Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells

Dominique Melck[a,1], Daniel Rueda[c,1], Ismael Galve-Roperh[c], Luciano De Petrocellis[b], Manuel Guzmán[c], Vincenzo Di Marzo[a,*]

[a] *Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072 Arco Felice, Italy*
[b] *Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072 Arco Felice, Italy*
[c] *Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, 28040 Madrid, Spain*

Received 10 September 1999; received in revised form 13 November 1999

Edited by Shozo Yamamoto

Abstract Anandamide (ANA) inhibits prolactin- and nerve growth factor (NGF)-induced proliferation of human breast cancer cells by decreasing the levels of the 100 kDa prolactin receptor (PRLr) and the high affinity *trk* NGF receptor, respectively, and by acting via CB_1-like cannabinoid receptors. However, the intracellular signals that mediate these effects are not known. Here, we show that, in MCF-7 cells: (i) forskolin and the mitogen-activated protein kinase (MAPK) kinase inhibitor PD098059 prevent, and the protein kinase A inhibitor RpcAMPs mimics, the inhibitory effects of ANA on cell proliferation and PRLr/*trk* expression and (ii) ANA inhibits forskolin-induced cAMP formation and stimulates Raf-1 translocation and MAPK activity, in a fashion sensitive to the selective CB_1 antagonist SR141716A. ANA stimulation of MAPK was enhanced by inhibitors of ANA hydrolysis. Forskolin inhibited MAPK and ANA-induced Raf-1 translocation. These findings indicate that, in MCF-7 cells, ANA inhibits adenylyl cyclase and activates MAPK, thereby exerting a down-regulation on PRLr and *trk* levels and a suppression of cell proliferation.

Key words: Cannabinoid; Receptor; *trk*; Prolactin; 2-Arachidonoyl glycerol; Cancer

1. Introduction

The finding of two receptor subtypes for (−)-Δ^9-tetrahydrocannabinol (THC), the CB_1 and the CB_2 receptor, led to the discovery of two endogenous ligands for such proteins (see [1] for review): anandamide (*N*-arachidonoyl-ethanolamine, ANA) [2] and 2-arachidonoyl glycerol (2-AG) [3,4] (hereafter referred to as 'endocannabinoids' [5]). The pharmacology and metabolism of these two metabolites have been studied (for reviews, see [5–7]), but their actual pathophysiological role(s) in vivo remain(s) to be elucidated. Most of the possible functions suggested so far for endocannabinoids are concerned with specific activities in particular cell types and tissues [5–8]. However, THC and endocannabinoids also modulate basic functions that are common to many cell types, such as energy metabolism (see [9] for review) and cell structure, proliferation and apoptosis [10–13]. We reported that ANA and the synthetic cannabinoid HU-210 inhibit the proliferation of human breast cancer cells (HBCCs) by down-regulating the levels of the ~100 kDa form of the receptor (PRLr) of prolactin, a hormone that is synthesized by these cells in culture and used as an autocrine mitogen [12]. We showed that ANA, 2-AG and HU-210 also potently inhibit the nerve growth factor (NGF)-induced proliferation of a HBCC line, the MCF-7 cells, by suppressing the levels of the high affinity *trk* NGF receptors [14]. We found that the anti-mitogenic effects of ANA, as well as its down-regulatory action on PRLr/*trk* proteins, were mediated by CB_1-like, and not CB_2-like, receptors [14]. In another study, we showed that THC causes an altogether unrelated effect, i.e. the apoptosis of glioma, astrocytoma and neuroblastoma cells, but not of neurons and astrocytes in primary culture, by acting through sphingomyelin hydrolysis in a fashion insensitive to the selective CB_1 antagonist SR141716A [13]. These studies point to the possible development of novel anti-cancer drugs from both synthetic and endogenous cannabinoids. To this end, it is of interest to identify the intracellular events leading to the down-regulation of the expression of PRLr and *trk* that is responsible for CB_1-mediated inhibition of HBCC proliferation. In fact, drugs capable of selectively interfering with such events may mimic the anti-cancer effects of cannabinoids without producing their undesired psychotropic actions. CB_1 receptor activation modulates the activity of adenylyl cyclase (AC) and mitogen-activated protein kinase (MAPK) (see [15] for review), thereby potentially regulating the expression of several genes. The cAMP/protein kinase A (PKA) pathway may also influence gene transcription by modulating the activity of MAPK [16]. *trk* receptors have been shown to be up-regulated by cAMP [17,18], but similar data for PRLr have not been reported yet. Based on this background, the present study was aimed at investigating the involvement of the cAMP/PKA and Raf-1/MAPK pathways in the inhibition of HBCC proliferation and PRLr/*trk* levels by ANA.

