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Dual Role of Fyn in the Regulation of FAK$^+$6,7 by Cannabinoids in Hippocampus*

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In hippocampus endocannabinoids modulate synaptic function and plasticity and increase tyrosine phosphorylation of several proteins, including focal adhesion kinase (FAK). Autophosphorylation of FAK on Tyr-397 is generally a critical step for its activation, allowing the recruitment of Src family kinases, and phosphorylation of FAK and associated proteins. We have examined the mechanisms of the regulation of FAK by cannabinoids in rat and mouse hippocampal slices. Anandamide and 2-arachidonoylglycerol, two endocannabinoids, and Δ9-tetrahydrocannabinol, stimulated tyrosine phosphorylation of FAK$^+$6,7, a neuronal splice isoform of FAK, on several residues including Tyr-397. Cannabinoids increased phosphorylation of p130-Cas, a protein associated with FAK, but had no effect on PYK2, a tyrosine kinase related to FAK and enriched in hippocampus. Pharmacological experiments and the use of knockout mice demonstrated that the effects of cannabinoids were mediated through CB1 receptors. These effects were sensitive to manipulation of cAMP-dependent protein kinase, suggesting that they were mediated by inhibition of a cAMP pathway. PP2, an Src family kinase inhibitor, prevented the effects of cannabinoids on p130-Cas and on FAK$^+$6,7 tyrosines 577 and 925, but not 397, indicating that FAK autophosphorylation was upstream of Src family kinases in response to CB1-R stimulation. Endocannabinoids increased the association of Fyn, but not Src, with FAK$^+$6,7. In hippocampal slices from Fyn −/− mice, the levels of p130-Cas were increased, and the effects of endocannabinoids on tyrosine phosphorylation, including of Tyr-397, were completely abolished. These results demonstrate the specific functional association of Fyn with FAK$^+$6,7 in a pathway regulated by endocannabinoids, in which Fyn may play roles dependent and independent of its catalytic activity.

Although cannabis has been used for several centuries as a therapeutic and recreational drug (1, 2), the mechanism of action of Δ9-tetrahydrocannabinol (THC),[1] its main active compound, is only beginning to be elucidated. THC acts through two G protein-coupled receptor subtypes, the CB1 and CB2 receptors (3, 4). CB1 receptors (CB1-R) are mainly expressed in the central nervous system and are particularly abundant in basal ganglia, hippocampus, and cerebellum (5). In contrast, CB2 receptors are expressed outside of the nervous system, mostly in lymphoid organs (4). Endogenous ligands of CB1-R (endocannabinoids) include anandamide and 2-arachidonoylglycerol (2-AG) (6–9). These arachidonic acid derivatives are not stored in vesicles, but their phospholipid precursors are synthesized and hydrolyzed following calcium influx into neurons or stimulation of neurotransmitter receptors (7, 9, 10). In hippocampus electrical stimulation of Schaeffer collaterals increases the production of 2-AG (9). CB1-R modulates synaptic plasticity in the hippocampus by decreasing long term potentiation (LTP) and depression (11). Recent work (12, 13) has demonstrated that endocannabinoids are retrograde synaptic messengers mediating depolarization-induced suppression of inhibition. The electrophysiological effects of endocannabinoids appear to result from the ability of CB1-R to decrease neurotransmitter release (11–15). In addition to the recent progress in understanding the action of endocannabinoids, the possible therapeutic effects of drugs affecting transmission by cannabinoids have received a great deal of interest in various conditions (see Ref. 16 for a recent review), underscoring the importance of identifying the actions of CB1-R.

The signal transduction pathways regulated by the activation of CB1-R include inhibition of adenylyl cyclase, activation of mitogen-activated protein kinases and phosphoinositide 3-kinase, induction of immediate early genes, and stimulation of nitric-oxide synthase (see Ref. 17 for a recent review). Many of these results were obtained in cells in culture, often non-neuronal, and the signaling pathways activated by CB1-R in the brain are still poorly characterized. We have shown previously that anandamide increases protein tyrosine phosphorylation in rat hippocampus (18). One of the proteins phosphorylated in response to anandamide was a splice isoform of FAK characterized by the presence of a 3-amino acid insertion in the C-terminal region, termed FAK$^+$ (18). Further work has revealed that the major neuronal isoform of FAK, termed FAK$^+$6,7, contains additional alternatively spliced exons, coding for two short peptides of 6 and 7 amino acids on either side of Tyr-397 (19). The presence of these latter peptides results in a dramatic increase in FAK autophosphorylation on Tyr-397

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[1] The abbreviations used are: THC, Δ9-tetrahydrocannabinol; ACSF, artificial cerebrospinal fluid; 2-AG, 2-arachidonoylglycerol; CB1-R, cannabinoid type 1 receptor; FAK, focal adhesion kinase; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; LTP, long term potentiation; p130-Cas, 130-kDa Crk-associated substrate; PKA, cAMP-dependent protein kinase; PYK2, proline-rich tyrosine kinase 2; TTX, tetrodotoxin; 8-Br-cAMP, 8-bromo-cAMP; mAb, monoclonal antibody.

(19, 20), a residue that is essential for FAK function.[2]

FAK transduces messages generated by integrin-mediated cell contacts and also by soluble factors acting on G protein-coupled receptors or on receptor tyrosine kinases (see Refs. 21 and 22 for reviews). FAK is upstream of several signaling pathways, and its functional roles include control of focal adhesions turnover, cell motility, and adhesion-dependent survival. In the context of the adult nervous system, several lines of evidence suggest that FAK may be involved in neuronal plasticity and survival (23). Although there are exceptions to this rule, tyrosine phosphorylation of FAK occurs generally in two steps. First, Tyr-397 is autophosphorylated, allowing the binding of SH2 domain-containing proteins including Src family kinases (24), phosphatidylinositol 3-kinase (25), and phospholipase Cγ (26). Src family kinases phosphorylate other residues in FAK and in neighboring proteins, including p130-Cas, a protein associated with FAK in many cell types (see Ref. 27 for a review). Thus, FAK behaves as an adapter protein regulated by autophosphorylation and structurally, as well as functionally, closely associated with Src family kinases.

