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# Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylethanolamide

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The human astrocytoma cell line CCF-STTG1 accumulates [<sup>3</sup>H]2-AG through an Na<sup>+</sup>- and energy-independent process, with a K<sub>m</sub> of 0.7 ± 0.1 μM. Non-radioactive 2-AG, anandamide or the anandamide transport inhibitor 4-hydroxyphenyl arachidonamide inhibit [<sup>3</sup>H]2-AG uptake with half-maximal inhibitory concentrations (IC<sub>50</sub>) of 5.5 ± 1.0 μM, 4.2 ± 0.3 μM and 1.8 ± 0.1 μM, respectively. A variety of lipid transport substrates and inhibitors interfere with neither [<sup>3</sup>H]2-AG nor [<sup>3</sup>H]anandamide uptake. These results suggest that 2-AG and anandamide are internalized in astrocytoma cells through a common carrier-mediated mechanism. After incubation with

[<sup>3</sup>H]2-AG, radioactivity is recovered in phospholipids, monoacylglycerols (unmetabolized [<sup>3</sup>H]2-AG), free fatty acids ([<sup>3</sup>H]arachidonate) and, to a minor extent, diacylglycerols and triacylglycerols. Arachidonic acid (100 μM) and triacsin C (10 μM), an acyl-CoA synthetase inhibitor, prevent incorporation of [<sup>3</sup>H]arachidonic acid in phospholipids and significantly reduce [<sup>3</sup>H]2-AG transport. Thus, the driving force for 2-AG internalization may derive from the hydrolysis of 2-AG to arachidonate and the subsequent incorporation of this fatty acid into phospholipids. *NeuroReport* 11:1231–1235 © 2000 Lippincott Williams & Wilkins.

**Key words:** 2-Arachidonylethanolamide; Anandamide; Endogenous cannabinoid; Human astrocytoma cells; Transport

## INTRODUCTION

Two endogenous cannabinoid compounds, anandamide (arachidonyl ethanolamide) and 2-arachidonylethanolamide (2-AG), have been identified in the brain [1–6]. Both compounds activate cannabinoid receptors producing a spectrum of cannabimimetic effects, which include movement inhibition, pain relief and vasodilation [7].

Anandamide is released from neurons in an activity-dependent manner [2,6] and is inactivated by transport into cells followed by intracellular hydrolysis. Anandamide transport is mediated by a high-affinity, Na<sup>+</sup>-independent carrier and is selectively inhibited by 4-hydroxyphenyl arachidonamide (AM404) [8,9]. Hydrolysis of anandamide to arachidonic acid and ethanolamine is catalyzed by a membrane-bound amidohydrolase enzyme [10–12], which has been purified and molecularly cloned [13,14]. 2-AG may be produced by cleavage of 1,2-diacylglycerol generated by phospholipase C (PLC) acting on phosphatidylinositol biphosphate [15]. The mechanisms mediating 2-AG inactivation are still only partially understood, but the ability of 2-AG to inhibit [<sup>3</sup>H]anandamide transport in human astrocytoma cells [16] and to serve as a substrate

for anandamide amidohydrolase [17,18] suggest that 2-AG and anandamide may share a common inactivation route.

As a test of this hypothesis, here we have investigated the biological disposition of [<sup>3</sup>H]2-AG in human astrocytoma cells. Our results suggest that 2-AG and anandamide may be internalized in these cells via a common carrier-mediated process. By contrast, the intracellular hydrolysis of 2-AG to arachidonic acid and glycerol may be catalyzed by an enzyme activity distinct from anandamide amidohydrolase.

## MATERIALS AND METHODS

**Chemicals:** Anandamide and palmitylethanolamide were prepared as described [1]; 2-AG was from Deva Biotech (Hartboro, PA); arachidonic acid from Nu Check-Prep (Elysian, MN); AM404 from Tocris (Ballwin, MO); triacsin C from Biomol (Plymouth Meeting, PA); prostaglandin E<sub>2</sub> and (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one from Cayman (Ann Arbor, MI); sulphobromophthalein, digoxin, taurocholic acid, quinidine, verapamil and phloretin from Sigma (St. Louis, MO). All organic solvents were from Burdick and Jackson.

