Identification and Quantification of a New Family of Peptide Endocannabinoids (Pepcans) Showing Negative Allosteric Modulation at CB₁ Receptors*

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Background: The α-hemoglobin-derived peptide RVDPNVFKLLSH was found to interact with cannabinoid CB₁ receptors. Results: We generated mAbs against RVDPNVFKLLSH and identified a new family of endogenous peptides (Pepcans) that act as negative allosteric modulators at CB₁ receptors. Conclusion: Allosteric ligands for CB₁ receptors are present in the brain. Significance: Pepcans are a novel class of endogenous modulators of endocannabinoid signaling.

The α-hemoglobin-derived dodecapeptide RVD-hemopressin (RVDPNVFKLLSH) has been proposed to be an endogenous agonist for the cannabinoid receptor type 1 (CB₁). To study this peptide, we have raised mAbs against its C-terminal part. Using an immunoaffinity mass spectrometry approach, a whole family of N-terminally extended peptides in addition to RVD-Hpα were identified in rodent brain extracts and human and mouse plasma. We designated these peptides Pepcan-12 (RVDPNVFKLLSH) to Pepcan-23 (SALSDLHAHKLRVDPVNFKLLSH), referring to peptide length. The most abundant Pepcans found in the brain were tested for CB₁ receptor binding. In the classical radioligand displacement assay, Pepcan-12 was the most efficacious ligand but only partially displaced both [³H]CP55,940 and [³H]WIN55,212-2. The data were fitted with the allosteric ternary complex model, revealing a cooperativity factor value α < 1, thus indicating a negative allosteric modulation. Dissociation kinetic studies of [³H]CP55,940 in the absence and presence of Pepcan-12 confirmed these results by showing increased dissociation rate constants by Pepcan-12. A fluorescently labeled Pepcan-12 analog was synthesized to investigate the binding to CB₁ receptors. Competition binding studies revealed Kᵢ values of several Pepcans in the nanomolar range. Accordingly, using competitive ELISA, we found low nanomolar concentrations of Pepcans in human plasma and ~100 pmol/g in mouse brain. Surprisingly, Pepcan-12 exhibited potent negative allosteric modulation of the orthosteric agonist-induced cAMP accumulation, [³⁵S]GTPγS binding, and CB₁ receptor internalization. Pepcans are the first endogenous allosteric modulators identified for CB₁ receptors. Given their abundance in the brain, Pepcans could play an important physiological role in modulating endocannabinoid signaling.

Cannabinoid CB₁ and CB₂ receptors are Grgyz, protein-coupled receptors with overall 48% amino acid sequence identity (1, 2). The CB₁ receptor is one of the most abundant receptors in brain (3) and the CB₂ receptor is primarily expressed in peripheral tissues (4). The known endogenous ligands for these receptors are the endocannabinoids derived from arachidonic acid, with arachidonylethanolamide (anandamide; AEA)² and 2-arachidonoylglycerol (2-AG) being the best characterized endogenous agonists (5). Endocannabinoids are pleiotropic and exert multiple physiological effects via CB₁ and CB₂ receptors but also activate other receptors, such as GPR55 (G protein-coupled receptor 55), TRPV1 (transient receptor potential cation channel subfamily V member 1), and PPARs (6). The endocannabinoid system is involved in inflammation, pain, and metabolic disorders, and the modulation of CB receptors is a promising strategy for pharmacotherapy (6, 7). In 2007, Heimann et al. (8) reported that the α-hemoglobin-derived peptide hemopressin (Hpα) with the amino acid sequence PVNFKGLSH competitively binds to the [³H]SR141716 binding site in isolated rat brain membranes. Based on in vitro func-

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EXPERIMENTAL PROCEDURES

Peptide Synthesis

Pepcans were synthesized using standard Fmoc solid-phase synthesis chemistry on a CS336 peptide synthesizer (CS Bio Co.) essentially as described before (19). Used resins were Rink Amide MBHA resin (peptides 2 and 4; Table 1) and Fmoc-His(Trt)-NovoSyn-TGT resin (peptides 1 and 3 and RVD-Hpα). Cysteine-containing Peptides were cleaved from the resin using TFA/trisopropylsilane/H₂O/1,2-ethanediethiol (94:1:2.5:2.5). Analytical and preparative HPLC analyses of the crude peptides were carried out on VWR HITACHI Elite LaChrom systems with symmetry C18 columns (3.5 μm, 4.6 × 100 mm for analytical HPLC and 5 μm, 19 × 100 mm for preparative HPLC) with flow rates of 1 and 25 ml/min, respectively. The peptides were identified by high resolution electrospray ionization mass spectrometry.

Peptide Coupling to Carrier Proteins and Generation of Fluorescently Labeled Peptides

A 5 mg/ml solution of peptide 1 (Table 1) in PBS, pH 7.2, was reduced with 5 mM tris(2-carboxyethyl)phosphine hydrochloride (Pierce) for 15 min at room temperature prior to incubation with either a 10 mg/ml solution of maleimide-activated mCKLH carrying a succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate cross-linker (Pierce) or a 10 mg/ml solution of ovalbumin (Pierce) preactivated with the heterobifunctional cross-linker N-(γ-maleimidobutyryloxy)succinimide ester (GMBS) for 2 h at room temperature. The ovalbumin-GMBS conjugate was synthesized using a 30-fold molar excess of sulfo-GMBS (Pierce) in activation buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) that was applied to a 5 mg/ml solution of ovalbumin and incubated for 60 min at room temperature. The conjugates were purified on an ÄKTA prime FPLC device with a 5-ml HiTrap desalting column (GE Healthcare), concentrated in Amicon-Ultra4 centrifugal filter units (Millipore), and analyzed with SDS-PAGE following Coomasie Brilliant Blue G-250 staining (Invitrogen). Introduction of fluorescein labels was done by a covalent postsynthetic coupling procedure of fluorescein 5-maleimide (Pierce) to the cysteine-containing peptides 1–4. Therefore, 2 mg of each peptide was dissolved in 200 μl of a 6.25 mg/ml solution of fluorescein 5-maleimide in N,N-dimethylformamide and incubated for 2 h in the dark at room temperature. After evaporation of N,N-dimethylformamide, the products were reconstituted in DMSO and purified and analyzed with preparative HPLC and electrospray ionization MS as described above.

Generation of Monoclonal Antibodies (mAbs)

Animals—For immunization experiments, three female 12-week-old (NZB×NZW)F1 hybrid mice (NZBNZW) from Harlan Laboratories were taken. Approval for animal experimentation was obtained from the responsible authorities, and all experiments were performed in strict accordance with the rules and regulations for the protection of animal rights laid down by the Swiss Bundesamt für Veterinärwesen.
**Pepcans Are Negative Allosteric Modulators of CB1 Receptors**

**Immunization, Generation of mAbs, and Antibody Purification**—Mice were immunized subcutaneously with a keyhole limpet hemocyanin (KLH)-peptide conjugate (Table 1) formulated in Sigma Adjuvant System (Sigma). Starting on day 0, they received three doses of 50 μg of conjugate at 3-week intervals. Blood was collected before each immunization and 2 weeks after the final injection. The blood was centrifuged for 10 min (1500 × g) at 4 °C, and the serum was stored at −80 °C. Three days before the cell fusion, the mouse received an intravenous booster injection with 50 μg of the KLH-peptide conjugate in PBS. The generation of peptide-specific clones was done as described before (20). Cells secreting peptide-specific IgG were identified and screened by ELISA. Four hybridoma cell lines were selected and cloned twice by limiting dilution. mAbs were purified from sterile filtered culture supernatant by protein A affinity chromatography (HiTrap rProtein A FF, Amersham Biosciences) for IgG2a, IgG2b, and IgG3 isotypes and by protein G affinity chromatography (HiTrap Protein G, Amersham Biosciences) for IgG1 isotypes. Purified mAbs were dialyzed against PBS with Slide-A-Lyzer dialysis cassettes (Pierce), aliquoted, and stored at −80 °C.

**Determination of Protein Concentration**

Protein determinations of samples were carried out in duplicates on Polysorp microtiter plates (Nunc) using the bicinchoninic acid (BCA) protein assay reagent kit according to the manufacturer’s protocol (Pierce). Calibration curves were prepared using defined concentrations of BSA. The protein complex absorption was detected at 562 nm.

**ELISA**

**Determination of Antibody Titers**—An ELISA was performed to test mouse sera for peptide-specific immune responses. Immulon 4 HBX microtiter plates (Dynex Technologies Inc.) were coated with 50 μl of a 10 μg/ml solution of an ovalbumin-peptide conjugate (Table 1) and ovalbumin (negative control) in PBS, pH 7.2, at 4 °C overnight. After washing once with PBS, 0.05% Tween, wells were blocked with 100 μl of 5% milk powder in PBS for 60 min at 37 °C. After another washing step, plates were incubated with 50 μl of eight 1:3 serial dilutions of mouse sera starting at 1:50 in PBS, 0.5% milk powder, 0.05% Tween by vacuum filtration (Merck Millipore). Finally, the formation of the antibody-peptide complexes was detected by incubation with 30 μl of alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain-specific) (Sigma), IgG1, IgG2a, IgG2b, IgG3, A, and κ (all from Southern Biotech), and plates were developed as described above.

**mAb Isotyping ELISA**—Plates were coated with 50 μl of a 10 μg/ml solution of an ovalbumin-peptide conjugate (Table 1). After blocking and washing, 100 μl of selected hybridoma supernatants were added in duplicates and incubated for 60 min at 37 °C. After washing, plates were incubated for 60 min with alkaline phosphatase-conjugated goat anti-mouse antibodies specific for IgG (γ-chain-specific) (Sigma), IgG1, IgG2a, IgG2b, IgG3, A, and κ (all from Southern Biotech), and plates were developed as described above.

**Preparation of Color-coded Bead-based Peptide Arrays**

To determine the minimal sequence required for antibody binding, a bead-based peptide array was prepared as previously described (22–24). The array consisted of the RVD-Hpα peptide (RVDVPNFKLLSH), a carboxamide version of the RVD-Hpα peptide, and 22 truncated peptides. Truncated peptides started from the C or the N terminus of the antigen. C-terminally truncated peptides were synthesized in the form of carboxamides, whereas N-terminal truncations had a carboxyl group at the C terminus. For immobilization purposes, two hydrophilic spacers (8-amino-2,6-dioxoacetoanoic acid) and a cysteine were added to the N terminus of all peptides. All peptides were purchased from Intavis. Each of the 24 peptides was conjugated onto a distinct color-coded bead population. For the epitope mapping, 500 beads of each population were mixed to give a peptide array. BSA-coated beads served as a negative control.

**Epitope Mapping of mAbs**

30 μl of the premixed bead-based peptide array (500 beads/population) were used to assess the minimal epitopes of the RVD-Hpα peptide-specific mAbs. The mixture of beads was transferred to 96-well filter plates (Merck Millipore) and incubated with 30 μl of the respective RVD-Hpα peptide-specific antibody (1 μg/ml) for 60 min at 23 °C and 650 rpm. Subsequently, beads were washed two times with 100 μl of PBS, 0.05% Tween by vacuum filtration (Merck Millipore). Finally, the formation of the antibody-peptide complexes was detected by incubation with 30 μl of anti-mouse IgG-phycoerythrin conjugate (2.5 μg/ml; Jackson ImmunoResearch Inc.) for another 45 min at 23 °C and 650 rpm. Following two further washing steps, the beads were resuspended in 100 μl of blocking reagent for ELISA (Roche Applied Science), 0.1% Tween. Read-out of the bead type-specific reporter fluorescence was performed on a Luminex IS 100 system (Luminex Corp.).

