# Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat

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Received 24 July 1996

**Abstract Anandamide (arachidonylethanolamide) is a novel lipid neurotransmitter first isolated from porcine brain which has been shown to be a functional agonist for the cannabinoid CB1 and CB2 receptors. Anandamide has never been isolated from human brain or peripheral tissues and its role in human physiology has not been examined. Anandamide was measured by LC/MS/MS and was found in human and rat hippocampus (and human parahippocampal cortex), striatum, and cerebellum, brain areas known to express high levels of CB1 cannabinoid receptors. Significant levels of anandamide were also found in the thalamus which expresses low levels of CB1 receptors. Anandamide was also found in human and rat spleen which expresses high levels of the CB2 cannabinoid receptor. Small amounts of anandamide were also detected in human heart and rat skin. Only trace quantities were detected in pooled human serum, plasma, and CSF. The distribution of anandamide in human brain and spleen supports its potential role as an endogenous agonist in central and peripheral tissues. The low levels found in serum, plasma, and CSF suggest that it is metabolized in tissues where it is synthesized, and that its action is probably not hormonal in nature.**

*Key words:* Anandamide; Cannabis; Cannabinoid receptor; Marijuana

## 1. Introduction

The psychotropic and therapeutic effects of cannabinoids, a class of compounds derived from the marijuana plant, have been known for centuries, but only relatively recently has the mechanism of their actions been elucidated. To date, three receptor subtypes have been identified which bind cannabinoids and related compounds with high affinity. The CB1 receptor [1] and splice variant, CB1a [2], are found predominantly in the central nervous system. The CB2 receptor is found predominantly in spleen and hemopoietic cells and has a 44% overall nucleotide sequence identity with the CB1 receptor [3–5]. In spite of these dissimilarities in structure and distribution, the CB1 and CB2 receptors bind most cannabinoid agonists with similar affinity [6]. The discovery of cannabinoid receptors suggested that an endogenous ligand may be found in areas of receptor expression.

The first endogenous cannabimimetic compound, anandamide (arachidonylethanolamide), was isolated from whole porcine brain and found to be a lipid of novel structure [7]. Anandamide displayed specific binding to the CB1 receptor and inhibited a prototypical twitch response in mouse vas deferens. Anandamide has also been shown to induce similar behavioral [8,9], pharmacological [10,11], and signal transduction effects [12] as classical cannabinoid agonists, but high concentrations were required to induce these effects. Levels of anandamide were first estimated to occur at 0.4 pmol/g (133 pg/g) in whole porcine brain [7], and recently quantitated in porcine and bovine brain at 173 pmol/g (60 ng/g) and 101 pmol/g (35 ng/g) respectively [13]. A recent study reports levels of anandamide in rat testis to be considerably lower (6 pmol/g, 2.1 ng/g) [14]. However, anandamide has never been isolated from human tissue or fluids. Furthermore, levels of anandamide have not been measured in regions of rat brain or in tissues such as spleen where CB2 receptors have been shown to be expressed at high levels. Studies of anandamide distribution should help elucidate the physiologic role of anandamide as a cannabimimetic eicosanoid and possibly broader functions. In this study we report the isolation and quantitation of anandamide by liquid chromatography/mass spectrometry in various tissues and fluids from postmortem human and rat.

## 2. Materials and methods

Anandamide (arachidonylethanolamide) and (5,6,8,9,11,12,14,15-$D_8$)anandamide were obtained from Biomol (Plymouth Meeting, PA). TLC plates (60 Å, 250 μm silica gel K6, 20×20 cm) were obtained from Whatman (Clifton, NJ). All other reagents were obtained from Sigma.

### *2.1. Isolation and extraction of rat and human tissues or fluids*

Normal human brain samples from individuals suffering acute cardiac failure (age 48 and 62) were obtained within 2 h post mortem and processed for routine histopathology and regions frozen at −70°C until use. Human serum, plasma, and cerebral spinal fluid were the generous gift from Dr. Robin Felder (Department of Pathology, University of Virginia, Charlottesville, VA) and were frozen at −70°C within 2 h of collection. Human serum, plasma, and cerebral spinal fluid were randomly pooled from at least 10 patients per sample. Female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) and anesthetized by brief $CO_2$ asphyxiation, decapitated, and tissues removed by surgical dissection within 5 min. All samples were snap frozen in liquid $N_2$ and stored at −70°C until use. All tissues and fluids were homogenized in buffer (200 mM sucrose, 50 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) containing 66% toluene (v/v) and then extracted at room temperature three times with 2 volumes, 2 volumes, and 1 volume toluene, respectively. Toluene extracts were pooled and dried under $N_2$ gas to a final volume of approximately 1 ml and streaked on TLC plates which were then

