Hemopressin is an inverse agonist of CB_1 cannabinoid receptors

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To date, the endogenous ligands described for cannabinoid receptors have been derived from membrane lipids. To identify a peptide ligand for CB_1 cannabinoid receptors, we used the recently described conformation-state sensitive antibodies and screened a panel of endogenous peptides from rodent brain or adipose tissue. This led to the identification of hemopressin (PVNFKFLSH) as a peptide ligand that selectively binds CB_1 cannabinoid receptors. We find that hemopressin is a CB_1 receptor-selective antagonist, because it is able to efficiently block signaling by CB_1 receptors but not by other members of family A G protein-coupled receptors (including the closely related CB_2 receptors). Hemopressin also behaves as an inverse agonist of CB_1 receptors, because it is able to block the constitutive activity of these receptors to the same extent as its well characterized antagonist, rimonabant. Finally, we examine the activity of hemopressin *in vivo* using different models of pain and find that it exhibits antinociceptive effects when administered by either intrathecal, intraplantar, or oral routes, underscoring hemopressin's therapeutic potential. These results represent a demonstration of a peptide ligand for CB_1 cannabinoid receptors that also exhibits analgesic properties. These findings are likely to have a profound impact on the development of novel therapeutics targeting CB_1 receptors.

G-protein-coupled receptors | rimonabant | inflammatory pain | drugs of abuse

The major psychoactive component of cannabis (also known as marijuana), delta-9-tetrahydrocannabinol, binds to at least two types of cannabinoid receptors, CB_1 and CB_2. Both of these receptors have been cloned and belong to the superfamily of G_i/G_o protein receptors (1). The CB_1 receptor is expressed primarily in the central nervous system (2), whereas the CB_2 receptor is expressed in immune cells (3), although recent studies have described the presence of detectable levels of CB_2 receptors in brain-stem neurons (4) and the spinal cord (5). These receptors are activated by endogenous ligands derived from membrane lipids termed "endocannabinoids" (6–8). Recent studies have proposed important roles for the endocannabinoid system (consisting of the receptors and endogenous ligands) in many physiological and pathophysiological processes. Thus, the development of agonists and antagonists, including those with marked selectivity for CB_1 or CB_2 receptors, is the focus of intense research (6–8).

There are many clinically relevant pathophysiological conditions where the endocannabinoid system has been demonstrated to play a role. These include Parkinson's disease, Alzheimer's disease, major depression, inflammation, neuropathic pain, and obesity (6, 8, 9). Because the endocannabinoid system is involved in a wide array of diseases that are of epidemiological relevance to the developed world, compounds that modulate cannabinoid receptors are good targets for the development of drugs that could be useful in the treatment of such diseases. Special interest has been recently devoted to cannabinoid-induced antinociception that shows clinical promise (9–11). In this study, we describe the identification and characterization of a natural peptidic cannabinoid receptor ligand that is able to block CB_1 receptor activity *in vitro* and *in vivo*. The identification of this cannabinoid peptide ligand provides an opportunity for the development of a class of therapeutic agents for the treatment of a number of disorders involving cannabinoid receptors.

Results and Discussion

To identify endogenous ligands modulating CB_1 receptor activity, we screened a panel of 17 peptides isolated from rodent tissues [supporting information (SI) Table 1]; these peptides were isolated by using a "substrate-capture" assay that used a catalytically inactive mutant of endopeptidease 24.15 to capture putative bioactive peptides from tissue extracts (12–14). Because this panel contains hemopressin (PVNFKFLSH) that was found to have nonopioid receptor mediated antinociceptive effects (15), we decided to screen CB_1 cannabinoid receptors. For screening the peptides, we used a recently described ELISA with anti-CB_1 receptor antibodies; these antibodies are sensitive to activity-mediated conformational changes in the receptors and thus are able to differentially recognize different activity states of the receptors (16). We found that among the peptides tested (SI Table 1), only hemopressin was able to substantially modulate antibody binding, suggesting it could be a potential ligand for CB_1 receptors (Fig. 1*A*). Next, we examined the selectivity of hemopressin for CB_1 receptors. For this, we used the conformation-sensitive antibodies to CB_2 cannabinoid receptors, μ and δ opioid, α_{2A} and β_2 adrenergic, angiotensin II types 1 and 2, and bradykinin B2 receptors. As expected, treatment with the agonist led to enhanced recognition of the receptors and thus increased binding by receptor specific antibodies (SI Table 2), suggesting that the antibodies were able to selectively detect changes induced by receptor-specific agonists. Interestingly, cotreatment with hemopressin led to the blockade of agonist-mediated increase in recognition of CB_1 receptors by the antibody. This was not observed with any other receptors tested, including the closely related CB_2 receptors (SI Table 2), suggesting that hemopressin is a highly selective ligand for CB_1 receptors. Next, we characterized the properties of hemopressin by comparing it to that of the well characterized CB_1 receptor antagonist, SR141716 (rimonabant). We found that the agonist-

