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# The Endocannabinoid Noladin Ether Acts as a Full Agonist at Human CB2 Cannabinoid Receptors

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## ABSTRACT

Noladin ether (NE) is a putative endogenously occurring cannabinoid demonstrating agonist activity at CB1 receptors. Because of reported selective affinity for CB1 receptors, the pharmacological actions of NE at CB2 receptors have not been examined. Therefore, the purpose of this study was to characterize the binding and functional properties of NE at human CB2 receptors stably expressed in Chinese hamster ovary (CHO) cells as well as in HL-60 cells, which express CB2 receptors endogenously. Surprisingly, in transfected CHO cells, NE exhibits a relatively high nanomolar affinity for CB2 receptors ($K_i$ = 480 nM), comparable to that observed for the endocannabinoid 2-arachidonoyl glycerol (2-AG) ($K_i$ = 1016 nM). Furthermore, NE activates G proteins and inhibits the intracellular effector adenylyl cyclase with equivalent efficacy relative to the full cannabinoid agonists 2-AG and CP 55,940 (CP) [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol]. The rank order of potency for G protein activation and effector regulation by the three agonists is similar to their apparent affinity for CB2 receptors; CP > NE ≥ 2-AG. Regulation of adenylyl cyclase activity by all agonists is inhibited by pertussis toxin pretreatment or by coincubation with AM630 [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)-methanone], a CB2 antagonist. Chronic treatment with NE or CP results in CB2 receptor desensitization and down-regulation. All agonists also inhibit adenylyl cyclase activity in HL-60 cells. Together, these data indicate that NE acts as a full agonist at human CB2 receptors and thus might have important physiological functions at peripheral cannabinoid receptors.

*Cannabis sativa* has been used both therapeutically and recreationally for centuries. $\Delta^9$-Tetrahydrocannabinol has been acknowledged to be the main psychoactive ingredient in marijuana and mediates its effects primarily through activation of two G protein-coupled receptors, CB1 and CB2 (Howlett, 1995). Identified in 1990 (Matsuda et al., 1990), the human CB1 receptor was found to be primarily localized in central and peripheral nervous tissue (Herkenham et al., 1990; Ishac et al., 1996). The CB1 receptor has been identified as a therapeutic target in a variety of disease states, such as obesity (Ravinet et al., 2002), alcohol dependence (Racz et al., 2003), Parkinson's disease (Brotchie, 2003), and pain (Iversen and Chapman, 2002). The second G protein-coupled cannabinoid receptor, CB2, was cloned 2 years later (Munro et al., 1993). These receptors are prevalently found in immune tissues, most abundantly in the spleen and leukocytes (Galiegue et al., 1995). As the localization of the CB2 receptors might indicate, selective CB2 receptor ligands have potential therapeutic use as immune modulators for tumor suppression (Klein et al., 2003) and inflammation (Conti et al., 2002). Recently, CB2 agonists have also been shown to produce potent and efficacious analgesia of neuropathic pain (Ibrahim et al., 2003; Scott et al., 2004). This finding is of particular benefit due to the localization of CB2 receptors outside of the CNS; therefore, agonists that selectively activate the CB2 receptor may produce effective analgesia with-

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**ABBREVIATIONS:** CB, cannabinoid receptor; CNS, central nervous system; 2-AG, 2-arachidonoyl glycerol [(5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester]; NE, noladin ether [2-[(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenyloxy]-1,3-propanediol]; DMEM, Dulbecco's modified Eagle's medium; CP, CP 55,940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol]; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)-methanone; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary; PTX, pertussis toxin; PMSF, phenylmethylsulfonyl fluoride; ANOVA, analysis of variance; AM1241, 2-iodo-5-nitrophenyl-(1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl)methanone.



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out the unwanted psychoactive CNS effects associated with CB1 agonists (Cravatt and Lichtman, 2004).

Recently the endogenous counterparts of $\Delta^9$-tetrahydrocannabinol have been revealed, and interest in investigating their pharmacology is increasing. The term *endocannabinoid* was coined in 1995 (Di Marzo and Fontana, 1995) to describe the function of this emerging class of innate signaling lipids that bind to cannabinoid receptors. To date, anandamide (Devane et al., 1992), 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995), noladin ether (NE) (Hanus et al., 2001), and virodhamine (Porter et al., 2002) have been included in this class. All bind to CB1 receptors with nanomolar affinity (Howlett et al., 2002), except virodhamine, which binds to both CB1 and CB2 receptors with micromolar affinity (Porter et al., 2002). Currently, no evidence indicates any endocannabinoid binds to CB2 receptors with submicromolar affinity. This disparity suggests that physiologically relevant CB2 endocannabinoids may yet be discovered.

NE was initially extracted from porcine (Hanus et al., 2001) and rat (Fezza et al., 2002) brain in moderate concentrations and identified as a putative endogenous cannabinoid agonist at CB1 receptors (Hanus et al., 2001). However, additional studies demonstrating low levels in the CNS of several mammalian species (Oka et al., 2003) indicate that NE might not be a physiologically relevant endogenous agonist at CB1 receptors in the brain. Because of the reported selective affinity for CB1 ($K_i$ = 21 nM) compared with CB2 receptors ($K_i > 3$ $\mu$M) (Hanus et al., 2001), the pharmacological actions of NE at CB2 receptors have not been examined. Therefore, the purpose of this study was to characterize the binding and functional properties of NE at human CB2 receptors stably expressed in CHO cells (e.g., CHO-CB2) as well as in HL-60 cells, which express CB receptors endogenously.

