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CB1 cannabinoid receptor-mediated tyrosine phosphorylation of focal adhesion kinase-related non-kinase

Dan Zhou, Z.H. Song *

Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, Louisville, KY 40292, USA

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Abstract The effect of cannabinoid on the tyrosine phosphorylation of focal adhesion kinase (FAK) and focal adhesion kinase-related non-kinase (FRNK) was investigated in differentiated mouse neuroblastoma N1E-115 cells. HU-210, a potent cannabinoid agonist, elicited a time-dependent enhancement of tyrosine phosphorylation of FRNK, but not FAK. Pretreatment of cells with antisense oligodeoxynucleotide targeting CB1 cannabinoid receptor abolished HU-210-induced FRNK tyrosine phosphorylation. In addition, pretreatment of cells with 8-Br-cAMP also blocked HU-210-induced FRNK tyrosine phosphorylation. These data demonstrated that HU-210 induces FRNK tyrosine phosphorylation by activating G_i-coupled CB1 cannabinoid receptor in N1E-115 cells. This newly discovered, cannabinoid-induced FRNK tyrosine phosphorylation might be a novel mechanism for cannabinoid-induced functional changes.

Key words: CB1 cannabinoid receptor; Mouse neuroblastoma N1E-115 cell; HU-210; Focal adhesion kinase-related non-kinase; Focal adhesion kinase; Phosphorylation

1. Introduction

The cannabinoid system, both the cannabinoid receptors and the endogenous cannabinoid ligands, has drawn much attention to cannabinoid research during the past 15 years [1–3]. So far, two cannabinoid receptors, CB1 and CB2, have been cloned and characterized [4,5]. Both of them belong to the superfamily of seven transmembrane domain G protein-coupled receptors, and couple to multiple signal transduction pathways, which include adenylyl cyclase, mitogen-activated protein kinase and ion channels [1–3]. The CB1 cannabinoid receptor is found in the central nervous and peripheral systems, whereas the CB2 cannabinoid receptor is expressed predominantly in the peripheral tissues such as the cells of the immune system [4–6]. In the brain, CB1 cannabinoid receptor is distributed predominantly in cortex, basal ganglia, cerebellum and hippocampus [6–9]. Based upon the anatomical distribution of CB1 cannabinoid receptors and the pharmacological effects of cannabinoid compounds, the endogenous cannabinoid system has been proposed to be involved in the control of movement, cognition, learning, memory and brain developments.

Focal adhesion kinase (FAK), one of the non-receptor protein tyrosine kinases, is a key tyrosine kinase that is involved in transmembrane transmission of signals from the extracellular milieu to the cell cytoskeleton and nucleus [10–13]. It has been demonstrated that FAK plays an important role in regulating cell morphology, migration, growth, and survival. FAK has been shown to associate with multiple signaling proteins such as Src family protein tyrosine kinases, $p130^{Cas}$, Shc, Grb2, and phosphatidylinositol 3-kinase. These associations enable FAK to function within a signaling network that stimulates a diversity of target pathways. Numerous studies have demonstrated that tyrosine phosphorylation of FAK plays an important role in regulating the functions of this enzyme. Focal adhesion kinase-related non-kinase (FRNK), the C-terminal non-catalytic domain of FAK, is expressed selectively in certain tissues. It has been suggested to be an endogenous regulatory mechanism that negatively regulates the tyrosine phosphorylation and the activity of FAK [14–16].

A number of cell surface receptors have been found to mediate tyrosine phosphorylation of FAK. These include integrins, receptor tyrosine kinases, receptors for neuropeptide, neurotransmitters, chemokines and bioactive lipids [17]. Previously, it has been shown that activation of CB1 cannabinoid receptors in rat hippocampal slices leads to enhanced tyrosine phosphorylation of FAK+, a FAK subtype [18]. Recently, we reported that activation of transfected CB1 cannabinoid receptors in HEK293 cells results in cell migration [19], and activation of endogenously expressed CB1 cannabinoid receptors in mouse neuroblastoma N1E-115 cells leads to neurite remodeling [20]. Taken together, these previous studies suggest that CB1 cannabinoid receptors may mediate cell cytoskeletal, morphological, and functional changes by coupling to FAK-related signaling pathways.

