

CB₁ Cannabinoid Receptor Activity Is Modulated by the Cannabinoid Receptor Interacting Protein CRIP 1a

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ABSTRACT

The CB₁ cannabinoid receptor is a G-protein coupled receptor that has important physiological roles in synaptic plasticity, analgesia, appetite, and neuroprotection. We report the discovery of two structurally related CB₁ cannabinoid receptor interacting proteins (CRIP1a and CRIP1b) that bind to the distal C-terminal tail of CB₁. CRIP1a and CRIP1b are generated by alternative splicing of a gene located on chromosome 2 in humans, and orthologs of CRIP1a occur throughout the vertebrates, whereas CRIP1b seems to be unique to primates. CRIP1a coimmunoprecipitates with CB₁ receptors derived

from rat brain homogenates, indicating that CRIP1a and CB₁ interact in vivo. Furthermore, in superior cervical ganglion neurons coinjected with CB₁ and CRIP1a or CRIP1b cDNA, CRIP1a, but not CRIP1b, suppresses CB₁-mediated tonic inhibition of voltage-gated Ca²⁺ channels. Discovery of CRIP1a provides the basis for a new avenue of research on mechanisms of CB₁ regulation in the nervous system and may lead to development of novel drugs to treat disorders where modulation of CB₁ activity has therapeutic potential (e.g., chronic pain, obesity, and epilepsy).

G protein-coupled receptors (GPCRs) provide a wide range of signaling capabilities to regulate the activity of downstream cellular targets. To signal efficiently, cells must be able to dynamically control the activity of GPCRs. Although

some regulatory pathways, such as desensitization and internalization mediated by β -arrestin (Benovic et al., 1986), are applicable to most GPCRs, specialized means of regulation for particular GPCRs have been identified. Because many GPCRs have been shown to have spontaneous basal activity, ancillary proteins that interact with GPCRs may prove to be specific modulators of this activity. A prominent protein-protein interaction site studied on GPCRs is the C-terminal tail; G-protein binding and post-translational modifications occur in this region in many GPCRs. The profound sequence variety of C-terminal tails provides a means for selectivity in G-protein interactions as well as diversity in receptor trafficking. The G-protein-coupled receptor-associated sorting protein GASPI interacts with the C-terminal tail of many GPCRs, including CB₁, resulting in down-regulation and degradation (Martini et al., 2007). The adaptor protein FAN is also able to interact with the CB₁ receptor

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CB₁, cannabinoid receptor subtype-1; CRIP1a, cannabinoid receptor interacting protein subtype 1a; CRIP1b, cannabinoid receptor interacting protein subtype 1b; SCG, superior cervical ganglion; WIN 55,212-2, [2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-(1-naphthyl)methanone; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; HEK, human embryonic kidney; CHO, Chinese hamster ovary; aa, amino acid(s); GST, glutathione transferase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; BSA, bovine serum albumin; PDZ, postsynaptic density 95/disc-large/zona occludens; HA, hemagglutinin.

(Sánchez et al., 2001). Regulation of basal activity of GPCRs by accessory proteins binding to the C-terminal tail has been described for metabotropic glutamate receptors (mGluRs). One of the members of the Homer protein family, Homer 1a, uncovers constitutive basal activity of group I mGluRs by competing for mGluR1/5 binding with other Homer isoforms that normally prevent constitutive signaling (Ango et al., 2001).

The CB₁ cannabinoid receptor, a GPCR, is activated by Δ^9 -tetrahydrocannabinol (Howlett, 1985), the primary psychotropic component of marijuana, as well as endocannabinoids such as anandamide (Devane et al., 1992) and 2-arachidonyl glycerol (Mechoulam et al., 1995). Endocannabinoids act as retrograde messengers mediating CB₁-dependent forms of short-term synaptic plasticity known as depolarization-induced suppression of inhibition or excitation (Diana and Marty, 2004) and longer-lasting forms of synaptic plasticity, such as long-term depression (Gerdeman and Lovinger, 2001; Robbe et al., 2002; Chevalyere and Castillo, 2003; Sjöström et al., 2003; Azad et al., 2004). Extinction of aversive memories is dependent on the endocannabinoid system (Marsicano et al., 2002). The endocannabinoid system also mediates a neuroprotective effect in models of excitotoxicity (Shen and Thayer, 1998; Abood et al., 2001), ischemia (Parmentier-Batteur et al., 2002) and seizure (Marsicano et al., 2003).

The complexity of CB₁ signaling is increased by the agonist-independent or ligand-free constitutive activity as measured by its reversal with the antagonist/inverse agonist SR141716 (Bouaboula et al., 1997). Application of SR141716 reverses the tonic inhibition of N-type voltage-gated Ca²⁺ channels, resulting in an increase in the Ca²⁺ current in superior cervical ganglion (SCG) neurons expressing CB₁ receptors (Pan et al., 1998). Deletion of the CB₁ C-terminal tail distal to the G-protein binding domain enhances the effect of SR141716. SR141716 produces a significantly larger increase in the Ca²⁺ current in neurons expressing C-terminally truncated CB₁ receptors (Nie and Lewis, 2001). Thus, deletion of the distal C-terminal region of CB₁ results in enhanced tonic inhibition of Ca²⁺ channels, suggesting that either this region constrains the receptor conformation or that accessory proteins binding to this region modulate CB₁ activity.

We report here the discovery of two cannabinoid receptor interacting proteins (CRIP), CRIP1a and CRIP1b, that interact with the distal C-terminal tail of CB₁. CRIP1a is expressed in the brain and is found throughout vertebrates, whereas CRIP1b seems to be unique to primates. CRIP1a coimmunoprecipitates with CB₁ from rat brain and colocalizes with CB₁ when heterologously expressed in neurons. Neither CRIP1a nor CRIP1b significantly alters the affinity of CB₁ for the antagonist/inverse agonist SR141716. However, CRIP1a, but not CRIP1b, significantly attenuates tonic inhibition of voltage-gated Ca²⁺ channels by CB₁ receptors.

Materials and Methods

Yeast Two-Hybrid Screening. The Matchmaker Two-Hybrid System (Clontech, Mountain View, CA) was used to screen a human brain cDNA library (Clontech) using a bait protein corresponding to the C-terminal tail of CB₁ (last 55 amino acids, 418–472, of human CB₁, excluding the G-protein binding region 400–417). The positive clone with the highest β -galactosidase activity as determined by

filter-lift assay (i.e., CRIP1b) was isolated and cotransformed with bait cDNA (CB₁ C-terminal tail) into yeast to confirm the interaction.

PCR Screening of Rat Brain cDNA Library. A rat brain cDNA library was constructed using a GeneRacer kit (Invitrogen, Carlsbad, CA). Primers to homologous regions of CRIP1b were used in combination with GeneRacer kit primers to determine the full coding region of CRIP1a. Full-length CRIP1a was then cloned using primers just upstream from the start site (5' primer: CTT CCT CCC TGC CTG TCT CTG) and downstream from the stop site (3' primer: GCT GTT TAT GTT ATT ACC TCT). Accession numbers for CRIP1a (AY883936) and CRIP1b (AY144596) nucleotide sequences have been deposited into GenBank.

In Vitro Binding Assay. The GST Gene Fusion System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to construct GST-CB₁ (C-terminal tail) fusion proteins using the pGEX-4T-1 vector. GST-CB₁ was expressed in *Escherichia coli*, isolated with glutathione-Sepharose beads, and incubated with lysate containing CRIP1a or CRIP1b S-tag fusion proteins subcloned into pET44a(+) or pET30c, respectively. Eluted proteins were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane. The S-tag [15 amino acids (aa)] was visualized by its interaction with ribonuclease S-protein conjugated to horseradish peroxidase (Novagen, San Diego, CA).