## Materials and Methods

**Materials.** Penicillin/streptomycin (10,000 IU/ml and 10,000 $\mu$g/ml, respectively), Geneticin (G418), fetal calf serum, RPMI 1640 medium, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Mediatech (Herndon, VA). The transfection agent lipofectin and serum-free medium Opti-MEM were obtained from Invitrogen (Carlsbad, CA). CP 55,950 (CP), NE, 2-AG, and AM630 were procured from Tocris Cookson Inc. (St. Louis, MO). [$^3$H]CP (168 Ci/mmol) and [$^{35}$S]GTP$\gamma$S (1250 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [$^3$H]Adenine (26 Ci/mmol) was purchased from Vitrax (Placenia, CA). Pertussis toxin was acquired from List Biological Laboratories Inc. (Campbell, CA). All other reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

**Cell Culture and Stable Transfection.** Chinese hamster ovary (CHO) cells were stably transfected with human CB2 receptor cDNA (Guthrie Research Institute, Sayre, PA) and were cultured in DMEM with 10% (v/v) fetal calf serum, 0.05 IU/ml penicillin, 50 $\mu$g/ml streptomycin, and 250 $\mu$g/ml of the selection antibiotic Geneticin (G418) and incubated in a humidified atmosphere of 5% $CO_2$, 95% $O_2$ at 37°C. Experiments were conducted with cells maintained between passages 4 and 18. In cases where pertussis toxin (PTX) and chronic drug treatments were examined, drugs were added to the medium for 24 h before the assays, and flasks were washed twice with warmed DMEM to remove residual drug or toxin before beginning an assay. Stable cell lines expressing CB2 receptors were created using the cationic-lipid lipofectin. CHO cells were cultured to 80% confluence ($3 \times 10^6$ cells in 100-mm dishes) and incubated for six h with 5 $\mu$g of pcDNA3.1 plasmids (Invitrogen) containing the cDNA encoding for the CB2 receptor, and 15 $\mu$g of lipofectin reagent in the serum-free Opti-MEM medium. Selective antibiotic (1 mg/ml G418) was added to the cell culture medium 48 h after transfection, and surviving colonies were picked 14 days after beginning selection. To confirm CB2 receptor expression, competition binding using whole cells obtained from each colony was performed with 0.2 nM of [$^3$H]CP displaced by nonradioactive CP (1 $\mu$M) as described below. The clone expressing the highest level of CB2 receptor binding (e.g., CHO-CB2) was selected for future studies. For all studies, CHO-CB2 cells were maintained in DMEM containing 250 $\mu$g/ml G418. HL-60 cells were a generous gift from P. Zimniak (University of Arkansas for Medical Sciences, Little Rock, AR) and were cultured in RPMI 1640 containing 10% fetal calf serum, 0.05 IU/ml penicillin and 50 $\mu$g/ml streptomycin.

**Membrane Preparation.** Brain tissue was collected from decapitated male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA). Specific brain regions were dissected from fresh rat brains while on ice. Tissue samples were then pooled before beginning homogenization. Pellets of frozen/thawed cells or freshly harvested brain tissue (Prather et al., 2000a) were resuspended in a homogenization buffer containing 50 mM HEPES, pH 7.4, 3 mM $MgCl_2$, and 1 mM EGTA. Using a 40-ml Dounce glass homogenizer (Wheaton, Philadelphia, PA), samples were subjected to 10 complete strokes and centrifuged at 18,000 rpm for 10 min at 4°C. After repeating the homogenization procedure twice more, the samples were resuspended in 50 mM HEPES buffer, pH 7.4, and subjected to 10 strokes using a 7-ml glass homogenizer. Membranes were stored in aliquots of approximately 1 mg/ml at −80°C.

**Saturation Binding.** Each binding assay contained 50 $\mu$g of membrane protein in a final volume of 1 ml in binding buffer (50 mM Tris, 0.1% bovine serum albumin, and 5 mM $MgCl_2$, pH 7.4), as described previously (Prather et al., 2000b). Membranes were incubated for 90 min at room temperature under gentle agitation with increasing concentrations of [$^3$H]CP (0.01–5 nM). Nonspecific binding was defined as binding observed in the presence of 10 $\mu$M nonradioactive CP. All binding experiments were performed in triplicate. Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters followed by two washes with ice-cold binding buffer. Binding data were analyzed using GraphPad Prism version 4.0b (GraphPad Software, Inc., San Diego, CA) by nonlinear regression to provide estimates of the apparent affinity ($K_d$) and receptor density ($B_{MAX}$) of [$^3$H]CP.

**Competition Binding.** Increasing concentrations of various nonradioactive cannabinoid ligands were incubated with 0.1 nM [$^3$H]CP in a final volume of 1 ml of binding buffer as described previously (Prather et al., 2000b). Each binding assay contained 50 $\mu$g of membrane protein and reactions were incubated for 90 min at room temperature with mild agitation. Nonspecific binding was defined as binding observed in the presence of 10 $\mu$M nonradioactive CP. Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters followed by two washes with ice-cold binding buffer. Analysis of the binding data were performed using the nonlinear regression (Curve Fit) function of GraphPad Prism version 4.0b to determine the concentration of the drug that displaced 50% of [$^3$H]CP ($IC_{50}$). A measure of affinity ($K_i$) was derived from the $IC_{50}$ values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**[$^{35}$S]GTP$\gamma$S Binding.** The [$^{35}$S]GTP$\gamma$S binding assay was performed as described previously (Prather et al., 2000a) in a buffer containing 20 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$ at pH 7.4. Each binding reaction contained 50 $\mu$g of CHO-CB2 membrane protein, the presence or absence of increasing concentrations of cannabinoid ligands, plus 0.1 nM [$^{35}$S]GTP$\gamma$S and 10 $\mu$M GDP to suppress basal G protein activation. Reactions were incubated for 1 h at 30°C. Nonspecific binding was defined by binding observed in the presence of 10 $\mu$M nonradioactive GTP$\gamma$S. The reaction was terminated by rapid vacuum filtration through glass fiber filters followed

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by two washes with ice-cold assay buffer. Four milliliters of Scintiverse (Fisher Scientific Co., Hampton, NH) was added to the filters, and the amount of radioactivity on the filters was determined by scintillation counting 12 h later.