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*PII* S0014-5793(96)00891-5

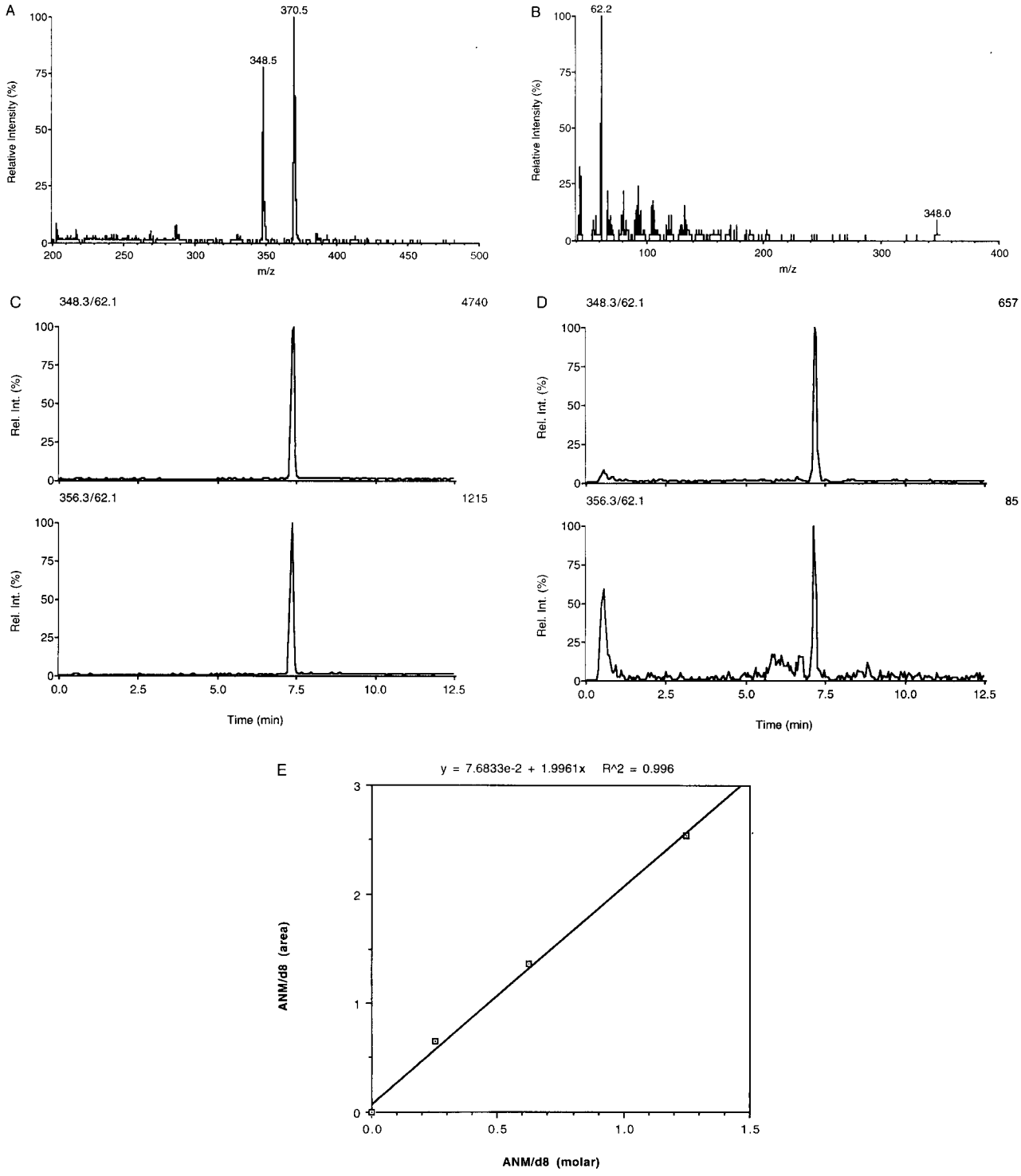


Fig. 1. A: ESI-MS spectrum of anandamide between m/z = 200 and m/z = 500. A solution of anandamide (0.1 mg/ml in methanol) was infused into the mass spectrometer. The ion at m/z = 348.5 represents the protonated molecular ion, $(M+H)^+$ (molecular weight of anandamide = 347.3) and the ion at m/z = 370.5 represents the sodium adduct of that ion $(M+H+Na)^+$. B: Product ion spectrum of m/z = 348.4 ion. A solution of anandamide (6.9 mg/ml in methanol) was infused into the mass spectrometer. The ion at m/z = 348.4 was selected as the precursor ion for MS/MS analysis and the resulting product ion spectrum was recorded between m/z = 40 and m/z = 500. C: LC/MS/MS chromatogram of a mixture of anandamide and deuterated anandamide standards. A solution containing anandamide (2.0 μM) and deuterated anandamide (1.6 μM) was injected onto the reversed-phase column and the column effluent was monitored in MRM mode as described in Section 2. The upper trace represents the anandamide response and the lower trace represents the deuterated anandamide response. D: LC/MS/MS chromatogram of a sample of an extract of rat striatum with deuterated anandamide added as an internal standard. Details of the chromatography and column monitoring are given in Section 2. The upper trace represents the anandamide response and the lower trace represents the deuterated anandamide response. E: Anandamide standard curve. Four standard mixtures containing anandamide and deuterated anandamide were prepared, each with a constant concentration of deuterated anandamide (1.6 μM) and increasing concentrations of anandamide (0, 0.4, 1.0, and 2.0 μM). Each was analyzed by the LC/MS/MS procedure described in Section 2. The data are displayed as the area ratio of anandamide to deuterated anandamide as a function of the molar ratio of anandamide to deuterated anandamide.

developed in the following organic solvent mixture (40:20:5, v/v/v, chloroform:hexane:methanol). The area of the TLC plate that migrated with an authentic anandamide standard was scraped and eluted with chloroform:methanol (85:15, v/v), dried under $N_2$, and resuspended in 50 μl methanol and stored at −70°C. 50 ng of anandamide-$D_8$ was added to all samples before the extraction procedure and final data normalized to anandamide-$D_8$ content. Recovery of [$^3$H]anandamide following these extraction procedures was greater than 80%.

