Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 1241–1245
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Cyclooxygenation of the arachidonoyl side chain of 1-arachidonoylglycerol and related compounds block their ability to prevent anandamide and 2-oleoylglycerol metabolism by rat brain in vitro

Christopher J. Fowler *, Gunnar Tiger

Department of Pharmacology and Clinical Neuroscience, Umeå University, SE-90187 Umeå, Sweden

Received 24 November 2004; accepted 28 January 2005

Abstract

In the present study, the abilities of cyclooxygenated derivatives of 1-arachidonoylglycerol and related compounds to prevent the metabolism of [^{3}H]2-oleoylglycerol and [^{3}H]anandamide by cytosolic and membrane fractions, respectively, have been investigated. For each compound, nine concentrations (range 0.2–100 μM) were tested. 1-Arachidonoylglycerol inhibited the hydrolysis of [^{3}H]2-oleoylglycerol with a pI_{50} value of 5.17 ± 0.04 (maximum attainable inhibition 88%). In contrast, the 1-glyceryl esters of prostaglandin D_2, E_2 and $F_{2\alpha}$ were very weak inhibitors of this hydrolysis. Similarly, prostaglandin D_2, prostaglandin D_2 ethanolamide and prostaglandin D_2 serinol amide produced <20% inhibition of [^{3}H]2-oleoylglycerol metabolism at any concentration tested, in contrast to previous data with arachidonic acid, anandamide and arachidonoyl serinol which are all able to inhibit metabolism of this substrate under the assay conditions used here. A similar pattern was seen for all the compounds with respect to the inhibition of [^{3}H]anandamide hydrolysis by the membrane fractions. Thus, cyclooxygenation of the arachidonoyl side chain greatly reduces the ability of 1-arachidonoylglycerol and related compounds to inhibit the hydrolysis of [^{3}H]2-oleoylglycerol and [^{3}H]anandamide.

Keywords: Monoacylglycerol lipase; Fatty acid amide hydrolase; Anandamide; 2-Arachidonoylglycerol; Endocannabinoid; Cyclooxygenase

1. Introduction

Since the discovery of the cannabinoid receptors in the early 1990s and the subsequent identification of the endogenous cannabinoid (endocannabinoid) agonists anandamide (AEA, arachidonoylethanolamide) and 2-arachidonoylglycerol (2-AG), the biology of the endocannabinoid system has been the subject of considerable study see, e.g. [1,2]. Both AEA and 2-AG are rather short-lived in the body due to effective metabolic pathways and it has been suggested by several authors that compounds blocking the removal of endocannabinoids may be useful for the treatment of a number of disorders as divergent as pain, stroke and cancer [1–4]. The principal enzyme responsible for the metabolism of AEA is fatty acid amide hydrolase (FAAH), and recent work with both genetically modified mice [5,6] and selective enzyme inhibitors [7,8] have identified possible therapeutic areas such as anxiety disorders and inflammatory pain where FAAH inhibitors could be useful. It has also been suggested that FAAH inhibition may contribute to the actions of clinically employed agents such as propofol [9] and indomethacin [10] (for a recent review on the pharmacology of FAAH, see [11]).

2-AG is also a substrate for FAAH [12,13], but recent data would suggest that a more important metabolic pathway, at least in the brain, is via other hydrolytic enzymes of which the enzyme monoacylglycerol lipase (MAGL) is the most important [14–16]. Thus, whilst AEA is primarily metabolised in membrane fractions by FAAH, 2-AG (or its close homologue 2-oleoylglycerol, 2-OG) are hydrolysed by both membrane and cytosolic fractions in a manner with rather different pharmacological properties. For example,

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; 1-AG, 1-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; 2-OG, 2-oleoylglycerol

* Corresponding author. Tel.: +46 90 7851510; fax: +46 90 7852752.
E-mail address: cf@pharm.umu.se (C.J. Fowler).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.01.016

the hydrolysis of [^{3}H]2-OG in the cytosolic fractions is not affected by concentrations of URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) and arachidonoylglycine that completely inhibit the hydrolysis of [^{3}H]AEA by FAAH in the membrane fractions [7,17]. As an initial step, it would be useful to the scientific community to identify compounds that do the reverse, i.e., completely block the hydrolysis of 2-OG (and hence 2-AG) without affecting the hydrolysis of AEA. So far, investigations in this respect are mainly confined to analogues of 2-AG with head group alterations rather than changes in the acyl side chain [14,15,17,18] and no potent selective inhibitors (i.e., $IC_{50} \leq 1$ μM) have yet been identified.