*Corresponding author. Fax: (39)-81-8041770.
E-mail: vdm@trinc.icmib.na.cnr.it

[1] These authors contributed equally to this work.

Abbreviations: HBCC, human breast cancer cell; ANA, anandamide; 2-AG, 2-arachidonoyl glycerol; PRLr, long form of prolactin receptor; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; *trk*, high affinity NGF receptor; AC, adenylyl cyclase; THC, (−)-Δ^9-tetrahydrocannabinol; PMSF, phenyl methyl sulfonyl fluoride

PII: S0014-5793(99)01639-7

2. Materials and methods

2.1. Materials

MCF-7 and EFM-19 cells were purchased from ATCC (USA) and DSM (Germany), respectively, and cultured as advised by the manufacturers. ANA, human prolactin, 8-Br-cAMP and β-NGF were purchased from Sigma and forskolin from Fluka. SR141716A was a gift from Sanofi Recherche, Montpellier, France, and HU-210 from Dr. R. Mechoulam, Hebrew University, Jerusalem, Israel. RpcAMPs was purchased from Biomol, USA, and PD098059 from Alexis Corp., USA. The concentrations of test substances (i.e. ANA, SR141716A, forskolin, RpcAMPs) used in different assays depended on the assay conditions, lower concentrations being used in long-incubation experiments (e.g. cell proliferation, assay of *trk*/PRLr levels).

2.2. Cell proliferation assays and ANA degradation by MCF-7 cells

Cell proliferation assays were carried out according to the method previously described [12,14] in six-well dishes containing sub-confluent cells (at a density of about 50 000 cells/well). With MCF-7 cells, test substances were introduced 3 h after cell seeding and then daily at each change of medium. With EFM-19 cells, test substances were introduced 24 h after cell seeding and then daily at each change of medium, in the presence or absence of 50 ng/ml of human prolactin. Cells were counted by a hemocytometer after 4 days from the addition of test substances. In order to study the effect of NGF on MCF-7 cell proliferation, we used a previously described procedure [19]. Twenty-four hours after cell seeding (50 000 cells/well), the medium was changed to serum-free medium and cells starved for 24 h. Cells were then treated with serum-free medium containing β-NGF (100 ng/ml) plus test substances or vehicle and counted after 48 h. The time-dependent degradation of [^{14}C]ANA (80 000 cpm, 1.5 μM in 8 ml of serum-free culture medium) by intact, sub-confluent MCF-7 cells was measured as described previously [20].

2.3. Effect on trk and PRLr levels

After treatment with test substances, which was carried out under the same conditions described above for cell proliferation assays, but in 100 mm Petri dishes, cells were washed twice with 137 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH = 7.4) and then lysed with a lysis buffer consisting of 50 mM Tris-HCl, pH = 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1% Triton X-100 and 1 μg/ml each of aprotinin, leupeptin and pepstatin A. Lysates were loaded onto gels containing 10% and 7.5% polyacrylamide for the blotting of PRLr or *trk*, respectively. Proteins were transferred to nitrocellulose membranes, which were then incubated first for 1 h at room temperature with the primary antibody, i.e. either anti-human prolactin receptor monoclonal antibody (U5, purchased from Affinity Bioreagents, Golden, USA, 1:1000) or anti-mouse *trk* monoclonal antibody (B-3, Santa Cruz Biotechnologies, USA, 1:500) and then with the appropriate horseradish peroxidase-labelled secondary antibody conjugates (1:5000, Bio-Rad, Hercules, USA). Bands were visualized by the ECL technique (Bio-Rad).

2.4. cAMP, MAPK and Raf-1 translocation assays

cAMP assays were performed on intact confluent MCF-7 cells plated in six-well dishes and stimulated for 10 min at 37°C with 1 μM forskolin in 400 μl of serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES, 0.1 mg/ml BSA, 0.1 mM 1-methyl-3-isobutylxanthine (Sigma). Cells were treated with either vehicle (ethanol) or ANA, at various concentrations, or ANA plus 0.5 μM SR141716A. After the incubation, cells were extracted and cAMP was determined by means of a cAMP assay kit (Amersham, UK). p42/p44 MAPK activity was determined in cell extracts as the incorporation of ^{32}P from [^{32}P]ATP into a MAPK substrate peptide [21]. Membrane-bound Raf-1 was determined by Western blot analysis with an anti-Raf-1 antibody (Santa Cruz Biotechnology, USA) as described before [21]. For both assays, cells were incubated with ANA for 30 min at 37°C. In some experiments, cells were pre-incubated with ANA hydrolysis inhibitors for 30 min at 37°C, followed by co-incubation with ANA for 30 min. In some experiments, SR141716A was co-incubated with ANA.