*2.2. Liquid chromatography/mass spectrometry*

An LC/MS/MS assay was utilized to quantify the amount of anandamide semi-purified from tissues and fluids because of its selectivity and sensitivity. The sample was injected, in a volume of 5 μl MeOH, onto a C-18 reversed-phase HPLC column (Hypersil C18, 1.0 mm×20 mm, Keystone Scientific, equilibrated in 20% methanol) flowing at 50 μl/min. The solvent composition was ramped from 20% to 99% methanol in 1 min and then held constant at 99% methanol for 14 min to elute the analyte from the column. The column effluent was directed through a fused silica capillary (50 μm in diameter) into the source of an electrospray ionization triple quadrapole mass spectrometer (API-III, PE SCIEX, Concord, Ontario). Mass spectrometer operating parameters were optimized before each set of samples was analyzed and were set to approximately the following values: orifice potential = +65 V; collision gas thickness = $250 \times 10^{12}$ molecules/cm$^2$. The instrument was operated in MRM (multiple reaction monitoring) mode whereby only two ions, m/z = 348.3 and m/z = 356.3, corresponding to the singly charged protonated molecular ions of anandamide and the deuterated form of anandamide, respectively, were allowed to pass into the collision chamber and were monitored simultaneously. A representative spectrum of anandamide standard is shown in Fig. 1A. Both forms of anandamide fragment to give a prominent product ion of m/z = 62.1 (Fig. 1B). Detection of anandamide, therefore, is based on the fragmentation of the precursor ion of m/z = 348.3 to yield a prominent product ion m/z = 62.1. The deuterated form of anandamide serves as an internal standard and its detection is based on the fragmentation of the precursor ion of m/z = 356.3 to yield m/z = 62.1. An example of a chromatogram showing the separation and detection of both anandamide and deuterated anandamide in a standard mixture and in rat striatum is shown in Fig. 1C,D. A standard curve (Fig. 1E) obtained by injecting mixtures of anandamide and deuterated anandamide in different ratios with constant deuterated anandamide shows a good linear relationship between the ratio of integrated peak areas and the ratio of molar amounts of the two components. The lower limit of detection by this assay was estimated to be approximately 150 nM in the injected sample.

