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The endocannabinoid nervous system: unique opportunities for therapeutic intervention

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Abstract

The active principle in marijuana, Δ^9 -tetrahydrocannabinol (THC), has been shown to have wide therapeutic application for a number of important medical conditions, including pain, anxiety, glaucoma, nausea, emesis, muscle spasms, and wasting diseases. Δ^9 -THC binds to and activates two known cannabinoid receptors found in mammalian tissue, CB1 and CB2. The development of cannabinoid-based therapeutics has focused predominantly on the CB1 receptor, based on its predominant and abundant localization in the CNS. Like most of the known cannabinoid agonists, Δ^9 -THC is lipophilic and relatively nonselective for both receptor subtypes. Clinical studies show that nonselective cannabinoid agonists are relatively safe and provide therapeutic efficacy, but that they also induce psychotropic side effects. Recent studies of the biosynthesis, release, transport, and disposition of anandamide are beginning to provide an understanding of the role of lipid transmitters in the CNS. This review attempts to link current understanding of the basic biology of the endocannabinoid nervous system to novel opportunities for therapeutic intervention. This new knowledge may facilitate the development of cannabinoid receptor-targeted therapeutics with improved safety and efficacy profiles. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Cannabinoid; Anandamide; Fatty acid amidohydrolase; Allosteric modulator

Abbreviations: cAMP, cyclic AMP; APCI, atmospheric pressure chemical ionization; FAAH, fatty acid amidohydrolase; GC, gas chromatography; GPCR, G-protein-coupled receptor; IOP, intraocular pressure; LC, liquid chromatography; MS, mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; THC, tetrahydrocannabinol; VR, vanilloid receptor.

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1. Introduction

The marijuana plant has been exploited since the early history of humans for the structural fibers in its stalk and stems and the oily resin in its leaves and flowers. Hemp rope and cloth are regaining popularity with renewed interest in natural fibers. Similarly, the popularity of natural medicines has focused new attention on the plethora of therapeutic indications ascribed to marijuana. However, refining the use of marijuana for medicinal purposes has been difficult due to the inability to separate the undesirable psychotropic side effects from the therapeutic benefits. Only very recently have significant strides been made in understanding the molecular and pharmacological mechanisms behind the physiological actions of active cannabinoid compounds found in marijuana. An endocannabinoid system has been identified in mammals that include two receptors for cannabinoids, CB1 and CB2, which are widely distributed in the CNS and peripheral tissues. More recently, two endogenous lipid agonists, anandamide and 2-arachidonylglycerol, have been identified that bind and activate cannabinoid receptors. A basic understanding of the synthesis, release, and disposition of endocannabinoids is beginning to emerge, suggesting that lipids, like peptides, amino acids, and biogenic amines, can act as neurotransmitters. This review will focus on potential therapeutic opportunities for cannabinoid compounds in the CNS. A brief historical background will be followed by a review of recent research findings on the endocannabinoid system. These emerging data should help build an understanding of the function of the anandaergic synapse and its relationship to the development of novel therapeutic interventions.

2. Historical background

Early marijuana use depended on the inhalation of volatile oils generated in the smoke. Attempts were initiated at the turn of the century to isolate the active principals in marijuana. In the late 1800s, Wood, Spivey, and Easterfield described purifying “an intoxicating red oil” from cannabis, and later determined the chemical formula of cannabinol, which was not actually the predominant active component (reviewed in Todd, 1946). In his 1942 Harvey

Lecture, Roger Adams reported the structure and pharmacological effects of the tetrahydrocannabinols, the active components of cannabis. After the work of the 1940s, it was assumed that the psychoactive properties of marijuana were due to a complex mixture of tetrahydrocannabinol compounds. It was not until 1965 that the predominant active principal found in marijuana, Δ^9 -tetrahydrocannabinol (THC), was identified (Mechoulam & Gaoni, 1965). This discovery led to the development of novel and significantly more potent cannabinoid agonists, which greatly facilitated further molecular pharmacological research (Melvin & Johnson, 1987).

Cannabinoid research was largely ignored in the first half of 20th century, influenced in part by political anti-marijuana sentiment that began in the United States with the Harrison Act in 1914 and that led to full prohibition 20 years later. The sudden increase in recreational use of marijuana and other psychoactive drugs during the 1960s resulted in public concern about the potential negative health effects of chronic marijuana use. This stimulated renewed scientific investigation into the chemical constituents of marijuana and their mechanism of action. The United States Congress responded in 1972 with the creation of the National Institute of Drug Abuse, which stimulated a comprehensive research program into the medicinal properties of marijuana and other drugs of abuse.

In the 1970s, pharmaceutical companies became involved in the development of cannabinoid analogs for the treatment of a variety of illnesses. Pfizer (New York, NY, USA) synthesized a series of compounds significantly more potent than Δ^9 -THC, such as levonantradol. Levonantradol was developed for the non-opioid treatment of postoperative pain and emesis associated with cancer chemotherapy. However, dose-limiting psychotropic side effects, including dysphoria, dizziness, thought disturbance, and somnolence, limited its use, and further development was discontinued. Eli Lilly and Company (Indianapolis, IN, USA) developed several cannabinoid analogs in the 1970s for the treatment of chronic pain. One compound, nabilone, was taken into Phase II clinical trials for emesis, anxiety, and other indications (Lemberger, 1999). Phase III trials were conducted on nabilone specifically for emesis associated with cancer chemotherapy treatment. Although data suggested that the abuse potential for nabilone was low, the United States Drug

Enforcement Agency insisted that it be listed as a Schedule II narcotic, resulting in restrictions on the way it was permitted to be prescribed. When the United States Food and Drug Administration requested further studies on the bioavailability of nabilone, the company decided to end its development. The British regulatory agency approved nabilone as an antiemetic without restrictions, and it has been in continual use since the 1970s, without drug abuse problems arising.

Over the last 20 years, the political and social attitude towards the medicinal use of marijuana has become more conducive to the therapeutic development of cannabinoids. Although still prohibited at the Federal level, medicinal use of smoked or ingested marijuana has been allowed through state referendums in Arizona, Nevada, Alaska, Washington, California, Oregon, Maine, and the District of Columbia in the last two years, and legislated in 2000 in Hawaii. In July 1999, marinol, an oral synthetic preparation of Δ^9 -THC, was reclassified from Schedule II narcotic to Schedule III drug, which eases restrictions on its prescription use. Recent deeper scientific understanding of the endocannabinoid system achieved over the last decade promises to improve our ability to develop more selective cannabinoid-based drugs. This review will provide a basis for understanding how selective targeting of specific physiological processes may offer unique opportunities for therapeutic interventions.