**Peptide Extraction from Brain and Plasma Samples**

**From Rodent Brain Tissues**—For brain peptide extractions female 12-week-old NMRI mice were used (Charles River). Hamster and rat brains were a kind gift from the group of Prof. Jennifer Keiser at the SwissTPH. From the sacrificed mice, brains were removed aseptically, immediately frozen in liquid nitrogen, and stored at −80 °C. Brains were homogenized (2 × 6000 rpm, 5-min break with cooling on ice) with a Precellys24 tissue homogenizer (Bertin Technologies) in 2-ml tubes containing 1.4-mm ceramic beads (Bertin Technologies) either
immediately in extraction buffer for Western blot and immunoaffinity MS experiments or first in double-distilled H₂O for spike and recovery experiments following an acetonitrile/H₂O (2:1) (ACN 66%) extraction for peptide quantification in competitive ELISA experiments. All brain extraction approaches contained 1× complete protease inhibitor mixture (Roche Applied Science). The different extraction buffers used were PBS, pH 7.2, ACN/H₂O 1:1 (ACN 50%), ACN 66%, 10 mM HCl, and 100 mM acetic acid (AcOH). Rodent brains were homogenized at 0.5 g wet weight/ml extraction buffer with subsequent centrifugation for 5 min at 12,000 × g. The supernatants of the ACN extractions were evaporated to dryness in a SpeedVac (CentriVap Concentrator and CentriVap Cold Trap, Labconco) at 60 °C. All extracts were stored at −80 °C.

From Human and Mouse Plasma—Human blood samples were drawn with the Vacuette blood collection system (Greiner Bio-One) from healthy female and male volunteers (age 25–30 years) with EDTA as anticoagulant. Mouse blood samples were collected by cardiac puncture with heparin as anticoagulant. Mouse brain extracts were reconstituted in 1 ml of PBS, pH 7.2) was washed four times with PBS, 0.3% N-octyl gluscolide, and 5 μl of the bead suspension were added to each sample. After a 60-min incubation at room temperature, the beads were washed four times and eluted in 1% formic acid as described previously (24, 25). 1 μl of each sample was spotted in triplicates onto a Prespotted HCCA AnchorChip (Bruker Daltonics). The MALDI MS readout was performed on an Ultraflex III MALDI-TOF/TOF (Bruker Daltonics) running in positive ion reflectron mode. Peptides were identified by de novo sequencing using tandem mass spectrometry (MS/MS). External calibration was automatically performed with the prespotted MALDI calibrants using the flexAnalysis version 3.0.92.0 software.

Cell Culture

All cell lines were cultured in medium supplemented with 10% FBS, penicillin, streptomycin, amphotericin B (all from Sigma) at 37 °C and 5% CO₂. CHO-K1 (CHO) and CHO-K1 cells stably transfected with human CB₁ (CHO-hCB₁) or human CB₂ receptors (CHO-hCB₂), were obtained from Dr. Michael Detheux ( Euroscreen S.A., Brussels, Belgium) and cultured in RPMI 1640 (Invitrogen) with 400 μg/ml G418 (Sigma). HL60 cells (human promyelocytic leukemia cells; ATCC CCL-240) were cultured in IMDM (ATCC). N18TG2 cells (ACC-103, DSMZ, Germany) were cultured in DMEM with 100 μM 6-thioguanine (Sigma). HEK293 cells stably transfected with human TRPV1 (a kind gift from Ken Mackie, Indiana University, Bloomington, IN) or human GPR55 receptors (a kind gift from Gábor Pethő, University of Pécs, Hungary) were cultured in RPMI 1640.

Receptor Binding Studies

Equilibrium Radioligand Binding Assays—Radioligand binding assays using [³H]CP55,940 and [³H]WIN55,212-2 were performed with CB₁ receptor membrane preparations as reported previously (26, 27). Briefly, 18 μg of crude membranes of CHO-hCB₁ cells (prepared as described previously (28)) were resuspended in 500 μl of binding buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl₂, 0.5 mg/ml fatty acid-free BSA, pH 7.4) in silanized glass tubes and co-incubated with different concentrations of Pepcans and 0.5 nM [³H]CP55,940 (168 Ci/mmol) or 2.5 nM [³H]WIN55,212-2 (40 Ci/mmol) for 60 min at 30 °C under shaking. Pepcans were directly diluted from the DMSO stock solutions in assay buffer (1:100) to the final concentrations. Because micromolar concentrations of Pepcans in buffer (fractional dilution of DMSO stock) may cause aggregation and loss of free peptides, the means of administration of Pepcans to cells was important. Nonspecific binding of the radioligand was determined in the presence of 10 μM WIN55,512-2 or CP55,940, respectively. Nonspecific binding was around 5%. After the incubation time, membrane suspensions were rapidly filtered through a 0.1% polyethyleneimine presoaked 96-well
microplate bonded with GF/B glass fiber filters (UniFilter-96 GF/B, PerkinElmer Life Sciences) under vacuum and washed 12 times with 167 μl of ice-cold washing buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl₂, 0.5% fatty acid-free BSA, pH 7.4). Filters were added with 40 μl of MicroScint20 scintillation liquid, and radioactivity was measured with the 1450 MicroBeta TriLux top counter (PerkinElmer Life Sciences). Data were collected from 3–5 independent experiments, each performed in triplicate, and the nonspecific binding was subtracted. The results are expressed as a percentage of vehicle–treated samples.

**Dissociation Kinetic Studies Using Isotopic Dilution**—Dissociation kinetics experiments were performed as described before (16, 29). Briefly, 18 μg of membranes of CHO- hCB1 cells were resuspended in 500 μl of binding buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl₂, 0.5 mg/ml fatty acid-free BSA, pH 7.4) in silanized glass tubes and incubated with 0.5 nM [3H]CP55,940 for 60 min at 30 °C under shaking. Radioligand dissociation was initiated by the addition of 1 μM WIN55,212-2 in the presence of 300 nM Pepcan-12 or solvent (DMSO) and incubated for 1–120 min, at 30 °C. Nonspecific binding of the radioligand was determined in the presence of 10 μM CP55,940. After the incubation time, membrane suspensions were rapidly filtered through a 0.1% polyethyleneimine presoaked 96-well microplate bonded with GF/B glass fiber filters and washed as described above.

**Equilibrium Binding Studies Using a Fluorescently Labeled Pepcan-12 Derivative**—The equilibrium dissociation constant (K_d value) of the fluorescein-labeled Pepcan-12 derivative Pepcan-F4 (Table 1) was determined using CHO- hCB, cells. Equilibrium saturation binding was determined experimentally, and maximal specific binding (saturation) was reached already at 5 min at 37 °C. Different concentrations of Pepcans and 100 nM Pepcan-F4 were incubated together with 10² cells suspended in 200 μl of binding buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl₂, and 0.5% fatty acid-free BSA, pH 7.4) in silanized plastic tubes, under shaking for 60 min at 37 °C. Af ter centrifugation at 4 °C and washing with ice-cold binding buffer, the cells were resuspended in ice-cold PBS and transferred into 96-well black plates, where the fluorescence intensities were measured using a Tecan Farcyte reader (485-nm excitation/535-nm emission).

The specific binding was determined after washing and subtracting the nonspecific fluorescence calculated in parallel experiments carried out in non-transfected CHO cells.

**Competitive Enzyme-linked Immunosor bent Assay (C-ELISA)**

**Characterization of mAb 1A12**—Binding affinity of the mab 1A12 toward the C-terminal truncated peptides RVDPVN-FKLL and RVDPVN; the N-terminal extended peptides KLRLVDPVNFKLSSH, HKLRLVDPVNFKLSSH, HAHLKLRLVDPVNFKLSSH, and SDLHAKLRVDPVNFKLSSH (all from Genscript); rat hemopressin (PVNFKLSSH) (Tocris Bioscience); the hemoglobin β-chain derived peptide HVDPVEN-FRLLLN (Genscript); and SDS-PAGE purified, 2× crystallized bovine hemoglobin (Sigma) was determined and compared with the binding affinity toward RVD-Hpα (RVDPVN-FKLSSH) with or without ACN extraction. As negative control, the peptide [Val⁵]angiotensin I (Sigma) was used. Maxisorp microtiter plates (Nunc) were coated with 50 μl of a 10 ng/ml solution of an ovalbumin-peptide conjugate (Table 1) as described above. After blocking and washing, 11:1:3 serial dilutions starting at a 10 μM concentration of the analytes in PBS, 0.5% BSA, 0.05% Tween (assay buffer) and each containing 20 ng/ml of mAb 1A12 were incubated for 2 h at room temperature. After washing, plates were incubated with 50 μl of goat anti-mouse IgG horseradish peroxidase-conjugated antibodies (Bio-Rad) for 60 min at room temperature. Plates were developed with 50 μl of 3,3',5,5'-tetramethylbenzidine substrate (Pierce) after washing. The reaction was stopped after 15 min with 50 μl of 2 m H₂SO₄, and OD of the reaction product was recorded at 450 nm. For ACN extraction, samples were processed as described above. Briefly, samples were incubated with ACN/H₂O in a ratio of 1:1 (ACN 50%) or 2:1 (ACN 66%), incubated, and centrifuged, and the supernatant was evaporated to dryness in the SpeedVac. Pellets were reconstituted in assay buffer.

**Peptide Quantification from Mouse Brain Tissue and Human Plasma Samples**—For peptide quantification, a C-ELISA as described above was used with ACN 66% extraction. Twelve calibrators containing 1000, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.16 nM RVD-Hpα were prepared in assay buffer and extracted with ACN/H₂O in a ratio 2:1 and processed as shown above. NMRI mouse brain extracts and human plasma samples for spike and recovery experiments were diluted 1:1 in assay buffer prior to ACN 66% extraction. Samples for quantification were treated the same way. The dried samples were reconstituted in the initial volume in assay buffer.

**Measurement of cAMP Accumulation**

For cAMP measurement, we used the GloSensor™ luminescence assay (Promega) for detecting cAMP levels in live cells. Therefore, CHO- hCB, and wild-type CHO cells were stably transfected with the pGloSensor™-2F plasmid using Lipofectamine 2000 in OptiMEM I reduced serum medium (both from Invitrogen). Screening of positive clones and assay development were according to the manufacturer’s instructions. For measurement of cAMP accumulation, 3 × 10⁵ cells were grown in RPMI 1640 medium overnight in white 96-well microtiter plates (Greiner, clear bottom). After incubation with 2% GloSensor cAMP reagent (Promega) in 200 μl of medium for 2 h at 37 °C, 1 μl of compounds diluted in DMSO were added, and luminescence was measured after 3 min of incubation on a 1450 MicroBeta TriLux counter. Competitors and agonists were applied in succession (3-min preincubation for SR141716). Results are expressed as percentages of vehicle control after division of preread responses, measured directly before adding compounds, to account for well-to-well variance.

**Agonist-stimulated[³²S]GTPγS Binding**

C57BL/6N male mice (3–6 months old) were anesthetized with isoflurane and decapitated. Brains were quickly removed, and cerebella were dissected on ice and homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 0.2 mM EGTA, 3 mM MgCl₂, 50 μM phenylmethanesulfonyl fluoride (PMSE), 1× Complete protease inhibitor (Roche Applied Science)) using a glass homogenizer. Protein concentration was determined by using a BCA assay (Thermo Scientific). Homo-
Pepcans Are Negative Allosteric Modulators of CB1 Receptors

The assessment of the surface expression of CB1 receptors was performed using CHO-K1 cells stably transfected with human CB1 receptors as described before (31). Compounds diluted in DMSO were added to 10⁵ cells and incubated for 60 min at 37 °C at a final concentration of 1 μM.