Generation of CRIP1a Antibodies. Rabbits were immunized with a conjugate of thyroglobulin and a peptide comprising the last 17 amino acids of rat/mouse/human CRIP1a, followed by affinity-purification of antibodies from antisera using the immunizing peptide.

Immunoblotting and Immunoprecipitation. Homogenates of mouse organs/tissues (10 μ g of protein per lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with CRIP1a antiserum (1:1000) or CRIPa antiserum (1:1000) that had been preabsorbed with the antigen peptide (20 μ M). Bound antibodies were revealed using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Vector Laboratories, Burlingame, CA).

The procedure for immunoprecipitation of CB₁ and associated proteins has been described previously (Mukhopadhyay and Howlett, 2001), as has the generation of antibodies in rabbits against the first 14 amino acids of CB₁ (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001). In brief, rat brain P2 membranes (5 mg of protein) were solubilized in 0.5 ml of buffer (30 mM Tris-Cl, pH 7.4, and 5 mM MgCl₂) containing 4 mg of CHAPS (Sigma, St. Louis, MO) and 20% glycerol on ice with gentle stirring for 30 min, followed by centrifugation at 100,000g for 40 min at 4°C. CHAPS solubilized proteins (100 μ l) were incubated with Sepharose beads coupled to anti-CB₁ antibodies (20 μ l) for 6 h at 4°C. The anti-CB₁ affinity matrix was then sedimented at 17,000g for 5 min and washed three times with 500 μ l of buffer (20 mM Tris-Cl, pH 7.4, 140 mM NaCl, and 0.1% Tween 20). Immunoprecipitated protein was eluted with 50 μ l glycine, pH 2.5 (100 mM) and immediately neutralized with 450 μ l of Tris-Cl, pH 8.0 (1.5 M). Protein from neutralized eluate was precipitated by addition of 8 volumes of CHCl₃/CH₃OH/H₂O (1:4:3), dissolved in Laemmli sample buffer, and heated at 65°C for 5 min. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, probed with CRIP1a antiserum (1:500) and CB₁ antibodies (1:1000, N-terminal), and detected using enhanced chemiluminescence.

[³H]SR141716 Binding Assay. Saturation analysis of [³H]SR141716 binding was performed by incubating 10 μ g of membrane protein with 0.01 to 5 nM [³H]SR141716 in 1 ml of buffer containing 0.5 g/liter bovine serum albumin (BSA) in the presence and absence of 5 μ M unlabeled SR141716 to determine nonspecific and specific binding, respectively. The assay was incubated for 90 min at 30°C and terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ) that were presoaked in Tris buffer containing 5 g/liter BSA (Tris-

BSA), followed by five washes with ice-cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after shaking of the filters for 1 h in 4 ml of ScintiSafe Econo 1 scintillation fluid. The presence of CB₁, CRIP1a, and CRIP1b in the appropriate membrane protein samples was verified by Western blot analysis.

Confocal Microscopy. SCG neurons were plated on poly-L-lysine-coated glass coverslips and microinjected with solutions containing plasmids containing HA-CB₁ cDNA (100 ng/ μ l) and CRIP1a cDNA (160 ng/ μ l) or FLAG-CRIP1b cDNA (170 ng/ μ l). Neurons were fixed in PBS containing 4% paraformaldehyde and 4% sucrose for 30 min, rinsed with excess PBS, and blocked with 5% nonfat dry milk, 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA), and 0.1% Triton X-100 in PBS for 1 h. Neurons were incubated with anti-HA monoclonal or polyclonal antibodies diluted 1:150 or 1:100, CRIP1a antiserum at 1:1000 or anti-FLAG M2 antibodies at 1:2000, as appropriate, for 1 h. Neurons were incubated with Alexa Fluor 488 (goat anti-rabbit) and Alexa Fluor 568 (goat anti-mouse) antibodies at 1:1000 for 1 h. SCG nuclei were identified using a nucleic acid stain, 4,6-diamidino-2-phenylindole (300 nM; Invitrogen, Carlsbad, CA). Excess antibodies were removed by PBS washes. Neurons were mounted on glass slides with ProLong antifade reagent (Invitrogen, Carlsbad, CA). Z-stack images were acquired on a Zeiss Axiovert LSM 510 META inverted confocal microscope using a 63 \times oil objective and documented using LSM510 software.

Electrophysiology. Rat SCG neurons were isolated, cultured, and microinjected as described previously (Nie and Lewis, 2001), with the following modifications. SCG were incubated with 0.32 mg/ml trypsin (Worthington Biochemical, Lakewood, NJ) and 0.52 mg/ml collagenase D (Roche, Palo Alto, CA) in Earle's balanced salt solution for 1 h at 35°C in a shaking water bath. During the last 10 min of incubation, 10 U (\sim 5 μ l) of DNase I (Worthington Biochemical, Lakewood, NJ) was added. Dissociated neurons were plated on precoated poly-L-lysine 35-mm culture dishes (BD Biosciences, San Jose, CA). Microinjection solutions contained plasmids with cDNA encoding CB₁ (100 ng/ μ l) and EGFP (10 ng/ μ l) with or without CRIP1a (160 ng/ μ l) or CRIP1b (340 ng/ μ l) cDNA.

Calcium current recordings were performed as described previously (Nie and Lewis, 2001). The extracellular recording solution consisted of 140 mM tetraethylammonium methanesulfonate, 10 mM HEPES, 15 mM glucose, 10 mM CaCl₂, and 0.1 μ M tetrodotoxin, pH 7.4 (adjusted with methanesulfonic acid). The intracellular solution contained 120 mM *N*-methyl-D-glucamine, 20 mM tetraethylammonium chloride, 10 mM HEPES, 11 mM EGTA, 1 mM CaCl₂, 4 mM Mg-ATP, 0.1 mM Na₂-GTP, and 14 mM phosphocreatine, pH 7.2 (adjusted with methanesulfonic acid). WIN 55,212-2 (Tocris Cookson, Ellisville, MO) or SR141716 (NIDA Drug Supply Program; www.nida.nih.gov/about/organization/DBNBR/CPSRB.html) were diluted fresh on the day of the experiment from 10 mM stock solutions in dimethylsulfoxide to 1 μ M in external solution and were briefly sonicated (20 s) to facilitate dispersion. Cumulative concentration-response experiments were performed by superfusing SCG neurons with progressively higher concentrations of WIN 55,212-2 after the response to the previous concentration had stabilized.

Statistics. Data are presented as means \pm S.E.M. Statistical significance was determined by Student's *t* test when comparing two conditions; analysis of variance with post hoc Bonferroni-adjusted *t* test or Dunnett's test were used when comparing three or more conditions. EC₅₀ values were calculated by unweighted least-squares nonlinear regression of log concentration values versus percentage effect (Prism; GraphPad Software, San Diego, CA). *B*_{max} and *K*_D values of [³H]SR141716 binding were similarly determined by nonlinear regression analysis of saturation curves using Prism. Differences were considered significant at *p* < 0.05.

