Cannabinoid Receptors Are Coupled to Nitric Oxide Release in Invertebrate Immunocytes, Microglia, and Human Monocytes*

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George B. Stefano†‡‡, Yu Liü†, and Michael S. Goligorsky§

From the †Neuroscience Research Institute, State University of New York, Old Westbury, New York 11568 and Departments of §Physiology and ¶Medicine, State University of New York, Stony Brook, New York 11794

The present study demonstrates that stereoselective binding sites for anandamide, a naturally occurring cannabinoid substance, can be found in invertebrate immunocytes and microglia. The anandamide-binding site is monophasic and of high affinity, exhibiting a $K_d$ of 34.3 nm with a $B_{max}$ of 441 fmol/mg protein. These sites are highly selective, as demonstrated by the inability of other types of signaling molecules to displace $[^3H]$anandamide. Furthermore, this binding site is coupled to nitric oxide release in the invertebrate tissues examined as well as in human monocytes. Interestingly, the cannabinoid-stimulated release of nitric oxide initiates cell rounding. Thus, these cannabinoid actions resemble those of opiate alkaloids. In this regard, we demonstrate that these signaling systems use the same effector system, i.e. nitric oxide release, but separate receptors. Last, the presence of a cannabinoid receptor in selected evolutionary diverse organisms indicates that this signaling system has been conserved for more than 500 million years.

δ-9-Tetrahydrocannabinol (THC) is the active ingredient of marijuana (1). In 1988 a receptor for this substance was identified in the rat brain (2) and subsequently cloned (3). An arachidonic acid derivative, anandamide (N-arachidonoyl-ethanolamine), was later identified as the endogenous ligand for this receptor (4). Extracellular anandamide is rapidly and selectively taken up in neurons, where its degradation to ethanolamine and arachidonate takes place (5). Thus, in a relatively short period of time, a great deal of information has been obtained on cannabinoid receptors and their ligands in mammals.

In this regard, it was of interest to determine if cannabinoid-binding sites are found in invertebrates. The present report demonstrates the existence of such a cannabinoid system for the first time. $[^3H]$Anandamide-binding sites are found in Mytilus edulis immunocytes and pedal ganglia microglia membrane homogenates. Furthermore, these high affinity sites appear to be coupled to NO release. Thus, both cannabinoids and opiates share the same effector system by engaging distinct receptors (6, 7).

MATERIALS AND METHODS

M. edulis, a marine bivalve mollusk, were harvested from Long Island Sound at Montauk and maintained in the laboratory for 3 weeks prior to dissection (9). Immunocytes and microglia were collected and processed as described elsewhere in detail (7, 10, 11).

Analysis of Anandamide Binding—Immunocytes (10) were homogenized in 50 volume of 0.32 M sucrose, pH 7.4, at 4°C by the use of a Brinkmann Instruments Polytron (30 s, setting 5). The crude homogenate was centrifuged at 900 × g for 10 min at 4°C, and the supernatant was preserved on ice. The whitish crude pellet was resuspended after homogenization (15 s, setting 5) in 30 volume of 0.32 M sucrose-Tris-HCl buffer, pH 7.4, and centrifuged at 900 × g for 10 min. The extraction procedure was repeated one more time, and the combined supernatants were centrifuged at 900 × g for 10 min. The resulting supernatant (S1) was used immediately. The S1 supernatant was centrifuged at 30,000 × g for 15 min, and the resulting pellet (P2) was washed once by centrifugation in 50 volume of the sucrose-Tris-HCl buffer. The P2 pellet was then homogenized with a Dounce hand-held homogenizer (10 strokes) and resuspended in 100 volume of buffer. Binding analysis was then performed on the immunocyte 1 membrane suspensions.

For displacement analysis, aliquots of membrane suspensions from these cells were incubated with nonradioactive compounds at five concentrations for 10 min at 22°C and then with $[^3H]$anandamide for 60 min at 4°C. One hundred percent binding is defined as bound $[^3H]$anandamide in the presence of 10 μM anandamide. $K_i$ is defined as the concentration of drug that elicits half-maximal inhibition of specific binding. The mean ± S.E. for three experiments is given. The displacement analysis data indicate the potency of various ligands in displacing $[^3H]$anandamide (34 Ci/mM; DuPont NEN, Boston, MA) and may give specific information on different receptor populations.

Immunocytes—Human blood was obtained from Long Island Blood Services (Melville, NY) and separated into a monocyte fraction using a Ficoll-Hypaque centrifugation kit (Accurate Chemical Scientific Inc., Westbury, NY) designed for rapid separation of monocytes from whole blood. Aliquots were placed on a slide and stained with Wright’s stain, followed by microscopic analysis to determine the purity of the preparations. The purity of the monocytes ranged from 94–98%. Following isolation from blood, the cells were suspended in phosphate-buffered saline and used immediately in assays.