In the present study, using morphologically differentiated mouse neuroblastoma N1E-115 cells, experiments were first performed to determine the effect of HU-210, a cannabinoid agonist, on tyrosine phosphorylation of FAK and FRNK. Furthermore, experiments were conducted to test the hypothesis that HU-210-induced tyrosine phosphorylation of FRNK is mediated by activation-specific CB1 cannabinoid receptors and a decrease of intracellular cAMP levels.

*Corresponding author. Fax: (1)-502-852 7868.
E-mail address: zhsong@louisville.edu (Z.H. Song).

Abbreviations: FAK, focal adhesion kinase; FRNK, focal adhesion kinase-related non-kinase; ODN, oligodeoxynucleotide; DMEM, Dulbecco's modified Eagle's medium

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2. Materials and methods

2.1. Materials

Mouse neuroblastoma N1E-115 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture medium and supplements were purchased from Biowhittaker (Walkersville, MD, USA). HU-210 was purchased from Tocris (Balwin, MO, USA). Goat and rabbit polyclonal anti-FAK antibodies (clone C-20) and mouse monoclonal anti-phosphotyrosine antibody (PY-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A-conjugated Sepharose CL-4B beads were purchased from Amersham Pharmacia Biotech AB (Piscataway, NJ, USA). Phosphorothioate oligodeoxynucleotides (ODN) were purchased from Integrated DNA Technologies (Coralville, IA, USA).

2.2. Cell culture and treatments

Mouse neuroblastoma N1E-115 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum supplemented with penicillin and streptomycin at 37°C in an atmosphere of humidified air and 5% CO_2. Cells were cultured in serum-free DMEM for 48 h before receiving cannabinoid treatment. More than 85% of the cells became flattened and about 40% extended neurite under these conditions.

Antisense pretreatment was performed according to a procedure published by Hunter and Burstein [21] using phosphorothioate ODN. The antisense probe is an 18-mer (5′-GTACTGAATGTCATTTGA-3′), complementary to positions 73–90 of the mouse CB1 cannabinoid receptor mRNA codon. The corresponding 18-mer sense fragment, 5′-TCAAATGACATTCAGTAC-3′, was synthesized and used as a control. The antisense sequence was compared with sequences deposited in the GenBank/EMBL data banks and revealed no identity with other rodent sequences. N1E-115 cells were pretreated with 3 μM antisense or sense control ODN for 48 h in serum-free DMEM at 37°C in an atmosphere of humidified air and 5% CO_2. Following antisense ODN pretreatment, the cells were used in cAMP accumulation and FAK immunoprecipitation experiments.

2.3. cAMP accumulation assay

cAMP accumulation assays were performed as described previously with slight modifications [22,23]. Briefly, HU-210 was diluted with DMEM medium containing 50 mg/ml fatty acid-free bovine serum albumin in silanized test tubes, and 25 μl solutions with different drug concentrations were added to individual tubes. Forskolin was also added to the test tubes in a volume of 25 μl to reach a final concentration of 1.0 μM. Confluent cells were lifted from culture plates with phosphate-buffered saline containing 0.5 mM EDTA. After washing with medium, the cells were incubated with phosphodiesterase inhibitor RO20-1724 for 10 min. The stimulation of cAMP synthesis was initiated by adding cells to test tubes that contain forskolin and HU-210. The final reaction volume was 250 μl with 1×10^6 cells per tube and the reaction mixture was incubated at 37°C for 5 min. The reaction was stopped with the addition of an equal volume of 0.1 N HCl. An aliquot of 50 μl reaction supernatant was removed for cAMP radioimmunoassay, using a kit from DuPont-NEN (DuPont-NEN, Wilmington, DE, USA).

2.4. Cell lysis, immunoprecipitation and Western blot

Cells were treated with 10 nM HU-210 for different time periods. The cells were then lysed on ice in cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM glycerophosphate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μM leupeptin and 1 mM Na_3VO_4. After centrifugation at $10\,000\times g$ for 20 min, the supernatants were collected. The anti-FAK immunocomplex was prepared by incubating the supernatants with a goat polyclonal anti-FAK antibody at 4°C overnight. The protein A-conjugated Sepharose beads (protein A Sepharose CL-4B) were added, and the mixture was incubated at 4°C for 2 h. Subsequently, the protein A beads containing the immunocomplex were washed twice with cell lysis buffer, and boiled with 2× Laemmli sample buffer for 5 min. After 10 min centrifugation at $5000\times g$, the supernatants were loaded to a SDS–polyacrylamide gel, electrophoresed, and transferred onto a nitrocellulose membrane. The tyrosine phosphorylation of FAK was detected with a mouse monoclonal anti-phosphotyrosine antibody (PY-20) as the first antibody and visualized using an ECL kit from Amersham. The loading of FAK protein was checked with a rabbit polyclonal anti-FAK antibody.

