MOL PHARM

MOLECULAR PHARMACOLOGY

Mechanism of Extracellular Signal-Regulated Kinase Activation by the CB$_1$ Cannabinoid Receptor

ISMAEL GALVE-ROPERH, DANIEL RUEDA, TERESA GÓMEZ DEL PULGAR, GUILLERMO VELASCO, and MANUEL GUZMÁN

Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Cannabinoids, the active components of marijuana and their endogenous counterparts, exert many of their actions in brain through the seven-transmembrane receptor CB$_1$. This receptor is coupled to the activation of the extracellular signal-regulated kinase (ERK) cascade. However, the precise molecular mechanism for CB$_1$-mediated ERK activation is still unknown. Here, we show that in U373 MG human astrocytoma cells, CB$_1$ receptor activation with the cannabinoid agonist Δ^8-tetrahydrocannabinol dimethyl heptyl (HU-210) was coupled to ERK activation and protection from ceramide-induced apoptosis. HU-210-induced ERK activation was inhibited by tyrphostin AG1478 and PP2, widely employed inhibitors of the epidermal growth factor receptor (EGF$_R$) and the Src family of cytosolic tyrosine kinases, respectively. However, HU-210 stimulation resulted in neither EGF$_R$ phosphorylation, Src tyrosine phosphorylation, nor increased Src activity. In addition, dominant-negative forms of both proteins were unable to prevent cannabinoid-induced ERK activation, thus excluding the existence of CB$_1$-mediated EGF$_R$ transactivation or Src activation. Wortmannin and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294,002), inhibitors of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, blocked cannabinoid-induced ERK activation. Likewise, HU-210 stimulated the PI3K downstream targets protein kinase B (PKB), as shown by its phosphorylation in Thr 308 and Ser 473 residues, and Raf-1. Moreover, $\beta\gamma$ subunit release mimicked ERK and PI3K/PKB activation, suggesting that activation of class IB PI3K mediates cannabinoid action. Pro-survival HU-210 action also required activation of both PI3K and ERK signaling pathways. In conclusion, CB$_1$-induced ERK activation was mediated by PI3K$_{IB}$ and this effect may have important consequences in the control of cell death/survival decision.

Cannabinoids, the active components of marijuana and their endogenous counterparts exert most of their actions through two well characterized seven-transmembrane receptors designated CB$_1$ and CB$_2$ (Matsuda et al., 1990; Munro et al., 1993). The CB$_1$ receptor is highly expressed in the central nervous system and is also present in peripheral and extraneural sites (Piomelli et al., 2000; Porter and Felder, 2001). In contrast, the CB$_2$ receptor is almost restricted to the immune system. The CB$_1$ receptor is known to be coupled to inhibition of adenylyl cyclase, inhibition of voltage-dependent Ca^{2+} channels, and activation of G-protein-regulated inwardly rectifying K$^+$ channels (Howlett, 1995; Porter and Felder, 2001). Besides these well established signal transduction events, novel possibilities have arisen to explain cannabinoid regulation of cell death/survival decision (Guzmán et al., 2001a,b). Thus the CB$_1$ receptor has been shown to regulate different members of the mitogen-activated protein kinase family, such as extracellular signal-regulated kinase (ERK) (Bouaboula et al., 1995; 1997; Sánchez et al., 1998), c-Jun N-terminal kinase (Liu et al., 2000; Rueda et al., 2000), and p38 (Galve-Roperh et al., 2000; Rueda et al., 2000). The CB$_1$ receptor can also activate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) signaling pathway (Gómez del Pulgar et al., 2000) and focal adhesion kinase (Derkinderen et al., 1996; 2001). In addition, the CB$_1$ receptor modulates sphingolipid metabolism, leading to increased ceramide levels by either activating sphingomyelin hydrolysis (Sánchez et al., 1998; 2001) or enhancing ceramide synthesis de novo (Gómez del Pulgar et al., 2002).

ABBREVIATIONS: ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PDGF, platelet derived growth factor; GST-Raf-RBD, glutathione *S*-transferase-Ras binding domain of Raf; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; AG1296, 6,7-dimethoxy-3-phenylquinoxaline; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; PD98059, 2′-amino-3′-methoxyflavone; LY294,002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; EGF$_R$, epidermal growth factor receptor; CP-55940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; WIN-55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; BB94, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(thiopen-2-ylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide.

A complex variety of mechanisms has been described to explain ERK regulation by seven-transmembrane receptors (reviewed in Gschwind et al., 2001; Marinissen and Gutkind, 2001; Pierce et al., 2001). In the case of G_i-coupled receptors such as CB_1, several mechanisms could be hypothesized to mediate ERK activation, including: 1) transactivation of growth factor receptors with intrinsic tyrosine kinase activity (Gschwind et al., 2001); 2) β-arrestin-mediated receptor internalization and recruiting of cytosolic tyrosine kinases of the Src family (Pierce et al., 2001; Marinissen and Gutkind, 2001); 3) G protein-dependent class IB PI3K activation (Lopez-Ilasaca et al., 1997; Marinissen and Gutkind, 2001). In the present work, we investigated how the CB_1 receptor is coupled to ERK activation and its implications in cannabinoid regulation of glial cell death/survival decision.

