Functional role of high-affinity anandamide transport, as revealed by selective inhibition.

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Anandamide, an endogenous ligand for central cannabinoid receptors, is released from neurons on depolarization and rapidly inactivated. Anandamide inactivation is not completely understood, but it may occur by transport into cells or by enzymatic hydrolysis. The compound N-(4-hydroxyphenyl)arachidonoylamine (AM404) was shown to inhibit high-affinity anandamide accumulation in rat neurons and astrocytes in vitro, an indication that this accumulation resulted from carrier-mediated transport. Although AM404 did not activate cannabinoid receptors or inhibit anandamide hydrolysis, it enhanced receptor-mediated anandamide responses in vitro and in vivo. The data indicate that carrier-mediated transport may be essential for termination of the biological effects of anandamide, and may represent a potential drug target.

Anandamide (arachidonylethanolamide) is an endogenous lipid that activates brain cannabinoid receptors and mimics the pharmacological effects of Δ2-tetrahydrocannabinol, the active principle of hashish and marijuana (1). In humans, such effects include euphoria, calmness, dream states, and drowsiness (2). Depolarized neurons release anandamide (3) through a mechanism that may require the calcium-dependent cleavage of a phospholipid precursor in neuronal membranes (4). Like other modulatory substances, extracellular anandamide is thought to be rapidly inactivated, but the exact nature of this inactivating process is still unclear. A possible pathway is hydrolysis to arachidonic acid and ethanolamine, catalyzed by a membrane-bound fatty acid amide hydrolase (FAAH) highly expressed in rat brain and liver (5). Nevertheless, the low FAAH activity found in brain plasma membranes indicates that this enzyme may be intracellular (5), a possibility that is further supported by sequence analysis of rat FAAH (6). Although anandamide could gain access to FAAH by passive diffusion, the transfer rate is expected to be low because of the molecular size of this lipid mediator (7). In that other lipids including polyunsaturated fatty acids and prostaglandin E1 (PGE1) enter cells by carrier-mediated transport (8, 9), it is possible that anandamide uses a similar mechanism. Indeed, the existence of a rapid, saturable process of anandamide accumulation into neural cells has been reported (3). This accumulation may result from the activity of a transmembrane carrier, which may thus participate in termination of the biological actions of anandamide. Accordingly, we developed drug inhibitors of anandamide transport and investigated their pharmacological properties in cultures of rat cortical neurons or astrocytes.

The accumulation of exogenous [3H]anandamide by neurons or astrocytes fulfills several criteria of a carrier-mediated transport (Fig. 1) (10). It is a rapid process that reaches 50% of its maximum within about 4 min (Fig. 1A). Furthermore, [3H]anandamide accumulation is temperature-dependent (Fig. 1A) and saturable (Fig. 1, B and C). Kinetic analyses revealed that accumulation in neurons can be represented by two components of differing affinities (lower affinity; Michaelis constant, $K_m = 1.2 \mu M$, maximum accumulation rate, $V_{max} = 90.9$ pmol/min per milligram of protein; higher affinity: $K_m = 0.032 \mu M$, $V_{max} = 5.9$ pmol/min per milligram of protein) (Fig. 1B). The higher affinity component may reflect a binding site, however, as it is displaced by the cannabinoid receptor antagonist, SR141716-A (100 nM) (11). In astrocytes, [3H]anandamide accumulation is represented by a single high-affinity component ($K_m = 0.32 \mu M$, $V_{max} = 171$ pmol/min per milligram of protein) (Fig. 1C). Such apparent $K_m$ values are similar to those of known neurotransmitter uptake systems (12) and are suggestive of high-affinity carrier-mediated transport.