3. The anandaergic synapse

The cannabinoid receptor family includes two subtypes of G-protein-coupled cannabinoid receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993), as well as a splice variant of the CB1 receptor (Shire et al., 1995; Rinaldi-Carmona et al., 1996). The CB1 receptor is widely distributed in the brain and the spinal cord, and is the only known subtype found to date in the CNS. CB1 is predominantly expressed presynaptically, and its primary action is to decrease the release of neurotransmitters, including dopamine, norepinephrine, glutamate, and serotonin (Ishac et al., 1996; Kathmann et al., 1999; Nakazi et al., 2000; Shen et al., 1996; Szabo et al., 1999). Endogenous cannabinoid agonists, including anandamide, have been identified that bind to and activate the cannabinoid receptors. These novel lipid compounds satisfy all of the criteria for a neurotransmitter, based on their synthesis and release from neurons, binding and activation of cannabinoid receptors, blockade of their actions by competitive antagonists, and the presence of a mechanism for the termination of their actions. A facilitated carrier-mediated process and cytoplasmic enzymatic degradation terminate the action of anandamide in the synapse. The endocannabinoid system is capable of interacting with several signal transduction pathways, as well as other receptor families, to affect a wide variety of physiological processes.

4. Cannabinoid receptors

A distinct cannabinoid-binding site was first proposed when behavioral pharmacological experiments showed that the (–)enantiomers of three different cannabinoid ligands, levonantradol, CP55,244, and CP55,940, were much more potent than their respective (+)enantiomers (Little et al., 1988). At the molecular level, Δ^9 -THC inhibited the activity of adenylate cyclase and decreased cellular levels of cyclic AMP (cAMP), indicating that its actions were mediated by a member of the G-protein-coupled receptor (GPCR) superfamily (Howlett, 1984; Howlett & Fleming, 1984). Sensitivity to pertussis toxin provided further confirmation that cannabinoid ligands were acting through a G_i -protein-coupled mechanism (Howlett et al., 1986). However, direct receptor-binding studies proved difficult due to the lipophilic nature of cannabinoid ligands. With the synthesis of more potent cannabinoid ligands (Melvin & Johnson, 1987) (Table 1), receptor-binding studies became possible and provided direct evidence for cannabinoid receptors in the brain (Devane et al., 1988).

The first cannabinoid receptor, CB1, was cloned from rat in 1990 using a screening approach based on sequence similarity with known GPCRs (Matsuda et al., 1990). Using a similar approach, a second cannabinoid receptor subtype, the human CB2, was cloned in 1993 (Munro et al., 1993). An amphibian cannabinoid receptor has been cloned recently, suggesting evolutionary conservation of the endocannabinoid system (Soderstrom et al., 2000). The pharmacology of cannabinoid ligands is strikingly similar between the two receptors, although their sequence homology at the proposed ligand-binding domains is only 68% (Munro et al., 1993). This is in contrast to other members of the GPCR family, where a single amino acid substitution within specific regions of the binding pocket can lead to significant changes in receptor pharmacology (Parker et al., 1993; Link et al.,

Table 1
Cannabinoid receptor ligands

	CB1	CB2
Endogenous agonists	Anandamide 2-arachidonylethanolamide	Anandamide palmitoylethanolamide
Receptor-selective agonists	Δ^9 -THC CP-55,940 WIN55212-2 HU210 Levonantradol Nabilone Methanandamide	Δ^9 -THC CP-55,940 WIN55212-2 HU210 Levonantradol Nabilone Methanandamide JWH-015 JWH-133
Receptor-selective antagonists	SR141716A AM630 LY320135	SR144528 AM630

1992). The pharmacology of cannabinoid receptors across species is just beginning to be investigated. Although the CB1 receptor sequence and pharmacology appears to be conserved across species, the CB2 receptor exhibits some pharmacological differences between rat, mouse, and humans (Griffin et al., 2000). Additional cannabinoid receptor family members have not been identified yet, but if found, they may have significantly different sequences from the CB1 and CB2 receptors.

The CB1 receptor originally was thought to be the predominant form found in the brain, while the CB2 was considered the peripheral cannabinoid receptor. Recently, however, questions are being raised about the absolute distribution of the two subtypes. The CB1 subtype is expressed in several brain regions associated with memory and movement, including hippocampus, cerebellum, and basal ganglia (Johnson et al., 1992). CB1 is also expressed in several peripheral tissues, including lung (Rice et al., 1997), presynaptic sympathetic nerve terminals (Ishac et al., 1996), mouse vas deferens (Pertwee et al., 1996), vascular endothelial cells (Liu et al., 2000), vascular smooth muscle cells (Holland et al., 1999), and hematopoietic cells (Bouaboula et al., 1993). CB2 receptors are associated primarily with the spleen and cells of the immune system (Munro et al., 1993; Lynn & Herkenham, 1994), and there is preliminary evidence for CB2 expression in brain-derived immune cells (Galve-Roperh et al., 2000).

There is growing evidence, based on expression patterns in key brain regions, that cannabinoid receptors play a role in the development of the brain (for a review, see Fernandez-Ruiz et al., 2000). Early studies described cannabinoid receptor binding in developing human (Mailleux & Vanderhaeghen, 1992) and rat (Rodriguez de Fonseca et al., 1993) brain. A detailed study of CB1 expression in human brain found that expression levels differ with developmental age. CB1 expression is substantially higher in the fetal and neonatal human brain when compared with the adult brain, and there are high levels of cannabinoid receptors in neonatal brain regions, which express very low levels during adulthood (Glass et al., 1997). In the rat, mRNA for CB1 was found very early in development at embryonic day 11 in the neural tube, and CB2 mRNA was found in the liver at embryonic day 13 (Buckley et al., 1998). Despite this evidence, no obvious developmental defects have been observed in CB1, CB2, or CB1/CB2 knockout mice (Zimmer et al., 1999; Jarai et al., 1999; Buckley et al., 2000). However, there was a significant increase in mortality in CB1 receptor knockout mice, the cause of which has yet to be determined (Zimmer et al., 1999).