In addition to the hydrolytic pathways described above, endocannabinoids can be metabolised by a cyclooxygenase-2 pathway [19]. In a recent study, it was reported that the cyclooxygenation of the acyl side chain of anandamide to give the prostaglandin D_2, E_2 and $F_{2\alpha}$ ethanolamides resulted in a loss of the ability to interact with FAAH [20]. It is not, however, known whether cyclooxygenation of the acyl side chain of arachidonoylglycerol produces a corresponding loss in their ability to inhibit 2-OG metabolism. This has been investigated in the present study. The regioisomer 1-AG has been used rather than 2-AG as the template molecule simply because the latter is rather unstable in biological solutions (and converts to 1-AG) [21,22], but the two compounds are equally efficacious as substrates for MAGL and FAAH [13,15].

2. Methods

2.1. Materials

Radiolabeled arachidonoylethanolamide [ethanolamine 1-^{3}H] ([^{3}H]AEA, 60 Ci mmol^{-1}) and 2-mono-oleoylglycerol [glycerol-1,2,3-^{3}H] ([^{3}H]2-OG, 20 Ci mmol^{-1}) were obtained from American Radiolabeled Chemicals, Inc. The compounds tested in the present study were all obtained from the Cayman Chemical Company. Their structures are shown in the figures to aid the reader. 1-Arachidonoylglycerol was dissolved in acetonitrile, and the other compounds were dissolved in ethanol. Solvent carrier concentrations were kept constant throughout the assays.

2.2. Assay of soluble [^{3}H]2-OG and membrane bound [^{3}H]AEA metabolism

Cerebella from adult Sprague–Dawley rats that had been obtained previously and stored frozen at −70 °C were thawed and homogenized at 4 °C in sodium phosphate buffer (50 mM, pH 8) containing 0.32 M sucrose. Homogenates were centrifuged at 100,000 × *g* for 60 min at 4 °C to give supernatants (“cytosol fractions”), which were collected. The pellets were suspended in sodium phosphate buffer (50 mM, pH 8) (“membrane fractions”). Samples were stored frozen in aliquots at −70 °C until used for assay. Protein concentration was determined [23], with bovine serum albumin as standard.

Assays of [^{3}H]2-OG and [^{3}H]AEA hydrolysis were essentially as described by Dinh et al. [14] and Omeir et al. [24] (and were the same as used in the study of Ghafouri et al. [17] although unfortunately in that study the assay concentration of fatty-acid free bovine serum albumin was not indicated). Briefly, aliquots (165 μl) of cytosol (1 μg/assay) or membrane (2 μg/assay) fractions in Tris–HCL buffer (10 mM, pH 7.2) containing 1 mM EDTA, unless otherwise stated, were added to glass tubes containing 10 μl of test compound. Blanks contained assay buffer instead of the cytosol or membrane fractions. Substrate (25 μl in 1% fatty acid-free bovine serum albumin, final substrate concentration 2 μM) was then added and the samples were incubated for 10 min at 37 °C. Reactions were stopped by the addition of 400 μl chloroform:methanol (1/1, v/v), vortex mixing the tubes twice and placing them on ice. The phases were separated by centrifugation (10 min, 2500 rpm) and aliquots (200 μl) of the methanol/buffer phase were taken and measured for tritium content by liquid scintillation spectroscopy with quench correction. Results were expressed as percent of controls and the pI_{50} values (and hence IC_{50} values) were calculated as described previously [17] using the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Inhibition of [^{3}H]2-OG and [^{3}H]AEA hydrolysis by 1-AG and its cyclooxygenated derivatives