**Measurement of cAMP Levels.** The conversion of [$^3$H]adenine-labeled ATP pools to cyclic AMP was used as a functional measure of cannabinoid agonist activity (Prather et al., 2000b). CHO-CB2 cells were seeded into 17-mm (24-well) plates ($4 \times 10^6$ cells/plate) and cultured to confluence. The day of the assay, an incubation mixture of DMEM containing 0.9% NaCl, 500 $\mu$M 3-isobutyl-1-methlyxanthine (IBMX), and 2 $\mu$Ci/well [$^3$H]adenine was added to the cells for 2 h at 37°C. The [$^3$H]adenine was removed, and the cannabinoid agonists were added to the cells for 15 min in a Krebs-Ringer-HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, and 10 mM HEPES, pH 7.4) containing 500 $\mu$M IBMX and 10 $\mu$M forskolin. The reaction was terminated with the addition of 50 $\mu$l of 2.2 N hydrochloric acid. [$^3$H]cAMP was separated by column chromatography. Ten milliliters of liquid scintillation cocktail was added to the final eluate before counting on a Packard Tri-Carb 2100TR liquid scintillation counter.

To measure intracellular cAMP levels in HL-60 cells cultured in suspension, approximately $5 \times 10^6$ cells were resuspended in an incubation mixture (DMEM containing 0.9% NaCl, 500 $\mu$M IBMX, and 4 $\mu$Ci/ml [$^3$H]adenine). Resuspended cells were incubated at 37°C in a 5% $CO_2$ incubator for 2 h and agitated every 30 min. After the [$^3$H]adenine incubation, cells were washed and resuspended in an ice-cold assay buffer (Krebs-Ringer-HEPES buffer, 500 $\mu$M IBMX, and 10 $\mu$M forskolin). Cannabinoid agonists were incubated with the cells for 15 min at 37°C. In selected experiments, 100 $\mu$M phenylmethylsulfonyl fluoride (PMSF) was added to the assay buffer and incubated with cells 10 min before the addition of agonists. The reaction was terminated with the addition of 50 $\mu$l of 2.2 N HCl. Cellular debris was removed via centrifugation at 1200 rpm for 5 min, and the supernatant containing the [$^3$H]cAMP was separated by column chromatography, as noted above.

**Statistics.** All data are expressed as mean ± S.E.M. For parameters estimated from the log concentration axis (e.g., $IC_{50}$, $K_i$, and $K_d$), averages were calculated as the geometric mean (Kenakin, 1977). Unless otherwise stated, data are represented by a minimum of three separate experiments, performed in triplicate. All curve-fitting and statistical analysis was conducted by using the computer program GraphPad Prism version 4.0b (GraphPad Software, Inc.). To compare three or more groups, statistical significance of the data was determined by a one-way ANOVA, followed by post hoc comparisons using a Tukey's or Dunnett's test. To compare two groups, the nonpaired Student's $t$ test was used. In some instances, a one-sample $t$ test was used to determine whether means were significantly different from 100%.

## Results

**NE Binds to Both CB1 and CB2 Receptors with High Affinity.** Saturation binding using [$^3$H]CP and CHO-CB2 membranes indicates the presence of a single, high-affinity binding site with a $K_d$ of 0.38 (0.32–0.45) nM and a receptor density ($B_{max}$) of 1.44 ± 0.24 pmol/mg protein ($n$ = 4; data not shown). Competition binding (Fig. 1A) demonstrates that all ligands fully displaced [$^3$H]CP from CB2 receptors with a rank order of affinity from highest to lowest of CP > NE ≥ 2-AG (Table 1).

To determine the affinity of the cannabinoid ligands for CB1 receptors, competition binding with [$^3$H]CP was conducted using membranes prepared from rat cerebellum. Cannabinoids bind to CB1 receptors in the cerebellum with a rank order of affinity of CP > NE > 2-AG (Fig. 1B; Table 1). In particular, as anticipated, NE demonstrates >50-fold higher affinity for CB1 receptors compared with CB2 receptors (9.14 versus 480 nM, respectively). Binding performed in the presence of 100 $\mu$M PMSF, a commonly used nonspecific enzymatic inhibitor to prevent degradation, did not affect the $K_i$ value of 2-AG (data not shown).