Hippocampus is an excellent model for studying the regulation of FAK by cannabinoids, since it is a brain region where CB1-R is abundantly expressed (14, 28, 29), and where their physiological effects are well characterized (11, 14, 30, 31). In addition, the use of hippocampal slices provides insights on the regulation and function of signaling pathways in the context of mature brain tissue. Therefore, the aim of the present study was to dissect the mechanism of FAK activation by cannabinoids in rat and mouse hippocampus. We studied the effects on protein tyrosine phosphorylation pathways of various cannabinoid agonists, including 2-AG, the major endocannabinoid in hippocampus (9), and THC. We examined the role of CB1-R and of inhibition of adenylyl cyclase in the activation of FAK and p130-Cas. Finally, we investigated the mechanisms of activation of FAK by cannabinoids in hippocampal slices, by assessing the contribution of FAK autophosphorylation and the role of Src family kinases.

EXPERIMENTAL PROCEDURES

Reagents—Anandamide, 8 bromo-3′-5′-cyclic adenosine monophosphate (8-Br-cAMP), THC, 3-isobutyl-1-methylxanthine (IBMX), lysophosphatidic acid (LPA), protein A-Sepharose, tetrodotoxin (TTX), WIN 55212-2, and 2-arachidonoylglycerol were from Sigma. CP 55940 was from Pfizer. PP2 was from Calbiochem. SR 141716A was from Sanofi (Sanofi Research, Montpellier, France). Artificial cerebrospinal fluid (ACSF) contained (in mM) NaCl, 125; KCl, 2.4; $MgCl_2$, 0.83; $CaCl_2$, 1.1; KH_2PO_4, 0.5; Na_2SO_4, 0.5; $NaHCO_3$, 27; glucose, 10; Hepes, pH 7.4, 110. Ca^{2+}-free ACSF had the same composition except for $MgCl_2$, 1.93 mM. The following commercially available phosphospecific antibodies were used for Western blotting: antiphospho-Tyr-577 FAK (BIOSOURCE, diluted 1:1,000) and antiphospho-Tyr-925 FAK (BIOSOURCE, diluted 1:1,000). Antiphospho-Tyr-397 FAK was produced and affinity-purified as described previously (20) or was from BIOSOURCE (diluted 1:1,000). Anti-Fyn antibodies were from Transduction Laboratories (mouse monoclonal for Western blot, 1:250) or from Santa Cruz Biotechnology (rabbit polyclonal for immunoprecipitation, 20 μl per sample). Anti-Src antibodies were from Calbiochem (rabbit polyclonal for Western blot, 1:500) or from Upstate Biotechnology, Inc. (mouse monoclonal for immunoprecipitation, 7 μl per sample). Anti-p130-Cas antibodies were from Transduction Laboratories (mouse monoclonal for Western blot, 1:500) or from Santa Cruz Biotechnology (rabbit polyclonal for immunoprecipitation, 20 μl per sample). For immunoprecipitation, polyclonal anti-FAK$^+$ and anti-PYK2 were produced as described previously (18, 32). Rabbit polyclonal antibody against CB1-R was a gift of Dr. Mackie. Monoclonal antibodies to phosphotyrosine (4G10) were from Upstate Biotechnology, Inc. (diluted 1:4,000). FAK-transfected COS-7 cells were prepared as described (20).

Rat and Mice Hippocampal Slices—Rat hippocampal slices (300-μm thick) were prepared from young male Harlan Sprague-Dawley rats (150–200 g) with a McIlwain tissue chopper, as described previously (33). Briefly, slices were dissected in ice-cold, Ca^{2+}-free ACSF and were placed for 10 min in polypropylene tubes (three slices per tube) containing 1 ml of Ca^{2+}-free ACSF at 35 °C, equilibrated at pH 7.4 in O_2/CO_2 (95:5, v/v). They were then incubated at 35 °C in 900 μl of ACSF containing 1.1 mM Ca^{2+} and 1 μM TTX for 50 min before pharmacological treatment. Treatment of control slices was carried out by the addition of vehicle. TTX was added to prevent indirect effects due to neuronal firing and had no effect on tyrosine phosphorylation by itself (data not shown). At the end of the experiment, ACSF was aspirated, and slices were immediately frozen on dry ice and kept at −80 °C. Mice hippocampal slices from wild type, CB1 knockout mice (34), and Fyn knockout mice (35) (The Jackson Laboratories) were prepared as rat slices except for the use of Vibratome instead of McIlwain chopper.

Immunoprecipitation—Protein A-Sepharose with 10% v/v Sephacryl was washed in PB+ buffer (2.7 mM KCl, 1.2 mM KH_2PO_4, 137 mM NaCl, 8.3 mM NaH_2PO_4, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 50 mM NaF) containing 10 mg/ml bovine serum albumin, equilibrated in PB+ buffer with 1% (w/v) Triton X-100 and used as a 50% (v/v) slurry. Homogenized samples (~500 μg of proteins in 1% SDS in the presence of 1 mM sodium orthovanadate) were equilibrated in PB+ buffer with 5% (w/v) Triton X-100 and precleared with 100 μl of Sephacryl G-10. Samples were then incubated for 2 h at 4 °C with 80 μl of protein A slurry and 25 μl of FAK$^+$, PYK2, or p130-Cas antisera. The protein A-Sepharose-bound immune complexes were washed three times in PB+ buffer as follows: once with Triton (1% w/v), once with Triton and 0.5 M NaCl, and once without Triton. For coimmunoprecipitation, slices (500–600 μg of protein) were homogenized with a Heidolph tissue homogenizer in ice-cold modified CRIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaF) containing 1 mM Na^+ orthovanadate, 1%(v/v) Triton X-100, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (complete, Roche Molecular Biochemicals). Samples were precleared with 100 μl of Sephacryl beads, and immunoprecipitations were carried out with polyclonal antibodies anti-Fyn or with monoclonal antibodies anti-Src preadsorbed, respectively, on protein A- or protein G-Sepharose beads. In all cases, immunoprecipitation pellets were heated at 100 °C for 5 min in 70 μl of sample buffer for SDS-polyacrylamide gel electrophoresis.

