Cannabinoid-receptor-independent cell signalling by *N*-acylethanolamines

Evgueni V. BERDYSHEV*, Patricia C. SCHMID*, Randy J. KREBSBACH*, Cecilia J. HILLARD†, Chuanshu HUANG*, Nanyue CHEN*, Zigang DONG* and Harald H. O. SCHMID*[1]

*The Hormel Institute, University of Minnesota, 801 16th Avenue N. E., Austin, MN 55912, U.S.A., and †Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.

Anandamide and other polyunsaturated *N*-acylethanolamines (NAEs) exert biological activity by binding to cannabinoid receptors. These receptors are linked to $G_{i/o}$ proteins and their activation leads to extracellular-signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAP kinase) activation, inhibition of cAMP-dependent signalling and complex changes in the expression of various genes. Saturated and monounsaturated NAEs cannot bind to cannabinoid receptors and may thus mediate cell signalling through other targets. Here we report that both saturated/monounsaturated NAEs and anandamide ($20:4_{n-6}$ NAE) stimulate cannabinoid-receptor-independent ERK phosphorylation and activator protein-1 (AP-1)-dependent transcriptional activity in mouse epidermal JB6 cells. Using a clone of JB6 P^+ cells with an AP-1 collagen–luciferase reporter construct, we found that 16:0, $18:1_{n-9}$, $18:1_{n-7}$, $18:2_{n-6}$ and $20:4_{n-6}$ NAEs stimulated AP-1-dependent transcriptional activity up to 2-fold, with maximal stimulation at approx. 10–15 μM. Higher NAE concentrations had toxic effects mediated by alterations in mitochondrial energy metabolism. The AP-1 stimulation appeared to be mediated by ERK but not JNK or p38 signalling pathways, because all NAEs stimulated ERK1/ERK2 phosphorylation without having any effect on JNK or p38 kinases. Also, overexpression of dominant negative ERK1/ERK2 kinases completely abolished NAE-induced AP-1 activation. In contrast with $18:1_{n-9}$ NAE and anandamide, the cannabinoid receptor agonist WIN 55,212-2 did not stimulate AP-1 activity and inhibited ERK phosphorylation. The NAE-mediated effects were not attenuated by pertussis toxin and appeared to be NAE-specific, as a close structural analogue, oleyl alcohol, failed to induce ERK phosphorylation. The data support our hypothesis that the major saturated and monounsaturated NAEs are signalling molecules acting through intracellular targets without participation of cannabinoid receptors.

Key words: anandamide, AP-1 activation, MAP kinase.

INTRODUCTION

It has long been known that long-chain *N*-acylethanolamines (NAEs) and their precursor phospholipids, *N*-acyl phosphatidylethanolamines (*N*-acyl PEs; which may consist of diacyl as well as alkylacyl- and alk-1-enylacyl subclasses), accumulate in mammalian cells and tissues as the result of injury (reviewed in [1,2]). More recently, it has become generally accepted that they occur as trace constituents in virtually all mammalian cells. NAE biosynthesis proceeds by the 'transacylation-phosphodiesterase pathway', a reaction sequence that starts with the generation of *N*-acyl PE by acyl transfer from the *sn*-1 position of a glycerophospholipid to the amino group of PE [3] and is followed by phosphodiesterase cleavage [4] of *N*-acyl PE to yield NAE and phosphatidic acid. NAE is catabolized by an amidase [5], which has been purified and cloned ([6,7], reviewed in [8]). We have recently shown that the constitutive N-acyl turnover via *N*-acyl PE and NAE can be substantially faster than the O-acyl turnover of cellular glycerophospholipids [9].

With the discovery that *N*-arachidonoylethanolamine ($20:4_{n-6}$ NAE, 'anandamide') is present in pig brain and binds to the central (CB1) cannabinoid receptor [10], research on the generation and function of polyunsaturated NAEs as second messengers has become of great interest (reviewed in [11–13]). It is now well established that anandamide and some other polyunsaturated NAEs bind to and activate both central (CB1) and peripheral (CB2) cannabinoid receptors, whereas saturated and monounsaturated NAEs are cannabinoid-receptor-inactive. However, saturated and monounsaturated congeners represent the predominant NAEs in most cells, whereas anandamide amounts to less than 1% of total NAE in brain ([14], but see [14a],[15]) and rarely exceeds 5% in other cells and tissues. Because in most glycerophospholipids only a small percentage of arachidonic acid is esterified at the *sn*-1 position, and because anandamide biosynthesis proceeds by the transacylation-phosphodiesterase pathway [11–13,15], the resulting amide-linked fatty acids of both *N*-acyl PE and NAE are primarily saturated and monounsaturated. There is also no evidence that agonist-induced NAE turnover can be selective for anandamide. It is therefore difficult to rationalize the generation and degradation of anandamide as a mechanism of cell signalling, as much larger amounts of receptor-inactive molecules are generated and degraded simultaneously by the same pathway. One explanation is the so-called 'entourage effect' hypothesis,

Abbreviations used: NAE, *N*-acylethanolamine; *N*-acyl PE, *N*-acyl phosphatidylethanolamine; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; WIN 55,212-2, *R*(+)-{2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl}-(1-naphthalenyl)methanone mesylate; SR141716A, *N*-piperidino-5-(5-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; SR144528, *N*-{1(*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl}-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt; AP-1, activator protein-1; ERK, extracellular-signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEM, minimum essential medium; FBS, fetal bovine serum; CP55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol.

[1] To whom correspondence should be addressed (e-mail hoschmid@smig.net).

which postulates that the majority of receptor-inactive NAEs protect the messenger molecule, anandamide, from degradation by amidase activity and thereby prolong and enhance its biological activity [16]. Another possibility would be selective transport of anandamide through the plasma membrane (reviewed in [17]) in order to reach cannabinoid receptors located at the cell surface. This view is supported by very recent evidence that cellular transport of *N*-palmitoylethanolamine is pharmacologically distinguishable from anandamide uptake [18].

We have proposed [19] that generation of the predominant saturated and monounsaturated NAEs could represent a cell-signalling system analogous to the sphingolipid signalling system by interacting either directly or indirectly with the targets of the ceramide signalling pathways [20]. This hypothesis is based on our observation that the levels of both *N*-acyl PEs and NAEs are tightly regulated in intact cells but increase in response to certain stress conditions [19]. These results were obtained with cultured mouse epidermal JB6 P^+ cells that are well characterized with respect to their cell-signalling systems and are extensively used for the study of neoplastic transformation [21–23]. We have shown that they contain *N*-acyl PE and NAE at levels of about 180 and 60 pmol/μmol of lipid phosphorus, respectively, and that arachidonic acid amounts to 0.9 % of the amide-linked fatty acids in *N*-acyl PE and 4.5 % in NAE [19]. Because agonist-induced neoplastic transformation in JB6 P^+ cells involves specific cellular responses such as the transcriptional activation of activator protein-1 (AP-1) and nuclear factor κB [21–23], it was of interest to test the ability of NAEs to modulate the activity of these nuclear factors.