3. Results

3.1. Effect of forskolin, RpcAMPs and PD098059 on basal and NGF-induced HBCC proliferation

ANA inhibits basal MCF-7 and EFM-19 cell proliferation with $IC_{50} = 1.4 \pm 0.9$ and 1.5 ± 0.3 μM ($n = 6$ and 7, respectively; see [12,14]). As shown in Fig. 1A, forskolin weakly inhibited MCF-7 cell proliferation ($-11 \pm 1\%$ inhibition at 10 μM) but significantly reduced ANA anti-proliferative effect on these cells. Forskolin also blocked the inhibition by ANA of both basal and prolactin-induced proliferation of EFM-19 cells (data not shown), which were even less sensitive than MCF-7 cells to forskolin alone (maximal inhibition of proliferation was $-6 \pm 1\%$ at 10 μM). Forskolin inhibited ANA anti-proliferative action on β-NGF-induced MCF-7 cell proliferation (estimated $IC_{50} = 1.2$ μM) and weakly inhibited

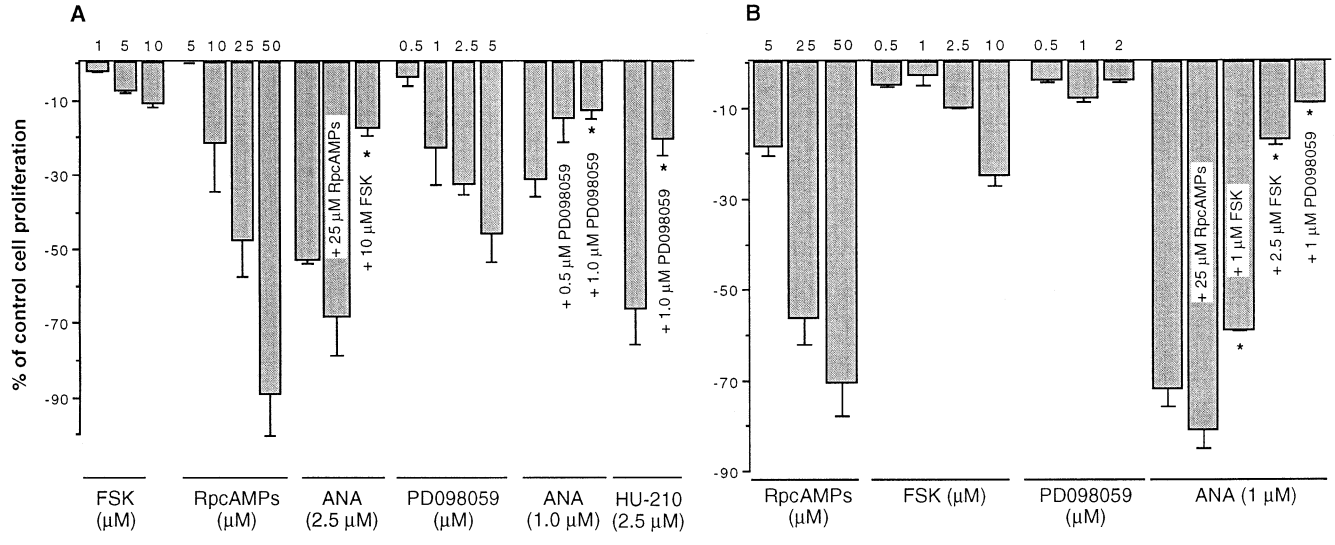

Fig. 1. Involvement of the cAMP/PKA and MAPK pathways in ANA anti-proliferative effects on MCF-7 cells. Effect of forskolin (FSK), RpcAMPs and PD098059 (at various concentrations) on (A) basal and (B) β-NGF-induced MCF-7 cell proliferation, as well as on the inhibition by ANA of (A) basal and (B) β-NGF-induced MCF-7 cell proliferation. Data are means ± S.D. of at least three separate experiments carried out in duplicate. *, $P < 0.05$ vs. ANA only, as assessed by ANOVA.

