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CB_1 cannabinoid receptor activity is modulated by the interacting protein CRIP1a

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Running Title: Cannabinoid receptor interacting proteins

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ABBREVIATIONS: CB_1, cannabinoid receptor subtype-1; CRIP1a, cannabinoid receptor interacting protein subtype 1a; CRIP1b, cannabinoid receptor interacting protein subtype 1b; GPCR, G protein-coupled receptor, 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-1H-pyrazole-3-carboxamide; HEK 293, human embryonic kidney cell line; CHO, Chinese hamster ovary cell line; aa, amino acid; GST, glutathione-S-transferase.

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ABSTRACT

The CB_1 cannabinoid receptor is a G-protein coupled receptor (GPCR) that has important physiological roles in synaptic plasticity, analgesia, appetite, and neuroprotection. Here we report the discovery of two structurally related CB_1 cannabinoid receptor interacting proteins (CRIP1a and CRIP1b) that bind to the distal C-terminal tail of CB_1. 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 appears to be unique to primates. CRIP1a co-immunoprecipitates with CB_1 receptors derived from rat brain homogenates, indicating that CRIP1a and CB_1 interact *in vivo*. Furthermore, in superior cervical ganglion neurons co-injected with CB_1 and CRIP1a or CRIP1b cDNA, CRIP1a, but not CRIP1b, suppresses CB_1-mediated tonic inhibition of voltage-gated Ca^{2+} channels. Discovery of CRIP1a provides the basis for a new avenue of research on mechanisms of CB_1 regulation in the nervous system and may lead to development of novel drugs to treat disorders where modulation of CB_1 activity has therapeutic potential (e.g., chronic pain, obesity and epilepsy).

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G protein-coupled receptors (GPCRs) provide a wide range of signaling capabilities to regulate the activity of downstream cellular targets. In order to signal efficiently, cells must be able to dynamically control the activity of GPCRs. While 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. As 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, as 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 GASP1 interacts with the C-terminal tail of many GPCRs including CB_1 resulting in downregulation and degradation (Martini et al., 2007). The adaptor protein FAN is also able to interact with the CB_1 receptor (Sanchez 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 CB_1 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

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(Mechoulam et al., 1995). Endocannabinoids act as retrograde messengers mediating CB_1-dependent forms of short-term synaptic plasticity known as depolarization-induced suppression of inhibition or excitation (DSI/DSE) (Diana and Marty, 2004) and longer-lasting forms of synaptic plasticity, such as long-term depression (LTD) (Azad et al., 2004; Chevaleyre and Castillo, 2003; Gerdeman and Lovinger, 2001; Robbe et al., 2002; Sjöström et al., 2003). 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 (Abood et al., 2001; Shen and Thayer, 1998), ischemia (Parmentier-Batteur et al., 2002) and seizure (Marsicano et al., 2003).

The complexity of CB_1 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 Ca^{2+} channels, resulting in an increase in the Ca^{2+} current in superior cervical ganglion (SCG) neurons expressing CB_1 receptors (Pan et al., 1998). Deletion of the CB_1 C-terminal tail distal to the G-protein binding domain enhances the effect of SR141716. SR141716 produces a significantly larger increase in the Ca^{2+} current in neurons expressing C-terminally truncated CB_1 receptors (Nie and Lewis, 2001). Thus, deletion of the distal C-terminal region of CB_1 results in enhanced tonic inhibition of Ca^{2+} channels, suggesting that either this region constrains the receptor conformation or that accessory proteins binding to this region modulate CB_1 activity.

We report here the discovery of CRIP1a and CRIP1b, novel proteins that interact with the distal C-terminal region of CB_1. CRIP1a is expressed in the brain and is found

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throughout vertebrates, whereas CRIP1b appears unique to primates. CRIP1a co-immunoprecipitates with CB_1 from rat brain and co-localizes with CB_1 when heterologously expressed in neurons. Neither CRIP1a nor CRIP1b significantly alter the affinity of CB_1 for the antagonist/inverse agonist SR141716. However, CRIP1a, but not CRIP1b, significantly attenuates tonic inhibition of voltage-gated Ca^{2+} channels by CB_1 receptors.

Materials and Methods

Yeast two-hybrid screening. The Matchmaker Two-Hybrid System (BD Biosciences Clontech, Mountain View, CA) was used to screen a human brain cDNA library (BD Biosciences Clontech) using a bait protein corresponding to the C-terminal tail of CB_1 (last 55 amino acids, 418-472, of human CB_1, 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 co-transformed with bait cDNA (CB_1 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).

***In vitro* binding assay.** The GST Gene Fusion System (Amersham Biosciences, Piscataway, NJ) was used to construct GST-CB_1 (C-terminal tail) fusion proteins using the pGEX-4T-1 vector. GST-CB_1 was expressed in *Escherichia coli*, isolated with

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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 PVDF membrane. The S-tag (15 aa) was visualized by its interaction with ribonuclease S-protein conjugated to HRP (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 (10µg protein per lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with CRIP1a antiserum (1:1000) or with CRIPa antiserum (1:1000) that had been pre-absorbed with the antigen peptide (20 µM). Bound antibodies were revealed using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and BCIP/NBT substrate (Vector Laboratories, Burlingame, CA).