Results

Discovery of CRIP1a and CRIP1b and Interaction with CB₁. Because the activity of CB₁ is influenced by its C-terminal tail, we used the last 55 amino acids of CB₁ that are distal to the G-protein binding region as bait in a yeast two-hybrid assay to screen a human brain cDNA library for potential interacting partners. Among several positive hits, a clone with no homology to other known proteins was sequenced and found to encode a 128-amino acid protein (Fig. 1a). Analysis of human genomic and cDNA sequence data revealed that this protein is encoded by a gene on human chromosome 2, which is alternatively spliced to generate mRNAs encoding a 164-amino acid protein (exons 1, 2, and 3a) and a 128-amino acid protein (exons 1, 2, and 3b), hereafter referred to as CRIP1a and CRIP1b, respectively (Fig. 1b). Neither CRIP1a nor CRIP1b interacted with the C-terminal tail of the CB₂ cannabinoid receptor in the yeast two-hybrid assay (data not shown). CRIP1a and CRIP1b were expected to be cytosolic proteins, because they contain no transmembrane domains, as predicted by hydropathy analysis. CRIP1a, but not CRIP1b, has a predicted palmitoylation site (palmitoylation sites prediction, http://bioinformatics.lcd-ustc.org/css_palm/) that may contribute to its localization at the plasma membrane, but neither splice variant possesses a myristoylation site (<http://ca.expasy.org/tools/myristoylator/>). In addition, CRIP1a, but not CRIP1b, contains a PDZ Class I ligand in its C-terminal tail, which could indicate a potential for interactions with proteins containing PDZ domains. Comparative genomic analysis and cDNA sequencing revealed that orthologs of CRIP1a are present throughout vertebrates, with CRIP1a orthologs in human, chicken, *X. laevis* and zebrafish sharing 96, 71, 66, and 59% sequence identity with rat CRIP1a, respectively. Orthologs of CRIP1b have thus far been discovered only in chimpanzee and macaque, indicating that exon 3b has evolved more recently than exon 3a and may be unique to primates. Furthermore, a mouse cerebellar cDNA sequence (accession number AK005381) derived from a 5' noncoding exon, exon 1, and a 3' extended variant of exon 2 indicates the existence of additional CRIP1a/b-like proteins generated by alternative mRNA splicing in rodents.

To identify the region of the CB₁ C-terminal tail necessary for interaction with CRIPs, we constructed and characterized several CB₁ mutants. Jin et al. (1999) identified two distinct regions of the CB₁ C-terminal tail that mediate desensitization or internalization. The CB₁ mutants we generated were designed to assess the importance of these regions in the CB₁-CRIP interaction. Note that aa numbering corresponds to the rat CB₁ sequence, which is one residue longer than the human CB₁ sequence because of an insertion at aa 74. Mutants lacking the desensitization domain (aa 419–438) or the internalization domain (aa 460–463) were nevertheless able to interact with CRIP1b at levels indistinguishable from the wild-type CB₁ C-terminal tail (Fig. 1c). The last nine amino acids of the CB₁ C-terminal tail, identical in rat (aa 465–473) and human (aa 464–472), comprised the minimal domain tested that was able to interact with CRIP1b. A complementary mutant lacking the last nine amino acids interacted only very weakly with CRIP1b, suggesting that the distal C-terminal region is the domain of CB₁ necessary for interaction with CRIP1b. Conversely, the region of CRIP1b necessary for

interaction with CB₁ was determined using deletion mutants of CRIP1b. No single exon of CRIP1b was sufficient to interact with the CB₁ C-terminal tail (Fig. 1d). A combination of exons 1 and 2 (aa 34–110) was the minimal domain tested that was able to interact with CB₁.

CRIP1a and CRIP1b Interacted with CB₁ in Vitro and in Vivo. To confirm the yeast two-hybrid data, we performed glutathione transferase (GST) pull-down assays with the C-terminal tail of CB₁ and either CRIP1a or CRIP1b. Bacterially expressed CRIP1a or CRIP1b bound to immobilized GST-CB₁ fusion proteins. The identity of CRIP1a or CRIP1b in the GST-column eluate was verified by Western blotting with ribonuclease S-protein conjugated to horseradish peroxidase that detected the S-tag peptide (15 aa) fused to CRIP1a (Fig. 2a) or CRIP1b (Fig. 2b). Neither CRIP1a nor CRIP1b was detected in eluate from the control GST-Sepharose column, indicating that CRIP1a and CRIP1b

bound specifically to CB₁ and not to the Sepharose in an in vitro interaction.

To investigate in vivo interaction of CB₁ and CRIP1a, we generated antibodies to the last 17 amino acids of CRIP1a. These CRIP1a antibodies labeled a single, intense band of the expected molecular mass (18 kDa) in Western blots of mouse brain homogenates. Preincubation of antibodies with the immunizing peptide prevented detection of the 18 kDa band, supporting the specificity of the antibodies for CRIP1a (Fig. 2c). Having developed specific CRIP1a antibodies, we investigated in vivo interaction between CB₁ and CRIP1a using a CB₁ N-terminal antibody (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001) to immunoprecipitate CB₁ and associated proteins from CHAPS solubilized rat brain membranes. Immunoblots probed with CRIP1a and CB₁ antibodies revealed that CRIP1a coprecipitated with CB₁ in membranes from rat brain (Fig. 2d) but not when the CB₁