Materials and Methods

Reagents. The following materials were kindly donated: pcDNA3-CD533 encoding truncated EGF_R by Dr. A. Ullrich (Max-Planck Institute, Martinsried, Germany), pcDNA3-Src dominant-negative K295R by Dr. F. Mayor Jr. (Autónoma University, Madrid, Spain), the fusion construct of glutathione *S*-transferase and the Ras binding domain of Raf (GST-Raf-RBD) by Dr. J.L. Bos (Utrecht University, Utrecht, The Netherlands), SR141716 by Sanofi Synthelabo (Montpellier, France), HU-210 by Dr. R. Mechoulam (Hebrew University, Jerusalem, Israel), Src substrate peptide by Dr. F. Barahona (Autónoma University, Madrid, Spain), and BB94 by Dr. C. López Otín (Oviedo University, Oviedo, Spain). Tyrphostins AG1296 and AG1478 were from Calbiochem (San Diego, CA); PD98059, wortmannin and LY294,002 from Alexis Biochemicals (San Diego, CA); polyclonal anti-Raf-1, Src, and Fyn antibodies as well as monoclonal anti-phospho-ERK antibody from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-EGF_R antibody and monoclonal anti-phosphoTyr1173-EGF_R (pY1173- EGF_R) and anti-α-tubulin antibodies from Upstate Biotechnology (Lake Placid, NY); anti-phosphotyrosine monoclonal antibody clone PY20 from BD Transduction Laboratories (Lexington, KY); polyclonal anti-phosphoThr308-PKB (pT308-PKB) and phosphoSer473-PKB (pS473-PKB) from Cell Signaling Technology (Beverly, MA); and anti-phosphoTyr 416-Src (pY416-Src) from Calbiochem.

Cell Culture and Transfection. Human astrocytoma U373 MG cells were cultured as described previously (Rueda et al., 2000). Cells were transferred to serum-free medium 12 h before the experiments. Stock solutions of cannabinoids were prepared in dimethyl sulfoxide and control incubations had the corresponding dimethyl sulfoxide content. No significant influence of the vehicle was observed at the final concentration used (0.1%, v/v) in any of the parameters determined. Cells were stably transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) with the aforementioned constructs, and transfected cells were selected and maintained in culture in the presence of 0.5 mg/ml G418. At least three different clones of stably transfected cells were assessed for each dominant negative construct, obtaining similar results.

Apoptosis and Cell Viability. U373 MG cells were incubated in the presence of 10 μM C_2-ceramide for 1 h, medium was removed, and cells were subsequently incubated for 15 h in the presence of cannabinoids with different pharmacological inhibitors. Apoptosis was quantified by determination of the content of oligonucleosomal DNA fragments, a hallmark of apoptotic cell death using an enzyme-linked immunosorbent assay according to manufacturer instructions (Roche Applied Science, Mannheim, Germany). Cell lysates were incubated for 90 min in a 96-well microtiter plate previously coated with an anti-histone antibody clone H11–4. After washing, the plate was incubated with a mouse anti-DNA-peroxidase–labeled antibody and peroxidase activity was determined by measuring absorbance at

405 nm after substrate incubation. In addition, for some experiments, cell viability was determined by Trypan blue exclusion.

Western Blot Analysis. Western blots were performed as described previously (Galve-Roperh et al., 2000). After stimulation, cells were washed with ice-cold phosphate-buffered saline (phosphate-buffered saline; 10 mM NaPi, 150 mM NaCl, pH 7.4) and scraped in lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 0.5 μM microcystin-LR, 17.5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and 5 μg/ml benzamidine. Cell lysates cleared by 15 min of centrifugation at 12,000g were employed for the different experimental procedures. After protein normalization samples were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Galve-Roperh et al., 2000). EGF_R blotting was performed using 8% SDS-PAGE and high-voltage transfer conditions to allow high-molecular weight proteins to be efficiently transferred. After incubation with primary antibodies (1:1000), blots were developed with appropriate horseradish peroxidase-coupled secondary antibodies (1:20,000) and enhanced chemiluminescence detection kit. Loading controls were performed with an anti-α-tubulin antibody. Densitometric analysis of the luminograms was performed using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and MultiAnalyst software. Relative values of optical density of the representative experiment shown are included in the figures.

Immunoprecipitation and Src Activity Assay. Cleared cell lysates (500 μg protein) were incubated with 2 μg of anti-EGF_R, Src, or Fyn antibodies precoupled to protein G-Sepharose. After washing with lysis buffer, EGF_R and Src phosphorylation status was determined in the immunoprecipitate by Western blot using the PY20 antibody. In addition, Western blot analyses of total cell extracts were performed with an anti-pY1173-EGF_R or anti-pY416-Src antibody. For Src activity assays, immunoprecipitated proteins were transferred to Src activity buffer consisting of 100 mM Tris-HCl, pH 7.2, 50 mM magnesium acetate, 10 mM $MnCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 0.5 μM microcystin-LR, 17.5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and 5 μg/ml benzamidine. Kinase activity was determined in the presence of 200 μM ATP, 5 μCi of $[\gamma-^{32}P]$ATP, and 500 μM substrate peptide corresponding to amino acids 6 to 20 of p34^{cdc2}. Endogenous phosphorylation was also determined in parallel. After 10 min, reactions were stopped with 75 mM orthophosphoric acid, samples were centrifugated, and supernatants spotted on Whatman P81 filter paper (Whatman, Maidstone, UK). Filters were washed and radioactivity was determined by liquid scintillation counting.

Ras Activation. The fusion construct GST-Raf-RBD was overexpressed in *E. coli* DH5α following standard procedures. Cleared cell lysates (250 μg of protein) were incubated in the presence of GST-Raf-RBD precoupled to agarose-glutathione complexes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) to detect activated Ras^{GTP}. Beads were extensively washed with phosphate-buffered saline and, after SDS-PAGE, immunoblotting was performed using monoclonal anti-pan-Ras antibody (Oncogene, Boston, MA).

Raf-1 Activity. Raf-1 activity assays were performed as described previously (Galve-Roperh et al., 2000). In summary, immunoprecipitated Raf-1 was incubated in kinase buffer in the presence of 1 μg of myelin basic protein and $[\gamma-^{32}P]$ATP, reactions were stopped with SDS sample buffer and substrate phosphorylation was determined after SDS-PAGE and autoradiography.

Statistical Analysis. Results shown represent the means ± S.D. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance. A post hoc analysis was made by the Student-Neuman-Keuls test.