Pharmacological and physiological evidence suggests that there may be other cannabinoid receptor subtypes that have yet to be cloned and characterized. For example, the vasodilatory actions of anandamide may be independent of the CB1 receptor in the mesenteric arterial bed (reviewed

in Hillard, 2000). The potent CB1 agonist WIN55212-2 causes vasodilatation in the cat cerebral artery (Gebremedhin et al., 1999). However, WIN55212-2 and HU210 do not cause vasodilatation in the rat mesenteric arterial bed, while the endogenous ligand anandamide does cause vasodilatation in this same location (Wagner et al., 1999). Moreover, in CB1 knockout mice, as well as CB1/CB2 double knockout mice, anandamide-induced mesenteric vasodilatation is intact (Jarai et al., 1999). Taken together, these data suggest that another receptor, distinct from CB1 and CB2, may mediate the endothelial cell-dependent vasorelaxation of anandamide. A possible candidate is the vanilloid receptor VR1, which is a nonselective cation channel activated by capsaicin. The VR1 ion channel has no homology to either CB1 or CB2 receptors, yet it is activated by anandamide, and this activation results in vasorelaxation (Zygmunt et al., 1999; Smart et al., 2000). The anandamide transport inhibitor AM404, which is structurally related to anandamide, has also been shown to activate VR1 receptors by a mechanism distinct from its ability to inhibit transport (Zygmunt et al., 2000). To date, no other cannabinoid ligands have been tested for their ability to activate VR1 receptors.

Additional evidence for non-CB1 or -CB2 cannabinoid receptors comes from studies with palmitoylethanolamide. It has been suggested that palmitoylethanolamide acts as a CB2-selective agonist to inhibit mast cell activation (Facci et al., 1995). In addition, local injection of palmitoylethanolamide has been shown to inhibit formalin-induced pain in mice, and this effect was reversed by the CB2 antagonist SR144528 (Calignano et al., 1998). However, *in vitro* data in CB2-expressing cell lines across different species, does not support this claim (Felder et al., 1992; Showalter et al., 1996; Sugiura et al., 2000). This and other evidence has led to the suggestion of the existence of a novel non-CB1, non-CB2 receptor subtype, designated CB_n (Di Marzo, 1998; Lambert & Di Marzo, 1999).

Knockout mice for the CB1 receptor (Ledent et al., 1999; Zimmer et al., 1999), the CB2 receptor (Buckley et al., 2000), and both CB1 and CB2 receptors (Jarai et al., 1999) have been generated. Cannabinoid modulation of helper T-cell function through macrophages is deficient in CB2 receptor knockout mice (Buckley et al., 2000). Loss of the CB1 receptor results in hypoalgesia, as well as hypoactivity, and increased mortality (Zimmer et al., 1999). Stress-induced release of endogenous opioids is decreased in CB1 knockout mice (Valverde et al., 2000), indicating a connection between the opioid and endocannabinoid systems. Morphine fails to induce dopamine release in the nucleus accumbens of CB1 knockout mice, suggesting that the endocannabinoid system may be involved in the rewarding properties of opioids (Mascia et al., 1999). Mice lacking the CB1 receptor also show an enhancement in long-term potentiation (Bohme et al., 2000), which may explain the detrimental effects of marijuana on memory and learning.

5. Signal transduction

CB1 and CB2 couple primarily to the inhibition of adenylate cyclase through the inhibitory G-protein, G_i . This coupling has been shown following the heterologous expression of each receptor in mammalian cell lines; however, it has been shown in native tissues or primary cell culture only for CB1 receptors.

The ability of cannabinoid receptor agonists to interact with ion channels was first demonstrated in 1992 (Caulfield & Brown, 1992; Mackie & Hille, 1992). Caulfield and Brown (1992) showed inhibition of voltage-activated Ca^{2+} currents by a pertussis toxin-sensitive mechanism. Mackie and Hille (1992) also showed that cannabinoids inhibit N-type Ca^{2+} channels in NG108 cells through a receptor-mediated mechanism requiring pertussis toxin-sensitive G-proteins. CB1 receptors have also been shown to inhibit N- and P/Q-type Ca^{2+} channels in cultured rat hippocampal neurons (Twitchell et al., 1997). Inhibition of Ca^{2+} channel activity provides a mechanism by which CB1 receptors reduce neurotransmitter release and decrease neuronal cell excitability. In the cerebral vasculature, anandamide inhibits L-type Ca^{2+} channels, resulting in vasorelaxation (Gebremedhin et al., 1999). Cannabinoid receptors activate inwardly rectifying K^+ channels (Mackie et al., 1995), as well as increase activation of the voltage-dependent K^+ A current (by decreased inactivation) (Deadwyler et al., 1993). Cannabinoid-receptor activation also reduces the amplitude of the K^+ D current (Mu et al., 1999). Ion channel regulation appears to be a feature associated exclusively with CB1 and not CB2 receptors (Felder et al., 1995).

Studies with cannabinoid receptor antagonists indicate that there may be a constant tonic level of signaling by both CB1 and CB2 receptors in the absence of agonist. For example, SR141716A (Landsman et al., 1997) and AM630 (Landsman et al., 1998) both act as inverse agonists at the CB1 receptor in a GTP-binding assay. SR144528 also acts as an inverse agonist at the CB2 receptor in a cAMP reporter assay (Portier et al., 1999). The constitutive activity of cannabinoid receptors could have important implications for cellular signaling. Chronic treatment with SR144528 increases the expression level of G_i protein (Bouaboula et al., 1999b). Chronic treatment with SR141716A increases CB1 receptor protein at the cell membrane, and sensitizes it to agonist (Rinaldi-Carmona et al., 1998). These data indicate that inverse agonists may be another class of ligands that could be utilized to modulate the endocannabinoid system.

The signaling pathways activated by cannabinoid receptors downstream of G_i are just beginning to be elucidated. Mitogen-activated protein kinase, which regulates proliferation and differentiation, is activated by CB1 receptors (Bouaboula et al., 1995). Stimulation of the Na^+/H^+ exchanger by CB1, through mitogen-activated protein kinase activation, alters cellular pH, and has been

hypothesized to lead to alterations in the excitability of neuronal cells (Bouaboula et al., 1999a). Stress-related kinases, p38, and c-jun amino terminal kinase are also activated by CB1 receptors (Liu et al., 2000). Stimulation of protein kinase B/Akt by CB1 receptors, which increases glycogen synthesis through activation of glycogen synthase, indicates that CB1 may play a role in cell proliferation and energy metabolism (Gomez del Pulgar et al., 2000). Immediate early gene expression in the striatum has been linked to cannabinoid receptor stimulation (Glass & Dragunow, 1995).