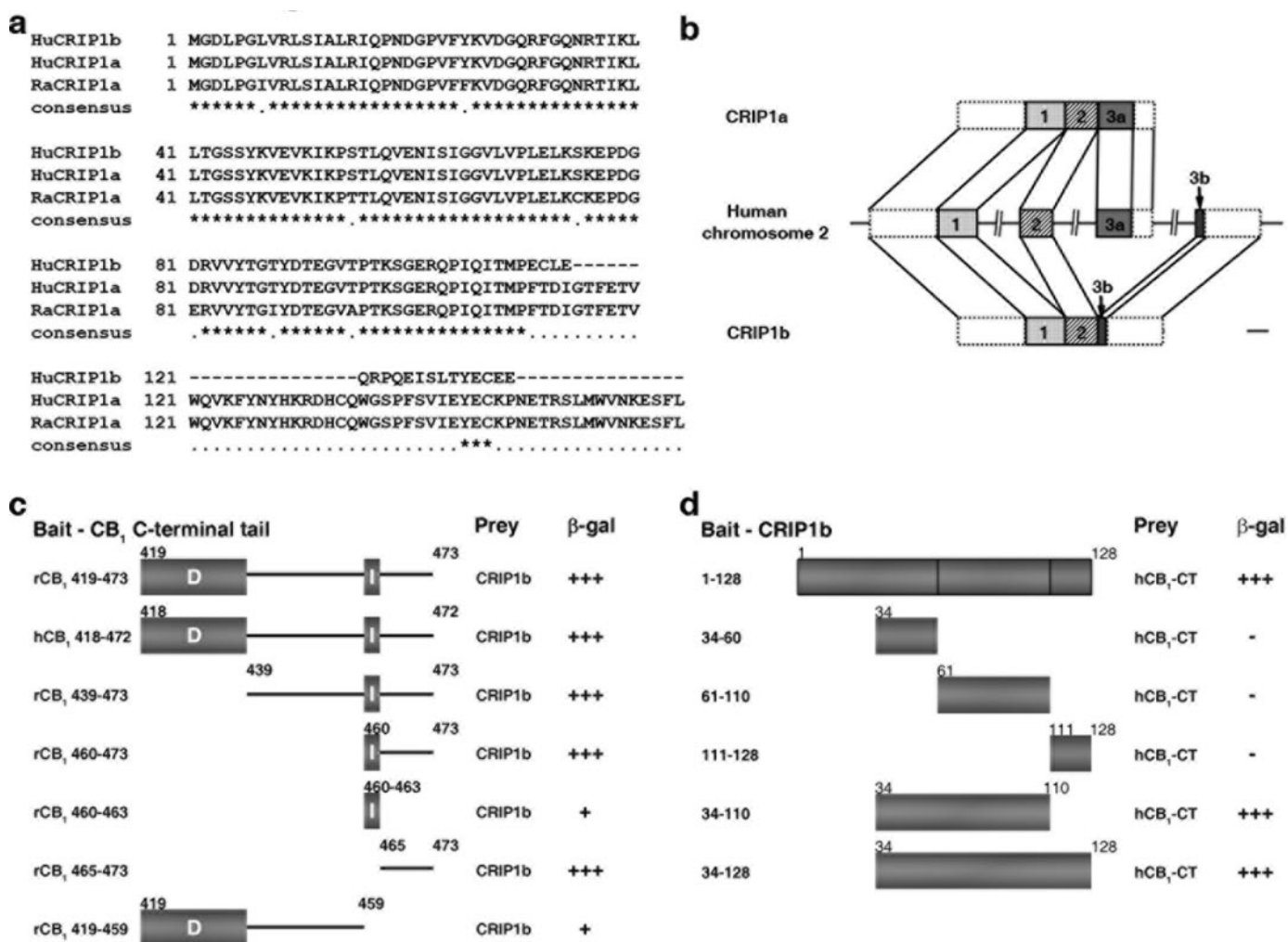


Fig. 1. Cannabinoid receptor interacting proteins, CRIP1a and CRIP1b. **a**, amino acid sequence alignment of human CRIP1b (HuCRIP1b), human CRIP1a (HuCRIP1a) and rat CRIP1a (RaCRIP1a). Identical amino acids are denoted (*); differences are denoted (.) in consensus line. Exon 1 is composed of amino acids 1–50; exon 2, amino acids 51–110; exon 3a, amino acids 111–164 in CRIP1a; exon 3b, amino acids 111–128 in CRIP1b. **b**, organization of human CRIP1 gene, which is alternatively spliced to yield CRIP1a and CRIP1b. Scale bar in lower right represents 180 base pairs. Scale breaks in human chromosome 2 correspond to 1.91, 23.13 and 8.53 kb spans, respectively. Exon 1 and exon 2 are conserved between CRIP1a and CRIP1b, while exon 3 is unique. Thus, exon 3a is present in CRIP1a mRNA and exon 3b is present in CRIP1b mRNA. **c**, the last nine amino acids of the CB₁ C-terminal tail were essential for interaction with CRIP1b. Desensitization (D) and internalization (I) regions (Jin et al., 1999) of CB₁ are depicted as boxes. Numbers indicate amino acid residues of rat (r) or human (h) CB₁ used as bait. **d**, interaction with wild type CB₁ C-terminal tail requires amino acids 34–110 of CRIP1b. Numbers refer to amino acid residues of CRIP1b used as bait. In **c** and **d**, yeast cells were cotransformed with plasmids encoding proteins fused with the Gal4 DNA binding domain (bait) or Gal4 DNA activation domain (prey). Transformed yeast cells were seeded on Leu⁻, Trp⁻, His⁻ plates and assayed for β-galactosidase activity.

antibody was omitted, indicating that endogenous CB₁ and CRIP1a interact *in vivo*.

Western blot analysis of a variety of different mouse tissues/organs indicate that CRIP1a is highly expressed in brain, but is also detected in heart, lung, intestine, kidney, testis, spleen, liver and muscle (Fig. 2e). CRIP1a was also detected by Western blot in cultured rat cerebellar granule neurons, SCG neurons, N18TG2 neuroblastoma and AtT-20 cells, but not in HEK 293 cells (data not shown).

CRIP1a and CRIP1b Did Not Alter the Expression or Affinity of CB₁. In data obtained from cell lines, CRIP1a did not change CB₁ receptor expression or protein maturation and membrane localization. In stable HEK 293 cell lines expressing CB₁ or CB₁ and CRIP1a or transiently cotransfected CHO cells, neither [³H]SR141716 binding affinity for CB₁ nor maximum binding was significantly affected by CRIP1a on total membrane fractions (Fig. 3; Table 1). These results demonstrate that CRIP1a does not affect the expres-

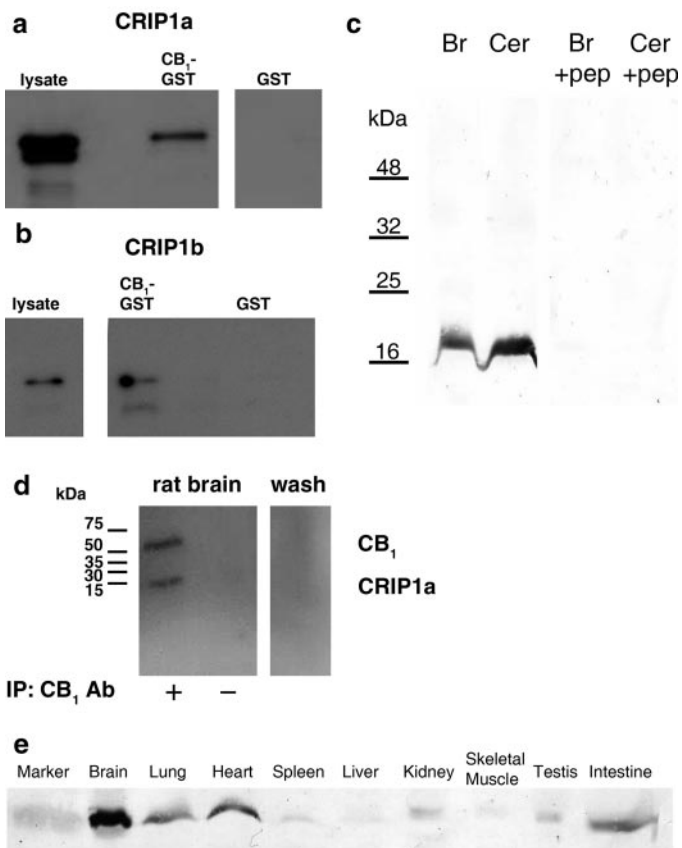


Fig. 2. CRIPs interact with CB₁ *in vitro* and *in vivo*. Bacterially expressed CRIP1a (a) or CRIP1b (b) bound specifically to immobilized GST-CB₁ C-terminal tail and not to the negative control, GST, in Western blot analyses of *in vitro* binding assay. Apparent molecular weight of CRIP1a or CRIP1b was determined by running a lysate sample. The experiments were repeated three times with similar results. c, CRIP1a antiserum specifically recognized a single immunoreactive band corresponding to the expected molecular mass of CRIP1a (18 kDa) in mouse brain (Br) or cerebellar (Cer) homogenates. Immunostaining was blocked by preabsorption of antisera with the immunizing peptide (pep). d, CRIP1a coimmunoprecipitated with CB₁ from rat brain in Western blot probed with anti-CB₁ and anti-CRIP1a antibodies. Neither CB₁ nor CRIP1a bound to Sepharose beads that were not conjugated with anti-CB₁ antibody. These experiments were repeated twice with similar results. e, CRIP1a is expressed in brain but is also detected in heart, lung, intestine, kidney, testis, spleen, liver, and muscle as shown in this Western blot of mouse tissues/organs.

sion of CB₁ receptors. Similar to CRIP1a, CB₁ expression in cell lines was not affected by coexpression with CRIP1b. In transfected CHO cells expressing CB₁ or CB₁ and CRIP1b, neither [³H]SR141716 binding affinity nor maximum binding was significantly altered (Table 1). Although there appeared to be approximately a 2-fold increase in [³H]SR141716 *K_D* value in CHO cells coexpressing CB₁ and CRIP1b compared with CHO cells expressing CB₁ and empty vector, this was only a nonsignificant trend (*p* = 0.086; *F* = 2.83).