The procedure for immunoprecipitation of CB_1 and associated proteins has been previously described (Mukhopadhyay and Howlett, 2001), as has the generation of antibodies in rabbits against the first 14 amino acids of CB_1 (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001). Briefly, rat brain P2 membranes (5 mg protein) were solubilized in 0.5 ml of TM buffer (30 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$) containing 4 mg CHAPS (Sigma) and 20% glycerol on ice with gentle stirring for 30 min, followed by centrifugation at 100,000 x *g* for 40 min at 4ºC. CHAPS solubilized proteins (100 µl) were incubated with sepharose beads coupled to anti-CB_1 antibodies (20µl) for 6

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h at 4ºC. The anti-CB_1 affinity matrix was then sedimented at 17,000 x *g* for 5 min and washed three times with 500 µl TBS-T buffer (20 mM Tris-Cl, pH 7.4; 140 mM NaCl; 0.1% Tween 20). Immunoprecipitated protein was eluted with 50 µl glycine, pH 2.5 (100 mM) and immediately neutralized with 450 µl Tris-Cl, pH 8.0 (1.5 M). Protein from neutralized eluate was precipitated by addition of 8 volumes of $CHCl_3/CH_3OH/H_2O$ (1:4:3), dissolved in Laemmli's sample buffer and heated at 65ºC for 5 min. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, probed with CRIP1a antiserum (1:500) and CB_1 antibodies (1:1000, N-terminal) and detected using enhanced chemiluminescence.

[^{3}H]SR141716 binding assay. Saturation analysis of [^{3}H]SR141716 binding was performed by incubating 10 µg membrane protein with 0.01-5 nM [^{3}H]SR141716 in 1 ml buffer containing 0.5 g/L bovine serum albumen (BSA) in the presence and absence of 5 µM unlabeled SR141716 to determine non-specific 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 that were pre-soaked in Tris buffer containing 5 g/L BSA (Tris-BSA), followed by five washes with cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after 1 hr shaking of the filters in 4 ml ScintiSafe Econo 1 scintillation fluid. The presence of CB_1, 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-CB_1 cDNA (100 ng/µl) and CRIP1a cDNA (160 ng/µl) or FLAG-CRIP1b cDNA (170 ng/µl).

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Neurons were fixed in PBS containing 4% paraformaldehyde (PFA) and 4% sucrose for 30 min, rinsed with excess PBS and blocked with 5% non-fat 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 568 (goat anti-mouse) antibodies at 1:1000 for 1 h. SCG nuclei were identified using a nucleic acid stain, DAPI (300 nM; Molecular Probes, Invitrogen, Carlsbad, CA). Excess antibodies were removed by PBS washes. Neurons were mounted on glass slides with ProLong antifade reagent (Molecular Probes, Invitrogen, Carlsbad, CA). Z-stack images were acquired on a Zeiss Axiovert LSM 510 META inverted confocal microscope using a 63X oil objective and documented using LSM510 software.

Electrophysiology. Rat superior cervical ganglion (SCG) neurons were isolated, cultured and microinjected as previously described (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 35ºC in a shaking water bath. During the last 10 min of incubation, 10 U (~5 µl) of DNase I (Worthington Biochemical, Lakewood, NJ) was added. Dissociated neurons were plated on pre-coated poly-L-lysine 35 mm culture dishes (BD Biosciences, San Jose, CA). Microinjection solutions contained plasmids with cDNA encoding CB_1 (100 ng/µl) and EGFP (10 ng/µl) with or without CRIP1a (160 ng/µl) or CRIP1b (340 ng/µl) cDNA.

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Calcium current recordings were performed as previously described (Nie and Lewis, 2001). The extracellular recording solution consisted of 140 mM tetraethylammonium methanesulfonate, 10 mM HEPES, 15 mM glucose, 10 mM $CaCl_2$, and 0.1 μM 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 $CaCl_2$, 4 mM Mg-ATP, 0.1 mM Na_2-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 μM in external solution and were briefly sonicated (20 sec) 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 ± s.e.m. Statistical significance was determined by Student's *t* test when comparing two conditions head-to-head; whereas, ANOVA with post-hoc Bonferroni-adjusted *t*-test or Dunnett's test were used when comparing three or more conditions. EC_{50} values were calculated by unweighted least squares nonlinear regression of log concentration values versus percent effect (GraphPad Prism). B_{max} and K_D values of [^{3}H]SR141716 binding were similarly determined by non-linear regression analysis of saturation curves using GraphPad Prism. Differences were considered significant at $p < 0.05$.

Results

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Discovery of CRIP1a and CRIP1b and interaction with CB_1. Because the activity of CB_1 is influenced by its C-terminal tail, we used the last 55 amino acids of CB_1 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 cannabinoid receptor interacting proteins CRIP1a and CRIP1b, respectively (**Fig. 1b**). Neither CRIP1a nor CRIP1b interacted with the C-terminal tail of the CB_2 cannabinoid receptor in the yeast two-hybrid assay (data not shown). CRIP1a and CRIP1b are expected to be cytosolic proteins, as 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/). Additionally, 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, *Xenopus* 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

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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' non-coding 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 CB_1 C-terminal tail necessary for interaction with CRIPs, we constructed and characterized several CB_1 mutants. Mackie and colleagues identified two distinct regions of the CB_1 C-terminal tail that mediate desensitization or internalization (Jin et al., 1999). The CB_1 mutants we generated were designed to assess the importance of these regions in the CB_1-CRIP interaction. Note that amino acid (aa) numbering corresponds to the rat CB_1 sequence, which is one residue longer than the human CB_1 sequence due to 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 CB_1 C-terminal tail (**Fig. 1c**). The last nine amino acids of the CB_1 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 CB_1 necessary for interaction with CRIP1b. Conversely, the region of CRIP1b necessary for interaction with CB_1 was determined using deletion mutants of CRIP1b. No single exon of CRIP1b was sufficient to interact with the CB_1 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 CB_1.