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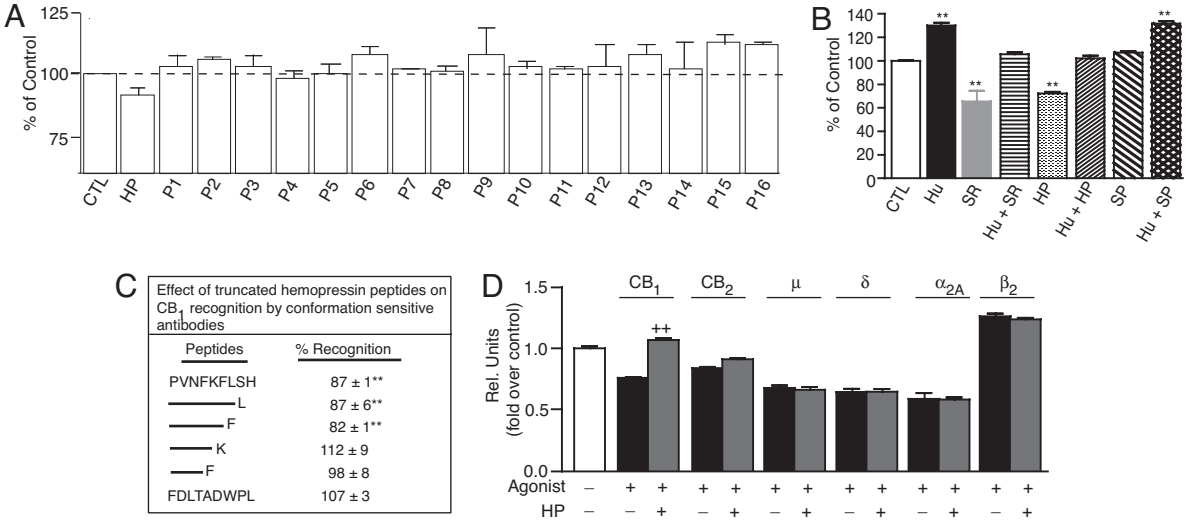

Fig. 1. Identification of hemopressin as a CB_1 receptor-modulating peptide. SKNSH cells (≈1 × 10^5 cells per well) were treated with (*A*) 1 μM various peptides (*B*) 1 μM Hu-210 (Hu) without or with hemopressin (HP), SR141716 (SR), a scrambled peptide (SP), or (*C*) C-terminally truncated hemopressin peptides and probed with anti-CB_1 receptor antibody by ELISA, as described in *Methods*. (*D*) HEK-293 cells (30,000 cells per well) coexpressing pCRE-SeAP and individual receptors were treated with 10 μM forskolin (except for β_2 adrenergic receptors) and its corresponding agonists (100 nM) in the absence or presence of 1 μM hemopressin and SeAP levels determined as described in *Methods*. Results are the mean ± SEM ($n = 6$). Statistically significant differences vs. control (*) and vs. agonist alone (+) are indicated; **, $P < 0.01$; ++, $P < 0.01$; one-way ANOVA and Dunnett's test.

induced increase in antibody recognition is significantly attenuated by hemopressin, and the extent of this decrease is the same as that of SR141716 (Fig. 1*B*); thus hemopressin behaves in a manner similar to SR141716, suggesting it could function as an antagonist of the CB_1 receptor. We also examined the structure–activity relationship of hemopressin by using peptides with C-terminal truncations. We found that the deletion of five, but not four, amino acids from the C terminus affects CB_1 receptor recognition, suggesting the requirement of N-terminal 5 aa for this activity (Fig. 1*C*). Taken together, these results suggest that hemopressin represents a natural peptide ligand that could function as an antagonist of the CB_1 receptor. In addition, these results demonstrate that a unique strategy for the identification of natural peptide ligands that uses a combination of "substrate-capture" assay (to isolate endogenous peptides) with a conformation-specific antibody-based assay can be used for the identification of peptide ligands for a variety of G protein-coupled receptors (GPCRs).

To directly examine the selectivity of hemopressin for CB_1 receptors and to characterize its effect on receptor activity, we used the secreted alkaline phosphatase (SeAP) assay, which indirectly measures the level of intracellular cAMP (that is decreased upon CB_1 receptor activation). In this assay, the levels of cAMP directly correlate with the cAMP-response element-mediated expression of SeAP activity. We find that hemopressin selectively blocks the CB_1 agonist-mediated decrease in SeAP levels but has no effect on agonist-induced changes in SeAP levels in cells expressing μ and δ opioid, α_{2A} and β_2 adrenergic, angiotensin II type 1, or CB_2 cannabinoid receptors (Fig. 1*D* and SI Table 3). These results indicate that CB_1 receptor-mediated signaling is blocked by hemopressin, and that it behaves as a receptor antagonist.