Results

CB$_1$-Induced ERK Activation Protects Astrocytoma Cells from Ceramide-Induced Apoptosis. To elucidate the mechanism of cannabinoid activation of the ERK pathway, we first examined cannabinoid-induced ERK activation in the presence of different pharmacological inhibitors using a monoclonal anti-phospho-Tyr 204 antibody that recognizes phosphorylated ERK. CB$_1$ stimulation with the cannabinoid agonist HU-210 resulted in a time-dependent ERK activation (data not shown) that peaked at 10 min (Fig. 1A). This effect was completely prevented by the selective CB$_1$ antagonist SR141716, thus evidencing the involvement of this receptor. To determine whether transactivation of receptors with intrinsic tyrosine kinase activity such as EGF$_R$ or platelet-derived growth factor receptor could be responsible for CB$_1$-induced ERK activation, we employed inhibitors of tyrosine kinase activity with different selectivity. Tyrphostin AG1478, a selective EGF$_R$ inhibitor, but not tyrphostin AG1296, a selective platelet-derived growth factor receptor inhibitor, abrogated HU-210–induced ERK activation (Fig. 1A). In addition, the pyrazolopyrimidine PP2 and herbimycin A, two inhibitors of the Src family of cytosolic tyrosine kinases, blocked cannabinoid-induced ERK activation (Fig. 1A). The concentration-dependent action of tyrphostin AG1478 and PP2 on HU-210-induced ERK activation was determined (data not shown), allowing us to calculate IC$_{50}$ values of 0.1 and 0.8 μM, respectively.

The important role of the mitogenic ERK cascade in the control of cell death/survival decision (Grewal et al., 1999; Kolch, 2000) prompted us to investigate the functional consequences of CB$_1$-induced ERK activation in astrocytoma cells. Cells incubated with the permeable ceramide analog C$_2$-ceramide underwent apoptosis (Fig. 1B), and HU-210 inhibited this process. Moreover, exposure to other synthetic cannabinoids, such as CP-55940 and WIN-55,212-2 mimicked the antiapoptotic action of HU-210 (Fig. 1C). Cannabinoid pro-survival action relied on CB$_1$ receptor activation, as evidenced by SR141716 antagonism (Fig. 1C). To investigate the potential involvement of the ERK cascade in CB$_1$-mediated protection from apoptosis, we employed the specific mitogen-activated protein kinase kinase inhibitor PD98059. This compound abrogated cannabinoid prosurvival action, thus indicating the involvement of the ERK signaling pathway. Moreover, like cannabinoid-induced ERK activation, cannabinoid prosurvival effect was also inhibited by tyrphostin AG1478. These data show that CB$_1$-induced ERK activation is functionally relevant in our experimental model and results in cell protection from apoptosis.

CB$_1$-Induced ERK Activation Does Not Involve EGF$_R$ Transactivation. Next, we examined whether CB$_1$-mediated transactivation of EGF$_R$ could be responsible for ERK activation. Although tyrphostin AG1478 prevented HU-210-induced ERK activation (Fig. 1A), HU-210 was not able to induce EGF$_R$ phosphorylation (Fig. 2A). Under the same experimental conditions, EGF potently increased tyrosine phosphorylation of immunoprecipitated EGF$_R$, and this effect was indeed blocked by tyrphostin AG1478. Similar results were obtained with an antibody against activated EGF$_R$ that specifically recognizes phosphorylated Tyr 1173. HU-210 was ineffective in eliciting EGF$_R$ phosphorylation, whereas EGF induced EGF$_R$ phosphorylation (Fig. 2B). The

apparent contradiction of the results obtained with tyrphostin AG1478 in ERK activation assays and EGF$_R$ phosphorylation experiments required further analysis. Therefore, we stably transfected U373 MG cells with CD533, a truncated form of EGF$_R$ that acts as a dominant negative form (Redemann et al., 1992). CD533-U373 MG cells did not show reduced cannabinoid-induced ERK activation (Fig. 2C), in line with the lack of involvement of EGF$_R$ in HU-210-induced ERK activation. In contrast, EGF-induced EGF$_R$ phosphorylation was effectively abrogated (Fig. 2D), evidencing the effectiveness of the stably generated CD533-U373 MG cells to block EGF signaling. In addition, BB94, an inhibitor of zinc-dependent metalloproteinases responsible for proheparin binding-EGF shedding required for G-protein-coupled receptor transactivation of EGF$_R$ (Prenzel et al., 1999), did not prevent HU-210-induced ERK activation (data not shown), supporting the absence of CB$_1$-mediated EGF$_R$ transactivation.

Lack of Involvement of Src Tyrosine Kinases in CB$_1$-Induced ERK Activation. To investigate the potential involvement of the Src family of tyrosine kinases in CB$_1$ signaling, kinase activity assays were performed. Immunoprecipitation from U373 MG cell extracts of Src, Fyn, and Yes using an anti-pan Src antibody did not reveal any increased activity of those kinases by HU-210 stimulation, whereas under the same conditions, PDGF induced a robust Src activation(Fig. 3A). Moreover, time course analysis from 5 to 60 min did not evidence stimulation of pan-Src activity by HU-210 (data not shown). As cannabinoid-induced focal adhesion kinase regulation in hippocampal neurons has been proposed to be specifically mediated by Fyn (Derkinderen et al., 2001), we examined Fyn kinase activity using a specific antibody against this protein. However, no changes in Fyn activity could be detected (Fig. 3A).

To fully address the question of cannabinoid regulation of Src, we have also analyzed its phosphorylation status. Immunoprecipitation with anti-PY20 antibody and subsequent detection with the Src antibody revealed an increased global tyrosine phosphorylation of Src induced by PDGF but not HU-210 (Fig. 3B). Because tyrosine phosphorylation can reflect either increased or blockade of Src activity (Tyr 416 versus Tyr 527; Martin, 2001) we have also employed a monoclonal antibody against phosphorylated Tyr 416 of Src that reflects the activation status of the protein. Again, Western blot results show the lack of phosphorylation/activation of Src by HU-210 stimulation (Fig. 3B). Moreover, enforced expression of dominant negative Src-K295R did not affect HU-210–induced ERK activation (Fig. 3C), whereas it efficiently blocked PDGF stimulation of Src activity (data not shown) because of its overexpression in transfected cells (Fig. 3D).