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CRIP1a and CRIP1b interact with CB_1 *in vitro* and *in vivo*. To confirm the yeast two-hybrid data, we performed glutathione-S-transferase (GST) pull-down assays with the C-terminal tail of CB_1 and either CRIP1a or CRIP1b. Bacterially expressed CRIP1a or CRIP1b bound to immobilized GST-CB_1 fusion proteins. The identity of CRIP1a or CRIP1b in the GST-column eluate was verified by Western blotting with ribonuclease S-protein conjugated to HRP 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 CB_1 and not to the sepharose in an *in vitro* interaction.

To investigate *in vivo* interaction of CB_1 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 CB_1 and CRIP1a using a CB_1 N-terminal antibody (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001) to immunoprecipitate CB_1 and associated proteins from CHAPS solubilized rat brain membranes. Immunoblots probed with CRIP1a and CB_1 antibodies revealed that CRIP1a co-precipitated with CB_1 in membranes from rat brain (**Fig. 2d**), but not when the CB_1 antibody was omitted, indicating that endogenous CB_1 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,

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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 HEK293 cells (data not shown).

CRIP1a and CRIP1b do not alter the expression or affinity of CB_1. In data obtained from cell lines, CRIP1a did not change CB_1 receptor expression or protein maturation and membrane localization. In stable HEK 293 cell lines expressing CB_1 or CB_1 and CRIP1a or transiently co-transfected CHO cells, neither [^{3}H]SR141716 binding affinity for CB_1 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 expression of CB_1 receptors. Similar to CRIP1a, CB_1 expression in cell lines was not affected by co-expression with CRIP1b. In transfected CHO cells expressing CB_1 or CB_1 and CRIP1b, neither [^{3}H]SR141716 binding affinity nor maximum binding was significantly altered (**Table 1**). Although there appeared to be approximately a 2-fold increase in [^{3}H]SR141716 K_D value in CHO cells co-expressing CB_1 and CRIP1b compared to CHO cells expressing CB_1 and empty vector, this was only a non-significant trend ($p=0.086$; $F=2.83$).

Expression and co-localization of CB_1 and CRIP1a and CRIP1b in SCG neurons. Since CRIP1a and CRIP1b interact with the C-terminal tail of CB_1, a domain known to affect the ability of the CB_1 receptor to tonically inhibit voltage-gated Ca^{2+} channels, we evaluated the potential for functional interaction between CB_1 and CRIP1a and CRIP1b by whole-cell patch clamp recordings from rat SCG neurons heterologously expressing CB_1 and CRIP1a or CRIP1b. N-type Ca^{2+} channels, which regulate excitability and neurotransmitter release, are homogeneously expressed and their modulation has been

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extensively studied in SCG neurons (Hille, 1994). Expression of CB_1 receptors in these neurons, results in tonic inhibition of N-type Ca^{2+} channels that can be reversed by the CB_1 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 Ca^{2+} channels and increasing the Ca^{2+} current. Since deletion of the C-terminal tail of CB_1 results in an increased tonic inhibition of Ca^{2+} currents in SCG neurons (Nie and Lewis, 2001), we hypothesized that CRIP1a or CRIP1b might serve as endogenous regulators of CB_1 activity. To verify that CB_1 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-CB_1 and CRIP1a or FLAG-CRIP1b. Both CRIP1a (**Fig. 4b**) and FLAG-CRIP1b (**Fig. 4e**) were enriched near the plasma membrane and overlapped with HA-CB_1 (**Fig. 4c, f**) indicating that CRIP1a and FLAG-CRIP1b were trafficked to the same subcellular compartment as HA-CB_1, (**Fig. 4a, d**), where they could affect CB_1 signaling. Although predicted to be cytosolic proteins, the expression pattern of CRIP1a and FLAG-CRIP1b in SCG neurons appeared to be membrane-associated rather than homogeneously distributed throughout the cytosol when expressed with or without HA-CB_1. These results agree with the finding that CRIP1a immunoreactivity was evident in Western blots of membrane preparations from CHO cells transiently expressing CB_1 receptors and CRIP1a (data not shown).