## 3. Results and discussion

Lipids from human and rat tissues or fluids were organic solvent extracted, semi-purified by thin layer chromatography, and analyzed for anandamide content by LC/MS/MS. The results of this study are the first report of anandamide in regions of human and rat brain (Fig. 2A,B). Relatively similar levels of anandamide were found in the four regions of rat brain studied (Fig. 2A). The highest level was found in the hippocampus (29 pmol/g) and the lowest level was found in the thalamus (20 pmol/g). The levels of anandamide in rat did not vary more than 10 pmol/g over the four brain regions evaluated. In contrast, considerable differences were seen in human brain distribution and may reflect regional differences in tissue samples selected. The five human brain regions selected for analysis ranged from 25 pmol/g in cerebellum to 148 pmol/g in hippocampus (Fig. 2B). Also reported in this study is the presence of anandamide in human and rat spleen suggesting that it may also play a role as an endogenous agonist in this organ (Figs. 2 and 3). Anandamide was also found in rat skin at levels comparable to those in rat brain.

The absence of anandamide in human serum, plasma, and CSF (Fig. 2B) suggests that anandamide is not hormonal in

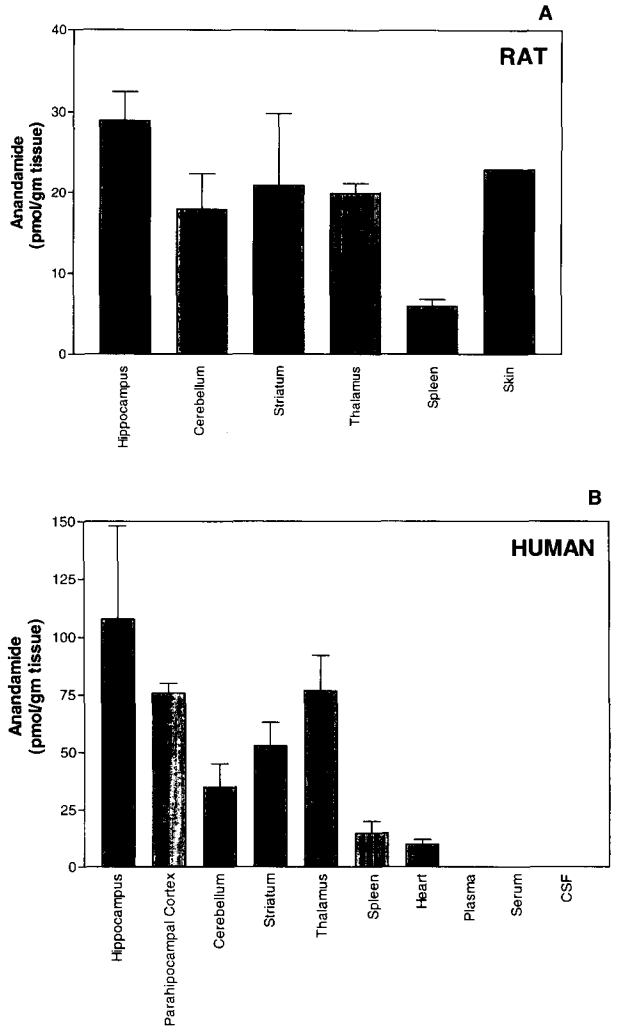


Fig. 2. A, B: Distribution of anandamide in selected regions of rat and human. Samples were homogenized and extracted in toluene in the presence of internal anandamide-$D_8$ standard, purified by TLC, and analyzed by LC/MS/MS as described in Section 2. Data from human samples are the mean ± SEM from at least two individuals and each sample was measured at least twice (except for hippocampus and parahippocampal cortex in which small tissue samples allowed only single determinations for each patient and therefore the data are the mean ± standard deviation). Rat tissue was pooled from six male rats and replicate measurements performed (data are the mean ± SEM). Anandamide was extracted from two pools of human serum, plasma, and CSF collected and combined from at least 10 patients in each pool. Total fluid volumes for both pools are as follows: serum (132 ml and 72 ml), plasma (36 ml and 41 ml), CSF (115 ml and 59 ml). Two replicate measurements were performed for each pool of plasma and CSF and one determination was performed for each pool of serum.