To characterize further this putative anandamide transport, we used cortical astrocytes in culture. As expected from a selective process, the temperature-sensitive component of [3H]anandamide accumulation was prevented by nonradioactive anandamide, but not by palmitoylethanolamide, arachidonate, prostanooids, or leukotrienes (Fig. 2A). Replacement of extracellular Na+ with N-dimethylglucosamine or choline had no effect (as percentage of control: N-dimethylglucosamine, 124 ± 12%; choline, 98 ± 14%; mean ± SEM, n = 6), suggesting that [3H]anandamide accumulation is mediated by a Na+-independent mechanism, which has been observed with other lipids (8, 9). Moreover, inhibition of FAAH activity by treating the cells with (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (25 μM) or linoleyl trifluoromethyl ketone (15 μM) (13, 14) had no effect (Fig. 2, B and C). This indicates that anandamide hydrolysis did not provide the driving force for anandamide transport into astrocytes within the
50 μM), phospholipids (verapamil, 100 μM; quinidine, 50 μM), or PGE₂ (bromocresol green, 0.1 to 100 μM) (15). Among the compounds tested, only bromocresol green interfered with anandamide transport, albeit with limited potency and partial efficacy (Fig. 3, A and B). Bromocresol green inhibited [3H]anandamide accumulation with an IC₅₀ (concentration needed to produce half-maximal inhibition) of 4 μM in neurons and 12 μM in astrocytes and acted noncompetitively (16). Moreover, bromocresol green had no significant effect on the binding of [3H]WIN-55212-2 to rat cerebral membranes (inhibition constant, Kᵢ = 22 μM), FAAH activity in rat brain microsomes (IC₅₀ > 50 μM), and uptake of [3H]arachidonate or [3H]ethanolamine in astrocytes (121 ± 13% and 103 ± 12%, respectively, at 50 μM bromocresol green, n = 3) (17). The sensitivity to bromocresol green, which blocks PGE₂ transport, raised the question of whether anandamide accumulation occurred by means of a PGE₂ carrier. That this is not the case was shown by the lack of [3H]PGE₂ accumulation in neurons or astrocytes (18) and by the inability of PGE₂ to interfere with [3H]anandamide accumulation (Fig. 2A). Previous results indicate that expression of PGE₂ transport may act synergistically in the brain to dispose extracellular anandamide and (ii) that the transport system in these two cell types may differ kinetically and pharmacologically (Fig. 1, B and C, and Fig. 3, C and D).

The identification of inhibitors allowed us to examine whether transmembrane transport participates in terminating anandamide responses mediated by cannabinoid receptor activation. Cannabinoid receptors of the CB₁ subtype are expressed in neurons (21) where they are negatively coupled to adenyl cyclase activity (22). Accordingly, in cultures of rat cortical neurons the cannabinoid receptor agonist WIN-55212-2 inhibited forskolin-stimulated adenosine 3′,5′-monophosphate (cAMP) accumulation (control: 39 ± 4 pmol per milligram of protein; 3 μM forskolin: 568 ± 4 pmol per milligram of protein; forskolin plus 1 μM WIN-55212-2: 220 ± 24 pmol per milligram of protein), and this inhibition was prevented by the antagonist SR-141716-A (1 μM) (555 ± 39 pmol/mg of protein, n = 9) (23). Anandamide produced a similar effect, but with a potency (IC₅₀, 1 μM) that was 1/20 of that expected from its binding to the cannabinoid receptor (20), suggesting that it may serve as a transport substrate or pseudosubstrate. In contrast, at the concentrations tested AM404 had no effect on FAAH activity (IC₅₀ > 30 μM) or on uptake of [3H]arachidonate or [3H]ethanolamine (102 ± 4% and 96 ± 14%, respectively, at 20 μM AM404, n = 6). Furthermore, a positional isomer of AM404, N-(3-hydroxyphenyl)arachidonoylamine (AM403), was significantly less effective than AM404 in inhibiting transport (Fig. 3, C and D). These data provide pharmacological evidence for the existence of a specific anandamide transporter and suggest (i) that neurons and astrocytes may act synergistically in the brain to dispose of extracellular anandamide and (ii) that the transport systems in these two cell types may differ kinetically and pharmacologically (Fig. 1, B and C, and Fig. 3, C and D).