Involvement of the PI3K/PKB Pathway in CB$_1$-Induced ERK Activation and Cell Survival. Because G protein $\beta\gamma$ subunit release may activate class IB PI3K leading to ERK activation (Lopez-Ilasaca et al., 1997; Marinissen and Gutkind, 2001), we examined whether this process occurred upon CB$_1$ activation by employing wortmannin and LY294,002, two PI3K inhibitors. As shown in Fig. 4A, HU-210-induced ERK activation was completely abrogated by PI3K inhibition. Moreover, mastoparan-induced G protein $\beta\gamma$ subunit release led to ERK activation, and such effect was not additive to HU-210 action (Fig. 4A). Support for the involvement of the PI3K signaling pathway was obtained by
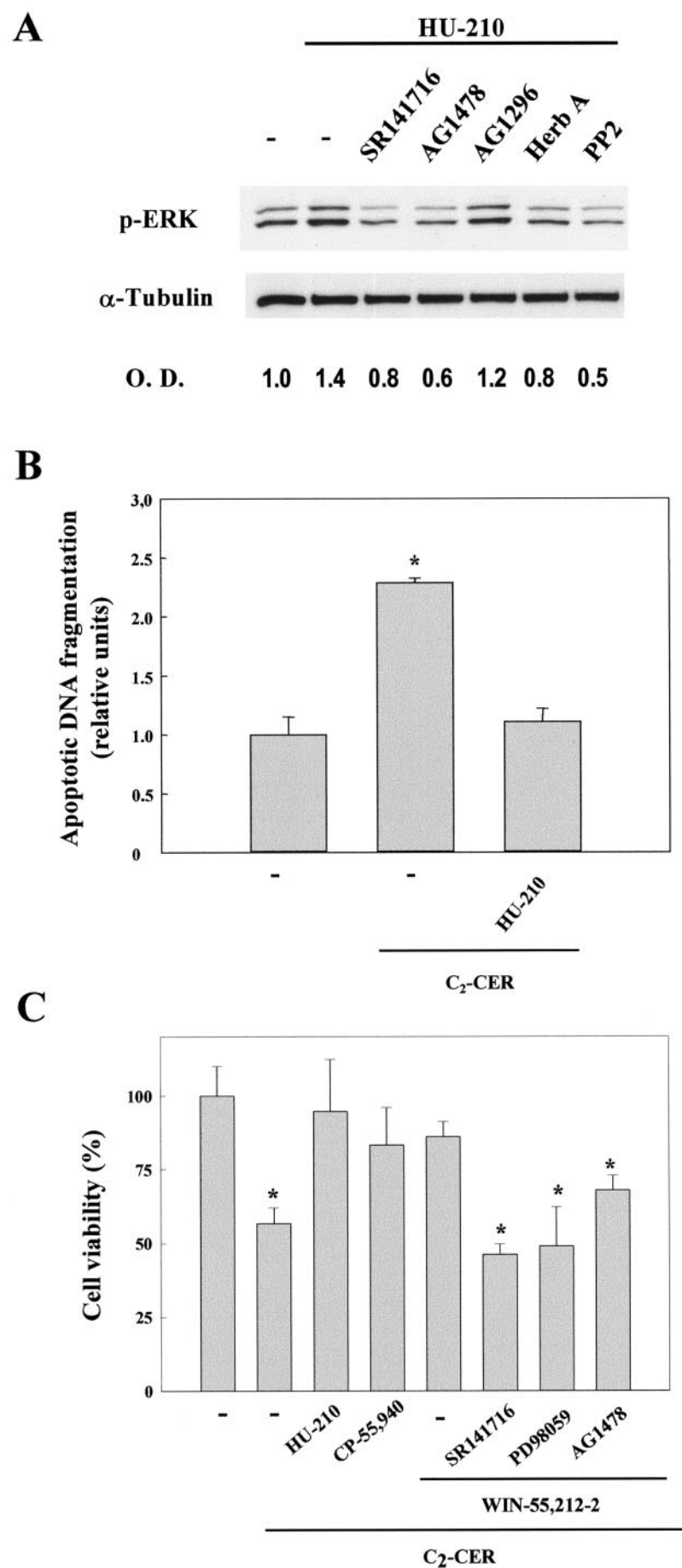
A

B

C

Fig. 1. CB$_1$-induced ERK activation protects astrocytoma cells from ceramide-induced apoptosis. A, U373 MG cells were incubated with 50 nM HU-210 for 10 min either alone or after 30-min preincubation with 1 μM SR141716, 1 μM tyrphostin AG1478, 30 μM tyrphostin AG1296, 10 μM herbimycin A (HerbA), or 10 μM pyrazolopyrimidine PP2. Cell lysates were subjected to Western blot analysis with an anti-phospho-ERK antibody. Loading control was carried out with an anti–α-tubulin antibody. O.D., optical density. B, U373 MG cells were incubated in the presence of 10 μM C$_2$-ceramide (C$_2$-CER) for 1 h, the medium was removed, and cells were incubated for 15 h in the presence of vehicle or 50 nM HU-210. Apoptosis was quantified by determination of oligonucleosomal DNA fragmentation. C, after C$_2$-ceramide–induced apoptotic stimulation, U373 MG cells were incubated in the presence of HU-210, CP-55940, or WIN-55,212-2 (50 nM). WIN-55,212-2 incubations were also performed in the presence of 1 μM SR141716, 25 μM PD98059, or 1 μM tyrphostin AG1478. Cell viability was determined 15 h later by Trypan blue exclusion. Results correspond in A to a representative experiment of six independent experiments and in B and C to three and four different experiments, respectively. *, $P < 0.01$, significantly different from vehicle incubations.