nature, requiring that it be synthesized in an endocrine organ and released to the circulation to act on distant tissues. However, pressor and depressor effects of anandamide on heart rate and blood pressure have been recently described [15]. The depressor effects were thought to be sympatho-inhibitory, augmenting noradrenaline release from sympathetic ganglion through CB1 receptors localized on the terminals of postganglionic sympathetic neurons (Lake et al., 1996, ICRS). However, in this study an additional pressor effect on peripheral vasculature could not be explained by this mechanism and a direct, non-CB1 receptor-mediated effect of anandamide

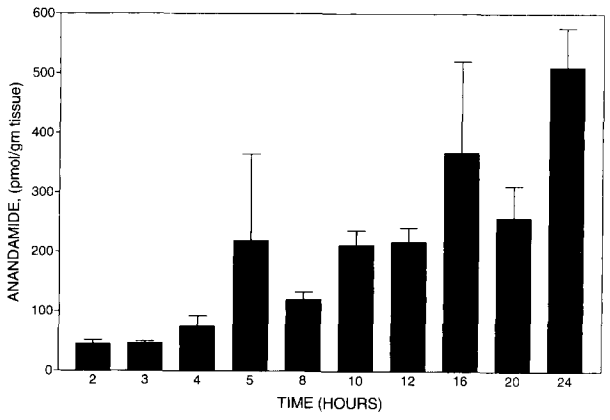


Fig. 3. Postmortem levels of anandamide in human cerebellum. A human brain was obtained less than 1 h post mortem and adjacent samples collected from cerebellar cortex at the times indicated. The tissue remained at room temperature during the entire collection period. Data are the mean ± standard deviation of duplicate determinations and are representative of two experiments.

on smooth muscle was proposed. We find small amounts of anandamide in human heart where a low level of CB1 mRNA has also been measured [5]. It is not clear if heart muscle synthesizes anandamide or if trace quantities of circulating anandamide accumulate in areas of CB1 receptor expression. It is possible that anandamide is synthesized and partly metabolized in circulating hemopoietic cells. In contrast, testis has been shown to contain significant levels of CB1 receptors [5], as well as anandamide and an anandamide containing *N*-acylphospholipids [14] suggesting that anandamide is generated near the location of the CB1 receptor in this tissue.

Recent evidence suggests that anandamide levels may increase significantly as postmortem collection time increases beyond 5 h in porcine and bovine brain [13]. We observed similar results in human cerebellum measured over a 24 h period (Fig. 3). However, all human samples in this study were collected within 2 h and rat samples within 5 min post mortem suggesting that postmortem delay would have little influence on anandamide levels in these studies. The increase in anandamide levels over postmortem time may result from activation of one or both currently proposed synthetic routes for anandamide formation. The most widely accepted biosynthetic pathway suggests that anandamide formation results from the degradation of a putative storage form of anandamide, *N*-arachidonylphosphatidylethanolamine, through activation of a sn-3 position phosphohydrolase such as phospholipase D [13,14,16]. Alternatively, a putative anandamide synthase enzyme would condense arachidonic acid and ethanolamine following their release from phospholipids by phospholipases A2 and D respectively [17,18]. It is not clear which of these two pathways or possible alternative pathways predominate during postmortem release of anandamide. Postmortem levels of anandamide may be determined by the equilibrium between the proposed routes of formation and its degradation by a partially characterized aminohydrolase [19]. More detailed study of the regulation of anandamide synthesis should help answer these questions.

The measurement of anandamide in human tissues expressing cannabinoid receptors supports the hypothesis that anandamide is an authentic endogenous agonist for this family of receptors. Previous reports have shown similarities in the behavioral responses resulting from cannabinoid agonists and anandamide in various well studied animal models [8,10]. Anandamide displays pharmacological and biochemical properties of a cannabinoid agonist for both the CB1 and CB2 receptors [6]. Furthermore, cannabinoid receptors show specificity for polyunsaturated fatty acylethanolamides with structural similarities to anandamide [12]. However, measurement of anandamide at significant levels in tissues such as heart and thalamus known to express few if any cannabinoid receptors and its relatively low affinity for both CB1 and CB2 receptors suggest that other receptors may exist for anandamide [20]. In addition, cannabinoid receptor-independent effects of anandamide have been reported [21], suggesting the presence of other anandamide binding sites.