Microglia—The pedal ganglia were removed from 3-week laboratory-maintained M. edulis (∼500) on ice and rinsed in artificial seawater (Instant Ocean, Inc., Boston, MA) and cell-free (verified by light microscopic examination) filtered and centrifuged hemolymph, 1:1 (v/v). This incubation medium also contained streptomycin (50 mg/100 ml), penicillin (30 mg/ml), and gentamycin (50 mg/ml) to further reduce any bacterial presence. The excised ganglia (10 per test tube × 50) were incubated for 24 h at room temperature. At the end of this time period, the ganglia were removed, and the remaining microglia (7, 12, 13) were rinsed three times in fresh incubation medium following centrifugation (900 × g for 15 min). These cells were then added to the NO detection vials and allowed to sit at room temperature for 40 min before the NO determination commenced. At this time, pharmacological agents were added to particular vials (see below). Other ganglia (five per treatment) were placed in a ring of Vaseline on a slide, and a coverslip was added to each slide. After 24 h of incubation at 20°C, the ganglia were examined for microglial egress number and conformation (see below) as described previously (7, 12, 13).

Pharmacological additions to the incubation media plus microglia or ganglia were added first to the artificial seawater before final adjustments, following the addition of the cell-free hemolymph. Examination of cell numbers in the incubation media on the slide preparations was...
performed by image analysis under \( \times 400 \) magnification, whereby the respective ganglia were placed in the center of the observation field, which was divided into four clocklike quadrants (i.e. 12:00, 3:00, 6:00, and 9:00). For each ganglia a quadrant was counted, and the mean was determined for all four quadrants. This mean value was evaluated with four other mean values (preparations) per treatment (±S.E.) and compared via one-tailed Student’s t test to vehicle-treated controls; the criterion for significance was \( p < 0.05 \). Vehicle controls were those containing all chemicals minus the noted pharmacological agent.

Morphological Analysis of Glia and Immunocytes—The morphological measurements of \( M. \) edulis microglia and immunocytes were based on cell area and perimeter determinations by the use of image analysis software (American Innovonix, Inc., San Diego, CA and Image Analyt- ics, Inc., Hauppauge, NY). Form factor calculations were performed as described previously (14). The observational area used for measurement determinations and frame grabbing of the respective ganglia was 0.4 m in diameter. The computer-assisted image analysis system (Zeiss Axio phot fitted with Nomarski and phase-contrast optics) was the same as described previously (15).

The cells were analyzed for conformational changes indicative of either activation (amoeboid and mobile) or inhibition (round and stationary). The lower the form factor number, the longer the perimeter and the more amoeboid the cellular shape. The proportion of activated cells was determined as described previously (10). The activated state (amoeboid conformation) of human and invertebrate cells is correlated to biochemical adhesion molecule alterations (16) as well as cytokine production (17, 18).

All pharmacological agents were purchased from Research Biochemicals Inc. (Natick, MA).

Monitoring of NO Release—Immunocytes or microglia were bathed (10\( \, ^{7} \) ml) in a 1:1 incubation medium composed of cell-free hemolymph and filtered and boiled seawater (9). NO release was monitored with a NO-selective microprobe manufactured by World Precision Instruments (Sarasota, Fl.). The redox current was detected by a current voltage converter circuit and continuously recorded. Tip diameter of the probe (25 \( \mu \) m) permitted the use of a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikom Diaphot) to position the sensor 10 \( \mu \) m above the cell surface. Calibration of the electrochemical sensor was performed by the use of different concentrations of nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine, as previously detailed (6).

The NO detection system was calibrated daily, and the probe was routinely cleaned in 0.1 N H\( _{2} \)SO\( _{4} \) to remove cellular debris, which tends to accumulate on the probe. The probe is allowed to equilibrate for 40 min in the incubation medium free of cells and tissues before being transferred to vials containing the tissue for another 30 min. Manipulations and handling of the cells were only performed with glass instruments. Each experiment was repeated four times, and the NO mean values obtained were either graphed to represent the actual NO release, or the values obtained at 5-min intervals were graphed (±S.E.). Each experiment was performed simultaneously with a control (vehicle minus drug) from the same tissue. Thus, the experiment was performed with four probes measuring the different experimental preparations (control, drug treated, drug in the presence of a NOS inhibitor, and drug plus antagonist). This strategy eliminated the probability of artifactual electrode drifts.