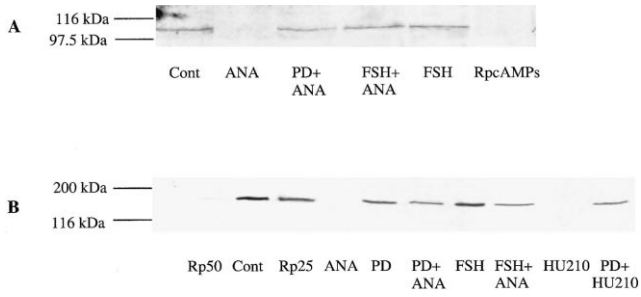

Fig. 2. Involvement of the cAMP/PKA and MAPK pathways in the down-regulatory effect of ANA on the levels of (A) the high molecular weight (~100 kDa) prolactin receptor (PRLr) and (B) the high affinity *trk* NGF receptors in MCF-7 cells. PRLr and *trk* were identified by Western immunoblotting by using the appropriate antibodies. The mobility of molecular weight markers is shown. In (A), the effect of RpcAMPs (50 μM) on PRLr levels and of forskolin (FSK, 10 μM) and PD098059 (PD, 1 μM) on ANA (1 μM) suppression of PRLr levels is shown. The same amount of protein (50 μg) was loaded onto each lane. In (B), the effect on PRLr and *trk* levels of forskolin (FSK, 10 μM) and PD098059 (PD, 1 μM) alone or in the presence of ANA (1 μM), or of RpcAMPs (Rp, 25 and 50 μM) is shown. Also the effect of HU-210 (2.5 μM) with or without PD098059 (PD, 1 μM) is shown. The anti-*trk* antibody cross-reacts with human *trk*. The same amount of protein (20 μg) was loaded onto each lane. Representative of three experiments yielding similar results. Cont, control.

β-NGF-induced MCF-7 cell proliferation (−25 ± 1% inhibition at 10 μM) (Fig. 1B). The PKA inhibitor RpcAMPs dose-dependently inhibited both basal and β-NGF-induced MCF-7 cell proliferation. The effect of a submaximal dose of this compound was not cumulative to that of a submaximal dose of ANA (Fig. 1A,B). This finding was confirmed in EFM-19 cells, where different concentrations of RpcAMPs were tested. The cytostatic effect of 2.5 μM ANA alone (−53 ± 2%) was not increased by 5 μM RpcAMPs (which alone produced −11 ± 2% inhibition), 10 μM RpcAMPs (which alone produced −20 ± 5% inhibition) or 25 μM RpcAMPs (which alone produced −55 ± 5% inhibition), since the cumulative effects in the three cases were −46 ± 4, −43 ± 4 and −63 ± 7%, respectively (means ± S.D., $n = 3$). We also tested two different doses (25 and 100 μM) of 8-Br-cAMP on cell proliferation. Unfortunately, this compound was toxic to HBCCs after long-term (>2 days) treatments and could not be used in experiments aimed at determining possible cytostatic effects. Finally, the MAPK kinase inhibitor PD098059 dose-dependently inhibited basal but not β-NGF-induced MCF-7 cell proliferation and, at a weakly active dose (1 μM), suppressed both ANA and HU-210 inhibition of MCF-7 cell proliferation (Fig. 1A,B). Interestingly, MCF-7 cells rapidly inactivate exogenous [^{14}C]ANA. After 10 min, more than half of the [^{14}C]ANA incubated with cells had already been cleared from the medium ($t_{1/2} = 7 \pm 1$ min). Hydrolysis to [^{14}C]ethanolamine proceeded more slowly ($t_{1/2} = 20 \pm 3$ min), as previously shown for neurons [22].

3.2. Effect of forskolin, RpcAMPs and PD098059 on MCF-7 cell trk and PRLr levels

As assessed by Western immunoblotting using the appropriate antibodies, MCF-7 cells express both PRLr and the ~140 kDa *trk* high affinity receptors for NGF (Fig. 2A,B). As reported previously [12,14], incubation of cells with ANA led to suppression of both PRLr and *trk* levels. Both forskolin and PD098059, at concentrations exerting only slight antiproliferative effects but a maximal prevention of ANA antimitogenic effects, antagonized the down-regulatory effect of ANA on PRLr and *trk* levels. PD098059 also attenuated the HU-210 effect on *trk* levels. RpcAMPs, at concentrations effecting strong inhibition of cell proliferation, decreased both PRLr and *trk* levels (Fig. 2A,B).

3.3. Effect of ANA on cAMP levels, MAPK and Raf-1 translocation in HBCCs

ANA dose-dependently inhibited forskolin-induced cAMP formation in MCF-7 cells (Fig. 3A). The effect was half-maximal with 3.5 μM ANA and was blocked by 0.5 μM SR141716A. Analogous results were obtained for EFM-19 cells (data not shown). Little inhibition by ANA (80 ± 1% of control, $n = 3$) of basal cAMP levels was observed in both HBCC lines. As shown in Fig. 3B, ANA induced a dose-dependent activation of MAPK activity. The stimulatory effect of ANA was half-maximal at around 10 μM and was antagonized by 5 μM SR141716A (Table 1). Forskolin and 8-Br-cAMP inhibited basal MAPK activity but did not prevent ANA activation of MAPK (Fig. 3, Table 1). However, MAPK activity in the presence of ANA and either forskolin or 8-Br-cAMP was significantly lower than that in the presence of ANA alone (Fig. 3, Table 1). By contrast, RpcAMPs stimulated MAPK. ANA-induced MAPK activation was potentiated by the ANA amidohydrolase irreversible inhibitor PMSF and by oleamide (Table 1), which is a substrate of the enzyme and a competitive inhibitor of ANA hydrolysis (for review, see [23]). PMSF and, particularly, oleamide per se also stimulated MAPK. Finally, ANA increased the amounts of membrane-bound Raf-1 at a concentration leading to full MAPK activation. This effect was prevented by forskolin (Fig. 3C).