Cross talk with other neurotransmitter receptor systems can expand the intracellular signal transduction pathways utilized by cannabinoid receptors. For example, when activated individually, both CB1 and the dopamine D_2 receptor couple to inhibition of adenylate cyclase and decrease cellular cAMP levels. However, when both types of receptors are activated simultaneously in striatal neurons in primary culture, there is an increase in cellular cAMP levels (Glass & Felder, 1997). In addition to coupling to G_s when co-stimulated with the D_2 receptor, the CB1 receptor is also able to couple to G_s when G_i is inactivated by pertussis toxin (Felder et al., 1998). The specific agonist occupying the receptor can also dictate coupling of cannabinoid receptors to specific types of G-proteins (Glass & Northup, 1999). In both active and inactive states, CB1 receptors are able to sequester $G_{i/o}$ proteins and to inhibit signal transduction by other families of receptors that couple to $G_{i/o}$ (Vasquez & Lewis, 1999). There is also evidence that other neurotransmitter systems can modulate endogenous cannabinoid release. Activation of D_2 receptors with dopamine agonists has been shown to increase release of anandamide, an endogenous cannabinoid ligand, in the striatum (Giuffrida et al., 1999). Thus, the local synaptic environment can provide additional levels of regulation beyond the known, direct-signaling pathways.

6. Endogenous cannabinoids

A search for endogenous agonists for the CB1 receptor followed closely behind the cloning of the receptor (Table 1). First identified and characterized by Devane et al. (1992), anandamide or arachidonyl ethanolamide was found to be an eicosanoid of novel structure. Anandamide was isolated from porcine brain and found to have properties similar to the plant-derived agonist THC (Devane et al., 1992). It binds both the CB1 and CB2 receptors, but has higher affinity for the CB1 receptor (Felder et al., 1995). Anandamide has been measured in human and rat brain, with the highest concentration in hippocampus, cortex, thalamus, and cerebellum (Felder et al., 1996).

Anandamide belongs to a family of nitrogen-containing fatty acid derivatives. Fatty acid amides were first discovered in the lipid fraction of chicken eggs, based on their anti-inflammatory properties (Kuehl et al., 1957). The

enzymatic synthesis of fatty acid amides was subsequently observed in liver microsomes, and this class of molecules was isolated, characterized, and quantitated in tissues (Bachur et al., 1965). Fatty acid amides were found to be elevated in canine myocardium following ischemia, although molecules containing arachidonic acid were not identified in the extracts, possibly due to their relatively low concentration (Epps et al., 1979). Arachidonylethanolamide was also discovered while investigating ligands that modify L-type voltage-sensitive ion channels (Johnson et al., 1993). It is not clear if receptors exist for all fatty acid amides or ethanolamides, or if only a specific subset acts in a signaling or transmitter role.

In addition to anandamide, other endogenous cannabinoid receptor agonists, all of which are fatty acid derivatives, have been identified. These include dihomogamma-linolenylethanolamide, adrenylethanolamide, and meadethanolamide (Hanus et al., 1993; Felder et al., 1993; Priller et al., 1995). More recently, 2-arachidonyl-glycerol, discovered in canine gut, has been shown to have cannabinoid agonist activity, but at more modest potencies and efficacies compared with anandamide (Mechoulam et al., 1995). 2-Arachidonyl-glycerol may be more selective for CB2 receptors under certain conditions (Sugiura et al., 2000). Palmitoylethanolamide has been proposed as a cannabinoid agonist (Facci et al., 1995), although it does not bind to either the CB1 or CB2 receptors (Felder et al., 1992; Showalter et al., 1996; Sugiura et al., 2000). However, cannabinoid-like physiological effects have been shown with palmitoylethanolamide, suggesting the existence of other cannabinoid receptor subtypes (Di Marzo & Deutsch, 1998).

7. Synthesis of endogenous cannabinoids

In order to establish that endogenous cannabinoids act as neurotransmitters, biosynthetic and degradative processes must be demonstrated. Because these ligands are very lipophilic, they would most likely not be stored in the aqueous interior of synaptic vesicles. It has been shown that phospholipid molecules found within the membrane phospholipid pool serve as precursor and storage depots for anandamide release (Di Marzo et al., 1994; Sugiura et al., 1996a, 1996b). Phosphatidylethanolamine is enzymatically converted to *N*-arachidonylphosphatidylethanolamine, the storage form of anandamide. This occurs through transfer of arachidonic acid from rare phospholipids containing arachidonic acid in the sn-1 position to the sn-3 position of phosphatidylethanolamine via *N*-acyltransferase, a Ca^{2+} -dependent enzyme. Anandamide is released from *N*-arachidonylphosphatidylethanolamine through cleavage of the phosphodiester bond by an as yet uncharacterized phospholipase D, which can also be activated by Ca^{2+} (Di Marzo et al., 1996). An alternative pathway has been postulated for the formation of anandamide from arachidonic acid and

ethanolamine by a synthase enzyme (Deutsch & Chin, 1993; Devane & Axelrod, 1994). A recombinant fatty acid amidohydrolase (FAAH), responsible for degradation of anandamide, has been shown to be capable of anandamide synthesis from arachidonic acid and ethanolamine in vitro, although high K_m values indicate that synthesis does not occur in vivo (Kurahashi et al., 1997; Katayama et al., 1999). FAAH, operating in reverse, is most likely responsible for the synthase activity previously described.

Release of endogenous cannabinoids into the synapse is regulated by several different signals. Depolarizing stimuli, such as ionomycin and high K^+ , induce release of anandamide (Di Marzo et al., 1994). Glutamate stimulation of cultured neuronal cells also increases formation of anandamide (Hansen et al., 1999). Agonist binding to a D_2 -like dopamine receptor and K^+ -stimulated depolarization increase the level of anandamide in the striatum (Giuffrida et al., 1999).

