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SHORT COMMUNICATION

A Fluorescence Displacement Assay for the Measurement of Arachidonoyl Ethanolamide (Anandamide) and Oleoyl Amide (Octadecenoamide) Hydrolysis

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ABSTRACT. We describe a simple fluorescence displacement assay to measure hydrolysis of arachidonoyl ethanolamide and oleoyl amide, two important pharmacological compounds. Hydrolysis at the amide linkage of these ligands releases a fatty acid as one of the products. The displacement of a fluorescent fatty acid analogue from rat liver fatty acid-binding protein by the released fatty acid can thus be measured as a decrease in fluorescence. This process is time- and concentration-dependent and shows hyperbolic enzyme kinetics. Electrospray ionisation mass spectrometry was used to validate the assay. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3: 433–435, 1997.

KEY WORDS. fatty acid-binding protein; arachidonoyl ethanolamide; anandamide; octadecenoamide; oleoyl amide; hydrolysis

Anandamide† is an endogenous cannabinoid agonist acting at the level of the cannabinoid receptor [1, 2], and oleoyl amide has recently been identified as a sleep inducer [3]. The potential clinical and pharmacological benefits of these compounds have therefore been established.

The hydrolytic cleavage of anandamide and oleoyl amide at the amide linkage yields fatty acids, i.e. arachidonic acid and oleic acid, and ethanolamine as products [4–9]. The enzyme hydrolysing anandamide has been referred to as either "anandamide amidohydrolase" or "anandamide amidase" [4, 5]. Most published methods utilise radiolabelled ligands to measure substrate hydrolysis and either TLC or HPLC to separate the products, processes that are both expensive and time consuming [7–10]. A recently published method uses a simple extraction procedure to separate radiolabelled ethanolamine from anandamide, but the authors had to initially synthesise anandamide by using radiolabelled ethanolamine [5]. Hydrolysis of phospholipids and triglycerides by phospholipases A_2 and triglyceride lipases can be monitored by a fluorescence displacement assay in which released fatty acids displace a fluorescent fatty acid analogue, DAUDA, from FABP with a resultant decrease in fluorescence [11, 12]. In this communication, we describe a similar protocol to assay for anandamide and oleoyl amide hydrolysis.

MATERIALS AND METHODS

Anandamide was obtained from Cascade Biochem (Reading, UK) and oleoyl amide from Alexis Corporation (Nottingham, UK). DAUDA was purchased from Molecular Probes (Eugene, OR, USA). Pentobarbitone was obtained from Rhône Mérieux (Essex, UK). All other chemicals were obtained from Sigma.

Purification and delipidation of recombinant rat liver FABP has been described [13–15]. For the preparation of subcellular fractions, rabbits were killed by an overdose of pentobarbitone (300 mg). The brains and livers were excised, and a 10% (w/v) homogenate was prepared in SET buffer (0.25 M sucrose, 1 mM EDTA, 20 mM Tris.HCl, pH 7.4). The homogenates were centrifuged at 800g for 5 min to remove cell debris. The supernatant fraction was centrifuged at 10,000g for 30 min before storage (−70°C). Protein concentrations were determined by the method of Bradford [16] using bovine serum albumin as a standard. All procedures were performed at 4°C.

TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 7.6) [17] contained 1 μM FABP, 1 μM DAUDA and protein solution. DAUDA fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 500 nm [18, 19]. The percentage of initial fluorescence was

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† *Abbreviations:* FABP, fatty acid-binding protein; DAUDA, 11-(5-dimethylaminonaphthalenesulphonyl)-undecanoic acid; anandamide, arachidonoyl ethanolamide; oleoyl amide, *cis*-9,10-octadecenoamide; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

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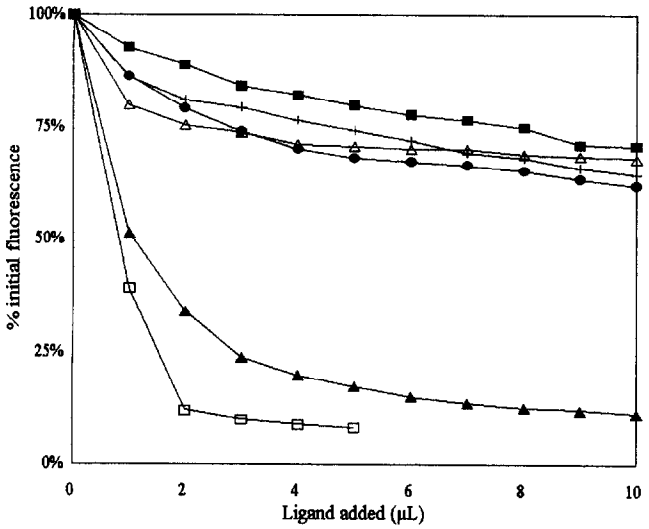

FIG. 1. Displacement of DAUDA from liver FABP by anandamide (●), oleoyl amide (△), arachidonic acid (▲), oleic acid (□), ethanolamine (+), and ethanol (■). Stock solutions of ligand (10 mM) were dissolved in ethanol. Methodology described in text.

