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Perspectives in Pharmacology

Mechanisms of Endocannabinoid Inactivation: Biochemistry and Pharmacology

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ABSTRACT

The endocannabinoids, a family of endogenous lipids that activate cannabinoid receptors, are released from cells in a stimulus-dependent manner by cleavage of membrane lipid precursors. After release, the endocannabinoids are rapidly deactivated by uptake into cells and enzymatic hydrolysis. Endocannabinoid reuptake occurs via a carrier-mediated mechanism, which has not yet been molecularly characterized. Endocannabinoid reuptake has been demonstrated in discrete brain regions and in various tissues and cells throughout the body. Inhibitors of endocannabinoid reuptake include *N*-(4-hydroxyphenyl)-arachidonylamide (AM404), which blocks transport with IC_{50} (concentration necessary to produce half-maximal inhibition) values in the low micromolar range. AM404 does not directly activate cannabinoid receptors or display cannabimimetic activity in vivo. Nevertheless, AM404 increases circulating anandamide levels and inhibits motor activity, an effect that is prevented by the CB1 cannabinoid antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A). AM404 also reduces behavioral responses to dopamine agonists and normalizes motor activity in a rat model of attention deficit hyperactivity disorder. The endocannabinoids are hydrolyzed by an intracellular membrane-bound enzyme, termed anandamide amidohydrolase (AAH), which has been molecularly cloned. Several fatty acid sulfonyl fluorides inhibit AAH activity irreversibly with IC_{50} values in the low nanomolar range and protect anandamide from deactivation in vivo. α-Keto-oxazolopyridines inhibit AAH activity with high potency (IC_{50} values in the low picomolar range). A more thorough characterization of the roles of endocannabinoids in health and disease will be necessary to define the significance of endocannabinoid inactivation mechanisms as targets for therapeutic drugs.

Cannabinoid receptors, the molecular targets of the active principle of cannabis Δ^9-tetrahydrocannabinol, are activated by a small family of naturally occurring lipids that include anandamide (arachidonylethanolamide) and 2-arachidonylglycerol (2-AG) (Devane et al., 1992; Di Marzo et al., 1994; Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). As in the case of other lipid mediators, these endogenous cannabis-like compounds (or "endocannabinoids") may be released from cells upon demand by stimulus-dependent cleavage of membrane phospholipid precursors (Di Marzo et al., 1994). After release, anandamide and 2-AG may be eliminated by a two-step mechanism consisting of carrier-mediated transport into cells followed by enzymatic hydrolysis (Fig. 1). Because of this rapid deactivation process, the endocannabinoids may primarily act near their sites of synthesis by binding to and activating cannabinoid receptors on the surface of neighboring cells (for review, see Pertwee, 2000; Piomelli et al., 2000).

The development of methods for endocannabinoid analysis (Giuffrida et al., 2000b; Schmid et al., 2000) and the availability of selective pharmacological probes for cannabinoid receptors (Pertwee, 2000) have allowed the exploration of the physiopathological functions served by the endocannabinoid system. Although still at their beginnings, these studies indicate that the endocannabinoids may significantly contrib-

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ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; AAH, anandamide amidohydrolase; AM374, palmitylsulfonyl fluoride; AM404, *N*-(4-hydroxyphenyl)-arachidonylethanolamide; CNS, central nervous system; OEA, oleylethanolamide; PAG, midbrain periaqueductal gray; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; GABA, γ-aminobutyric acid; SHR, spontaneously hypertensive rats.