8. Termination of endocannabinoid activity through high-affinity transport

Signal termination for anandamide follows a mechanism similar to that for classical neurotransmitters, including reuptake across the plasma membrane. However, due to their hydrophobic properties, endocannabinoids appear to follow two routes of cellular reuptake, involving both facilitated and passive diffusion. A specific transporter or facilitative carrier protein, which provides a mechanism for high-affinity uptake of endocannabinoids, has been characterized, but has not been isolated or cloned yet.

Initial studies on the fate of anandamide and palmitoylethanolamide showed that these endocannabinoids were taken up by neuroblastoma and glioma cells in culture and that they are rapidly degraded by a membrane-associated amidase that is inhibited by phenylmethylsulfonyl fluoride (PMSF) (Deutsch & Chin, 1993). A subsequent study showed that anandamide uptake by central neurons is rapid, saturable, and temperature dependent, implicating a specific carrier protein (Di Marzo et al., 1994). Anandamide transport is not coupled to ion gradients, and does not require ATP (Hillard et al., 1997). The K_m and V_{max} values for anandamide transport are similar in both rat mast cells (Rakhshan et al., 2000) and cerebellar granule cells (Hillard et al., 1997). 2-Arachidonylglycerol, (*R*)-methanandamide, and Δ^9 -THC have been shown to block anandamide transport, indicating that other cannabinoid ligands may also be substrates for the anandamide transporter (Rakhshan et al., 2000). Inhibitors of known, well-characterized transporter proteins, including bromocresol green, cocaine, and verapamil, did not inhibit anandamide uptake in RBL-2H3 cells, indicating that the transporter may be a novel protein (Rakhshan et al., 2000). Competitive blockade of high-affinity anandamide transport by AM404 in rat cortical neurons was shown to enhance the

receptor-mediated effects of anandamide, suggesting a potential for therapeutic intervention through transporter blockade (Beltramo et al., 1997).

Despite all of the evidence for its existence, an anandamide transport protein has yet to be cloned. Barker and co-workers suggested a number of known proteins that may function as the anandamide transporter, including fatty acid translocases, plasma membrane fatty acid-binding proteins, and fatty acid transport proteins (Rakhshan et al., 2000). It is also possible that the anandamide transporter will be a unique molecule, unrelated to previously described fatty acid transport proteins.

9. Metabolic disposal of anandamide via fatty acid amidohydrolase

Termination of endocannabinoid activity appears to be a two-step process that involves facilitated transport followed by enzymatic degradation by FAAH (reviewed in (Di Marzo & Deutsch, 1998)). An enzyme responsible for anandamide hydrolysis was partially characterized and found to be membrane-associated, pH-dependent, selective, and sensitive to blockade by PMSF (Desarnaud et al., 1995; Hillard et al., 1995; Maurelli et al., 1995; Ueda et al., 1997; Watanabe et al., 1998). This enzyme was cloned in 1996 and was characterized as an FAAH. Molecular characterization of FAAH revealed a catalytic domain similar to other amidases, a transmembrane domain, and an SH₃ protein–protein interaction domain (Cravatt et al., 1996). In addition to its amidase activity, FAAH possesses esterase activity that may be responsible for hydrolysis of 2-arachidonoylglycerol (Patricelli & Cravatt, 1999; Di Marzo et al., 1998). In rat hippocampal slices, the ability of anandamide to inhibit acetylcholine release was enhanced by inhibition of FAAH activity with AM374 (Gifford et al., 1999b) in much the same manner as inhibition of the transporter enhanced anandamide activity in another study (Beltramo et al., 1997).

Both recombinant FAAH (Kurahashi et al., 1997; Arreaza et al., 1997) and crude enzyme extract preparations (Ueda et al., 1995; Schmid et al., 1985; Devane & Axelrod, 1994; Sugiura et al., 1996a) have been shown to have anandamide synthase activity *in vitro*. However, the synthase activity was dependent on very high concentrations of arachidonic acid and ethanolamine, with K_m values in the high micromolar to low millimolar range. Therefore, the enzyme probably acts primarily through its hydrolase, not synthase, activity under physiological conditions (Ueda & Yamamoto, 2000).

A recent study suggests a connection between the transport process and the enzymatic degradation of endocannabinoids. PMSF, arachidonyl trifluoromethyl ketone, and methyl arachidonyl fluorophosphonate, inhibitors of FAAH activity, reduce anandamide uptake in RBL-2H3 cells (Rakhshan et al., 2000). The most potent FAAH inhibitor,

methyl arachidonyl fluorophosphonate, only inhibited transport by 50% at a concentration well above its K_i value, indicating that FAAH alone does not facilitate anandamide transport (Rakhshan et al., 2000).

10. Measurement of endogenous cannabinoids

Since the early 1990s, reports of the measurement of endogenous cannabinoids in brain, as well as peripheral tissues, have been published (Tables 2 and 3). However, the lipophilic nature and relatively short half-life of these compounds have made measurement challenging. Techniques for extraction, partial purification, and quantitation of endogenous cannabinoids vary widely, and some procedures may introduce artifacts in the estimation of tissue levels. Anandamide has been extracted from tissue or bodily fluids using a variety of organic solvents, including acetone, toluene, ethyl acetate, chloroform, or a mixture of chloroform and methanol. It has been reported that anandamide can be generated chemically during the extraction procedure in the presence of acid or base (Yang et al., 1999) and that anandamide levels increase as postmortem collection time increases (Schmid et al., 1995; Felder et al., 1996).

Following solvent extraction, anandamide has been partially purified using thin-layer chromatography or solid phase extraction. Several different techniques have been used to measure anandamide after extraction and partial purification, including gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), and atmospheric pressure chemical ionization (APCI) LC/MS, as well as LC/MS/MS. Each of these methods has associated limitations. For example, GC/MS requires derivatization of anandamide prior to analysis. LC/MS is less selective than LC/MS/MS, where an initial molecular ion is selected and then fragmented to give a characteristic second molecular ion. However, MS/MS requires specialized and relatively expensive instrumentation. The inclusion of an internal standard allows measure-

Table 2
Measurement of anandamide in brain tissue

Species and tissue	Anandamide		Reference
	pmol/g	Method	
Pig brain	380	GC/MS	Devane et al., 1992
Pig brain	17±2.9	GC/MS	Schmid et al., 1995
Rat brain	N.D.	GC/MS	Kempe et al., 1996
Rat brain	4.3±1.1	GC/MS	Sugiura et al., 1996a
Rat thalamus	20	LC/MS/MS	Felder et al., 1996
Rat hippocampus	29		
Human cerebellum	30		
Human hippocampus	110		
Sheep	N.D.	GC/MS	Schmid et al., 1996
Cow	9–11		
Rat brain	11±7	GC/MS	Cadas et al., 1997
Rat hippocampus	45.8±2.6	GC/MS	Bisogno et al., 1999
Rat brain	N.D.	GC/MS	Yang et al., 1999

N.D., not detectable.