Assessment of Peptide Aggregation

Peptide aggregation was measured by exploiting the competitive ELISA for peptide quantification described above, however without ACN extraction. For each peptide, a low (10 nm) and a high concentration (1 μM) were incubated in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and amphotericin B for 60 min at 37 °C and subsequently centrifuged in ultrafiltration tubes with 2,000 (Vivaspin 2 Hydroph, GE Healthcare) molecular weight cut-off, respectively, at 40,000 rpm for 30 min at 30 °C. Nonspecific binding was determined in the presence of 10 μM GTPγS, and specific binding was defined as total binding minus nonspecific binding. Bound [35S]GTPγS was harvested by vacuum filtration over Whatman GF/B filters using a Brandel Cell Harvester and transferred to scintillation vials containing Aquasafe 300 plus (Zinsser Analytic) scintillation mixture. Radioactivity was measured by liquid scintillation counting using Tri-Carb 2800 TR (PerkinElmer Life Sciences). Data are expressed as percentage of basal specific binding.

Receptor Internalization Studies

The assessment of the surface expression of CB1 receptors was performed using CHO-K1 cells stably transfected with human CB1 receptors as described before (31). Compounds diluted in DMSO were added to 10⁵ cells and incubated for 60 min at 37 °C at a final concentration of 1 μM.

AEA and 2-AG Hydrolysis Assays

[ethanolamine-1-3H]Anandamide (40–60 Ci/mmol) and [1,2,3-3H]2-arachidonyl glycerol (20–40 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. (Saint Louis, MO). Different concentrations of Pepcan-12 or 1 μM PMSF (as the positive control) in a 2.5-μl volume (DMSO) were preincubated at 37 °C for 20 min with 195 μl of diluted pig brain homogenate in 10 mM Tris-HCl and 1 mM EDTA, pH 7.6, corresponding to 200 μg of total protein and 1% (w/v) fatty acid-free BSA (Sigma). Successively, 2.5 μl of non-radioactive anandamide and [ethanolamine-1-3H]anandamide (40–60 Ci/mmol) mixture were added to the homogenate (final concentration in the assay was 2 μM) and incubated for 10 min at 37 °C under shaking. After the incubation time, 400 μl of a methanol/chloroform mixture (1:1, v/v) was added at each sample, vigorously vortexed, and centrifuged at 10,000 rpm for 10 min at 4 °C to separate aqueous and organic phases. The radioactivity associated with the hydrolytic formed [3H]ethanolamine was measured upon the addition of 3 ml of Ultima Gold scintillation liquid (PerkinElmer Life Sciences) to the aqueous phase, using a Tri-Carb 2100 TR scintillation counter (PerkinElmer Life Sciences). Data were collected, and FAAH activity was expressed as a percentage of vehicle-treated samples. For 2-AG hydrolysis, 195 μl (corresponding to 200 μg of total protein) of diluted pig brain homogenate in 10 mM Tris-HCl, 1 mM EDTA plus 0.1% (w/v) fatty acid-free BSA, pH 7.4, was preincubated for 30 min at 37 °C with different concentrations of Pepcan-12 and 1 μM JZL184 as the positive control for the maximal 2-AG hydrolysis inhibition via MAGL. After the preincubation, 2.5 μl of nonradioactive 2-AG and [1,2,3-3H]2-AG (60 Ci/mmol) mixture were added to the homogenate (final concentration in the assay was 10 μM) and incubated for 10 min at 37 °C under shaking. Successively, 400 μl of a methanol/chloroform mixture (1:1, v/v) were added and, after vigorous vortexing, aqueous and organic phases were separated by centrifugation at 10,000 rpm for 10 min at 4 °C. The radioactivity associated with the [3H]glycerol formation was measured upon the addition of 3 ml of Ultima Gold scintillation liquid (PerkinElmer Life Sciences) to the aqueous phase, using a Tri-Carb 2100 TR scintillation counter (PerkinElmer Life Sciences). Data were collected from different experiments, and results were expressed as [3H]glycerol formation in a percentage of vehicle-treated samples.

Data Analysis

All data analyses were done by using GraphPad Prism 5 software.

Radioligand Binding Assays—Competitive binding curves of orthosteric ligands were analyzed using a three-parameter one-site competitive binding equation.

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{[\log IC_{50} - Y]}^{[\Delta]}}
\]

(Eq. 1)

A relationship between the IC₅₀ and the Kᵢ value is given by the Cheng-Prusoff equation (32),

\[
K_i = \frac{IC_{50}}{1 + \frac{[R]}{K_d}}
\]

(Eq. 2)

where Y represents fractional specific binding, [A] is the logarithm of the concentration of cold orthosteric competitor, Top and Bottom represent plateau values in units of the y axis, [R] is the concentration of the hot ligand used (0.5 nM for [3H]CP55,940 and 2.5 nM for [3H]WIN55,212-2), and K₅₀ values are equilibrium dissociation constants of the hot ligand determined in former saturation binding experiments (0.5 nM for [3H]CP55,940 and 2.5 nM for [3H]WIN55,212-2). The Kᵢ values were determined by nonlinear regression.

The interaction between radioligands and Pepcan-12 (modulator titration curves) was analyzed using the following allosteric TCM equation as introduced before (16),

\[
Y = \frac{[A]}{K_a(1 + \frac{[B]}{K_b})} + \frac{1 + \alpha[B]}{[A] + \frac{1 + \alpha[B]}{K_b}}
\]

(Eq. 3)
where \([A]\) and \([B]\) represent concentration of orthosteric and allosteric ligands, respectively; \(\alpha\) is the cooperativity factor; \(K_A\) and \(K_B\) are the respective equilibrium dissociation constants; \([A]\) and \(K_A\) were fixed as constants (see above for values); and \(\alpha\) and \(K_B\) were determined by nonlinear regression.

**Dissociation Kinetics**—The dissociation kinetic experiment was analyzed using the following two-phase exponential decay model introduced before (16),

\[
Y = \text{Plateau} + \text{Span1} \times e^{-kt} + \text{Span2} \times e^{-kt} \quad (\text{Eq. 4})
\]

where \(\text{Span1}\) and \(\text{Span2}\) represent the percentage of each phase; \(k_1\) and \(k_2\) are the dissociation rate constants for the components defined by \(\text{Span1}\) and \(\text{Span2}\), respectively; and \(\text{Plateau}\) is the minimal asymptotic value. Values were determined by nonlinear regression with constraint of the minimal plateau value to 0 to account for the subtraction of non-specific binding.

**Equilibrium Binding Studies Using a Fluorescently Labeled Pepcan-12 Derivative**—The \(K_d\) value of Pepcan-F4 for \(hCB_1\) was determined by a nonlinear fit using the following one-site specific binding model,

\[
Y = \frac{B_{\max} \times [X]}{K_d \times [X]} \quad (\text{Eq. 5})
\]

where \(B_{\max}\) represents maximum specific binding, and \([X]\) is the concentration of Pepcan-F4. \(IC_{50}\) values were determined by a four-parameter dose-response curve with variable slope (here the \(IC_{50}\) values used were the concentrations of competitors displacing 50% of Pepcan-F4 (33). Derived from the \(K_d\) value of Pepcan-F4 for \(hCB_1\) (37 nM) and the \(IC_{50}\) value, the \(K_i\) value of each competitor was calculated by using the Cheng-Prusoff equation (see above).

**Functional Data Analysis**—\(EC_{50}\) (half-maximal effective concentration) values from competitive ELISA, cAMP assay, and \(^{[35]}\)GTP\(\gamma\)S binding assay data were obtained by using a four-parameter dose-response (stimulation or inhibition) model with variable slope,

\[
Y = \text{basal} + \frac{(E_{\max} - \text{basal})}{(1 + 10^{logEC_{50} - [X] \times n_H})} \quad (\text{Eq. 6})
\]

where \([X]\) represents the logarithm of the concentration of drug used; \(n_H\) is Hill slope, and \(E_{\max}\) is maximal efficacy/maximal response. Depending on the assay, pEC\(_{50}\) or pIC\(_{50}\) (negative logEC\(_{50}\) or negative logIC\(_{50}\)) values are shown.

The Schild analysis was chosen to analyze competitive antagonism in the cAMP accumulation assay and was done as described in literature (15, 34, 35). There is a relationship between the dextral shift of agonist curves incubated with increasing concentrations of competitor (expressed as concentration ratio (CR) values defined by dividing \(EC_{50}\) values of agonist curves with increasing competitor concentrations by the \(EC_{50}\) value of the agonist curve without competitor) and the concentration of competitor, \([A]\), and its equilibrium dissociation constant \(K_A\) by the Schild equation,

\[
\log(CR - 1) = \log[A] - \log K_A \quad (\text{Eq. 7})
\]

Linear regression delivers estimates about the slope (unity slope for competitive antagonists) and the \(K_A\) value of the competitor (35).

**Statistics**

Results are expressed as mean values ± S.D. or mean values ± S.E. for each examined group. Statistical significance of differences between groups was determined by the unpaired Student’s \(t\) test or one-way analysis of variance followed by Dunnett’s post hoc test with the GraphPad Prism 5 software. Outliers in a series of identical experiments were determined by Grubb’s test (extreme studentized deviate (ESD) method) with a set to 0.05. As described before (16), an extra-sum-of-squares test (F test) was used to determine if the exponential two-phase decay model is superior to a corresponding one-phase decay model.

For the quantification of Pecpans from brain tissue and plasma, descriptive statistics (mean, S.D. values, percentage coefficients of variation, differences from theoretical, and percentage recovery values) were calculated for the calibrators and the samples used for spike and recovery experiments.

**RESULTS**

**Generation and Characterization of mAbs Specific for CB Receptor Binding Peptides**—Our initial strategy was to raise mAbs recognizing the reported endogenous brain peptides RVD-Hp\(_{\alpha}\) (RVDPVNFKLLSH), VD-Hp\(_{\alpha}\), and Hp\(_{\alpha}\) (hemo-pressin), which differ only in their N termini. We therefore generated mAbs against the C-terminal part with the intention of detecting all three peptides. Mice were immunized with a peptide conjugate in which RVD-Hp\(_{\alpha}\) was N-terminally coupled to KLH (Table 1). We have chosen the mouse generating the highest affinity antibodies, reported to be capable of generating high affinity antibodies against self-antigens (36) were chosen for immunization. To select for the mouse generating the highest affinity antibodies, we determined the avidity index with an NH\(_4\)SCN competition ELISA (Fig. 1A). All mice showed affinity maturation in the

**TABLE 1**

| Name | Sequence | \(m/z\) [MH\(^+\)]* |
|------|----------|---------------------|
| 1    | C-Aca\(^{34}\)Aca-RVDPVNFKLLSH | 1753.97 |
| 2    | RVDPVNFKLLSH-Aca-Aca-C  | 1752.99 |
| 3    | C-RVDPVNFKLLSH  | 1527.81 |
| 4    | RVDPVNFKLLSH-C  | 1526.83 |
| RVD-Hp\(_{\alpha}\) (Pepcan-12) | RVDPVNFKLLSH | 1424.79 |
| Pepcan-F1 | KLH\(^{37}\)C-Aca-Aca-RVDPVNFKLLSH | 1954.87 |
| Pepcan-F2 | RVDPVNFKLLSH-C-FL  | 1953.89 |
| Pepcan-F3 | FL-C-Aca-Aca-RVDPVNFKLLSH | 2181.04 |
| Pepcan-F4 | RVDPVNFKLLSH-Aca-Aca-C-FL | 2180.06 |

* Measured mass from ESI-MS (charge +1).
** Aca, aminocaproic acid.
*** Keyhole limpet hemocyanine.
**** Ovalbumin.
***** FL, fluorescein.
Pepcans Are Negative Allosteric Modulators of CB1 Receptors

Results of four mAbs in epitope mapping and C-ELISA experiments

| mAb     | Isotype | Epitope | Neoepitope | pIC50 | S.E. |
|---------|---------|---------|------------|-------|------|
| 1A12    | IgG2a, κ| FKLLSH  | FKLLSH     | 8.02  | 0.02 |
| 2C11    | IgG2b, κ| FKLLSH  | FKLLSH     | 7.33  | 0.12 |
| 7A5     | IgG2a, κ| KLLSH   | FKLLSH     | 6.33  | 0.07 |
| 8D4     | IgG1, κ | FKLLSH  | FKLLSH     | 6.84  | 0.02 |

*Neoepitopes contain the charge of the C terminus as an essential part of their epitope. mAbs 7A5 and 8D4 recognized RVD-Hpα with a free C terminus but not the carbamidomethyl derivative (Fig. 1). pIC50 values were estimated by nonlinear regression analysis of the synthetic peptide RVDPVNFKLLSH competing for the binding of the mAb toward the corresponding ovalbumin-peptide conjugate (Table 1) in C-ELISA under optimized conditions (2C11 (2 ng/ml), 7A5 (10 ng/ml), 8D4 (50 ng/ml); for experiments with 2C11 and 7A5, 50 μl of 15 ng/ml ovalbumin-peptide conjugate were coated, and for 8D4, 50 μl of 150 ng/ml were used). The avidity index corresponds to the molar NH4SCN concentration that displaces 50% of binding toward coated ovalbumin-C-Aca-Aca-RVDPVNFKLSSH (Table 1) of sera used at half-maximal titer concentrations of reagent control. Representative results for mAb 1A12 (Fig. 2B) and mAb 7A5 (Fig. 2C) are shown.