2.5. Data analysis

Results from cAMP accumulation assays represent the mean ± S.E.M. values obtained from three independent experiments. Statistical differences between different treatment groups were determined by one-way ANOVA. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of HU-210 on tyrosine phosphorylation of FAK and FRNK

Using the approaches of immunoprecipitation and Western blot, we determined the effect of HU-210 treatment on tyrosine phosphorylation of FAK and FRNK in differentiated mouse neuroblastoma N1E-115 cells. The anti-FAK antibody (C-20) recognizes both FAK and FRNK in the immunoprecipitation experiments. As shown in Fig. 1, no significant changes of tyrosine phosphorylation of FAK were found after HU-210 treatment. However, in a time-dependent manner, HU-210 induced an increase in tyrosine phosphorylation of FRNK.

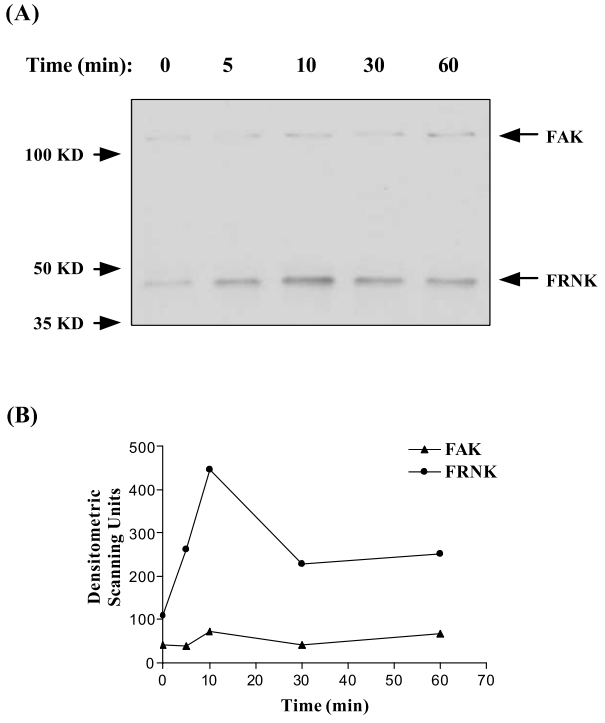

Fig. 1. Time course of the effect of HU-210 on tyrosine phosphorylation of FAK and FRNK in differentiated N1E-115 cells. Differentiated N1E-115 cells were incubated with 10 nM HU-210 for the time period indicated, and the tyrosine phosphorylation levels of FAK and FRNK were examined by immunoprecipitation combined with Western blot. The immunocomplexes of FAK/FRNK mixture were obtained using an antibody against the C-terminal end of FAK (C-20) that hence recognizes both FAK and FRNK in the cell lysate. The tyrosine phosphorylation levels of FAK and FRNK were detected with Western blot using an anti-phosphotyrosine antibody (PY-20). Molecular masses are indicated on the left. The Western blot shown in A is representative of three independent experiments. The results from densitometric scanning of the Western blot are shown in B.