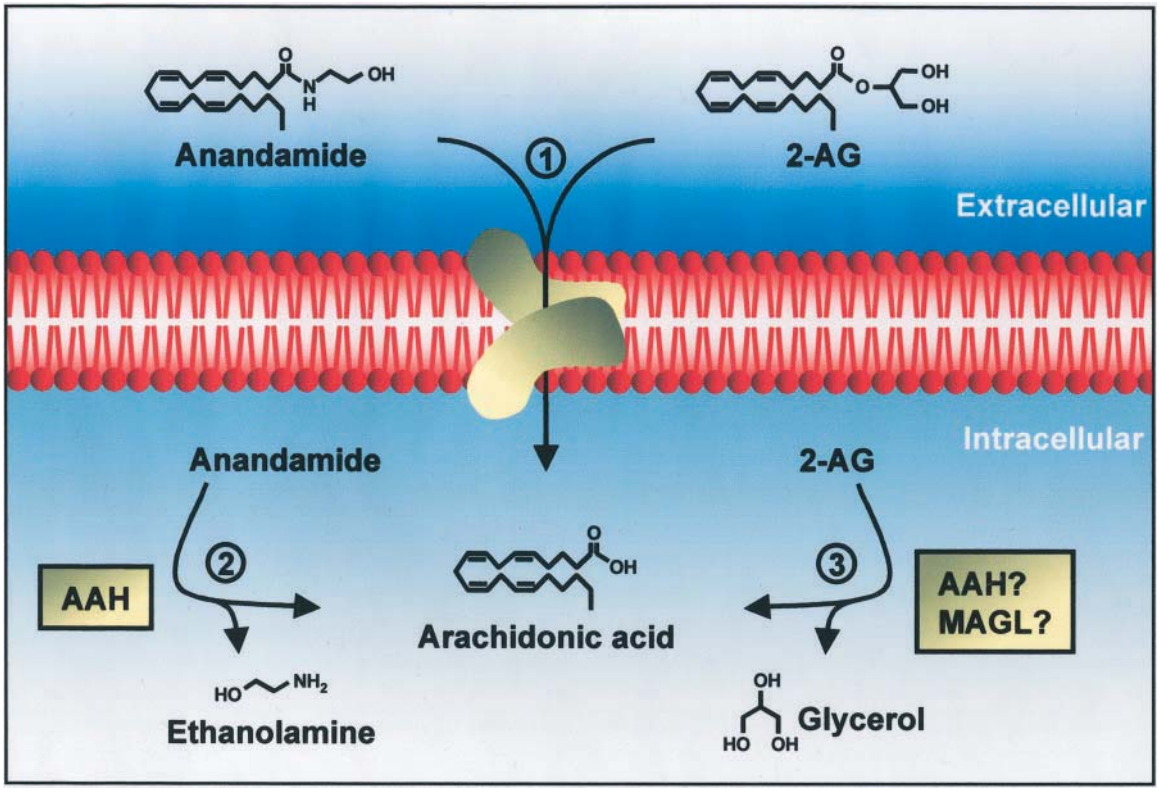


Fig. 1. Hypothetical pathways of endocannabinoid inactivation. Anandamide and 2-AG may be removed from their sites of action through a common carrier-mediated transport mechanism (1). Once inside the cell, anandamide may be hydrolyzed to arachidonic acid and ethanolamine by AAH (2); intracellular 2-AG may be cleaved to arachidonic acid and glycerol by an uncharacterized esterase such as AAH or monoacylglycerol lipase (MAGL) (3).

ute to the regulation of pain processing (for review, see Walker et al., 1999; Calignano et al., 2000), motor activity (Giuffrida et al., 1999), blood pressure (Wagner et al., 1998; Hillard, 2000), and tumor cell growth (Galve-Roperh et al., 2000; Melck et al., 2000). Furthermore, these investigations point to the endocannabinoid system—with its network of endogenous ligands, receptors, and inactivating mechanisms—as a potentially important arena for drug discovery. In this context, emphasis has been especially placed on the possible roles that CB1 and CB2 receptors (the two cannabinoid receptor subtypes identified so far) may play as drug targets (for review, see Piomelli et al., 2000). Here, we focus our attention on another facet of endocannabinoid pharmacology: the mechanisms by which anandamide and 2-AG are deactivated. We summarize current knowledge on how these mechanisms may function, describe pharmacological agents that interfere with their actions, and highlight the potential applications of these agents to medicine.

Endocannabinoid Transport

Mechanism and Kinetics. Extracellular anandamide is rapidly recaptured by neuronal and non-neuronal cells through a mechanism that meets four key criteria of carrier-mediated transport: fast rate, temperature dependence, saturability, and substrate selectivity (Beltramo et al., 1997; Hillard et al., 1997). Importantly, and in contrast with transport systems for classical neurotransmitters, [^{3}H]anandamide reuptake is neither dependent on external Na^+ ions nor affected by metabolic inhibitors, suggesting that it may be mediated by a process of carrier-facilitated diffusion (Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999).

How selective is anandamide reuptake? Cis-inhibition studies in a human astrocytoma cell line have shown that [^{3}H]anandamide accumulation is not affected by a variety of amino acid transmitters (such as glutamate or γ-aminobutyrate) or biogenic amines (such as dopamine or norepinephrine). Furthermore, [^{3}H]anandamide reuptake is not prevented by fatty acids (such as arachidonate), neutral lipids (such as ceramide), saturated fatty acyl ethanolamides (such as palmitylethanolamide, an endogenous cannabinoid-like molecule), prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, and epoxyeicosatetraenoic acids. Even further, [^{3}H]anandamide accumulation is insensitive to substrates or inhibitors of fatty acid transport (such as phloretin), organic anion transport (such as *p*-aminohippurate and digoxin), and P-glycoproteins (verapamil, quinidine) (Piomelli et al., 1999). By contrast, in the same cells, [^{3}H]anandamide reuptake is competitively blocked by either of the two endogenous cannabinoids, anandamide or 2-AG (Piomelli et al., 1999; Beltramo and Piomelli, 2000). Similar selectivity profiles are observed in primary cultures of rat cortical neurons or astrocytes (Beltramo et al., 1997) and rat brain slices (Beltramo et al., 2000).

The fact that both anandamide and 2-AG prevent [^{3}H]anandamide transport in cis-inhibition studies suggests that the two compounds compete for the same transport system. This possibility is further supported by three observations: 1) anandamide and 2-AG can mutually displace each other's transport (Beltramo and Piomelli, 2000); 2) [^{3}H]anandamide and [^{3}H]2-AG are accumulated with similar kinetic

properties (Piomelli et al., 1999); and 3) the transports of both compounds are prevented by the endocannabinoid transport inhibitor, *N*-(4-hydroxyphenyl)-arachidonylamide (AM404) (Fig. 2) (Beltramo and Piomelli, 2000). Together, these findings indicate that anandamide and 2-AG may be internalized via a common carrier-mediated process, which displays a substantial degree of substrate and inhibitor selectivity. The molecular structure of this hypothetical transporter remains, however, unknown.