Next, the ligand-binding properties of hemopressin were examined and compared with the properties of SR141716. For these studies, striatal membranes were chosen to examine whether hemopressin is able to bind to endogenous receptors, because striatum has been reported to contain a relatively pure population of CB_1 receptors [because, to date, CB_2 receptors have been convincingly shown to be present only in brainstem neurons and spinal cord (4, 5)]. Hemopressin is able to displace [^{3}H]SR141716 binding with an affinity in the subnanomolar range, whereas a scrambled peptide is not (Fig. 2*A*). Interestingly, the apparent affinity of hemopressin is very similar to that of SR141716, suggesting that hemopressin exhibits relatively high affinity for CB_1 receptors. Next, we characterized the ability of hemopressin to block CB_1 receptor-mediated signaling in striatal membranes using a variety of assays. In the GTPγS-binding assay, hemopressin is able to block the agonist- (Hu-210) mediated increase with a potency similar to that of SR141716 (Fig. 2*B*). In the adenylyl cyclase assay, hemopressin is able to block agonist-mediated decreases in adenylyl cyclase activity with a potency similar to that of SR141716 (Fig. 2*C*). To ensure that the effects seen with hemopressin in striatal membranes were mediated through CB_1 receptors (and not other related receptors), we examined the effect of hemopressin on heterologously expressed recombinant CB_1 receptors. In the MAPK assay, hemopressin is able to block Hu-210-mediated increases in phospho ERK1/2 levels to the same extent as SR141716 (Fig. 2*D*) in HEK cells expressing CB_1 receptors. These results demonstrate that hemopressin binds and signals in a manner similar to that of the synthetic CB_1 receptor antagonist, SR141716, and thus represents a natural peptide antagonist of CB_1 cannabinoid receptors.

Next, we examined the selectivity of hemopressin for CB_1 receptors by comparing the effect of hemopressin on GTPγS-binding and adenylyl cyclase activity in HEK cells individually expressing CB_1 or CB_2 receptors. We find that in both assays, hemopressin attenuates the signaling of CB_1 but not CB_2 receptors (Fig. 3 *A* and *B*). Interestingly, hemopressin is able to decrease basal levels of signaling in a manner similar to SR141716, which has been shown to have inverse agonist activity [Fig. 3 (17)]. Thus, hemopressin is able to block the constitutive activity of CB_1 but not CB_2 receptors (Fig. 3). Next, we examined the effect of hemopressin on agonist-mediated decrease in cAMP levels (using SeAP levels as a readout) in Neuro 2A cells expressing either CB_1 or CB_2 receptors. We find that hemopressin significantly blocks agonist-mediated decreases in SeAP levels in cells expressing CB_1 receptors. Furthermore, hemopressin reduces the basal activity supporting its inverse agonist nature (Fig. 3*C*). Finally, to investigate this inverse agonist