Table 1
Effect of modulators of AC, PKA and ANA hydrolysis on ANA stimulation of MAPK in MCF-7 cells

Additions	MAPK activity (%)
None	100 ± 13
10 μM ANA	142 ± 9*
25 μM ANA	201 ± 29*
25 μM forskolin	64 ± 15*
25 μM ANA+25 μM forskolin	155 ± 16*#
50 μM 8-Br-cAMP	76 ± 8*
25 μM ANA+50 μM 8-Br-cAMP	158 ± 8*#
25 μM RpcAMPs	148 ± 19*
5 μM SR141716A	101 ± 3
25 μM ANA+5 μM SR141716A	100 ± 4#
50 μM oleamide	176 ± 24*
10 μM ANA+50 μM oleamide	301 ± 44*#
1 mM PMSF	138 ± 6*
10 μM ANA+1 mM PMSF	205 ± 31*#

Cells were exposed to the different agents for 30 min. In the case of incubations with PMSF and oleamide, cells were pre-incubated with these compounds for 30 min before ANA was added. Data are means ± S.D. of four separate experiments. *, $P < 0.01$ vs. control incubations as assessed by the paired Student's t test. #, $P < 0.01$ vs. ANA only. The effect of oleamide on MAPK activity is in agreement with our previous observation that this compound per se also inhibits HBCC proliferation by enhancing endocannabinoid levels [20].