A primary criterion for defining carrier-mediated transport is pharmacological inhibition. To identify inhibitors of anandamide transport, we first examined compounds that prevent the cellular uptake of other lipids, such as fatty acids (phloretin, lorlopamidene (120 μM)), leukotriene C₄ (LTC₄; 1 μM), leukotriene B₄ (LTB₄; 1 μM), PGE₂ (100 μM), or thromboxane B₂ (TXB₂; 50 μM). The broken line indicates nonspecific [3H]anandamide accumulation in cells measured at 0°C to 4°C (43 ± 3% of total accumulation, which in these experiments was 43,104 ± 1249 dpm per well). Results are expressed as mean ± SEM (n = 6 to 9). Effects of FAAH inhibitors on (B) FAAH activity and (C) [3H]anandamide accumulation in cortical astrocytes. Cells were incubated for 10 min with (-)-6-(bromomethyl)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP, 25 μM) or linoleyl trifluoromethylketone (Lyn-TFK, 15 μM), and then with the same drugs plus [3H]anandamide for an additional 20 min. The total radioactivity in cell lipid extracts (to measure [3H]anandamide transport) (10) and radioactivity in nonesterified arachidonate (to measure FAAH activity) (13) were measured separately in lipid extracts prepared from the same cultures.

![Fig. 2. (A) Selectivity of [3H]anandamide accumulation in cortical astrocytes. Accumulation was measured after a 4-hr incubation with [3H]anandamide at 37°C, in the absence (control) or presence of nonradioactive anandamide (100 μM), N-palmitoylethanolamide (100 μM), arachidonate (100 μM), leukotriene C₄ (1 μM), leukotriene B₄ (1 μM), PGE₂ (100 μM), or thromboxane B₂ (15 μM). The broken line indicates nonspecific [3H]anandamide accumulation in cells measured at 0°C to 4°C (43 ± 3% of total accumulation, which in these experiments was 43,104 ± 1249 dpm per well). Results are expressed as mean ± SEM (n = 6 to 9). Effects of FAAH inhibitors on (B) FAAH activity and (C) [3H]anandamide accumulation in cortical astrocytes. Cells were incubated for 10 min with (-)-6-(bromomethyl)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP, 25 μM) or linoleyl trifluoromethylketone (Lyn-TFK, 15 μM), and then with the same drugs plus [3H]anandamide for an additional 20 min. The total radioactivity in cell lipid extracts (to measure [3H]anandamide transport) (10) and radioactivity in nonesterified arachidonate (to measure FAAH activity) (13) were measured separately in lipid extracts prepared from the same cultures.](image-url)
constant for CB1 cannabinoid receptors (Kᵢ ≈ 50 nM) (1) (Fig. 4A). The transport inhibitor AM404 bound to CB1 receptors with low affinity (Kᵢ = 1.8 μM) (19) and did not reduce cAMP concentrations when applied at 10 μM (Fig. 4B). Nevertheless, the drug enhanced the effects of anandamide, increasing the potency (by a factor of 10) and decreasing the threshold (by a factor of 1/100), an effect that was prevented by SR-141716-A (Fig. 4A). Thus, a concentration of anandamide that was below threshold when applied alone (0.3 μM) produced an almost maximal effect when applied with AM404 (Fig. 4B). Bromocresol green and AM403, which were less effective than AM404 in inhibiting anandamide transport (Fig. 4B), produced an almost maximal effect when applied at 10 μM (Fig. 4B). Nevertheless, AM404 (10 mg/kg, intravenously) had no antinociceptive effect within 60 min of injection and was prevented by SR-141716-A (Fig. 4C). Administration of AM404 (10 mg/kg, intravenously) had no antinociceptive effect within 60 min of injection but significantly enhanced and prolonged anandamide-induced analgesia (Fig. 4C) (P < 0.01, Student’s t test).