BSA-coated beads served as control. Representative results for mAb 1A12 (Fig. 2B) and mAb 7A5 (Fig. 2C) are shown.

Error bars, S.D., AU, arbitrary units.

Additional experiments with 2C11 and 7A5, 50 μl of 15 ng/ml ovalbumin-peptide conjugate were coated, and for 8D4, 50 μl of 150 ng/ml were used. The avidity index corresponds to the molar NH4SCN concentration that displaces 50% of binding toward coated ovalbumin-C-Aca-Aca-RVDPVNFKLSSH (Table 1) of sera used at half-maximal titer concentrations of individual animals. The line displays the geometric mean of IC50 values for mAbs 7A5 and 8D4 that were determined in triplicate experiments. B–D, minimal epitopes of RVD-Hpα-specific mAbs. Color-coded bead-based peptide arrays were employed to determine the minimal epitopes of the RVD-Hpα-specific mAbs. The array was fabricated from a set of N-terminally and C-terminally truncated versions of the synthetic peptide. Mean fluorescence intensities (MFI) (n = 3) are plotted against the respective peptide sequences in a bar chart. Results for C-terminally truncated peptides are displayed in light gray (left) and for N-terminally truncated peptides in dark gray (right). BSA-coated beads served as control. Representative results for mAb 1A12 (8) and mAb 7A5 (C) are shown. Results for reagent control are shown in D. Results indicate an epitope of mAb 1A12 containing the peptide sequence FKLLSH (8). C, epitope mapping of mAb 7A5 revealed a neoepitope bearing the sequence KLLSH. Minimal epitopes of four mAbs are indicated in Table 2. Error bars, S.D., AU, arbitrary units.

FIGURE 1. A, peptide-specific immune responses to antigen KLH-C-Aca-Aca-RVDPVNFKLSSH (Table 1) of three NZBNZW mice (after the third immunization) determined by ELISA (n = 1). Bottom, results from NH4SCN elution ELISA
**Pepcans Are Negative Allosteric Modulators of CB1 Receptors**

Identification of N-terminally Extended Peptides by Immunoaffinity MS Experiments—To identify the antigens of the bands stained in Western blots with mouse brain extracts, we performed immunoaffinity MS experiments. A mass spectrum of the mAb 1A12 with mouse brain extract after ACN extraction is shown in Fig. 3A. Mass spectra of mouse brains after PBS and acidic extractions showed essentially similar results (Fig. 4). The prominent peak at m/z 1424.8 represents the singly protonated peptide RVD-Hpα that was identified in all mouse brain extracts independently of the extraction buffer used. Intriguingly, we identified not only RVD-Hpα but also several N-terminally extended peptides, which are listed in Table 3. Additionally, we investigated hamster and rat brain ACN extracts. In hamster brain extracts, we obtained similar results (Fig. 3B), whereas in rat brain extracts, no peaks were visible in the corresponding mass range (Fig. 3C) possibly because the rat sequence has a Leu → Phe amino acid substitution in the C-terminal part located within the epitope of the mAb 1A12 (Table 2). Reconfirmation of the identification of the peptides by MS/MS fragmentation is representatively shown for the peptides at m/z 1424.8 and 2011.1 in Fig. 3, E and F, respectively. The reported brain peptides VD-Hpα and Hpα (8, 10, 12) could not be detected in the extracts despite the fact that, according to our epitope mapping (Fig. 1), the mAb 1A12 was *per se* able to recognize the mouse/human Hpα (PVNFKLSSH) and VD-Hpα (VDPVFKLSSH) peptides. This is in line with the insight thatHpα is a hot acid extraction artifact (11) and does not occur endogenously. Because RVDPVFKLSSH together with the newly identified N-terminally extended peptides represents a whole peptide family, and hemopressin is reported to be an artifact and was not found in this study, we propose the new nomenclature Pepcan-X, with X referring to the number of amino acids. Pepcan is the abbreviation for “peptide endocannabinoids” because several identified peptides also showed significant CB1 receptor binding and functional activities (see below). No peaks corresponding to the 6 kDa band observed in Western blot experiments of brain extracts were found in immunoaffinity MS experiments (data not shown). Apparently, the AcOH extraction was more efficient for Pepcan-17 compared with Pepcan-12, as shown in Fig. 4C, which demonstrates the importance of which extraction buffer is used. Several members of the Pepcan family were also identified in mouse (Fig. 3D) and human plasma samples (data not shown). Altogether, we identified 11 Pepcans in mouse and hamster brain tissues, and five of them, Pepcan-12, -14, -15, -17, and -20, were also detected in mouse and human plasma (Table 3).

**CB1 Receptor Binding Interactions of Pepcans**—Pepcans were investigated for CB1 receptor binding properties by applying classical radioactivity-based assays and a new fluorescence-based assay. We measured Pepcan-12, -14, -15, -17, and -20, which were detected in rodent brain and mouse/human plasma samples, and the two C-terminally truncated peptides RVD-PVNFKL and RVDVPNF, which were previously reported to occur in mouse blood (12). First, we carried out conventional radioligand binding assays using [3H]CP55,940 and [3H]WIN55,212-2, which both have low nanomolar affinities toward CB1 receptors (3, 37). The results showed a weak to moderate radioligand displacement for Pepcan-12, -14, and -20.

Entire Western blots displayed in Fig. 2, D and E, of an acidic and an ACN extraction, respectively, illustrate the selectivity of the mAb 1A12 for the low molecular weight band.

**FIGURE 2.** A–C, Western blots of mouse brain extracts with the mAb 1A12. A, Western blots of mouse brain extracts using five different extraction buffers: PBS, pH 7.2, ACN/H2O 1:1 (ACN 50%), ACN/H2O 2:1 (ACN 66%), 10 mM HCl, and 100 mM AcOH. B, mAb 1A12 recognized the synthetic peptide RVD-Hpα (1423.8 Da) at two different antibody concentrations (10 and 1 μg/ml). C, competitive Western blot of the ACN 66% brain extract with the mAb 1A12 using increasing concentrations of the synthetic RVD-Hpα as competitor. 10 μM [Val5]angiotensin I was taken as negative control peptide. For A and C, the mAb 1A12 was incubated at 10 μg/ml. Entire Western blots of the 10 mM HCl and ACN 66% mouse brain extracts using six different anti-RVD-Hpα mAbs (1A10 (lane 1), 1A12 (lane 2), 2C11 (lane 3), 7A5 (lane 4), 2C1 (lane 5), and 8D4 (lane 6)) are shown in D and E, respectively. In both blots, only mAb 1A12 stained a band in the mass range of RVD-Hpα. The 50 kDa band of the HCl extract (D) is stained by the secondary antibody (goat anti-mouse IgG y-chain, lane 7). In blot E, this band is not visible because ACN efficiently precipitates high molecular weight proteins, such as immunoglobulins. Representative blots of three independent experiments are shown.
at 1 μM, whereas no significant displacement was observed for Pepcan-15 and -17 (Fig. 5, A and B). Pepcan-12 most efficiently displaced both radioligands by reaching ~50% binding at 1 μM. Therefore, we next characterized Pepcan-12 receptor binding potency toward CB1 in a concentration-dependent assay. As shown in Fig. 5, C and D, Pepcan-12 was able to potently but only partially displace both [3H]CP55,940 and [3H]WIN55,212-2. This saturable but incomplete displacement indicated an allosteric rather than a competitive binding interaction. Therefore, we analyzed the Pepcan-12 data with the allosteric TCM model, which is suitable to describe an allosteric interaction between two ligands that bind to topographically distinct binding sites at the same receptor (38). This model has been used previously to confirm the allosteric interaction of the synthetic Organon compounds with the CB1 receptor (16). Pepcan-12 induced a similar maximal displacement (around 45–50%), of [3H]CP55,940 and [3H]WIN55,212-2 with similar pKB values. In both cases, the calculated cooperativity constant was less than 1, indicating a negative allosteric modulation (Table 4). As shown below, Pepcans can form aggregates (dimers or oligomers) at high concentrations (>1 μM); thus, we cannot exclude the possibility that oligomerization may potentially contribute to the partial displacement of the orthosteric ligands.

FIGURE 3. Mass spectra of different rodent brain and a mouse plasma sample extract with immunoaffinity enrichment using the mAb 1A12. A–C, results from mouse, hamster, and rat brains extracted with ACN, respectively. D, mass spectrum of mouse plasma extracted with ACN 66%. The identified peptides are displayed in gray. E and F, MS/MS fragmentation spectra of the peaks at m/z 1424.8 (RVDVPNFKLLSH) and m/z 2011.1 (HAHKLRVDPNFKLLSH). a-, b-, and y-ion series are displayed in the tandem mass spectra.
CP55,940 was added to the CB₁ receptor-bound [³H]CP55,940 when incubated in the presence of Pepcan-12 compared with vehicle. Data analysis shows higher values of both fast $k_1$ and slow $k_2$ dissociation rate constants (Table 5). This enhancement of [³H]CP55,940 dissociation from CB₁ receptors is in line with the partial displacement of the radioligands by Pepcan-12 in the equilibrium binding experiments. Therefore, both results clearly indicate that Pepcan-12 binds to the CB₁ receptor at an allosteric binding site.

With the intention to further characterize the peptide binding site, we generated four fluorescently labeled Pepcan-12 conjugates (Pepcan-F1 to -F4; Table 1). Labeling was done by coupling fluorescein to either the N-terminal or C-terminal part of Pepcan-12 with and without a chemical spacer. The equilibrium binding experiments revealed that N-terminal labeling (with and without spacer) had a negative effect on CB₁ receptor binding properties, whereas C-terminal fluorescein-labeled Pepcan-12 derivatives showed potent receptor binding (Fig. 6A). Among all of the derivatives tested, the strongest binding interaction was detected for Pepcan-F4 carrying a C-terminal label, including two aminocaproic acid spacers. We therefore used Pepcan-F4 to further characterize the binding of Pepcans to CB₁ receptors. The apparent $K_d$ value for Pepcan-F4 at human CB₁ receptors (37 ± 0.9 nM) was calculated from concentration-dependent curves (Fig. 6B). Additionally, we analyzed the Pepcan-F4 binding selectivity for other receptors activated by endocannabinoids (i.e. CB₂, TRPV1, and GPR55). As shown in Fig. 6C, Pepcan-F4 showed selective binding to CB₁ and CB₂ receptors but not to GPR55 or TRPV1 receptors. As negative controls, non-transfected CHO cells and CB₁/₂ receptor-negative HL60 cells (27) were used. Pepcan-F4 only stained CB receptor-transfected CHO cells and not non-transfected CHO cells, as illustrated in Fig. 6, C and D. Intriguingly, staining of cells with Pepcan-F4 occurred immediately, and only one washing step with PBS was necessary to obtain clear images (Fig. 6D). The results demonstrate the selectivity of Pepcan-F4 for CB receptors and are in agreement with the results previously published at the functional level for RVDPVFKLLSH (Pepcan-12) using HEK293 cells stably transfected with CB₁, CB₂, μ-opioid, δ-opioid, α₂-adrenergic, β₂-adrenergic, or AT1 receptors, respectively (9).