Expression and Colocalization of CB₁ and CRIP1a and CRIP1b in SCG Neurons. Because CRIP1a and CRIP1b interact with the C-terminal tail of CB₁, a domain known to affect the ability of the CB₁ receptor to tonically inhibit voltage-gated Ca²⁺ channels, we evaluated the potential for functional interaction between CB₁ and CRIP1a and CRIP1b by whole-cell, patch-clamp recordings from rat SCG neurons heterologously expressing CB₁ and CRIP1a or CRIP1b. N-type Ca²⁺ channels, which regulate excitability and neurotransmitter release, are homogeneously expressed, and their modulation has been extensively studied in SCG neurons (Hille, 1994). Expression of CB₁ receptors in these neurons results in tonic inhibition of N-type Ca²⁺ channels

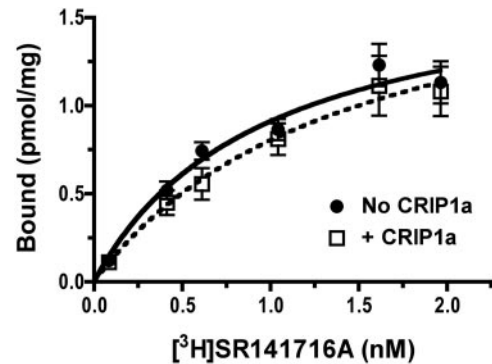


Fig. 3. [³H]SR141716 saturation binding in membranes from hCB₁-HEK cells with and without stable coexpression of CRIP1a. Membranes were incubated with the indicated concentrations of [³H]SR141716, and binding was assessed as described under *Materials and Methods*. Values shown are mean picomoles of [³H]SR141716 specifically bound per milligram of membrane protein ± S.E. (*n* = 4).

TABLE 1

CRIP1a or CRIP1b did not significantly alter the maximum binding (*B_{max}*) or [³H]SR141716 binding affinity (*K_d*) for CB₁ in HEK 293 or CHO cells

Membranes were prepared from HEK cells stably expressing CB₁ receptors with or without CRIP1a or from CHO cells that were transiently transfected with CB₁ receptor cDNA and an empty vector, CRIP1a or CRIP1b. Membranes were incubated with varying concentration of [³H]SR141716 as described under *Materials and Methods*. Data are mean *B_{max}* and *K_D* values ± S.E. from nonlinear regression analysis of the saturation binding curves. Statistical significance of differences in *B_{max}* or *K_D* values between HEK cells stably expressing CB₁ or CB₁ + CRIP1a was determined by the two-tailed non-paired *t* test; statistical differences among these parameters in the three transiently transfected CHO cell preparations were determined by analysis of variance with post hoc Bonferroni-adjusted *t* test or Dunnett's test. None of these inferential statistical tests revealed any significant differences in *B_{max}* or *K_D* values between these data sets.

	<i>n</i>	<i>B_{max}</i> pmol/mg protein	<i>K_d</i> nM
CB ₁ stable HEK 293	4	1.87 ± 0.26	1.05 ± 0.19
CB ₁ + CRIP1a stable HEK 293	4	2.01 ± 0.26	1.52 ± 0.20
CB ₁ transfected CHO	7	1.82 ± 0.34	0.75 ± 0.10
CB ₁ + CRIP1a transfected CHO	7	1.85 ± 0.35	0.80 ± 0.20
CB ₁ + CRIP1b transfected CHO	7	1.74 ± 0.40	1.64 ± 0.47

that can be reversed by the CB₁ antagonist/inverse agonist SR141716 (Pan et al., 1998). SR141716 stabilizes the inactive state of the receptor (Hurst et al., 2002), relieving the tonic inhibition of Ca²⁺ channels and increasing the Ca²⁺ current. Because deletion of the C-terminal tail of CB₁ results in an increased tonic inhibition of Ca²⁺ currents in SCG neurons (Nie and Lewis, 2001), we hypothesized that CRIP1a or CRIP1b might serve as endogenous regulators of CB₁ activity. To verify that CB₁ and CRIP1a or CRIP1b had the potential to interact in SCG neurons, we investigated the extent of overlap in neurons microinjected with cDNA encoding HA-CB₁ and CRIP1a or FLAG-CRIP1b. Both CRIP1a (Fig. 4b) and FLAG-CRIP1b (Fig. 4e) were enriched near the plasma membrane and overlapped with HA-CB₁ (Fig. 4, c and f) indicating that CRIP1a and FLAG-CRIP1b were trafficked to the same subcellular compartment as HA-CB₁ (Fig. 4, a and d), where they could affect CB₁ signaling. Although predicted to be cytosolic proteins, the expression pattern of CRIP1a and FLAG-CRIP1b in SCG neurons seemed to be membrane-associated rather than homogeneously distributed throughout the cytosol when expressed with or without HA-CB₁. These results agree with the finding that CRIP1a immunoreactivity was evident in Western blots of membrane preparations from CHO cells transiently expressing CB₁ receptors and CRIP1a (data not shown).

CRIP1a and CRIP1b Did Not Alter Agonist-Dependent CB₁ Signaling. Activation of CB₁ by the agonist WIN 55,212-2 inhibits Ca²⁺ currents, and WIN 55,212-2 inhibition of Ca²⁺ currents was unaltered by coexpression with CRIP1a. The EC₅₀ response of Ca²⁺ current inhibition to the agonist WIN 55,212-2 in SCG neurons expressing CB₁ receptors was not significantly altered by coexpression of CRIP1a (Fig. 5). The EC₅₀ was 37 nM for CB₁-expressing neurons (*n* = 5) and 32 nM for CB₁- and CRIP1a-expressing neurons (*n* = 6). The maximal Ca²⁺ current inhibition was also not

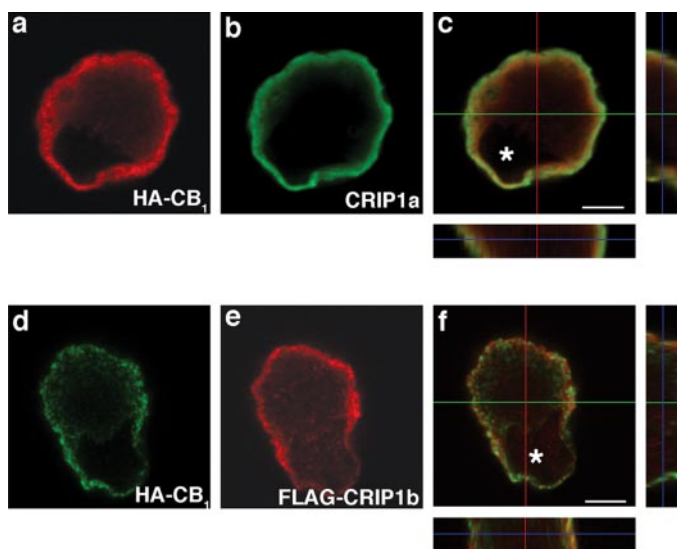


Fig. 4. CRIP1a and CRIP1b are enriched near the plasma membrane in SCG neurons. SCG neurons microinjected with HA-CB₁ and CRIP1a cDNA (a–c) or HA-CB₁ and FLAG-CRIP1b cDNA (d–f) were immunolabeled with antibodies as described in methods. HA-CB₁ (a and d) was detected at the plasma membrane. Both CRIP1a (b) and CRIP1b (e) are enriched near the plasma membrane. CB₁ and CRIP1a (c) and CB₁ and CRIP1b (f) overlap in confocal photomicrographs showing dual-labeling, including orthogonal perspectives (adjacent panels). Scale bar in c and f, 5 μm. Asterisk (*) indicates position of nucleus.

affected by coexpression with CRIP1a. The maximal Ca²⁺ current inhibition was 61% with CB₁ expression and 60% with coexpression of CB₁ and CRIP1a. In a separate set of experiments, WIN 55,212-2 (1 μM) decreased Ca²⁺ currents 44.2 ± 7.6% (*n* = 7) in neurons expressing only CB₁ (Fig. 6, a, b, and e) and 48.7 ± 3.3% (*n* = 14) in neurons coexpressing CB₁ and CRIP1a (Fig. 6, c–e). The time course of Ca²⁺ current inhibition in the presence of CRIP1a tended to be slower (*p* = 0.09), but recovery from inhibition was not significantly altered in the presence of CRIP1a (Table 2). WIN 55,212-2 inhibition of Ca²⁺ currents was similarly unaffected by coexpression of CRIP1b and CB₁. WIN 55,212-2 decreased Ca²⁺ currents 42.2 ± 6.0% (*n* = 7) in neurons expressing only CB₁ (Fig. 7, a, b, and e) and 42.6 ± 6.6% (*n* = 6) in neurons coexpressing CB₁ and CRIP1b (Fig. 7, c–e). Neither the time course of inhibition nor recovery from inhibition was significantly altered in the presence of CRIP1b (Table 2).