Our findings indicate that a high-affinity transport system present in neurons and astrocytes has a role in anandamide inactivation by removing this lipid mediator from the extracellular space and delivering it to intracellular metabolizing enzymes such as FAAH (5, 6). Therefore, the identification of selective inhibitors of anandamide transport should be instrumental in understanding the physiological roles of the endogenous cannabinoid system and may lead to the development of therapeutic agents.

REFERENCES AND NOTES

1. W. A. Devane et al., Science 258, 1946 (1992); R. Mechoulam, L. Harus, B. R. Martin, Biochem. Pharmacol. 48, 1537 (1994).

2. W. L. Dewey, Pharmacol. Rev. 38, 151 (1986).

3. V. Di Marzo et al., Nature 372, 686 (1994).

4. H. Cadas, S. Gaillet, M. Beltramo, L. Venance, D. Piomelli, J. Neurosci. 16, 3934 (1996); T. Sugitara et al., Eur. J. Biochem. 240, 53 (1996); H. Cadas, E. D’Iorio, D. Piomelli, J. Neurosci., 17, 1292 (1997).

5. D. G. Deutsch and S. Chih, Biochem. Pharmacol. 46, 791 (1993); F. Desarnaud, H. Cadas, D. Piomelli, J. Biol. Chem. 270, 6598 (1995); N. Ueda, Y. Kurasaki, S. Yamamoto, T. Tokunaga, ibid., p. 23823.

6. E. Cravatt et al., Nature (London) 384, 53 (1996).

7. W. D. Stein, Channels and Pumps. An Introduction to Membrane Transport, (Academic Press, San Diego, 1990), pp. 53–57.

8. L. Z. Bito, Nature 256, 1234 (1975); U. E. Schaffer and H. F. Lodish, Cell 79, 427 (1994); I. N. Bojesen and E. Bojesen, Acta Physiol. Scand. 156, 501 (1996).

9. N. Kanai et al., Science 266, 866 (1995).

10. Cultures of cortical neurons [N. Stella, L. Pellerin, P. Magistretti, J. Neurosci. 15, 3307 (1995)] or astrocytes (13) were prepared from rat embryos and were used after 4 to 6 days and 21 to 25 days in vitro, respectively. Accumulation of [3H]anandamide (221 Ci/mmol, New England Nuclear, Wilmington, DE) was measured by incubating the cells (six-well plates) for various times in Krebs buffer [116 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 10 mM glucose, and 20 mM Trizma base; (pH 7.4), at 37°C] containing [3H]anandamide (0.45 μM, brought to 100 μM with nonradioactive anandamide). Incubations were stopped by aspirating the media, and cells were rinsed with Krebs buffer containing bovine serum albumin (BSA, 0.1% w/v) and subjected to extraction with methanol and chloroform. Radioactivity in the extracts was measured directly or after fractionation of cell lipids by thin-layer chromatography (11). For kinetic analyses, the neurons were incubated for 4 min at 37°C in the presence of 10 to 500 nM anandamide containing 0.05 to 2.5 nM [3H]anandamide. We subtracted nonspecific accumulation (measured at 0° to 4°C) before determining kinetic constants by Lineweaver-Burk analysis.

11. M. Beltramo and D. Piomelli, unpublished data.

12. E. L. Barker and R. D. Blakely, in Psychopharmacology: The Fourth Generation of Progress, F. E. Bloom and D. J. Kupfer, Eds. (Raven, New York, 1995), pp. 321–334. In a previous study with mixed cultures of rat cortical neurons and astrocytes, a Kᵢ of 30 μM for [3H]anandamide accumulation was obtained (9). Such a high value likely resulted from the low specific radioactivity (0.3 mCi/mmol) of the [3H]anandamide used.

