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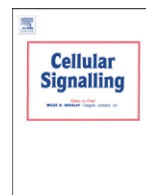

CB_1 cannabinoid receptors promote maximal FAK catalytic activity by stimulating cooperative signaling between receptor tyrosine kinases and integrins in neuronal cells

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Tyrosine phosphorylation (Tyr-P) of focal adhesion kinase (FAK) regulates FAK activation. Phosphorylated FAK Tyr 397 binds Src family kinases (Src), which in turn directly phosphorylate FAK Tyr 576/577 to produce maximal FAK enzymatic activity. CB_1 cannabinoid receptors (CB_1) are abundantly expressed in the nervous system and influence FAK activation by presently unknown mechanisms. The current investigation determined that CB_1-stimulated maximal FAK catalytic activity is mediated by $G_{i/o}$ proteins in N18TG2 neuronal cells, and that $G_{12/13}$ regulation of Rac1 and RhoA occurs concomitantly. Immunoblotting analyses using antibodies against FAK phospho-Tyr 397 and phospho-Tyr 576/577 demonstrated that the time-course of CB_1-stimulated FAK 576/577 Tyr-P occurred in three phases: Phase I (0–2 min) maximal Tyr-P, Phase II (5–20 min) rapid decline in Tyr-P, and Phase III (>20 min) plateau in Tyr-P at submaximal levels. In contrast, FAK 397 Tyr-P was monophasic and significantly lower in magnitude. FAK 397 Tyr-P and Phase I FAK 576/577 Tyr-P involved protein tyrosine phosphatase (PTP1B and Shp1/Shp2)-mediated Src activation, Protein Kinase A (PKA) inhibition, and integrin activation. Phase I maximal FAK 576/577 Tyr-P also required cooperative signaling between receptor tyrosine kinases (RTKs) and integrins. The integrin antagonist RGDS peptide, Flk-1 vascular endothelial growth factor receptor (VEGFR) antagonist SU5416, and epidermal growth factor receptor (EGFR) antagonist AG 1478 blocked Phase I FAK 576/577 Tyr-P. CB_1 agonists failed to stimulate FAK Tyr-P in the absence of integrin activation upon suspension in serum-free culture media. In contrast, cells grown on the integrin ligands fibronectin and laminin displayed increased FAK 576/577 Tyr-P that was augmented by CB_1 agonists and blocked by the Src inhibitor PP2 and Flk-1 VEGFR antagonist SU5416. Taken together, these studies have identified a complex integrative pathway utilized by CB_1 to stimulate maximal FAK 576/577 Tyr-P in neuronal cells.

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1. Introduction

CB_1 receptors are predominantly expressed in the nervous system and mediate many of the neuronal effects produced by the major psychoactive component of *Cannabis sativa* Δ^9-THC, the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG), and synthetic cannabinoid drugs (e.g., CP55940 and WIN55212-2) (see [1] for review). CB_1 is a G protein-coupled receptor (GPCR) that associates with pertussis toxin-sensitive $G_{i/o}$ proteins to regulate a variety of signal transduction pathways including inhibition of adenylyl cyclase, inhibition of L-, N-, and P/Q-type Ca^{2+} channels, induction of immediate early gene expression, stimulation of nitric oxide production, activation of members of the mitogen-activated protein kinase (MAPK) family, and activation of FAK [1,2]. FAK is a ubiquitously expressed nonreceptor protein tyrosine kinase that localizes to multi-protein complexes found at the cell membrane called focal adhesions (FAs) in which integrins link the actin cytoskeleton to proteins of the extracellular matrix (ECM) [3]. Activated FAK mediates many of the downstream signaling events emanating from FAs that regulate cell proliferation, survival, migration, and adhesion [3,4]. FAK activation occurs through Tyr-P and begins with FAK phosphorylation at Tyr 397 which creates a high affinity binding

Abbreviations: 2-AG, 2-arachidonoylglycerol; BSA, bovine serum albumin; CB_1, CB_1 cannabinoid receptor; Csk, C-terminal Src kinase; DAGL, diacylglycerol lipase; DTT, dithiothreitol; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinases 1 and 2; EGFR, epidermal growth factor receptor; FA, focal adhesion; FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; PKA, Protein Kinase A; RTK, receptor tyrosine kinase; SDS, sodium dodecyl sulfate; Sp-cAMPS, adenosine 3′,5′-cyclic phosphorothioate-Sp; Src, Src family kinase; THL, tetrahydrolipstatin; Tyr-P, tyrosine phosphorylation; VEGFR, vascular endothelial growth factor receptor.