Western Blot Analysis—Slices were lysed by sonication in 1% SDS (v/v) containing 1 mM Na^+ orthovanadate at 100 °C. Equal amounts of slices lysates (60 μg) were separated by SDS-polyacrylamide gel electrophoresis (8% weight/vol) prior to electrophoretic transfer onto nitrocellulose membrane (Hybond Pure, Amersham Pharmacia Biotech). Membranes were blocked 1 h at room temperature in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH 7.5) with 0.1% Tween 20 or 5% nonfat dry milk for the detection of phosphorylated and non-phosphorylated proteins, respectively. The membranes were then incubated overnight at 4 °C with the primary antibodies. After three short washes, membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham Pharmacia Biotech, diluted 1:4,000). Bound antibodies were visualized by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech). When necessary, membranes were stripped in buffer (containing 100 mM glycine pH 2.5, 200 mM NaCl, 0.1% Tween 20 (v/v) and 0.1% (v/v) β-mercaptoethanol) for 45 min at room temperature, followed by extensive washing in TBS before reblocking and reprobing. Quantification was achieved by scanning the autoradiograms and measurement of relative optical density with NIH Image.

RESULTS

Effects of 2-AG and THC on Tyrosine Phosphorylation of FAK in Rat Hippocampal Slices Are Mediated through CB1 Receptors—We compared the effects on FAK of 2-AG, the endocannabinoid produced in hippocampal slices following electrical stimulation (9), to those of anandamide previously shown to increase protein tyrosine phosphorylation in rat hippocampal slices (18). We also examined the effects of THC, the widely

[2] FAK$^+$ corresponds to the presence of three additional residues inserted at position 903; FAK6 corresponds to six additional residues at position 392; FAK7 corresponds to seven additional residues at position 411 and a frameshift mutation that causes a Thr to Ala residue substitution (19). To avoid confusion, in this report we use the numbering of FAK tyrosine residues corresponding to the "standard" FAK isoform, without additional exons, although tyrosines 397, 576, 577, and 925 correspond to positions 403, 589, 590, and 941, respectively, in FAK$^+$6,7.

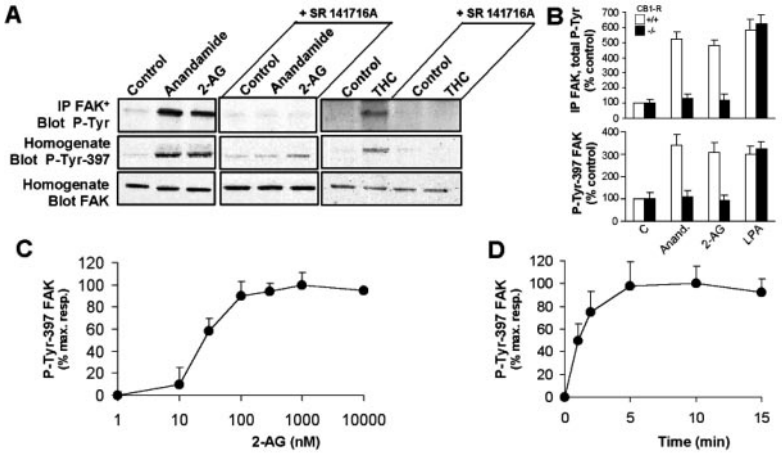

FIG. 1. **Effects of 2-AG and THC on FAK in hippocampal slices from rat and wild type or CB1-R knockout mice.** *A,* rat hippocampal slices were incubated in ACSF at 35 °C for 50 min before the addition of either vehicle (*Control*), 1 μM anandamide, 1 μM 2-AG, or 0.1 μM THC for 5 min, in the absence or in the presence of 100 μM SR 141716A. Hippocampal slices homogenates (60 μg of protein per sample) were subjected to immunoprecipitation with serum against FAK⁺ followed by anti-phosphotyrosine immunoblotting (*IP-FAK⁺ Blot P-Tyr*) or to direct immunoblotting with antibodies against the phosphorylated form of Tyr-397, the autophosphorylation site (*Homogenate Blot P-Tyr-397*). After stripping, the membranes were reprobed with anti-FAK antibody (*Homogenate Blot FAK*). The data presented are representative of three independent experiments. *B,* similar experiments were carried out using hippocampal slices from wild type (+/+) and CB1-R knockout (−/−) mice. Total FAK⁺ phosphotyrosine and phospho-Tyr-397 were quantified by scanning the autoradiograms and densitometry and are expressed as percentages of values in unstimulated controls. Data correspond to means + S.E. of three independent experiments. The effects of anandamide (*Anand.*) and 2-AG were absent in CB1-R knockout mice. In contrast those of LPA (0.2 μM), used as a positive control, were unaltered. *C* and *D*, quantification of the effects of 2-AG on FAK phosphorylation on Tyr-397: concentration response (treatment for 5 min, *C*) and time course (2-AG concentration 1 μM, *D*). Experiments were as in *A*, and immunoreactivity was quantified by scanning the autoradiograms and densitometry. Values are means + S.E. of 4–8 independent experiments and are expressed as percentages of the maximal increase above unstimulated control values.

abused cannabinoid agonist. Immunoprecipitation with antibodies specific for FAK⁺ revealed that 2-AG (1 μM) and THC (0.1 μM) increased its tyrosine phosphorylation in rat hippocampal slices, as anandamide (1 μM) (Fig. 1*A*). Since autophosphorylation of FAK on Tyr-397 is the critical step in its activation (24, 36, 37), we measured the phosphorylation of this residue with a phosphorylation state-specific antibody (19, 20). The increased phosphorylation of Tyr-397 in response to cannabinoid agonists matched that of FAK total tyrosine phosphorylation (Fig. 1*A*).

