^6$/well) were labeled with [^{3}H]AA and washed as described in Section 2. They were incubated with 2.5 μM indomethacin at 37°C for 15 min, and/or with 4.8 μM A23187 in 50% DMSO as shown. After 30 min, media were sampled and radioactive content was determined by liquid scintillation counting (light bars). ANA was determined from combined extracts of cells and media as described in Section 2 (dark bars). The values shown are the means of three determinations ± S.E. Mean values for the controls are: AA; vehicle control, 1977 ± 32 dpm/ml; ANA, vehicle control, 445 ± 12 dpm. *95% confidence limit vs. control.

treated samples indicating that stimulation of ANA synthesis had occurred. At the final step, about a 10–15-fold purification of ANA was achieved for both of the samples when compared to the Sep-Pak media extract.

3.3. A possible role for the CB2 receptor in the effects of THC

CB2 has been shown to be the predominant cannabinoid receptor in certain cells of the immune system [23]. In order to find out whether THC exerted its effects on RAW cells through the CB2 receptor, we used the recently reported CB2 antagonist SR144528 [15]. This antagonist brought about a significant decrease in the THC-stimulated AA release at all of the concentrations of THC used (Fig. 3A). However, ANA synthesis was significantly inhibited only at 8 μM THC (Fig. 3B). While the full implications of these results are not as yet clear, it is interesting that the antagonist by itself also stimulated AA release and ANA synthesis (data not shown) suggesting that it is a mixed agonist–antagonist. The CB1 antagonist, SR141716A, did not affect either release of AA or ANA levels excluding the possible mediation of this receptor (S.A. Hunter and S.H. Burstein, unpublished work).

3.4. Calcium ionophore together with indomethacin augments ANA levels

ANA synthesis is induced by calcium-mobilizing agents in cells of neuronal origin [24] and certain cells of immune system origin [25]. In RAW cells, the calcium ionophore A23187 alone stimulated AA release; however, it could induce ANA synthesis only in combination with indomethacin, an inhibitor of cyclooxygenase [26] (Fig. 4A,B). This may be due to elevated intracellular calcium levels that could lead to AA release which in turn may contribute to higher ANA synthesis. However, ANA could subsequently be metabolized by cyclooxygenase-2 leading to a decrease in its levels. Another reason for low levels of ANA could be its sensitivity to hydrolysis of the amide bond. Inhibitors of ANA amidohydrolase, phenylmethylsulfonylfluoride and arachidonyl trifluoromethyl ketone are available. These compounds are reported to inhibit the amidase in N18TG2 cells [16,27] and in cells of the immune system [25]. Using either THC or LPS as the agonist we did not find a significant difference in ANA synthesis in the presence or absence of these inhibitors at concentrations of 1.5 mM and 12 μM, respectively (data not shown).

4. Discussion

The ability of the cannabinoids, ANA and THC to stimulate ANA synthesis in the peripheral cell line RAW264.7 described here (Fig. 1B,D) is similar to the effect of THC on the neuronally derived N18 cells [8]. The resemblance between the ability of these agonists to mobilize AA (Fig. 1A,C) and their effect on ANA synthesis suggests a possible relationship between the two processes. This is not too surprising since AA is one of the precursors, either immediate or distal, for ANA synthesis. On the other hand, such a relationship may be empirical and due to similar, but independent, mechanisms for the actions of these agonists. Our observation that ANA can

enhance its own synthesis (Fig. 1B) is important because it indicates a positive feedback mechanism whereby cells could rapidly and efficiently respond to a signal from some other agonist for a surge of ANA. Similar mechanisms have precedent elsewhere in the eicosanoid field [28,29].

Although receptors for cannabinoids have been discovered, given the strongly hydrophobic nature of the cannabinoids, an important question is whether their effects on AA metabolism are mediated through either of the cannabinoid receptors. Earlier work from our laboratory [9,11] and others [12,18] suggested that this was the case. In studies presented here, we have used SR144528, a CB2 receptor antagonist, which was reported to display nanomolar affinity for the cloned human CB2 receptor or the binding site in rodent immune tissue [15]. It has low affinity for the human CB1 receptor and, over the range of 1 nM to 1 μM, antagonizes several effects of the cannabinoid agonist, CP55940 [15]. In the present study, 1 μM SR144528 (the reported maximal effective concentration), inhibited 8 μM THC-induced ANA synthesis by about 50%. The reason for the lack of antagonism to ANA synthesis at lower concentrations of THC is not clear; however, it raises the possibility that a non-CB2-mediated pathway may operate in this dose range. The CB1 antagonist, SR141716A, at 1 μM had no effect on ANA synthesis. Recent experiments, using antisense oligonucleotides, indicate that in N18 cells the CB1 receptor mediates cannabinoid induced AA release while in RAW264.7 cells the CB2 receptor is primarily involved [9]. Moreover, the CB1 specific receptor antagonist, SR141716A, inhibits the release effect in N18 [9] cells, but not in RAW264.7 cells (S.A. Hunter, and S.H. Burstein, unpublished work). Thus, it seems that the cannabinoid effect on ANA synthesis in macrophages, at certain concentrations, is mediated via the CB2 receptor.