**Transport assay:** Human astrocytoma CCF-STTG1 cells (American Type Culture Collection, Rockville, MD) were grown in 24-well plates with RPMI 1640 medium containing FBS (10%) and glutamine (1 mM) [16]. Confluent cultures were rinsed with Tris–Krebs' buffer at 37°C, incubated for 4 min with buffer containing [<sup>3</sup>H]2-AG (for kinetic analyses: 28–1600 nM; for inhibition assays: 30 nM; 100 mCi/mmol; New England Nuclear, custom synthesized) either at 37°C or 0–4°C, rinsed with buffer containing bovine serum albumin (BSA, 0.1%), and disrupted by sonication in buffer containing Triton X-100 (1%). Radioactivity in samples was measured by liquid scintillation counting. In some experiments, anandamide transport was measured using [<sup>3</sup>H]anandamide (30 nM, 220 Ci/mmol, New England Nuclear). For inhibition assays, the cells were preincubated for 10 min with test compounds at appropriate concentrations. The same concentrations of test compounds were also added to the final incubations, which were carried out for an additional 4 min period. Concentrations required for half-maximal inhibition of transport (IC<sub>50</sub>) were determined by non-linear least square fitting of the data, using the software GraphPad Prism (GraphPad Software, San Diego, CA).

**Hydrolysis assay:** Following incubations with [<sup>3</sup>H]2-AG or [<sup>3</sup>H]anandamide, the cells were rinsed and disrupted by sonication in methanol at 0–4°C for 30 s. Lipids were extracted with chloroform (chloroform/methanol/buffer, 2:1:1; vol./vol./vol.). The lipid extracts were analyzed by thin-layer chromatography (TLC) on silica gel G plates (Analtech). TLC analyses were carried out using either ethylacetate/methanol/water/ammonium hydroxide (20:1:10:1) or chloroform/methanol/ammonium hydroxide (80:20:1) as solvent system. The lipids were visualized with a 10% solution of phosphomolybdic acid (Sigma, Saint Louis, MO) in ethanol followed by heating at 250°C. Bands with the mobility of authentic standards were scraped off the TLC plates for radioactivity determination.

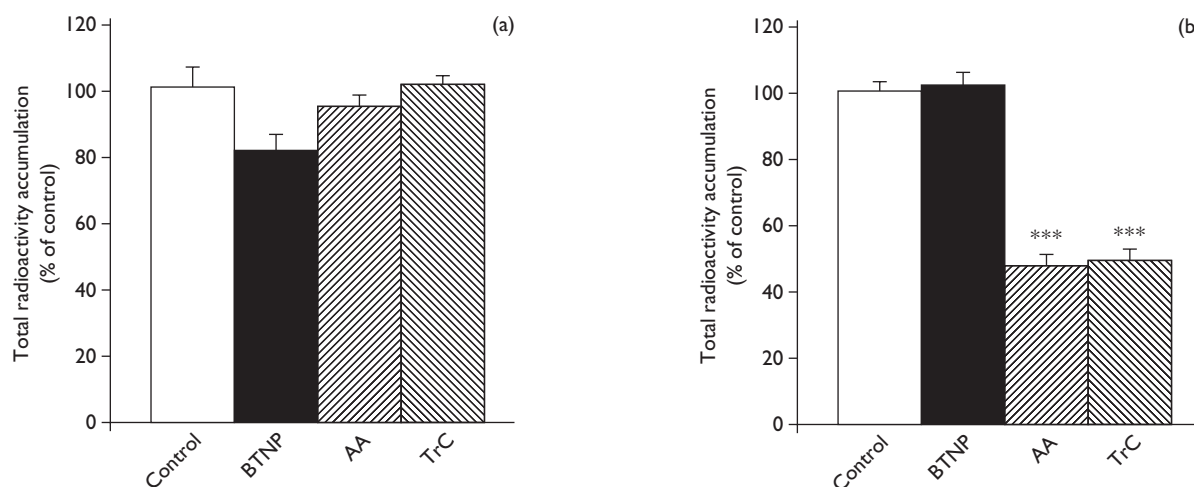
**Statistical analyses:** Statistically significant differences were determined by ANOVA, followed by Bonferroni's multiple comparison test.