Here we report for the first time that the predominant, saturated and monounsaturated, NAEs can elicit biological effects through targets other than cannabinoid receptors and that these effects include enhanced AP-1 transcriptional activity mediated by the extracellular-signal-regulated protein kinase (ERK) mitogen-activated protein kinase (MAP kinase) pathway.

EXPERIMENTAL

Materials

Anandamide was from Matreya (Pleasant Gap, PA, U.S.A.). *N*-palmitoylethanolamine (16:0 NAE), *N*-oleoylethanolamine ($18:1_{n-9}$ NAE), *N*-vaccenoylethanolamine ($18:1_{n-7}$ NAE) and *N*-linoleoylethanolamine ($18:2_{n-6}$ NAE) were synthesized and purified as described in [14,14a]. *N*-Piperidino-5-(5-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A) and *N*-{1(*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl}-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl]-pyrazole-3-carboxamide (SR144528) were a generous gift from Sanofi Recherche (Montpellier, France). *R*(+)-{2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl}-(1-naphthalenyl)methanone mesylate (WIN 55,212-2) and oleyl ($18:1_{n-9}$) alcohol were obtained from Sigma-RBI (Natick, MA, U.S.A.). All compounds were initially dissolved in DMSO, the final concentration of which in the incubation medium did not exceed 0.1 %.

Cell cultures

JB6 P^+ mouse epidermal cells (Cl41), P^- cells (Cl30.7b) and their subclones stably transfected with AP-1 luciferase reporter (Cl41 AP-1 $mass_1$, Cl30.7b AP-1 $mass_3$) [24], as well as Cl30.7b AP-1 $mass_3$ cells stably co-transfected with a wild-type ERK2 (Cl30.7b MAPK WT $mass_2$) [24], were cultured in monolayers at 37 °C in Eagle's minimum essential medium (MEM) containing 5 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5 % CO_2. Each dish contained approx. 10^6 cells, representing a volume of about 40–50 μl.

Assay for AP-1 activity

Confluent monolayers of stably transfected JB6 P^+ or P^- cells were trypsinized, and 5×10^3 cells suspended in 200 μl of MEM/5 % FBS were added to each well of a 96-well plate. Cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were starved 24–48 h later by culturing in 100 μl of MEM/0.1 % FBS for 12–18 h before treatment. After treatment in a total volume of 200 μl for 8–24 h, cells were extracted with lysis buffer and luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI, U.S.A.) and a Monolight 2010 luminometer. The results are expressed as relative AP-1 activity or relative luminescence units (RLU) [25,26]. Relative AP-1 activity is presented as the luciferase activity in treated cells relative to that in cells exposed to 0.1 % DMSO in MEM (control treatment).

MAP kinase analysis

ERK, c-Jun N-terminal kinase (JNK) and p38 kinase activation was determined by immunoblotting with phospho-specific antibodies (all antibodies were from Cell Signalling Technology, Beverly, MA, U.S.A.). Cell extracts were analysed for ERKs using antibodies against ERK1 and ERK2. Phosphorylated ERK1/ERK2 were detected with phospho-specific antibodies raised against p44 and p42 ERK1/ERK2 phosphorylated at threonine-202 and tyrosine-204. Phosphorylated JNK1/JNK2 were detected with phospho-specific antibodies raised against p46 and p54 JNK1/JNK2 phosphorylated at threonine-183 and tyrosine-185. Phosphorylated p38 kinase was detected with phospho-specific antibodies raised against p38 kinase phosphorylated at threonine-180 and tyrosine-182. Antibodies bound to proteins were detected by chemiluminescence (ECF™ substrate; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) using a Storm 840 imaging system (Molecular Dynamics).

Phase-contrast microscopy

Intact cells were observed during the course of experiments using a Leica DMIRB inverted microscope with an NPlan 40× objective. Pictures were acquired digitally using a LEI-750D CCD camera and Image-Pro Plus image-analysis software.

Determination of mitochondrion-mediated NAE toxicity

JB6 P^+ Cl41 cells (2×10^3) in 100 μl of MEM/5 % FBS were seeded into each well of a 96-well plate and allowed to grow for 24 h. Then cells were treated with different NAE concentrations or DMSO (0.1 % final concentration) in a total volume of 200 μl for 3–8 h. Before (1 h) termination of treatment, 20 μl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt; Promega Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega] were added to each well, and the NAD(P)H-dependent transformation of soluble tetrazolium MTS salt into its formazan product was evaluated spectrophotometrically at 492 nm using a Multiscan MS microplate reader (Labsystems). Data were expressed as a percentage of MTS transformation by control cells.

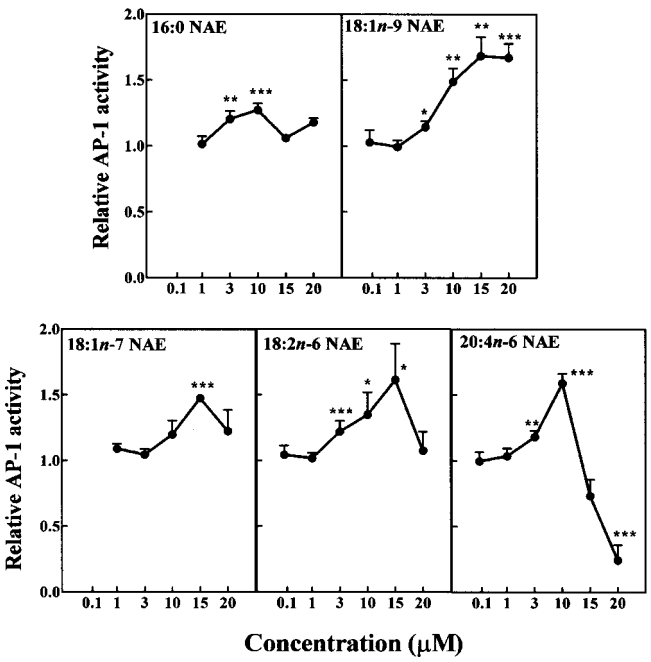

Figure 1 Activation of AP-1-dependent transcriptional activity by NAEs

JB6 P^+ Cl41 AP-1 $mass_1$ cells (5×10^3) were seeded into a 96-well plate and grown in MEM/5% FBS for 48 h. Then the medium was replaced with MEM/0.1% FBS, and cells were starved for 12 h before stimulation with different NAEs. DMSO (0.1% final concentration) was used as a control. After 18 h of stimulation, cells were lysed and luciferase activity was measured using the Promega Luciferase Assay Kit. Data represent relative AP-1-dependent luciferase activity as compared with corresponding controls (means ± S.E.M. of at least three independent experiments each done in triplicate); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Radioligand binding assay