The effects of endocannabinoids and THC on total tyrosine phosphorylation of FAK and on phosphorylation of Tyr-397 were completely prevented by a pretreatment of slices with a high concentration (100 μM) of SR 141716A, a specific inhibitor of CB1-R (38) (Fig. 1*A*). In contrast, the effects of lysophosphatidic acid (LPA), a lipidic messenger unrelated to cannabinoids and known to increase the tyrosine phosphorylation of FAK in hippocampus (32), persisted in the presence of SR 141716A (data not shown). In addition, synthetic CB1 agonists CP 55940 and WIN 55212-2 also increased tyrosine phosphorylation of FAK, and their effects were blocked by the specific CB1 antagonist SR 141716A (data not shown). However, endocannabinoids have a potential for acting on other targets beside CB1 and CB2 receptors (17), and the concentrations of WIN 55212-2 and SR 141716A required to achieve their effects were high (100 μM), raising the possibility that they could exert unspecific effects. To assess the exact contribution of CB1-R in the effects of anandamide and 2-AG, we used hippocampal slices prepared from genetically altered mice lacking CB1-R (34). In wild type mice, as in rats, anandamide and 2-AG increased FAK total tyrosine phosphorylation and Tyr-397 phosphorylation (Fig. 1*B*). These effects were completely prevented in CB1-R −/− mice, although the expression levels of FAK were unchanged (Fig. 1*B*). However, FAK could still be activated by LPA in CB1-R −/− mice, demonstrating that the other components of the pathway were not altered in these mutant mice (Fig. 1*B*). Combined with the pharmacological experiments described above, the results in CB1-R knockout mice demonstrate that the effects of endocannabinoids on FAK in hippocampus are mediated through activation of CB1-R.

Finally, we examined the concentration and time dependence of the effects of 2-AG on phosphorylation of FAK on Tyr-397. The effects of 2-AG were detected at very low concentration (half-maximal effect at ~20 nM, Fig. 1*C*) and were rapid (half-maximal effect at ~1 min, Fig. 1*D*).

Endocannabinoids Increase Tyrosine Phosphorylation of the Neuronal Isoform of FAK, FAK⁺6,7, and p130-Cas but Are Inactive on PYK2—We have shown previously (18–20) that FAK⁺6,7 is the major isoform of FAK expressed in forebrain. Several lines of evidence indicate that the isoform of FAK in hippocampal slices was indeed FAK⁺6,7. It was recognized by anti-FAK⁺ antibodies (Fig. 1*A*). In SDS-polyacrylamide gel electrophoresis it migrated with an apparent molecular weight slightly higher than FAK⁺, FAK⁺6, or FAK⁺7 expressed in COS-7 cells, and comigrated with FAK⁺6,7 (Fig. 2*A*). It was not recognized by monoclonal antibody 77 (mAb 77, Fig. 2*A*) whose interaction with FAK is prevented by the presence of box 7 (20). On the basis of its apparent molecular weight and of the lack of detection by mAb77, this isoform of FAK was found in neurons but not in astrocytes or microglial cells in culture (Fig. 2*B*). The form of FAK phosphorylated on Tyr-397 in response to anandamide had the same characteristics (Fig. 2*C*). These results provide strong evidence that the isoform of FAK regulated by cannabinoids in hippocampal slices was FAK⁺6,7 and that the observed regulations were taking place in neurons.

FAK belongs to a family of two highly related kinases, including PYK2/RAFTK/cell adhesion kinase β/CADTK (see Ref. 39 for a review), which is highly expressed in hippocampus (40) and is involved in LTP (41). In PC12 cells and in other cell lines, both kinases can be activated by the same extracellular messengers (42, 43). In contrast, in hippocampal slices, FAK and PYK2 are differentially regulated; FAK is activated by neurotransmitters such as acetylcholine and glutamate, whereas PYK2 is sensitive to depolarization and osmotic shock (32, 44). Such a specificity in the regulation of FAK and PYK2 in hippocampus was also observed with endocannabinoids,

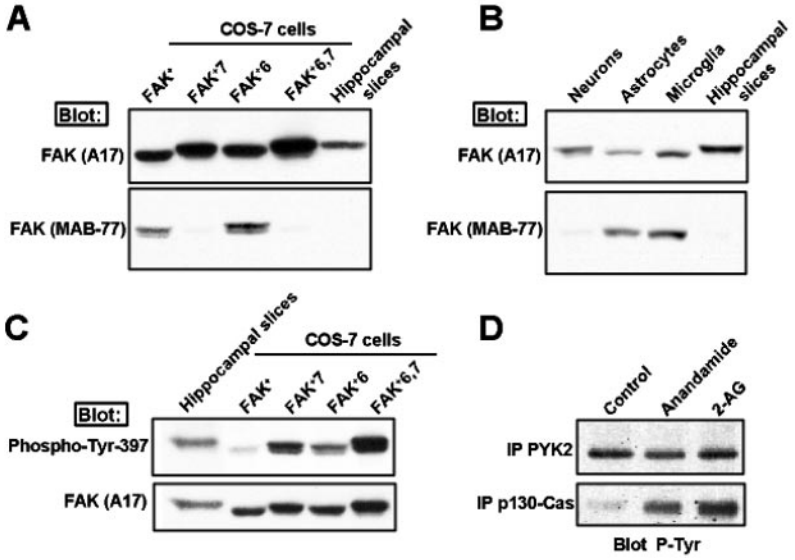

FIG. 2. **In hippocampal slices, endocannabinoids increase tyrosine phosphorylation of FAK$^+$6,7 and p130-Cas but not PYK2.** *A,* comparison of the apparent M_r of FAK in rat hippocampal slices and of FAK splice isoforms expressed in COS-7 cells. Immunoblotting was done with two different antibodies: serum A17 that reacts with all isoforms and mAb-77 whose interaction with FAK is impaired by the presence of box 7 (20). The apparent M_r of FAK isoforms is altered by the presence of alternative exons: the fastest migrating species correspond to FAK$^+$, and the slowest to FAK$^+$6,7, whereas FAK$^+$6 and FAK$^+$7 have intermediate migrations. Hippocampal FAK migrated as FAK$^+$6,7 and was not recognized by mAb-77. *B,* comparison of immunoblots of hippocampal slices and cell cultures shows that the M_r of FAK is higher in hippocampal slices and in neurons than in astrocytes or microglia. mAb-77 recognizes FAK in astrocytes and in microglia but not in hippocampal slices or neurons in culture. *C,* blotting with antibodies specific for phospho-Tyr-397 shows that the isoform of FAK phosphorylated in anandamide-treated hippocampal slices has the same migration as FAK$^+$6,7 expressed in COS-7 cells. Note that the presence of "box 7" and to a lesser degree "box 6" increases phosphorylation of FAK on Tyr-397, as reported previously (20). *D,* rat hippocampal slices were incubated for 5 min in the presence of vehicle (*Control*), anandamide (1 μM), or 2-AG (1 μM). Slice homogenates (500 μg of protein per sample) were subjected sequentially to immunoprecipitation with serum against PYK2 (*IP PYK2*) and p130-Cas (*IP p130-Cas*) followed by antiphosphotyrosine immunoblotting (*Blot P-Tyr*). The data presented are representative of at least three independent experiments.