The effects of 1-AG and its cyclooxygenated derivatives upon the hydrolysis of [^{3}H]2-OG and [^{3}H]AEA by the soluble and membrane fractions, respectively, are shown in Fig. 1. The data for 1-AG, prostaglandin D_2-1-glyceryl ester and prostaglandin $F_{2\alpha}$-1-glyceryl ester were obtained concomitantly. As expected, 1-AG inhibited the hydrolysis of [^{3}H]2-OG with an pI_{50} value of 5.17 ± 0.04 (max attainable inhibition 88 ± 3%), corresponding to an IC_{50} value of 7 μM. This is in reasonable agreement with our previous study, where complete inhibition was seen, with an IC_{50} value of 17 μM [17]. In that study, 1-AG was a rather poor inhibitor of [^{3}H]AEA hydrolysis, producing only 42% inhibition at a concentration of 100 μM. However, it was argued that the presence of MAGL in the membrane fractions would metabolise the compound and thereby reduce its observed effects upon FAAH, a result supported by the ability of the α-methyl derivative of 1-AG to inhibit [^{3}H]AEA metabolism with an IC_{50} value of 33 μM [17]. In the present study, we used a lower protein concentration per assay (2 μg versus 4 μg), thereby reducing the rate of MAGL-catalysed 1-AG metabolism and

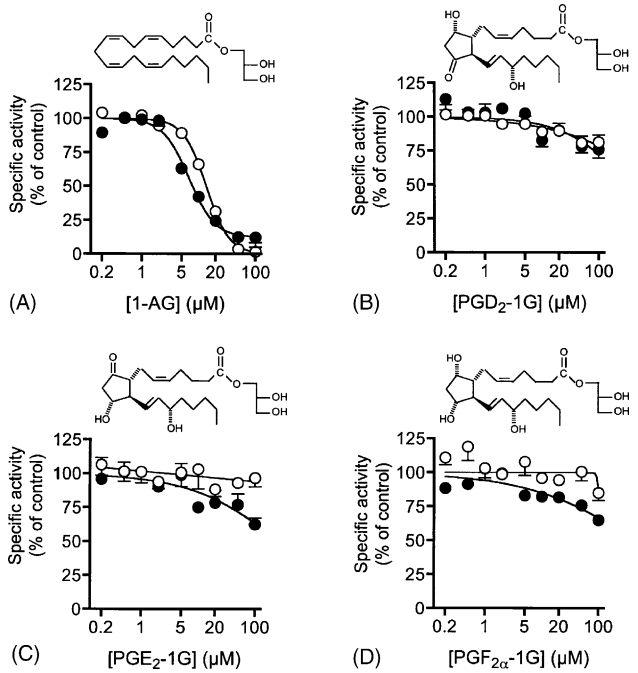

Fig. 1. Effects of 1-AG (Panel A), prostaglandin D_2-1-glyceryl ester (Panel B), prostaglandin E_2-1-glyceryl ester (Panel C) and prostaglandin $F_{2\alpha}$-1-glyceryl ester (Panel D) upon the hydrolysis of [^{3}H]2-OG (●) and [^{3}H]AEA (○) by cytosolic and membrane fractions, respectively. Data are mean ± S.E.M., where $n = 3$. The substrate concentrations were 2 μM.