13. M. Beltramo, E. di Tomaso, D. Piomelli, FEBS Lett. 403, 263 (1997).

14. B. Koutek et al., J. Biol. Chem. 269, 22397 (1994).

15. L. Z. Bito, H. Davison, E. V. Salvador, J. Physiol. 256, 257 (1976); W. Stremmel and P. D. Berk, Proc. Natl. Acad. Sci. U.S.A. 83, 3086 (1986); S. Ruetz and P. Gros, Cell 77, 1071 (1994).

16. In astrocytes, Vₘₐₓ values for [3H]anandamide accumulation were 200 pmol/mg protein per minute (Vₘₐₓ) with bromocresol green, and 111 pmol/mg per milligram of protein with bromocresol green (10 μM). Apparent Kᵢ values were 0.24 and 0.25 μM, respectively (n = 6).

17. Displacement of [3H]WIN-55212-2 binding (40 to 60 Ci/mmol; New England Nuclear) to rat cerebellar
membranes (0.1 mg/ml) was determined as described [J. E. Kuster et al., J. Pharmacol. Exp. Ther. 264, 1352 (1993)]. Non-specific binding was measured in the presence of 1 μM non-radioactive WIN-55212-2. FAAH activity was measured in rat brain particulate fractions as described (13). The uptake of [3H]arachidonate (Amer sham, 200 Ci/mmol; 5 nM brought to 100 nM) and [3H]J-hemanolamine (Amer sham, 50 Ci/mmol; 20 nM brought to 100 nM) was determined on cortical astrocytes for 4 min as described (10). The control uptake for [3H]arachidonate was 16729 ± 817 dpm per well and for [3H]J-hemanolamine it was 644 ± 100 dpm per well (n = 6).

18. Neurons or astrocytes were incubated for 4 min at 37°C in Krebs buffer containing [3H]PGE2. [3H]PGE2 brought to 100 nM with non-radioactive PGE2 (171 Ci/mmol, New England Nuclear). After rinsing with Krebs buffer containing BSA, we subjected the cells to lipid extraction and counted radioactivity in the extracts. On average, neurons contained 245 ± 65 dpm per well and astrocytes 302 ± 20 dpm per well; non-specific accumulation in astrocytes at 0°C to 4°C was 355 ± 28 dpm per well (n = 6).

19. A. Khanolkar et al., J. Med. Chem. 39, 4515 (1996).

20. In astrocytes, apparent 

K∞ values for [3H]J-hanadamide accumulation were 0.11 μM without AM404 and 0.27 μM with AM404 (10 μM). U∞ values were 29 pmol/min per milligram of protein without AM404 and 26 pmol/min per milligram of protein with AM404, respectively (n = 6).

21. M. Herkenham et al., Proc. Natl. Acad. Sci. U.S.A. 90, 1352 (1990); L. A. Matsuda, T. I. Bonner, S. J. Lollak, J. Comp. Neurol. 327, 539 (1993). 22. L. Pre´zeau et al., Proc. Natl. Acad. Sci. U.S.A. 89, 8040 (1992).

23. Cortical neurons were prepared in 12-well plates and used after 4 to 6 days in vitro. Incubations were carried out in the presence of forskolin (3 μM) and isobutyl methyl xanthine (1 mM). The CAMP concentrations were measured by radioimmunoassay with a commercial kit (Amer sham, Arlington, IL) and following manufacturer’s instructions.

24. J. E. Kuster et al., J. Neurosci. 14, 6754 (1994).

25. The amounts of cAMP in the presence of a concentration of WIN-55212-2 below threshold (1 nM, determined in preliminary experiments) were 96.7 ± 2.5% of forskolin alone and not significantly affected by 10 μM AM404 (98.9 ± 2.6%), 10 μM AM403 (92.4 ± 2.3%), or 10 μM bromocriptine green (92.9 ± 2.3%) in (n = 3). In the presence of a concentration of glutamate below threshold (3 μM) (24), cAMP concentrations were 91.6 ± 2.0% of forskolin alone and were not significantly affected by AM404 (84.4 ± 4.9%), AM403 (89.5 ± 2.4%), or bromocriptine green (84.4 ± 3%) (n = 3).