since they had no effect on PYK2 tyrosine phosphorylation (Fig. 2*D*). In contrast, endocannabinoids increased the tyrosine phosphorylation of p130-Cas (Fig. 2*D*), an adapter protein associated with FAK (27). The protein levels of PYK2 and p130-Cas were not affected by treatment with cannabinoids (data not shown).

Role of cAMP in the Effects of Endocannabinoids on FAK and p130-Cas Tyrosine Phosphorylation—CB1-R inhibits adenylyl cyclase via a G_i protein (45, 46). Our previous results suggested that inhibition of cAMP signaling was involved in the action of CB1-R on FAK tyrosine phosphorylation (18, 32). We examined the consequences of manipulating the cAMP pathway on the effects of cannabinoids on FAK$^+$6,7 phosphorylation on Tyr-397 and on p130-Cas, the FAK-associated adapter protein. Pretreatment of slices with both 8-Br-cAMP, a non-hydrolyzable cell-permeant analog of cAMP, and IBMX, an inhibitor of phosphodiesterase, completely prevented the effects of endocannabinoids on p130-Cas and on FAK$^+$6,7 tyrosine phosphorylation, including on Tyr-397 (Fig. 3*A*). We then examined the effects of two unrelated inhibitors of PKA, H-89, which acts on the catalytic subunit (47), and (R_p)-cAMPS, which prevents cAMP binding to the regulatory subunit (48). Treatment of slices with either inhibitor mimicked the effects of endocannabinoids on FAK$^+$6,7 and p130-Cas tyrosine phosphorylation (Fig. 3*B*). These results suggest that the effects of endocannabinoids on FAK$^+$6,7 and p130-Cas tyrosine phosphorylation were mediated, at least in part, through inhibition of adenylyl cyclase.

Role of Src Family Kinases and Fyn in the Effects of Endocannabinoids—FAK is closely associated with the tyrosine kinases Src and Fyn, which bind with a high affinity to phospho-Tyr-397 (24, 36). These kinases phosphorylate multiple residues in the kinase domain, including Tyr-576 and Tyr-577 in the activation loop, which are responsible for an increased catalytic activity of FAK. Src also phosphorylates Tyr-925 in the C-terminal region of FAK and several tyrosine residues in associated proteins, including p130-Cas, and thus can trigger several signaling pathways (see Ref. 22 for a review). To determine the role of Src family kinases in the effects of endocannabinoids, we used PP2, a compound that inhibits this group of kinases (49) and has also been reported recently to inhibit platelet-derived growth factor receptor (50). Pretreatment of slices with PP2 strongly diminished basal protein tyrosine phosphorylation and virtually abolished the response induced by endocannabinoids (data not shown), demonstrating that PP2-sensitive kinases, most probably Src family kinases, are critical for the effects of cannabinoids on tyrosine phosphorylation in hippocampus. Similarly, the endocannabinoid-induced total tyrosine phosphorylation of FAK$^+$6,7 was dramatically decreased in the presence of PP2 (Fig. 4), showing the contribution of Src family kinases to the phosphorylation of FAK. By using site-specific antibodies that recognize the phosphorylated forms of these residues, we found that PP2 decreased markedly phosphorylation of FAK$^+$6,7 on Tyr-577 and Tyr-925 (Fig. 4), two residues that are known substrates for Src family kinases (51, 52). In contrast PP2 had no effect on phosphorylation of Tyr-397 (Fig. 4). These results support the notion that the phosphorylation of FAK$^+$6,7 on Tyr-397 results primarily from autophosphorylation and is independent of both Src family kinases and tyrosine phosphorylation of FAK activation loop. Moreover, PP2 completely prevented the phosphorylation of p130-Cas (Fig. 4), indicating that, although this protein is a good substrate for FAK *in vitro* (53), its phosphorylation in cells requires the action of Src family kinases. Thus, Src family kinases appear to be responsible for most of the tyrosine phosphorylation induced by endocannabinoids in hippocampal slices, whereas the autophosphorylation of FAK occurs independently and presumably upstream of Src family kinases activation.