CRIP1a Attenuated CB₁-Mediated Constitutive Inhibition of Ca²⁺ Channels. CB₁ expression in SCG neurons regulates Ca²⁺ channels constitutively as evidenced by the ability of SR141716 to enhance Ca²⁺ currents. Expression of CRIP1a with CB₁ receptors reduced this response. The antagonist/inverse agonist SR141716 increased the Ca²⁺ current 47.9 ± 9.1% (*n* = 7) in neurons expressing CB₁ alone (Fig. 6, a, b, and f). In contrast, SR141716 increased Ca²⁺ currents only 10.5 ± 5.5% (*n* = 14) in neurons coexpressing CB₁ and CRIP1a (Fig. 6, c, d, and f). This pronounced decrease in response to SR141716 is evident in representative Ca²⁺ current traces (Fig. 6, compare a and c) and time course plots (Fig. 6, compare b and d). The results suggest that CRIP1a attenuates CB₁-mediated tonic inhibition of Ca²⁺ channels. Deletion of the last 9 amino acids of CB₁ restored the effect of SR141716 in the presence of CRIP1a. SR141716 increased the Ca²⁺ current 40.3 ± 7.4% (*n* = 7) in SCG neurons coexpressing CRIP1a and CB₁ receptors in which the last nine amino acids were deleted (data not shown) suggesting that the last nine amino acids of the CB₁ C-terminal tail is a critical CRIP1a interaction domain.

CRIP1a expressed in the absence of CB₁ had no effect on Ca²⁺ currents compared with uninjected SCG neurons, indicating that CRIP1a does not directly affect Ca²⁺ currents. Ca²⁺ currents were not affected by WIN 55,212-2 (6.8 ±

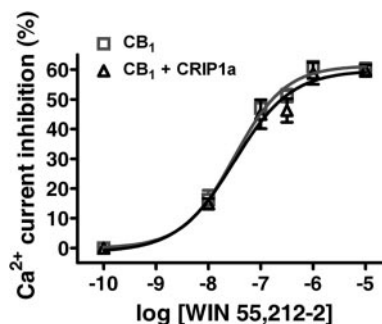


Fig. 5. CRIP1a did not shift the cumulative concentration-response curve for WIN 55,212-2 inhibition of Ca²⁺ channels in SCG neurons expressing CB₁ receptors. The EC₅₀ was 37 nM in SCG neurons expressing CB₁ (*n* = 5) and 32 nM in SCG neurons coexpressing CB₁ and CRIP1a (*n* = 6). The smooth curves were obtained by fitting the data to a sigmoid dose-response curve (variable slope) with nonlinear regression (GraphPad Prism). Each point represents the mean Ca²⁺ current inhibition (percentage) calculated at each concentration of WIN 55,212-2. Data are plotted as mean ± S.E.M.

3.0%; $n = 9$) or SR141716 ($4.8 \pm 3.9\%$; $n = 9$) in neurons injected with CRIP1a cDNA without CB₁ cDNA as the amplitude of Ca²⁺ currents was not significantly different from uninjected neurons [WIN 55,212-2, $7.5 \pm 1.7\%$ ($n = 7$) and SR 141716, $1.4 \pm 1.1\%$ ($n = 7$)].

Support for a role of CRIP1a in CB₁-mediated tonic inhibition of Ca²⁺ channels is strengthened by the Ca²⁺ current facilitation ratio, which is the ratio of the Ca²⁺ current amplitudes elicited before and after a strongly depolarizing voltage step. The current amplitude after the depolarization is facilitated because the G protein-dependent inhibition of Ca²⁺ channels is relieved by the strongly depolarizing voltage step (Elmslie et al., 1990). Thus, the Ca²⁺ current amplitude after depolarization (Fig. 6a, second pulse) is larger than the amplitude before depolarization (Fig. 6a, first pulse). A larger facilitation ratio indicates a greater tonic inhibition of Ca²⁺ channels. Expression of CB₁ tonically inhibits Ca²⁺ channels and thereby results in a larger basal facilitation ratio. The Ca²⁺ current basal facilitation ratio in uninjected SCG neurons is 1.24 ± 0.04 ($n = 8$) and increases to 1.46 ± 0.08 ($n = 8$) in SCG neurons expressing CB₁, indicating enhanced tonic inhibition of Ca²⁺ channels by

CB₁. The facilitation ratio decreases to 1.25 ± 0.03 ($n = 14$) in SCG neurons coexpressing CRIP1a and CB₁. Thus, a significant decrease in tonic inhibition of Ca²⁺ channels was observed in neurons coexpressing CB₁ and CRIP1a.

CRIP1b Does Not Affect CB₁-Mediated Tonic Inhibition of Ca²⁺ Channels. Unlike CRIP1a, coexpression of CRIP1b with CB₁ in SCG neurons did not significantly alter the tonic regulation of Ca²⁺ currents by CB₁ receptors. SR141716 increased Ca²⁺ currents $54.7 \pm 8.8\%$ ($n = 7$) in neurons expressing CB₁ alone (Fig. 7, a, b, and f) but also increased Ca²⁺ currents in neurons coexpressing CB₁ and CRIP1b (Fig. 7, c, d, and f) $35.9 \pm 6.7\%$ ($n = 6$). These data suggest that CRIP1b is not directly involved in regulation of CB₁-mediated tonic inhibition of Ca²⁺ channels. Taken together, these results indicate that CRIP1a, but not CRIP1b, is able to suppress the tonic inhibition of voltage-gated Ca²⁺ channels by CB₁ receptors.

Discussion

By screening a human brain cDNA library with the C-terminal region of the CB₁ cannabinoid receptor, we