## RESULTS

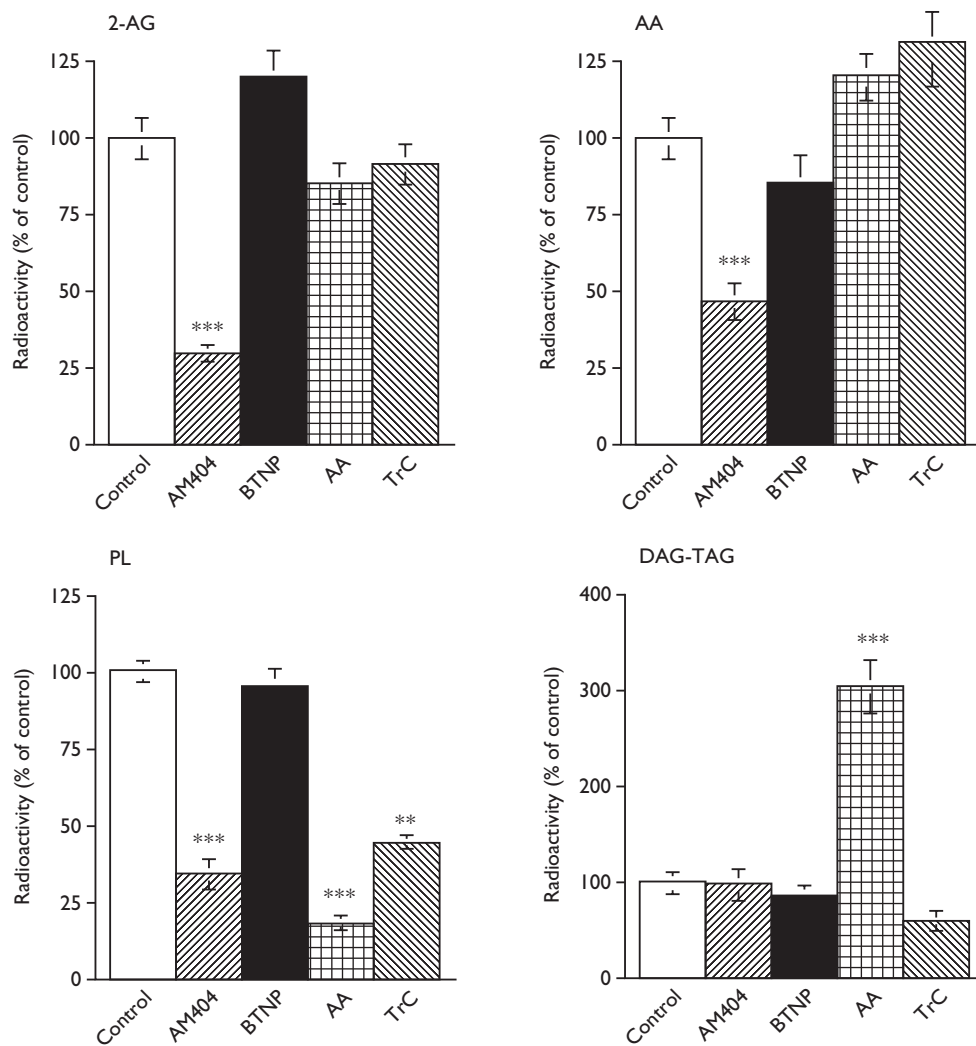
[<sup>3</sup>H]2-AG accumulation in astrocytoma cells is rapid and saturable: Lineweaver–Burk analyses of this accumulation yield a Michaelis constant (K<sub>m</sub>) of 0.7 ± 0.1 μM and a maximal accumulation rate (V<sub>max</sub>) of 28 ± 6 pmol min<sup>-1</sup> mg<sup>-1</sup> protein (n = 9).