Structure-Activity Relationship Studies. The structures of anandamide and 2-AG contain three potential pharmacophores: 1) the hydrophobic carbon chain; 2) the carboxamido/carboxyester group; and 3) the polar head group (Scheme 1).

Systematic modifications in the carbon chain suggest that the structural requisites for substrate recognition by the putative endocannabinoid transporter may be different from those of substrate translocation. Substrate recognition appears to require the presence of at least one *cis* double bond in the middle of the fatty acid chain, indicating a preference for substrates (or competitive inhibitors) whose hydrophobic tail can adopt an extended U-shaped conformation. By contrast, a minimum of four *cis* nonconjugated double bonds may be required for translocation, suggesting that substrates need to adopt a closed "hairpin" conformation to be transported across the membrane (Piomelli et al., 1999). In agreement with this hypothesis, molecular modeling studies show that transport substrates (such as anandamide and 2-AG) have both extended and hairpin low-energy conformers (Piomelli et al., 1999). By contrast, extended, but not hairpin, conformations may be thermodynamically favored in pseudosubstrates such as oleylethanolamide (OEA, 18:1 Δ^9), that displace [^{3}H]anandamide from transport without being themselves internalized (Piomelliet al., 1999).

The impact that modifications of the polar head group exert on endocannabinoid transport has also been investigated (Piomelli et al., 1999; Jarrahian et al., 2000). The available data suggest that ligand recognition may be favored 1) by a head group of defined stereochemical configuration containing a hydroxyl moiety at its distal end; and 2) by replacing the ethanolamine group with a 4-hydroxyphenyl or 2-hydroxyphenyl moiety. The latter modification leads to compounds, such as AM404 (Beltramo et al., 1997), that are competitive transport inhibitors of reasonable potency and efficacy (Fig. 2).

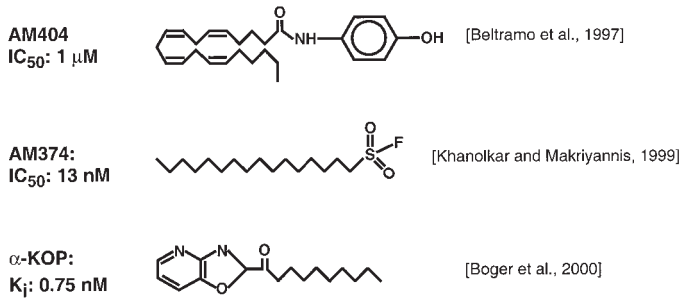
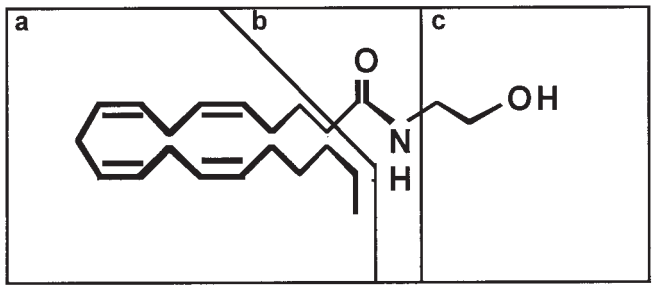


Fig. 2. Inhibitors of endocannabinoid inactivation. Shown are the chemical structures of the transport inhibitor AM404 and two AAH inhibitors representative of the fatty acid sulfonylfluoride group (AM374) and the α-keto-oxalopyridine group (α-KOP). IC_{50}, concentration required to produce half-maximal inhibition of endocannabinoid transport or hydrolysis. K_i, inhibition constant.



Scheme 1.

Distribution of Endocannabinoid Transport in the CNS. Anatomical studies of endocannabinoid transport are greatly limited by the lack of transporter-specific markers. Nevertheless, biochemical experiments have documented the existence of [^{3}H]anandamide uptake in primary cultures of rat cortical neurons and astrocytes (Beltramo et al., 1997), rat cerebellar granule cells (Hillard et al., 1997), human neuroblastoma cells (Maccarrone et al., 1998), and human astrocytoma cells (Piomelli et al., 1999; Beltramo and Piomelli, 2000). The CNS distribution of endocannabinoid transport was investigated by exposing metabolically active rat brain slices to [^{14}C]anandamide and analyzing the distribution of radioactivity in the tissue by autoradiography (Fig. 3). A receptor antagonist was included in the incubations to prevent the binding of [^{14}C]anandamide to CB1 receptors, which are very numerous in certain brain regions (Herkenham, 1995), and AM404 was used to differentiate transport-mediated [^{14}C]anandamide reuptake from nonspecific binding (Beltramo and Piomelli, 2000). Substantial levels of AM404-sensitive [^{14}C]anandamide reuptake were observed in the somatosensory, motor, and limbic areas of the cortex and in the striatum. Additional brain regions showing detectable [^{14}C]anandamide accumulation included the hippocampus, thalamus, septum, substantia nigra, amygdala, and hypothalamus (Fig. 3) (M. Beltramo and D. Piomelli, unpublished observations). Thus, endocannabinoid transport may be present in discrete regions of the rat brain that also express CB1 receptors (Herkenham, 1995).