Since PP2 inhibits both Src and Fyn, we examined the pos-

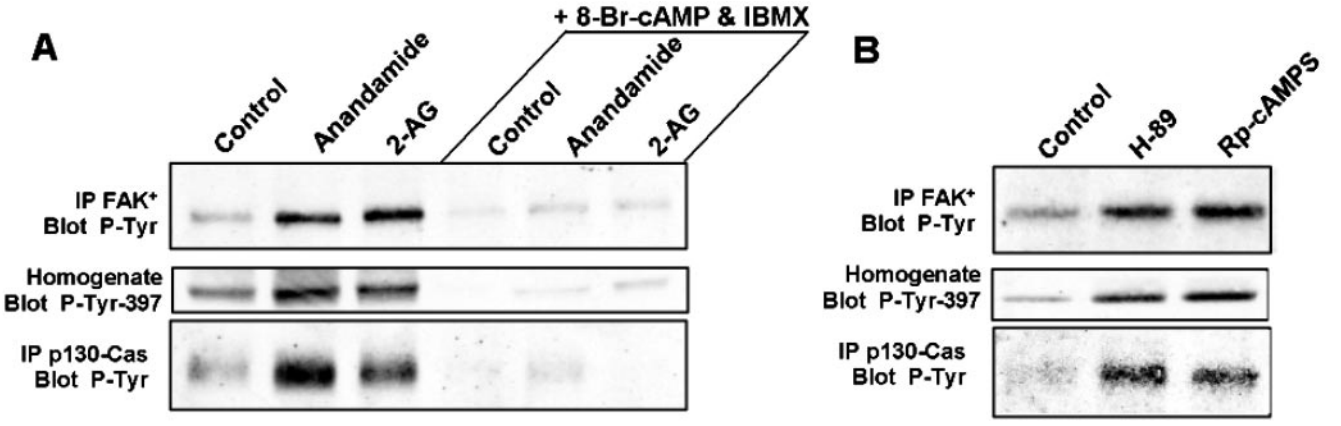

FIG. 3. **Role of cAMP in the effects of endocannabinoids in rat hippocampal slices.** *A,* rat hippocampal slices were incubated as described in the legend to Fig. 1 in the presence or in the absence of 4 mM 8-Br-cAMP and 100 μM IBMX for 45 min before the addition of either vehicle (*control*), 1 μM anandamide, or 1 μM 2-AG for 5 min. *B,* rat hippocampal slices were incubated in the presence of PKA inhibitors, H-89 (100 μM) or (R_p)-cAMPS (1 mM) for 20 min. Total tyrosine phosphorylation of FAK and p130-Cas were assayed by phosphotyrosine immunoblotting after immunoprecipitation, and phosphorylation of FAK on Tyr-397 was measured by immunoblotting of total homogenates with a specific antibody, as described in the legend to Fig. 1. The data presented are representative of at least two independent experiments.

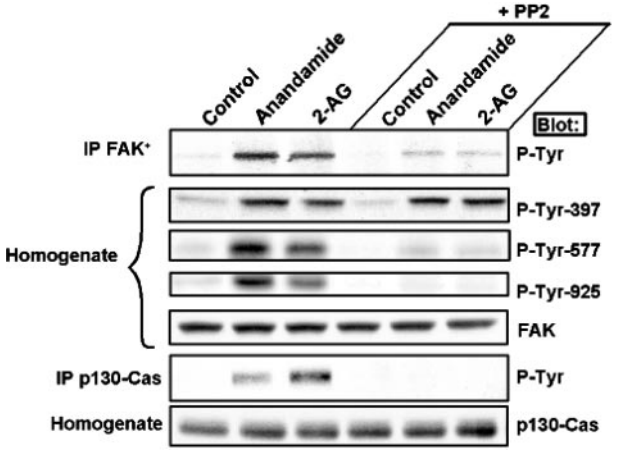

FIG. 4. **Role of Src family kinases in the effects of endocannabinoids in rat hippocampal slices.** Rat hippocampal slices were incubated as described in the legend to Fig. 1 before the addition of either vehicle (*Control*), 1 μM anandamide, or 1 μM 2-AG for 5 min in the absence or in the presence of 5 μM PP2, an inhibitor of Src family kinases. PP2 was added 30 min before cannabinoid agonists. Total tyrosine phosphorylation of FAK⁺6,7 and p130-Cas was measured following immunoprecipitation. Phosphorylation of FAK⁺6,7 on Tyr-397, Tyr-577, and Tyr-925 was assayed by immunoblotting from homogenates. The data presented are representative of three independent experiments.

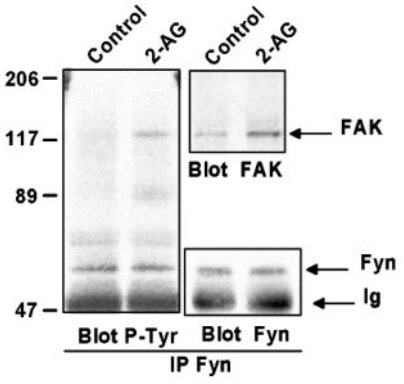

FIG. 5. **2-AG increases the association of FAK with Fyn in hippocampal slices.** Rat hippocampal slices were incubated as described in the legend to Fig. 1 before the addition of either vehicle (*Control*) or 1 μM 2-AG for 5 min. Slices were homogenized in ice-cold modified RIPA buffer as described under "Experimental Procedures" and subjected to immunoprecipitation with antibodies against Fyn (*IP Fyn*). Immune precipitates were analyzed by phosphotyrosine immunoblotting (*Blot P-Tyr*). The presence of a phosphorylated band of the expected apparent M_r of FAK⁺6,7 was detected in Fyn immune precipitates from 2-AG-treated slices. The immune precipitates were also blotted with antibodies against FAK (*Blot FAK*, *upper part*) or Fyn (*Blot Fyn, lower part*), showing that 2-AG treatment did not alter the recovery of Fyn immunoprecipitation but increased the amount of FAK associated with Fyn. The data presented are representative of three independent experiments.

sible association of each of these kinases with FAK⁺6,7. No change in tyrosine-phosphorylated proteins was detected in Src immune precipitates following treatment of hippocampal slices with 2-AG (data not shown). In contrast, 2-AG induced the coimmunoprecipitation with Fyn of an ~130-kDa protein phosphorylated on tyrosine (Fig. 5). This phosphoprotein corresponded to FAK⁺6,7, as shown by blotting with anti-FAK antibodies (Fig. 5). In fact, some FAK was associated with Fyn in untreated slices, and this amount was markedly increased by 2-AG (Fig. 5). Thus, in hippocampal slices, endocannabinoids increased specifically the association of FAK⁺6,7 with Fyn.