Non-radioactive 2-AG inhibits [<sup>3</sup>H]2-AG accumulation in a concentration-dependent manner, with a half-maximal inhibitory concentration (IC<sub>50</sub>) of 5.5 ± 1.0 μM (n = 6). At 100 μM, non-radioactive 2-AG reduces [<sup>3</sup>H]2-AG accumulation in cells to 24 ± 1% of control (control: 8381 ± 393 d.p.m./well; 2-AG: 2017 ± 87 d.p.m./well; n = 6). We considered this value to be an estimate of the non-specific association of [<sup>3</sup>H]2-AG with the cells, and used it in subsequent experiments as a background value to calculate potency and efficacy of test compounds.

Non-radioactive anandamide inhibits [<sup>3</sup>H]2-AG accumulation with an IC<sub>50</sub> of 4.2 ± 0.3 μM and a maximal effect of 100% (n = 6). The anandamide transport inhibitor AM404 is also very effective in interfering with [<sup>3</sup>H]2-AG uptake (IC<sub>50</sub> = 1.8 ± 0.1 μM, n = 6). Conversely, a variety of substrates and inhibitors of lipid transport systems (including prostaglandin E<sub>2</sub>, sulphobromophthalein, digoxin, taurocholic acid, quinidine, verapamil and phloretin) have no effect on the accumulation of [<sup>3</sup>H]2-AG (data not shown). On the other hand, arachidonic acid (100 μM) substantially reduces the accumulation of [<sup>3</sup>H]2-AG in astrocytoma cells (Fig. 1b). TLC analyses revealed that astrocytoma cells rapidly metabolize [<sup>3</sup>H]2-AG (Fig. 2). After a 4 min incubation, cell-associated radioactivity is distributed among TLC fractions migrating with monoacylglycerols (unmetabolized [<sup>3</sup>H]2-AG; 1384 ± 46 d.p.m./well), free fatty acids (probably [<sup>3</sup>H]arachidonic acid, 560 ± 64 d.p.m./well), phospholipids (6928 ± 504 d.p.m./well), diacylglycerols and triacylglycerols (370 ± 52 d.p.m./well; results are from



**Fig. 1.** Effects of various compounds on [<sup>3</sup>H]2-AG and [<sup>3</sup>H]anandamide accumulation by astrocytoma cells. Intracellular accumulation of [<sup>3</sup>H]anandamide (a) and [<sup>3</sup>H]2-AG (b) was measured in cells at confluence in the presence of the anandamide amidohydrolase inhibitor BTNP (5 μM), the acyl-CoA synthetase inhibitor triacsin C (10 μM), or the hydrolysis product, arachidonic acid (100 μM). Results represent the mean ± s.e.m. (n = 6–9). \*\*\* p < 0.001 (ANOVA followed by Bonferroni multiple comparison test).



**Fig. 2.** Effects of various compounds on  $[^3\text{H}]2\text{-AG}$  metabolism in astrocytoma cells. Cells at confluence were incubated in Tris-Krebs' buffer containing  $[^3\text{H}]2\text{-AG}$  with or without one of the following compounds: AM404, an anandamide transport inhibitor ( $10\ \mu\text{M}$ ); BTNP, an anandamide amidohydrolase inhibitor ( $5\ \mu\text{M}$ ); arachidonic acid (AA,  $100\ \mu\text{M}$ ); and triacsin C, an acyl CoA synthetase inhibitor ( $10\ \mu\text{M}$ ). Reactions were stopped by addition of cold methanol, and the lipids were extracted and analyzed by TLC. The lipids were stained with phosphomolybdic acid, identified by comparison with authentic standards and quantified by liquid scintillation counting. PL, phospholipids; DAG, diacylglycerols; TAG, triacylglycerols. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (ANOVA followed by Bonferroni multiple comparison test).

one experiment representative of three). AM404 ( $10\ \mu\text{M}$ ), significantly reduces the incorporation of radioactivity in the TLC fractions migrating with  $[^3\text{H}]2\text{-AG}$  ( $413 \pm 54$  d.p.m./well),  $[^3\text{H}]$ arachidonic acid ( $203 \pm 26$  d.p.m./well) and phospholipids ( $2906 \pm 141$  d.p.m./well). These results are in agreement with the ability of AM404 to inhibit  $[^3\text{H}]2\text{-AG}$  accumulation in astrocytoma cells, and underscore the intracellular localization of  $[^3\text{H}]2\text{-AG}$  hydrolysis. Conversely, the anandamide amidohydrolase inhibitor, (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP;  $5\ \mu\text{M}$ ) [19] has no effect on  $[^3\text{H}]2\text{-AG}$  accumulation (Fig. 1b) or metabolism (Fig. 2).