Distribution of Endocannabinoid Transport Outside the CNS. The endocannabinoid system is not confined to the brain, and it is reasonable to anticipate that mechanisms of endocannabinoid inactivation may also exist in peripheral tissues. In keeping with this expectation, carrier-mediated [^{3}H]anandamide transport was demonstrated in J774 macrophages (Bisogno et al., 1997), RBL-2H3 cells (Bisogno et al., 1997; Rakhshan et al., 2000), and human endothelial cells (Maccarrone et al., 2000a). Although the kinetic and pharmacological properties of endocannabinoid uptake in peripheral cells appear to be generally similar to those reported in the CNS, some important difference have been observed. For example, in contrast to neurons, [^{3}H]anandamide uptake in RBL-2H3 cells is inhibited by arachidonic acid (Rakhshan et al., 2000). Such disparities might reflect the existence in non-neural tissues of mechanisms of endocannabinoid internalization that are distinct from those found in the CNS.

Inhibition of Endocannabinoid Transport: Molecular Tools. A variety of compounds have been tested for their ability to interfere with [^{3}H]anandamide internalization (Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999; Jarrahian et al., 2000; Rakhshan et al., 2000). Among

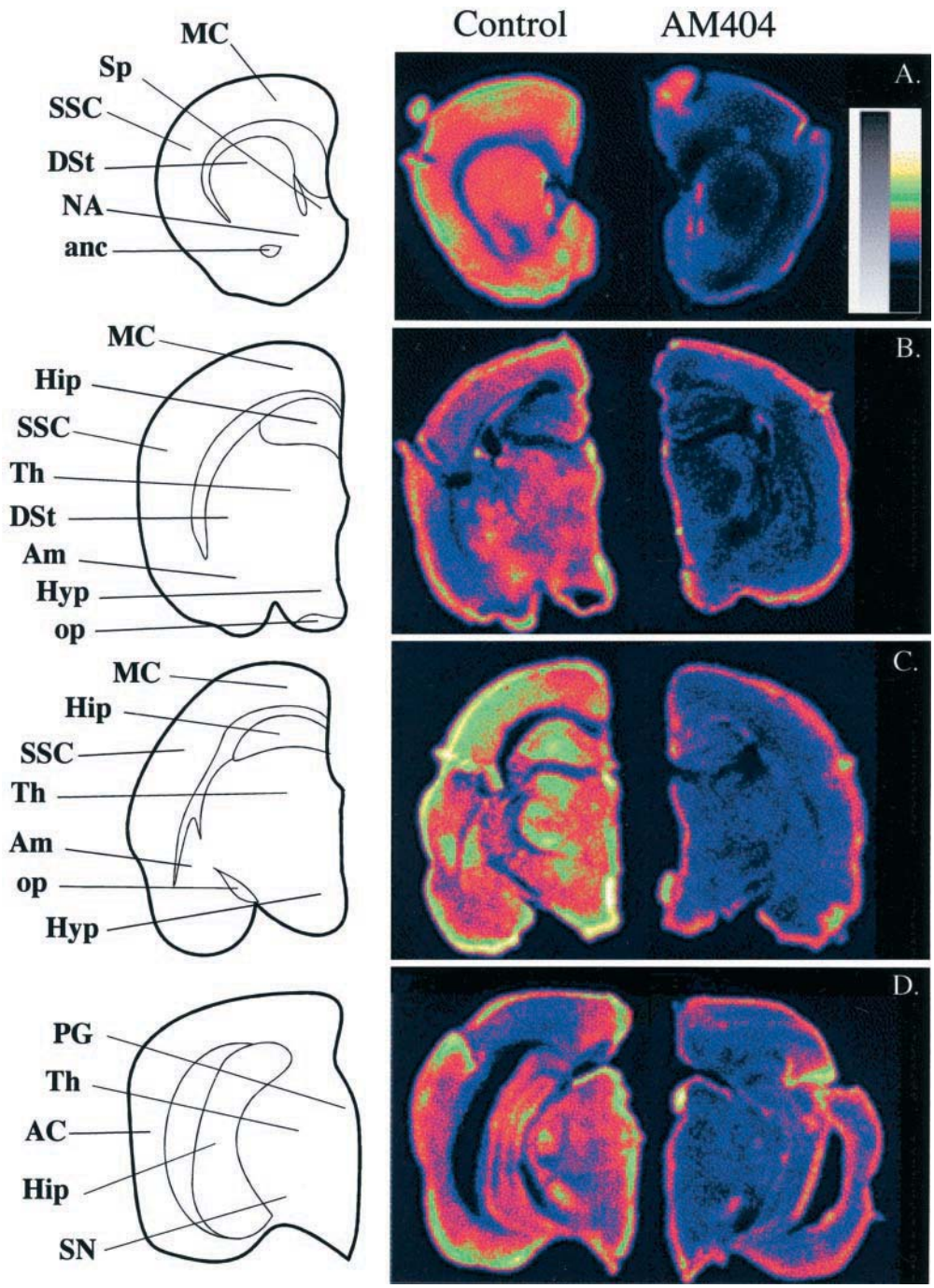


Fig. 3. Distribution of AM404-sensitive [^{14}C]anandamide accumulation in the rat brain. MC, motor cortex; Sp, septum; SSC, somato-sensory cortex; DSt, dorsal striatum; NA, nucleus accumbens; anc, anterior commissure; Hip, hippocampus; Th, thalamus; Am, amygdala; Hyp, hypothalamus; op, optic tract; PG, periaqueductal gray; AC, auditory cortex; SN, substantia nigra. Coronal slices were incubated in the presence of [^{14}C]anandamide (0.5 μM, 8×10^4 dpm/μl, 55 mCi/mmol) and SR141716A (0.5 μM) and fixed for 10 min in ice-cold paraformaldehyde (4%, v/v), and the distribution of radioactivity was determined by exposure to a Hyperfilm Betamax (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 to 3 weeks.

them, the anandamide analog AM404 (Fig. 2) stands out for its relatively high potency and its ability to block endocannabinoid transport both in vitro and in vivo. AM404 inhibits [^{3}H]anandamide uptake in rat brain neurons and astrocytes (Beltramo et al., 1997), human astrocytoma cells (Piomelli et al., 1999), rat brain slices (Beltramo and Piomelli, 2000), and RBL-2H3 cells (Rakhshan et al., 2000).

AM404 does not directly activate cannabinoid receptors in vitro (Beltramo et al., 1997; Beltramo and Piomelli, 2000), but it augments several CB1 receptor-mediated effects of anandamide. For example, AM404 enhances anandamide-evoked inhibition of adenylyl cyclase activity in cortical neurons, an effect that is reversed by the CB1 antagonist SR141716A (Beltramo et al., 1997). Likewise, AM404 potentiates the inhibitory actions of anandamide on GABA-ergic neurotransmission in the periaqueductal gray matter (PAG) (Vaughan et al., 2000). These findings are consistent with the hypothesis that AM404 protects anandamide from inactivation and, by doing so, magnifies the biological effects of this short-lived lipid mediator. It is important to point out, how-