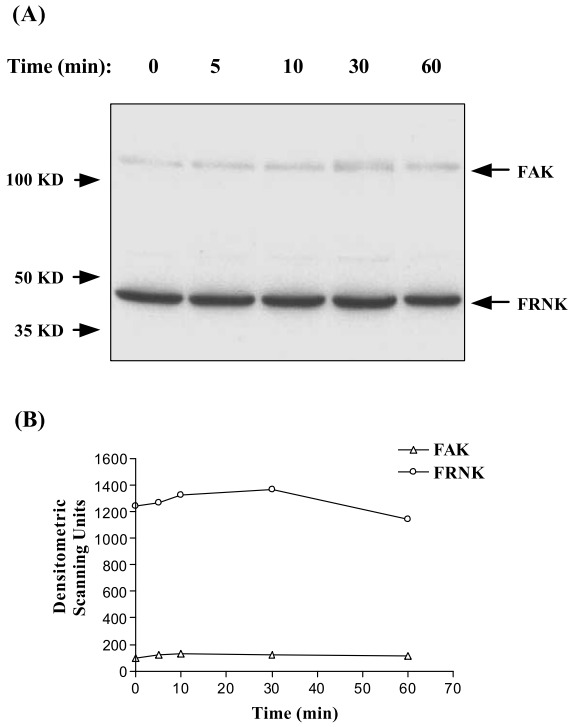

Fig. 2. HU-210 induces neither FAK nor FRNK expression in differentiated N1E-115 cells. Differentiated N1E-115 cells were incubated with 10 nM HU-210 for the time period indicated, and the expression levels of FAK and FRNK were examined by Western blot. The cell lysate was separated by SDS–PAGE and FAK and FRNK protein levels were detected with Western blot using an antibody against the C-terminal end of FAK (C-20) that hence recognizes both FAK and FRNK. Molecular masses are indicated on the left. The Western blot shown in A is representative of three independent experiments. The results from densitometric scanning of the Western blot are shown in B.

3.2. Lack of effect of HU-210 on FAK and FRNK protein expression levels

The possible effects of HU-210 on FAK and FRNK protein expression levels in differentiated mouse neuroblastoma N1E-115 cells were evaluated with cell lysate by Western blot. As shown in Fig. 2, the expression level of neither FAK nor FRNK was changed by HU-210 treatment.

Table 1
Effect of antisense ODN pretreatment on HU-210-induced inhibition of forskolin-stimulated cAMP accumulation

Treatment	cAMP accumulation (%)
Control	100
HU-210	52.80 ± 19.53
Sense plus HU-210	60.50 ± 17.32
Antisense plus HU-210	90.20 ± 12.34*

Adenylate cyclase activity was determined in the presence of 10 μM forskolin or in the presence of 10 μM forskolin plus 10 nM HU-210. The level of forskolin-stimulated cAMP accumulation was assigned a value of 100%. In some experiments, N1E-115 cells were pretreated for 48 h with 3.0 μM antisense ODN targeting CB1 cannabinoid receptor or corresponding sense control ODN before the cAMP accumulation assay.
*Significantly different from the HU-210 group by one-way ANOVA at $P < 0.05$.

3.3. Effect of CB1 antisense ODN treatment on HU-210-induced cAMP accumulation and HU-210-induced tyrosine phosphorylation of FRNK

An antisense 'knock-down' strategy was used to examine the involvement of CB1 cannabinoid receptors in mediating the HU-210-induced tyrosine phosphorylation of FRNK. As shown in Table 1, HU-210 inhibited forskolin-stimulated cAMP accumulation in differentiated mouse neuroblastoma N1E-115 cells. In sense control ODN-pretreated cells, HU-210 retained its effect in inhibiting forskolin-stimulated cAMP accumulation. In contrast, in CB1 antisense ODN-pretreated cells, HU-210 failed to inhibit forskolin-stimulated cAMP accumulation.

In the series of experiments using antisense strategy to study HU-210-induced tyrosine phosphorylation of FRNK, the cells that were pretreated with CB1 antisense ODN demonstrated no tyrosine phosphorylation in response to HU-210 treatment (Fig. 3). In contrast, no significant suppression of HU-201-induced tyrosine phosphorylation of FRNK was found in the sense ODN pretreatment group.