The Effects of Endocannabinoids on Hippocampal Signaling Pathways Are Severely Altered in Fyn Knockout Mice—To investigate further the role of Fyn in the effects of endocannabinoids on tyrosine phosphorylation, we used hippocampal slices from mice in which the Fyn gene was inactivated by homologous recombination. These mice are apparently normal but display some anomalies in hippocampal development and have a markedly altered synaptic plasticity (35). When compared with their wild type littermates Fyn −/− mice had normal levels of Src, FAK, and CB1-R (Fig. 6*A*). However, they displayed consistently an increase in p130-Cas levels (Fig. 6*A*, 379 ± 47% of wild type levels). In hippocampal slices from Fyn −/− mice, 2-AG did not increase FAK and p130-Cas tyrosine phosphorylation, an observation reminiscent of the effects of PP2 (Fig. 6*B*). However, the absence of Fyn had more profound consequences than its acute pharmacological blockade by PP2, since the phosphorylation of FAK Tyr-397 was also abolished (Fig. 6*B*). These results show that Fyn is necessary for establishment of the pathway linking CB1-R to FAK autophosphorylation. Since the acute pharmacological blockade of Fyn activity did not mimic all these effects, our results indicate that Fyn may play a role independently of its catalytic activity.

DISCUSSION

The present study demonstrates that 2-AG, the endocannabinoid produced endogenously in hippocampus following

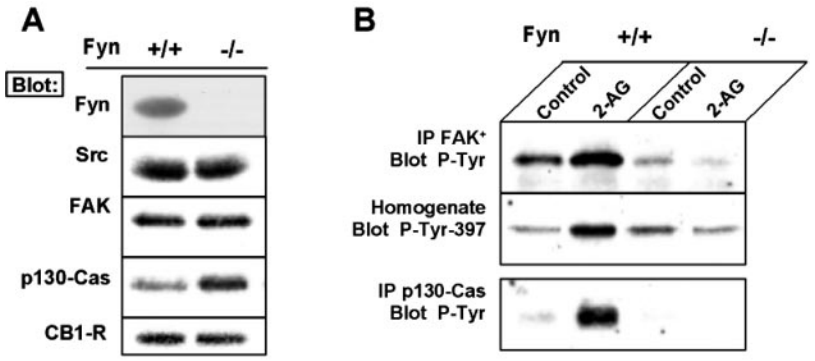

FIG. 6. **The effects of 2-AG on FAK$^+$6,7 and p130-Cas are absent in slices from Fyn −/− mutant mice.** *A,* the amounts of Fyn, Src, FAK, p130-Cas, and CB1-R were measured in hippocampal slices from wild type (Fyn +/+) or knockout mice (Fyn −/−). *B*, hippocampal slices from wild type (Fyn +/+) or knockout mice (Fyn −/−) were incubated as described in the legend to Fig. 1 in the presence of either vehicle (*Control*) or 1 μM 2-AG for 5 min. FAK$^+$6,7 total tyrosine phosphorylation (*IP FAK$^+$ Blot P-Tyr*), phosphorylation on Tyr-397 (*Homogenate Blot P-Tyr-397*), and p130-Cas tyrosine phosphorylation (*IP p130-Cas Blot P-Tyr*) were assayed as described in the legend to Fig. 1. The data presented are representative of three independent experiments.

electrical stimulation (9), activates a protein tyrosine phosphorylation pathway in rat hippocampal slices. We also show that THC, a cannabinoid agonist that is the major psychoactive compound in cannabis, has similar effects. Cannabinoids stimulated the phosphorylation of FAK on Tyr-397, the autophosphorylation site (24, 36), and on other tyrosine residues phosphorylated by Src family kinases, including Tyr-577 and Tyr-925 (51, 52). Cannabinoids also stimulated phosphorylation of p130-Cas, an adapter protein associated to FAK by its Src homology 3 domain (54). Pharmacological experiments and use of CB1-R knockout mice demonstrated that these effects were mediated through activation of CB1-R.

The phosphorylation of FAK induced by CB1-R stimulation was specific, since the closely related tyrosine kinase PYK2 (39) was not activated, although it is highly enriched in hippocampus (40). The differential regulation of the two related tyrosine kinases FAK and PYK2 by cannabinoids is consistent with our previous observations (32, 44) showing that FAK was activated by glutamate, acetylcholine, and LPA, whereas PYK2 was only sensitive to depolarization and hyperosmolarity. The clear-cut difference in the regulation of FAK and PYK2 in hippocampal slices, in contrast to cells in culture in which their regulation by several extracellular stimuli clearly overlaps (55, 56), suggests that they have distinct functions in mature nervous tissue (see Ref. 23).

The isoform of FAK activated by cannabinoids was FAK$^+$6,7, the major isoform of FAK in hippocampus and other forebrain regions (19, 20). This isoform was found in neurons but not in astrocytes or microglial cells in culture. Although we cannot rule out that non-neuronal cells express some levels of FAK$^+$6,7 in adult hippocampus, this finding strongly indicates that the regulation of protein tyrosine phosphorylation by CB1-R takes place in neurons. FAK$^+$6,7 differs from the standard isoform of FAK by a dramatically increased autophosphorylation in transfected cells and in immune precipitates kinase assays (19, 20). We do not know whether this distinct property of neuronal FAK plays a role in its regulation by cannabinoids.

CB1-R inhibits adenylyl cyclase (46). The present results, extending our previous findings (18, 32), suggest a role for the inhibition of cAMP production in the regulation of FAK and p130-Cas by cannabinoids in hippocampal slices, since the effects of endocannabinoids were prevented in the presence of a non-hydrolyzable cAMP analogue and were mimicked by two unrelated PKA inhibitors. Interestingly, an increasing amount of evidence suggests a close antagonistic relationship between FAK-mediated pathways and a cAMP-mediated pathway, since agents that raise cAMP levels decrease FAK phosphorylation in mesangial cells (57), astrocytes (58), and adrenocortical cells (59) in culture. In this latter system, the effects of cAMP may be mediated through activation of SHP2 (60), a tyrosine phosphatase that controls FAK tyrosine phosphorylation (61, 62). In fibroblasts or endothelial cells in culture, cAMP and PKA appear to oppose cell cycle progression and survival in the absence of cell-matrix adhesion (see Ref. 63), and induces tyrosine dephosphorylation of FAK and paxillin following cell suspension (64). Our results in hippocampal slices suggest that in a differentiated tissue changes in cAMP levels induced by a G_i-coupled receptor or pharmacological manipulation of PKA activity is sufficient to activate a signaling pathway involving FAK, similar to that triggered by integrin-dependent cell adhesion.