CRIP1a and CRIP1b do not alter agonist-dependent CB_1 signaling. Activation of CB_1 by the agonist WIN 55,212-2 inhibits Ca^{2+} currents, and WIN 55,212-2 inhibition of Ca^{2+} currents was unaltered by co-expression with CRIP1a. The EC_{50} response of Ca^{2+} current inhibition to the agonist WIN 55,212-2 in SCG neurons expressing CB_1 receptors

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was not significantly altered by co-expression of CRIP1a (**Fig. 5**). The EC_{50} was 37 nM for CB_1 expressing neurons (n = 5) and 32 nM for CB_1 and CRIP1a expressing neurons (n = 6). The maximal Ca^{2+} current inhibition was also not affected by co-expression with CRIP1a. The maximal Ca^{2+} current inhibition was 61% with CB_1 expression and 60% with co-expression of CB_1 and CRIP1a. In a separate set of experiments WIN 55,212-2 (1 μM) decreased Ca^{2+} currents 44.2 ± 7.6% (n = 7) in neurons expressing only CB_1 (**Fig. 6a,b,e)** and 48.7 ± 3.3% (n = 14) in neurons co-expressing CB_1 and CRIP1a (**Fig. 6c,d,e**). The time course of Ca^{2+} 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 Ca^{2+} currents was similarly unaffected by co-expression of CRIP1b and CB_1. WIN 55,212-2 decreased Ca^{2+} currents 42.2 ± 6.0% (n = 7) in neurons expressing only CB_1 (**Fig. 7a,b,e**) and 42.6 ± 6.6% (n = 6) in neurons co-expressing CB_1 and CRIP1b (**Fig. 7c,d,e**). Neither the time course of inhibition nor recovery from inhibition was significantly altered in the presence of CRIP1b (**Table 2**).

CRIP1a attenuates CB_1–mediated constitutive inhibition of Ca^{2+} channels. CB_1 expression in SCG neurons regulates Ca^{2+} channels constitutively as evidenced by the ability of SR141716 to enhance Ca^{2+} currents. Expression of CRIP1a with CB_1 receptors reduced this response. The antagonist/inverse agonist SR141716 increased the Ca^{2+} current 47.9 ± 9.1% (n = 7) in neurons expressing CB_1 alone (**Fig. 6a,b,f**). In contrast, SR141716 increased Ca^{2+} currents only 10.5 ± 5.5% (n = 14) in neurons co-expressing CB_1 and CRIP1a (**Fig. 6c,d,f**). This pronounced decrease in response to SR141716 is evident in representative Ca^{2+} current traces (compare **Fig. 6a,c**) and time course plots (compare **Fig. 6b and d**). The results suggest that CRIP1a attenuates CB_1-

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mediated tonic inhibition of Ca^{2+} channels. Deletion of the last 9 amino acids of CB_1 restored the effect of SR141716 in the presence of CRIP1a. SR141716 increased the Ca^{2+} current 40.3 ± 7.4% (n=7) in SCG neurons co-expressing CRIP1a and CB_1 receptors in which the last 9 amino acids were deleted (data not shown) suggesting that the last 9 amino acids of the CB_1 C-terminal tail is a critical CRIP1a interaction domain.

CRIP1a expressed in the absence of CB_1 had no effect on Ca^{2+} currents, when compared to uninjected SCG neurons, indicating that CRIP1a does not directly affect Ca^{2+} currents. Ca^{2+} currents were not affected by WIN 55,212-2 (6.8 ± 3.0%; n = 9) or SR141716 (4.8 ± 3.9%; n = 9) in neurons injected with CRIP1a cDNA without CB_1 cDNA as the amplitude of Ca^{2+} currents was not significantly different from uninjected neurons (WIN 55,212-2, 7.5 ± 1.7% (n = 7) and SR 141716, 1.4 ± 1.1% (n = 7)).

Support for a role of CRIP1a in CB_1-mediated tonic inhibition of Ca^{2+} channels is strengthened by the Ca^{2+} current facilitation ratio, which is the ratio of the Ca^{2+} 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 Ca^{2+} channels is relieved by the strongly depolarizing voltage step (Elmslie et al., 1990) . Thus, the Ca^{2+} current amplitude following 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 Ca^{2+} channels. Expression of CB_1 tonically inhibits Ca^{2+} channels, and thereby results in a larger basal facilitation ratio. The Ca^{2+} 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 CB_1, indicating enhanced tonic inhibition of Ca^{2+} channels by CB_1. The facilitation ratio decreases to 1.25 ± 0.03 (n

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= 14) in SCG neurons co-expressing CRIP1a and CB_1. Thus, a significant decrease in tonic inhibition of Ca^{2+} channels was observed in neurons co-expressing CB_1 and CRIP1a.

CRIP1b does not affect CB_1–mediated tonic inhibition of Ca^{2+} channels. Unlike CRIP1a, co-expression of CRIP1b with CB_1 in SCG neurons did not significantly alter the tonic regulation of Ca^{2+} currents by CB_1 receptors. SR141716 increased Ca^{2+} currents 54.7 ± 8.8% (n = 7) in neurons expressing CB_1 alone (**Fig. 7a,b,f**), but also increased Ca^{2+} currents in neurons co-expressing CB_1 and CRIP1b (**Fig. 7c,d,f**) 35.9 ± 6.7% (n = 6). These data suggest that CRIP1b is not directly involved in regulation of CB_1–mediated tonic inhibition of Ca^{2+} channels. Taken together, these results indicate that CRIP1a, but not CRIP1b, is able to suppress the tonic inhibition of voltage-gated Ca^{2+} channels by CB_1 receptors.

Discussion

By screening a human brain cDNA library with the C-terminal region of the CB_1 cannabinoid receptor, we have identified two proteins that interact with CB_1. 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. Interestingly, while 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-terminal region of CB_1, with the last nine amino acids of CB_1 comprising the minimal domain tested that strongly interacted with CRIP1b. These nine amino acids are well conserved

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between mammals and fish, suggesting that acquisition of the distal C-terminal region of CB_1 may have been a vital step in the co-evolution of these interacting proteins.