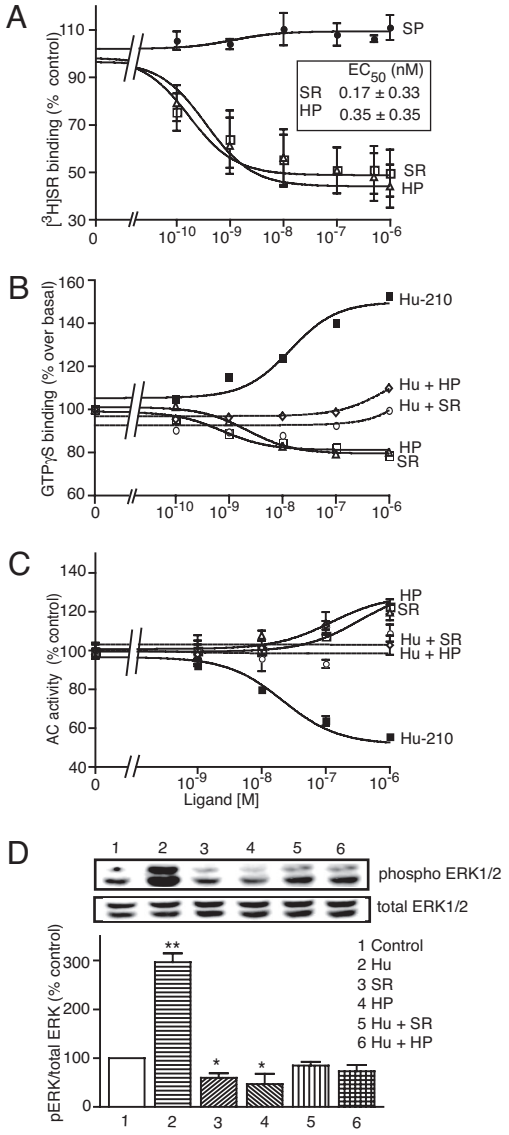

Fig. 2. Hemopressin is an antagonist of the CB_1 cannabinoid receptor. (*A*) Striatal membranes (10 μg) were incubated with 3 nM [^{3}H]SR141716 in the absence or presence of increasing concentrations (0–1 μM) of hemopressin (HP), SR141716 (SR), or scrambled peptide (SP) and the ligand-binding analysis carried out as described (36). (*B* and *C*) Striatal membranes (10 μg) were subjected to a GTPγS-binding assay (*B*) or an adenylyl cyclase assay (*C*) using increasing concentrations (0–1 μM) of Hu-210 (Hu), HP, or SR or increasing concentrations of Hu in the absence or presence of 10 μM HP or SR, as described (36, 37). (*D*) HEK-293 cells expressing Flag-tagged CB_1 receptors were treated for 5 min with 100 nM Hu-210 in the absence of presence of 10 μM SR or HP and levels of phosphorylated MAP kinase determined as described (36). Values obtained in the absence of drug treatment were taken as 100%. Results are mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; one-way ANOVA and Dunnett's test.

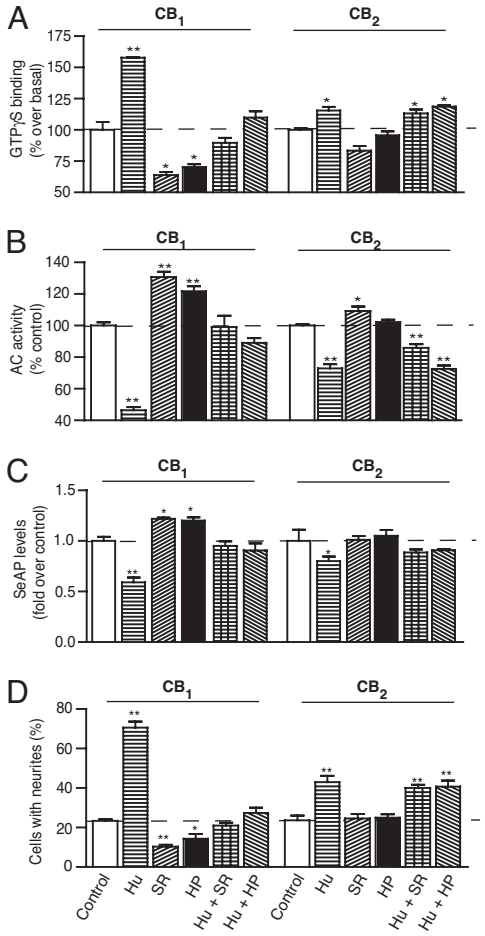

Fig. 3. Hemopressin functions as an inverse agonist of CB_1 but not CB_2 receptors. Membranes (10 μg) from striatum (CB_1 receptors) or spleen (CB_2 receptors) were subjected to a GTPγS-binding assay (*A*) or an adenylyl cyclase assay (*B*) using 1 μM of Hu-210 (Hu), hemopressin (HP), SR141716 (SR), Hu + SR or Hu + HP, as described (36, 37). Results are mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; one-way ANOVA and Dunnett's test. (*C*) Neuro 2A cells (30,000 cells per well) coexpressing pCRE-SeAP and CB_1 or CB_2 cannabinoid receptors were treated with 10 μM forskolin and 100 nM Hu-210 (cannabinoid receptor agonist) in the absence or presence of 1 μM hemopressin and probed for SeAP levels, as described in *Methods*. Results are the mean ± SEM of sextuplicate determinations ($n = 2$). Statistically significant differences from control (*) and from agonist alone (+) are indicated, *, $P < 0.05$; **, $P < 0.01$; ++, $P < 0.01$; one-way ANOVA and Dunnett's test. (*D*) Neuro 2A cells expressing CB_1 or CB_2 receptors were treated for 16 h with 100 nM Hu, 10 μM SR or HP, 100 nM Hu + 10 μM SR, or 100 nM Hu + 10 μM HP, and percentage of cells extending neurites was determined as described (19). Values obtained in the absence of drug treatment were taken as 100%. Results are mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; one-way ANOVA and Dunnett's test.

activity in a functional assay, we examined its effect on neurite outgrowth in Neuro 2A cells. We have shown that activation of cannabinoid receptors leads to neuritogenesis in these cells (18, 19). As expected, hemopressin treatment essentially blocked the agonist-mediated increase in the number of cells with neurites in cells expressing CB_1 receptors but not in cells expressing CB_2 receptors (Fig. 3*D*). Hemopressin treatment alone (in the absence of agonist) led to a significant decrease in the number of cells with neurites in a manner similar to SR141716 (Fig. 3*D*). Taken together, these results indicate that hemopressin, like SR141716, is an inverse agonist at CB_1 (but not at CB_2) receptors.