The expression of cannabinoid receptors in JB6 P^+ Cl41 cells was determined using a radioligand-binding assay with [^{3}H]CP55,940 {(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol} as a probe and WIN 55,212-2 (CB1/CB2 receptor ligand), SR141716A (CB1 receptor antagonist) and SR144528 (CB2 receptor antagonist) as competitive ligands. JB6 P^+ cell membranes or rat forebrain membranes were obtained by centrifugation of cell lysates in TME buffer (50 mM Tris/HCl/3 mM $MgSO_4$/1 mM EDTA, pH 7.4) at 10000 ***g*** for 20 min. Membranes (75–100 μg of protein) were incubated with 1.5–3.2 nM [^{3}H]CP55,940 (120 Ci/mmol; NEN DuPont, Boston, MA, U.S.A.) for 60 min at 30 °C in TME buffer containing 1 mg/ml fatty acid-free BSA. The total volume of the incubation was 250 μl. Incubates also contained 5 μM of one of three unlabelled ligands (diluted from 1 mg/ml DMSO stocks) or an equivalent amount of DMSO (total binding). Specific binding was calculated as the difference between total binding and the binding in the presence of the unlabelled ligands. The binding was terminated by filtration through glass-fibre filters presoaked in polyethyleneimine followed by washing (three times by 3 ml) with wash buffer (incubation buffer without BSA).

RESULTS

Both saturated and unsaturated NAEs stimulate AP-1-dependent transcriptional activity

AP-1-dependent transcriptional activity was measured in JB6 P^+ Cl41 AP-1 $mass_1$ cells that had been transfected with a luciferase reporter gene [24]. In order to decrease background AP-1 activity, cells were initially serum deprived for 12–18 h and then stimulated with 1–20 μM NAEs or DMSO. As shown in Figure 1, all NAEs, at concentrations of 3–10 μM, stimulated AP-1-dependent transcriptional activity 1.5–2-fold compared with DMSO-treated controls. This activity was even higher at 15 μM, except for anandamide ($20{:}4_{n-6}$ NAE), which caused inhibition of AP-1 activity at this or higher levels. The observed stimulation or inhibition of AP-1 activity was accompanied by morphological changes in JB6 P^+ cells (Figure 2). Concentrations of NAEs which stimulated AP-1-dependent transcriptional activity caused early cell elongation that disappeared with time. In contrast, higher toxic NAE concentrations caused cell elongation with

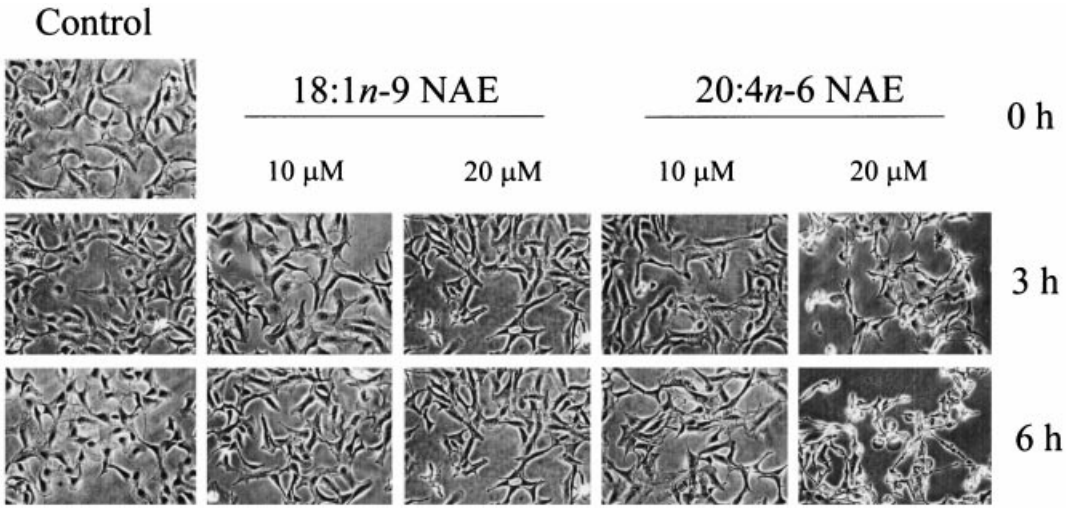

Figure 2 Morphological changes in JB6 P^+ Cl41 cells stimulated with $18{:}1_{n-9}$ NAE and anandamide

JB6 P^+ Cl41 cells (1×10^5) were seeded into a 6-well plate and grown for 24 h in MEM/5% FBS. Then the medium was replaced with MEM/0.1% FBS 4 h before treatment with the indicated concentrations of compounds in MEM/0.1% FBS. DMSO (0.1% final concentration) was used as a control. Phase-contrast micrographs were taken 3 and 6 h after the beginning of cell treatment.