The data obtained was then evaluated by the Student’s t test. Data acquisition was by the computer interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma-Plot and Stat-Graph-Stat (Jandel Scientific, San Rafael, CA) for graphic representation and evaluation. It is of importance to note that the data gatherers were unaware of the experimental conditions they were monitoring.

RESULTS

Membrane homogenates of \( M. \) edulis immunocytes revealed monophasic and specific anandamide binding sites (Fig. 1 and Table I). Scatchard analysis showed a single, relatively high affinity, binding site with a \( K_{d} \) of 34.3 \( \mathrm{nm} \) and a \( B_{\text{max}} \) of 441 fmol/mg membrane protein for the immunocytes (Figs. 1 and 2). Nonspecific binding increased linearly with respect to the concentration of the binding ligand (not shown). Mytilus microglia exhibited the same binding profile (\( K_{d} \) 32.7 \( \mathrm{nm} \); \( B_{\text{max}} \) 458 ± 28 fmol/mg membrane protein), suggesting that they originate from this animal’s immunocytes. Furthermore, a variety of diverse signal molecules were ineffective in displacing specifically bound [\( ^{3} \mathrm{H} \) ]anandamide (Table I). In this regard, the cannabinoid agonist CP 55940 and antagonist SR 141716A were quite potent in displacing [\( ^{3} \mathrm{H} \) ]anandamide, whereas Win 55212–2, another agonist, was less efficacious.

Given the fact that cannabinoid signal molecules have immune-altering actions (see ‘Discussion’), it was of interest to determine if anandamide would induce the formation of NO, as does morphine, which exhibits similar immunocyte-suppres- sive actions (6, 7).\(^2\) Anandamide and CP 55940 initiate the release of NO in a concentration-dependent manner from Mytilus immunocytes, microglia, and human macrophages (Figs. 2 and 3). This process can be antagonized by coincubating the cells with the nitric oxide synthase inhibitor N\(-\)omega-nitro-L-arginine methyl ester (L-NAME), as well as by exposing the cells to the cannabinoid antagonist SR 141716A (Figs. 3 and 4 and Table II). Interestingly, the level of NO release following exposure of the cells to \( 10^{-6} \) m anandamide (approximately 14.4 ± 4.7 (S.E.) for all tissues combined) is lower than that of an equivalent concentration of morphine (37 \( \mathrm{nm} \); Ref. 6).\(^2\) It is even lower than that produced by \( 10^{-6} \) m morphine (Fig. 5). Prior treatment of the cells with SR 141716A (\( 10^{-6} \) m) blocked the
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NO release was recorded using an NO-specific amperometric probe (25 μM; World Precision Instruments). NO release values were obtained 10 min after the addition of the various ligands. Control preparations received vehicle and exhibited no substantial release of NO. The THC receptor antagonist SR 141716A did not induce NO release from these cells. Each experiment was replicated five times, and means ± S.E. of NO release are shown. Anan, anandamide; CP, CP 55940; SR, SR 141716A.

DISCUSSION

The present study demonstrates the following: 1) stereoselective binding sites for anandamide can be found in invertebrate immunocytes and microglia; 2) these binding sites are monophasic and of high affinity; 3) these sites are highly selective, as demonstrated by the inability of other types of signaling molecules to displace [3H]anandamide; 4) these binding sites are selective for anandamide; 5) the cannabinoid-stimulated release of NO leads to immunocyte, microglia, and human macrophage cell rounding; and 6) the presence of a cannabinoid receptor in selected evolutionary diverse organisms indicates that this signaling system has been conserved for more than 500 million years.

With regard to immunocytes, THC inhibits macrophage cell line contact-dependent cytolysis of tumor cells (19). THC also appears to alter antigen processing (20) and the expression of select proteins, the induction of which is associated with macrophage activation, as well as the expression of tumor necrosis factor (21, 22). THC was found to increase supernatant interleukin 1 bioactivity in cultures of mouse resident peritoneal

Fig. 2. Concentration dependence of NO release from human macrophages and M. edulis immunocytes. NO release was recorded using an NO-specific amperometric probe (25 μM; World Precision Instruments). NO release values were obtained 10 min after the addition of the various ligands. Control preparations received vehicle and exhibited no substantial release of NO. The THC receptor antagonist SR 141716A did not induce NO release from these cells. Each experiment was replicated five times, and means ± S.E. of NO release are shown. Anan, anandamide; CP, CP 55940; SR, SR 141716A.