Next, the antagonistic activity of hemopressin was examined using *in vivo* models of hyperalgesia. We used the paw-pressure assay to test the effect of hemopressin on carrageenan (Cg)-induced hyperalgesia. We found that an intraplantar injection of hemopressin reduced inflammatory pain to the same extent as the CB_1 antagonist, AM251 (Fig. 4*A*). In this pain model (paw-pressure test), hemopressin on its own has no antihyperalgesic action (15). We also found that hemopressin administered intrathecally (Fig. 4*B*) or orally (Fig. 4*C*) is able to efficiently block the carrageenan (Cg)-induced hyperalgesia. We also examined the effect of hemopressin in additional models of

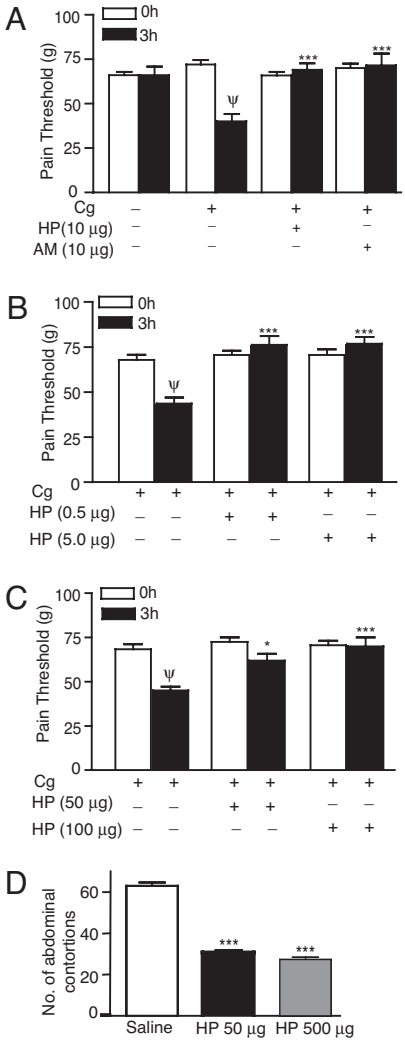

Fig. 4. *In vivo* hemopressin antihyperalgesic activity. (*A*) Comparative analyses of intraplantar (i.pl.) administration of hemopressin and AM251 on the hyperalgesia induced by carrageenan. Rats were treated i.pl. with hemopressin (HP, 10 μg per paw) or AM251 (AM, 10 μg per paw) immediately before the i.pl. injection of carrageenan (Cg, 200 μg per paw) and were evaluated before (0 h, empty bars) and 3 h after Cg injection (black bars). Rats administered i.pl. with vehicle (saline) were subjected to the same protocol (control group). Results are presented as mean ± SEM, $n = 6–8$ (Ψ, $P < 0.001$ vs. initial measurement; *, $P < 0.05$ vs. control group; and ***, $P < 0.001$ vs. control group, ANOVA with Bonferroni post hoc test). (*B*) Effect of intrathecal (i.t.) administration of hemopressin. Rats received HP i.t. (0.5 or 5 μg/kg) immediately before i.pl. injection of Cg (200 μg per paw) and were evaluated before (0 h, empty bars) and 3 h after treatment (black bars). Rats administered i.t. with saline were submitted to the same protocol (control group). Results are presented as mean ± SEM, $n = 6–8$ [Ψ, $P < 0.001$ vs. initial measurement, *, $P < 0.05$ vs. control group (3 h) and ***, $P < 0.001$ vs. control group (3 h); ANOVA with Bonferroni post hoc test]. (*C*) Effect of oral administration of hemopressin on carrageenan-induced hyperalgesia. Rats were administered with saline (control group) or hemopressin (HP, 50 or 100 μg/kg) *per os* (p.o.) immediately before the i.pl. injection of carrageenan (Cg, 200 μg per paw), and the nociceptive threshold measured by using an Ugo Basile pressure apparatus was evaluated before (0 h, empty bars) and 3 h after Cg injection (black bars), as described in detail in *Methods*. Results are presented as mean ± SEM, $n = 6–8$ [Ψ, $P < 0.001$ vs. initial measurement; *, $P < 0.05$ vs. control group (3 h) and ***, $P < 0.001$ vs. control group (3 h), ANOVA with Bonferroni post hoc test]. (*D*) Effect of i.p. administration of hemopressin on writhing test. Abdominal contortions resulting from i.p. injection of 0.6% (vol/vol) acetic acid, at a dosage of 60 mg/kg body weight, are contractions of the abdominal muscles with a stretching of hind limbs. The number of abdominal contortions was counted cumulatively over a period of 20 min after acetic acid injection. Hemopressin (HP, 50 or 500 μg/kg) was injected i.p. 1 h before the acetic acid administration. Antinociceptive activity was expressed as the reduction in the number of abdominal contortions between hemopressin- and vehicle-treated animals. Results are presented as mean ± SEM, $n = 6–8$ (***, $P < 0.001$ vs. control group; ANOVA with Bonferroni post hoc test).

