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 CB1-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 PD98059 prevent, and the protein kinase A (PKA) 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 CB1 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 adenyl cyclase and activates MAPK, thereby exerting a down-regulation on PRLr and trk levels and a suppression of cell proliferation.

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

1. Introduction

The finding of two receptor subtypes for (−)-Δ⁹-tetrahydrocannabinol (THC), the CB1 and the CB2 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 CB1-like, and not CB2-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 CB1 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 CB1-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. CB1 receptor activation modulates the activity of adenyl 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.

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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, adenyl cyclase; THC, (−)-Δ⁹-tetrahydrocannabinol; PMSF, phenyl methyl sulfonyl fluoride

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PHI: 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. Rp-cAMPs was purchased from Biomol, USA, and PD908059 from Alexis Corp., USA. The concentrations of test substances (i.e. ANA, SR141716A, forskolin, Rp-cAMPs) 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 [125I]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 Na2HPO4, 2 mM KH2PO4 (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 Na3VO4, 1 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1% Triton X-100 and 1 μM 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 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, Rp-cAMPs and PD908059 on basal and NGF-induced HBCC proliferation

ANA inhibits basal MCF-7 and EFM-19 cell proliferation with IC50 = 1.4 ± 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 ± 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 ± 1% at 10 μM). Forskolin inhibited ANA anti-proliferative action on β-NGF-induced MCF-7 cell proliferation (estimated IC50 = 1.2 μM) and weakly inhibited ANA, 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 horse-radish peroxidase-labelled secondary antibody conjugates (1:5000, Bio-Rad, Hercules, USA). Bands were visualized by the ECL technique (Bio-Rad).

Fig. 1. Involvement of the cAMP/PKA and MAPK pathways in ANA anti-proliferative effects on MCF-7 cells. Effect of forskolin (FSK), Rp-cAMPs and PD098059 (at various concentrations) on the basal proliferation of MCF-7 cells (data not shown), which were even less sensitive than MCF-7 cells to forskolin alone (maximal inhibition of proliferation was ~6 ± 1% at 10 μM). Forskolin inhibited ANA anti-proliferative action on β-NGF-induced MCF-7 cell proliferation (estimated IC50 = 1.2 μM) and weakly inhibited ANA, 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 horse-radish peroxidase-labelled secondary antibody conjugates (1:5000, Bio-Rad, Hercules, USA). Bands were visualized by the ECL technique (Bio-Rad).


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PRLr and trk were iden-
tified by Western immunoblotting by using the appropriate antibod-
ies. 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
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% inhibi-
tion 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-cell proliferation (Fig. 1A,B). Interestingly, MCF-7
cells rapidly inactivate exogenous [14C]ANA. After 10 min,
more than half of the [14C]ANA incubated with cells had already
been cleared from the medium (t1/2 = 7 ± 1 min). Hyd-
drolysis to [14C]ethanolamine proceeded more slowly
(t1/2 = 20 ± 3 min), as previously shown for neurons [22].

3.2. Efect 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 anti-
proliferative effects but a maximal prevention of ANA mit-

Table 1

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

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].
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 CB1-like receptors, can inhibit both basal (i.e. endogenous prolactin-induced) and exogenous prolactin or L-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 RpeAMPS 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]. CB1-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 RpeAMPS (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 a recent study in which over-expression of mutant-activated Gs 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 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 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/S/001798), to M.G.

References


An additional factor that might have led to observe relatively high IC50 and EC50 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 CB1-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.