If arachidonic acid directly interfered with  $[^3\text{H}]2\text{-AG}$  transport it should produce an effect similar to that of AM404 (i.e. a complete inhibition of  $[^3\text{H}]2\text{-AG}$  transport). In contrast with this prediction, arachidonic acid ( $100\ \mu\text{M}$ ) does not affect radioactivity in the TLC fractions migrating

with  $[^3\text{H}]2\text{-AG}$  ( $1222 \pm 152$  d.p.m./well) and  $[^3\text{H}]$ -arachidonic acid ( $631 \pm 75$  d.p.m./well), but drastically reduces the radioactivity in phospholipids (to  $1369 \pm 287$  d.p.m./well). Arachidonic acid also augments incorporation of radioactivity in diacylglycerols and triacylglycerols (to  $1335 \pm 42$  d.p.m./well; Fig. 2). However, this increase is not sufficient to compensate for the substantial loss of radioactivity in the phospholipid fraction and we did not investigate further its molecular basis. These findings suggest that arachidonic acid inhibits  $[^3\text{H}]2\text{-AG}$  accumulation indirectly, possibly by hindering the esterification into phospholipids of the  $[^3\text{H}]$ arachidonate produced by the hydrolysis of  $[^3\text{H}]2\text{-AG}$ .

To test this possibility, we investigated the effects of triacsin C, a selective acyl-coenzyme A synthetase inhibitor [20], on  $[^3\text{H}]2\text{-AG}$  uptake and metabolism. Incubation of astrocytoma cells with triacsin C ( $10\ \mu\text{M}$ ) significantly

reduces both [ $^3\text{H}$ ]2-AG uptake (Fig. 1b) and incorporation of [ $^3\text{H}$ ]arachidonate in phospholipids (Fig. 2). By contrast, the inhibitor has no effect on the levels of radioactivity in [ $^3\text{H}$ ]2-AG, [ $^3\text{H}$ ]arachidonic acid, diacylglycerols and triacylglycerols (Fig. 2). In addition, triacsin C does not affect [ $^3\text{H}$ ]anandamide accumulation (Fig. 1a) or its intracellular hydrolysis (data not shown).

## DISCUSSION

Human astrocytoma CCF-STTG1 cells internalize [ $^3\text{H}$ ]anandamide by a transport mechanism functionally indistinguishable from that found in rat brain neurons and astrocytes [8,16]. Therefore, we used these cells to test the hypothesis that [ $^3\text{H}$ ]2-AG may be a substrate for the anandamide transporter. The kinetic of [ $^3\text{H}$ ]2-AG accumulation in astrocytoma cells is consistent with a single high-affinity transport process. In the same cells and under identical conditions, [ $^3\text{H}$ ]anandamide transport displays similar characteristics ( $K_m$  of  $0.6 \pm 0.1 \mu\text{M}$  and  $V_{max}$  of  $14.7 \pm 1.5 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ ) [16]. Several lines of evidence indicate that [ $^3\text{H}$ ]2-AG accumulation in astrocytoma cells is mediated by a transport system akin to that involved in anandamide internalization. First, non-radioactive anandamide inhibits [ $^3\text{H}$ ]2-AG accumulation while, conversely, 2-AG inhibits [ $^3\text{H}$ ]anandamide transport ( $IC_{50}$  of  $18.5 \pm 0.7 \mu\text{M}$ ) [16]. Second, the anandamide transport inhibitor AM404 is also effective, and even more potent in interfering with [ $^3\text{H}$ ]2-AG uptake than anandamide or 2-AG. Third, a variety of substrates and inhibitors of lipid transport systems have no effect on the accumulation of either [ $^3\text{H}$ ]2-AG or [ $^3\text{H}$ ]anandamide [8,16]. Finally, anandamide and 2-AG transport are both  $\text{Na}^+$  and energy independent [8,16].