showing that its primary downstream target PKB was regulated in a similar manner. Phosphorylation of PKB occurs in different residues that represent progressive steps in its activation mechanism (Brazil and Hemmings, 2001). Thus, HU-210 challenge induced PKB Thr 308 phosphorylation (Fig. 4B), which reflects PI3K/PDK1-mediated phosphorylation. HU-210 also induced PKB Ser 473 phosphorylation (Fig. 4B), indicative of the last activation step upon PI3K stimulation. Phosphorylation of both residues was antagonized by SR141716, pointing to the involvement of CB$_1$ receptor. In addition, PI3K inhibition by wortmannin and LY294,002 abrogated HU-210–induced phosphorylation of both PKB residues and therefore validated ERK data obtained with these inhibitors. Moreover, mastoparan-induced $\beta\gamma$ subunit release increased PKB phosphorylation in both residues, and this effect was not additive to that of HU-210 (Fig. 4B). We next determined whether Ras was involved in ERK activation given that PI3K-dependent ERK activation might be mediated by this monomeric G protein (Lopez-Ilasaca et al., 1997). However, HU-210 stimulation did not induce Ras activation in U373 MG cells (Fig. 4C), pointing to a PI3K-dependent mechanism of ERK activation that does not involve Ras activation. A similar mechanism has been proposed for PI3K activation of the ERK cascade by direct PAK-mediated Raf-1 phosphorylation (King et al., 1998; Chaudhary et al., 2000; Zang et al., 2001). Likewise, CB$_1$ receptor activation enhances Raf-1 activity in U373 MG cells (Fig. 4D), similarly to what occurs in primary astrocytes (Sánchez et al., 1998) and C6 glioma cells (Galve-Roperh et al., 2000).

Confirmation of the functional relevance of PI3K-dependent ERK activation was investigated in the U373 MG cell model of cannabinoid anti-apoptotic action. Cannabinoid-mediated protection from ceramide-induced cell death was abrogated by wortmannin and LY294,002 (Fig. 4E). Thus, cannabinoids exert an effective pro-survival action because of their ability to activate in a concerted manner the classical
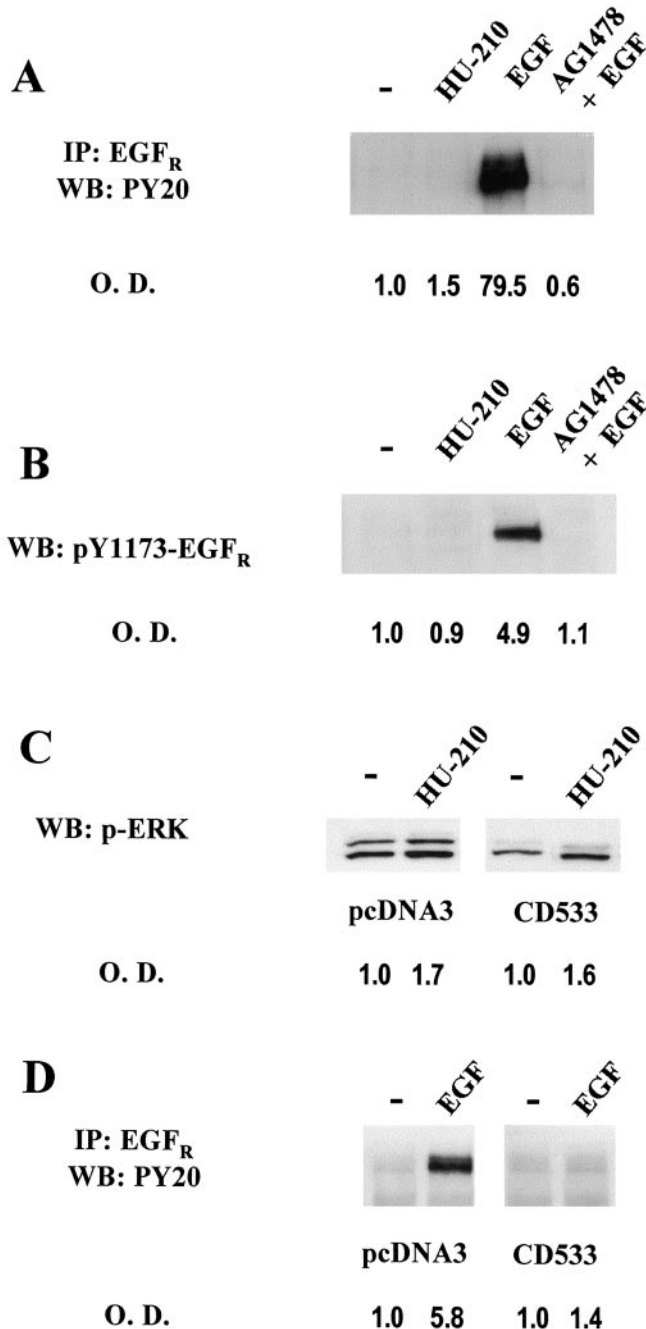

Fig. 2. CB$_1$-induced ERK activation does not involve EGF$_R$ transactivation. O.D., optical density. A, after stimulation with 50 nM HU-210 or 100 ng/ml EGF in the absence or presence of 1 μM tyrphostin AG1478, U373 MG cell extracts were immunoprecipitated (IP) with an anti-EGF$_R$ antibody and Western blot (WB) was performed using anti-phosphotyrosine PY20 antibody. B, cell lysates from stimulated cells were subjected to Western blot analysis using an anti-phospho-Tyr-1173-EGF$_R$ antibody. C, Western blot analysis of phospho-ERK in vehicle or HU-210–stimulated cell lysates from vector- and CD533-stably transfected U373 MG cells. D, tyrosine phosphorylation status of immunoprecipitated EGF$_R$ in vector- and CD533-U373 MG cells after EGF stimulation. Representative luminograms are shown. Similar results were obtained in at least two other experiments for each.
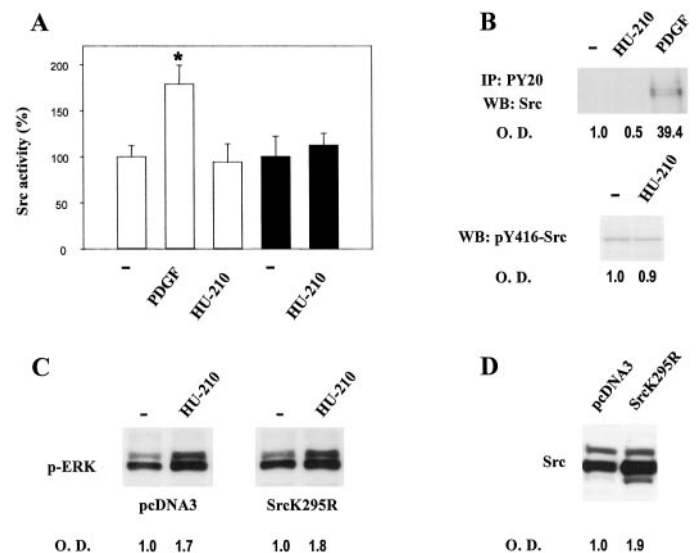