We next exploited Pepcan-F4 binding properties to perform competitive displacement experiments in the presence of different concentrations of all Pepcans that we measured ex vivo. Pepcan-12, -14, -15, and -17 showed similar CB₁ receptor binding affinities in the nanomolar range (Fig. 7, A–D), whereas the longest peptide tested (Pepcan-20) showed no significant Pepcan-F4 displacement up to 10 μM (Fig. 7E). We also measured the CB₁ receptor binding affinity of the two C-terminally truncated versions of Pepcan-12 (RVDPVFKLL and RVDPVFN) that were reported to occur in mouse blood (12). The displacement curves clearly showed only a weak and partial receptor binding to the Pepcan-F4 binding site (Fig. 7, F and G). In all experiments, the nonspecific binding of Pepcan-F4 was calculated by performing parallel experiments in non-transfected CHO cells. In this case, no synthetic (i.e. CP55,940 or WIN55,212-2) or endogenous (i.e. AEA or 2-AG) CB₁ receptor ligands could be used to determine the nonspecific binding.
already at 1 SR141716 significantly increased the fluorescence signal of Pepcan-12, and from five independent experiments. The corresponding equations for all models mentioned are given under “Experimental procedures.”

induced a strong concentration-dependent increase in fluorescence of CP55,940 was ineffective (Fig. 7).

Ligands CP55,940 and WIN55,212-2 differed in their effects. Allosteric modulation of Pepcan-F4 to orthosteric cannabinoid binding site.

We further measured the effects of the endocannabinoids AEA and 2-AG and the synthetic high affinity ligands CP55,940, SR141716A, and WIN55,212-2 on Pepcan-F4 binding to CB1 receptors (Fig. 7, H–K). Based on fluorescence, AEA and 2-AG increased Pepcan-F4 binding to CB1 receptors only at the highest concentration tested (10 μM) (Fig. 7H), whereas SR141716 significantly increased the fluorescence signal already at 1 μM (Fig. 7J). The two high affinity CB receptor ligands CP55,940 and WIN55,212-2 differed in their effects. CP55,940 was ineffective (Fig. 7J), whereas WIN55,212-2 induced a strong concentration-dependent increase in fluorescence intensity (Fig. 7K). Altogether, these results suggest an allosteric modulation of Pepcan-F4 to orthosteric cannabinoid ligands rather than a competitive interaction on the same binding site.

Quantification of Pepcans by Competitive ELISA—According to the results of the binding experiments, not only Pepcan-12 but also Pepcan-14, -15, and -17 strongly bound to CB receptors (Ki values of 2–29 nM). Thus, although Pepcan-12 may be the major peptide endocannabinoid in the brain, the other Pepcans could also play a physiological role. To estimate the absolute levels of Pepcans in mouse brain tissue and human plasma samples, we developed a C-ELISA for their quantification. In a first step, the relative affinity of four mAbs toward the synthetic Pepcan-12 was determined using Pepcan-12 as competitor for the binding of the mAbs toward a coated ovalbumin-peptide conjugate (Table 1). The pIC50 values of the mAbs are listed in Table 2. The mAb 1A12, which already showed the best results in Western blot and immunoaffinity MS experiments, also had the highest affinity toward Pepcan-12 in C-ELISA experiments and was therefore chosen for establishing the quantification assay.

The αβ2 tetramer hemoglobin (Hb) was reported to dissociate into αβ dimers or even further into monomers when released from red blood cells (40, 41), potentially uncovering parts of the epitope of the Pepcan-12 sequence in the protein (Fig. 8, A and C). We therefore investigated whether Hb interferes in the C-ELISA. The results demonstrated that interference is only seen at very high Hb concentrations; interference was seen above 2 μM (130 ng/ml) without extraction and above 20 μM (1.3 mg/ml) and 50 μM (3.3 mg/ml) after ACN 50% and ACN 66% extractions, respectively. BSA did not have any effect on the pIC50 of Pepcan-12 at different concentrations (Table 6), indicating that the decrease in signal intensity by Hb was probably of a specific nature and not just caused by increasing protein concentration.
For the subsequent quantification, we decided to use the ACN 66% extraction, which was found to be equally efficient for Pepcans of different length (Table 6 and Fig. 9C). Furthermore, ACN nonspecifically precipitates protein, including Hb, and can be easily evaporated, eliminating an additional neutralization step that would be mandatory after an acidic extraction.

All Pepcans tested showed similar affinity to the mAb 1A12 after ACN extraction (pIC50 values from 8 to 8.3), whereas the C-terminally truncated peptides had much lower affinity (Fig. 9, B and C, and Table 6), thus confirming the epitope mapping results. Because VD-Hpβ (VDPFNRLGNNM) of the Hb β chain was also reported to occur in mouse brain extracts (10) and closely resembles the Pepcan-12 sequence (RVDPVFNLKILLS), we performed experiments with the most analogous synthetic Hb β chain peptide HVDPENFRLLGNM and observed a very low binding affinity (pIC50 = 4.79 ± 0.04; Fig. 9B). The linearity of the quantification assay was evaluated by analysis of 11 calibrators using a four-parameter logistic nonlinear regression model to fit the response (42, 43). A representative stan-

### TABLE 5

Dissociation rate constants for [3H]CP55,940 at hCB1 receptors in the presence and absence of the allosteric modulator Pepcan-12, respectively

|            | k1 (fast) | Half-life (fast) | k2 (slow) | Half-life (slow) |
|------------|-----------|-----------------|-----------|-----------------|
| Vehicle    | 0.67 ± 0.18 | 1.03 (0.67–2.24) | 0.72 ± 0.14 | 96.2 (69.14–158.0) |
| Pepcan-12 (300 nM) | 0.84 ± 0.32 | 0.82 (0.47–3.46) | 1.46 ± 0.23* | 47.6 (36.0–70.0) |

* p < 0.05, Student’s unpaired t test.

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**FIGURE 6.** Pepcan-F4 receptor binding properties and selectivity studies.

**A.** 100 nM of each of the fluorescein-labeled Pepcan-12 derivatives, Pepcan-F1 to F4 (Table 1), was incubated with 1 × 10⁵ CHO-hCB1 cells for 60 min at 37 °C and then washed twice with ice-cold binding buffer. Fluorescence intensities were compared with non-transfected CHO cells. Data show means ± S.E. (error bars) (n = 6) from two independent experiments. **B.** total, nonspecific, and specific saturation binding curves of Pepcan-F4. The Kd value was obtained by nonlinear fit (one-site specific binding model) of the specific binding curve, which was obtained by subtracting the fluorescence intensity measured in untransfected CHO cells (nonspecific binding) from the fluorescent signal measured in CHO-hCB1 cells (total binding). Data show means ± S.E. (error bars) (n = 6) from three independent experiments. **C.** binding interactions of Pepcan-F4 with different human receptor (CB1, CB2, TRPV1 and GPR55)-transfected and untransfected cell lines. A 250 nM concentration of Pepcan-F4 was incubated with 2 × 10⁶ detached cells for 60 min at 37 °C under shaking and washed twice with PBS, 0.5% BSA, 5% FCS prior to measurement by FACS on a FACScan BD equipped with a solid state laser (Cytek). Data were analyzed on the CellQuest software. FACS experiments were carried out as described before (72). The CB receptor-negative HL60 cell line was described previously (72). Data show mean fluorescence values (geo mean) ± S.D. (n = 9) from three independent experiments. **D.** representative images of three independent experiments of wild-type CHO, CHO-hCB1, and N18TG2 cells with Pepcan-F4 (100 nM for CHO and CHO-hCB1, cells; 1 μM for N18TG2). Cells were incubated for 60 min at 37 °C and washed once with PBS. Images were obtained with a Nikon D501 camera mounted on a Nikon Eclipse TS100 microscope equipped with a Tripleband DAPI/FITC/TRITC HC filter set (AHF, Tübingen, Germany). Bar, 10 μm.
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FIGURE 7. Pepcan-induced Pepcan-F4 displacement from human CB₁ receptors. Different concentrations (1 nM to 10 μM) of Pepcan-12 (A), -14 (B), -15 (C), -17 (D), -20 (E), RVDPVNF (F), and RVDPVNF (G) were incubated with 1 × 10⁶ CHO-hCB1 cells in the presence of a 100 nM concentration of the fluorescently labeled Pepcan-F4 for 60 min at 37 °C under shaking. The IC₅₀ values ("absolute" IC₅₀ values representing the concentration of competitor displacing 50% of Pepcan-F4 (33)) of Pepcans were estimated from fitted nonlinear curves and were used to calculate the Kᵢ values by applying the Cheng-Prusoff equation as described under "Experimental Procedures." Data show means ± S.E. (error bars) (n = 6–9) from 2–3 independent experiments. 1 × 10⁶ CHO-hCB1 cells were incubated with 100 nM Pepcan-F4 in the presence of different concentrations of AEA or 2-AG (H), SR141716 (I), CP55,940 (J), and WIN55,212-2 (K) for 60 min at 37 °C under shaking. 2-AG and AEA weakly increased the Pepcan-F4 fluorescence intensity only at 10 μM, whereas SR141716 and WIN55,212-2 (1 and 10 μM) induced a moderate and high fluorescence increase, respectively (*, p < 0.05, Student's unpaired t test of competitor versus vehicle control). Data show means ± S.E. (n = 6–9) from 2–3 independent experiments.

The standard curve of Pepcan-12 after ACN 66% extraction is shown in Fig. 9D. In order to determine the endogenous amounts of peptides in mouse brain and human plasma, we performed spike and recovery experiments in both matrices. The results are shown in Table 7 together with the quantification results of Pepcans in physiological samples. The recoveries varied from 73 to 98% in the relevant concentration range for mouse brain samples and from 44 to 100% for human plasma samples.

The average endogenous amount of quantified Pepcans in three mouse brains was 92 ± 7 pmol/g brain tissue (22.7 ± 1.7 nm in C-ELISA). The average endogenous concentration of Pepcans in human plasma of four healthy volunteers (three
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FIGURE 8. Structural representations of the α,β2 tetramer Hb (Protein Data Bank entry 2DN2) that display the location of the α-chain-derived Pepcan-12 (RVPDVNFKLSSH) sequence (colored in red) until Pepcan-23 (SALSDLHAHKL) colored in green. A and B, images of Hb with Hb α-chains colored in blue and β-chains in yellow. C and D, surface plots of Hb show the internal location of Pepcan-12 and demonstrate that the N-terminal part of Pepcan-23 reaches the protein surface, possibly exposing it for a proteolytic attack. Images were generated using PyMOL software.