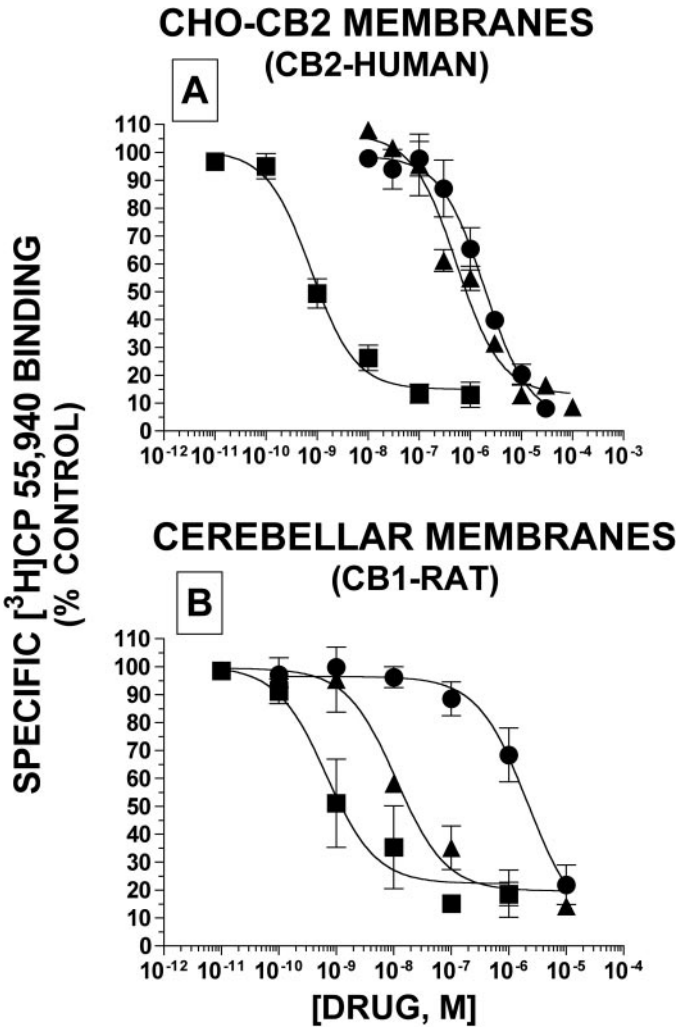


**Fig. 1.** Competition binding between [$^3$H]CP 55,940 and cannabinoid agonists in membranes prepared from CHO-CB2 cells (A) or rat cerebellum (B). Receptor binding experiments were performed as described under *Materials and Methods*. CHO-CB2 membranes (A) and rat cerebellar membranes (B) were incubated with 0.1 nM [$^3$H]CP 55,940 and increasing concentrations of nonradioactive CP 55,940 (■), noladin ether (▲), or 2-arachidonoyl glycerol (●). Nonspecific binding was defined by the addition of 10 $\mu$M of nonradioactive CP 55,940. Data are presented as the percentage of specific [$^3$H]CP 55,940 binding observed in the presence of increasing concentrations of the nonradioactive ligands, compared with the specific binding of [$^3$H]CP 55,940 alone (e.g., percentage of control). Receptor affinity ($K_i$) values for each cannabinoid ligand were derived from the $IC_{50}$ values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are presented in Table 1. Data represent the mean ± S.E.M for three or four independent experiments performed in triplicate.

**NE Activates G Proteins with Similar Efficacy Relative to Other Full CB2 Cannabinoid Agonists.** To determine whether NE acted as an agonist or antagonist at CB2 receptors, the ability of cannabinoids to activate G proteins was examined. All agonists produce a concentration-dependent increase in [$^{35}$S]GTP$\gamma$S binding in CHO-CB2 membranes (Fig. 2A; Table 1). Furthermore, NE activates G proteins with equivalent efficacy relative to the full cannabinoid agonists 2-AG and CP. The rank order of potency for G

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TABLE 1

Receptor affinity, G protein activation, and adenylyl cyclase regulation by cannabinoid agonists

Competition binding was performed with 0.1 nM [$^3$H]CP 55,940 in the presence of increasing concentrations of the competing drugs. The $ED_{50}$ and $E_{MAX}$ values for stimulation of [$^{35}$S]GTP$\gamma$S binding and the $IC_{50}$ and $I_{MAX}$ values for inhibition of adenylyl cyclase activity were derived by nonlinear regression analysis of full concentration-effect curves. Data are presented as the mean (±S.E.M.) determined from three to nine separate experiments, each performed in triplicate.

| Drug | Cerebellum (CBI-Rat) | Transfected CHO-CB2 Cells (CB2-Human) | | | | |
|---|---|---|---|---|---|---|
| | | | | [$^{35}$S]GTP$\gamma$S Binding | | Adenylyl Cyclase |
| | $K_i$ | $K_i$ | $ED_{50}$ | $E_{MAX}$ | $IC_{50}$ | $I_{MAX}$ |
| | *nM* | *nM* | *nM* | % | *nM* | % |
| CP | 0.55$^a$ (0.31–0.98) | 0.58$^a$ (0.42–0.79) | 0.54$^a$ (0.41–0.67) | 33.1$^a$ (31.0–35.5) | 0.39$^a$ (0.25–0.60) | 56.8$^a$ (52.0–61.6) |
| NE | 9.14$^b$ (7.10–11.78) | 480$^{b,**}$ (408–564) | 221$^b$ (156–286) | 44.3$^a$ (41.4–47.2) | 35.0$^b$ (26.5–46.1) | 47.3$^a$ (42.8–51.8) |
| 2-AG | 1750$^c$ (1183–2588) | 1016$^b$ (736–1403) | 253$^b$ (208–298) | 43.2$^a$ (37.8–48.6) | 230$^c$ (207–255) | 48.5$^a$ (43.7–53.3) |

$^{a-c}$ Values in the same column designated with different letters are significantly different, $P < 0.01$ (one-way ANOVA followed by Tukey's post hoc comparison).
** Significantly different from the $K_i$ value determined in cerebellum, $P < 0.01$ (unpaired Student's $t$ test).

protein activation by the three agonists is similar to their apparent affinity for CB2 receptors: CP > NE ≥ 2-AG.

**NE Acts as a Full Agonist to Regulate the Intracellular Effector Adenylyl Cyclase.** To determine whether the G proteins activated by NE proceed to regulate intracellular effectors, the ability of cannabinoids to regulate adenylyl cyclase activity in whole CHO-CB2 cells was evaluated. All three cannabinoid agonists examined produce a concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in whole CHO cells expressing CB2 receptors. The rank order of potency is similar to their CB2 receptor affinity: CP > NE > 2-AG (Fig. 2B; Table 1). Agonists are also equally efficacious, producing similar maximal reductions in cAMP levels of approximately 50% (Table 1). Addition of 100 $\mu$M PMSF does not alter the amount of 2-AG required to produce half-maximal inhibition of adenylyl cyclase activity in whole CHO-CB2 cells (e.g., $IC_{50}$; data not shown).

**Adenylyl Cyclase Inhibition by NE Is Mediated through CB2 Receptors Coupled to $G_i/G_o$ Proteins.** To determine whether the inhibition of adenylyl cyclase activity produced by NE is specifically mediated through the activation of CB2 receptors, AM630, a specific CB2 receptor antagonist/inverse agonist, was used (Ross et al., 1999). It was first determined that AM630 binds to CB2 receptors in CHO-CB2 cells with an affinity of 22.5 (21.1–24.0) nM (data not shown). When cells are incubated with a receptor saturating concentration of AM630 (10 $\mu$M) 5 min before the administration of agonist concentrations previously shown to produce half-maximal effects, the inhibition of adenylyl cyclase by all ligands is virtually eliminated (Fig. 3A). To determine whether adenylyl cyclase inhibition produced by the cannabinoid agonists is mediated through CB2 receptors coupling to $G_i/G_o$ proteins, functional assays were conducted after overnight treatment with 200 ng/ml pertussis toxin. This PTX pretreatment eliminates the ability of receptor-saturating concentrations of CP (1 $\mu$M), NE (10 $\mu$M), or 2-AG (10 $\mu$M) to produce inhibition of adenylyl cyclase activity (Fig. 3B).