ever, that AM404 is readily transported inside cells (Piomelli et al., 1999), where it can reach concentrations that may be sufficient to inhibit anandamide hydrolysis (Jarrahian et al., 2000; M. Beltramo and D. Piomelli, unpublished observations). To what extent this effect contributes to the ability of AM404 to prolong anandamide's life span is at present unclear.

The selectivity of AM404 for endocannabinoid transport has been the object of investigation. An initial screening found that AM404 has no affinity for a panel of 36 different pharmacological targets, including G protein-coupled receptors (such as cannabinoid and dopamine receptors) and ligand-gated ion channels (such as $GABA_A$ chloride channels) (Beltramo et al., 2000). However, additional studies revealed that AM404 activates capsaicin ("vanilloid") receptor channels at concentrations similar to those necessary to inhibit endocannabinoid transport (Smart and Jerman, 2000). The fact that AM404 can produce undesired effects underscores the need to introduce appropriate controls in the design of in vivo experiments with this compound. In particular, the effects of a cannabinoid receptor antagonist should be routinely tested to verify that endogenously produced anandamide and 2-AG are involved in the response to AM404 (Beltramo and Piomelli, 2000).

Inhibition of Endocannabinoid Transport: Functional Studies. AM404 does not display a typical cannabimimetic profile when administered in vivo; this is consistent with its poor affinity for cannabinoid receptors. For example, AM404 has no antinociceptive effect in mice (Beltramo et al., 1997) or rats (Beltramo et al., 2000) and causes no hypotension in guinea pigs (Calignano et al., 1997a). Nevertheless, in the same models, AM404 increases the responses elicited by exogenous anandamide, and this potentiation is reversed by the CB1 antagonist SR141716A (Beltramo et al., 1997; Calignano et al., 1997a).

Despite the absence of overt cannabimimetic properties, AM404 resembles anandamide and other cannabinoid receptor agonists in certain respects. For example, when administered alone, AM404 causes a reduction in motor activity, which is prevented by the CB1 antagonist SR141716A (Beltramo et al., 2000; Giuffrida et al., 2000a). Furthermore, AM404 reduces the yawning evoked by low doses of the mixed D_1/D_2 dopamine agonist apomorphine and inhibits the hyperactivity elicited by the selective D_2 agonist quinpirole (Beltramo et al., 2000). AM404 also decreases the levels of circulating prolactin, but the role of CB1 receptors in this response is unknown (González et al., 1999). Can the effects of AM404 be explained by its in vitro affinity for vanilloid receptors (Smart and Jerman, 2000)? The fact that SR141716A, a selective CB1 antagonist, blocks the motor inhibitory effects produced by AM404 argues against this possibility. Furthermore, vanilloid agonists such as capsaicin have very different, in some cases even opposite, effects. For example, capsaicin causes hyperkinesia and pain (Szallasi and Blumberg, 1999), whereas AM404 elicits hypokinesia and enhances anandamide's analgesic properties (Beltramo et al., 2000). Therefore, a more plausible interpretation of the available data is that, by inhibiting anandamide clearance, AM404 may cause this lipid to accumulate outside cells and activate local cannabinoid receptors. In further support of this possibility, the systemic administration of AM404 in rats was found to cause a time-dependent increase in circulating anandamide levels (Giuffrida et al., 2000a).