We also report the effects on ANA synthesis of two physiologically related agonists, bacterial LPS and PAF. As in the case of the agonists discussed above, PAF also is able to promote AA release presumably by mediation of its G-protein coupled, seven transmembrane receptor [19]. This results in a number of physiological and pathophysiological events, such as platelet aggregation, acute inflammation, neuronal cell differentiation, etc. If, as our results indicate, ANA is a product of PAF action (Fig. 1F), then it may have an important role in many of the processes attributable to PAF action. How ANA may participate in these effects is not known at this point; however, our findings may suggest new areas for studying its actions and could, perhaps, help to better understand some of its known activities.

Additional evidence that the macrophage cell line, RAW264.7 is capable of ANA synthesis in response to stimulation by LPS was obtained by the use of radiochemically pure [^{3}H]ANA standard in combination with successive TLC analyses. This involves the addition of the standard to incubation media samples from [^{14}C]AA labeled cells followed by extraction and TLC. The attainment of a constant isotope ratio is considered evidence for the identity of ANA as a metabolite of AA. In our experimental model, a relatively constant tritium to carbon-14 ratio for ANA over three consecutive chromatographic separations was obtained (Table 2). The modest recoveries at each step could be due to the lability of ANA; however, this would not affect the ratio values. The mean ratio for ANA from the LPS-stimulated cells is about three times lower than the control value. This is due to the proportionately higher carbon-14 content found in the ANA TLC region of the media extract from LPS-treated cells supporting our other data (Fig. 1H) that LPS stimulates ANA synthesis. The mobilization of AA and its subsequent conversion to eicosanoids by LPS has been widely observed [19,20], so that the observations we report here would seem to be a logical extension of what has been reported by others. As with the cannabinoids, there is a correlation between the release of arachidonate and the synthesis of ANA suggesting that the two events are related. LPS stimulation of AA mobilization appears to involve the participation of a G-protein [20]; however, the identity of the related receptor is not well understood. The precise role of ANA in the cellular response to endotoxins is a matter for speculation at this time. In this context, it is interesting that endogenous as well as exogenous cannabinoids have been suggested to have effects on the immune response [30] and may even play a role in HIV induced pathogenesis [31]. In fact, a negative effect of cannabinoids on immune responses to bacterial infection in mice and antagonism of this effect by prior exposure to pertussis toxin has been

reported [32]. It was suggested that the decreased immunity observed was due to increased mobilization of acute phase cytokines such as IL-1 and TNF, and that AA metabolites are involved in mediating these effects. Possibly ANA is one of the mediators responsible.

Work done by others has implicated calcium as an important factor in stimulation of ANA synthesis in intact rat central neurons [14], rat basophilic leukemia (RBL-2H3) cells [25], rat blood macrophages [33] and J774 macrophages [25]. This is suggested to occur through induction of hydrolysis of a membrane phospholipid, NAPE and the subsequent release of ANA. In RAW264.7 cells the calcium ionophore A23187 stimulated significant AA release; however, the ANA levels increased only when the cells were pretreated with indomethacin (Fig. 4). Since indomethacin is a known cyclooxygenase inhibitor, we suggest that one of the pathways for ANA metabolism in RAW264.7 cells is via oxidation by cyclooxygenase-2. Recently reported evidence for the existence of a cyclooxygenase-2-mediated pathway for ANA metabolism [34,35] supports this notion. It is interesting to note that in J774 cells ANA hydrolysis is a relatively slow process [25] suggesting that other routes of metabolism for ANA may be of greater physiological importance. It is, of course, also possible that the indomethacin effect on ANA synthesis could be due to a shunting of free AA away from prostaglandin synthesis. The present evidence does not distinguish between these two possibilities.

In conclusion, we have shown that several diverse agonists for the mobilization of AA also promote the synthesis of ANA resulting in dramatic increases in ANA levels. These observations may reflect an empirical relationship or, may possibly, have a mechanistic basis. This point remains to be determined. THC-mediated AA release and to some extent ANA synthesis appear to be CB2 receptor-mediated processes in RAW264.7 cells. ANA synthesis by these cells is upregulated by combined treatment with calcium ionophore and indomethacin, suggesting cyclooxygenase-mediated metabolism of ANA. Our findings on the synthesis of ANA by a macrophage derived cell line indicate a possible role for ANA when the immune system is challenged by agents such as PAF and the bacterial endotoxins.

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