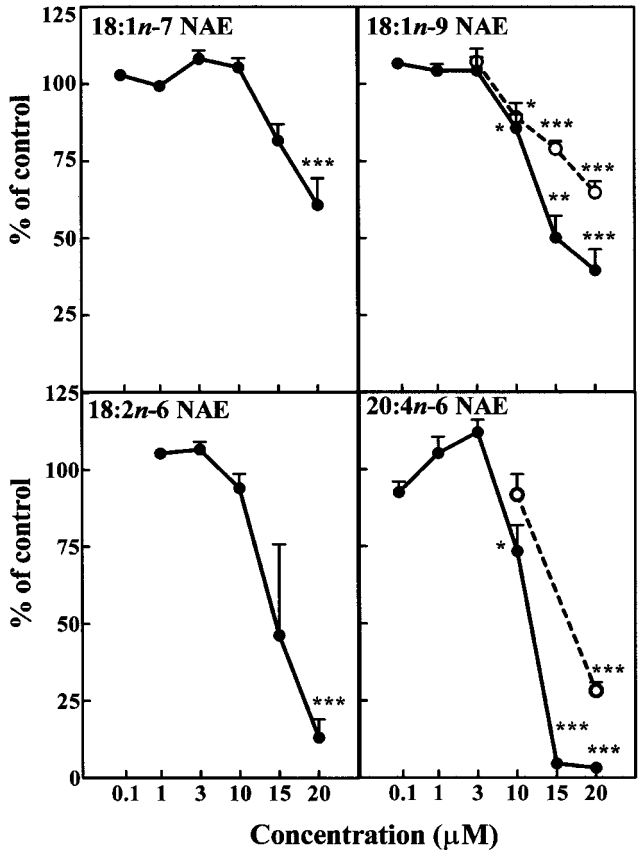

Figure 3 Mitochondrial toxicity induced by NAEs

JB6 P^+ Cl41 cells (2×10^3) were seeded into a 96-well plate, grown for 24 h and then treated with different concentrations of NAEs or DMSO (0.1% final concentration) as a control. Then, 2 h (○) or 7 h (●) after the beginning of cell treatment, 20 μl of MTS reagent (Promega Cell Titer 96® Aqueous One Solution Cell Proliferation Assay) was added and the degree of NAD(P)H-dependent MTS transformation into its soluble formazan product was evaluated spectrophotometrically at 492 nm 1 h later. Data are expressed as percentages of corresponding controls (means ± S.E.M. from two to four independent experiments, each done in quadruplicate); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

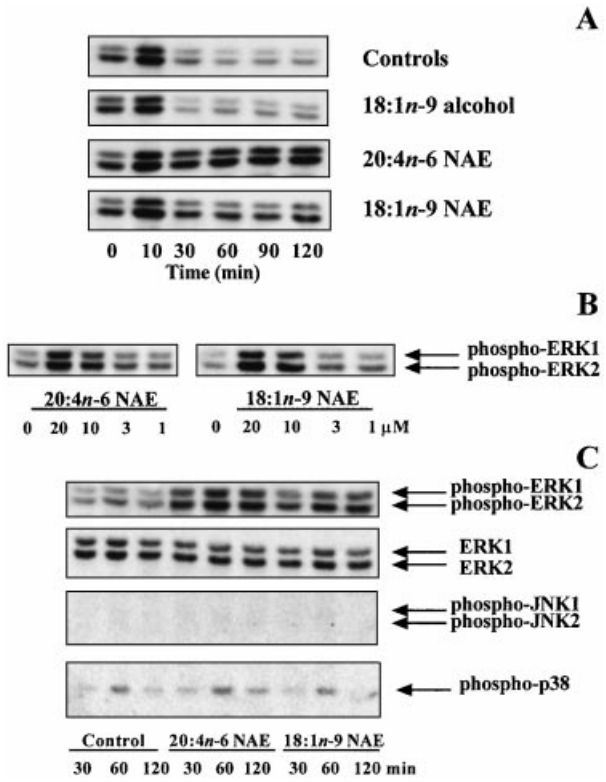

Figure 4 Stimulation of ERK, but not JNK or p38 kinase, phosphorylation by NAEs in JB6 P^+ cells

(**A**) Time-dependent ERK phosphorylation by anandamide and $18:1_{n-9}$ NAE, but not by oleyl alcohol. JB6 P^+ Cl41 cells (1×10^5) were seeded into a 6-well plate. After 48 h the medium was replaced with MEM/0.1% FBS and cells were starved for 12–24 h before treatment with 15 μM NAEs or oleyl alcohol in MEM/0.1% FBS for 10–120 min. For the 0 min time point, 0.1% DMSO solution was added to the cells and cells were immediately lysed. Cell lysates were processed for phospho-ERK determination by Western blotting with antibodies specific for phosphorylated ERK1/ERK2. (**B**) Dose-dependent ERK phosphorylation by anandamide and $18:1_{n-9}$ NAE. JB6 P^+ Cl41 cells were grown as described for (**A**). After 48 h the medium was replaced with MEM/0.1% FBS and cells were starved for 12–24 h before treatment with NAEs in MEM/0.1% FBS. After stimulation (90 min) cells were processed for phospho-ERK determination by Western blotting with antibodies specific for phosphorylated ERK1/ERK2. (**C**) Time-dependent phosphorylation of ERK but not JNK or p38 kinases by anandamide and $18:1_{n-9}$ NAE. Cells were grown and treated for 30–120 min with 10 μM NAEs as described in (**A**). Cells were lysed and processed for determination of ERKs, phosphorylated ERKs, JNKs and p38 kinase as described in the Experimental section. Non-phosphorylated ERKs were detected as a control for total protein level.

subsequent cell shrinking, detachment, rounding and final decomposition.

NAE toxicity is linked to alterations in energy production

Because NAE production and degradation occur in both endoplasmic reticulum and mitochondria [1], it was logical to propose that mitochondria could serve as physiological targets and that their functions might be altered by high NAE concentrations. To determine possible effects of NAE on mitochondrial energy metabolism, we measured the transformation of MTS to its formazan product, which depends on mitochondrial NAD(P)H production. When used in short-term experiments, this NAD(P)H-dependent MTS transformation reflects the ability of mitochondria to generate and provide NAD(P)H for oxidative phosphorylation and ATP production [27]. We found that cellular NAD(P)H production begins to decrease at about 10 μM NAE and is almost completely blocked by anandamide and $18:2_{n-6}$ NAE at 15 and 20 μM, respectively (Figure 3). This effect was seen not only after long-term (8 h) NAE treatment but also as early as 3 h after incubating cells in the presence of NAE, well before cell shrinking and decomposition occurred.

NAEs stimulate ERK, but not JNK or p38 kinases

AP-1 activation is induced by a variety of stimuli and is mediated by different MAP kinase signalling pathways [28,29]. To determine whether any one of these pathways is involved in NAE-induced signalling, we checked the phosphorylation of ERK, JNK and p38 kinases using an immunoblot approach. As shown in Figure 4(A), serum-deprived JB6 P^+ cells exhibited a very high sensitivity to the vehicle (0.1% DMSO) in terms of immediate ERK activation. However, this vehicle-induced activation disappeared within the first 30 min, and during that time NAE did not exert significant additional stimulation. At time periods of 30 min or longer, both anandamide and $18:1_{n-9}$ NAE stimulated ERK1/ERK2 phosphorylation. The effect of both NAEs was dose-dependent (Figure 4B) and reached a maximum 1–2 h after the beginning of NAE treatment (Figures 4A and 4C). The effect appeared to be specific for NAE, because a close structural