**Chronic Exposure to NE Results in CB2 Receptor Desensitization and Down-Regulation.** Chronic exposure of cells expressing cannabinoid and other G protein-coupled receptors to agonists results in a loss of response when cells are subsequently challenged after drug washout (Breivogel et al., 1999). This adaptation to prolonged drug exposure is known as receptor desensitization and occurs in part due to uncoupling of receptors from their downstream G proteins and/or effectors. CHO-CB2 cells were exposed for 24 h to receptor saturating concentrations of CP (1 $\mu$M) or NE (10 $\mu$M) and subsequently challenged acutely with each drug to determine the effect on the regulation of adenylyl cyclase activity. After chronic exposure to CP or NE, inhibition of adenylyl cyclase activity elicited by acute challenge with either agonist is eliminated (Fig. 4A).

Down-regulation is another adaptation of G protein-coupled receptors occurring in response to chronic agonist exposure (Breivogel et al., 1999). Overnight pretreatment of CHO-CB2 cells with receptor saturating concentrations of either CP (1 $\mu$M) or NE (10 $\mu$M) decreases specific [$^3$H]CP binding by 93 and 42%, respectively (Fig. 4B).

**NE Acts as a Full Agonist at Endogenously Expressed CB2 Receptors in HL-60 Cells.** The regulation of adenylyl cyclase activity by cannabinoid agonists was measured in HL-60 cells that endogenously express CB2, but not CB1, receptors (Bouaboula et al., 1996). Surprisingly, CP and NE, but not 2-AG, produce inhibition of adenylyl cyclase activity in HL-60 cells (Fig. 5A). Although the inhibition produced by NE is minimal (e.g., 15.6 ± 2.6%), it nevertheless produces the greatest effect of any agonist tested under these conditions. Most importantly, pretreatment with the selective CB2 antagonist AM630 (10 $\mu$M) completely reverses the ability of NE to inhibit adenylyl cyclase activity.

It is possible that the unanticipated lack of effect by 2-AG in these cells might be due to its rapid metabolism, preventing CB2 receptor activation. As such, the ability of NE and 2-AG to inhibit adenylyl cyclase activity was examined in the presence of 100 $\mu$M of the nonselective enzymatic inhibitor PMSF (Fig. 5B). Under these conditions, NE and 2-AG produce equivalent levels of adenylyl cyclase inhibition (e.g., NE, 34.5 ± 4.4%; 2-AG, 34.3 ± 4.4%). Interestingly, it seems that the addition of PMSF also enhances the effect of NE, resulting in over twice the level of inhibition relative to that produced in untreated cells.

## Discussion

Our results demonstrate that the putative CB1-selective endocannabinoid NE also binds to human CB2 receptors with relatively high nanomolar affinity. Furthermore, NE activates G proteins and produces inhibition of the intracellular effector adenylyl cyclase in CHO-CB2 cells with equivalent efficacy relative to the full cannabinoid agonists CP and 2-AG. The rank order of potency for G protein activation and effector regulation by the three agonists is similar to their

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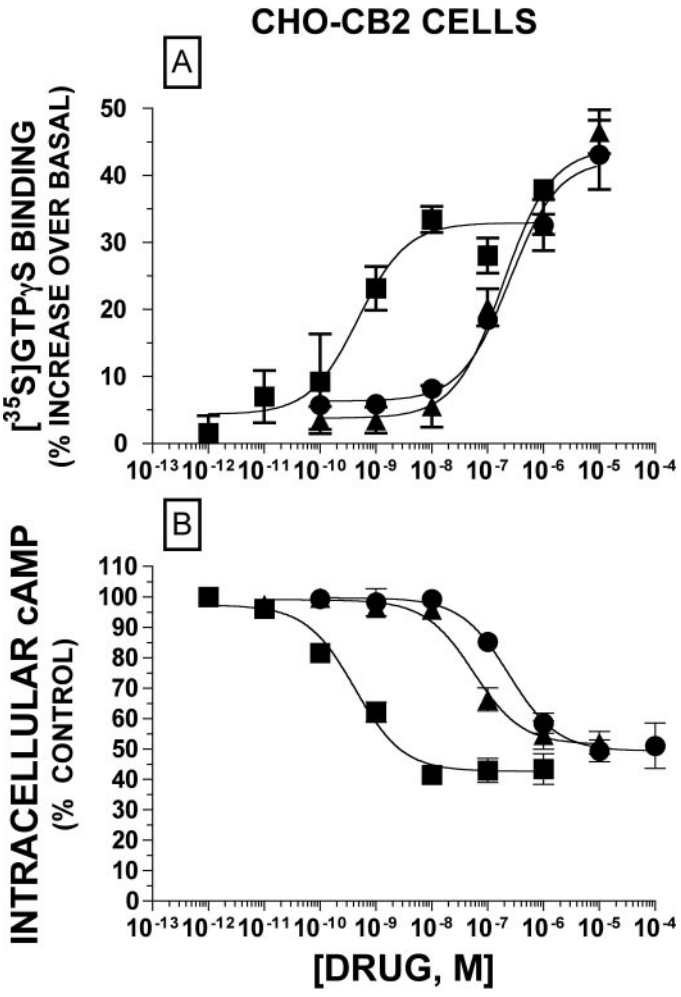