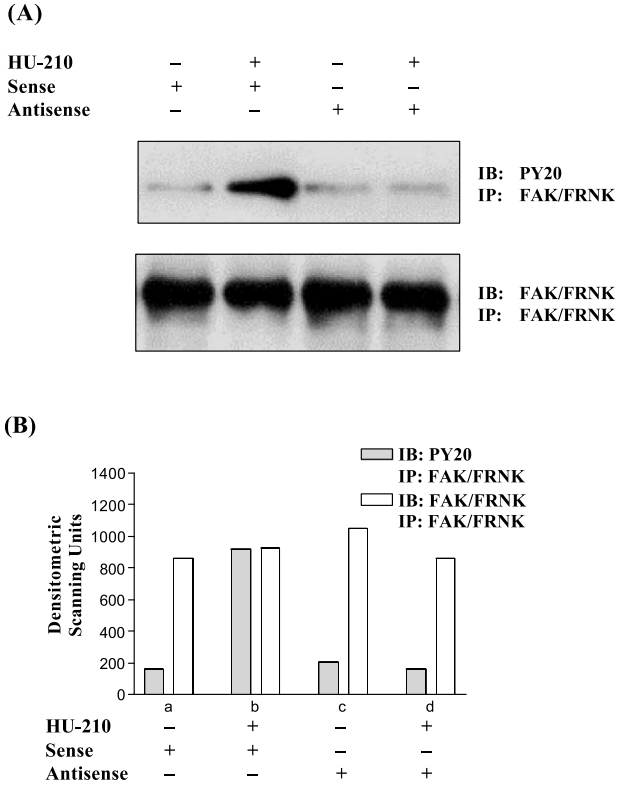

Fig. 3. Antisense ODN pretreatment blocks HU-210-induced tyrosine phosphorylation of FRNK in differentiated N1E-115 cells. N1E-115 cells were pretreated for 48 h with either 3 μM antisense ODN targeting CB1 cannabinoid receptor, or sense control ODN, and followed by 10 nM HU-210 treatment for 10 min. The immunocomplex of FRNK was obtained using a goat antibody against the C-terminal end of FAK (C-20) that hence recognizes both FAK and FRNK in the cell lysate. The tyrosine phosphorylation levels of FRNK were detected with Western blot using an anti-phosphotyrosine antibody (PY-20). The upper panel represents the anti-phosphotyrosine antibody (PY-20) Western blot, and the lower panel shows rabbit anti-FAK antibody Western blot after stripping the same membrane. The Western blot shown in A is representative of two independent experiments. The results from densitometric scanning of the Western blot are shown in B.

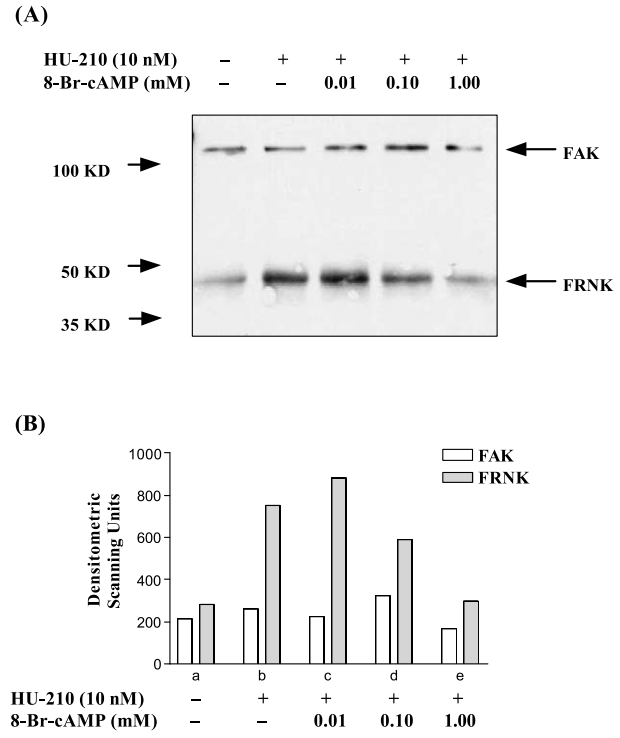

Fig. 4. 8-Br-cAMP pretreatment blocks HU-210-induced tyrosine phosphorylation of FRNK in differentiated N1E-115 cells. N1E-115 cells were pretreated with 8-Br-cAMP for 30 min, followed by 10 nM HU-210 treatment for 10 min. The immunocomplexes of FAK/FRNK mixture were obtained using an antibody against the C-terminal end of FAK (C-20) that hence recognizes both FAK and FRNK in the cell lysate. The tyrosine phosphorylation levels of FAK and FRNK were detected with Western blot using an anti-phosphotyrosine antibody (PY-20). Molecular masses are indicated on the left. The Western blot shown in A is representative of three independent experiments. The results from densitometric scanning of the Western blot are shown in B.