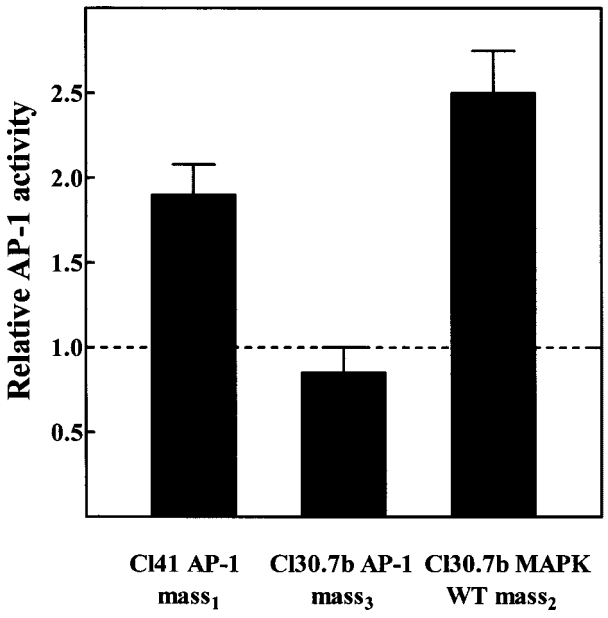

Figure 5 The importance of ERK for the induction of AP-1 activity in JB6 cells

5×10^3 JB6 P^+ Cl41 AP-1 $mass_1$, CL 30.7b AP-1 $mass_3$ (ERK-depleted) and Cl30.7b MAPK WT $mass_2$ (ERK2-transfected) cells were seeded into a 96-well plate, grown and treated with 10 μM anandamide as described in the legend for Figure 1. Data represent relative AP-1 activity as compared with corresponding controls (means $\pm$ S.E.M. from three independent experiments, each done in triplicate).

analogue, oleyl ($18:1_{n-9}$) alcohol, did not induce ERK activation at the same concentration and time periods (Figure 4A). Neither anandamide nor $18:1_{n-9}$ NAE induced JNK or p38 kinase phosphorylation (Figure 4C), suggesting that NAE-induced AP-1 activation is mediated by the ERK MAP kinase pathway rather than JNK or p38 kinases.

To confirm that AP-1 activation is mediated by the ERK MAP kinase pathway, we investigated the ability of anandamide to stimulate AP-1-dependent transcriptional activity in JB6 P^- cells transfected with AP-1 luciferase reporter (Cl30.7b AP-1 $mass_3$ [24]). JB6 P^- cells have less than half the level of ERKs that JB6 P^+ cells have [24], and this was expected to decrease the effect of NAE as compared with JB6 P^+ cells. In fact, the stimulatory effect of anandamide on AP-1 activation was completely abolished in JB6 P^- cells (Figure 5). Moreover, the overexpression of a wild-type ERK2 protein in JB6 P^- cells significantly increased AP-1 activity in comparison with Cl30.7b AP-1 $mass_3$ cells (Figure 5), confirming once again the importance of ERK MAP kinase signalling pathway for NAE-induced AP-1 activation.

JB6 P^+ cells express CB1 cannabinoid receptors

In spite of the well-established fact that saturated/mono-unsaturated NAEs cannot bind to and activate cannabinoid receptors [30], the participation of these receptors in anandamide-induced AP-1 activation could not be ruled out, because anandamide is a potent ligand for both CB1 and CB2 receptors [31]. Also, it was shown that cannabinoid-receptor activation may lead to ERK activation [32–35]. Therefore it was important to know whether or not JB6 cells express cannabinoid receptors. When we compared the binding of the radioactive CB1/CB2 receptor ligand [^{3}H]CP55,940 to membranes prepared from JB6 P^+ Cl41 cells in the presence or absence of specific antagonists for

Table 1 Specific binding of [^{3}H]CP55,940 in the presence of different cannabinoid-receptor ligands

JB6 P^+ Cl41 cell membranes or rat forebrain membranes (100 μg of protein/tube) were incubated with 1.5–3.2 nM [^{3}H]CP55,940 (120 Ci/mmol) for 60 min at 30 °C in TME buffer (pH 7.4) containing 1 mg/ml fatty acid-free BSA. Incubations also contained 5 μM of one of three unlabelled ligands or an equivalent amount of DMSO (total binding). The binding was terminated by filtration through glass-fibre filters presoaked in polyethyleneimine, followed by washing three times with 3 ml of TME buffer without BSA. Specific binding was calculated as the difference between total binding and the binding in the presence of the unlabelled ligands.

	Specific binding (fmol/mg of protein)		
	WIN 55,212-2	SR141716A	SR144528
Mouse brain*	581 $\pm$ 95	560 $\pm$ 187	354 $\pm$ 202
JB6 P^+ cells†	106 $\pm$ 22	99 $\pm$ 11	63 $\pm$ 2

* Means $\pm$ S.E.M. from two independent experiments.
† Means $\pm$ S.E.M. from three independent experiments.

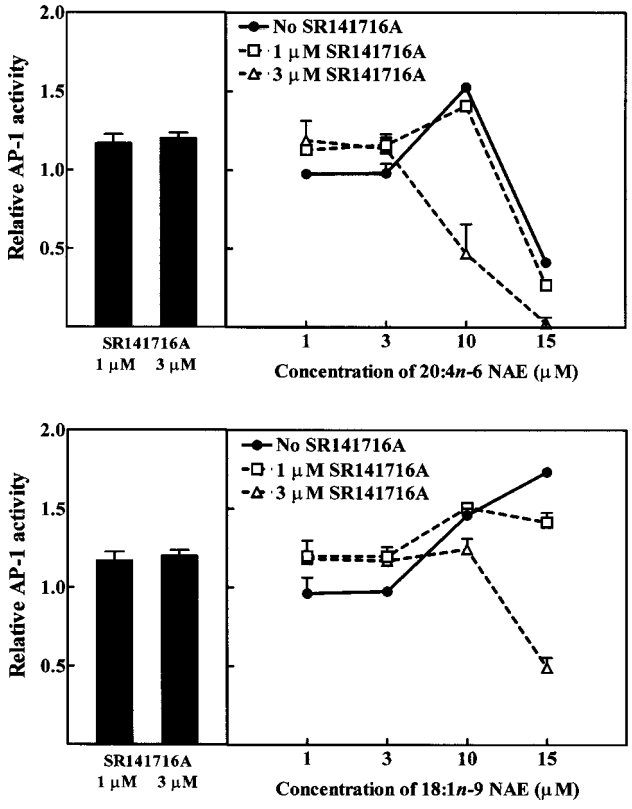

Figure 6 The effect of SR141716A on anandamide- and $18:1_{n-9}$ NAE-induced AP-1 activation in JB6 P^+ cells

JB6 P^+ Cl41 AP-1 $mass_1$ cells (5×10^3) were seeded into a 96-well plate, grown and prepared for treatment as described in the legend for Figure 1. Cells were treated with SR141716A for 15 min, then stimulated with anandamide or $18:1_{n-9}$ NAE in MEM/0.1% FBS for 18 h. Luciferase activity was measured using the Promega Luciferase Assay Kit. Data represent relative AP-1 activity as compared with controls (0.2% DMSO). Data from one of three representative experiments, each done in quadruplicate, are presented (means $\pm$ S.E.M.).