**Fig. 2.** [$^{35}$S]GTP$\gamma$S binding (A) and inhibition of adenylyl cyclase activity (B) by cannabinoid agonists in CHO-CB2 cells. A, amount of [$^{35}$S]GTP$\gamma$S binding occurring in the presence of increasing concentrations CP 55,940 (■), noladin ether (▲), or 2-arachidonoyl glycerol (●) was used as a measure of G protein activation. Nonspecific binding was determined by the inclusion of 10 $\mu$M of nonradioactive GTP$\gamma$S. Data are presented as the percentage of increase in [$^{35}$S]GTP$\gamma$S binding in the presence of the indicated drug compared with basal binding in the absence of any agonist (e.g., percentage of increase over basal). The $ED_{50}$ and $E_{MAX}$ values determined for each agonist are presented in Table 1. Data represent the mean ± S.E.M. for four experiments performed in triplicate. B, 10 $\mu$M forskolin stimulated adenylyl cyclase assays were conducted in whole cells as described under *Materials and Methods*. The level of intracellular cAMP was measured in response to increasing concentrations of CP 55,940 (■), noladin ether (▲), or 2-arachidonoyl glycerol (●) in whole CHO-CB2 cells. All agonists produced concentration-dependent inhibition of adenylyl cyclase activity, resulting in maximal reductions of cAMP levels of approximately 50%. Data are presented as the percentage of cAMP levels measured in the presence of the indicated drug concentrations, compared with that observed in the absence of any drug (i.e., percentage of control). Data represent the mean ± S.E.M. from four to nine experiments performed in triplicate. The $IC_{50}$ and $I_{MAX}$ values for each ligand were derived by nonlinear regression analysis and are presented in Table 1.

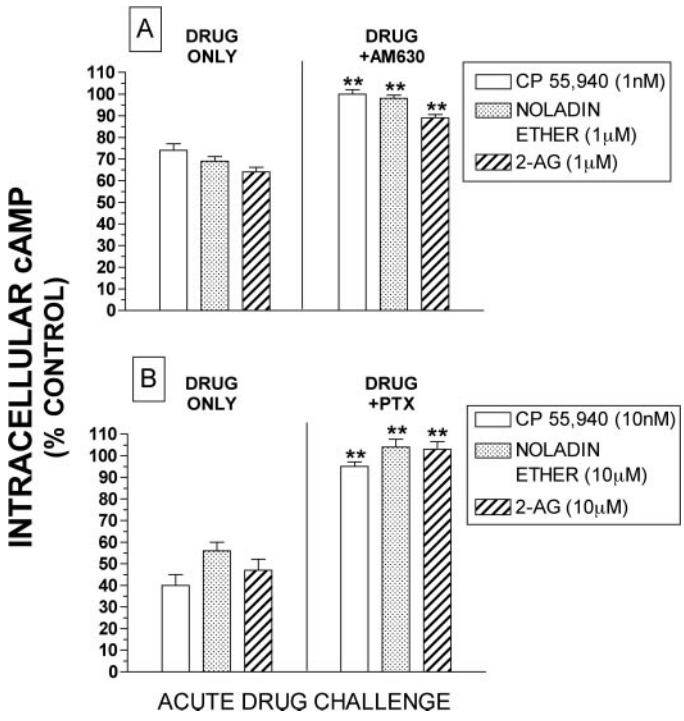


**Fig. 3.** The effect of a pretreatment with a CB2 antagonist (A) or pertussis toxin (B) on cannabinoid agonist mediated inhibition of adenylyl cyclase activity in CHO-CB2 cells. A, 10 $\mu$M forskolin-stimulated adenylyl cyclase assays were conducted in whole cells as described under *Materials and Methods*. CHO-CB2 cells were incubated with the CB2-selective antagonist AM630 (10 $\mu$M) or vehicle for 5 min before the addition of the indicated agonist. The inhibition of adenylyl cyclase activity produced by all three agonists was significantly reversed by pretreatment with AM630. Data are presented as the mean ± S.E.M. for three independent experiments performed in triplicate. **, significantly different from the percentage of inhibition produced by the indicated agonist in the absence of AM630 (unpaired Student's $t$ test, $P < 0.01$). B, CHO-CB2 cells exposed to PTX overnight (200 ng/ml) and washed twice before adenylyl cyclase assays as described under *Materials and Methods*. Pretreatment with pertussis toxin completely blocked the inhibition of adenylyl cyclase activity produced by all three agonists tested. Data are presented as the mean ± S.E.M. for three to five independent experiments performed in triplicate. **, significantly different from the percentage of inhibition produced by the indicated agonist in cells not pretreated with pertussis toxin (unpaired Student's $t$ test, $P < 0.01$).

apparent affinity for CB2 receptors; CP > NE ≥ 2-AG. The ability of NE and other agonists examined to reduce cAMP levels is mediated specifically through activation of CB2 receptors and requires the participation of pertussis toxin-sensitive $G_i/G_{o\alpha}$ proteins. Chronic exposure of CHO-CB2 cells to NE produces CB2 receptor adaptations similar to that produced by prolonged administration of the full agonist CP, including desensitization and down-regulation. Last, the agonist activity of NE is not only limited to transfected cells but also occurs in HL-60 cells expressing endogenous CB2 receptors. Collectively, these results indicate that NE acts as a full agonist at human CB2 receptors.

To date, most research into the signaling properties of endocannabinoids focuses on their neuromodulatory functions mediated by CB1 receptors. In contrast, little is known about the peripheral effects of endocannabinoids mediated by CB2 receptors. In this study and others (Hanus et al., 2001; Steffens et al., 2005), NE exhibits high nanomolar affinity for the CB1 receptors. Conversely, NE has been shown to have only low micromolar affinity for CB2 receptors (Hanus et al., 2001). This degree of CB1 receptor selectivity indicates that NE should preferentially, and possibly only, activate CB1 receptors. However, after initial identification of low-to-moderate levels of NE in porcine (Hanus et al., 2001) and rat (Fezza et al., 2002) brain, subsequent studies indicate a relative absence of NE (Oka et al., 2003) in CB1-rich brain tissue (Herkenham et al., 1990). If NE levels in the CNS are indeed proven to be negligible, this brings into question the physiological relevance of the action of NE at CB1 receptors.

