The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide

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Abstract Anandamide is an endogenous ligand for Δ^9-tetrahydrocannabinol (THC) receptors. Incubation of cultured cells with anandamide or THC causes increased arachidonic acid release and eicosanoid biosynthesis. Here we demonstrate that the MAP kinase signal transduction pathway contributes to this response. Treatment of WI-38 fibroblasts with anandamide causes increased MAP kinase activity and increased phosphorylation of the arachidonate-specific cytoplasmic phospholipase A_2 ($cPLA_2$). Significantly, MAP kinase phosphorylates and activates $cPLA_2$ [Lin, et al., *Cell*, 72 (1993) 269–278]. The MAP kinase signal transduction pathway may therefore mediate the effects of anadamide on $cPLA_2$ activation and arachidonic acid release.

Key words: Anandamide; Cannabinoid; Phosopholipase A_2; MAP kinase

1. Introduction

The major active constituent of marijuana, Δ^9-Tetrahydrocannabinol (THC), and its derivatives have potential clinical use because these drugs cause analgesia, immunosuppression, and reduced nausea in addition to psychoactive effects [1,2]. The actions of THC are thought to be mediated, in part, by specific receptors that are expressed on the surface of responsive cells. Two proteins with seven transmembrane-spanning domains typical of G protein-coupled receptors have been identified as putative THC receptors by molecular cloning [3,4]. The existence of receptors for the drug THC suggests that a physiologically relevant ligand may exist. This possibility has recently been confirmed by the identification of arachidonylethanolamide (*anandamide*) as an endogenous cannabimimetic that binds and activates THC receptors [5]. It is therefore likely that anandamide is an important regulatory molecule. However, the physiological role of anandamide has not been established.

The results of previous studies have indicated that the mechanism of signal transduction by cannabinoids may be mediated, in part, by cAMP accumulation and an inhibition of N-type Ca^{2+} channels [6–12]. However, it has also been proposed that several actions of cannabinoids may result from the increased level of tissue eicosanoids that occur in humans and experimental animals treated with cannabinoids [2]. These include the effect of THC on time estimation, heart-rate acceleration, and the subjective 'high' in humans; the induction of catalepsy in mice; and the hypotensive response in dogs [2]. The increased level of eicosanoids has been attributed to the ability of cannabinoids to mobilize arachidonic acid from cellular phospholipid storage sites by activation of phospholipase A_2 (PLA_2), whereupon it is rapidly metabolized into various eicosanoids [13,14]. Although evidence has been presented indicating that the brain-type THC receptor does not mediate arachidonic acid release when expressed in Chinese hamster ovary cells [10], THC-induced release of arachidonic acid is receptor-mediated in mouse peritoneal cells [15].

Recently, a hormonally-responsive, arachidonic acid-specific, cytoplasmic PLA_2 ($cPLA_2$) has been identified by molecular cloning [16,17]. We have demonstrated that $cPLA_2$ is phosphorylated and activated by MAP kinase [18]. The identification of $cPLA_2$ as a target of the MAP kinase signal transduction pathway [19] suggests a possible mechanism of signaling by cannabinoids. Here, we demonstrate that the endogenous cannabinoid anandamide activates $cPLA_2$ and eicosanoid production via the MAP kinase signal transduction pathway.

2. Materials and methods

2.1. Tissue culture

WI-38 human fetal lung fibroblasts (American Type Culture Collection, passage 17–21) were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL).

2.2. Arachidonic acid release

The release of [^{3}H]arachidonic acid (and metabolites) was measured as described by Lin et al. [18] Briefly, the cells were seeded into 12-well dishes, grown to confluency and then incubated for 18 h with 0.5 μCi of [^{3}H]arachidonic acid (Dupont-NEN) in 0.5 ml of serum-free modified Eagle's medium. The cell monolayers were rinsed three times with Krebs-Ringer HEPES-buffered saline containing 0.1% bovine serum albumin (KRH/BSA) and pre-incubated for 30 min at 37°C. The cells were then incubated with KRH/BSA (0.5 ml) together with the indicated concentrations of agonists (5 μl) for 10 min. The medium was removed and the released radioactivity was measured by liquid scintillation counting. PGE_2 synthesis was measured after 30 min of incubation by radio-immunoassay [13].