CB1 and CB2 receptors (SR141716A and SR144528, respectively), we observed the inhibition of [^{3}H]CP55,940 binding by SR141716A, and very low, if any, inhibition of its binding by SR144528 (Table 1). This indicates a predominant expression of

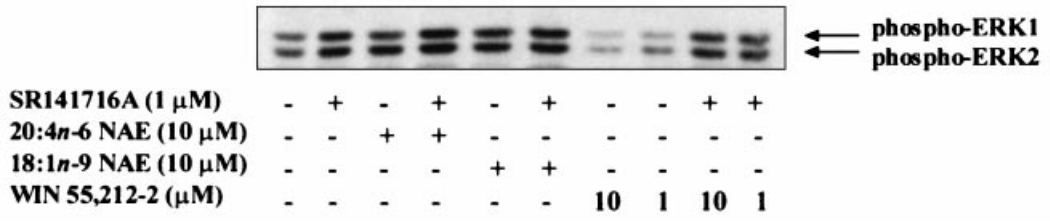

Figure 7 SR141716A activates ERK together with NAEs and antagonizes the effect of WIN 55,212-2 in JB6 P$^+$ cells

JB6 P$^+$ Cl41 cells (1×10^5) in a 6-well plate were grown for 24 h. Then cells were starved for 24 h by replacing the medium with MEM/0.1% FBS and finally treated for 90 min with 1 μM SR141716A, 10 μM anandamide, 10 μM 18:1$_{n-9}$ NAE or 1–10 μM WIN 55,212-2, separately or in combination with SR141716A. DMSO (0.1% final concentration) was used as a control. After treatment, cells were lysed and processed for determination of phosphorylated ERKs as described in the Experimental section.

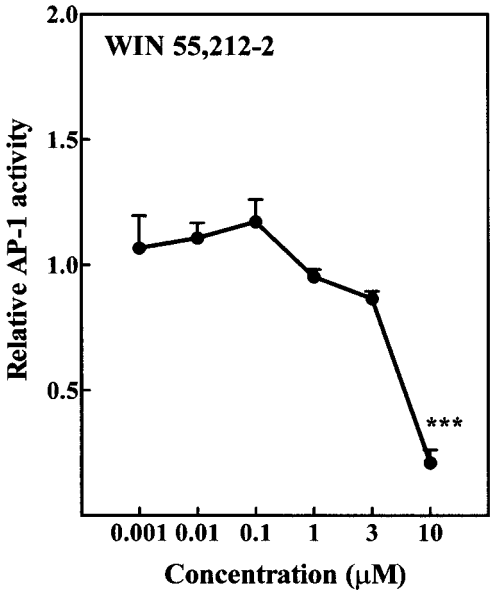

Figure 8 Effect of cannabinoid WIN 55,212-2 on AP-1 activity in JB6 P$^+$ cells

JB6 P$^+$ Cl41 AP-1 mass$_1$ cells (5×10^3) were seeded into a 96-well plate, grown and prepared for treatment as described in the legend for Figure 1. Cells were treated with WIN 55,212-2 or DMSO (0.1% final concentration) in MEM/0.1% FBS for 18 h, and luciferase activity was measured by Promega Luciferase Assay Kit. Data represent relative AP-1 activity as compared with control (means ± S.E.M. from three independent experiments, each done in triplicate).

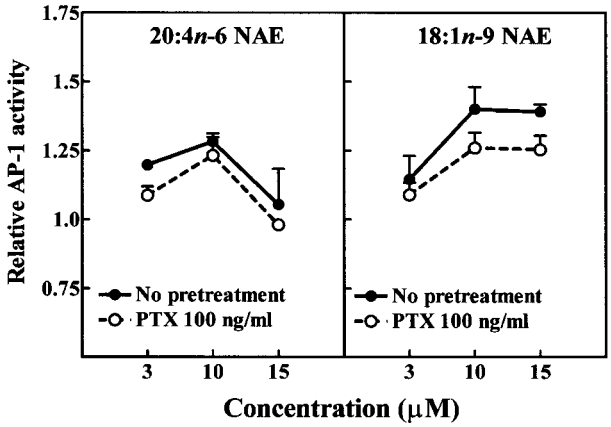

Figure 9 Pertussis toxin cannot block NAE-induced AP-1 activation in JB6 P$^+$ cells

JB6 P$^+$ Cl41 AP-1 mass$_1$ cells (5×10^3) were grown in a 96-well plate for 48 h. Then cells were starved by changing the medium to 0.1 ml of MEM/0.1% FBS, with or without 100 ng/ml pertussis toxin (PTX), for 12 h and then cells were stimulated with anandamide or 18:1$_{n-9}$ NAE for 18 h. Luciferase activity was measured by Promega Luciferase Assay Kit. Data represent relative AP-1 activity as compared with controls (0.1% DMSO). Data for one of three representative experiments, each done in triplicate, are presented (means ± S.E.M.).

CB1 receptors by JB6 P$^+$ cells and is consistent with the fact that CB2 receptors are primarily present in cells of the immune system (reviewed in [36]). However, JB6 P$^+$ cells expressed only one-fifth of the CB1 receptors present in mouse forebrain membranes, as seen from the data on the specific binding of [^{3}H]CP55,940 to these two preparations. We therefore investigated to what extent cannabinoid receptors mediate anandamide-induced ERK phosphorylation and AP-1 activation and mitochondrial toxicity.

Cannabinoid receptors do not mediate NAE-induced AP-1 activation, ERK phosphorylation or mitochondrial toxicity

One approach to establish the potential role of cannabinoid receptors in mediating NAE-induced effects was to use the CB1 receptor antagonist SR141716A. Surprisingly, SR141716A slightly stimulated AP-1 activity by itself (Figure 6). When co-incubated with anandamide, the inhibitory effect of high anandamide concentrations (15 μM) on AP-1 activity was even increased by SR141716A. The stimulatory effect of 10 μM anandamide was unaffected by SR141716A at 1 μM, but rendered inhibitory at 3 μM (Figure 6). SR141716A at 1 μM also did not inhibit 10 μM 18:1$_{n-9}$ NAE-induced AP-1 activation, but, in contrast with SR141716A/anandamide combinations, the combination of SR141716A (3 μM)/18:1$_{n-9}$ NAE (10–15 μM) happened to be less toxic to cells (Figure 6).