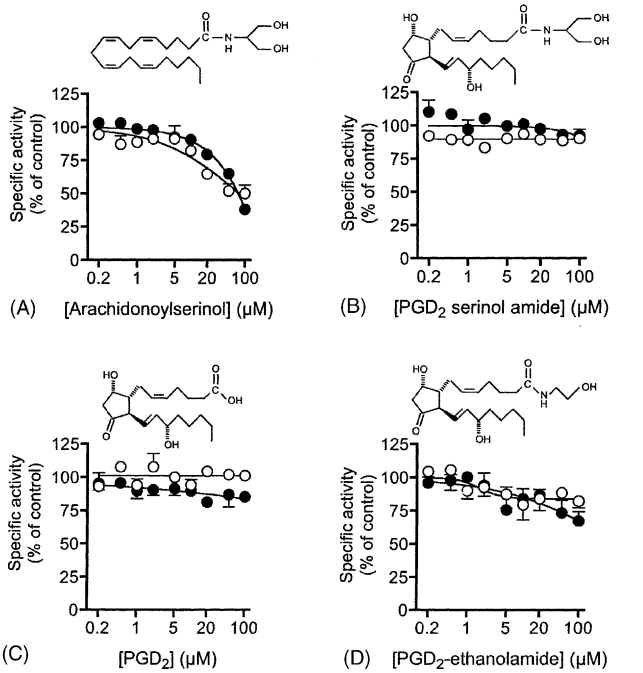

Fig. 2. Effects of arachidonoyl serinol (Panel A), prostaglandin D_2 serinol amide (Panel B), prostaglandin D_2 (Panel C) and prostaglandin D_2 ethanolamide (Panel D) upon the hydrolysis of [^{3}H]2-OG (●) and [^{3}H]AEA (○) by cytosolic and membrane fractions, respectively. Data are mean ± S.E.M., where $n = 3$, with the exception for the 1 and 100 μM concentrations of arachidonoyl serinol and [^{3}H]AEA as substrate, where $n = 2$. The substrate concentrations were 2 μM. The pI_{50} and IC_{50} values for arachidonoyl serinol calculated from the data shown in Panel A have been reported elsewhere [17].

found a complete inhibition of [^{3}H]AEA metabolism (pI_{50} value 4.86 ± 0.02, IC_{50} value 14 μM). A second factor that may be involved in the differences in potency for 1-AG is the lipophilicity of this compound, and hence its solubility at high concentrations in an aqueous environment. This problem was recently highlighted for non-radioactive AEA as an inhibitor of [^{3}H]AEA metabolism, where there was evidence of limited (and variable) solubility at high concentrations [17].

In contrast to the inhibitory effect of 1-AG, the three cyclooxygenase derivatives of this compound had very weak effects upon either the metabolism of [^{3}H]2-OG or [^{3}H]AEA by the soluble and membrane fractions, respectively (Fig. 1, Panels B–D). Thus, the highest concentrations tested (100 μM) of prostaglandins D_2, E_2 and $F_{2\alpha}$ glyceryl esters gave 24 ± 6, 37 ± 5 and 35 ± 2% inhibition of [^{3}H]2-OG metabolism and 18 ± 5, 3 ± 6 and 15 ± 5% inhibition of [^{3}H]AEA metabolism (mean ± S.E.M., $n = 3$).

3.2. Inhibition of [^{3}H]2-OG and [^{3}H]AEA hydrolysis by the cyclooxygenase derivatives of arachidonoyl serinol, arachidonic acid and AEA

The data shown in Fig. 1, indicate that cyclooxygenation of the arachidonoyl side chain of 1-AG reduces the ability of the compounds to inhibit [^{3}H]2-OG and [^{3}H]AEA metabolism by the soluble and membrane fractions, respectively. In order to determine whether this was also seen for other arachidonoyl compounds, prostaglandin D_2 derivatives of arachidonoyl serinol, arachidonic acid and AEA were investigated (Fig. 2, Panels B–D). Arachidonoyl serinol and arachidonic acid inhibit [^{3}H]2-OG and [^{3}H]AEA metabolism with IC_{50} values of ~70 μM [17], (see Fig. 2A for the original data for arachidonoyl serinol that were used to calculate the IC_{50} values in that study). However, the prostaglandin D_2 derivatives of these two compounds were without any effect on the metabolism of [^{3}H]2-OG and [^{3}H]AEA by the soluble and membrane fractions, respectively, over the concentration range tested (Figs. 2B and C). AEA will of course inhibit the metabolism of [^{3}H]AEA (IC_{50} value ~4 μM) but can also inhibit [^{3}H]2-OG metabolism at higher concentrations (IC_{50} value 60 μM) [17]. Once again, the prostaglandin D_2 analogue of AEA was a very weak inhibitor of [^{3}H]2-OG and [^{3}H]AEA metabolism, producing 33 ± 7 and 18 ± 5% inhibition, respectively, at the highest concentration tested (100 μM) (Fig. 2D), the latter result being entirely consistent with the recent literature [20].