Finally, it is important to point out that several anandamide responses are not affected by AM404. One example is the inhibition of intestinal motility, which anandamide may produce in rodents by activating CB1 receptors on the surface of enteric neurons (Calignano et al., 1997b; Colombo et al., 1998). This effect is not enhanced by AM404, suggesting that the predominant pathway of endocannabinoid inactivation in the intestine may be through enzymatic hydrolysis, not transport (Calignano et al., 1997b). The fact that rat intestinal tissue contains high AAH levels is in agreement with this possibility (Katayama et al., 1997). Alternatively, anandamide transport may occur in the intestine through transport mechanisms that are insensitive to AM404.

Endocannabinoid Hydrolysis

Mechanisms and Kinetics. Long before the discovery of anandamide, Schmid and coworkers identified in rat liver an amidohydrolase activity, which catalyzes the hydrolysis of fatty acid ethanolamides to free fatty acid and ethanolamine (Natarajan et al., 1984). That anandamide may serve as a substrate for this activity was first suggested on the basis of biochemical evidence (Deutsch and Chin, 1993; Di Marzo et al., 1994; Désarnaud et al., 1995; Ueda et al., 1995) and then demonstrated by molecular cloning and heterologous expression of the enzyme involved (Cravatt et al., 1996).

AAH (also called fatty acid amide hydrolase) is an intracellular membrane-bound protein whose primary structure displays significant similarities with a group of enzymes known as "amidase signature family" (Cravatt et al., 1996; Giang and Cravatt, 1997). AAH may act as a general hydrolytic enzyme not only for fatty acid ethanolamides (such as anandamide or OEA) but also primary amides (such as oleamide, a biologically active lipid of unclear function) (Cravatt et al., 1995) and even esters (such as 2-AG) (Goparaju et al., 1998; Lang et al., 1999). Site-directed mutagenesis experiments indicate that this unusually wide substrate preference may be underpinned by a novel catalytic mechanism involving the amino acid residue lysine 142. This residue may act as a general acid catalyst, favoring the protonation and consequent detachment of reaction products from the enzyme's active site (Patricelli and Cravatt, 1999). Three serine residues that are conserved in all amidase signature enzymes (S241, S217, and S218 in AAH) may also be essential for enzymatic activity: serine 241 may serve as the enzyme's catalytic nucleophile, while serine 217 and 218 may modulate catalysis through an as-yet-unidentified mechanism (Patricelli et al., 1999).

Like other hydrolase enzymes, AAH may act in reverse, catalyzing the synthesis of anandamide from free arachidonate and ethanolamine (Arreaza et al., 1997). The high K_M values reported for anandamide synthase activity suggest, however, that under normal circumstances AAH acts predominantly as a hydrolase. One exception is represented by the rat uterus, where substrate concentrations in the micromolar range are required for the synthase reaction to occur, implying that in this tissue AAH could contribute to anandamide biosynthesis (Schmid et al., 1997).

In addition to AAH, other ill-characterized enzyme activities may participate in the breakdown of anandamide and

2-AG. A fatty acid ethanolamide-hydrolyzing activity catalytically distinct from AAH was described in rat brain membranes (Désarnaud et al., 1995) and human megakaryoblastic cells (Ueda et al., 1999). Furthermore, evidence indicates that 2-AG degradation may be predominantly catalyzed by an enzyme different from AAH, possibly a monoacylglycerol lipase (Goparaju et al., 1999; Beltramo and Piomelli, 2000).

Structure-Activity Relationship Studies. Modifications in three potential pharmacophores (Scheme 1) have helped define several general requisites for endocannabinoid hydrolysis by AAH. First, reducing the number of double bonds in the hydrophobic carbon chain causes a gradual increase in metabolic stability (Désarnaud et al., 1995; Ueda et al., 1995). Thus, [^{3}H]anandamide hydrolysis is inhibited by fatty acid ethanolamides in the 20 carbon atom series with the following rank order of potency: 20:4 (anandamide) > 20:3 > 20:2 > 20:1 > 20:0 = no effect (Désarnaud et al., 1995). Second, replacing the ethanolamine moiety with a primary amide leads to good AAH substrates. For example, the rate of hydrolysis of arachidonylamide is approximately twice that of anandamide (Lang et al., 1999). Third, anandamide congeners containing a tertiary nitrogen in the ethanolamine moiety are poor AAH substrates (Lang et al., 1999). Fourth, introduction of a methyl group at the C2, C1′, or C2′ positions of anandamide yields analogs that are resistant to hydrolysis, likely as a result of increased steric hindrance around the carbonyl group (Abadji et al., 1994; Lang et al., 1999). Fifth, substrate recognition at the AAH active site is stereoselective, at least with fatty acid ethanolamide congeners containing a methyl group in the C1′ or C2′ positions (Abadji et al., 1994; Lang et al., 1999). Finally, as a result of AAH's remarkable "directed nonspecificity" (Patricelli and Cravatt, 1999), fatty acid esters also serve as substrates for this enzyme. Thus, 2-AG is hydrolyzed by AAH at a rate that is about 4 times faster than anandamide is (Goparaju et al., 1998).