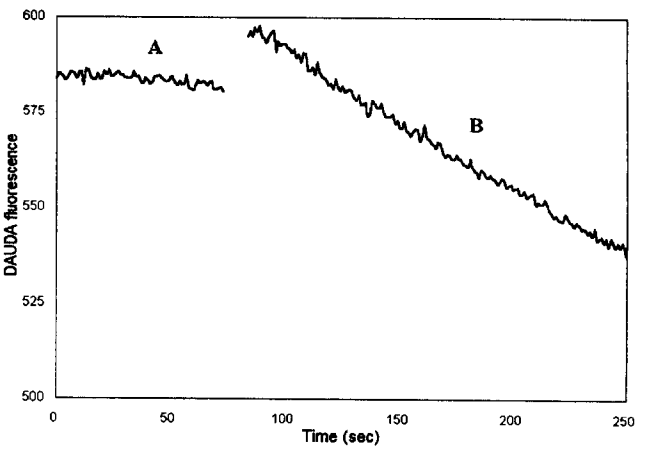

FIG. 2. The time-dependent hydrolysis of anandamide by rabbit brain 100,000g microsomes. The sample contained (A) buffer, 1 μM FABP, 1 μM DAUDA 10 μM arachidonoyl ethanolamide and (B) 100,000g microsomes (0.012 mg protein). The rate of hydrolysis displayed pseudo first-order kinetics, with $t_{1/2}$ ~ 5 min.

calculated as (fluorescence in the presence of ligand) ÷ (fluorescence in the absence of added ligand). Hydrolysis assays were started by addition of protein, and rates were calibrated with arachidonic acid or oleic acid (for anandamide and oleoyl amide, respectively). All ligands, with the exception of DAUDA, were dissolved in ethanol.

Electrospray mass spectrometry was performed on a VG Quattro II mass spectrometer (Fisons Instruments) using methanol as solvent (10 μL/min). Voltages were capillary 2.50 kV, HV lens 0.20 kV, cone 40 V. Spectra were collected over 1 sec with 0.1-sec intervals. Arachidonic acid was detected as a negative ion and anandamide as the positive ion. In the case of arachidonic acid, a 1-μM sample was used for calibration.

RESULTS AND DISCUSSION

The hydrolysis of anandamide and oleoyl amide releases fatty acids that can bind to liver FABP and displace the fluorescent fatty acid probe DAUDA [11]. The two substrates investigated, i.e. anandamide and oleoyl amide, show low affinity for liver FABP, whereas the respective two fatty acids produced in the hydrolysis, i.e. arachidonic acid and oleic acid, bind with high affinity (Fig. 1). Therefore, the hydrolysis of anandamide and oleoyl amide can be measured by using a displacement assay with calibrations using the appropriate fatty acid.

The hydrolysis of anandamide and oleoyl amide is time, protein and substrate dependent. Pseudo first-order kinetics are observed when adding protein (Fig. 2) hyperbolic kinetics are obtained with substrate variation and K_M (ap-

TABLE 1. Kinetic parameters for the hydrolysis of anandamide and oleoyl amide by various tissue fractions

Substrate	K_M (μM)	V_{max}†	Comments
Anandamide	2.8 ± 1.1	5.5 ± 1.3 (6)	Rabbit brain 100,000g microsomes‡
Anandamide	0.7 ± 0.2	0.96 ± 0.04 (8)	Rabbit brain 10,000g cytosol‡
Anandamide	12.7	5.6	Rat brain microsomes [9]
Anandamide	15	2.3	Mouse neuroblastoma 10,000g pellet [10]
Anandamide	6.9	0.95	Mouse neuroblastoma microsomes [10]
Anandamide	3.4	2.2	Rat brain membranes [7]
Anandamide	60	0.37	Porcine brain partially purified amidohydrolase [8]
Anandamide	30	3.3	Bovine brain homogenate [5]
Oleoyl amide	2.2 ± 0.9	5.8 ± 0.8 (6)	Rabbit brain 100,000g microsomes‡
Oleoyl amide	5.3 ± 0.7	1.0 ± 0.8 (8)	Rabbit brain 10,000g cytosol‡
Oleoyl amide	9.0	0.94	Mouse neuroblastoma 10,000g pellet [10]
Oleoyl amide	14.4	0.34	Mouse neuroblastoma microsomes [10]

† Nmoles ligand hydrolysed per minute per milligram of protein.