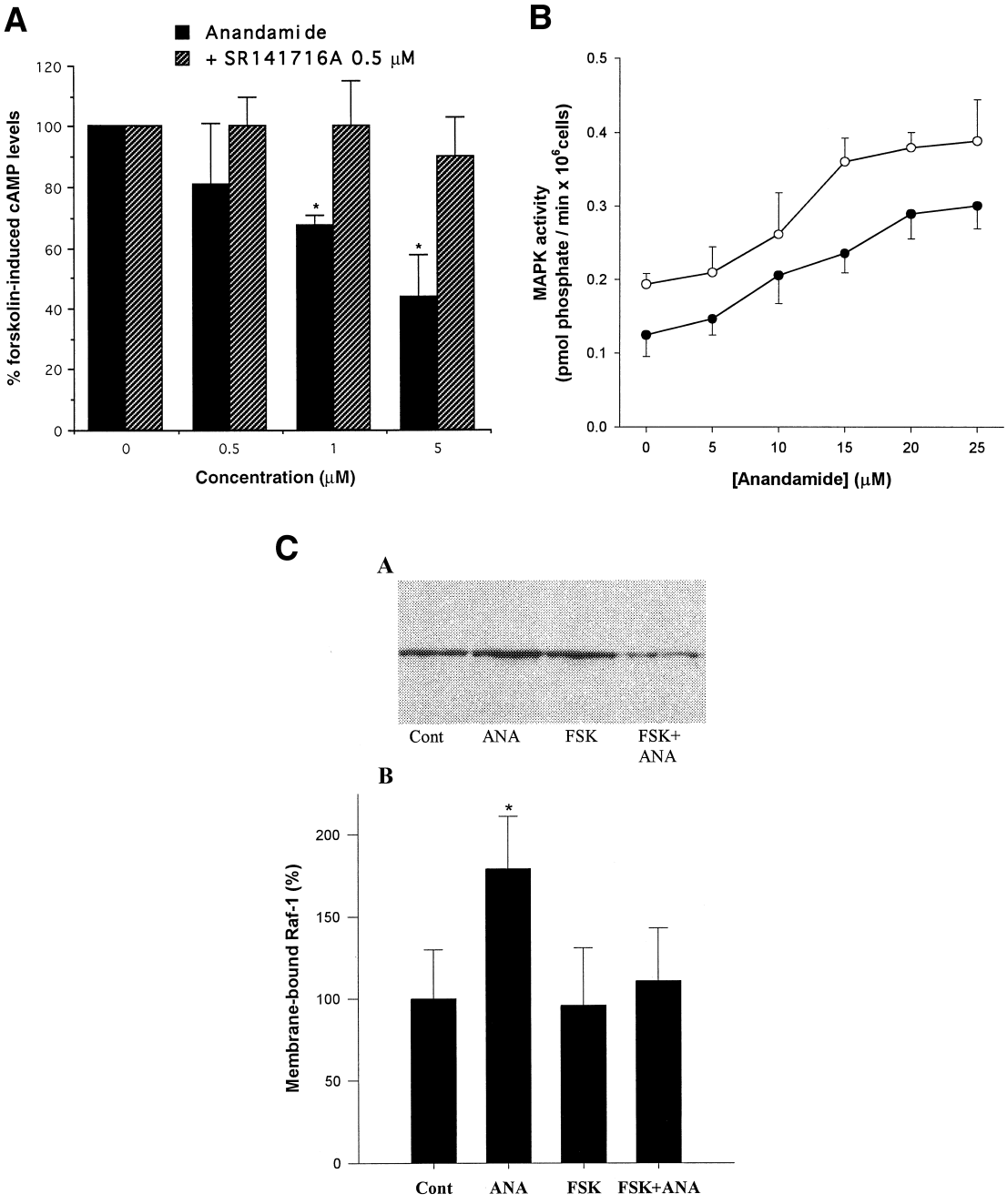

Fig. 3. Effect of ANA on intracellular signals in MCF-7 cells. (A) Effect on forskolin-induced cAMP formation. Data are means ± S.D. of three separate experiments. Control levels of cAMP after 10 min stimulation of cells with 1 μM forskolin were 63.2 ± 5.5 pmol/well, whereas basal cAMP levels were 8.4 ± 1.5 pmol/well ($n = 6$). *, $P < 0.05$ vs. ANA plus SR141716A, as assessed by ANOVA. (B) Effect of ANA on MAPK activity in MCF-7 cells. Cells were exposed for 30 min to different concentrations of ANA in the absence (open circles) or presence (filled circles) of 25 μM forskolin. Data are means ± S.D. of four separate experiments. (C) Effect of ANA on Raf-1 translocation to the membrane fraction in MCF-7 cells. Cells were incubated for 30 min in the absence or presence of ANA (25 μM) and/or forskolin (FSK, 25 μM). Upper panel: representative luminogram. Lower panel: relative values of membrane-bound Raf-1 protein. Data are means ± S.D. of six separate experiments. *, $P < 0.01$ vs. control incubations as assessed by the paired Student's t test.

4. Discussion

It is known that HBCCs synthesize prolactin and use it as an autocrine growth factor [12,24]. We showed that ANA, by acting at CB_1-like receptors, can inhibit both basal (i.e. endogenous prolactin-induced) and exogenous prolactin or β-NGF-induced HBCC proliferation by suppressing the levels of PRLr and the high affinity *trk* NGF receptors, respectively [12,14]. Here, we studied the molecular mechanisms by which ANA exerts these effects. We found that ANA stimulated MAPK and inhibited AC in a fashion sensitive to SR141716A. Drugs interfering with either the cAMP/PKA or the MAPK pathways affected in a similar manner both the inhibition of cell proliferation and the suppression of PRLr/*trk* levels by ANA. In particular, we observed that the AC activator, forskolin, and the MAPK kinase inhibitor, PD098059, counteracted, whereas the PKA inhibitor, RpcAMPs, mimicked these two effects of the endocannabi-

noid, thus suggesting the involvement of the cAMP/PKA and MAPK signalling cascades in ANA inhibition of PRLr/*trk* levels and cell proliferation.

We found that RpcAMPs stimulated, whereas forskolin inhibited, basal MAPK activity. However, forskolin alone did not inhibit Raf-1 translocation to the cell membrane. This suggests that, in MCF-7 cells, MAPK is constitutively down-regulated only in part through the PKA-catalyzed phosphorylation, and subsequent inhibition, of Raf-1 [16]. CB_1-mediated inhibition of AC by ANA may release this inhibitory tone and potentially restores MAPK activity by enhancing Raf-1 translocation, an effect that is reversed by forskolin. It is likely that ANA activates MAPK also independently from its effect on AC (as shown for synthetic cannabinoids in astrocytoma cells [25]) and Raf-1 translocation, thus explaining why forskolin did not prevent ANA-induced MAPK stimulation. However, only the putative cAMP-dependent activation of MAPK by ANA would lead to inhibition of cell proliferation. In fact, if also the 'direct' effect of ANA on MAPK resulted in the inhibition of cell proliferation, there should have been a cumulative cytostatic action with submaximal doses of RpcAMPs (which mimics only the cAMP-dependent effect on the enzyme) and ANA, which we did not observe (Fig. 1 and Section 3). This latter finding may also indicate that, additionally, ANA reduces the proliferation of MCF-7 cells by inhibiting cAMP formation without passing through MAPK activation. Indeed, inhibition of cAMP formation by peptide YY was recently shown to lead to inhibition of MCF-7 cell growth in vitro and in vivo [26].