TABLE 6

| Displacement of the binding of the mAb 1A12 toward a synthetic ovalbumin-Pepcan-12 conjugate (Table 1) by selected competitors |
|---|
| Extraction method |
| No extraction | ACN 66% |
| Hemoglobin | 4.45 ± 0.01 | 3.61 ± 0.04 |
| PC-12 | 8.02 ± 0.02 | 8.0 ± 0.03 |
| PC-14 | 8.25 ± 0.02 | 8.23 ± 0.01 |
| PC-15 | 8.4 ± 0.03 | 8.2 ± 0.02 |
| PC-17 | 8.36 ± 0.01 | 8.17 ± 0.01 |
| PC-20 | 8.42 ± 0.02 | 8.28 ± 0.02 |
| PVNFKLSSH | 7.26 ± 0.05 | 6.85 ± 0.02 |
| RVPDVNFKL | 6.15 ± 0.05 | 5.82 ± 0.02 |
| RVPDVNF | <4 | <4 |
| HVDPENRLLGN | 4.87 ± 0.08 | 4.79 ± 0.04 |
| [Val5]Angiotensin I | <4 | <4 |
| PC-12 + 3.5 mg/ml BSA | 8.11 ± 0.02 | |
| PC-12 + 5 mg/ml BSA | 8.08 ± 0.02 | |
| PC-12 + 7.5 mg/ml BSA | 8.12 ± 0.01 | |
| PC-12 + 12.5 mg/ml BSA | 8.13 ± 0.02 | |

a Averaged value from n = 8 in four independent experiments.
b Averaged value from n = 6 in three independent experiments.

toxin (PTX)-sensitive G\(_{i/o}\) proteins (48–51), they have also been shown to positively couple to adenylyl cyclase via PTX-insensitive G\(_i\) proteins with or without forskolin stimulation (52–55). We were able to measure cAMP accumulation upon agonist stimulation without forskolin stimulation and also without significant inhibition of the G\(_{i/o}\) signaling pathway by PTX (Fig. 10B). This putative G\(_i\)-mediated pathway of the CB1 receptor is in agreement with the literature, where a PTX-insensitive adenylyl cyclase stimulatory pathway upon agonist exposure without forskolin stimulation has been also shown (54, 56). The synthetic cannabinoid WIN55,212-2 and the endocannabinoid 2-AG acted as full agonists in comparison with CP55,940 (Fig. 10A and Table 8), which is in line with a previous report (55). Importantly, SR141716 efficiently decreased basal cAMP levels down to 14% (Fig. 10A). Pepcan-12 did not elicit any changes in basal CAMP levels up to 1 \(\mu\)M (Fig. 10A). None of the tested compounds showed any change in basal cAMP levels in the wild-type CHO cells, indicating a strictly CB1-receptor mediated cAMP accumulation in the CHO-hCB1 cells. Next, we decided to investigate whether Pepcan-12 would exert negative or positive allosteric effects on the efficacy and/or potency of cAMP accumulation induced by the orthosteric ligands. Because the extent and direction of an allosteric interaction can vary between different orthosteric ligands due to the so-called “probe dependence” (38, 57), we chose two distinctly different orthosteric ligands for the interaction studies: 2-AG, representing the most abundant endogenous CB1 receptor agonist in brain tissue, and the synthetic agonist WIN55,212-2. Pepcan-12 concentration-dependently reduced the efficacy of cAMP accumulation induced by both WIN55,212-2 and 2-AG without significantly affecting the potency (Fig. 10, C and F, and Table 8). Pepcan-12 was able to reduce the maximal efficacy (\(E_{\text{max}}\)) of these cannabinoids from 20–30% at 1 nm up to 45–47% at 1 \(\mu\)M. The same experiments were also conducted in the presence of different concentrations of the inverse agonist SR141716 (Fig. 10, D and G). Unlike Pepcan-12, SR141716 did not affect the efficacy of orthosteric agonists but concentration-dependently decreased their potency represented by decreasing pEC\(_{50}\) values (Table 8). The interaction of different SR141716 concentrations on WIN55,212-2-induced cAMP accumulation was analyzed using a Schild plot (see “Experimental Procedures” and Fig. 10E), where competitive and non-competitive ligands can be distinguished. The slope near unity (1.19 ± 0.12) clearly indicates competitive interaction of SR141716 with WIN55,212-2, and also the calcu-
lated $K_d$ value of 2.5 nM derived from the $x$ intercept at $y = 0$ is in accordance with literature (3, 58). In summary, the Pepcan-12-induced decrease of efficacy and concomitant lack of a right shift of the WIN55,212-2 and 2-AG cAMP accumulation curves indicate a negative allosteric modulation of the orthosteric binding site, thus confirming the receptor binding data. Importantly, with both ligands, the same effects were obtained, and therefore no “probe dependence” was observed.

The next step in our study was the confirmation of these results in a more physiological system as both experiments, the receptor binding as well as the cAMP accumulation assay, were performed using recombinant cells with artificially high expression of CB$_1$ receptors. Therefore, we performed $[^35S]GTP\gamma S$ binding assays using mouse cerebellar membranes in which a CB$_1$ receptor-dependent Gs signaling response is known to be prevalent (51, 59). The species difference in this case was irrelevant because the mouse and human sequence of Pepcan-12 are identical. Intriguingly, we observed the same negative allosteric modulation seen with Pepcan-12 on HU-210-induced $[^35S]GTP\gamma S$ binding (Figs. 10, H and I). Pepcan-12 was ineffective by itself in changing basal $[^35S]GTP\gamma S$ binding but significantly decreased by 20% the maximal efficacy of HU-210 without affecting potency, which is in good agreement with its allosteric binding. SR141716 was used to demonstrate the distinctly different behavior between the non-competitive allosteric modulator Pepcan-12 and the competitive inverse agonist at the orthosteric binding site. As shown, SR141716 induced a decreased potency of HU-210 without altering its efficacy (Fig. 10I and Table 8). It is noteworthy that in both cAMP accumulation and $[^35S]GTP\gamma S$ binding assays, all responses induced by agonists could be fully inhibited by higher concentrations of SR141716, thus demonstrating the CB$_1$ receptor specificity for the observed responses (Fig. 10, D, G, and I). For $[^35S]GTP\gamma S$ binding using 2-AG, the effect plateau was not achieved up to 50 $\mu M$, probably due to a strong and multienzymatic 2-AG degradation present in the cerebellar preparation, despite the pretreatment with the serine hydrolase inhibitor PMSF. However, Pepcan-12 almost completely abolished $[^35S]GTP\gamma S$ binding induced by 80 nM 2-AG to basal levels and significantly reduced the effects induced by 10 and 50 $\mu M$ 2-AG (Fig. 10J and Table 8).

In order to profile the other Pepcans in cAMP accumulation and $[^35S]GTP\gamma S$ binding assays, we incubated 1 and 100 nM concentrations of the Pepcans with the same CB$_1$ receptor agonists used for Pepcan-12 interaction studies. The concentrations of the agonists were chosen at maximal response, and the impact of Pepcans on the efficacy was investigated. As shown in Fig. 11, A and B, Pepcan-12 was still the most efficient negative allosteric modulator, but Pepcan-14, -17, and -20 also showed a significant partial inhibition, especially of 2-AG signaling. It is noteworthy that, although a dose-response was observed in mouse brain and human plasma. pIC$_{50}$ values of compounds with and without ACN extraction were obtained from nonlinear regression (shared bottom values for graphs A and B) and are listed in Table 6. The corresponding graphs for peptide competitors without ACN extraction are not shown because the pIC$_{50}$ values did not differ substantially from the ones obtained with ACN extraction. Data show means ± S.D. (error bars) ($n = 2–3$) in A–C and a cumulated standard curve of two independent experiments in duplicates in D.
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Results of spike and recovery experiments and quantification results for mouse brain and human plasma samples

| Spike (pmol/g) | Mean | S.D. | CV (%) | Recovery (%) | Sample | Results ± S.D. |
|---------------|------|------|--------|-------------|--------|---------------|
| Brain 0       | 16.9 | 1.1  | 6.3    | NMRI mouse brain 1⁺ | 26.1 ± 1.29⁺ |
| 12.5          | 29.1 | 10.1 | 34.7   | NMRI mouse brain 2  | 18.3 ± 2.6⁻  |
| 25            | 35.0 | 5.6  | 16.1   | NMRI mouse brain 3  | 23.6 ± 1.3²  |
| 100           | 56.2 | 7.6  | 13.4   | Human plasma, female 1 | 1.31 ± 0.6⁻  |
| Plasma 0      | <LLOQ |      |        | Human plasma, female 2 | 6.25 ± 0.6⁻  |
| 0.78          | 1.11 | 0.19 | 17.7   | Human plasma, female 3 | 5.05 ± 2.1¹  |
| 1.56          | 1.56 | 0.39 | 25.1   | Human plasma, male 1  | <LLOQ²  |
| 12.5          | 5.5  | 0.6  | 10.6   |                      |        |
| 25            | 14.4 | 2.4  | 16.3   |                      |        |
| 100           | 50.9 | 4.7  | 9.3    |                      |        |

⁻ Coefficient of variation
⁺ Percentage recovery was calculated by subtracting the endogenous amount (spike 0 pmol) from the measured value, divided by the nominal spike value multiplied by 100. For plasma samples, no endogenous amount could be subtracted because it was below the lower limit of quantification (~1 nM).
⁻ NMRI mouse brains 1, 2, and 3 = 0.49, 0.49, and 0.50 g wet weight, respectively.
⁻ These values correspond to 107 ± 5 pmol of Pepcans/g of mouse brain, 75 ± 11 pmol of Pepcans/g of mouse brain, and 94 ± 5 pmol of Pepcans/g of mouse brain (calculated for 1:1 diluted samples and 1 ml of total brain extract).
⁻ LLOQ, lower limit of quantification.
⁻ These values correspond to 2.6 ± 1.3, 12.5 ± 1.3, and 10.1 ± 4.2 nM (calculated from the values obtained for 1:1 diluted samples in the competitive ELISA). For the calculation of the average endogenous amounts of Pepcans in plasma, we adapted a concentration of 0 nM for the male sample.

cAMP accumulation experiments using CHO-hCB₁ cells, no dose response could be achieved in [³⁵S]GTPγS binding assays in mouse cerebellar homogenates. We assume that differences in the cell preparation (living cells versus homogenates) and the incubation times (3 min at 30–37 °C in the cAMP assay versus 60 min at 37 °C in the [³⁵S]GTPγS binding assay) play a role in Pepcan aggregation. In order to experimentally address the aggregation phenomenon, we used an ultrafiltration approach combined with a subsequent quantification of Pepcans by C-ELISA to estimate the degree of peptide aggregation at low and physiological concentrations. As shown in Fig. 11C, by using ultrafiltration tubes with a molecular weight cut-off of 2000, Pepcan-12 and -20 showed a significant loss of recovery (~40%) at 1 μM concentration after 1 h of incubation at 37 °C relative to 10 nM concentrations. This indicates that aggregation occurs in a peptide concentration-dependent manner. On the contrary, when we used ultrafiltration tubes with a molecular weight cut-off of 3000, the recovery was almost fully restored. This difference suggests that at high concentrations, Pepcans form lower molecular weight aggregates, such as dimers or trimers.

Finally, we addressed the question whether Pepcans can also activate CB₁ receptor as reported before for Pepcan-12 (RVD-Hpα) (10). Pepcan-12 was reported to induce internalization of the CB₁ receptor in CHO cells expressing myc-tagged CB₁ receptors (10). Thus, we assessed the effects of Pepcans on CB₁ receptor internalization/externalization in CHO-hCB₁ cells using the FACS-based method reported previously (31). As shown in Fig. 11D, at 1 μM, Pepcan-12 induced the strongest receptor internalization (21%), followed by Pepcan-14 (20%), Pepcan-15 (12%), Pepcan-17 (10%), and Pepcan-20 (7%), thus confirming the results in the literature (10). As positive controls, we used the agonists WIN55,212-2 (36% internalized receptors) and 2-AG (22% internalized receptors) and the inverse agonist (antagonist) SR141716 (15% externalized receptors). Intriguingly, when WIN55,212-2 and 2-AG were co-incubated with Pepcan-12, the latter exerted an antagonistic effect by partially reversing the CB₁ receptor internalization induced by the CB₁ receptor agonists. No significant effects were observed when Pepcan-12 was co-incubated with SR141617. Altogether, these data support the negative allosteric mechanism of Pepcan-12 at CB₁ receptors. To ensure that Pepcan-12 does not indirectly affect CB₁ receptors through modulation of AEA and 2-AG levels, we measured the effect of Pepcan-12 on AEA and 2-AG hydrolysis in pig brain homogenates, as previously described (28). As shown in Fig. 12, Pepcan-12 did not affect AEA and 2-AG degradation up to 10 μM.