2.3. MAP kinase assay

WI-38 lung fibroblasts were seeded into 6-well plates, grown to confluency and then incubated in serum-free medium for 18 h. The cells were then incubated with vehicle, anandamide, or PMA. The cell monolayers were washed with KRH and harvested in hypotonic lysis buffer (25 mM HEPES, pH 7.4, 5 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na-orthovanadate, 10 μg/ml leupeptin). The cell extracts were clarified by centrifugation for 10 min at 12,000 × *g*. The MAP kinase activity in the supernantant fraction was measured using a synthetic peptide substrate based on the sequence surrounding the EGF receptor phosphorylation site Thr-669 [20]. MAP

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kinase activity was also examined using an in-gel kinase assay with myelin basic protein (MBP) as the substrate [21].

2.4. Western blotting

Immunoblot analyses were performed after electrophoretic transfer onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The membranes were probed consecutively with the monoclonal anti-phosphotyrosine antibody PY-20 (ICN) and the monoclonal ERK1/2 antibody #107 [22]. Immunecomplexes were detected by enhanced chemiluminescence (Amersham International PLC).

3. Results

We investigated the effect of anandamide on arachidonic acid release using WI-38 human fetal lung fibroblasts pre-labeled with [^{3}H]arachidonic acid. Treatment with anandamide caused a concentration-dependent increase in the release of [^{3}H]arachidonic acid and its metabolites (Fig. 1A). A similar increase was not observed when the cells were incubated with the anandamide analog ethylarachidonate or arachidonic acid (Fig. 1C). Arachidonic acid is a precursor for eicosanoid biosynthesis. Previous studies have demonstrated that prostaglandin E_2 (PGE_2) is the major eicosanoid synthesized by WI-38 cells [13]. We therefore examined the effect of anandamide on PGE_2 biosynthesis. It was observed that anandamide caused a concentration-dependent increase in PGE_2 production by the cells (Fig. 1B). Together, these observations establish that anandamide increases de novo eicosanoid biosynthesis.

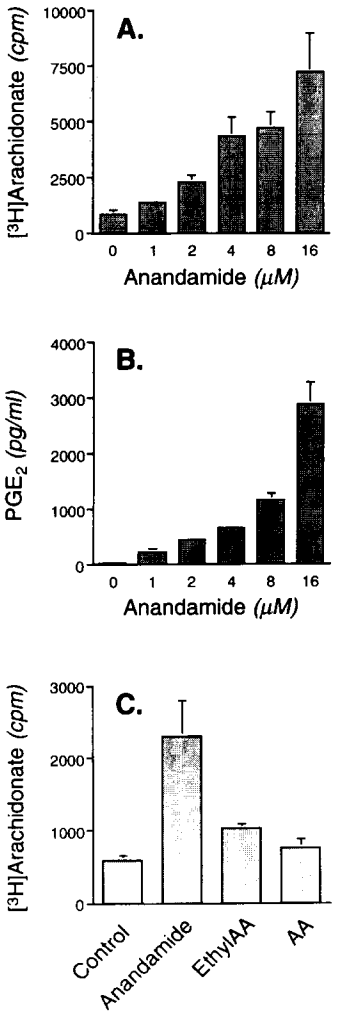

Fig. 1. Anandamide stimulates the production of arachidonic acid and prostaglandin E_2 (PGE_2) in human WI-38 lung fibroblasts. The fibroblasts were incubated for 10 min with vehicle (DMSO) or with increasing concentrations of anandamide for 10 min prior to measurement of the release of arachidonic acid (A) and PGE_2 (B). The effect of treatment (10 min) with vehicle (DMSO), or with 8 μM anandamide, ethylarachidonate (EthylAA) or arachidonic acid (AA) on [^{3}H]arachidonic acid release is shown (C). The data are presented as the mean ± S.D. of triplicate determinations.