pain. In the acetic acid-induced visceral nociception model, we found that hemopressin exhibited a marked antinociceptive effect (Fig. 4*D*). Administration of hemopressin (500 μg/kg) did not impair motor activity or alter pentobarbital-induced sleeping time, indicating the absence of sedative or motor abnormalities that could account for the appearance of antinociceptive action (SI Fig. 5).

Our findings are consistent with recent reports showing that CB_1 receptor antagonists can exhibit antihyperalgesic and antinociceptive effects mediated via CB_1 receptors in certain pain models. For example, studies have shown that repeated administration of the CB_1 receptor antagonist, SR141716, relieved neuropathic pain after sciatic nerve ligature (20, 21). These effects required the presence of CB_1 receptors, because SR141716 was not antinociceptive in a similar pain model in CB_1 knockout mice (20). In addition, repeated oral administration of SR141716 reduced sensory hypersensitivity associated with complete Freund's adjuvant-induced arthritic pain (22). Because a large body of evidence has clearly demonstrated the antinociceptive action of CB_1 receptor agonists, these results are paradoxical (in that receptor antagonists also have antinociceptive effects; reviewed in refs. 23–26). A possible explanation for these paradoxical *in vivo* effects (by CB_1 agonists and antagonists) could be that endocannabinoids activate distinct signal transduction pathways under different paradigms used to measure pain leading to distinct and opposing effects. It is also likely that, after CB_1 receptor blockade by the antagonist, the released endocannabinoids could induce antinociception by affecting other pain transmission mechanisms. Recent findings showing an up-regulation of endocannabinoids (anandamide and 2-arachidonoylglycerol) in neuropathic pain models (27) and findings showing this in turn could cause the desensitization of transient receptor potential vanilloid type I leading to blockade of pain transmission (28) would support such a hypothesis.

An intriguing finding of the current study is that hemopressin is orally active. A number of reports have documented that bioactive peptides retain biological activity after oral administration. These include peptides C111 and C112 from bonito liver (29, 30) and peptides IPP and VPP as well as tryptic peptides from casein (31–33). It is likely that a similar mechanism is involved in maintaining the biological activity of hemopressin after oral administration, although not much is known about it at this time. Another possibility is that hemopressin is further processed to shorter bioactive peptides, some of which may be responsible for additional biological activity. This is based on preliminary results showing that shorter forms of hemopressins are able to bind to and signal via CB_1 receptors.

Hemopressin was originally isolated from a peptide-enriched fraction obtained from rat brain extracts using an inactive mutant peptidase (12). To further examine whether hemopressin exists as a native peptide *in vivo*, we carried out mass spectrometric analysis of extracts from brain regions subjected to treatments that minimize nonspecific postmortem proteolyses (34, 35) and were able to identify hemopressin and longer hemopressin-containing peptides (data not shown). Additional studies using hemopressin-specific reagents are needed to document the endogenous nature of hemopressin. The N-terminal 7 aa of the hemopressin sequence are entirely conserved in turtle, crocodile, bird, marsupial, rodent, and mammal (I.G. and L.A.D., unpublished work), suggesting this peptide is evolutionarily conserved and hence could have important and conserved

biological function. It will be interesting to examine how the generation of hemopressin is regulated, sequestered, and/or released from a specific organelle or cell compartment. Taken together, these data are consistent with the notion that hemopressin represents a neuromodulatory peptide with CB_1 receptor activity.

In this study, we describe a peptide with inverse agonist activity with respect to G protein and ERK1/2 signaling for CB_1 cannabinoid receptors. Although the modulation of CB_1 receptors by peptides has been indirectly suggested by functional studies, identification of such ligands has been elusive until now, and the endogenous ligands of CB_1 receptor identified thus far have been lipid derivatives. Our finding that hemopressin represents a peptide ligand with inverse agonist activity at CB_1 receptors is exciting, although further studies are needed to evaluate whether it is an endogenous ligand CB_1 receptors. Taken with the fact that hemopressin exhibits antihyperalgesic activity when administrated systemically or locally, this makes it a strong candidate for a pain therapeutic in the near future.