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site for Src that then phosphorylates FAK on five additional Tyr residues (Tyr 407, Tyr 576/577, Tyr 861, and Tyr 925) [5–7]. Tyr 576/577 are located in the activation loop of the FAK central catalytic domain and their phosphorylation is required for maximal FAK catalytic activity. Studies have shed minimal light on the cellular mechanisms that regulate CB_1-mediated FAK activation which appears to involve integrin activation, PKA inhibition, and Src activation [8–10]. During development of the central nervous system, endocannabinoid signaling networks regulate proliferation, migration, specification, and survival of neural progenitors [11,12]. Given the crucial role of FAK in these biological processes, it is important to gain a better understanding of the cellular and molecular mechanisms that regulate CB_1-FAK signaling pathways in neuronal cells [4].

The aim of the present study was to investigate the signaling pathways that regulate CB_1-stimulated maximal FAK catalytic activation in neuronal N18TG2 cells that express endogenous CB_1 receptors. To accomplish this, immunoblotting analyses were conducted using phosphorylation site-specific antibodies against FAK Tyr 576/577 and Tyr 397. Our results revealed the time-course of CB_1-mediated FAK 397 and 576/577 Tyr-P are markedly different in N18TG2 cells. FAK 576/577 Tyr-P occurred in three phases: Phase I (0–2 min) involved maximal Tyr-P, Phase II (5–20 min) involved a rapid decline in Tyr-P, and Phase III (>20 min) involved a plateau in Tyr-P at submaximal levels. In contrast, FAK 397 Tyr-P was monophasic and significantly lower in magnitude. CB_1-mediated FAK 397 Tyr-P and Phase I FAK 576/577 Tyr-P involved protein tyrosine phosphatase (PTP1B and Shp1/Shp2)-mediated Src activation, PKA inhibition, integrin activation, and were adhesion-dependent. Phase I FAK 576/577 Tyr-P also involved cooperative signaling between RTKs (Flk-1 VEGFRs and EGFRs) and integrins. These studies have identified a novel cellular mechanism by which CB_1 induces maximal FAK enzymatic activity in neuronal cells that involves crosstalk between CB_1, RTKs, and integrins.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. CP55940 ((−)-*cis*-3R-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4*R*-3(3-hydroxypropyl)-1*R* cyclohexanol) and SR141716A (*N*-(piperidin-1yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*H*-pyrazole-3-carboxamide) were provided by the National Institute of Drug Abuse drug supply program. Methanandamide [(R)-(+)-Arachidonyl-1′-hydroxy-2′-propylamide], 2-AG, WIN55212-2, and tetrahydrolipstatin (THL, Orlistat) were from Cayman Chemical (Ann Arbor, MI, USA). Acrylamide, *N*,*N*,*N*′, *N*-tetramethylethylene diamine (TEMED), and sodium dodecyl sulfate (SDS) were from BioRad Laboratories, Inc. (Hercules, CA, USA). Integrin α5 siRNA (mouse), control siRNA-A, anti-integrin β1 (M-106), anti-p-FAK (2D11), anti-integrin α7 (H-40), anti-p-FAK (Tyr 576/577), anti-FAK (H-1), anti-FAK (A-17), anti-GAPDH (A-3), and anti-$G_{12/13}$ (H-300) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-integrin α5 and anti-integrin α6 were from Cell Signaling Technology (Danvers, MA, USA). VEGF and EGF were from R&D Systems (Minneapolis, MN, USA). AG 1478, SU5416, PP2, NSC87877 (Shp1/Shp2 inhibitor), Sp-cAMPS (adenosine 3′,5′-cyclic phosphorothioate-Sp), and PTP1B inhibitor were from EMD Biosciences (San Diego, CA, USA). RGDS and RGES peptides were from Abbiotec, LLC (San Diego, CA, USA). Anti-Rac1, anti-RhoA, and laminin (mouse) were from BD Biosciences (Bedford, MA, USA). Odyssey Blocking buffer, nitrocellulose membranes, IRDye 800CW goat anti-rabbit secondary antibody, and IRDye 680CW goat anti-mouse secondary antibody were from LI-COR Biosciences (Lincoln, NE, USA). BD Falcon 6-well multiwell plates were from VWR International (Suwanee, GA, USA).