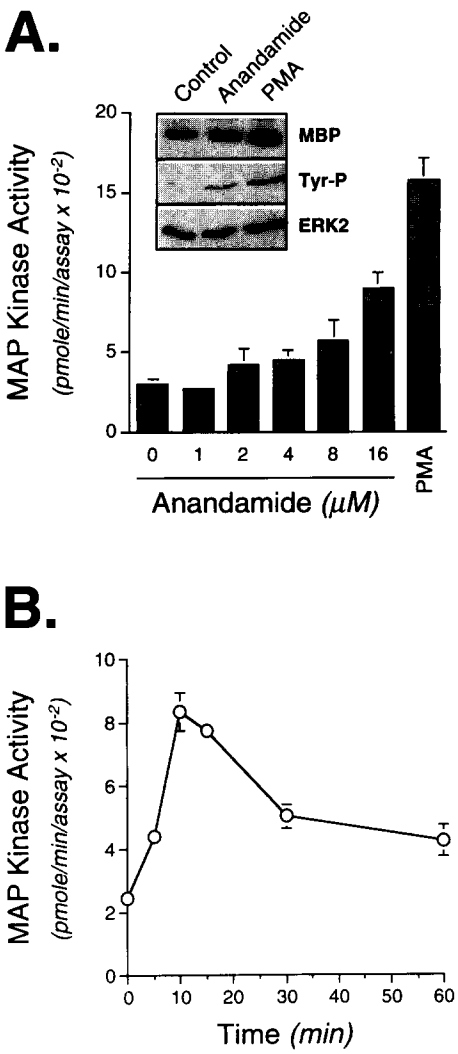

Fig. 2. Anandamide stimulates MAP kinase activity. WI-38 fibroblasts were incubated for 10 min with vehicle (DMSO), the indicated concentrations of anandamide or 100 nM PMA (A) or with 8 μM anandamide for the indicated times (B). MAP kinase activity was measured using a synthetic peptide substrate. The data are presented as the mean ± S.D. of triplicate determinations. Insert: the cells were incubated for 10 min with vehicle (DMSO), 8 μM anandamide, or 100 nM PMA. MBP, MAP kinase activity was measured using an in-gel kinase assay with myelin basic protein (MBP) as a substrate. Tyr-P, Western blot analysis using an anti-phosphotyrosine antibody. ERK2, Western blot analysis using an anti-ERK1/2 antibody.

The effect of anandamide on MAP kinase activity was examined using a synthetic peptide substrate based on the sequence surrounding the EGF receptor phosphorylation site Thr-669

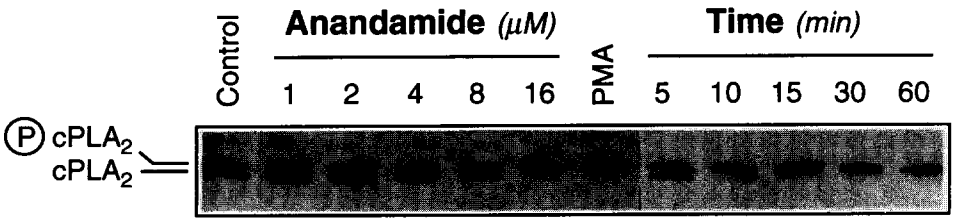

Fig. 3. Anandamide activates $cPLA_2$. The electrophoretic mobility shift associated with $cPLA_2$ activation was examined by Western blot analysis using an anti-$cPLA_2$ antibody. The anandamide dose response was investigated by incubation of WI-38 fibroblasts with increasing concentrations of anandamide or with 100 nM PMA for 10 min. The time course was examined by treatment of the cells with 8 μM anandamide for the indicated times.

[20]. Treatment of WI-38 fibroblasts with increasing doses of anandamide caused MAP kinase activation (Fig. 2A). At the highest concentration of anandamide tested, MAP kinase activity was approximately 60% of that found with 100 nM phorbol myristate acetate (PMA). MAP kinase activation by anandamide was rapid with maximal activity at 10 min followed by a decline at later times (Fig. 2B). The stimulation of MAP kinase by anandamide was confirmed using an in-gel kinase assay with myelin basic protein as a substrate (Fig. 2A, inset). Immunoblot analysis demonstrated that the major MAP kinase isoform expressed by WI-38 cells is ERK2 and that anandamide increased ERK2 tyrosine phosphorylation (Fig. 2A, inset). As tyrosine phosphorylation is required for ERK2 activation [19,23], the observation of increased ERK2 tyrosine phosphorylation in anandamide-treated cells provides strong additional evidence for MAP kinase activation.

Anandamide is rapidly metabolized by a membrane-associated amidase [24]. A significant question is therefore whether anandamide or a metabolite of anandamide mediates the effects on arachidonic acid release and MAP kinase activation (Figs. 1 and 2). To examine this question we investigated the effect of phenylmethylsulfonyl fluoride, a potent inhibitor of the amidase that degrades anandamide [24]. Treatment of WI-38 cells with 1 mM phenylmethylsulfonyl fluoride was found to potentiate the stimulation of arachidonic acid release, PGE_2 production, and MAP kinase activity by anandamide (data not shown). This observation is consistent with the hypothesis that there is a direct signaling role for anandamide.