The wide distribution of anandamide in brain and in peripheral tissues suggests that anandamide represents a new class of lipid neurotransmitter with little resemblance to well-studied biogenic amines, amino acids, or peptides. Its lipid character and ability to permeate membranes suggest that vesicular storage is unlikely, and that storage would more likely occur in the lipid bilayer as components of phospholipids. The levels of anandamide measured in human brain are not unlike those previously reported for dopamine and serotonin [22], but at least 10-fold lower than those reported for GABA and glutamate [23]. However, our measurements of anandamide most likely represent a combination of basal levels as well as the anandaergic nervous system in various states of stimulation and down regulation. The regulation and physiological relevance of anandamide as a novel neurotransmitter await further investigation.

## References

[1] Matsuda, L.A., Lolait, S.J., Brownstein, M., Young, A. and Bonner, T.I. (1990) Nature 346, 561–564.
[2] Shire, D., Carillon, C., Kaghad, M., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Caput, D. and Ferrara, P. (1995) J. Biol. Chem. 270, 3726–3731.
[3] Munro, S., Thomas, K.L. and Abu-Shaar, M. (1993) Nature 365, 61–65.
[4] Lynn, A.B. and Herkenham, M. (1994) J. Pharmacol. Exp. Ther. 268, 1612–1623.
[5] Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G. and Casellas, P. (1995) Eur. J. Biochem. 232, 54–61.
[6] Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Mackie, K., Lai, Y. and Ma, A.L. (1995) Mol. Pharmacol. 48, 443–450.
[7] Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Science 258, 1946–1949.
[8] Crawley, J.N., Corwin, R.L., Robinson, J.K., Felder, C.C., Devane, W.A. and Axelrod, J. (1993) Pharmacol. Biochem. Behav. 46, 967–972.
[9] Souilhac, J., Poncelet, M., Rinaldi-Carmona, M., Le Fur, G. and Soubrie, P. (1995) Pharmacol. Biochem. Behav. 51, 3–7.
[10] Fride, E. and Mechoulam, R. (1993) Eur. J. Pharmacol. 231, 313–314.
[11] Smith, P.B., Compton, D.R., Welch, S.P., Razdan, R.K., Mechoulam, R. and Martin, B.R. (1994) J. Pharmacol. Exp. Ther. 270, 219–227.
[12] Felder, C.C., Briley, E.M., Axelrod, J., Simpson, J.T., Mackie, K. and Devane, W.A. (1993) Proc. Natl. Acad. Sci. USA 90, 7656–7660.
[13] Schmid, P.C., Krebsbach, R.J., Perry, S.R., Dettmer, T.M., Maasson, J.L. and Schmid, H.H.O. (1995) FEBS Lett. 375, 117–120.
[14] Sugiura, T., Kondo, S., Sukagawa, A., Tonegawa, T., Nakane,

S., Yamashita, A. and Keizo, W. (1996) Biochem. Biophys. Res. Commun. 218, 113–117.
[15] Varga, K., Lake, K., Martin, B.R. and Kunos, G. (1995) Eur. J. Pharmacol. 278, 279–283.
[16] Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C. and Piomelli, D. (1994) Nature 372, 686–691.
[17] Devane, W.A. and Axelrod J (1994) Proc. Natl. Acad. Sci. USA 91, 6698–6701.
[18] Kruszka, K.K. and Gross, R.W. (1994) J. Biol. Chem. 269, 14345–14348.
[19] Desarnaud, F., Cadas, H. and Piomelli, D. (1995) J. Biol. Chem. 270, 6030–6035.
[20] Ueda, N., Kurahashi, Y., Yamamoto, S. and Tokunaga, T. (1995) J. Biol. Chem. 270, 23823–23827.
[21] Venance, L., Piomelli, D., Glowinski, J. and Giaume, C. (1995) Nature 376, 590–594.
[22] Mackay, A.V., Yates, C.M., Wright, A., Hamilton, P. and Davies, P. (1978) J. Neurochem. 30, 841–848.
[23] Perry, T.L., Berry, K., Hansen, S., Diamond, S. and Mok, C. (1971) J. Neurochem. 18, 513–519.