The indirect stimulation of MAPK by ANA is also strongly supported by (1) a recent study in which over-expression of mutant-activated Gαs subunits or incubation with 8-Br-cAMP were found to decrease the intrinsic activity of ERK-1 and ERK-2 kinases in MCF-7 cells [27] and (2) our present observation that ANA affects cAMP levels at doses slightly lower than those required to stimulate MAPK. The fact that the IC_{50} and EC_{50} values for these two effects are in the low μM range of concentrations should not lead to the conclusion that ANA action on the two systems is non-physiological or is not mediated by cannabinoid receptors. In fact, both effects were blocked by SR141716A and the relative IC_{50} and EC_{50} values were only 3–6-fold higher than the K_i values for ANA displacement of [^{3}H]SR141716A from HBCC membranes (0.85–1.50 μM) or the IC_{50} values for ANA inhibition of HBCC proliferation (1.4–1.5 μM, see [12,14]). Furthermore, ANA effect on cAMP levels and MAPK activity was very probably blunted by the occurrence of enzymatic hydrolysis during the assays. Indeed, we have shown here and previously [12,20] that HBCCs rapidly hydrolyze [^{14}C]ANA. Accordingly, inhibitors of the enzymatic hydrolysis of ANA (e.g. PMSF and oleamide) significantly enhanced its effect both on cell proliferation and MAPK activity (this study and [12]). Interestingly, PMSF and, particularly, oleamide per se also induced MAPK activation. These effects may be due to the enhancement of endogenous ANA levels and are in agreement with our previous finding that oleamide per se also inhibits HBCC proliferation (in fact, the anti-proliferative effect of oleamide, which does not bind to CB_1 receptors, was blocked by SR141716A [20]). Nevertheless, oleamide may also exert part of its effects independently from ANA hydrolysis [28].

An additional factor that might have led to observe relatively high IC_{50} and EC_{50} values for ANA inhibition of cAMP formation and stimulation of MAPK is that short incubation times are normally used in the assay protocols for these two effects. It is possible that longer incubations (such as those used to observe the anti-mitogenic and anti-PRLr/*trk* effects of the lipid) might have led to an action on cAMP levels or, particularly, MAPK activity with lower ANA concentrations and, subsequently, to a counteraction of these effects with doses of forskolin and SR141716A closer to those required to antagonize ANA inhibition of cell proliferation. Also a more potent inhibition of the basal cAMP formation by ANA might have been observed with longer incubations. It is possible that only after prolonged treatment, low doses of ANA lead to effects on basal cAMP levels or MAPK activity strong enough to inhibit PRLr/*trk* levels and MCF-7 cell proliferation. This hypothesis agrees with previous observations that sustained, as opposed to transient, activation of MAPK is necessary to observe inhibition, instead of activation, of MCF-7 cell proliferation [29]. In fact, both activation and inhibition of MAPK have been previously shown to lead to inhibition of HBCC growth, depending on the culturing conditions and on the presence and concentration of growth factors. For example, incubation of MCF-7 cells with basic fibroblast growth factor at concentrations that effected growth inhibition resulted in activation of both ERK-1 and ERK-2 [30]. On the other hand, inhibition of MAPK activity by tumor necrosis factor-α was described to lead to growth inhibition in epidermal growth factor-stimulated cells [31]. Thus, our finding of the inhibitory effect on basal MCF-7 cell proliferation by PD098059 and of the counteraction by the latter compound of ANA anti-proliferative effects both agree with previous data. It is possible that a finely regulated tone of MAPK activity is necessary to these cells for normal proliferation. Disruption of this tone by either blockade (with PD098059) or sustained dis-inhibition (by CB_1-mediated inhibition of cAMP levels) may lead to decreased proliferation.

In conclusion, we have shown here that the suppression of PRLr and *trk* levels by ANA and its inhibition of prolactin and NGF-induced MCF-7 cell proliferation may be due, at least in part, to AC inhibition and MAPK activation. Our data are in agreement with reports indicating that the transcription of *trk* genes is enhanced by the cAMP/PKA cascade [17,18]. However, to the best of our knowledge, ours is the first report of the suppression of both PRLr and *trk* levels following inhibition of the cAMP/PKA pathway and stimulation of MAPK.