26. The hot plate test (55°C) was carried out on male Swiss mice (25 to 30 g, Noissan, Italy) according to standard procedures [F. Porreca, H. L. Mosberg, R. H. Unger, V. J. Hruby, T. F. Burks, J. Pharmacol. Exp. Ther. 230, 341 (1984)]. Anandamide and AM404 were dissolved in 0.9% NaCl solution containing 20% dimethyl sulfoxide and injected intravenously at 20 mg/kg and 10 mg/kg, respectively. To determine whether cannabinoids receptors participate in the effect of anandamide, we administered anandamide (20 mg/kg intravenously) or anandamide plus SR141716-A (2 mg/kg, subcutaneously) to two groups of six mice each. In mice that received anandamide alone, latency to jump increased from 21.7 ± 1.5 to 30.7 ± 0.8 s (P < 0.05; ANOVA) 20 min after injection. In contrast, in mice that received anandamide plus SR141716-A, the latency to jump was not affected (19.6 ± 3.1 s).

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An NGF-TrkA–Mediated Retrograde Signal to Transcription Factor CREB in Sympathetic Neurons

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Nerve growth factor (NGF) is a neurotrophic factor secreted by cells that are the targets of innervation of sympathetic and some sensory neurons. However, the mechanism by which the NGF signal is propagated from the axon terminal to the cell body, which can be more than 1 meter away, to influence biochemical events critical for growth and survival of neurons has remained unclear. An NGF-mediated signal transmitted from the terminals and distal axons of cultured rat sympathetic neurons to their nuclei regulated immediate early genes and delayed transcripts, which is regulated innervation of sympathetic and some sensory neurons (1). The growth and survival of many populations of neurons depends on trophic support provided by their target tissue (1). NGF is secreted by targets of sympathetic and some sensory neurons, and it is also expressed within discrete regions of the central nervous system (1, 2). NGF belongs to a family of structurally related neurotrophic factors termed neurotrophins; this family includes brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (2). Two cell surface receptors for NGF have been identified: a receptor tyrosine kinase, TrkA, and the low-affinity neurotrophin receptor, p75NTR. NGF exerts its growth- and survival-promoting effects on neurons through activation of TrkA and subsequent biochemical events that ultimately influence the expression of various genes, including those encoding ion channels, neurotransmitter-synthesizing enzymes, and cytoskeletal components (3).

NGF stimulates dimerization and autophosphorylation of TrkA and initiation of intracellular signaling cascades that propagate the signal to the nucleus (4). One transcription factor that is a key target of an NGF-stimulated signaling pathway is CREB (5). Upon exposure of peptidergic cell lines PC12 to NGF, CREB becomes phosphorylated on its transcriptional regulatory site Ser133 (5), and this phosphorylation event promotes NGF activation of transcription of the immediate early gene c-fos. Because many NGF-regulated immediate early genes and delayed-response genes contain CREB binding sites within their upstream regulatory regions (5), CREB is likely to be a mediator of the general nuclear response to neurotrophins.

Because NGF is internalized and retrogradely transported from the axon terminal to the cell body (6), NGF itself may carry signals from the axon terminal to the nucleus. Alternatively, TrkA or p75NTR, an NGF-receptor complex, or a terminally derived second messenger molecule might serve as a retrograde messenger (7). To address questions of retrograde NGF signaling, we used compartmentalized cultures of sympathetic neurons (8) and antibodies that distinguish between the Ser133-phosphorylated and unphosphorylated states of CREB (anti–CREB9 (9) and TrkA (anti-P-Trk) (Fig. 1A). In these cultures, the cell bodies are separated from the axon terminals and distal processes by a distance of either 1 or 2 mm, and the cell bodies and distal processes are located in separate fluid compartments (Fig. 1B). This system enables us to expose isolated terminals and distal axonal processes to NGF and then to assess the phosphorylation of CREB in cell bodies of sympathetic neurons.

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