We performed a series of experiments to investigate the *in vitro* and *in vivo* interaction between CRIP1a and CRIP1b and CB_1. GST pull-down assays confirmed that CRIP1a and CRIP1b interact with the C-terminal tail of CB_1 *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 CB_1 in co-immunoprecipitation experiments using a CB_1 N-terminal antibody to isolate CB_1 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 CB_1 C-terminal nine residues that interact with CRIP1a and CRIP1b. Nonetheless, CRIP1a contains a 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 CB_1 receptors with CRIP1a or CRIP1b. We found that CRIP1a, but not CRIP1b, suppressed the tonic inhibition of voltage-gated Ca^{2+} channels by CB_1 receptors. CB_1 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 CB_1 receptors in check in neurons in which CRIP1a and CB_1 receptors are co-localized. Thus, the presence or absence of CRIP1a may determine whether basal CB_1 activity is modulated in specific neurons.

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The mechanisms by which CRIP1a inhibits basal CB_1 modulation of Ca^{2+} channels are unknown. Binding data indicate that CRIP1a and CRIP1b do not affect the expression of CB_1 receptors. Our data showing that CRIP1a does not change the EC_{50} 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 CB_1 receptors available to the agonist. However, we cannot exclude the possibility that CRIP1a might have an effect on CB_1 receptor trafficking. A small reduction in CB_1 trafficking to the plasma membrane could decrease the effect of SR141716 by reducing the number of tonically active CB_1 receptors. Alternatively, an inhibition of CB_1 constitutive activity by CRIP1a might increase the number of CB_1 receptors on the plasma membrane.

Leterrier et al. found that inhibition of CB_1 constitutive activity increased the number of CB_1 receptors on the somatodendritic membrane and blocked the axonal targeting of CB_1 receptors in hippocampal neurons (Leterrier et al., 2006). However, McDonald et al. showed that axonal targeting of CB_1 receptors in hippocampal neurons was not dependent on constitutive activity but on constitutive endocytosis (McDonald et al., 2007). These authors suggested that anchoring proteins that interact with CB_1 receptors might contribute to their axonal localization. CRIP1a through its C-terminal PDZ ligand domain may act as a scaffolding protein to anchor CB_1 receptors by interacting with proteins containing PDZ domains. A scaffolding function of CRIP1a could contribute to the subcellular localization of CB_1 receptors. Further research will be needed to determine the subcellular localization of CRIP1a and whether CRIP1a participates in axonal targeting of CB_1 receptors.

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Our finding that CRIP1a selectively blocks CB_1 basal activity but not agonist activation of CB_1 receptors suggests the possibility that CRIP1a may block coupling to specific $G_{i/o}$ proteins that are responsible for tonic inhibition of Ca^{2+} channels but not to other $G_{i/o}$ proteins that inhibit Ca^{2+} channels in response to agonist activation. This dissociation between CB_1 basal activity that causes tonic inhibition of Ca^{2+} channels and agonist-induced CB_1 activation that also inhibits Ca^{2+} channels is not a unique effect of CRIP1a. We have previously reported that a D164N point mutation of the CB_1 receptor blocks tonic inhibition of Ca^{2+} channels without affecting agonist-dependent Ca^{2+} channel inhibition in SCG neurons (Nie and Lewis, 2001). The idea that CRIP1a may block coupling of CB_1 to specific $G_{i/o}$ proteins that are responsible for tonic inhibition of Ca^{2+} channels comes from $G_{i/o}$ protein reconstitution experiments. We found that reconstitution of tonic inhibition of Ca^{2+} channels by CB_1 receptors in SCG neurons with pertussis toxin insensitive $G\alpha_{i3}(C351G)/G\beta_1\gamma_2$ significantly enhanced the tonic inhibition of Ca^{2+} channels; whereas, $G\alpha_{oA}(C351G)/G\beta_1\gamma_2$ abolished the tonic inhibition of Ca^{2+} channels (Anavi-Goffer et al., 2007). These results suggest that a specific disruption in the coupling of CB_1 to $G\alpha_{i3}$ by CRIP1a would abolish the tonic inhibition of Ca^{2+} channels without disrupting coupling to $G\alpha_{oA}$ that supports Ca^{2+} channel inhibition by agonist stimulation of CB_1 receptors.

In conclusion, our discovery of the cannabinoid receptor interacting proteins CRIP1a and CRIP1b initiates a new avenue for research on regulation of CB_1 receptor function and may provide a basis for development of novel drugs to treat disorders where modulation of CB_1 activity has therapeutic potential (e.g. chronic pain, obesity, epilepsy).

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Accession numbers: CRIP1a (AY883936) and CRIP1b (AY144596) nucleotide sequences have been deposited into GenBank.

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Figure Legends

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 comprised 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 bp. 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 CB_1 C-terminal tail were essential for interaction with CRIP1b. Desensitization (D) and internalization (I) regions (Jin et al., 1999) of CB_1 are depicted as boxes. Numbers indicate amino acid residues of rat (r) or human (h) CB_1 used as bait. (**d**) Interaction with wild type CB_1 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 co-transformed 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.