Methods

Cell Culture and Transfection. HEK-293 and SK-N-SH cells were grown in DMEM containing 10% FBS and 1% penicillin-streptomycin. Neuro 2A cells were grown in DMEM and F12 media (50:50) containing 10% FBS and 1% penicillin–streptomycin. HEK-293 or Neuro 2A cells were transfected with Flag-tagged wild-type CB_1 or CB_2 receptors (2 μg) using Lipofectamine per the manufacturer's protocol (Invitrogen). For the SeAP assay, HEK-293 or Neuro 2A cells were transfected with 5 μg of pCRE-SeAP and 1 μg of either Flag-tagged μ opioid receptor, δ opioid receptor, α_{2A} adrenergic receptor, β_2 adrenergic receptor, AT1 angiotensin receptor, CB_1 or CB_2 cannabinoid receptor using Lipofectamine per the manufacturer's protocol (Invitrogen).

Screening Using Conformation-Sensitive Anti-CB_1 Receptor Antibodies. Activation-sensitive antibodies were generated and characterized for their specificities as described (16). The anti-CB_2 cannabinoid, -AT2 angiotensin, and -B2 bradikynin receptor antibodies were generated in rats as described (16). For screening compounds, SK-N-SH cells ($\approx 1 \times 10^5$ cells per well) were plated on 96-well Nunc-Immuno plates (Nalge Nunc) and air-dried at room temperature. The cells were washed with PBS and incubated without or with different concentrations of ligands, and the extent of receptor recognition by the antibodies was assayed by ELISA as described (16). The characterization of the antibodies for μ opioid, δ opioid, α_{2A} adrenergic, β_2 adrenergic, AT1 angiotensin, and CB_1 cannabinoid receptor has been reported (16). We find that the AT2 angiotensin, CB_2 cannabinoid, and B2 bradikynin receptor antibodies are also highly selective, in that they exhibit $<$10% cross-reactivity for all of the above receptors (A.S.H. and L.A.D., unpublished work). To characterize the specificity of hemopressin (Proteimax Biotechnology) toward CB_1 receptors, SK-N-SH cells plated as described above were treated without or with 1 μM hemopressin in the absence or presence of 1 μM agonists for μ opioid receptor (DAMGO), δ opioid receptor (Deltorphin II), α_{2A} adrenergic receptor (Clonidine), β_2 adrenergic receptor (Isoproterenol), AT1 angiotensin receptor (Angiotensin II), AT2 angiotensin receptor (Angiotensin II), B2 bradykinin receptor (Bradykinin), or CB_1 cannabinoid receptor (WIN 55,212-2). The extent of recognition by various GPCR antibodies was probed by ELISA as described (16). The recognition by CB_2 cannabinoid receptor antibody in the absence or presence of the agonist (WIN 55,212-2) was examined by using spleen membranes by the ELISA, as described (16).

Ligand-Binding and Signaling Studies. Membranes (5 μg per well) from C57BL/6 mice or Long–Evans rat striatum were prepared as described in ref. 36, plated onto a 96-well Nunc-Immuno plate, treated with ligands, and the extent of antibody recognition assayed by ELISA as described (16).

For ligand binding, membranes from rat striatum (10 μg) were incubated with 3 nM [^{3}H]SR141716A in the absence or presence of increasing concentrations (0–1 μM) of SR141716, hemopressin or scrambled peptide, as described (36). For GTPγS binding or adenylyl cyclase assay, membranes from striatum or spleen (10 μg) were incubated with increasing concentrations (0–1 μM) of Hu-210 (cannabinoid agonist), SR141716, hemopressin, or a combination of different concentrations of Hu-210 without or with 10 μM hemopressin or SR141716 as described (36, 37).

Phosphorylation of MAPK. HEK-293 cells expressing Flag-tagged CB_1 receptors (2×10^6 cells per well) were treated for 5 min with 100 nM Hu-210 in the absence or presence of 10 μM SR141716 or hemopressin and levels of phosphorylated MAP kinase determined as described (36).