3.4. *Effect of 8-Br-cAMP pretreatment on HU-210-induced tyrosine phosphorylation of FRNK*

CB1 cannabinoid receptors are negatively coupled to adenylyl cyclase via a G_i protein. To investigate whether the HU-210-induced tyrosine phosphorylation of FRNK involves a reduction of intracellular cAMP levels, we used 8-Br-cAMP, a cell membrane-permeable analogue of cAMP. Pretreatment of differentiated N1E-115 cells with 8-Br-cAMP for 30 min inhibited HU-210-induced tyrosine phosphorylation of FRNK in a concentration-dependent manner (Fig. 4).

4. Discussion

FAK is enriched in brain, especially in hippocampal formation and young neurons, where it is located in the growth cones [24–27]. The activity of FAK is regulated by integrin engagement and stimulation of various membrane receptors via the phosphorylation of its tyrosine residues [10–13]. Although it has been shown that many factors induce tyrosine phosphorylation of FAK or its subtype FAK+ in the central nervous system, the function and regulation of FAK in neurons are still poorly understood.

Many studies have demonstrated that transfected FRNK was able to regulate the activity and tyrosine phosphorylation of FAK [14–16]. FRNK has been found to act as an inhibitor of FAK by blocking the formation of focal adhesions and constitutively reducing tyrosine phosphorylation of FAK. These inhibitory effects of FRNK were reversed by co-expression of FAK, or a point mutation of FRNK that prevents its localization to the focal contact sites. This suggests that FAK and FRNK compete for common binding proteins whose association with FAK is necessary for signaling by FAK. Furthermore, activated caspases have been shown to cleave FAK to generate FRNK during apoptotic processes. Thus, FRNK is believed to function as an endogenous regulator of FAK that regulates both tyrosine kinase activity and cellular functions [14–16].

In the current study, HU-210 was found to induce tyrosine phosphorylation of FRNK, but not FAK in differentiated mouse neuroblastoma N1E-115 cells. To study the possible involvement of CB1 cannabinoid receptors in the HU-210-induced tyrosine phosphorylation of FRNK, a specific CB1 antisense ODN was employed. Functional knocking down of CB1 cannabinoid receptors was achieved by the antisense pretreatment, since the inhibition of forskolin-stimulated cAMP accumulation by HU-210 was blocked by antisense but not sense control ODN pretreatment. Pretreatment with antisense, but not sense, control ODN also blocked HU-210-induced tyrosine phosphorylation of FRNK in these cells. These data indicate that HU-210-induced tyrosine phosphorylation of FRNK was mediated by CB1 cannabinoid receptors endogenously expressed in the differentiated N1E-115 cells. One of the major signaling pathways of cannabinoid receptors is the inhibition of adenylate cyclase. It has been reported previously that intracellular cAMP is an important factor in regulating neurite formation and stability [28,29]. In the current study, 8-Br-cAMP pretreatment was found to inhibit HU-210-induced tyrosine phosphorylation of FRNK. These data suggest that the induction of tyrosine phosphorylation of FRNK is mediated by HU-210-induced reduction of intracellular cAMP levels.

To our knowledge, this study is the first to report that endogenous FRNK can be tyrosine-phosphorylated, and the tyrosine phosphorylation of FRNK can be regulated by a membrane receptor-mediated mechanism. At present, the functional significance of HU-210-induced tyrosine phosphorylation of FRNK is not clear. In previous studies, it has been demonstrated that FAK is crucial for neurite outgrowth and the FAK activation-induced neurite outgrow can be blocked by overexpression of FRNK [30,31]. In a recent study, we discovered that HU-210 induces neurite retraction in differentiated N1E-115 cells [20]. Based on these previous findings, one could propose that by inducing the tyrosine phosphorylation of FRNK, HU-210 might regulate the activity of FAK, thereby cause neurite remodeling in differentiated NIE-115 cells. Obviously, this hypothesis needs to be tested in future studies.

In summary, in the present study we have found HU-210 treatment of differentiated N1E-115 cells induces the tyrosine phosphorylation of FRNK. Using an antisense strategy and 8-Br-cAMP, we have demonstrated that CB1 cannabinoid receptors and decreases of intracellular cAMP levels are involved in HU-210-induced tyrosine phosphorylation of FRNK. The functional significance of this newly discovered CB1 cannabinoid receptor-mediated tyrosine phosphorylation of FRNK remains to be elucidated.

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