DISCUSSION

Identification of Pepcans in Brain and Plasma Samples Using High Affinity mAbs—The previous report on potentially endogenous peptide agonists for the CB₁ cannabinoid receptor (10) motivated us to raise mAbs against the peptide RVDVPNFKLLS. Because we failed to generate suitable mAbs against the highly conserved peptide sequence in a conventional mouse strain (data not shown), we decided to use the autoimmune-prone (NZB×NZW)F₁ hybrid mouse strain. We were able to generate mAbs recognizing C-terminal epitopes and neo-epitopes of the peptide RVDVPNFKLLS, the sequence of which is buried in its source protein Hb (Fig. 8, A and C). The mAb 1A12 recognized the synthetic peptide as well as a broader band in the same molecular weight range in different mouse brain extracts in Western blot experiments. The extended molecular weight range of these bands was due to the fact that the mAb recognized, in addition to RVDVPNFKLLS, several N-terminally extended peptides from 1.4 to 2.6 kDa, as demonstrated by immunoaffinity MS experiments. Because hemo-pressin is not found endogenously using the mAbs and the soft extraction protocols (i.e. it is an extraction artifact), the newly identified peptides together with RVDVPNFKLLS (Pepcan-12) were designated Pepcans (peptide endocannabinoids). The term “peptide endocannabinoids” was already mentioned previously by Gomes et al. (10). In immunoaffinity MS experiments, we could not detect a peak corresponding to the 6 kDa band observed in Western blots of mouse brain extracts, suggesting either that these antigens are only generated in the Western blot experiments or that they have reduced affinity to the mAbs and that binding interactions are lost during the
Pepcans Are Negative Allosteric Modulators of CB₁ Receptors

FIGURE 10. Negative allosteric modulation of cannabinoid signaling at the human CB₁ receptor by Pepcan-12. All data were analyzed by using a four-parameter dose-response model with variable slope described under "Experimental Procedures." A, measurement of cAMP accumulation induced by 2-AG, WIN55,212-2, SR141716, and Pepcan-12 (no significant change of basal cAMP accumulation) at hCB₁ receptors. Results show means ± S.E. (error bars) (n = 6–15) from 2–5 independent experiments. B, preincubation for 16 h with 5 ng/ml PTX did not significantly alter the cAMP accumulations induced by WIN55,212-2 and 2-AG. Data show means ± S.D. (n = 4) of two independent experiments. C and D, WIN55,212-2-stimulated cAMP accumulation in the presence of different concentrations of Pepcan-12 (C) and SR141716 (D) (0 nM (vehicle control): pEC₅₀ = 6.88 ± 0.15; Eₘₐₓ = 506.7 ± 36.6; 1 nM: pEC₅₀ = 6.72 ± 0.10; Eₘₐₓ = 477.9 ± 28.1; 10 nM: pEC₅₀ = 6.36 ± 0.09; Eₘₐₓ = 527.7 ± 35.7; 25 nM: pEC₅₀ = 5.68 ± 0.05***; 50 nM: pEC₅₀ = 5.08 ± 0.09***; Eₘₐₓ values for the nonlinear fits of the 25 and 50 nM curves were constrained to 501.1; representing the average Eₘₐₓ values of the other curves. *, p < 0.05; ***, p < 0.001, one-way analysis of variance followed by Dunnett’s post hoc test). Data show means ± S.E. (n = 9) from three independent experiments. Results from D were used to generate a Schild plot (E) (see "Experimental Procedures") for the analysis of competitive binding of SR141716. Shown is 2-AG-stimulated cAMP accumulation in the presence of different concentrations of Pepcan-12 (F) and SR141716 (G). Pepcan-12 did not elicit a significant change of basal cAMP accumulation. Results show means ± S.E. (n = 6–30) from 2–10 independent experiments. pEC₅₀ and Eₘₐₓ values of the curves displayed in C and F–I are shown in Table 8. J, partial reduction of the [³⁵S]GTPγS binding by Pepcan-12 measured at three different concentrations of 2-AG. Data show means ± S.E. (n = 9–30) from 3–10 independent experiments. **, p < 0.01; ***, p < 0.001, one-way analysis of variance followed by Dunnett’s post hoc test.
**Pepcans Are Negative Allosteric Modulators of CB1 Receptors**

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**Table 8**

| Assay (agonist) + parameter | Competitor | 0 nM | 1 nM | 100 nM | 1 μM |
|-----------------------------|------------|------|------|--------|------|
| **cAMP (WIN55,212-2)**     |            |      |      |        |      |
| pEC$_{50}$                  | 7.07 ± 0.05$^a$ | 6.99 ± 0.06$^a$ | 6.98 ± 0.12$^a$ | 7.02 ± 0.08$^a$ |
| F$_{max}$                   | 468.2 ± 16.3$^a$ | 370.1 ± 14.6$^***$ | 301.3 ± 22.1$^***$ | 255.1 ± 11$^***$ |
| **cAMP (2-AG)**            |            |      |      |        |      |
| pEC$_{50}$                  | 7.08 ± 0.15$^a$ | 7.33 ± 0.15$^a$ | 7.30 ± 0.38$^a$ | 7.78 ± 0.06$^a$ |
| F$_{max}$                   | 620.8 ± 39.8$^a$ | 420.9 ± 23.7$^***$ | 435.3 ± 59.7$^***$ | 328 ± 5.8$^***$ |
| **[^35]S[GTPyS (HU-210)]** |            |      |      |        |      |
| pEC$_{50}$                  | 7.79 ± 0.25$^a$ | 7.79 ± 0.17$^a$ | 7.91 ± 0.26$^a$ | 8.5$^a$ |
| F$_{max}$                   | 265.6 ± 16.6$^a$ | 214.0 ± 7.2$^***$ | 221.5 ± 8.2$^a$ | - |
| **[^3H]CP55,940**          |            |      |      |        |      |
| pEC$_{50}$                  | 8.61 ± 0.9$^a$ | 7.5 ± 0.23$^a$ | 6.21 ± 0.15$^a$ | - |
| F$_{max}$                   | 187.6 ± 8.5$^a$ | 186.2 ± 9.5$^b$ | 186.9$^a$ | - |

$^a$ Pepcan-12.

$^b$ SR141716.

$^c$ Constrained F$_{max}$ value for nonlinear fitting procedure.

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immunoaffinity MS assay procedure. Furthermore, we were not able to detect the N-terminally truncated peptide VDPVNFKLLSH reported to occur in the mouse brain (10, 12). The identification of the Pepcan family in hamster brain extracts confirmed our results obtained with mouse brain tissue. That we did not detect any Peps in rat brain tissue using this immunoaffinity MS method was most likely due to the decreased binding affinity of our mAbs toward the rat Peps, as indicated by a 14-fold decreased binding affinity of the mAb 1A12 for the rat sequence after ACN extraction (Table 7).

Because of the immunoaffinity enrichment step, we were able to identify, for the first time, Pepcan-14 to -23 in brain extracts and plasma samples. It seems as if Pepcan-12, -14, -15, -17, and -20 are the most relevant endogenous Peps occurring in mouse brain and mouse/human plasma samples. In comparison with conventional LC-MS/MS methods, the immunoaffinity step provides a significant enrichment of Peps and increased signal/noise ratios, resulting in increased sensitivity and in the reduction of ion suppression mediated by highly abundant proteins and peptides (60–64).

**Cannabinoid Receptor Binding Interactions of Peps**—The Peps identified ex vivo were investigated for their CB$_1$ binding properties. We used both a classical radioligand assay and a newly developed assay with the Pepcan-12 derivative Pepcan-F4, containing a C-terminally attached fluorescein via spacer molecules. At 1 μM, Pepcan-12, -14, and -20 were found to moderately displace[^3H]CP55,940 and[^3H]WIN55,212-2 from CB$_1$ receptors. Pepcan-12 showed the strongest displacement of both radioligands characterized by a saturable but only partial receptor binding, thus suggesting an interaction with an allosteric binding site. Binding data were fitted with the allosteric TCM model, revealing similar pK$_B$ values of Pepcan-12 for both radioligands at the putative allosteric binding site. The cooperativity factor $\alpha$, which describes the magnitude of the allosteric change in ligand affinity that occurs between two conformationally linked sites when both ligands are bound (39), was in both cases less than 1, indicating a negative allosteric modulation. Although these results could theoretically also be in agreement with our aggregation theory, at higher Pepcan concentrations, the dissociation kinetic data clearly favor an allosteric binding site for Pepcan-12. Indeed, only ligands that bind to a topographically distinct site at the receptor can enhance (or reduce) the dissociation of an orthosteric ligand in the isotopic dilution approach used (39). The biphasic dissociation kinetics of[^3H]CP55,940 also indicates the presence of at least two receptor conformations (65) and was already discussed in detail by Price et al. (16). Pepcan-12 increased the dissociation rate constants for both the fast and slow components of[^3H]CP55,940 binding. This is in line with a conformational change induced by Pepcan-12 binding to an allosteric site that decreases the agonist (CP55,940) affinity for the orthosteric binding site at the CB$_1$ receptor.

Experiments with the fluorescently labeled Pepcan-F1 to -F4, which were derived from Pepcan-12, show that N-terminal derivatization of Pepcan-12 abolished CB receptor binding. In contrast, C-terminal derivatization had comparatively little effect, as long as the fluorophore was not attached to the peptide directly but rather via a spacer molecule. These results suggest that the N terminus of the peptide is of particular importance for CB receptor binding interactions. Thus, the differential interactions of Pepcan-12 to -20 may be determined by particular amino acid residues. Nonetheless, the less strong and only partial displacement by the two C-terminally truncated versions of Pepcan-12, RVDPVN and RVDPVNFKL, indicates that the whole peptide sequence, including the C terminus, is probably involved to different extents in CB$_1$ receptor binding interactions. Whereas Pepcan-12, -14, -15, and -17 interacted strongly with the Pepcan-F4 binding site in CB$_1$ receptors, the longest Pepcan tested (Pepcan-20) did not displace Pepcan-F4. Although the nonspecific Pepcan-F4 binding was subtracted in all of the Pepcan inhibition curves, a residual 15–25% of fluorescence remained. Possible explanations for this may either be in vitro peptide aggregation at higher concentrations or that the fluorescent label, including spacer molecules, modifies the Pepcan-12 binding behavior by increasing its binding affinity for CB$_1$ receptors. Alternatively, potential peptide aggregation at the CB$_1$ receptor may play a role. Because of conformational linkage between the two binding

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**Table 8**

| Assay (agonist) + parameter | Competitor | 0 nM | 1 nM | 100 nM | 1 μM |
|-----------------------------|------------|------|------|--------|------|
| **cAMP (WIN55,212-2)**     |            |      |      |        |      |
| pEC$_{50}$                  | 7.07 ± 0.05$^a$ | 6.99 ± 0.06$^a$ | 6.98 ± 0.12$^a$ | 7.02 ± 0.08$^a$ |
| F$_{max}$                   | 468.2 ± 16.3$^a$ | 370.1 ± 14.6$^***$ | 301.3 ± 22.1$^***$ | 255.1 ± 11$^***$ |
| **cAMP (2-AG)**            |            |      |      |        |      |
| pEC$_{50}$                  | 7.08 ± 0.15$^a$ | 7.33 ± 0.15$^a$ | 7.30 ± 0.38$^a$ | 7.78 ± 0.06$^a$ |
| F$_{max}$                   | 620.8 ± 39.8$^a$ | 420.9 ± 23.7$^***$ | 435.3 ± 59.7$^***$ | 328 ± 5.8$^***$ |
| **[^35]S[GTPyS (HU-210)]** |            |      |      |        |      |
| pEC$_{50}$                  | 7.79 ± 0.25$^a$ | 7.79 ± 0.17$^a$ | 7.91 ± 0.26$^a$ | 8.5$^a$ |
| F$_{max}$                   | 265.6 ± 16.6$^a$ | 214.0 ± 7.2$^***$ | 221.5 ± 8.2$^a$ | - |
| **[^3H]CP55,940**          |            |      |      |        |      |
| pEC$_{50}$                  | 8.61 ± 0.9$^a$ | 7.5 ± 0.23$^a$ | 6.21 ± 0.15$^a$ | - |
| F$_{max}$                   | 187.6 ± 8.5$^a$ | 186.2 ± 9.5$^b$ | 186.9$^a$ | - |

$^a$ Pepcan-12.