4. Discussion

The aim of the present study was very simple, namely to determine the effect of cyclooxygenation of the arachidonoyl side chain of 1-AG and related compounds upon their abilities to inhibit the metabolism of [^{3}H]2-OG and

[^{3}H]AEA by soluble and membrane fractions, respectively. The data indicate clearly that the affinities of the compounds for the enzymes involved in these processes are reduced upon cyclooxygenation of the acyl side chain. This would suggest that a strategy based upon using prostaglandin analogues in the search for selective inhibitors of 2-OG (and hence 2-AG) metabolism relative to AEA metabolism is unlikely to be fruitful.

In addition to providing new data on the pharmacology of 2-OG metabolism, the present results also have some physiological implications. It is now established that cyclooxygenase-2 can metabolise both AEA and 2-AG see [19] and that inhibition of this enzyme can potentiate endocannabinoid actions [25]. Cyclooxygenase-2 derived metabolites of AEA and 2-AG have also been shown to possess biological activity, including activation of protein kinase C and peroxisome proliferator-activated receptor γ, as well as effects upon the contractility of a variety of smooth muscle preparations [20,26–28]. Given that the cyclooxygenase-2 pathway may be of some importance for endocannabinoid metabolism and function, it could be hypothesised that the cyclooxygenase-2 derived prostaglandin ethanolamides and glyceryl esters might act as feedback regulators of the other endocannabinoid metabolising enzymes. The present study would suggest that this is unlikely to be the case.

Acknowledgements

The authors are grateful to Britt Jacobsson for her excellent technical assistance. This study was supported by grants from the Swedish Research Council (Grant no. 12158, medicine), Konung Gustav V's and Drottning Victorias Foundation, Gun and Bertil Stohne's Foundation, Stiftelsen för Gamla Tjänarinnor and the Research Funds of the Medical Faculty, Umeå University.