Table 3
Measurement of anandamide in brain microdialysate

Species and brain region	Basal anandamide	Stimulation % of basal	Method	Reference
Rat periaqueductal gray	2.79 ± 0.27 fmol 15 min 1 µL/min	Electrical (140%) Pain(140%)	LC/APCI-MS	Walker et al., 1999
Rat dorsal striatum	1.5 ± 0.3 pmol 30 min 10 µL/min	KCl (175%) D ₂ agonist (800%)	GC/MS	Giuffrida et al., 1999

ment data to be normalized to a ratio of anandamide to internal standard, thereby increasing measurement accuracy. The use of APCI-MS has been reported to increase the sensitivity of anandamide measurement down to the femtomolar level (Koga et al., 1997). Ultimately, it will be necessary to achieve a balance between selectivity and sensitivity for the measurement of endocannabinoids.

There is a large disparity in the measurement of anandamide levels reported in the literature. Several groups have found undetectable levels of anandamide in rat brain (Kemp et al., 1996; Yang et al., 1999). Other groups have found levels ranging from 4.3 pmol/g (Sugiura et al., 1996a) to 11 pmol/g (Cadas et al., 1997) in whole brain, 20 pmol/g in rat thalamus, and 29 pmol/g in rat hippocampus (Felder et al., 1996). Table 2 shows the levels of anandamide measured in the brains of various species. Anandamide has also been measured in peripheral tissues, including kidney (Deutsch et al., 1997), uterus (Schmid et al., 1997b), spleen (Yang et al., 1999), macrophages (Schmid et al., 1997a), blood (Giuffrida & Piomelli, 1998), and skin (Felder et al., 1996).

Similar to developmental expression patterns of the cannabinoid receptors, there appear to be differences in anandamide levels in rat brain at different developmental stages (for a review, see Fernandez-Ruiz et al., 2000). Koga et al. (1997) measured anandamide levels in the cerebrum, cerebellum, and hippocampus, along with several peripheral tissues at different postnatal developmental stages. Very low levels of anandamide were detected in the cerebrum, cerebellum, and hippocampus in 2-week-old rats. The levels increased across all brain regions at 6 weeks, and further increased at 12 weeks. By 24 weeks, the levels of anandamide had markedly decreased over all 3 brain regions. This pattern mirrors what has been observed for the expression pattern of the CB1 receptor during development. Fatty acid amides of varying chain length and degrees of saturation are released along with anandamide. Further research is necessary to determine if these molecules work alone or in concert to regulate development.

Recently, two groups have measured anandamide under basal and stimulated conditions in microdialysate from rat brains (Table 3). Walker et al. (1999) measured anandamide by LC/APCI-MS in rat periaqueductal gray. In response to painful stimuli, there was a 140% increase in basal anandamide levels. This report suggests that attomolar levels of anandamide could be detected in microdialysate; however, LC/MS was used with no internal standard.

Giuffrida et al. (1999) measured anandamide in the dorsal striatum using GC/MS, with a limit of detection in the picomolar range. They report a 175% increase in basal levels of anandamide in response to KCl stimulation and an 800% increase in response to the dopamine D₂ agonist quinpirole. The reason for the large difference in anandamide levels with the two stimuli is not clear.

The ability to measure anandamide levels in the brains of freely moving rats subjected to a variety of stimuli will enable us to further characterize the involvement of cannabinoid signaling in numerous physiological pathways. Currently, there is substantial variation in the literature regarding anandamide levels in brain and peripheral tissues, challenging even the most parsimonious conclusions. Ultimately, selective and sensitive measurement will improve, and along with it our understanding of the storage, release, and reuptake of this and other endogenous cannabinoids.

11. Therapeutic manipulation of the endocannabinoid system

The ability of the endocannabinoid system to modulate other neurotransmitter systems could be leveraged using several approaches. One obvious approach is activation or antagonism of the CB1 receptor. Dose-limiting psychotropic side effects have restricted the use of direct-acting receptor agonists. However, development of partial receptor agonists might alleviate side effects, and the large CB1 receptor reserve in the brain makes a partial agonist a viable therapeutic approach (Gifford et al., 1999a). Partial agonists have the added advantage of decreased receptor down-regulation, and, therefore, patients may be less likely to develop tolerance to treatment.

There are two potential mechanisms of action of cannabinoid antagonists. Because there seems to be a tonic, constitutive level of cannabinoid receptor activity in the absence of agonist, both a neutral antagonist, as well as an inverse agonist, have potential as therapeutic agents. Neutral antagonists would block increased activation by agonist. Inverse agonists have the ability to decrease the constitutive level of receptor activation in the absence of agonist.

A higher degree of therapeutic specificity may be achieved through the use of receptor subtype-selective potentiators or allosteric modulators. Modulators have been identified for many GPCRs. These compounds can increase

or decrease the response to agonists, and are devoid of intrinsic agonist activity (Lazareno et al., 2000; Tian et al., 2000). The use of a potentiator for the CB1 receptor has the advantage of affecting only the receptors that are being activated by endogenous ligand. This may alleviate many of the side effects associated with administration of exogenous cannabinoid ligands that act at both active and inactive anandaergic synapses.

Recent information concerning the release, reuptake, and degradation of endogenous cannabinoids introduces new areas of therapeutic manipulation. Reuptake inhibitors have been used clinically for a number of neurotransmitter systems, including serotonin and norepinephrine, to treat depression, anxiety, and other psychiatric disorders (Wong et al., 1995; Kent, 2000). Selective reuptake inhibitors of anandamide transport have already been identified (Beltramo et al., 1997). These inhibitors would function in much the same way as receptor potentiators, in that only the receptors exposed to increases in endogenous ligand would be affected, decreasing the chances of undesirable side effects. In addition, inhibitors of FAAH, the enzyme responsible for anandamide degradation, have also been identified (Boger et al., 1999, 2000). This class of compounds would increase the time of activation of cannabinoid receptors in a manner similar to cholinesterase inhibitors, which currently are being used to increase the half-life of acetylcholine in the synapse to treat Alzheimer's Disease (VanDenBerg et al., 2000; Emilien et al., 2000). However, the broad distribution of FAAH in both the CNS and peripheral tissues raises concerns about the specificity of this approach.