2.2. Cell culture

N18TG2 neuroblastoma cells (passage numbers 25–50) were maintained at 37 °C under a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM):Ham's F-12 (1:1) complete with GlutaMax, sodium bicarbonate, and pyridoxine–HCl, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco Life Technologies, Gaithersburg, MD, USA) and 10% heat-inactivated bovine serum (JRH Biosciences, Lenexa, KS, USA). An aliquot of cannabinoid drug stocks (stored at −20 °C as 10 mM solutions in ethanol) or ethanol (control) was air-dried under sterile conditions in trimethylsilyl-coated glass test tubes and taken up in 100 volumes of 5 mg/ml fatty acid-free bovine serum albumin (BSA) and serially diluted before being added to cells. Where indicated, N18TG2 cells were pretreated with receptor antagonists or other inhibitors prior to addition of CB_1 agonists. Pertussis toxin (List Biological Laboratories, Campbell, CA, USA) was added to cells (100 ng/mL) for 16–20 h before addition of agonists.

2.3. Immunoblot analysis

Because N18TG2 cells can produce 2-AG [13], cells at 90% confluency were serum-starved (20–24 h) and pretreated with the diacylglycerol lipase (DAGL) inhibitor THL (1 μM, 2 h) prior to stimulation with cannabinoid agonists. Following indicated drug treatments, cells were harvested with PBS–EDTA (2.7 mM KCl, 138 mM NaCl, 10.4 mM glucose, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, and 0.625 mM EDTA, pH 7.4). Cells were resuspended for 20 min on ice in cold NP-40 lysis buffer that contained 10 mM NaHEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 100 μM EDTA, 250 μM Na orthovanadate, 1 mM Na fluoride, 1% NP-40, 1 μM DTT, and a protease inhibitor cocktail (EMD Biosciences, La Jolla, CA, USA) with broad specificity for the inhibition of aspartic, cysteine, and serine proteases as well as aminopeptidases. Lysates were clarified by centrifugation at 20,000 *g* at 4 °C and supernatants were stored at −80 °C. Protein concentrations were determined using the Bradford method with BSA as the standard [14]. Lysates were taken up in Laemmli's sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromophenol blue, 100 mM DTT) and heated at 95 °C for 5 min. Cell lysates were resolved by 7.5% SDS-PAGE gels run at 125 V for 90 min. Gels were pre-equilibrated in Towbins buffer (25 mM Tris Base, 192 mM glycine, and 20% methanol; pH 8.3) for 30 min and proteins were transferred to nitrocellulose membranes for 5 h at 35 V on ice using a BioRad Trans-Blot Cell. Blots were rinsed one time with Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.4, 137 mM NaCl), blocked with Odyssey Blocking buffer, and then incubated with primary antibodies overnight at 4 °C. Blots were washed four times with TBST (TBS containing 0.1% Tween-20), incubated with IRDye 800CW goat anti-rabbit or IRDye 680CW goat anti-mouse secondary antibodies (1:15,000) for 1 h at room temperature, followed by three washes with TBST and one wash with TBS. Immunoblots were imaged and bands were quantified by densitometry using Odyssey Infrared Imaging System software (LI-COR Biosciences, Lincoln, NE, USA).

2.4. RhoA and Rac1 activity assays

GST-fusion proteins that contained the isolated GTP-dependent binding domains of the Rac1 effector PAK1 or the RhoA effector rhotekin were a generous gift from Dr. Keith Burridge (University of North Carolina at Chapel Hill) or were purchased from Cytoskeleton Inc. (Denver, CO, USA). RhoA and Rac1 activities were measured as described previously [15,16]. Briefly, N18TG2 cells were lysed following treatment with cannabinoid agonists. Cell lysates were clarified by centrifugation and were incubated at 4 °C with GST-PAK1-binding domain (PBD) fusion proteins or GST-rhotekin-binding domain (RBD) fusion proteins that were immobilized on Glutathione-Sepharose beads.

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