AAH Distribution in the CNS. AAH is widely distributed in the brain, with particularly high levels in cortex, hippocampus, cerebellum, amygdala, thalamus, and pontine nuclei (Désarnaud et al., 1995; Thomas et al., 1997; Egertova et al., 1998). Immunohistochemical studies suggest that neurons, not glia, are the predominant cell type expressing AAH (Egertova et al., 1998), although astrocytes in primary culture have been shown to contain AHH activity (Beltramo et al., 1997). CB1 cannabinoid receptors are present in various brain regions that also express AAH, but there appears to be no direct correlation between the concentrations of these two proteins (Egertova et al., 1998). This discrepancy may reflect the participation of AAH in the degradation of noncannabinoid lipid amides, such as oleamide and OEA.

AAH Distribution outside the CNS. AAH mRNA and enzyme activity have been measured in a variety of nonneural cells lines, including lung carcinoma (Deutsch and Chin, 1993), human breast carcinoma (Bisogno et al., 1998), leukemia basophils (Bisogno et al., 1997), human monocytic leukemia (U937) (Maccarrone et al., 1998), rat renal endothelial and mesangial cells (Deutsch et al., 1997a), rat macrophages (Di Marzo et al., 1999), human platelets (Maccarrone et al., 1999), and human lymphocytes (Maccarrone et al., 2000b). Furthermore, high AAH levels have been found in rat liver, testis, kidney, lung, spleen, uterus, small intestine, and stomach; whereas lower levels were observed in heart and skeletal muscle (Désarnaud et al., 1995; Cravatt et al., 1996). The distribution of AAH in human tissues is somewhat different from the rat, with expression levels that are reportedly higher in pancreas, brain, kidney, and skeletal muscle than in liver (Giang and Cravatt, 1997).

Inhibition of AAH Activity: Molecular Tools. The armamentarium of AAH inhibitors available to the experimentalist (for review, see Khanolkar and Makriyannis, 1999) has been recently enriched by two important groups of molecules. The first are fatty acid sulfonyl fluorides, such as the compound AM374 (Fig. 2). AM374 irreversibly inhibits AAH activity with an IC_{50} value in the low nanomolar range and displays a 50-fold preference for AAH inhibition versus CB1 cannabinoid receptor binding (Deutsch et al., 1997b). In superfused hippocampal slices, AM374 augments anandamide's ability to inhibit [^{3}H]acetylcholine release, although it does not affect release when it is applied alone (Gifford et al., 1999). The second group of AAH inhibitors is represented by a series of substituted α-keto-oxazolopyridines (Fig. 2), which are reversible and extremely potent (Boger et al., 2000). Little information is as yet available on the pharmacological selectivity and in vivo properties of these interesting compounds.

AAH Inhibition: Functional Studies. Systemic administration of the potent AAH inhibitor AM374 does not produce clear cannabimimetic effects in rats (for example, it does not inhibit motor activity) but enhances the operant lever-pressing response evoked by anandamide administration (Salamone et al., 2000). These results suggest that AM374 protects exogenous anandamide from degradation (possibly by blocking its first-pass liver metabolism) but does not cause a significant accumulation of endogenously generated anandamide. This idea is consistent with the finding that, in contrast to the transport inhibitor AM404 (Giuffrida et al., 2000a), AM374 does not increase circulating anandamide levels in rats (A. Giuffrida, F. Nava, A. Makriyannis, and D. Piomelli, unpublished observations). Further studies will be required to fully evaluate the behavioral impact of AAH inhibitors and to assess the biological availability and pharmacokinetics of these molecules.

Therapeutic Perspectives

In Search of a Role. What place will inhibitors of endocannabinoid clearance occupy in medicine, if any, will largely depend on the answers to two key questions. The first is whether endogenously produced anandamide and 2-AG participate in the modulation of specific disease states. Drugs that block endocannabinoid inactivation should magnify this adaptive function in the same way as serotonin reuptake or monoaminooxidase (MAO) inhibitors heighten the mood-regulating actions of endogenous biogenic amines. The second question is whether inhibiting endocannabinoid clearance provides a therapeutic advantage over direct activation of cannabinoid receptors with agonist drugs. The latter approach has been generally favored thus far, and several classes of subtype-selective cannabinoid agonists are already available for preclinical use (for review, see Pertwee, 2000). Thus, demonstrating that inhibitors of endocannabinoid inactivation possess a unique pharmacological profile is essential to justify the substantial efforts associated with the development of a new class of drugs. In the following sections, we illustrate with some examples the endocannabinoids' role

in pathology and discuss the potential therapeutic value of drugs that target endocannabinoid inactivation.