FAK is autophosphorylated on Tyr-397, allowing the recruitment of Src family kinases that phosphorylate several additional tyrosines in FAK and in associated proteins, including p130-Cas (see Ref. 22 for a review). Cannabinoids induced tyrosine phosphorylation of FAK including Tyr-397, Tyr-577, Tyr-925, and p130-Cas. Src family kinases played an important role in these responses to cannabinoids since total tyrosine phosphorylation of FAK was reduced in the presence of PP2, an inhibitor of these kinases. PP2 dramatically reduced tyrosine phosphorylation of Tyr-577 and Tyr-925, in agreement with the notion that these residues are phosphorylated by Src (51, 52). PP-2 also virtually abolished phosphorylation of p130-Cas. This suggests, in agreement with previous reports (65), that although p130-Cas is a good substrate for FAK *in vitro*, it is mostly phosphorylated by Src family kinases in intact cells. Alternatively, it could be possible that phosphorylation of p130-Cas by FAK in cells requires phosphorylation of its activation loop by Src family kinases. In contrast, phosphorylation of Tyr-397 was unaltered by PP2, showing a clear distinction between the regulation of the autophosphorylation site and the other phosphorylated tyrosines. These observations indicate that stimulation of CB1-R leads first to autophosphorylation of FAK and then to activation of Src family kinases. It has recently been proposed that, following integrin engagement, the phosphorylation of the activation loop of FAK kinase domain by Src family kinases on Tyr-576 and Tyr-577 stimulates intermolecular phosphorylation at Tyr-397, leading to signal amplification and maximal activation of FAK (66). However, the lack of effects of PP2 on phosphorylation at Tyr-397, although it strongly inhibited the phosphorylation at Tyr-577, does not support such a reciprocal catalytic activation of FAK and Src family kinases in response to cannabinoids in hippocampus. Accordingly, it has been shown recently (67) in fibroblasts that

FAK (auto)phosphorylation on Tyr-397 induced by G protein-coupled receptor agonists is independent of Src family kinases, whereas FAK activation following integrin engagement is dependent on these enzymes.

Although FAK can interact with either Src or Fyn (24), which are both abundant in hippocampus, we found by coimmunoprecipitation that Fyn, but not Src, was associated with FAK and that CB1-R stimulation increased this association. A preferential association of FAK with Fyn has also been observed following high frequency stimulation of hippocampal slices, whereas in the same conditions PYK2 was associated with Src (41, 68). Similarly, we have observed a depolarization-induced association of PYK2 with Src in hippocampal slices.[3] The preferential association between FAK and Fyn in hippocampus cannot be simply accounted for by the expression of FAK$^+$6,7, since this isoform interacts apparently equally well with Fyn, c-Src, and v-Src in transfected cells (19, 20). Interestingly, in *fyn* −/− mice tyrosine phosphorylation of several proteins, including FAK, was altered (69). These knockout mice display a slightly abnormal development of hippocampus, with an increased number of cells in the dentate gyrus and an impairment of LTP (35). The synaptic plasticity deficit was rescued by re-expression of Fyn, showing that it was independent of developmental problems (70). In contrast, no molecular, developmental, or electrophysiological phenotype was observed in *src* −/− mice (35, 69), although the role of Src in synaptic plasticity is supported by other types of evidence (41, 71). The impairment of FAK signaling was more severe in hippocampal slices from Fyn knockout mice than in PP2-treated slices; phosphorylation of Tyr-397 in response to cannabinoids was unaltered by PP2, whereas it was completely blocked in *fyn* −/− mice. This result indicates that Fyn plays a crucial role in FAK-related signaling in the hippocampus. A possible explanation for the discrepancy between the consequences of the acute blockade of kinase activity by PP2 and those of Fyn knockout would be that Fyn has functions independent of its kinase activity. In support of this hypothesis, it has been reported that the abnormal phenotype of *src* −/− mice, a functional deficit of osteoclasts, was rescued by a kinase-dead mutant of Src (72). These results suggest that some non-redundant functions of Src family kinases in cells are independent of their kinase activity. In the case of Fyn in hippocampus, such a role may involve the autophosphorylation of FAK. It is noteworthy that the levels of p130-Cas were markedly increased in the hippocampus of *fyn* −/− mice. The change in p130-Cas levels may not be related to its decreased tyrosine phosphorylation, since this phosphorylation is markedly altered in both *fyn* −/− hippocampal slices (present study) and *FAK* −/− cells in culture (66), whereas p130-Cas levels were unchanged in this latter system. Although the mechanism of this increase is not known, it underlines the importance of Fyn in the regulation of the FAK pathway.

What are the possible roles of the tyrosine phosphorylation pathways regulated by CB1-R in hippocampus? In this brain region, CB1-R is highly concentrated at the presynaptic level, on nerve terminals from interneurons (15, 28), and exerts a negative effect on γ-aminobutyric acid release (14). The reported effects of CB1-R on synaptic plasticity are complex. On the one hand endocannabinoids act as retrograde messengers in depolarization-induced suppression of inhibition, presumably by inhibiting γ-aminobutyric acid release (12, 13), and thus have a positive role in synaptic plasticity. On the other hand, stimulation of cannabinoid receptors impairs LTP and long term depression (73, 74), apparently by inhibiting glutamate release (11). These effects on neurotransmitter release may result, at least in part, from the inhibition of P/Q Ca^{2+} currents (75, 76) and the increase of I_A K^+ current (77). Interestingly a role of tyrosine kinases, and especially Fyn, is well documented in LTP (35, 70, 78). It is tempting to speculate that activation of a FAK/Fyn pathway by endocannabinoids participates in the control of synaptic plasticity by this new class of messengers. Our results also raise the possibility of a regulation by endocannabinoids, through CB1-R and FAK, of known targets of Fyn in the nervous system, including the *N*-methyl-D-aspartate subtype of glutamate receptor (79), and control of cell-cell interaction by cadherin-related neuronal receptors (80).

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[3] J. C. Corvol, D. Hervé, and J.-A. Girault, unpublished observations.

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