Fig. 2. CRIPs interact with CB_1 *in vitro* and *in vivo*. Bacterially expressed CRIP1a (**a**) or CRIP1b (**b**) bound specifically to immobilized GST-CB_1 C-terminal tail and not to the negative control, GST, in Western blot analyses of *in vitro* binding assay. Apparent

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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 co-immunoprecipitated with CB_1 from rat brain in Western blot probed with anti-CB_1 and anti-CRIP1a antibodies. Neither CB_1 nor CRIP1a bound to sepharose beads that were not conjugated with anti-CB_1 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.

Fig 3. [^{3}H]SR141716 saturation binding in membranes from hCB_1-HEK cells with and without stable co-expression of CRIP1a. Membranes were incubated with the indicated concentrations of [^{3}H]SR141716A, and binding was assessed as described in Methods. Values shown are mean pmol of [^{3}H]SR141716A specifically bound per mg of membrane protein ± SE (n=4).

Fig. 4. CRIP1a and CRIP1b are enriched near the plasma membrane in SCG neurons. SCG neurons microinjected with HA-CB_1 and CRIP1a cDNA (**a-c**) or HA-CB_1 and FLAG-CRIP1b cDNA (**d-f**) were immunolabeled with antibodies as described in methods. HA-CB_1 (**a, d)** was detected at the plasma membrane. Both CRIP1a (**b**) and CRIP1b (**e**) are enriched near the plasma membrane. CB_1 and CRIP1a (**c**) and CB_1 and

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CRIP1b (**f**) overlap in confocal photomicrographs showing dual-labeling, including orthogonal perspectives (adjacent panels). Scale bar in (**c**) and (**f**) is 5μm. Asterisk (*****) indicates position of nucleus.

Fig. 5. CRIP1a did not shift the cumulative concentration-response curve for WIN 55,212-2 inhibition of Ca^{2+} channels in SCG neurons expressing CB_1 receptors. The EC_{50} was 37 nM in SCG neurons expressing CB_1 (n = 5) and 32 nM in SCG neurons co-expressing CB_1 and CRIP1a (n = 6). The smooth curves were obtained by fitting the data to a sigmoid dose-response curve (variable slope) with non-linear regression (GraphPad Prism). Each point represents the mean Ca^{2+} current inhibition (%) calculated at each concentration of WIN 55,212-2. Data are plotted as mean ± SEM.

Fig. 6. CRIP1a decreases CB_1–mediated tonic inhibition of voltage-gated Ca^{2+} channels. (**a**) *Top*, Voltage-step protocol utilized to elicit Ca^{2+} current. *Bottom*, Superimposed Ca^{2+} current traces during perfusion of control solution (middle trace), 1μM WIN 55,212-2 (top trace) or 1 μM SR141716 (bottom trace) for a representative SCG neuron expressing CB_1. (**b**) Ca^{2+} current amplitude from a SCG neuron expressing CB_1 plotted over the time course of a representative experiment. Application of the CB_1 agonist WIN 55,212-2 decreased Ca^{2+} current, whereas the CB_1 inverse agonist SR141716 increased Ca^{2+} current. (**c**) *Top*, Voltage-step protocol utilized to elicit Ca^{2+} current. *Bottom*, Superimposed Ca^{2+} current traces during perfusion of control solution (middle trace), 1μM WIN 55,212-2 (top trace) or 1 μM SR141716 (bottom trace) for a representative SCG neuron co-expressing CB_1 and CRIP1a. (**d**) Ca^{2+} current amplitude from a SCG

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neuron co-expressing CB_1 and CRIP1a plotted over the time course of a representative experiment. Application of the CB_1 agonist WIN 55,212-2 decreased Ca^{2+} current; however, the ability of the CB_1 inverse agonist SR141716 to increase Ca^{2+} current was impaired. (**e**) The ability of CB_1 agonist WIN 55,212-2 to inhibit Ca^{2+} currents is unaffected by CRIP1a. (**f**) However, CB_1-mediated enhancement of Ca^{2+} current by antagonist/inverse agonist SR141716 is significantly attenuated by CRIP1a (* $p < 0.05$). Scale bars in (**a**) and (**c**): 500 pA, 25 ms.

Fig. 7. CRIP1b does not alter CB_1–mediated tonic inhibition of voltage-gated Ca^{2+} channels. (**a**) *Top*, Voltage-step protocol utilized to elicit Ca^{2+} current. *Bottom*, Superimposed Ca^{2+} current traces during perfusion of control solution (middle trace), 1μM WIN 55,212-2 (top trace) or 1μM SR141716 (bottom trace) for a representative SCG neuron expressing CB_1. (**b**) Ca^{2+} current amplitude from a SCG neuron expressing CB_1 plotted over the time course of a representative experiment. Application of the CB_1 agonist WIN 55,212-2 decreased Ca^{2+} current, whereas the CB_1 inverse agonist SR141716 increased Ca^{2+} current. (**c**) *Top*, Voltage-step protocol utilized to elicit Ca^{2+} current. *Bottom*, Superimposed Ca^{2+} current traces during perfusion of control solution (middle trace), 1μM WIN 55,212-2 (top trace) or 1μM SR141716 (bottom trace) for a representative SCG neuron co-expressing CB_1 and CRIP1b. (**d**) Ca^{2+} current amplitude from a SCG neuron co-expressing CB_1 and CRIP1b plotted over the time course of a representative experiment. Application of the CB_1 agonist WIN 55,212-2 decreased Ca^{2+} current, whereas the CB_1 inverse agonist SR141716 increased Ca^{2+} current. (**e**) The ability of CB_1 agonist WIN 55,212-2 to inhibit Ca^{2+} currents is unaffected by CRIP1b. (**f**)

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Similarly, CRIP1b failed to affect CB_1-mediated enhancement of Ca^{2+} current by inverse agonist SR141716 ($p = 0.13$). Scale bars in (**a**) and (**c**): 500 pA, 25 ms.