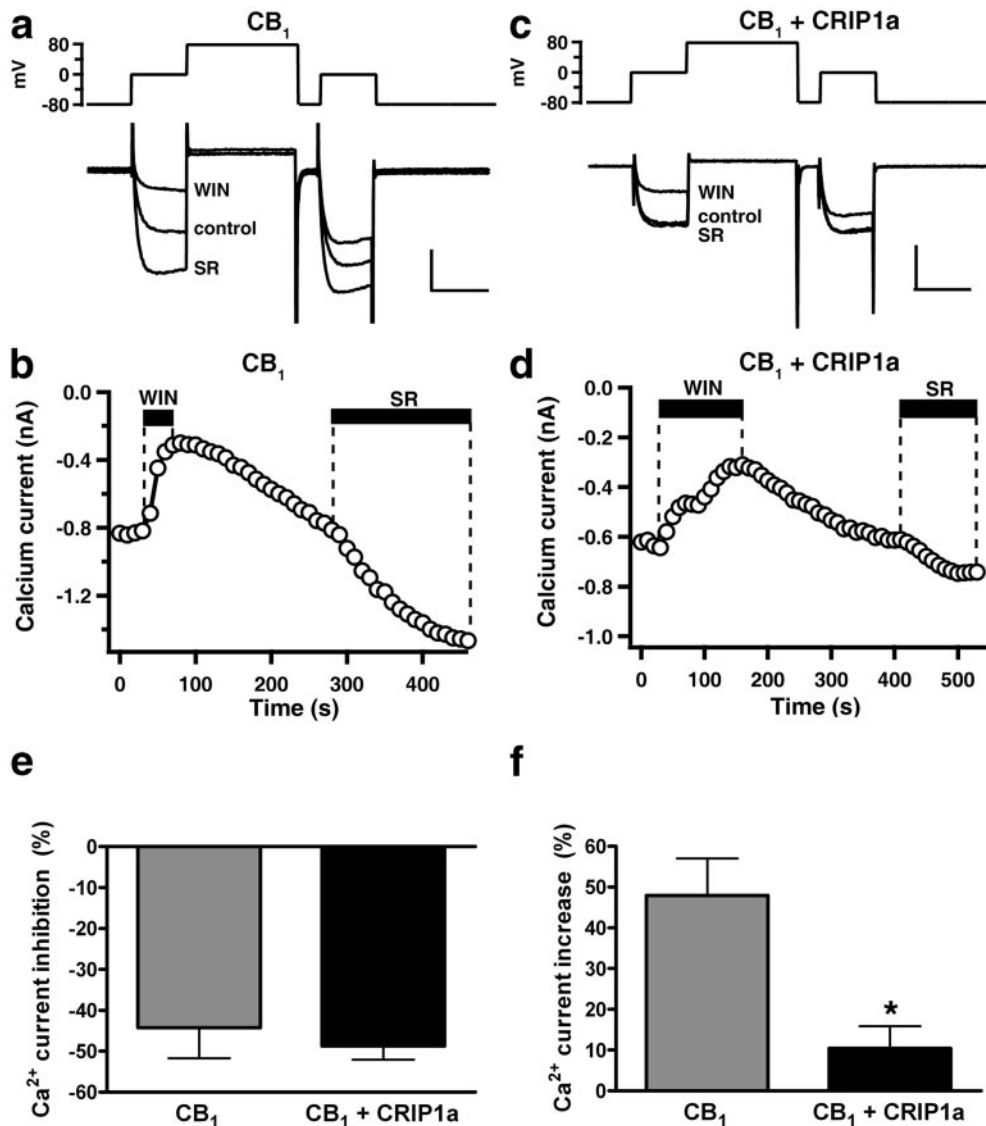


Fig. 6. CRIP1a decreases CB₁-mediated tonic inhibition of voltage-gated Ca²⁺ channels. **a**, top, voltage-step protocol used to elicit Ca²⁺ current. Bottom, superimposed Ca²⁺ current traces during perfusion of control solution (middle trace), 1 μM WIN 55,212-2 (top trace), or 1 μM SR141716 (bottom trace) for a representative SCG neuron expressing CB₁. **b**, Ca²⁺ current amplitude from a SCG neuron expressing CB₁ plotted over the time course of a representative experiment. Application of the CB₁ agonist WIN 55,212-2 decreased Ca²⁺ current, whereas the CB₁ inverse agonist SR141716 increased Ca²⁺ current. **c**, top, voltage-step protocol used to elicit Ca²⁺ current. Bottom, superimposed Ca²⁺ current traces during perfusion of control solution (middle trace), 1 μM WIN 55,212-2 (top trace), or 1 μM SR141716 (bottom trace) for a representative SCG neuron coexpressing CB₁ and CRIP1a. **d**, Ca²⁺ current amplitude from a SCG neuron coexpressing CB₁ and CRIP1a plotted over the time course of a representative experiment. Application of the CB₁ agonist WIN 55,212-2 decreased Ca²⁺ current; however, the ability of the CB₁ inverse agonist SR141716 to increase Ca²⁺ current was impaired. **e**, the ability of CB₁ agonist WIN 55,212-2 to inhibit Ca²⁺ currents is unaffected by CRIP1a. **f**, however, CB₁-mediated enhancement of Ca²⁺ current by antagonist/inverse agonist SR141716 is significantly attenuated by CRIP1a (* $p < 0.05$). Scale bars in **a** and **c**, 500 pA, 25 ms.

have identified two proteins that interact with CB₁. We have named these cannabinoid receptor interacting proteins, CRIP1a and CRIP1b, with the *a* and *b* nomenclature corresponding to the different exons that encode the alternatively spliced C-terminal region of these proteins in the human genome. It is noteworthy that although CRIP1a occurs throughout vertebrates, CRIP1b has so far been identified only in human, chimpanzee, and macaque genomes. CRIP1a and CRIP1b interact at the distal C-ter-

minial region of CB₁, with the last nine amino acids of CB₁ comprising the minimal domain tested that strongly interacted with CRIP1b. These nine amino acids are well conserved between mammals and fish, suggesting that acquisition of the distal C-terminal region of CB₁ may have been a vital step in the coevolution of these interacting proteins.

We performed a series of experiments to investigate the *in vitro* and *in vivo* interaction between CRIP1a and CRIP1b and CB₁. GST pull-down assays confirmed that CRIP1a and CRIP1b interact with the C-terminal tail of CB₁ *in vitro*. We generated a CRIP1a antibody that recognizes a single protein band of approximately 18 kDa in Western blots of mouse brain. The CRIP1a antibody was used to identify CRIP1a as an interaction partner with CB₁ in coimmunoprecipitation experiments using a CB₁ N-terminal antibody to isolate CB₁ and associated proteins from rat brain *in vivo*. Our data do not exclude the possibility that CRIP1a and CRIP1b interact with other G protein-coupled receptors or other proteins, although *in silico* searching did not reveal any GPCR with homology to the CB₁ C-terminal nine residues that interact with CRIP1a and CRIP1b. Nonetheless, CRIP1a contains a

TABLE 2

Neither CRIP1a nor CRIP1b altered the time course of Ca²⁺ current inhibition or the recovery from inhibition by the CB₁ agonist WIN 55,212-2 in SCG neurons microinjected with cDNA encoding CB₁, CB₁ and CRIP1a, or CB₁ and CRIP1b

SCG neurons expressing	N	Time to 90% Maximal Inhibition	Time to 50% Recovery
		<i>s</i>	<i>s</i>
CB ₁	8	40 ± 4	71 ± 7
CB ₁ + CRIP1a	14	59 ± 11	87 ± 6
CB ₁	7	42 ± 6	63 ± 8
CB ₁ + CRIP1b	6	39 ± 4	82 ± 21