Fig. 3. Anandamide stimulates NO release from invertebrate immunocytes, microglia, and human macrophages that is abrogated by L-NAME. NO release was recorded using an NO-specific amperometric probe (25 μM; World Precision Instruments). NO release was recorded for 12 min (end point, arrow) to establish baseline values, followed by addition of anandamide (arrow) and recording of NO release for an additional 25 min, and maximal NO release in response to (10−6 M) anandamide was determined. Treatment with 0.01 μM anandamide did not result in a significant increase in NO release, whereas treatment with 0.1, 1.0, or 10 μM anandamide resulted in release of NO that was increased significantly over baseline values. For experiments in the presence of L-NAME, this agent was preincubated with the cells for 5 min prior to anandamide (1 μM) addition. Control preparations received vehicle at the respective times intervals. Pretreatment with L-NAME resulted in concentration-dependent inhibition of anandamide-induced NO release that was similar for all the cells. Each experiment was replicated five times, and means ± S.E. of NO release are shown. p < 0.05 from 8 to 14 min when compared with L-NAME-pretreated cells. Anan, anandamide; Im, immunocytes; Mic, microglia; Mac, macrophages.

Fig. 4. Real time analysis of cannabinoid-induced NO release by invertebrate immunocytes. Cells were stimulated (arrow) with CP 55940 (CP, 1 μM) and evaluated for NO release over 25 min, as described in Fig. 2. For experiments in the presence of SR 141716A (SR, 10−6 M), the cells were preincubated with this cannabinoid antagonist for 5 min prior to CP 55940 addition. The representative experiments shown have been replicated three to five times with similar results. Add, drug added.
inhibit human peripheral blood macrophage spreading and phagocytosis (24). In earlier reports, THC was found to inhibit P388D1 macrophage cell cultures with THC results in a rounded cell shape, indicating this cell rounding (6, 29). Given the fact that naloxone does not antagonize or bind to the anandamide receptor, these signal systems appear to be distinct. Thus, naturally occurring cannabinoids may share the NO-producing effector system with opiate alkaloids (6, 7).2

The cannabinoid-signaling system exhibits many biological similarities to that of opiate molecules. As with morphine, this psychoactive agent has been used by man for thousands of years. Biomedical properties it shares with morphine include analgesia, anti-inflammation, and immunosuppression (see Ref. 30). Another similarity this compound shares with morphine is that its receptors are found on neurons and immunocytes, suggesting autoimmune and neuroimmune actions. In this regard, the cloning of a receptor for cannabinoids includes one found in macrophages (30). The significance of this finding in light of the actions of cannabinoids on the immune system appears unsettled (see Ref. 31). However, combined with the information presented in this report, that significance becomes more apparent.

Other common effects of cannabinoid mimetic agents and opiates are in the inhibition of N18TG2 neuroblastoma cell adenylyl cyclase (1). The δ-opioid receptor subtype on the N18TG2 membranes is unaltered by cannabinoid mimetic drugs. Furthermore, opioid and opiate agents also inhibit this enzyme in N18TG2 cells. Therefore it was concluded that both molecules were using diverse receptors but the same effector process, since naloxone only blocked the opioid action (1). This observation is supported in the present study; naloxone, an opioid receptor antagonist, does not block the invertebrate cannabinoid receptor, nor does it antagonize cannabinoid release of NO. This link between opioids and cannabinoids is further strengthened by the observation that both bind to G-protein-coupled receptors to inhibit adenylyl cyclase in neurons (32). In cerebellar granule cells, both cannabinoid and opioid receptors appear to exist on the same cells, and their respective activation produces similar biological responses (32). A recent report (33) provides evidence for a possible link with κ receptors; however, the exact nature of the interaction of the cannabinoids and the κ receptors to be elucidated.

Given the above remarkable parallelism between opiate- and cannabinoid-signaling cascades and the overall array of physiological systems they affect, including NO release, we must ask why two such “redundant” systems are required. Again, both types of compounds have analgesic, anti-inflammatory, and immunosuppressive properties (see Ref. 30). Based on the results of the present study, namely, that both compounds can release NO by separate processes, we speculate that the answer may be found in the degree of this action. Morphine in diverse tissues and animals appears to be a more potent stimulator of NO release than cannabinoids on a same dose basis (6, 7).2 Cannabinoids also exert this action, NO release, in other...
mammalian systems. We surmise that the cannabinoid system is "activated" at times requiring a milder analgesic, anti-inflammatory, or immunosuppressive action. This hypothesis is supported by the findings concerning cannabinoid tolerance and addiction (8). O'Brien summarizes that tolerance to cannabinoids disappears rapidly and without withdrawal symptoms, and "few patients seek treatment for marijuana addiction" (8). Indeed, this hypothesis can also be used to explain the presence of the cannabinoid receptor in invertebrates.

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