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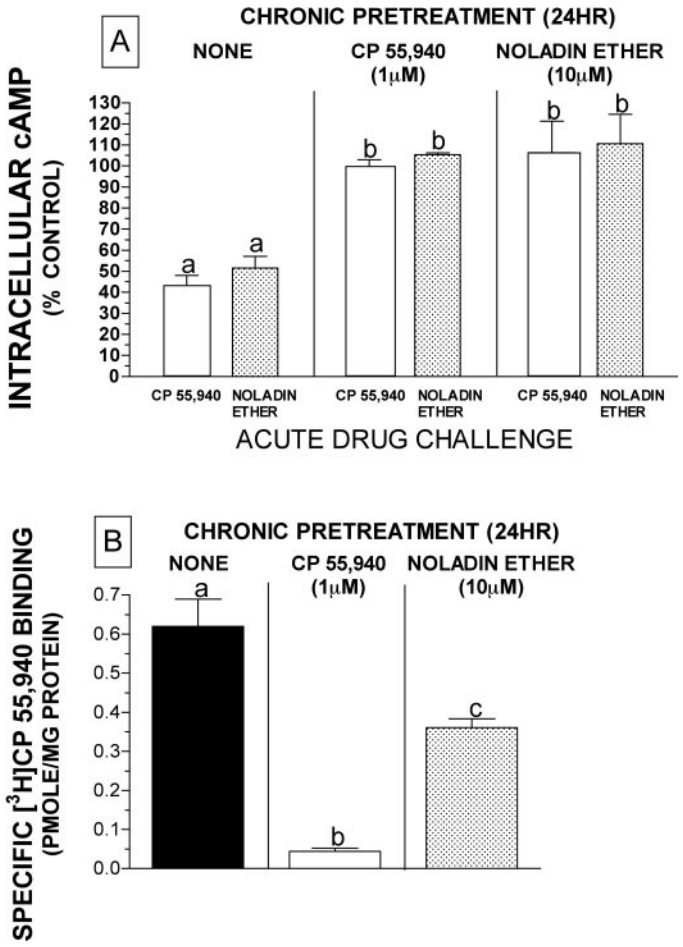


**Fig. 4.** Chronic exposure (24 h) of CHO-CB2 cells to receptor saturating concentrations of noladin ether or CP 55,940 produces CB2 receptor desensitization (A) and down-regulation (B). A, CHO-CB2 cells cultured for 24 h in the presence of receptor saturating concentrations of vehicle (left), CP 55,940 (1 $\mu$M; middle), or noladin ether (10 $\mu$M; right). After extensive washing to remove residual agonist, 10 $\mu$M forskolin-stimulated adenylyl cyclase assays were conducted in whole cells as described under *Materials and Methods*. Overnight exposure to either cannabinoid agonist eliminated the ability of the agonists to produce inhibition of adenylyl cyclase activity upon acute challenge after chronic agonist washout. Data are presented as the mean ± S.E.M. for three experiments performed in triplicate. a and b, values designated with different letters above the error bars are significantly different (one-way ANOVA followed by a Tukey's post hoc comparison, $P < 0.01$). B, CHO-CB2 cells cultured for 24 h in the presence of receptor saturating concentrations of vehicle (left), CP 55,940 (1 $\mu$M; middle), or noladin ether (10 $\mu$M; right). After extensive washing to remove residual agonist, membranes were prepared, and receptor binding was conducted as described under *Materials and Methods*. Chronic exposure to CP 55,940 or noladin ether produced 92 or 43% reductions in specific [$^3$H]CP 55,940 binding, respectively. Data represent the mean ± S.E.M. for three to six experiments performed in triplicate. a–c, values designated with different letters above the error bars are significantly different (one-way ANOVA followed by a Tukey's post hoc comparison, $P < 0.01$).