References

[1] Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Dis 2004;3:771–84.
[2] Piomelli D, Giuffrida A, Calignano A, Rodriguez de Fonseca F. The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol Sci 2000;21:218–24.
[3] Fowler CJ. Plant-derived synthetic and endogenous cannabinoids as neuroprotective agents. Non-psychoactive cannabinoids, "entourage" compounds and inhibitors of *N*-acyl ethanolamine breakdown as therapeutic strategies to avoid pyschotropic effects. Brain Res Rev 2003;41:26–43.
[4] Bifulco M, Laezza C, Valenti M, Ligresti A, Portella G, Di Marzo V. A new strategy to block tumor growth by inhibiting endocannabinoid inactivation. FASEB J 2004;18:1606–8.
[5] Lichtman AH, Shelton CC, Advani T, Cravatt BF. Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. Pain 2004;109:319–27.
[6] Cravatt BF, Saghatelian A, Hawkins EG, Clement AB, Bracey MH, Lichtman AH. Functional disassociation of the central and peripheral fatty acid amide signaling systems. Proc Natl Acad Sci USA 2004; 101:10821–6.
[7] Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, et al. Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 2003;9:76–81.
[8] Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL, et al. Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. J Pharmacol Exp Ther 2004;311: 441–8.
[9] Patel S, Wohlfeil ER, Rademacher DJ, Carrier EJ, Perry LJ, Kundu A, et al. The general anesthetic propofol increases brain *N*-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase. Br J Pharmacol 2003;139:1005–13.
[10] Gühring H, Hamza M, Sergejeva M, Ates M, Kotalla CE, Ledent C, et al. A role for endocannabinoids in indomethacin-induced spinal antinociception. Eur J Pharmacol 2002;454:153–63.
[11] Fowler CJ. Metabolism of the endocannabinoids anandamide and 2-arachidonoyl glycerol, a review, with emphasis on the pharmacology of fatty acid amide hydrolase, a possible target for the treatment of neurodegenerative diseases and pain. Curr Med Chem Cent Nerv Sys Agents 2004;4:161–74.
[12] Goparaju SK, Ueda N, Yamaguchi H, Yamamoto S. Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. FEBS Lett 1998;422:69–73.
[13] Di Marzo V, Bisogno T, Sugiura T, Melck D, De Petrocellis L. The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. Biochem J 1998;331:15–9.
[14] Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci USA 2002;99:10819–24.
[15] Saario SM, Savinainen JR, Laitinen JT, Järvinen T, Niemi R. Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in rat cerebellar membranes. Biochem Pharmacol 2004;67:1381–7.
[16] Dinh TP, Kathuria S, Piomelli D. RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. Mol Pharmacol 2004;66:1260–4.
[17] Ghafouri N, Tiger G, Razdan RK, Mahadevan A, Pertwee RG, Martin BR, et al. Inhibition of monoacylglycerol lipase and fatty acid amide hydrolase by analogues of 2-arachidonoylglycerol. Br J Pharmacol 2004;143:774–84.
[18] Cascio MG, Bisogno T, Matias I, De Petrocellis L, Orlando P, Di Marzo V. Enzymes for 2-AG biosynthesis and metabolism in cell lines, and their pharmacological inhibition. In: 2004 Symposium on the Cannabinoids, International Cannabinoid Research Society. 2004.110.
[19] Kozak KR, Marnett AB. Oxidative metabolism of endocannabinoids. Prostaglandins Leukot Essent Fatty Acids 2002;66:211–20.
[20] Matias I, Chen J, De Petrocellis L, Bisogno T, Ligresti A, Fezza F, et al. Prostaglandin ethanolamides (prostamides): in vitro pharmacology and metabolism. J Pharmacol Exp Ther 2004;309:745–57.
[21] Savinainen JR, Järvinen T, Laine K, Laitinen J. Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB_1 receptor-dependent G-protein activation in rat cerebellar membranes. Br J Pharmacol 2001;134:664–72.
[22] Rouzer CA, Ghebreselasie K, Marnett LJ. Chemical stability of 2-arachidonoyl-glycerol under biological conditions. Chem Phys Lipids 2002;119:69–82.
[23] Harrington CR. Lowry protein assay containing sodium dodecyl sulfate in microtiter plates for protein determination on fractions from brain tissue. Anal Biochem 1990;186:285–7.
[24] Omeir R, Chin S, Hong Y, Ahern D, Deutsch D. Arachidonyl ethanolamide-[1,2-^{14}C] as a substrate for anandamide amidase. Life Sci 1995;56:1999–2005.

[25] Kim J, Alger BE. Inhibition of cyclooxygenase-2 potentiates retrograde endocannabinoid effects in hippocampus. Nat Neurosci 2004; 7:697–8.

[26] Ross RA, Craib SJ, Stevenson LA, Pertwee RG, Henderson A, Toole J, et al. Pharmacological characterization of the anandamide cyclooxygenase metabolite: prostaglandin E_2 ethanolamide. J Pharmacol Exp Ther 2002;301:900–7.

[27] Rockwell CE, Kaminski NE. A cyclooxygenase metabolite of anandamide causes inhihbition of interleukin-2 secretion in murine splenocytes. J Pharmacol Exp Ther 2004;311:683–90.

[28] Nirodi CS, Crews BC, Kozak KR, Morrow JD, Marnett LJ. The glyceryl ester of prostaglandin E2 mobilizes calcium and activates signal transduction in RAW264.7 cells. Proc Natl Acad Sci USA 2004; 101:1840–5.