12. Therapeutic indications

The molecular basis for the physiological effects of cannabinoids and endocannabinoids is just beginning to be elucidated. One of the major mechanisms whereby the endocannabinoid system exerts its physiological effects is through interaction with, and modulation of, other neurotransmitter systems. Activation of cannabinoid receptors affects a number of intracellular signaling pathways, and leads to activation of K^+ channels and inhibition of adenylate cyclase and voltage-dependent Ca^{2+} channels. Ultimately, in the CNS, the cannabinoid system decreases the release of neurotransmitters into the synapse, modulating synaptic transmission. Cannabinoids act through cannabinoid receptors to modulate dopamine, glutamate, opiate, adrenergic, and possibly other systems.

There is anecdotal evidence for the therapeutic benefit of cannabinoid agonists in a variety of human disease conditions over many centuries. Recently, however, in depth research efforts have begun to document the biological mechanisms involved. In addition, it is becoming clear that antagonism of cannabinoid receptors may also have important therapeutic applications. Several well-recognized side effects of cannabinoid agonists have hampered the use of

these compounds in treatment protocols. These include sedation, cognitive dysfunction, tachycardia, postural hypotension, dry mouth, ataxia, and immunosuppressant effects, as well as psychotropic effects. The side effect profile of cannabinoid antagonists is less well understood. The physiological mechanisms responsible for generating the known side effects associated with either agonists or antagonists have not been established. Lack of pharmacological selectivity may contribute to these side effects, since previously developed cannabinoid agonists are relatively equiselective for CB1 or CB2 receptors. Therefore, it may be possible to avoid many, if not all, of the undesirable side effects through novel mechanistic approaches based on recent discoveries. These approaches include targeting a single cannabinoid receptor subtype, as well as targeting release, reuptake, or degradation of endogenous cannabinoids.

12.1. Emesis

One of the earliest and most widely studied therapeutic benefits of cannabinoids is the treatment of nausea and vomiting. Nausea is a devastating side effect of cancer chemotherapy. Studies in the 1970s and early 1980s with synthetic cannabinoids, as well as smoked marijuana, provided proof of their effectiveness as antiemetic agents. Eli Lilly and Company's synthetic cannabinoid, nabilone, originally was developed as an antiemetic for co-administration with cancer chemotherapeutic agents. It has been used for this purpose for over 20 years in the United Kingdom (Lemberger, 1999). Nabilone has also been shown to be useful in treating nausea and vomiting associated with anesthesia after abdominal surgery (Lewis et al., 1994), as well as radiation therapy (Priestman et al., 1987).

12.2. Analgesia

Cannabinoids have the potential to be useful as pain medications. Cannabinoid agonists elicit antinociception by spinal routes measured by the tail-flick assay and supraspinal routes measured by the hot-plate assay. These effects can be blocked by specific CB1 antagonists (reviewed in Martin & Lichtman, 1998; Manzanares et al., 1999). Reports indicate that cannabinoids can act independently and/or in concert with opioids to modulate the pain response (Welch & Stevens, 1992; Smith et al., 1994; Meng et al., 1998). Also, cannabinoid compounds have been shown to be effective in a rat model of allodynic pain (pain resulting from normally innocuous stimuli) (Martin et al., 1999). Other types of pathological pain, which are resistant to all current therapies and represent a significant unmet health need, can also be treated with cannabinoids (Mao et al., 2000). The peripheral CB2 receptor may be involved in pain modulation at the site of injury through inhibition of the inflammatory response (Jaggar et al., 1998; Calignano et al., 1998). There is also evidence that cannabinoids are effective in the treatment of migraine headaches (Russo,

1998). Therefore, several opportunities exist for modulation of pain by cannabinoids, especially through novel mechanistic approaches.

12.3. Anxiety

Studies in humans and animals indicate that cannabinoid agonists have an anxiolytic effect. This effect appears to be dose dependent, with low doses being anxiolytic and higher doses increasing anxiety in some cases. For example, low doses of HU210 have been shown to have anxiolytic properties (Rodriguez de Fonseca et al., 1997), whereas high doses are anxiogenic (Giuliani et al., 2000). The inverse agonist SR141716A (Landsman et al., 1997) induces anxiety-like responses in rats (Navarro et al., 1997; Akinshola et al., 1999), possibly by inhibition of the tonic level of CB1 receptor activity in the absence of agonist. Cannabidiol, the nonpsychoactive cannabinoid, also has anxiolytic properties in rats, although over a relatively limited dose range (Guimaraes et al., 1990).

Nabilone has been studied for its anxiolytic properties. In one double-blind placebo-controlled study, patients suffering from anxiety showed a “dramatic improvement” after nabilone treatment for 28 days (Fabre & McLendon, 1981). A single-dose regimen of treatment with nabilone in anxious patients was less efficacious; however, a small percentage of patients did show improvement in this study (Glass et al., 1980). Some side effects, including orthostatic hypotension and drowsiness, were reported in both studies.

12.4. Feeding behavior

Therapeutic manipulation of the endogenous cannabinoid system has the potential to increase or decrease feeding. Increased feeding is desirable in acquired immunodeficiency syndrome–wasting syndrome, and marijuana is effective for this purpose (Mechoulam, 1999). It has been suggested that cannabinoid agonists could also be effective for the pain and anxiety associated with acquired immunodeficiency syndrome–wasting syndrome (Watson et al., 2000). Anandamide, the endogenous cannabinoid, also increases feeding through a CB1 receptor-mediated mechanism (Williams & Kirkham, 1999).

Therapeutic manipulation of the endocannabinoid system may also be useful in the treatment of obesity. The CB1 selective antagonist SR141716A blocks the increase in feeding associated with administration of anandamide (Williams & Kirkham, 1999). SR141716A alone caused appetite suppression and weight loss, and although tolerance developed to the appetite suppression, the body weight of treated animals remained below that of control animals for the duration of the study (Colombo et al., 1998). The effect of SR141716A could be related to its activity as an inverse agonist at the CB1 receptor (Landsman et al., 1997),

indicating that a tonic level of CB1 receptor activation may be an important factor in feeding behavior.