Fig. 3. Lack of involvement of Src tyrosine kinases in CB$_1$-induced ERK activation. O.D., optical density. A, Src family (Src, Fyn, and Yes, $\square$) or Fyn activity ($\blacksquare$) as determined by immunoprecipitation (IP) with an anti-pan-Src antibody or anti-Fyn antibody from lysates of cells stimulated with 50 ng/ml PDGF for 5 min or 50 nM HU-210 for 10 min. B, tyrosine phosphorylation status of Src after cell stimulation. Top, Src Western blot of PY20 immunoprecipitates; bottom, phosphorylated Tyr 416 of Src in total cell extracts using an specific antibody. C, Western blot analysis with anti-phospho-ERK antibody in vector and dominant-negative Src-K295R stably transfected U373 MG cells after vehicle or HU-210 stimulation. D, overexpression of Src in stable Src-K295R-transfected cells as evidenced by Western blot with an anti-Src antibody. Results correspond to six different experiments (A), and representative luminograms of three (B), six (C), and three (D) independent experiments.
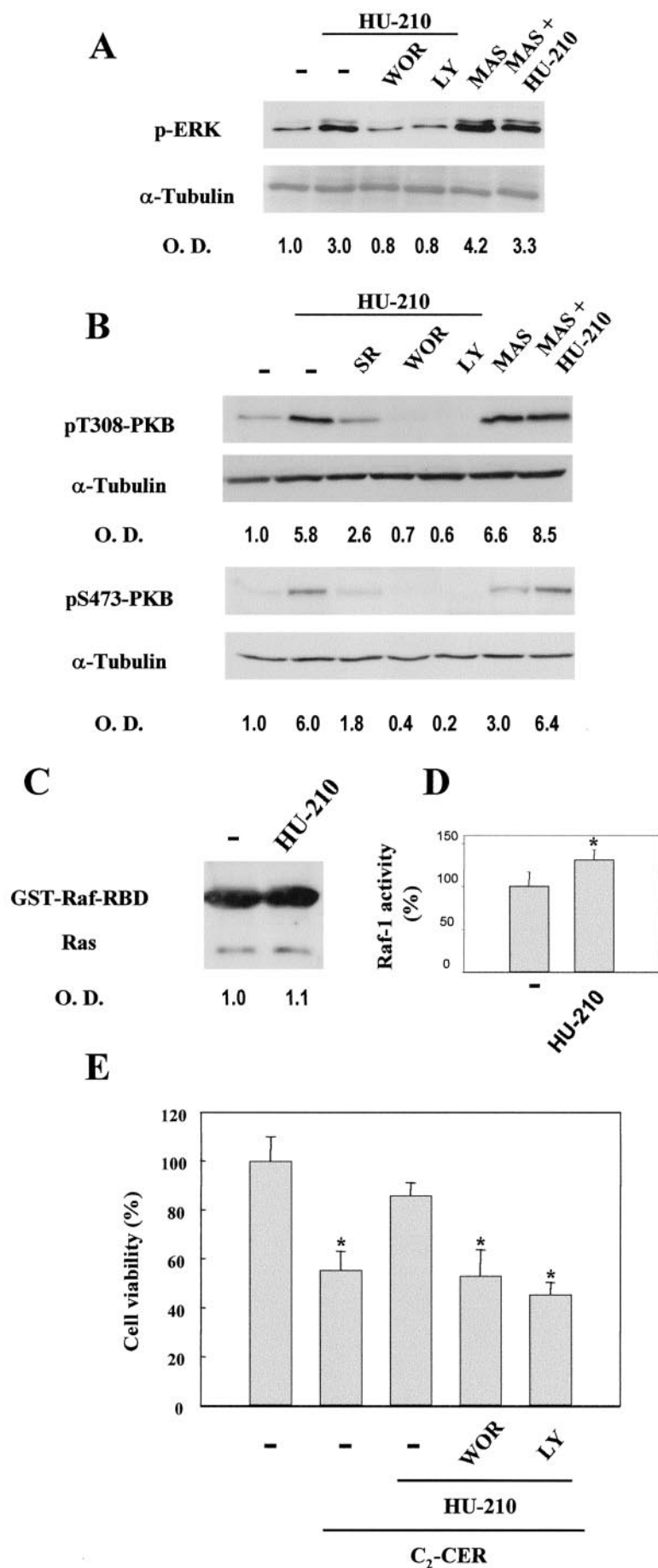
A

HU-210

— — WOR LY MAS MAS + HU-210

p-ERK

α-Tubulin

O. D.   1.0 3.0 0.8 0.8 4.2 3.3

B

HU-210

— — SR WOR LY MAS MAS + HU-210

pT308-PKB

α-Tubulin

O. D.   1.0 5.8 2.6 0.7 0.6 6.6 8.5

pS473-PKB

α-Tubulin

O. D.   1.0 6.0 1.8 0.4 0.2 3.0 6.4

C

— HU-210

GST-Raf-RBD

Ras

O. D.   1.0 1.1

D

Raf-1 activity (%)

— HU-210

E

Cell viability (%)