SeAP Assay. HEK-293 or Neuro 2A cells coexpressing pCRE-SeAP and either μ opioid, δ opioid, α_{2A} adrenergic, β_2 adrenergic, AT1 angiotensin, CB_1 or CB_2 cannabinoid receptors (30,000 cells per well) were plated on a 96-well poly-L-lysine-coated plate. The next day, the cells were serum-starved for 16–18 h. Cells were then stimulated with 10 μM forskolin (except for β_2 adrenergic receptors) in the absence or presence of hemopressin or SR141716A (1 μM) and agonists for each individual receptor (100 nM) for 6 h in media without serum. The supernatant (15 μl) was transferred into a second 96-well plate (Luminunc, Nunc) diluted in dilution buffer (150 mM NaCl, 40 mM Tris·HCl, pH 7.2), and heated at 65°C for 20 min. Plates were cooled to room temperature. The amount of alkaline phosphatase present in the supernatant was determined by adding 50 μl of assay buffer (2 M diethanolamine, 10 mM $MgCl_2$, 20 mM L-homoarginine, pH 9.8), incubating for 5 min at room temperature, and adding 50 μl of reaction mix (32.5 μl of water, 15 μl of Enhancer solution, and 2.5 μl of CSPD substrate; Tropix, Applied Biosystems). After 20-min incubation at room temperature, luminescence was quantified (Packard).

Neurite Outgrowth Assays. Neuro 2A cells expressing CB_1 or CB_2 receptors were treated for 16 h in medium containing 0.1% FBS with 100 nM Hu-210, 10 μM SR, 10 μM HP, 100 nM Hu-210 + 10 μM SR, or 100 nM Hu-210 + 10 μM HP, and neurite length was determined as described (19).

***In Vivo* Studies.** Male Wistar rats (200 g) and C57BL/6 mice (20 g), used in these studies, were maintained on a 12-h light/dark cycle at 22 $\pm$ 2°C, with free access to food and water. Throughout the experiments, the animals were handled in accordance with the principles and guidelines for the care of laboratory animals in studies involving pain (38). Institute for Animal Care and Use Committee approval was obtained for the nociceptive pain assays from the Institute of Biomedical Sciences, University of São Paulo, and the Center for Applied Toxinology, Butantan Institute, São Paulo, Brazil.

Assessment of Nociception (Paw-Pressure Test). The nociceptive threshold was measured by using a Ugo Basile pressure apparatus, essentially as described (39). Briefly, a force of increasing magnitude (16 *g*/s) was applied to the paw. When the rat reacted by withdrawing the paw, the force (in *g*) required to induce this response represented the pain threshold. Antinociceptive activity was expressed as the increase in the force needed to induce the withdrawal response in treated rats compared with control rats that received only saline. Nociceptive tests were applied immediately before (0 h) and 3 h after drug administration.

Inflammatory Hyperalgesia. Hyperalgesia was induced by the intraplantar administration of 0.1 ml of sterile saline containing carrageenan (200 μg per paw; Sigma) into the right hind paw. Concomitant with carrageenan, rats were injected with hemopressin intraplantar (10 μg per paw), oral (50 or 100 μg/kg), intrathecal (0.5 or 5 μg/kg), or AM251 (10 μg per paw). The pain threshold was measured by the paw-pressure test immediately before (0 h) and 3 h after carrageenan, hemopressin, and/or AM251 injection.

Writhing Test for Pain Sensitivity. The writhing test was performed in mice, based on procedures described (39). Abdominal contortions resulting from i.p. injection of 0.6% (vol/vol) acetic acid (Merck), at a dosage of 60 mg/kg of body weight, were taken as contraction of the abdominal muscles together with a stretching of hind limbs. The number of abdominal contortions was counted cumulatively over a period of 20 min after acetic acid injection. Hemopressin (50 or 500 μg/kg) was injected i.p. 1 h before acetic acid administration. Antinociceptive activity was expressed as the reduction in the number of abdominal contortions by comparing hemopressin- vs. vehicle-treated animals.

Spontaneous Motor Activity and Pentobarbital Hypnosis. Possible changes in motor activity provoked by hemopressin were investigated in an open-field arena as described (40). Each animal was individually placed in the center of the open field and behavioral parameters recorded for 3 min. Hand-operated counters were used to score ambulation (locomotion) frequency (number of floor units entered), and rearing frequency (number of times the animal stood on hind legs). The open field was washed with alcohol (5%) before the animals

were placed in it, to avoid possible biasing effects due to odor clues left by previous individuals. This test was applied 1 h after hemopressin was injected i.p. (50 μg/kg). The effect of hemopressin on pentobarbital-induced sedation was determined after animals were injected i.p. with hemopressin (500 μg/kg) and a single i.p. dose of 45 mg/kg of sodium pentobarbitone (Cristália). Sedation was determined by measuring the duration of sleep time defined as the period from the moment of loss of the righting reflex until its return (41).

Statistical Analysis. Results were expressed as the mean ± SEM. Statistical comparisons were done by using ANOVA followed by the Tukey test. A value of $P < 0.05$ indicated a significant difference. Statistical analyses of data were generated by using GraphPad Prism, version 4.02 (GraphPad).

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