Pain. Considerable evidence indicates that the endocannabinoid system plays an essential role in pain regulation (Walker et al., 1999; Calignano et al., 2000). For example, in vivo microdialysis experiments have shown that peripheral injections of the chemical irritant formalin are accompanied by increases in anandamide outflow within the PAG, a brain region intimately involved in pain processing (Walker et al., 1999). Since activation of CB1 receptors in the PAG causes profound analgesia, it has been argued that inhibitors of anandamide inactivation "may form the basis of a modern pharmacotherapy of pain, particularly in instances where opiates are ineffective" (Walker et al., 1999). The fact that the endocannabinoid transport inhibitor AM404 has no antinociceptive effect in models of acute pain seems to contradict this possibility (Beltramo et al., 1997, 2000). It should be noted, however, that neither AM404 nor any other inhibitor of anandamide clearance has yet been tested in animal models that are directly relevant to pathological pain states in humans. In models that mimic such states (for example, constriction nerve injury or chronic inflammation models), the CB1 receptor antagonist SR141716A exacerbates pain when administered alone, suggesting that inflammation and nerve injury may be associated with compensatory increases in cannabinergic activity (Martin et al., 1999). If this hypothesis is correct, one would expect endocannabinoid inactivation inhibitors to alleviate inflammatory or neuropathic pain. This possibility has not yet been tested, however.

Hypotensive Shock. During hemorrhagic and septic shock, anandamide and 2-AG may be released from macrophages and platelets, activate CB1-type receptors on the surface of vascular smooth muscle cells, and produce vasodilatation (Wagner et al., 1997, 1998). The physiological significance of this response is still unclear. Nevertheless, the fact that a CB1 antagonist reduces survival time in "shocked" rats suggests that activation of the endocannabinoid system may have beneficial effects, possibly by redistributing cardiac output to or improving microcirculation in vital organs such as the kidneys (Wagner et al., 1997, 1998). If this is true, inhibitors of endocannabinoid inactivation that do not appear to exert direct vasoactive effects (Calignano et al., 1997a) could be used to prolong life expectancy in hemorrhagic and septic shock.

Disorders of Dopamine Transmission. Functional interactions between dopamine and endocannabinoids are well documented. CB1 receptors are highly expressed in CNS regions that are innervated by dopamine-releasing neurons (Herkenham, 1995). In one of these regions, the striatum, anandamide release is stimulated by activation of dopamine D_2-family receptors (Giuffrida et al., 1999). Furthermore, the CB1 antagonist SR141716A, which has no effect on motor activity when administered alone, enhances the motor hyperactivity elicited by D_2-family agonists (Giuffrida et al., 1999). These findings suggest that one role of the endocannabinoid system in the CNS may be to act as an inhibitory feedback mechanism countering dopamine-induced facilitation of psychomotor activity (Giuffrida et al., 1999). A corollary of this idea is that drugs that prevent endocannabinoid clearance should antagonize dopamine-mediated responses. As a test of this hypothesis, the endocannabinoid transport inhibitor AM404 was injected into the cerebral ventricles of rats that were then systemically treated with the mixed D_1/D_2 dopamine agonist apomorphine or the selective D_2-family agonist quinpirole. AM404 blocked the yawning evoked by apomorphine and reduced the motor stimulation elicited by quinpirole. By contrast, when administered alone, AM404 produced only a mild hypokinesia, not other cannabinoid actions such as catalepsy (Beltramo et al., 2000). The effects of AM404 were also studied in juvenile spontaneously hypertensive rats (SHR). Juvenile SHR are not yet hypertensive but are hyperactive and show a number of attention deficits, which have been linked to alterations in mesocorticolimbic dopamine transmission and dopamine receptor expression (Beltramo et al., 2000). Systemic administration of AM404 normalizes the behavior of juvenile SHR without affecting that of control rats (Beltramo et al., 2000). These findings suggest that inhibitors of endocannabinoid inactivation may be used to alleviate certain symptoms of dopamine dysfunction. Clinical data showing that Δ^9-tetrahydrocannabinol ameliorates tics in Tourette's syndrome patients lend further support to this possibility (Müller-Vahl et al., 1999).

Future Challenges. In conclusion, three major challenges lie before the pharmacologist interested in the mechanisms of endocannabinoid inactivation from the perspective of drug discovery. The first is the need for a deeper molecular understanding of these mechanisms. Considerable insight has been gained in the last few years on the structure and catalytic properties of AAH, but many questions remain unanswered, including the identity of the putative endocannabinoid transporter and the existence of additional hydrolytic enzymes for anandamide and 2-AG. The second challenge lies in the development of potent and selective inhibitors of endocannabinoid inactivation. Future AAH inhibitors should combine the potency of those currently available with greater pharmacological selectivity and biological availability. A second generation of endocannabinoid transport blockers that overcome the limitations of AM404 and its congeners is also needed. The third challenge is the validation of endocannabinoid mechanisms as targets for therapeutic drugs. This task is intertwined, of course, with that of understanding the endocannabinoids' roles in normal physiology, one on which much research is currently focused.

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