The inability of SR141716A to counteract the effects of NAEs was also demonstrated at the level of NAE-induced ERK phosphorylation. Interestingly, SR141716A stimulated ERK phosphorylation by itself (Figure 7). This observation complicated the interpretation but indicated a possible mechanism for the observed SR141716A-induced AP-1 activation. When JB6 P$^+$ cells were treated with both 1 μM SR141716A and 10 μM anandamide or 18:1$_{n-9}$ NAE, their combined effect was slightly additive rather than counteracting. Thus SR141716A failed to block NAE-induced AP-1 activation and ERK phosphorylation,

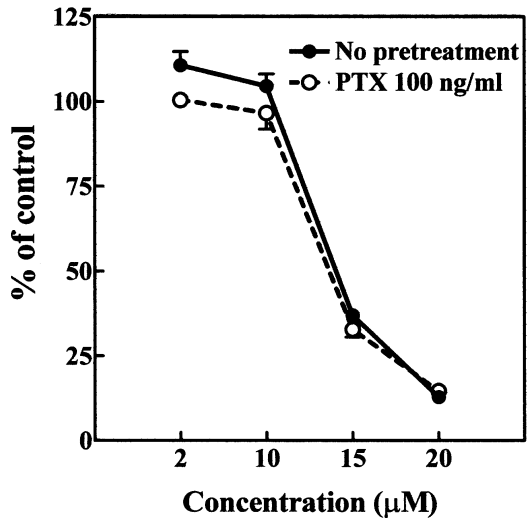

Figure 10 Pertussis toxin cannot block anandamide-induced toxicity in JB6 P$^+$ cells

JB6 P$^+$ Cl41 cells (2×10^3) were seeded into each well of a 96-well plate and grown for 24 h in the presence or absence of 100 ng/ml pertussis toxin (PTX) for the last 12 h of growth. Then cells were treated with the indicated concentrations of anandamide or DMSO (0.1% final concentration). Later (6 h), 20 μl of MTS reagent (Promega Cell Titer 96® Aqueous One Solution Cell Proliferation Assay) was added, and 2 h later the absorbance at 492 nm was measured. Data are expressed as the percentage of corresponding control MTS transformation (means $\pm$ S.E.M. of three independent experiments, each done in triplicate).

and was able to stimulate AP-1 activity by itself, possibly through the ERK pathway.

If cannabinoid receptors were somehow implicated in the stimulatory effect of anandamide, in spite of the failure of SR141716A to block it, the action of anandamide should be mimicked by synthetic cannabinoids such as WIN 55,212-2. However, 0.001–1 μM WIN 55,212-2 failed to stimulate AP-1 activity (Figure 8) and showed an inhibitory effect on ERK phosphorylation at higher concentrations (Figure 7). Once again, high concentrations of WIN 55,212-2 (10 μM) appeared to be toxic to JB6 P$^+$ cells, as the observed inhibition of AP-1 activity by this cannabinoid was accompanied by cell decomposition. The inhibition of ERK phosphorylation by WIN 55,212-2 was blocked by SR141716A (Figure 7). However, we cannot conclude whether it was due to antagonism at the level of the CB1 cannabinoid receptor or just the result of adding opposite independent signals. Nevertheless, this experiment showed a clear difference between the effects of NAEs and WIN 55,212-2 and, most importantly, between anandamide and WIN 55,212-2, in their ability to modulate AP-1 activity and the ERK MAP kinase signalling pathway in JB6 P$^+$ cells.

Finally, we observed that pertussis toxin failed to block any effects of NAEs. Pertussis toxin is an inhibitor of $G_{i/o}$ proteins because it ADP-ribosylates the α-subunit of the $G_{i/o}$ protein $\alpha\beta\gamma$ trimer [37,38]. As both CB1/CB2 cannabinoid receptors are linked to $G_{i/o}$ proteins [39–41], cell preincubation with pertussis toxin should block the effects mediated by any of the cannabinoid receptor subtypes. However, cell pretreatment with 100 ng/ml pertussis toxin did not block AP-1 activation induced by either anandamide or $18:1_{n-9}$ NAE (Figure 9), and the mitochondrial toxicity of anandamide was also not affected (Figure 10). Only ERK phosphorylation by anandamide was partially attenuated by pertussis toxin pretreatment, with $18:1_{n-9}$ NAE-induced stimulation being unaffected (Figure 11A). This finding indicates that the anandamide-induced effect on ERK phosphorylation might be partially cannabinoid-receptor-mediated, but this signal was not strong enough to seriously influence AP-1 activation. Surprisingly, pertussis toxin failed to block the inhibitory effect of WIN 55,212-2 on ERK phosphorylation (Figure 11B), while having a noticeable down-regulatory effect on background ERK phosphorylation during all experiments.

DISCUSSION

To our knowledge, these data represent the first demonstration that saturated and monounsaturated NAEs can function as intracellular signalling molecules. Specifically, we have shown

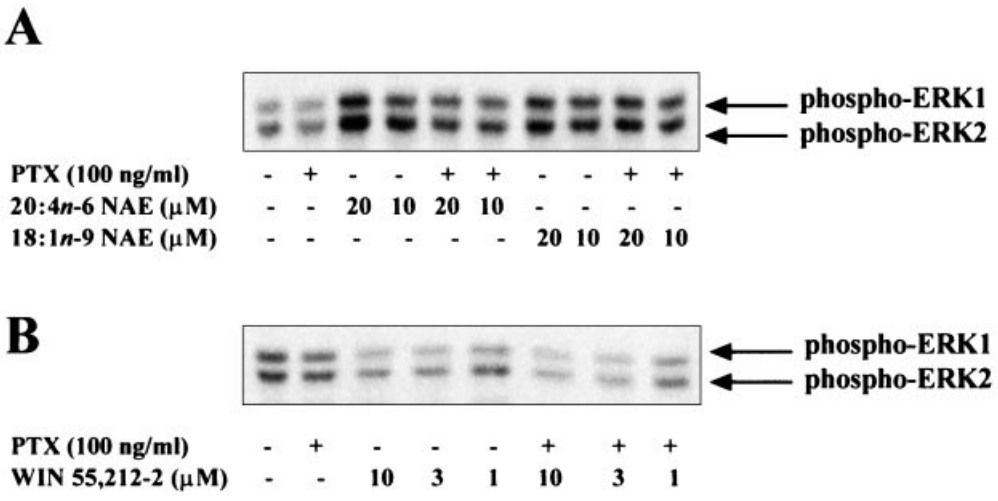

Figure 11 Pertussis toxin cannot block NAE-induced ERK activation and WIN 55,212-2-induced ERK inhibition in JB6 P$^+$ cells