‡ Initial rates of hydrolysis were measured by fluorescence displacement and converted to units of nmoles of fatty acid released per minute by using the appropriate calibration curve. The data were fitted to a hyperbolic equation to determine the apparent K_M and V_{max} values (number of data points).

parent) and V_{max} (apparent) values are shown in Table 1. Although it is difficult to compare kinetic parameters from different tissue fractions, the kinetic parameters obtained with this described fluorescence displacement method are equivalent to values obtained by other laboratories (Table 1). To validate the production of arachidonic acid and ethanolamine from anandamide, an incubated sample was analysed by electrospray ionisation mass spectrometry. A value of approximately 0.5 nmol arachidonic acid released per minute per milligram of protein was obtained by using this method, which correlates very well with the value obtained in Table 1 (0.96 nmol/min/mg protein).

In conclusion, a simple fluorescence assay has been described that allows rapid measurement of enzymatic acyl ethanolamide hydrolysis. Although only anandamide and oleoyl amide were used to validate the displacement assay, other long-chain acyl ethanolamides, such as palmitoyl ethanolamide, could also be used because long-chain fatty acids are ligands for liver FABP [11]. This method is comparable to other methods that use radiolabelled ligands and TLC or HPLC but has distinct advantages because it is relatively inexpensive and fast.

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References

1. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R, Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258:** 1946–1949, 1992.
2. Vogel Z, Barg J, Levy R, Saya D, Heldman E and Mechoulam R, Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J Neurochem* **61:** 352–355, 1993.
3. Cravatt BF, Prosperogarcia O, Siuzdak G, Gilula NB, Henriksen SJ, Boger DL, and Lerner RA, Chemical characterization of a family of brain lipids that induce sleep. *Science* **268:** 1506–1509, 1995.
4. Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC and Piomelli D, Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372:** 686–691, 1994.
5. Omeir RL, Chin S, Hong Y, Ahern DG and Deutsch DG, Arachidonoyl ethanolamide [1,2-^{14}C] as a substrate for anandamide amidase. *Life Sci* **56:** 1999–2005, 1995.
6. Campbell ID and Dwek RA, *Biological Spectroscopy*, pp. 91–125, The Benjamin/Cummings Publishing Company, London, 1993.
7. Hillard CJ, Wilkison DM, Edgemond WS and Campbell WB, Characterization of the kinetics and distribution of *N*-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim Biophys Acta* **1257:** 249–256, 1995.
8. Ueda N, Kurahashi Y, Yamamoto S and Tokunaga T, Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem* **270:** 23823–23827, 1995.
9. Desarnaud F, Cadas H and Piomelli D, Anandamide amidohydrolase activity in rat brain microsomes. Identification and partial characterization. *J Biol Chem* **270:** 6030–6035, 1995.
10. Maurelli S, Bisogno T, Depetrocellis L, Diluccia A, Marino G and Di Marzo V, Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma "anandamide amidohydrolase." *FEBS Lett* **377:** 82–86, 1995.
11. Wilkinson TCI and Wilton DC, Studies on fatty acid-binding proteins. The binding properties of rat liver fatty acid-binding protein. *Biochem J* **247:** 485–488, 1987.
12. Wilton DC, A continuous fluorescence displacement assay for the measurement of phospholipase A_2 and other lipases that release long-chain fatty acids. *Biochem J* **266:** 435–439, 1990.
13. Wilton DC, Studies on fatty acid-binding proteins. The purification of rat liver fatty-acid-binding protein and the role of cysteine-69 in fatty acid binding. *Biochem J* **261:** 273–276, 1989.
14. Worrall AF, Evans C and Wilton DC, Synthesis of a gene for rat liver fatty-acid-binding protein and its expression in *Escherichia coli*. *Biochem J* **278:** 365–368, 1991.
15. Glatz JFC and Veerkamp JH, A radiochemical procedure for the assay of fatty acid binding by proteins. *Anal Biochem* **132:** 89–95, 1983.
16. Bradford M, A rapid and sensitive method for the quantitiation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
17. Deutsch DG and Chin SA, Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **46:** 791–796, 1993.
18. Wilkinson TCI and Wilton DC, Studies on fatty acid-binding proteins. The detection and quantification of the protein from rat liver by using a fluorescent fatty acid analogue. *Biochem J* **238:** 419–424, 1986.
19. Haughland RP, *Handbook of Fluorescent Probes and Research Chemicals*, pp. 249–259. Molecular Probes Inc., Eugene, OR, 1992.