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Table 1. CRIP1a or CRIP1b did not significantly alter the maximum binding (B_{max}) or [^{3}H]SR141716 binding affinity (K_d) for CB_1 in HEK 293 or CHO cells.

	n	B_{max} (pmol/mg protein)	K_d (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

Membranes were prepared from HEK cells stably expressing CB_1 receptors with or without CRIP1a or from CHO cells that were transiently transfected with CB_1 receptor cDNA and an empty vector, CRIP1a or CRIP1b. Membranes were incubated with varying concentration of [^{3}H]SR141716 as described in Methods. Data are mean B_{max} and K_D values ± SE 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 CB_1 or CB_1 + CRIP1a was determined by the two-tailed non-paired *t*-test; whereas, statistical differences among these parameters in the three transiently transfected CHO cell preparations were determined by ANOVA with post-hoc Boferroni-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.

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Table 2. Neither CRIP1a nor CRIP1b altered the time course of Ca^{2+} current inhibition or the recovery from inhibition by the CB_1 agonist WIN 55,212-2 in SCG neurons microinjected with cDNA encoding CB_1, CB_1 and CRIP1a, or CB_1 and CRIP1b.

SCG neurons expressing	*n*	Time to 90% maximal inhibition (sec)	Time to 50% recovery (sec)
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