$^b$ SR141716.

$^c$ Constrained F$_{max}$ value for nonlinear fitting procedure.
sites, the interaction between the orthosteric endocannabinoid binding site and the allosteric Pepcan binding site should be reciprocal (39). Therefore, we would expect to observe a similar partial displacement of Pepcan-F4, at least for WIN55,212-2 and CP55,940, both used in radioligand displacement experiments. However, we did not observe an increased binding of Pepcan-F4 to its binding site when co-incubated with cannabinoids. On the contrary, SR141716 induced an increase of Pepcan-F4 binding. This could be explained by probe dependence. The synthetic Organon compounds have been reported to show positive allosteric modulation of CP55,940 and negative allosteric modulation of SR141716 (16). The strong increase in Pepcan-F4 fluorescence intensity mediated by WIN55,212-2 might be due to a prominent allosteric effect, but it could also be caused by proximity interactions between the fluorophore in Pepcan-F4 and the conjugated electron system present in WIN55,212-2. Considering this together with the results obtained for CP55,940, which showed no effect on Pepcan-F4 binding, and for the endocannabinoids AEA and 2-AG, which showed only a weak increase of Pepcan-F4 binding at 10 μM, we conclude that the fluorescence labeling of Pepcan-12 might modify its binding affinity to CB₁ receptor, or there is residual
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The hydrolytic products \([^{3}H]\)ethanolamine from AEA breakdown and \([^{3}H]\)glycerol from 2-AG breakdown were quantified. The positive controls URB597 (fatty acid amide hydrolase inhibitor) and JZL184 (monoacylglycerol lipase inhibitor) showed significant inhibition of AEA and 2-AG breakdown, respectively. Data show means ± S.D. (error bars) \((n = 3)\) (*, \(p < 0.05\); **, \(p < 0.01\); Student’s unpaired \(t\) test of compounds versus vehicle control).

![Graph: Effect of Peptcan-12 on AEA and 2-AG hydrolysis in pig brain homogenate](image)

Quantification of Peptcans by Competitive ELISA—Because our Peptcan-specific mAbs bind to multiple members of the Peptcan family, only concentrations for these Peptcans together could be determined. The measured average Peptcan plasma levels of 6 nM were in the range of the obtained \(K_i\) values. For quantification of Peptcans from brain tissues (92 pmol/g of brain tissue) we were concerned about potential postmortem cleavage of Hb or Peptcan fragments. Already after a few min post mortem, several proteins and peptides were reported to be enzymatically degraded, resulting in a different composition of the peptidome and proteome compared with the real situation in vivo (67). Gomes et al. (10) and Gelman et al. (12) used microwave-irradiated mice to prevent postmortem protein and neuropeptide degradation and suggested a possible role of global ischemia in the up-regulation of VDPVNFKLLSH and Peptcan-12 (10, 12, 68, 69). Additionally, they reported VDPVNFKLLSH to be much more abundant in brain extracts that were not subjected to microwave irradiation (10, 68). Sköld et al. (67) reported several drawbacks of microwave irradiation and introduced a novel method using rapid and conductive heat transfer on brain tissue prior to the extraction procedure. For our quantification experiments, brain tissues from conventionally sacrificed mice were frozen in liquid nitrogen within 3 min post mortem.

Although Peptcan-12, -14, -15, -17, and -20 were also detected in plasma, we cannot exclude the possibility that Peptcans that showed weak signal/noise ratios in immunoaffinity MS spectra, such as Peptcan-16, -18, and -19, may be post-mortem cleavage artifacts of longer Peptcan precursor molecules. However, indirect confirmation for the existence of N-terminal extended Peptcans is also provided by the fact that Hb \(\alpha\) chain fragments, such as AGHLDLGALSA, AGHLDLGALSA, ANAAGHLDDL, ANAAGHLDDL, ADAALANAAAGHLDDL, and ADAALANAAGHLDL, have been reported to occur in mouse brain and blood (10, 12), indicating the presence of N-terminal cleavage sites for Peptcan-20 and -21 (Table 3). Additionally, LSDLHAHKL was detected, supporting our hypothesis that longer Peptcans (in this case Peptcan-21) could be processed to Peptcan-12. Because our mAbs are not specific for any single Peptcan, the developed competitive ELISA is not suitable for individual quantifications. Therefore, we have considered developing a quantitative immunoaffinity MS/MS approach for the quanti-
Pepcans are negative allosteric modulators of CB₁ receptors

It is tempting to speculate that the different N-terminally truncated Pepcan species found endogenously in rodent brain tissues as well as mouse and human plasma represent precursor molecules of Pepcan-12, which is apparently the most abundant and functionally most efficacious member. However, different Pepcans may exert additional yet unknown effects at CB₁ receptors and possibly also CB₂ receptors (studies on CB₂ receptors are ongoing in our laboratory). Whereas Pepcan-12 appears to be abundant in brain tissue, based on our immunoaffinity MS experiments, and is detectable by conventional less sensitive LC-MS/MS approaches (10, 12), comparable amounts of all major Pepcans were found to occur in plasma. Using our mAbs, we will be able to address the issue of whether Pepcans are synthesized within the brain or are transported there from the periphery. Gelman et al. (12) have suggested that the Hb-derived peptides described by them are specifically produced in the brain. Because we did not use perfused mouse brains, we cannot draw conclusions from the presented data but favor the hypothesis that Pepcans in the brain are produced there. The fact that we identified a broader range of Pepcan species in brain extracts than in plasma could be due to the overall higher concentration of Pepcans in the brain extracts or differential processing.

**Pepcans Act as Negative Allosteric Modulators at CB₁ Receptors**—It is known that the way in which an allosteric ligand modulates the affinity of an orthosteric ligand does not necessarily coincide with its effects on orthosteric ligand efficacy. In agreement with the CB₁ receptor binding data, Pepcan-12 acted as a negative allosteric modulator of cannabinoid and endocannabinoid efficacy in assays investigating CB₁ receptor-induced G protein signaling activation. We obtained conclusive data in both cAMP accumulation and [35S]GTPγS binding assays using CHO-hCB₁ cells and mouse cerebellar extracts. Although the inverse agonist SR141716 competitively inhibited the potency of different cannabinoid agonists, Pepcan-12 reduced the efficacy without altering potency, thus clearly indicating a negative allosteric interaction. Why only a maximal effect of 20–50% could be achieved remains to be elucidated. A possible explanation may be a “ceiling effect,” which has been reported for allosteric modulators, where the saturation of the allosteric site is determined by the cooperativity between the orthosteric and allosteric ligands. Therefore, beyond a specific threshold concentration, allosteric modulators cannot produce any further modulatory effects of the orthosteric ligands. Furthermore, the possibility cannot be excluded that Pepcan-12 only targets a subpopulation of CB₁ receptors (e.g. homodimers or oligomers).

Interestingly, although Pepcan-20 was not able to displace the fluorescent derivative Pepcan-F₄ from CB₁ receptors, it induced a significant displacement of [³H]CP55,940 and [³H]WIN55,212-2, thus showing that Pepcan-F₄ binds differently to CB₁ receptors than native Pepcans. Additionally, Pepcan-20 decreased the agonist-induced cAMP accumulation and [³⁵S]GTPγS binding like Pepcan-12, although less strongly. Why Pepcan-20 was not able to displace Pepcan-F₄ from CB₁ receptors is not yet clear. A possible explanation could be that Pepcan-20 binds to a distinct or partially overlapping binding site from Pepcan-F₄. This may suggest that the peptide elongation by three additional amino acids from Pepcan-17 (which displace Pepcan-F₄ very efficiently) has a great impact on the binding affinity and/or site. This needs to be investigated using a radiolabeled Pepcan-12 molecule. Nonetheless, Pepcan-20 may play a physiological role because its functional effect is qualitatively identical to that of Pepcan-12.

In both assays investigating G protein signaling, Pepcans did not elicit any response by themselves but only modulated the effects induced by orthosteric ligands. Because Pepcan-12 was reported to act as an agonist (10) and hemopressin as an inverse agonist (8, 9) at CB₁ receptors, we also investigated a potentially G protein-independent pathway. We decided to investigate the agonist-induced receptor internalization of CB₁ receptors in a transfected cell line because this mechanism may be independent of G proteins (31, 70). Although Pepcan-12, -14, -15, -17, and -20 apparently behaved as partial agonists in the CB₁ receptor internalization assay using CHO-hCB₁ cells, they reversed the 2-AG- and WIN55,212-2-induced internalization by 25–35%. Whether this Pepcan-induced receptor internalization is merely an artificial in vitro effect or has some relevance in vivo remains to be elucidated. If Pepcans behave as partial agonists in other CB₁ receptor signaling pathways (e.g. via arrestins) needs to be investigated in further studies. Pepcan-12 has been shown to mediate a sustained increase in Ca²⁺ levels and neurite outgrowth in Neuro2A cells and to increase ERK phosphorylation in HEK293 cells expressing myc-tagged CB₁ receptors (10). In our laboratory, we were unable to detect significant Ca²⁺ transients with Pepcans in CHO-hCB₁ and Neuro2A cells using fluorescent imaging plate reader calcium assays (data not shown). On the other hand, G₁₅₀ and G₁₃ pathways are well established and predominant in CB₁ receptors (73). In our hands, Pepcans clearly behaved as negative allosteric modulators on the orthosteric binding site for these pathways in two different experimental setups. From our data, we can conclude that the actions of Pepcans are exerted via a novel allosteric binding site at CB₁ receptors.

**Conclusions**—The discovery of a family of endogenous peptides generated from α-hemoglobin that act as negative allosteric modulators at CB₁ receptors provides new evidence for additional players in the endocannabinoid system. Importantly, the negative allosteric effects mediated by Pepcans occur at nanomolar concentrations, which are in agreement with the concentrations detected in mouse brain and human plasma samples. As previously emphasized, allosteric modulation heralds a new approach to the manipulation of the endocannabinoid system for therapeutic benefit; it promises to augment the existing portfolio of known CB₁ receptor ligands, such as direct agonists and competitive antagonists. Because Pepcans are the first endogenous allosteric modulators described, it will be interesting to see whether the small synthetic allosteric modulators reported previously (15–18) target the same binding sites as Pepcans at CB₁ receptors. Finally, the anti-Pepcan mAbs and ELISA described in this study represent first important tools for studying the role of Pepcans in cellular and physi-
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ioleological systems as well as for their quantification in disease models.

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