One substrate for activated MAP kinase is $cPLA_2$ [19]. We have demonstrated that MAP kinase phosphorylates $cPLA_2$ at a single site, Ser-505, and that this phosphorylation increases the enzymatic activity of $cPLA_2$ and results in a marked electrophoretic mobility-shift during SDS-PAGE [18]. The stimulation of both MAP kinase and arachidonic acid release (Figs. 1 and 2) suggests that anandamide activates $cPLA_2$. We therefore examined $cPLA_2$ in cells treated with anandamide or PMA. Fig. 3 shows that anandamide caused a time- and concentration-dependent redistribution of $cPLA_2$ from the basal state to the phosphorylated and activated form that exhibits reduced electrophoretic mobility [18]. The conversion of $cPLA_2$ to the activated form caused by anandamide was similar to that caused by 100 nM PMA (Fig. 3).

The cloned THC receptors have seven transmembrane-spanning domains typical of G protein-coupled receptors [3,4]. Activation of MAP kinase and increased arachidonic acid release caused by receptors coupled to heterotrimeric G proteins has been shown in previous studies to be inhibited by pertussis toxin [25–28]. We therefore investigated the effect of pertussis toxin on WI-38 fibroblasts. No significant effect of pertussis toxin on EGF-stimulated MAP kinase activation was detected (data not shown). However, treatment of the cells with pertussis toxin was found to block the effect of anandamide on MAP kinase activation and arachidonic acid release (Fig. 4). This sensitivity to pertussis toxin has also been observed for the regulation of cAMP levels and N-type calcium channels by anandamide [6,10]. Together, these data are consistent with the hypothesis that the cellular response to anandamide is mediated by a G protein-coupled receptor.

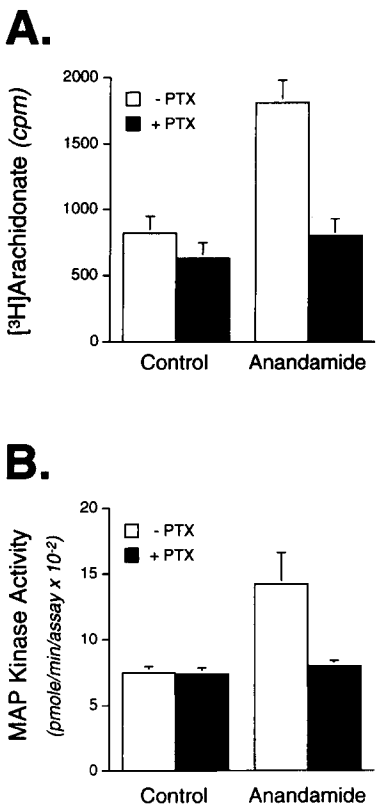

Fig. 4. A G protein pathway mediates the activation of MAP kinase and $cPLA_2$ by anandamide. The effect of pertussis toxin-treatment on anandamide-stimulated arachidonic acid release (A) and MAP kinase activity (B) was examined. Confluent WI-38 cells were incubated without (−PTX) or with (+PTX) 200 ng/ml pertussis toxin (Calbiochem-Novabiochem Corp.) for 18 h. The cells were then incubated for 10 min with vehicle (Control) or 8 μM anandamide. The data are presented as the mean ± S.D. of triplicate determinations.

4. Discussion

4.1. Signal transduction by anandamide mediated by a G protein-coupled receptor

Two cannabinoid receptors have been molecularly cloned. The amino acid sequence of these receptors indicates that they are members of the seven transmembrane -spanning domain class of G protein-coupled receptors [3,4]. Interestingly, the tissue distribution of these receptors is distinct for each subtype (highest expression in the brain [4] and spleen [3]). Additional receptor sub-types expressed in other tissues remain to be identified.

Examination of the function of the brain receptor [4] expressed in heterologous cells indicates that it does not mediate the effects of anandamide on arachidonic acid metabolism [10,11]. The effect of anandamide on arachidonic acid metabolism in this particular model could therefore be mediated by a non-receptor mechanism. However, the finding that pertussis toxin inhibits anandamide-stimulated arachidonic acid release indicates that a G protein-coupled receptor is likely to mediate the effects of anandamide in human WI-38 fibroblasts (Fig. 4). Further studies are required to molecularly characterize the receptor(s) that mediates the effects of anadamide on MAP kinase activation and arachidonic acid release in human WI-38 fetal lung fibroblasts.

4.2. Anandamide activates the MAP kinase signal transduction pathway

The newly described endogenous cannabimimetic anandamide is likely to be a physiologically significant regulatory molecule [5]. The finding that anandamide activates the MAP kinase signal transduction pathway is consistent with this hypothesis (Fig. 2). Importantly, MAP kinase activation provides a mechanism for signal transduction from cell surface THC receptors to targets located within multiple cellular compartments, including the cell surface, the cytoskeleton, and the nucleus [19]. MAP kinase activation may therefore represent a primary response of cells to anandamide. Indeed, it is likely that the MAP kinase signal transduction pathway mediates the effects of anandamide on arachidonic acid metabolism (Fig. 3).