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Figure 1

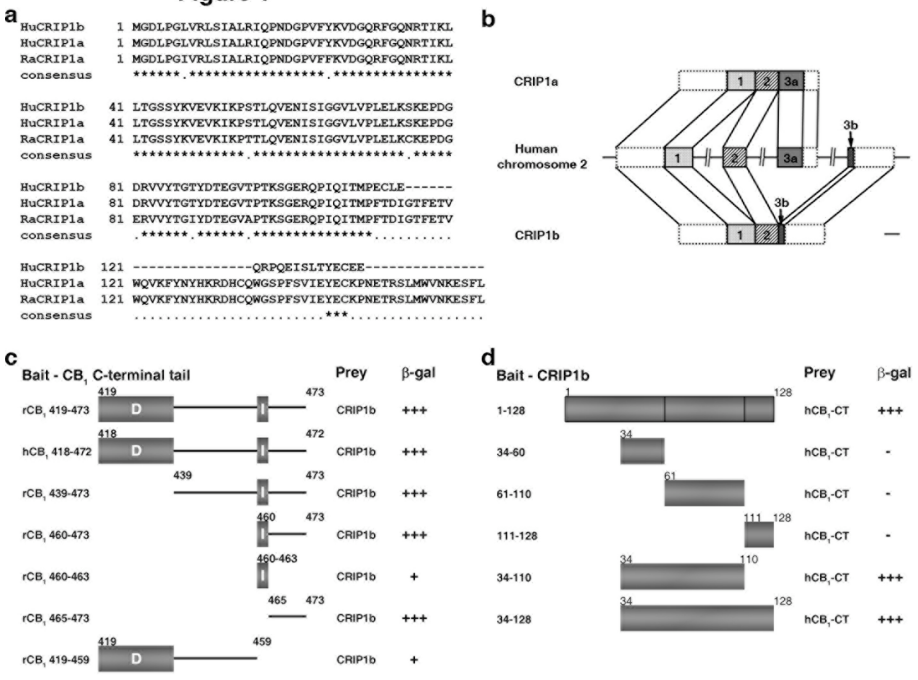

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Figure 2

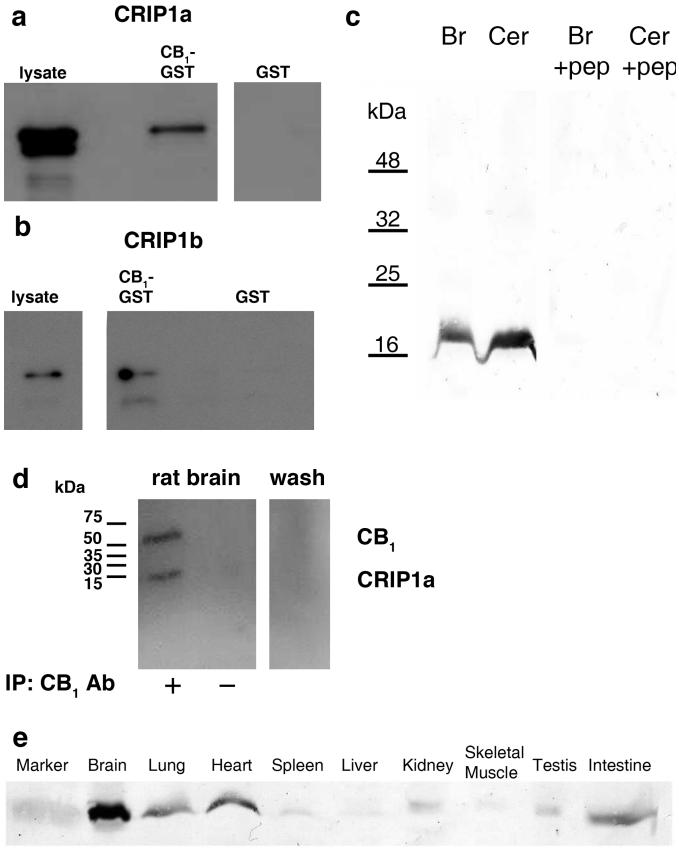

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Figure 3

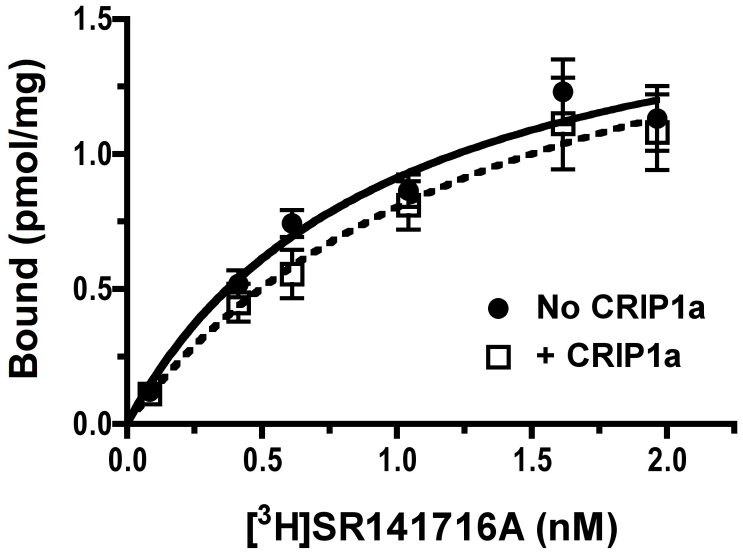

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Figure 4

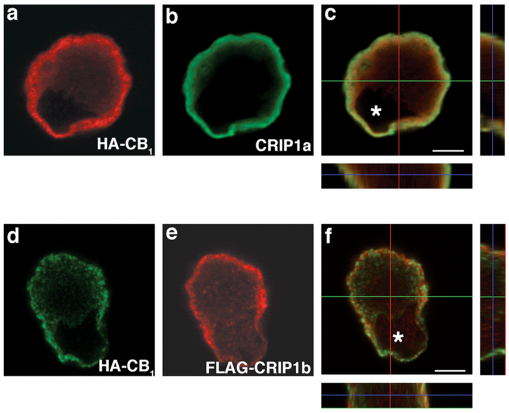

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Figure 5

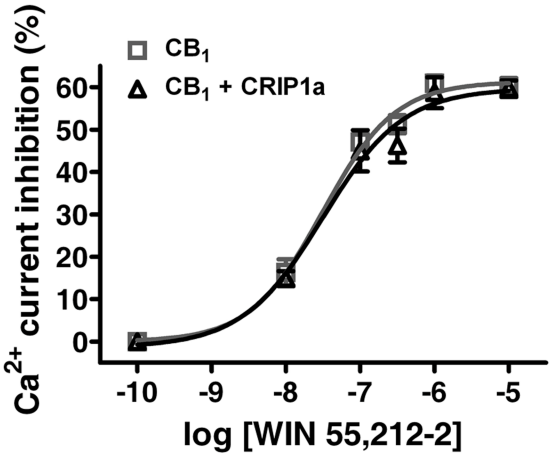

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Figure 6

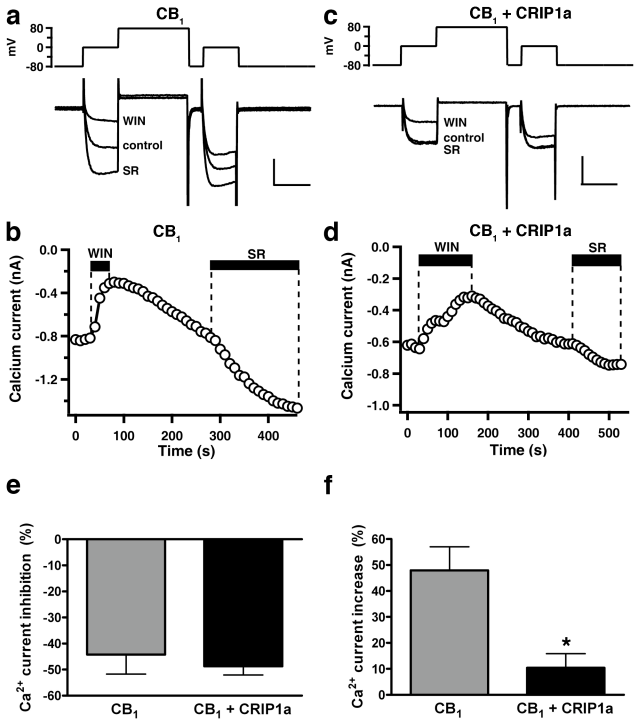

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Figure 7