12.5. Movement disorders

The endogenous cannabinoid system plays a regulatory role in the basal ganglia, an area thought to be important in the control of movement. It has been suggested that the endocannabinoid system establishes a “set point” of excitatory and inhibitory inputs within the basal ganglia (Rodriguez de Fonseca et al., 1998). Parkinson’s disease (Sanudo-Pena et al., 1998), dyskinesias, dystonias, as well as Huntington chorea (Glass et al., 1993; Richfield & Herkenham, 1994), Gilles de Tourette syndrome (Muller-Vahl et al., 1998), and multiple sclerosis (Baker et al., 2000) should be considered candidates for cannabinoid-based therapy (reviewed in Rodriguez de Fonseca et al., 1998).

12.6. Glaucoma

The ability of smoked marijuana to lower intraocular pressure (IOP) was first noted in the early 1970s (Hepler & Frank, 1971). At the time, the mechanism of action of marijuana was not clear. However, recent studies into the expression of cannabinoid receptors in the eye, as well as the effects of novel cannabinoid agonists, are beginning to elucidate the mechanism of action. Immunohistochemical analysis of the anterior eye has shown expression of CB1 receptors in the ciliary pigment epithelium, where they could have an effect on aqueous humor production, and the trabecular meshwork and Schlemm’s canal, where they could have an effect on aqueous humor outflow (Straiker et al., 1999). Topical application of three different cannabinoid agonists has been shown to lower IOP. WIN55212-2 decreased IOP in rabbits, and this effect was blocked by the CB1 antagonist SR141716A, suggesting that it is CB1 receptor-mediated (Song & Slowey, 2000). Anandamide has been shown to lower IOP in normotensive rabbits in a dose-dependent manner (Pate et al., 1995). CP55940 has also been shown to be effective for lowering IOP (Pate et al., 1998). Several cannabinoid compounds without systemic effects are currently being developed for topical application to treat glaucoma (Buchwald et al., 2000).

12.7. Neuroprotection

Cannabinoids have been implicated as neuroprotective agents. However, it is not clear whether or not this is a CB1 receptor-dependent process. WIN55212-2 and CP55940 have been shown to protect cultured rat hippocampal neurons from glutamate-induced death by a CB1 receptor-mediated process that is blocked by SR141716A (Shen et al., 1996; Shen & Thayer, 1998). WIN55212-2 is also neuroprotective in a rat model of ischemic brain injury, and this effect is also blocked by SR141716A

(Nagayama et al., 1999). In contrast, Δ^9 -THC, as well as cannabidiol, which has very low affinity for the CB1 receptor, both decrease glutamate toxicity in rat cortical neuron cultures by a receptor-independent process that is not blocked by SR141716A (Hampson et al., 1998). HU211, a nonpsychotropic cannabinoid that also has very low affinity for the CB1 receptor, acts as a neuroprotective agent after closed-head injury in rats (Shohami et al., 1993) and in cultured neuronal preparations after glutamate toxicity (Nadler et al., 1993). HU211 is also neuroprotective after ischemic insult in rodents (Bar-Joseph et al., 1994; Belayev et al., 1995). The neuroprotective effects of HU211 probably are due to its ability to block *N*-methyl-D-aspartate receptors (Feigenbaum et al., 1989).

12.8. Cardiovascular disease

Modulation of the endocannabinoid system has potential in the treatment of certain forms of hypertension, as well as shock-related hypotension (reviewed in Hillard, 2000; Randall & Kendall, 1998). Recent reports indicate that cannabinoid compounds cause vasodilation and hypotension by a CB1 receptor-mediated process. The anandamide-induced decrease in systemic blood pressure in anesthetized guinea pigs is blocked by CB1 receptor antagonists and is augmented by the reuptake inhibitor AM404 (Calignano et al., 1997). The CB1 agonist HU210 also causes hypotension in anesthetized rats (Vidrio et al., 1996). The CB1 antagonist SR141716A inhibits the hypotension associated with endotoxin-induced shock in rats, and increases the rate of survival (Varga et al., 1998). However, SR141716A shortened survival after hemorrhagic shock (Wagner et al., 1997). A decrease in vasodilation after administration of SR141716A may further compromise tissue perfusion in cases of hemorrhagic shock, and may explain the decreased survival under these conditions (Varga et al., 1998). CB1-dependent hypotension may be due to decreased sympathetic outflow through inhibition of norepinephrine release from sympathetic nerve terminals (Ishac et al., 1996). Cannabinoid compounds also have effects on vascular tone (Gebremedhin et al., 1999) and directly on the vascular endothelium (Jarai et al., 1999) that appear to be CB1 receptor-independent. A CB1 agonist could be useful for hypertension resulting from aberrant sympathetic outflow, and a CB1 antagonist might be useful for the treatment of hypotension associated with endotoxin-induced shock.

13. Summary

Evidence beginning with the early history of humans indicates that there is a potential for the therapeutic use of cannabinoids for a variety of disorders. Recent scientific investigations have begun to elucidate the mechanisms of

action responsible for these effects. Two subtypes of cannabinoid receptors have been identified, and there are indications that other family members may exist. In addition, several endogenous ligands for the cannabinoid receptors have been documented, with the possibility that others also exist. Endogenous cannabinoids act as neurotransmitters and appear to have a modulatory role in the CNS. Therapeutic potential exists for the use of cannabinoid compounds as neuroprotective agents; to alleviate pain; and to treat anxiety, emesis, obesity, movement disorders, and glaucoma. Subtype-specific ligands, as well as the use of potentiators and partial agonists, may help to eliminate side effects associated with classical cannabinoids. In addition, research identifying mechanisms of endogenous cannabinoid release, reuptake, and degradation is providing new targets for therapeutic intervention. Although psychotropic side effects and public opinion have limited therapeutic development in this area, our current state of understanding has renewed interest in the therapeutic potential of the endocannabinoid system. New targets for intervention have been identified that may help to limit side effects and enable us to develop novel and effective endocannabinoid-based treatments for a variety of intractable disorders in the near future.

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