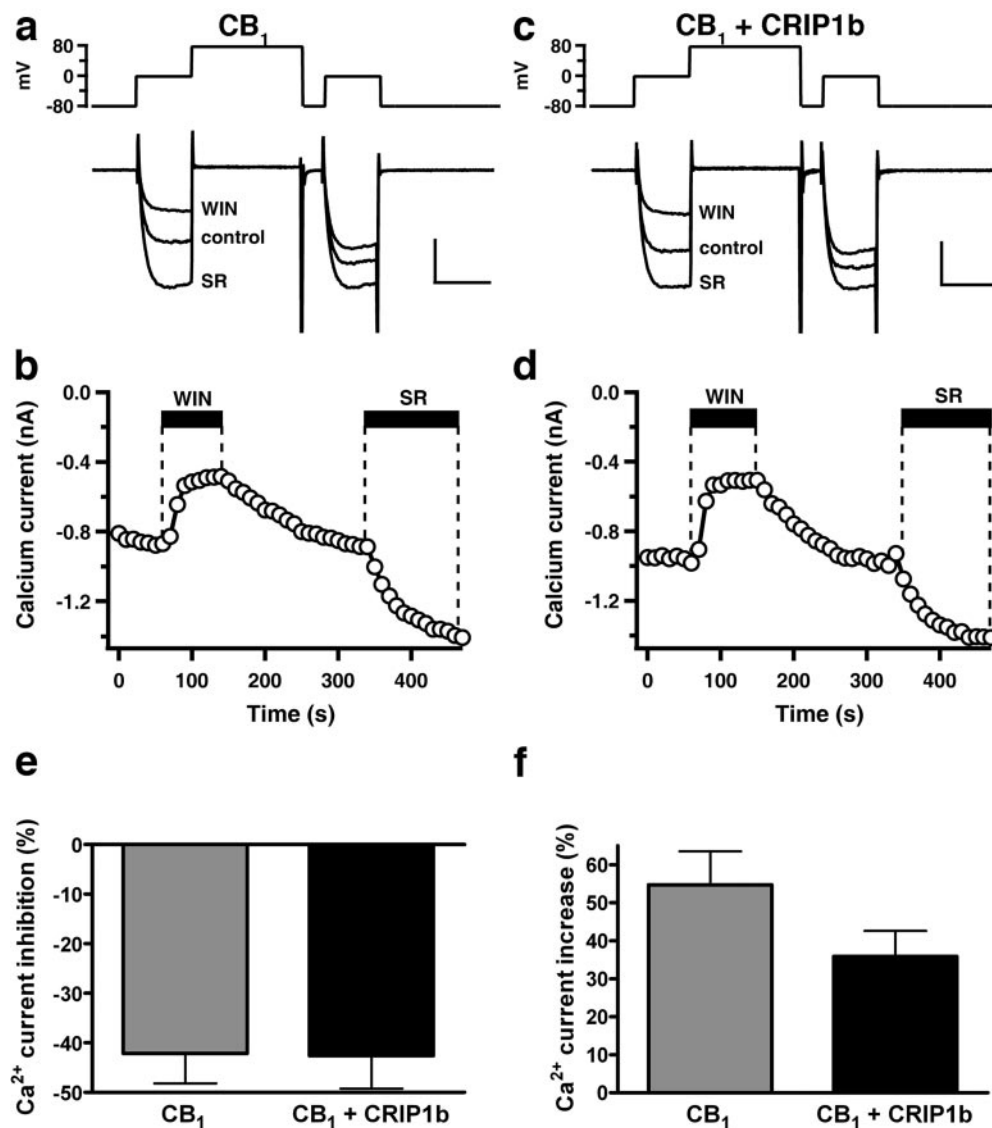


Fig. 7. CRIP1b does not alter CB₁-mediated tonic inhibition of voltage-gated Ca²⁺ channels. **a**, top, voltage-step protocol used to elicit Ca²⁺ current. Bottom, superimposed Ca²⁺ current traces during perfusion of control solution (middle trace), 1 μM WIN 55,212-2 (top trace) or 1 μM SR141716 (bottom trace) for a representative SCG neuron expressing CB₁. **b**, Ca²⁺ current amplitude from a SCG neuron expressing CB₁ plotted over the time course of a representative experiment. Application of the CB₁ agonist WIN 55,212-2 decreased Ca²⁺ current, whereas the CB₁ inverse agonist SR141716 increased Ca²⁺ current. **c**, top, voltage-step protocol used to elicit Ca²⁺ current. Bottom, superimposed Ca²⁺ current traces during perfusion of control solution (middle trace), 1 μM WIN 55,212-2 (top trace), or 1 μM SR141716 (bottom trace) for a representative SCG neuron coexpressing CB₁ and CRIP1b. **d**, Ca²⁺ current amplitude from a SCG neuron coexpressing CB₁ and CRIP1b plotted over the time course of a representative experiment. Application of the CB₁ agonist WIN 55,212-2 decreased Ca²⁺ current, whereas the CB₁ inverse agonist SR141716 increased Ca²⁺ current. **e**, the ability of CB₁ agonist WIN 55,212-2 to inhibit Ca²⁺ currents is unaffected by CRIP1b. **f**, likewise, CRIP1b failed to affect CB₁-mediated enhancement of Ca²⁺ current by inverse agonist SR141716 (*p* = 0.13). Scale bars in **a** and **c**, 500 pA, 25 ms.

PDZ ligand in its C-terminal tail that could interact with proteins containing PDZ domains.

We also performed functional experiments using SCG neurons to heterologously express CB₁ receptors with CRIP1a or CRIP1b. We found that CRIP1a, but not CRIP1b, suppressed the tonic inhibition of voltage-gated Ca²⁺ channels by CB₁ receptors. CB₁ receptors exhibit agonist-independent activity in some signal transduction studies but not others (Pertwee, 2005). CRIP1a may function to keep agonist-independent regulation of voltage-gated ion channels by CB₁ receptors in check in neurons in which CRIP1a and CB₁ receptors are colocalized. Thus, the presence or absence of CRIP1a may determine whether basal CB₁ activity is modulated in specific neurons.

The mechanisms by which CRIP1a inhibits basal CB₁ modulation of Ca²⁺ channels are unknown. Binding data indicate that CRIP1a and CRIP1b do not affect the expression of CB₁ receptors. Our data showing that CRIP1a does not change the EC₅₀ or the maximal response to WIN 55,212-2 are consistent with the interpretation that CRIP1a does not greatly affect the number of G protein-coupled CB₁ receptors available to the agonist. However, we cannot exclude the possibility that CRIP1a might have an effect on CB₁ receptor trafficking. A small reduction in CB₁ trafficking to the plasma membrane could decrease the effect of SR141716 by reducing the number of tonically active CB₁ receptors. Alternatively, an inhibition of CB₁ constitutive activity by CRIP1a might increase the number of CB₁ receptors on the plasma membrane.

Leterrier et al. (2006) found that inhibition of CB₁ constitutive activity increased the number of CB₁ receptors on the somatodendritic membrane and blocked the axonal targeting of CB₁ receptors in hippocampal neurons. However, McDonald et al. (2007) showed that axonal targeting of CB₁ receptors in hippocampal neurons was not dependent on constitutive activity but on constitutive endocytosis. These authors suggested that anchoring proteins that interact with CB₁ receptors might contribute to their axonal localization. CRIP1a through its C-terminal PDZ ligand domain may act as a scaffolding protein to anchor CB₁ receptors by interacting with proteins containing PDZ domains. A scaffolding function of CRIP1a could contribute to the subcellular localization of CB₁ receptors. Further research will be needed to determine the subcellular localization of CRIP1a and whether CRIP1a participates in axonal targeting of CB₁ receptors.

Our finding that CRIP1a selectively blocks CB₁ basal activity but not agonist activation of CB₁ receptors suggests the possibility that CRIP1a may block coupling to specific G_{i/o} proteins that are responsible for tonic inhibition of Ca²⁺ channels but not to other G_{i/o} proteins that inhibit Ca²⁺ channels in response to agonist activation. This dissociation between CB₁ basal activity that causes tonic inhibition of Ca²⁺ channels and agonist-induced CB₁ activation that also inhibits Ca²⁺ channels is not a unique effect of CRIP1a. We have previously reported that a D164N point mutation of the CB₁ receptor blocks tonic inhibition of Ca²⁺ channels without affecting agonist-dependent Ca²⁺ channel inhibition in SCG neurons (Nie and Lewis, 2001). The idea that CRIP1a may block coupling of CB₁ to specific G_{i/o} proteins that are responsible for tonic inhibition of Ca²⁺ channels comes from G_{i/o} protein reconstitution experiments. We found

that reconstitution of tonic inhibition of Ca²⁺ channels by CB₁ receptors in SCG neurons with pertussis toxin insensitive Gα_{i3}(C351G)/Gβ₁γ₂ significantly enhanced the tonic inhibition of Ca²⁺ channels, whereas, Gα_{oA}(C351G)/Gβ₁γ₂ abolished the tonic inhibition of Ca²⁺ channels (Anavi-Goffer et al., 2007). These results suggest that a specific disruption in the coupling of CB₁ to Gα_{i3} by CRIP1a would abolish the tonic inhibition of Ca²⁺ channels without disrupting coupling to Gα_{oA} that supports Ca²⁺ channel inhibition by agonist stimulation of CB₁ receptors.

In conclusion, our discovery of the cannabinoid receptor interacting proteins CRIP1a and CRIP1b initiates a new avenue for research on regulation of CB₁ receptor function and may provide a basis for development of novel drugs to treat disorders where modulation of CB₁ activity has therapeutic potential (e.g., chronic pain, obesity, epilepsy).

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