— — — WOR LY

HU-210

C$_2$-CER

Fig. 4. Involvement of the PI3K/PKB pathway in cannabinoid-induced ERK activation and cell survival. O.D., optical density. A, Western blot analysis of phospho-ERK in cells stimulated with vehicle or 50 nM HU-210 alone or in the presence of 200 nM wortmannin (WOR), 25 μM LY294,002 (LY), or 15 μM mastoparan (MAS). B, phosphorylation status of PKB as determined with antibodies against Thr 308 (top) or Ser 473 (bottom). Loading controls were carried out with an anti–α-tubulin antibody. C, Ras activation was determined after 50 nM HU-210 stimulation in cell extracts by affinity-precipitation of active Ras with GST-Raf-RBD precoupled to GSH-agarose and subsequent Western blot with an anti-Ras antibody. D, Raf-1 activity was determined by immunoprecipitation of Raf-1 from HU-210–stimulated cells. E, cell viability of U373 MG cells after 10 μM C$_2$-ceramide (C$_2$-CER) challenge and subsequent exposure to 50 nM HU-210 alone or in the presence of 200 nM wortmannin (WOR) or 25 μM LY294,002 (LY). Results correspond to four different experiments for each. *, $P <$ 0.01, significantly different from vehicle incubations.

survival signaling pathways PI3K/PKB and ERK via the CB$_1$ receptor.

Discussion

Results presented herein show that CB$_1$ receptor coupling to ERK activation depends on Gi protein dissociation and subsequent PI3K$_{IB}$ activation. Although many G protein-coupled receptors activate different members of the mitogen-activated protein kinase family by transactivation of tyrosine kinase receptors (Gschwind et al., 2001), CB$_1$-mediated activation of ERK occurs by an EGF$_R$-independent mechanism. In addition, seven-transmembrane receptors have been shown to recruit Src family members during the β-arrestin–mediated internalization process, which in certain cases is coupled to ERK activation (Marinissen and Gutkind, 2001; Pierce et al., 2001). Regarding the CB$_1$ receptor, our results exclude the involvement of the Src family of cytosolic tyrosine kinases in ERK activation, in line with data showing that internalization-defective CB$_1$ receptors exhibit correct ERK activation capacity (Roche et al., 1999).

As previously highlighted by others (e.g., Davies et al., 2000), the study of cannabinoid-induced ERK activation has taught us the necessity for caution when interpreting data obtained using pharmacological inhibitors claimed in the literature to be highly selective. Thus, whereas experiments conducted with pharmacological inhibitors suggested the involvement of certain mechanism for CB$_1$-mediated ERK activation, detailed study of such pathways (EGF$_R$ transactivation or Src involvement) was fruitless and pointed in different directions. It should be noted that the IC$_{50}$ values for AG1478 and PP2 action obtained in this study (0.1 and 0.8 μM, respectively) indicate that the concentrations employed (1 and 10 μM) are high enough to inhibit the desired target (EGF$_R$ and Src) but not so massive as to explain the nonspecific actions observed. The existence of potential targets for tyrphostins different from the paradigmatic ones can be exemplified by tyrphostin AG213, which, although claimed to be specific for the EGF$_R$, also exerts complex actions on the EGF-ERK signaling pathway (Levitzki, 1999). Paradoxical stimulation of the ERK pathway by tyrphostins has been described and attributed to a potential inhibitory effect on tyrosine phosphatase activity (Nowak et al., 1997). In addition, different tyrphostins (AG213, AG34, and AG82) have been shown to alter Src-initiated signaling and cellular transformation, as well as to inhibit Bcr-Abl tyrosine kinase (Agbotounou et al., 1994; Levitzki, 1999). Moreover, in astrocytes, tyrphostin AG1478 was able to prevent thrombin-induced ERK activation, but EGF$_R$ transactivation could not be evidenced (Wang et al., 2002). Like quinoline-derived tyrphostins, pyrazolopirimidine-derived inhibitors of the Src family, such as PP2, act as ATP binding competitors. In this regard, interference of both types of inhibitors toward unrelated postulated targets has been described. Thus, in certain cases, tyrphostins may inhibit Src family-mediated actions (Agbotounou et al., 1994; Levitzki, 1999) and pyrazolopirimidines may inhibit tyrosine kinase receptors (Susa et al., 2000).

Our results highlight the existence of cannabinoid-induced activation of survival signaling pathways in a coordinated manner. Thus, CB$_1$ activation results in PI3K/PKB stimulation (Gómez del Pulgar et al., 2000; this study) in concert with the ERK pathway (Guzmán et al., 2001b; this study). The important role of the mitogenic ERK cascade (Grewal et al., 1999; Kolch, 2000) as well as of the anti-apoptotic PI3K/PKB pathway (Brazil and Hemmings, 2001) in the control of the cell death/survival decision is well known. In general, activation of these signaling pathways leads to cell protection from death stimuli by acting either independently or coordinately. Thus, CB$_1$-mediated acute ERK and PI3K/PKB activation protects glial cells from ceramide induction of the apoptotic program. On the other hand, CB$_1$-activation can also promote apoptosis, particularly in transformed cells, owing to its ability to induce sustained ceramide generation and ERK activation (Galve-Roperh et al., 2000; Guzmán et al., 2001b). In fact, recent research has evidenced growth arrest and deleterious actions of the ERK pathway when activated in a sustained manner. For example, sustained ERK activation is in part responsible for neural cell death after brain ischemia or glutamate excitotoxicity (Alessandrini et al., 1999). Similarly, in PC12 neuronal-like cells, whereas short-term ERK activation promotes proliferation, sustained ERK activation results in cell cycle arrest and neuronal differentiation (Grewal et al., 1999). Regarding glial cells, different magnitude and kinetics of CB$_1$-induced ERK activation produces opposite cellular outcomes (Guzmán et al., 2001a). Thus, whereas short-term ERK activation protects glial cells from ceramide-induced apoptosis (this study), sustained ERK activation promotes apoptosis or growth arrest (Galve-Roperh et al., 2000; Fanton et al., 2001). Current research in our laboratory focuses on the identification of the downstream targets of these signaling pathways that would be ultimately responsible for CB$_1$-mediated cell protection or death.

References

Agbotounou WK, Levitzky A, Jacquemin-Sablon A, and Pierre J (1994) Effects of tyrphostins on the activated c-src protein in NIH/3T3 cells. *Mol Pharmacol* **45:** 922–931.