There are, however, a number of differences between anandamide and 2-AG inactivation. [ $^3\text{H}$ ]Anandamide transport is not affected by arachidonic acid (Fig. 1a) and, *vice versa*, [ $^3\text{H}$ ]arachidonic acid transport is not affected by anandamide (data not shown), indicating that distinct membrane mechanisms mediate the internalization of these two lipid compounds [8,16]. By contrast, arachidonic acid substantially reduces the accumulation of [ $^3\text{H}$ ]2-AG (Fig. 1b). A possible interpretation of this finding is that arachidonic acid may directly interfere with the putative carrier involved in [ $^3\text{H}$ ]2-AG uptake, implying the existence of different transport mechanisms for [ $^3\text{H}$ ]2-AG and [ $^3\text{H}$ ]anandamide (sensitive and insensitive, respectively, to arachidonic acid). An alternative possibility is that arachidonic acid may inhibit [ $^3\text{H}$ ]2-AG accumulation indirectly, for example by interfering with the intracellular disposition of [ $^3\text{H}$ ]2-AG. To evaluate these alternative possibilities, we investigated the fate of [ $^3\text{H}$ ]2-AG in astrocytoma cells. The routes of 2-AG metabolism in cells are still only partially understood, but two enzymes are thought to be primarily involved: anandamide amidohydrolase and monoacylglycerol lipase [17,18,21]. Both enzymes catalyze the hydrolytic cleavage of 2-AG to arachidonate and glycerol. Free arachidonate is short-lived in cells, being rapidly converted to arachidonyl-coenzyme A (CoA) and then incorporated into phospholipids [22]. Blockade of anandamide amidohydrolase activity completely abrogates [ $^3\text{H}$ ]anandamide degradation [8,16], but it does not affect [ $^3\text{H}$ ]2-AG hydrolysis, suggesting that monoacylglycerol lipase may be a major

player in 2-AG degradation in intact cells [23]. The transport inhibitor AM404 dramatically reduces the intracellular accumulation of [ $^3\text{H}$ ]2-AG whereas administration of arachidonic acid has no effect on intracellular [ $^3\text{H}$ ]2-AG levels. These findings suggest that arachidonic acid may inhibit [ $^3\text{H}$ ]2-AG accumulation indirectly, possibly by hindering the esterification into phospholipids of the [ $^3\text{H}$ ]arachidonate produced by the hydrolysis of [ $^3\text{H}$ ]2-AG. These results imply that arachidonate esterification in membrane phospholipids may be a primary driving force for [ $^3\text{H}$ ]2-AG transport.

## CONCLUSIONS

Our findings suggest that 2-AG and anandamide are internalized in astrocytoma cells via a common carrier-mediated transport system. Four key observations support this hypothesis: (1) the kinetic properties of [ $^3\text{H}$ ]2-AG and [ $^3\text{H}$ ]anandamide accumulation are very similar; (2) 2-AG and anandamide mutually displace each other's accumulation; (3) [ $^3\text{H}$ ]2-AG uptake is blocked by the anandamide transport inhibitor, AM404; (4) [ $^3\text{H}$ ]2-AG and [ $^3\text{H}$ ]anandamide uptake are insensitive to several substrates and inhibitors of lipid transporters and  $\text{Na}^+$ - and energy-independent. By contrast, the intracellular degradation of 2-AG may differ substantially from that of anandamide: whereas anandamide hydrolysis is blocked by BTNP, an inhibitor of amidohydrolase activity, 2-AG hydrolysis is insensitive to this drug. This indicates that 2-AG breakdown in intact cells may depend on other hydrolase enzymes, such as monoacylglycerol lipase [23]. The role of hydrolysis in 2-AG disposition may differ from anandamide in another important way. We found that treating the cells with arachidonic acid or the acyl-CoA synthetase inhibitor, triacsin C, prevents the transport of 2-AG, but not that of anandamide. A plausible interpretation of these results is that 2-AG hydrolysis into free arachidonic acid and the subsequent incorporation of arachidonic acid into membrane phospholipids may be a primary driving force for 2-AG internalization. This difference between the biological dispositions of anandamide and 2-AG might be exploited for the development of selective inactivation inhibitors.

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