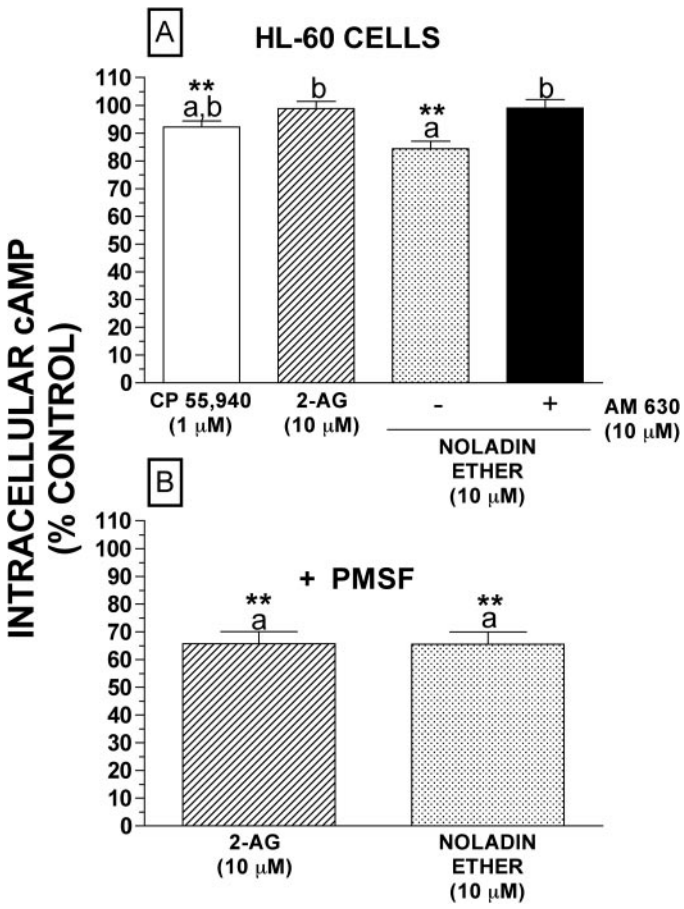


**Fig. 5.** Inhibition of adenylyl cyclase activity by cannabinoid agonists in HL-60 cells endogenously expressing CB2 receptors. A, 10 $\mu$M forskolin-stimulated adenylyl cyclase assays conducted in whole HL-60 cells as described under *Materials and Methods*. Receptor saturating concentrations of CP 55, 950 (1 $\mu$M) and noladin ether (10 $\mu$M), but not 2-arachidonoyl glycerol (10 $\mu$M), produced a significant reduction in intracellular cAMP levels. The inhibition produced by noladin ether was completely reversed when cells were preincubated with the CB2-selective antagonist AM630 (10 $\mu$M) for 5 min. Data are presented as the mean ± S.E.M. for four to 10 independent experiments performed in triplicate. B, 10 $\mu$M forskolin-stimulated adenylyl cyclase assays were conducted in whole HL-60 cells in the presence of 100 $\mu$M of the nonspecific enzymatic inhibitor PMSF as described under *Materials and Methods*. Receptor saturating concentrations of noladin ether (10 $\mu$M) and 2-arachidonoyl glycerol (10 $\mu$M) produced a significant reduction in intracellular cAMP levels. Data are presented as the mean ± S.E.M. for four independent experiments performed in triplicate. a and b, values designated with different letters above the error bars are significantly different (one-way ANOVA followed by a Tukey's post hoc comparison, $P < 0.01$). **, significantly different from 100% (one-sample $t$ test, $P < 0.01$).

Although currently unknown, if NE is found to be present in the CB2-rich periphery in physiologically relevant amounts, it might instead serve a physiologically important role at CB2 receptors. Support for this hypothesis is provided by the relatively high affinity of both NE and 2-AG for CB2 receptors reported in the present study. Not only does NE bind to CB2 receptors with high affinity, but evidence provided here indicates that NE also acts as a full agonist at CB2 receptors compared with other well characterized CB2 agonists. It is also possible that NE may not be found in appreciable amounts in peripheral tissues. Such observations in the periphery, combined with negligible levels in the CNS, would bring into question the classification of NE as an endocannabinoid with physiological relevance.

Endocannabinoid signaling is altered in several acute and chronic pathological conditions (Di Marzo et al., 2004). Overproduction of endocannabinoids such as 2-AG occurs in models of inflammation and is associated with the induction of chemokines through CB2 receptor activation (Sugiura et al., 2004). In contrast, other endogenous cannabinoids seem to exhibit anti-inflammatory and antinociceptive properties (Calignano et al., 1998; Conti et al., 2002). Although little is yet known concerning the involvement of NE in such disease states, CB2 antagonists have been shown to produce signif-

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icant hyperalgesia in models of pain initiated by tissue injury (Calignano et al., 1998). It has been suggested that the hyperalgesia produced in these models may occur due to a reduction of endogenous cannabinoid tone in cutaneous tissues. Although the endogenous ligands responsible for maintaining such tone have yet to be identified, a selective CB2 agonist, AM1241, is effective in blocking neuropathic pain (Ibrahim et al., 2003). The authors suggest that CB2 receptor activation by AM1241 may reduce the release of inflammatory mediators or inhibit input to the CNS. If the levels of 2-AG, NE, anadamide, virodhamine, or other as yet unidentified endocannabinoids are regulated in response to pathological conditions occurring in the periphery, they might serve as important modulators for the development of inflammation or chronic nociception through their action at CB2 receptors. As such, development of CB2 agonists as potential pharmacological agents for pain management in such conditions is attractive due to the relative absence of unwanted, adverse CNS side effects that are often observed with the most efficacious analgesics currently available (Cravatt and Lichtman, 2004).

The action of endocannabinoids at CB2 receptors might also be important in modulating the development of neuroinflammatory states. Postmortem analysis of the brains of Alzheimer's patients show that central CB2 receptors are overexpressed in neuritic plaque associated astrocytes and microglia (Benito et al., 2003). This relationship indicates a potential protective role for endocannabinoids in such disease states. The endocannabinoid 2-AG has been shown to activate microglial cells, the immune cells of the CNS, in models of neuroinflammation (Walter et al., 2003). Furthermore, these authors showed that microglial activation occurs through activation of CB2 receptors that are not expressed under basal conditions. Although the level of NE present in the intact mammalian brain is controversial (Hanus et al., 2001; Fezza et al., 2002; Oka et al., 2003), the amount of NE or other endocannabinoids might be augmented, in a manner similar to that of 2-AG (Walter et al., 2003) when exposed to inflammatory stimuli. As such, NE as well as 2-AG might participate in the development of such neuroinflammatory disease states. Regardless, it remains to be determined whether enhanced levels of NE or 2-AG would have a beneficial or harmful action. The general consensus is that cannabinoids are initially protective, but continuous activation leads to negative events (Di Marzo et al., 2004).

Many studies indicate that chronic exposure to cannabinoids may trigger adaptations of CB1 and CB2 receptors, including desensitization and down-regulation. It has been suggested that these adaptations may contribute to the development of tolerance and/or dependence (Breivogel et al., 1999; Howlett et al., 2002). The fact that prolonged exposure to NE produces receptor adaptations similar to CP provides further support for the suggestion that NE acts as an agonist at CB2 receptors. Interestingly, it seems that whereas chronic NE and CP treatment produce equivalent levels of desensitization, prolonged NE exposure results in significantly less receptor down-regulation than CP. This indicates that CB2 receptor adaptations to prolonged endocannabinoid exposure may be less pronounced and thus result in development of less tolerance and dependence.

It might be suggested that the relatively high receptor affinity and full agonist activity of NE at CB2 receptors reported in the present study may result from the overexpression of CB2 receptors in the cellular model used. This explanation seems unlikely for the following reasons. First, the original study by Hanus et al. (2001), reporting negligible binding of NE to CB2 receptors, used transfected COS7 cells also overexpressing human CB2 receptors as their cellular model. Second, the affinity of CP and 2-AG for CB2 receptors reported in the present study using transfected CHO-CB2 cells are similar to those determined previously in physiological systems (Rinaldi-Carmona et al., 1994; Mechoulam et al., 1995). Last and most importantly, NE produces equivalent adenylyl cyclase inhibition relative to the full agonist 2-AG in HL-60 cells expressing physiological levels of CB2 receptors. Together, these results strongly support the hypothesis that the relatively high receptor affinity and functional regulation produced by NE at CB2 receptors expressed in CHO-CB2 cells is not only due merely to high receptor expression levels but also occurs in tissues that contain physiological densities of CB2 receptors.

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