JB6 P$^+$ Cl41 cells (1×10^5) were seeded into a 6-well plate and grown for 24 h. Then cells were starved by changing the medium to MEM/0.1% FBS for 24 h, with or without 100 ng/ml pertussis toxin (PTX) for the last 12 h of growth. Finally, cells were treated with the indicated concentrations of NAEs (**A**), WIN 55,212-2 (**B**) or DMSO (0.1% final concentration) for 90 min. After treatment, cells were lysed and processed for determination of phosphorylated ERKs as described in the Experimental section.

that they stimulate AP-1 activity in mouse epidermal JB6 P^+ cells through the ERK MAP kinase pathway. Even though these cells possess CB1 cannabinoid receptors, NAE-mediated signalling is cannabinoid-receptor-independent, because NAE-induced AP-1 activation and ERK phosphorylation were not inhibited by the $G_{i/o}$-protein blocker pertussis toxin or the CB1 cannabinoid receptor antagonist SR141716A (Figures 6–9 and 11). Furthermore, the effects elicited by NAEs were different from those elicited by the synthetic cannabinoid WIN 55,212-2, which failed to activate AP-1 (Figure 8) and inhibited the background phosphorylation of ERKs (Figure 11). Finally, the endocannabinoid anandamide and the cannabinoid-receptor-inactive $18:1_{n-9}$ NAE had similar potencies in stimulating AP-1 activity and ERK phosphorylation (Figures 1 and 4). Taken together, our data present strong evidence for the signalling function of the major cellular saturated and monounsaturated NAEs being through intracellular targets rather than through cannabinoid receptors. In addition, our data suggest that anandamide, a known cannabinoid-receptor agonist, also elicits its signalling effect in JB6 P^+ cells largely by a cannabinoid-receptor-independent pathway while a cannabinoid-receptor-mediated pathway may be involved as well. Apparently, both pathways result in ERK phosphorylation, but anandamide-induced cannabinoid-receptor-mediated signalling was responsible for only a part of the total ERK phosphorylation, as shown by pertussis toxin pretreatment (Figure 11), and had no measurable effect on AP-1 activation (Figures 6 and 9). This is in accordance with previous observations showing a positive link between cannabinoid-receptor activation and MAP kinase signalling [32–35,42–44] but also suggests that, in contrast with classical cannabinoids, at least some of the anandamide-induced effects on MAP kinases are cannabinoid-receptor-independent ([32,45], reviewed in [46]). It is thus logical to assume that at least some of the known anandamide-induced biological effects can be mediated through the cannabinoid-receptor-independent pathway common to all NAEs, and therefore can be elicited by the predominant, saturated and monounsaturated, NAEs as well. In this context, the observation that anandamide can increase the phosphatidylserine-induced activation of protein kinase C by a cannabinoid-receptor-independent mechanism [47] appears to be of interest. It is possible that among the primary targets of saturated and monounsaturated NAEs are certain protein kinase isoforms which, in turn, could mediate in the observed ERK activation.

NAEs are generated by membrane-bound enzymes located in both the endoplasmic reticulum and mitochondria [1,3,4]. We have shown recently that their levels can increase several-fold in intact cells as the result of stress [19]. Because exogenous NAEs are readily taken up into cells [17,18], we assume that the biological effects reported here resemble those elicited by endogenously synthesized NAEs. This requires that NAEs taken up by the cells from the media reach intracellular concentrations comparable with those achieved by endogenously generated NAEs. Endogenous NAEs occur in JB6 P^+ cells at a base level of about 60 pmol/μmol of lipid phosphorus and can reach 250 pmol/μmol of lipid phosphorus under stress [19]. This is equivalent to 12 and 51 μmol/10^6 cells, respectively, representing a volume of about 40–50 μl. Hence, the endogenous 'concentration' of NAE ranges between 0.26 and 1.0 μM, i.e. up to the lowest exogenous NAE concentration tested. Although we did not measure intracellular NAE level at any time point, we have to consider that uptake of saturated and monounsaturated NAEs may be relatively slow [48] and subject to hydrolysis by intracellular fatty acid amidohydrolase [8]. It is therefore likely that the NAEs applied in our experiments at concentrations of 15 μM or less generate intracellular NAE levels comparable with those endogenously generated under physiological conditions. In this context, anandamide represents a special case because it represents less than 5% of cellular NAE [19], its uptake is expected to be relatively fast [48] and it appears to be the most toxic (Figure 1).

Our attempt to block the effects of anandamide by the cannabinoid-receptor antagonist SR141716A revealed its own signalling potential. We have observed that SR141716A can stimulate ERK phosphorylation (Figure 7) and slightly stimulate AP-1 activity (Figure 6). These findings suggest that precautions should be taken when using this compound. Whereas the inverse agonist properties of SR141716A are well documented [49,50], the effect elicited by SR141716A in our experiments was opposite to that of pertussis toxin pretreatment. Therefore, we believe that a cannabinoid-receptor-independent target also mediates the observed SR141716A-induced signalling in JB6 P^+ cells.

When administered in relatively high amounts, NAEs, especially anandamide, have profound cytotoxic effects on JB6 P^+ cells (Figures 1–3). Our data suggest that these effects are brought about by a collapse of mitochondrial energy metabolism, thus compromising mitochondrial functions. Whether or not mitochondria are subject to physiological regulation by endogenous NAEs remains to be elucidated. However, our finding may be of particular interest to endocannabinoid researchers who often use anandamide concentrations of 20 μM and higher.

Finally, our present results on the messenger function of saturated and monounsaturated NAEs raise questions regarding their use as ceramidase inhibitors. Because $18:1_{n-9}$ NAE has long been known to inhibit an alkaline ceramidase *in vitro* [51], it is often used in experiments intended to increase cellular ceramide levels [52–54]. Various biological effects have thus been ascribed to ceramide-mediated signalling without considering possible effects of the added NAE itself. It is possible that NAE signalling involves intracellular targets analogous to those responding to ceramide and that cross-talk between NAE and ceramide may occur. This possibility deserves further study.

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