Acknowledgements: The authors are grateful to Dr. T. Bisogno for assistance. This work was supported by Grants from the INTAS (97/1297), to V.D.M., and CICYT (PM 98/0079) and CAM (08.5/0017/98), to M.G.

References

[1] Pertwee, R.G. (1997) Pharmacol. Ther. 74, 129–180.
[2] 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.
[3] Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., Pertwee, R.G., Griffin, G., Bayewitch, M., Barg, J. and Vogel, Z. (1995) Biochem. Pharmacol. 50, 83–90.

[4] Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A. and Waku, K. (1995) Biochem. Biophys. Res. Commun. 215, 89–97.
[5] Di Marzo, V., Melck, D., Bisogno, T. and De Petrocellis, L. (1998) Trends Neurosci. 21, 521–528.
[6] Mechoulam, R., Fride, E. and Di Marzo, V. (1998) Eur. J. Pharmacol. 359, 1–18.
[7] Hillard, C.J. and Campbell, W.B. (1997) J. Lipid Res. 38, 2382–2398.
[8] Wagner, J.A., Varga, K. and Kunos, G. (1998) J. Mol. Med. 76, 824–836.
[9] Guzman, M. and Sanchez, C. (1999) Life Sci. 65, 657–664.
[10] Schwarz, H., Blanco, F.J. and Lotz, M. (1994) J. Neuroimmunol. 55, 107–115.
[11] Wilson Jr., R.G., Tahir, S.K., Mechoulam, R., Zimmerman, S. and Zimmerman, A.M. (1996) Cell Biol. Int. 20, 147–157.
[12] De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (1998) Proc. Natl. Acad. Sci. USA 95, 8375–8380.
[13] Sanchez, C., Galve-Roperh, I., Canova, C., Brachet, P. and Guzman, M. (1998) FEBS Lett. 436, 6–10.
[14] Melck, D., De Petrocellis, L., Orlando, P., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (1999) Endocrinology (in press).
[15] Di Marzo, V. (1998) Biochim. Biophys. Acta 1392, 153–175.
[16] Morrison, D.K. and Cutler, R.E. (1997) Curr. Opin. Cell Biol. 9, 174–179.
[17] Ehrhard, P.B., Ganter, U., Stalder, A., Bauer, J. and Otten, U. (1993) Proc. Natl. Acad. Sci. USA 90, 5423–5427.
[18] Rosenbaum, T., Vidaltamayo, R., Sanchez-SotoMa, C., Zentella, A. and Hiriart, M. (1998) Proc. Natl. Acad. Sci. USA 95, 7784–7788.
[19] Descamps, S., Lebourhis, X., Delehedde, M., Boilly, B. and Hondermarck, H. (1998) J. Biol. Chem. 273, 16659–16662.
[20] Bisogno, T., Katayama, K., Melck, D., Ueda, N., De Petrocellis, L., Yamamoto, S. and Di Marzo, V. (1998) Eur. J. Biochem. 254, 634–642.
[21] Sanchez, C., Galve-Roperh, I., Rueda, D. and Guzman, M. (1998) Mol. Pharmacol. 54, 834–843.
[22] Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C. and Piomelli, D. (1994) Nature 372, 686–691.
[23] Ueda, N., Goparaju, S.K., Katayama, K., Kurahashi, Y., Suzuki, H. and Yamamoto, S. (1998) J. Med. Invest. 45, 27–36.
[24] Fuh, G. and Wells, J.A. (1995) J. Biol. Chem. 270, 13133–13137.
[25] Bouaboula, M., Bourrie, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G. and Casellas, P. (1995) J. Biol. Chem. 270, 13973–13980.
[26] Moody, T.W., Leyton, J., Gozes, I., Lang, L. and Eckelman, W.C. (1998) Ann. N.Y. Acad. Sci. 865, 290–296.
[27] Chen, J., Bander, J.A., Santore, T.A., Chen, Y., Ram, P.T., Smit, M.J. and Iyengar, R. (1998) Proc. Natl. Acad. Sci. USA 95, 2648–2652.
[28] Boger, D.L., Henriksen, S.J. and Cravatt, B.F. (1998) Curr. Pharm. Des. 4, 303–314.
[29] Liu, J.F., Issad, T., Chevet, E., Ledoux, D., Courty, J., Caruelle, J.P., Barritault, D., Crepin, M. and Bertin, B. (1998) Eur. J. Biochem. 258, 271–276.
[30] Fenig, E., Wieder, R., Paglin, S., Wang, H., Persaud, R., Haimovitz-Friedman, A., Fuks, Z. and Yahalom, J. (1997) Clin. Cancer Res. 3, 135–142.
[31] Flury, N., Eppenberger, U. and Mueller, H. (1997) Eur. J. Biochem. 249, 421–426.