Incubation of cultured cells with anandamide causes increased arachidonic acid release. The mechanism of this response can be accounted for by the activation of one or more PLA_2 isoforms [29]. One of these isoforms, $cPLA_2$ [16,17], is activated by the MAP kinase signal transduction pathway [18]. Significantly, the addition of anandamide to WI-38 fibroblasts causes activation of MAP kinase (Fig. 2) and $cPLA_2$ (Fig. 3). Together, these observations suggest that MAP kinase and $cPLA_2$ mediate, at least in part, the effect of anandamide to cause increased arachidonic acid release.

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References

[1] Pertwee, R.G. (1988) Pharmacol. Ther. 36, 189–261.
[2] Burstein, S.H. (1992) in: Marijuana/Cannabinoids (L. Murphy and A. Bartke eds.) pp. 73–91, CRC, Boca Raton, FL.
[3] Munro, S., Thomas, K.L. and Abu-Shaar, M. (1993) Nature 365, 61–65.
[4] Matsuda, L.A., Lolait, S.J., Brownstein, B.J., Young, A.C. and Bonner, T.I. (1990) Nature 346, 561–564.
[5] 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.
[6] Mackie, K., Devane, W.A. and Hille, B. (1993) Mol. Pharmacol. 44, 498–503.
[7] Mackie, K. and Hille, B. (1992) Proc. Natl. Acad. Sci. USA 89, 3825–3829.
[8] Caufield, M.P. and Brown, D.A. (1992) Br. J. Pharmacol. 106, 231–232.
[9] Vogel, Z., Barg, J., Levy, R., Saya, D., Heldman, E. and Mechoulam, R. (1993) J. Neurochem. 61, 352–355.
[10] 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.
[11] Felder, C.C., Veluz, J.S., Williams, H.L., Briley, E.M. and Matsuda, L.A. (1992) Mol. Pharmacol. 42, 838–845.
[12] Howlett, A.C., Evans, D.H. and Houston, D.B. (1992) in: Marijuana/Cannabinoids, (L. Murphy and A. Bartke eds.) pp. 35–72, CRC, Boca Raton, FL.
[13] Burstein, S.H., Hunter, S.A., Sedor, C. and Shulman, S. (1982) Biochem. Pharmacol. 31, 2361–2365.
[14] Audette, C.A., Burstein, S.H., Doyle, S.A. and Hunter, S.A. (1991) Pharmacol. Biochem. Behav. 40, 559–563.
[15] Burstein, S., Budrow, J., Bedatis, M., Hunter, S.A. and Subramanian, A. (1994) Biochem. Pharmacol. (in press).
[16] Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Miona, N. and Knopf, J.L. (1991) Cell 65, 1043–1051.
[17] Sharp, J.D., White, D.L., Chioux, X.G., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoskin, J., Skatrud, P.L., Kang, L.H., Robert, E.F. and Kramer, E.M. (1991) J. Biol. Chem. 266, 14850–14853.
[18] Lin, L.-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R.J. (1993) Cell 72, 269–278.
[19] Davis, R.J. (1993) J. Biol. Chem. 268, 14553–14556.
[20] Gonzalez, F.A., Raden, D.L. and Davis, R.J. (1991) J. Biol. Chem. 266, 22159–22163.
[21] Northwood, I.C., Gonzalez, F.A., Wartmann, M., Raden, D.L. and Davis, R.J. (1991) J. Biol. Chem. 266, 15266–15276.
[22] Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R.J. and Kelly, K. (1994) Nature 367, 651–654.
[23] Cobb, M.H., Boulton, T.G. and Robbins, D.J. (1991) Cell Regul. 2, 965–978.
[24] Deutsch, D.G. and Chin, S.A. (1993) Biochem. Pharmacol. 46, 791–796.
[25] Gupta, S.K., Diez, E., Heasley, L.E., Osawa, S. and Johnson, G.L. (1990) Science 249, 662–666.
[26] Gupta, S.K., Gallego, C., Johnson, G.L. and Heasley, L.E. (1992) J. Biol. Chem. 267, 7987–7990.
[27] Anderson, N.G., Kilgour, E. and Sturgill, T. (1991) J. Biol. Chem. 266, 10131–10135.
[28] Howe, L.R. and Marshall, C.J. (1993) J. Biol. Chem. 268, 20717–20720.
[29] Dennis, E.A. (1994) J. Biol. Chem. 269, 13057–13060.