Alessandrini A, Namura S, Moskowitz MA, and Bonventre JV (1999) MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proc Natl Acad Sci USA* **96:**12866–12869.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, et al. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–2233

Bouaboula M, Poinot Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, and Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312:**637–641. 9.

Brazil DP and Hemmings B (2001) Ten years of protein kinase B signaling: a hard Akt to follow. *Trends Biochem Sci* **26:**657–664.

Chaudhary A, King WG, Mattaliano MD, Frost JA, Día B, Morrison DK, Cobb MH, Marshall MS, and Brugge JS (2000) Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. *Curr Biol* **10:**551–554.

Davies SP, Reddy H, Caivano M, and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351:**95–105.

Derkinderen P, Toutant M, Burgaya F, Le Bert M, Siciliano JC, de Franciscis V, Gelman M, and Girault J (1996) Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science (Wash DC)* **273:**1719–1723.

Derkinderen P, Toutant M, Kadaré G, Ledent C, Parmentier M, and Girault JA (2001) Dual role of Fyn in the regulation of FAK$^+$6, 7 by cannabinoids in hippocampus. *J Biol Chem* **276:**38289–38296.

Fanton CP, McMahon M, and Pieper RO (2001) Dual growth arrest pathways in

astrocytes and astrocytic tumors in response to Raf-1 activation. *J Biol Chem* **276:**18871–18877.

Galve-Roperh I, Sánchez C, Cortés ML, Gómez del Pulgar T, Izquierdo M, and Guzmán M (2000) Antitumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med* **6:**313–319.

Gómez del Pulgar T, Velasco G, and Guzmán M (2000) The CB_1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem J* **347:**369–373.

Gómez del Pulgar T, Velasco G, Sánchez C, Haro A, and Guzmán M (2002) De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis. *Biochem J* **363:**183–188.

Grewal SS, York RD, and Stork PJS (1999) Extracellular-signal regulated kinase signalling in neurons. *Curr Opin Neurobiol* **9:**544–553.

Gschwind A, Zwick E, Prenzel N, Leserer M, and Ullrich A (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* **20:**1594–600.

Guzmán M, Galve-Roperh I, and Sánchez C (2001a) Ceramide, a new second messenger of cannabinoid action. *Trends Pharmacol Sci* **22:**9–22.

Guzmán M, Sánchez C, and Galve-Roperh I (2001b) Control of the cell survival/death decision by cannabinoids. *J Mol Med* **78:**613–625.

Howlett AC (1995) Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35:**607–634.

King AJ, Sun H, Díaz B, Barnard D, Miao W, Bagrodia S, and Marshall MS (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature (Lond)* **396:**180–183.

Kolch W (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* **351:**289–305.

Levitzki A (1999) Protein tyrosine kinase inhibitors as novel therapeutic agents. *Pharmacol Ther* **82:**231–239.

Liu J, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyanis A, and Kunos G (2000) Functional CB_1 cannabinoid receptors in human vascular endothelial cells. *Biochem J* **346:**835–840.

Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS, and Wetzker R (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ. *Science (Wash DC)* **275:**394–397.

Marinissen MJ and Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* **22:**368–376.

Martin GS (2001) The hunting of the Src. *Nat Rev Mol Cell Biol* **2:**467–473.

Matsuda LA, Lolait SJ, Brownstein M, Young A, and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346:**561–564.

Munro S, Thomas KL, and Abu Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365:**61–65.

Nowak F, Jacquemin-Sablon A, and Pierre J (1997) Epidermal growth factor receptor signalling cascade as target for thyrphostin (RG50864) in epithelial cells. *Biochem Pharmacol* **53:**287–298.

Piomelli D, Giuffrida A, Calignano A, and Rodríguez de Fonseca F (2000) The endocannabinoid system as a target for therapeutic drugs. *Trends Pharmacol Sci* **21:**218–224.

Pierce KL, Luttrell LM, and Lefkowitz RJ (2001) New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene* **20:**1532–1539.

Porter AC and Felder CC (2001) The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacol Ther* **90:**45–60.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, and Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature (Lond)* **402:**884–888.

Redemann N, Holzmann B, von Ruden T, Wagner EF, Schlessinger J, and Ullrich A (1992) Anti-oncogenic activity of signalling-defective epidermal growth factor receptor mutants. *Mol Cell Biol* **12:**491–498.

Roche JP, Bounds S, Brown S, and Mackie K (1999) A mutation in the second transmembrane region of the CB1 receptor selectively disrupts G protein signaling and prevents receptor internalization. *Mol Pharmacol* **56:**611–618.

Rueda D, Galve-Roperh I, Haro A, and Guzmán M (2000) The CB_1 cannabinoid receptor is coupled to the action of c-Jun N-terminal kinase. *Mol Pharmacol* **58:**814–820.

Sánchez C, Galve-Roperh I, Rueda D, and Guzmán M (1998) Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Δ^9-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. *Mol Pharmacol* **54:**834–843.

Sánchez C, Rueda D, Segui B, Galve-Roperh I, Levade T, and Guzmán M (2001) The CB_1 cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolisis through the adaptor protein Fan. *Mol Pharmacol* **59:**955–959.

Susa M, Missbach M, and Green J (2000) Src inhibitors: drugs for the treatment of osteoporosis, cancer or both? *Trends Pharmacol Sci* **21:**489–495.

Wang H, Ubl JJ, Stricker R, and Reisser G (2002) Thrombin (PAR-1)-induced proliferation in astrocytes via MAP kinase involves multiple signalling pathways. *Am J Physiol Cell Physiol* 10.1152/ajpcell. 00001.2002.

Zang M, Waelde CA, Xiang X, Rana A, Wen R, and Luo Z (2001) Microtubule integrity regulates Pak leading to Ras-independent activation of Raf-1. *J Biol Chem* **276:**25157–25165.

Address correspondence to: Dr. Ismael Galve-Roperh, Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, 28040 Madrid, Spain. E-mail: igr@bbm1.ucm.es